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JOURNAL of FOOD SCIENCE

BASIC SCIENCE

633 Preparation of yeast protein isolate with low nucleic acid by succinylation—*K.J. Shetty and J.E. Kinsella*
639 Preliminary identification of volatile flavor compounds in the neutral fraction of roast beef—*D.B.S. Min, K. Ina, R.J. Peterson and S.S. Chang*
643 Dye binding procedure for the estimation of protein content of meat components and sausage emulsions—*G.J. Seperich and J.F. Price*
646 *Clostridium perfringens* population dynamics during constant and rising temperatures in beef—*D.R. Thompson, R.R. Willardsen, F.F. Busta and C.E. Allen*
652 Retention and stability of volatile sulfur-containing compounds in chicken meat—*M.I. Farbood and J.H. MacNeil*
656 Ion-exchange chromatography and electrophoresis of egg yolk proteins—*L.E. Mc Bee and O.J. Cotterill*
661 Textural deterioration of red hake and haddock muscle in frozen storage as related to chemical parameters and changes in the myofibrillar proteins—*T.A. Gill, R.A. Keith and B. Smith-Lall*
668 Sodium nitrite and sorbic acid effects on *Clostridium botulinum* toxin formation in chicken frankfurter-type emulsions—*J.N. Sofos, F.F. Busta, K. Bhothipaksa and C.E. Allen*
673 Characteristics of some radiation-resistant hemolytic micrococci isolated from chicken—*A.B. Welch and R.B. Maxcy*
676 Fatty and amino acid composition of salted mullet roe—*J.Y. Lu, Y.M. Ma, C. Williams and R.A. Chung*
678 Chemical composition of chops from pale, soft, exudative (PSE) and normal pork loins—*R.C. Ewan, D.G. Topel and K. Ono*
681 Gamma-irradiation of 5′-inosinic acid in fish meat cake—*S. Uchiyama and M. Uchiyama*
685 Fluorescence changes resulting from methemoglobin and fatty-acid interaction—*S. Grossman, I.S. Hammerman and T. Schaap*
690 Protein quality of mechanically processed (species) product and bone residue—*R.A. Field, Y.-O. Chang and W.G. Kruggel*
693 Fat cell rupture in a comminuted meat batter as a determinative factor of heat stability—*B.J. Tinbergen and W.J. Olsman*
696 Frozen concentrated cultures of *Kluyveromyces Fragilis*—*M. Bostian and S.E. Gilliland*
700 Effect of oxidation-reduction potential on the outgrowth and chemical inhibition of *Clostridium botulinum* 10755A spores—*L.A. Smoot and M.D. Pierson*
705 Activation of *Bacillus stearothermophilus* spores at low pH—*Y.E. El-Mabsout and K.E. Stevenson*
710 Inhibition of *Staphylococcus aureus* growth by combinations of butylated hydroxyanisole, sodium chloride, and pH—*N.J. Stern, L.A. Smoot and M.D. Pierson*
713 Thermal destruction of folacin: Effect of ascorbic acid, oxygen and temperature—*T.-S. Chen and R.G. Cooper*
717 Thermal destruction of folacin: Effect of pH and buffer ions—*B. Paine-Wilson and T.-S. Chen*
723 Vitamin analysis by microbial and protozoan organisms: response to food preservatives and neutralization salts—*M.N. Voigt, R.R. Eitenmiller and G.O. Ware*
729 Comparison of protozoan and conventional methods of vitamin analysis—*M.N. Voigt, R.R. Eitenmiller and G.O. Ware*
735 Comparison of the abilities of trichloroacetic, picric, sulfosalicylic, and tungstic acids to precipitate protein hydrolysates and proteins—*N.A. Greenberg and W.F. Shipe*
738 Zinc interference with copper, iron and manganese in young Japanese quail—*R.P. Hamilton, M.R.S. Fox, B.E. Fry Jr., A.O.L. Jones and R.M. Jacobs*
742 Effect of glucose and fructose on incubation glycogenic capacity in tissue from mice previously consuming various carbohydrates—*P.E. Araujo*
745 Influence of dietary fiber on the growth and protein metabolism of the rat—*M.Z.A. Nomani, E.F. Fashandi, G.K. Davis and C.J. Bradac*
748 Relative sweetness of fructose and sucrose in model solutions, lemon beverages and white cake—*A.V. Cardello, D. Hunt and B. Mann*
752 Influence of pH on the binding of copper, zinc and iron in six fiber sources—*S.A. Thompson and C.W. Weber*
755 Sporulation of *Clostridium perfringens* in meats supplemented with soy materials—*S.E. Craven and A.J. Mercuri*
758 Gas chromatographic characterization of sages of various geographic origins—*H.Y. Rhyu*
763 Evaluation of a fluorometric technique for quantitative determination of soy flour in meat-soy blends—*A.C. Eldridge and L.G. Holmes*
765 Salt concentration, pH and flour concentration effects on nitrogen solubility and emulsifying properties of peanut flour—*K.H. McWatters and M.R. Holmes*
770 Influence of pH and salt concentration on nitrogen solubility and emulsification properties of soy flour—*K.H. McWatters and M.R. Holmes*

Contents continued on the inside of the front cover

IFT
A PUBLICATION OF
THE INSTITUTE OF FOOD TECHNOLOGISTS

774 Influence of moist heat on solubility and emulsification properties of soy and peanut flours—*K.H. McWatters and M.R. Holmes*

777 Orange pyruvate decarboxylase: Isolation and mechanistic studies—*W.R. Raymond, J.B. Hostettler, K. Assar and C. Varsel*

782 Factors affecting the threshold of methyl and ethyl oleate emulsions in raisins and raisin paste—*D.G. Guadagni and A.E. Stafford*

785 Heat inactivation of peroxidase in corn-on-the-cob—*Y.C. Lee and J.K. Hammes*

788 Water-soluble products from patulin during alcoholic fermentation of apple juice—*E.E. Stinson, S.F. Osman and D.D. Bills*

790 Effect of storage time and conditions on the hard-to-cook defect in cowpeas (*Vigna unguiculata*)—*S. Sefa-Dedeh, D.W. Stanley and P.W. Voisey*

797 Pasting behavior of rice: A new method of viscography—*K.R. Bhattacharya and C.M. Sowbhagya*

APPLIED SCIENCE AND TECHNOLOGY

801 Sonication effect on potato starch and sweet potato powder—*A. Azhar and M.K. Hamdy*

805 Composition of raw and cooked potato peel and flesh: Proximate and vitamin composition—*J. Augustin, R.B. Toma, R.H. True, R.L. Shaw, C. Teitzel, S.R. Johnson and P. Orr*

807 Changes in the nutrient composition during commercial processing of frozen potato products—*J. Augustin, B.G. Swanson, C. Teitzel, S.R. Johnson, S.F. Pometto, W.E. Artz, C.P. Huang and C. Schomaker*

810 Sensory properties of high fructose corn syrup ice cream formulations—*A.M. Pearson and D.M. Ennis*

813 Sweetness of lactulose relative to sucrose—*F.W. Parrish, F.B. Talley, K.D. Ross, J. Clark and J.G. Phillips*

816 Production of precooked corn flours for Arepa making using an extrusion cooker—*O. Smith, T.S. de Buckle, A.M. de Sandoval and G.E. González*

820 Peanut-fortified food blends—*G.N. Bookwalter, K. Warner, R.A. Anderson and E.B. Bagley*

826 Production of textured vegetable protein from cottonseed flours—*J. Cabrera, L.E. Zapata, T.S. de Buckle, I. Ben-Gera, A.M. de Sandoval and I. Shomer*

831 Heating characteristics of cream-style corn processed in a steritort: Effects of headspace, reel speed and consistency—*M.R. Berry Jr., R.A. Savage and I.J. Pflug*

836 Sweet corn: Varietal and developmental differences in amino acid content and composition of grain—*J.E. Sanderson, J.W. Paulis, F.N. Porcuna and J.S. Wall*

839 Baking characteristics and protein quality of soy-whole egg, soy-egg yolk and soy-egg white supplemented breads—*M.P. O'Connor, J.W. Erdman Jr. and A.I. Nelson*

843 Effects of storage conditions on firmness of brined cucumbers—*R.L. Thompson, H.P. Fleming and R.J. Monroe*

847 Sensory quality of cucumbers before and after brining—*D.M. Ennis and J.O'Sullivan*

850 Premiums and coupons as a potential source of objectionable flavor in cereal products—*M.G. Heydanek Jr., G. Woolford and L.C. Baugh*

854 Preparation of a shelf-stable, intermediate moisture food product from muscadine grape skins—*L.F. Flora, L.R. Beuchat and V.N.M. Rao*

857 Prevention of toughening of chicken breast muscle cut immediately after slaughter by control of pH decline—*D.W. Peterson and A.L. Lilyblade*

859 Consumer acceptability and retail yield in beef after pelvic suspension and delayed chilling—*E.D. Aberle and M.D. Judge*

863 Shelf-life and quality characteristics of poultry parts dipped in potassium sorbate—*F.E. Cunningham*

865 Effects of oilseed proteins, at two replacement levels, on chemical, sensory and physical properties of frankfurters—*R.N. Terrell, J.A. Brown, Z.L. Carpenter, K.F. Mattil and C.W. Monagle*

869 Effects of precooking beef patties on palatability, cooking properties and storage stability—*H.R. Cross, D. Muse and E.C. Green*

873 Vitamin B_{12} and ascorbic acid in Kimchi inoculated with *Propionibacterium freudenreichii ss. shermanii*—*S.L. Ro, M. Woodburn and W.E. Sandine*

878 Discrimination of fish and seafood quality by consumer populations—*J.B. Wesson, R.C. Lindsay and D.A. Stuiber*

883 Stability of refrigerated mullet sausage—*L.H. Daley, J.C. Deng and J.L. Oblinger*

887 Essential elements in fresh and in canned onions—*A. Lopez and H.L. Williams*

892 Application of HPLC to characterization of individual carbohydrates in foods—*W.J. Hurst, R.A. Martin Jr. and B.L. Zoumas*

896 Optimization of a lactose hydrolysis process—*F.C. Knopf, M.R. Okos, D.A. Fouts and A. Syverson*

901 Use of graphic linear scales to measure rates of staling in beer—*D.J. Schmitt and J.T. Hoff*

905 Analysis of continuous sterilization processes for Bingham plastic fluids in laminar flow—*C. Guariguata, J.A. Barreiro and G. Guariguata*

RESEARCH NOTES

911 Influence of electrical stimulation on certain characteristics of heavy-weight beef carcasses—*J.W. Savell, G.C. Smith, Z.L. Carpenter and F.C. Parrish Jr.*

914 Quality of boneless dry-cured ham produced with or without nitrate, netting or potassium sorbate—*J.D. Kemp, B.E. Langlois, M.B. Solomon and J.D. Fox*

916 Estimation of fresh mutton color by reflectance spectrophotometry—*C.D.P. Attrey and T.R. Sharma*

918 Connective tissue profiles of various raw sausage materials—*E.L. Wiley, J.O. Reagan, J.A. Carpenter and D.R. Campion*

920 Relationship between methylmercury and total mercury in blue crabs (*Callinectes sapidus*)—*D.R. Ward, R. Nickelson II and G. Finne*

922 Amine content of toxic and wholesome canned tuna fish—*I.-S. Kim and L.F. Bjeldanes*

Research Notes (continued from preceding page)

924 Enzyme neutral detergent fiber analysis of selected commercial and home-prepared foods—*W.-L.J. Jwuang and M.E. Zabik*
926 High-pressure liquid chromatography of cranberry anthocyanins—*A.L. Camire and F.M. Clydesdale*
928 Microbiological and toxicological evaluation of fermented cowpeas (*Vigna sinensis*) and chickpeas (*Cicer arientinum*)—*A.F. Zamora and M.L. Fields*
930 Sensory evaluation and nutritive value of soups and chips made from fermented and nonfermented beans—*A.F. Zamora and M.L. Fields*
932 Ascorbic acid in peas cooked by microwaves—*L.B. Mabesa and R.E. Baldwin*
934 Loss of green color of refrigerated and nonrefrigerated southern peas—*W.C. Hurst and G.A. Schuler*
936 Germination and relative nutritive value of corn meal and corn chips—*N.B. Hashim and M.L. Fields*
938 Intensification of fruit-flavors by aspartame—*R.E. Baldwin and B.M. Korschgen*
940 Extruded corn flour as an alternative to lime-heated corn flour for tortilla preparation—*C.D. Bazúa, R. Guerra and H. Sterner*
942 A comparison of the suitability of three hydrogen donors in the determination of peroxidase activity—*M. Marshall and G.W. Chism*
944 Effects of peanut shell flour on firmness, color and acceptance of peanut butter—*J.L. Collins and J.F. Sanchez*
946 Studies on kinetics of inosine 5′-monophosphate instability—*J.H. Lee*
948 Erratum notice
ii Letters

AUTHOR INDEX

Volume 44: Number 3

Aberle, E.D., 859
Allen, C.E., 646, 668
Anderson, R.A., 820
Araujo, P.E., 742
Artz, W.E., 807
Assar, K., 777
Attrey, D.P., 916
Augustin, J., 805, 807
Azhar, A., 801

Bagley, E.B., 820
Baldwin, R.E., 932, 938
Barreiro, J.A., 905
Baugh, L.C., 850
Bazua, C.D., 940
Berry, M.R. Jr, 831
Beuchat, L.R., 854
Bhattacharya, K.R., 797
Bhothipaksa, K., 668
Bills, D.D., 788
Binte Hasim, N., 936
Bjeldanes, L.F., 922
Bookwalter, G.N., 820
Bostian, M., 696
Bradac, C.J., 745
Brown, J.A., 865
Busta, F.F., 646, 668

Cabrera, J., 826
Camire, A.L., 926
Campion, D.R., 918
Cardello, A.V., 748
Carpenter, J.A., 918
Carpenter, Z.L., 865, 911
Chang, S.S., 639
Chang, Y-O., 690
Chen, T.-S., 713, 717
Chism, G.W., 942
Chung, R.A., 676
Clark, J., 813
Clydesdale, F.M., 926
Collins, J.L., 944
Cooper, R.G., 713
Cotterill, O.J., 656
Craven, S.E., 755
Cross, H.R., 869
Cunningham, F.E., 863

Daley, L.H., 883
Davis, G.K., 745
de Buckle, T.S., 816, 826
de Sandoval, A.M., 816, 826
Deng, J.C., 883

Eitenmiller, R.R., 723, 729
Eldridge, A.C., 763
El-Mabsout, Y.E., 705
Ennis, D.M., 810, 847
Erdman, J.W. Jr, 839
Ewan, R.C., 678

Farbood, M.I., 652
Fashandi, E.F., 745
Field, R.A., 690
Fields, M.L., 928, 930, 936
Finne, G., 920
Fleming, H.P., 843
Flora, L.F., 854
Fouts, D.A., 896
Fox, J.D., 914
Fox, M.R.S., 738
Fry, B.E. Jr, 738

Gill, T.A., 661
Gilliland, S.E., 696
Gonzalez, G.E., 816
Green, E.C., 869
Greenberg, N.A., 735
Grossman, S., 685
Guadagni, D.G., 782
Guariguata, C., 905
Guariguata, G., 905
Guerra, R., 940

Hamdy, M.K., 801
Hamilton, R.P., 738
Hammerman, I.S., 685
Hammes, J.K., 785
Harper, J.M., ii
Heydanek, M.G. Jr, 850
Hoff, J.T., 901
Holmes, L.G., 763
Holmes, M.R., 765, 770, 774
Hostettler, J.B., 777
Huang, C.P., 807
Hunt, D., 748
Hurst, W.J., 892
Hurst, W.C., 934

Ina, K., 639

Jacobs, R.M., 738
Johnson, S.R., 805, 807
Jones, A.O.L., 738
Judge, M.D., 859
Jwuang, W-L.J., 924

Keith, R.A., 661
Kemp, J.D., 914
Kim, I-S., 922
Kinsella, J.E., 633
Knopf, F.C., 896
Korschgen, B.M., 938
Kruggel, W.G., 690
Langlois, B.E., 914
Lee, J.H., 946
Lee, Y.C., 785
Lilyblade, A.L., 857
Lindsay, R.C., 878
Lopez, A., 887
Lu, J.Y., 676

McBee, L.E., 656
MacNeil, J.H., 652
McWatters, K.H., 765, 770, 774
Ma, M., 676
Mabesa, L.B., 932
Man, B., 748
Marshall, M., 942
Martin, R.A. Jr, 892
Mattil, K.F., 865
Maxcy, R.B., 673
Mercuri, A.J., 755
Min, D.B.S., 639
Monagle, C.W., 865
Monroe, R.J., 843
Muse, D., 869

Nelson, A.I., 839
Nickelson, R. II, 920
Nomani, M.Z.A., 745

Oblinger, J.L., 883
O'Connor, M.P., 839
Okos, M.R., 896
Olsman, W.J., 693
Ono, K., 678
Orr, P., 805
Osman, S.F., 788
O'Sullivan, J., 847

Paine-Wilson, B., 717
Parrish, F.W. Jr, 813, 911
Paulis, J.W., 836
Pearson, A.M., 810
Peterson, D.W., 857
Peterson, R.J., 639
Pflug, I.J., 831
Phillips, J.G., 813
Pierson, M.D., 700, 710
Pometto, S.F., 807
Porcuna, F.N., 836
Price, J.F., 643

Rao, V.N.M., 854
Raymond, W.R., 777
Reagan, J.O., 918
Rhyu, H.Y., 758
Ro, S.L., 873
Ross, K.D., 813

Sanchez, J.F., 944
Sanderson, J.E., 836
Sandine, W.E., 873
Savage, R.A., 831
Savell, J.W., 911
Schaap, T., 685
Schmitt, D.J., 901
Schomaker, C., 807
Schuler, G.A., 934
Sefa-Dedeh, S., 790
Seperich, G.J., 643
Sharma, T.R., 916
Shaw, R.L., 805
Shetty, K.J., 633
Shipe, W.F., 735
Shomer, I., 826
Smith, G.C., 911
Smith, O., 816
Smith Lall, B., 661
Smoot, L.A., 700, 710
Sofos, J.N., 668
Solomon, M.B., 914
Sowbhagya, C.M., 797
Stafford, A.E., 782
Stanley, D.W., 790
Stern, N.J., 710
Sterner, H., 940
Stevenson, K.E., 705
Stinson, E.E., 788
Stuiber, D.A., 878
Swanson, B.G., 807
Syverson, A., 896

Talley, F.B., 813
Teitzel, C., 805, 807
Terrell, R.N., 865
Thompson, D.R., 646
Thompson, R.L., 843
Thompson, S.A., 752
Tinbergen, B.J., 693
Toma, R.B., 805
Topel, D.G., 678
True, R.H., 805

Uchiyama, M., 681
Uchiyama, S., 681

Varsel, C., 777
Voigt, M.N., 723, 729
Voisey, P.W., 790

Wall, J.S., 838
Ward, D.R., 920
Ware, G.O., 723, 729
Warner, K., 820
Weber, C.W., 752
Welch, A.B., 673
Wesson, J.B., 878
Wiley, E.L., 918
Willardsen, R.R., 646
Williams, C., 676
Williams, H.L., 887
Woodburn, M., 873
Woolford, G., 850

Zabik, M.E., 924
Zamora, A.F., 928, 930
Zapata, L.E., 826
Zoumas, B.L., 892

Letters

EXTRUDER NOT PREREQUISITE FOR TEXTURE FORMATION

The recent article by Taranto et al. (1978) reported the differences between soy and cottonseed flours texturized by a laboratory extruder and heated press. The authors work again emphasized that the extruder is not a prerequisite for the formation of texture. As additional support for this position, these authors cited prior work using a heated press reported by McAnelly (1964), Touba (1970), and Sterner and Sterner (1976).

Other nonextrusion low moisture texturization processes exist and are worth noting. Dunning, et al. (1972) and Valentas and Tsuchiya (1976) reported that steam heated protein particles would texturize when released rapidly to the atmosphere. The steam texturization process has been commercialized (Lemaire, 1977) and now accounts for a significant portion of texturized vegetable protein production.

Since it has been clearly demonstrated that extrusion is not necessary to develop texture, then the significant question is what are the requisite environments for intermediate moisture texturization and how might these be varied to control or optimize the process? Considerable evidence exists that changes in the flow induced shear environment while the temperature is being controlled has a major effect on the texturization process. A shear field can align long protein molecules, necessary for fiber formation, while very high shear rates and stresses may actually rupture bonds that cross link proteins, reducing product stability.

Flow induced shear occurs in both the extrusion screw and die. Smith and Crocco (1975) have reported on a dual extrusion process where cooled texturized soy protein is extruded a second time in a low shear screw to enhance layered fiber formation and meat-like texture. Feldbrugge et al. (1978) have shown a textured product can be made in a heated extrusion screw under low rates of shear with very little backpressure. The use of a long temperature controlled die to achieve product texturization and retort stability has been reported by Crocco (1976) and Sair and Quass (1976). Aguilera et al. (1976) showed that the most extensive fiber formation in texturized soy came after the product had expanded through the extrusion die.

Sterner and Sterner (1976) have demonstrated how time, temperature and pressure affect texturization in a heated press. The flow induced shear on these products occurs when the product violently expands from the press when the pressure is released. Due to the nature of the expansion process, it is difficult to characterize the shear rate and stresses in the rapidly expanding product coming from a heated press, extrusion die or steam texturizing gun. If the heated product in a press is rapidly cooled and not released or allowed to expand, the resulting product is normally very polymerized but does not seem to contain oriented fibers. This observation provides further evidence that some sort of shear field is required for the orientation of fibers in texturized products.

Because texturization only occurs at relatively high temperatures with very viscous doughs, experimentation on the effect of a controlled shear environment on fiber formation will be difficult but I expect very fruitful.

—*Judson M. Harper,* Dept. of Food Engineering & Biotechnology, Technion-Israel Institute of Technology, Technion City, Haifa

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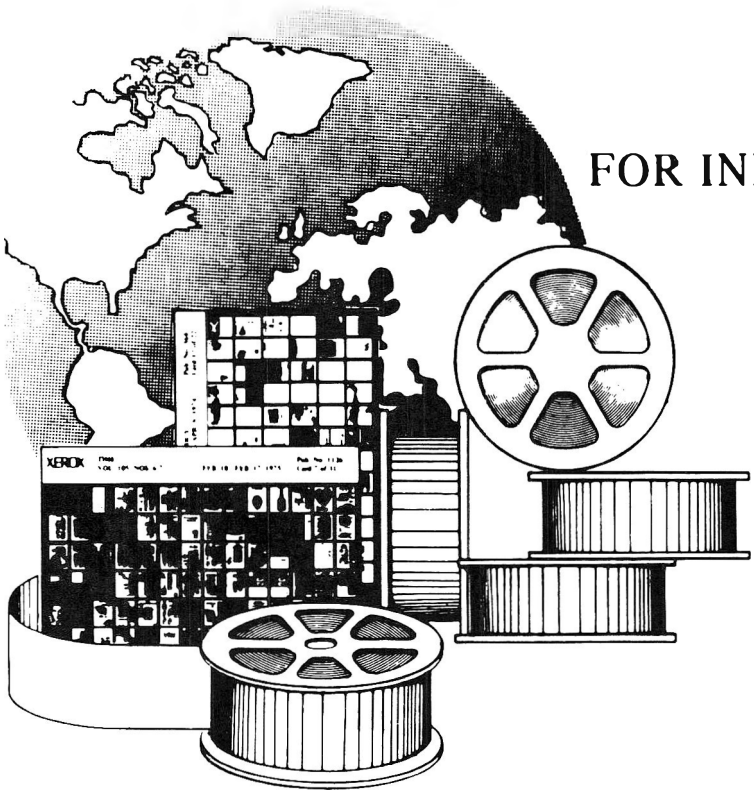

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PREPARATION OF YEAST PROTEIN ISOLATE WITH LOW NUCLEIC ACID BY SUCCINYLATION

K. J. SHETTY and J. E. KINSELLA

ABSTRACT

Succinylation after cell disruption facilitates the isolation of yeast protein in high yields with less than 2% nucleic acid. The method involves the addition of excess succinic anhydride at pH 8 immediately after cell disruption followed by isoelectric precipitation of the modified protein at pH 4.2. Most of the nucleic acid remains in the protein free supernatant. Succinylation increased extractability of the protein, decreased proteolysis and improved thermal stability. The precipitated protein contained 15.61, 1.8 and 7.0% nitrogen, nucleic acid and carbohydrate, respectively.

INTRODUCTION

AS THE WORLD population expands toward 6 billion by the 21st century, the need for more food protein will become accentuated (Waterlow and Payne, 1975; Sukhatme, 1972). Proteins from microbial cells, particularly yeast, could significantly supplement those produced from conventional sources which will become increasingly limited by the prevailing constraints on land, energy and cost of production by conventional agriculture. The advantages of yeast protein, a source which can complement conventional methods of food production, have been well documented (Cooney et al., 1975; Lipinsky and Litchfield, 1970, 1974; McLaren, 1975, Tannenbaum and Wang, 1975; Tannenbaum, 1971, 1977; Tannenbaum et al., 1973; Kinsella and Shetty, 1978). However, a number of problems associated with microbial protein have to be solved to make it an acceptable, safe and inexpensive source of protein for human consumption.

Cell wall material in unfractionated microbial cells is undesirable because it reduces the bioavailability of proteins (Kihlberg, 1972; Worgan, 1975) and may contain antigenic, allergenic agents and factors causing nausea, gastrointestinal disturbances (flatulence, diarrhea) (Scrimshaw, 1975; Young et al., 1976; Young and Scrimshaw, 1975). For these reasons and other potential applications of these protein(s), it is desirable to separate cell wall material from the protein. The various methods used to rupture yeast cell walls have been discussed by Mogren and Lindblom, (1974); Vananuvat and Kinsella, (1975a); Kinsella and Shetty, (1978). Treatment of yeast cells or disrupted yeast cells with alkali weakens the cell wall and facilitates the solubilization of proteins (Hedenskog and Mogren, 1973; Lindblom, 1974; Vananuvat and Kinsella, 1975a). However, alkaline treatment of food grade proteins is undesirable because of hydrolysis of proteins, depolymerization, racemization of component amino acids, β-elimination reactions, cross-linking and denaturation (Cheftel, 1977; Friedman, 1977; DeGroot et al., 1977).

Another problem associated with consumption of microbial protein is its high content of nucleic acid (NA) which ranges from 8–25g per 100g protein. Most of the NA is present as RNA (Sinskey and Tannenbaum, 1975). Before yeast can be used as a major source of protein for human consumption, the content of NA must be reduced, so that daily intake of NA from yeast would not exceed 2g on dry weight basis. High quantities cause uricemia and continued ingestion of yeast may also result in gout (Miller, 1968; Waslien, et al., 1970). The current methods used for the reduction of RNA include enzymatic and/or chemical treatments during extraction of the proteins (Sinskey and Tannenbaum, 1975; Lindblom, 1974). However, the enzymatic processes result in concurrent proteolysis of the protein during treatment, with significant reduction in yields of protein (Sinskey and Tannenbaum, 1975; Lindblom, 1977). The chemical processes with alkali cause chemical alteration, denaturation and destruction of desirable physicochemical properties of extracted proteins (DeGroot et al., 1977; Vananuvat and Kinsella, 1978). Thus, the existing methods for the recovery of the proteins and the reduction of the NA result in low yields of protein, impairment of desirable functional characteristics and destruction of some essential amino acids.

Functional properties are important in determining the potential uses of new proteins for the development of new food products (Kinsella, 1976, 1977; Feeney, 1977). There is limited information available concerning the functional properties of yeast proteins (Akin, 1976; Newell et al., 1975). Tannenbaum (1971) alluded to the importance of functional properties and need for the study of physical properties of SCP and Vananuvat and Kinsella (1975b) reported some of the functional properties of proteins isolated from Saccharomyces fragilis.

Chemical derivatization to modify the functional properties of proteins from seeds and yeast for food applications has been reviewed by Feeney (1977) and Kinsella and Shetty, (1979). Chemical modification may be used for several purposes in relation to food proteins, to block reactive groups involved in deteriorative reactions, to improve nutritional properties, to enhance digestibility, to facilitate the study of structure-function relationship and to aid the separation, processing and refining of proteins (Kinsella, 1976, 1977; Feeney, 1977).

In an earlier study a decrease in the nucleic acid content of yeast protein was observed following succinylation (Vananuvat and Kinsella, 1978). In this paper we describe how treatment of yeast cell homogenates with succinic anhydride immediately after cell disruption facilitates the isolation of intact yeast proteins in high yields with low nucleic acid level. The effects of succinylation on protein extractability, proteolytic enzymes, ribonuclease, on the heat coagulability of proteins and nucleic acid content of protein isolated from mechanically disrupted yeast cells are reported.

MATERIALS & METHODS

Preparation of disrupted yeasts

Brewer's yeast obtained from Genesee Brewing Company, New York, was washed three times with distilled water, freeze-dried and stored at 3–5°C. The freeze-dried yeasts were disrupted in a Braun

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mechanical cell homogenizer (Braun-Melsung, West Germany). For disruption of the cells, equal volumes (50 ml) of cells suspended in water (5% level) and glass beads (0.45–0.50 mm diameter) were placed in the disruption flask and homogenized for 3 min. Licuid CO_2 was passed continuously through the system to cooling during homogenization. Disrupted cells were freeze-dried and stored at 3–4°C.

Derivatization of disrupted yeast cells (homogenate)

A 5% (w/v) suspension of the disrupted yeast cells was prepared in distilled water and adjusted to pH 8.5. Succinic anhydride (Eastman) was added with stirring to the suspension over a 1-hr period (at different concentrations) while the pH was maintained between 8.0 and 8.5 by the addition of NaOH (3.5N). After the pH was stabilized, the suspension was stirred for another 30 min and soluble materials were separated by centrifuging at 28,000 × G for 45 min at 5°C. The pH of the supernatant was then adjusted to 4.2. The precipitated protein was separated, washed once with water (pH 4.2), dissolved in water at pH 8.5, dialyzed against distilled water and lyophilized. When a protein concentrate was required, the pH of the succinylated homogenate was decreased to 4.2 before removal of cellular debris by centrifugation.

Determination of extent of chemical modification

The extent of succinylation was determined by tri-nitrobenzenesulphonic acid (TNBS) method as described by Hall et al. (1973). Protein (<5 mg) in 0.8 ml of aqueous solution was added to 1 ml of 4% $NaHCO_3$ followed by the addition of 0.2 ml of 2,4,6-trinitrobenzenesulphonic acid (12.5 mg/ml). The reaction mixture was incubated at 40°C for 2 hr and 3.5 ml of concentrated HCl was added. Tubes were stoppered and kept at 110°C for 4 hr and then after cooling the volume of the solution was made up to 10 ml with distilled water and extracted twice with anhydrous diethyl ether. The tubes were unstoppered and held at 40°C to allow the residual ether to evaporate. The absorbance of the yellow (ϵ - TNP lysine) solution was measured at 415 nm against a blank. The amount of unreacted lysine in the unsuccinylated and succinylated yeast protein thus determined from the standard curve constructed using lysine.

Methods of analysis

Total lipid content of the protein preparations were determined by the method of Folch et al. (1957). Nitrogen was estimated by the Micro Kjeldahl method (AOAC, 1975) and protein by the method of Lowry et al. (1951). The method of Montgomery (1961) was used to determine the carbohydrate content of protein preparations.

Yeast nucleic acids which consist mainly of ribonucleic acid (RNA) were determined by the method of Herbert et al. (1971). RNA was extracted from samples with aqueous perchloric acid (0.5M) at 37°C for 2 hr. The RNA content of the extract obtained was determined spectrophotometrically with orcinol reagent. Purified yeast RNA (Sigma, St. Louis, MO) was used to prepare the standard curve.

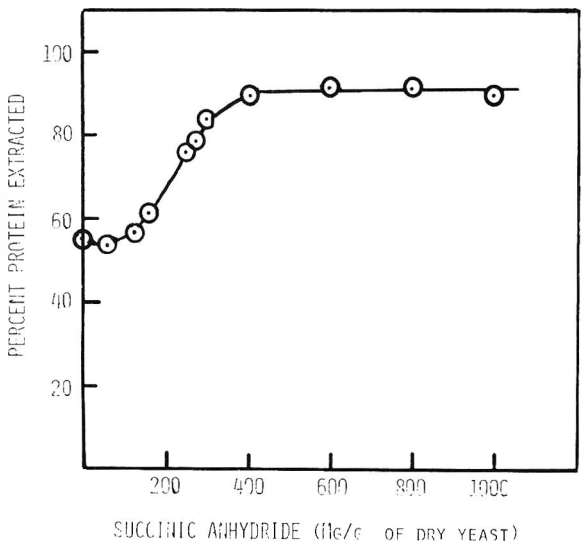

Fig. 1—Effect of succinic anhydride on the extractibility of proteins from mechanically disrupted yeast cells at pH 8.5.

Assay of ribonuclease (RNase) activity

The RNase activity in the yeast suspension and the succinylated yeast suspension was assayed according to the procedure described by Lindblom (1977) in order to check if succinylation affected the endogeneous RNase activity of yeast. Normal, unsuccinylated and succinylated yeast suspension were incubated at 52°C at pH 5.8 for 30 min. The reaction was stopped by adding an equal volume of 0.4% uranyl acetate in 12.5% perchloric acid. After cooling on ice for 15 min the solution was filtered. The filtrate was then diluted with water and the absorbance at 260 nm was determined to quantify extent of hydrolysis by RNase.

Determination of proteolysis during incubation of yeast suspension for RNA reduction

The proteins extracted at pH 9.0 from disrupted cells were succinylated to get more than 90% modification of lysine ϵ-NH_2 groups. The pH was then decreased to 5.8 and the mixture incubated at 52°C in a water bath to activate endogenous RNase and reduce RNA (Lindblom, 1977). For comparison, proteins extracted at pH 9.0 without succinylation were incubated under identical conditions. At different intervals of time, a known volume of the sample was pipetted into a centrifuge tube, cooled to 4°C and the pH was decreased to 4.2 (isoelectric point of the proteins). The precipitated protein was separated by centrifugation, washed once with water (pH 4.2), dissolved in 0.1N NaOH and quantified. The percent protein precipitated in each case was calculated and compared with the concentration in the unsuccinylated extract.

Amino acid analyses

Amino acid analyses were performed on unsuccinylated and succinylated yeast protein isolate to determine whether the succinylation altered the amino acid composition of these proteins. Amino acid analysis was carried out by the procedure of Spackman et al. (1958) and a Beckman automatic amino acid analyzer model 119CL was used.

RESULTS & DISCUSSION

Effect of succinylation on protein extraction from disrupted yeast cells

A plot of the amount of protein extracted from disrupted yeasts as affected by succinic anhydride concentration (Fig. 1) revealed that succinylation of yeast homogenate (suspension prepared from disrupted yeast cell in water, 1:20 w/v) caused a marked increase in the quantity of protein extracted at pH 8.5. The extractability of protein increased with increasing amount of succinic anhydride up to a ratio of 0.4 (succinic anhydride to yeast, g/g). Approximately 55% of the total protein was extractable from disrupted yeast cells at pH 8.5 and this was increased to 90% when the ratio of succinic anhydride to yeast cells was 0.4 (g/g) and above.

Succinylation increases the nitrogen solubility of peanut and wheat flour (Beuchat, 1977 and Grant, 1973). During succinylation positively charged amino groups of the proteins are converted to negatively charged residues (Means and Feeney, 1971). The increased net negative charge may be responsible for increasing solubility of proteins. During extraction of yeast protein under alkaline conditions, the protein may tend to form complexes with insoluble carbohydrate of the yeast cells through carbonyl-amine interactions (Feeney et al., 1975). This may account for the lower recovery of the protein when alkaline extraction is used compared to the present method.

Succinylation immediately following cell rupture, may protect the protein from this kind of complexation and in addition it may induce the release of intracellularly bound protein from insoluble components of the yeast cell, resulting in increased protein extraction. This conclusion is corroborated by the observation of the increased extractability of proteins with increasing concentration of succinic anhydride.

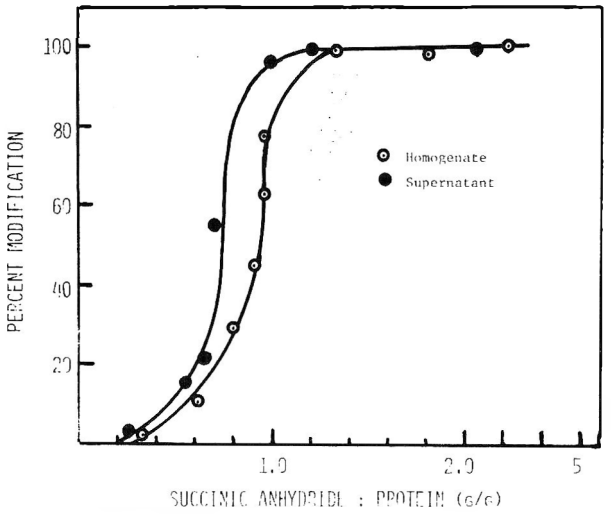

Fig. 2—Effect of succinic anhydride concentration on the extent of modification of ϵ-NH_2 groups of lysine of the extracted yeast proteins.

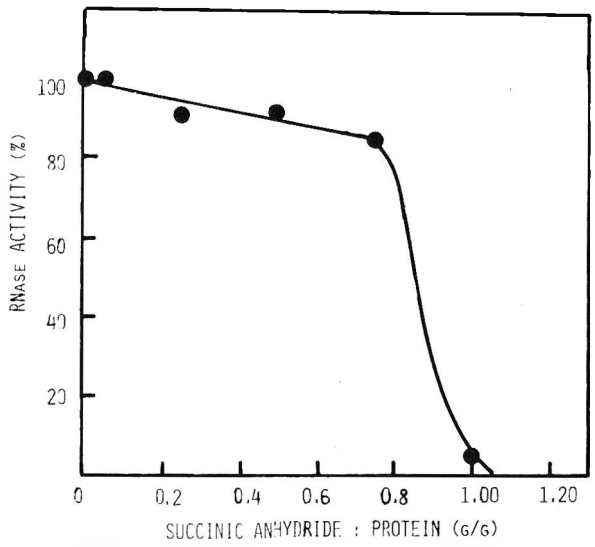

Fig. 3—Effect of succinylation on the RNase activity of yeast.

Extent of succinylation

The extent of derivatization obtained for the cell homogenate depended upon the concentration of the succinic anhydride (Fig. 2). Succinylation increased with the increasing concentration of succinic anhydride and more than 90% modification of the ϵ-NH_2 groups of lysine in the protein was obtained at 1:1 ratio of succinic anhydride to protein.

Yeast cell homogenate contains mainly carbohydrates, proteins and nucleic acids. It is possible that the succinic anhydride reacted with all of these constituents. The extent of succinylation of the proteins may have depended on the presence and reactivity of these other materials, i.e. cell wall and nucleic acids. Hence succinylation studies were carried out on the extracted solubles after separating the cell wall materials by centrifugation. As observed with the cell homogenate, succinylation increased with increasing concentration of succinic anhydride but the extent of modification of available ϵ-NH_2 groups of proteins was greater in the supernatant fraction than in the complete homogenate for the same ratio of succinic anhydride to protein (Fig. 2). Thus, to obtain more than 90% succinylation of available ϵ-NH_2 groups, a ratio of 0.9 and 1.1 (succinic anhydride to protein, g/g) was required for the supernatant and homogenate respectively, the difference reflecting the succinylation of the cell wall materials and possibly carbohydrates.

Effect of succinylation on the endogeneous RNase activity of yeast

The high content of nucleic acid in microbial proteins limit their application in foods for human uses (Scrimshaw, 1975). Several methods have been developed to reduce nucleic acid content of yeast protein (Sinskey and Tannenbaum, 1975). Endogeneous RNase of yeast cell is activated when the disrupted yeast cells are incubated at 55°C at pH 5.8–6.0 (Robbins et al. 1975; Lindblom, 1977). The activation of endogenous RNase during protein isolation is used to reduce RNA content of yeast protein preparation (Lindblom, 1977; Ohta et al., 1971). Hence the effect of succinylation on RNase was examined. Succinylation progressively inactivated RNase (Fig. 3). Complete inactivation occurred when more than 90% of ϵNH_2 groups of the proteins were modified. Succinylation of yeast cell homogenate either altered the enzymatically active structure of RNase or made the substrate resistant to hydrolysis.

Effect of succinylation on heat coagulation of proteins

Normally during extraction of yeast proteins under mild conditions when accompanied by incubation at 55°C (pH 5.8–6.0) to reduce nucleic acid, there is a progressive precipitation of proteins as hydrolysis of RNA proceeds (Robbins et al. 1975; Lindblom and Morgren, 1974; Lindblom, 1977). This coagulated protein is very difficult to resolubilize and has limited functional properties. Furthermore, during this incubation, proteolysis by activated proteases significantly reduces the total yield of proteins (Lindblom, 1977). Both of these undesirable events limit this incubation step as a practical method for reducing RNA in yeast protein. Therefore, the effect of succinylation on the susceptibility of protein to coagulation during incubation (pH 6, 55°C for 30 min) was studied. The soluble materials were extracted at pH 8.5 from mechanically disrupted yeast cells and succinylated to different levels. The pH of each of the succinylated extracts was adjusted to 5.8 and the mixture incubated at 55°C for 30 min. The reaction mixture cooled immediately in an ice-water bath to arrest the enzyme reactions and the protein which spontaneously coagulated was recovered by centrifugation, dissolved in known volume of 0.1N NaOH and the protein was quantified. Coagulation during incubation varied with the extent of succinylation (Fig. 4). Thus up to 20% succinylation tended to enhance the amount of protein precipitation but above this it rapidly decreased. Apparently the increased negative charge on the protein molecules increased the solvent chain interaction leading to the solubilization and minimization of protein-protein interaction and coagulation (Klotz and Keresztes–Nagy, 1962).

Effects of succinylation on proteolytic enzymes

Yeast cells contain intracellular proteolytic enzymes and three of them (protease A, B and C) have been studied in detail (Pringle, 1975). These are liberated after cells are broken by autolysis (Hough and Maddox, 1970; Shetty and Kinsella, 1978) or by mechanical disruption (Follows et al., 1971). These liberated enzymes, unless inactivated during isolation and purification of yeast proteins, hydrolyze the proteins, and result in poor recovery of proteins (i.e. poor yields). In fact, intracellular proteases in yeast play an important role in autolysis in commercial production of yeast extract. Special precautions must therefore be taken during isolation of yeast proteins to minimize proteolysis.

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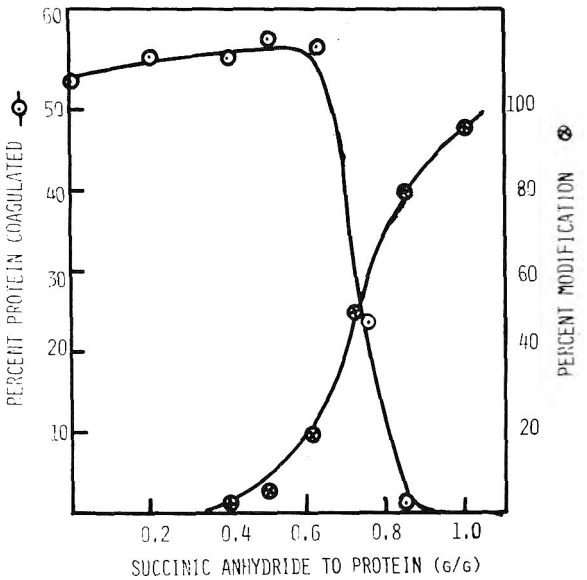

Fig. 4—Effect of succinylation on the percent coagulation of yeast proteins during incubation (pH 6.0, at 55°C for 30 min) for RNA reduction.

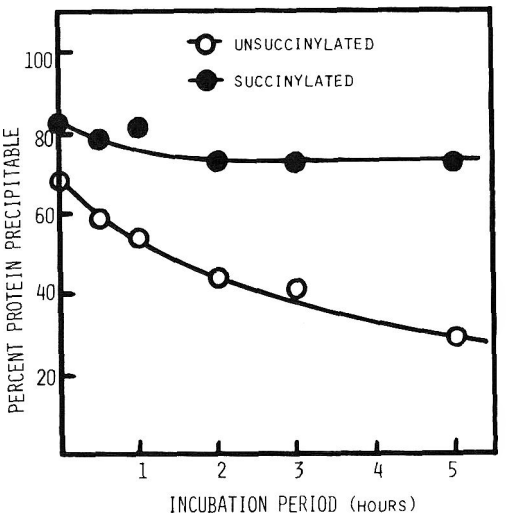

Fig. 5—Effect of succinylation on proteolysis by intracellular proteolytic enzymes of yeast during incubation for RNA reduction (pH 6.0, at 55°C for 30 min).

Potent inhibitors of these proteolytic enzymes, i.e. phenylmethylsulphonyl fluoride and diisopropylfluorophosphate have been reported (Pringle, 1975), but these reagents are highly toxic and not usable in food applications. Thus inhibition of these enzymes through succinylation of yeast proteins was studied.

Succinylation markedly decreased the activity of intracellular proteolytic enzymes of the yeast. The relative rates at which the extracted proteins and succinylated proteins from yeast were hydrolyzed by intracellular proteases of yeast are summarized in Figure 5. About 70% of the extracted protein was initially preciptible from the unsuccinylated supernatant. However, this decreased gradually with increasing incubation period and only 30% of the protein was precipitable after incubation for 5 hr, indicating the extensive hydrolysis of protein by endogenous proteolytic enzymes during incubation. Lindblom (1977) reported that up to 70% of the yeast proteins were degraded during incubation at 55°C, pH 6, for 5 hr to reduce RNA.

Significant reduction in proteolysis was observed when the supernatant proteins were succinylated before incubation (Fig. 5). The precipitation of the protein was increased to 82% from 70% when the proteins were succinylated before incubation. After 5-hr incubation it had decreased only to 75% indicating the inhibition of the proteolytic enzymes activity by succinylation. Inhibition of proteolytic enzymes during extraction may have resulted from inactivation of the proteases by succinylation, nonsusceptibility of succinylated hydrolytic products. Thus, the inhibition of proteolytic enzymes through succinylation may provide a useful approach for obtaining maximum yields of food grade yeast protein.

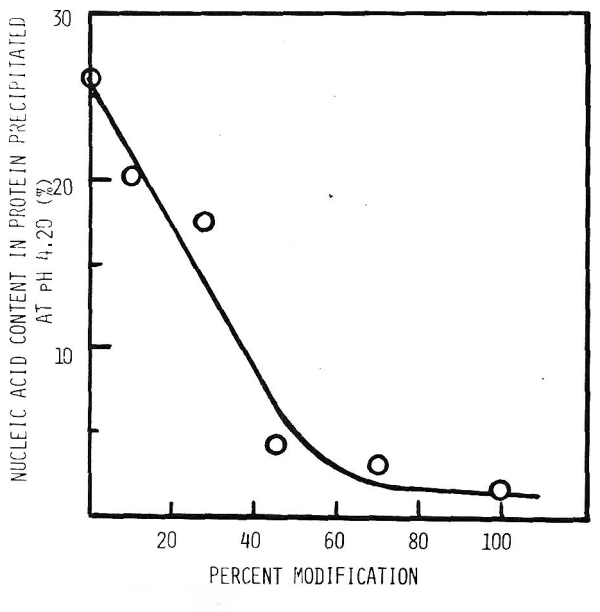

Fig. 6—Effect of succinylation on nucleic acid content in the precipitated protein (pH 4.2) from the succinylated yeast protein extract.

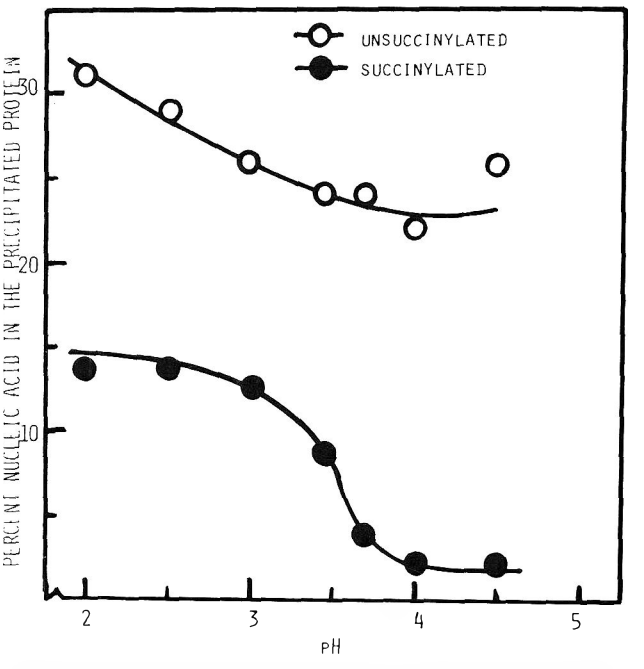

Fig. 7—Effect of pH on the nucleic acid content in the protein precipitated from succinylated and unsuccinylated yeast extract.

Extent of succinylation on nucleic acid reduction

The proteins obtained after isoelectric precipitation from succinylated supernatant were analyzed for nucleic acid content (Fig. 6). Protein obtained from unsuccinylated supernatant contained a very high amount of nucleic acid (~25%) and it decreased steadily with increasing succinylation. A significant decrease was obtained when the proteins were modified more than 90% (Fig. 6). Hence succinylation apparently altered the relative solubilities of modified proteins and nucleic acids at pH 4.2. Therefore, the effect of pH of precipitation on the nucleic acid content of the succinylated (>90%) protein was studied.

A significant decrease in nucleic acid content was obtained for the protein precipitated from the succinylated extract at pH 4.2 compared to the protein precipitated under identical conditions from unsuccinylated extract (Fig. 7). The nucleic acid content in the precipitated protein from nonmodified controls increased with decreasing pH. At pH 4.5, it was around 26% and it increased gradually up to 32% at pH 2.0, whereas protein precipitated from succinylated extract contained significantly less nucleic acid at all pH levels compared to the controls. Protein precipitated around pH 4.5 from the succinylated supernatant contained only 1.8–2.0% nucleic acid. The increased nucleic acid content of proteins precipitated at lower pH values in both cases was attributed to the smaller quantities of proteins precipitated at the lower pH values.

Thus succinylation of yeast proteins by increasing their net electronegativity accentuated the small differences in the total charges between the derivatized proteins and the nucleic acids facilitated the almost exclusive precipitation of proteins at pH 4.5 leaving soluble nucleic acid in the supernatant. This procedure greatly simplified the separation of nucleic acid from yeast proteins which are recovered in high yields in a highly soluble state.

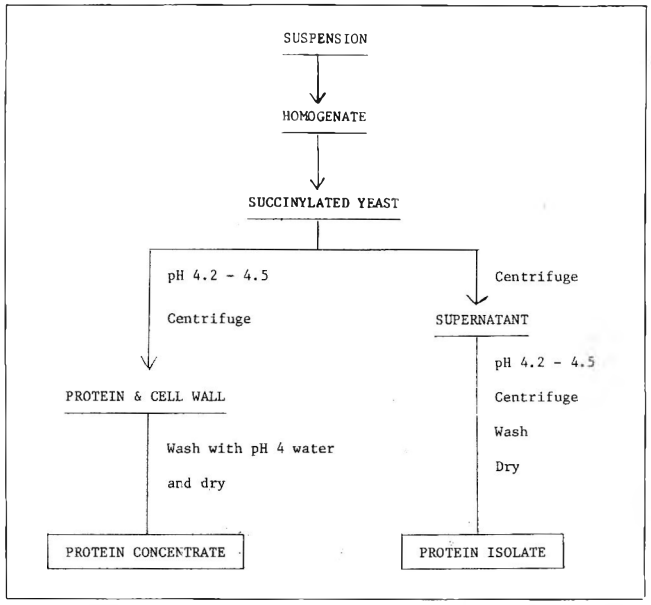

Fig. 8—Flow chart for processing succinylated yeast protein concentrate and succinylated yeast protein isolate.

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Table 1—Chemical composition of whole yeast, succinylated yeast protein concentrate and succinylated yeast protein isolate (expressed as g/100g)[a]

Sample	Total nitrogen	Nucleic acid	Protein nitrogen	Carbohydrate	Crude lipid	Moisture
Yeast cell	8.0	7.90	6.71	35.40	2.38	3.03
Succinylated yeast Homogenate (Protein conc)	11.61	2.0	11.28	24.80	4.45	1.56
Succinylated yeast protein isolate	15.61	1.80	15.32	7.0	3.47	1.54

[a] Mean values from three separate samples

Table 2—Amino acid composition of yeast protein isolated by different methods (expressed as g/16g nitrogen)

Amino acid	Whole yeast	Succinylated yeast protein concentrate	Succinylated yeast protein isolate	Yeast protein isolated by the method of Hedenskog and Mogren (1973)	Yeast protein isolated by the method of Robbins et al. (1975)
Aspartic Acid	7.98	8.58	10.14	9.12	8.01
Threonine	3.81	4.26	4.28	4.00	4.23
Serine	3.74	3.89	3.87	3.64	3.15
Glutamic Acid	10.81	13.18	12.41	12.52	11.39
Proline	4.50	4.76	4.19	4.49	3.67
Glycine	3.08	3.14	3.47	3.26	3.40
Alanine	4.43	4.93	5.05	4.80	4.73
Half cystine	–	1.83	1.48	1.40	–
Valine	4.96	5.55	6.08	5.90	5.88
Methionine	1.82	2.04	2.09	1.90	2.11
Isoleucine	4.46	4.74	5.44	5.07	5.44
Leucine	6.59	7.46	7.94	7.40	7.93
Tyrosine	3.20	3.66	4.08	3.80	4.00
Phenylalanine	4.02	4.50	4.93	4.58	4.89
Lysine	5.72	6.18	7.65	6.96	6.46
Histidine	1.94	2.15	2.42	2.28	2.40
Ammonia	2.69	2.25	2.05	2.24	1.76
Arginine	4.17	4.55	5.27	4.93	4.90

A flow chart showing the procedure for the production of single-cell protein concentrate and isolate is given in Figure 8.

Chemical composition

Crude fat, carbohydrate, total nitrogen and nucleic acid content of yeast, succinylated yeast protein concentrate and isolate are given in Table 1. The values obtained are within the range of the values reported by others (Robbins et al. 1975; Newell et al. 1975; Vananuvat and Kinsella, 1978). The carbohydrate content in succinylated yeast homogenate was less than the total yeast; the difference reflected the soluble carbohydrate content. The protein nitrogen was obtained by subtracting the nitrogen contributed by the nucleic acid from the total nitrogen.

Amino acid composition

Amino acid analyses of yeast protein and succinylated yeast proteins were determined (Table 2). For comparison yeast protein isolate by the methods described by Robbins et al. (1975) and Hedenskog and Mogren (1973) were also analyzed. There was slight improvement in the content of each amino acid in the protein isolated by the present succinylation method. This indicated that succinylation of yeast homogenate during extraction of proteins protected some amino acids from partial destruction caused either by alkaline extraction followed by heat precipitation (Hedenskog and Mogren, 1973) or extraction of proteins from mechanically disrupted yeast cells followed by incubation for RNA reduction and acid precipitation (Robbins et al., 1975). Succinylation did not reduce the lysine levels of the hydrolyzed protein.

In conclusion succinylation enhances protein recovery from yeast cells while minimizing nucleic acid contamination. The properties of the succinylated yeast proteins are presently being determined.

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PRELIMINARY IDENTIFICATION OF VOLATILE FLAVOR COMPOUNDS IN THE NEUTRAL FRACTION OF ROAST BEEF

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ABSTRACT

The volatile flavor compounds (VFC) in the neutral fraction of roast beef flavor isolate were identified in this study. The VFC from 150 lb of eye round roast beef were isolated and separated into acidic, basic and neutral fractions. Since the latter fraction was the only one to have a pleasant roast beef-like aroma, it was the only fraction further analyzed. The components of the neutral VFC fraction were separated into relatively pure subfractions by repetitive gas chromatography and collection in "hairpin" cold traps. The relatively pure subfractions were then analyzed by GC-MS combination. More than 120 compounds were identified including a number of lactones, substituted aromatic compounds, furan compounds, and sulfur-containing compounds. The aforementioned classes of compounds may play significant roles in the roast beef flavor.

INTRODUCTION

DUE TO BOTH economic importance and academic interest, considerable research has been carried out on the identification of volatile flavor compounds (VFC) in beef. The VFC of boiled beef were studied by Hornstein and Crowe (1960), Yueh and Strong (1960), Kramlich and Pearson (1960), Bender (1961), Bender and Ballance (1961), Self et al. (1963), Sanderson et al. (1966), Chang et al. (1968), and Hirai et al. (1973). Tonsbeek et al. (1968, 1969, 1971) and Brinkman et al. (1972) studied flavor compounds in beef broth. Canned beef flavor has been extensively studied by Persson and von Sydow (1973) and Peterson et al. (1975). Watanabe and Sato (1971, 1972) investigated flavor compounds in shallow fried beef. Wilson et al. (1973) and Mussinan et al. (1973) studied the volatile compounds in pressure-cooked beef. Liebich et al. (1972) reported VFC in roast beef and also suggested that further investigation was needed to understand the nature of roast beef flavor due to its complexity.

This paper reports the systematic characterization of the volatile compounds in the neutral fraction of eye round roast beef and speculates on the possible contribution of some of these compounds to roast beef flavor.

EXPERIMENTAL

Raw material and roast conditions

Eye round roasts weighing 7 lb each were roasted in a gas oven at 182°C for 4 hr. A total of 150 lb was roasted. After cooking, the roast beef was cooled at room temperature for about 1 hr. The roast beef was then wrapped with aluminum foil and the drippings from the roasting pan were transferred to an amber glass bottle and both were stored at −20°C until used for flavor isolation. The roast beef was sliced to a thickness of about 5 mm with a meat slicer. The combined sliced roast beef and roast beef drippings were then made into a fine slurry with the addition of distilled water, using a Waring Blendor.

Isolation of volatile flavor compounds (VFC)

The volatile flavor compounds were isolated from the slurry with the apparatus shown in Figure 1. The slurry in the sample flask (B) was maintained at 80°C with a water bath and was purged continuously with high purity nitrogen (99.999%) at a flow rate (J) of 60 ml/min. The volatile flavor compounds and water, swept out by nitrogen, were condensed in a series of cold traps (G through I) which were cooled with a dry ice and acetone slurry. The condensate which collected in the traps was combined, saturated with sodium chloride and extracted with double distilled ethyl ether. It was first separated into acidic and nonacidic fractions by extraction with 10% aqueous sodium bicarbonate solution, and the nonacidic fraction was further separated into basic and neutral fractions by extraction with 5% HCl solution. All ether solutions of acidic, basic and neutral compounds were dried with anhydrous Na_2SO_4 and concentrated to 5 ml with a 30 plate Oldershaw distillation column. Blanks consisting of distilled ether and extracting chemical solutions were analyzed to insure the adequate purity of the ether and extracting chemical solutions. A flow diagram indicating the steps taken in the analysis of roast beef flavor volatiles is given in Figure 2.

Gas chromatography

The neutral fraction was separated into 13 broad fractions on a Beckman GC-5 gas chromatograph with a thermal conductivity detector. A 12 ft × 1/8 in. o.d. stainless steel column packed with 10% SE-30 on 60/70 mesh Anakrom ABS (Supelco, Inc., Bellefonte, PA) was used. The flow rate was 20 ml/min with a column temperature program of 60°C held for 10 min then 4.5°C/min to a holding temperature of 220°C. The gas chromatography was repeated until the sample was exhausted. Each fraction was accumulatively collected in a "hairpin" cold trap according to the method of Thompson et al. (1978). The 13 fractions thus collected were each chromatographed for a second time, using a 10 ft × 1/8 in. o.d. stainless steel column packed with 10% OV-101 on 60/80 mesh Chromosorb W AW, DMCS (Johns-Manville, Denver, CO).

Identification of the gas chromatographic fractions

The subfractions obtained from the repeated gas chromatography were analyzed by a DuPont Model 21-490 mass spectrometer with a jet separator interfaced with a Varian Moduline 2700 gas chromatograph with flame ionization detector. A 10 ft × 1/8 in. o.d. stainless steel column packed with 10% Carbowax 20M on 60/80 mesh Chromosorb W AW, DMCS was used. The flow rate was 30 ml/min and the column temperature was programmed to provide maximum resolution of each fraction analyzed. Conditions for the mass spectral scans were: ionization voltage 70 eV, ion source temperature 270°C, ion source pressure $<10^{-6}$ Torr, and jet separator temperature 250°C.

Compounds were identified by comparing the mass spectra obtained with the reference spectra in the literature, and a check on the identity of the compound indicated by the spectrum was made by comparing the component gas chromatographic retention time with that of the authentic compound when available.

RESULTS & DISCUSSION

IN PRELIMINARY experiments, the volatile flavor compounds (VFC) of roast beef were isolated by either vacuum distillation or vacuum, steam distillation. In neither case did the isolated flavor compounds give the characteristic, desirable odor of roast beef as judged by four experienced flavor researchers. This may have been due, in part, to the formation of artifacts during the flavor isolation.

—Text continued on page 640

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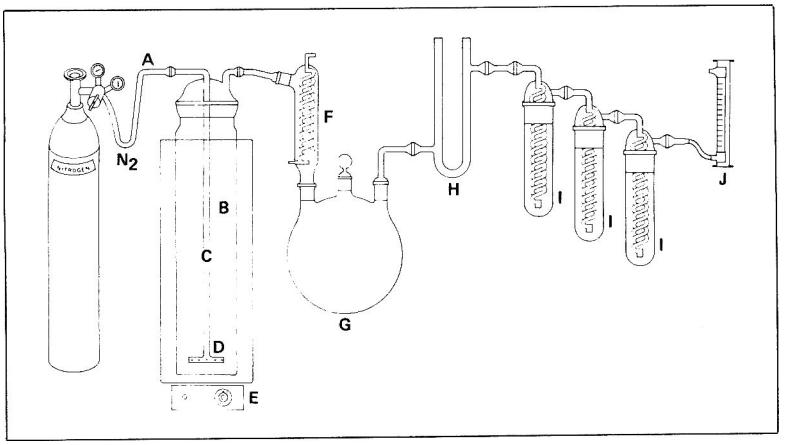

Fig. 1—Apparatus used for the isolation of the volatile flavor compounds of roast beef.

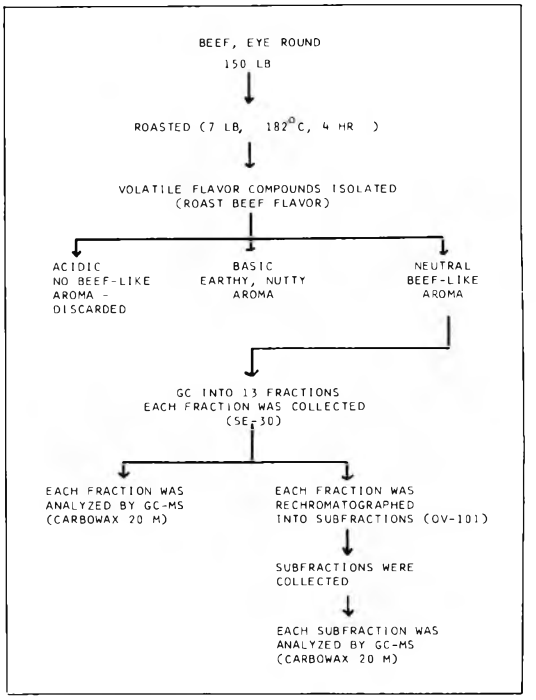

Fig. 2—Flow diagram of the steps taken in the analysis of the roast beef flavor volatiles.

The odor of the volatile flavor isolate of roast beef, obtained with the gas entrainment apparatus (Fig. 1), was judged to have a desirable roast beef flavor very reminiscent of the original roast beef sample. The amount of flavor volatiles isolated by the gas entrainment method is less than the amount obtained by either vacuum distillation technique.

It is interesting to note that the neutral fraction of the roast beef flavor isolate had a pleasant beef-like aroma, while the basic fraction had an earthy, nutty and roasted type odor, and the acidic fraction had a weak, objectionable, sour odor which was uncharacteristic of roast beef. Only those compounds identified in the neutral fraction of roast beef flavor volatiles are reported in this paper.

In order to ascertain if any of the volatile compounds of the neutral fraction, which are important to the beef-like odor, would decompose or irreversibly adsorb under the GC conditions, an aliquot of the neutral fraction was injected and all peaks except the solvent peak were collected in a "hairpin" trap cooled in a dry ice-acetone bath. The contents collected in this trap were evaluated for odor by a number of experienced flavor researchers. They agreed that the odor of the trap was still beef or meat-like, but could not be considered identical to the original neutral flavor isolate. This suggests that the majority of constituents involved in the beef-like odor can be collected intact under the GC conditions.

Although the neutral fraction itself possessed a beef-like odor, no individual peak eluting from the GC exit port had this beef-like aroma. Several peaks did, however, have odors with notes reminiscent of cooked meats in general.

The compounds identified in the neutral fraction of the roast beef flavor isolate are listed in Table 1. The identified compounds included: saturated and unsaturated hydrocarbons, aromatic compounds, ketones, aldehydes, alcohols, esters, lactones, furans, and sulfur-containing compounds.

The basic fraction of the volatile flavor constituents of roast beef has been reported to contain pyrazine, oxazole, and thiazole compounds which generally have nutty, roasted and green type odor (Deck et al., 1973; Mussinan et al., 1976). The basic fraction obtained in this study does have such odors. It may contribute to the overall roast beef aroma.

Compounds of lesser importance

The saturated and unsaturated hydrocarbons do not play a significant role in the roast beef flavor, since they possess relatively weak, nonbeef-like odors. Most of these hydrocarbons have been previously identified in boiled beef (Hirai et al., 1973) or roast beef (Liebich et al., 1972). The large number of alkyl substituted benzenes identified also seem to play a minor role in roast beef flavor. The occurrence of the alkyl substituted benzenes in roast beef has been previously discussed by the authors (Min et al., 1977).

A relatively small number of aliphatic alcohols, aldehydes, esters and ketones were identified in this study. One possible explanation of this is the eye rounds used in this investigation were lean with external fat trimmed off before cooking. Since the above-mentioned compounds are principally oxidative decomposition products of fats (Watanabe and Sato, 1971), one would expect a smaller amount of these compounds from a lean piece of meat. With the exception of 2,3-butanedione (diacetyl) and 3-hydroxy-2-butanone (acetoin) these aliphatic compounds probably are of minor significance in the flavor of roast beef (Chang and Peterson, 1977). The diacetyl:acetoin system has been identified in many food products including canned beef stew (Peterson et al., 1975) and boiled beef (Hirai et al., 1973) where it was considered to contribute a buttery note to the cooked meat.

Substituted aromatic compounds

A relatively large number of carbonyl, hydroxy and/or alkoxy substituted aromatic compounds were identified in this study. Many of these compounds are reported for the first time in cooked meat, including: acetophenone, benzyl methyl ketone, 4-phenyl-2-butanone and anisole. These aromatic compounds may play a role in the overall flavor of roast beef although no individual compound of this group has a meat-like odor itself. It is interesting to note the identification of BHT and BHA which were probably used as antioxidants in the feed of the animals. Hirai and coworkers (Hirai et al., 1973) have previously found BHT in boiled beef.

Some of the γ-lactones identified in this study have previously been identified in roast beef (Liebich et al., 1972) or heated beef fat (Watanabe and Sato, 1968). Since the γ-lactones are strong, pleasant odored compounds, they probably play a role in the roast beef flavor, especially in

the fatty notes of the flavor. A number of patents have been issued on the use of various lactones in improving the flavor of margarine (Wade and Holm, 1959) and for imparting deep-fat fried flavor to foods (Chang and May, 1973).

Furan compounds

A relatively large number of furan compounds were identified in the present study. Furan compounds have been previously found in a number of cooked meats and meat products. Tonsbeek and co-workers (Tonsbeek et al., 1968; 1969) identified 4-hydroxy-5-methyl-3(2H)-furanone and 4-hydroxy-2,5-dimethyl-3(2H)-furnaone in the flavor isolate of beef broth. Hirai et al. (1973) identified 2-pentylfuran and 5-thiomethylfurfural in boiled beef. Liebich et al. (1972) identified 2-pentyl-, 2-hexyl-, 2-heptyl-, and 2-octyl-furans in roast beef. Persson and von Sydow (1973) reported furan, 2-methyl, 2-propyl, 2-butyl, 2-pentyl, and 2-hexylfurans, 3-methyl-furan, 2,5-dimethylfuran, and 2-methyl-5-ethylfuran in canned beef. Peterson et al. (1975) reported 2-pentylfuran, furfuryl alcohol, 2-furaldehyde, 5-methyl-2-furaldehyde, 2-acetylfuran, and 5-methyl-2-acetylfuran in canned beef stew. Ouweland and Peer (1975) reported that the reaction of 4-hydroxy-5-methyl-3(2H)-furanone and H_2S produced a roasted meaty flavor. A number of meat flavor patents have been issued which further suggest the importance of furan compounds in beef flavor. May (1960) described a process for the preparation of meat flavor by reacting a furan, either furan itself or a furan preferably substituted in the 2-position, with cysteine or cystine. Ouweland and Peer (1970) and Katz et al. (1971) produced meaty flavors by the reaction of a dimethylfuranone with H_2S. Evers (1972) produced a meat-like flavor by reacting 2,5-dimethylfuran with sulfur dichloride. DeGroot and Hobbs (1973) reported the improvement of meaty flavor by incorporating either 2,5-diethyl or 2-methyl-5-ethyl-4-hydroxy-2,3-dihydrofuran-3-one in a meaty foodstuff.

Sulfur containing compounds

The importance of sulfur containing compounds to the flavor of meat is also indicated in some of the aforementioned patents. A relatively large number of sulfur containing compounds were identified in the present study. Sulfur compounds have been previously identified in a number of studies concerning the flavor of meat or meat products. Brinkman et al. (1972) found 1-methylthio-ethane-thiol in beef broth. Chang et al. (1968), Brinkman et al. (1972), Persson and von Sydow (1973) and Wilson et al. (1973) reported an additional 45 sulfur compounds in pressure cooked beef. Hirai et al. (1973) reported methyl propyl sulfide, methyl allyl sulfide, dimethyl disulfide, diallyl sulfide, 5-thiomethyl furfural, thiophene-2-carboxaldehyde, and 3,5-dimethyl-1,2,4-trithiolane in boiled beef. Liebich et al. (1972) identified dimethyl disulfide, and dimethyl sulfone in roast beef. Mussinan and Katz (1973) reported 34 sulfur compounds in two model systems approximating cooked meat. These same authors stated that one of the major difficulties in meat flavor research is the interference of the large amounts of lipid derived aldehydes and ketones with the analysis of the small quantities of sulfur compounds present. One reason for the identification of the relatively large number of sulfur compounds in this study is the large amount of roast beef used for flavor isolation, which made the analysis of the relatively small amounts of sulfur compounds more easy. Schutte (1974) reviewed thoroughly the precursors of sulfur compounds in meat and suggested the important role of sulfur compounds in meat flavor. Ouweland and Peer (1975) showed that the reaction product of 4-hydroxy-5-methyl-3(2H)-thiophenone with H_2S gave a flavor resembling the roast meat flavor.

The importance of sulfur compounds in meat flavor is

Table 1—Volatile compounds identified in the neutral fraction of roast beef flavor isolate

Aromatic compounds	
Acetophenone	Methoxybenzaldehyde
Benzyl methyl ketone	Benzyl alcohol
Tolyl methyl ketone	Phenol
4-Phenyl-2-butanone	Cresol
Acetylnaphthalene	Anisole
Benzaldehyde	Methoxytoluene
Methylbenzaldehyde	Butylated hydroxytoluene
Ethylbenzaldehyde	Butylated hydroxyanisole
Furans	
2-Ethylfuran	Dihydrofuran
2-Propylfuran	2-Acetylfuran
2-Butylfuran	2-Furaldehyde
2-Pentylfuran	5-Methyl-2-furaldehyde
2-Heptylfuran	(2-Furyl) acetaldehyde
Dimethylfuran	3-(2-Furyl) propionaldehyde
2-Methyl-5-ethylfuran	4-(2-Furyl) butan-2-one
Trimethylfuran	2,2′-Difurfuryl ether
	2-Methyldihydrobenzofuran
Sulfur compounds	
2-Methylthiophene	2-Hexylthiophene
2-Ethylthiophene	2-Heptylthiophene
2-Propylthiophene	2-Octylthiophene
2-Butylthiophene	2,5-Dimethylthiophene
2-Pentylthiophene	Dimethyl sulfide
Methyl pentyl sulfide	2-Acetylthiophene
Propyl isopropyl sulfide	Ethyl thiopropionate
Dimethyl disulfice	Dipropyl disulfide
Diethyl disulfide	
Hydrocarbons	
n-Hexane	2-Hexene
n-Heptane	1-Heptene
n-Octane	2-Heptene
n-Nonane	1-Octene
n-Undecane	3-Octene
n-Dodecane	1-Nonene
n-Tridecane	2-Decene
n-Tetradecane	1-Dodecene
n-Pentadecane	2,4-Hexadiene
n-Hexadecane	2-Methyl-1-pentene
n-Heptadecane	Limonene
Aromatic hydrocarbons	
o-Xylene	1,2,3,4-Tetramethylbenzene
m-Xylene	1,2,4,5-Tetramethylbenzene
p-Xylene	Benzene
1,2,4-Trimethylbenzene	Toluene
1,3,5-Trimethylbenzene	n-Ethylbenzene
1-Ethyl-2-methylbenzene	n-Propylbenzene
n-Butylbenzene	n-Nonylbenzene
n-Pentylbenzene	n-Decylbenzene
n-Hexylbenzene	Styrene
n-Heptylbenzene	Naphthalene
n-Octylbenzene	
Alcohols	
3-Methyl-1-butanol	3-Hexen-1-ol
1-Heptanol	3-Methyl-2-hexen-1-ol
Hexenol	
Aldehydes	
2-Methylbutanal	2-Hexenal
Hexanal	2-Heptenal
Heptanal	2,4-Hexadienal
4-Pentenal	
Ketones	
2-Pentanone	2-Tridecanone
2-Hexanone	Octenone
2-Nonanone	2,3-Butanedione
4-Undecanone	3-Hydroxy-2-butanone
Esters and lactones	
Ethyl acetate	γ-Hexalactone
Pentyl acetate	γ-Heptalactone
Methyl palmitate	γ-Octalactone
γ-Pentalactone	γ-Dodecalactone

further substantiated by a number of patents utilizing sulfur compounds for the production or enhancement of synthetic meat flavor. Giacino (1968, 1970), May (1970), Soeters (1970), Katz et al. (1971), Mussinan et al. (1972) and others have produced meat flavor compositions by the reaction of sulfur compounds such as cysteine, cystine, taurine, thiamine, H_2S and cycloalkylmercaptans with other compounds such as furans and reducing sugars.

The identification of the large number of volatile flavor compounds in the neutral fraction of roast beef flavor isolate confirms previous work indicating the complex nature of meat flavor chemistry. Because of the powerful odor characteristics and relatively large numbers of lactones, substituted aromatics, furans and sulfur containing compounds, found, it is felt that these classes of compounds are important in the roast beef flavor. Due to the complexity of roast beef flavor, further work is now under way to uncover additional important compounds and to more precisely define the importance of those that have been identified here.

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DYE BINDING PROCEDURE FOR THE ESTIMATION OF PROTEIN CONTENT OF MEAT COMPONENTS AND SAUSAGE EMULSIONS

G. J. SEPERICH and J. F. PRICE

ABSTRACT

The dye binding technique was investigated for its ability to determine the protein content in a processed meat product. Frankfurter emulsions were prepared with varying protein content by adjusting the fat content of the emulsions. The unprocessed (raw) emulsion and the processed (cooked, smoked) emulsion were tested with the dye binding technique using Acid Orange 12 (C.I. 15870). In both the processed and unprocessed emulsion products it was found that the bound dye parameter alone could be used to monitor the protein content. A regression equation containing two variables, the reciprocal of the dye binding capacity and the bound dye, would account for over 90% of the variation in the protein content of a processed emulsion product.

INTRODUCTION

HIGH SPEED METHODS for preparing processed meats necessitate the use of rapid forms of analysis for the individual components. The dye binding procedure was tested for its ability to determine the protein content of a processed meat product.

Bunyan (1959) attempted to analyze crude protein in "meat" meals and fish meals using Orange G (Acid Orange-10), but noted large variations in the ability of the dye to bind the proteins. Torten and Whitaker (1964) worked with Amido Black 10B (Acid Black-1) and Acid Orange 10 (AO-10). They compared the ability of these two dyes and the biuret method against the Kjeldahl method to determine the protein content in meat types ranging from beef and pork to chicken and cod. The biuret method was found comparable to the Kjeldahl method and the method using AO-10 was found sufficient as compared to Kjeldahl method if the preparations and procedures were carefully standardized and if the protein content did not vary more than a few percent. Acid Black-1 was found to be too dependent upon sample size. Moss and Keilsmeier (1967) working with meat slurries reported results similar to Torten and Whitaker.

Ashworth (1971) found the dye binding method with AO-12 to be rapid and useful for composition control in ground meats, eggs and prepared mixes. Udy (1971) suggested the use of dye binding with AO-12 for a variety of food products including meat products.

The cautions of early investigators coupled with recent papers seemed to indicate that the dye binding method could be applied to a sausage emulsion product.

EXPERIMENTAL

Experimental design

The ability of the dye, Acid Orange-12 (C.I. 15970) to bind proteins was determined with three test groups: (1) The major muscle protein fractions, myofibrillar, sarcoplasmic and stroma were investigated for their dye binding capability. (2) A model emulsion system using two of the muscle protein fractions, myofibrillar and sarcoplasmic, with a polyunsaturated oil was used to determine if the presence of a fat or oil would interfere with the dye binding capacity of the proteins. And (3) frankfurter emulsions, processed (cooked and smoked) and unprocessed (raw) were tested for the ability of the proteins present to bind with Acid Orange-12.

Sausage emulsion preparation

Sausage emulsions of three different levels of fat and protein were prepared from meat available at Michigan State University. Lean beef, fat beef and fat pork were represented by beef rounds, plates and pork jowls, respectively. Each type of meat was ground three times, twice through a plate with 1.9 cm holes and once through a plate with 0.54 cm holes, packed in Cryovac bags in 4.5–5.5 kg units and frozen until 2 days before its use.

Using the formulation equations of Cunningham (1971) three different frankfurter emulsions were formulated and prepared to yield products with approximately 10, 12 and 14% protein. The frankfurter emulsions (10.9 kg batches) were prepared in the normal manner using a pilot plant scale vertical cutter-mixer and emulsion mill. The unprocessed emulsion samples were removed before stuffing the emulsion into cellulosic casings (26 mm diam). The frankfurters were smoked, cooked (internal temperature, 70°C), cooled and samples were taken for protein analysis. The samples were segregated into two lots. One lot was prepared for analysis on the next day and the other lot was frozen for later work.

Chemical analysis

Moisture, crude fat and protein (N × 6.25) were determined in triplicate in the processed and unprocessed samples according to the AOAC (1965).

Dye binding procedure

The dye binding procedure was modified from Ashworth (1971). A 3.75-g sample of the emulsion was added to 51.0 ml, citric acid-phosphate buffer (0.1M citric acid, 0.2M dibasic phosphate), pH 5.5 in a Virtis homogenizing cup (capacity 150 ml) and homogenized for 2 min. Two, 5 ml aliquots were taken for Kjeldahl analysis and the remaining mixture was mixed with 80 ml, 0.56M AO-12 and transferred to a 250 ml centrifuge bottle. The sample was agitated for 30 min at ambient temperature, centrifuged at 5,680 × G (R.C.F.) for 5 min in a Sorvall Superspeed RC2-B automatic refrigerated centrifuge at 2°C and filtered through 11 cm, S&S No. 597 filter paper. The supernatant was measured at 475 nm on a Beckman D U Spectrophotometer.

Protein extraction

To ascertain the dye binding capabilities of the major fractions of muscle proteins and model emulsion systems, the protein fractions were extracted by a modification of the method proposed by Holander (1957).

Thirty grams of ground beef or pork were blended in a 210 ml 0.03M phosphate buffer, pH 7.4, at a low and high speed setting for 1 min each. The slurry was transferred to a 500 ml Erlenmeyer flask with 75 ml 0.03M phosphate buffer, pH 7.4, and gently agitated for 4 hr at 4°C. The mixture was transferred to a 250 ml centrifuge bottle and centrifuged at 1400 × G (R.C.F.) for 20 min in a Sorvall Superspeed RC2-B automatic refrigerated centrifuge set at 2°C. The supernatant was filtered through cheese cloth and retained. The residue was resuspended with 250 ml 0.03M phosphate buffer, pH 7.4, reextracted for 4 hr, centrifuged and filtered. The fraction separated was the "sarcoplasmic" fraction.

The residue was resuspended in 1.1M KI, 0.1M phosphate buffer, pH 7.4, and extracted for 2 hr, centrifuged, filtered, resuspended and the extraction repeated. The combined supernatants represented the "myofibrillar" fractions and the insoluble residue was the "stroma" fraction.

–Text continued on page 644

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Table 1—Effect of varying the lipid concentration upon the DBC of the myofibrillar beef proteins at two dye concentrations, pH 5.0 in a 9% protein solution[a]

Dye conc (ng/ml)	Lipid free system	Dye binding capacity (ng/dye bound/mg protein) 20% Fat	25% Fat	30% Fat	35% Fat	40% Fat
80	—	13.6a	13.4a	13.2a	13.2a	13.2a
200	36.0b	33.8b	33.9b	33.9b	34.1b	34.1b

[a] Means carrying different letters are significantly different from one another ($P \leq 0.01$).

Table 2—Effect of species upon the DBC of myofibrillar and sarcoplasmic proteins in a 30% lipid emulsion with a 9% protein solution[a]

	Protein fraction					
	Myofibrillar			Sarcoplasmic		
Species	DBC	Std dev	n	DBC	Std dev	n
Porcine	112.4a	±16.6	9	83.8b	± 8.5	4
Bovine	105.7a	±17.0	7	83.0b	±10.8	5

[a] Means carrying different letters are significantly different from one another ($P \leq 0.01$).

Model emulsion system

Utilizing the crude protein extracts, a model emulsion system was prepared by adding a volume of the respective extract to a 50 ml volumetric flask and filling to volume with a 0.03M phosphate buffer, pH 7.4. Different protein concentrations were handled in this manner. The protein solution (50 ml) and 1.547g NaCl were added to a Virtis homogenizing cup and mixed for 15 sec. Twenty-one and one-half milliliters of Wesson Oil (a soybean-cottonseed oil) was added to the mixture and homogenized for 2 min at maximum speed. A 5-ml aliquot was taken for Kjeldahl analysis and the rest of the system was used for dye binding analysis.

Statistical analysis

A randomized block design was used. Regression equations were calculated via a least square analysis utilizing stepwise addition or deletion of parameters from the equations according to pre-determined statistical parameters (Draper and Smith, 1966). All analyses were conducted with standard computer library programs on a CDC 3600.

RESULTS & DISCUSSION

THE CRUDE PROTEIN fractions, i.e., myofibrillar, sarcoplasmic and stroma proteins in concentrations ranging from 0.1–1.0 mg/ml were exposed to AO-12. The concentration of AO-12 was 200 ng/ml. The average dye binding capacity (DBC, ng AO-12 dye bound/mg protein) for each of the protein fractions was as follows: myofibrillar 36.0 (±2.8), sarcoplasmic 27.0 (±2.1) and stroma protein 14.4 (±2.0). This established the average DBC for each of the protein fractions when not present in an emulsion. Both beef and pork protein fractions were used with no significant difference in the dye binding capacity. The results support the work of Ashworth (1971) who found that pork bound the same amount of dye as beef.

Before investigating into the model emulsion system and its dye binding properties, it was imperative to determine if the presence of lipid material in an emulsion would alter the DBC of the protein fraction. A number of investigators have demonstrated in sausage emulsions that the myofibrillar protein fraction is the primary contributor to the membrane surrounding the fat globules; therefore, the effect of lipid material on the myofibrillar DBC was determined. Table 1 illustrates that for the same dye concentration (200 ng/ml) the average value in an emulsion was 34.0 (±1.3). There was no significant difference in the DBC of either the free or the emulsified myofibrillar protein fraction. Table 1 further illustrates that the DBC of a protein fraction will increase or decrease commensurately with the concentration of the dye but will remain constant at a given protein and dye concentration, even though the lipid level may vary.

For the model emulsion system, both the myofibrillar and the sarcoplasmic protein fractions were emulsified in a 30% lipid system. For this system the AO-12 dye concentration was increased from 200 ng/ml to 1,300 ng/ml (0.56M–3.64M). The increased dye concentration was suggested by Ashworth (1971) and Sherbon (1970) and was used in the analysis of the sausage emulsion. Table 2 presents the results of the model emulsion system with two of the major protein fractions. Analysis of variance of the results in Table 2 demonstrated that there was no significant species difference with either the sarcoplasmic or the myofibrillar proteins. Increasing the dye concentration from 200 (Table 1) to 1,300 ng/ml resulted in an increase in the DBC of both protein fractions although the myofibrillar proteins bound a significantly greater amount of dye.

Table 3 contains the regression equations calculated from data collected on the model system, the processed (cooked) sausage, and the unprocessed (raw) batter by the methods of stepwise addition and deletion of variables. The equation from the model system data contains the reciprocal of the dye binding capacity (DBC^{-1}) along with the bound dye parameter (BD) and the intercept value which represents the mean of all DBC values. The DBC^{-1} can be experimentally determined for a product and substituted into the equation. Thus, by using essentially two means to stabilize the equation, a high coefficient of determination (0.999) resulted.

The regression equations calculated for the sausage emulsion data for both the processed and unprocessed categories involved fewer sets of observations (12 and 15 vs 30) than did the previous equation (Table 3), but had very similar high coefficients of determination. The equation for the processed product is very similar to that for the model system with the selection of identical variables. A third variable was added in the case of the unprocessed emulsion making the equation somewhat unwieldy. Table 4 shows the correlations that were found between the individual dye parameters and the protein content in the various systems. The same dye concentration, 1,300 ng/ml (3.64M) of AO-12 was used in the sausage emulsion samples. The sausage emulsions were analyzed in two states, processed, i.e., cooked and smoked, and unprocessed, the raw emulsion. It was found in working with the model system that the myofibrillar proteins bound a greater amount of dye than the sarcoplasmic proteins irrespective of species (Table 2). Thus

Table 3—Regression equations calculated for three emulsion systems

Emulsion type	Coefficient of detm	Std dev of the means	Regression equation[a]
Model emulsion system (Myofib.)	0.9993	0.038	$Y = -3.07 + 0.012DBC^{-1} + 0.002BD$
Processed sausage	0.9998	0.046	$Y = -8.65 + 1.28DBC^{-1} + 0.01BD$
Unprocessed sausage	0.9988	0.056	$Y = -7.67 + 1.52DBC^{-1} + 0.004BD + 0.01DBC$

[a] Y = the protein percentage predicted; DBC^{-1} = the reciprocal of the dye binding capacity;
BD = the bound dye; DBC = the dye binding capacity.

Table 4—Correlation coefficients of four dye parameters with the protein content of three emulsion systems

Emulsion type	Dye parameters			
	DBC	DBC^{-1}	BD	OD
Model emulsion	−0.68	0.99	−0.44	−0.12
Proc. sausage	−0.97	0.99	0.21	−0.25
Unproc. sausage	−0.96	0.99	0.84	−0.84

it was felt that the dye binding procedure could possibly be used in protein assessment.

Table 4 demonstrates that the dye parameters generally followed the same pattern of relationship to protein content in all three emulsion systems. However, it is evident that the dye parameter, bound dye (BD) was highly correlated with the protein content of an unprocessed emulsion, i.e., it was able to account for 71% of the variation in the protein content of the emulsion. The DBC and its reciprocal were highly correlated with the protein content in both the processed and unprocessed emulsions.

As in the multiple regression equations, the DBC^{-1} seemed to be preferable (more accurate) as an index of protein content than DBC from which it is derived. This constituted a somewhat mysterious trend since each parameter measured essentially the same thing. Apparently the precision of the estimate is improved by coding the number as a reciprocal. On the other hand, the BD and OD parameters measure essentially the same thing but differ from the DBC units in that they are not units relative to protein concentration.

The relationship between the DBC and the protein content in the two emulsion states is illustrated in Figure 1. The trend for each emulsion state appeared to indicate that as the protein content increased the DBC decreased. This trend was maintained in both the processed and unprocessed emulsions. The DBC of the unprocessed product was greater than its processed counterpart, even though the processed product possessed a greater amount of protein. The decrease in the DBC of the processed emulsion might be explained by the aggregation of the protein components during the processing procedure. This aggregation would reduce the number of available sites to which the dye could bind and thus reduce the DBC. Furthermore, Saffle (1968) demonstrated that the method of protein membrane formation around the fat globules in an emulsion is through the accretion of one protein layer upon another. In the unprocessed product this layering may be loose or netlike with access to the binding sites still available to the dye: however, upon processing this network may aggregate upon itself thus reducing the number of available binding sites. Under this hypothesis the greater the protein content in the product the greater the amount of protein accretion on the fat globule. The greater the amount of layering the less

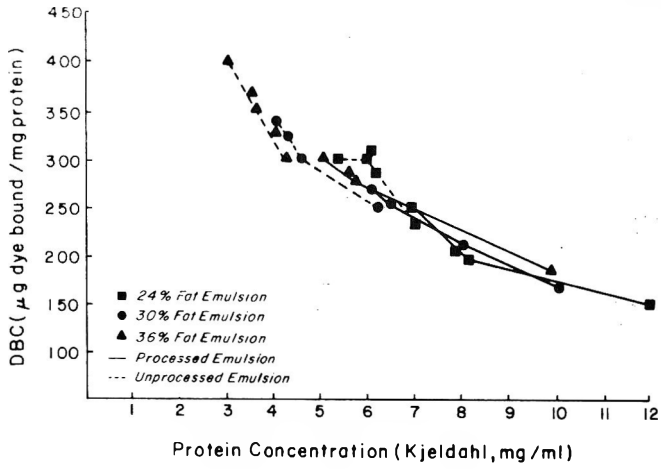

Fig. 1—Relationship of dye binding capacity and protein content of sausage emulsions.

available the binding sites of the inner layer protein become. This might also account for the decrease in DBC with increasing protein content. It was demonstrated in the model emulsion system that the fat component of the emulsion did little to interfere with the DBC of the protein.

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Clostridium perfringens POPULATION DYNAMICS DURING CONSTANT AND RISING TEMPERATURES IN BEEF

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ABSTRACT

The population dynamics of *C. perfringens* organisms in autoclaved ground beef heated at 16 constant- or rising-temperature conditions has been mathematically modeled. The basic model is a rate equation determined from the 16 experimental conditions. The specific reaction rate is based on an effective temperature rather than the instantaneous environmental temperature. The effective temperature for both growth and death was found to be a time average of the previous environmental temperatures. The concentration term includes both an initial lag and a maximum population concentration for continued growth as reported by Kono (1968). Between these limits and after the effective temperature is above the death initiation temperature, the equation is first-order in concentration. The model has been verified by experimental data taken both from constant-temperature growth conditions and from rising-temperature growth and death conditions.

INTRODUCTION

ILLNESS from *Clostridium perfringens* in meat products is a major concern in the food industry (Bryan and Kilpatrick, 1971; Hall and Angelotti, 1965). Long time, low temperature (LTLT) cookery for beef or other meat in the processing industry, food service establishments and the home creates the potential for substantial growth of *C. perfringens* during cooking. The frequent contamination of raw beef (Hall and Angelotti, 1965) and the potential of *C. perfringens* for rapid growth at relatively high temperatures is of primary concern. The combination of LTLT cookery and processes such as mechanical tenderization which contaminate the interior of beef roasts present an ideal situation for excessive growth. However, the increased product yield and final product tenderness support the use of LTLT cookery.

Mathematical modeling of physical and biological mechanisms is a common and useful practice (Wolberg, 1976; Pirt, 1975). Effective mathematical models can be a valuable process design tool and can provide valuable assistance in understanding the system being studied. The mechanisms that are operational during the growth and inactivation of a population of organisms cannot be studied independently. A model facilitates the concurrent study of the mechanisms and their interdependence. When additional experimentation is anticipated, a mathematical model can be utilized to improve the efficiency of the design for future experiments.

Kinetic models based on the Arrhenius equation have been used not only for chemical reactions, but also for microorganism growth (Crozier, 1926; Pirt, 1975; Scott, 1937) and inactivation (Pflug, 1973). However, an important result of this study is the failure of these models to explain the response of *C. perfringens* to constant and rising temperatures spanning both growth and inactivation. This study clearly shows that kinetic models for this organism can not be developed solely from constant temperature experiments. Varying temperature experiments are necessary for identifying the correct model and for estimating parameters.

The objective of this study was to develop and test a mathematical model of the assumed mechanisms of growth and inactivation for *C. perfringens* during constant and rising temperature processing of beef. Constant and rising temperatures were selected because these approximate normal cooking conditions. The model was tested and incorrect assumptions about mechanisms of growth and inactivation were eliminated by estimating parameter values and comparing the predicted results with experimental data of Willardsen et al. (1978). The reported model explains over 90% of the experimental variation reported by Willardsen et al. (1978) with 156 degrees of freedom for error.

MODEL DEVELOPMENT

Experimental data

The experimental data utilized for estimating model parameters and evaluating the mathematical model have been previously reported (Willardsen et al., 1978). These data were based on the growth of a composite of eight strains of *C. perfringens*: ATCC 3624, NCTC 8238, NCTC 8239, NCTC 8797, NCTC 8798, NCTC 10240, S-40 and S-45. The composite strain was inoculated into a growth medium of autoclaved ground beef. The inoculated samples were heated at a constant rate of temperature rise or held at a constant temperature. Periodically, sub-samples were removed for enumeration by plating in Tryptose Sulfite Cycloserine (TSC) medium (Speck, 1976). The sample temperatures were controlled by immersion in a water bath (Blue M Model MW-1140 E-1) supplemented with a variable wattage cirulating heater (Haake Model E-52). The sample temperatures were monitored by an electronic thermometer (YSI Telethermometer Model 425C). Constant temperatures of 15, 26, 33, 37, 41, 45, 49, 51 and 53°C and rising temperature rates of 4.1, 6.0, 7.5, 8.5, 9.5, 10.5, 12.5 and 17°C/hr were studied.

The model was further verified by comparing predicted populations with six new experimental curves. The new experimental curves included both changing and constant rates of temperature rise. Three sampling times with three replicates at each time were nested in each constant rate experimental curve. Four sampling times with three replicates at each time were nested in each changing rate experimental curve—thus a total of 60 new data points were utilized for verification. The sampling times were selected to be near the time of the greatest organism population. The rising temperature conditions in this study were 5, 6, 9, 9, 4.5 changing to 6.6 and 6 changing to 8.2°C/hr. The other procedures for the verifying experiment were the same as for the original experiments.

Model description

The basic mathematical model is given by Eq (1):

$$\frac{dC}{dt} = k*f \qquad (1)$$

where: C is the organism concentration (cells/g); t is time (hr); k is the specific reaction rate constant and is a function of both time and temperature (hr^{-1}); f is a function of concentration and time (cells/g).

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This standard reaction equation is utilized by the model for all phases of growth and death.

Specific reaction rate

Unlike earlier work done at constant temperatures, (Hall and Angelotti, 1965; Rey et al., 1975) our data from linearly rising temperatures indicated that the specific reaction rate constant is dependent on a time average of the temperatures the organism has been experiencing. This average temperature is calculated by integrating the environmental temperature over a period of time and dividing by that time interval. A similar technique has been discussed by Otterman (1960) except an exponential weighting function was utilized.

The functional definition of the specific reaction rate constant depends on the phase of the microorganism growth or death. The model utilizes four phases for the definition of the specific reaction rate constant: induction, growth$^+$, growth$^-$, and inactivation. The induction phase is a time interval prior to the start of organism growth. Variation in the number of organisms enumerated during the induction phase was assumed to be negligible. During the growth$^+$ phase, the growth rate increases with increasing temperature. This is the growth phase in which the Arrhenius model was utilized. The growth$^-$ phase demonstrates a declining growth rate with increasing temperature. The growth$^-$ phase may reflect either a disruption of metabolic regulation or inactivation of cells (Pirt, 1975). Both assumptions were evaluated in the model. The minimum variance for the disruption assumption was less than half the minimum variance for the inactivation assumption. Because of this and the lack of any experimental evidence of high total cell concentrations generated during growth$^-$, the metabolic disruption assumption was utilized. During the inactivation phase the rate of decrease of viable cells increases with increasing temperature according to the Arrhenius model.

Earlier studies of the induction phase of other organisms (Hartsell et al., 1959; Penfold, 1914) have shown a logarithmic relationship between temperature and induction time. Our data indicated a similar effect, but because of the time dependence of temperature, an integral equation (2) was utilized to calculate the duration of induction.

$$\int_0^t \ln (T - T_b)\, d\tau < T_i \qquad (2)$$

where: ln is the natural logarithm function and is given the value of 0.0 for all temperatures, T less than $T_b + 1$; T is the current environmental temperature (°K); T_b is a base temperature, one degree less than the maximum no growth temperature (°K); t,τ is time (hr); T_i is the integral value corresponding to the end of induction (hr). The specific reaction rate constant does not need to be defined during induction because the concentration function is zero.

The specific reaction rate constant during the growth$^+$ phase was based on the Arrhenius model.

$$k = k_{300} * \exp\left[\frac{-E_g}{R}\left(\frac{1}{T_g} - \frac{1}{300}\right)\right] \qquad (3)$$

where: k is the specific reaction rate constant at an effective temperature, T_g (hr^{-1}); k_{300} is the specific reaction rate constant at 300°K (27°C) (hr^{-1}); exp is the exponential function; E_g is the activation energy for growth (J/mol); R is the gas constant (8.31431 J/K · mol); T_g is the effective growth temperature (°K). The effective growth temperature (T_g) is equivalent to the average environmental temperature of the preceding t_1 hours:

$$T_g = \frac{\int_{t-t_1}^{t} T(\tau)\, d\tau}{t_1} \qquad (4)$$

where: T_g is the effective growth temperature (°K); $T(\tau)$ is the cell environment temperature at time τ (°K); t,τ is the time (hr); t_1 is the time interval for averaging the effective growth temperature (hr).

For temperature curves that are linear in time, this definition of effective growth temperature is equivalent to the environmental temperature at time $t - t_1/2$. The end of the growth$^+$ phase also depends on the effective growth temperature for the cells, T_g. In the case of very rapid temperature increases, the growth$^+$ phase was found to terminate when the environmental temperature reached a specified temperature value even though the effective growth temperature had not reached the terminal temperature found for slower rates of growth. Thus, the end of growth$^+$ was expressed mathematically by either of two inequalities being satisfied.

$$T > T_1$$

$$T_g > T_2 \qquad (5)$$

where: T is the cell environmental temperature (°K); T_1 is the maximum environmental temperature at which cells can maintain growth$^+$ characteristics (°K); T_g is the effective growth temperature (°K); T_2 is the maximum average environmental temperature for continued growth$^+$ (°K).

The growth$^-$ phase was characterized by a linear reduction in growth rate with increasing temperature. The linear reduction was calculated from the specific reaction rate constant at the end of growth$^+$ phase and a reaction rate of zero at the start of the inactivation phase. The initiation of inactivation and the rate of inactivation both showed a significant dependence on the rate of temperature change which could be accounted for by a time average of temperature (an effective inactivation temperature), T_d. Thus, the effective inactivation temperature was utilized in the linear reduction of growth rate during the growth phase.

$$k = k_+\left(\frac{T_3 - T_d}{T_3 - T_{d_+}}\right) \qquad (6)$$

where: k is the specific reaction rate during the growth$^-$ phase (hr^{-1}); k_+ is the specific reaction rate at the end of the growth$^+$ phase (hr^{-1}); T_3 is the minimum inactivation effective temperature to initiate the inactivation phase (°K); T_d is the inactivation effective temperature (°K); T_{d_+} is the inactivation effective temperature at the end of the growth$^+$ phase (°K).

The specific reaction rate constant was mathematically modeled by the Arrhenius equation.

$$k = -k_{350} * \exp\left[\frac{-E_d}{R}\left(\frac{1}{T_d} - \frac{1}{350}\right)\right] \qquad (7)$$

where: k is the negative specific reaction rate constant for inactivation of *C. perfringens* at an effective temperature, T_d (hr^{-1}); k_{350} is the reference rate for inactivation at 350°K (77°C) (hr^{-1}); exp is the exponential function; E_d is the activation energy for inactivation of *C. perfringens* (J/mol); R is the gas constant (8.31431 J/K · mol); T_d is the effective inactivation temperature (°K).

The effective inactivation temperature was utilized both for calculating the inactivation specific reaction rate constant and for determining the boundary between growth$^-$ and death phases. Separate integration steps were included in the model for the rate constant and boundary, but the best estimates for integration times were identical. Therefore, the inactivation effective temperature was calculated by Eq (8):

$$T_d = \frac{\int_{t-t_2}^{t} T(\tau)\, d\tau}{t_2} \qquad (8)$$

where: t_2 is the time interval for averaging the effective temperature for inactivation; t,τ is time (hr); T_d is the effective inactivation temperature (°K); $T(\tau)$ is the ambient temperature at time τ (°K).

Concentration effects

The concentration function was based on the concepts presented by Kono (1968). The function includes five distinct phases: induction, transient, exponential, decline and inactivation (death). The induction phase for concentration is defined by the same limits as for the specific reaction rate. The value of the concentration function during induction is zero.

The transient phase accounts for the gradual start of growth under constant temperature conditions. This effect cannot be distinguished from the change in specific reaction rate constant with temperature during the rising temperature experiments. However, the constant temperature experiments also show a smooth transition from no growth to growth which cannot be accounted for simply by the temperature effects included in the specific reaction rate constant.

—Text continued on page 648

The value of the concentration function during the transient phase and the inequality defining the time limits of the transient phase are:

$$f = C - [1 - \alpha(t - t_o)]\, C_o$$

$$\text{for } t_o \leq t < \frac{1}{\alpha} + t_o \qquad (9)$$

where: f is the concentration function value (cells/g); C is the cell concentration in the growth medium (cell/g); C_o is the initial cell concentration in the growth medium (cell/g); α is the mean rate of increase in the fraction of the inoculated cells which have the ability to consume substrate and propagate themselves (hr^{-1}); t is time (hr); t_o is the time at the end of the induction phase (hr). The functional form for the concentration term given in Eq (9) is identical to the one proposed by Kono (1968) with his term Φ', the fraction of inoculated cells which have the ability to consume substrate and propagate themselves, given by the linear function $\alpha(t - t_o)$.

During the exponential phase the concentration function assumes the value of the current concentration. This indicates that the growth is first-order in cell concentration.

The decline phase is characterized by a smooth transition from exponential growth to no growth caused by a limiting concentration of organisms in the growth medium. This phase was mathematically defined by Kono (1968) by the expression:

$$f = \left[\frac{C_m - C}{C_m - C_c}\right] C_c$$

where: f is the concentration function value (cell/g); C is the cell concentration in the growth medium (cells/g); C_m is the maximum possible cell concentration (cells/g); C_c is the critical cell concentration at which growth rate decline is initiated (cells/g). This decline in growth rate was clearly distinguishable in the experimental data reported by Willardsen et al. (1978).

The inactivation phase in the concentration function occurs simultaneously with the inactivation phase defined for the specific reaction rate. The concentration function is equal to the current concentration during the inactivation phase. Inactivation due to accumulated toxin products in the growth medium is not considered in the model. This type of inactivation was not evident in any of our constant temperature experiments and the rising temperature experiments provided less opportunity for toxic product accumulation.

The parameters utilized in this mathematical model are summarized in Table 1. The number of parameter utilized by this model to characterize the growth and inactivation of a particular organism is substantially greater than in previous models of organism growth or death. Most previous models (Kono, 1968; Collee et al., 1961) have been based on constant temperature experiments. Thus, the time averaging functions for determining the effective temperature for growth and death were not required. Powers et al. (1965) studied the growth of *Aorobactes aerogenes* and *Staphylococcus aureus* in cyclic temperatures. However, the effective temperature effect, if present, was masked by the rapid temperature cycles and inadequate analysis. Ng et al. (1962) studied the growth rate of *Escherichia coli* subjected to step temperature changes but found no time lag comparable with the results of Willardsen et al. (1978). Both the Ng and Powers studies were restricted to the growth$^+$ phase. Many previous models (Kono, 1968; Nishida et al., 1969; Powers et al., 1965) were restricted to either growth or death which further decreases the number of parameters required.

The parameters utilized in this model do exhibit an interdependence. A number of subsets of the experimental conditions considered in this study were found that would permit the elimination of one or more of these parameters. However, the complete set of parameters were required when experimental conditions including constant temperatures from a low value with no growth to a high value causing death and rising temperatures with a range of rates from 4.1°C/hr to 17°C/hr were considered.

The model was solved on a CDC Cyber 74 computer. The differential equation was solved by a Runge-Kutta-Gill procedure (Gill, 1951). The integrations were numerically approximated by the Trapezoidal Rule. The parameter estimation was attempted with a nonlinear least squares program (Brown and Dennis, 1972), but was ultimately based on least sum of squares without a formal procedure. The combination of the interdependence and the staged or phased characteristics of the model favored less formalized parameter estimation.

Table 1—Model Parameters for C. perfringens

Parameter label	Parameter name	Estimated value	Approx 95% confidence interval
C_c	Critical cell conc	2×10^7 cells/g	$\pm 4. \times 10^7$
C_m	Maximum possible cell conc	3×10^8 cells/g	$- 2.5 \times 10^8$
E_d	Activation energy for death	260000 J/mol	± 9000.
E_g	Activation energy for growth	114000 J/mol	± 6000.
k_{300}	Specific reaction rate for growth at 300 K	0.72 hr^{-1}	± 0.05
k_{350}	Specific reaction rate for death at 350 K	2400. hr^{-1}	± 450.
T_b	Base temp for induction	290.2° K (17° C)	± 6.
T_i	Integrated-log-temp-difference for initiation of growth	2.0 hr	± 0.2
T_1	Effective mean temp for transition from growth$^+$ to growth$^-$	313.2° K (40° C)	± 0.9
T_2	Max possible environment temp to maintain growth$^+$	323.3° K (50.1° C)	± 2.
T_3	Minimum death effective temp to terminate the growth phase and initiate the death phase	325.2° K (52° C)	± 1.0
t_1	Time for integrating effective growth temp	2.0 hr	± 0.2
t_2	Time for integrating effective death temp	0.61 hr	±0.06
α	Rate of increase of fraction of cells which have the ability to propagate	2.5 hr^{-1}	− 1.6

RESULTS & DISCUSSION

ESTIMATES of the parameter values and their accuracy for prediction of the population dynamics of *C. perfringens* in constant and rising temperature environments are given in Table 1. These estimated values are based on the sum of squares between the mathematical model predictions and the experimental results published by Willardsen et al. (1978). The accuracy or 95% confidence limits in Table 1 are based on the linear assumption (Draper and Smith, 1968).

The value estimates were accepted on the graphical comparison of predicted and experimental values (see Fig. 1 through 3 for examples), the variance of 0.071 with 156 degrees of freedom between the ten base logarithm values of the predicted and experimental values and an approximate overall r^2 value of 0.92. This variance corresponds to a 95% confidence interval for the ten base logarithm of the mathematically predicted values of ± 0.52. The r^2 estimate suggests that the mathematical model is explaining 92% of the variation in experimental measurements. Because the model is nonlinear in the parameters, the r^2 value is not exact. The r^2 and variance values are the best indicators of overall fit. However, for improving the estimates of parameter values the plots were essential. From the plots the environment conditions and phase of the population dynamics exhibiting the poorest fit could be identified. From this information, improved parameter estimates could be selected.

The 95% confidence interval for each parameter was determined by independently varying each parameter around its optimum value. The amount of positive and negative variation required to satisfy Eq (11) (Draper and Smith, 1968) was averaged for most parameters and recorded as the approximate 95% confidence interval. Two parameters (C_m and α) exhibited very little influence on the error sum of squares for variation in the negative direction. For these variables the 95% confidence interval is based on variation in the positive direction only.

$$S(\theta) = S(\hat{\theta}) \left[1 + \frac{p}{n - p} F(p, n - p, 0.95) \right] \qquad (11)$$

where: $S(\theta)$ is the error sum of squares for a parameter set with one variable varied from the optimum; $S(\hat{\theta})$ is the error sum of squares for the optimum parameter set; θ is the p dimensional vector of parameter values; p is the number of parameters, 14; n is the number of data points, 180; $F(p, n - p, 0.95)$ is the statistical F – distribution for the upper 5% with p and n – p degrees of freedom.

These confidence limits are not exact because of the nonlinearity of the parameters in the model. A p dimensional plot of all parameter combinations, θ, satisfying Eq (11) would show greater confidence intervals when dependent parameters were varied simultaneously.

The variance between the model and the six verifying experimental curves was 0.11. This is greater than the variance between the model and the previous data (0.071). A comparison of the variance about the model and the mean square error for the verifying experimental data (0.013) shows a highly significant lack of fit. The populations observed during the verifying experiments were consistently higher than predicted by the model and higher than similar experimental results reported by Willardsen et al. (1978). For example, considering the experimental data after the induction phase of the 6°C/hr experiment of Willardsen, five data points are below the model prediction and four are above. By contrast, all nine of the comparable points of the verifying experiment are above the model prediction. The apparent block effect between the two groups of experimental data is also statistically highly significant. One of the changing rate-verifying experiments is compared with the model prediction in Figure 4.

The population dynamics during a constantly rising environmental temperature are characterized by the three-dimensional plot in Figure 5. This plot was generated by

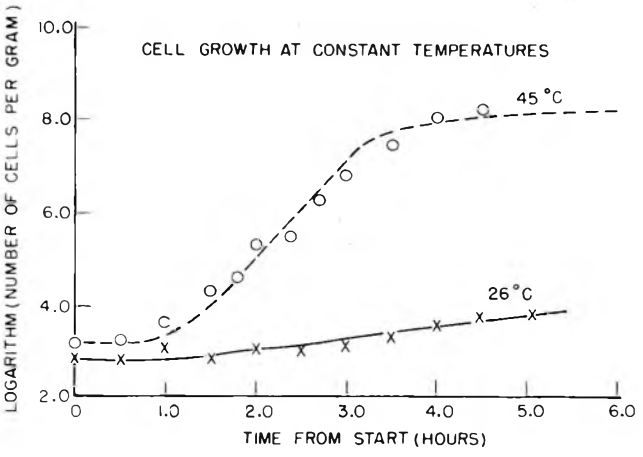
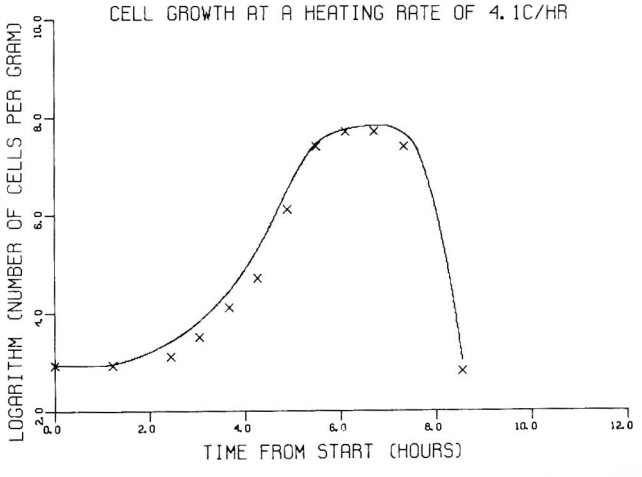


Fig. 1—C. perfringens cell numbers in meat held at 26 and 45° C. Experimentally measured values (x and o) and the model prediction (——and– – –).

predictions from the mathematical model using the parameter values listing in Table 1. The important influence of rate of temperature rise on maximum organism population is evident from this plot. Each curve in this plot assumes a starting environmental temperature of 298.15°K (25°C) and an initial cell concentration of 1000 viable organisms per gram of meat.

Predicted population dynamics under constant temperature growing conditions are illustrated in Figure 6. This figure assumes that meat was inoculated with 1000 organisms per gram at time 0. The influence of temperature on growth rate is evident in Figure 5 and 6. Under changing temperature conditions (Fig. 5) the growth rate at a given temperature does not correspond to the growth rate under constant temperature (Fig. 6) because of the time averaging for the effective temperature for growth. This influence of changing temperatures on growth and inactivation is further illustrated by effective decimal reduction times, D = 2.303/k, and specific reaction rate constants, k, in Table 2. The values in this table have been calculated and are used by the model for the specified conditions. The high plateau of no growth in Figure 5 for temperatures in the optimum growth range illustrates the decline phase of the concentra-



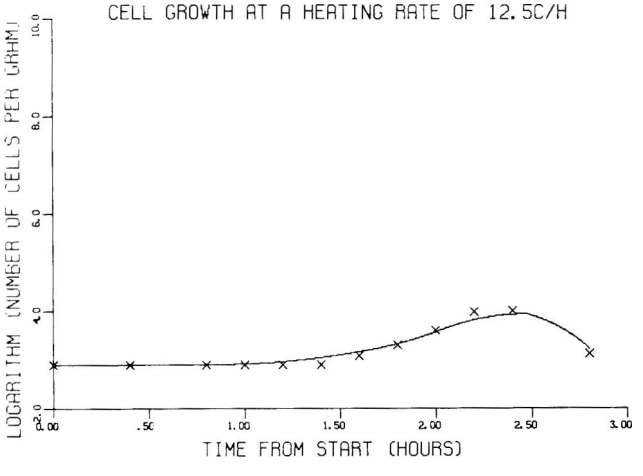


Fig. 2 & 3—C. perfringens cell numbers in meat subjected to constantly rising temperatures at rates of 4.1° C/hr and 12.5° C/hr. Experimentally measured values (x) and the model prediction (——).

tion function. By contrast, the ridge in Figure 5 was caused by temperatures exceeding the growth range and initiating death. None of the constant temperatures simulated in Figure 6 are sufficiently high to initiate death.

Table 2—Influence of rate of temperature change on growth and inactivation

Rate of temp change (°C/hr)	Specific reaction rate constants (k) and decimal increase (K) or reduction (D) times			
	Growth (40°C)		Inactivation (60°C)	
	K (min)	k (hr^{-1})	D (min)	k (hr^{-1})
0	28.	4.9	5.3	26
5	57.	2.4	8.2	17
10	119	1.2	13	11
15	255	0.54	20	6.9

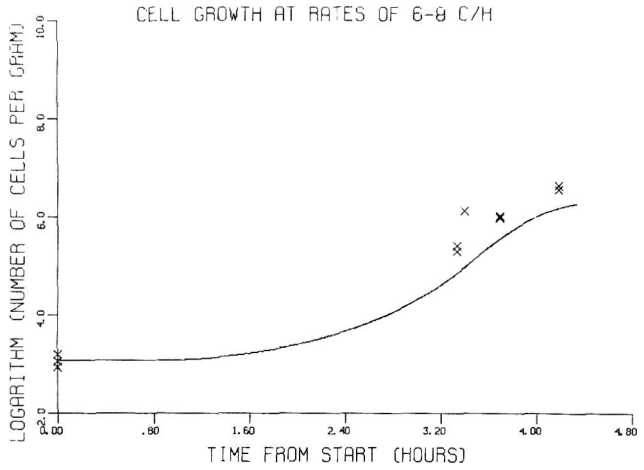


Fig. 4—C. perfringens cell numbers in meat subjected to temperatures rising at 6° C/hr followed by 8.2° C/hr. Experimentally measured values (x) and the model prediction (——).

The reference reaction rates and activation energies for growth and inactivation deserve special attention. The equivalent D and z values, calculated by the equations of Lund (1975), and the activation enthalphy and entropy, calculated by the equations of Glasstone et al. (1941), are given in Table 3. The growth activation energy is within the range of previously reported values (Pirt, 1975; Garrett et al., 1966) for *E. coli* and *Pseudomonas pseudomonad*. Very few researchers have reported kinetic parameters for the inactivation of vegetative cells capable of forming spores. However, the activation energy determined in this study is in the range of values for inactivation of vegetative cells (Tomlins and Ordal, 1976) or spores (Russell, 1971). The entropy-enthalpy values fall very close to the temperature compensation line discussed by Rosenberg et al. (1931) and Banks et al. (1972).

The reference reaction rates and activation energies reported in this paper are for a composite of eight strains. Inactivation of composite strains was computer simulated with both constant and rising temperature assumptions to better understand the relation of the composite kinetic parameters to the individual strain parameters. As expected, the reference reaction rates for inactivation of composites at constant temperatures are strongly influenced by the most heat resistant strain. The dominance of the most heat resistant strain is accented by individual strain activation energies that vary inversly with reference reaction rate and increasing number of strains in the composite. The apparent activation energy of the composite was always near the average activation energy for the individual strains.

The same effects were observed for constantly rising temperatures, but the effects were more pronounced. The apparent reference reaction rate constant for the composite can be less than the rate for any strain in a rising temperature environment if there is a negative correlation between the reference reaction rates and the activation energies for the individual strains. This means that a composite of several strains could appear to be more heat resistant than any of the individual strains in an increasing temperature environment. The composite activation energies varied more from the mean, but always remained in the range of the

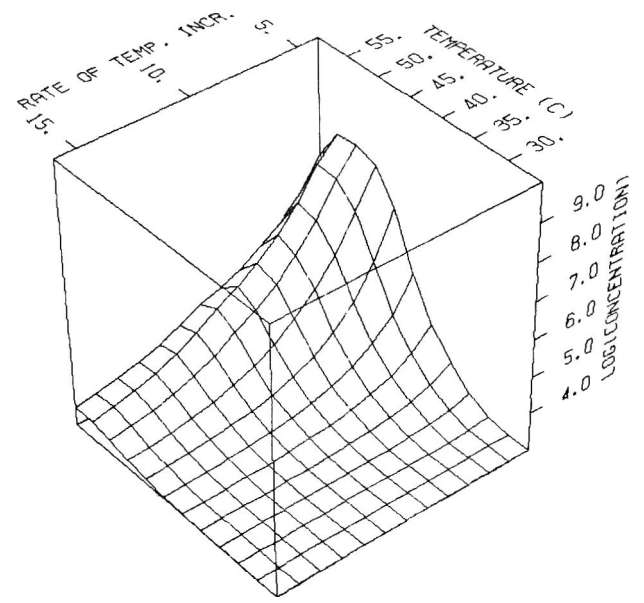


Fig. 5—C. perfringens population dynamics in meat subjected to constantly rising temperature at rates from 4—16° C/hr as predicted by the model.

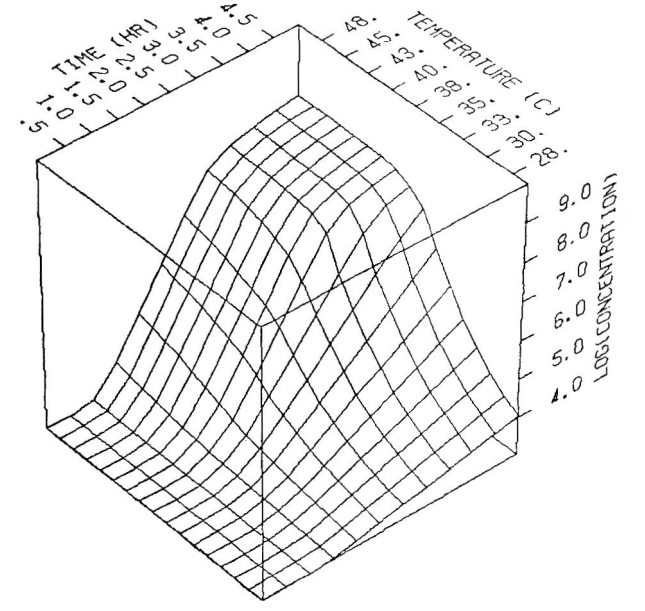


Fig. 6—C. perfringens population dynamics in meat held at constant temperatures between 26 and 50° C as predicted by the model.

Table 3—Kinetic parameters for growth and death of C. perfringens

	Activation energy (J/mol)	z (C)	Reference reaction rate (hr^{-1})	D (min)	Entropy of activation (J/mol·K)	Enthalpy of activation (kJ/mol)
Growth	114,000	15.8	0.72 @ 300° K	14.1 @ 45° C	36.4	111.
Death	260,000	8.5	2400 @ 350° K	5.28 @ 60° C	466.	257.

individual strain energies in rising temperature conditions. The simulation results clearly indicate that individual strain characteristics cannot be inferred from measurements on a composite of several strains.

Other parameter values also deserve attention. The model evaluated maximum possible cell concentration, C_m, found in this study agrees well with the 10^8 cells/g reported by Rey et al. (1975). The integrated-log-temperature-difference designated by the model for growth initiation appears to correspond with the induction time at optimum growth temperature reported by Collee et al. (1961). The integrated or averaged time for effective growth temperature, 2.0 hr, is substantially longer than the time for determining effective death temperature, 0.62 hr. In an environment in which temperature is linear in time, the effective growth and death temperatures are occurring 60 and 18 min prior to the current time, respectively. Interestingly, the low activation energy for growth suggests that the process depends on a few low energy bonds (Brown and Melling, 1971), but the long time lag for effective growth temperature suggests a long chain reaction. By contrast the values for inactivation indicate high energy bonds with very little chain reaction. Inactivation is much more temperature sensitive than growth.

CONCLUSIONS

THE RESULTS indicate that *C. perfringens* has different effective growth rates at the same temperature if the rate of temperature change is varied. This is because of the organism's response lag in recognition of its real environmental temperature during constantly rising temperature conditions. The differential between the effective temperature interpreted by the organism and the real environmental temperature is greater when the cooking rate is more rapid. Thus, our data suggest that if a roast is cooked rapidly to a minimum internal temperature where microbiological safety is desired, it would be necessary to hold it longer at that temperature than if cooked slowly to that same minimum temperature. This emphasizes that both time and temperature are important considerations in establishing the conditions for microbiological safety in long-time, low-temperature cooking of meat and similar processes for other products.

The results also show that organism response cannot be predicted in changing temperature conditions based on constant temperature experiments alone. This is in agreement with the findings of Howell et al. (1971).

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RETENTION AND STABILITY OF VOLATILE SULFUR-CONTAINING COMPOUNDS IN CHICKEN MEAT

M. I. FARBOOD and J. H. MacNEIL

ABSTRACT

Methionine-^{35}S was used to generate radioactive volatile sulfur-containing compounds. The degree of retention and stability of these compounds in a cold chicken meat slurry was determined. It was found that substantial amounts of radioactive volatile sulfur-containing compounds were trapped in the slurry (.71 μci). It was also concluded that 44.0% of the total methanethiol generated was trapped in the slurry. Further investigation revealed that the major portion of these highly volatile sulfur-containing compounds, which were trapped in the slurry, was no longer volatile and would not be released upon heating. Subsequent heating of the slurry for 4 hr under reflux and in an open vessel resulted in a 32.3% and 33.8% loss of radioactivity, respectively. However, the major loss of radioactivity occurred within the first hr of cooking with little loss of activity after that period. The results of a chloroform-methanol fractionation showed that 90.7% of the trapped radioactive sulfur-containing compounds were associated with the protein fraction of meat. The association of these compounds with the protein fraction was confirmed using picric acid precipitation. The remaining radioactive sulfur-containing compounds retained in the aqueous and lipid fraction, 8.95% and 0.35% respectively, could also play an important role in flavor.

INTRODUCTION

THE MAIN FLAVOR characteristics of foods are derived primarily from volatile components, although nonvolatiles also contribute to the flavor of foods. The role of nonvolatiles is generally restricted to taste sensations like sweet, sour, salt, and bitter. Examination of the literature shows that the majority of studies on flavor chemistry have been of a qualitative nature: flavor compounds isolated and identified in a particular food are simply reported with little information given about their relative importance in a total flavor response (Wilson and Katz, 1972). Sulfur-containing flavor compounds usually make a dominant contribution to the overall flavor impression of foods in which they are present because of their strong characteristic odor and their very low threshold values (on the order of a few μg/kg food). Using chicken meat and radioactive methionine, a study was undertaken to measure retention and distribution of various sulfur compounds in individual chicken meat fractions (i.e. lipid, protein, etc.). The stability of volatile sulfur compounds retained in chicken meat after heating was also investigated.

Origin of flavor compounds in poultry meat

Precursor(s) of meaty flavor can be extracted from meat fibers by steeping meat in water or by extraction of the meat after suitable denaturation of the protein (Bouthilet, 1951; Pippen et al., 1954). Pippen et al. (1954) have shown that water-extracted chicken meat lost much of its ability to impart flavor to broth. Keohler and Jacobson (1967) reported, that upon heating the dialysate of water-extracts of ground white and dark chicken muscle produced significantly stronger chicken flavor than filtrates of nondialyzable material.

Contribution of fat to the aroma of chicken broth is indirect and passive, it dissolves and retains aroma components formed during cooking (Pippen et al., 1954, 1969). These results were confirmed by the finding of Wasserman and Talley (1968) and Wasserman and Spinelli (1972) that characteristic aromas of heated beef, lamb and pork adipose tissues are in the water-wash of chloroform:methanol extracts.

Minor et al. (1965a) studied the carbonyl and sulfur containing compounds in the volatile fraction of cooked chicken meat. They concluded that carbonyl and sulfur containing compounds are of major importance in flavor. This conclusion was later confirmed by Minor et al. (1965b). They demonstrated that the removal of sulfur containing compounds resulted in an almost complete loss of the meaty odor in the broth of dark and light meat, and the removal of the carbonyls from the volatile fraction resulted in a loss of "chickeny" flavor and intensification of the meaty or beef like odor.

Importance of hydrogen sulfide and methanethiol in chicken flavor

It has been demonstrated that hydrogen sulfide and methanethiol are evolved continuously during the simmering of chicken meat (Gordon, 1972). However, when the meat commences to cool before being consumed, hydrogen sulfide and methanethiol are probably produced at a slower rate, and there may also be an appreciable loss of these volatiles due to their volatility and reactivity. The result is there is no assurance that the level of these volatiles present in poultry meat when consumed plays a significant part in the aroma (Gordon, 1972). Pippen and Mecchi (1969) have looked into this aspect in some depth. They reported a 10 PPB odor threshold for hydrogen sulfide in water and pointed out that hydrogen sulfide may also contribute indirectly to cooked poultry flavor by the formation of secondary products.

Methanethiol is also present in relatively high concentrations in cooked beef. Like hydrogen sulfide, this compound is formed readily under very high heat conditions and thus gives rise to off-flavor (Ziemba and Malkki, 1971). The major route of methanethiol formation is the Strecker degradation of methionine (Ballance, 1961).

Finally, in 1974 Boelens et al. studied reaction products of aldehydes with hydrogen sulfide and methanethiol. The major reaction products of saturated aldehydes with hydrogen sulfide and thiols at room temperature were 1,1-dialkylthioalkanes and 1-alkylthio-1-alkanethiols. Upon heating 1-alkylthio-1-alkanethiols were converted into dialkyl-disulfide and 1-alkylthio-alkyl-aklyl disulfides. Dialkyl disulfides occur in the flavor complex of boiled meat (Wilson et al., 1973). The major products of the reaction of unsaturated aldehyde, hex-2-enal, with hydrogen sulfide and methanethiol were 3-methylthio-hexanal, 1,1-dimethylthio-hex-2-ene, and 1,3-dimethylthio-hex-1-ene (Boelens et al., 1974). 3-methylthiohexanal has been reported to be used in preparing flavor compositions (Givaudan, 1971). The principal reaction products of the deca-2,4-dienal with a mixture of

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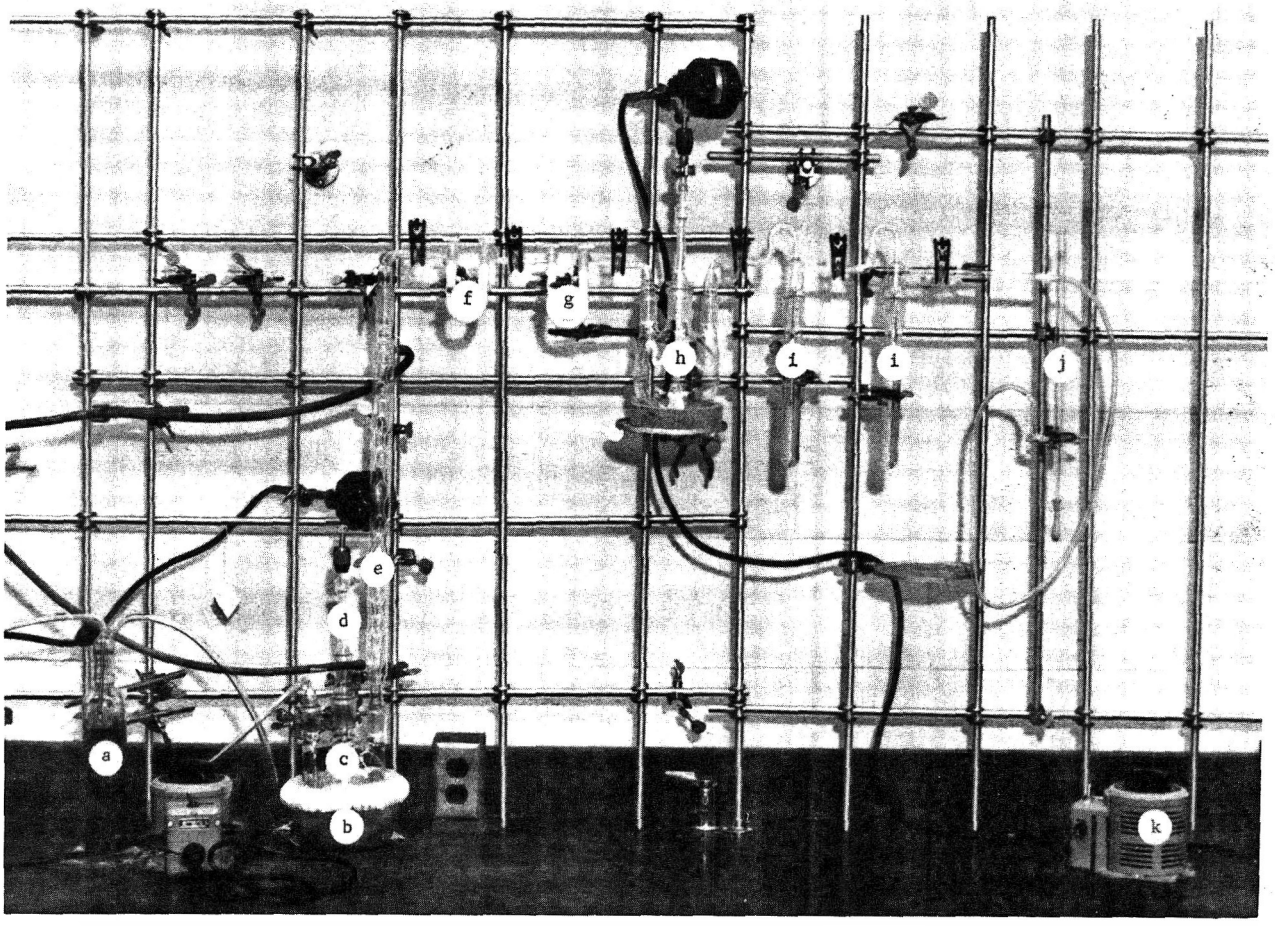


Fig. 1—Distillation apparatus for retention studies: a—500 ml gas wash bottle containing a mixture of 5.0% (w/v) Pyrogallol in 50.0% (w/w) aqueous potassium hydroxide; b—Heating mantles; c—Three-neck distillation flask (generator flask); d—Sleeved glass stirrer with mechanical stirrer; e—Condensor; f—Schwartz absorption tube containing granular calcium chloride (12 mesh); g—Schwartz absorption tube containing 25g crystalline lead acetate; h—Three-neck distillation flask containing cold chicken meat slurry (retention flask); i—Vacuum traps containing DNFB-reagent; j—Soap bubble flow meter; k—Variac.

hydrogen sulfide and methanethiol were 1,1-dimethylthiodeca-2,4-diene, 1,3-dimethyl-thiodeca-1, 4-diene, 1,5-dimethylthiodeca-1, 3-diene and a little 2-hexylthiophene. Thus, 2-alkylthiophenes, which occur in the flavor of boiled meat (Wilson et al., 1973) and cranberries (Anjou and von Sydow, 1967), may be formed from unsaturated aldehydes, hydrogen sulfide and methanethiol.

EXPERIMENTAL

Distillation and retention

The procedures for preparation of chicken meat and purification of methionine-^{35}S were those outlined by Farbood and MacNeil (1978).

The components of the distillation apparatus used for studying the retention of volatile sulfur compounds in chicken meat are shown in Figure 1. This apparatus consisted of a pair of 2-liter, 3-neck distillation flasks (c,h). These flasks were equipped with a glass sleeve mechanical stirrer. The first flask (generator flask) contained 450g of chicken meat slurry (1 part ground meat, 2 part distilled water and 240 μci Met-^{35}S. The second flask (retention flask) contained only 300g of a cold chicken meat slurry. Components e, f and g (Fig. 1) remove the moisture and hydrogen sulfide content of volatiles produced. The vacuum traps (i) contain 1-fluoro-2,4-dinitrobenzene (DNFB) reagent which would react with thiols emerging from the retention flask.

To measure the retention of the volatile sulfur containing compounds in chicken meat radioactive volatile sulfur compounds were generated from a chicken meat slurry containing methionine-^{35}S. These compounds generated were then passed through a cold chicken meat slurry. Any hydrogen sulfide that might have been present was trapped in a crystalline lead acetate trap. The distillation procedure was also the same as that reported by Farbood and MacNeil (1978). At the end of a 6-hr distillation period samples of the meat slurry obtained from the retention flask were digested using Protosol (New England Nuclear, Boston, MA). After addition of 10 ml Aquasol (New England Nuclear, Boston, MA) the radioactivity of these samples was determined using a Packard Tri-Carb liquid scintillation spectrometer model 3375 (Packard Instrument Co., Inc., Downers Grove, IL). The 2,4-dinitrophenyl (DNP) derivatives of thiols produced in DNFB reagent were purified and identified using descending paper chromatography on Whatman chromatographic paper No. 1 against known DNP derivatives of thiols (Libbey and Day, 1963). The DNFB reagent was also analyzed for total radioactivity and radioactive CH_3SH according to Farbood and MacNeil (1978).

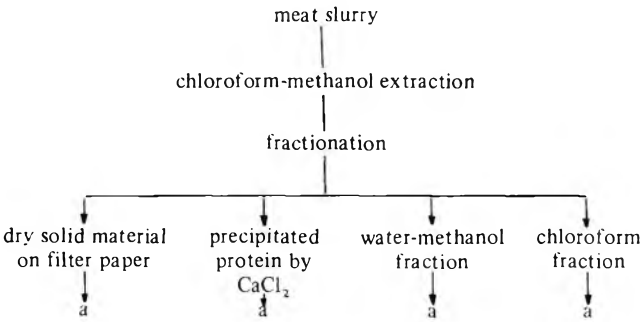
Stability of radioactive volatile sulfur compounds in cold chicken meat

The effect of heating on the reduction of radioactive levels in the meat was determined as follows: At the end of the 6 hr distillation period the contents of the retention flask were divided into two equal portions. One portion was placed into a beaker and the contents of the beaker heated for 4 hr at 101°C using a hot plate. Samples of the meat slurry were obtained at 0, 0.5, 1, 2, 3 and 4 hr

of cooking. The radioactivity of each sample was determined using previously described methods.

The second portion was heated at 101°C under reflux for 4 hr using a nitrogen flow rate of 20–25 ml/min. The effluent was trapped in a DNFB reagent. The DNFB traps were analyzed for radioactivity and methanethiol. The total radioactivity of the meat slurry was also determined.

A chloroform-methanol extraction was performed on a sample of the cooked meat slurry using a modified method of Bligh and Dyer (1959) shown below. The activity of each fraction was determined. The dried solid material, collected on filter paper after extraction with chloroform-methanol, was further analyzed as described below.



a = radioactivity determined

Picric acid treatment

A sample of dried solid material was soaked in 10 ml distilled water overnight at 4°C. The sample was then centrifuged at 6000 rpm for 30 min followed by dividing the supernatant solution into two equal portions. One portion was designated as the control sample and its radioactivity was determined without further treatment. The second portion of supernatant solution was deproteinated using picric acid (Beckman Instructional Manual, 1965). The radioactivity of the deproteinated sample was determined. All experiments were done in duplicate.

RESULTS & DISCUSSION

In the study on the retention of volatile sulfur containing compounds in chicken meat, it was found that a substantial amount of volatile sulfur containing compounds generated were trapped in the cold chicken meat slurry. An analysis of the DNFB trap, which collected the volatile compounds lost during heating, showed that methanethiol constituted 5.3% of the sulfur compounds trapped. This also represented 18% of the methanethiol determined to be present in the starting meat slurry. The results of these experiments support the theory that volatile sulfur containing compounds contribute to the flavor profile either directly as odorous compounds or indirectly by formation of secondary reaction products (Pippen and Mecchi, 1969; Parr and Levett, 1969). The occurrence of saturated and unsaturated aldehydes in chicken meat has been demonstrated by Nonaka et al. (1967) and many others. Boelens et al. (1974) have shown that the reaction products of hydrogen sulfide and methanethiol with unsaturated aldehydes resulted in the formation of compounds which occurs in the flavor profile of boiled meat, namely 2-alkylthiophenes. Therefore, it is possible that a portion of the methanethiol trapped in the chicken meat slurry reacted with an unsaturated aldehyde and hydrogen sulfide. As a result, compounds like dialkyl disulfides which occurs in the flavor complex of boiled meat may have been produced.

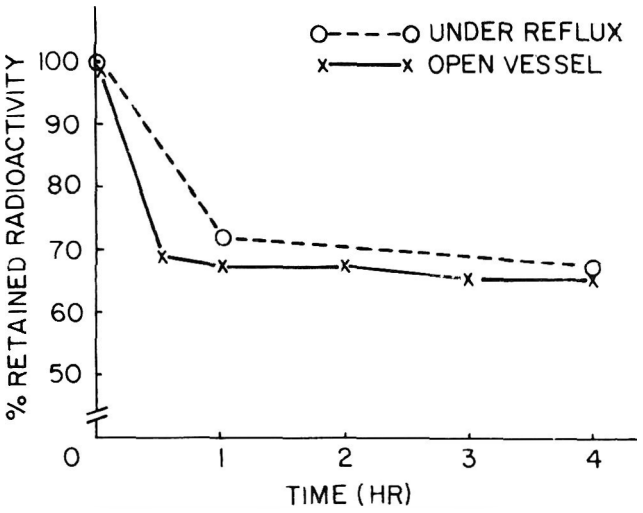


Fig. 2—Retention of trapped radioactive sulfur compounds in chicken meat slurry during 4 hr of cooking under reflux and cooking in an open vessel.

Stability of trapped volatile sulfur compounds in chicken meat

The stability of trapped radioactive volatile sulfur-containing compounds in a cold chicken meat slurry was evaluated while heating the slurry under reflux and in an open vessel. The loss of trapped radioactive volatile sulfur-containing compounds in a chicken meat slurry during 4 hr of heating under reflux and heating in an open vessel is illustrated in Figure 2. The loss of trapped radioactive volatile sulfur-containing compounds during 4 hr of heating under reflux was 32.3%. Heating the sample in an open vessel resulted in 33.8% loss of radioactivity (Fig. 2). The maximum loss of radioactivity occurred within the first hr of heating with very little loss of activity after that period. The loss of radioactivity at the beginning of heating could be due to the portion of volatile sulfur containing compounds which did not react with meat constituents. This unreacted portion was possibly absorbed by the constituents of the meat slurry and lost upon heating.

Paper chromatography and maximum absorption of purified DNP derivatives obtained from heating the meat under reflux confirmed the release of radioactive methanethiol. The chromatogram also showed a new radioactive spot (Fig. 3) located at the original spot. The R_f value of the DNP derivative of hydrogen sulfide and the new radioactive spot are similar. The wavelength of maximum absorption of this spot was the same as the known DNP of hydrogen sulfide namely, 327 nm in absolute ethanol. Therefore, the formation of hydrogen sulfide might be attributed to secondary decomposition of volatile sulfur containing compounds.

Association of trapped volatile sulfur compounds with protein

Further investigation was conducted to determine the distribution of radioactivity in different fractions of the meat obtained from a chloroform-methanol extraction (Bligh and Dyer, 1959). It was found that most of the radioactivity was associated with the dry residue (59.25%) and the calcium chloride precipitated fraction (31.45%). The calcium chloride functions as a demulsifier. Thus the emulsion of the water soluble protein and water was broken and the protein precipitated (Folch et al., 1957). The radioactivity of fat fraction was only 0.35% of the total (Table 1).

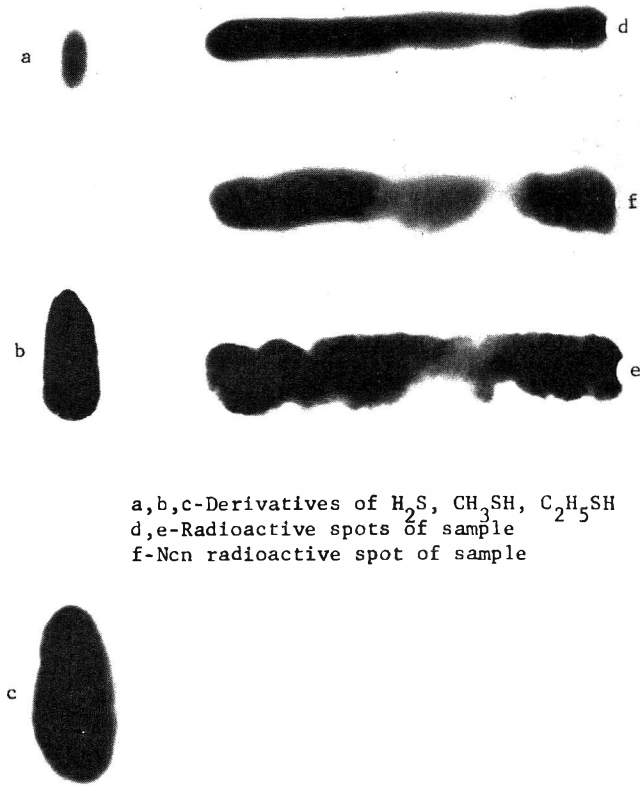
To confirm the association of radioactive volatile sulfur compounds with the protein fraction, the dry residue obtained from chloroform-methanol fractionation was suspended in distilled water overnight and then centrifuged. The

Table 1—Percent distribution of radioactivity in different fractions of chicken meat slurry after chloroform-methanol extraction

Fraction	% Radioactivity
Fat	0.35
Water	8.95
Dry residue of meat	59.25
Fraction precipitated with $CaCl_2$	31.45

supernatant was removed and divided into two samples. One sample was used directly for radioactivity determinations and was found to contain 42.0% of the total activity. The second sample was treated with picric acid and resulted in the loss of 95.5% of the activity mentioned above. Picric acid treatment removes the proteins of a sample; therefore, a 95.5% decrease in radioactivity of the picric acid-treated samples would indicate that sulfur compounds were associated with the protein fraction of the meat. It is speculated that the sulfur compounds retained in meat, in addition to contributing directly to chicken meat flavor, act as precursors of sulfur-containing flavor compounds. It should also be pointed out that methanethiol could reduce and react with protein disulfide bonds and become incorporated as part of the protein molecule.

Although a total of 90.7% of the volatile sulfur compounds retained in the chicken meat was associated with the protein fraction, the remaining percentages found in the water (8.95%) and lipid fraction (0.35%) could also be responsible for significant flavor responses. This speculation is supported by the findings of Batzer et al. (1960), Koehler and Jacobson (1967), Macy et al. (1964a, b), Wasserman and Gray (1965) and Wasserman and Spinelli (1970).



a,b,c-Derivatives of H_2S, CH_3SH, C_2H_5SH
d,e-Radioactive spots of sample
f-Ncn radioactive spot of sample

Fig. 3—Paper chromatography of the DNFB-derivatives obtained from chicken meat while cooking under reflux.

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ION-EXCHANGE CHROMATOGRAPHY AND ELECTROPHORESIS OF EGG YOLK PROTEINS

L. E. MC BEE and O. J. COTTERILL

ABSTRACT

Pure egg yolk was fractionated using DEAE ion-exchange cellulose. Eluted components were simultaneously detected by fluorescence and ultra-violet absorption. Selected chromatographic fractions were subjected to disc gel electrophoresis and assayed for total lipids, amino acids and phosphorus. Native egg yolk was separated into 18 peaks chromatographically and 23 bands electrophoretically. Multicomponent chromatographic peaks were demonstrated by electrophoresis. Chromatographic peaks and corresponding electrophoretic bands were identified by literature reference for lipovitellin, livetins, phosvitin and a low density fraction. A direct relationship existed between chromatographic position and electrophoretic mobility. Lipid and phosphorus concentrations were high in the same fractions.

INTRODUCTION

MANY METHODS have been employed to isolate and study egg yolk (EY) proteins. Many early techniques involved organic solvent fractionation, heat, pH extremes and salts which tended to modify the structural integrity of the components. The milder procedures such as centrifugation, electrophoresis and ion-exchange chromatography not only privide better resolution of components but are less damaging. Ultracentrifugation produced fractions which contain mixtures of EY protein (Vandegaer et al., 1956; Schjeide and Urist, 1959) and is used for the primary separation of yolk components for future study (Garland and Powrie, 1978). Electrophoresis and chromatography have been used mostly as secondary procedures. Paper, starch gel and polyacrylamide gel were used by Evans and Bandemer (1957), Steven (1961) and Chang et al. (1970) to separate EY components. Shantz and Dawson (1974) obtained 29 bands from EY with disc gel electrophoresis.

Ion-exchange has several unique features when compared to the harsh methods used earlier: (1) Fractionation is on the basis of charge, (2) slight differences in charge are sufficient to change separational patterns, (3) alterations caused by reagents are minimized, (4) cellulose has little filtering effect, and (5) wide ranges of pH and ionic strength can be used. Many researchers have used ion-exchange chromatography to study various isolates from EY, but only limited use has been made of this technique for primary separation of egg yolk proteins. Seideman et al. (1969) obtained five fractions from native egg yolk (NEY) with carboxymethyl-cellulose by eluting with decreasing pH and increasing NaCl concentration gradients.

This study further characterized NEY and EY fractions by diethylaminoethyl (DEAE)-cellulose ion-exchange chromatography and disc gel electrophoresis.

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METHODS & MATERIALS

Chromatographic procedure

A continuous gradient system was formed by permitting buffer from an upper reservoir to flow into a closed mixing chamber at the same rate it was removed. This elution buffer passed consecutively (3 ml per min) through a pH monitor, a UV reference flow cell, metering pump, ion-exchange column, UV monitoring cell, fluorometric monitoring cell, volumeter and then to a fraction collector. Aliquots of 20 ml were collected. An ISCO Model UA-2, double beam, ultraviolet absorbance monitor (Instrumentation Specialties Co., Lincoln, NB) was used to detect absorbance at 280 nm.

Fluorescence was measured and recorded with a Model 111 fluorometer (G.K. Turner Associates, Palo Alto, CA) with a #7–60 primary filter (excitation from 320 nm to 380 nm with a maximum at 360) and a #3 secondary filter (passing fluorescence greater than 450 nm). A 0.73 ppm solution of quinine sulfate produced 100 scale units of fluorescence.

The chromatographic scheme of Mandeles (1960) was used with minor modification in flow rate and buffer volumes. DEAE-cellulose anionic exchanger, coarse mesh, 0.95 meq/g exchange capacity was obtained from Sigma Chemical Co., St. Louis, MO. After use, the DEAE was regenerated by suspension in 0.1M HCl, distilled water, 0.1M NaOH and distilled water, in succession. Liquid was removed with a Büchner funnel. DEAE-cellulose was prepared and packed in a column (2 × 30 cm) according to Mandeles, and equilibrated to pH 4.5 with 0.02M glycine (first buffer). Samples were applied and eluted with buffers of increasing ionic strength and decreasing pH as shown in Figure 2. Buffers were formulated as follows:

Stock solutions. 0.1M glycine, 1M KH_2PO_4, 1M NaCl, and 1M HCl.

Working buffers. (1) First buffer (pH 4.5, μ = 0.04) – 100 ml 0.1M glycine, and 400 ml distilled water (300 ml as first buffer in mixing reservoir). (2). Second buffer (pH 4.6, μ = 0.12) – 50 ml 0.1M glycine, 12.5 ml 1M KH_2PO_4, 12.5 ml 1M NaCl, and 175 ml distilled water (placed in upper reservoir). (3) Third buffer (pH 4.5, μ = 0.19) – 50 ml 0.1M glycine, 25 ml 1M KH_2PO_4, 25 ml 1M NaCl, and 150 ml distilled water (replaced second buffer when exhausted). (4) Fourth buffer (pH 2.75, μ = 0.19) – 50 ml 0.1M glycine, 25 ml 1M KH_2PO_4, 25 ml 1M NaCl, 6 ml 1M HCl, and 144 ml distilled water (replaced third buffer). (5) Fifth buffer (pH 1.0, μ = 0.17) - 25 ml 1M HCl, and 125 ml distilled water (replaced fourth buffer).

Electrophoresis procedure

Disc-gel electrophoresis was performed with Canalco Research Disc Equipment (Model 1200, Rockville, MD) using their RDS Reagent Kit. Gels were fixed with 12% trichloroacetic acid and stained with a 0.025% solution of Coomassie Brilliant Blue R in 7% acetic acid.

Fluorescence spectrum

The fluorescence spectrum of selected chromatographic fractions was obtained with a spectrophotofluorometer (Baird-Atomic, Inc., Model SF/1, Bedford, MD) standardized with quinine sulfate solutions.

Other analyses

The sulfo-phosphovanillin method described by Frings and Dunn (1970) was used to estimate total lipids. Protein concentration was determined by micro-Kjeldahl (AOAC, 1970) using the factor 6.25 for converting N to protein. The amino acid analyses were performed by automated cation-exchange chromatography after hydrolysis of the samples with 6N HCl for 22 hr at 110° C (Benson and Patterson, 1971). The method of Fisk and Subbarow (1925), as described by Hawk et al. (1954), was used to determine total phosphorus.

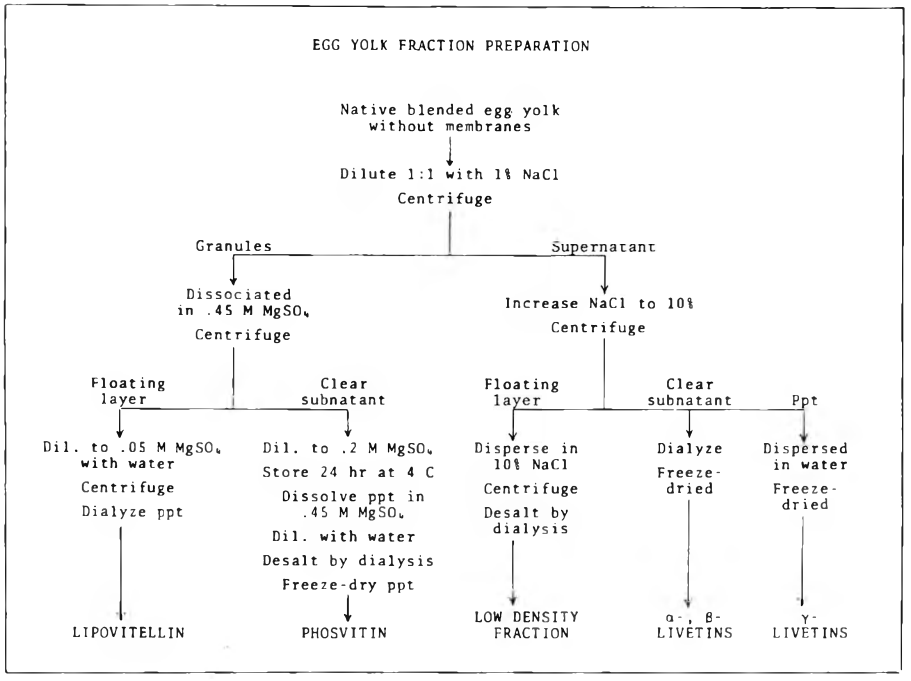

Fig. 1—Egg yolk fraction preparation.

Native egg yolk

Native egg yolk (NEY) was prepared from one day old eggs produced by a genetically closed flock of Single-Comb White Leghorn hens. After removal of adhering albumen, yolk membranes and germinal discs, the yolk contents from the least six eggs were pooled and mixed.

Egg yolk fraction preparation (see Fig. 1)

Granules, the source of phosvitin (PV) and lipovitellin (LV), were prepared by diluting NEY at a ratio of 1:1 with 1% NaCl. Centrifugation at 4°C with 10,000 RCF for 15 min formed a firm pellet of granules on the bottom and an opaque orange supernatant which was kept for further fractionation. LV was obtained from these granules by dissociation in 0.45M $MgSO_4$. After centrifuging for 12 hr with 31,000 RCF, a floating layer of crude LV formed which was removed and precipitated by diluting to 0.05M $MgSO_4$ with water. This LV precipitate was centrifuged again, dialyzed against distilled water, and stored at 4 C. The clear subnatant remaining after LV removal was diluted to 0.2M $MgSO_4$. The PV which precipitated during 24 hr storage at 4°C was then redissolved in 0.45M $MgSO_4$ and precipitated again with water (Joubert and Cook, 1958). After desalting by dialysis against distilled water, the clear dialysate of PV was freeze-dried. Also, PV, as prepared by the method of Sandararajan et al. (1960), was obtained from Sigma Chemical Co., St. Louis, MO.

The supernatant remaining after granule preparation was the source of the α-, β- and γ-livetins and the low density fraction (LDF) lipoproteins (Bernardi and Cook, 1960). The salt concentration of this supernatant was increased to 10%, then centrifuged at 4°C with 31,000 RCF for 18 hr. The LDF (floating layer) was dispersed in 10% KaCl, recentrifuged, dialyzed, and stored at 4°C for 48 hr or less before usage. The γ-livetin was precipitated as a firm pellet during the first centrifugation of the supernatant containing 10% NaCl. This pellet was dispersed in distilled water and freeze-dried. The remaining clear subnatant, containing the α- and β-livetins, was dialyzed and freeze-dried.

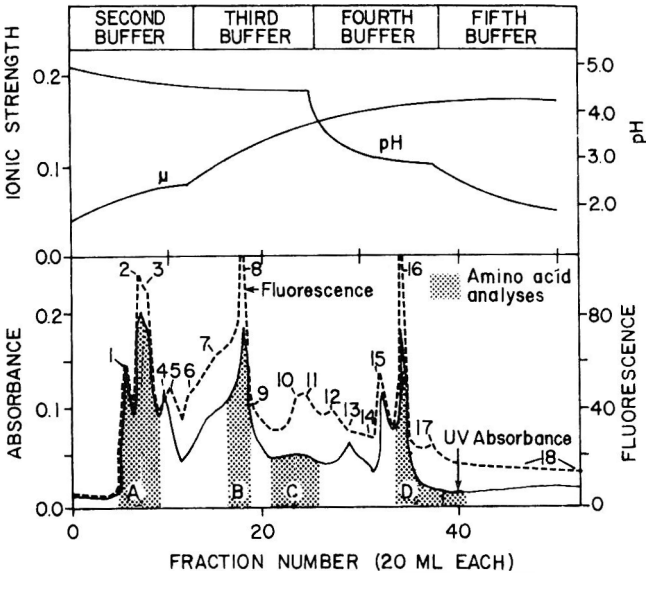

Fig. 2—Ionic strength of buffer and eluent pH with resulting chromatogram of native egg yolk from DEAE-cellulose separation.

RESULTS & DISCUSSION

Analysis of native egg yolk

Chromatographic Separation of Native Egg Yolk. When NEY (0.5g) was chromatographed on DEAE (Fig. 2), it was possible to separate ten major absorbance peaks and eight minor peaks for further evaluation. Although the mild buffers did not cause alteration of most proteins, the macrostructure of the granules was disrupted by a combination of low pH and increased ionic strength (Bernardi and Cook, 1960), thus permitting chromatographic separation of subcomponents (Fig. 2 and 4). The fluorometric tracing indicated that compounds other than protein were present, since fluorescence from protein was excluded by wavelength selection.

The pH dropped slowly with the addition of the second buffer. The first of the six peaks, eluted before fraction 15, appeared with the void volume of the column which had reacted loosely with the absorbant. The second and third peaks were very close together. The fourth peak absorbed at 280 nm but did not fluoresce while the fifth peak fluoresced but did not absorb at 280 nm. The sixth peak was overlapped by peak seven.

The ionic strength was increased by the third buffer causing peak eight to elute quickly. The ninth peak appeared as a shoulder on peak eight. Four additional peaks were eluted between fractions 22 and 30. Peaks 10 and 11 overlapped with the first absorbing weakly and the second

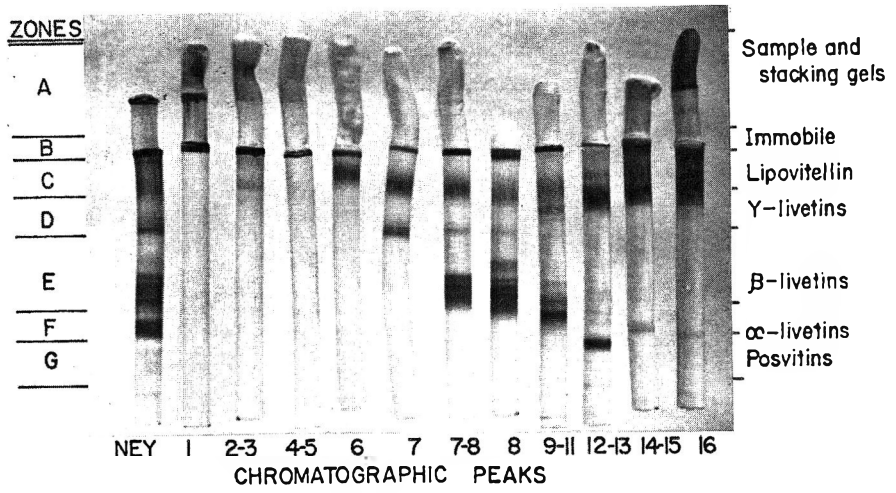

Fig. 3—Electrophoretograms of Native Egg Yolk and DEAE-Cellulose Fractions of Native Egg Yolk.

fluorescing more strongly. Peak 12 fluoresced strongly; while peak 13 absorbed but did not fluoresce.

The fourth buffer created a sharp drop in pH resulting in quick elution of peak 15. Peak 16 appeared to contain multiple compounds which fluoresced strongly and was followed by a minor fluorescent peak.

Further elution by the fifth buffer produced only a broad peak from which no electrophoretic bands were observed.

The removal of lipids from NEY prior to chromatographing on DEAE-cellulose is not necessary as stated by Mandeles (1960). Perhaps the reduced elution volume and increased flow rate were sufficient to permit fractionation of NEY without lipid removal.

Electrophoresis. NEY and DEAE fractions of NEY were further characterized by electrophoresis (Fig. 3). NEY was separated into 18 protein bands. A portion of NEY was immobile and remained in the sample and stacking gels, zones A and B. The immobile material probably had a very large molecular size, as in a protein aggregate, or had a nearly neutral charge. Immobile protein in zone B was considered a separated band because it had sufficient solubility and mobility to pass through the sample and stacking gels. Based on its apparent large molecular size and lipid staining characteristics, zone B appeared to be LDF lipoproteins. Zone C of NEY was diffuse and, also, stained with Sudan Black, a lipid stain. LV dissociated from PV would also have appeared in zone C. Three well defined bands appeared in zone D. Chang et al. (1970) stated that compounds in this area consisted of livetins. More specifically, γ-livetin appeared in this zone because its molecular weight was higher than β- or α-livetin, which appeared in zones E and F, respectively. The many livetin bands, which were present within each group, corresponded to similar bands in serum protein identified by Mok and Common (1964). The three faint bands appearing in zone G were PV. The high electrophoretic mobility, known multiple forms and level of occurrence concur with the literature. The light band below zone G was the bromphenol blue tracking dye.

Chromatographic fractions from NEY (Fig. 3) contained proteins which increased in electrophoretic mobility as the pH of the eluting buffer decreased. Fractions 6, 8 and 10 formed the first major group of chromatographic peaks. There was a gradual reduction in the quantity of immobile lipoprotein of zone B through peak 7. The only other bands visible in these gels were a group of three bands in zone C of peaks 2–3 which also appeared faintly in peaks 4–5. These peaks were high in lipid and the particle size was large. At this pH and ionic strength, the granules would not have been dissociated and livetins would have been attached

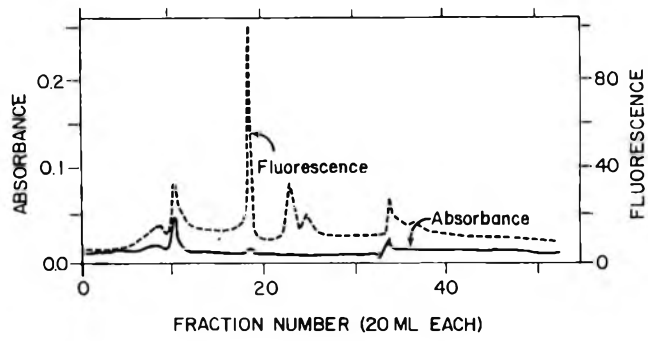

Fig. 4—Chromatogram of DEAE-cellulose separation of egg yolk granules.

to the exchange sites. Therefore, the first group of chromatographic peaks contained primarily lipoproteins from the LDF.

A broad band appeared in zone C in gels from peak 6 and continued through peaks 9–11. This component was not identified, but may have been related to the multicomponent LDF. Burley and Sleigh (1971) experimented with a variety of apoproteins with behaviors different from that of LDF which might be related to this unknown component.

A group of three bands appeared in zone D in the gel from peak 7. These bands had the same mobilities as γ-livetin in NEY. β-livetin appeared in zone E in gels from peaks 7–8, 8 and 9–11. Peak 8 had an additional band of unknown identity. Also, an unidentified band appeared in the gel from peaks 9–11 and other β-livetin bands disappeared.

LV and PV appeared in the gel of peaks 12–13. These components of granules were physically retarded at the chromatographic cellulose surface until conditions existed for dissociation, thus permitting elution from the column. LV appeared in zone C, overlapping into zone D, while PV appeared as two faint bands in zone G. The dense band at the top of zone G was α-livetin. The mobility of this band corresponded to that expected from the literature.

Peaks 14–15 and 16 had similar electrophoretic gel patterns. Because of their diffuse nature and slight immobility, the proteins in zone C appeared to have high molecular weights or composed of mildly denatured proteins eluted with the low pH buffer. Other protein bands were not visible.

Observations of electrophoretic patterns support a view that chromatographic separation may not alter the natural structure of the yolk proteins. For example, electrophoretic

bands from chromatographed fractions of NEY had the same appearance and mobility as corresponding electrophoretic bands from NEY which had not been chromatographed. The electrophoretic migration rates for PV and for α-, β- and γ-livetin compared well with literature values. Electrophoretic patterns of NEY resembled those obtained by Chang et al. (1970). The observed migration rates for LV and LDF were less than those based on non-restrictive moving boundary electrophoresis. The molecular sieving effect of polyacrylamide gel retards migration of large molecules to a greater extent than smaller molecules of equal charge.

Fluorescence Spectrum. Spectrographic evaluation of all chromatographic peaks, except the first, was prevented since the level of fluorescence was below the detection limits of the spectrophotofluorometer. Analysis of this peak from NEY (chromatographic fraction 6) indicated two fluorescent compounds. First, a pattern characteristic of protein with an excitation maximum at 288 nm and emission maximum at 350 nm was observed. A second pattern, different from protein, had a weak excitation maximum at 374 nm and emission maximum at 425 nm. This second compound was responsible for the fluorescence observed upon elution from the column. The identity of fluorescent compounds observed in fractions chromatographed on DEAE-cellulose was uncertain. Unsaturated fatty acids possess double bonds and therefore may have fluoresced weakly. Steroids may have produced fluorescence because of unsaturation and multiple ring structure. Vitamins, particularly riboflavin, fluoresce when in pure form. Fluorescent bands were not observed in electrophoretically separated chromatographic fractions nor were additional protein-staining bands apparent.

Strongly fluorescent chromatographic peaks appeared soon after addition of buffer. Lipid and phosphorus concentrations were highest in the same areas. Thus, the observed fluorescence was not due to protein, but was possibly due to certain lipids and vitamins closely associated with protein.

The simultaneous monitoring of UV absorbance and fluorescence of column effluent was useful in resolving shoulders on peaks and hidden sub-components within peaks. Many unaltered compounds fluoresce weakly, thus, a sensitive fluorometer should be used to detect fluorescent compounds in chromatographic fractions. Excitation and emission at narrow wavelength bands increases specificity but reduces the detection sensitivity.

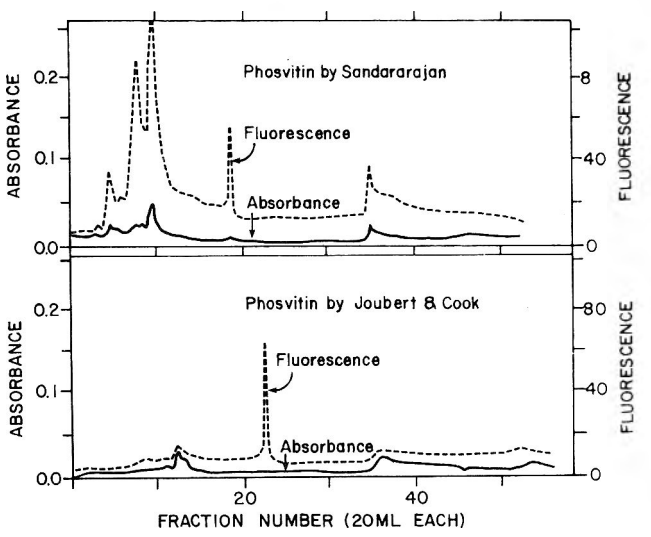

Fig. 5—Chromatogram of DEAE-cellulose separation of phosvitin prepared by two methods.

Lipids. Lipid content was determined of all fractions (20 ml each) obtained by DEAE separation of NEY' Ether-extractable EY lipids were used as standards. The pattern of lipid elution was diffuse due to the averaging effect of analyzing 20 ml aliquots. Lipid present in NEY was primarily eluted in two major areas in Figure 2: Peaks 1 through 5 contained 35% of the total separated lipid, peaks 6 through 9 contained 42% of the lipid from separated NEY. Remaining lipid was eluted slowly with a small increase in peak 15. Lipid data were verified using Sudan Black in conjunction with protein staining of electrophoretic gels. The magnitude of staining and appearance of bands containing lipids corresponded to areas of high lipid concentration in the chromatographic fractions.

Phosphorus. The phosphorus content of NEY fractions separated by DEAE-cellulose was determined. Phosphorus in buffers was replaced with citrates to reduce the high level of phosphorus. Substitution had little effect on the pH gradient or resulting chromatogram. Phosphorus was eluted in three areas. The first and second areas corresponded to lipoprotein elution. A third area, of greater magnitude, corresponded with phosvitin bands observed in peaks 12–13 of Figure 2.

Amino acids. NEY protein fractions selected for amino acid analyses are shaded in Figure 2. Fractions were collected and dialyzed exhaustively to remove the glycine buffer.

Area A contained the highest quantities of aspartic acid and leucine, and the lowest amounts of cystine, histidine and glutamic acid when compared to the other areas. Area B components differed in having the lowest quantities of arginine and glutamic acid, and the highest cystine content of any area analyzed. Compounds from area C were high in cystine, while tyrosine and serine concentrations were lower than the other three areas. Area D proteins contained smaller quantities of cystine and higher glutamic acid than did proteins from other areas. None of the different amino acid profiles indicated the presence of single pure proteins when compared to literature data.

Analysis of egg yolk fractions

The granules, PV, LV, livetins and LDF fractions were chromatographed on DEAE-cellulose using the same elution scheme as for NEY.

Granules. Figure 4 is a chromatogram of the NEY granules. Initially, a layer of the insoluble granules formed on the cellulose surface and did not dissociate in the low ionic strength buffer used for sample application. Most of the granules were later solubilized and components eluted between fractions 33 and 36 when the buffer had reached pH 3.0 and $\mu \simeq 0.175$. This resulted in dissociated single components. The peaks at fractions 10, 12 and 20 were composed of material more soluble than the granules. A small amount of LV and γ-livetin may have been soluble in the first buffer and, thus, detectable in these fractions. The soluble PV was eluted with the PV which was released after the granules dissociated. The first peak of the group was probably LV and the second peak PV.

Phosvitin. PV, prepared by two methods, was evaluated chromatographically. Fluorescence monitoring enhanced the detection of contaminating compounds present in these samples of PV.

The top chromatogram in Figure 5 is a separation of PV obtained commercially which was prepared by the method of Sandararajan et al. (1960). Fluorescent peaks were observed between fractions 5 and 20. This early eluted material could have been PV which was altered by adverse preparation procedures, or contaminates from the granules assoc-

iated with PV. Phosvitin was eluted in a small trailing peak in fraction 35. This behavior was expected due to the high content of serine and other charged groups which compose this protein.

Figure 5 also shows a chromatogram of PV prepared in this laboratory by the method of Joubert and Cook (1958). A series of small peaks were detected between fractions 5 and 15 in the same area of those observed from the commercial phosvitin. A sharp fluorescent peak was observed at fraction 22. A broad peak, between fractions 35 and 55, was again observed. This peak appeared to divide into two wide peaks at fraction 45. Connelly and Taborsky (1961) separated PV into at least two chromatographic fractions.

Lipovitellin. A chromatogram of LV obtained from granules (Fig. 6) had two peaks near fraction 10. The magnitude of these peaks was in agreement with the ratio of their occurrence as reported by Bernardi and Cook (1960). Thus, the first peak appeared to be β-lipovitellin and the second α-lipovitellin. LV is associated with PV in the granules and elutes poorly unless dissociated or separated. Fluorescent components were present in fractions 15 through 25. Fluorescent compounds were also observed in the same chromatographic fractions from PV and from granules. The presence of multiple peaks with similar appearance in chromatograms of PV and LV was expected due to methods of purification used. Recent literature indicate that previous preparations, thought to be pure, contain additional definable components (Garland and Powrie, 1978).

Livetins. The γ-livetin was more concentrated in the precipitate than in the subnatant which contained higher amounts of α- and β-livetins. The two fractions were chromatographed separately (Fig. 7). The components eluted between fractions 10 and 15 were γ-livetins. β-livetins eluted in fractions 15 through 20, while α-livetins appeared in fractions 30–35. This sequence of elution from the column and order of migration electrophoretically was the same. The large fluorescent peak at fraction 25 was not identified. The multi-peak patterns provide further evidence for the heterogeneity of the livetin proteins. More electrophoretic livetin components were observed than could be accounted for chromatographically. The ratio of combined major peak areas from both chromatograms of Figure 7 (2.5:5.1:1.5) was near that reported by Martin et al. (1964).

Low density fraction. The LDF chromatogram (Fig. 8) had many components. The major portion of material was eluted during the first one-third of the chromatogram. Augustyniak et al. (1964), Burley (1973), and others have reported that the structure of the LDF is a lipid core surrounded by a phospholipid and protein shell similar to a micelle. As such, the number of charged groups exposed may vary with change in the lipid composition of the micelle.

In conclusion, evidence presented in this paper shows that DEAE-cellulose ion-exchange chromatography is a useful technique for separation and characterization of egg yolk components. When supplemented with electrophoresis, fluorescence, and other analytical techniques, detailed evaluation of various processing and handling treatments is possible.

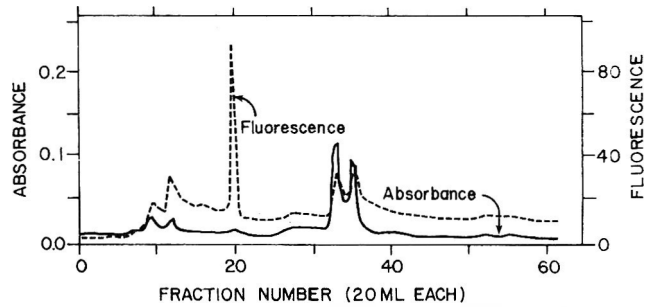

Fig. 6—Chromatogram of DEAE-cellulose separation of lipovitellin from granules.

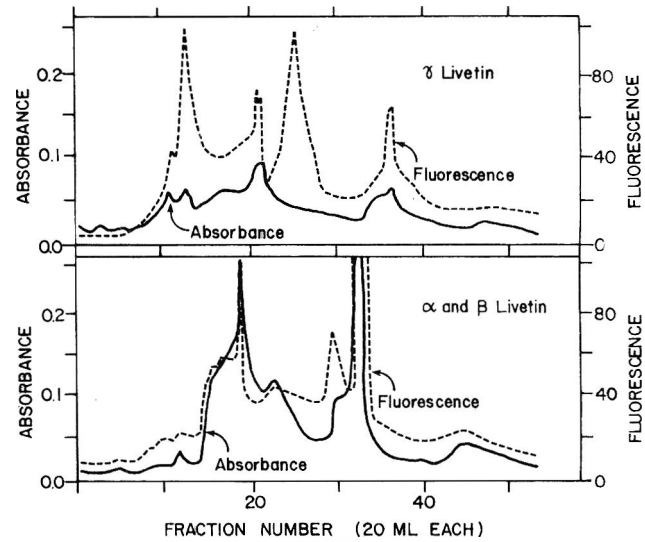

Fig. 7—Chromatograms of DEAE-cellulose separation of γ-livetin and of α- and β-livetin.

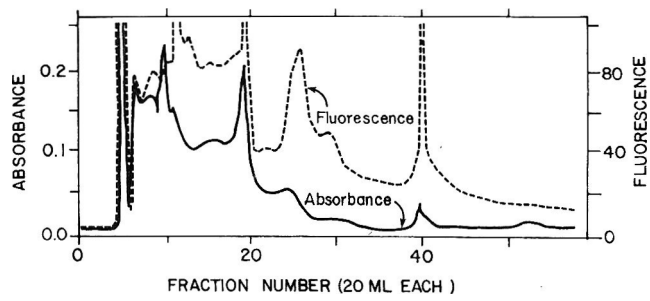

Fig. 8—Chromatogram of DEAE-cellulose separation of low density fraction.

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TEXTURAL DETERIORATION OF RED HAKE AND HADDOCK MUSCLE IN FROZEN STORAGE AS RELATED TO CHEMICAL PARAMETERS AND CHANGES IN THE MYOFIBRILLAR PROTEINS

T. A. GILL, R. A. KEITH and B. SMITH LALL

ABSTRACT

The present study involved the measurement of fish muscle texture by both objective and subjective means. Reliable methods for the evaluation of fish texture as well as interpretation of results are discussed. The study demonstrated that significant correlations between the methods could be achieved if carefully controlled conditions were maintained. The presence of dimethylamine in frozen hake (*Urophycis chuss*) appeared to be a good chemical indicator of toughness whereas the extractable protein nitrogen was not as reliable. Data suggest that although the enzymatic formation of formaldehyde was a major factor in the toughening of red hake, other factors probably contribute to the textural deterioration observed during cold storage. It is evident that haddock (*Melanogrammus aeglefinus*) toughens when stored at relatively high temperatures (−5°C) and like hake, experiences a loss of water-holding capacity although no formaldehyde accumulates in the tissues. The molecular basis of toughening in fish was examined by SDS gel electrophoresis. Apparently, the formaldehyde produced by the TMAO-ase enzyme system in red hake resulted in the covalent cross linking of troponin and myosin light chains, forming higher molecular weight aggregates. Changes at the molecular level were not detected by this method in haddock. Textural changes in this species are not as pronounced as those of hake and are most likely due to secondary bonds such as hydrogen or electrostatic.

INTRODUCTION

THE TEXTURAL deterioration of frozen stored fish fillets is usually accelerated at higher temperatures. It has been known for some time that toughening of fish muscle from the Gadidae family occurs more quickly and at lower storage temperatures than most other types of fish. This is believed to be due to the TMAO-ase enzyme which catalyzes the decomposition of trimethylamine oxide (TMAO) to form formaldehyde and dimethylamine (DMA), (Babbitt et al., 1972; Castell et al., 1973; Tokunaga, 1974; Dingle et al., 1977). It has been demonstrated that when formaldehyde is added to fresh cod muscle in the concentrations normally found in poor quality frozen stored fillets the salt-extractable protein nitrogen (EPN) content diminishes rapidly (Castell, 1971; Castell et al., 1973). It is believed by some that the toughening of the muscle of frozen-stored gadoid species is due to the ability of formaldehyde to induce intermolecular linkages between adjacent protein molecules (Castell et al., 1973; Dingle et al., 1977).

Since textural changes often occur during frozen storage of species devoid of the TMAO-ase enzyme system, it would seem reasonable to assume that a mechanism for toughening is also present which does not involve the three-dimensional cross-linking induced by formaldehyde. The present study was initiated in order to gain more knowledge of the mechanisms of the two types of toughening in a species of fish containing high levels of the TMAO-ase enzyme (red hake) and in haddock, which produces almost no formaldehyde. The study was designed to test the ability to predict subjective toughness scores from objective texture evaluation as well as the prediction of gadoid texture from the commonly used chemical parameters: DMA, HCHO and EPN. It was also anticipated that any molecular alterations to the myofibrillar proteins due to frozen storage could be detected by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS).

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EXPERIMENTAL

BOTH HADDOCK (*Melanogrammus aeglefinus*) and hake (*Urophycis chuss*) samples were caught by trawl set, gutted, iced and held 2–3 days to permit resolution of rigor. At this time the fish were filleted and the fillets vacuum sealed in plastic bags such that the thickness of each sample bag was approximately 3 cm. The fillets were frozen in a plate freezer at −40°C for 3–4 hr and then placed in low temperature storage cabinets at −5°C and −17°C. Samples of fish were removed periodically, thawed at room temperature and their quality assessed both objectively and subjectively.

Chemical evaluation of frozen stored fillets

The chemical analyses were included to assess the effect of the TMAO-ase system on deterioration of texture. Formaldehyde and DMA assays were performed in duplicate for each sampling period. The thawed fillets from each storage cabinet were sampled as follows. The fish were cut into 1 cm^3 pieces, mixed and 30-g samples homogenized in 60 ml 6% perchloric acid. Homogenization was accomplished with a Lourdes Multi-Mixer set at maximum speed for a period of 1.5 min. The sample cup was cooled with crushed ice to control overheating. The homogenates were centrifuged at about 2000 × G for 15 min at 4°C and aliquots of the supernatant liquid adjusted to pH 6.5–7.0 with 30% KOH. DMA and formaldehyde assays were carried out on two samples for each sampling period by the methods of Dyer and Mounsey (1945) and of Nash (1953), respectively.

Duplicate EPN extractions were carried out on 30-g samples of chopped fillet as described by Dyer et al. (1950, 1964) using 430 ml 0.6M NaCl–0.003M $NaHCO_3$. An appropriate amount of the extract was centrifuged for 20 min at 1000 × G and 4°C and protein concentration determined by the biuret procedure of Snow (1950).

Preparation of myofibrils for electrophoresis

Twenty grams frozen finely divided muscle were minced in 5 volumes ice cold 100 mM KCL–20 mM potassium phosphate buffer (pH 7.0)–1 mM EDTA–1 mM NaN_3 as suggested by Olson et al. (1976). The samples were homogenized in the Lourdes Multi-Mixer at high speed for 15 sec (0°C) using the small stainless steel cup. Homogenates were centrifuged at 600 × G for 15 min at 4°C. The pellet was formed in two layers: a highly compacted solid matrix on the bottom and a finely divided upper layer which was carefully removed with a Pasteur pipette. The finely divided sediment was washed and centrifuged six times in cold homogenization buffer as above, while the bottom layer from the first centrifugation was discarded. The myofibrillar material was then washed thrice in cold 100 mM NaCl to remove potassium ions. The white myofibrils were then dissolved by suspending the sediment from the last centrifugation in 2 volumes 8M urea–2.5% SDS–5 mM EDTA–1% 2-mercaptoethanol and 100 mM Tris/glycine (pH 8.80). The suspension was heated in a boiling water bath for 5 min to prevent proteolytic degradation (Weber et al., 1972), and dialyzed overnight against 25 mM Tris/HCl–0.1% 2-mercaptoethanol–0.2% SDS (pH 7.4). The reduced, dissociated myofibrillar proteins were then subjected to SDS – disc gel electrophoresis as described by Porzio and Pearson (1977).

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SDS polyacrylamide gel electrophoresis

Electrophoresis was carried out in a Model GE-4 gel electrophoresis apparatus (Pharamacia Fine Chemicals) at 1 mA per tube for 15 min and then at 2 mA per tube for the duration of the run. The molecular weight markers used in preparation of standard curves were as follows: ribonuclease, 13,700 (Weber and Osborn, 1969); bovine serum albumin (BSA) monomer, 68,000; BSA dimer, 136,000; BSA trimer, 204,000; BSA tetramer, 272,000. Gels were stained overnight in Coomassie brilliant blue (Weber and Osborn, 1969) and destained in a Model GD-4 gel destainer (Pharamacia) with 5% methanol–7.5% acetic acid. Gels were scanned in a Beckman Model R-112 scanning densitometer.

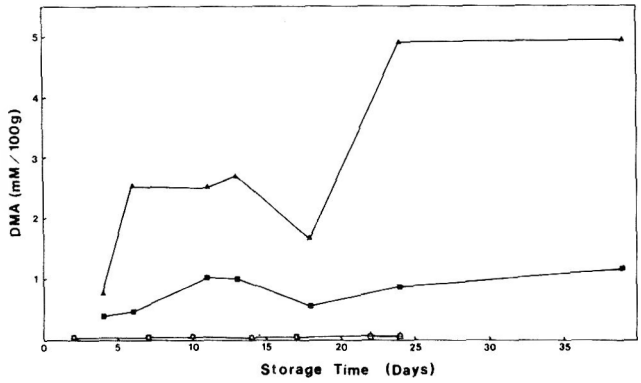

Fig. 1–DMA content of frozen stored hake and haddock fillets (▲ hake at −5°C, ■ hake at −17°C, Δ haddock at −5°C, □ haddock at −17°C).

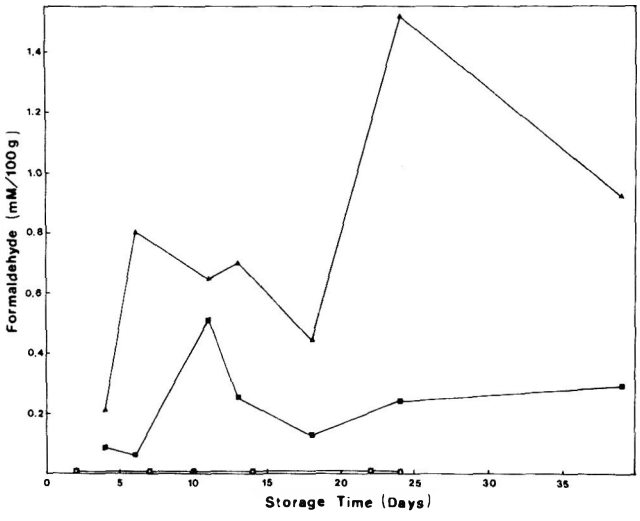

Fig. 2–Formaldehyde content of frozen stored hake and haddock fillets (▲ hake at −5°C, ■ hake at −17°C, Δ haddock at −5°C, □ haddock at −17°C).

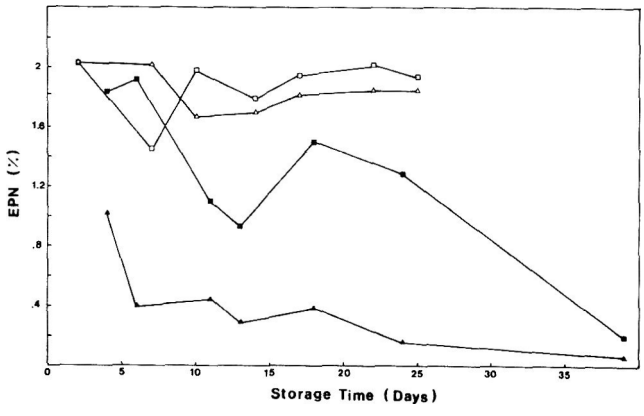

Fig. 3–Extractable protein nitrogen of frozen stored hake and haddock fillets (▲ hake at −5°C, ■ hake at −17°C, Δ haddock at −5°C, □ haddock at −17°C).

Objective evaluation of texture

Objective texture measurements were performed with the Instron Universal Testing Machine Model TM. Several geometries were tested for the evaluation of fish texture at the Engineering Research Service, Research Branch, Agriculture Canada in Ottawa and the two most useful designs were found to be the 10 cm^2 Ottawa Texture Measuring wire shear cell (Voisey, 1972) and a Kramer Shear-Compression Cell (66 mm × 66 mm) used at diminshed capacity (66 mm × 28 mm, 4 blades) by insertion of two aluminum plates as suggested by Bilinski et al. (1977). The latter geometry was chosen and the 4-bladed Kramer cell further modified by the replacement of the aluminum cell front with a plexiglass window which was fabricated at a local machine shop. This second modification was suggested by Voisey (1977). Both haddock and hake fillets were tested in the raw state as well as cooked. Samples were cooked by baking in a 230°C oven for 12 min. After cooking, the fillets were cut into approximately 1 cm^3 pieces and 50-g samples placed in sealed plastic containers and cooled to 22 ± 2°C. The Instron Model TM was operated with a cross head speed of 10 cm min^{-1} while a recorder speed of 20 cm min^{-1} was found convenient. The return gauge was set at 8.35 cm so that the compression cycle was terminated just after the blades emerged from the bottom of the test cell. Peak heights and slopes were tabulated usually for 8–10 samples of each type of fish, for each treatment temperature. The events occurring during cycles of compression and shear were recorded photographically to aid in the interpretation of the force-deformation traces.

Subjective evaluation of texture

Panelists were trained by tasting cod fillets which had been soaked in aqueous solutions of formaldehyde ranging from 0–1800 ppm. Each panelist was asked to rank the cooked sample of formaldehyde-treated fish relative to a standard. The standards were prepared by rehydration of unflavored pieces of texturized soy protein (Mira-Tex TVP, A.E. Staley Mfg. Co., Decatur, IL) in 70°C water for 10 min. The standards were anchored with a vertical stroke, 7.5 cm from the left end of an unstructured 10.0 cm horizontal line. Each sample was ranked relative to the standard by placing a vertical stroke on the unstructured line, labelled only by the terms 'tender' and 'tough' on left and right-hand ends respectively.

Experimental samples of stored hake and haddock were cooked as for the objective evaluation and presented as above. In most cases, each panel session involved ranking the toughness of only 2 samples marked with 3 digit random numbers. Every effort was made to present samples and standards at the same temperature. The panel consisted of eight regular judges plus three people who attended most but not all of the sessions. Panelists were given water to clear the palate and fruit juice as a reward at the end of each session.

Correlation of subjective and objective data

Stanley et al. (1972), pointed out that nonparametric statistics must be used for the type of data derived from such an experiment.

Raw data from the texture panels were calculated as follows. The distance between the left (most tender) end of the unstructured line and the vertical mark placed on the line by the panelist for each sample was interpreted as degree of toughness. The average degree of toughness of a sample was calculated as the mean for all of the panelists able to attend every session. The averages for the samples were then assigned ranks, a rank of 1 representing most tender. Means for objective data, peak force and peak slope were ranked in a similar manner and the Spearman rank correlation coefficient (r_s) calculated for each parameter.

RESULTS & DISCUSSION

THE DMA, formaldehyde and EPN levels of fillets which were stored at the two temperatures are illustrated with relation to time in Figures 1, 2 and 3, respectively. The species differences are perhaps the most notable features of all three figures. Haddock was chosen for the study because

of its relatively low level of TMAO-ase activity and so that the effects of frozen storage alone could be compared as a control against red hake.

Hake stored at −5°C showed a rapid increase in DMA content after 4 days, whereas fillets stored at −17°C increased very little in a period of 39 days. For some reason, hake samples demonstrated an initial increase in both DMA and formaldehyde, followed by an apparent decrease which reached a minimum after 18 days of frozen storage although the decrease was not significant ($P < 0.01$) when compared to values obtained on days 6, 11 and 13. It may be that the production of end products was minimal between the sixth and eighteenth days. The initial rise observed between the fourth and sixth days could have been due to cell disruption by ice crystals whereas resistance to diffusion could be one explanation for the apparent leveling off of DMA production observed between the sixth and eighteenth day. There is some evidence that very little enzyme activity is found in unfrozen hake carcases stored on ice at 0°C (J.R. Dingle, personal communication). It may be that this type of deterioration is enhanced to a large degree by ice recrystallization rather than ice crystal formation. It is not unlikely that the rate of recrystallization and therefore the ultimate size of the crystals could be related to the temperature of frozen storage.

It is important to note that the data obtained for Figures 1, 2 and 3 were mean values derived from two extractions. Sampling problems were most evident with pieces of hake muscle containing high levels of end products. The major site of activity was located in the red muscle particularly subsutaneously along the lateral line. This observation has also been confirmed in studies of Pacific hake (Babbitt et al., 1972) and Alaska pollock (Tokunaga, 1974). The toughening, presumably due to the evolution of formaldehyde, progressed from the lateral line toward the peritoneum. Although the data would suggest that more samples are required when dealing with muscle containing high levels of TMAO-ase, there was a highly significant correlation between the means of DMA and formaldehyde levels in stored hake samples ($r = 0.936$, $n = 12$).

It has been suggested that the action of the TMAO-ase system in several species of the family Gadidae is responsible for rapid textural deterioration and it is also believed that equimolar quantities of DMA and formaldehyde are formed from TMAO (Dingle et al., 1977). Since formaldehyde binds to protein in a complex manner (Galembeck et al., 1977) the molar quantities of recovered formaldehyde are considerably less than those of DMA. In order to investigate the hypothesis of formaldehyde-induced toughening, both objective and subjective methods of texture evaluation were carried out.

Before an objective test could be used to determine or predict perceived toughness, it was necessary to establish a relationship between Instron and panel scores. Typical force-deformation traces for "tough" and "tender" fish samples (as determined by 8 panelists) are illustrated in Figure 4. The upper trace was derived from a sample of hake stored for 24 days at −5°C whereas the lower curve was obtained from a test of 4 day hake which had been stored at −5°C. The corresponding mean texture panel scores were 11 and 1 for the upper and lower fish samples respectively. These scores were ranked on an 11-point scale where 1 represented most tender and 11 represented tough. In general, the samples ranked most tender by panelists could be differentiated from tougher samples by examination of the Instron force-deformation curves similar to those in Figure 4. The tougher samples yielded both higher peak forces and steeper slopes in the region of compression without shear (28–34 sec). This portion of the trace represents a time interval in which there was a constant change

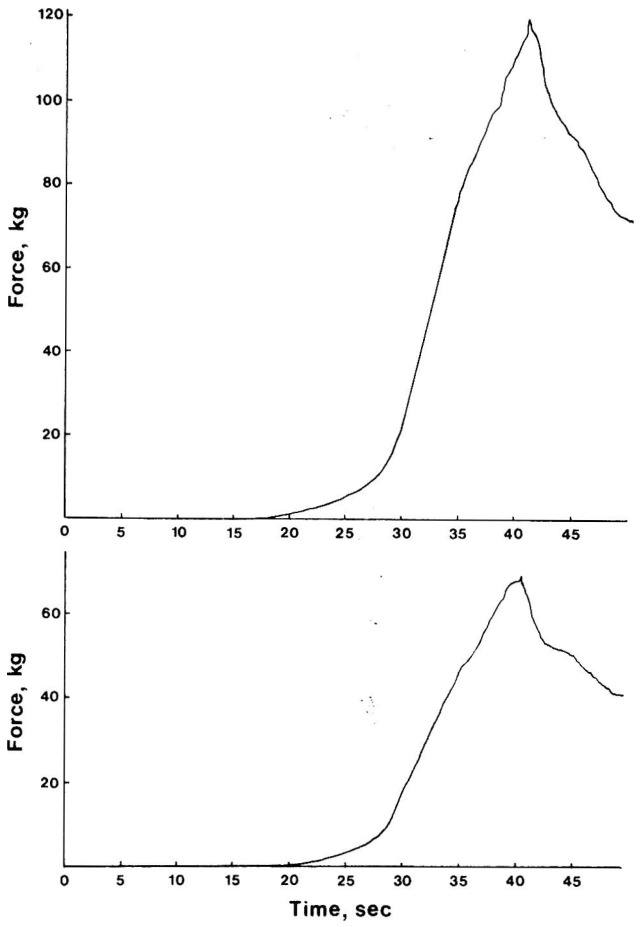

Fig. 4—Typical Instron force deformation curves for 'tough' (upper) and 'tender' (lower) samples of cooked hake muscle. Crosshead speed was 10 cm min^{-1} and tests were run on 50-g samples using a modified 4-bladed Kramer cell.

in force per unit crosshead movement and could perhaps be regarded as a measure of 'chewiness' since it monitored resistance to compression alone. The point at which maximum peak forces were achieved represented a region in which the blades passed through the floor of the cell. At this point, compression, shear and extrusion appeared to occur simultaneously, forcing the sample to move in several directions at the same time. Since this was the point at which most of the sample failed, it could be termed 'cohesiveness'. The sensations of 'chewiness' and 'cohesiveness' as defined here were not evaluated separately by the panelists, however both parameters correlated significantly with subjective 'toughness' scores. The Spearman rank correlation coefficients (r_s) were calculated as outlined by Siegel (1956) for panelists evaluating all samples of hake and haddock. Since relatively large sample sizes (50g) were used for the texture tests, less error due to sampling was encountered as compared to chemical analyses. The average coefficients of variation of the peak heights were 9.22% and 7.64% for cooked haddock and hake samples respectively. An exception to this trend was observed with the hake which had been stored at −17°C for 11 days. At the time of sampling, it was noted that the fillets used for Instron testing contained larger quantities of red muscle, than those used for panel evaluation. Since Instron readings for this sampling period were much higher than those for the same muscle sampled before and after the eleventh day, correlation coefficients were calculated both with and without the data derived from the 11-day (−17°C) samples. The correlation coefficients are listed in Table 1. All coefficients were

Table 1—Correlations of texture panel results with maximum peak force and with initial peak slope of Instron force-deformation traces

	Peak height	Peak slope
All data used (14 samples)	0.576	0.699**
All data except 11 day hake (−17°C) (13 samples)	0.708*	0.879**

* $P < 0.05$
** $P < 0.01$

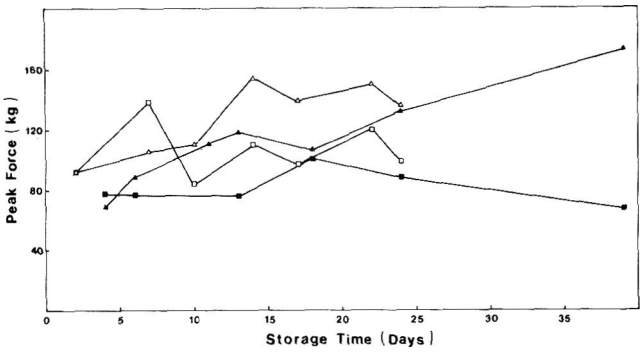

Fig. 5—Peak Instron force measured for samples of frozen stored hake and haddock fillets (▲ hake at −5°C, ■ hake at −17°C, Δ haddock at −5°C, □ haddock at −17°C).

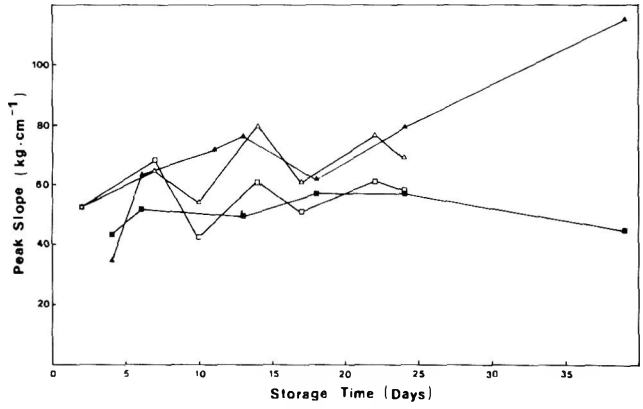

Fig. 6—Peak slope for frozen stored hake and haddock fillets (▲ hake at −5°C, ■ hake at −17°C, Δ haddock at −5°C, □ haddock at −17°C).

significant; however, it would appear that peak slope was a slightly more reliable indicator of subjective toughness values in cooked fillets than peak height. Scores were not included if panelists were not present at all of the sessions since the mean panel reaction to any one sample was strongly influenced by the use of data from 'spare' panelists.

The Instron peak heights and slopes for cooked haddock and hake fillets are presented in Figures 5 and 6 as functions of time of frozen storage at either −5°C or −17°C. Each data point represents a mean of 6–10 determinations. Both Figures 5 and 6 illustrate that the toughness ratings for cooked haddock were initally higher than the corresponding values for hake although the relative increases in toughness were greater for hake during the storage period. Also, as would be expected, the samples stored at −5°C toughened more quickly than those stored at −17°C.

Although several reports have linked formaldehyde formation with toughening in gadoids (Babbit et al., 1972; Castell et al., 1973; Tokunaga, 1974; Dingle et al., 1977) little statistical evidence for such a relationship nor reliable estimation of degree of toughening have been presented. It has generally been assumed that EPN could be directly related to both formaldehyde content and toughness of frozen stored gadoid fillets. The correlation matrix illustrated in Table 2 shows the simple correlation coefficients for the various parameters measured on stored hake. It was apparent that DMA was by far the best chemical index for textural deterioration in both raw and cooked hake fillets. EPN was the poorest chemical index of ultimate cooked texture although it was somewhat better indicator of the toughness observed in raw hake muscle. It should be remembered however, that it would be difficult to directly relate the objective data for raw fish with the subjective responses of a panel. It is not possible to explain all of the toughening in the hake samples by the 3 chemical indicators used here. Stepwise multiple regression of the three independent variables, DMA, HCHO and EPN, indicated that DMA and HCHO were useful for hake texture prediction but EPN was not, ($p < 0.05$). It is true that additions of HCHO to fresh fish muscle can bring about marked decreases in extractable protein (Castell et al., 1973) and that toughening is evident at the surface of tissues placed in dilute formaldehyde solutions (unpublished observation). The present data tend to suggest a more complex mechanism of toughening. Decreases in EPN need not necessarily correspond directly with the rate of formation of DMA and HCHO, which are relatively good predictors of cooked textural quality in red hake. Another factor which has been implicated in protein denaturation in both gadoids and non-gadoids during frozen storage is the production of free fatty acids by hydrolysis of muscle lipid. It has been hypothe-

Table 2—Correlation matrix of chemical data and objective indices of toughness in raw and cooked hake fillets

				Peak height		Peak slope	
	DMA	HCHO	EPN	Raw	Cooked	Raw	Cooked
DMA	1	0.936**	−0.768**	0.797**	0.866**	0.860**	0.874**
HCHO		1	−0.728**	0.670*	0.696*	0.670*	0.688*
EPN			1	−0.744**	−0.551	−0.680*	−0.585*
Peak height (raw)				1	0.764**	0.910**	0.747**
Peak height (Cooked)					1	0.899**	0.969**
Peak slope (Raw)						1	0.896**
Peak slope (Cooked)							1

$r^2 = 0.963$**

* $P < 0.05$
** $P < 0.01$

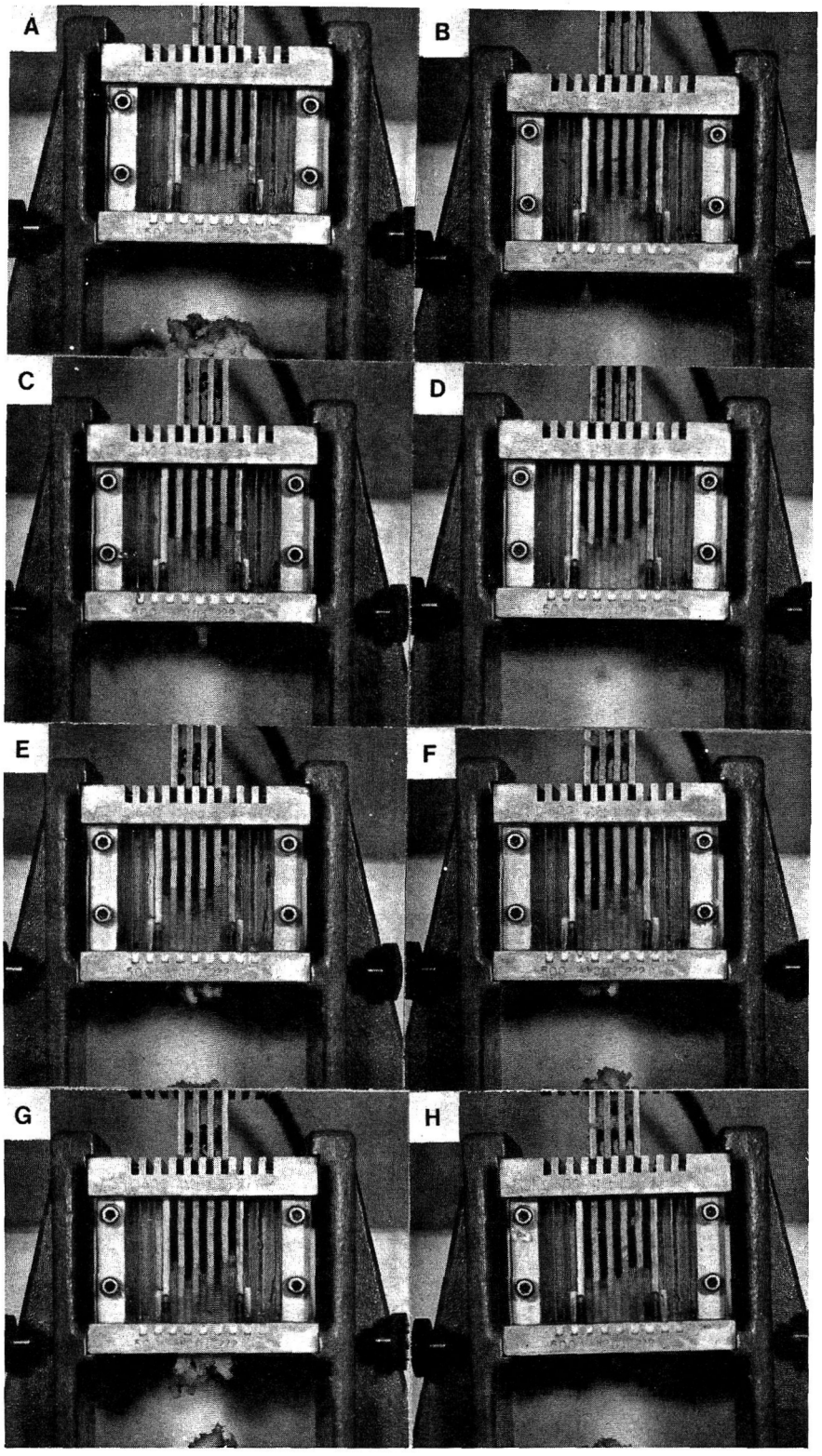

Fig. 7—Comparison of compression and shear of 'tough' and 'tender' samples of cooked hake fillets. A, C, E and G illustrate tests of tender samples which had been stored at −17°C for 39 days. B, D, F and H illustrate tests of tough samples which had been stored at −5°C for 39 days. The photographs show the various stages of: compression (A, B); compression and shear (C, D); sample failure (E, F); and extrusion (G, H). The apparatus illustrated is a Universal Testing Machine equipped with a modified Kramer Shear-Compression Cell. Crosshead speed was 10 cm · min^{-1}.

sized that these free fatty acids react with the proteins to form insoluble complexes; however, cooking alone results in more than a 95% decrease in EPN without producing the same degree of toughness found in fillets which have been stored improperly in the frozen state (Castell et al., 1973). The influence of lipid hydrolysis on toughening remains debatable.

A correlation matrix was prepared from haddock chemical data and the objective texture evaluations of the cooked fillets. Although significant correlations were found between peak height and DMA, HCHO and EPN levels, stepwise multiple regression indicated that none of these parameters could be used for prediction of haddock texture, ($p < 0.05$). It would appear also that EPN in the haddock samples was not related to the formaldehyde content of the muscle although marginally correlated to the DMA content. Certainly the lack of the TMAO-ase system in haddock would readily explain the lack of agreement between the three chemical indicators and objective texture measurements.

In an attempt to visualize qualitative differences as clues to the mechanism of toughening, objective texture tests of

the fillets were recorded photographically on the 39th day of storage. The force-deformation curves (Fig. 4) could be clearly divided into 5 distinct regions. Photographs recorded for hake stored at $-5^{\circ}C$ and $-17^{\circ}C$ are illustrated in Figures 7A and B, respectively. The first stage was observed from 20–28 sec of crosshead movement (Fig. 4). The force-deformation curve showed a rapid increase in slope as the blades began to compress the sample. There was little difference observed between treatments at this point. Stage 2 refers to the region of constant compression without shear, previously used to define the term 'chewiness'. Figures 7A and B illustrate that at this stage, compression resulted in weeping of the $-5^{\circ}C$ samples (arrows) which were sponge-like when squeezed between the fingers. Loss of water holding capacity was evident. The inability to hold water was also indicated by considerably higher water loss on cooking (31.8%) for the hake stored at $-5^{\circ}C$, compared with that of the $-17^{\circ}C$ sample (15.7%). It should be noted that although water-holding capacity was affected, the difference between EPN values was not large: 0.19% and 0.064% for the $-17^{\circ}C$ and $-5^{\circ}C$ samples respectively. The third stage (34–40 sec) was defined by the changing slope, a region in which both compression and shear resulted in water loss in both samples (Fig. 7C and D). It was at this point that the $-17^{\circ}C$ sample (C) began to extrude through the cell floor. The fourth stage was typified by maximum force and was previously used to define the term "cohesiveness." Movement of the sample within the cell was complex and represented maximum sample disruption. The sample stored at $-17^{\circ}C$ (Fig. 7E) extruded easily through the bottom of the cell, the extruded material conformed to the shape of the slots and emerged as strings of minced fish. Figure 7F demonstrates that the $-5^{\circ}C$ sample had undergone extensive compression but maintained a relatively high resistance to extrusion and shear; the material emerging from the test cell did not conform to the slots in the floor. The fifth stage was identified with a decrease in recorded force. Differences between samples stored at the two temperatures were much the same as those noted in stage 4.

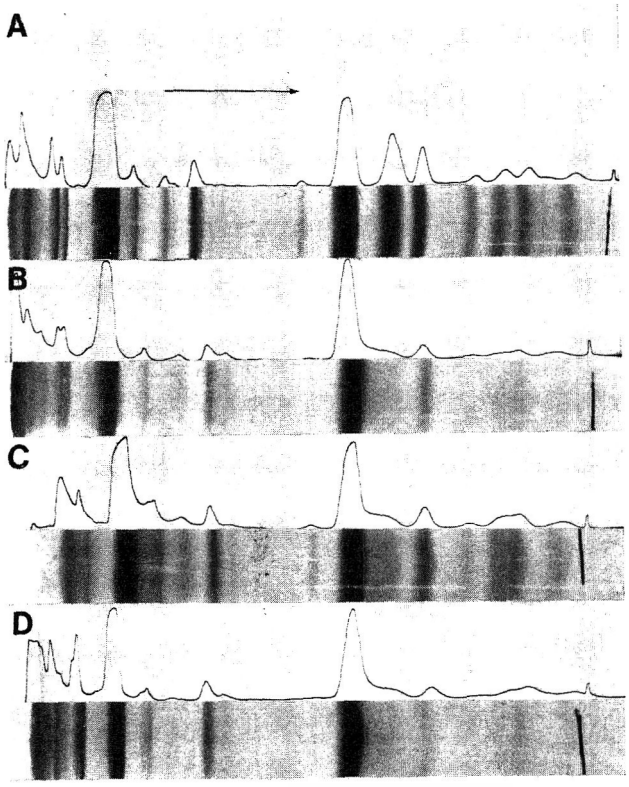

Fig. 8–Densitometric scans of SDS electrophoretic gels: A–fresh hake myofibrils, B–hake myofibrils prepared from HCHO-soaked muscle, C–hake myofibrils prepared from muscle stored at $-17^{\circ}C$ for 35 days, D–hake myofibrils stored at $-5^{\circ}C$ for 35 days. Arrow indicates direction of electrophoretic migration.

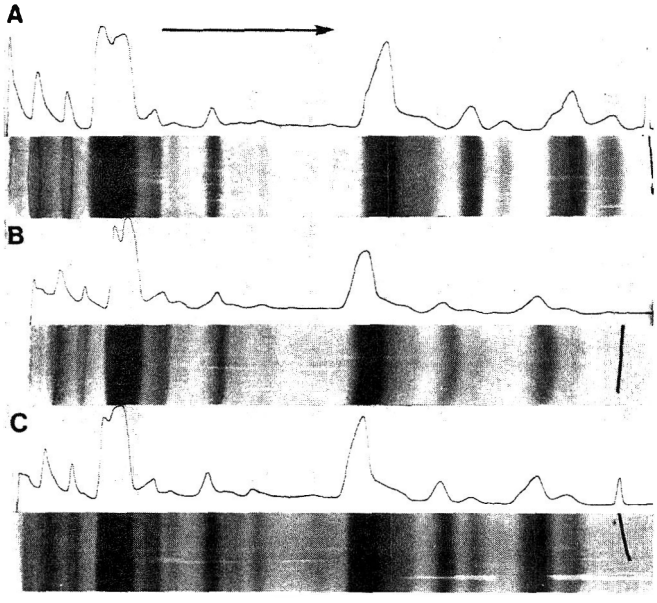

Fig. 9–Densitometric scans of SDS electrophoretic gels: A–fresh haddock myofibrils, B–haddock myofibrils prepared from muscle stored at $-17^{\circ}C$ for 37 days, C–haddock myofibrils prepared from muscle stored at $-5^{\circ}C$ for 37 days. Arrow indicates direction of electrophoretic migration.

It is evident that toughening of hake muscle during frozen storage coincided with two separate events often related to protein denaturation: loss of salt-soluble protein (EPN) and water-holding capacity (WHC). It would also appear that these parameters were to a certain extent independent of one another since large differences in WHC were monitored at a time when corresponding large differences in EPN were not observed. A loss in water-holding capacity was also observed in haddock samples subjected to high temperature ($-5^{\circ}C$) storage. For example, 17 day haddock fillets which had been stored at $-17^{\circ}C$ and $-5^{\circ}C$ lost 7.97% and 14.6% (w/w) of their weight in thaw drip respectively. An explanation for this phenomenon was beyond the scope of the present study, but future work will attempt to separate the effects of EPN and WHC on the ultimate texture of the product.

SDS electrophoresis of the hake and haddock myofibrillar proteins was carried out to gain some knowledge of the molecular events resulting in toughening during frozen storage. This technique has found wide application in the detection of covalent interaction between food proteins. The method of Porzio and Pearson (1977) is particularly useful for muscle components since it yields excellent resolution of the high molecular weight subunits. The deterioration of the native structural proteins of hake is illustrated in Figure 8. The electrophoretic patterns of freshly extracted hake myofibrils (Fig. 8A) are compared with those of hake myofibrils that had received an overnight soak in 1200 ppm formaldehyde, 35 days storage at $-17^{\circ}C$ and at $-5^{\circ}C$ in Figures 8B, C and D respectively. The most obvious changes in gels B, C and D occurred in the lower molecular weight proteins. Decreases in components with apparent molecular weights of 32,000, 27,000, 20,000, 17,000 and 15,000 were evident and the relative abundances of the higher molecular weight components increased. These changes came

about under conditions considered to be inhibitory to proteolysis (0.02% NaN_3 was used in all steps of myofibril preparation and all freshly prepared myofibrils immediately heated to 100°C in the presence of 8M urea, SDS and 2-mercaptoethanol). The similarity between myofibrillar patterns from muscle which was soaked in aqueous formaldehyde and that stored at −5°C for 35 days is striking. The muscle which had been stored at −17°C yielded myofibrillar banding patterns somewhat intermediate between the fresh controls and the muscle stored at −5°C. These observations are further evidence that the changes associated with toughening in frozen stored red hake muscle are chiefly due to the covalent cross linking of the structural proteins by formaldehyde. The proteins involved have been tentatively identified as troponin and the myosin light chains. Figures 9A, B and C illustrate myofibrillar subunit patterns of fresh haddock, and haddock fillets which had been stored at −17°C and −5°C respectively. The patterns show little change and suggest that although toughening and loss of water-holding capacity occur as a result of "high temperature" frozen storage, the changes are most likely due to weak bond interaction (i.e. hydrogen or electrostatic forces) or disulfide links which were servered druing preparation for electrophoresis. Future work will involve the investigation of the nature of the toughening of species devoid of the TMAO-ase enzyme system.

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SODIUM NITRITE AND SORBIC ACID EFFECTS ON *Clostridium botulinum* TOXIN FORMATION IN CHICKEN FRANKFURTER-TYPE EMULSIONS

J. N. SOFOS, F. F. BUSTA, K. BHOTHIPAKSA and C. E. ALLEN

ABSTRACT

C. botulinum growth and toxin production and residual nitrite depletion were studied in a mechanically deboned chicken meat (MDCM) frankfurter-type product, during 27°C temperature abuse. A series of three trials were conducted in order to determine the effects of sodium nitrite and sorbic acid on the above parameters. Low nitrite concentrations (20 and 40 μg/g), did not influence *C. botulinum* growth and toxin production. The addition of sorbic acid (0.2%) to these nitrite levels resulted in a significant extention of the time necessary for toxin to develop. The 0.1% sorbic acid level was ineffective. Nitrite concentrations of 156 μg/g doubled the time necessary for botulinal toxin production, as did 0.2% sorbic acid alone. The magnitude of toxin production delay was increased five fold when 156 μg/g nitrite and 0.2% sorbic acid were combined. Nitrite depletion was rapid during processing and 27°C incubation. Low residual nitrite concentrations were present for a longer time in nitrite-sorbic acid combination treatments, indicating that the presence of sorbic acid may delay depletion of residual nitrite. Gas production and spoilage of the product followed the same rate of development as toxin.

INTRODUCTION

THE USE OF NITRITE in the curing of meat is presently under review because of the implication of nitrite as a precursor of carcinogenic nitrosamines in cured meat products. The Expert Panel on Nitrites, Nitrates and Nitrosamines in its final report to the Secretary of Agriculture is recommending lowering of the nitrite levels permitted, or entirely removing it from some cured meat products (Anon., 1978).

In the event that the ingoing nitrite levels are lowered or its use is completely discontinued, some nitrite supplements or replacers, or some processing and handling changes, will be necessary for the cured meat products to assure their botulinal safety.

Sorbic acid and its alkaline salts are being used as spoilage preservatives (yeast and mold inhibitors) in various commercially prepared foods (Deuel et al., 1954; Costilow et al., 1957; Perry et al., 1964). Recently, some reports dealing with the use of sorbic acid or its potassium salt in preventing botulinal toxicity in cured meats have been published or circulated (Tanaka et al., 1977; Ivey and Robach, 1978; Ivey et al., 1978; Robach et al., 1978). However, studies of this type are few and limited. The lack of extensive research or information on the use of sorbic acid as a botulinal inhibitor might have been the result of earlier work (Emard and Vaughn, 1952; York and Vaughn, 1954; Hansen and Appleman, 1955) recommending sorbate as a selective agent for the isolation of certain clostridia.

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Comminuted, cured poultry products have been experiencing increased popularity and consumption in recent years. A request for an Interim Food Additive Regulation for the use of nitrite in poultry products has been filed with the Food and Drug Administration (FDA), including a report of a study on the effect of nitrite on botulinal toxicity and other characteristics of cured poultry products (Christiansen et al., 1977).

The controversy over the use of nitrite in cured meat products, the indications of a possible sorbic acid effect in inhibiting botulinal toxin production, and the increased consumption of cured poultry products, were the reasons for conducting the following research.

The objective of the study was to determine the effect of nitrite and sorbic acid, used individually or in combination, on *C. botulinum* toxin production in an emulsified, cured poultry product abused at elevated temperatures.

MATERIALS & METHODS

THE WORK was performed in a biohazard area of the Andrew Boss Laboratory-Meat Science of the University of Minnesota.

Three trials (A, B and C) were conducted based on varying sodium nitrite (0, 20, 40, 156 μg/g) and sorbic acid (0, 0.1, 0.2%, w/w) concentrations alone and in assorted combinations (Fig. 1 and 2).

Spore inoculum

The *C. botulinum* spore inoculum used was a composite of equal numbers of five type A (36A, 52A, 62A, 77A, and 12885A) and five type B (ATCC 7949, 41B, 53B, 213B, and Lamanna B) strains. The original cultures of each strain were provided as frozen spore suspensions by the Swift and Co. Research Center (Oak Brook, IL). Sporulation of the strains and preparation of the spore crops were performed according to the method described by Christiansen et al. (1973). Spore counts were determined by a five-tube Most Probable Number (MPN) procedure in modified peptone colloid medium (Greenberg et al., 1966). The same MPN procedure was used to determine spore levels in the cooked emulsions after a heat shock at 80°C for 15 min. The composite spore suspension contained a calculated equal number of spores of each strain and it was heat-shocked (80°C, 15 min) before inoculation of the meat.

Ingredients

Mechanically deboned chicken meat (MDCM) provided by Horace W. Longacre, Inc. (Franconia, PA) was used to form all the emulsions. The frozen MDCM was received and kept frozen until used (within 10–40 days). Thawing of MDCM, before each trial, took place in a 4°C cold room for a period of 36–48 hr.

Proximate composition of MDCM, as determined by standard AOAC methods was: protein, 12.3%; fat, 19.2%; and, moisture, 66.8%.

Microbiological counts of raw MDCM were: aerobic total plate count (APC), 1.07×10^6 CFU/g; heat-shocked (80°C, 15 min) APC, <10 CFU/g; and, heat-shocked (80°C, 15 min) anaerobic MPN, <2 spores/g.

Trials A and B were based on different nitrite and sorbic acid concentrations and formulated with the same lot of MDCM. Trial C was a replication of trial B formulated with a second lot of MDCM. All treatments of all trials were formulated with the following common ingredients and concentrations: MDCM, 93.63%; salt, 2.5%; corn syrup solids (Star-Dri, 35C, A.E. Staley Manufacturing Co., Decatur, IL), 2.0%; dextrose (Mallinckrodt, Inc., St. Louis, MO), 1.0%, white pepper, 0.25%; nutmeg, 0.07%; ascorbic acid (Mallin-

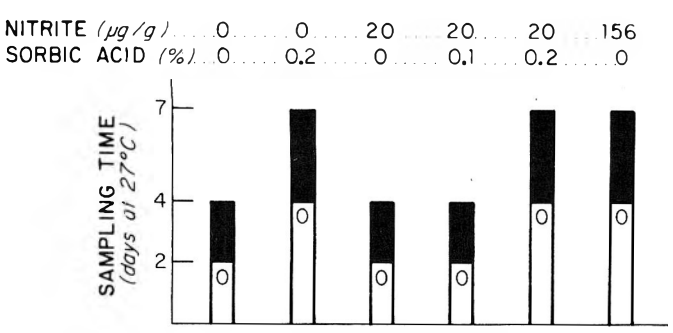

Fig. 1—Effect of sodium nitrite and/or sorbic acid on toxin production in Clostridium botulinum inoculated chicken frankfurter-type emulsions during temperature abuse (Trial A). Numbers show toxic samples out of five tested, and dark bars indicate that all samples (5) tested were toxic.

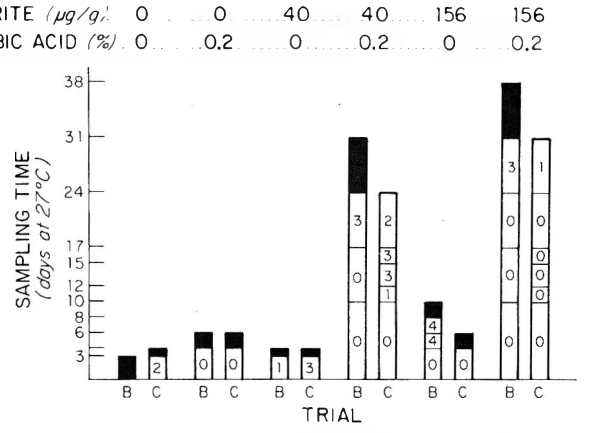

Fig. 2—Effect of sodium nitrite and/or sorbic acid on toxin production in Clostridium botulinum inoculated chicken frankfurter-type emulsions during temperature abuse (Trials B and C). Numbers show toxic samples out of five tested, and dark bars indicate that all samples (5) tested were toxic.

ckrodt, Inc., St. Louis, MO), 0.05%; and liquid smoke (Charoil, Red Arrow Products Co., Manitowoc, WI), 0.5%.

Based on the total batch weight (1200–2000g), treatment combinations containing sodium nitrite (Mallinckrodt, Inc., St. Louis, MO) and sorbic acid (Monsanto Co., St. Louis, MO) were formulated at the levels shown in Figures 1 and 2.

A total of 10% water was added to each treatment in order to incorporate the spore inoculum and nitrite. All treatments were inoculated with the same volume of the composite spore suspension. The average anaerobic MPN spore counts (*C. botulinum*) for the cooked emulsions were: 608, 1054, 111 spores/g for trials A, B, and C, respectively.

Product formation and processing

A Hobart KitchenAid mixer was used to mix the ingredients in the following sequence: MDCM, spices, sugars, ascorbic acid, liquid smoke; heat-shocked (80°C, 15 min) spore inoculum; sodium nitrite and/or sorbic acid as applicable (Fig. 1 and 2); and, salt. The salt was added last to avoid MDCM salt-soluble protein extraction and stickiness of the mixture, and thus to secure good mixing of the spore suspension and other ingredients with the MDCM. The total mixing time was 5 min.

After mixing, emulsification took place in a Waring Blendor-type emulsifier developed by Johnson et al. (1977) and modified for microbiological work. The emulsions were directly extruded into test tubes (18 × 150 mm) through the side-bottom extrusion tube of the emulsifier. Visual examination of the cooked emulsions indicated acceptable emulsion stability.

All samples (tubes) of all treatments of each trial were heat processed at the same time in a forced air oven immediately after emulsification. Oven and internal product temperatures were monitored with thermocouples. The oven temperature was gradually increased to 80°C and the products were cooked to an internal final temperature of 68.5°C over a 2-hr period. After cooking the tubes were cooled by immersing in cold tap water, capped with vaspar, and incubated at 27°C.

Sampling of the treatments

All tubes of each treatment were checked for gas formation at specified time intervals (Table 1). Six tubes for each treatment were

Table 1—Gas production in Clostridium botulinum inoculated chicken frankfurter-type emulsions containing sodium nitrite and/or sorbic acid during temperature abuse[a]

Nitrite (μg/g)	Sorbic acid (%)	Trial	Days at 27°C												
			2	3	4	5	7	8	10	12	15	17	24	31	38
0	0	A	11.9	–[b]	100.0*										
		B	–	100.0*											
		C	–	100.0*											
20	0	A	4.4	–	100.0*										
40	0	B	–	62.3*	100.0										
		C	–	100.0*											
156	0	A	0	–	60.5	–	100.0*								
		B	–	23.0	54.1	100.0*									
		C	–	41.2	77.9	100.0*									
0	0.2	A	0	–	0	–	100.0*								
		B	–	0	0	2.6*	–	100.0							
		C	–	0	0	14.3*	–	100.0							
20	0.1	A	0	–	26.3*	–	100.0								
20	0.2	A	0	–	0	–	22.6*	–	100.0						
40	0.2	B	–	0	0	0	–	0	0	–	–	0	27.0*	100.0	
		C	–	0	0	0	–	25.6	46.5	62.8*	86.0	97.7	100.0		
156	0.2	B	–	0	0	0	–	0	0	–	–	0	0	23.5*	100.0
		C	–	0	0	0	–	14.6	45.8	66.7	81.3	95.8	100.0	100.0*	

[a] Data expressed as percent of incubated samples showing gas

[b] Not checked for gas

* Toxin was first detected

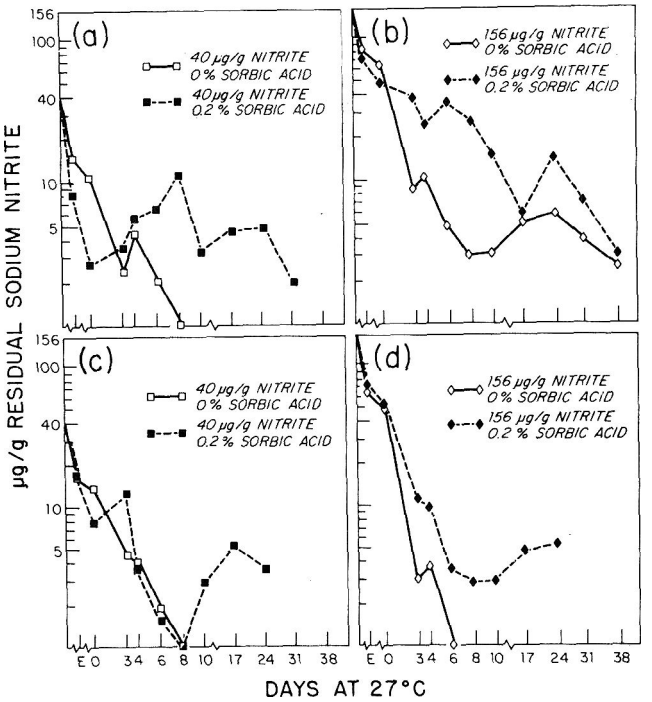

Fig. 3—Sodium nitrite depletion in Clostridium botulinum inoculated chicken frankfurter-type emulsions during processing and temperature abuse. (a), (b):trial (B); (c), (d):trial (C). □, 40 µg/g nitrite – 0% sorbic acid; ■, 40 µg/g nitrite – 0.2% sorbic acid; ◇, 156 µg/g nitrite – 0% sorbic acid; ◆, 156 µg/g nitrite – 0.2% sorbic acid; (E) uncooked emulsion; (0) after cooking and before incubation.

removed at each sampling time: five for toxicity testing and spoilage assessment; and, one for residual nitrite determination. Tubes with gas were preferentially selected. All nitrite analyses were performed on the specified day of sampling. Generally, the remaining samples were frozen and toxicity tested at a later time.

Spoilage assessment

The following scale was used for subjective spoilage assessment of the samples: N, intact sample; S_1, slightly digested; S_2, moderately digested; S_3, highly digested; and, S_4, completely digested sample. With increasing digestion, the putrefactive odor of the samples increased also. However, this was not as evident in samples containing nitrite and/or sorbic acid.

Toxicity testing

For botulinal toxicity testing the basic Center for Disease Control (CDC) procedure was followed (Anon., 1974). After the vaspar was removed, each sample was aseptically removed from the test tube, mixed with approximately twice its weight of gel-phosphate buffer (0.2% gelatin; 0.4% Na_2HPO_4, adjusted to pH 6.2 with HCl) in a blendor jar and blended for 1 min in an Osterizer blender. The blend was centrifuged at 4°C for 20 min at 12000 × G, and the supernatant was collected into sterile vials. A portion (0.5 ml) of the supernatant was intraperitoneally injected into each of two mice (Swiss, white, male, or female, 18–22g). Death of at least one mouse, after appearance of the specific botulism symptoms (Anon., 1974) during the 4 to 96-hr observation period, was used as evidence of sample toxicity. A botulinal toxin confirmation test was run on one toxic sample for each treatment and testing time. For confirmation, a portion of the sample was boiled for 10 min and each of two mice was injected with 0.5 ml boiled sample. Another portion (1.2 ml) of the unboiled sample supernatant was mixed with 0.3 ml of a mixture of equal volumes of botulinal antitoxins A and B, provided by CDC. After a half hour incubation at room temperature, each of two mice was injected with 0.5 ml of the detoxified sample. Survival of these mice confirmed the botulinal toxicity of the samples.

Residual nitrite analysis

Residual nitrite, after emulsification, cooking, and at the specified times was determined according to the method employed by Ashworth and Spencer (1972), using a saturated solution of potassium aluminum sulphate to precipitate soluble proteins, which could interfere with absorbance readings. The absorbance values were converted to µg/g (ppm) of sodium nitrite. Duplicate determinations were performed and the average values were plotted vs time in semi-logarithmic paper (Fig. 3).

RESULTS

Toxin production

The data of Figure 1 indicate, that under the conditions tested, two days of temperature abuse were inadequate for toxin to be formed in the control treatment. Control samples and samples from treatments containing low nitrite concentrations (20 or 40 µg/g) became toxic during 3 or 4 days of incubation at 27°C (Fig. 1 and 2). The higher nitrite concentration (156 µg/g) doubled the time necessary for toxin to develop, resulting in toxic samples after 6 days of incubation. Other researchers have also found that nitrite concentrations in the range of 156 µg/g extend the time necessary for botulinal toxin production under temperature abuse of similar products (Christiansen et al., 1977; Robach et al., 1978). However, low initial nitrite concentrations (less than 50 µg/g) have been reported to be ineffective against *C. botulinum* growth and toxin inhibition in various types of cured meat products or meat systems (Christiansen et al., 1973; Bowen et al., 1974; Rhodes and Jarvis, 1976; Roberts et al., 1976; Ivey and Robach, 1978; Tanaka et al., 1977; Tompkin et al., 1977; 1978a). Data in Figures 1 and 2 support these reports, since products containing 20 or 40 µg/g of nitrite did not differ from no-nitrite control products in botulinal toxin development. However, 156 µg/g nitrite was effective in delaying toxin development.

A delay in toxin production, similar to that observed with 156 µg/g nitrite, was observed in treatments formulated with 0.2% sorbic acid alone (Fig. 1 and 2). Toxic samples were detected after 6 or 7 days at 27°C. The results of other researchers with similar or other meat products generally agree with the findings reported here (Ivey and Robach, 1978; Ivey et al., 1978; Robach et al., 1978; Tanaka et al., 1977).

The low sorbic acid concentration (0.1%) in combination with 20 µg/g nitrite showed no effect on toxin production (Fig. 1). Since 20 and 40 µg/g nitrite alone were also ineffective, it can be assumed, that in this system, 0.1% sorbic acid alone would have also been ineffective. Addition of 20 µg/g nitrite to the treatment formulated with 0.2% sorbic acid did not change its effect against botulinal toxin formation (Fig. 1). Increasing the nitrite level to 40 µg/g in combination with 0.2% sorbic acid, produced a marked increase in inhibition of botulinal toxin production. No toxin was detected through 10 to 17 days of incubation (Fig. 2), depending on the trial (replicate). Toxic samples were found after 12 or 24 days at 27°C. Compared to the controls, this sorbic acid-nitrite combination (0.2%–40 µg/g) extended from four to almost eight times the incubation period necessary for toxin formation. With the higher nitrite concentration (156 µg/g) and 0.2% sorbic acid, an even greater effect on inhibition of toxicity was found (Fig. 2). Toxic samples were detected after 31 days of temperature abuse. Similar effects of nitrite-sorbate combinations in chicken meat, pork macerate, comminuted pork and bacon were found by Ivey and Robach, (1978), Ivey et al. (1978), Tanaka et al. (1977), and Robach et al. (1978). Generally, nitrite-sorbate combinations have been found more effective in retarding *C. botulinum* growth and toxin production than nitrite or sorbate used singly.

Residual nitrite

Treatments formulated without nitrite were analyzed and found to contain less than 1 µg/g residual nitrite. Gen-

Table 2—Spoilage of Clostridium botulinum inoculated chicken frankfurter-type emulsions containing sodium nitrite and/or sorbic acid during temperature abuse[a]

Nitrite (μg/g)	Sorbic acid (%)	Trial	Days at 27°C 3	4	6	8	10	12	17	24	31	38
0	0	B	S_1T	S_4								
		C	S_1T	S_4								
40	0	B	S_1T	S_2	S_4							
		C	S_1T	S_4								
156	0	B	S_1	S_2	S_4T							
		C	S_1	S_2	S_4T							
0	0.2	B	N	N	S_1T	S_2	S_3					
		C	N	N	S_1T	S_2	S_3					
40	0.2	B	N	N	N	N	N	—[b]	N	S_1T	S_3	
		C	N	N	N	S_1	S_2	S_2T	S_2	S_3		
156	0.2	B	N	N	N	N	N	—	N	N	S_1T	S_3
		C	N	N	N	S_1	S_2	S_2	S_2	S_2	S_2T	S_3

[a] N = Intact sample; S_1 = Slightly digested; S_2 = Moderately digested; S_3 = Highly digested; S_4 = Completely digested; T = Toxin was first detected.
[b] Not checked for spoilage

erally residual nitrite was depleted rapidly during 27°C incubation (Fig. 3). The low nitrite concentration (20 μg/g) was entirely lost during processing (data not shown). It was observed that 20 μg/g nitrite gave a cured meat color inferior to that of samples with 40 and 156 μg/g nitrite, which were similar in color. This observation of color differences was done after cooking and cooling, but before any storage or temperature abuse of the products.

At the 40 μg/g nitrite input, a 66–93% loss was recorded during processing while a 48–69% loss occurred at the 156 μg/g level. Similarly rapid losses have been reported by Pivnick et al. (1969) in luncheon meat; Hustad et al. (1973) in wieners; Christiansen et al. (1974) in bacon; and, Christiansen et al. (1977) in chicken frankfurters. A large amount of the nitrite remaining after processing was depleted during the first three days of incubation (Fig. 3), which is in agreement with losses observed by the other workers.

Figure 3 indicates that the presence of sorbic acid in the formulation decreased the disappearance rate of nitrite in the product. The 40 μg/g nitrite-0.2% sorbic acid treatment of trial B and 156 μg/g nitrite-0.2% sorbic acid treatments of trials B and C clearly demonstrated the effect of sorbic acid in delaying nitrite depletion (Fig. 3). This is not evident in part (c) of Figure 3 which presents data on the 40 μg/g nitrite-0.2% sorbic acid treatment of trial C. Data presented in Figure 2 indicated that this combination treatment in trial C was not as effective in delaying botulinal toxin production when compared to the same treatment of trial B. However, sorbic acid (0.2%) seemed to delay nitrite depletion, especially when used in combination with the higher levels (156 μg/g) of nitrite. This delay in nitrite depletion was associated with the slower rate of toxin production.

Gas and spoilage

Gas formation and spoilage preceded the development of botulinal toxicity (Tables 1 and 2). In all treatments and trials with nitrite or sorbic acid, the effects on gas formation and spoilage were similar to those observed for toxin formation. No toxin was detected before part or all of the samples of each treatment showed gas and started demonstrating spoilage. All the samples found toxic showed gas and spoilage. No toxin was detected in samples without gas or spoilage, but not all gassy and spoiled samples were toxic. It is significant from the present data that sorbic acid and nitrite did not prevent gas formation and spoilage more than they did toxin formation.

Christiansen et al. (1973) reported that not all toxic cans of comminuted cured meat were swollen and not all swollen cans were toxic, but all toxic samples were putrid and proteolyzed. Greenberg et al. (1959) demonstrated toxicity before the occurrence of organoleptically detectable spoilage. Even though gas formation and spoilage are not always associated with botulinal growth and toxin formation, they can be helpful indicators of growth in many cases.

DISCUSSION

COMPARED TO RESULTS from studies with other frankfurter-type products, the MDCM used in our studies appeared to support faster *C. botulinum* growth and earlier toxin production than that in pork and beef products. Large variations due to the type of meat tested were reported by Tompkin et al. (1978b). They found a range from 5–55 days in swelling time with 156 μg/g nitrite in meats ranging from pork and beef hearts to pork ham. They also showed that the nitrite effect on botulinal inhibition was lost when hemoglobin was added to the formulation and suggested that a higher level of available iron in heart meat caused the loss of inhibition. Another suggestion is that the level of residual nitrite remaining after processing influences the rate of *C. botulinum* growth (Tompkin et al., 1978b,c). Thus, the additional hemoglobin reacted with nitrite and decreased the residual nitrite, which in turn was associated with the loss in inhibition. Variations such as the above (in muscle pigmentation and available iron) could also explain variations in the effect of nitrite against *C. botulinum* growth reported in the literature, as well as the lower effectiveness of nitrite in inhibiting botulinal toxin production in chicken frankfurters compared to other meat products. An important aspect is whether mechanical deboning of the chicken meat used in the studies reported here increased the level of available iron, and thereby increased the rate of botulinal toxin development. In such a case, the theory of decreased botulinal inhibition with increased available iron (Tompkin et al., 1978b) would readily explain the lower, compared to other meats, nitrite effectiveness in controlling botulinal growth and toxin production in the chicken frankfurters made with MDCM and used in our studies.

Variations among laboratories or different trials by the same laboratory, even when the same product was tested, are common when studying the effect of nitrite on botulinal toxin formation (Hustad et al., 1973; Bowen et al.,

1974; Tompkin et al., 1977). Rhodes and Jarvis (1976) suggested that variations in the effectiveness of nitrite against botulinal growth and toxin formation can be due to differences in the lots of meat used. The variation in the toxicity results of trials B and C (Fig. 2) for the 40 μg/g nitrite-0.2% sorbic acid treatments may be related to the use of different lots of MDCM in these two trials.

One of the first indications of the possible value of sorbic acid for securing botulinal safety of meat products was a report by Tompkin et al. (1974). They demonstrated that 0.1% potassium sorbate retarded the growth and toxin production by *C. botulinum* in an uncured sausage product. Our data reported here with an emulsified MDCM product, support the results of Tompkin et al. (1974) that sorbic acid (0.2%) delayed botulinal toxin development. Other studies with culture media have not shown that sorbic acid has any effect in controlling *C. botulinum* growth (Emard and Vaughn, 1952; York and Vaughn, 1954; 1955; Hansen and Appleman, 1955). However, the very low solubility of sorbic acid in water, the fact that these studies were performed in aqueous laboratory media at near neutral pH, and possibly other factors could have been responsible for these negative results. It is also possible that the effect of sorbic acid might very well be different in laboratory media and in food.

The pH of the treatments reported here was determined after emulsification and found to be in the range 5.8–6.4. The sorbic acid-containing treatments showed the lower values. Subsequent observations revealed that sorbic acid not only decreased the pH of the emulsions, but such decreases varied depending on how long after emulsification the pH was measured. Systematic studies were therefore conducted by adjusting and standardizing the pH in order to determine its effects on delaying toxin production and/or sorbic acid activity. The results of these studies will be reported in the future.

The mechanism for the effectiveness of nitrite and sorbic acid combinations in substantially extending the safety of meat products from botulinum toxicity reported here and elsewhere in the literature is not known. Speculations include the possibility of an additive nitrite-sorbic acid effect and the production of a more inhibitory substance(s) through reaction of nitrite and sorbic acid.

Tompkin et al. (1978c) related swelling of the cans of a perishable cured meat product with residual nitrite concentrations, indicating that the lower the residual nitrite at the time of incubation the faster the rate of swelling. The possibility exists that sorbic acid, as our results indicate (Fig. 3), somehow delays nitrite disappearance from the product. The slower rate of toxin production in nitrite-sorbic acid treatments could be partially explained by the premise that residual nitrite or a compound related to nitrite is effective against *C. botulinum* growth (Tompkin et al., 1978c). Another possible mechanism of inhibition could be based on Gould's findings (1964) with *Bacillus* sp. spores that sorbate may also depress the rate of germination of *C. botulinum* spores. The germination of the spores and the depletion of nitrite would be depressed by the presence of sorbic acid. Since residual nitrite has been implicated in delaying toxin production, a larger amount of residual nitrite would be present for a longer time to prevent growth of the fewer and slower germinating spores.

Namiki and Kada (1975) reported formation of ethylnitrolic acid when a solution of sodium nitrite and sorbic acid was heated at 90°C for 1 hr. Ethylnitrolic acid was found inhibitory against *Escherichia coli* (Namiki and Kada, 1975) and against *Micrococcus* sp., *Enterobacter liquefaciens, E. coli,* and *Staphylococcus aureus* (Mirna and Coretti, 1977). Whether compounds such as ethylnitrolic acid can be formed with the sorbic acid and nitrite concentrations that were used in cured meat products is unknown. The possibility, however, exists and might explain the nitrite-sorbic acid effectiveness against botulinal toxicity in cured meat products.

SUMMARY

IN MECHANICALLY DEBONED chicken meat (MDCM) frankfurter-type emulsions, nitrite concentrations of 20 or 40 μg/g were ineffective in controlling *C. botulinum* growth and toxin production during 27°C temperature abuse. The 20 μg/g nitrite level gave inadequate cured meat color compared to 40 and 156 μg/g nitrite and it was entirely depleted during processing. Compared to no-nitrite control treatments, the highest nitrite level (156 μg/g), doubled the time necessary for botulinal toxin to be detected.

Addition of 0.1% sorbic acid to 20 μg/g nitrite did not extend the time necessary for toxin to be formed, whereas increasing the sorbic acid concentration to 0.2% and using it alone or in combination with 20 μg/g nitrite gave an effect similar to that of 156 μg/g nitrite. The 0.2% sorbic acid concentration was found much more effective when used in combination with 40 or 156 μg/g nitrite compared to sorbic acid or nitrite used singly. The botulinal safety of the combination nitrite-sorbic acid (0.2%) treatments increased four- to ten-fold depending on the nitrite concentration and the replicate.

The depletion of residual nitrite was slower in treatments formulated with both nitrite and sorbic acid when compared to treatments containing only nitrite. The slower residual nitrite disappearance in nitrite-sorbic formulations was associated with the longer stability of those treatments.

Gas production and product breakdown were effected in a manner similar to toxin production by nitrite and/or sorbic acid, but preceded the detection of botulinal toxin.

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—Continued on page 675

CHARACTERISTICS OF SOME RADIATION-RESISTANT HEMOLYTIC MICROCOCCI ISOLATED FROM CHICKEN

ARDYCE B. WELCH and R. B. MAXCY

ABSTRACT

Radiation-resistant micrococci isolated from chicken were studied because of their hemolytic properties. Morphology, growth, and physiological characteristics were similar in most ways to the common micrococci. There were some differences in pigmentation, nitrate reduction, hydrolysis of proteins, and salt tolerance. None of these characteristics appeared to be uniquely linked to radiation resistance of micrococci. Present knowledge does not indicate these bacteria to be of public health significance or to pose an organoleptic problem in quality protection.

INTRODUCTION

HIGHLY RADIATION-RESISTANT asporogenous bacteria that were hemolytic were isolated from chicken (Maxcy and Rowley, 1978; Welch and Maxcy, 1979). Radiation-resistant bacteria are common in food (Ingram and Farkas, 1977); however, reports of hemolysis have been limited to slight hemolytic activity by *Micrococcus radiodurans* (Anderson et al., 1956). Hemolysis on a blood agar plate is associated with pathogenicity of many bacteria of public health significance (Bailey and Scott, 1974).

Since these bacteria are radiation resistant and are of potential public health significance, additional knowledge of their nature is required in order to evaluate their significance in irradiation processing of food before a commercial process can be approved.

MATERIALS & METHODS

Cultures and media

Three highly radiation-resistant isolates, C-3, C-4 and C-7, that showed hemolysis on blood agar were obtained from chicken (Maxcy and Rowley, 1978; Welch and Maxcy, 1979). *Micrococcus radiodurans* ATCC 13939 and *Micrococcus radiophilus* (supplied by Dr. N.F. Lewis, Bhabha Atomic Research Center, Bombay 85, India) were included for comparative purposes. C-3, C-4, C-7 and *M. radiodurans* were propagated on plate count agar (PCA). *M. radiophilus* was propagated on tryptic soy agar (TSA), because it grew more luxuriantly than on PCA. Young cultures (<24 hr) were used for inocula.

Growth characteristics

Extent of growth was determined both under quiescent conditions and on an incubator-shaker at 32°C. Gross changes were determined by comparison with McFarland nephelometer standards. Tubes and flasks without visible growth were plated to determine the fate of the inocula.

Influence of sodium chloride in various concentrations on growth was determined by inoculation into TGY broth (0.5% tryptone, 0.1% glucose, 0.3% yeast extract; Anderson et al., 1956). Appropriate media and plating were used to determine the fate of the various inocula at 32°C.

Range of growth temperatures was determined by inoculating appropriate broths to obtain a concentration of approximately 2×10^4 CFU/ml followed by incubation (Temperature Gradient Incubator Model TN-3, Toyo Kagaku Sangyo Co., LTD, Japan). Visible growth was determined by comparison with turbidity standards, while cultures not showing visible change at 9 days were evaluated by plate counts.

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Table 1–Days required for detectable growth in broth at 32°C

Media	Organism	Shaker	Stationary
Plate count broth	C-3	1	1
	C-4	1	3
	C-7	1	1
	M. radiodurans	1	1
	M. radiophilus	4	4
Tryptic soy broth	C-3	1	1
	C-4	15[a]	–[b]
	C-7	1	1
	M. radiodurans	1	1
	M. radiophilus	1	1
Nutrient broth	C-3	1	1
	C-4	3	2
	C-7	1	1
	M. radiodurans	1	1
	M. radiophilus		7
Brain heart infusion	C-3	6[c]	1
	C-4	15[a]	–[b]
	C-7		2
	M. radiodurans	1	1
	M. radiophilus	2	2
Micro inoculum broth	C-3	2	1
	C-4	15[a]	–[b]
	C-7	1	2
	M. radiodurans	1	1
	M. radiophilus	15[a]	–[b]

[a] Terminated when volume evaporated to 5 ml. No visible growth. No viable bacteria recovered.

[b] No visible growth during 33 days incubation. No viable bacteria recovered.

[c] Terminated when volume evaporated to 5 ml. No visible growth.

Physiological characteristics

Standard methods (*Manual of Clinical Microbiology*, 1974) were used to determine the presence of oxidase, catalase, nitrate reductase, urease and arginine dihydrolase. Further tests were made to determine citrate utilization, indole production, H_2S production, penicillin sensitivity, action in litmus milk, hydrolysis of skim milk in PCA, gelatin liquefaction, reaction in triple sugar iron (TSI), aerobic and anaerobic utilization of dextrose (Hugh-Leifson O/F medium, API system, and Roche Diagnostics Oxi-Ferm tubes), and production of acid from dextrose, mannitol, xylose, lactose, maltose, salicin, trehalose, or dulcitol in both cystine trypticase semisolid agar (CTA) and phenol red agar (PRA).

RESULTS

Morphological characteristics

The highly radiation-resistant isolates were nonmotile cocci, gram-positive to gram-variable tending toward variability in older cultures. Cells occurred primarily in pairs with some tetrads and clusters. Isolates C-4 and C-7 were approximately 1 μm in diameter; C-3 was larger (approximately 1.5 μm). Clonal growth was raised, circular, opalescent, and exhibited a soft creamy consistency. Pigmentation of surface colonies was rose-buff to pink depending on stage of growth and medium.

Growth characteristics

The isolates showed various growth responses on common laboratory media (Table 1). There were both similari-

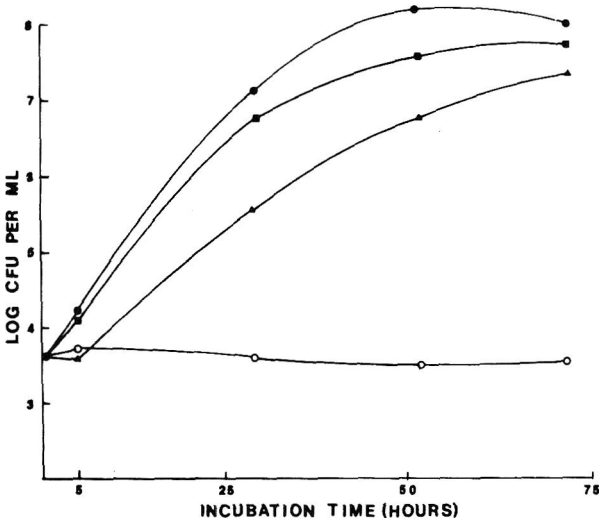

Fig. 1—Average results of growth of C-3, C-7 and M. radiodurans in TGY broth + NaCl. ● = 0%, ■ = 0.5%, ▲ = 1%, ○ = 2% or greater.

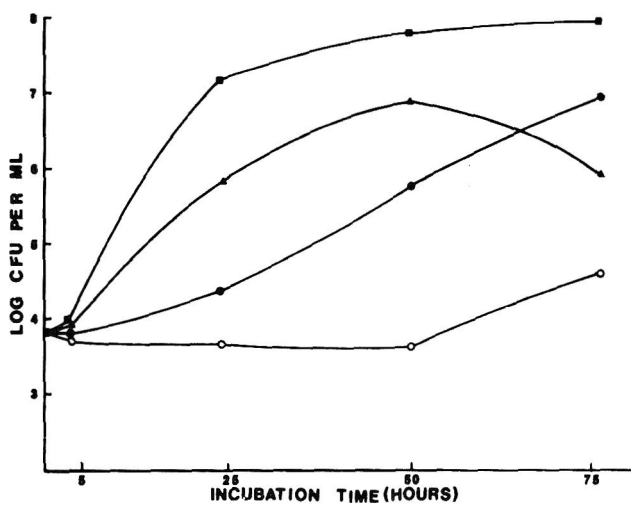

Fig. 2—Effect of various salt concentrations on growth of M. radiophilus in TGY broth. ● = 0%, ■ = 0.5–3.0%, ▲ = 4%, ○ = 5% or greater.

Table 2—Tests for growth of radiation-resistant micrococci at various temperatures in m plate count broth, except for M. radiophilus in tryptic soy broth

Temp (°C)	Microorganism				
	C-3	C-4	C-7	*M. radiodurans*	*M. radiophilus*
4.5	a	a	a	b	a
6.5	a	a	a	b	a
8.5	5	a	5	a	a
10	4	a	4	8	a
12	4	8	4	7	a
13	5	3	3	7	a
14	3	3	3	5	a
15.5	2	3	2	2	a
25	1	3	1	1	1
30.5	1	1	1	1	1
36	1	1	1	1	7
39	1	3	1	1	b
41.5	3	b	3	2	b
42.5	3	b	3	b	b
44.5	b	b	b	b	b

[a] = No visible growth during 9 days incubation, but viable cells remained.

[b] = No visible growth and no viable cells were indicated by plating after 9 days incubation.

Table 3—Physiological characteristics of radiation-resistant micrococci[a]

Isolate	Nitrate-to-nitrite	TSI[b]	1% Dextrose		1% Mannitol	
			CTA	PRA[b]	CTA	PRA[b]
C-3	–	K/N	N	K/K	K	K/K
C-4	+	N/N	N	N/N	N	K/K
C-7	–	K/N	N	K/K	K	N/N
M. radiodurans	–	N/N	N	K/K	K	K/K
M. radiophilus	–	K/N	Sl. A (15)	A/A (7)	Sl. A (15)	A/A (7)

[a] TSI = Triple sugar iron agar; CTA = Cystine trypticase agar (semi-solid); PRA = Phenol red agar; N = Neutral; K = Alkaline reaction; A = Acid reaction; Sl. = Slight; (#) = Number indicates days required for reaction to occur.

[b] Reactions in slant agar tubes reported in the form surface reaction/butt reaction.

ties and differences between the growth of each of these isolates compared with *M. radiodurans* and *M. radiophilus*. Growth was more rapid on a shaker incubator.

Growth of C-3, C-7 and *M. radiodurans* was similarly influenced by the presence of sodium chloride. Results were therefore combined and presented in Figure 1. Inhibition was complete at 2% or more. C-4 was most sensitive to salt in that it grew in 0 and 0.5% but did not grow in 1% NaCl.

Salt tolerance of *M. radiophilus* was unique among these radiation-resistant bacteria. Growth was enhanced by the presence of 0.5–3.0% NaCl. At 4% NaCl inhibition was moderate, and beyond this level inhibition was essentially complete for the test period (Figure 2). These radiation-resistant bacteria grew over a wide range of temperatures (Table 2) with optima in the generally recognized mesophilic range.

Physiological characteristics

These radiation-resistant bacteria, with the exception of salt tolerance, showed similar reactions that were typical of micrococci. Exceptions in similarity of reactions among these bacteria are shown in Tables 3 and 4. These differences, though of potential taxonomic interest, would not be important in identifying radiation-resistant bacteria in foods and evaluating safety of food processing operations.

Table 4—Hydrolysis of gelatin and skim milk by radiation-resistant micrococci

Isolate	Gelatin[a]				
	22°C		32°C		
	Growth	Liquefaction	Growth	Liquefaction	Skim Milk[a]
C-3	+ (5)	+ (5)	– (25)	– (25)	+ (2)
C-4	Sl. (5) + (8)	Sl. (5) + (8)	– (25)	– (25)	+ (2)
C-7	+ (5)	+ (5)	+ (8)	+ (8)	+ (2)
M. radiodurans	Sl. (8) + (15)	Sl. (8) + (15)	Sl. (5)	– (25)	+ (2)
M. radiophilus	Sl. (8) + (15)	Sl. (15)	– (25)	– (25)	Partial (4)

[a] + = Positive; – = Negative; Sl. = Slight; (#) = Number indicates days after inoculation until reaction occurred.

DISCUSSION

THESE RADIATION-RESISTANT hemolytic isolates have the common characteristics of micrococci and previously isolated radiation-resistant *M. radiodurans* and *M. radiophilus*. However, differences were apparent in pigmentation, nitrate reduction, hydrolysis of protein, and particularly in salt tolerance. With exception of *M. radiophilus*, these radiation-resistant bacteria were strikingly intolerant of NaCl in comparison to the commonly observed high NaCl tolerance by micrococci. *M. radiophilus* was inhibited at levels beyond 4% NaCl, although its growth was stimulated with up to 3% NaCl. Another radiation-resistant pigmented micrococcus showing moderate salt tolerance has been observed (Davis et al., 1963).

Although the hemolytic as well as the nonhemolytic radiation-resistant micrococci have many properties in common, none of these or combinations thereof appear unique for taxonomic purposes. Furthermore, none of the presently recognized characteristics can be linked to radiation resistance. This is true also for other highly radiation-resistant vegetative bacteria characterized by Welch and Maxcy (1975). Apparently the radiation-resistant bacteria commonly occur in foods (Ingram and Farkas, 1977), but these bacteria are recognized only as a result of applying unusual selective pressures. Results published elsewhere (Welch and Maxcy, 1979) indicate these bacteria are not of public health significance. Present knowledge does not indicate them to be a problem in protection of quality of food.

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FATTY AND AMINO ACID COMPOSITION OF SALTED MULLET ROE

J. Y. LU, Y. M. MA, C. WILLIAMS and R. A. CHUNG

ABSTRACT

Salted mullet roe was prepared by soaking in brine, pressing and air drying. The prepared roe product was high in protein (35.5%) and lipids (25.7%). The GLC analysis showed that the major saturated fatty acid was 16:0 and the major unsaturated fatty acids were 16:1 and 18:1 in the roe lipids. The analysis of the roe protein indicated that the major amino acids were glutamic acid, proline and lysine. The roe protein is well balanced with essential amino acids, and appears to be a high quality protein.

INTRODUCTION

MULLET (Mugil cephalus Linnacus) roe is often regarded as a delicacy and the use of roe by salting or pickling is many centuries old. In Florida alone nearly 40 million pounds of mullet are caught annually (Orthoefer, 1973). During the spawn season almost 20% of the mullet caught have roe. The value of the roe is not fully recognized. It is consumed locally; some is used as bait, and some simply thrown away by the people who do not know the value of the roe.

Pickled mullet roe is called "Botargo" by the Italians on the Mediterranean. In Japan the salted mullet roe is known as "Karasumi" and regarded as a highly prized delicacy. Various processing conditions of salted mullet roe were reported recently by Hsu and Deng (1978).

Little information is available regarding the composition of salted mullet roe. In this study, salted mullet roe was prepared, and its proximate, fatty and amino acid composition was determined.

EXPERIMENTAL

Material

Several pounds of fresh mullet roe ranging from 1/4 to 1 lb were purchased from a seafood store at St. Petersburg, FL. Salted mullet roe was prepared according to the traditional method as follows: roe was soaked in 5–6 volumes of brine (15% NaCl) overnight. Excess salt was removed by rinsing and washing the roe 4–5 hr the following day. The roe was placed between two wood boards and pressed for 1–2 days, then air-dried in the shade for 3–5 days until properly dried. The operation temperatures were 10–15°C. The product thus prepared had a yellow-reddish color, slightly rubbery texture, and a characteristic mullet roe flavor. The yield was approximately 60%. Several salted roe were cut, mixed well, and a portion taken for analysis.

Methods

Moisture, protein, ash, and NaCl were determined in triplicate according to AOAC (1970). The lipid content was determined by extraction with $CHCl_3$:MeOH (2:1), followed by evaporation of the solvent. An aliquot of the lipid extract was taken and analyzed for cholesterol (Sperry and Webb, 1950). A portion of lipid was saponified, and after acidification, fatty acids were extracted by hexane. Fatty acids were converted into methyl esters by the BF_3-methanol method (Metcalfe and Schmitz, 1961). A Hewlet-Packard 7610 gas chromatograph equipped with a flame ionization detector was employed. The column material was 10% SP-PS, 100/200 mesh (Supleco, Inc., Bellefonte, PA) packed in a 6-ft U-shaped glass tube (2 mm diam). Carrier gas was N_2 (20 ml/min). The injection and detector temperature was 250°C, and the operation temperature was 197°C. The fatty acid ester standards, RM-1, RM-3, and PUFA-1 (Supleco, Inc., Bellefonte, PA) were employed for identification and quantification of the sample fatty acids.

For amino acid analysis, 0.1-g sample was hydrolyzed with 3 ml of 6N HCl in a sealed tube at 100–110°C for 18 hr. Amino acids were purified by passing thru the Dowex-1 column, then eluted with 1N NH_4OH, and evaporated to dryness under vacuum. The purified amino acids were prepared as N-heptafluorobutyl n-heptyl ester derivatives (Coulter and Hann, 1968), and analyzed by the gas chromatographic method described by Moss et al. (1971). A Hewlet-Packard 7610 gas chromatography was employed. The amino acid ester samples were analyzed on a 1.75 m × 4 mm (i.d.) glass column packed with 3% OV-7 and 1.5% OV-22 (Applied Science Lab., State College, PA). The other conditions were: column temperature programmed from 75°C to 225°C at the increase rate of 2°C/min. Detector temperature was 230°C, and the carrier gas, N_2 at the rate of 42 ml/min. The air and hydrogen flow rates to the detector were 475 ml/min and 30 ml/min, respectively. Amino acids were identified and quantified by comparison with pure amino acids of known concentrations prepared by the same methods and run thru the column under the same conditions.

Tryptophan was analyzed separately in alkaline hydrolyzates (Inglis and Leavev, 1964).

Table 1—Proximate analysis of mullet roe[a]

	% Protein	% Lipid	% Moisture	% Ash	% NaCl	Cholesterol mg/100g
Fresh	22.6	13.7	61.5	1.8	–	440
Salted & dried	35.5	25.7	30.5	5.4	5.2	–

[a] Average of 3–5 determinations

RESULTS & DISCUSSION

TABLE 1 shows that the fresh mullet roe had a high level of protein (22.6%) and lipids (13.7%). The proximate composition resembles that of other fish roe such as salmon, sturgeon, and turbot—moisture 61%, protein 25.3%, oil 10.0%, and ash 1.7% (Watts and Merril, 1963). Like some other seafoods such as lobster and caviar, the mullet roe was high in cholesterol—440 mg/100g. Because of drying, the protein and lipid content of the salted roe increased to 35.5% and 25.7%, respectively. The dried product appeared relatively stable against bacterial spoilage possibly because of salt content and lower water activity. The water activity of salted, dried mullet roe (4% NaCl) was reported to be 0.85 (Hsu and Deng, 1978).

Table 2 shows the fatty acids from the mullet roe lipids. The lipids included wax esters, triglycerides, phospholipids, and some cholesterol esters. The major saturated fatty acids was 16:0, and the major unsaturated fatty acids were 16:1 and 18:1. Appreciable quantities of polyunsaturated fatty acids, 20:5, 22:5, and 22:6, were also present. This observation agrees with the report of Iyengar and Schlenk (1967) who analyzed the fatty acid composition of wax, triglycerides and free fatty acids of mullet roe. The fatty acid com-

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Table 2—Fatty acid composition of mullet roe lipids[a]

Fatty acid	%	Fatty acid[b]	%
14:0	5.60	18:4	4.16
15:0	1.76	NK	trace
16:0	11.63	NK	0.32
16:1	18.12	20:4	1.61
17:0	4.50	20:5	6.37
18:0	4.44	NK	0.19
18:1	18.27	NK	trace
18:2	6.14	22–5	5.00
20:1	trace	22–6	8.37
18:3	3.44		

[a] Average of 5 determinations
[b] NK - not known

Table 3—Amino acid composition of mullet roe

Amino acid	Protein g/100g	Amino acid	Protein g/100g
Alanine	6.91	Aspertic acid	7.67
Glycine	5.18	Phenylalanine[a]	4.78
Valine[a]	4.42	Glutamic acid	12.76
Threonine[a]	4.27	Lysine[a]	9.85
Serine	5.40	Tyrosine	4.13
Leucine[a]	7.95	Arginine	5.46
Isoleucine[a]	4.95	Cystine	1.56
Proline	10.39	Tryptophane[a]	1.54
Methionine[a]	2.32		

[a] Essential amino acid

position of mullet flesh oil has been extensively studied (Gruger et al., 1964; Orthoefer, 1973; Sen and Schlenk, 1964; and Orthoefer et al., 1976). Their reports indicate that the fatty acid composition of mullet varies somewhat depending upon location of catch, season, sex, and the age of the fish. It is recognized, however, that the unique characteristics of the mullet flesh oil is its high odd-chain, 15 and 17, fatty acid content. The fatty acid composition of the roe generally resembles that of the flesh oil. Some differences observed were that the roe has higher 18:1 and lower 15 than the flesh oil. It was reported that the mullet flesh oil contained mostly triglycerides whereas the roe oil contained 67–70% wax esters (Iyengar and Schlenk, 1967). This indicates that the nutritional requirements of the larvae apparently differ from that of the fish.

The amino acid composition of mullet roe is shown in Table 3. It shows that the roe contained a high portions of glutamic acid, lysine, and proline. The fish protein is known for high lysine content, but it is interesting to note that the mullet roe had a high level of proline. The high proline and wax content of the roe may be related to somewhat "rubbery" and "gummy" texture of salted roe. Table 3 also shows that the mullet roe protein is well balanced with essential amino acids, and is a high quality protein.

During the season mullet roe is available in large quantities on the East and the Gulf of Mexico coast. The roe could be developed into an economically valuable product. The result of this study may be of value to nutritionists and food scientists who are interested in mullet roe as food.

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CHEMICAL COMPOSITION OF CHOPS FROM PALE, SOFT, EXUDATIVE (PSE) AND NORMAL PORK LOINS

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ABSTRACT

Chops from normal or pale, soft and exudative (PSE) pork loins were displayed under simulated retail conditions, and drip was collected. During display, drip from chops was greater for PSE than normal loins, but the drip from both types of chops contained similar concentration of protein, free amino acids, total amino acids, sodium, potassium, calcium and magnesium. However, loss per kg lean was higher in the PSE chops for protein ($P < 0.01$) and potassium, calcium and magnesium ($P < 0.05$). The lean tissue from PSE chops contained more protein but less ether extract and energy than did lean from normal chops. Differences between PSE and normal chops were not significant for dry matter, ash, sodium, potassium, calcium, magnesium, phosphorus, iron, copper, zinc, free amino acids or total amino acids.

INTRODUCTION

FEW SCIENTIFIC REPORTS compare the chemical composition of chops from pale, soft, exudative (PSE) and normal pork loins from stress-susceptible and stress-resistant pigs, respectively. Lawrie (1960) and Wismer-Pedersen and Briskey (1961a,b) found no significant difference in percentage moisture, ether extract or protein between normal and pale, watery longissimus muscle. Merkel (1971) found less fat and more protein in PSE than normal colored longissimus muscle, but the differences were not significant ($P < 0.05$).

Swift and Berman (1959) found a significant correlation in pork muscle between water retention and zinc content and an inverse relationship between water retention and either calcium or magnesium content. Topel et al. (1967) reported no significant differences in sodium or potassium levels between PSE and dark colored M. longissimus. Dahl (1962) compared the levels of 19 amino acids between one PSE and one normal M. longissimus, but no other comparison of amino acid composition has been reported.

The composition of the drip has not been compared between chops from PSE and normal pork loins. Topel et al. (1975) compared sodium, potassium and specific gravity in the drip from hams prepared from PSE and normal pork and reported considerable variation. We wanted to determine the chemical composition of chops from normal and PSE pork and the composition of the drip from the tissue and to determine whether or not PSE characteristics affected either the loss of nutrients during retail storage or the final nutritive value of the pork.

MATERIALS & METHODS

WHOLESALE LOINS were selected on the basis of percentage reflectance (Marple et al., 1969) at a commercial packing plant. Values of 25 or higher represented the PSE group (10 loins) and 15–25 represented the normal colored group (10 loins). The loins were stored at 2°C for 9–10 days before being cut into chops 1.9 cm thick, between the 7th and last rib. Two packages of chops (3 chops per package) were stored at 5°C in an open display case under soft white fluorescent lights to simulate retail conditions. After 96 hr, the drip from the chops was collected by decantation into a graduated cylinder and frozen until analyzed. Each chop was physically separated into fat, bone and lean. The lean tissue, which was defined as muscles devoid of fat, except marbling, was ground in a small Hobart Silent Cutter, and an aliquot was homogenized with deionized water in a Waring Blendor and lyophilized. Expressible juice was determined by pressing 300g of meat between filter papers at 136 kg on a Carter press. Expressible juice values are expressed as the ratios of moisture area to meat area.

The dry matter was determined by drying the lyophilized tissue in a forced-air oven at 100°C for 24 hr. The values represent the dry matter remaining after lyophilization and oven drying. Nitrogen, ether extract and ash were determined by AOAC (1975) methods. Energy was determined by bomb calorimetry as described by the Parr Instruments Co. (1960). Lean tissue for mineral analysis was wet ashed with nitric and perchloric acids by the procedure of Johnson and Ulrich (1959). After appropriate dilution, sodium, potassium, calcium, magnesium, iron, copper and zinc were measured by atomic absorption spectrophotometry (Varian Tectron AA-5). Copper was chelated and extracted into isomethyl butyl ketone before measurement by atomic absorption. Phosphorus was measured by the phosphomolybdovandate reaction as outlined by AOAC (1975). The amino acids were analyzed by ion-exchange chromatography with a Technicon TSM Autoanalyzer. The protein content of the drip was measured by the method of Lowry et al. (1951). The mineral contents of the drip were determined by atomic absorption after appropriate dilution.

Data were analyzed statistically by the method of least-squares (Harvey, 1960), and the variation between loins was used to determine significance of differences between types of muscle.

Table 1—Expressible juice, pH and percentage reflectance of chops from PSE and normal loins after display for 96 hr at 5° C under soft white fluorescent light[a]

Item	Normal		PSE	
	Mean	S.E.	Mean	S.E.
pH	5.74	0.06	5.34	0.04
Expressible juice[b]	2.54	0.09	3.76	0.11
Percentage reflectance[c]	16.59	0.57	27.80	0.47

[a] All comparisons are significantly different ($P < 0.01$).
[b] Expressed as ratio of moisture area to meat area
[c] Scale calibration: >25, pale; 15–24, normal; <15, dark

RESULTS & DISCUSSION

PERCENTAGE REFLECTANCE, pH and expressible juice differed significantly ($P < 0.01$) between PSE and normal chops (Table 1). Those differences confirmed that the two groups of chops were from different populations.

In the drip, protein, sodium, potassium, calcium and magnesium, expressed as mg/ml, did not differ between PSE and normal chops (Table 2). Mean volume of drip was 2.97 ml for normal and 6.36 ml for PSE chops. Therefore, total losses of nutrients (protein, sodium, potassium, cal-

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Table 2—Composition of drip from pork chops[a]

Item	Units	Normal Mean	Normal S.E.	PSE Mean	PSE S.E.
		Composition			
Volume[b]	ml	2.97	0.33	6.36	0.85
Protein	mg/ml	56.2	10.4	56.8	7.8
Sodium	mcg/ml	1775	454	1706	335
Potassium	mcg/ml	113	27	169	24
Calcium	mcg/ml	3093	419	3532	439
Magnesium	mcg/ml	149	19	240	63
		Nutrients lost per kg of lean			
Protein[c]	mg	357	93	801	145
Sodium	mcg	11285	2987	27224	7160
Potassium[b]	mcg	19148	3350	49599	7372
Calcium[b]	mcg	666	176	2412	415
Magnesium[b]	mcg	913	143	2822	440

[a] 1.9 cm thick, 3 chops per package
[b] Significant difference ($P < 0.01$)
[c] Significant difference ($P < 0.05$)

cium and magnesium) in drip were greater from PSE than from normal chops.

The levels of free amino acids did not differ significantly between the drip from PSE and the normal chops (Table 3). Because the volume of drip was greater from PSE than from normal chops, losses of free amino acids also were greater, but the variation between loins was high, and differences were not significant. Nor were the differences significant if the data were expressed as milligrams of amino acid lost per kilogram of lean tissue. Total amino acids in the drip (mg/ml) did not differ between normal and PSE chops (Table 4).

The percentages of lean, fat or bone did not differ significantly between chops from normal or PSE chops (Table 5). Muscle from normal chops was lower in protein ($P < 0.05$) and ash ($P < 0.05$) and higher in ether extract ($P < 0.05$) and energy ($P < 0.01$) than muscle from PSE chops. These data agree with those of Merkel (1971) but not with those of Lawrie (1960) and Wismer-Pederson and Briskey (1961a,b). Mineral composition was similar in the two muscle types and to that reported by Topel et al. (1967). Unexplained variations, however, were high among loins, especially for calcium, iron and copper.

Relative amounts of free amino acids did not differ significantly between normal and PSE chops (Table 6). The amounts of free amino acids were similar in drip and muscle.

Total amino acids were greater for PSE than for normal chops, but because of the high variation among loins, the differences were not significant (Table 7). Our data are similar to the data of Dahl (1962) who reported no difference

Table 3—Free amino acids in pork chop drip[a]

Amino acid	Normal Mean	Normal S.E.	PSE Mean	PSE S.E.
	mg/100 ml			
Aspartic acid	4.23	1.61	3.79	1.35
Threonine	16.06	6.38	10.72	6.99
Serine	6.86	0.11	9.96	1.54
Asparagine	1.69	0.67	2.52	0.81
Glutamic acid	24.79	8.18	20.12	3.90
Glutamine	33.92	7.74	52.68	19.59
Proline	12.64	4.93	12.28	4.28
Glycine	19.74	5.86	18.81	3.69
Alanine	31.08	99.18	25.85	4.83
Citrulline	0.00	0.81	1.45	0.92
Cystine	0.24	0.10	1.42	0.81
Valine	7.56	1.68	9.95	1.82
Methionine	6.52	2.18	5.22	1.40
Isoleucine	9.34	3.03	7.36	1.89
Leucine	14.62	4.74	11.08	1.97
Tyrosine	5.75	1.40	6.51	1.16
Phenylalanine	8.81	2.76	8.19	3.14
Ornithine	0.00	0.00	0.18	0.18
Lysine	7.54	1.14	8.82	2.30
Histidine	12.61	6.41	6.36	1.46
Arginine	4.07	0.94	5.52	1.21
Total	228.08	53.45	228.79	41.80

[a] Means of 16 observations for normal and 20 for PSE samples

Table 4—Total amino acids in pork chop drip[a]

Amino acid	Normal Mean	Normal S.E.	PSE Mean	PSE S.E.
	mg/ml			
Aspartic acid	8.44	2.46	6.49	0.99
Threonine	4.43	1.65	3.09	0.57
Serine	3.49	1.30	2.54	0.54
Glutamic acid	9.85	3.00	7.50	1.22
Proline	5.98	1.81	4.83	0.81
Glycine	4.82	1.39	3.39	0.39
Alanine	3.75	1.06	3.17	0.46
Cystine	0.60	0.12	0.71	0.13
Valine	5.11	1.31	4.75	0.74
Methionine	2.04	0.61	1.54	0.19
Isoleucine	4.52	1.42	3.72	0.64
Leucine	6.30	1.58	5.42	0.77
Tryrosine	2.65	0.77	2.34	0.50
Phenylalanine	3.65	0.87	2.95	0.41
Lysine	7.49	2.17	5.87	0.98
Histidine	5.31	1.40	4.74	0.85
Arginine	4.35	1.48	3.47	0.50
Total	82.78	24.20	66.54	10.52

[a] Means of 20 observations

Table 5—Composition of lean tissue from pork chops[a]

Item	Units	Normal Mean	Normal S.E.	PSE Mean	PSE S.E.
		Separable composition			
Lean	%	57.5	1.8	60.4	1.4
Fat	%	20.7	1.8	16.5	1,6
Bone	%	21.1	0.8	22.3	1.4
		Proximate analysis of lean tissue			
Dry matter	%	31.3	0.6	30.0	0.6
Nitrogen[b,c]	%	10.7	0.3	11.6	0.3
Protein (N X 6.25)[b,c]	%	67.1	1.6	72.4	1.9
Ether extract[b,c]	%	31.6	1.3	25.3	1.8
Ash[b]	%	3.3	0.1	3.6	0.1
Energy[b,d]	kcal/g	6.65	0.06	6.37	0.07
		Mineral composition of lean tissue[b]			
Sodium	mcg/g	1989	65	2087	71
Potassium	mcg/g	10884	300	11559	284
Calcium	mcg/g	764	75	888	79
Magnesium	mcg/g	663	18	698	24
Phosphorus	mcg/g	6357	180	6785	154
Iron	mcg/g	28.4	2.4	36.6	5.6
Copper	mcg/g	6.4	1.0	7.9	1.5
Zinc	mcg/g	59.8	3.2	66.5	4.1

[a] Means of 20 observations
[b] Dry matter basis
[c] Significance difference ($P < 0.05$)
[d] Significance difference ($P < 0.01$)

Table 6—Free amino acids in lean tissue from pork chops[a]

Amino acid	Normal		PSE	
	Mean	S.E.	Mean	S.E.
	mg/100g fresh tissue			
Aspartic acid	7.19	1.28	5.87	1.00
Threonine	16.56	4.19	10.65	1.01
Serine	12.04	2.75	13.32	1.17
Asparagine	3.45	0.83	4.66	0.69
Glutamic acid	32.81	8.53	21.78	1.03
Glutamine	77.83	21.76	111.73	18.44
Proline	18.68	6.54	10.51	2.36
Glycine	19.40	2.09	15.61	1.08
Alanine	28.83	6.08	25.46	4.84
Citrulline	3.79	2.00	0.81	0.64
Cystine	0.38	0.14	0.15	0.10
Valine	16.37	2.88	13.48	1.33
Methionine	7.44	0.97	7.14	0.67
Isoleucine	9.91	1.14	10.34	1.64
Leucine	16.35	2.29	15.80	1.16
Tyrosine	5.97	1.35	9.08	0.76
Phenylalanine	10.94	1.91	10.92	1.22
Ornithine	0.22	0.13	0.07	0.06
Lysine	5.08	1.78	9.75	3.49
Histidine	5.27	2.44	5.33	2.03
Arginine	15.39	3.86	13.81	2.72
Total	313.90	31.50	316.29	14.38

[a] Means of 20 observations

Table 7—Total amino acids in lean tissue from pork chops[a]

Amino acid	Normal		PSE	
	Mean	S.E.	Mean	S.E.
	mg/g fresh tissue			
Aspartic acid	14.68	2.16	19.06	2.65
Threonine	7.56	1.03	9.87	1.37
Serine	6.50	1.02	8.36	1.31
Glutaminc acid	23.07	2.70	31.43	4.73
Proline	10.36	0.61	11.72	0.95
Glycine	6.23	0.34	7.92	0.40
Alanine	7.68	0.94	8.57	0.99
Cystine	1.05	0.12	1.00	0.15
Valine	9.78	1.04	11.36	0.08
Methionine	5.05	0.66	5.77	0.63
Isoleucine	8.89	0.52	10.24	0.76
Leucine	14.32	0.63	15.84	1.11
Tyrosine	7.18	1.08	8.52	1.51
Phenylalanine	9.88	1.91	12.35	2.63
Lysine	13.30	0.99	15.08	2.52
Histidine	11.09	2.10	15.16	3.71
Arginine	12.75	1.17	15.78	1.54
Total	169.35	15.89	208.05	23.29

[a] Means of 20 observations

in amino acid composition between normal and PSE muscle and to those of Williams et al. (1954) who reported the amino acid composition of the entire pig carcass.

In our study, PSE muscle lost more exudate during a simulated retail holding period than normal muscle and, therefore, tended to lose more of all nutrients. The losses, however, represented a small portion of the total nutrients present in the pork chop and would not markedly diminish the nutritive value of the chop.

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γ-IRRADIATION OF 5′-INOSINIC ACID IN FISH MEAT CAKE

SADAO UCHIYAMA and MITSURU UCHIYAMA

ABSTRACT

The radiolysis of 5′-inosinic acid (5′-IMP) was studied to evaluate the wholesomeness of γ-irradiated fish meat cake. 5′-IMP in a phosphate buffer (pH 6.5) decomposed at 3.12 of G(−M) by γ-irradiation to hypoxanthine. Hypoxanthine, purine base, decomposed almost at the same rate as 5′-IMP. Protein and unsaturated fatty acid had a markedly protective effect on 5′-IMP against γ-irradiation, and metal ions like iron were somewhat protective. In an actual fish meat cake, Kamaboko, 5′-IMP was kept stable even at a high dose of γ-ray. The solvent extract from irradiated Kamaboko with 5′-IMP and hypoxanthine or without them showed no mutagenecity in the mutation assay using *Salmonella typhimurium*.

INTRODUCTION

MUCH INTEREST has been accorded γ-irradiation as a food sterilization method, in view of the tendency in Japan to prohibit use of antibacterial agents such as 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide for fish meat products. (Ministry of Health and Welfare of Japan, 1973). The wholesomeness of the irradiated food has therefore been assessed prior to its use (Joint FAO/IAEA/WHO, 1976; Fujimaki, 1976).

5′-Inosinic acid (5′-IMP), a compound related to nucleic acid, is known to be susceptible to γ-ray (Sholes and Weiss, 1954), and is also added to fish meat products in comparatively large quantities as a flavoring.

In animal tissues or microorganisms, 5′-IMP decomposes enzymatically to inosine and subsequently to hypoxanthine (Hx). On the other hand, according to Terada et al. (1970) using paper chromatography, the radiolysis of 5′-IMP led to the direct formation of Hx in the neutral solution by scission of the bond between Hx and phosphoribose. There remains, however, the possibility that Hx might decompose in different ways such as radiolysis of adenine (Ponnamperuma et al., 1963) from the degradation in the lives, producing a mutagenic base analogue of nucleic acid such as 2-aminopurine (Ames et al., 1972) and acting as a radiosensitizer to worsen the quality of food.

The present study was conducted first to determine the stability of 5′-IMP to γ-irradiation and the effect of coexisting substances in Kamaboko (a fish meat cake produced in Japan) mainly in a model system by use of high performance liquid chromatography, and second, to test for mutagenicity of irradiated Kamaboko containing 5′-IMP and Hx.

EXPERIMENTAL

Preparation of sample solution and γ-irradiation

Sample solutions were prepared as solutions containing 5′-IMP, 4×10^{-3} M, and co-existing substances of various concentration in a phosphate buffer of pH 6.5. 5′-IMP sodium salt derived from a muscle was obtained from Sigma Chemicals. As co-existing substances, aqueous solutions of ferrous, ferric and calcium chlorides were used for metallic ions and soybean protein (Wako Pure Chemical Industries) was utilized for protein. The potassium salts of stearic, linoleic and linolenic acids were also added to the system as fatty acids. These mixtures in 4-ml glass tubes were irradiated in the range 0.22–1.08 Mrad by a ^{60}Co-irradiator at a dose rate of 12.1 krad/hr.

To the homogenate prepared from 10g of Kamaboko and 15 ml of distilled water was added 5′-IMP of 2.5×10^{-4} mol. The mixtures in 25-ml glass tubes were irradiated at 0.49, 4.13 and 10.79 Mrad by a ^{60}Co-irradiator at a dose rate of 635 krad/hr. Kamaboko consisted of protein, 11.2%; lipid, 0.6%; sugariness, 12.5%; ash, 3.0%; and water, 72.7%.

All irradiations were accomplished in sealed glass tubes at ambient temperature, average 20°C.

High performance liquid chromatography (HPLC)

Pretreatment of the sample solution was conducted as follows: solutions in the absence of protein and fatty acid were injected into a column of HPLC without any treatment. Mixtures with soybean protein were ultrafiltered using a Centry flow-25 (Amicon) and the filtrates were injected in the same manner. In the case of fatty acid, 1 ml of 10% phosphoric acid was added into 4 ml of the mixture. After fatty acids were eliminated by extraction with five 2-ml portions of ether, the aqueous solution was adjusted to pH 6.5 by addition of 1N KOH and the total volume was then made up to 7 ml with distilled water. The slight ether residue in the solution was eliminated by heating at 40°C.

HPLC was carried out with a Hitachi Liquid Chromatograph, Model 634 A, under the following conditions: Gel, Hitachi Gel 2610 (cation exchange resin); Column, 3 × 300 mm, glass; Column temperature, 50°C; Eluent, 0.05M Na_2HPO_4; Pressure, 58 kg/cm^2; Flowrate, 0.29 ml/min; Detector, UV 254 nm, 0.08 AUFS; Injection volume, 5 μl.

Thin-layer chromatography (TLC)

The concentrated solution in the radiolysis of 5′-IMP was chromatographed on PEI-Cellulose F (Merck) plate by the author's method (Uchiyama et al., 1974). Nucleotides, nucleosides or nucleic base were detectable as a dark spot on the plate under UV light of 254 nm.

Mutation assay

The preparation of the sample for mutation assay was carried out as follows. Kamaboko sealed with cellophane was irradiated at 0.49, 4.13 and 10.79 Mrad. The irradiated Kamaboko, 10g, was homogenized and extracted with a mixture of chloroform and methanol (2:1). The extract was tested for the mutation assay using *Salmonella typhimurium*. At the same time, the homogenate containing 10g of Kamaboko and 2.5×10^{-4} mol of 5′-IMP or 10g of Kamaboko and 2.5×10^{-4} mol of Hx was irradiated under the same conditions. The extract from the irradiated homogenate was prepared in the same manner.

The mutation assay was carried out using the procedure of Ames et al. (1975), as modified by Yahagi et al. (1977). Strain TA 100 of *Salmonella typhimurium* was used to count the colonies of revertants from histidine requirement to histidine prototrophy. S9 mix was prepared to investigate the metabolic activation for mutation. It contained S9 fraction obtained from PCB-treated rat liver (Wister rat, male) by centrifugation at 9000 × G for 20 min, and other chemicals.

G(−M) value

G(−M) for the decomposition of 5′-IMP of 4×10^{-3} M was measured in a phosphate buffer (pH 6.5) at 20°C without the elimination of air. The value represents the mean in the range 0.22–1.08 Mrad.

—Text continued on page 682

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Degradation and protection constant

The degradation constant k of 5′-IMP of Hx by γ-radiolysis was expressed as follows:

$$k = \frac{2.303}{t} \cdot \log \frac{X_o}{X}$$

where t = Dose of γ-ray (Mrad); X_o = Initial amount; X = Remaining amount.

The protection constant p of co-existing substance against the radiolysis of 5′-IMP was exprssed as follows:

$$p\ (\%) = \frac{Ix - Ii}{I_o - Ii} \times 100$$

where I_o = Initial amount of 5′-IMP; Ii = remaining amount of 5′-IMP on radiolysis without co-existing substance; Ix = Remaining amount of 5′-IMP on radiolysis with co-existing substance.

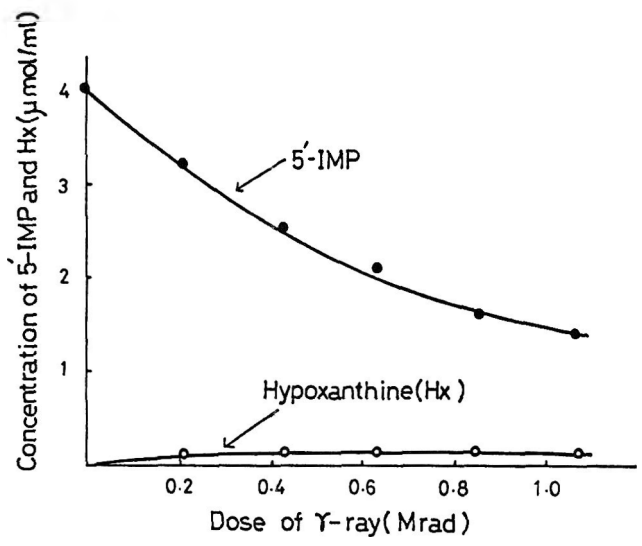

Fig. 1—Degradation of 5′-IMP by γ-irradiation. Each point represents the mean of the data of three experiments. Each SD was less than 0.1 μmol/ml.

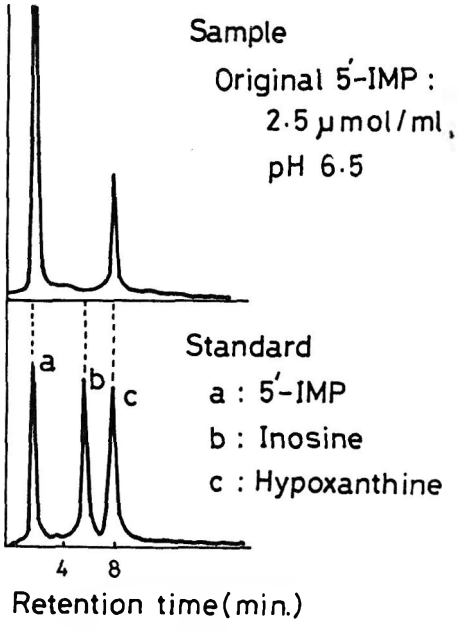

Fig. 2—High performance liquid chromatogram of degradation products of 5′-IMP by γ-irradiation at 0.918 Mrad.

RESULT & DISCUSSION

Radiolysis of 5′-IMP in an aqueous solution

The first approach to investigate the stability of 5′-IMP to γ-irradiation was undertaken in an aqueous solution of a phosphate buffer, pH 6.5, normal pH of Kamaboko. As shown in Figure 1, 5′-IMP decreased with an increased dose of γ-ray and this decrease obeyed a good first-order rate in the range 0.22–1.08 Mrad. In the reaction system, a small amount of Hx was found and remained almost constant even when the dose increased. On the other hand, inosine residue was not identified even by HPLC similar to Terada's findings (Terada et al., 1970) (Fig. 2). In addition, G(−M) for the decomposition of 5′-IMP was 3.12. This suggested that 5′-IMP itself, in an aqueous, solution is not as sensitive to radiolysis compared with nucleic base (Holian and Garrison, 1967) and other chemicals (Ikebuchi et al., 1977).

The additional experiment by thin-layer chromatography using PEI-Cellulose F showed that there was not any main product with absorption at 254 nm except for Hx in the irradiated system. This also suggested that the other products besides Hx might be deficient of the aromatic skeleton.

Comparison between radiolysis of 5′-IMP and Hx

The radiolysis of Hx, a substantial base of 5′-IMP, was conducted to examine the probability of alteration to another compound. Hx was also found to decompose at a good first-order rate. The degradation constants of 5′-IMP and Hx were 1.0429 ± 0.049 (n=6) and 0.943 ± 0.053 (n=6), respectively. This means that the purine skeleton occurring from γ-radiolysis of 5′-IMP decomposed almost at the same rate as 5′-IMP and, as it were, is as fragile as the purine mononucleotide (Fig. 3).

Influence of co-existing substances on radiolysis of 5′-IMP

Soybean protein was added to the system containing 5′-IMP solution at pH 6.5. Protection against radiolysis of 5′-IMP was 55% even in the 0.3% concentration of soybean protein (Fig. 4). With fatty acid, linoleic acid and linolenic

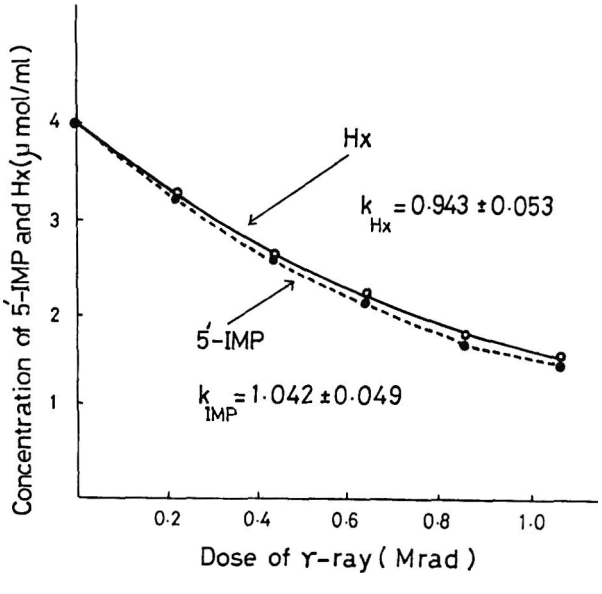

Fig. 3—Degradation of 5′-IMP and hypoxanthine (Hx) by γ-irradiation. Each point represents the mean of the data of three experiments. Each SD was less than 0.1 μmol/ml.

acid, assumed to facilitate the decomposition of 5′-IMP since they are susceptible to irradiation and may play some catalytic role, had a markedly protective effect as shown in Figure 5. On the other hand, stearic acid, the saturated fatty acid, had no protective effect.

The data of co-existing substances involving protein and fatty acids are summarized in Table 1. The data on saccharides were neglected since detailed data in which saccharide had a protective effect against radiolysis of 5′-IMP had been reported previously by Terada et al. (1970); also, the author's objective concerned protein-abundant food. In the table, the protection represents a positive value and the decomposition negative.

Protection up to 44.1% was observed in the co-existence of 2.5 mg/ml of soybean protein in the system. While the co-existence of 1.67 mg/ml of unsaturated fatty acids led to 64.8% of protection in linoleic acid and 57.6% in linolenic acid. This indicated that the protective effect of protein was lower than that of unsaturated fatty acids. It was, however, interesting that these protein and indispensable fatty acids, playing an important role in a living body, effectively protected the mononucelotide from γ-irradiation.

Both ferric and ferrous ions, contained so much in food comparatively, were assumed to promote degradation of 5′-IMP due to their oxidative or reductive action but actually showed small protective effect. These ions might make 5′-IMP stable due to the formation of salts.

Trimethylamine oxide (TMAO) contained uniquely in fish meat had no protective effect, and even the co-existence of TMAO more than 500 times to 5′-IMP did not form any products, although TMAO was expected to concern the methylation of 5′-IMP.

These results suggested that 5′-IMP was actually protected against γ-irradiation in food consisting of protein and lipid.

Radiolysis of 5′-IMP in Kamaboko

Since the absolute amount of 5′-IMP in Kamaboko is small, a 40% homogenate of Kamaboko in which 5′-IMP was mixed additionally was irradiated in the range of 0.45–10.79 Mrad. As shown in Figure 6, the stability of 5′-IMP was maintained even at a high dose of γ-ray.

This suggests that protein, lipid, metal ions, saccharide and other constituents in Kamaboko protect 5′-IMP against γ-irradiation as shown in the previous model system.

Mutation assay

Although it was clear that 5′-IMP was comparatively stable on radiolysis, there still remained the possibility of the

Table 1—Effect of co-existing substances on γ-irradiation of 5′-IMP at 0.918 Mrad

Additive	mg/ml	Protection (%)
Fe^{+++}	1.40	10.1
Fe^{+++}	2.79	17.7
Fe^{++}	1.40	5.1
Fe^{++}	2.79	11.5
Ca^{++}	1.00	4.2
Ca^{++}	2.00	9.3
Soybean protein	2.50	44.1
Soybean protein	5.00	53.7
Stearic acid	1.67	0
Stearic acid	3.33	0.6
Linoleic acid	1.67	64.8
Linoleic acid	3.33	67.6
Linolenic acid	1.67	57.6
Linolenic acid	3.3	64.8
Trimethylamine oxide	2.55	0.4
Trimethylamine oxide	25.50	8.5

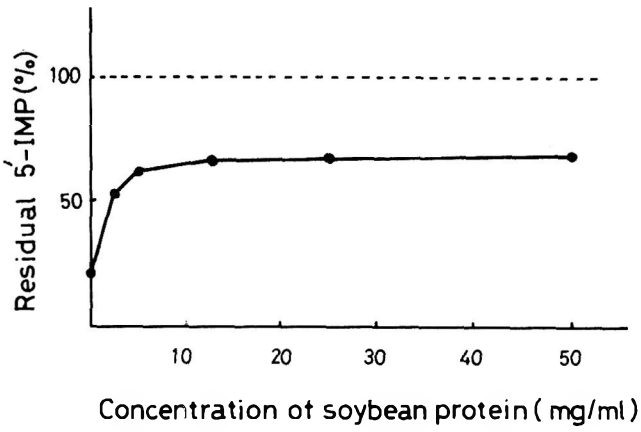

Fig. 4—Effect of soybean protein on γ-irradiation of 5′-IMP at 0.918 Mrad.

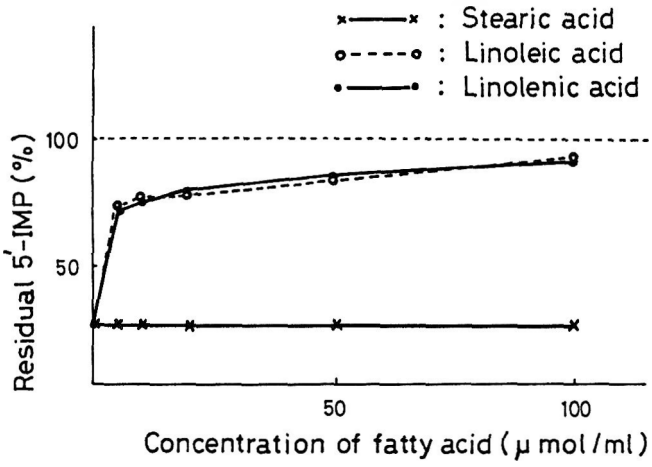

Fig. 5—Effect of stearic, linoleic and linolenic acids on γ-irradiation of 5′-IMP at 0.918 Mrad.

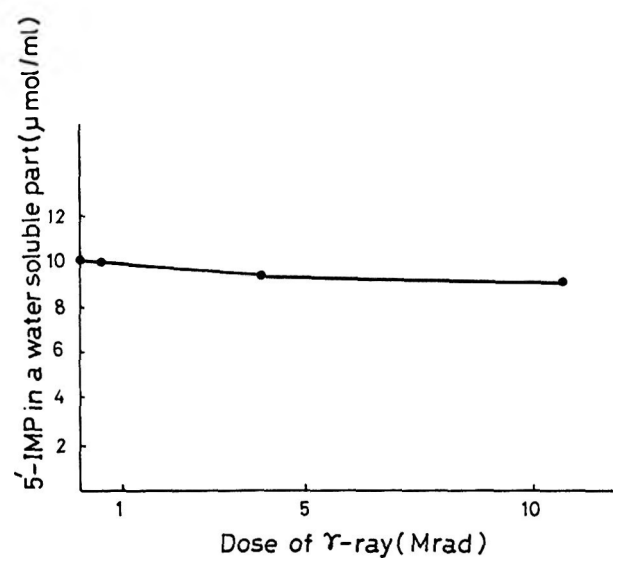

Fig. 6—Degradation of 5′-IMP in Kamaboko homogenate by γ-irradiation.

Table 2—Mutation assay of the extracts from irradiated Kamaboko using Salmonella typhimurium

Extract	Dose (μg/plate)	Number of revertant per plate +S9	Number of revertant per plate −S9
Kamaboko	113	116	104
Intact	225	111	96
	450	104	84
Irradiated	163	134	94
at 0.49	325	97	79
Mrad	650	134	70
Irradiated	163	106	74
at 4.13	325	138	50
Mrad	650	152	52
Irradiated	150	117	79
at 10.79	300	117	76
Mrad	600	121	68
Kamaboko homogenate			
Irradiated	75	90	104
at 10.79	150	92	72
Mrad	300	108	77
Kamaboko homogenate with Hx			
Irradiated	113	124	105
at 0.49	225	98	103
Mrad	450	115	71
Irradiated	75	106	111
at 4.13	150	97	91
Mrad	300	96	77
Irradiated	70	78	97
at 10.79	140	107	86
Mrad	280	110	74
Kamaboko homogenate with 5′-IMP			
Irradiated	75	112	86
at 0.49	150	116	91
Mrad	300	99	62
Irradiated	125	79	75
at 4.13	250	94	76
Mrad	500	95	76
Irradiated	90	116	81
at 10.79	180	89	88
Mrad	360	95	70

formation of poisonous products in small quantity from 5′-IMP and by the interaction between 5′-IMP and other compounds in Kamaboko.

In order to estimate the toxicity of irradiated Kamaboko preliminarily, kamaboko itself and the kamaboko homogenate containing 5′-IMP and Hx were irradiated. The extracts from these irradiated samples with a mixture of chloroform and methanol (2:1) were tested for the mutation assay using *Salmonella typhimurium*. Table 2 showed that the irradiated Kamaboko had no mutagenicity and that the same held true when an excess of 5′-IMP and Hx was added to Kamaboko. This assay dealt with only the extract of samples with a mixture of chloroform and methanol. The real assessment of the toxicity in the irradiated food containing 5′-IMP should be conducted also for other fractions and finally for the whole animals like those done on irradiated milk powder (Renner and Reichelt, 1973).

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FLUORESCENCE CHANGES RESULTING FROM METHEMOGLOBIN AND FATTY-ACID INTERACTION

SHLOMO GROSSMAN, IRA S. HAMMERMAN and TOVA SCHAAP

ABSTRACT

Interaction between methemoglobin and saturated and unsaturated fatty acids was studied experimentally by fluorescence and absorption spectrum techniques, affinity chromatography and sedimentation coefficient measurements. Buffered solutions of linoleate, solubilized with Tween 20, when added to beef methemoglobin, and fresh rabbit oxyhemoglobin, caused a substantial increase in tryptophan fluorescence around 334 nm for an excitation frequency of 280 nm. At pH 7.8, at 25°C, 1×10^{-4}M linoleate added to 1.1×10^{-6}M methemoglobin increased the quantum yield from 1.5% of that of the equivalent amount of tryptophan to 3.8%, and when added to the same concentration of oxyhemoglobin increased the quantum yield from 0.14% to 2.1%. Below pH 4 linoleate did not increase the fluorescence. The percentage increase in fluorescence is an exponential function of the number of carbon atoms in the saturated fatty acids. For linoleate the fluorescence dependence on linoleate concentration yielded $K_m = 1.8 \times 10^{-4}$M. The increase in fluorescence was about three times greater from unsaturated than from saturated fatty acids. Methemoglobin in 5mM acetate buffer bound specifically to sepharose-linoleate at pH 5 at 4°C and was eluted with 0.2M acetate buffer. The change in the absorption spectrum of the methemoglobin indicated a reduction of the methemoglobin upon the addition of linoleate. Band sedimentation in 0.5M KCl of methemoglobin to which was added linoleate yielded $s_{20,w} = 2.58$S compared to $s_{20,w} = 3.51$S for the untreated methemoglobin. The results may be explained by a conformational change or dissociation of the methemoglobin tetramer.

INTRODUCTION

IN ADDITION TO its role in oxygen and carbon monoxide transport, hemoglobin also functions as a catalyst for the peroxidation of lipids and olefins (Tappel, 1962). This function is also shared by other heme-proteins (myoglobin, cytochromes, catalase, peroxidase) and by the non-heme iron-containing enzyme lipoxygenase (E.C.1.13.11.12) (Eskin et al., 1977).

The transition metals also serve as peroxidation catalysts, but the heme-proteins and lipoxygenase have the highest such catalytic activity. The understanding of lipid peroxidation by heme-proteins is of practical importance because: (1) it is a principal cause of deterioration of stored meat and fish (Govindarajan et al., 1977); (2) it appears to cause damage to subcellular structures in vitamin E deficient animals; (3) it may be involved in human atherosclerosis and cause damage to mitochondria (Di Luzio, 1973; McKnight et al., 1965; Clark et al., 1969); (4) it appears to be a cause of stored whole blood deterioration due to red cell membrane lipid peroxidation and subsequent lysis (Tappel, 1962; Goldstein and Harber, 1972).

In the course of studying the peroxidation of fatty acids by iron-containing proteins (Pinsky et al., 1971; Shahin et al., 1978; Grossman and Zakut, 1978) we found it necessary to distinguish between the effects of lipoxygenase and of the heme-proteins. It was reported by Finazzi-Agro et al. (1973) and Egmond et al. (1975) that in the case of lipoxygenase, the addition of linoleic acid causes a decrease in the fluorescence and an increase in the ultraviolet absorbance. Here we have followed the change in intrinsic fluorescence, absorbance and other physical properties of oxyhemoglobin and methemoglobin in the presence of linoleate.

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MATERIALS & METHODS

HEMOGLOBIN from beef blood (Type I) (Sigma Chemical Co., St. Louis, MO) containing 99% methemoglobin by absorption spectroscopy measurements, (the rest being oxyhemoglobin) and fresh oxyhemoglobin from rabbits were used in this study. Also the following saturated fatty acids were used: formic acid and acetic acid (Frutarom, Haifa, Israel), n-butyric acid and palmitic acid (Sigma), hexanoic acid, lauric acid and myristic acid (Fluka, Switzerland), octanoic acid and decanoic acid (B.D.H., England), and stearic acid (E. Merck, W. Germany).

In addition the following unsaturated fatty acids were used: oleic acid and linolenic acid (Fluka, Switzerland) and linoleic acid (Sigma, Grade III, 99% from sealed ampule). Buffered linoleate solutions were obtained with the aid of Tween 20 according to Ben Aziz et al. (1968). The weight ratio between linoleate and Tween 20 was kept constant (approximately 1:1) during the addition of this linoleate-Tween 20 stock to the protein solution. In the comparison of the effects of various fatty acids, the acids were first dissolved in absolute ethyl alcohol (0.12mM) and 10μl solutions were added to 3 ml of methemoglobin.

Linoleate oxidation

Linoleate (the salt of linoleic acid) oxidation was assayed by two different techniques: (a) oxygen absorption, measured polarographically (Grossman and Zakut, 1978), and (b) absorption spectra measurement of conjugated diene formation (Ben Aziz et al., 1970).

Affinity chromatography of methemoglobin on linoleyl aminoethyl sepharose

The linoleyl aminoethyl sepharose was prepared essentially as described by Grossman et al. (1972). Five mg methemoglobin [homogenous according to sodium dodecyl-sulfate polyacrylamide gel electrophoresis (Weber and Osborn, 1969)] dissolved in 5 mM sodium acetate buffer, pH 5.0, were applied to a linoleyl aminoethyl sepharose column (10 × 2 cm) at 4°C and washed with the same buffer, until no absorption at 280 nm was detected. Most of the methemoglobin was subsequently eluted with 0.2M acetate buffer, pH 5.0.

Fluorescence spectroscopy

Corrected fluorescence emission spectra were measured with a Perkin-Elmer MPF-44A Fluorescence Spectrophotometer attached to emission and excitation correction units. The spectrophotometer was equipped with a thermostated quartz cuvette. All measurements were made at 25°C. The entrance and exit slits were set at 7 nm.

Ultracentrifugation

The sedimentation coefficient for methemoglobin following the addition of linoleate was measured in the Model E Beckman ultracentrifuge. 25 μl of 1M methemoglobin in a 0.2M sodium-phosphate buffer containing 7.5 mM linoleate was layered onto a 0.5M KCl solution in a 12 mm-thick band-forming cell (Vinograd et al., 1963). Sedimentation of the band at 24°C at an angular velocity of 56,000 rev/min was followed with an absorption scanner at 280 nm and 415 nm, near the peaks for globin and heme absorbance, respectively. Solutions containing the same concentration of methemoglobin but without linoleate were run as controls.

—Text continued on page 686

RESULTS

Affinity of methemoglobin to linoleate

The affinity of methemoglobin to linoleate is demonstrated by the affinity chromatography experiment presented in Figure 1.

Effect of linoleate on hemoglobin fluorescence and absorbance

It is seen (Fig. 2) that methemoglobin shows fluorescence emission in the region of 300–400 nm upon excitation at 280 nm. The addition of linoleate to this solution resulted in a pronounced increase in the fluorescence for both methemoglobin and oxyhemoglobin (not shown).

The influence of linoleate on the absorbance of methemoglobin in the visible region is shown in Figure 3. It is seen that the absorbance in the Soret region of heme absorption is reduced by the addition of linoleate and the peak is shifted to higher wavelength, while for oxyhemoglobin no change was observed. In contrast to linoleate, stearic acid had no effect on the absorption spectra in the Soret region of either methemoglobin or oxyhemoglobin.

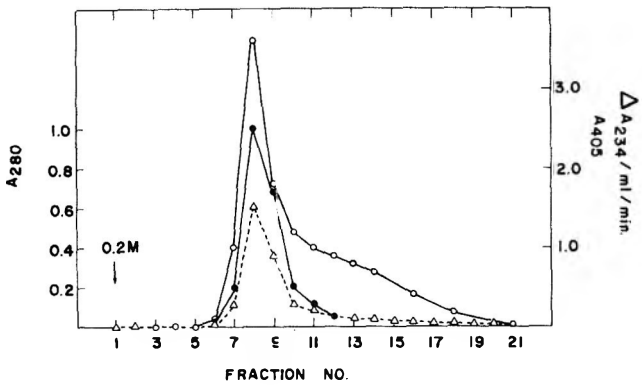

Fig. 1—Affinity chromatography elution pattern of methemoglobin on linoleyl aminoethyl sepharose. A solution of methemoglobin was chromatographed as described in Methods. Amount of protein was followed by absorption at 280 nm (Δ– –Δ), amount of methemoglobin at 405 nm (●——●), and linoleate oxidation by conjugated diene formation at 234 nm (○——○).

Effect of methemoglobin concentration on fluorescence

A linear relation was found between the amount of methemoglobin fluorescence at 330 nm and the concentration of methemoglobin at least up to a concentration of 50 $\times 10^{-8}$ M (Fig. 4). Upon the addition of linoleate the fluorescence increased at all methemoglobin concentrations.

Effect of linoleate concentration on fluorescence

Increasing the concentration of linoleate resulted in a significant increase in methemoglobin fluorescence (Fig. 5), following well the Michaelis-Menten equation with a K_m of 1.8×10^{-4} M.

Fluorescent yield

The fluorescent yield of oxy- and methemoglobin before and after the addition of linoleic acid was measured relative to that of tryptophan. A solution of hemoglobin and a solution of tryptophan of six times the molarity were used to which was added linoleate at a concentration of 1×10^{-4} M. The quantum yield of a solution relative to tryptophan is given by the following equation:

$$\frac{\phi \text{ sol}}{\phi \text{ try}} = \frac{F_{sol}}{F_{try}} \times \frac{\text{O.D.}_{try}}{\text{O.D.}_{sol}}$$

where F is the integral of the fluorescent spectra and ϕ is the quantum efficiency. Assuming the value of 14% for the quantum yield of tryptophan, we can obtain the absolute quantum yield of the solution (Table 1).

Effect of pH

The pH profile of methemoglobin fluorescence between

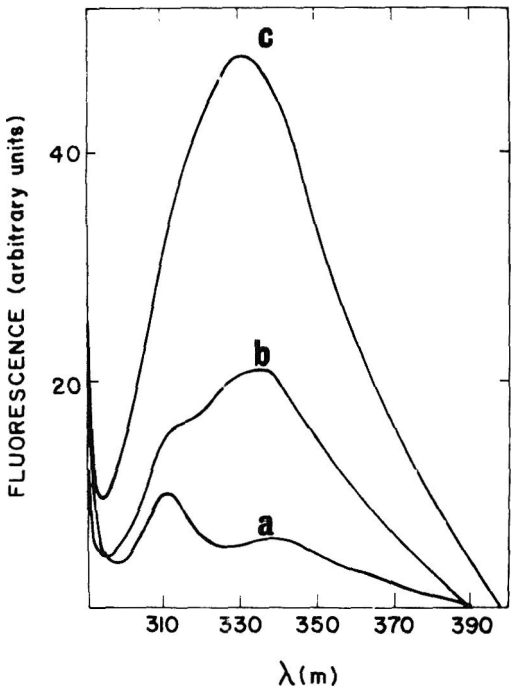

Fig. 2—Effect of linoleate on the fluorescence of methemoglobin under aerobic conditions. (a) 1×10^{-4} M linoleate in 3 ml of 0.2M sodium phosphate buffer, pH 6.5; (b) 100 μg methemoglobin dissolved in 3 ml of same buffer; (c) 1×10^{-4} M linoleate added to 100 μg methemoglobin in 3 ml of same buffer. All measurements were performed at 25°C. $\lambda_{excitation}$ = 280 nm. The peak at 312 nm is due to Raman scattering.

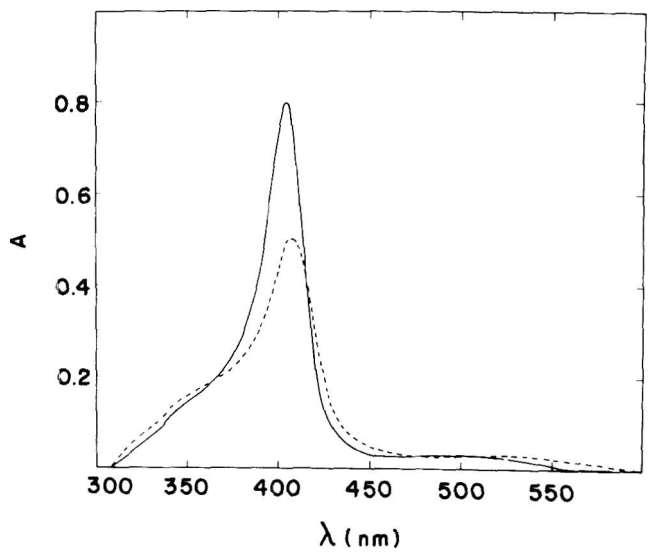

Fig. 3—Effect of linoleate on the absorbance of methemoglobin under aerobic conditions. The absorbance of 3.2×10^{-5} M methemoglobin in 0.2M sodium phosphate buffer, pH 6.5 was followed: in the absence (——), and in the presence of 1.9×10^{-4} M linoleate (---).

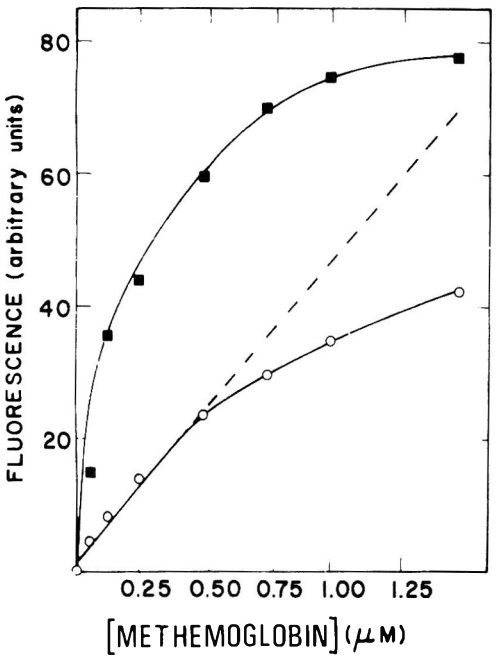

Fig. 4—Relation of fluorescence to methemoglobin concentration with and without addition of linoleate. Methemoglobin in 0.2M sodium phosphate buffer pH 6.5 (○—○). Methemoglobin in the presence of 10^{-4}M linoleate in the same buffer (■—■). The detergent Tween 20 in the linoleate solution caused at most a 7% increase in the fluorescence. This effect has been subtracted.

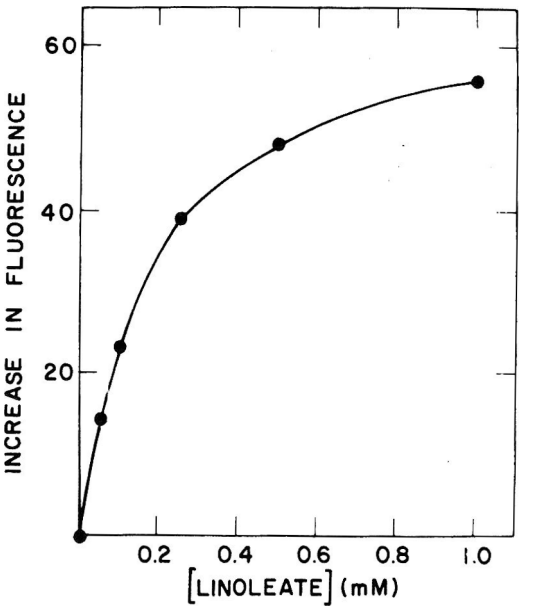

Fig. 5—Percent increase in methemoglobin fluorescence as a function of linoleate concentration. Linoleate at various concentrations was added to a 0.5 μM solution of methemoglobin in 0.2M sodium phosphate buffer, pH 6.5.

pH 3 and 5 shows a steep increase in methemoglobin fluorescence with a pK of about 4.1 (Fig. 6). The pronounced effect of linoleate in increasing methemoglobin fluorescence is limited to pH values above 4.

Effect of different fatty acids

The increase of methemoglobin fluorescence depends on the hydrocarbon chain length of the fatty acids used (Fig. 7). For saturated fatty acids the percent increase in fluorescence varies as $10^{0.092C}$, where C is the number of carbon atoms in the molecule. The percent increase in fluorescence in unsaturated fatty acids is three to four times higher than that for the saturated in the case of the 18-carbon chain.

Sedimentation studies

The concentration profiles of solutions of methemoglobin in a band-forming cell with and without the addition of linoleate were obtained. In both conditions one prominent peak is seen with a small heterogeneous heavier component which sediments faster. A peak at the meniscus due to the linoleate is also seen. The observed sedimentation coefficients and those corrected to standard conditions are given in Table 2. Similar concentration profiles were obtained at $\lambda = 280$ nm and at $\lambda = 418$ nm.

Table 1—Quantum yield of hemoglobins, hemoglobins with linoleate and tryptophan with linoleate[a]

Solution	λ_{max}	Relative yield	Absolute quantum yield[b] (%)
Tryptophan	358	1	14
Tryptophan + linoleic acid	358	0.892	12.5
methemoglobin	334	0.0151	0.21
methemoglobin + linoleic acid	334	0.0378	0.55
oxyhemoglobin	334	0.0014	0.02
oxyhemoglobin – linoleic acid	334	0.0210	0.29

[a] The measurements were for solutions of oxy- and methemoglobin and tryptophan (with the number of moles of tryptophan equal to the number of moles of tryptophan residues in the hemoglobin), at 25°C, pH 7.8, 0.2M phosphate buffer. The linoleate was added at a concentration of 1×10^{-4}M. Measurements were made immediately upon addition of the linoleate. The optical density of all solutions was 0.135 at 280nm.

[b] Assuming 14% for tryptophan

DISCUSSION

Fluorescent quenching

Hemoglobin and other heme-proteins are fluorescent in the ultraviolet region although their intrinsic fluorescence is highly quenched by the heme group to which excitation energy of the aromatic residues is transferred (Weber and Teale, 1959). Nevertheless, the fluorescence of hemoglobin, although small, is measurable as shown in this study (Fig. 2 and Table 1).

The fluorescence of hemoglobin is primarily due to the six tryptophan residues (two on each β chain, and one on each α chain), but its quantum yield is very low due to the quenching effect of the heme group (Weber and Teale, 1959). The critical distance for the transfer of energy from the tryptophan residues to the heme groups is 6 nm (Weber and Teale, 1959), a distance comparable to the size of the hemoglobin tetramer. Thus, it is expected that energy may be transferred from a tryptophan in one monomer to a heme group in another monomer of the tetramer (Morrison, 1965; Treffry and Ainsworth, 1974; Cassoly et al., 1967; Leonard et al., 1974).

Because of the heme quenching the hemoglobin quantum yield is low as can be seen from our results (Table 1), which are consistent with previous data: 0.5% (Konev et al., 1965), 0.6% (Kinderlerer et al., 1973), 0.1% (Cassoly et al.,

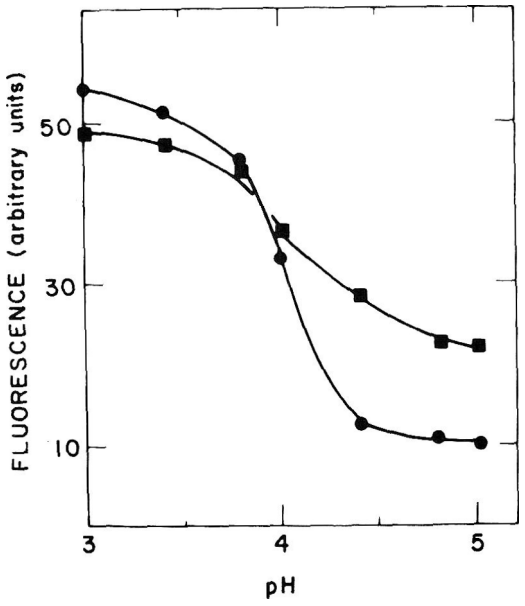

Fig. 6—pH profile of methemoglobin fluorescence and effect of linoleate. The fluorescence of 0.2 μM methemoglobin solution in 0.2M citrate-phosphate buffer was followed as pH was changed from pH 9 to pH 3. Between pH 9 and pH 5 (not shown) the fluorescence is unchanged within 10% of the value at pH 5. Methemoglobin solution alone (●——●); methemoglobin solution with 1 X 10^{-4} M linoleate (■——■).

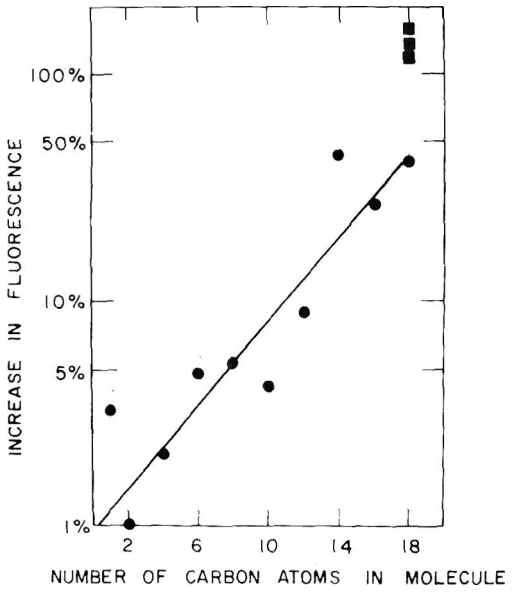

Fig. 7—Effect of hydrocarbon chain length of fatty acids on increase in methemoglobin fluorescence. To a 0.5 μM methemoglobin solution in 3 ml of 0.2M sodium phosphate buffer, pH 6.5, were added 0.12 mM ethanolic solution of fatty acids (10 μl ethanol in the medium). The ethanol itself had no effect on the fluorescence. The saturated acids tested (●) were: formic acetic, n-butyric, hexanoic, octanoic, decanoic, lauric, myristic, palmitic, and stearic (in order of increasing chain length). The unsaturated acids (■) were: linoleic, oleic, and linolenic (in order of increase in methemoglobin fluorescence). The line is the least squares fit to the logarithm of the % increase in methemoglobin fluorescence as a function of the number of carbon atoms in the lipid molecule.

Table 2—Sedimentation coefficients from band sedimentation experiment in analytical ultracentrifuge[a]

Solution in lamina	Temp (°C)	Obs. sedimentation coefficient of band, s_{obs}	Corrected sedimentation coefficient, $s_{20,w}$
metHb	21	3.10S	3.51S
metHb + linoleate	24	2.46S	2.58S

[a] The solution through which all the bands sedimented consisted of 0.5M KCl in 0.2M sodium phosphate buffer pH 7. 25μl of 1.3 X 10^{-5} M Hb solution with and without 7.5 X 10^{-3} M linoleate in the above buffer was layered in a lamina upon the KCl solution

1967), and less than 0.2% in the earlier measurements (Weber and Teale, 1959). This is to be compared to a quantum yield of 14.5% for the apohemoglobin (Cassoly et al., 1967).

The change in hemoglobin fluorescence as a consequence of fatty acid binding may be due to a change in the heme environment. The conformation of the protein and environment of the heme can affect the extent of quenching as can be seen from the fact that, while in most heme-proteins the quenching is by a factor of 100 or more, in horseradish peroxidase the quenching is only by a factor of 7 (Weber and Teale, 1959; Rosen, 1970).

The pronounced increase in methemoglobin fluorescence caused by the addition of linoleate (Fig. 2) may be explained by the partial decrease in the quenching effect of the heme due to dissociation of the hemoglobin tetramer or by a conformational change of the protein (Antonini and Chiancone, 1977). Dissociation would remove the quenching effect of two or three heme groups. The fact that fluorescence yield after the addition of linoleate is still an order of magnitude smaller than that of free tryptophan indicates that the heme group is still attached to the globin.

The possibility of a conformation change being responsible for the increase in fluorescence is supported by the effects of n-butanol on myoglobin fluorescence. n-Butanol causes a pronounced increase in fluorescence from 0.7% to 30% which, however, does not reach the free tryptophan levels, indicating that the heme is not detached (Brunori et al., 1972). In the same experiment the extinction coefficient at 412 nm decreased by a factor of two upon denaturation. The changes in fluorescence and absorption in that experiment are in the same direction as in the current results (Fig. 2 and 3). Brunori et al. (1972) attribute the effects to a change in the heme binding site, either in heme orientation or in the relative distance between donor groups and the heme. In the current experiment no wavelength shift is seen in the peak of the fluorescence spectrum upon the addition of the linoleate indicating that whatever structural changes may occur, they are not so drastic as to expose the tryptophan to the polar medium.

In our experiment there is an increase in methemoglobin fluorescence at acidic pH (Fig. 6) similar to that seen by Kirby and Steiner (1970) in apomyoglobin. However, in our case no increase in fluorescence was seen at neutral or alkaline pH. Upon the addition of linoleate we see an increase in fluorescence for pH greater than 4, while below this pH there is no increase in the already enhanced methemoglobin fluorescence. This behavior can be explained if the effect of linoleate on the methemoglobin conformation (even at neutral or high pH) is similar to that of the protons at low pH. The decrease in absorbance and shift in the absorption spectrum of methemoglobin is apparently due to the reduction of the heme group accompanying the linoleate peroxidation, since stearic acid which cannot undergo peroxidation has no effect on the absorption spectrum.

Moreover, the absorbance of oxyhemoglobin (already in the reduced state) was not affected by the fatty acids.

Acetic acid, a short fatty acid, is known to facilitate the dissociation of hemoglobin into subunits (Chiancone and Gilbert, 1965), but when the detergent SDS is added to human methemoglobin at concentrations sufficiently low so as not to cause dissociation or monomer formation, there is an increase in fluorescence and a decrease in absorption, very similar to the results of our study (Rogers, 1970). According to Rogers (1970), the fluorescent enhancement must be due to a decrease in the nonradiative energy transfer, and the environment around the residues might become more non-polar due to the combination with the detergent. A similar explanation may be proposed for the changes in spectra which we see, since the hydrocarbon chain of SDS is similar to that of long-chain fatty acids.

Hemoglobins peroxidize unsaturated fatty acids, such as linoleic acid. The resulting lipid peroxide and transient free-radicals which are produced are highly reactive and may react with and damage the hemoglobin molecule itself (Nishida and Nishida, 1965; Chio and Tappel, 1969). This mechanism may partly explain our results in the case of unsaturated fatty acids. However, there must be an additional major mechanism, since substantial changes in the fluorescent spectra are seen when saturated fatty acids are used (Fig. 7) which cannot undergo peroxidation.

We have seen in this study an increase in methemoglobin fluorescence and a decrease in absorption upon the addition of linoleate. This contrasts with the results in lipoxygenase (Finazzi-Agro et al., 1973; deGroot et al., 1975) where the fluorescence was markedly quenched by the addition of linoleate, while absorption between 300 nm and 450 nm was increased. Similar results were obtained with affinity-purified lipoxygenase in our laboratory (unpublished data). The different effects of linoleate on the fluorescence and absorption spectra of these two fatty acid peroxidation catalysts, provide the basis for development of a system which will distinguish between these two peroxidation catalysts in tissues and food.

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PROTEIN QUALITY OF MECHANICALLY PROCESSED (SPECIES) PRODUCT AND BONE RESIDUE

R. A. FIELD, YET-OY CHANG and W. G. KRUGGEL

ABSTRACT

Six samples of mechanically processed (species) product (MPP) from a Beehive deboner equipped with 0.46 mm holes in the cylinder, one sample of beef semimembranosus muscle and three samples of bone residue were analyzed. Protein efficiency ratios (PER) for MPP ranged from 1.44–2.90 compared to 2.91 for beef semimembranosus muscle. None of the bone residue samples supported rat growth giving a PER of ≤0. Regression equations to predict the PER of MPP were developed using the data obtained on the six MPP samples and the three bone residue samples described in this paper plus PER and amino acid data obtained on nine other samples of MPP. The regression equations were then tested using 24 different lots of MPP. Multiple regression equations using leucine and proline or hydroxyproline and isoleucine as independent variables were suitable for use in categorizing MPP on the basis of protein value. An alternative method of controlling protein quality in MPP by limiting the hydroxyproline content was suggested.

INTRODUCTION

PROTEIN QUALITY of meat is excellent as long as collagen which is high in hydroxyproline and other nonessential amino acids, is kept to a minimum (Bender and Zia, 1976; Noda et al., 1977; Lee et al., 1978). Protein quality of meat varies inversely with the amount of hydroxyproline present. The protein quality:hydroxyproline relationship exists because of a close negative correlation between the amount of all essential amino acids and the amount of hydroxyproline in meat (Dvorák and Vognarová, 1969). The FAO (1972) reported that the protein efficiency ratio (PER) of beef and veal ranged from 1.92–2.68 and Dvorák (1972) reported that net protein utilization of meat by rats ranged from 49–89%.

Alsmeyer et al. (1974) used eight samples of beef tissue to develop three equations for predicting PER from amino acid analysis. The three equations gave good results for meat and poultry samples where amino acid composition and actual PER values were available. The USDA (1977) used the three equations of Alsmeyer et al. (1974) to predict PER of mechanically processed (species) product (MPP) and found that while one equation gave estimated PER values which were close to the actual values, the other two equations of Alsmeyer et al. (1974) overestimated actual PER values of MPP.

Protein efficiency ratios for MPP, like those for hand boned meat, vary considerably (Chang and Field, 1977) and the USDA (1978) states that the PER of MPP must equal or exceed 2.5. However, the expense and time required for the assay make PER impractical for regulatory control. Equations such as those developed by Alsmeyer et al. (1974) appear to be the most practical solution. Nevertheless, Alsmeyer's equations were developed on beef muscle and collagen and it was thought that the equations may not apply to MPP which contains bone powder and bone marrow in addition to muscle and collagen. This study was undertaken to further investigate the amino acid composition and PER values of MPP and to determine if new or existing regression equations could be used to predict PER of MPP.

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EXPERIMENTAL

SIX SAMPLES of MPP from a Beehive mechanical deboner equipped with 0.46 mm holes in the cylinder, one sample of beef semimembranosus muscle and three samples of bone residue were evaluated for moisture, fat, ash, calcium, iron, amino acid composition and rat PER. The MPP samples were from: (1) Choice beef plates which had been trimmed of meat except for that between the ribs; (2) lamb neck bones made up of the ribs and the cervical and thoracic vertebrae from square-cut lamb shoulders; (3) all bones from carcasses of 1– to 2–wk old veal calves; (4) closely trimmed cow wholesale ribs from the ribs and thoracic vertebrae; (5) pork neck bones removed by normal packing plant procedures; and (6) pork neck bones similar to those in number five which were hand trimmed to remove some of the lean. Beef semimembranosus muscle from a Good grade steer was included for comparative purposes. Three samples of bone residue (bone and other tissue discarded during the mechanical deboning process) from Utility cow neck bones, Choice beef neck bones and Choice lamb neck bones were also studied.

Percent moisture in the MPP and bone residue samples was determined by oven drying at 100°C to a constant weight. Fat and ash were obtained by standard methods (AOAC, 1970). Calcium and iron were determined by atomic absorption spectrophotometry as outlined by the Perkin-Elmer Corp. (1964). Amino acid analysis and the PER study were carried out as outlined by Chang and Field (1977) except 10 male albino rats of the Sprague-Dawley strain per protein source were used for PER.

Regression equations to predict the PER of MPP were developed using the data obtained on the six MPP samples and the three bone residue samples described in this paper plus PER and amino acid data obtained in our laboratory on nine other samples of MPP. The additional data for developing prediction equations were those described by Chang and Field (1977).

Prediction equations for estimating PER were then applied to 24 different MPP samples (USDA, 1977) on which complete amino acid analysis, calcium, iron and PER were available. Specific PER values for the lots of MPP, not averages as is described by the USDA (1977), were furnished through personal correspondence with Elizabeth Murphy of FSQS. Differences in PER were analyzed by least-squares analysis of variance (Steel and Torrie, 1960). Prediction equations, along with multiple correlation coefficients and standard errors of estimate, were also obtained as outlined by Steel and Torrie (1960). Simple correlations between actual PER on the 24 MPP samples studied by the USDA (1977) and PER of the same samples as estimated from prediction equations were obtained.

RESULTS & DISCUSSION

MOISTURE, fat, ash and calcium percentages for the six samples of MPP in Table 1 were quite variable and typical of other MPP samples (Field, 1976). MPP always has higher values for ash and calcium than hand boned meat such as beef semimembranosus muscle because of bone powder which is present. Higher iron values for MPP when compared to beef muscle is due in part to red marrow. Sanchez et al. (1978) found that red marrow contains 12.5 mg of iron/100g compared to 3.2 mg of iron/100g for the beef muscle in this study.

Bone residue samples contained less moisture and fat and much more ash and calcium than MPP reflecting the high bone content. Protein content of bone residue can be approximated at 20–25% by subtracting the moisture, fat and ash percentages from 100%. The high protein content is a result of high amounts of collagen in bone and other tissue discarded during mechanical deboning (Field, 1976).

Amino acid composition of MPP in Table 2 is similar to other amino acid values for MPP (USDA, 1977) and for hand boned meat (Happich et al., 1975). There is a striking increase in the amount of proline, glycine and hydroxyproline when these amino acids in bone residue are compared to the same amino acids in MPP. In addition, cystine and methionine are much lower in bone residue protein than in MPP. Since collagen is high in proline, glycine and hydroxyproline and low in the sulfur containing amino acids (Osborne et al., 1971; Eastoe et al., 1973) the data in Table 2 furnish good evidence that the major protein in bone residue is collagen. The amino acid composition of bone residue is similar to that of residue from mechanically desinewed meat as reported by Hendricks et al. (1977). Collagen in bone residue comes from bone and from tendon and other connective tissue which the mechanical deboner removes from the meat. Amount of collagen present in bone residue would be expected to vary with yield of MPP, with the item mechanically deboned and with type of equipment used.

Growth response, food intake and PER for rats fed MPP at the 9% protein level are presented in Table 3. Lamb neck bones produced MPP which was similar in PER to beef semimembranosus muscle. Lamb neck bones, because of their smaller size, have more surface area per unit weight of bone on which lean meat is attached after hand boning. The higher proportion of lean on lamb neck bones when compared to the other bones listed in Table 3 is undoubtedly related to the higher PER. Veal bones and untrimmed pork neck bones are next to lamb neck bones in terms of the amount of lean left on the bones and they are also next in line in terms of PER. Although the PER for MPP from beef bones in Table 3 is low, beef bones often yield meat with a high PER (USDA, 1977). A high PER in MPP is usually obtained by keeping the calcium content below the maximum limit of 0.75% (USDA, 1978) or by leaving greater amounts of lean on the bones prior to mechanical deboning. The effect of amount of lean on the bone in relation to PER values is illustrated by the PER of 2.60 in MPP from regular pork neck bones when compared to the PER of 2.00 in MPP from closely trimmed pork neck bones.

Values for bone residue are not listed in Table 3 because the protein in bone residue failed to promote growth in rats. All three bone residue samples for which composition (Table 1) and amino acid values (Table 2) are given had PER values of $\leqslant 0$. PER values of $\leqslant 0$ for bone residue were expected since bone residue is high in collagen which is lacking the essential amino acid tryptophan in addition to being low in most other essential amino acids. When collagen is fed to rats as the only protein source the PER is always $\leqslant 0$.

Four equations for predicting PER developed from MPP and bone residue produced by a Beehive mechanical deboner at the University of Wyoming and one equation developed by Alsmeyer et al. (1974) on eight samples of beef

Table 1–Composition of mechanically processed (species) product and bone residue

Protein source	Moisture (%)	Fat (%)	Ash (%)	Calcium (%)	Iron (mg/100g)
MPP					
Choice beef plates	52.7	22.1	3.9	1.15	8.0
Lamb necks	56.3	22.5	2.1	.54	5.0
Cow ribs	46.7	31.6	3.5	.94	5.0
Veal bones	64.7	7.6	3.4	1.08	6.0
Pork neck (regular)	56.6	25.6	2.0	.46	3.8
Pork neck (trimmed)	53.6	27.7	2.2	.54	4.5
Beef semimembranosus	70.9	6.7	1.0	.01	3.2
Bone residue					
Cow neck	41.9	3.7	31.5	12.4	–
Choice beef neck	41.0	4.9	30.1	11.9	–
Lamb neck	49.4	2.7	26.2	8.3	–

Table 2–Amino acid composition of mechanically processed (species) product and bone residue discarded from the mechanical deboner[a]

Amino acid	MPP source[b]					Bone residue source[b]		
	BP	LN	CR	PN	PB	CN	BN	LN
Asp	8.02	7.24	7.64	8.14	8.17	7.47	7.89	7.91
Thr	3.82	4.74	3.84	3.92	3.93	2.51	2.29	2.93
Ser	4.26	4.37	4.69	3.95	4.24	3.29	3.34	2.94
Glu	12.91	11.63	12.26	12.94	12.02	11.58	11.00	11.72
Pro	6.53	5.60	8.34	5.62	5.61	11.93	10.26	10.56
Gly	7.61	5.08	7.60	6.62	6.59	14.16	14.68	14.42
Ala	7.31	5.18	6.51	6.10	6.48	8.26	8.55	9.01
Cys½	1.20	1.50	1.10	1.50	1.40	.20	.10	.22
Val	7.24	7.01	7.89	7.28	7.67	4.85	4.29	4.15
Met	2.15	3.18	2.02	2.94	2.91	1.66	1.80	1.51
Ile	3.69	4.39	3.78	5.07	5.09	2.61	2.89	2.62
Leu	7.20	7.70	6.20	8.09	6.15	5.40	5.92	5.19
Tyr	2.45	3.25	2.72	3.54	4.02	1.90	1.62	1.41
Phe	4.56	4.49	4.35	4.70	5.02	3.74	3.60	3.35
Lys	6.71	7.41	8.15	8.02	7.24	4.98	5.50	5.26
His	3.28	3.54	3.03	3.73	3.87	1.65	1.40	1.61
Arg	7.06	7.49	7.74	7.91	6.76	8.03	8.65	8.21
Hyp	3.85	1.81	4.48	2.32	3.05	10.86	10.07	10.21

[a] Each value in the table represents an average of duplicate analyses and is expressed as g/100 protein.

[b] BP = Choice beef plates trimmed of meat; LN = lamb neck bones; CR = Cow wholesale rib bones; PN = pork neck bones (regular trim); PB = pork neck bones (close trim); CN = cowneck bones; BN = Choice beef neck bones.

Table 3–Protein efficiency ratios for rats fed mechanically processed (species) product, hand boned beef and casein

Protein sources	Initial wt, g	Final wt, g	Total gain, g	Total food consumed, g	Total protein eaten, g	PER g gained/g protein eaten[a]
Choice beef plates	56.1^{cd}	110.6^{d}	54.0^{e}	313.9^{ef}	28.4^{de}	1.90^{d}
Lamb neck bones	61.9^{b}	151.8^{bc}	90.0^{cd}	344.8^{cde}	31.0^{cd}	2.90^{b}
Cow rib bones	57.4^{cd}	99.8^{e}	42.4^{f}	324.9^{de}	29.2^{d}	1.44^{e}
Veal bones	56.4^{cd}	153.9^{b}	97.5^{bc}	404.4^{b}	36.4^{b}	2.67^{c}
Beef semimembranosus	55.9^{cd}	147.0^{bc}	91.1^{cd}	347.4^{cd}	31.3^{cd}	2.91^{b}
Pork neck bones (reg)	56.6^{cd}	143.3^{c}	85.7^{d}	364.5^{c}	32.8^{c}	2.60^{c}
Pork neck bones (Close trim)	58.1^{c}	109.5^{b}	51.4^{e}	285.7^{f}	25.7^{e}	2.00^{d}
Casein (control)	54.6^{d}	155.8^{b}	101.2^{b}	377.4^{bc}	34.0^{bc}	2.97^{b}

[a] Ten rats per group. Rats were fed for 5 wk.

[b,c,d,e,f] Means within columns having a common superscript letter are not significantly different ($P \leqslant 0.05$).

tissue are shown in Table 4. The PER data obtained at the University of Wyoming ranged from 0 on bone residue samples to 2.90 on MPP from lamb neck bones. When hydroxyproline was used as the only independent variable the correlation coefficient was −0.82 confirming the work of Dvorák (1972) who found a close negative relationship between the amount of hydroxyproline in meat and net protein utilization. When equation one utilizing hydroxyproline was applied to the 24 MPP samples described by the USDA (1977) the actual PER determined from rat growth was underestimated (2.38 vs 2.63) and the simple correlation between the actual and estimated PER was 0.48. The relatively low correlation between the actual and estimated PER is not surprising when the narrow range of 2.3–3.0 for PER of the USDA (1977) data is considered. Regression equation two using leucine and proline as the independent variables (R = 0.86, SE = 0.45) and regression equation three using hydroxyproline and isoleucine (R = 0.83, SE = 0.49) as independent variables also underestimated the actual PER values of the USDA (1977) data. Nevertheless, these two equations were more suitable for use in categorizing MPP on the basis of protein value than the others listed because simple correlations between estimated PER and actual PER were higher. Isoleucine may be useful in equation three as an independent variable because isoleucine is low in the bone marrow found in MDM (Field et al., 1978). Leucine (equations 2 and 5) is low in collagen but marrow as well as lean meat are an excellent sources of leucine (Field et al., 1978). Collagen accounts for 3.26% of the fresh weight of commercial samples of MPP when the average hydroxyproline level of 0.45% (USDA, 1977) is multiplied by a factor of 7.25 (Eastoe and Leach, 1958) to obtain collagen content. Therefore, the low level of leucine in collagen should not be a factor influencing the PER of MPP in samples where the collagen content is low. However, leucine was useful in developing equations two and five where some MPP samples which were high in collagen were included (Table 4). Simple correlations between leucine, proline and isoleucine with PER in the 18 samples of MPP used to develop equations one through four were 0.51, −0.79 and 0.51 respectively indicating that these amino acids, as well as hydroxyproline (r = −0.82) are related to PER values.

Equation four in Table 4, which utilized iron, leucine and calcium as independent variables to predict PER, had a high multiple correlation coefficient and a low standard error of estimate but when the equation was applied to the USDA (1977) data a low correlation of 0.28 between the actual and estimated PER was obtained. The 24 MPP samples studied by the USDA (1977) were obtained from several different sources and from different makes of mechanical deboners. Apparently calcium and iron content were of value in estimating PER values when the MPP came from one source but calcium and iron were of little value in estimating PER on the MPP studied by the USDA (1977) which came from different makes of machines in different areas.

Equation five in Table 4 was developed by Alsmeyer et al. (1974) using eight samples of beef tissue which varied greatly in PER. The multiple correlation coefficient for PER with leucine and proline was higher (R = 0.99) on the eight beef tissue samples than any of the multiple correlation coefficients obtained in the present study with MPP. Nevertheless, the simple correlation between the actual and estimated PER of 0.48 was the same as that for hydroxyproline alone.

Most commercial lots of MPP have high PER values typical of those studied by the USDA (1977). It is possible that because of the many variables of meat source, connective tissue amount, marrow content and type of equipment used, no one equation will be completely satisfactory for estimating PER values of commercial samples of MPP. However, lots of MPP with low PER values should easily be identified using the equation of Alsmeyer et al. (1974) or equations one, two or three in Table 4. Since PER values of MPP may be over or underestimated using the equations and since the usefulness of PER as an indicator of protein quality is questionable (Hegsted and Chang, 1965; Pellett, 1978), alternative methods of controlling protein quality in MPP should be considered. One simple inexpensive method which could easily be used in regulatory control would be to place a maximum value on the amount of hydroxyproline allowed in MPP. An additional precaution based upon our study would be to require lots of MPP to have minimum values for isoleucine or leucine. Perhaps the most logical approach would be to drop all PER and amino acid

–Continued on page 695

Table 4–Means and correlations obtained when prediction equations for estimating PER were applied to amino acid composition and PER values of 24 MPP samples not used in developing the equations[a]

Equation no.		Means for PER: Estimated from equation	Means for PER: Rat growth	Correlation between actual and estimated PER
1	PER[b] = 3.28 − 0.27 (Hyp) R = −0.82 SE = 0.49	2.38	2.63	0.48*
2	PER[b] = 1.06 + 0.35 (Leu) −0.24 (Pro) R = 0.86 SE = 0.45	2.40	2.63	0.60**
3	PER[b] = 2.64 − 0.25 (Hyp) + 0.14 (Ile) R = 0.83 SE = 0.49	2.35	2.63	0.56**
4	PER[b] = 1.29 − 0.10 (Fe,mg/100g) + 0.24 (Leu) −0.30 (Ca, %) R = 0.95 SE = 0.28	2.57	2.63	0.28
5	PER[c] = −0.684 + 0.456 (Leu) − 0.047 (Pro) R = 0.99 CV = 98.41%	2.70	2.63	0.48*

[a] Amino acids used to derive the equations were expressed in g/100g protein.

[b] The regression equation along with the R (multiple correlation coefficient) and the SE (standard error of estimate) were developed on 18 lots of MDM and bone residue from beef, pork and lamb with amino acid composition and PER values typical of those listed in Tables 1 and 2 respectively.

[c] The regression equation, multiple correlation coefficient and CV (coefficient of variation) was derived by Alsemeyer et al. (1974) using eight samples of beef tissue.

* $P < 0.05$

** $P < 0.01$

FAT CELL RUPTURE IN A COMMINUTED MEAT BATTER AS A DETERMINATIVE FACTOR OF HEAT STABILITY

B. J. TINBERGEN and W. J. OLSMAN

ABSTRACT

A method was developed for the selective extraction of fat from ruptured fat cells in comminuted sausage batters. It was found that over a wide range of chopping temperatures (4–28°C) the level of extractable fat in an unheated meat batter is significantly correlated ($P < 0.001$) with the percentage of fat separation after heating to 80°C or 120°C. The results indicate that the integrity of the fat cells in a sausage batter rather than the availability of released fat for emulsification determines the heat stability of the batter.

INTRODUCTION

THE TERM 'meat emulsion' is widely used for the description of various comminuted meat systems (Swift et al., 1961; Carpenter and Saffle, 1964; Inklaar and Fortuin, 1969; Acton and Saffle, 1970, 1972). However, these systems not always possess structures akin to that of a true and simple emulsion: one liquid phase finely dispersed in another. For emulsification of fat in comminuted meat mixtures, it has to be released from the adipose tissue cells and should be in the liquid state. The latter is not achieved at the currently used chopping temperatures. Furthermore, commercial fat emulsifiers have been found to decrease rather than increase fat binding in meat products (Meyer et al., 1970). Recent studies show that, apart from the raw material composition, the heat stability of meat products depends on chopping conditions (speed, time and temperature) and on the extent of breakdown of the adipose tissue cells (Evans and Ranken, 1975b).

Increasing amounts of free fat in the mixture reduce its heat stability (v.d. Oord and Visser, 1973). Microscopic examination of heat-processed sausages showed that under optimal conditions most of the fat cells maintain their integrity despite extensive grinding and chopping. After comminution the major part of the fat is dispersed in the mixture as intact cells or cell clusters (Evans and Ranken 1975a; Tinbergen et al., 1977). Therefore Brown and Toledo (1975) proposed to discontinue the use of the word 'emulsion' and replace it by the term 'batter'. The resistance of the fat cells to damage during comminution was found to depend largely on the type of fat tissue and on its storage history (e.g. frozen vs unfrozen).

It is unknown, however, to what extent the fat cell rupture is related to the fat separation after heating. Microscopic examination only allows a rough impression of the degree of fat cell damage in the meat batter. This study represents a method for the selective extraction of the exposed fat from ruptured cells in the meat batter. A relationship between the amount of extracted fat before and separated fat after heating implied that a measurable property had been found that is predictive of the heat stability of a critical product.

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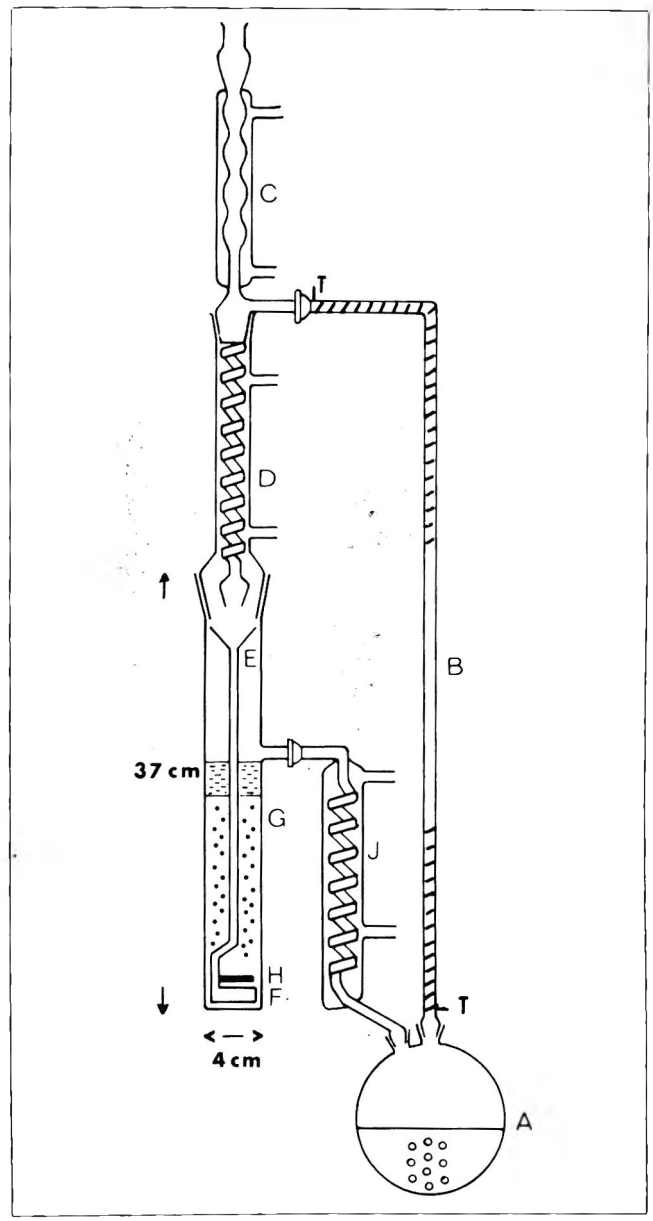

Fig. 1–Extraction apparatus for the selective extraction of fat from ruptured fat cells.

EXPERIMENTAL

Extraction of free fat

A slurry of 6.0g meat batter in 150 ml water was prepared with caution by gentle stirring with a glass rod to avoid any damage to the fat cells due to shear forces. The slurry was quantitatively transferred to the extraction chamber G of the all-glass percolation-extraction apparatus (Fig. 1). The slurry must remain 2–3 cm below the side arm which is connected to the cooler (J) by means of a ball joint. The solvent collection tube (EF), with a magnetic stirring bar

–Text continued on page 694

Table 1—Product formulae and proximate analytical data for three batter groups

Batter group symbol (see Fig. 2)	% lean beef	% lean pork	% back fat	% added ice/water	% salt + seasonings	% fat	% protein	% moisture
▲	49.8	25.0	–	21.7	3.5	5.9	9.0	43.0
o	29.9	15.0	40.0	13.0	2.1	39.6	10.3	48.0
X	28.0	28.0	31.0	10.8	2.2	35.5	11.9	50

(H) placed on top of the hollow glass disk (F), is inserted into (G). The extraction chamber (G) is mounted onto the apparatus. The vapor from boiling n-hexane contained in flask (A) rises in tube (B), which is heated by electric heating tape spirally wound around it. The vapor condenses in the water-cooled bulb-cooler (C) or helix-cooler (D). The condensed hexane drops into funnel (E) which is connected to the glass tube debouching into disk (F). The hexane penetrates the meat batter slurry via the topside of (F) which is perforated with 1 mm wide holes. The magnetic stirring bar (H) keeps the slurry in motion and dissipates the hexane droplets more or less at random over the slurry. The hexane rises to the top of the aqueous phase. Fat-containing hexane accumulates there until it reaches the side-arm and flows back into the boiling flask (A) by way of the helix-cooler.

The extraction is terminated after 3 hr. The hexane is completely evaporated and the remaining fat residue is weighed. The mean percentage of extractable fat from the raw meat batter was calculated from duplicate determinations. In Table 2 both extractable and separated fat percentages are given for 20 samples. The extraction procedure could become a practical tool for the determination of the heat stability of meat batters, provided a substantial reduction in extraction time can be achieved by a more efficient procedure.

Sample material

Samples were taken from three groups of batters of different composition. Product formulas and analytical data are given in Table 1. To obtain marginal compositions with regard to heat stability, binders such as (poly)phosphates and starch were deliberately omitted. All batters were prepared in a 40L Kilia laboratory bowl chopper with three knives (rotation speed: knives 2500 rpm, bowl 20 rpm).

Table 2—Chopping times, final temperatures and percentages of free extractable fat in unheated meat batters and corresponding percentages of fat separated after heating in cans for 60 min at 80°C

Batter group symbol (see Fig. 2)	Batter no.	Chopping time in min	Attained temp °C in chopper	% fat extracted from meat batter	% fat separation after heating
▲	1	10	15	3.1	0.0
▲	2	8	5[a]	6.1	0.0
▲	3	5	4	6.4	0.0
X	4	10	20	3.3	0.0
X	5	20	22	4.0	0.3
X	6	10	20	4.7	0.5
X	7	10	23	20.0	19.0
X	8	12	25	26.2	22.1
o	9	12	25	21.0	2.5
o	10	6	7	8.7	3.5
o	11	8	10	14.0	7.8
o	12	11	15	9.4	11.8
o	13	14	25	16.6	14.8
o	14	16	28	15.0	17.0
o	15	13	25	24.0	18.3
o	16	14	25	27.1	19.0
o	17	14	11[a]	23.8	20.9
o	18	17	19	27.7	24.0
o	19	20	28	35.0	27.6
o	20	22	20[a]	41.2	31.0

[a] Reduced final chopping temperature obtained by using crushed ice instead of water (+ 2°C)

The degree of comminution was varied by different chopping times. This resulted in different final temperatures of the batters (see Table 2). In some experiments the final temperature after a certain chopping time was lowered by chopping with crushed ice instead of water (compare batters 2 vs 11 and 16 vs 17) to obtain normal chopping temperatures in the range 5–11°C.

Determination of separated fat

All batters were filled into cans (approx 330g net weight, Φ 76 mm, height 88 mm) and pasteurized for 60 min at 80°C to a core temperature of 73°C. In most cases the same batters were also sterilized for 60 min at 120°C (F_o = 1.6), representing a very intensive heat treatment. After cooling the cans to 23°C and decantation of the jelly, the remaining separated fat was weighed.

For each product the mean percentage of separated fat was calculated from the results obtained on five pasteurized cans.

RESULTS & DISCUSSION

FIGURE 2 SHOWS the correlation between the percentages on sample weight basis of the free extractable fat and the separated fat after heating at 80°C. The regression equations are

$$Y_{\text{extr. fat\%}} = 0.977\, x_{\text{separated fat\%}} + 5.14 \qquad (1)$$

and

$$X_{\text{separated fat\%}} = 0.864\, y_{\text{extr. fat\%}} - 2.57 \qquad (2)$$

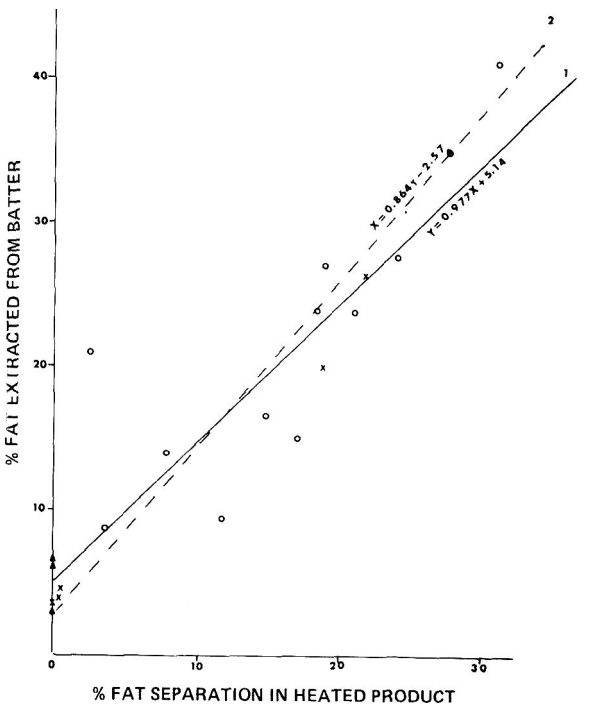

Fig. 2—Correlation between the amounts of free extractable fat in a batter and fat separated after heating at 80°C.

The correlation coefficient is 0.915 which is highly significant ($P < 0.001$). A similar correlation was found after sterilization at 120°C: The regression line had practically the same slope, but was shifted to higher fat separation percentages:

$$Y_{\text{extr. fat\%}} = 0.992\, x_{\text{separated fat\%}} + 2.23$$

$$X_{\text{separated fat\%}} = 0.825\, y_{\text{extr. fat\%}} + 2.05$$

Microscopic examination of the batter residue after extraction revealed that all remaining fat was enclosed in intact cells that evidently had resisted the mild extraction treatment.

The results strongly support that the fat separated upon heating was present as free fat (i.e. exuded from ruptured tissue cells) in the batter.

If availability of free fat for emulsification would be a prerequisite to prepare a stable product, one would expect that stability increases with the extent of fat cell damage. This is opposite to what we actually found. Evidently, the meat proteins are unable to emulsify the released fat in our products. Only in the presence of emulsifying additives, such as polyphosphates, stable products were obtained from the batter of which no free fat could be extracted (Tinbergen, 1978). Our results indicate that the integrity of the fat cells in the processed meat batter rather than the availability of released fat for emulsification determines its heat stability.

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PROTEIN QUALITY OF MPP . . . From page 692

requirements since MPP is limited to 20% of the product to which it is added (USDA, 1978). According to the expert panels' protein advisor, (USDA, 1978) low levels of collagen in MPP would be supplementary to other proteins and collagen could increase the overall nutritional value of MPP. Another reason for dropping all PER and amino acid requirements for MPP is that most of us consume about twice as much protein as we need and if we have a shortage in our diet, it certainly is not in the essential amino acids.

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FROZEN CONCENTRATED CULTURES OF Kluyveromyces Fragilis

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ABSTRACT

Concentrated cultures of *K. fragilis* Y-1156, prepared by suspending the yeast cells in 10% nonfat milk solids (NFMS) survived better at −19°C than those held in liquid nitrogen (−196°C). Cells from the mid-exponential growth phase survived better at −19°C than those from the stationary phase. In contrast yeasts frozen at −196°C exhibited highest levels of survival at the mid-stationary phase. The addition of glucose to the suspending medium, in amounts up to 30%, resulted in increased storage stability.

INTRODUCTION

A LIMITED AMOUNT of work has been reported on the freezing and frozen storage of yeast cultures, particularly in liquid nitrogen. The rate at which the cells are frozen appears to be one of the most important factors in determining survival. When *Saccharomyces cervisiae* was cooled rapidly to −30°C or below, less than 0.01% of the cells survived (Mazur, 1961a). However, when these cells were slowly cooled to the same temperature, as many as 50% survived. Hwang (1966) found that he could preserve pathogenic strains of yeast in liquid nitrogen by slowly freezing the cultures to −35°C (1°C/min) and then using an accelerated rate to lower the temperature to −196°C.

The temperature and time of yeast culture storage is also important. Wood (1957) and Mazur and Schmidt (1968) report little inactivation of yeast cultures frozen and stored at temperatures above −10°C. They found that survival was not due to the time a culture was held at a particular temperature. However, Meryman (1966) stated that survival of cells frozen at temperatures above −70°C is affected by storage time, the extent of damage to the cells being dependent on the suspending medium. Mazur (1961b) reported that damage to cells exposed to low temperatures occurred almost immediately when cells were rapidly cooled to −30°C. Under such conditions survival after a 1 min exposure was 1%. With extended exposure of up to 90 min, there was little additional damage.

The objective of this study was to find a means of storing concentrated yeast cultures in a frozen state, at either −19°C or −196°C, in such a manner that the culture would retain its viability and activity.

EXPERIMENTAL

Source and maintenance of cultures

Kluyveromyces fragilis Y-1156, Y-1171, Y-108, and Y-1196 were obtained from the Northern Regional Research Laboratory (Peoria, IL), and *K. fragilis* 50-16, 50-29, 55-1, 72-297, and C-106 were obtained from the Dept. of Food Science & Technology, University of California at Davis. All stock cultures were maintained by inoculating lactose agar slants (2.5% agar, 2.0% lactose, 1.0% peptone, and 0.1% yeast extract) and incubating them at 35°C for 18 hr. The cultures were stored at 6°C between weekly subcultures. One loop of growth was inoculated into 10 ml of the appropriate sterile broth and incubated 18 hr at 35°C. An additional subculture was made using 1% inoculum into the broth prior to experimental use.

Determination of yeast viability and activity

Colony counts of yeasts were determined using a pour plate method. Dilutions were prepared using peptone dilution blanks containing 0.1% peptone and 0.001% Antifoam A Emulsion (Sigma Chemical Co.). The initial dilution for the concentrated yeast cultures was made by weighing 1g of the concentrated culture into an empty sterile dilution bottle and aseptically pouring 99 ml of sterile diluent into the bottle. Additional dilutions were made as described in *Compendium of Methods for the Microbiological Examination of Foods* (Speck, 1976). Duplicate plates were poured with melted Sabouraud Dextrose Agar and incubated for 48 hr at 35°C. All colonies visible with the aid of a Quebec colony counter were counted.

An activity test was employed to compare the abilities of the strains of *K. fragilis* to utilize the lactose in whey. Cottage cheese whey was heated in a boiling water bath for 30 min to precipitate whey proteins. It was then cooled and centrifuged at 3000 × G for 10 min. Fifty ml of the supernatant solution was aseptically transferred to a sterile 250 ml Erlenmeyer flask and inoculated at 1% with a freshly prepared *K. fragilis* lactose broth culture. The flask was incubated in a 35°C reciprocating shake water bath (82 strokes of 40 mm per min) for 16–18 hr. This served as the inoculum for the activity test. For each *K. fragilis* culture, 60 ml of unheated cottage cheese whey was inoculated (1%) with the protein-free whey culture and incubated in the 35°C shake water bath. Five ml samples were taken at predetermined times and the yeasts enumerated by colony counts. The samples were assayed for lactose by an anthrone method similar to that described by Morris (1948).

Preparation of concentrated cultures

K. fragilis was subcultured twice in pepsinized whey prior to use in preparing the concentrated cultures. The pepsinized whey was prepared by reconstituting Krafen dried, sweet whey (Kraft Industrial Foods Division, Chicago, IL) as a 5% aqueous solution. The mixture was adjusted to pH 3.0 with 30% citric acid. Pepsin (Sigma Chemical Co.) was added to the whey (25 mg/100 ml), and the medium incubated for 30 min in a 37°C water bath. The digested whey was adjusted to pH 6.5 with 20% NaOH and autoclaved for 15 min at 121°C. The resulting medium had no precipitate.

The prescribed amount of growth medium was inoculated with a fresh pepsinized whey culture of the yeast at a level of 1%. It was placed in the 35°C reciprocating shake water bath and incubated for 15 hr. The cells were harvested by centrifuging at 15000 × G for 10 min at 10°C, resuspended in cold sterile 10% nonfat milk solids (NFMS) and dispensed into sterile plastic freezing vials. The vials were immediately placed in a freezer (−19°C) or submerged in liquid nitrogen (−196°C) for storage.

Effect of added glucose on yeast cell survival

To determine the effect of various glucose concentrations in the suspending medium on the survival of yeasts over prolonged storage periods, experiments were conducted in which glucose in amounts of 0, 10, 20 and 30% were added to the NFMS. The milk and glucose were autoclaved separately, combined, and heated in a boiling water bath to dissolve the sugar. The mixtures were chilled in an ice bath prior to using them for preparing concentrated cultures. The mixtures containing the yeast cells were held in an ice bath at 0°C for 10–15 min prior to freezing. Vials of each concentrated culture were frozen at −19°C and −196°C, and samples were plated before freezing and after 1, 7, 14 and 21 days of frozen storage.

Effect of a dual-freezing treatment on yeast cell survival

To test the effect of a freezing at −19°C followed by frozen

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Table 1—Growth of Kluyveromyces fragilis in cottage cheese whey

Strain	Count/ml[a] 11 Hr	14 Hr
Y-1156	2.4×10^8	3.2×10^8
Y-1171	3.2×10^8	3.5×10^8
Y-108	3.6×10^8	3.9×10^8
Y-1196	3.2×10^8	2.9×10^8
50-16	1.8×10^8	2.6×10^8
50-29	1.7×10^8	2.6×10^8
55-1	9.1×10^7	2.0×10^8
72-297	2.6×10^8	3.1×10^8
C-106	1.2×10^8	2.0×10^8

[a] Each count represents the average of two trials

storage at −196°C on the survival of the concentrated cultures, additional experiments were conducted. The protocol was the same as that described in the previous section except that additional concentrates were prepared so some samples could be frozen at −19°C for 24 hr and then transferred to liquid nitrogen for storage.

Statistical analyses

An analysis of variance procedure was used to determine if significant differences occurred among glucose concentrations for cell concentrates frozen at −19°C for 21 days, and to determine if there was significant variation among replicates of these same experiments. The least significant difference procedure was used to compare mean cell survivals of yeasts frozen and stored for extended storage periods with the 0-time count for each glucose preparation (Steel and Torrie, 1960).

RESULTS

THE IDENTITIES of the yeasts used in this study were confirmed as *K. fragilis* according to procedures outlined by Lodder (1970). The data comparing the abilities of the nine strains of *K. fragilis* to grow in cottage cheese whey are presented in Table 1. The amount of growth based on colony counts was similar for all strains after 14 hr. When the yeasts were compared for their ability to utilize the lactose in whey, numerous differences were observed (Fig. 1). The data are presented graphically as percent lactose remaining after 8, 11 and 14 hr. *K. fragilis* 50-29, 50-16, and 55-1 used less than 30% of the lactose in a 14-hr period, whereas approximately 90% or more of the lactose was used in the whey cultured with *K. fragilis* 72-297, Y-1156, Y-108, Y-1171, and Y-1196 in 14 hr. *K. fragilis* C-106 was intermediate in lactose utilization, with 45% remaining at the end of the test period. From the data collected in this phase of the study, *K. fragilis* Y-1156 was chosen for further experimentation in culture concentrate preparation and freezing trials. Its ability to grow in whey and to readily utilize most of the lactose were the determining factors.

Initial studies comparing the effect of freezing at −19°C and −196°C on *K. fragilis* Y-1156 revealed a significant difference in survival. Those frozen at −19°C survived much better than did those at −196°C after 24 hr storage (Table 2). Damage to cultures frozen at −19°C appeared to occur gradually during 21 days, whereas damage suffered by those frozen in liquid nitrogen occurred almost immediately and the viability of the cells changed little during extended storage (Fig. 2).

Because freezing at −19°C was found to be less damaging to yeast cells than freezing rapidly at −196°C, another freezing and storage process was evaluated to determine whether a slow freezing at −19°C followed by storage at ultra low temperatures (−196°C) could improve survival of yeast cells. Cultures frozen and stored at −19°C for the total storage period exhibited the highest survival levels (Table 3). Storage in the liquid nitrogen resulted in the lowest

Table 2—Survival of Kluyveromyces fragilis Y-1156 during freezing at −19° C and −196° C for 24 hr[a]

Temperature	Count/g[b] 0-Time	24 Hr	% Survivors
−19°C	1.2×10^9	1.0×10^9	83
−196°C	1.2×10^9	9.9×10^7	8

[a] Cells were suspended in sterile 10% nonfat milk solids for freezing and storage.

[b] Each count represents the average of four trials.

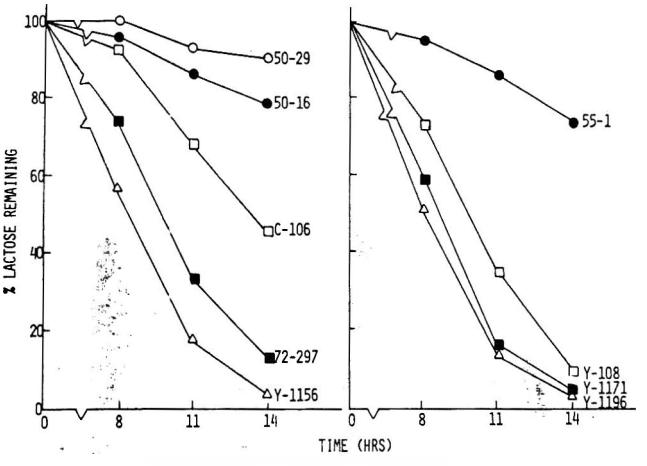

Fig. 1—Lactose utilization by different cultures of Kluyveromyces fragilis in cottage cheese whey.

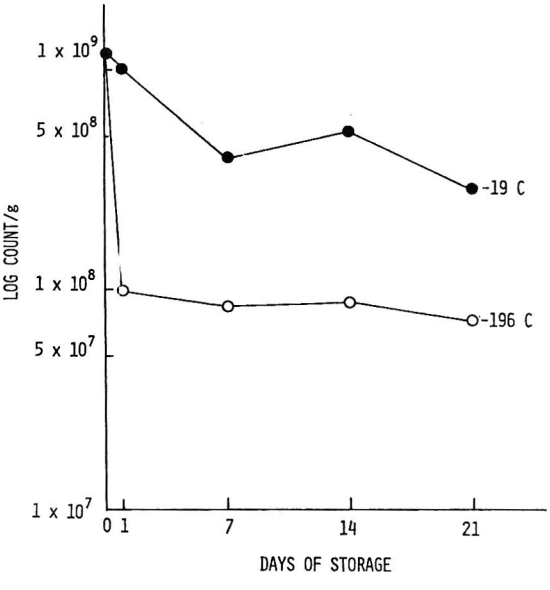

Fig. 2—Survival of Kluyveromyces fragilis Y-1156 during extended storage at −19° C and −196° C.

percentage of survivors. The dual-freezing treatment resulted in increased survival as compared to results for freezing and storage in liquid nitrogen alone, but not as high as for those cultures frozen and stored at −19°C.

Various concentrations of glucose (0, 10, 20 and 30%)

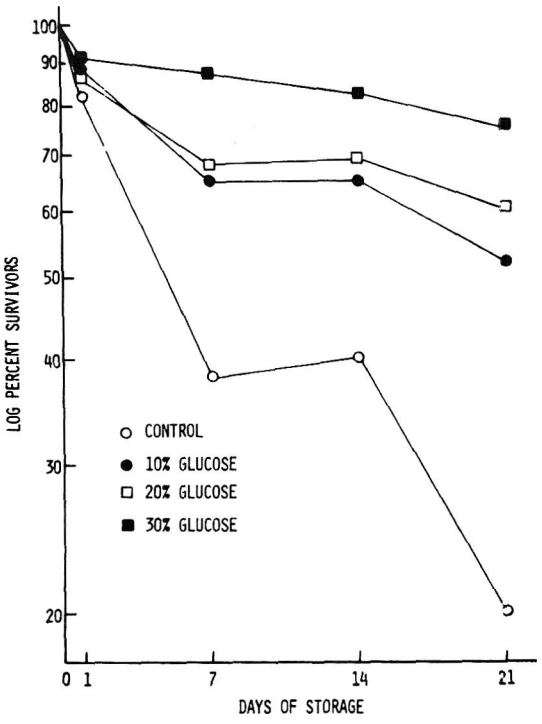

Fig. 3—Effect of glucose concentration and storage time on survival of Kluyveromyces fragilis Y-1156 at −19° C.

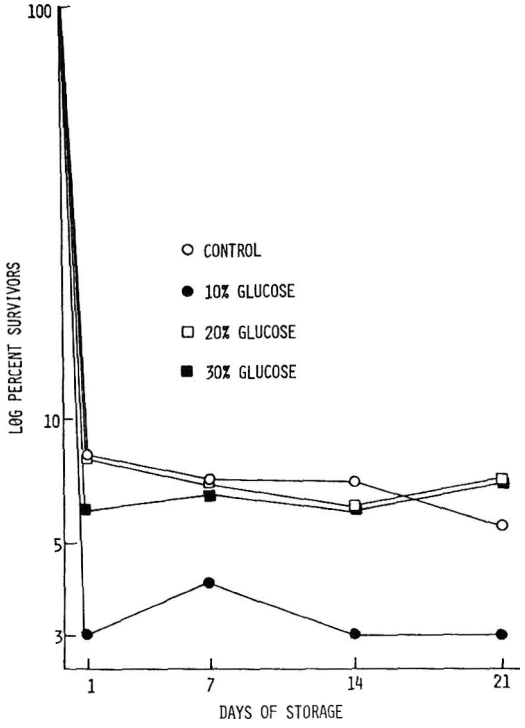

Fig. 4—Effect of glucose concentration and storage time on survival of Kluyveromyces fragilis Y-1156 at −196° C.

were added to concentrated cultures of *K. fragilis* Y-1156 in attempts to increase survival during freezing and frozen storage. The addition of glucose to cultures frozen at −19°C resulted in increased percentages of survivors (Fig. 3). While the glucose appeared to have little or no beneficial effect on survival after one day of storage at −19°C, during extended storage periods of 7–21 days the number of yeast cells surviving increased as the amount of glucose in the medium increased. The concentrated cultures frozen at −196°C exhibited much lower survival than those frozen at −19°C. The addition of glucose to the suspending medium provided no apparent protection to the cells during freezing and frozen storage (Fig. 4).

To test for any significant differences among the percentages of survivals of *K. fragilis* in concentrated cultures containing various glucose concentrations, the data for cultures frozen at −19°C for 21 days was subjected to statistical analysis. While there were significant differences ($P < 0.005$) among the effects of the glucose concentrations on survival at −19°C, no significant variations occurred among trials ($P > 0.1$).

In order to determine at what point in the storage of the concentrated cultures (at −19°C) a significant drop in survival occurred, data for each glucose concentration was analyzed separately with percentages of survival being compared to day zero (100%), using a least significant difference test. For those cultures frozen in 0, 10, or 20% glucose, a significant drop in survival occurred after 1 day of frozen storage ($P < 0.01$ for 0 and 20% glucose; $P < 0.05$ for 10% glucose). However, cultures frozen in 30% glucose did not show a significant drop in cell survival until they were stored for 14 days ($P < 0.01$).

DISCUSSION

WHEY DISPOSAL is a major concern of dairy processing plants. One possible alternative to dairy processors in treating cottage cheese whey is to culture it with *K. fragilis*. Such a fermentation has been reported to reduce the biochemical oxygen demand of the liquid (Knight et al., 1972), and a high protein material can be recovered which could be used as a nutritious food supplement. A convenient means of handling the yeast culture in the dairy plant is needed for this process. Frozen concentrated cultures of *K. fragilis* could help eliminate the problems involved in a continuous transfer of the culture and save time, labor, and storage space.

It was important to be selective in choosing a strain of *K. fragilis* for culturing cottage cheese whey. The abilities to grow in the whey and utilize the lactose varied considerably among the nine cultures. Those yeasts which grew best in the whey did not necessarily reduce the lactose content sufficiently during the test period. *K. fragilis* Y-1156 was chosen for further study in the preparation of culture concentrates and for freezing trials because it grew well in the whey and utilized the lactose quite readily.

In preliminary freezing trials, it was found that the rate at which the cells were frozen appeared to be one of the most important factors determining the survival of the culture. Cultures frozen slowly (−19°C) survived much better

Table 3—Effect of freezing and storage on percentages of survivors[a] of Kluyveromyces fragilis Y-1156

Days of storage	Freezing treatment		
	−19°C	−196°C	−19°C/−196°C
1	76	18	–
7	44	14	22
14	36	19	31
21	22	15	18

[a] Results are the average of two trials; cells were suspended in sterile 10% nonfat milk solids for freezing and storage.

than those frozen at −196°C. It was also observed that the survivals of the concentrated cultures frozen at −19°C decreased gradually over the study period as a whole. Damage to cultures frozen at −196°C was evident within 1 day and there was little or no additional loss of viability for the rest of the storage period. There appeared to be a possible advantage in freezing the yeast cells slowly at first, where they would undergo minimal damage, and then transferring them to liquid nitrogen for storage. Hopefully, at this stage the cultues would receive no additional damage. Yeasts undergoing the dual-temperature treatment survived better than those frozen and stored in liquid nitrogen, but not as high as those frozen and stored at −19°C. This method of freezing appeared to be superior to freezing and frozen storage in liquid nitrogen, but temperatures of −19°C still seemed optimal. These findings are in general agreement with previous reports in the literature in which other yeast were studied. The highest survivals of brewing yeasts frozen in liquid nitrogen were obtained using a freezing rate of 1°C/min (Wellman and Stewart, 1973). The mechanisms by which yeast cells are damaged during cooling has been explained by Mazur and Schmidt (1968) and Bank and Mazur (1973). Cells which are frozen slowly have time to equilibrate with the external medium. Intracellular water moves out of the cell, making the formation of internal ice crystals less likely. With a rapid cooling rate, the yeast cells have less time to equilibrate with the environment. Therefore, the cells lose less water, and the probability of internal ice formation increases. Internal ice crystals are the principle cause of damage to rapidly frozen cells, while the concentration of cellular solutes causes damage to slowly frozen cells over extended periods of storage.

Survival of the concentrated yeast cultures suspended in 10% nonfat milk solids (NFMS) did not appear to be sufficiently high for use in culturing cottage cheese whey, particularly for those stored at −196°C. Glucose in various concentrations was added to the NFMS, and these suspending media were tested for the possibility of increasing the survival of yeast cells. The addition of glucose appeared to have a beneficial effect on the survival of the concentrated cultures stored at −19°C but not at −196°C. This difference, along with the differing effects of freezing mid-exponential phase cells and stationary phase cells at −19°C and at −196°C, suggests that different mechanisms may be involved in damage resulting from freezing at the two temperatures. Allowing additional time for the yeast cells to equilibrate in the milk-glucose mixtures might improve their resistance to freezing. This would depend on the mechanism of the cryoprotective effect. Additional studies would be needed to determine this.

Although freezing the concentrated cultures in 30% glucose at −19°C yielded the highest survival levels, a storage period longer than 14 days is needed for this process to be practical for use by dairy processors. The decrease in survival of these cultures was significant at 14 days. Other methods need to be investigated to increase the storage period for the cultures if frozen concentrated cultures are to be prepared that can be successfully utilized in industry. Other cryoprotective agents such as glycerol may be useful in improving stability of these yeast cultures during freezing and frozen storage.

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EFFECT OF OXIDATION-REDUCTION POTENTIAL ON THE OUTGROWTH AND CHEMICAL INHIBITION OF Clostridium botulinum 10755A SPORES

L. A. SMOOT and M. D. PIERSON

ABSTRACT

The influence of oxidation-reduction potential (Eh) on the outgrowth, toxin production, and chemical inhibition of *C. botulinum* (10755A) was investigated. Strict anaerobic procedures and prereduced media were utilized in all growth experiments. No difference in growth or toxin formation in Trypticase Soy Broth (TSB) at an Eh7 of -60 mv or in TSB at an Eh7 of -145 mv was observed. However, growth and toxin production were delayed and/or decreased in the higher Eh medium as compared to the lower Eh medium by the addition of sodium chloride (5.0% and 6.0%), hydrogen ion concentration (pH 5.2 and 5.3), and sucrose (30%) to TSB.

INTRODUCTION

AN IMPORTANT and often overlooked consideration in assessing the growth and toxin production of *C. botulinum* in media and foods is the oxidation-reduction potential (Eh) of the system. The Eh is possibly even more critical when growth is being restricted or inhibited by chemicals such as salt and sucrose. Ando and Iida (1970) found that the outgrowth of type E spores was significantly affected by the Eh of the growth medium while germination was virtually unaffected. In a review by Rowley and Anellis (1976) it was reported that an Eh of +150 mv or greater will inhibit outgrowth of types A, B, and E spores when all other parameters are held optimum.

Several studies have found the Eh of various media to have a significant effect on the chemical inhibition of several clostridial species. Hanke and Bailey (1945) demonstrated that the growth limiting Eh for *C. welchii, C. sporogenes,* and *C. histolyticum* was affected by hydrogen ion concentration. More recently Smith and Pierson (1976) demonstrated that differences in medium Eh have an effect on growth limiting concentrations of chemical inhibitors for type E spores of *C. botulinum.*

To date, there are very few data available for *C. botulinum* growth and toxin production where Eh has been used as a variable, particularly in concert with microbial inhibitors commonly found in foods such as salt, sucrose, and hydrogen ions. The objectives of this investigation were to obtain outgrowth and toxin production of *C. botulinum* 10755A spores under optimum growth conditions in low and high Eh media, and to determine the effect of the Eh of these media on growth limiting levels of sodium chloride, sucrose and hydrogen ion concentration.

MATERIALS & METHODS

Test strain

A lyophilized culture of *C. botulinum* type A, 10755A (NCTC 2021), was obtained from the VPI&SU Anaerobe Laboratory, Blacksburg, Va. The test strain identity was confirmed by biochemical tests, gas chromatographic analysis of volatile fermentation products and toxicity tests as described by Holdeman and Moore (1975).

Inocula for all growth studies consisted of spores of the 10755A strain prepared as outlined by Rowley and Feeherry (1970). The washed spores were suspended in sterile distilled water at a concentration of 5.5×10^8 spores per ml and stored at 4°C. The spores were heat shocked at 80°C for 10 min prior to inoculating the growth medium.

Growth media

The basal assay medium used in all growth, toxin formation, and Eh7 experiments consisted of Trypticase Soy Broth (TSB, BBL), 0.05% cysteine, and one of the following chemicals added at the indicated levels: sucrose (0, 30, and 35% w/v); sodium chloride (0, 5, 6, and 7% w/v); and hydrochloric acid adjusting pH to 5.1, 5.2 and 5.3. All media were prepared according to the general anaerobic methods of Holdeman and Moore (1975). A 1-L round bottom distilling flask (Corning Glass Works, Corning, NY) was filled with 3N hydrochloric acid for 16 hours and rinsed with distilled water to remove any substances which might produce variations in the Eh7 readings. The dry ingredients for the basal medium were added to the flask and rehydrated in 750 ml of distilled water. In order to prevent foaming during sparging and growth, a silicone antifoaming agent, "Siliclad" (Becton, Dickinson, and Co., Parsippany, NJ) was added to the medium to produce a final concentration of 300 ppm. For the experiments on inhibition by sucrose or sodium chloride, the compounds were added to the medium at this point in their specified amounts. To eliminate traces of oxygen and other dissolved gases, the medium was boiled and refluxed for 15 min in the flask fitted with a refluxing chimney (Holdeman and Moore, 1975). The low Eh medium was cooled in ice water while being sparged with prepurified nitrogen which was passed through a copper-filled furnace (Sargent-Welch, Skokie, IL) to insure oxygen free nitrogen (OFN). Cysteine hydrochloride was then added to the cooled medium to a concentration of 0.05% and the pH was adjusted to pH 7.0 ± 0.1 with 5N hydrochloric acid or 8N sodium hydroxide. The prereduced medium was stoppered, placed in a press clamp and autoclaved at 121°C for 15 min. The medium was cooled at room temperature or placed in a cold water bath. The high Eh medium was prepared as above, but sparged with compressed air rather than oxygen-free nitrogen during cooling after refluxing. To induce further oxidation of the medium, the stoppered flask was agitated at 180 rpm in a cold water bath to cause mixing of the air in the headspace with the medium. Media utilized in experiments on inhibition by high hydrogen ion concentration were adjusted to a pH value of 5.7 with concentrated hydrochloric acid prior to sterilization. The desired pH values for the media were obtained by aseptically adding 5N hydrochloric acid to the medium after it had been sterilized and dispensed in the electrode vessel (described below).

Electrode vessel

All growth, toxin, and Eh7 experiments were performed in a 1,000 ml 4-necked distilling flask (Corning No. 400764; Corning Glass Works, Corning, NY) as described by Smith (1975). One neck of the electrode vessel (Fig. 1) was fitted with a combination pH electrode (Corning No. 476051) to measure pH; the second with a platinum inlay electrode (Corning No. 476060) to measure Eh; the third with cotton plugged glass tubing for the exchange and mixing of various gases. The electrodes and glass tubing were placed in rubber stoppers to insure airtight connections to the electrode vessel. The fourth neck was arranged similarly with an airtight screw cap tube which was utilized for dispensing media into the vessel and sample collecting. Flow meters (Air Products and Chemicals, Inc., Allentown, PA) were arranged in a series in order to mix and control the gases. All gases used (i.e. prepurified nitrogen and anaerobe grade carbon dioxide) were obtained from Industrial Gas and Supply, Bluefield, WV.

The platinum and pH electrodes were connected to a Model 801 Digital pH/mv Meter (Orion Research Inc., Cambridge, MA), and Model 855 Automatic Electrode Switch (Orion), and a Model 851

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Digital Printer (Orion), which allowed continuous monitoring and automatic recording of each electrode potential. To obtain maximum sensitivity in measuring Eh, the platinum electrode was polished with a jeweler's rouge polishing cloth (Bulova Watch Co., Flushing, NY) prior to each experiment.

Preceding each experiment, the flask with attached tubing was sterilized by autoclaving at 15 psi for 15 min while the electrodes were sterilized in 0.15% sodium hypochlorite for 20 min and rinsed with sterile distilled water prior to aseptic insertion into the sterile flask.

Media dispensing and equilibration

The anaerobic medium was aseptically dispensed into the electrode vessel as described by Smith (1975), upon completion of preparation, sterilization and cooling. Prior to dispensing the medium, OFN was used to flush the electrode vessel and apparatus for 1 hr at at rate of 100 cc per min, to remove any air present which might oxidize the medium. The temperature of the medium was maintained at 35°C during equilibration and growth experiments by immersing the growth flask in an insulated water bath equipped with a Haake model E52 constant temperature circulator. Once the growth medium had been dispensed into the vessel, it was sparged with the OFN throughout the equilibration period and growth experiment. A period of 2–4 hr was necessary for the Eh of the medium to come to equilibrium. Anaerobe grade CO_2 was mixed with the sparging OFN to maintain a medium pH that was 0.3 units lower than the OFN equilibrated medium. Carbon dioxide served to promote spore germination when the flask was inoculated. The CO_2 sparging was terminated upon germination of the spores as seen by phase contrast microscopy.

Eh and pH calculations

Measurement of pH values were recorded in millivolts rather than pH units to facilitate continuous, noninterrupted data collection. Calibration of pH was achieved by use of a standard curve of pH vs millivolts prepared by using the following standard buffers (pH 4.02, 6.98, 9.94, obtained from Harleco Co., Philadelphia, PA) at 35°C.

Standardization of the platinum electrode was performed in a potassium phthalate buffer, pH 4.0, saturated with quinhydrone at 35°C; this system has an Eh of +447 mv. The platinum electrode was standardized prior to each set of experiments to insure accuracy and consistency of the Eh measuring system.

All Eh values recorded were adjusted to pH 7.0 (Eh7) to eliminate the effect of pH on the Eh of the system by use of the equation developed by Leistner and Mirna (1959):

$$Eh7 = E_{obs} + E_{ref} + 2.303\ (RT/F)\ (pHX - 7.0)$$

E_{obs} is the measured potential of the system; E_{ref} is the reference electrode potential; 2.303 (RT/F) equals 61.1 at 35°C; and (pHX–7.0) is the pH corrective term with pHX being the measured pH of the system.

Growth studies

The electrode vessel containing the growth medium was inoculated anaerobically with the heat-shocked spore suspension (80°C, 10 min) to produce a final concentration of 1,000 spores per ml. Spore outgrowth in all experiments was quantitatively determined by duplicate anaerobic roll tube counts in peptone yeast extract agar (PYA) incubated at 35°C for 48 hr as described by Pierson et al. (1974) and Shoemaker and Pierson (1976). Dilution media consisted of 0.1% peptone blanks containing 0.05% cysteine. Samples were taken at appropriate times over a 144 hr period to monitor growth and toxin production.

Toxin studies

At selected times during all growth studies except those with sodium chloride as the chemical inhibitor, 4.0 ml samples were aseptically removed from the electrode vessel for toxicity testing. The samples were centrifuged at 20,000 × G for 10 min at 4°C to remove all vegetative cells and debris. One ml of the sample supernatant fluid was then serially diluted in 9.0 ml of a gelatin-phosphate buffer (0.1% gelatin in 0.05M sodium phosphate at pH 6.2). Quantitation of the toxin produced by the test strain was estimated by the quantal mouse assay method for determining LD_{50}'s (Tint and Gillen, 1961). White male ICR mice weighing 18–20g were each injected intraperitoneally with 0.5 ml of the diluted sample. Six mice were used per group and deaths were recorded over 96 hr.

In the particular experiments where growth was expected to be inhibited, toxin screening was performed on the samples by injecting four mice each with 0.5 ml of sample preparation diluted to 1/10 of the original. The mice were observed for 24 hr and if death occurred, the sample which had been stored at 4°C was quantitated as previously described.

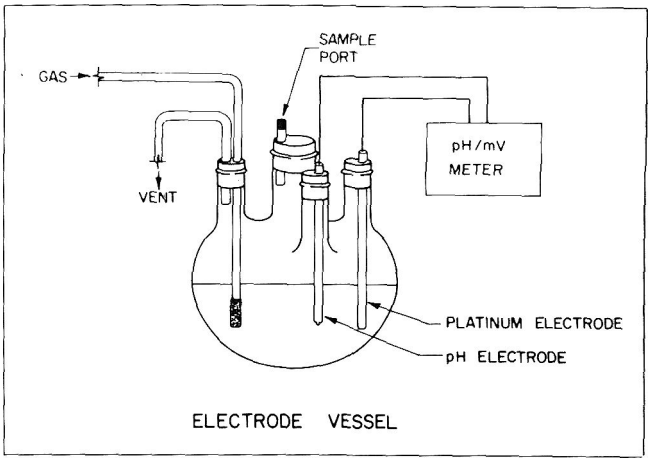

Fig. 1—Electrode - Growth Vessel.

For those growth studies in which sodium chloride was used as the growth inhibitor, 12.0 ml samples were taken for toxicity testing and centrifuged at 20,000 × G for 10 min at 4°C. The samples were prepared by four different treatments in order to determine if sodium chloride affected toxin production or the preformed toxin. All samples were quantitated as outlined above. First, 6.0 ml of the supernatant was dialyzed (Dialyzer tubing, Fisher Scientific Products, Raleigh, NC) in 1L of sterile phosphate buffer (0.05M sodium phosphate at pH 6.2) for 2 hr at 4°C to remove the NaCl. One ml of the dialysate was serially diluted and injected. Another 4.5 ml of this treatment was mixed with 0.5 ml of a 1.0% solution of trypsin (Difco 1:250, Difco Laboratories, Detroit MI) and incubated at 35°C for 30 min before serially diluting and injecting. Second, from the remaining 6.0 ml of non-dialyzed sample supernatant fluid, 1.0 ml was serially diluted and injected while another 4.5 ml was trypsin treated as described previously before dilution and injection.

RESULTS & DISCUSSION

THE RESULTS of the growth studies in TSB at an Eh7 of −60 mv, pH 6.8, (high Eh7 medium) and at an Eh7 of −145 mv, pH 6.8, (low Eh7 medium) showed no apparent difference in growth and toxin production of the *C. botulinum* 10755A spores (Fig. 2). A maximum cell population of approximately 2.5×10^9 cells/ml was observed after 20 hr of incubation at 35°C for both the low and high Eh7 TSB. This lack of difference in growth of the test strain in these media, which differ only in Eh, is consistent with an earlier study showing that the Eh7 of the medium, ranging from −40 mv to −185 mv, does not have a significant effect on the growth of *C. botulinum* type E spores (Smith, 1975). Also, in these media, a corresponding decrease in the Eh7 was observed during the growth phase, while a slight increase in the Eh7 was noticeable during the death phase. This finding is in agreement with similar experiments where hydrogen gas production by type E vegetative cells was found to lower the Eh7 of the growth medium (Smith, 1975). Toxin was first detected in both media after 24 hr of incubation. A maximum toxin titer of 66,000 LD_{50}/ml was obtained at 72-hr incubation in the low Eh7 TSB and 54,000 LD_{50}/ml in the high Eh7 TSB.

The comparison of growth and Eh7 changes for the low (−145 mv) and high (−60 mv) Eh7 TSB (pH 6.8) containing 5% (w/v) added NaCl is shown in Figure 3. In the low Eh7 TSB, growth and Eh7 reduction started 30–40 hr after inoculation. In the high Eh7 TSB, growth and Eh7 changes were initiated after approximately 48 hr incubation. The high Eh7 TSB plus 5% (w/v) NaCl induced a slightly increased lag phase in growth; however, a higher maximum

cell number was eventually attained in the high Eh7 TSB as compared to the low Eh7 TSB. This may occur once the Eh7 of the medium has been sufficiently reduced to a favorable level by growth of the culture. This is in agreement with data reported by Boyd and Reed (1931a, b), where they demonstrated that the Eh of a culture medium may be lowered by the evolution of hydrogen gas produced by an organism with an actively reducing metabolism.

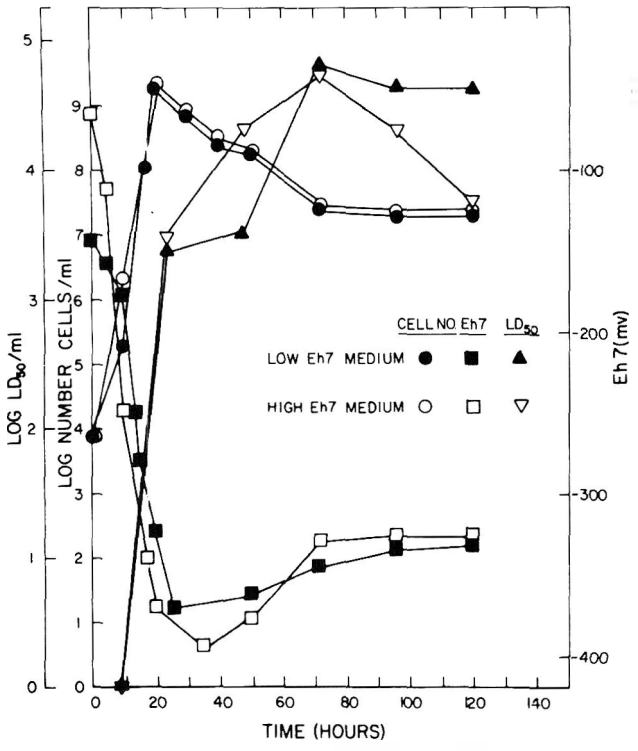

Fig. 2—Eh7 changes and growth and toxin production by C. botulinum 10755A at 35°C in low and high Eh7 Trypticase Soy Broth (pH 6.8).

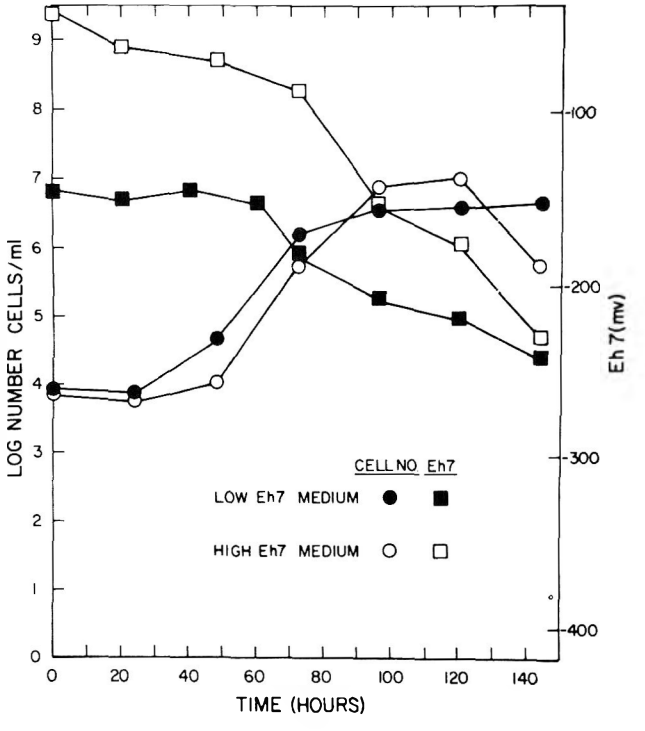

Fig. 3—Eh7 changes and growth of C. botulinum 10755A at 35°C in low and high Eh7 Trypticase Soy Broth (pH 6.8) plus 5% (w/v) added NaCl.

For analysis of toxin formation in these two media, the culture supernatant fluid samples were given four different treatments (no treatment, trypsinized, dialyzed, and dialyzed-trypsinized) in order to determine if the 5% (w/v) NaCl had an effect on toxin formation or the preformed toxin. The results of these treatments on the quantitation of toxin for the low Eh7 TSB plus 5% (w/v) NaCl is presented in Figure 4. Toxin formation was not detected until 72 hr after inoculation of the vessel. Maximum toxin strength was 6320 LD_{50}/ml at the end of 144 hr of growth for the dialyzed-trypsinized samples. Figure 5 illustrates the effects of the four treatments on toxin quantitation in the high Eh7 TSB plus 5% (w/v) NaCl. Due to delay in growth in this medium, toxin was not detected until 96 hr with the maximum toxin titer of 7000 LD_{50}/ml attained at 144 hr. No significant differences in toxin quantitations in these media indicate that NaCl at this concentration had no direct effect on toxin formation or preformed toxin, but rather an indirect effect through its growth inhibiting properties on *C. botulinum*.

To investigate the levels of NaCl necessary to completely inhibit growth of the test strain, 6% (w/v) NaCl was added to both high Eh7 (−60 mv) and low Eh7 (−145 mv) TSB (pH 6.8). In the low Eh7 TSB, growth and Eh7 changes began at 100 hr with a maximum cell number of 5.0×10^5 cells/ml at 144 hr. Neither changes in cell number or Eh7 reduction due to growth occurred in the high Eh7 TSB through 144 hr. The spores remained phase bright and heat resistant. Toxin was not detected in either trypsinized, dialyzed, dialyzed-trypsinized or untreated culture supernatant for both the high and low Eh7 and TSB.

Complete inhibition of the test strain in low Eh7 TSB (pH 6.8) was achieved when 7.0% (w/v) NaCl was added to the medium. Neither germination, toxin production, or Eh7 reduction occurred through 144 hr. The initial spore inocu-

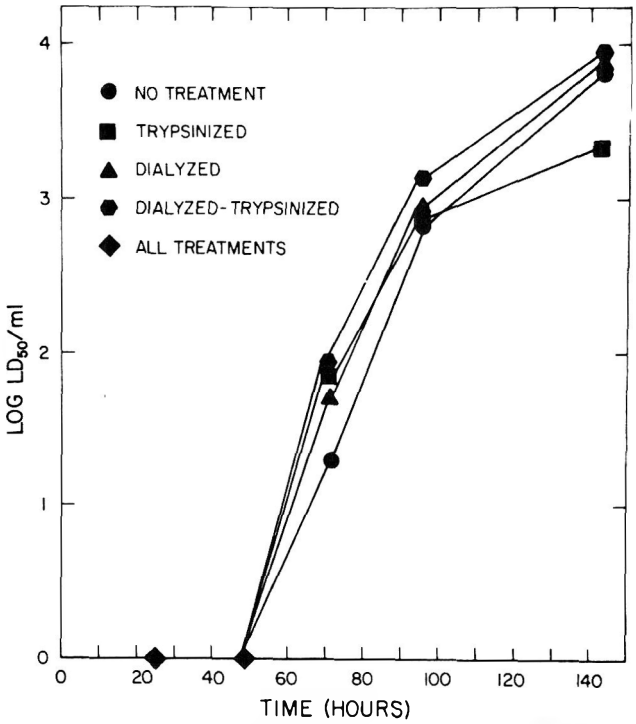

Fig. 4—Toxin production by C. botulinum 10755A at 35°C in low Eh7 Trypticase Soy Broth (pH 6.8) plus 5% (w/v) added NaCl. Toxicity of the culture was assayed in samples which had been centrifuged only; trypsinized; dialyzed; and dialyzed and trypsinized.

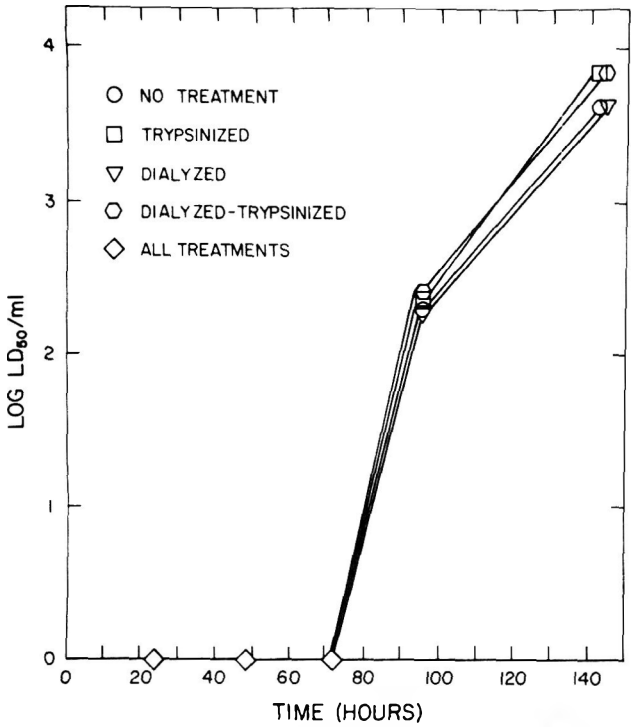

Fig. 5—Toxin production by C. botulinum 10755A at 35° C in high Eh7 Trypticase Soy Broth (pH 6.8) plus 5% (w/v) added NaCl. Toxicity of the culture was assayed in samples which had been centrifuged only; trypsinized; dialyzed; and dialyzed and trypsinized.

lum remained phase bright and heat resistant. This inhibitory level is in agreement with published data (Spencer, 1967; Greenberg et al., 1959).

These results show that in determining the concentration of NaCl needed for inhibition of the test strain, the Eh7 has a definite influence on the inhibitory levels. For complete inhibition of *C. botulinum* in a low Eh7 medium as compared to a high Eh7 medium, more NaCl may be necessary. Previous work by Mead (1969) demonstrated similar results in that the growth limiting concentrations of salt for *C. welchii* was reduced as the medium Eh was increased.

Growth, toxin formation, and Eh7 changes by the test strain in low Eh7 (−145 mv) TSB and high Eh7 (−60 mv) TSB at pH 5.3 are presented in Figure 6. A maximum cell population of 5.5×10^8 cells/ml was attained in the low Eh7 TSB after 48 hr while 4.3×10^8 cells/ml was detected in the high Eh7 TSB after the same period of time. Also, there appeared to be a slightly longer lag phase for growth in the high Eh7 medium at this hydrogen ion concentration. Eh7 reduction began within 20 hr after inoculation for both media. Toxin formation in the low Eh7 TSB and high Eh7 TSB at pH 5.3 was found to be similar (Fig. 6). In both media, toxin was not detected until 48 hr of incubation. Maximum toxin formation in the high Eh7 medium occurred at 72 hr with 20,000 LD_{50}/ml while in the low Eh7 medium the maximum titer of 20,000 LD_{50}/ml was not detected until after 96 hr.

When the pH of the low and high Eh7 TSB was lowered to 5.2, further inhibition of growth and toxin production by the test strain was observed (Fig. 7). Growth and Eh7 changes in the low Eh7 TSB began about 10–20 hr after inoculation. A maximum cell population of 3.8×10^8 cells/ml attained after 48 hr. Growth was not initiated in the high Eh7 TSB until 24 hr of incubation and the maximum cell number of 5.7×10^6 cells/ml was not detected until 48 hr later. The observed gradual increase in the Eh7 of both media at 72 hr may be attributed to the decrease in

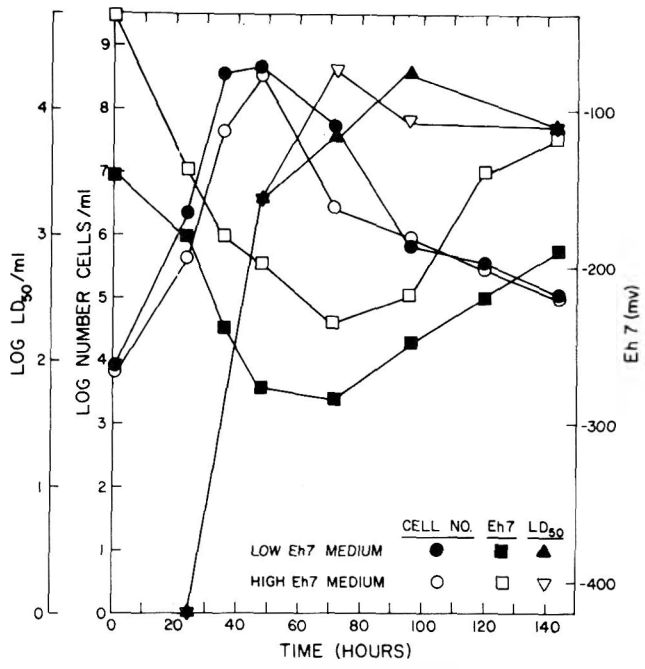

Fig. 6—Eh7 changes and growth and toxin production of C. botulinum 10755A at 35° C in low and high Eh7 Trypticase Soy Broth (pH 5.3).

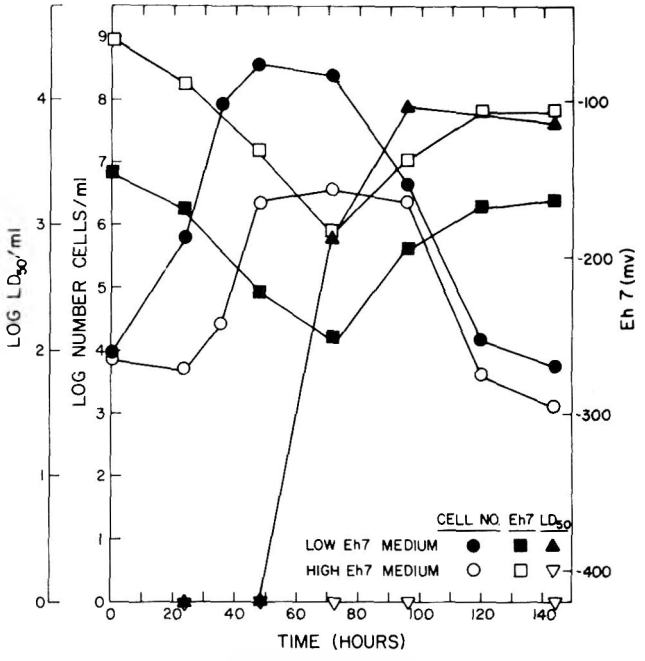

Fig. 7—Eh7 changes and growth and toxin production of C. botulinum 10755A at 35° C in low and high Eh7 Trypticase Soy Broth (pH 5.2).

metabolic production of hydrogen gas during the death phase of the microorganism.

Toxin formation in the low Eh7 TSB, pH 5.2, was first detected at 72 hr and a maximum toxin titer of 9,000 LD_{50}/ml was found at 96 hr of incubation (Fig. 7). Toxin was not detected in the high Eh7 TSB, pH 5.2, through 144 hr.

Upon lowering the pH of the low Eh7 TSB to 5.1, inhibition of germination for the test strain was observed. Through 144 hour of incubation, neither growth, toxin production, nor Eh7 changes were detected. The initial spore inoculum remained phase bright and heat resistant.

—Text continued on page 704

This group of experiments is in agreement with an earlier study (Smith, 1975), where the Eh of the system was reported to have a marked effect on the limiting pH for growth of *C. botulinum* type E spores. Other investigators have reported the outgrowth of type A and B spores was inhibited at pH 4.8 to 5.0 depending upon the substrate (Townsend et al., 1954). However, most studies in which pH is considered to be a controlling factor in the growth of *C. botulinum*, ignore Eh. This research shows that the growth limiting effects of pH is dependent upon the Eh of the system even though there may be a negative Eh.

The effect of Eh7 and 30% (w/v) sucrose on the inhibition of *C. botulinum* 10755A is presented in Figure 8. The low Eh7 (−195 mv) TSB (pH 6.8) showed an increase in cell numbers within 24 hr and a maximum cell population of 6.0×10^7 cells/ml was detected after 72 hr. The Eh7 began to drop as growth was initiated and continually decreased throughout the growth phase. The addition of 30% (w/v) sucrose to this same medium at an Eh7 of −105 mv resulted in an extended lag phase of growth and a maximum cell number of 8.5×10^5 cells/ml at 144 hr. Toxin was not detected in the low Eh7 TSB until after 72 hr of incubation with the culture reaching maximum toxicity of 8300 LD_{50}/ml at 96 hr (Fig. 8). Toxin formation in the high Eh7 TSB was less than the detection level of 20 LD_{50}/ml.

Differences in growth and toxin production between the two media are attributed to their method of preparation resulting in high and low Eh7 values since both contained the same amount of sucrose. In a previous investigation with similar media, it was reported that growth differences for *C. botulinum* type E was not the result of inhibitory substances formed during the oxidation of the medium components, but rather the redox potential of the medium (Smith, 1975).

Upon increase of the sucrose concentration to 35% (w/v), growth in the low Eh7 TSB was sufficiently inhibited that no toxin was detected for up to 144 hr. The maximum cell number attained over this period was 4.8×10^4 cells/ml which was less than a one log cycle increase over the initial inoculum of 7.3×10^3 cells/ml.

There is very little data on sucrose inhibition of *C. botulinum* type A spores. Bever and Halvorson (1948) reported that 50–55% sucrose was necessary for inhibition of types A and B vegetative cells. In another study, a sucrose concentration of greater than 31% was found necessary to inhibit germination of type B spores (Beers, 1957). In comparison of results from this study to previous investigations, the variations in strains used and conditions (i.e. pH and Eh) under which they were tested should be considered.

Results from this study indicate that the oxidation-reduction potential of the system should be considered when determining the growth limiting concentrations of various microbial inhibitors (i.e. sodium chloride, hydrogen ion, sucrose) for *C. botulinum*. These data suggest that the manipulation of the Eh may prove useful in the control of toxic anaerobic microorganisms in food systems. However, further investigation with other strains of *C. botulinum* is warranted. Also, growth studies should be conducted in food systems to determine if they have the ability to poise the redox potential at inhibitory levels. Use of such a phenomenon in a food may very well depend on the chemical composition of that food since various reducing compounds may be present.

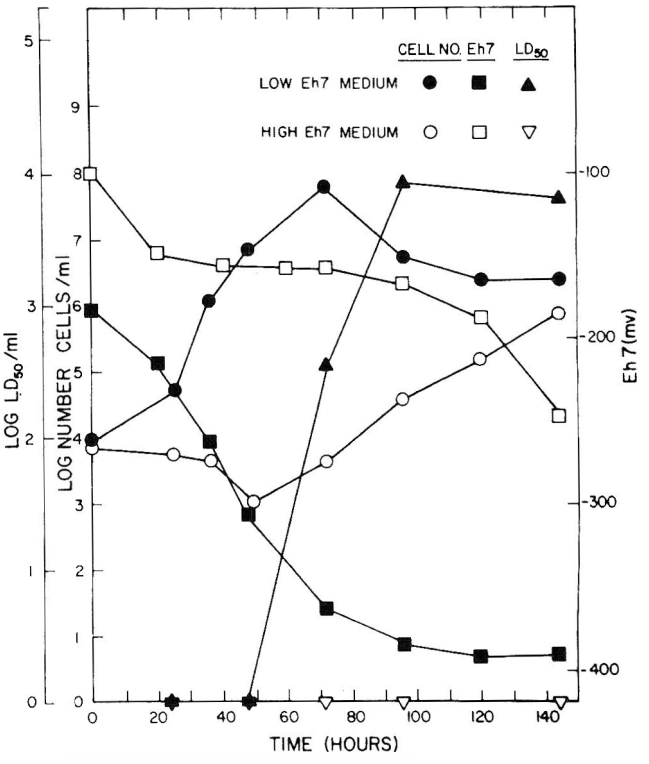

Fig. 8—Eh7 changes and growth and toxin production of C. botulinum 10755A at 35°C in low and high Eh7 Trypticase Soy Broth (pH 6.8) and 30% (w/v) sucrose.

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ACTIVATION OF Bacillus stearothermophilus SPORES AT LOW pH

Y. E. EL-MABSOUT and K. E. STEVENSON

ABSTRACT

Activation and decrease in heat resistance of *B. stearothermophilus* E-2 spores were studied after mild heat treatments at low pH. Activated spores were measured by colony formation on Nutrient Agar + 0.03% $MnSO_4$. Under the conditions used in this investigation activation of spores paralleled transformation to a heat-sensitive form (D_{86} = 7 min at pH 7.0). Activation was accelerated at pH 1.1–2.0 and 60–70°C. Prolonged low pH-mild heat treatments resulted in decreased counts due to the inability of some spores to germinate. *B. stearothermophilus* spores present in selected food ingredients were activated in a similar manner. Under certain conditions, a low pH-mild heat treatment may be used as a substitute for a severe heat-shock to activate spores of *B. stearothermophilus*.

INTRODUCTION

SPORES which fail to germinate, even under apparently favorable conditions, are said to be dormant (Murrell, 1961). "Activation" is the process by which these dormant spores are converted into spores that have the ability to germinate. Several treatments can be used to activate spores, although application of heat (i.e. heat shock) is the simplest and most widely used method.

Activation of spores by heat is influenced by time and temperature (Busta and Ordal, 1964). Spores of *B. stearothermophilus* exhibit a high degree of dormancy and require a severe heat shock with temperatures of 105–115°C for activation (Brachfeld, 1955; Titus, 1957; Finely and Fields, 1962). Furthermore, if carbohydrates (Fields and Finely, 1964) and salts (Halmann and Keynan, 1962; Keynan et al., 1965) are present during heat activation there is a reduction in counts due to inhibition of activation and/or germination. Some spore destruction occurs during the heat shock even under optimal conditions. When the heat treatment is not carefully controlled, artificially low spore counts will be obtained due to inadequate heat activation or excessive spore destruction. Obviously, a chemical treatment which results in spore activation could provide a means for improved assays of *B. stearothermophilus* spores.

Various reports have shown that heat activation of bacterial spores is pH-dependent. Moreover, low pH activates spores under certain conditions. For instance, Lewis et al. (1965) demonstrated that *B. stearothermophilus* spores were partially activated by treatment at pH 1.5 for 80 min at 25°C. They also showed that dormancy could be restored by treatment with 0.02M Ca^{++} ions at pH 9.7. Brown et al. (1968) also reported activation of *B. stearothermophilus* spores in 0.5N HCl at 25°C although counts decreased after approximately 30 min. The purpose of this investigation was to study the combined effects of low pH and mild heat on the activation of *B. stearothermophilus* spores.

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MATERIALS & METHODS

Preparation of spores

The organism used in this study was *B. stearothermophilus* E-2 obtained from the culture collection of the Department of Food Science & Human Nutrition at Michigan State University. It was maintained in nutrient broth (Difco).

One-ml portions of 48-hr nutrient broth cultures were used to inoculate double-strength nutrient agar + 0.03% $MnSO_4$ (NAM) in plates or 32-oz prescription bottles which were incubated for 3 days at 55°C. Spores were harvested in 10 ml of sterile, distilled water, centrifuged, and washed three times with sterile, distilled water. The washed spores were treated with 0.1 mg/ml lysozyme, 3x crystalline (Nutritional Biochem. Corp., Cleveland, OH), at 37°C for 2 hr, and cleaned three times by centrifugation with sterile, deionized water. The spore slurry was suspended in water and stored at 4°C.

Formation of heat-sensitive spores

Heat-sensitive (H-form) spores were prepared by mixing normal spores in a solution described by Alderton and Snell (1969a) containing 1% tryptone, 0.5% glucose and 0.1% soluble starch. The pH of the solution was adjusted to 1.1 with 3N HCl. The spore suspension was incubated overnight at 20°C, centrifuged, washed three times and suspended in sterile, deionized water.

Formation of heat-resistant spores

Heat-resistant spores (Ca-form) were prepared by mixing normal spores in a solution described by Alderton and Snell (1969a) containing 0.02M calcium acetate adjusted to pH 9.7 with aqueous $Ca(OH)_2$. The suspension was incubated overnight at 20°C and 10 ml aliquots were heated in a steam cabinet for 15 min. The suspension was centrifuged, washed three times and suspended in sterile deionized water.

Activation of spores

In one set of experiments, five 10-ml portions of spore suspensions were pipetted into 16 × 125-mm screw-capped test tubes. Each tube contained 10 ml of 0.2N HCl, giving a final pH of 1.1. These suspensions were stored at 50°C for 0, 30, 60, 90 or 120 min.

For determining the effect of temperature, four 10-ml samples of spore suspension were added to screw-capped test tubes containing 10 ml of 0.2N HCl. These suspensions were incubated at 40, 50, 60 or 70°C for 1 hr.

For determining the effect of pH, four 10-ml aliquots of spore suspension were added to screw-capped test tubes containing acid-buffer solutions adjusted to pH 1.1, 2.0, 3.0 or 4.0. Each tube contained 10 ml of the spore suspension. These sample suspensions were incubated for 1 hr in a water bath adjusted to 60°C.

Heat treatment at 86°C

One-milliliter aliquots of samples treated at low pH with mild heat were added to 9 ml of phosphate buffer, pH 7.6, in screw-capped test tubes. The tubes were heated in a water bath at 86°C and consecutive dilutions were made from suspensions which had been heated for various time intervals.

Plating procedures

In most cases, appropriate dilutions of samples were surface plated onto NAM agar plates. The plates were inverted and incubated at 55°C for 48 hr. Other methods used in this study which combined activation and plating included the standard method for the determination of thermophilic flat-sour spores described by the American Public Health Association (APHA, 1966) and the following method used by Gerber Products Co. (J.C. Canada, personal communication) which is used for examining raw materials for heat-resistant aerobic thermophiles and flat-sour spores.

A sample of sugar or flour was weighed and dispersed in H_2O by blending or shaking at 1:10. Twenty milliliters of the suspension were pipetted into a 250-ml Erlenmeyer flask containing 100 ml

Table 1—Activation of B. stearothermophilus spores at pH 2.0 and 50° C as determined on NAM agar after incubation for 48 hr at 55° C

	Time of activation treatment (min)				
	0	30	60	90	120
Colonies/plate[a]	9	19	74	55	51

[a] Numbers represent the average count on duplicate plates at a 10^{-6} dilution.

Table 2—Activation of B. stearothermophilus spores at pH 2.0 and 60° C as determined on NAM agar after incubation for 48 hr at 55° C

	Time of activation treatment (min)				
	0	30	60	90	120
Colonies/plate[a]	10	31	83	67	59

[a] Numbers represent the average count on duplicate plates of a 10^{-6} dilution.

Table 3—Survival of activated (treated at pH 2.0 and 60° C for 60 min) spores at 86° C as determined on NAM agar after incubation for 48 hr at 55° C

	Heating time at 86° C					
	0	5	10	15	20	30
Colonies/plate[a]	300	249	183	63	38	5

[a] Numbers represent the average count on duplicate plates at a 10^{-5} dilution.

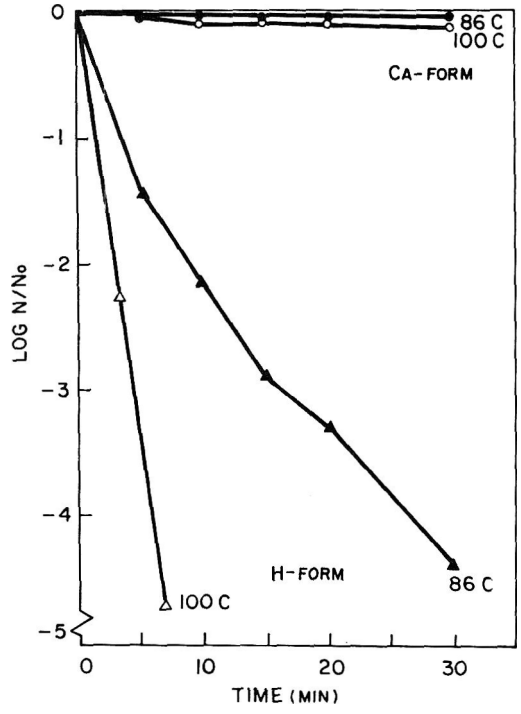

Fig. 1—Survivor curves of heat-sensitive (H-form) and heat-resistant (Ca-form) B. stearothermophilus spores heated at 86 and 100° C.

sterile dextrose-tryptone agar with bromcresol purple. The flask was cotton plugged and swirled to mix contents and autoclaved at 108° C (5 psi) for 10 min. The autoclave was previously standardized to give a heat treatment equivalent to $F_0^{1.8} = 0.74$ when heating was completed with the autoclave set for slow exhaust. The flask was immediately removed and the contents were cooled to 43–45°C in running water. The contents were poured into 4–5 petri dishes and the plates were inverted and incubated at 55° C for 48 hr.

RESULTS

Growth and sporulation

Preliminary investigation showed that *B. stearothermophilus* strain E-2 grew poorly and failed to sporulate on several media such as dextrose tryptone bromcresol purple agar (DTBA), plate count agar (PCA), and nutrient agar (NA). When 0.03% $MnSO_4$ was incorporated into NA in this investigation, better growth and sporulation was observed. Approximately 80% sporulation was observed when *B. stearothermophilus* strain E-2 was cultured on NAM for 2 days at 55° C. Thus, NAM was used in subsequent experiments.

Measurement of activation by plating

Table 1 shows average plate counts of spores which have been activated at pH 2.0 and 50° C for different periods vs. nonactivated spores. The total count of spores, as determined by direct microscopic count, was 9×10^7 spores/ml.

These results indicated that activation was occurring during incubation since the plate counts increased from 9×10^6 spores/ml at 0 min (untreated control) to 7.4×10^7 spores/ml at 60 min, which represented the optimal time for activation under these conditions. The counts decreased slightly during extended treatment.

When activation was carried out at 60° C the same trend was observed. However, higher activation was obtained with the 60° C treatment (Table 2). After 60 min plate counts increased more than eightfold, and the numbers of activated spores which formed colonies increased to at least 90% of the total spore count (9.0×10^7 spores/ml).

Heat resistance of H-form and Ca-form spores

Data presented in Figure 1 show survivor curves for heat-sensitive (H-form) and heat-resistant (Ca-form) spores when heated at 86 and 100° C. The Ca-form spores were extremely heat resistant and the total remained virtually unchanged when heated for 30 min at 86 or 100° C. On the other hand, 99.95% of the H-form spores were killed after the first 5 min of heating at 100° C. In addition, when the H-form spores were heated at 86° C, over 99.99% of the total were destroyed after 30 min.

Heat resistance of activated spores

Spores subjected to an activation treatment for 60 min at pH 2.0 and 60° C showed a marked decrease in their heat resistance. Table 3 shows the number of treated spores which survived heating at 86° C. These data indicated that over 90% of the spores were heat-sensitive. This is comparable to the percentage of spores activated during treatment at pH 2.0 and 60° C for 60 min (Table 2). Thus, during activation under these conditions (60° C and pH 2.0) spores were transformed to a heat-sensitive state. Data obtained on the heat resistance of untreated spores at 86° C indicated that *B. stearothermophilus* spores were very heat-resistant. This confirmed the previous conclusion that under the conditions used in this investigation activation of spores was accompanied by their sensitization to heat.

In measuring activation, the use of plate counts (before and after activation) is limited to values up to ~80–90% of the total count. However, at higher levels, activation can be approximated by measuring the proportion of heat-sensitive spores. Thus, heating at 86° C was used in the following

Table 4—Enumeration of thermophilic flat-sour spores in flour and sugar samples using various methods

	Methods[a]		
	Low pH-Mild heat (spores/g)	APHA (spores/g)	Gerber (spores/g)
Flour[b]	54	7	10
Sugar[b]	70	44	—[c]

[a] See text for details.
[b] Inoculated with approximately 80 *B. stearothermophilus* spores/g
[c] Not tested

experiments to monitor the proportion of spores which were activated, i.e. heat-sensitive.

Effect of temperature on activation

Figure 2 shows survivor curves for spores which were treated at pH 1.1 for 60 min at temperatures of 40, 50, 60 or 70°C, and then heated at 86°C. Results indicate that at least 90% of the spores were activated (heat-sensitive) during treatment at 40 and 50°C. Activation increased to approximately 99.9% and 99.99% for treatment at 60 and 70°C, respectively.

Rate of activation at 50°C

Figure 3 shows survivor curves during heating at 86°C for spores treated at 50°C and pH 1.1 for various periods of time. The data show that 55% of the total spores were heat sensitive (activated) when treated for 30 min under the aforementioned conditions. Similarly over 90% of the spores were activated by treatment for 60 and 90 min. The 120-min treatment gave approximately 99.9% activation.

Effect of pH on activation of spores

For spores activated for 60 min at 60°C, maximum activation occurred at pH 1.1–2.0. At higher pH values (3.0 and 4.0), no significant activation was observed. Figure 4 shows survivor curves for spores heated at 86°C after treatment for 60 min at 60°C and pH values of 1.1, 2.0, 3.0 and

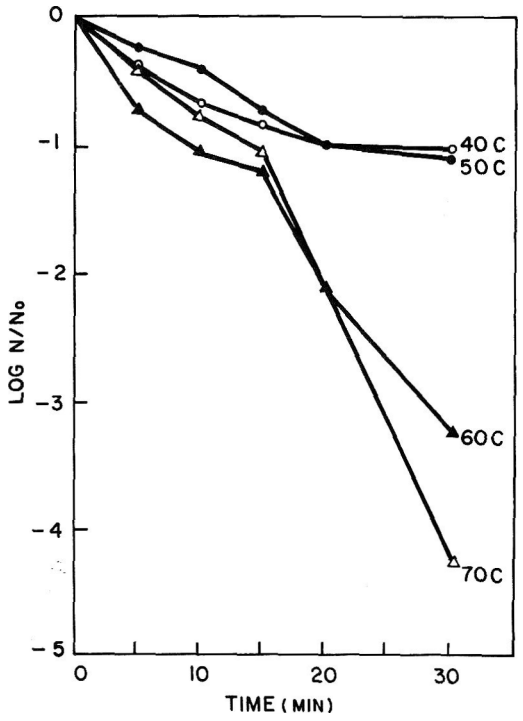

Fig. 2—Survivor curves of B. stearothermophilus spores activated by treatment at pH 1.1 for 60 min at 40, 50, 60 or 70° C and heated at 86° C.

4.0. These data indicate that over 99.9% of the spores were activated at pH 1.1 and 98% at pH 2.0. At pH values of 3.0 and 4.0 activation was minimal.

The effect of pH was also shown by the fact that over 99.99% activation was obtained when the spores were treated at pH 1.1 and 20°C overnight (Fig. 1). On the contrary, an overnight treatment of spores at pH 9.7 and 20°C resulted in essentially no activation.

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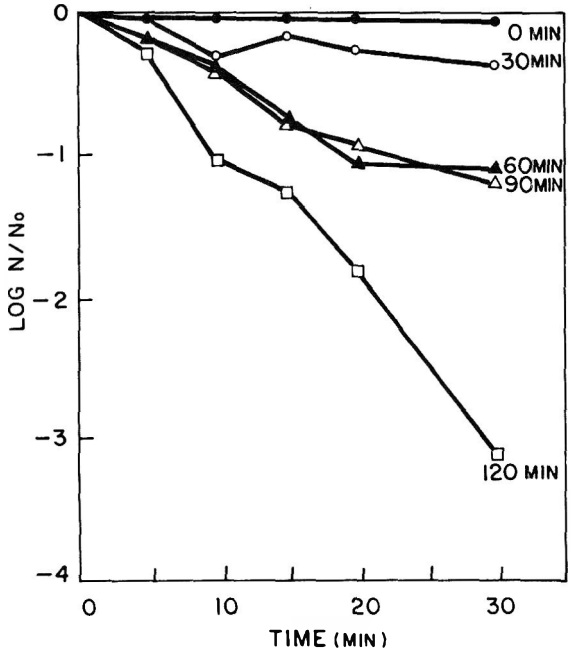

Fig. 3—Survivor curves of B. stearothermophilus spores activated by treatment at 50° C and pH 1.1 for various periods of time and heated at 86° C.

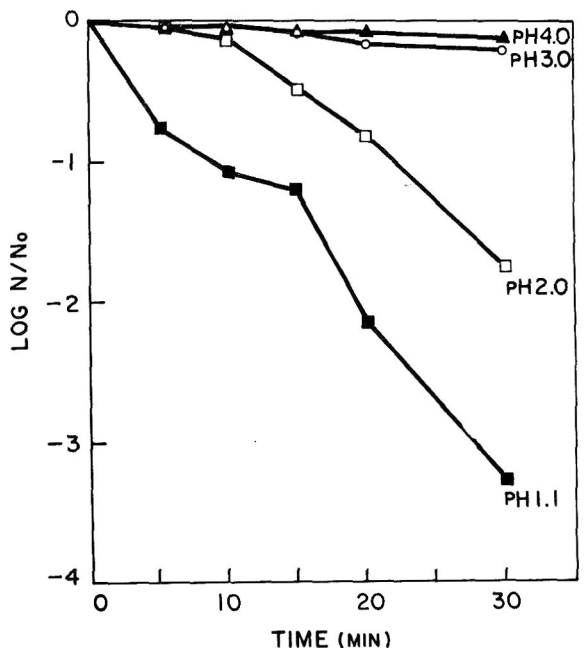

Fig. 4—Survivor curves of B. stearothermophilus spores activated by treatment for 60 min at 60° C and pH values of 1.1, 2.0, 3.0 or 4.0 and heated at 86° C.

Activation of spores in flour and sugar samples

When *B. stearothermophilus* spores were inoculated into flour and sugar samples, the spores were activated by the low pH-mild heat (pH 1.1 and 60°C) treatment. Higher plate counts were obtained with the low pH-mild heat treatment than with other methods which combined activation and plating procedures (Table 4).

DISCUSSION

PRELIMINARY INVESTIGATIONS suggested than Mn^{++} was necessary at a certain level in the plating medium for improved growth and sporulation of *B. stearothermophilus* strain E-2. Similar situations were encountered by other workers. Rowe et al. (1975) found that manganese was required at relatively high concentrations (1.0 μM) for the growth of *B. stearothermophilus* 1503 in a defined liquid medium. The addition of 15–30 ppm of manganese (Mn^{++}) to a defined liquid medium containing tryptone was found to stimulate sporulation of *B. stearothermophilus* (Thompson and Thames, 1967). Addition of Mn^{++} to nutrient agar for increased sporulation of *B. stearothermophilus* has been utilized by various investigators (Schmidt, 1950; Titus, 1957; Kim and Naylor, 1966; Alderton and Snell, 1969a,b).

The D_{86}-value (decimal reduction time, or the time required to destroy 90% of the spores at 86°C) obtained when *B. stearothermophilus* strain E-2 spores were heated at pH 7.0 and 60°C indicated significant differences between spores activated by low pH-mild heat treatments and normal spores. These differences were dependent on the conditions (pH, time and temperature) under which the spores were activated. The minimum value (D_{86} = ~7 min) was observed when the spores were activated at pH 1.1 and 70°C for 60 min. When the temperature of activation was decreased to 60°C and 50°C, the D_{86}-value increased to 9 and 20 min, respectively. However, similar D-values were shown for spores which were treated overnight at pH 1.1 and 20°C or for 1 hr at pH 1.1 at 60°C. Thus, the higher D_{86}-value for spores treated at 50°C probably represents partial activation or a partial change to the heat-sensitive state.

On the other hand, the Ca-form spores have shown extreme heat resistance when heated below 100°C at pH 7.0, indicating a D_{36}-value of over 1000 min, which is similar to the D-value obtained with natural (untreated) spores heated under the same conditions.

Difficulties were encountered when attempts were made to compare these findings to those of other workers. That is, the conditions under which their experiments were carried out were different from those which were employed in this study. However, the D-values obtained with natural spores are comparable to those obtained by other workers. When *B. stearothermophilus* NCIB 8919 spores were heated in water at 100°C, a D_{100}-value of 3000 min was obtained (Briggs, 1966). For spores of *B. stearothermophilus* ATCC 7953, two D-values (D_{100} = 459 and 714 min) were reported (Murrell and Warth, 1965). The difference between the two values was attributed to differences in Ca:DPA ratios of the spores. A comparison between these findings and the data obtained with *B. stearothermophilus* E-2 native spores would indicate a fairly good agreement, and the discrepancies are quite explainable in light of the various factors which affect heat resistance.

The effect of an acid wash (Slepecky and Foster, 1959) on spore heat resistance was investigated by Murrell and Warth (1965). They reported little effect for such treatment (at 5°C) on the heat resistance of four *Bacillus* species. However, when spores of *B. stearothermophilus* strain NCA 1518 were treated in a liquid culture medium, previously adjusted to pH 3.0 with HCl, for 60 min at 70°C, the heat resistance was reduced greatly as compared to the untreated spores. Alderton and Snell (1969b) found there was a several hundred-fold difference between the heat resistance of heat-sensitive and heat-resistant forms of *B. stearothermophilus*. Thus the data obtained for the heat resistance of H-form and natural spores of *B. stearothermophilus* E-2 are generally comparable to those obtained by other workers.

Various studies have shown that activation of *Bacillus stearothermophilus* spores will take place primarily at temperatures above 100°C. With spores of *B. stearothermophilus* NCA 1518, Brachfeld (1955) found that maximum activation was obtained when the spores were heated for 5 min at 105°C. He also found that heating at lower temperatures (55–85°C) resulted in heat-induced dormancy and lower plate counts. Furthermore, his data have shown that even after heating the spores at 105°C for 5 min, complete activation was not obtained and a portion of the spores were unable to form colonies.

Lewis et al. (1965) demonstrated that activation of *B. stearothermophilus* spores was possible during prolonged incubation at 25°C and pH 1.5. The results of this study indicated that *B. stearothermophilus* E-2 spores were activated by a relatively mild heat treatment (50–70°C) under controlled pH conditions (1.1–2.0). At higher pH values (pH $\geq$ 3.0) and lower temperature (40°C) little activation was observed. The optimal time for activation under these conditions (pH 1.1–2.0 and 50–60°C) was found to be approximately 60 min.

Activation of dormant spores in this investigation was measured by two methods: (1) plating before and after treatment, and (2) by measuring the proportion of heat-sensitive spores. The assumption was that if the spores were sensitized to heat by the acid-low heat treatment, even if the acid was removed and heating was carried out at pH 7.0 then the amount of activated spores should be comparable to the amount of heat-sensitized ones. The data presented earlier (Tables 2 and 3) indicated that this relationship existed under the conditions used in this investigation. At higher levels of activation (>90% of the total count of spores) the plate count method becomes less sensitive in measuring activation. However, under these circumstances the amount of low pH-mild heat activation was more accurately measured by determining the proportion of heat-sensitive spores.

Lewis et al. (1965) found that the treatment of *B. stearothermophilus* spores at pH 1.5 and 25°C for 80 min increased the colony count from 18% of total count of spores to 80%, and the heat resistance of spores was greatly reduced. When these activated spores were exposed to 0.02M Ca^{++} at pH 9.7, dormancy and heat resistance was restored. Results obtained with *B. stearothermophilus* E-2 revealed over 99.9% activation (of total count) when the spores were treated at pH 1.1 and 60°C for 60 min. The higher amount of activation found in our experiments was probably due to the combined effects of higher temperature (60°C) and lower pH (1.1). However, temperature had a profound effect since treatment at 60°C and pH 2.0 yielded greater activation of *B. stearothermophilus* E-2 spores than was obtained by Lewis et al. (1965).

In another investigation, Brown et al. (1968) reported that the presence of 0.5N hydrochloric acid at 25°C increased the colony count of *B. stearothermophilus* (NCIB 8919) spores to the total microscopic count. Although the time of exposure was not stated in their work, their data show that maximum activation occurred after 28–30 min, and that longer exposure to the acid reduced the colony count. However, 60 min were required to obtain maximum

activation at pH 1.1–2.0 in this study. This difference is probably due to the higher acid concentration, resulting in a lower pH, used by Brown et al. (1968). The reduced colony counts which were observed when the spores of *B. stearothermophilus* E-2 spores were treated over 60 min may be due to inhibition or loss of certain factor(s) which affect activation, germination or outgrowth. When the amount of activation was determined by measuring the proportion of heat-sensitive spores, no reduction of activation over time was observed. This suggests that under the conditions used in this investigation activation was not inhibited but the ability of spores to form colonies on NAM was impaired. The effect is presumably associated with germination since microscopic examination showed that <1.0% of the spores were converted to phase dark spores even after 120 min at pH 2.0 and 60°C. When *Bacillus cereus* spores were heated at pH 1 and 65°C the spores were activated by short (0–30 min) exposures, but longer (45–90 min) exposures blocked their ability to germinate in a solution containing L-alanine and adenosine; this was interpreted as a specific inhibition of the alanine-adenosine germination system since the spores were able to germinate and form colonies on a rich medium (Keynan et al., 1965). Other investigators have also reported that heating impaired germination systems of *Bacillus subtilis* (Edwards et al., 1965; Busta and Adams, 1972). Since we did not study germination per se, it is impossible to determine whether the decrease in colony counts was due to inactivation of germination systems and/or outgrowth processes of germinated spores.

Results on enumeration of spores inoculated into flour and sugar samples indicated that activation occurred when the spores were incubated for 60 min at pH 1.1 and 60°C. Higher plate counts were obtained with the low pH-mild heat treatment than with a recommended method for enumeration of spores (APHA, 1966). Similar low results were obtained using the "Gerber" method described previously. Apparently heat activation and germination of some of the spores were partially inhibited while activation by low pH was not affected. Some of the components of flour, such as minerals and salts, reduce germination (Halmann and Keynan, 1962 and Keynan et al., 1965) and carbohydrates interfere with heat activation (Fields and Finely, 1964). Further investigations are being planned to assess the feasibility of utilizing low pH-mild heat treatments for enumerating thermophilic flat-sour spores in food ingredients. Additional experiments are underway to determine the nature of the type(s) of injury induced by prolonged exposure to low pH-mild heat environments.

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INHIBITION OF Staphylococcus aureus GROWTH BY COMBINATIONS OF BUTYLATED HYDROXYANISOLE, SODIUM CHLORIDE, AND pH

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ABSTRACT

Trypticase Soy Broth containing butylated hydroxyanisole (BHA; 0, 50 or 100 ppm) and sodium chloride (NaCl; 3, 5, or 7%) at pH 5.0, 6.0, or 7.0 was inoculated with *Staphylococcus aureus* S-6 to evaluate the antimicrobial effectiveness of these chemicals in concert. BHA at the levels tested became more effective in preventing growth as the pH of the medium was decreased and the NaCl concentration was increased. The combination of 100 ppm BHA with 5 or 7% NaCl at all three pH levels tested had the strongest bactericidal effect.

INTRODUCTION

DIFFERENT antimicrobial agents that prevent growth and toxin production by *Staphylococcus aureus* have been studied. Nunheimer and Fabian (1940), reported that 15–20% salt was inhibitory for growth, while 20–25% was found to be germicidal. Lechowich et al. (1956), studied the effects of NaCl and pH on the growth of food poisoning staphylococci in culture media and in meat products. They found growth inhibition below a pH of 4.8. Genigeorgis and Sadler (1966) reported that *S. aureus* S-6 produced enterotoxin B at pH 6.9 with 10% salt, at pH 5.5 with 8% salt, and at pH 5.1 with up to 4% salt. Increased inhibition of growth of unstressed *S. aureus* cells by NaCl at pH levels above or below the optimum was demonstrated by Iandolo et al. (1964). Other investigators (Minor and Marth, 1971; Tatini, 1973; Smolka et al., 1974) further reviewed the inhibitory effects of pH and NaCl on *S. aureus.*

The antimicrobial effects of butylated hydroxyanisole (BHA), which is currently added to foods as an antioxidant, have been studied by Chang and Branen (1975), Robach et al (1977), Shih and Harris (1977) and VanTassell et al. (1978). These investigators have shown and confirmed that various strains of *S. aureus* are inhibited at 150–200 ppm BHA in synthetic media. Minor and Marth (1976) stated that "the behavior of staphylococci and their significance in acidic environments with another form of stress is unpredictable." Therefore, the purpose of this study was to determine the influence of BHA, pH and NaCl, in concert, on *S. aureus* S-6 growth.

MATERIALS & METHODS

Culture

Staphylococcus aureus S-6 was obtained from R. Bennett (FDA, Washington, DC). A stock culture was prepared by inoculating a tube of Trypticase Soy Broth (TSB; BBL), incubating for 24 hr at 37°C and transferring at weekly intervals. The inocula for growth studies were prepared by transferring a loopful of the stock culture to a tube containing 5 ml of TSB and incubating the tube for 12 hr at 37°C. The culture was diluted 1:100 in sterile 0.1% peptone and 1.0 ml was inoculated into 50 ml of sterile experimental medium described below, to obtain approximately 10^5 cells per ml.

Growth inhibition studies

BHA was obtained from the Eastman Chemical Co., Kingsport, TN. One gram of BHA was dissolved in 95% ethanol to obtain a 2% w/v final solution. The solution was filter sterilized with a 0.22μ Millipore filter (Millipore Corp., Bedford, MA). The sterile solution was refrigerated at 4°C throughout the investigation.

The experiments were performed with 250 ml screw cap Erlenmeyer flasks containing 50 ml of TSB. Prior to sterilization, NaCl was added to the TSB to produce concentrations of 3, 5 and 7% (w/v). The acidity of these media was adjusted to pH 5, 6 or 7 by the addition of 5N HCl. Appropriate amounts of the filter sterilized BHA were aseptically added to the sterile, cooled TSB to give final concentrations of 0, 50 and 100 ppm BHA. All flasks containing the various inoculated test media were incubated at 37°C, 180 rpm in a shaker waterbath (Fermentation Design, Inc., Allentown, PA). Each experiment had one flask for each variable.

Enumeration

Cell numbers in the TSB were determined by the use of Trypticase Soy Agar (TSA; BBL) pour plates of samples taken after 0, 6, 12, 18, 24, 36 and 48 hr incubation. The samples were serially diluted with 0.1% peptone blanks before plating. The TSA plates were incubated at 37°C for 36–48 hr at which time the colonies were counted and their numbers recorded. Reported values are the average from duplicate experiments. The data for 48 hr growth were analyzed statistically by Duncan's Multiple Range Test (Duncan, 1955).

RESULTS & DISCUSSION

THE GROWTH of *S. aureus* in TSB containing 3, 5 or 7% NaCl and 0, 50 or 100 ppm BHA at pH 7.0 is shown in Figure 1. In the absence of BHA, there was relatively little effect of NaCl on the growth response of this microorganism. The log number of cells obtained after 48 hr growth was not significantly different at all three salt concentrations. In the presence of 50 ppm BHA, there was an increased lag phase of growth as the salt concentration was increased from 3 to 5%. With 7% NaCl and 50 ppm BHA, a bacteriostatic effect was obtained. After 48-hr incubation in the presence of 50 ppm BHA the numbers of cells were significantly less ($P \leqslant 0.05$) in the 7% NaCl medium as compared to the 3 and 5% NaCl media. At 100 ppm BHA, a bactericidal action was observed and as the salt concentration was increased from 3 to 7% this action became more pronounced. The log number of cells obtained after 48 hr incubation in the presence of 100 ppm BHA was significantly less ($P \leqslant 0.05$) than 0 and 50 ppm BHA at all salt levels.

Chang and Branen (1975) suggested that the phenolic nature of BHA might allow it to be used as an antistaphylococcal agent since other phenolic compounds have been used for this purpose. They found that 150 ppm BHA was necessary to obtain a bactericidal effect on the growth of *S. aureus.* More recently, Shih and Harris (1977) reported that 150–200 ppm BHA only produced a bacteriostatic effect on the growth of *S. aureus,* while 400 ppm was required to observe bactericidal activity. Differences between BHA levels necessary to inactivate cells of *S. aureus* in these investigations might be attributed to the strains of organisms and culture media used (Sands and Bennett, 1966). However, when reporting the concentra-

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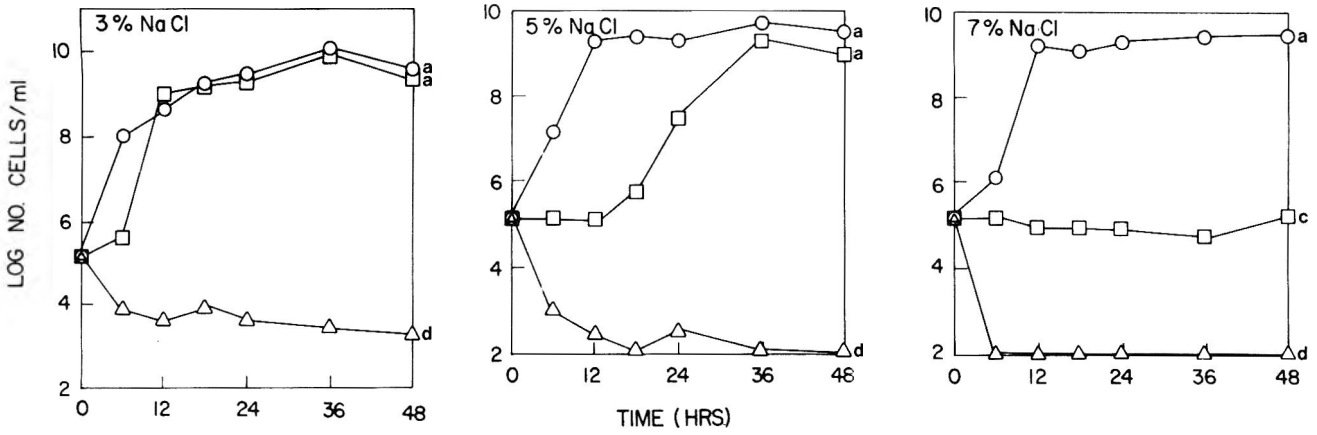

Fig. 1—Effect of 0 (○), 50 (□) and 100 (Δ) ppm butylated hydroxyanisole (BHA) on the growth of Staphylococcus aureus S-6 in Trypticase Soy Broth containing either 3, 5 or 7% sodium chloride at pH 7.0. Log cell numbers at 48 hr that have different letters are significantly different at the level P ≤ 0.05 according to Duncan's multiple range test (Duncan, 1955).

tions of BHA required to inhibit growth of *S. aureus* the additional stress afforded by the NaCl and pH must be considered.

Figure 2 shows the effect of pH 6.0 on the growth response of *S. aureus* in TSB containing 3, 5 or 7% sodium chloride and 0, 50 or 100 ppm BHA. Again, there was little effect of increasing salt concentration upon the growth of *S. aureus* in the absence of BHA. When a concentration of 50 ppm BHA was added to the growth medium with 3% NaCl, there was an increased lag phase of growth. As the concentration of salt was increased to 5%, growth was only observed after 36 hr of incubation. In 7% NaCl and 50 ppm BHA, a bacteriostatic effect was observed throughout the incubation period. The log number of cells obtained after 48 hr growth in the presence of 50 ppm BHA significantly decreased ($P \leq 0.05$) as the concentration of salt was increased from 3 to 5 to 7%. Inactivation of *S. aureus* became more distinct in TSB at pH 6.0 with 100 ppm BHA when the NaCl level was increased from 3% to 7%, producing results similar to those observed in TSB at pH 7.0 with 100 ppm BHA.

Figure 3 gives the growth curves for *S. aureus* at 3, 5 and 7% NaCl and 0, 50 and 100 ppm BHA at pH 5.0. In the absence of BHA, the lag phase of growth increased as the salt concentration increased from 3 to 7%. However, at 48-hr incubation, the log number of cells was not significantly different at all levels of NaCl. With 50 ppm BHA, a reduction in cell numbers was noticeable throughout the incubation period and this decrease became more evident as the salt concentration was increased. Again, in the presence of 100 ppm BHA, a bactericidal effect was obtained and this effect was markedly increased as the salt level was raised. In comparing the effect of 50 ppm BHA at different pH values, inhibition became more pronounced as the pH was decreased. In fact, there were significantly less cells ($P \leq 0.05$) after 48 hr at pH 5.0 with 50 ppm BHA than at pH 6.0 or pH 7.0 with 50 ppm BHA for all salt concentrations. However, in the absence of BHA at pH 5.0, 6.0, and 7.0, there were no significant differences between final cell populations at all levels of NaCl.

To date, reports on the effects of antioxidants on *S. aureus* have been concerned with BHA alone or BHA in combination with other antioxidants and have neglected interactions with NaCl and/or pH. Data obtained in this study indicate that the addition of BHA to growth media at various pH values, allows lower concentrations of NaCl to inhibit growth of *S. aureus* when compared to other investigations using NaCl and pH alone. For example, Genigeorgis et al. (1971), reported limiting pH values of 4.5 and 8.0 at 12% NaCl. Tompkin et al. (1973) found growth of *S. aureus* to occur at pH 4.9 with 3% NaCl. In our experiments, growth was inhibited with 3% NaCl at pH 5.0 and

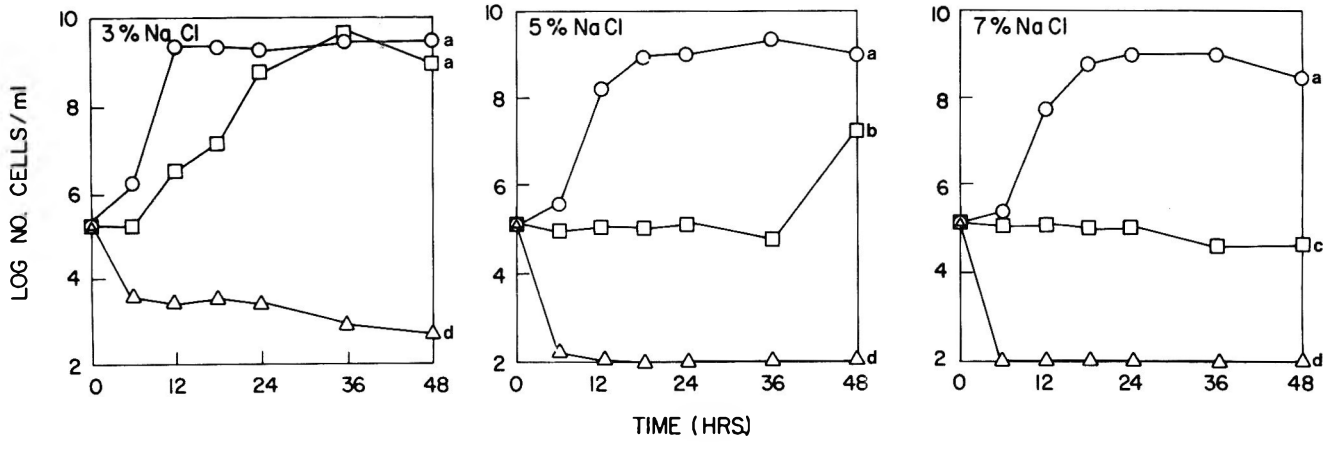

Fig. 2—Effect of 0 (○), 50 (□) and 100 (Δ) ppm butylated hydroxyanisole (BHA) on the growth of Staphylococcus aureus S-6 in Trypticase Soy Broth containing either 3, 5 or 7% sodium chloride at pH 6.0. Log cell numbers at 48 hr that have different letters are significantly different at the level P ≤ 0.05 according to Duncan's multiple range test (Duncan, 1955).

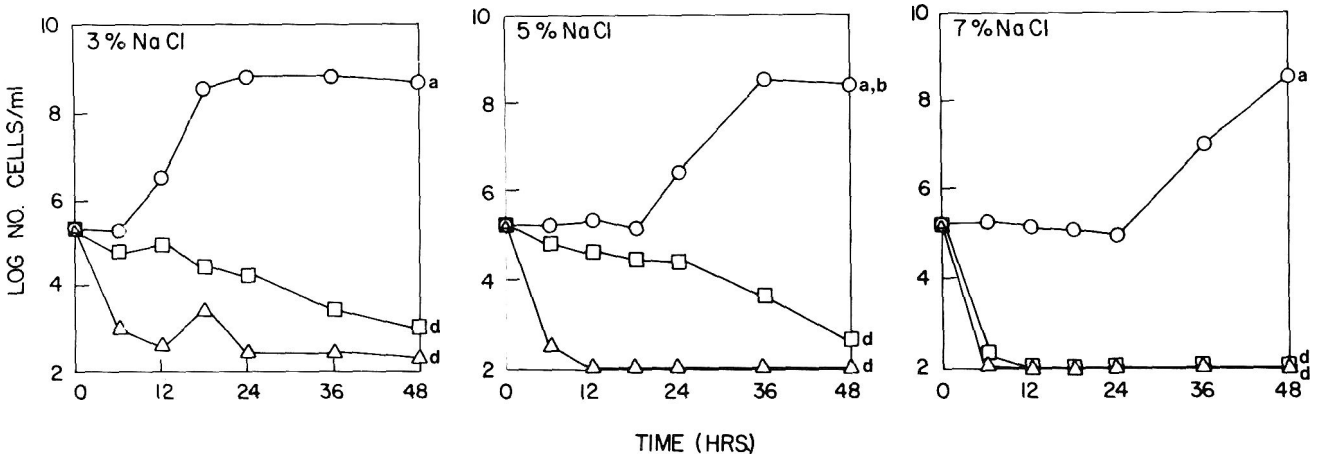

Fig. 3—Effect of 0 (○), 50 (□) and 100 (△) ppm butylated hydroxyanisole (BHA) on the growth of Staphylococcus aureus S-6 in Trypticase Soy Broth containing either 3, 5 or 7% sodium chloride at pH 5.0. Log cell numbers at 48 hr that have different letters are significantly different at the level $P \leq 0.05$ according to Duncan's multiple range test (Duncan, 1955).

pH 6.0 in the presence of either 50 ppm or 100 ppm BHA. Growth was also prevented at pH 7.0 with 3% NaCl in the presence of 100 ppm BHA. In general, BHA became more effective as the pH of the medium was decreased and the NaCl concentration was increased. Genigeorgis and Sadler (1966) and Smolka et al. (1974) reported that cell numbers were found to decrease as NaCl levels were increased at any pH value tested.

The recognized function of BHA in foods is the prevention of oxidative rancidity (Stuckey, 1972). However, results from the present study as well as past investigations indicate a potential use for BHA as an anti-staphylococcal agent. BHA used in combination with known antimicrobial agents (i.e. esters of p-hydroxybenzoic acid) and chemicals commonly found in foods (i.e. NaCl and hydrogen ion) may aid in decreasing the concentrations of these chemicals presently used to inhibit pathogens in food systems.

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THERMAL DESTRUCTION OF FOLACIN: EFFECT OF ASCORBIC ACID, OXYGEN AND TEMPERATURE

T.-S. CHEN and R.G. COOPER

ABSTRACT

The effects of temperature and the presence of oxygen and reducing agent on the thermal stability of two naturally occurring labile folates were investigated. Microbiological assay of samples heated from 49–100°C for various lengths of time revealed that the loss of 5-methyltetrahydrofolic acid in aqueous solution followed first order kinetics with a low Arrhenius activation energy of 9.5 kcal/mole. Tetrahydrofolic acid was extremely labile during heating and the stability of both forms of folate was increased in the presence of ascorbate and nitrogen atmosphere.

INTRODUCTION

THE GROUP of compounds denoted by the term folacin is a heterogeneous group of derivatives with a similar basic structure and biological function. Pteroylglutamic acid (PteGlu), or folic acid, is the basic structural unit in these compounds. Other monoglutamate folates are formed when the pteridine moiety of this basic molecule is reduced or substituted in the 5-nitrogen or 10-nitrogen position. In addition, all of these monoglutamate folates may be transformed into polyglutamates of various length by the addition of glutamic acid residues to the basic molecule.

The existence of folacin in numerous forms coupled with the lack of differentiation between forms has led to complications in characterizing the vitamin and establishing meaningful tables for folacin content of foods. Improved methods have recently come into use for determining the specific forms of folacin present in foods (Chan et al., 1973). The value derived from separation and quantification of folates has been limited due to the lack of information concerning the stability characteristics of various folate forms. Indeed, various monoglutamate forms of folacin have been found to vary tremendously in their thermal stability (Cooper et al., 1978; Paine-Wilson and Chen, 1979).

Some forms of folacin are subject to greater deterioration than other forms. This has been concluded from research on pure folates in solution at room temperature (O'Broin et al., 1975) as well as from studies on purified and naturally occurring folates exposed to heat (Ghitis, 1966; Cooper et al., 1978). Tetrahydrofolic acid (H_4PteGlu), and 5-methyl-tetrahydrofolic acid (5-CH_3H_4PteGlu) have been shown to be moderately labile derivatives under all conditions of testing, yet the nature and rate of the degradation reactions for these nutrients have not been characterized.

The presence of oxygen has been implicated as a possible factor in the degradation of folates during heating. Heat processing of canned and sealed foods has resulted in markedly higher folacin retention than home cooking methods (Huskisson and Retief, 1970; Hellendorn et al., 1971; Lin et al., 1975; Suckewer et al., 1971). In addition, Rolls and Porter (1973) found that the percentage of folacin destroyed during heat processing of milk is greatly affected by the level of residual oxygen in the milk. Ascorbic acid has a protective effect on folates possibly due to its action as a reducing agent (O'Broin et al., 1975).

The response of folacin to changes in temperature has not been well documented. PteGlu has been the most studied folate form in this regard. Dick et al. (1948) undertook a study to determine the thermal stability of PteGlu. Their results showed that PteGlu is stable up to 1 hr when heated at 100°C in solutions with pH above 5. Destruction occurred at lower pH. The reaction was shown to be unimolecular, although the initial rate was frequently erratic.

Another study concerning kinetics of PteGlu destruction by heat involved heating a multivitamin preparation containing PteGlu at various temperatures (Garrett, 1956). The pH of the vitamin preparation was 3.2 and experimental temperatures ranged from 50–70°C. It was found that the rate of PteGlu destruction increased with increasing temperature. After 10 days, loss of PteGlu ranged from approximately 22% at 50°C to 90% at 70°C. The initial rate of loss of PteGlu was described as being pseudo zero order which subsequently becomes pseudo first order with the extent of such zero order degradation increases with decreasing temperature. Reaction rates for other folates have not been reported.

Two labile folates were investigated in this study with respect to the conditions affecting thermal degradation. The folates used were H_4PteGlu and 5-CH_3H_4PteGlu. These forms, along with derivatives of these forms are widely distributed in biological materials. The effects of temperature and the presence of oxygen and reducing agent were investigated.

MATERIALS & METHODS

Sample preparation

Two crystalline folacin derivatives were used, including dl,L-tetrahydrofolic acid (H_4PteGlu), (Sigma Chemical Co., St. Louis, MO, 76% purity), and DL-N-5-methyltetrahydrofolic acid (5-CH_3H_4PteGlu), (Barium salt, Sigma Chemical Co., 90% purity). A stock solution of 5-CH_3H_4PteGlu (37.5 μg/ml) was prepared and frozen in 5 ml aliquotes. A stock solution of H_4PteGlu (34 μg/ml) was prepared immediately prior to the experiment, due to its reported lability upon exposure to air. This form was dissolved under bubbling nitrogen in deaerated, deionized water. Folacin stock solutions were diluted 100-fold at the start of an experiment, effecting final folacin concentration of 567 nM for 5-CH_3H_4PteGlu and 431 nM for H_4PteGlu. Diluting solution consisted of deionized water, with or without 0.1% ascorbate (0.0056M).

Exposure to heat

Diluting solutions were preheated and maintained at the temperature under investigation. Ninety-nine milliliters of each solution were heated in a triple neck flask connected to a reflux condenser. The flask and condenser were foil covered to prevent photo-degradation of folacin. Flasks were heated over a heating mantle for experiments at 100°C and in water baths for experiments at temperatures lower than 100°C. One ml of the folacin stock solution was added to the preheated diluting solution. The resulting experimental solution was agitated and sampled immediately for the zero time folacin

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concentration. One ml samples were then withdrawn at intervals for assay and quenched by transferring to test tubes immersed in an ice bath.

Those solutions heated under nitrogen atmosphere were treated as described above. Nitrogen gas was passed through glass tubing inserted through a rubber stopper to the bottom of the flask. The top of the condenser was covered lightly with foil, while the third neck of the flask was sealed. Flasks were opened to remove samples of the solution.

Folacin determination

Folacin concentration of experimental solutions was determined through microbiological assay using *Lactobacillus casei* (ATCC 7469). This folacin assay was essentially the procedure used by Waters and Mollin (1961), with revisions reported by Tamura et al. (1972). A higher level of ascorbate was used in the assay medium for H_4PteGlu, due to its greater lability. A level of 0.3% ascorbate (0.0168M) was used in place of the usual 0.05% (0.0028M) for this assay method. Five or ten microliters of the cooled samples were aseptically micropipetted into the sterilized assay medium and then inoculated with *L. casei*. Growth of the microorganism was measured by changes in optical density. This was compared to a standard curve of *L. casei* growth in medium containing PteGlu as the sole source of folacin available to the microorganism. The results are expressed in PteGlu activity in ng per 5 ml tube. Each sample was assayed in triplicate.

RESULTS & DISCUSSION

Effect of ascorbate

The presence of 0.1% ascorbate was found to have a marked effect on the stability of 5-CH_3H_4PteGlu at 100°C. As shown in Figure 1, this form lost only 20% of its activity through 3 hr of heating, in the presence of ascorbate. In contrast, under the same conditions without ascorbate, 5-CH_3H_4PteGlu lost 90% of its activity in 65 min of heating. The pH of the aqueous 5-CH_3H_4PteGlu solution was altered by the addition of ascorbate, dropped from 7 to 3 when ascorbate was added. However, the greater stability of 5-CH_3PteGlu in the presence of ascorbate cannot be attributed to pH, as 5-CH_3H_4PteGlu is more stable at neutral pH than at acidic pH (O'Broin et al., 1975; Paine-Wilson and Chen, 1979). A level of 0.1% ascorbate in the present study was sufficient to afford protection for 5-CH_3H_4PteGlu for up to 3 hr at boiling. O'Broin et al. (1975) reported that a level of 0.2% ascorbate afforded protection for a routine autoclave cycle (121°C, 12–15 min) at pH 6.1. They also found that 5-CH_3H_4PteGlu is quite stable above neutrality, but unstable at more acidic pH, resulting in the formation of an unidentified, nutritionally inactive derivative. Results of the present study show that the degradation product of 5-CH_3H_4PteGlu when heated in neutral solution is nutritionally inactive as determined by the *L. casei* assaying method.

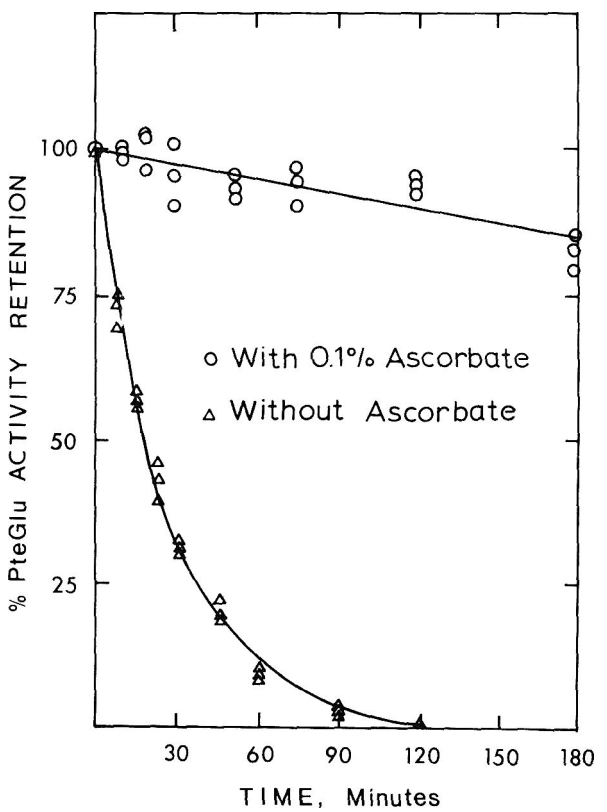

Fig. 1—Effect of 0.1% ascorbate on thermal stability of 5-CH_3H_4PteGlu at 100° C in aqueous solution.

Effect of nitrogen atmosphere

To investigate the role of atmospheric oxygen in folacin thermal degradation, nitrogen was bubbled through the solution in the heating vessel. Both 5-CH_3H_4PteGlu and H_4PteGlu were considerably stabilized when heated at 100°C under nitrogen atmosphere (Fig. 2). 5-CH_3H_4PteGlu lost little activity through 1 hr of heating under nitrogen when the system was disturbed only every 20 min for sampling. Preliminary studies showed an equal degree of 5-CH_3H_4PteGlu destruction when heated with and without nitrogen atmosphere. However, in these preliminary studies, the flask was opened for sampling at frequent intervals. The slight destruction which occurred in the final study may still be partially attributed to oxygen entering the flask during sampling. Stability afforded by the nitrogen atmosphere was comparable to the stability afforded by a level of 0.1% ascorbate (Fig. 1).

H_4PteGlu showed considerable stability under nitrogen through 2 hr of heating at 100°C. Although the level did decrease during the heating period, the extent of loss was not nearly as great as during heating without nitrogen where most of the *L. casei* activity was lost within 12 min of heating (Fig. 2). The degradation observed during heat-

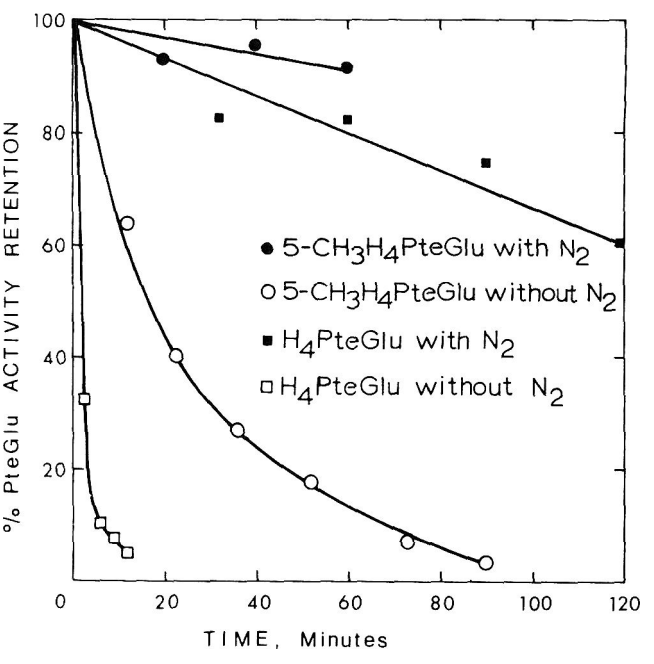

Fig. 2—Effect of nitrogen atmosphere on folacin stability at 100° C. (Each point represents the mean of triplicates).

ing under nitrogen atmosphere may also be attributed to the sampling process.

These data indicate that the major portion of degradation of H_4PteGlu and 5-CH_3H_4PteGlu at elevated temperatures is due to an oxidative process, requiring the presence of molecular oxygen. These data also suggest the possibility of using nitrogen storage to protect labile folates in food products.

Effect of temperature

The effects of temperature on folacin stability were investigated in order to establish kinetic data on folacin destruction by heat. Effect of temperature on degradation of H_4PteGlu is shown in Figure 3. The same initial concentration of H_4PteGlu was used for each temperature, as 1 ml was taken from the H_4PteGlu stock solution under bubbling nitrogen and added to 99 ml deionized water preheated to each of the temperature under investigation. Samples taken from flasks held at temperatures lower than 100°C had virtually leveled off in *L. casei* activity by the time the 3-min sample was taken. Folacin levels in these flasks were erratic and showed no correlation to temperature. H_4PteGlu in the 100°C flask showed a more gradual decrease in activity. Temperature effects alone cannot account for these data.

Bakerman (1961) also noted an ambiguity during heat studies on H_4PteGlu. Samples of a H_4PteGlu solution with ascorbate which was heated initially showed a markedly slower degradation over a period of days than samples of the solution which had not been heated. The suggested mechanism for this observation was that heating effected a decrease in dissolved oxygen in the solution, thereby slowing the rate of oxidation. This mechanism may be a factor in the greater stability of H_4PteGlu observed at boiling temperatures in the present study, with erratic results at lower temperatures.

Destruction of 5-CH_3H_4PteGlu in aqueous solution is remarkably temperature dependent, as shown in Figure 4. The rate of destruction increased with increasing temperature.

Ghitis (1966) divided the folates in milk into three fractions based on thermal stability. One fraction was described as resembling 5-CH_3H_4PteGlu in its lability to heat. This fraction was able to resist pasteurization at 56°C, but could not resist a temperature of 100°C, whether for 5 sec or 5 min.

Although the present data do indicate the lability of 5-CH_3H_4PteGlu at 100°C, the lability is not nearly great enough to result in loss of this folate form after only 5 sec of boiling, as the half-life is considerably greater. It might be expected that this form of folate would be even more stable in a food system such as milk, in the neutral pH range with the presence of buffering components and some reducing agents. However, the effects of folate concentration, level of dissolved oxygen, and presence of other food components would have to be investigated to make definite conclusions in comparing the two sets of data.

Kinetics of 5-CH_3H_4PteGlu degradation

The rate of degradation of 5-CH_3H_4PteGlu by heat was ascertained by computerized regression analysis of data on folate concentration versus time at constant temperature. This analysis resulted in a straight line when the logarithm of the concentration is plotted against time (Fig. 4). Considering the inherent error in microbiological assays, the extremely good fit of the data points to regression line indicates that the thermal degradation of 5-CH_3H_4PteGlu under the conditions studies is first order at temperatures ranging from 65–100°C. At 49°C the reaction order describing the rate of folate loss cannot be ascertained since the experiment was not carried out for a peroid longer than

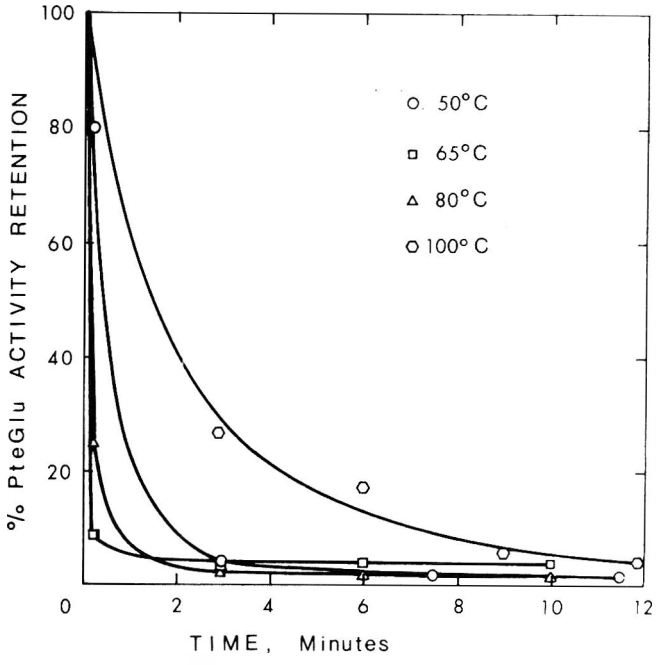

Fig. 3—Effect of temperature on stability of H_4PteGlu in aqueous solution. (Each point represents the mean of triplicates).

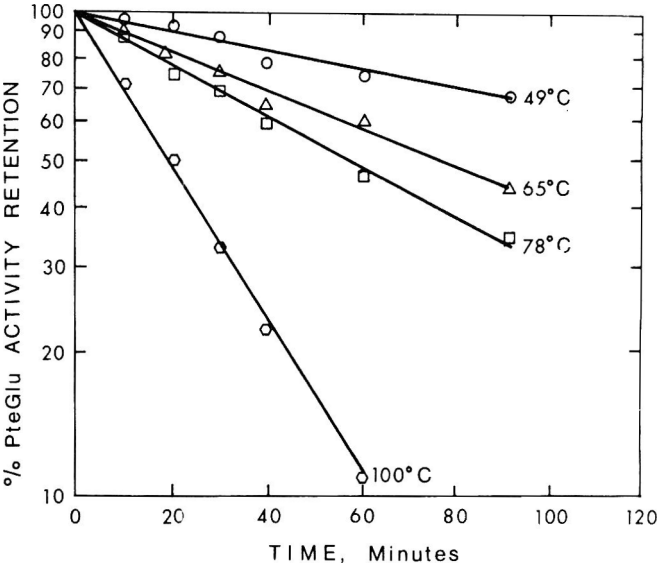

Fig. 4—Effect of temperature on stability of 5-CH_3H_4PteGlu in aqueous solution. (Each point represents the mean of triplicates).

one half-life. The slope of the regression line represents the first-order rate constant (k), in reciprocal minute for the specific temperature. From k, the half-life of the compound can be calculated by the equation: $t_{1/2} = \log_e 2/k$. The rate constants and half-lives for 5-CH_3H_4PteGlu at various temperatures tested are presented in Table 1. Comparing the half-life of 5-CH_3H_4PteGlu at 100°C to that of other heat-labile vitamins, it appears 5-CH_3H_4PteGlu is considerably less heat stable than other vitamins. For example, the half-life of thiamine, the most heat sensitive of the B vitamins, as calculated from the data reported by Goldblith et al. (1968) is 63 min at 101.5°C, compared to 21 min for 5-CH_3H_4PteGlu at 100°C.

The temperature dependence for 5-CH_3H_4PteGlu de-

Table 1—First-order reaction rate constants (k) and half-lives (t½) for 5-CH_3H_4PteGlu destruction as a function of temperature

Temp (°C)	k (min^{-1})	t½ (min)
49	0.0043	163.1
65	0.0085	80.8
78	0.0145	47.8
100	0.0323	21.4

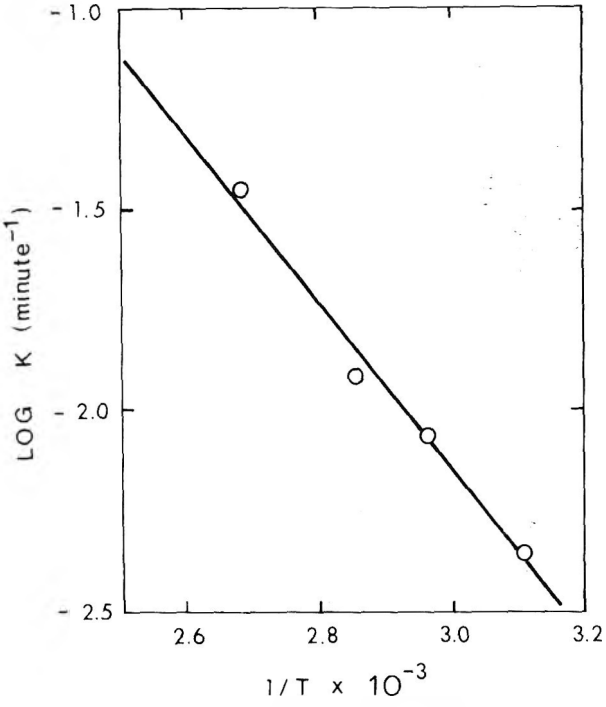

Fig. 5—Arrhenius plot for thermal destruction of 5-CH_3H_4PteGlu in aqueous solution.

struction was further analyzed according to the Arrhenius equation:

$$k = A \exp(-E_a/RT)$$

where k = first-order rate constant; A = Arrhenius pre-exponential; E_a = Arrhenius activation energy (cal/mole); R = gas constant and T = absolute temperature (K°) Conformity of the temperature data for 5-CH_3H_4PteGlu to the Arrhenius equation is shown graphically in Figure 5 where log k is plotted against 1/T. The linearity of the plot supports the use of the Arrhenius model for expressing the temperature dependence of the reaction rate constant for 5-CH_3H_4PteGlu. Among vitamins that have been shown to conform to the Arrhenius equation in their degradation reactions are thiamine, ascorbic acid (Labuza, 1972), and pantothenic acid (Hamm and Lund, 1978).

The Arrhenius activation energy (E_a) for the 5-CH_3H_4PteGlu degradation reaction was calculated to be 9.5 kcal/mole, from the slope of the regression line in Figure 5. Only one reported E_a for a folacin compound was found in the literature. Garrett (1956) reported the E_a for PteGlu degradation at pH 3.2 to be 16.8 kcal/mole. Some E_a for nutrient destruction and other food related reactions have been reported in the literature. For example, the E_a for ascorbic acid may vary from 10–30 kcal/mole, while that for lipid oxidation ranges from 15–25 kcal/mole (Labuza, 1972), and the E_a for thiamine degradation ranges from 27–29 kcal/mole. From these data, it appears that the destruction of 5-CH_3H_4PteGlu has a relatively low E_a in comparison with other nutrients.

SUMMARY

UNDER THE CONDITIONS of this study:

1. Among the two naturally occurring folates studied, H_4PteGlu is extremely heat-labile. The half-life of H_4PteGlu at 100°C was found to be 2.25 min while that of 5-CH_3H_4PteGlu was 21.4 min.

2. The stability of both forms of folate at 100°C was drastically increased in the presence of ascorbate or under nitrogen atmosphere. These data indicate that degradation of these folates at elevated temperatures is due to an oxidative process requiring the presence of molecular oxygen.

3. The rate of 5-CH_3H_4PteGlu destruction is temperature dependent conforming to the Arrhenius equation with a low activation energy (E_a) of 9.5 kcal/mole. This is consistent with first order kinetics.

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THERMAL DESTRUCTION OF FOLACIN: EFFECT OF pH AND BUFFER IONS

B. PAINE-WILSON and T. -S. CHEN

ABSTRACT

Thermal stability of four folacin derivatives at 100°C was determined as a function of pH and buffer ions. The degradation reaction at 100°C for all the folate forms studied followed first order kinetics. Under identical heating conditions, folic acid (PteGlu) and 5-formyltetrahydrofolic acid (5-$CHOH_4$ PteGlu) were quite stable whereas 5-methyltetrahydrofolic acid (5-CH_3H_4 PteGlu) and tetrahydrofolic acid (H_4 PteGlu) were very labile. Both PteGlu and 5-$CHOH_4$ PteGlu were stable up to 10 hr of heating in pH 4–12, but unstable in more acidic conditions. 5-CH_3H_4 PteGlu showed greatest stability at pH 7. The rate constant for 5-CH_3H_4 PteGlu destruction is directly proportional to hydrogen ion concentration between pH 2.6 and 7.0 and inversely proportional to hydrogen ion concentration between pH 7–12. The stability of H_4 PteGlu at 100°C was found to decrease linearly with the hydrogen ion concentration in the pH range 4–12. Universal buffer was found to cause higher thermal destruction rate only for 5-CH_3H_4 PteGlu and H_4 PteGlu than the other buffers tested.

INTRODUCTION

RECENT FINDINGS of both low dietary intake of folacin (Walker and Page, 1975) and low serum folate levels (Baker et al., 1975) have prompted a reevaluation of the stability of naturally occurring folacin compounds. Early research which indicated that folic acid is stable to heat in a pH range 4.0–10.0 was based on experimentation with pteroylglutamic acid (PteGlu)(Dick et al., 1948). However, it has been established that the predominant forms of folacin in most foods are polyglutamates (total folates) and mono- and di-glutamates (free folates) of tetrahydropteroylglutamic acid (H_4 PteGlu), 5-formyl-tetrahydropteroylglutamic acid (5-$CHOH_4$ PteGlu), 5-methyl-tetrahydropteroylglutamic acid (5-CH_3H_4 PteGlu), and 10-formyl-tetrahydropteroylglutamic acid (10-$CHOH_4$ PteGlu) (Butterworth, 1968; Perry, 1971; Santini et al., 1964). It is therefore of interest to compare the thermal stability of PteGlu with that of the naturally occurring folates.

In both food and model systems large losses of free and total folates have been reported to occur as a result of heat treatment (Malin, 1975). However, due to variations in folate retention reported, it is probable that factors other than heat may modify the degradation reaction. Among the factors which have been implicated as contributing to the nutritional stability of food folates are temperature and oxygen (Chen and Cooper, 1979); source of heat (Cooper et al., 1978); sunlight and artificial light (Stokstad et al., 1947); chemical environment (O'Broin et al., 1975; Rabinowitz, 1960); pH (Blakley, 1969; Rabinowitz, 1960; Stokstad and Thenen, 1972); and leaching loss (Malin, 1975).

The importance of pH in the oxidative destruction of folacin compounds has been reported by a number of researchers and summarized by Blakley (1969), Rabinowitz (1960), and Stokstad and Thenen (1972). There is general agreement that PteGlu is stable under neutral and alkaline conditions and less stable under acidic conditions (Blakley, 1969; Dick et al., 1948; Stokstad and Thenen, 1972). There is little information, however, concerning the relative thermal stability of the naturally occurring folates over a wide range of pH. In addition, variations in percent destruction reported have suggested that the chemical environment may also play a role in the thermal stability of these compounds (Dick et al., 1948; O'Broin et al., 1975; Rabinowitz, 1960; Ramasastri and Blakley, 1964). This study was undertaken in order to determine the effect of pH and buffer ions on the thermal stability of naturally occurring folacin compounds in buffered solutions. The folates used were PteGlu, 5-$CHOH_4$ PteGlu, 5-CH_3H_4 PteGlu, and H_4 PteGlu.

MATERIALS & METHODS

Preparation of samples

PteGlu was obtained from ICN Pharmaceuticals (Cleveland, OH); dl-5-$CHOH_4$ PteGlu (Calcium Salt) was a gift of Lederle Division, American Cyanamid Co. (Pearl River, NY); dl-5-CH_3H_4 PteGlu (Barium Salt, 90% purity) and dl-L-H_4 PteGlu (76% purity) were purchased from Sigma Chemical Co. (St. Louis, MO).

Stock solutions (56.7 mM) of PteGlu, 5-$CHOH_4$ PteGlu, and 5-CH_3H_4 PteGlu, prepared by dissolving each folate compound in deionized water, were stored in 8 ml aliquots in the dark at −15°C until used. The stock solution of H_4 PteGlu (43.1 mM) was prepared immediately prior to the experiment by dissolving it under bubbling nitrogen in deaerated, deionized water, due to its reported lability upon exposure to air. The experimental folate solutions were prepared by diluting 1 ml of the stock solution with 99 ml of an appropriate buffer solution.

A stock solution of PteGlu for use in the establishment of standard growth curves for *Lactobacillus casei* was made by dissolving PteGlu in 0.01N NaOH in 20% ethanol and stored in the same manner as the experimental folate solutions.

Buffer systems

The buffer solutions used for diluting the folacin stock solutions were Universal buffer (pH 2.6–12.0), HCl/KCl buffer (pH 1.1, 2.0, and 3.0), Citrate Phosphate buffer (pH 3.0), and citrate buffer (pH 3.0). These were prepared according to the instructions of Dawson and McKenzie (1969).

Thermal processing system

The experiments were all carried out at 100°C in tripleneck round bottom flasks fitted with a condenser and a thermometer. The flasks were heated over a heating mantle. All glassware was covered with foil to protect the folacin solution from light. On the day of the experiment, the frozen folate stock solution was thawed and 1 ml was added to 99 ml of preheated buffer solution in the flask to give a final concentration of 0.567 mM (0.431 mM for H_4 PteGlu) at zero-time. Flasks were swirled to insure thorough mixing of the contents. Samples of 1 ml each were withdrawn at pre-determined time intervals, immediately chilled in an ice bath and protected from the light before they were assayed for folacin content.

Folacin determination

Folacin content of experimental solutions was determined through microbiological assay using *Lactobacillus casei* (ATCC 7469) as described by Waters and Mollin (1961) with revisions re-

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ported by Tamura et al. (1972). Bacto Folic Acid Casei Medium (Difco Laboratories, Detroit, MI) was used as the growth medium.

Chilled experimental samples were micropipetted in triplicate into 5.0 ml sterilized assay medium containing 0.05% ascorbate to protect the folacin during the assay. Exceptions were made for H_4 PteGlu samples where 0.3% ascorbate was used in the culture medium as recommended by O'Broin et al. (1975). Assay tubes were inoculated with washed *L. casei* cells and incubated at 37°C for 20 hr. Microbial growth was determined by measuring optical density at 640 nm using a Beckman Spectrophotometer Model 24. This was compared to a standard growth curve of *L. Casei* grown in medium containing PteGlu as the sole folate source available to the microorganism. The results of the folate assay were expressed in ng of PteGlu equivalents.

Treatment of data

The order of the degradation reaction of folacin by heat in the various systems under study was ascertained graphically by plotting concentration against time of heating. This was done at each pH level at which each of the four folate compounds was heated. Average values of the triplicate samples were plotted and lines of best fit were calculated by linear regression analysis using a computer program CURFIT. The straight line which resulted from plotting logarithm of concentration against time indicated a first order reaction.

The first-order reaction rate constant (k) was determined for each experiment by the formula:

$$k = \frac{2.303}{t} \log \frac{a}{a - x}$$

where a is the initial folate concentration and x is the amount destroyed in time t (measured in hr for PteGlu and 5-$CHOH_4$ PteGlu and in min for 5-CH_3H_4 PteGlu and H_4 PteGlu). The half-life of folacin at each pH level was calculated by the equation, $t_{1/2} = 0.693/k$.

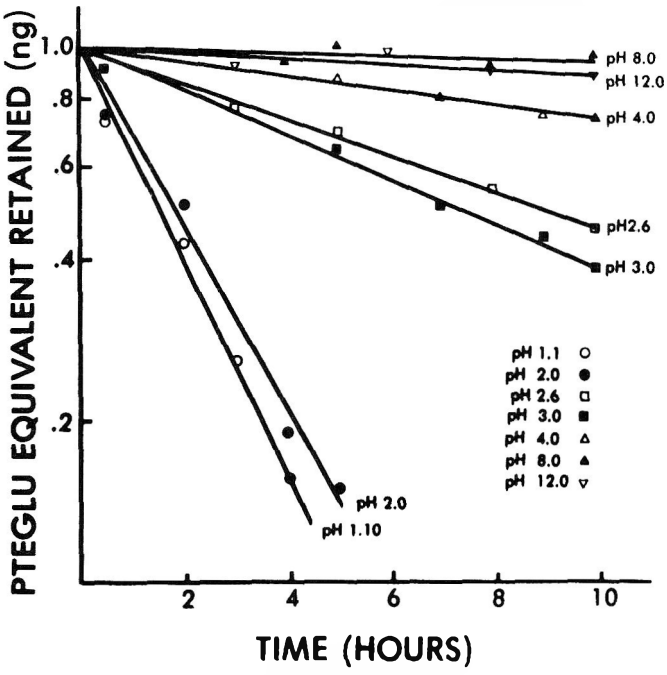

Fig. 1—Rate of destruction for PteGlu at 100° C as a function of pH.

Table 1—Effect of pH and buffer ions on reaction rate constant (k) and half-life (t½) for PteGlu at 100° C

Buffer system	pH	k (hr^{-1})	t½ (hr)
HCl/KCl	1.1	0.470	1.5
HCl/KCl	2.0	0.397	1.7
Universal	2.6	0.079	8.8
Universal	3.0	0.095	7.3
HCl/KCl	3.0	0.079	8.8
Citrate	3.0	0.071	9.8
Citrate/phosphate	3.0	0.069	10.0
Universal	4.0	0.031	22.4
Universal	5.0	0.010	69.3
Universal	6.0	0.006	115.5
Universal	7.0	−0.006	–
Universal	8.0	0.007	99.0
Universal	9.0	0.008	86.6
Universal	10.0	0.019	36.5
Universal	11.0	0.001	693.0
Universal	12.0	0.011	63.0

RESULTS & DISCUSSION

PteGlu

The rate of destruction curves at 100°C for PteGlu at various pH levels may be seen in Figure 1. Data for pH 5.0, 6.0, 7.0, 9.0, 10.0 and 11.0 were not plotted in Figure 1 since they demonstrated a rate of destruction similar to that observed at pH 8.0 and 12.0. The reaction rate constant and half-life for PteGlu heated at 100°C are presented in Table 1. These results indicate that PteGlu was stable at 100°C for 10 hr in a pH range 5.0–12.0; however, the stability decreased below pH 5.0. This is in agreement with previous findings. Dick et al. (1948), after holding PteGlu for 1 hr at 100°C reported 50% destruction at pH 1.0, 20% at pH 2.0 and 5–10% at pH 3.0 and 4.0. Daniel and Kline (1947) and Dick et al. (1948) reported a similar pattern of pH contingent destruction of the vitamin at 121°C; however, the rate of degradation was greater at an elevated temperature.

Examination of the rate constants (Table 1) confirms the pH contingent lability of the vitamin. The contingency of k upon pH is in agreement with previous findings (Dick et al., 1948).

As presented in Table 1, the t½ for PteGlu at 100°C increased markedly from 1.5 hr to 22 hr as the pH increased from 1.1 to 4.0. Above pH 5.0, the t½ are generally greater than 60 hr. O'Broin et al. (1975) reported that the time required for 50% destruction of PteGlu at pH 5.0 or above, at room temperature, was more than 700 hr. However, it decreased sharply to 64–24 hr when the pH dropped below 4.0. These findings are in good agreement.

The effect of buffer ions on the rate of PteGlu destruction at 100°C is shown in Figure 2. It was found that at pH 3.0 the four buffer systems tested resulted in similar thermal stability of PteGlu. The rate constants ranged from 0.07–0.09 hr^{-1} and the half-lives varied slightly from 7–10 hr (Table 1). These findings are in agreement with that of Dick et al. (1948), who found that variation of thermal destruction of PteGlu is a function of pH only and does not depend on the constituents of the buffer solutions. O'Broin et al. (1975) compared stability of PteGlu in different buffer systems and found that in the presence of phosphate buffer at pH 6.0 and 8.0, PteGlu underwent considerably more thermal destruction as compared to the other buffer systems tested at the same pH levels. Although the mechanism for this buffer ion contingent stability pattern is unclear, it may be an important finding since phosphate buffer is used in some routine folate assays. However, phosphate buffer was not tested in the present study.

5-$CHOH_4$ PteGlu

The effect of pH on the rate of 5-$CHOH_4$ PteGlu destruction at 100°C is shown in Figure 3. The data satisfactorily conform to the first order function for the degradation of 5-$CHOH_4$ PteGlu at all pH levels tested. The rate constants and half-lives calculated (Table 2) show that the stability of 5-$CHOH_4$ PteGlu increased drastically when pH increased from 1.1 to 3.0. The vitamin was stable for 10 hr at boiling temperature at pH ranging from 4.0–8.0. Above pH 8.0,

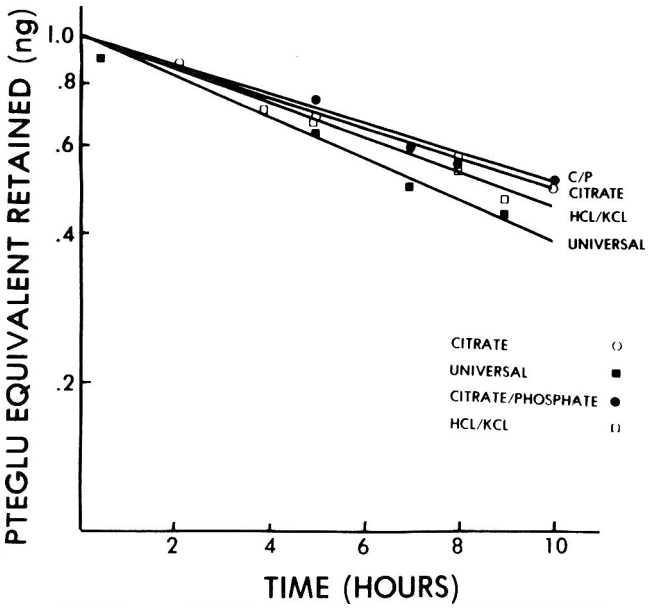

Fig. 2—Effect of buffer ions on the stability of PteGlu at pH 3 during heating at 100° C.

Table 2—Effect of pH and buffer ions on reaction rate constant (k) and half-life (t½) for 5-CHOH$_4$ PteGlu at 100° C

Buffer system	pH	k (hr^{-1})	t½ (hr)
HCl/KCl	1.1	0.721	0.96
HCl/KCl	2.0	0.199	3.48
Universal	2.6	0.041	16.90
Universal	3.0	0.034	20.38
HCl/KCl	3.0	0.052	13.33
Citrate	3.0	0.033	21.00
Citrate/phosphate	3.0	0.071	9.76
Universal	4.0	−0.014	–
Universal	5.0	−0.027	–
Universal	6.0	−0.028	–
Universal	7.0	−0.013	–
Universal	8.0	−0.003	–
Universal	9.0	0.009	77.00
Universal	10.0	0.010	69.30
Universal	11.0	0.027	25.67
Universal	12.0	0.030	23.10

5-CHOH$_4$ PteGlu showed slight destruction during heating. The stability of 5-CHOH$_4$ PteGlu in neutral or mildly alkaline solution to oxygen has been attributed to the presence of the formyl group on the 5-nitrogen which stabilizes the reduced pyrazine ring (Stokstad and Thenen, 1972).

The thermal lability of 5-CHOH$_4$ PteGlu (Fig. 3) is in general agreement with previous reports. May et al. (1951) reported a t½ of 1 hr at pH 1.0 and 17 hr at pH 2.0 while the t½ calculated in the present study are 0.96 hr and 3.47 hr at pH 1.1 and 2.0, respectively (Table 2). Under acidic pH, 5-CHOH$_4$ PteGlu loses a molecule of water to form 5,10-methenyl-H$_4$ PteGlu, especially at elevated temperatures (May et al., 1951).

A comparison of the stability of 5-CHOH$_4$ PteGlu with that of PteGlu indicates that at neutral pH the compounds are equally stable to thermal degradation. However, at pH 1.1 and 2.0, 5-CHOH$_4$ PteGlu was less stable than PteGlu.

It is interesting to note that under slight acidic conditions (pH 4.0–7.0), holding 5-CHOH$_4$ PteGlu at 100°C resulted in a small but consistent increase in its biological activity (Fig. 3). Since it has been reported (Butterworth,

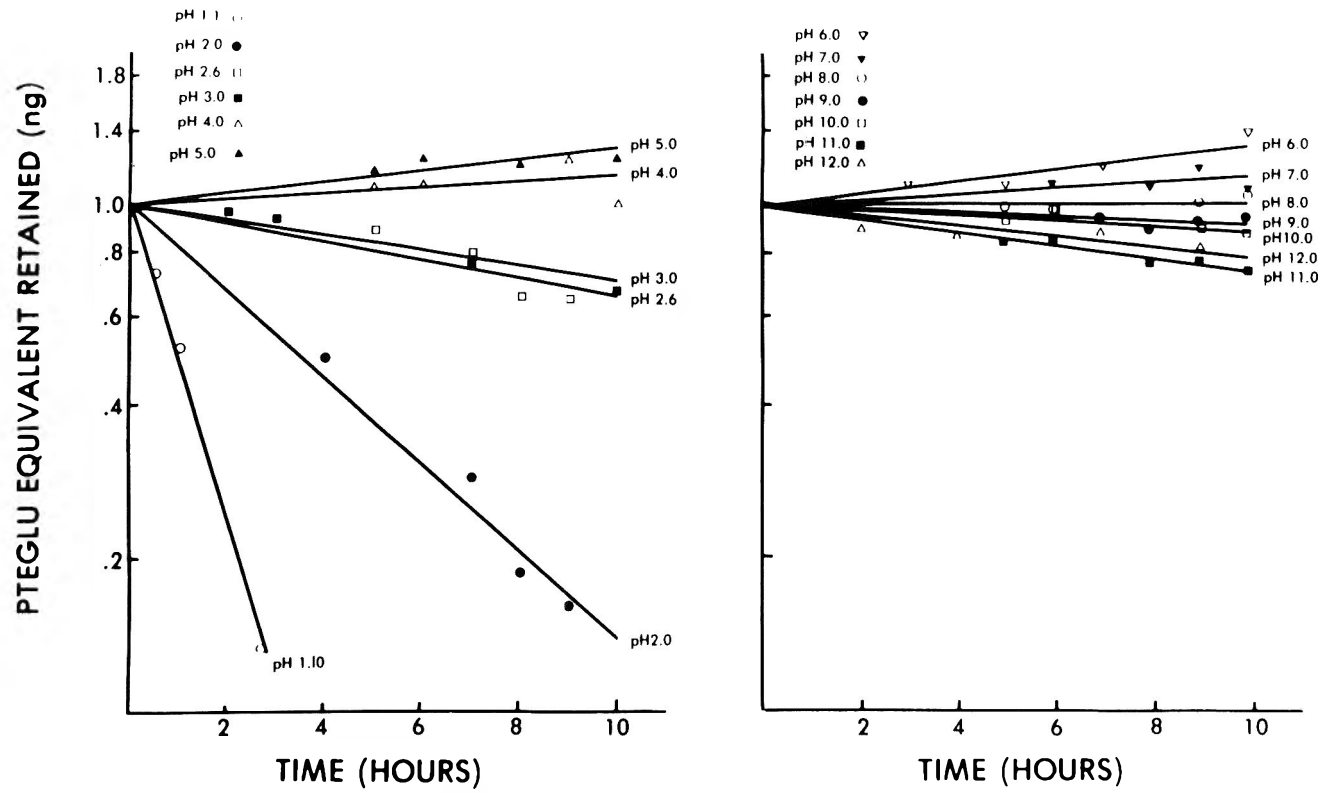

Fig. 3—Rate of destruction for 5-CHOH$_4$ PteGlu at 100° C as a function of pH.

1968) that synthetic 5-$CHOH_4$ PteGlu is only half as active microbiologically due to its diastereoisomeric nature as the natural material, the increase in *L. casei* microbiological activity for 5-$CHOH_4$ PteGlu observed might be attributed to the isomerization occurring at this pH range. Further research is needed to clarify this point.

There was no significant difference in stability of 5-$CHOH_4$ PteGlu when heated at pH 3.0 in various buffer systems. The compound was relatively stable in all buffer systems tested (Fig. 4).

Rabinowitz (1960) reported that at pH 7.0 the compound is relatively stable in maleate buffer; however, it is rapidly hydrolyzed in the presence of phosphate, pyrophosphate and arsenate buffers. O'Broin et al. (1975) who reported a decreased stability of PteGlu and 5-CH_3H_4 PteGlu in the presence of phosphate buffer ions, did not observe a phosphate ion contingent variation in stability for 5-$CHOH_4$ PteGlu at room temperature. He did however report a slightly lower stability at pH 10.0 in the presence of NaOH as compared with the same pH in carbonate/bicarbonate.

5-CH_3H_4 PteGlu

As shown in Figure 5, the destruction of 5-CH_3H_4 PteGlu at 100°C occurred very rapidly and followed first order kinetics at all pH levels. The greatest thermal stability was displayed at pH 7.0. However, O'Broin et al. (1975) reported that at room temperature the compound had maximum stability at pH 9.0 and was relatively stable in alkaline conditions.

A comparison of the reaction rate constants at the pH levels tested indicates that k is directly proportional to hydrogen ion concentration between pH 2.6 and 7.0 and inversely proportional to hydrogen ion concentration between pH 7.0 and 12.0 (Table 3).

Larrabee et al. (1961) showed that when 5-CH_3H_4 PteGlu is shaken in air or oxygen at pH 8.7, it is converted to 5-CH_3H_2 PteGlu. The dihydroPteGlu compound was further identified by Gupta and Huennekens (1967) to be

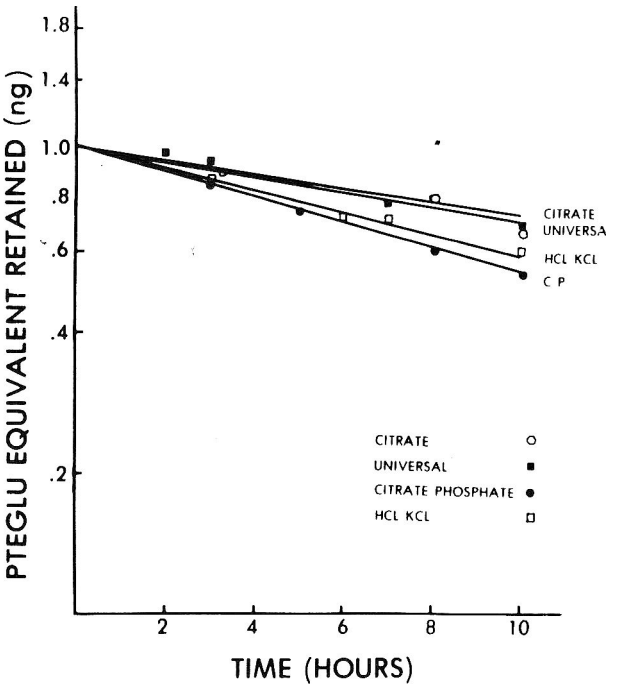

Fig. 4—Effect of buffer ions on the stability of 5-$CHOH_4$ PteGlu at pH 3 during heating at 100° C.

5-CH_3,5,6-dihydrofolate which is not effective in supporting the growth of *L. casei* (Blakley, 1969). The destruction of 5-CH_3H_4 PteGlu at alkaline pH in the present study may be explained by this oxidative mechanism involving the formation of 5-CH_3H_2 PteGlu. At acidic pH, the loss of microbiological activity for 5-CH_3H_4 PteGlu during heating may be due to the formation of an unrecognized nutritionally

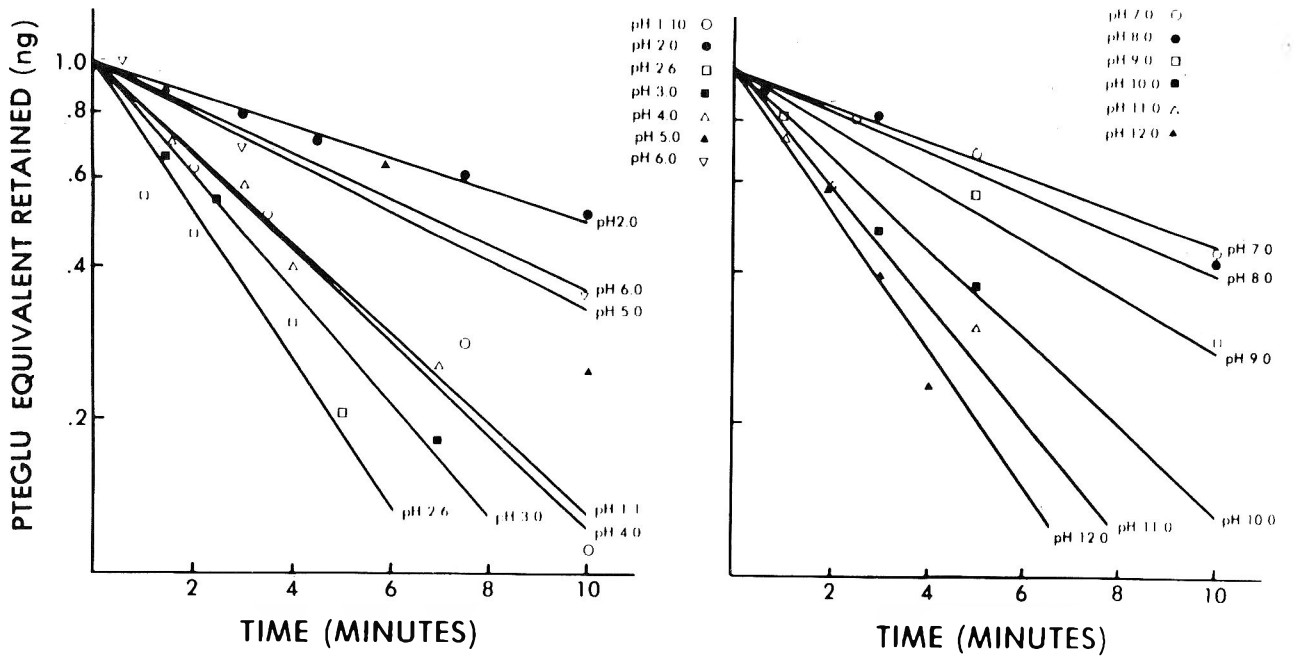

Fig. 5—Rate of destruction for 5-CH_3H_4 PteGlu at 100° C as a function of pH.

inactive derivative as suggested by O'Broin et al. (1975), rather than the oxidative cleavage at the C9–N10 bond resulting in the separation of the pteridine and p-aminobenzoyl ring. The substitution in the 5-position would be expected to be resistant to such a cleavage reaction (Rabinowitz, 1960).

Table 3–Effect of pH and buffer ions on reaction rate constant (k) and half-life (t½) for 5-CH_3H_4 PteGlu at 100° C

Buffer system	pH	k (min^{-1})	t½ (min)
HCl/KCl	1.1	0.205	3.38
HCl/KCl	2.0	0.070	9.90
Universal	2.6	0.336	2.06
Universal	3.0	0.254	2.73
HCl/KCl	3.0	0.053	13.08
Citrate	3.0	0.083	8.35
Citrate/phosphate	3.0	0.081	8.56
Universal	4.0	0.207	3.35
Universal	5.0	0.110	6.30
Universal	6.0	0.103	6.73
Universal	7.0	0.079	8.77
Universal	8.0	0.092	7.53
Universal	9.0	0.128	5.41
Universal	10.0	0.201	3.45
Universal	11.0	0.264	2.63
Universal	12.0	0.313	2.21

Table 4–Effect of pH on reaction rate constant (k) and half-life (t½) for H_4 PteGlu at 100° C

Buffer system	pH	k (min^{-1})	t½ (min)
HCl/KCl	1.1	0.113	6.13
HCl/KCl	2.0	0.111	6.24
Universal	4.0	0.567	1.22
Universal	6.0	0.207	3.35
Universal	8.0	0.145	4.78
Universal	12.0	0.042	16.50

An unexpected finding was that at pH 1.1 and 2.0, 5-CH_3H_4 PteGlu demonstrated an irregularly high stability pattern similar to that observed at neutral pH levels. Table 3 shows that the t½ at pH 1.1 is approximately one-half that observed at pH 9.0 and that the t½ at pH 2.0 is even higher than that at pH 9.0. This is in contrast to that reported by O'Brcin et al. (1975) who found that holding the compound at pH 1.0 resulted in a 15-fold greater decrease in activity as compared with holding the compound at pH 9.0. This finding may be related to increased protection offered the compound by variations in buffer ions since the thermal stability of 5-CH_3H_4 PteGlu was found to be affected by manipulation of buffer ions (Fig. 6). 5-CH_3H_4 PteGlu demonstrated similar stability in citrate, citrate/phosphate and HCl/KCl buffers at pH 3.0. However in the presence of universal buffer (pH 3.0), its stability was greatly reduced. O'Broin et al. (1975) too found variation in rate of destruction of 5-CH_3H_4 PteGlu as a function of buffer ions. At pH 6.0 and 7.0 the compound was less stable in the presence of phosphate buffer as compared to citrate-phosphate buffer; however, the difference was not as great as that observed for PteGlu.

The half-life of 5-CH_3H_4 PteGlu in the present study ranged from 2–9 min (Table 3) while that reported by O'Broin et al. (1975) ranged from 21–329 hr. This difference is due to the difference in temperature used in the two studies. The present study was conducted at 100°C while room temperature was employed in the experiments conducted by O'Broin et al. (1975). Chen and Cooper (1979) have shown that the rate of thermal destruction of 5-CH_3H_4 PteGlu is temperature dependent conforming to the Arrhenius equation with a low activation energy (Ea) of 9.5 Kcal/mole.

H_4 PteGlu

The degradation of H_4 PteGlu followed first order reaction kinetics as shown in Figure 7. It is highly unstable at 100°C as compared to other folates and the lability is greatly influenced by pH and the buffer ions. The first-order reaction rate constant decreased with increasing pH in

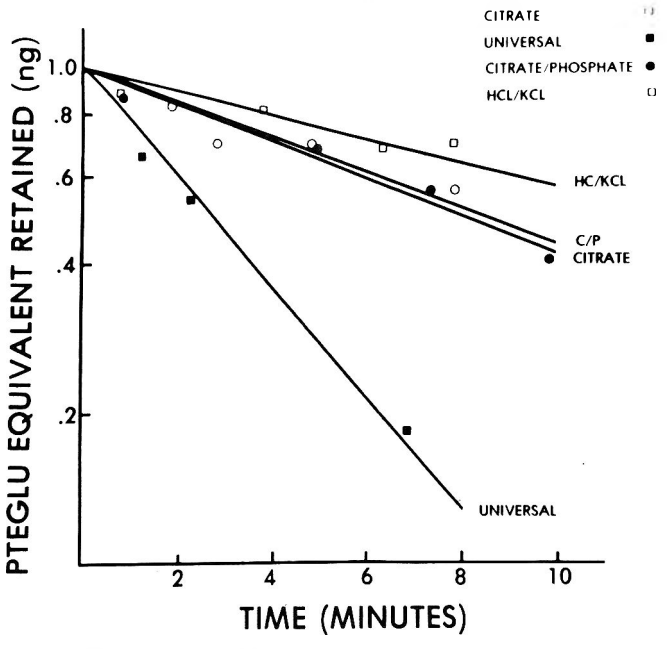

Fig. 6–Effect of buffer ions on the stability of 5-CH_3H_4 PteGlu at pH 3 during heating at 100° C.

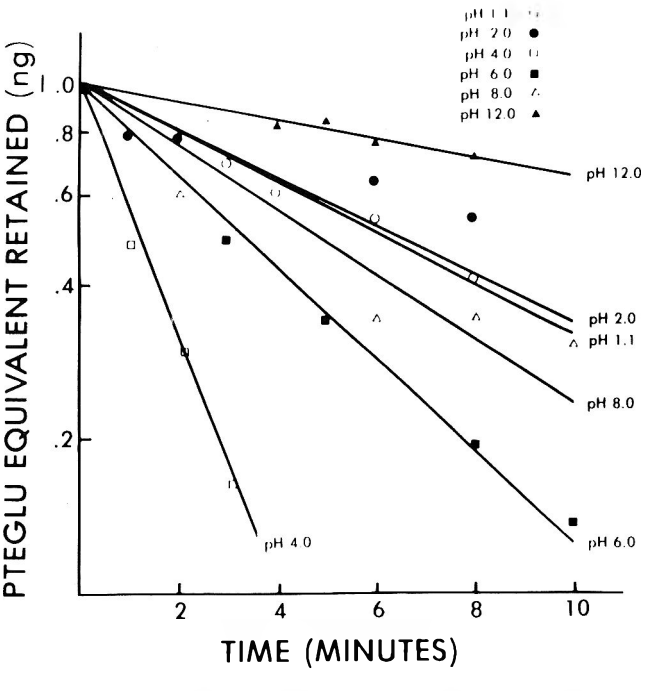

Fig. 7–Rate of destruction of H_4 PteGlu at 100° C as a function of pH.

the pH range of 4.0–12.0 (Table 4), with irregularly low values at pH 1.1 and 2.0. This irregularity observed in strong acidic pH might be attributed to the difference in the buffer systems used. HCl/KCl buffer was used for pH 1.1 and 2.0 while universal buffer was used for all the other pH levels. The effect of buffer ions on the stability of H_4 PteGlu was not further investigated in this study due to the extreme lability of the compound. However, universal buffer could have resulted in the lower stability of H_4 PteGlu during heating similar to that shown for 5-CH_3H_4 PteGlu (Fig. 6). This could also explain the unusual high stability of H_4 PteGlu in acidic pH reported by O'Broin et al. (1975) since they used HCl/KCl buffer for this pH range.

It has been established that H_4 PteGlu is extremely labile. It is rapidly oxidized by air in neutral solution with the formation of p-aminobenzoylglutamic acid and a number of pterins. Some reoxidation to H_2 PteGlu and PteGlu also occurs at neutral pH. In acid solution, however, cleavage to p-aminobenzoylglutamic acid is quantitative in absence of protective agents (Blakley, 1969). On the other hand, there is no general agreement on the effect of pH on the stability of H_4 PteGlu. Ramasastri and Blakley (1964) found that neutral pH (7.4) afforded the maximum stability for H_4 PteGlu at 0–5°C while O'Broin et al. (1975) reported that H_4 PteGlu was least stable at pH 6.0 at room temperature. These variations may be related to the differences in the ionic species of the buffer used. In the present study, when the same buffer (universal) was used in a wide range of pH (4.0–12.0), the first-order rate constant of H_4 PteGlu destruction is directly proportional to the hydrogen ion concentration. This indicates that the stability of H_4 PteGlu at 100° is decreased linearly with the hydrogen ion concentration, in the absence of variations in ionic species of the buffer.

SUMMARY

THE THERMAL DEGRADATION reaction at 100°C for all the folate forms studied followed first order kinetics. Under identical heating conditions, PteGlu and 5-$CHOH_4$-PteGlu were quite stable with half-lives in hours whereas 5-CH_3H_4 PteGlu and H_4 PteGlu were very labile with half-lives of only minutes.

pH was found to have profound influence on the thermal stability of folacin. Both PteGlu and 5-$CHOH_4$ PteGlu were stable up to 10 hr of heating in pH 4.0–12.0, but the rate of destruction increased rapidly with decreasing pH below 4.0. 5-CH_3H_4 PteGlu showed greatest stability at pH 7.0 and exhibited parallel patterns of decreasing stability with increasing acidity and alkalinity. The first order rate constant for 5-CH_3H_4 PteGlu destruction is directly proportional to hydrogen ion concentration between pH 2.6 and 7.0 and inversely proportional to hydrogen ion concentration between pH 7.0–12.0. The stability of H_4 PteGlu at 100°C was found to decrease linearly with the hydrogen ion concentration in the pH range 4.0–12.0

The difference in the ionic constituents of the four buffer systems tested at pH 3.0 did not result in any significant difference in the thermal stability of PteGlu and 5-$CHOH_4$-PteGlu. However, universal buffer was found to cause higher thermal destruction rate for 5-CH_3H_4 PteGlu than the HCl/KCl, citrate/phosphate, and citrate buffers. Similar effect was also observed for H_4 PteGlu.

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VITAMIN ANALYSIS BY MICROBIAL AND PROTOZOAN ORGANISMS: RESPONSE TO FOOD PRESERVATIVES AND NEUTRALIZATION SALTS

M. N. VOIGT, R. R. EITENMILLER and G. O. WARE

ABSTRACT

Vitamin analyses using protozoa (*Tetrahymena pyriformis, Ochromonas danica* and *Ochromonas malhamensis)* as test organisms were found to be affected by lower concentrations of neutralization salts than were the vitamin analyses which used bacteria and yeast as test organisms. Neutralization salts are formed during vitamin extraction procedures. Growth of protozoa and yeast was affected by the food preservatives—sodium sorbate, sodium propionate and sodium benzoate. Except for sodium benzoate inhibition of *Lactobacillus leichmannii,* the growth of bacterial test cultures was not affected by these chemicals. Levels of the neutralization salts and food preservatives in vitamin assays that are required to affect the growth of the test organisms were calculated. These levels were compared to the concentrations of salts formed by accepted vitamin extraction procedures to determine the dilutions of sample extracts necessary to avoid interference. Similar calculations were completed to determine minimal dilutions of foods necessary to avoid the effects of food preservatives on the test organisms. Acids and bases that neutralized to form salts that had the least effect on the growth of the test cultures were determined.

INTRODUCTION

VITAMIN ANALYSIS methods that utilize bacteria, yeast and chemical reagents are the routine techniques employed to quantify vitamins in foods. These procedures are more rapid than the methods which use protozoa as the test organisms; however, they are not as accurate in determining the vitamin levels in foods that are useful in mammalian metabolism (Baker and Frank, 1968).

Many analytical difficulties are encountered in the determination of the vitamin contents in foods, thus an understanding of the types of interferences that may occur with any given food is necessary for successful vitamin analysis. No sample preparation method is applicable to all types of food. Vitamin analysis methods are subject to interference by a variety of substances that may be carried through and/or added by the sample preparation procedure. Official methods of sample preparation have been developed for the vitamin analysis procedures which use bacteria, yeast and chemical reagents, but not for the techniques which employ protozoa. Research completed in this laboratory has shown that the sample preparation procedures described in the *Official Methods of Analysis* (AOAC, 1975) for the commonly used methods of assaying vitamins are not suitable when using protozoa as the test organisms (Voigt et al., 1979a).

Quantitative vitamin assays depend upon the relationship between vitamin dosage and test organism response. To develop calibration curves, graded levels of a vitamin are added to separate vessels containing basal media followed by inoculation with the test organism. An adequate assay of a sample requires the addition of at least three levels of the sample extract to separate vessels containing basal media; however, five levels are preferable. Two dosage-response curves are obtained, one for the standard and one for the sample. After the sigmoidal dosage-response curves are converted into linear regressions by the logit transformation, intercept and slope coefficients are calculated for the two curves. Comparison of the two slope coefficients for equality determines whether the dosage-response of the sample is free from interference, i.e. whether the relative potency of the sample is independent of its dosage level. This is equivalent to determining whether the dosage-response curves for the standard and sample are parallel within the sampling error (Bliss and White, 1967). An alternative technique of evaluating for the proportional increase in organism response with increasing addition of sample is to determine whether the sample potencies calculated for the 3–5 additions agree within ± 10% of their common mean (Strohecker and Henning, 1966). If these conditions are not fulfilled, the determination is not valid. If the slopes of the standard and sample dosage-response curves differ significantly, the direction of the divergence is then determined. A sample dosage-response curve with a slope greater than the standard curve contains a substance(s) other than the vitamin in the sample extract that is stimulating the growth of the test organism, while a lesser slope indicates the presence of an inhibitor(s). This phenomenon is known as "drift" and it can be caused by many factors (Association of Vitamin Chemists, 1966; Voigt and Eitenmiller, 1978). Substances structurally related to the vitamin or incompletely hydrolyzed vitamin complexes stimulate the growth of the test organism, but not to the same degree as the vitamin. Compounds chemically unrelated to the vitamin may stimulate or inhibit the growth of the organism. For example, salts formed during sample preparation may be in sufficient concentration to inhibit growth of the test organism. If a significant level of drift is present, the analysis is not valid. The interfering substance(s) must either be removed or a different sample preparation procedure used. A statistical method to evaluate drift was developed by Bliss and White (1967) and has been used by Voigt et al. (1978a) in evaluating vitamin analysis data.

The objective of this research was to determine the effects of food preservatives and neutralization salts on test organisms used in vitamin analysis. Information generated by this study was necessary in order to develop optimal methods of preparing extracts from foods to be analyzed for vitamin content by protozoa. After these sample preparation procedures are developed, the vitamin assay techniques employing protozoa can be compared to the commonly used methods which employ bacteria, yeast and chemical reagents to determine if the methods employing protozoa indicate significantly different levels of the vitamins in foods. This is of importance to food scientists, since they are responsible for presenting accurate data in food consumption tables and on nutritional panels of product labels.

—Text continued on page 724

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Table 1—B-vitamin additive study conditions

Vitamin assay	Vitamin level (ng/ml)	Additive range (mM)								
		Sodium sorbate	Sodium propionate	Sodium benzoate	Potassium chloride	Sodium chloride	Potassium sulfate	Sodium acetate	Potassium citrate	Potassium phosphate[a]
Thiamine										
L. viridencens	4	0.018–0.9	0.31–15.6	0.07–3.5	66.8–1334	84.8–1708	28.8–575	36.8–735		
O. danica	30	0.018–0.9	0.31–15.6	0.07–3.5	66.8–1334	84.8–1708	28.8–575	36.8–735		
Riboflavin										
L. casei	30	0.018–0.9	0.31–15.6	0.07–3.5	66.8–1334	84.8–1708	28.8–575	36.8–735		
T. pyriformis	50	0.018–0.9	0.31–15.6	0.07–3.5	13.3–267	17.0–339	5.75–115	7.35–147		
Vitamin B_6										
S. uvarum	2	0.018–5.4	0.31–15.6	0.07–3.5	66.8–1334	84.8–1708	28.8–575	36.8–735		
T. pyriformis	5	0.018–0.9	0.31–15.6	0.07–3.5	13.3–267	17.0–339	5.75–115	7.35–147		
Vitamin B_{12}										
L. leichmannii	0.03	0.018–0.9	0.31–15.6	0.07–3.5	66.8–1334	84.8–1708	28.8–575	36.8–735		
O. malhamensis	0.1	0.018–0.9	0.31–15.6	0.07–3.5	13.3–267	17.0–339	5.75–115	7.35–147		
Biotin										
L. plantarum	0.06	0.018–0.9	0.31–15.6	0 07–3.5	66.8–1334	84.8–1708	28.8–575	36.8–735		
O. danica	0.1	0.018–0.9	0.31–15.6	0 07–3.5	13.3–267	17.0–339	5.75–115	7.35–147		9.2–147
Niacin										
L. plantarum	60	0.018–0.9	0.31–15.6	0 07–3.5	66.8–1334	84.8–1708	28.8–575	36.8–735		
T. pyriformis	30	0.018–0.9	0.31–15.6	0.07–3.5	66.8–1334	84.8–1708	28.8–575	36.8–735	3.85–61.5	0.46–9.2
Pantothenate										
L. plantarum	10	0.018–0.9	0.31–15.6	0.07–3.5	66.8–1334	84.8–1708	28.8–575	36.8–735		
T. pyriformis	100	0.018–0.9	0.31–15.6	0.07–3.5	13.3–267	17.0–339	28.8–575	7.35–147		

[a] Monobasic salt (KH_2PO_4)

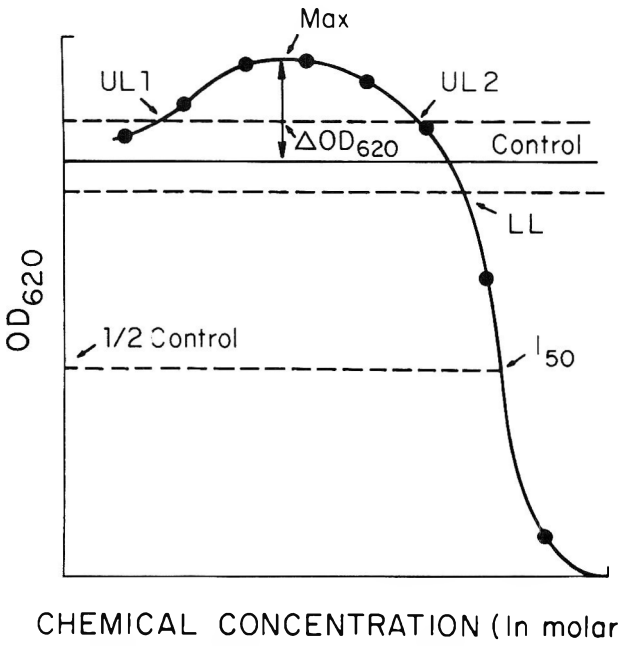

Fig. 1—Inhibition and/or stimulation effects of the chemical additives on the vitamin assays were evaluated at the points indicated in this diagram. [The solid line labeled "CONTROL" is the measured growth in the vitamin assays containing no added chemical. The confidence limits for the control are indicated by the dashed lines enclosing the "CONTROL" line. "UL1" and "UL2" are the additive concentration limits for stimulation. "MAX" is the additive concentration at maximum stimulation. "ΔOD_{620}" is the measured optical density increase at maximum stimulation. "LL" is the maximum additive concentration that had no effect; however, if stimulation was detected, the no effect level is defined by "UL1". "I_{50}" is the additive concentration that inhibited the growth 50%.]

MATERIALS & METHODS

THE EFFECTS of food preservatives and neutralization salts on commonly used microbial and protozoan methods of vitamin analysis were examined. The test organisms used in assaying for the B-vitamins are listed in Table 1. Each vitamin assay was prepared with a given level of the vitamin being examined which was less than the upper response limit of the test organism (Table 1; Voigt et al., 1978b). This design allowed measurement of both stimulation and inhibition of the test organisms by the added chemicals. Table 1 also lists the ranges of concentrations of the chemicals that were added to each type of vitamin assay. The highest chemical concentration added to a vitamin assay corresponded to the level that could be expected to be contributed to an assay by a food extract, due either to the inherent amount in food or to the level of that chemical formed by a corresponding method of vitamin extraction (Table 2). Decreasing concentrations of the chemicals were added until no measurable difference between a blank (water) addition and the chemical addition was apparent. This level was not always reached. Assays utilizing bacteria or yeast were completed following the procedures outlined by Difco (1968). Protozoan assays were performed according to procedures of Baker and Frank (1968) as modified by Voigt et al. (1978b). Optical densities of the growth of the test organisms were measured at 620 nm.

Figure 1 illustrates a theoretical dosage-response curve for the effect of increasing addition of a chemical on the optical density of the growth of a test organism. Initially, low levels of the chemical produced stimulation, which was followed by inhibition at higher levels of addition. Specific chemical concentrations of this dosage-response curve are of interest: (1) the concentration limits for stimulation ("UL1" to "UL2"); (2) the concentration at maximal stimulation ("MAX") and its corresponding increase in optical density ("Δ OD_{620}"); (3) the maximal concentration that had no detectable effect ("LL or "UL1" if stimulation was present); and (4) the concentration that inhibited the growth of the test organism 50% ("I_{50}"). These parameters were calculated by fitting a second degree polynomial to the dosage-response data followed by calculating the chemical levels corresponding to these points on the curves. The concentration at "MAX" was evaluated by placing the first derivative of the quadratic equation equal to zero, since the rate of slope change at this point equals zero. The concentration at the no effect level was defined as being the highest concentration at which the

Table 2—Extraction media used for water-soluble vitamin analysis[a]

Assay	Extraction media	Reference
Thiamine		
L. viridescens	water adjusted to pH 2.5 with HCl	Horwitz et al. (1975)
O. danica	water adjusted to pH 2.5 with HCl	Horwitz et al. (1975)
Riboflavin		
L. casei	water adjusted to pH 2.5 with HCl	Horwitz et al. (1975)
T. pyriformis	water adjusted to pH 2.5 with HCl	Horwitz et al. (1975)
Vitamin B_6		
S. uvarum	0.55N HCl	Horwitz et al. (1975)
T. pyriformis	sodium phosphate, monobasic (2.76 g liter), pH 4.5	Baker and Frank (1968)
Biotin		
L. plantarum	2N H_2SO_4	Strohecker and Henning (1966)
O. danica	citric acid · H_2O (92 g liter); sodium phosphate, dibasic (0.4 g liter), pH 4.5	Baker and Frank (1968)
Niacin		
L. plantarum	1N H_2SO_4	Horwitz et al. (1975)
T. pyriformis	citric acid (1.07 g liter)-monobasic, sodium phosphate, (1.26 g liter), pH 4.5	Baker and Frank (1968)
Pantothenate		
L. plantarum	water adjusted to pH 5.6 with acetic acid	Horwitz et al. (1975)
T. pyriformis	sodium citrate (3.3 g liter) citric acid (1.2 g liter) pH 5.0	Baker and Frank (1968)

[a] Sample preparation methods are detailed in Voigt et al., 1979a.

chemical was present without increasing or decreasing the measured growth of the test organism beyond the confidence limits of the control. The confidence limits corresponded to ± 15% of the optical density of the growth of the organism in the control. The controls contained no added chemicals. R^2 values, a commonly used measure of goodness of fit for the quadratic functions fitted to the dosage-response curves, were always greater than 0.80 for the regressions used in data calculations ($P \leq 0.0001$). A computer program was written to complete these evaluations (Voigt et al., 1979b).

RESULTS & DISCUSSION

THE COMMONLY USED bacterial and yeast, as well as the protozoan test organisms employed in vitamin analyses were subjected to graded concentrations of sodium sorbate, sodium propionate, sodium benzoate, potassium chloride, sodium chloride, potassium sulfate, sodium acetate, potassium citrate and potassium phosphate. A representative dosage-response curve of the effect of these chemicals on the optical density of a test culture is illustrated in Figure 2. The vitamin assay illustrated is the *Tetrahymena pyriformis* assay for riboflavin. The concentrations of the chemicals that gave no effect, 50% inhibition and stimulation of the vitamin assay cultures are given in Tables 3 and 4. The concentration limits of the chemicals that induced stimulation of the test organism are given in Table 5. Sodium propionate and sodium benzoate had no detected effect on the growth of the bacterial test cultures. Sodium sorbate inhibited *Lactobacillus leichmannii* at the 0.08 mM level. *Lactobacillus casei* showed no growth response to the neutralization salts added to the riboflavin assay media, while the other bacteria were inhibited by these chemicals. Growth of *Lactobacillus viridescens* was initially stimulated by sodium acetate. Stimulation occurred between 1.3 and 52 mM and peaked at 47 mM. This stimulation was probably the result of the formation of a buffer system which neutralized acids formed by metabolism. The inhibition of the growth of the bacterial test cultures by potassium chloride, sodium chloride, potassium sulfate and sodium acetate occurred over similar concentration ranges. Inhibition thresholds and I_{50} levels were usually lower for potassium chloride than for sodium chloride, while potassium sulfate usually had the highest thresholds. Thresholds for sodium acetate were more variable.

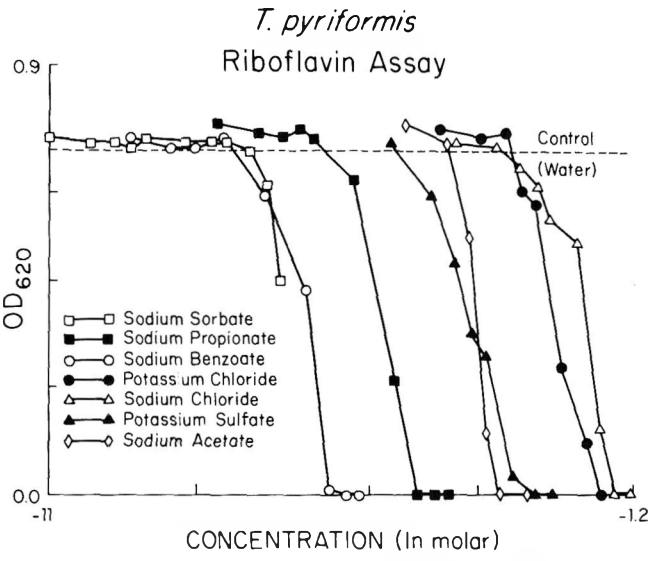

Fig. 2—Effect of chemicals on the growth of T. pyriformis in a riboflavin-limited medium. Riboflavin and chemical concentrations are given in Methods.

The growth of the yeast *Saccharomyces uvarum* was affected by all of the chemicals added to the media. As expected, *S. uvarum* was inhibited by low levels of sodium sorbate, sodium benzoate and sodium propionate. The thresholds for inhibition were in the same respective order. Inhibition thresholds for potassium chloride and sodium chloride were about equal; however, concentrations of sodium chloride below the inhibition thresholds and doses to 39 mM caused stimulation while potassium chloride did not

Table 3—Effects of food preservatives on growth of organisms used microbial vitamin assays[a]

Vitamin assay	Sodium sorbate (mM)			Sodium propionate (mM)			Sodium benzoate (mM)		
	NE[b]	I_{50}[c]	S[d]	NE	I_{50}	S	NE	I_{50}	S
Thiamine									
L. viridescens	0.45 N[b]	NI[e]	NS[d]	8 N	NI	NS	1.8 N	NI	NS
O. danica	0.02	0.40	NS	0.16 E[b]	13	NS	0.035 N	6	NS
Riboflavin									
L. casei	0.45 N	NI	NS	8 N	NI	NS	1.8 N	NI	NS
T. pyriformis	0.15	0.45	NS	0.15	2.5	0.35	0.2	0.65	NS
Vitamin B_6									
S. uvarum	.01 E	0.37	.004	0.15 E	170	0.35	0.24	1.7	NS
T. pyriformis	0.045	NI	NS	1.3	19	NS	0.3	6	NS
Vitamin B_{12}									
L. leichannii	0.08	1.4	NS	8 N	ND	NS	1.8 N	ND	NS
O. malhamensis	0.01	0.2	.02	0.22	2	ND[e]	0.15	0.7	NS
Biotin									
L. plantarum	0.045 N	ND	NS	8 N	ND	NS	1.8 N	ND	NS
O. danica	0.005	0.23	NS	0.45	ND	NS	0.02	ND	NS
Niacin									
L. plantarum	0.045 N	ND	NS	8 N	ND	NS	1.8 N	ND	NS
T. pyriformis	0.24	ND	NS	0.19	4.4	0.5	0.4	1.1	0.13
Pantothenate									
L. plantarum	0.45	ND	NS	8 N	ND	NS	1.8 N	ND	NS
T. pyriformis	0.015	0.12	NS	0.17	0.6	NS	0.35 E	0.46	0.07

[a] Responses were fitted to the quadratic function. R^2 values were greater than 0.80 for the regressions used in data interpretation ($P \leq 0.0001$).
[b] "NE" maximum additive concentration that had no effect, "N" indicates that the given concentration was the highest added but still had no observed effect; "E" indicates that the given concentration was the lowest added but still had an effect.
[c] Additive concentration that inhibited the growth response 50%
[d] Concentration (mM) where maximum stimulation occurred, NS: No Stimulation.
[e] "ND" — Not Determined, "NI" — No Inhibition detected.

Table 4—Effects of neutralization salts on growth of organisms used in microbial vitamin assays[a]

Vitamin assay	Potassium chloride (mM)			Sodium chloride (mM)			Potassium sulfate (mM)			Sodium acetate (mM)		
	NE[b]	I_{50}[c]	S[d]	NE	I_{50}	S	NE	I_{50}	S	NE	I_{50}	S
Thiamine												
L. viridescens	1.00	350	NS[d]	90	550	NS	13	ND[e]	NS	1.3	370	47
O. danica	34 E	60	ND	21	85	ND	15 E	22	ND	20	40	ND
Riboflavin												
L. casei	650	ND	NS	850 N	NI[e]	NS	290 N	NI	NS	370 N	NI	NS
T. pyriformis	24	44	NS	25	85	NS	8.5	44	11	3.7 E	13	3.7
Vitamin B_6												
S. uvarum	620	ND	NS	39	ND	140	15 E	ND	180	13	310	29
T. pyriformis	24	40	NS	27	55	NS	17	3.2	NS	39	100	NS
Vitamin B_{12}												
L. leichannii	55	140	ND	70	120	ND	16	81	ND	55	210	ND
O. malhamensis	8	23	ND	8.5	32	ND	0.05	8.5	ND	2.5	7.5	ND
Biotin[f]												
L. plantarum	27	380	NS	270	650	NS	290	ND	NS	240	ND	NS
O. danica	6.5	100	20	6.5	120	26	5	50	12	19	60	NS
Niacin[g]												
L. plantarum	140	310	NS	100	405	NS	140	ND	NS	140	330	NS
T. pyriformis	31	60	NS	34	65	ND	23	75	ND	11	20	ND
Pantothenate												
L. plantarum	120	470	NS	250	850	NS	240	ND	NS	220	ND	NS
T. pyriformis	65 E	50	6.5	8.5 E	50	13	15 E	33	ND	3.7 E	9	3.7

[a] Responses were fitted to the quadratic function. R^2 values were greater than 0.80 for the regressions used in data interpretation ($P \leq 0.0001$).
[b] "NE" maximum additive concentration that had no observed effect, "N" indicated that the given concentration was the highest added but still had no observed effect, "E" indicates that the given concentration was the lowest added but still had an effect.
[c] Additive concentration that inhibited the growth response 50%.
[d] Concentration (mM) where maximum stimulation occurred, NS — No stimulation.
[e] "ND" not determined, "NI" no inhibition detected.
[f] Effects of potassium phosphate on biotin assay by *O. danica:* NE = 29, I_{50} = 60, S = NS.
[g] Effects of potassium phosphate on niacin assay by *T. pyriformis:* NE = 0.23, I_{50} = 3.5, S = 0.9, Potassium citrate: NE = 9, I_{50} = 32, S = ND.

Table 5—Concentration (mM) limits of the additive induced stimulation in the microbial vitamin assays[a]

Vitamin assay	Sodium sorbate	Sodium propionate	Sodium benzoate	Potassium chloride	Sodium chloride	Potassium sulfate	Sodium acetate
Thiamine							
L. viridescens	NS[b]	NS	NS	NS	NS	NS	1.3 – 52
Riboflavin							
T. pyriformis	NS	0.15 – 0.8	NS	NS	NS	8.5 – 14	ND – 7
Vitamin B_6							
S. uvarum	ND – 0.6	0.01 – 0.04	NS	NS	39 – 500	ND – 290	13 – 65
Vitamin B_{12}							
O. malhamensis	ND – 0.045	NS	NS	ND	ND	ND	ND
Biotin							
O. danica	NS	NS	NS	6.5 – 34	6.5 – 34	5 – 13	NS
Niacin							
T. pyriformis	NS	0.19 – 1.4	0.05 – 0.33	NS	ND	ND	ND
Pantothenate							
T. pyriformis	NS	ND	ND – 0.13	ND – 25	ND – 23	ND – 13	ND – 5

[a] Responses were fitted to the quadratic function. R^2 values were greater than 0.80 for the regressions used in data interpretation ($P \leq 0.0001$

[b] "ND" not determined, "NS" no stimulation detected.

induce this effect. Potassium sulfate induced stimulation at the concentrations examined. Low concentrations (13–65 mM) of sodium acetate stimulated *S. uvarum*, whereas, concentrations above 65 mM inhibited this organism.

The growth of protozoan cultures were inhibited by the added chemicals. Thresholds of growth inhibition were usually in the following order: sodium sorbate, sodium benzoate, sodium propionate, potassium phosphate, potassium sulfate, sodium chloride and potassium chloride. At low levels, potassium chloride, sodium chloride and potassium sulfate all induced growth stimulation in the biotin assay by *Ochromonas danica*. Similar results were not obtained from *O. danica* and *Ochromonas malhamensis* used in the assay of thiamine and vitamin B_{12}, respectively.

None of the added chemicals stimulated *T. pyriformis* growth in the vitamin B_6 assays and the inhibition thresholds were at higher concentrations of the added chemicals than for the other vitamins assayed by this organism. Growth of *T. pyriformis* was stimulated by low concentrations of sodium propionate in the riboflavin assay and by low concentrations of sodium propionate and sodium benzoate in the niacin assay. Each of the chemicals, except sodium sorbate, stimulated the growth of *T. pyriformis* at low concentrations in the pantothenate assay. Table 6 compares the concentrations of the salts that had no effect on the growth of the test organisms used in vitamin assays (Tables 3 and 4) to the levels of neutralization salts that are formed in the procedures used for vitamin extraction (Table 2). The only bacterial vitamin assay method that required dilution of the extract solution before addition to the vitamin assay media was the niacin determination by *Lactobacillus plantarum*. Most of the sample preparation solutions for the vitamin assays which employed yeast and protozoa as the test organisms required dilution to avoid interference by neutralization salts. Usually, it is necessary to dilute extracts of food samples to bring the vitamin level within the sensitivity limits of the vitamin assay. Sufficient dilution must also be used to avoid salt interference. If this dilution lowers the vitamin level below the sensitivity limit of the vitamin assay method, a different sample preparation method must be employed. Preparations of extracts of samples usually require a minimum of three pH adjustments. The neutralization salt levels indicated in Table 6 assume that the target pH was attained without overshooting and readjustment. The cited salt levels do not include the levels of salts formed when overcoming natural food buffering capacities, which may be considerable. Thus, actual minimal dilutions necessary to overcome the total neutralization salts present in a sample solution would be larger than those indicated in Table 6. The problem of interference can be overcome by proper design of the sample preparation method; that is, to employ, acids, bases and buffer systems that neutralize to form salts that have the highest threshold levels to effect the growth of the test organism and to tailor the vitamin extraction procedure to the type of food being examined. On a practical basis, this is primarily a problem encountered when employing protozoa as the test organisms, since they are more sensitive to the neutralization salts than bacteria and yeast. A study in which the vitamin contents in foods were determined using protozoa as the test organisms confirmed this observation (Voigt et al., 1979a).

Table 7 lists acids and bases which when neutralized resulted in salts that were required to be present in the highest concentrations to induce an effect on the growth of each test organism. The table is based on equal equivalents and not equal molar levels, although in some cases they are the same. The acids examined included hydrochloric, sulfuric, citric, phosphoric, and acetic, while the bases were potassium and sodium hydroxides. Only the *L. viridescens* assay for thiamine and the *S. uvarum* assay for vitamin B_6 tolerated higher levels of salts from potassium hydroxide than from sodium hydroxide. Vitamin assays employing *Ochromonas* were more tolerant to salts from hydrochloric acid. Vitamin assays by *L. plantarum* and *T. pyriformis* were less susceptible to salts from sulfuric acid, except for the vitamin B_6 assay that employed *T. pyriformis*, which was less affected by salts from acetic acid.

Limiting levels of four common food additives were evaluated to determine the minimal dilutions of food extracts necessary to avoid the influence of these compounds on the growth of test organisms used in vitamin assays. Results of these calculations are listed in Table 8. The calculations assume that the levels of these compounds in foods are within normal limits, that is, sodium sorbate 0.01%, sodium propionate 0.1%, sodium benzoate 0.1%, and sodium chloride 1%. Except for *L. leichmannii*, the vitamin assays that employed bacteria were not affected by these levels, while the assays employing yeast and protozoa were affected and thus would require dilution of the food extracts (from 2.4 to 180) to avoid interference.

It is postulated that the mode of inhibition for acetic acid on bacteria is due to the undissociated acetic acid molecule permeating the cell membrane, dissociating at the

Table 6—Minimum vitamin extract dilutions required to eliminate salt inteference in the microbial vitamin assays, the assay limiting salt concentrations and the salt levels contributed to the assays by the extraction methods

Vitamin assay	Neutralization salt	Salt conc (mM) Extraction[a]	Limiting	Minimum dilution[b]
Thiamine				
L. viridescens	Potassium chloride	8.8	100[4]	0
O. danica	Potassium chloride	8.8	6.3[4]	1.4
Riboflavin				
L. casei	Potassium chloride	8.8	1700	0
T. pyriformis	Potassium chloride	8.8	24	0
Vitamin B_6				
S. uvarum	Potassium chloride	140	55	2.6
T. pyriformis	Potassium chloride	140	24	5.8
Biotin				
L. plantarum	Potassium sulfate	500	290	1.7
O. danica	Potassium sulfate	500	5	100
Niacin				
L. plantarum	Potassium sulfate	250	143	1.8
T. pyriformis	Potassium sulfate	250	23	11
T. pyriformis	C/P[c]	8.8/2.5	9/0.23[d]	11
Pantothenate				
L. plantarum	Sodium acetate	3.8	220	0
T. pyriformis	Sodium acetate	3.8	3.7[d]	1.03
T. pyriformis	Sodium citrate	0.25	9[c]	0

[a] Assumes target pH attained without overshooting. Does not include salts formed while overcoming natural food buffering capacities. Corresponds to the 1.25 ml addition of sample extract to an assay total volume of 5 ml and does not include any extract dilutions. Extraction procedures are listed in Table 2.

[b] Minimum extract dilution to avoid the limiting effects induced by the neutralization salts

[c] mM ratio of sodium citrate to potassium phosphate

[d] Estimated from available data

Table 7—Acids and bases showing the least effect on the various microbial vitamin assays[a]

Vitamin assay	Acid	Base
Thiamine		
L. viridescens	hydrochloric	potassium hydroxide
O. danica	hydrochloric	sodium hydroxide
Riboflavin		
L. casei	hydrochloric	sodium hydroxide
T. pyriformis	sulfuric	sodium hydroxide
Vitamin B_6		
S. uvarum	hydrochloric	potassium hydroxide
T. pyriformis	acetic	sodium hydroxide
Vitamin B_{12}		
L. leichmannii	hydrochloric	sodium hydroxide
O. malhamensis	hydrochloric	sodium hydroxide
Biotin		
L. plantarum	sulfuric	sodium hydroxide
O. danica	hydrochloric	sodium hydroxide
Niacin		
L. plantarum	sulfuric	sodium hydroxide
T. pyriformis	sulfuric	sodium hydroxide
Pantothenate		
L. plantarum	sulfuric	sodium hydroxide
T. pyriformis	sulfuric	sodium hydroxide

[a] Interpretation of tolerances listed in Tables 3 and 4. Possible acids: hydrochloric, sulfuric, citric, phosphoric, or acetic; possible bases: potassium hydroxide or sodium hydroxide.

higher internal pH of the cell and then causing protein denaturation in proportion to the amount of acetic acid absorbed. In some bacteria, this effect is 10–100 times greater than for the same hydrogen ion concentration from hydrochloric acid (Reynolds, 1974). Results of this investigation generally agree with the previous observation. Sodium acetate has a stimulation effect on the growth of certain *Lactobacilli* apart from its buffering capacity (Association of Vitamin Chemists, 1966). Ruthven and Cairns (1973) indicated that the toxicity of acids to ciliates—such as *T. pyriformis*—depends on the pH, osmic pressure, cell surface changes, effects on enzymes and lipid solubility. *T. pyriformis* requires potassium ions, and probably sodium and chloride ions for growth (Hill, 1972). This ciliate maintains a high intracellular level of potassium ions and a lower level of sodium ions, at about a 3 to 1 ratio. Increasing the extracellular potassium level increases the internal level, but equilibrium is not reached rapidly, especially at high levels. On the other hand, the extracellular sodium level up to 20 mM does not increase the internal level, suggesting the presence of a sodium elimination system. At higher levels of sodium, the elimination system is overcome. The sodium and potassium ion regulatory systems are apparently independent. This explains why the vitamin assays employing *Tetrahymena* were less susceptible to salts of sodium hydroxide than salts from potassium hydroxide. The chloride ion content in *Tetrahymena* is normally maintained at a lower level in the cells than in the environment—the internal level does not increase until a saturation point is reached. Perhaps the chloride ion saturation point is below that of the sulfate ion, which is physically larger, thus explaining the greater tolerance of *Tetrahymena* to the sulfate ion.

CONCLUSION

Tetrahymena is susceptible to interference by low levels of citrate ion. This should be kept in mind when assaying foods which inherently contain this ion, e.g. fruit juices. Likewise, sodium citrate should not be used as an anticoagulant for blood when blood is to be analyzed for vitamin content by *Tetrahymena*. Formulations of basal media for the vitamin analysis methods which use protozoa need to be examined for the effects of varying certain constituents,

—*Continued on page 737*

Table 8—Minimum sample preparation dilutions necessary to avoid the effects of sorbate, propionate, and benzoate[a]

Vitamin	Assay culture	Minimum sample dilutions Sodium sorbate	Sodium propionate	Sodium benzoate	Sodium chloride
Thiamine	*L. viridescens*	NL[b]	NL	NL	0
	O. danica	46	64[c]	20[c]	8.2
Riboflavin	*L. casei*	NL	NL	NL	NL
	T. pyriformis	6	66	3.6	6.8
Vitamin B_6	*S. uvarum*	90[c]	66[c]	3	4.4
	T. pyriformis	20	8	2.4	6.4
Vitamin B_{12}	*L. leichmannii*	11	NL	NL	2.4
	O. malhamensis	90	46	4.8	20
Biotin	*L. plantarum*	NL	NL	NL	0
	O. danica	180	22	36	26
Niacin	*L. plantarum*	NL	NL	NL	0
	T. pyriformis	2.8	52	2.8	5
Pantothenate	*L. plantarum*	NL	NL	NL	0
	T. pyriformis	60	60	20[c]	20[c]

[a] Assumes food levels: sorbate (0.01%), propionate (0.1%), benzoate (0.1%), and sodium chloride (1%)

[b] NL—The compound did not affect growth of the microorganism at the concentrations studies, Tables 4 and 5.

[c] Estimated

COMPARISON OF PROTOZOAN AND CONVENTIONAL METHODS OF VITAMIN ANALYSIS

M. N. VOIGT, R. R. EITENMILLER AND G. O. WARE

ABSTRACT

Data obtained from commonly used methods of assaying for riboflavin, vitamin B_6, niacin, pantothenic acid, thiamine, biotin and vitamin B_{12} were compared to data obtained from vitamin analysis procedures employing protozoa as the test organisms. The protozoa were of the genera *Tetrahymena* and *Ochromonas*. Generally, the procedures employing *Tetrahymena* indicated higher levels of the vitamins in the foods analyzed, while the procedures employing *Ochromonas* indicated lower vitamin levels than the corresponding commonly used methods of vitamin analysis. Both protozoan and the commonly used methods for analysis of vitamins indicated higher levels of vitamins in food samples when the sample extracts received enzymatic as well as acid hydrolysis treatments. Neutralization salts formed during vitamin extraction procedures were found to interfere with both the conventional and the protozoan methods. High-acid foods (e.g., orange and tomato juices) were found to inhibit the growth of *Tetrahymena*. Further studies are required to develop optimal methods of vitamin extraction and analysis in order to quantify the biologically available vitamin contents of foods.

INTRODUCTION

VITAMIN ASSAY methods using protozoa as test organisms have been reported to be more specific for vitamin forms utilized by man (Voigt and Eitenmiller, 1978). However, few food scientists have employed protozoa in determining the vitamin content in foods.

Tetrahymena pyriformis is a heterotrophic protozoa which utilizes carbon dioxide, acetate, lipid, lactate, carbohydrate and amino acids as carbon sources, and requires nitrogen from ten amino acids, guanine and uracil or cytidine. Most strains specifically require thiamine, riboflavin, vitamin B_6, niacin, pantothenic acid and lipoic acid (Sleigh, 1973). A few clones require biotin and choline. Generally, *T. pyriformis* is as capable as the rat or chick in using conjugates of the B-complex vitamins (Hutner, 1964). *Tetrahymena* does not require vitamin B_{12}, vitamin C and the fat-soluble vitamins (Hill, 1972). The inhibitory effect of visible light on the growth of *Tetrahymena* is due to the photo-decomposition of thiamine, riboflavin, vitamin B_6 and folic acid. *Tetrahymena* obtains nutrients by diffusion through the cell membrane, pinocytosis and phagocyotosis (Sleigh, 1973). Ingested food particles collect in food vacuoles that fuse with lysosomes which contain alpha-glucosidase, phosphatase, deoxyribonuclease, alpha-amylase, beta-N-acetylglucosaminidase, ribonuclease, protease and conjugase.

Ochromonas malhamensis and *Ochromonas danica* are photoflagellates that obtain energy by a heterotrophic and photoautotrophic combination. *O. malhamensis* requires both light and complex organic nutrients for optimum growth. Photosynthesis in this organism is low and does not compensate for respiration. There is a net consumption of oxygen even in the presence of light. *O. malhamensis* will survive anaerobically in the light, producing alcohol and carbon dioxide from carbohydrate, but not in the dark (Chen, 1967). It is not able to fix enough energy by photosynthesis to grow autotrophically. Carbon dioxide, starch, saccharides and glycerol can act as carbon sources while ammonia is sufficient as a nitrogen source. Thiamine, biotin and vitamin B_{12} complete its requirements, except for trace minerals (Sleigh, 1973). *O. danica* requirements are the same, except that it does not require vitamin B_{12}. *O. danica* is more active photosynthetically than is *O. malhamensis*. *Ochromonas* species obtain nutrients by diffusion through the cell membrane, pinocytosis and phagocytosis. Since *O. danica* is phagotrophic, it can ingest and digest low-molecular forms of the vitamins that are complexed; for example, it can release and utilize biotin from biocytin and other peptide bound biotins (Baker and Frank, 1973). Lactobacilli do not possess this capability for releasing and utilizing complexed nutrients.

The methods of vitamin analysis utilizing protozoa have not been systematically compared to the commonly used microbial assays in quantifying vitamins in foods. The objective of this research was to compare data obtained from vitamin assays using protozoa as test organisms to data obtained from methods recommended by Horwitz et al. (1975) in the *Official Methods of Analysis* by the Association of Official Analytical Chemists.

MATERIALS & METHODS

Assay cultures

The nine test organisms used for assaying vitamins in this study are listed in Table 1. The two maintenance media and basal media and incubation temperatures corresponding to each culture are also listed in Table 1. The first maintenance medium was used to prepare the inocula for vitamin assays, while the second medium was used to carry the stock cultures. All media and cultures were held in the dark to prevent photo-inactivation of vitamins. Cultures were activated by a minimum of three transfers, allowing 24 hr between each transfer for the lactobacilli and *Saccharomyces* and 72 hr for the *Tetrahymena* and *Ochromonas*. Table 1 indicates the number of washings the harvested test organisms received, the solutions used for cell washings and dilutions and also the ratio to which the cells were diluted to prepare the inocula. The media used in this study (Table 2) were prepared as described in the indicated reference. Maintenance media were dispensed in 10 ml portions into test tubes and sterilized at 121°C for 10 min. Test tube diameters were: 18 mm for the lactobacilli, *Saccharomyces* and the *Tetrahymena* stock culture, while 25 mm tubes were used for the growth of *Tetrahymena* and *Ochromonas* cells to be used for inoculation of vitamin assays.

Extraction methods

Table 3 lists the extraction methods employed in this investigation. In each procedure, an aliquot of a food sample estimated to contain a vitamin level within the sensitivity range of the assay method was weighed or pipetted into a 250 ml beaker. Sample aliquots varied from 0.5–7.5 ml. Final volumes of extraction solutions were 100 ml. If a 0.5-g or ml sample contained a vitamin level greater than the assay methods sensitivity (Voigt et al., 1978), the extract was diluted with distilled water. Extracts from Methods 1, 5, 6, 10 and 12 were adjusted to the pH of the added buffer when the

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Table 1—B-vitamin assay conditions

Vitamin	Assay method[a]	Extraction method (Table 3)	Standard curve levels (ng/ml)[b]	Culture dilution[c]	Media (Table 2)[d]	Assay pH	Incubation temp (C)	References[e]
Thiamine	*L. viridescens* (12706)	2	0.2 – 5	1:99	1,2;10	6.0	30	1,4
	O. danica (30004)	1	0.1 – 100	1:6.5	5,6;11	5.5	30	2,4
	Thiochrome	1	–	–	–	3.5	–	3
Riboflavin	*L. casei* (7469)	1	5 – 100	1:19	1,2;12	6.8	37	1
	T. pyriformis WH_{14} (30008)	1	1 – 200	1:9	7,8;13	6.1	30	2
B_6[f]	*S. uvarum* (9080)	4,5	0.1 – 4	1:1	3,4;14	4.5	30	1
	T. pyriformis WH_{14} (30008)	4,5	0.1 – 30	1:6.5	7,8;15	6.1	30	2
B_{12}	*L. leichmannii* (7830)	6	0.005 – 0.1	1:1	1,2;16	6.1	37	1
	O. malhamensis (11532)	6	0.003 – 1	1:6.5	5,6;17	5.5	30	2
Biotin	*L. plantarum* (8014)	7,8	0.003 – 1	1:99	1,2;18	6.8	37	1
	O. danica (30004)	7,8	0.003 – 3	1:6.5	5,6;11	5.5	30	2,4
Niacin[g]	*L. plantarum* (8014)	9,10	1 – 200	1:99	1,2;19	6.8	37	1
	T. pyriformis WH_{14} (30008)	9,10	1 – 3000	1:6.5	7,8;13	6.1	30	2
Pantothenate	*L. plantarum* (8014)	11,12	2 – 20	1:99	1,2;9	6.7	37	1
	T. pyriformis WH_{14} (30008)	11,12	3 – 1000	1:6.5	7,8;13	6.1	30	2

[a] Cultures obtained from ATCC, Rockville, MD 20852, except *T. pyriformis* (Drs. H. Baker and O. Frank, College of Medicine and Dentistry, East Orange, New Jersey 07017). ATCC strain number is in parenthesis. Genera: *L.* = *Lactobacillus; O.* = *Ochromonas; S.* = *Saccharomyces;* and *T.* = *Tetrahymena.*

[b] Assay volumes were 5 ml.

[c] Wash and diluting solutions: saline for bacteria and yeast, distilled water for protozoa. No. of times washed: four times for lactobacilli, once for yeast and *Ochromonas,* none for *Tetrahymena.*

[d] Maintenance media are the first two media (weekley, monthly transfers), assay media follows semicolon.

[e] References: 1. Difco (1968); 2. Baker and Frank (1968); 3. Horwitz et al. (1975); 4. Strohecker and Henning (1966)

[f] *S. uvarum* (pyridoxine), *T. pyriformis* (pyridoxine, -al, -amine; equal molar amounts of each isomer)

[g] Nicotinic acid + nicotinamide; equal molar amounts of each.

addition of the food sample resulted in a pH different from that of the added buffer. At the completion of the extraction procedures, the solutions were adjusted to the pH of the vitamin assay medium to be used (Table 1). Sets of extractions were also completed without these adjustments being made. Extraction method 3 (enzymatic digestion) was only run on samples previously digested by one of the other extraction methods. Suspended particles were removed from the final extracts by centrifugation and, if necessary, filtration.

Assay methods

Vitamin concentrations used in preparing the standard curves are given in Table 1. Standard and unknown solutions were added to assay vessels, brought to 2.5 ml with distilled water and then mixed with 2.5 ml double strength basal medium. The prepared assays were autoclaved at 121°C for 5 min; except for the *Saccharomyces uvarum* assays for pyridoxine, which were steamed for 10 min. Incubation times for the lactobacilli and *S. uvarum* were 16–20 hr, while vitamin assays using *Tetrahymena* and *Ochromonas* were incubated 5 days. Vitamin assays using *Ochromonas* as the test organisms were incubated in an illuminated chamber (Baker and Frank, 1968). After incubation, all assays were autoclaved at 121°C for 5 min. Optical density of the assay solutions was measured at 620 nm with a Bausch and Lomb Spectronic 20 against an uninoculated control.

Comparison of vitamin analysis methods

Vitamin contents in tomato juice, orange juice, blood, yogurt, round steak and spinach were determined by a commonly used microbial or yeast method and by a protozoan method of vitamin assay. Different extraction procedures are recommended for the preparation of samples to be analyzed by the conventionally used methods of vitamin analysis and the protozoan methods of vitamin analysis. These two types of food extracts were analyzed by both methods of vitamin analysis to determine if differences in the indicated vitamin levels were due to the method of vitamin extraction. Since the growth of protozoa is inhibited by certain salts and ionic strength of the media, vitamin extracts of foods were also prepared without adjusting the pH of the extract solution (Voigt et al., 1979a). These variations of the assay procedure were termed "microbial vitamin assays, microbial vitamin assays on protozoan extracts, protozoan vitamin assays on pH-adjusted extracts and protozoan vitamin assays on pH-not-adjusted extracts." Both enzymatically and nonenzymatically digested samples were analyzed by both types of vitamin analysis procedures (common vs protozoan). Enzymatic digestions were completed on aliquots of samples previously digested by the nonenzymatic methods. These digestions were termed acid digests and enzymatic digests. Five extract aliquots (0.25, 0.5, 0.75, 1.0 and 1.25 ml) were assayed in duplicate. Two separate runs were completed with all methods, except there was only one run on the "protozoan vitamin assays on pH-not-adjusted extracts."

Data analysis

Sigmoidal dosage-response curves from both the standards and the unknown tests were transformed by the logistic function into linear regressions. Measures of the goodness of the least-squares-fits (R^2 values) of the logistic transformations to the linear model were usually greater than 0.95 ($P \leq 0.0001$). Application of the logistic transformation to bioassays was discussed in a monograph by Ashton (1972). Validity of the test responses was determined by evaluating test and standard curves for parallelism; that is, statistically, the null hypothesis tested was whether the difference in slopes of the test and standard curves equalled zero. The Student t-test was employed at the 95% level to evaluate the null hypothesis. Confidence limits for the mean values of the vitamin contents of the food samples were obtained by using the likelihood function as demonstrated by Schatzki and Keagy (1975). Mean values of vitamin content obtained from valid replicate runs were pooled. When all experimental runs for a particular analysis were evaluated to be invalid, the symbol "NV" (data not valid) was reported. These invalid runs were caused by the presence of excess inhibition or stimulation in the vitamin assay. The mean values of the vitamin contents of food samples represent the geometric means of lognormal distributions and not the arithmetic means of normal distributions. Percent standard errors for each analysis were also calculated. Ideally, they should be less than 10 percent (Bliss and White, 1967). Confidence limits at the 95 percent level were calculated for the mean vitamin contents. These limits are skewed due to the bias resulting from using logarithms, which gave lognormal vs normal distributions. Log bias can be corrected (Beauchamp and Olson, 1973; Thoni, 1969). Significant differences between vitamin levels found in a given food by two different assay methods required the 95% confidence limits not to overlap. Significant differences between experimental and literature values could not be calculated, since standard errors were not available for vitamin levels in foods obtained from food composition

Table 2—Culture maintenance and vitamin assay media

Medium no.	Culture medium	Reference[a]
1.	*Lactobacillus* Broth AOAC (Difco)	1
2.	*Lactobacillus* Agar AOAC (Difco)	1
3.	Malt Extract, 3%, pH 5.5	5
4.	Malt Extract Agar (Difco)	5
5.	Fluid Thioglycolate Medium (Difco)	4
6.	*Ochromonas* Maintenance Medium	3
7.	*Tetrahymena* Maintenance Medium	4
8.	ATCC *Tetrahymena* Medium	2
9.	Pantothenate Medium USP (Difco)	1
10.	Thiamine Assay Medium LV (Difco)	1
11.	*Ochromonas* Biotin and Thiamine Assay Medium	5
12.	Riboflavin Assay Medium (Difco)	1
13.	*Tetrahymena* Niacin, Riboflavin, and Pantothenate Assay Medium[b]	3
14.	Pyridoxine Y Medium (Difco)	1
15.	*Tetrahymena* B_6 Assay Medium[b]	3
16.	B_{12} Assay Medium USP (Difco)	1
17.	*Ochromonas* B_{12} Assay Medium	3
18.	Biotin Assay Medium (Difco)	1
19.	Niacin Assay Medium (Difco)	1

[a] References: 1. Difco (1968); 2. Anon. (1974); 3. Baker and Frank (1968); 4. Conner and Cline (1964); 5. Strohecker and Hennine (1966)

[b] Trisodium guanylate was guanosine 2′ + 3′ monophosphoric acid mixed isomers (Sigma). Yeast adenylic acid was adenosine 2′ – 3′ monophosphoric acid mixed isomers (Sigma). DL-thioctic acid was oxidized form (Sigma). Vitamin free casamino acids (Difco). EDTA, HEDTA, methionine, guanine, sodium glycerophospate, and hydrolyzed yeast nucleic acid were omitted. Soluble starch (Eastman), 1% was added (starch was dissolved by autoclaving 10 min in 1 liter water and then added to cold base after pH adjustment). Hypozanthine, 2 mg %, and Tween 80, 5 mg %, were added to the B_6 medium. Other changes: adenine to 5 mg %, uracil to 10 mg %, L-tryptophan to 0.02%, glycine to 0.02%, thiamine to 0.1 mg %, biotin to 0.1 ug %, metal mix to 5 mg %, diacetin to 0.02 %. Personal communications with Drs. H. Baker and O. Frank (College of Medicine and Dentistry, East Orange, NJ 07017).

Table 3—B-vitamin extraction methods

Method no.	Extract solution	Temperature (°C/min)	Reference[a]
1.	Water, pH 2.5 (hydrochloric acid)	121/30	2, 3, 4
2.	Aconitate buffer[b], 1:5[c], after autoclaving dilute 1:9[d]	121/15	1
3.	Papain Type II, (Sigma), 20mg; Diastase® (Fisher), 20 mg; acid digest[e], 100 ml; pH 4.5 (potassium hydroxide, sodium acetate)	45/180	2, 3, 4
4.	0.55N Hydrochloric acid	121/120	2
5.	Phosphate buffer[f], 1:1[b] after autoclaving dilute 1:4[d]	121/30	1
6.	Sodium phosphate, monobasic (anhy), 2.6g; citric acid (monohydrate), 2.4g; sodium metab sulfite (anhy), 30 mg; sample + water to 200 ml	121/10	1, 2
7.	2N Sulfuric acid	121/120	4
8.	Citrate buffer[g], 1:4[c], after autoclaving dilute 1:3	121/10	1
9.	1N Sulfuric acid	121/30	2
10.	Citrate-phosphate buffer[h], 1:20[c], pH 4.5	121/30	1
11.	Water, pH 5.6–5.7 (acetic acid, sodium acetate)	121/7	2
12.	Citrate buffer[i], 1:20[c]	121/5	1

[a] References: 1. Baker and Frank (1968); 2. Horwitz et al. (1975); 3. Saarivirta (1969); 4. Strohecker and Henning (1966)

[b] Trans-aconitic acid, 1g; water, 200 ml; pH 4 (potassium hydroxide)

[c] Sample:buffer

[d] Extract:water

[e] Acid digest was any of the other extract solutions after autoclaving.

[f] Sodium phosphate, monobasic (monohydrate), 2.76g; water, 1 liter, pH 4.5

[g] Citric acid·H_2O, 92 mg; sodium phosphate, dibasic, 0.4g; water, 1 liter, pH 4.5

[h] Sodium phosphate, monobasic (anhy), 1.26g; citric acid (anhy), 1.07g; water, 1 liter, pH 4.5

[i] Sodium citrate (dihydrate), 3.3g; citric acid (monohydrate), 1.2g; water, 1 liter, pH 5

tables cited in the literature. Computer programs employing the IBM 370/158 and Cyber 74 were developed to evaluate the data (Voigt et al., 1979b).

RESULTS & DISCUSSION

THE FOOD SAMPLES were first digested by extraction methods 1–2, 4–13, (Table 3), these preparations were designated as the acid digests. Aliquots of these extracts were then digested enzymatically as described in extraction method 3. Almost all vitamin B_6 analyses completed on the acid digests indicated lower levels of the vitamins in the sample than did analyses on the corresponding enzymatically digested extracts (Table 4). In Tables 5–7, only the vitamin contents obtained from the analyses of the enzyme digestions of the samples are reported; however, values from the enzymatic digestions that were significantly different than the values obtained from the analyses of the acid digestions are identified. The only acid digested samples with higher values without enzymatic hydrolysis were the riboflavin analyses on tomato juice, orange juice and blood.

Assays for vitamins using protozoa as the test organisms were completed on sample extracts prepared as described for analyses by the corresponding bacterial and yeast methods of vitamin determination. Data from these analyses are not reported in Table 4–8, since the levels of salts formed by neutralizing the high concentrations of acids used in these extraction methods nearly completely inhibited the growth of the protozoa. These vitamin extraction methods are not suitable when employing protozoa as the test organisms.

Tetrahymena pyriformis was employed as the assay organism for riboflavin, vitamin B_6, niacin and pantothenic acid. Data from the various sample preparation methods are only presented for vitamin B_6 (Table 4) since most of the trends observed in these data occurred in the analysis of the other vitamins. Values followed by the superscripts "e or f" indicated that slight innibition or stimulation not due to vitamin content was detected in the test responses. *S. uvarum* assays for vitamin B_6 consistently showed the presence of stimulation. In contrast, the commonly used methods for determining riboflavin (*Lactobacillus casei*), niacin (*L. plantarum*) and pantothenic acid (*L. plantarum*) consistently showed inhibition. More inhibition was detected in the pH-adjusted assays using *T. pyriformis* than in the pH-not adjusted. Inhibition was most frequent in the bacterial and *T. pyriformis* analyses for riboflavin, niacin and pantothenic acid contents in tomato juice, orange juice and blood. A combination of neutralization salts formed by the pH adjustments and the organic acids present in these samples had an inhibitive effect on the growth of these test cultures. Citric acid was used as the anticoagulant in blood. *T. pyriformis* assay for vitamin B_6 usually indicated higher levels of vitamin B_6 in the foods than did *S. uvarum*, although the differences were not always significant at the 5% level. Vitamin levels in yogurt and round steak may be

Table 4—Vitamin B_6 in the acid and enzyme hydrolysates compared to literature values

Sample	Vitamin B_6 (ng/g or ml)[a]				
	S. uvarum		*T. pyriformis*		
	Microbial[c]	Protozoan[c]	pH-not-adjusted	pH-adjusted	Literature[d]
Acid hydrolysate					
Tomato juice	420 A^b	1100 B^f	1200 B	1600 C	1900
Orange juice	330 A^b	110 B^b	450 C	400 D	350
Blood	37 A^b	0 $B^{b,e}$	ND[g]	170 C	20 to 180
Yogurt	310 A^b	340 A	1400 B	1200 B	460
Round steak	4800 A^b	11000 $B^{b,f}$	11000 B	4500 A^b	4400
Spinach	1300 A^b	2000 $B^{b,f}$	ND	ND	2800
Enzyme hydrolysate					
Tomato juice	1000 $A^{b,f}$	1000 A^f	1200 B	1300 B	1900
Orange juice	810 $A^{b,f}$	280 B^b	440 C	570 D	350
Blood	110 $A^{b,e}$	58 B^b	170 C	190 C^e	20 to 180
Yogurt	650 $A^{b,f}$	420 A^f	1600 C	1600 C	460
Round steak	11000 $A^{b,f}$	16000 $B^{b,f}$	12000 AC	13000 C	4400
Spinach	1900 $A^{b,f}$	2900 $B^{b,f}$	ND	1900 $A^{b,e}$	2800

[a] Values for the same sample followed by a different capital letter are significantly different ($P \leq 0.05$).
[b] Significantly different ($P \leq 0.05$) than the vitamin content determined in the acid hydrolysis.
[c] Table 3: Microbial = Method 4, Protozoan = Method 5.
[d] Acid and enzyme hydrolysates not distinguished (Baker and Frank, 1968; Robinson, 1972)
[e] Inhibition detected ($P \leq 0.05$)
[f] Stimulation detected ($P \leq 0.05$)
[g] "ND" calculation not determined

considerably under estimated in the literature, since *T. pyriformis* has not been used as the test organism. Data from the other vitamin analyses (riboflavin, niacin and pantothenic acid) of the low-acid foods by *T. pyriformis* also consistently yielded higher values than did the corresponding commonly used methods (Table 8). *T. pyriformis* has the potential of giving higher values for blood if heparin is used as an anticoagulant. Analyses of the vitamin extracts prepared for assay by *T. pyriformis* but assayed by *S. uvarum* did not yield the higher estimate of vitamin content indicated by *T. pyriformis*, i.e., without interference due to stimulation being detected. *Tetrahymena* has a more developed ingestive and digestive system than the yeast and lactobacilli, which gives it the ability to respond to conjugated forms of vitamins. The pH adjustments of the sample solutions allowed the Diastase ®-papain enzymatic digestions to more efficiently hydrolyze the samples; however, this digestion is not sufficient to make the commonly used bacterial and yeast methods equivalent to using *T. pyriformis* as the test organism. The problem of interference due to inhibition or stimulation of the test organisms might be overcome by developing vitamin extraction methods which consider the type of food being analyzed and the assay method being used. Vitamin data should not be reported without evaluating for the presence of inhibition or stimulation of the test organism.

Comparisons of the thiamine contents found in the food samples are presented in Table 5. Thiamine levels quantitated by *Lactobacillus viridescens* agreed with literature values, except for values obtained from the enzymatic digestions of steak and spinach, which were about twice as high. Vitamin levels determined using *O. danica* as the test organism were usually lower than those determined by either *L. viridescens* or thiochrome or those reported in the literature. Significantly larger thiamine levels were indicated by the thiochrome method when the acid hydrolyzed samples were digested enzymatically before analysis. All three meth-

Table 5—Thiamine contents in the acid and enzyme hydrolysates compared to literature values

Sample	Thiamine (ng/g or ml)[a]			
	L. viridescens	*O. danica*	Thiochrome	Literature[c]
Enzyme hydrolysate				
Tomato juice	480 A	310 B	550 C^b	500
Orange juice	870 A^d	610 B	630 B^b	900
Blood	150 A^b	15 B^b	28 C	20 to 300
Yogurt	520 A	610 $A^{b,e}$	410 B^b	400
Round steak	1700 A^b	790 B^e	540 C^b	900
Spinach	1600 A^b	720 B^b	1100 C^b	700

[a] Values for the same sample followed by a different capital letter are significantly different ($P \leq 0.05$).
[b] Significantly different ($P \leq 0.05$) than the vitamin content determined in the acid hydrolate
[c] Acid and enzyme hydrolysates not distinquished (Baker and Frank, 1968; Church and Church, 1970)
[d] Inhibition detected ($P \leq 0.05$)
[e] Stimulation detected ($P \leq 0.05$)

Table 6—Biotin in the acid and enzyme hydrolysates compared to literature values

Sample	Biotin (ng/g or ml)[a]						
	L. plantarum						
	Microbial[c]		Protozoan[c]		*O. danica*		Literature[d]
Enzyme hydrolysate							
Tomato juice	39	A^e	110	B^f	21	C^b	40
Orange juice	19	A	51	B^f	2.7	C^b	10
Blood	4.6	A^e	7.2	$B^{b,f}$	1.3	C^b	0.2–5
Yogurt	57	A	49	A	13	B^b	43
Round steak	53	A	48	$A^{b,f}$	15	B^b	26
Spinach	124	$A^{b,e}$	88	$B^{b,f}$	5.6	C^b	69

[a] Values for the same sample followed by a different capital letter are significantly different ($P \leq 0.05$).
[b] Significantly different ($P \leq 0.05$) than the vitamin content determined in the acid hydrolysate
[c] Table 3: Microbial = Method 7, Protozoan = Method 8.
[d] Acid and enzyme hydrolysates not distinquished (Baker and Frank, 1968; Cooper et al., 1943)
[e] Inhibition detected ($P \leq 0.05$)
[f] Stimulation detected ($P \leq 0.05$)

ods of thiamine analysis usually gave significantly ($P \leq 0.05$) different thiamine contents in the food samples. The difference being the largest with blood, round steak and spinach. Higher thiamine levels indicated by *L. viridescens* is not surprising, since this organism produces extracellular lysolytic enzymes, which free bound thiamine. The lysolytic enzymes of *O. danica* are associated with its internal food vacuoles. Gregory and Kirk (1978) found that the thiochrome procedure indicates higher thiamine values than the rat bioassay.

Comparisons of the biotin contents found in the food samples are presented in Table 6. Each of the three assay methods usually gave significantly different results The lowest levels were indicated by *O. danica*. Except for the biotin level in blood, all of the biotin contents in the foods assayed by *O. danica* were considerably lower than the values cited in the literature. *L. plantarum* analysis of the food extracts prepared for analysis by *O. danica* gave test results which consistently showed the presence of stimulation not due to vitamin content; thus, this extraction method is unsuitable for the preparation of samples for biotin analysis using *L. plantarum*. However, these data show that the lower levels of biotin indicated by *O. danica* are due to the test organism and not the extraction method, since both *L. plantarum* assays indicated higher biotin levels. The lower biotin values indicated by *O. danica* reflect the greater specificity of the organism for forms of biotin that are clinically active. Several nonclinically active biotin compounds have the ability to promote growth of *L. plantarum*, for example, oxybiotin, biotin sulfone and sulfoxide, fatty acids and lipids (Strohecker and Henning, 1966). The literature values were probably determined using the *L. plantarum* assay method for biotin, and thus are overestimates.

Comparisons of the vitamin B_{12} contents found in the food samples are presented in Table 7. The commonly used method with *Lactobacillus leichmannii* was compared to the protozoan method which employed *O. malhamensis*. *L. leichmannii* assays for vitamin B_{12} indicated the presence of low vitamin B_{12} levels in the enzymatic hydrolysates of tomato juice and orange juice and the acid and enzyme hydrolysates of spinach. *L. leichmannii* is stimulated by purines, pyrimidines and clinically inactive cobalamins (Castor, 1953; Baker and Frank, 1968). Since vitamin B_{12} was not indicated by this test organism in the acid digests of tomato and orange juices, the indicated vitamin activity was a result of the enzymatic digestion. Vitamin B_{12} activity was indicated by *L. leichmannii* but not by *O. malhamensis*, which indicated nonspecific stimulation of *L. leichmannii*.

Table 9 presents pooled distributions of the percent standard errors (% SE) from the vitamin analysis data. Bliss and White (1967) indicated that a good % SE for a vitamin assay was 10% or less. This appears to be an empirical limit, and reflects a limit arrived at from their experience in conducting vitamin assays. On a practical basis, a lower % SE represents a more precise assay (a smaller standard error and hence less data scatter). However, a vitamin assay method that yields data with little scatter is not necessarily indicating the clinically-active vitamin content in the sample. A vitamin estimate of low precision but with a reasonable assurance of accuracy is preferable to one of higher precision that is irrelevant because of inaccuracy. The data in Table 9 indicate that 73% of the bacterial and yeast assay had % SE's $\leq$ 10%, while the remaining 27% were between 10 and 30%. This was expected, since the bacteria and yeast used for analysis of food contents of vitamins are more tolerant to neutralization salts (Voigt et al., 1979a). Also, the assay incubation periods are 2–4 days shorter than the protozoan incubations. Data from the vitamin assays employing *Ochromonas* test organisms had the fewest % SE's $\leq$ 10% and the most between 10 and 30%. *Ochromonas* assays for vitamins are more accurate assays, especially for biotin and vitamin B_{12}, but at this stage of development are less precise than the corresponding bacterial methods. The pH-not-adjusted food extracts assay by *Tetrahymena* for vitamin content had less scatter in their data than did the corresponding assays on pH-adjusted food extracts. This scatter was induced by the neutralization salts contributed to the assay extracts by pH-adjustments. This problem may be overcome by the proper selection of acids, bases, buffers and enzymes for vitamin extraction procedures.

Table 7—Vitamin B_{12} in the acid and enzyme hydrolysates compared to literature values

Sample	Vitamin B_{12} (ng/g or ml)[a]		
	L. leichmannii	*O. malhamensis*	Literature[c]
Enzyme hydrolysate			
Tomato juice	0.25 A^b	0.0 B^d	0
Orange juice	0.091 A^b	0.0 B^b	0
Blood	0.51 $A^{b,d}$	0.17 B^b	0.12–0.5
Yogurt	2.2 $A^{b,d}$	3.5 B	1.1
Round steak	19 A^d	12 A	18
Spinach	0.02 A^d	0.0 $B^{b,d}$	0

[a] Values for the same sample followed by a different capital letter are significantly different ($P \leq 0.05$).
[b] Significantly different ($P \leq 0.05$) than the vitamin content determined in the acid
[c] Acid and enzyme hydrolysates not distinguished (Baker and Frank, 1968; Robinson, 1972)
[d] Inhibition detected ($P \leq 0.05$)

CONCLUSIONS

VITAMIN LEVELS of clincial specimens have been routinely determined using protozoa as the organisms (Baker

Table 8—Comparison of the riboflavin, niacin and pantothenate levels in food determined by T. pyriformis and the corresponding microbial methods

Sample	Riboflavin[a] (ng/g or ml)		Niacin[a] (ng/g or ml)		Pantothenic acid[a] (ng/g or ml)	
	L. casei	*T. pyriformis*	*L. plantarum*	*T. pyriformis*	*L. plantarum*	*T. pyriformis*
Tomato juice	250 A^b	260 A^c	5000 A	2700 B^b	400 A^b	10000 B^b
Orange juice	170 A^b	68 B	1700 A	740 B	1000 A	3000 B^c
Blood	340 A^b	250 B	7700 A^c	14000 B	340 A^b	120 B
Yogurt	5800 A	8000 B	780 A^b	1700 B	2900 A	9400 B^c
Round steak	6500 A	7400 B	61000 A	73000 A	5900 A	16000 B^c
Spinach	4300 A	5900 B	3700 A	13000 B	2000 A	1700 A^b

[a] Values for the same vitamin and sample followed by a different capital letter are significantly different ($P \leq 0.05$).
[b] Inhibition detected ($P \leq 0.05$)
[c] Stimulation detected ($P \leq 0.05$)

and Frank, 1968). Those studies indicated that the protozoa were more accurate in determining clinical vitamin status. Little research has been completed to determine whether there is a significant descrepancy between the vitamin contents in foods indicated by the protozoan vs the commonly used methods of vitamin analysis. This investigation was conducted to provide an answer to this question. Interpretation of data from this study must consider that sample preparation techniques for the commonly used vitamin assay procedures have been well developed through use while there are no established methods of sample preparation for the protozoan analyses of the vitamin contents in food samples. Initially, in this study, sample preparation methods developed for the conventionally used bacterial and yeast assays for the vitamins were employed. These sample extracts inhibited the growth of the protozoan test organisms, due to their high content of neutralization salts. Sample preparation procedures developed by Baker and Frank (1968) for clincial specimens were then tried. It was found that the buffers used in the sample extractions did not maintain the same pH when food samples were added; however, it was not known whether the protective action of the buffers added were entirely pH dependent. These two alternatives were examined. Generally, the pH-not-readjusted to the buffer pH values gave test responses showing less evidence of inhibition or stimulation, because of the lower levels of neutralization salts in the food extracts. However, the pH adjusted extracts from the enzymatically digested samples usually indicated the highest levels of the vitamins, since the Diastase®-papain digestion was more active at its optimum pH and less vitamin was rendered inactive by being subjected to heat at a less than ideal pH for stability. Further work is required to develop optimal methods of vitamin extraction to be used to prepare food samples for assay of vitamin contents by protozoa.

This research indicated that the commonly used vitamin assay methods consistently over-estimate the levels of thiamine, biotin and vitamin B_{12}, but consistently under-estimate the levels of riboflavin, vitamin B_6, niacin and pantothenic acid when compared to corresponding protozoan analyses. This bias is reflected in the values published in food composition tables. This problem will continue to grow because of the expanding use of automated chemical techniques of vitamin analyses, since they are selective for particular forms of vitamins and do not respond to all of the clinically active forms (Baker and Frank, 1968). After satisfactory methods of vitamin extraction have been developed for the preparation of samples for the protozoa techniques of vitamin analysis, the protozoan techniques could become accepted methods of determining the vitamin contents in foods. Currently, the *O. malhamensis* assay for determining the vitamin B_{12} content in clinical samples is the official method in Great Britain.

Table 9—Distribution of the percent standard errors (% SE) from the various vitamin assay methods

Assay condition	Relative distribution of % SE		
	10%	11–30%	30%
Bacteria and yeast – pooled	0.73	0.29	0.00
Ochromonas – pooled	0.05	0.81	0.14
Tetrahymena – pH-not-adjusted	0.42	0.58	0.00
Tetrahymena – pH-adjusted	0.24	0.54	0.22

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COMPARISON OF THE ABILITIES OF TRICHLOROACETIC, PICRIC, SULFOSALICYLIC, AND TUNGSTIC ACIDS TO PRECIPITATE PROTEIN HYDROLYSATES AND PROTEINS

NORMAN A. GREENBERG and W. F. SHIPE

ABSTRACT

The abilities of picric, sulfosalicylic (SSA), trichloroacetic (TCA), and tungstic acids to precipitate a partial hydrolysate of egg albumin and several proteins were examined. After precipitation of the hydrolysate with SSA, TCA, and tungstic acid respectively, it was found that 88%, 79%, and 69% of the nitrogen remained in solution. The average size of the peptides in the supernatants varied from 330 to 380 daltons. The ability to precipitate native proteins varied with the precipitant and the protein. Tungstic acid precipitated both bovine serum albumin and β-lactoglobulin completely at low concentration (0.5% final concentration, w/v). Bovine serum albumin was precipitated by 3% TCA or SSA but, β-lactoglobulin was not completely precipitated until the concentrations had been increased to 10% and 20%, respectively. Pretreating β-lactoglobulin with sodium dodecylsulfate increased the amount precipitated by SSA and TCA.

INTRODUCTION

THE EFFECT of precipitating agents upon proteins varies with the protein, precipitant, and their respective concentrations. For example, precipitants are used to separate digested protein from undigested protein in various in vitro protein evaluation procedures, yet no investigator has compared these precipitants with respect to the size of peptides left in solution and the efficacy of intact protein precipitation. Knipfel et al. (1969), claimed that it may be difficult to compare published data on amino acid levels of body tissues when different deproteinating agents are used.

Various workers have compared different precipitating agents by examining the resulting "protein-free" plasma of blood for free amino acid composition (Hamilton, 1962; Dickinson et al., 1965; Block et al., 1966; Jones and Belling, 1970). The published literature pertaining to the effectiveness of the precipitants, does not reveal any clear cut advantage of one precipitant over another. The study reported here was designed to provide a direct comparison of precipitating agents. First, it compares the effects of TCA, picric, tungstic, and SSA acids upon a partial pepsin hydrolysate of egg albumin and secondly, it examines the concentration of precipitant required for precipitation of various proteins.

MATERIALS & METHODS

Preparation of hydrolysate

Thirty-five grams of egg albumin (ICN Life Sciences Group), were dissolved in water and brought to 3.5L with the final pH adjusted to 1.6 by addition of HCl. Then 350 mg of pepsin (3X crystallized, ICN Life Sciences Group) was added. This was agitated for 4 hr at 37°C at which time the pH was adjusted to 7 with 1N NaOH. This material was freeze dried and stored in a desiccator until needed.

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General precipitation procedures

All centrifuged samples were centrifuged at 15°C in an International Refrigerated Centrifuge (Model B-20). All centrifugal forces are given for the bottom of the centrifuge tube. The term hydrolysate supernatant refers to the soluble fraction obtained by centrifugation or filtration of the hydrolysate after treatment with a precipitant. Similarly, protein supernatant refers to the soluble fraction after treatment of a protein with a precipitant. Duplicate hydrolysate and protein preparations were analyzed, and the results averaged. This procedure was very reproducible and the duplicates were always in good agreement.

Hydrolysate precipitation

Picric acid procedure (adapted from Akeson and Stahlman, 1964). A 200-mg aliquot of hydrolysate was added to 20 ml of water and mixed with 100 ml of 1% picric acid (Matheson, Coleman and Bell Manufacturing Chemists, Norwood, OH). The mixture was centrifuged for 30 min at 1,000 × G. The supernatant was freeze dried.

Tungstic acid procedure (adapted from Sheffner et al., 1956). A 1.00-g portion of the hydrolysate was dissolved in 30 ml of water, then 3.75 ml of 10% (w/v) sodium tungstate (General Chemical Company, New York, NY) and 3.75 ml of 0.67N H_2SO_4 were added. It was allowed to stand for 10 min and then filtered through Whatman #1 filter paper. The filtrate was freeze dried.

Sulfosalicylic acid procedure (adapted from Mondino et al., 1972). A 1.05-g aliquot of hydrolysate was dissolved in 15 ml of water and mixed with 60 ml of 3.75% SSA (Fisher Scientific Co., Fair Lawn, NJ) in 0.3N lithium citrate (pH 2.8). The final pH was adjusted to 1.8 by addition of concentrated LiOH. The mixture was centrifuged for 10 min at 10,000 × G. The supernatant was freeze dried.

Trichloroacetic acid procedure A 400-mg aliquot of hydrolysate and 40 ml of water were placed in a centrifuge bottle and then 40 ml of 20% TCA (Mallinckrodt, Inc., St. Louis, MO) were added. This was held for 10 min before centrifuging for 30 min at 10,000 × G. The supernatant was then freeze dried.

Protein precipitation

Aliquots (25 mg) of bovine serum albumin, β-lactoglobulin, keratin (ICN Life Sciences Group, Cleveland, OH), and hemoglobin (BBL, Division of Bioquest, Cockeysville, MD) were weighed into 13 ml centrifuge tubes. For TCA precipitation, 4 ml of water were added except for keratin which was dissolved in 4 ml of 0.1N NaOH instead of water due to the insolubility of keratin in acid and neutral liquids (Merck Index, 8th ed., 1968). For the SSA and tungstic acid precipitations, the proteins were dissolved in 2.5 ml and 3.0 ml of water respectively (the latter 2 precipitants were used only with the bovine serum albumin and β-lactoglobulin). Before adding the precipitant, some aliquots of β-lactoglobulin were treated with sodium dodecylsulfate (SDS) to yield a final concentration of 0.4% SDS. Solutions of chicken breast muscle (Kendrick-Jones et al., 1970) containing 25 mg of protein in 4 ml and chicken breast muscle natural actomyosin (NAM) (Galluzo, 1977) containing 25 mg of protein in 2 ml were used since these protein preparations were not stored in a dry form. An aliquot of TCA or SSA was added to each tube to obtain the desired final concentration. This was 1–10% for TCA and 1–20% for SSA. Sheffner et al. (1956) used equal volumes of 10% sodium tungstate and 0.69N H_2SO_4. These proportions were used and each was added in 0.38 ml of liquid. The tubes were vortexed, held for 10 min and then centrifuged for 10 min at 12,500 × G for the TCA and SSA treatments. The tungstic acid treated samples were filtered with Whatman #1 filter paper (Sheffner et al., 1956).

Analysis

Aliquots of each hydrolysate supernatant were analyzed for Kjeldahl nitrogen amino acid composition, and put on a calibrated

Table 1—Effect of TCA concentration on precipitation of various proteins[a]

Final % TCA	β-lacto-globulin	Bovine serum albumin	Kera-tin	Hemo-globu-lin	Myosin	Natural acto-myosin
0	90	92	99	100	85	14
1	86	60	52	12	48	38
2	80	8	16	3	45	32
3	77	0	16	4	36	27
4	59	0	18	3	16	24
5	12	0	14	4	1	21
6	17	–	14	4	4	4
7	6	–	14	3	–	1
8	–	–	–	3	3	1
9	–	–	–	–	5	1
10	2	–	11	3	5	2

[a] Expressed as percentages of nitrogen remaining in the supernatant. Calculated as follows: %N = (mg N in supernatant/mg N in protein added) X 100.

Sephadex G-15 column (1 cm × 100 cm) to estimate the molecular weight of the molecules left in solution. The aliquots for amino acid analysis were hydrolyzed for 22 hr at 110°C in evacuated vials. Norleucine was added as an internal standard. It was dried and placed in 4 ml at pH 2.2 sodium citrate buffer. This was then analyzed on a Beckman-Spinco Model 120C Amino Acid Analyzer. The procedure of Spies (1967) was used for trytophan analysis. The Sephadex column was calibrated with oxidized glutathione (612.6 daltons), hexaglycine (360.3 daltons), reduced glutathione (307.3 daltons) and L-glutamic acid diethyl ester HCl (239.7 daltons). The eluant was 0.1N sodium phosphate buffer (pH 8.0), with 0.02% sodium azide added as a preservative. The outlet was attached to the sample side of a Varian Aerograph Refractive Index Detector (Varian Aerograph, Walnut Creek, CA). The reference side of the cell had a flow of eluant from the eluant reservoir. The protein supernatants were analyzed for Kjeldahl nitrogen after they had been dialyzed to remove the precipitants. This was necessary since high concentrations of TCA (and presumably other acids as well) interferes with Kjeldahl nitrogen determinations. The increase in solutes raises the temperature of the digestion mixture and an excessive amount of H_2SO_4 is lost. To be consistent, all protein supernatants were dialyzed.

RESULTS & DISCUSSION

Hydrolysates

After precipitation with SSA, TCA, and tungstic acid respectively, it was found that 88%, 79% and 69% of the nitrogen from the hydrolysate remained in solution. The picric acid supernatant is not included since the micro-Kjeldahl method did not fully recover all the nitrogen in the picric acid supernatant. This became apparent when the nitrogen content of an aliquot of picric acid could not be reproducibly determined.

Overall, the differences in amino acid composition were minor. Bito and Dawson (1969) found that histidine coprecipitated with proteins when tungstic acid was the precipitant. The tungstic acid supernatant did have a low histidine value (about 50% less) as compared to the albumin hydrolysate. Based on percentage composition, histidine is a minor amino acid. Therefore, the overall pattern was only slightly affected. The important message is that these precipitants did not selectively precipitate any one group of amino acids e.g. basic.

The Sephadex separation did not reveal any significant differences in the size of peptides left in solution by the four precipitating agents. The average molecular weights of the soluble fraction ranged from 330 to 380 daltons. This indicates that regardless of the precipitant used most of the peptides left in solution contained three or four amino acid residues.

Table 2—Comparison of the precipitation of β-lactoglobulin and bovine serum albumin by tungstic, sulfosalicylic and trichloroacetic acids[a]

Acid conc (%)	β-lactoglobulin			Bovine serum albumin		
	TCA	SSA	Tung-stic	TCA	SSA	Tung-stic
0	90		92			
0.1	–	–	38	–	–	1
0.3	–	–	2	–	–	1
0.5	–	–	0	–	–	0
1.0	86(48)[b]	79(1)[b]	0	60	5	0
3.0	77	78	–	0	3	–
5.0	12	53	–	0	2	–
10.0	2	38	–	–	0	–
15.0	–	13	–	–	0	–
20.0	–	3	–	–	0	–

[a] Expressed as percentage of nitrogen remaining in the supernatant.
[b] Numbers in parenthesis indicate the %N remaining in samples treated with 0.4% SDS

Sephadex columns have been commonly used to estimate the molecular weight of proteins but they have not been used to determine the molecular weight of small peptides. In view of the simplicity of Sephadex separations, this technique was applied to the partially hydrolyzed proteins. The fact that the observed molecular weights did not vary much between samples may partially be due to the limitations of the method. However, since all samples were subjected to 4-hr hydrolysis there may not have been much variation in the average molecular weights. Additional research is needed to determine the limits of the Sephadex separation of peptides.

Protein precipitation

The data presented in Table 1 show the effect of different precipitants and their ability to precipitate different proteins. For all the proteins, a concentration greater than 10% TCA did not cause an increase in precipitation. Due to the fluffy nature of some of the pellets formed after centrifugation, some protein was recovered in the supernatant. The bovine serum albumin formed a very firm pellet and thus very little material was stirred up when the supernatant was decanted. By contrast keratin and hemoglobin formed fluffy pellets. At 4% TCA, bovine serum albumin was completely precipitated while β-lactoglobulin was the least precipitated protein. Therefore, it was decided to determine if bovine serum albumin and β-lactoglobulin showed similar responses to SSA and tungstic acid.

The results in Table 2 reveal that these three precipitants vary in their ability to precipitate a given protein. Bovine serum albumin is readily precipitated by all of the precipitants. β-lactoglobulin is quite soluble in low concentrations of SSA and TCA but, not in tungstic acid. This knowledge could be used to separate different proteins. By using 3% SSA or TCA, one could precipitate the bovine serum albumin and leave the majority of the β-lactoglobulin in solution. Fox et al. (1967), used 3% TCA to separate β-lactoglobulin from the other milk serum proteins. Tungstic acid is obviously the wrong choice for that kind of procedure.

As may be seen, 0.4% sodium dodecylsulfate had a marked effect on β-lactoglobulin precipitation. This pretreatment increased the efficiency of precipitation by both TCA and SSA. This indicates that the secondary and/or teritary structure, of β-lactoglobulin and perhaps other proteins, influences the ability of a precipitant to precipitate a protein.

The choice of precipitants is also influenced by what the researcher requires of the protein. Saidel and Modapalli-

mattam (1970), showed that α-chymotrypsin precipitated with 7% TCA, 5% SSA, and 0.9% picric acid maintained its proteolytic activity while tungstic acid decreased its activity to about one-eighth of its starting activity. In other situations, the minimum concentration required for precipitation should be used. Alfred and Narasinga Roo (1971), found that up to 5% TCA did not inactivate pepsin. When TCA was used in excess of the 5% level, denaturation and inactivation occurred.

It should be emphasized that this study involved a comparison of published precipitation methods. These methods varied with respect to concentration of precipitant and separation procedures as well as the type of precipitant. Consequently, the observed differences between methods may not be solely due to the choice of precipitant. (Additional studies are needed to compare different precipitants under identical conditions). However, it is believed that the differences observed in this study are primarily due to the precipitants per se. Therefore, these findings should aid in selecting a precipitant for a specific purpose. For example, if complete precipitation of a protein or mixture of proteins is required, then it would seem logical to choose tungstic acid. On the other hand TCA or SSA would be preferable for differential precipitation.

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VITAMIN ANALYSIS USING PROTOZOA . . . From page 728

such as sodium acetate, sodium citrate, potassium phosphate and hydrochloric acid salts of component compounds. The adjustments of the pH of basal media and sample preparation solutions should be made with acids and bases that neutralize to form salts which have the highest threshold levels to effect the growth of the test organisms. Water used in media and sample preparation should be distilled to remove chlorine. Dilutions of extracts of foods must be sufficient to avoid neutralization salt interference. The presence of such interference can be determined by evaluating the data for drift as described by Bliss and White (1967). An expanded version of this study is required to develop optimal methods of preparing food samples for the analysis of vitamin content using protozoa as the test cultures. The expanded version should employ several types of foods and a series of different sample preparation methods for assay of vitamin content by the protozoa. Acids, bases, buffer systems, and enzymes used in the preparation methods should be varied to obtain the optimal extraction methods.

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ZINC INTERFERENCE WITH COPPER, IRON AND MANGANESE IN YOUNG JAPANESE QUAIL

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ABSTRACT

To identify the most sensitive adverse effects of high dietary zinc, day-old Japanese quail (*Coturnix coturnix japonica*) were fed diets marginally deficient, adequate or slightly elevated in copper with supplements of 15.6–2000 ppm zinc. By 14 days of age, sensitive zinc antagonisms of copper, iron and manganese were evident. The supplemental zinc caused depressed growth, decreased feather pigmentation, mild perosis and anemia; the first three generally being more severe when dietary copper was marginally deficient. The duodenum and liver accumulated zinc in relation to the dietary zinc level. Supplemental zinc caused decreased levels of iron, manganese and copper in the liver. The adequacy of copper intake is important when zinc intake is increased by use of dietary supplements or fortification of foods.

INTRODUCTION

THE INCREASED fortification of individual human foods with zinc and the consumption of zinc supplements in concentrated form raise questions about the need and safety of additional zinc. It is well recognized that very high levels of zinc are toxic; however, less is known about the minimal levels that may adversely affect metabolism of other minerals and produce undesirable health effects.

High levels of dietary zinc interfere with the normal absorption and metabolism of several minerals (Underwood, 1977). Stewart and Magee (1964) reported that when rats were fed 7500 ppm zinc in a purified diet the concentrations of calcium and phosphorus in bone were decreased. Settlemire and Matrone (1967a, b) and Coleman and Matrone (1969) showed that rats fed this same level of zinc were anemic and had deformed, fragile erythrocytes. The livers contained reduced amounts of ferritin and lower concentrations of iron in the ferritin. In rats fed 2000 and 4000 ppm zinc, liver copper concentration was decreased (Whanger and Weswig, 1971). Huber and Gershoff (1973) reported decreased hematocrit values in rats fed 1200 ppm zinc. A dietary level of 4000 ppm zinc fed to pregnant or lactating rats produced changes in zinc, iron, copper and calcium concentrations in the tissues of the pups (Cox et al., 1969; Chu and Cox, 1972). Similar results have been reported by other investigators (Grant-Frost and Underwood, 1958; Magee and Matrone, 1960; Cox and Hale, 1962).

In the above studies zinc was fed at relatively high levels, in the range of 1000–8000 ppm. Dietary levels of other essential minerals were typically in excess of requirement. The most sensitive responses to zinc have been obtained with animals receiving minimally required or suboptimal levels of copper (Hill and Matrone, 1962; Campbell and Mills, 1974; Murthy et al., 1974).

The purpose of the present experiments was to identify the minimal level of excess dietary zinc that would produce physiological and metabolic deviations from normal and to define some of the most sensitive zinc-mineral interactions. Because other workers have found a sensitive zinc-copper antagonism, the effects of supplemental zinc were studied at copper levels of marginally deficient to above requirement.

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Table 1–Diet composition[a]

Ingredient	(g/kg)
Casein, vitamin-free[b]	280.0
Gelatin[c]	70.0
L-Arginine hydrochloride, NRC[d]	6.0
Glycine[d]	5.0
DL-Methionine, NRC[d]	6.0
Choline dihydrogen citrate[d]	6.5
Mineral mixtures[e]	58.4
Corn oil[f]	40.0
Glucose monohydrate[g]	528.1

[a] The B vitamins were mixed with 10g glucose/kg diet and the fat-soluble vitamins were mixed with 5g corn oil/kg diet. Vitamins were present as follows (mg/kg diet): thiamine-HCl 8, riboflavin 8, D-Ca pantothenate 40, nicotinic acid 100, pyridoxine-HCl 8, folic acid 3, vitamin B_{12} 0.02, biotin 0.6, retinyl acetate 6, menadione 1, vitamin D_3 0.02, DL-α-tocopherol 25, DL-α-tocopheryl acetate 25.

[b] Teklad Test Diets, Madison, WI

[c] Wilson Pharmaceutical, Calumet City, IL

[d] Grand Island Biological Co., Grand Island, NY

[e] Except for Mg, the macroelements and I were supplied as follows (g/kg diet): $CaHPO_4$ 28.39, $CaCO_3$ 10, NaCl 4, Na_2HPO_4 7, KCl 7 plus KIO_3 0.0099. Se (0.2 mg) was added as Na_2SeO_3. For other elements the dietary totals by analysis were (ppm): Zn 25, Fe 95, Mn 6.8, Mg 300. Cu content of the basal diet was 1.0 ppm. Individual mineral powders were prepared from glucose monohydrate and zinc carbonate, ferric citrate, manganese sulfate, copper sulfate or magnesium sulfate.

[f] Mazola, Best Foods, Englewood Cliffs, NJ

[g] Cerelose, Corn Products, Englewood Cliffs, NJ

EXPERIMENTAL

NEWLY HATCHED Japanese quail (*Coturnix coturnix japonica*) of both sexes from our stock colony were assigned to experimental groups of 9 or 10 birds each. The birds were housed in suspended stainless steel cages under conditions that minimized environmental contamination. They received continuous light and adequate heat. Diet and deionized water were available at all times.

The birds were fed purified casein-gelatin diets that were adequate, but not excessive in all nutrients, insofar as requirements were known. Details regarding diet composition and preparation are given in Table 1. In experiment 1 supplemental zinc was supplied in the diet as zinc carbonate at levels of 62.5, 125, 250, 500, 1000 and 2000 ppm. The copper concentrations of these diets were either marginally low (1.0 ppm) or slightly more than twice the copper requirement of 1.5 ppm (Fox et al., 1977). In experiment 2 supplemental zinc at 15.6, 31.2, 62.5 and 125 ppm was fed in diets containing 1.0, 1.5 or 3.6 ppm copper. Unless otherwise noted, levels of zinc fed will refer to zinc added as a supplement, in excess of that in the basal diet (25 ppm, see Table 1, footnote e). The zinc requirement in this diet has since been determined to be 20 ppm (Fox et al., 1977). The birds were fed the experimental diets from hatching to 14 days.

Birds were wing-banded at 7 days of age and weighed on days 7 and 14. The occurrence and severity of perosis and decreased wing feather pigmentation were evaluated on a scale of 0 to 4 with 4 representing maximal abnormality and 0 no defect. Wing feathers were examined against a light beige background. At 2 wk of age, hemoglobin by the cyanmethemoglobin method (Drabkin and Austin, 1932) and microhematocrits were measured on blood collected from the median wing vein.

Table 2–Effects of copper content of the casein-gelatin diet on response of Japanese quail to graded levels of excess dietary zinc by 2 wk of age[a]

Dietary copper (ppm)	Excess zinc (ppm)	Mortality[b]	Body wt (g)	White feather score[c]	Perosis score[c]	Hemoglobin (g/dl)	Hematocrit (%)
				Experiment 1			
1.0	0	1/10	45.6 ± 0.73	0.0	0.0	12.9 ± 0.24	45 ± 0.5
	62.5	1/10	41.5 ± 1.61a	0.2 ± 0.22	0.0	12.9 ± 0.28	43 ± 0.9
	125	0/10	42.3 ± 1.00a	0.8 ± 0.22b	0.0	12.3 ± 0.25	44 ± 0.7
	250	1/10	34.6 ± 1.86b	1.7 ± 0.17b	0.2 ± 0.15	10.5 ± 0.53b	41 ± 0.9b
	500	2/9	34.9 ± 1.95b	2.3 ± 0.29b	0.0	10.7 ± 0.51b	38 ± 1.1b
	1000	2/10	27.2 ± 1.86b	2.5 ± 0.19b	0.2 ± 0.14	6.3 ± 0.86b	28 ± 2.2b
	2000	6/10	19.9 ± 1.88b	2.5 ± 0.29b	0.0	4.4 ± 0.32b	21 ± 1.5b
3.6	0	0/9	47.7 ± 1.23	0.0	0.0	14.3 ± 0.20d	48 ± 0.6d
	62.5	1/10	40.2 ± 1.14a	0.0	0.0	13.8 ± 0.21c	47 ± 1.0c
	125	0/10	45.1 ± 0.93	0.0d	0.0	12.9 ± 0.24b	45 ± 0.8b
	250	0/9	40.5 ± 1.46ac	0.0d	0.1 ± 0.08	11.9 ± 0.72a	43 ± 1.4b
	500	0/10	43.6 ± 1.22d	0.1 ± 0.07d	0.1 ± 0.07	9.8 ± 0.59b	38 ± 2.0b
	1000	0/9	37.4 ± 1.32bd	0.1 ± 0.06d	0.0	7.6 ± 0.60b	33 ± 1.4b
	2000	5/9	27.8 ± 1.27bc	1.2 ± 0.48b	0.2 ± 0.16	4.2 ± 0.22b	26 ± 0.8bc
				Experiment 2			
1.0	0	1/10	42.6 ± 1.57	0.4 ± 0.22	0.1 ± 0.08	12.4 ± 0.20	44 ± 0.4
	15.6	0/10	40.5 ± 1.79	0.6 ± 0.26	0.2 ± 0.11	11.8 ± 0.30	42 ± 2.1
	31.2	0/10	42.2 ± 1.36	1.8 ± 0.40a	0.8 ± 0.19b	12.8 ± 0.15	46 ± 0.8
	62.5	0/10	41.3 ± 1.73	1.6 ± 0.24b	0.6 ± 0.26	12.2 ± 0.29	45 ± 0.8
	125	2/10	36.1 ± 1.99a	1.8 ± 0.37b	0.6 ± 0.26	11.7 ± 0.40	45 ± 1.1
1.5	0	0/10	42.5 ± 2.57	0.0	0.0	12.5 ± 0.19	46 ± 0.5c
	15.6	0/10	47.8 ± 1.51d	0.0c	0.1 ± 0.07	12.4 ± 0.18	45 ± 0.5
	31.2	0/10	43.6 ± 1.26	0.0d	0.0d	12.0 ± 0.24c	44 ± 0.9a
	62.5	0/10	44.9 ± 1.57	0.2 ± 0.11d	0.1 ± 0.07	12.4 ± 0.27	45 ± 1.1
	125	0/10	44.4 ± 0.89d	0.5 ± 0.22d	0.0c	11.8 ± 0.25	43 ± 0.9b
3.6	0	0/10	44.8 ± 1.84	0.0	0.0	12.6 ± 0.22	44 ± 0.8
	15.6	1/10	47.8 ± 1.09d	0.0c	0.0	12.4 ± 0.18	43 ± 0.6
	31.2	0/10	45.6 ± 1.20	0.0d	0.0d	12.5 ± 0.17	44 ± 0.6
	62.5	0/10	44.9 ± 1.62	0.0d	0.0c	12.4 ± 0.24	44 ± 0.7
	125	0/10	44.8 ± 1.27d	0.0d	0.0c	12.6 ± 0.26e	44 ± 0.7

[a] Mean values ± S.E. Mean values were significantly different within experiments from the control with no excess zinc: a: $P < 0.05$; b: $P < 0.01$; significantly different from groups receiving 1.0 ppm Cu and the same level of excess zinc: c: $P < 0.05$, d: $P < 0.01$; significantly different from groups receiving 1.5 ppm Cu and the same level of excess zinc: e: $P < 0.05$, f: $P < 0.01$.

[b] Number of deaths/number of birds started

[c] Scored on a basis of 0 to 4, with 0 as normal and 4 as the maximal abnormality

At the end of experiment 1 the birds were killed by decapitation and the liver and duodenum were immediately excised. The duodenum consisted of the section of small intestine that was distal to the gizzard and that encircled the pancreas. The duodenal loop was removed, freed from pancreatic tissues, placed on a piece of rigid plastic and cut open longitudinally. The serosal surface adhered to the plastic while the mucosal surface was washed with a stream of 0.75% sodium chloride solution. The liver was also rinsed in saline solution. Both tissues were blotted dry and placed in tightly sealed, preweighed plastic bags. Samples were weighed and stored at −15°C until analysis.

The tissues were wet digested in a 5:1 (v/v) mixture of nitric and perchloric acids. The zinc, iron, manganese and copper contents of the tissues were determined by conventional flame atomic absorption spectrophotometry (Perkin-Elmer Corp., Model No. 503). To minimize phosphate interference and control viscosity, the final sample dilutions contained 10% glycerol and 0.7% perchloric acid by volume (Rains et al., 1963). Using these analytical procedures, values for these elements in the National Bureau of Standards Liver Reference Material fell within the certified ranges. Mineral values were expressed on the basis of fresh weight. Diets (0.5g) and mineral premixes (0.1g) were assayed by the same technique.

Student's *t* test (Steel and Torrie, 1960) was used to evaluate the data.

RESULTS

DATA for mortality, body weight, white feather and perosis scores, hemoglobin and hematocrit are shown in Table 2. Birds receiving 1 ppm dietary copper and no supplemental zinc grew normally. They had neither white feathers nor perosis in experiment 1 and had very low scores in experiment 2. In experiment 1, birds receiving 3.6 ppm copper had higher hemoglobin and hematocrit values than birds receiving 1.0 ppm copper. This difference did not occur in experiment 2.

Mortality was low for all groups except those receiving 2000 ppm zinc. Growth was consistently depressed with 125 ppm zinc and 1 ppm copper, whereas with 3.6 ppm copper higher levels of zinc were required to depress growth rate severely.

The incidence of white feathers was consistently higher than that of the controls beginning with 125 ppm zinc for birds fed 1 ppm copper; however, the birds fed the higher levels of copper (1.5 and 3.6 ppm) did not show white feathers significantly different from their controls except for 2000 ppm zinc with 3.6 ppm copper. Figure 1 shows normal feathering plus varying degrees of white feathers resulting from supplemental zinc.

In experiment 2, with 1 ppm copper, the perosis score for birds fed 31.2 ppm zinc was different from the control value; no other levels of zinc had a significant effect. However, in experiment 2 the lack of perosis at the two higher copper levels (1.5 and 3.6 ppm) was significantly different than that which occurred with the low copper diet beginning at 31.2 ppm zinc.

In experiment 1, hemoglobin and hematocrit values were inversely related to dietary zinc levels of 250 ppm or more with both levels of copper. Almost no decrease in hemoglobin or hematocrit was observed in experiment 2, to some

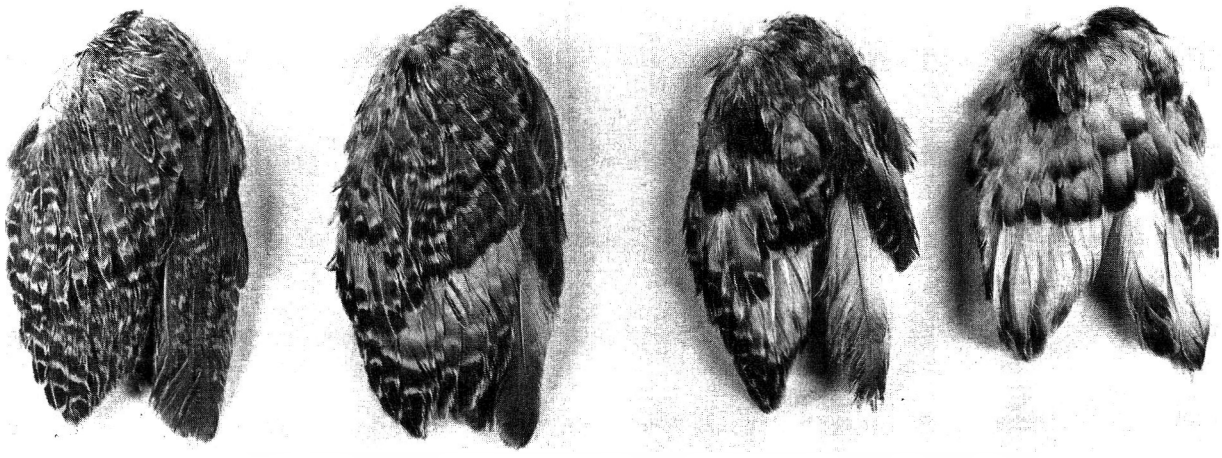

Fig. 1—Wing feathers from 2-wk-old Japanese quail. The one on the far left is from a control bird. The other three were taken from birds fed supplemental dietary zinc. They represent white feather scores (left to right) of 0, 1.0, 2.0 and 3.0.

extent reflecting lower values for the control group with 3.6 ppm copper.

Concentrations of zinc, iron, manganese and copper in the duodenum and liver are reported in Table 3. With both levels of dietary copper, the lowest level of zinc, 62.5 ppm caused a significant increase in duodenal zinc concentration, which rose progressively with increasing zinc levels. At 125 ppm or more dietary zinc, the duodenal zinc concentrations were significantly greater in birds fed 3.6 than those fed 1 ppm copper. Duodenal iron was consistently reduced in relation to the zinc level at the highest concentrations of zinc (1000 and 2000 ppm). Manganese concentration in the duodenum significantly decreased with 250 ppm or more zinc in the low copper diet and with 500 ppm or more zinc in the higher copper diet. The highest level of zinc markedly reduced duodenal copper concentrations.

With both levels of copper, addition of 125 ppm or more zinc caused increased zinc concentrations in the liver. Dietary zinc, at concentrations of 250 ppm or more, caused decreased concentrations of iron in the liver with each copper level. With the control diet and most levels of zinc, iron concentration in the liver was lower with 3.6 than with 1 ppm copper. As with iron, manganese concentrations in the liver were decreased by 250 ppm or higher zinc; however, dietary copper level had little effect on this relationship. With marginally deficient dietary copper (1 ppm), 125 ppm zinc caused a significant decrease in liver copper that was progressively more marked as the dietary zinc level increased. A similar effect was observed with the higher level of copper, except that the first significant decrease in liver copper occurred with 250 ppm zinc. All groups fed the higher copper level had higher liver copper concentrations than corresponding groups receiving the lower level of copper.

DISCUSSION

EVIDENCE for antagonism of copper, iron and manganese by low levels of zinc was obtained in these experiments. The decreases in feather pigmentation and tissue copper concentrations, particularly the marked increases in sensitivity with diets marginally low in copper, support an interpretation of copper antagonism by zinc. The decreased concentrations of iron in both the duodenum and liver and the decreased hemoglobin concentrations are indicative of an iron antagonism. The effect of copper on iron mobilization cannot be excluded; however, the higher level of dietary

Table 3—Effects of copper content of the casein-gelatin diet on mineral concentrations in the duodenum and liver of Japanese quail fed graded levels of excess zinc to 2 wk of age (experiment 1)[a]

Dietary copper (ppm)	Excess zinc (ppm)	Duodenum				Liver			
		Zn (ppm)	Fe (ppm)	Mn (ppm)	Cu (ppm)	Zn (ppm)	Fe (ppm)	Mn (ppm)	Cu (ppm)
1.0	0	32 ± 1.3	60 ± 9.7	1.9 ± 0.16	1.6 ± 0.05	20 ± 0.8	132 ± 18.0	4.0 ± 0.17	3.3 ± 0.30
	62.5	71 ± 11.7a	44 ± 10.8	2.0 ± 0.15	1.8 ± 0.10	23 ± 2.7	123 ± 13.8	3.8 ± 0.15	2.7 ± 0.11
	125	143 ± 13.7b	43 ± 6.8	2.0 ± 0.14	1.8 ± 0.08	23 ± 0.9a	97 ± 11.7	3.5 ± 0.21	2.3 ± 0.11b
	250	173 ± 12.7b	65 ± 12.5	1.3 ± 0.09b	1.4 ± 0.07a	46 ± 8.8b	59 ± 6.5b	2.7 ± 0.23b	1.7 ± 0.11b
	500	369 ± 22.3b	47 ± 7.8	0.9 ± 0.10b	1.5 ± 0.10	70 ± 19.8a	48 ± 4.5b	2.3 ± 0.25b	1.6 ± 0.19b
	1000	506 ± 42.9b	25 ± 2.6b	1.1 ± 0.11b	1.5 ± 0.13	84 ± 18.8b	48 ± 4.5b	2.0 ± 0.20b	1.2 ± 0.16b
	2000	574 ± 64.2b	18 ± 1.0b	1.1 ± 0.18a	0.9 ± 0.10b	156 ± 11.0b	41 ± 3.4b	1.2 ± 0.09b	0.7 ± 0.17b
3.6	0	32 ± 0.9	55 ± 8.6	1.9 ± 0.12	4.8 ± 0.52d	20 ± 0.6	76 ± 4.0d	4.1 ± 0.15	5.1 ± 0.28d
	62.5	68 ± 7.1b	32 ± 5.0a	2.3 ± 0.18	6.9 ± 0.60ad	22 ± 0.8	63 ± 6.7d	3.8 ± 0.16	4.6 ± 0.24d
	125	197 ± 20.9bc	44 ± 8.0	1.9 ± 0.09	6.6 ± 0.82d	29 ± 3.5a	73 ± 7.0	3.9 ± 0.20	4.6 ± 0.19d
	250	354 ± 37.4bd	34 ± 5.2ac	1.6 ± 0.15	4.2 ± 0.27d	33 ± 2.4b	37 ± 4.1bd	3.4 ± 0.20bc	3.7 ± 0.25bd
	500	510 ± 43.3bc	35 ± 4.4	1.3 ± 0.09bc	4.4 ± 0.22d	38 ± 4.3b	37 ± 1.8b	2.8 ± 0.22b	3.9 ± 0.25bd
	1000	701 ± 35.1bd	30 ± 2.7a	1.1 ± 0.06b	3.4 ± 0.25ad	47 ± 6.7b	32 ± 1.2bd	2.0 ± 0.16b	2.8 ± 0.21bd
	2000	762 ± 36.5bc	21 ± 2.2b	1.1 ± 0.07b	2.3 ± 0.32ad	91 ± 33.4	30 ± 2.2bc	1.2 ± 0.02b	1.6 ± 0.18bd

[a] Mean values ± S.E. Mean values were significantly different from the control with no excess zinc: a: $P < 0.05$, b: $P < 0.01$; significantly different from groups receiving 1 ppm copper and the same level of excess zinc: c: $P < 0.05$, d: $P < 0.01$. Mineral values are on the basis of the fresh weight.

copper did not decrease the effect of excess dietary zinc on hemoglobin and hematocrit values. This finding does not agree with the observations of Grant-Frost and Underwood (1958), Cox and Harris (1960) and Hill and Matrone (1962). Decreased manganese concentrations in the duodenum and liver occurred with elevated zinc; however, these changes were not related to perosis.

The effects of marginally low, adequate and a small excess of copper on the responses to supplemental zinc were investigated because of the reported sensitivity of the zinc-copper antagonism (Hill and Matrone, 1962; Campbell and Mills, 1974; Murthy et al., 1974; Hamilton et al., 1976). Although higher dietary copper protected against the growth depression, decreased feather pigmentation and perosis caused by zinc, it is possible that similar functional ranges in dietary levels of iron and manganese might reveal similar or other sensitive effects of supplemental zinc.

The decrease in most levels of liver iron with 3.6 ppm dietary copper as compared with 1 ppm copper may indicate a sensitive copper-iron anatagonism. Since the required amount of copper (1.5 ppm) was not fed in this experiment, it is not clear whether iron was abnormally high with 1 ppm copper or whether 3.6 ppm copper decreased iron or both. Copper antagonism of iron is well established at much higher levels of copper intake (Suttle and Mills, 1966; Gipp et al., 1973, 1974).

Tissue mineral levels were measured in these experiments to obtain evidence for zinc-essential element antagonisms and to assess the sensitivity of the zinc effects. The duodenum is known to be an important site of mineral absorption in most species. We generally have observed a good correlation between abnormalities in individual duodenal mineral concentrations and changes in nutritional status with respect to a given element (Fox et al., 1973). In these experiments the duodenum accumulated zinc to a greater degree than the liver. Concentrations of iron in the duodenum reflected status less accurately and less sensitively than iron in the liver or changes in hemoglobin and hematocrit. The decreases of manganese in the duodenum and liver were of a similar order of magnitude. From this limited assessment of the effects of supplemental zinc, the most sensitive and useful indices include body weight, feather pigmentation, hemoglobin, duodenal zinc and liver concentrations of zinc, iron, copper and manganese.

The young quail responded more sensitively to supplemental zinc than has been reported for other avian species (Roberson and Schiable, 1960, Johnson et al., 1962; Vohra and Kratzer, 1968; Kincaid et al., 1976). In the earlier studies at least 1500–4000 ppm zinc were the minimal levels that produced adverse physiological changes. The young quail is usually sensitive to nutrient imbalances due to its rapid growth rate; however, it is thought that the major contributory factor to sensitivity in this study was the use of a purified diet in which minerals were supplied at required or slightly higher levels, except for copper in some groups. In the previous avian studies, diets of natural ingredients were used; they may have contained components other than minerals that protected against zinc toxicity.

These data show that adequacy of copper intake is important when supplemental zinc is consumed either as a dietary supplement or in foods fortified with zinc. The results may be important for the general human population, whose dietary intake of many minerals, including copper, does not usually exceed the requirement or may be marginally deficient (Milne et al., 1978; Harland et al., 1978; Klevay, 1978).

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EFFECT OF GLUCOSE AND FRUCTOSE ON INCUBATION GLYCOGENIC CAPACITY IN TISSUE FROM MICE PREVIOUSLY CONSUMING VARIOUS CARBOHYDRATES

PAUL E. ARAUJO

ABSTRACT

The effect of various carbohydrates upon liver glycogen production was investigated. Fructose enhanced in vitro glycogen production when having been given previously as a pretreatment in the drinking water of the mice. This enhancement was not increased when fructose was the in vitro substrate for liver slices. Response to a sucrose solution varied but generally was similar to that for fructose. Dietary studies were also carried out to eliminate uncertainty caused by the carbohydrate content of the laboratory chow fed while the mice were drinking the sweetened solutions. In these studies, the livers from mice consuming the sucrose-based diet did not differ greatly from those of mice consuming the starch-based diet. Addition of cellulose to these diets heightened the glycogen levels found in vitro, however.

INTRODUCTION

THERE HAVE BEEN many reports of various effects of sucrose on lipogenesis (Bender and Damji, 1972). Fewer investigators have been concerned with the effects that this sugar might have on glycogenesis. Dietary data indicate that water is being replaced by carbonated beverages in the dietary patterns of many Americans (Anon., 1976). Moreover, the possible loss of saccharine as a sweetener has motivated some manufacturers of diet drinks to seek alternative formulations utilizing fructose as the sweetening agent.

This experiment was undertaken to compare effects of imbibing sucrose and fructose as compared both to tap water and glucose. It was hypothesized that the reported lipogenic effects of both sucrose and fructose would be reflected by a metabolic adaptation to these carbohydrates. A concomitant decrease in glycogenic capability in the tissues of organisms exposed to these sugars was also expected since fructose does not require insulin to enter the cell.

MATERIALS & METHODS

MICE of the C57Bl/Fn strain were placed in individual hanging wire-bottomed cages and exposed to the various pretreatments for 3 wk as described below. The rooms were air conditioned with 12 hr photoperiod. The first set of pretreatments consisted of dividing the mice into four equal groups. One group was allowed to consume tap water but the other three groups had glucose, fructose or sucrose (10g/100 ml) added to their drinking water. All solutions were consumed ad libitum for 5 wk.

As a test of the role of sucrose in the diet, this carbohydrate was compared with starch as a glycogenic substrate. Since some reports lay the metabolic changes associated with increased dietary sucrose not with the nature of the available carbohydrate but with the lack of dietary fiber in a purified diet, cellulose was added to each diet. Thus, one-fourth of the animals consumed a diet with 77% sucrose without fiber and another fourth of the mice consumed such a diet to which 10% cellulose had been added. Similarly, a control starch diet was fed with one group of mice receiving the diet without and another group with the added cellulose. Details of the diets are presented in a previous publication (Araujo, 1978).

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Table 1—Tissue glycogen accumulation after pretreatment with sweetened drinking solutions in vivo

Tissue/Pretreatment	Glycogen μg/mg protein[a] Substrate: Glucose	Substrate: Fructose
Liver		
Tap water	19.7 ± 6.3a	24.9 + 5.3ab
Glucose solution	33.6 ± 10.3b	18.1 ± 4.4a
Fructose solution	53.3 ± 14.2c	50.8 ± 14.0c
Sucrose solution	9.3 ± 2.5d	49.4 ± 9.7bc
Kidney		
Tap water	71.0 ± 25.0ab	80.7 ± 12.7be
Glucose solution	11.1 ± 3.0c	34.1 ± 8.0d
Fructose solution	84.5 ± 20.6be	105.3 ± 14.0e
Sucrose solution	13.0 ± 4.8c	46.7 ± 10.1ad

[a] Means ± SEM, means followed by the same letter are not statistically significantly different ($p \leq 0.05$) for comparisons between treatments among data from each tissue. Eight mice per treatment.

All tests described below were performed upon the mice in the morning after the 18 hr fast. During the fourth week, the blood glucose was analyzed by means of a glucose oxidase procedure on serum samples obtained from the suborbital sinus of unanesthetized mice at 0, 1/2, 1, 2 and 4 hr after gastric loading of a glucose solution (1.0 mg/g body wt). The following week, liver, kidney, epididymal fat pad and diaphraghm were taken from these mice which had been killed by cervical dislocation. Liver and kidney were sliced by means of a Stadie-Riggs device. Diaphragm was dissected from the rib cage and cut in half, fat pads were separated from the testes and epididymus and minced. Standard manometric procedures were used (Umbreit et al., 1957). Glycogen was assayed by the anthrone procedure after digestion of the tissue in KOH and precipitation overnight in methanol. Protein was assayed by the Biuret method with bovine albumin as a standard. Data were statistically analyzed using nonparametric methods.

RESULTS

IN VITRO GLYCOGENIC levels found after the incubation are presented in Table 1. In both liver and kidney, pretreatment with a fructose drinking solution increased the glycogen level at the end of the incubation period. This higher glycogen level was found whether the in vitro substrate was glucose or fructose. A similar glycogen response was found in the liver of sucrose drinking mice whose tissues were incubated in a fructose containing medium. The Qo_2 data presented in Table 2 show that kidney was respiring at a greater rate than liver but neither tissue showed clear differences between pretreatments and substrates. The lowest rates of respiration were found in two sets of glucose metabolizing liver slices, one from animals which had been drinking tap water and the other set from animals drinking the sucrose solution.

Figure 1 contains the curves which express the glucose tolerance of the mice on the various diets. It can be observed that the 18-hr fasting blood glucose levels did not vary greatly among the groups. In general, the points for the mice consuming the sucrose-cellulose range higher than the other groups.

The in vitro data from the various tissues of the mice on

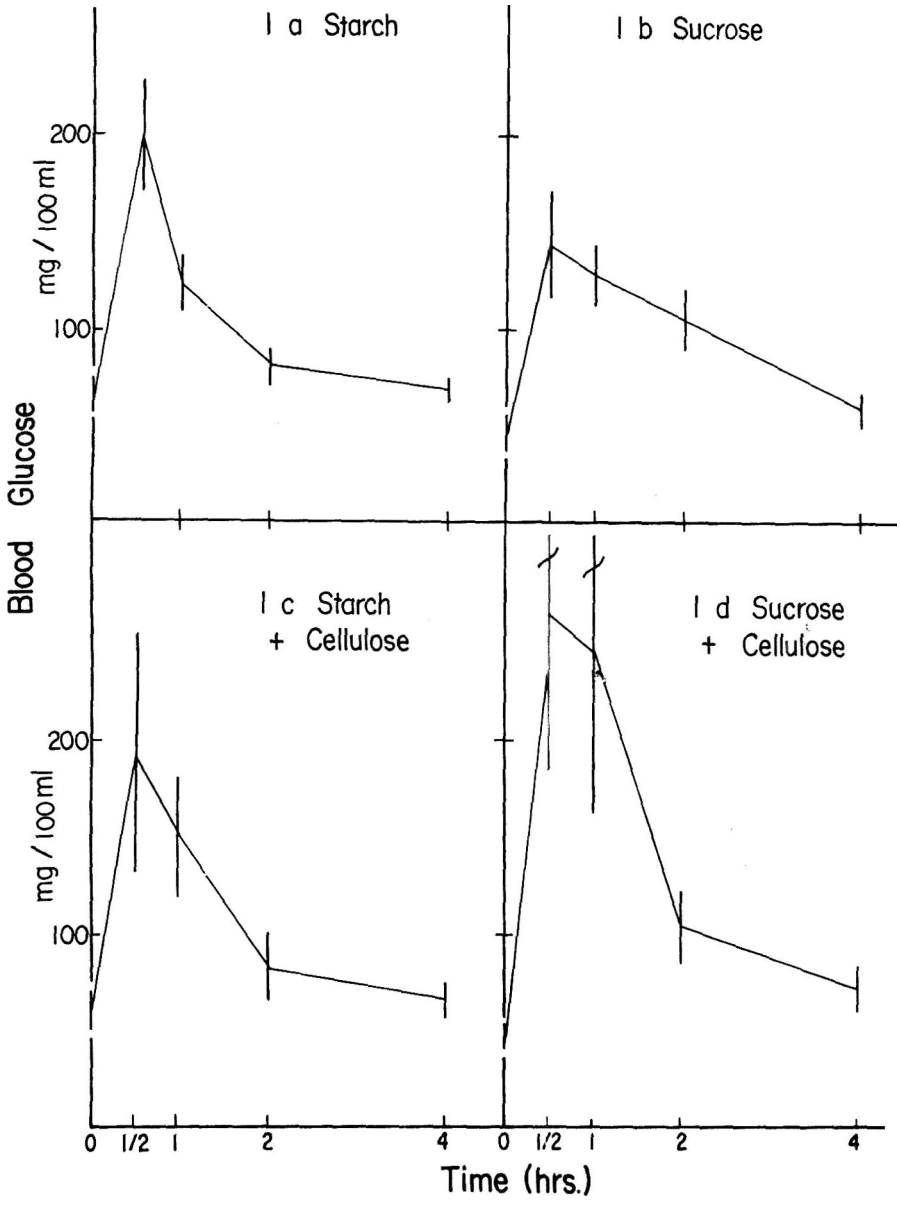

Fig. 1—Glucose tolerance test curves from fasting mice with dietary pretreatments. Bars indicate standard error of the mean at each data point. Eight mice per diet.

these diets are presented in Table 3. Here it is clear that the higher glycogen levels are found in tissues from mice consuming the starch-cellulose diet. The greater amount of glycogen is found in liver, kidney, fat pad and diaphragm from mice on these treatments whether the incubating media contained glucose or fructose. The sucrose-cellulose based diet showed variability with higher levels only in a few trials. Again kidney slices respired at the greatest rate and no obvious patterns were revealed upon statistical analysis (Table 4).

DISCUSSION

THE METABOLIC EFFECTS of sucrose have been reported at length (Bender and Damji, 1972). While some investigators feel that it is sucrose itself that exerts these effects (Michaelis and Szepesi, 1974) others report that it is the metabolism of the fructose portion of that disaccharide that is causing the effects (Pereira and Jangaard, 1971). In this series of experiments, evidence is presented that it is the fructose that exerts the primary stimulus to a metabolic adaptation.

The data may be interpreted with an unified hypothesis which depends upon the relative rates of glycogenesis and

Table 2—Tissue respirating rates after pretreatment with sweetened drinking solutions in vivo

	Qo_2 (μl O_2/mg protein/hr)[a]	
	Substrate	
Tissue/Pretreatment	Glucose	Fructose
Liver		
Tap water	4.6 ± 0.9a	8.5 ± 0.7bc
Glucose	10.8 ± 1.9bc	8.0 ± 0.8b
Fructose	9.6 ± 2.0bc	13.8 ± 2.1c
Sucrose	5.1 ± 0.7a	11.1 ± 1.2c
Kidney		
Tap water	37.0 ± 3.6ac	49.2 ± 6.1df
Glucose	34.6 ± 10.5abc	31.6 ± 3.6bce
Fructose	39.4 ± 11.7acd	47.2 ± 5.9df
Sucrose	29.2 ± 5.1bce	56.0 ± 6.6f

[a] Means ± SEM, means followed by the same letter are not statistically significantly different ($p \leq 0.05$) for comparisons made between treatments among data from each tissue. Eight mice per treatment.

Table 3—Tissue glycogen response after dietary pretreatments

	Glycogen μg/mg protein[a]	
	Substrate	
	Glucose	Fructose
Liver		
Starch	10.4 ± 1.6ab	7.3 ± 1.4a
Sucrose	9.3 ± 1.4ab	9.2 ± 1.1ab
Starch & cellulose	27.9 ± 6.8cd	36.1 ± 9.6cd
Sucrose & cellulose	15.6 ± 5.2bc	9.6 ± 3.1ab
Kidney		
Starch	16.3 ± 3.4ab	14.4 ± 3.2ab
Sucrose	14.2 ± 2.4ab	14.6 ± 3.9ab
Starch & cellulose	39.0 ± 14.3ab	47.2 ± 16.3a
Sucrose & cellulose	41.7 ± 25.7ab	10.3 ± 1.8b
Fat pad		
Starch	8.9 ± 1.8a	5.4 ± 1.2a
Sucrose	16.0 ± 5.2ab	24.1 ± 13.4bc
Starch & cellulose	56.7 ± 29.5c	55.9 ± 32.1bc
Sucrose & cellulose	10.1 ± 2.2a	14.0 ± 2.3ab
Diaphragm		
Starch	8.2 ± 1.6abc	11.6 ± 4.2abc
Sucrose	9.2 ± 1.8abc	6.4 ± 2.3a
Starch & cellulose	21.1 ± 0.2bc	35.8 ± 25.0c
Sucrose & cellulose	6.4 ± 1.7a	8.0 ± 2.1ab

[a] Mean ± SEM, means followed by the same letter are not statistically significantly different ($p \leq 0.05$) for comparisons made between treatments among data from each tissue. Eight mice per treatment.

Table 4—Tissue respiratory rate after dietary pretreatment

	Qo_2 (μl O_2/mg protein/hr)[a]	
	Substrate	
Tissue/Pretreatment	Glucose	Fructose
Liver		
Starch	13.8 ± 4.6	13.2 ± 2.8a
Sucrose	20.8 ± 5.0a	21.8 ± 6.6a
Starch & cellulose	10.0 ± 2.2a	14.2 ± 3.6a
Sucrose & cellulose	22.2 ± 7.5a	12.5 ± 2.4a
Kidney		
Starch	27.0 ± 4.9ab	40.3 ± 12.1b
Sucrose	37.2 ± 6.6ab	35.8 ± 7.4ab
Starch & cellulose	22.9 ± 4.4a	27.4 ± 1.8ab
Sucrose & cellulose	34.1 ± 6.8ab	36.6 ± 7.7ab
Fat pad		
Starch	26.8 ± 6.2ac	29.2 ± 8.8a
Sucrose	14.3 ± 4.1bc	24.0 ± 11.5ac
Starch & cellulose	21.0 ± 7.1abc	21.7 ± 5.9abc
Sucrose & cellulose	8.3 ± 1.8b	11.1 ± 3.5bc
Diaphragm		
Starch	9.7 ± 3.0a	9.8 ± 3.4ab
Sucrose	26.5 ± 3.7ab	31.5 ± 12.1b
Starch & cellulose	15.2 ± 2.2ab	22.8 ± 3.4ab
Sucrose & cellulose	22.3 ± 2.9ab	17.7 ± 3.9ab

[a] Mean ± SEM, means followed by the same letter are not statistically significantly different ($p \leq 0.05$) for comparisons made between treatments among data from each tissue. Eight mice per treatment.

lipogenesis. The dietary studies indicate that a starch based purified diet with added cellulose has the greatest glycogenic capacity. This capacity is unaffected by a glucose or fructose substrate in vitro at least. Thus, it may be that the drinking of a fructose beverage by the mice consuming a laboratory chow (Purina Laboratory Chow®) diet mimicks the starch fed-fructose media incubations. This combination would be similar since the starch diet's composition is quite similar to that of the laboratory chow.

The mechanism by which the cellular metabolic apparatus is capable of converting fructose to glycogen is not difficult to postulate. Fructose enters the cell and is phosphorylated. Thereupon, as Pereira and Jangaard (1971) have indicated there proceeds a more rapid metabolism of that carbohydrate than of glucose. The pathways open for fructose metabolism have been shown to be dependent on the metabolic state of the organism (Sillero et al., 1969). The liver can convert fructose to glucose by the formation of glyceraldehyde-3-phosphate (G-3-P) and dehydroxyacetone phosphate. The former compound can be condensed to give fructose 1,6-diphosphate. The fructose diphosphate can then be metabolized to glucose. The formation of G-3-P is favored at physiological fructose concentrations (Sillero et al., 1969). It is hypothesized that glycogen formation by this mechanism could occur under the conditions of these experiments.

This series of reactions would be much enhanced in the situation with fructose as substrate and a starch based diet as overriding carbohydrate source. It might be supposed that a sucrose based diet should further enhance the metabolic effects observed since the glucose: fructose ratio is higher. Indeed, drinking the sucrose solution produces the highest predisposition as measured in vitro. Perhaps this effect is not observed with dietary pretreatment because under these conditions the lipogenic effects of fructose predominate, and there are less carbon skeletons available for entry into gluconeogenesis and carbohydrate storage. This biasing of the metabolic apparatus for various substrates may have far reaching consequences. Yudkin (1963) has proposed that such chronic disease states as diabetes and ischemic heart disease result from a maladoption to sucrose. Response of the kidney to this carbohydrate may be particularly important since hypertension has also been reported in populations consuming large amounts of sucrose. Thus rapid change in the nature of dietary sweetening agents should be accompanied by careful study of the metabolic response of the organism to the various carbohydrates.

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INFLUENCE OF DIETARY FIBER ON THE GROWTH AND PROTEIN METABOLISM OF THE RAT

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ABSTRACT

Growth study was made on 96 male rats with treatments (15g diet, 03.g N/day): wheat bran (B) 5%, 2.1% neutral (NDF) and acid (ADF) detergent fibers, pectin (P), cellulose (C) and no-fiber (No-F) at two levels of energy (57 and 67 Kcal/day) for 4 wk and extended period, II, until 8 wk. In Period II, B and NDF diet resulted in significantly higher ($P < 0.05$) weight gain and protein efficiency ratio than No-F, in spite of increased fecal N excretion. Urinary N excretion was the most for No-F and the biological value the least ($P < 0.05$). Data suggest, at marginal intake of protein and energy closer to the requirement, dietary fiber has a role in the rat's growth and improved utilization of absorbed N.

INTRODUCTION

IN RECENT YEARS, much interest has been focused on the possible physiological and beneficial effects of dietary fiber on humans (Kelsay, 1978). Earlier studies (Osborne and Mendel, 1919) indicated little nutritive value of wheat bran which is an excellent source of dietary fiber. McLaren and his group (McLaren et al., 1973, 1974; Fahey et al., 1976) indicated growth promoting influence on ruminants and nonruminants by acid resistant hemicellulose fractions of dietary fiber. The current interest in dietary fiber also supports the hypothesis that indigestible fiber may be nutritively beneficial because of microflora influence on food residue and endogeneous nitrogeneous compounds (Salter, 1973). Present world supply of protein and energy is limited, especially in underdeveloped countries. Therefore, the objective of the experiment was to study the role of dietary fiber on growth and nitrogen metabolism at marginal intake of protein and energy using a rat model.

EXPERIMENTAL

Dietary fibers

Feed grade wheat bran from Southern States Cooperative was obtained; pectin (polygalacturonic acid methyl ester) was purchased from Sigma Chemical Company, and cellulose (alphacelhydrolyzed) from ICN, Incorporated.

Neutral and acid detergent fibers (NDF and ADF) from wheat bran were prepared by the method described by Goering and Van-Soest (1970), but modified for larger quantities. The quantity of detergent solutions was increased from 100 to 2,000 ml to accommodate 100g of the sample. After 1 hr of digestion the residue was filtered through four folds of cheesecloth and dialyzed against water for 48 hr, then rinsed with acetone and air dried. Percent yields of NDF and ADF fractions from bran were 42.0 and 13%, respectively.

Preparation of diets

The experimental diets were semipurified in nature and isonitrogenous (2.044% N). The constituents of the diets are given in Table 1. Bran contained 42% neutral detergent fiber, therefore to make the diets isofibrous the fractions were kept at 2.1% level. The USP salt mixture XIV (General Biochemical) was supplemented with 2 mg of Cu/kg diet as $CuSO_4$ and 4 mg of Zn/kg diet as $ZnCO_3$. The vitamin mixture was prepared as described by Rao et al. (1959). Fish oil contained not less than 2500 IU vitamin A and 250 IU vitamin D per gram. The energy level was obtained by interchange of coconut oil and cornstarch. Energy content of the diet was determined by calculation, and nitrogen by Kjeldahl method (AOAC, 1970).

Growth and nitrogen metabolism trials

Four feeding trials with the six treatments were conducted using 24 growing male albino rats of the Wister strain (Hilltop Laboratory Animal, Incorporated) in each. In trial 1 the feeding period was 4 wk. In trial 2, the period was extended to 7 wk. In trial 3 and 4, after 4 wk on 6% fat diet, the energy level was changed by elevating the fat content to 18%. The period after 4 wk was designated as period II, and the 18% fat diet as energy level II.

The rats were weighed, placed in individual stainless steel cages with raised wire floors in a constant temperature room (24.5°C) and under a controlled lighting regime (14 hr light and 10 hr dark). The initial weight of the rats averaged 60g. They were randomly assigned to the experimental diets. Each rat was fed 15g of feed (0.3g N) per day throughout the trial and tap water was provided ad libitum. Food consumption was recorded. The initial weight of the rats was recorded and then they were weighed weekly. Weight gain per day during each trial was obtained and Protein Efficiency Ratio (PER = weight gain/protein intake) calculated, for period I and II.

During the fourth week of each period in trial 3 and 4, daily total feces and urine were collected from each rat for 7 days and composited. From fecal samples of each rat all recognizable hairs were removed. The samples were dried at 60°C for 3 days and dry matter estimated. Dried fecal sample was then ground into powder with a mortar and pestle.

To prevent ammonia loss and bacterial growth in urine, sulfuric acid (2.5 ml, 8N) was added to the collection jar at the start of each day. Urine was refrigerated until nitrogen analysis was performed. Before analysis, urine was filtered and diluted with distilled water to 250 ml then 1 ml of diluted urine was analyzed in duplicate for total nitrogen by the micro-Kjeldahl method (AOAC, 1970).

Statistical analysis

Analysis of variance was performed on the data collected. Mean values of the six treatments at the two levels of energy intake were tested for significance by least significant difference method at a probability of 0.05 (Steel and Torrie, 1960).

RESULTS & DISCUSSION

Weight gain

In period I and corresponding energy Level I (57 Kcal/-day/animal) mean weight gain and Protein Efficiency Ratio (PER) (Table 2) were not significantly different. However, higher values for fiber diets were observed. Significant differences were not noticed, probably because during the earlier period of growth dietary protein level was enough or close to the requirement (NRC, 1972), Table 3.

Table 4 shows weight gain and PER at the two levels of energy intake within the same period, II. Dietary fiber, especially B and its fiber fractions, NDF and ADF, and P improved ($P < 0.05$) weight gain and PER at higher energy level over lower energy intake. Energy level II was closer to the NRC requirement (67 Kcal vs 72) than the energy level I (57 vs 72), Table 3. The differences among treatments within level of energy were not significant, probably due to

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Table 1—Composition of experimental diets

Constituents	Wheat bran (B)	Neutral detergent fiber (NDF)	Acid detergent fiber (ADF)	Pectin (P)	Cellulose (C)	No fiber (No-F)
Casein	14.4	15.0	15.1	15.2	15.2	15.2
DL Methionine	0.3	0.3	0.3	0.3	0.3	0.3
USP Salt Mixture XIV[a]	4.0	4.0	4.0	4.0	4.0	4.0
Vitamin Mixture[b]	5.0	5.0	5.0	5.0	5.0	5.0
Coconut Oil[c]	6.0/18	6.0/18	6.0/18	6.0/18	6.0/18	6.0/18
Fish Oil[d]	1.2	1.2	1.2	1.2	1.2	1.2
Cornstarch[c]	64.1/52.1	66.4/54.4	66.3/54.3	66.2/54.2	66.3/54.2	68.3/56.3
Bran	5.0	–	–	–	–	–
NDF	–	2.1	–	–	–	–
ADF	–	–	2.1	–	–	–
Pectin	–	–	–	2.1	–	–
Cellulose	–	–	–	–	2.1	–
TOTALS	100	100	100	100	100	100

[a] Supplemented with 0.2 mg Cu + 0.4 mg Zn/100g diet
[b] Rao et al. (1959)
[c] Energy level adjusted by starch and coconut oil variation
[d] Fish oil 1 + Cholesterol 0.2, not less than 2500 IU vitamin A and 250 IU vitamin D/g fish oil

not having enough population in each group, four rats in each. However, in energy level II, there was a tendency among fiber groups for higher weight gain and PER than No-F. With eight rats in each group (Table 6), B, NDF, ADF and P resulted in significantly ($P < 0.05$) higher weight gain than No-F. PER was significantly improved in all of the fiber diets.

Nitrogen metabolism

Fecal N excretion was consistently higher in B and NDF in period I and II (Tables 5 and 6) than No-F (0.026g, 0.025 and 0.018; 0.038, 0.034 and 0.02). The percent digestibility of N was also significantly ($P < 0.05$) lower than No-F (90.9, 91.7 and 93.9; 88.5, 89.5 and 93.5). The observations are in agreement with the report by Spiller and Amen (1976). They noted higher fecal N excretion in monkeys with nonpurified fiber diet than no-fiber. The fiber part in bran and NDF contain cell-wall constituents. Higher fecal N in B and NDF may be due to cell-all-bound N and lignified N (Van Soest, 1966).

In period II, when the protein was more limiting than period I, urinary N excretion was the highest ($P < 0.05$) with No-F (36.80, 35.99, 37.99, 41.66, 35.95 and 43.65%). N balance for ADF and C was significantly ($P < 0.05$) higher than No-F. Biological value of protein in the experimental diet was calculated by means of the Thomas-Mitchell equation (Mitchell, 1928) and values used to estimate the metabolic fecal and endogenous urinary N given by McLaren et al. (1974). In period I, the biological values (Table 7) were not significantly different. In period II, when the rats were older and received higher energy, biological values in B (69.6), NDF (71.3), ADF (70.7) and C (74.4) were improved ($P < 0.05$) over No-F (63.9).

The mode of action of dietary fiber in the digestive tract depends upon its chemical nature. Neutral detergent fiber mainly consists of cellulose, hemicellulose and lignin (Van Soest, 1966). Acid detergent fiber is ligno-cellulose in nature. Isolated cellulose is the least complex form of fiber. Bran contains neutral detergent fiber in natural form. Pectin is a soluble polysaccharide resistant to animal enzymes, but relatively more fermentable through bacteria in the

Table 2—Body weights (g) and protein efficiency ratio (PER) of rats fed experimental diets in period I (1–4 wk) and energy level I[a]

Diet	Initial wt	Wt on day 28	Wt gain/day	PER
Bran	60.24 ± 3.21	213.7 ± 9.37	5.48 ± 0.31	3.05 ± 0.18
NDF	59.64 ± 3.49	212.8 ± 10.05	5.47 ± 0.37	2.95 ± 0.18
ADF	60.59 ± 3.03	211.3 ± 9.06	5.38 ± 0.29	2.93 ± 0.18
Pectin	59.8 ± 3.72	212.0 ± 10.13	5.43 ± 0.31	2.95 ± 0.13
Cellulose	58.54 ± 4.78	206.7 ± 14.21	5.29 ± 0.44	2.95 ± 0.25
No-F	59.75 ± 3.69	209.6 ± 8.99	5.35 ± 0.31	2.91 ± 0.18

[a] Trial 1, 2, 3 and 4 combined, each value mean of 16 rats with standard deviation; total number of rats (N) = 96.

Table 3—Energy and protein intake and requirement (NRC) for male rats

Period	Experimental	NRC
Period I (1–4 wk)		
Body wt (g)	60–211	55–220
M E energy Kcal/day	57	54–76 Avg 65
Protein (net) g/day	1·3	1.1–2.5 Avg 1.8
Period II (5–7/8 wk)		
Body wt (g)	211–322	220–385
M E energy Kcal/day	57/67	76–72 Avg 74
Protein (net) g/day	1.3	2.5–2.4 Avg 2.45

Table 4—Weight gain and PER, period II (5–7 wk) at two levels of energy (N=48)

Diet	Weight Gain (g/day) at Energy level I	Weight Gain (g/day) at Energy level II	PER at Energy level I	PER at Energy level II
Bran	2.88	4.24*	1.65	2.07*
NDF	2.79	4.05*	1.53	2.04*
ADF	2.92	4.09*	1.64	2.12*
Pectin	2.76	4.10*	1.53	2.02*
Cellulose	2.93	3.82	1.63	1.91
No-F	3.07	3.78	1.70	1.85

* Significantly different ($P < 0.05$) from energy level I

Table 5—Weight gain and nitrogen metabolism data of experimental diet, period I (energy level I), trial 3 and 4 (N=48)

	Bran	NDF	ADF	Pectin	Cellulose	No-F
N intake (g)	0.287	0.297	0.289	0.288	0.287	0.289
Fecal N (g)	0.026*	0.025*	0.022*	0.024*	0.017	0.018
Urine N (g)	0.080	0.085	0.090	0.097	0.106	0.096
N balance (g)	0.185	0.187	0.182	0.182	0.165	0.181
Digested (%)	90.90*	91.68*	92.38	91.59*	94.26	93.95
Urinary N (%)	27.64	28.56	30.52	32.89	36.95	32.71
Initial wt (g)	60.28	59.14	60.24	59.83	60.04	59.53
Final wt (g)	212.99	205.59	213.37	212.46	208.01	209.06
Wt gain (g)	5.45	5.39	5.47	5.45	5.28	5.32
PER	3.03*	2.91	2.98*	2.96	2.95	2.89

* Significant difference $P < 0.05$ from No-F

Table 6—Weight gain and nitrogen metabolism data of experimental diet, period II (energy level II), trial 3 and 4 (N=48)

	Bran	NDF	ADF	Pectin	Cellulose	No-F
N intake (g)	0.325	0.332	0.321	0.316	0.316	0.319
Fecal N (g)	0.038*	0.034*	0.025	0.025	0.021	0.020
Urine N (g)	0.120*	0.118*	0.122*	0.132	0.114*	0.139
N balance (g)	0.168	0.172	0.174*	0.159	0.186*	0.154
Digested (%)	88.46*	89.76*	92.16	92.22	93.41	93.63
Urinary N (%)	36.80*	35.99*	37.99*	41.66	35.95*	43.65
Initial wt (g)	212.99	205.59	213.37	212.46	208.01	209.06
Final wt (g)	329.58*	323.49	324.79	325.48	317.79	313.54
Wt gain (g)	4.20*	4.05*	3.98*	4.18*	3.92	3.73
PER	2.07*	1.96*	1.99*	2.11*	1.98*	1.86

* Significant difference $P < 0.05$ from No-F

large intestine than NDF, ADF or cellulose. Further, pectin may coat the absorptive lining of the gut altering the absorption of nutrients (Van Soest and Robertson, 1977).

In the present study low biological value of P and No-F corresponded with increased urinary N excretion. Weight gain or PER of P was not consistent with the biological value. As pectin has the property of coating the absorptive lining of the alimentary tract, it may have altered the absorption of nutrients and water. Among the fiber diets P contributed the highest digestible dry matter (^{e}93.6 B, ^{d}94.26 NDF, ^{c}94.86 ADF, ^{b}95.84 P, ^{c}94.73 C and ^{a}96.53 No-F, means with different prefix are significantly different, $P < 0.05$). The liver triglyceride was also the highest in P fed rats (Davis, 1977), indicating higher body fat. Further studies are suggested for better understanding of the action of pectin in the digestive system and metabolism of the mongastric.

The results of the nonsoluble fibers can be better understood than P. B, NDF, ADF and C contributed more bulk than P or No-F and may have resulted in more endogenous protein. Meyer (1956) noted higher metabolic fecal N in rats with increased dietary fiber, cellulose, intake. Nasset (1965) reported on the significance of endogenous protein-cell debris, enzyme secretions, etc, in the maximal efficiency of absorption and utilization of amino acid. Southgate (1973) suggested dietary fiber may contribute the rapid turnover of mucosal cells in the gastrointestinal tract. Part of the dietary fiber may also serve as a source of energy after microbial fermentation in the large intestine (Van Soest and Robertson, 1977). The growth promoting factor (McLaren et al., 1976) in hemicellulatic fraction of dietary fiber increased ($P < 0.05$) rumen microbial protein synthesis, in vitro. The inclusion of hemicellulose degradation products from feed grade bran and cereal all bran at the level of 0.05% to a 14.4% casein diet significantly improved ($P < 0.05$) the growth rate of male weanling rats (Williams et al., 1977). In view of the above reports and the results of the present experiment, the influence of insoluble fiber, NDF, on protein metabolism may be related to the endogenous protein and growth promoting factor. As ADF and cellulose do not contain hemicellulose, their main influence may be on endogenous protein. In summary the present study indicates the role of dietary fiber in the improved utilization of absorbed nitrogen and growth in the rat, at marginal level of dietary protein and energy intake closer to the requirement.

Table 7—Biological value of protein in experimental diets

	Bran	NDF	ADF	Pectin	Cellulose	No-F
Period I (Energy I)	80.1	78.0	77.7	78.2	70.2	75.4
Period II (Energy II)	69.3*	71.3*	70.7*	66.4	74.4*	63.9

* Significant different ($P < 0.05$) from No-F

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—Continued on page 751

RELATIVE SWEETNESS OF FRUCTOSE AND SUCROSE IN MODEL SOLUTIONS, LEMON BEVERAGES AND WHITE CAKE

ARMAND V. CARDELLO, DEBORAH HUNT and BRUCE MANN

ABSTRACT

The relative sweetness of fructose and sucrose was evaluated at various concentrations in distilled water, in citric acid solutions (pH = 2.35 and 3.0), in lemon flavored beverages (pH = 2.35 and 2.7), and in vanilla cake. In distilled water and in the less acidic beverage and citric acid solutions, magnitude estimates of sweetness revealed fructose to be sweeter than sucrose by a factor of 1.6–1.9 at lower sugar concentrations. However, the greater relative sweetness of fructose declined with increasing sugar concentration. In the more acidic beverage and citric acid solutions, no difference in perceived sweetness between fructose and sucrose was found at any sugar concentration. Similarly, no difference in the perceived sweetness of the two sugars was found when each was used as a sweetener in vanilla cake. These data suggest that the greatest sweetening advantage of fructose over sucrose occurs at the lower concentrations of the two sugars. However, when used in certain beverages or foods, concomitant tastes may mask or mitigate this differential sweetness.

INTRODUCTION

IT HAS BEEN REPORTED by numerous investigators that D-fructose is 1.1–2.0 times as sweet as sucrose [Sale and Skinner, 1922; Biester et al., 1925; Willaman et al., 1925; Dahlberg and Penczek, 1941; Cameron, 1943; Nieman, 1958 (cited in Amerine et al., 1965) Shallenberger, 1963; Stone and Oliver, 1969; Moskowitz, 1971a]. The variability in these reported relative sweetness values may be due to a number of subject and experimental variables, including differences in the sensory methods used to evaluate sweetness in these studies and the fact that relative sweetness has been shown to vary as a function of sugar concentration (Dahlberg and Penczek, 1941; Cameron, 1943; Tsuzuki and Yamazaki, 1953; Hyvonen et al., 1977).

Recently, Harris et al. (1978) reported on the relative sweetness of sucrose, D-fructose, and 90% fructose corn syrup when used as sweeteners in an orange-flavored dry beverage base. Using a ranking procedure they found that the sucrose sweetened beverage was sweeter than either of the two fructose sweetened beverages. These data are similar to those reported by Pangborn (1963) who found sucrose to be sweeter than fructose when tested in pear nectar. Since these data contradict most other published results on the relative sweetness of these sugars, further analysis of the problem is needed.

The most obvious factor distinguishing the studies of Harris et al. (1978) and Pangborn (1963) from other studies examining the relative sweetness of sugars is that they were conducted using beverage media that possessed concurrent flavors, as compared to previous studies which have used primarily distilled water solutions. In fact, Pangborn (1963), in the same study reported above, found fructose to be sweeter than sucrose when mixed in distilled water rather than pear nectar. Secondly, the beverages used by Harris et al. (1978) and Pangborn (1963) were acidic, and acids have been shown to have a wide variety of effects on subjective sweetness (Fabian and Blum, 1943; Anderson, 1950; Sjostrom and Cairncross, 1955; Pangborn, 1960, 1961, 1965; Kamen et al., 1961; Stone et al., 1969; Moskowitz, 1971a, b, 1972). Yet, as representative examples of applied uses of sugar sweeteners, these studies have important implications for the generalization of model solution data to real food systems, and for the economics of sweetening agents.

The tests reported are an attempt to replicate the findings of Harris et al. (1978), to compare these findings to relative sweetness in distilled water solutions, in another dry beverage base, and in a solid food system, and to assess the contribution of the acidity of the solution to these findings.

EXPERIMENTAL

THREE sensory tests were conducted.

Test 1

The first test compared the relative sweetness of fructose and sucrose at six different sugar concentrations (1.4, 2.1, 3.5, 5.7, 9.1 and 14.3% wt/wt) when mixed with distilled water, a lemon-flavored beverage or a lemonade flavored kool-aid drink. Sugar concentrations were chosen to encompass a wide range of concentrations used previously in the literature, as well as to include real product use levels. Beverages were chosen to both replicate Harris et al. (1978) and to provide a difference in pH for comparison purposes. For purposes of replication the crystalline fructose used in these tests was obtained from N. Harris of the U.S. Army Natick R & D Command and was the same as used by Harris et al. (1978). The crystalline sucrose in these tests was reagent grade. The three test media were prepared by reconstituting the dry beverage bases as per label instructions. The appropriate weights of fructose and sucrose were then added to each medium. The 36 solutions (2 sugars × 6 concentrations × 3 media) were stored at 4°C for 24 hr before sensory testing. The degree of hydrolysis in the sucrose solutions was assessed by liquid chromatography after 24 hr and no hydrolysis was observed. The pH of each solution was tested immediately after use. The pH of the distilled water solutions was 6.30 ± 0.10, that of the lemon-flavored beverage solutions was 2.35 ± 0.05, and that of the kool-aid beverage was 2.70 ± 0.05.

Test subjects were 14 employees of the U.S. Army Natick R & D Command, all of whom were well experienced in sensory testing and all of whom had used the psychophysical method of magnitude estimation previously. Testing was conducted in sensory testing booths. Since the beverages possessed a slight yellow color, a small amount of yellow food coloring was added to the distilled water solutions to render them subjectively equivalent in color to the beverage solutions. To further mitigate any possible color effects, red flourescent lighting was used in the testing booths.

Testing was conducted during a single afternoon session. Solutions were allowed to warm to room temperature (25°C) immediately prior to testing. Ten milliliter samples of solution were presented to subjects in small plastic cups, and all solutions were presented in random order. Subjects were instructed to assess the intensity of perceived sweetness in each of the solutions using the method of magnitude estimation. No modulus was used. Subjects judged each sample only once and were instructed to judge only the sweetness of the solutions and to ignore other tastes. After tasting each solution, subjects rinsed with distilled water. A 1-min interstimulus interval was maintained.

Test 2

The same concentrations of sucrose and fructose were used as in Test 1, except that the lowest sugar concentration (1.4% wt/wt) was eliminated because a small number of subjects in Test 1 could not

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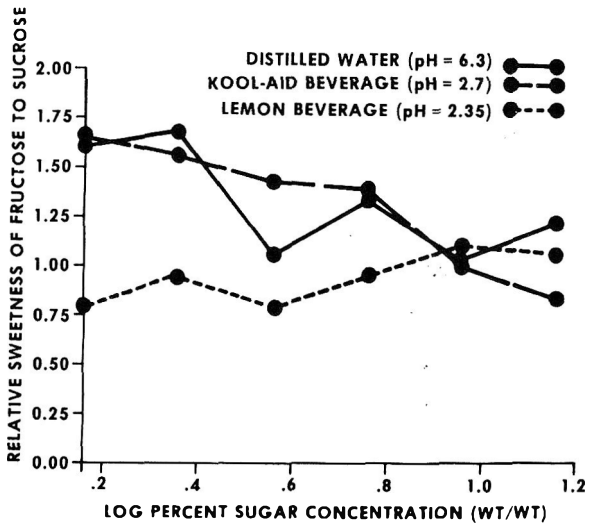

Fig. 1—Relative sweetness of fructose to sucrose as a function of sugar concentration for the media of Test 1.

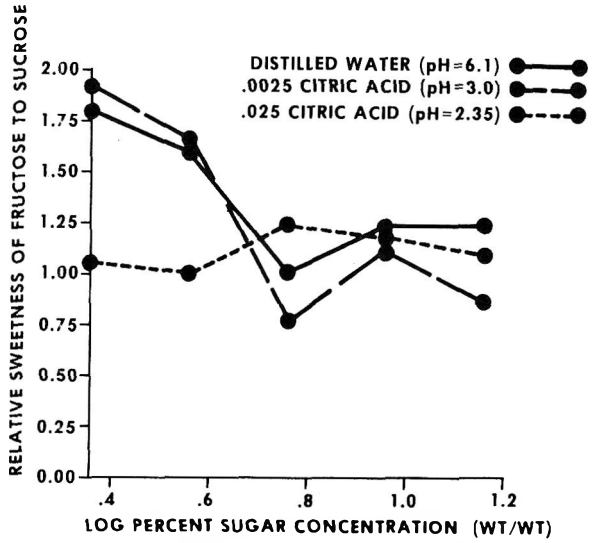

Fig. 2—Relative sweetness of fructose to sucrose as a function of sugar concentration for the media of Test 2.

perceive any sweetness at this concentration. Test media consisted of distilled water and two citric acid solutions (0.0025M and 0.025M). These media were used to assess the effect of pH on the relative sweetness of fructose and sucrose without confounding by minor ingredient differences that were present in the beverage bases used in Test 1. The same procedures were used to prepare, store and present solutions as were used in the first test, although food color was not added, since all test solutions were colorless and did not differ in appearance. Hydrolysis of sucrose was again assessed and no hydrolysis was observed. The pH of the distilled water solutions was 6.10 ± 0.10, that of the 0.0025M citric acid solutions was 3.00 ± 0.05, and that of the 0.025M citric acid solution was 2.35 ± 0.05.

A different random sample of 20 experienced subjects made the same sensory judgments as in the first test. All other aspects of procedure were identical to Test 1.

Test 3

The final test compared the sweetness of two vanilla cakes with equal amounts-by-weight of sucrose or fructose. Cake was chosen as a test medium to provide relative sweetness data in a commonly used solid food system.

The cakes were prepared using 275g of self-rising flour, 240g of milk, 120 ml of shortening, 2 medium sized eggs, 262g of sucrose or fructose and 5 ml of vanilla extract. The cakes were baked in a conventional electric oven for 20 min at 177°C. The cakes were allowed to remain at room temperature for 24 hr and were then sliced into 5 cm × 5 cm × 5 cm pieces (edges removed) for presentation to subjects.

A random sample of 15 experienced subjects was used. Order of presentation of the two samples was counterbalanced and judgments of sweetness intensity were again made using the method of modulus-free magnitude estimation. A 90-sec interstimulus interval was used and subjects rinsed with distilled water before and after each presentation.

RESULTS

Tests 1 and 2

Data were analyzed by subjecting the magnitude estimates to a normalization procedure (Stevens, 1971) and then determining the median of the normalized magnitude estimates assigned to each solution. The ratio of the medians for the fructose and sucrose solutions were calculated for each concentration and medium, and these served as the relative sweetness measures.

Figure 1 shows the results from Test 1. As is evident from the figure, the relative sweetness of fructose to sucrose was approximately 1.6 at the lowest sugar concentrations in distilled water and in the higher pH beverage. This relative sweetness declined to approximately 0.9–1.2 with increasing sugar concentration. In the lower pH beverage the relative sweetness of fructose to sucrose was approximately 0.8–1.0 at the lowest sugar concentrations and varied only slightly with concentration.

Figure 2 shows a similar set of results obtained during Test 2. Here the relative sweetness of fructose to sucrose declines from values of approximately 1.8–1.9 to 0.9–1.2 with increasing sugar concentration in distilled water and the higher pH citric acid solutions. In the lower pH citric acid solution the relative sweetness of fructose to sucrose remains slightly above 1.0 at all sugar concentrations.

In order to assess the underlying relationship between perceived sugar sweetness and sugar concentration, psychophysical power functions of the form $S = kI^n$ (where S is perceived sweetness, I is sugar concentration, n is the exponent of the function and k is the intercept value) were fitted by a least-squares regression procedure to the relationship between median magnitude estimates and concentration. Table 1 shows the values of n and k determined for sucrose and fructose in the three media of Test 1, and Table 2 shows these same two parameters for the media of Test 2.

A repeated measures analysis of variance on the normalized magnitude estimates of Test 1 showed a significant main effect for concentration ($F = 31.31$, $df = 5/65$, $p < 0.05$) and a significant sugar × concentration × beverage effect ($F = 2.13$, $df = 10/130$, $p < 0.05$). A similar analysis of variance on the data of Test 2 showed a significant main effect for concentration ($F = 11.37$, $df = 4/76$, $p < 0.05$), a significant concentration × pH effect ($F = 2.83$, $df = 8/152$, $p < 0.05$) and a significant sugar × concentration × pH effect ($F = 1.98$, $df = 8/152$, $p < 0.05$).

Test 3

The magnitude estimates of sweetness obtained in response to the two cakes were normalized and a matched t-test was used to assess the difference between the two sets of normalized magnitude estimates. No significant difference was found between the sweetness judgments for the two cakes.

DISCUSSION

THE DATA from the first test (Fig. 1) demonstrate that fructose is sweeter than sucrose by a factor of approximately 1.6 when these two sugars are mixed with distilled water at low concentrations. This fact is also reflected in the higher intercept value (k) for fructose over sucrose in

Table 1—Parameters of the psychophysical power function relating perceived sweetness to sugar concentration for the sugars and media of Test 1

	Sucrose		Fructose	
	n	k	r	k
Distilled water (pH = 6.3)	1.51	1.41	1.35	2.32
Kool-Aid drink (pH = 2.7)	1.41	1.49	1.13	2.90
Lemon-flavored drink (pH = 2.35)	0.88	4.24	1.00	3.27

Table 2—Parameters of the psychophysical power function relating perceived sweetness to sugar concentration for the sugars and media of Test 2

	Sucrose		Fructose	
	n	k	n	k
Distilled water (pH = 6.1)	1.46	0.82	1.38	1.24
0.0025M Citric acid (pH = 3.0)	1.43	0.90	1.02	2.21
0.025M Citric acid (pH = 2.35)	1.28 (0.94)	0.81 (1.78)	1.33 (0.80)	0.80 (2.82)

distilled water (Table 1). However, at higher sugar concentrations, the more rapid rate of growth of sucrose, as reflected by the larger exponent in Table 1, reduced the relative sweetness of fructose to a value just greater than 1.0 (Fig. 1).

Although the relative fructose-sucrose sweetness values observed in the distilled water solutions of Test 1 approximate previously published values obtained with distilled water solutions, the direction of change with concentration is contrary to that found by Dahlberg and Penczek (1941) and Cameron (1943). These investigators found that fructose sweetness increased with increased sugar concentration relative to sucrose sweetness, while the current data show a decrease in relative fructose sweetness with concentration. However, Tsuzuki and Yamazaki (1953) found a decrease in relative fructose sweetness with increasing concentration from 3% to 20% wt/wt. More recently, Moskowitz (1970) has found the sweet taste of sucrose and fructose to "grow at about the same rate" with concentration, and Hyvonen et al. (1977) have again shown that the relative sweetness of fructose to sucrose is lower at higher concentrations when the solutions are tested at room temperature, a condition which prevailed throughout these tests.

Similar results as were found for distilled water were found for the less acidic beverage. The relative sweetness values closely approximate those found with distilled water (Fig. 1) and the *relative* values of k and n for sucrose and fructose are the same (Table 1). The fact that the absolute values of the sweetness exponents are lower than in distilled water is consistent with data presented by Moskowitz (1971b), which indicate that the sweetness exponent for sugar is reduced in sugar-acid mixtures (Moskowitz, 1971b; Table 4). In addition, the fact that the absolute values of the intercepts are greater suggests that the lowered exponents are due to an enhancement of the sweet taste at lower concentrations in both sugars. Such enhancement of sugar sweetness by citric acid has been demonstrated previously by Fabian and Blum (1943), Anderson (1950), and Kamen et al. (1961). The fact that Pangborn (1960, 1961, 1965) has found suppression of sweetness by citric acid has been attributed to both individual subject differences (Gregson and McCowen, 1963) and to the confusion of sourness with sweetness in moderate pH citric acid solutions (Stone et al., 1969).

In the more acidic beverage, the relative sweetness data (Fig. 1) depart from that found in the other two test media. No large sweetening advantage of fructose over sucrose is evident at any sugar concentration. This fact is reflected in both the larger percentage decrease in the exponent for sucrose (41% vs 26%) and the larger percentage increase in the intercept value for sucrose (200% vs 40%) with decreasing pH (Table 1). Both of these factors combine to produce a larger exponent (1.00 vs 0.88) and lower intercept value (3.27 vs 4.24) for fructose relative to sucrose in this medium.

The recent failure by Harris et al. (1978) to find a differential sweetness between fructose and sucrose when each is substituted as a sweetener in a kool-aid beverage, is confirmed by the data in Figure 1. When this beverage base is mixed with sugar according to label directions, as in the Harris et al. study, the concentration of sugar in the beverage is 8.7%. This corresponds to an abscissa value of 0.94 in Figure 1. At this concentration, the relative fructose-sucrose sweetness value is close to 1.0, and no sweetening advantage of fructose would be expected.

The relative sweetness data obtained in Test 2 (Fig. 2) is almost identical to that obtained in Test 1. The distilled water and less acidic citric acid media show a relatively large sweetening advantage (1.8–1.9) of fructose over sucrose at low sugar concentrations, but this advantage dissipates with increasing concentration. In the more acidic solutions, no advantage of fructose over sucrose is found at any sugar concentration. These data are again reflected in lower intercept and higher exponent values for sucrose in the distilled water and 0.0025M citric acid media, compared to the 0.025M citric acid medium.

While the net effect of the obtained parameter values for the psychophysical functions governing the growth of sweetness in the media of Test 2 is the same as Test 1, the change in parameter values with change in pH is more complex. Although the intercept values (k) increase and exponent values (n) decrease in going from the distilled water solution to the higher pH citric acid solution, similar changes are not observed with the still lower pH citric acid solution. Here both intercepts *decline* to their lowest values, while the exponent for sucrose continues to decrease and that for fructose *increases*.

Although the parameter changes which occur in the psychophysical sweetness function in the low pH medium (0.025M citric acid) of this test are somewhat confusing, they are not inexplicable. The 0.025M citric acid solution was described by subjects as being very tart or sour. Moskowitz (1972), in his work on sugar mixtures, has observed that "on occasion, some severe changes occurred in the exponent (of the psychophysical function), particularly in the presence of a high amount of a second taste material." He goes on to suggest that these marked departures may result from "too few points and possible masking effects that selectively affected the more dilute concentrations of sugar." Analysis of the raw magnitude estimates reported by subjects for the low pH solutions in Test 2 support the notion of masking as the cause of the inconsistent exponent and intercept values in this medium. At the two lowest sugar concentrations (2.1 and 3.5%) over 40% of the subjects gave zero magnitude estimates in the low pH medium. No other sugar concentrations in any of the media used in the two tests had zero magnitude estimates totaling 15%. The effect of these zero magnitude estimates is to depress the median magnitude estimates at these concentrations, producing a lowering of the intercept and raising of the exponent. Ignoring these two concentrations, the empirically determined intercepts for sucrose and fructose are 1.78 and 2.82, respectively, and the exponents are 0.94 and 0.80, respectively. These parameter values are reported in parentheses at the bottom of Table 2, and it can be seen

that these values are consistent with an increase in intercept and decrease in exponent with decreasing pH.

Thus, the data from Tests 1 and 2 show that, in model distilled water solutions and in low acidic media, fructose has a distinct sweetening advantage (1.6–1.9) over sucrose at low sugar concentrations. At higher sugar concentrations, this advantage is lost. Furthermore, the sweetening advantage at low concentrations is not observed in highly acidic solutions.

The failure to find a differential sweetening effect of the two sugars in vanilla cake (Test 3) suggests that the loss of sweetening advantage of fructose at the lower sugar concentrations of Tests 1 and 2 may not be strictly dependent on the dynamics of the acid-sugar interactions. The fact that concurrent flavors can mask sweetness at low sugar concentrations (Moskowitz, 1972) suggests that any food product with a strong flavor may do so. Thus, the moderately strong vanilla flavor of the cakes of Test 3 may have masked any differential sweetness in the sugars at the concentration tested. The fact that only 1 level of sugar was used in Test 3, precludes an in-depth analysis of the data, although the results do provide an example of the failure to find a sweetening advantage of fructose over sucrose when used in a solid food system, rather than in model solutions or acid-sugar beverages.

As far as the economics of alternative sweeteners is concerned, the combined test data suggest that the relative sweetening advantage of fructose is best utilized in products which contain low concentrations of sugar and in those products which do not have a strong concurrent taste. Thus, the economic sweetening advantage of fructose must be considered within the context of its specific application. Broad generalizations of its sweetening power may be inappropriate or even misleading.

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INFLUENCE OF DIETARY FIBER ON GROWTH . . . From page 747

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INFLUENCE OF pH ON THE BINDING OF COPPER, ZINC AND IRON IN SIX FIBER SOURCES

S. A. THOMPSON and C. W. WEBER

ABSTRACT

The binding of endogenous copper, zinc and iron in six fiber sources was examined via incubation under three pH conditions: (1) pH 0.65; (2) pH 6.8; and (3) a sequential treatment of pH 0.65, neutralization, then pH 6.8. After both the pH 6.8 and sequential treatments, most of the minerals remained bound in the residues, while little remained bound after the acidic treatment. This indicated that the minerals were rebound when the pH was raised from very acidic to only slightly acidic. Titrations of the untreated fiber sources showed that protein influenced the hydrogen ion buffering capacity. After enzymatic proteolysis, the buffering capacities were reduced and other physicochemical properties appeared to gain influence.

INTRODUCTION

INTEREST in dietary fiber and its physiological effects is growing rapidly. Much of the recent research has been excellently reviewed (Kelsay, 1978). The positive effects attributed to increased dietary fiber intakes include: reduction of diverticulosis symptoms, increase in fecal bile acid excretion, and control of diabetic symptoms. There may be negative effects of increasing dietary fiber intakes. One of these effects may be the reduction in absorption of trace minerals to the body from the gastrointestinal tract.

All the physiological effects of dietary fiber are a function of the physical and chemical properties of the various fiber sources. Some of these properties are beginning to be elucidated (Parrott and Thrall, 1978). And just as some of the positive symptom-relief effects may be correlated to particle size or water binding capacity, the reduction of mineral absorption may be correlated to the cation exchange activities of various fibers. One example of this is the correlation of calcium binding to unsubstituted uronic acid content of hemicelluloses (James et al., 1978; Branch et al., 1975). Differences in the chemical and physical properties also may account for variation in abilities to buffer hydrogen ions by various cereal grains (Jalan et al., 1977).

The many fiber sources warrant examination with regard to their physical and chemical properties. A wide range of fiber sources have already been evaluated as supplements in layer cakes and breads (Zabik et al., 1977; Shafer and Zabik, 1978; Colmey, 1978). It is important to know the properties of the fiber sources not only because of their functional effects on the product but also because of their possible physiological effects. High extraction, fiber-rich, unleavened breads have been blamed for the zinc deficiency symptoms discovered in Iran (Reinhold et al., 1975). This may not be due entirely to fiber (phytic acid may have an effect on mineral absorption from these breads); however, the contribution of fiber must be investigated (Davis et al., 1977).

During the process of digestion the gastrointestinal environment undergoes a dramatic change in pH from very acidic, pH 1.0, to near neutral, pH 6.8–7.2 (Harper, 1975; Sturkie, 1965). It has been substantiated that changes in pH affect mineral binding capacity. Therefore, it is important to investigate the mineral binding capacity of various fiber sources under conditions which parallel physiological pH conditions. It was for this reason that the present study was initiated.

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EXPERIMENTAL

Fiber sources

The six fiber sources used were: wheat bran (Gooch Milling and Elevator Co.); corn bran (Tabor Milling Co.); soy bran (ADM Co.); oat hulls (National Oat Co.); rice bran (Riviana Foods Inc.); and cellulose (ICN Pharmaceuticals). The fiber sources were analyzed for moisture, protein, fat and ash by standard AOAC (1970) procedures, in duplicate. Fiber content was determined by both the acid detergent method and the neutral detergent method of Van Soest (1963).

Incubations for endogenous mineral binding

The fiber sources were incubated in a shaker-water bath at 40°C using three pH treatments: (1) 0.2N HCl, pH 0.65; (2) 0.1N phosphate buffer, pH 6.8; and (3) a sequential treatment of 0.2N HCl, pH 0.65, neutralization, then 0.1N phosphate buffer, pH 6.8. Three time periods of 10, 30 and 120 min were used for each pH treatment. After incubation, the samples were centrifuged at 15,000 rpm for 15 min. An aliquot of the supernatant was taken from each sample for mineral analysis. The residues were dried at 45°C and then ashed in a muffle furnace at 450°C. The ash was dissolved in 5% HCl and diluted for analysis of copper, zinc, and iron via atomic absorption spectrophotometry.

Titrations for hydrogen ion buffering capacity

The six crude fiber sources were first titrated as received and then were titrated again after an enzymatic treatment. The enzymatic treatment was a modified pepsin-pancreatin method which digested protein and other components (Hellendoorn et al., 1975). This method was chosen because of its similar pH properties as compared to both the prior incubation treatments and physiological changes during digestion.

For the titrations, 1.0g of air-dried fiber sample was suspended in 30 ml of deionized H_2O and stirred. The initial pH was recorded after a time period of 2 min. Increments of 0.1021N HCl or 0.0824N NaOH were added at 3-min intervals and the pH was recorded after it had stabilized. The fiber samples were titrated with HCl to an endpoint of pH 2.0, and with NaOH to an endpoint of pH 10.0.

Statistical analysis

Analysis of variance and Pearson's correlations were accomplished using the SPSS-computer program (Nie et al., 1970).

RESULTS & DISCUSSION

THE RESULTS of the proximate analysis of the fiber sources are listed in Table 1. The rice bran had the highest protein content at 20%, while oat hulls and cellulose had the lowest protein content at 4% and 0%, respectively. The endogenous mineral contents of the untreated fiber are given in Table 2. This table shows the wide range of absolute amounts of endogenous copper, zinc, and iron. Due to the large variation in the mineral contents of the fiber sources, the data analysis was based upon percent change in concentration of minerals.

The data for the combined mineral values for the residues from the three incubation time periods are graphically

Table 1—Results of analysis for the six fiber sources expressed on a dry weight basis

Fiber source	Protein (%)	Fat (%)	Carbohydrate[a] (%)	Moisture (%)	Ash (%)	ADF[b] (%)	NDF[c] (%)
Wheat bran	17.8	3.5	48.4	7.9	7.0	15.5	45.4
Corn bran	10.8	5.1	67.0	8.8	2.0	6.4	26.0
Soy bran	17.3	3.6	28.6	0.5	5.8	38.1	50.8
Oat hulls	4.4	0.5	41.0	5.0	7.2	42.1	80.1
Rice bran	20.3	2.5	48.9	5.3	13.0	10.0	27.0
Cellulose	0.0	0.1	18.4	4.3	0.2	76.9	93.8

[a] Carbohydrate = 100 − (Protein + Fat + Moisture + Ash)
[b] Acid detergent fiber
[c] Neutral detergent fiber

Table 2—Endogenous mineral content of the six fiber sources expressed on wet weight basis

Fiber source	Copper (ppm)	Zinc (ppm)	Iron (ppm)
Wheat bran	15.3 ± 0.68[a]	106.3 ± 1.13	160.8 ± 3.06
Corn bran	3.9 ± 0.30	38.7 ± 1.31	73.8 ± 2.09
Soy bran	9.0 ± 0.72	46.6 ± 2.17	395.6 ± 6.35
Oat hulls	17.0 ± 3.42	31.8 ± 5.85	176.0 ± 4.01
Rice bran	7.8 ± 0.96	81.8 ± 6.29	132.8 ± 4.93
Cellulose	2.2 ± 0.43	1.8 ± 0.24	114.3 ± 6.26

[a] mean ± standard deviation

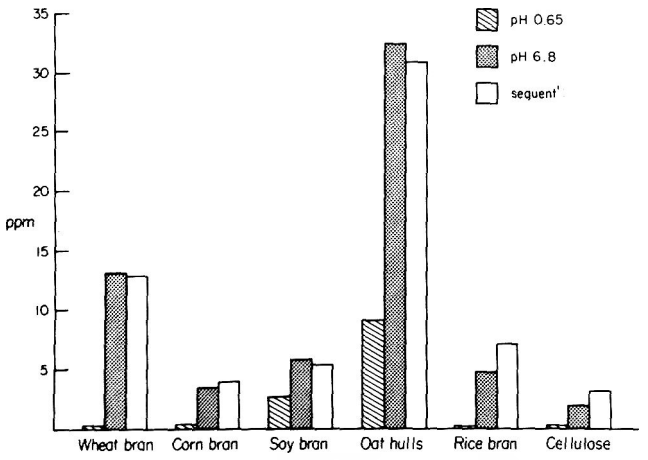

Fig. 1—Effect of pH treatment on copper in residues.

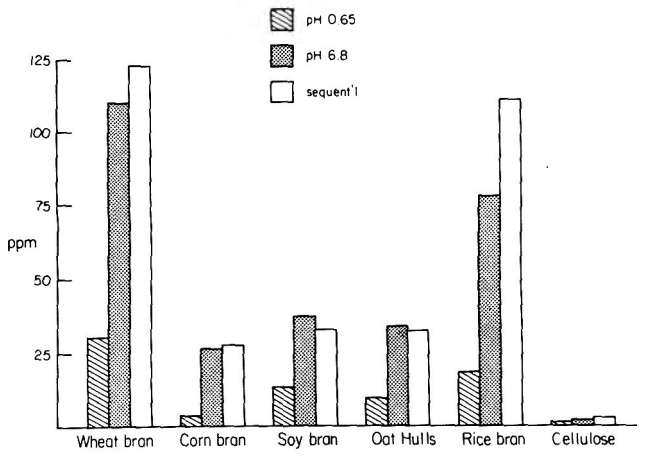

Fig. 2—Effect of pH treatment on zinc in residues.

represented in Figures 1, 2 and 3. The three time periods were combined because there were no statistically significant differences between the time periods in the amount of mineral released. Average values for copper are illustrated in Figure 1, zinc in Figure 2, and iron in Figure 3. Supernatant values are not given as they were reciprocal to the residue values.

The pH treatments had significant effects on the binding of the endogenous minerals to the fiber sources as can be seen in Figures 1, 2 and 3. Most of the minerals were still bound after the pH 6.8 incubation, but were released into solution after the pH 0.65 incubation. After the sequential treatment, the residues had mineral contents close to those from the pH 6.8 incubation. This indicated that while the endogenous minerals were not bound at the very acidic pH, they were rebound when the pH was raised. If the endogenous minerals can be rebound when the pH is changed from very acidic to slightly acidic, then the possibility of other minerals present in the diet being bound to the fiber material must be considered. However, these untreated fiber sources contain protein and other chemical components, which may affect mineral binding in these in vitro analyses, but most likely would be removed during the digestion process. The contribution of digestible components to cation binding was partially examined in the titration part of this study.

The sequential treatment showed that the three minerals were bound differently to the various fiber sources. The very low copper and zinc levels in cellulose lead to a large magnitude of error and therefore have not been included. Analysis of the data showed that the relative amounts of each mineral remaining in the residues were as follows (lowest to highest): (1) for copper, soy bran-oat hulls < wheat bran-rice bran < corn bran; (2) for zinc, soy bran-corn bran < oat hulls < wheat bran-rice bran; and (3) for iron, oat hulls < corn bran < soy bran < wheat bran-cellulose < rice bran. The differences in the fiber sources' abilities to retain the minerals was obvious. Corn bran had a high relative amount of residual copper but was low in zinc and iron, while oat hulls had low amounts of iron, but higher amounts of zinc. The cellulose had high residual iron which was unexpected as was the original high iron content. No speculation on the cause of this was formulated. The correlations of copper, zinc and iron in the residues compared to chemical constituents such as protein, acid detergent fiber, enzymatic indigestible residue and phytic acid, were analyzed. Of the three minerals, only zinc had strong significant correlations, which were to protein (0.766) and to phytic acid (0.885), at the 0.05% level.

Protein is known to have a good buffering capacity. Since zinc was strongly correlated to protein content of the

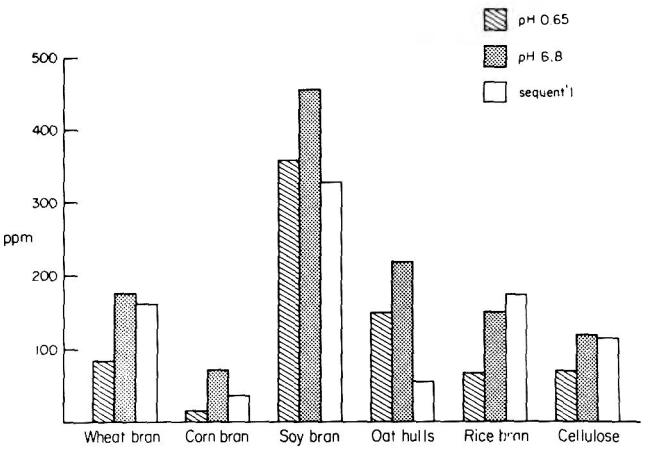

Fig. 3—Effect of pH treatment on iron in residues.

untreated fiber sources, the titrations of the untreated and the enzymatically treated fiber sources were undertaken. The titration curves for the untreated and treated sources are shown in Figures 4 and 5, respectively. The cellulose which contained no protein acted as a nonbuffering control. The slope of the titration curves indicates buffering capacity, with the greater buffering capacity resulting in a less vertical slope. The untreated rice bran had the greatest ability to buffer the hydrogen ions and had the highest protein level of all the fiber sources (Table 1). Wheat bran and soybran had protein contents similar to each other and had similar abilities to buffer hydrogen ions.

The titration curves in Figure 5 show that all treated fiber sources had decreased buffering capabilities, with the exception of the cellulose control, which did not change. The protein content of the treated fiber sources are given in Table 3. The protein was reduced 76% or more from the original levels. The buffering capacities of the treated fiber sources did not related to protein content as closely as they had for the untreated fiber sources. For example, treated rice bran had a higher protein content than treated soybran, but its buffering capacity was less than that for the soybran. These results suggest that when protein is reduced in the fiber sources, that other physicochemical properties gain in their influence on buffering capacity. Thus, care must be taken when extrapolating from in vitro analyses to physiological conditions, where protein and other digestible components are removed during digestion. In light of the present results, it would have been interesting if Jalan et al. (1977) had presented protein contents of their cereal grains while discussing hydrogen ion buffering capacity. Physiological conditions may not have been accurately represented in their analyses, thus making the extrapolation to human duodenal cancers more difficult.

The results of this study indicate that changes in pH, which parallel physiological conditions, do affect mineral binding capacities of various fiber sources. The endogenous minerals were not bound in the very acidic condition, but became rebound when the pH was raised. The fiber sources had differing abilities to rebind the endogenous minerals. While iron and copper were present in the residues, their levels did not correlate to any of the constituents examined. Zinc, however, did correlate to protein and phytic acid. The titrations showed that protein influenced buffering capacity. However, when protein and other digestible constituents were removed, other physicochemical properties appeared to influence the buffering. The binding of trace minerals by fiber sources is a complex mechanism, and understanding this mechanism will require further elucidation of the influence of physicochemical properties.

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Table 3—Protein content of enzymatically treated fiber sources

Fiber source	EIR[a] (as % of original)	Protein (as % of EIR)	% Reduction in protein from original
Wheat bran	48	4.2	76.2
Corn bran	60	1.8	83.5
Soy bran	57	3.6	79.4
Oat hulls	83	0.6	87.2
Rice bran	29	4.0	80.5
Cellulose	100	0.0	–

[a] EIR = enzymatic indigestible residue left after enzymatic treatment.

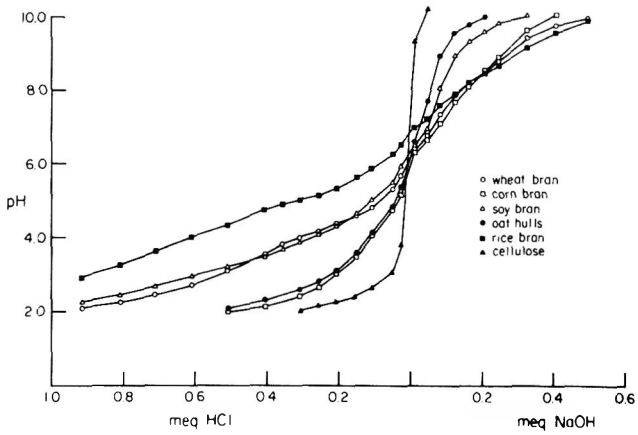

Fig. 4—Titration curves for untreated fiber sources.

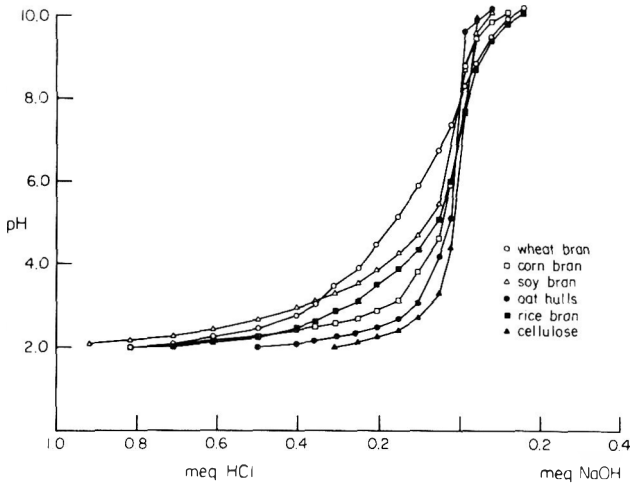

Fig. 5—Titration curves for enzymatically treated fiber sources.

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SPORULATION OF Clostridium perfringens IN MEATS SUPPLEMENTED WITH SOY MATERIALS

S. E. CRAVEN and A. J. MERCURI

ABSTRACT

Cooked meat samples and meat supplemented with 30% hydrated isolated soy protein (ISP), soy protein concentrate (SPC), or textured soy protein (TSP) were inoculated with vegetative cells of *Clostridium perfringens* and incubated anaerobically at 37°C for 24 hr. Of six *C. perfringens* strains, six sporulated in chicken thigh meat, two in beef, and none in pork sausage. Sporulation was significantly less ($P < 0.01$) in chicken thigh meat or beef supplemented with SPC or TSP than in chicken or beef controls. Sporulation was significantly higher ($P < 0.01$) in beef supplemented with ISP than in beef. Of the three pork sausage-soy protein combinations, sporulation was infrequently detected in pork sausage-ISP samples. The changes in sporulation in chicken or beef by addition of ISP, SPC and TSP were apparently due to a pH effect. With the pH adjusted to 7.5, sporulation of *C. perfringens* in Duncan-Strong (D-S) sporulation medium with added ISP, SPC, or TSP was similar to or greater than that in D-S medium.

INTRODUCTION

SOY PROTEINS are increasingly used to supplement meat systems or to simulate meat products. From the standpoint of food safety, the effects on growth or enterotoxin production of food-poisoning bacteria should be compared between these soy products and the meat product they replace.

The influence of protein supplements in foods on staphylococcal enterotoxin production was studied by Fung (1972), Tatini et al. (1976) and Craven et al. (1978). Soy proteins stimulated growth rates of *Clostridium perfringens* in turkey loaf but not in beef loaf (Busta and Schroder, 1971; Schroder and Busta, 1971). Soy flour had no effect and cottonseed protein had a negative effect on growth of *C. perfringens* in beef, chicken, and turkey (Kokoczka and Stevenson, 1976).

Sporulation of *C. perfringens* in foods can contribute to a food-poisoning hazard because many more spores than vegetative cells survive low-temperature storage conditions (Strong and Canada, 1964; Fruin and Babel, 1977; Trakulchang and Kraft, 1977) and can be activated to germinate by normal heating or cooking conditions (Bryan, 1969; Craven and Lillard, 1974). Also, *C. perfringens* that sporulates in foods can form enterotoxin (Naik and Duncan, 1977) that is ingested with the food. *C. perfringens* sporulated to a limited extent in pea puree and tuna (Kim et al., 1967) but not in beef (Barnes et al., 1963). Sporulation was high in turkey and chicken (Mead, 1969) and in vacuum-sealed meats (Dework, 1972).

Experiments were conducted to determine the effect of commercially prepared soy materials on sporulation of *C. perfringens* in meat-soy mixtures.

EXPERIMENTAL

Meat sample preparation

Chicken samples were prepared by combining chicken thigh muscle (65%), chicken skin (22%), and chicken fat (13%) obtained from a local processing plant. Beef was purchased as boneless round from a local supermarket. Because soy products often are added to sausage preparations, pork was obtained as a breakfast sausage mixture from a local processing plant. No spices were added other than NaCl which was added at 1.75 g/100g meat as recommended for some breakfast sausage formulations (Kramlich et al., 1973). Each meat type was ground with a Sears meat grinder (400.8260).

The commercially prepared soy proteins used were: isolated soy protein (ISP, Promine-D, Central Soya), 90% protein dry weight; soy protein concentrate (SPC, A.E. Staley Manufacturing Co.), 70% protein dry weight; and textured soy protein (TSP, Ultra Soy, Far-Mar-Co.), 56% protein dry weight. The TSP contained ferrous sulfate, niacinamide, calcium pantothenate, vitamin B_6, riboflavin, vitamin B_1, and vitamin B_{12} as additives. The soy materials were hydrated (2.5 ml of distilled water per gram of soy) and combined with the ground meats to give a 30% (wt/wt) soy level in the product.

In a separate experiment, the pH of hydrated SPC or hydrated ISP was adjusted to the pH of the raw beef or chicken with 5.0N NaOH or HCl. The volume of the water mixture was reduced after pH adjustment so that the ratio when added to soy was 2.5:1.

Portions of the meat-soy combinations and of their corresponding meat controls were ground and 30-g samples of each weighed into 50-ml pyrex beakers and covered with aluminum foil. The samples were cooked to 57°C in a Despatch rotary oven set at 191°C, and cooking juices were discarded. After the samples cooled to room temperature, they were autoclaved at 121°C for 10 min. After shrinkage, only the bottom meat surface was in contact with the beaker.

Preparation of sporulation media

In a separate set of experiments, the effect on sporulation of soy materials adjusted to pH 7.5 in liquid media was determined. Each soy material was added to attain a protein level from soy of 1.5% in 10 ml of (1) Duncan and Strong (D S) sporulation medium (Duncan and Strong, 1968), (2) D S medium without proteose peptone, and (3) D S medium without proteose peptone or yeast extract. The media containing soy materials were soaked at 4°C for 24 hr, the pH was adjusted, and the samples were autoclaved at 121°C for 15 min.

Inoculation

C. perfringens strains 7947 (Hobbs type 1), 7948 (Hobbs type 2), PS17, PS18, PS44, and PS57 were obtained from the Center for Disease Control, Atlanta, GA. These strains had been associated with food-poisoning incidents. Two transfers into 10 ml of thioglycollate medium without indicator (Difco) by use of a 0.1 ml inoculum and 24 hr incubation at 37°C were made from stock cultures in cooked meat medium (Difco). From the last 24 hr culture, 0.1 ml was inoculated into 10 ml of thioglycollate medium in screw cap test tubes and incubated at 37°C for 16 hr. After incubation the contents of four tubes were combined and centrifuged at 12,000 × G for 15 min. The supernatant was decanted and the cells were resuspended in 40 ml of 0.85% sodium chloride and diluted to attain the desired number of vegetative cells. Inocula when heat shocked at 75°C for 20 min contained essentially no spores (<10/ml).

The autoclaved meat samples were incubated at 4°C anaerobically in Gas Pak anaerobic jars (BBL) with Gas Pak hydrogen + carbon dioxide generator envelopes (BBL) for 42–44 hr prior to inoculation. The aluminum foil covering was vented to allow exchange of gases in the beakers without contamination of samples. Tubes of soy protein-sporulation medium were heated to eliminate oxygen.

Each autoclaved meat or meat-soy sample and each soy protein-sporulation medium sample was inoculated with 0.01 ml per g or ml of sample, respectively. Beakers containing inoculated meat samples were vented and incubated anaerobically in the Gas Pak anaerobic system (BBL) at 37°C for 24 hr. For those studies involving an-

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aerobic incuabtion for less than 24 hr the Gas Pak disposable anaerobic system (BBL) was used. Anaerobiosis, as indicated by complete color change for methylene blue indicators, was reached within 2 hr and 1 hr, respectively, for the two methods of incubation. Tubes of soy proteins in sporulation medium were incubated aerobically at 37°C for 24 hr.

Sampling

Meat samples were transferred from beakers into sterile polyethylene bags containing sufficient sterile 0.1% peptone water to give a 1/10 dilution and macerated with a Colworth stomacher 400 for 1 min. For spore counts, 5 ml of the 1/10 dilution or 5 ml of the liquid sporulation medium was heat shocked at 75°C for 20 min in screw cap test tubes. Serial dilutions in peptone water were pour-plated with Shahidi Ferguson perfringens (SFP) agar base (Difco).

Colony-forming units were counted on plates that were incubated anaerobically in desiccators at 37°C for 18–24 hr. A Gas Pak catalyst (BBL) contained in a 50 ml beaker was placed in a 100 ml beaker for heat insulation and, with two Gas Pak hydrogen + carbon dioxide generator envelopes (BBL), provided anaerobic conditions in each desiccator. A reduced pressure of 130 mm of mercury was used to prevent gas release from lifting the top of the desiccator.

Measurement of pH

A Corning 112 digital pH meter equipped with an Ingold #6162 pH electrode was used to measure sample pH.

Statistical analysis

Data from plate counts were treated by analysis of variance and Duncan's new multiple-range test (1955).

Table 1—Sporulation of six strains of Clostridium perfringens in cooked ground meat incubated anaerobically at 37° C for 24 hr

Strain	Inoculum (log_{10} vegetative cells/g)	Log_{10} spores/g[a] Chicken thigh	Beef	Pork sausage
7947	5.11	4.30	<1.00	<1.00
7948	5.18	4.58	<1.00	<1.00
PS17	5.11	6.23	3.73	<1.00
PS18	4.91	5.20	<1.00	<1.00
PS44	5.11	6.68	2.58	<1.00
PS57	4.93	6.00	<1.00	<1.00

[a] Results reported as the mean of duplicate samples from one trial.

Table 2—Growth and sporulation of Clostridium perfringens after anaerobic incubation for 24 hr at 37° C in cooked meats supplemented with 30% hydrated soy proteins

Meat-soy combination	Log_{10} spores/g (Log_{10} total viable cells/g)[a] Strain[b] 7947	PS17	PS44
Chicken	4.56a[c] (7.76)	ND[d]	6.91a (8.85)
Chicken + ISP	4.01a (7.88)		6.92a (8.95)
Chicken + SPC	<1.00b (7.30)		5.48b (8.91)
Chicken + TSP	<1.00b (8.23)		5.11b (9.20)
Beef	ND	4.94b (8.89)	3.15b (9.06)
Beef + ISP		6.23a (8.99)	4.26a (9.06)
Beef + SPC		<1.00[c] (8.79)	<1.00[c] (8.93)
Beef + TSP		<1.00[c] (9.11)	<1.00[c] (9.07)
Pork sausage	ND	<1.00 (7.96)	<1.00 (<4.00)
Pork + ISP		<1.33[e] (8.62)	<1.00 (5.57)
Pork + SPC		<1.00 (8.13)	<1.00 (<4.00)
Pork + TSP		<1.00 (8.56)	<1.00 (5.89)

[a] Values represent the mean of 4 samples from 2 replicate trials.
[b] Inocula (log_{10} vegetative cells/g): 7947, 3.73; PS17, 4.38; PS44, 4.51.
[c] For each meat type, values within a column with the same letter are not significantly different.
[d] Not determined.
[e] Mean of 4 samples: <1.00, <1.00, 1.18, 2.13.

RESULTS & DISCUSSION

OF THE SIX STRAINS of *Clostridium perfringens* tested, six sporulated at high levels in chicken thigh meat after 24 hr at 37°C, two sporulated at lower levels in beef and none sporulated at detectable levels in pork sausage (>log_{10} 1.00 spores/g) (Table 1). These results are similar to those of Mead (1969) who showed log 1.0–6.7 spores/g for 4 strains of *C. perfringens* in cooked chicken leg muscle. Barnes et al. (1963) found no sporulation of a *C. perfringens* strain in raw beef stored at 37°C for 15 hr. Dework (1972) found sporulation of *C. perfringens* in vacuum-sealed packages of cooked hamburger after 24 hr at 37°C at higher levels (log 4.67–5.94) than those of beef in this study. For further studies with chicken-soy products, the strains producing in chicken the greatest number of spores, PS44, and the fewest number of spores, 7947, were chosen. For studies of sporulation in beef-soy products, the two strains (PS17 and PS44) that sporulated in beef were selected. These latter two strains were also used for studies of sporulation in pork-soy products.

Addition of 30% hydrated soy materials to ground meat products significantly ($p < 0.01$) affected sporulation after 24 hr (Table 2). In chicken thigh meat supplemented with SPC or TSP, *C. perfringens* spore counts for both the low-sporulating strain (7947) and the high-sporulating strain (PS44) were lower ($p < 0.01$) than in chicken controls or chicken with ISP. Compared to counts in beef controls, spore counts for *C. perfringens* strains PS17 and PS44 were lower in beef with added SPC or TSP and higher with added ISP ($p < 0.01$). Of the pork sausage and pork-soy mixtures, strain PS17 sporulated at low levels in 2 of 4 pork-ISP samples. Strain PS44 did not sporulate at detectable levels in any pork samples. Even the total viable cells were at low numbers for strain PS44 after 24 hr in pork products.

The poor sporulation of *C. perfringens* strains in pork sausage and pork-soy products was due to the level of salt (1.75%). In separate experiments, strain PS17 formed log$_{10}$6.7 spores/g of cooked pork sausage without salt after 24 hr at 37°C. Kang et al. (1969) reported that sporulation of *C. perfringens* dropped from about log 6.0 spores/ml to essentially none when the water activity of Ellner's sporulation medium was decreased from 0.993 to 0.98 by addition of sodium chloride. Wynne (1948) found that 1.0% sodium chloride severely depressed sporulation of *Clostridium botulinum* in brain-heart infusion broth.

Since *C. perfringens* enterotoxin can be preformed in foods (Naik and Duncan, 1977) and enterotoxin production coincides with spore formation (Duncan et al., 1972), conditions that increase the onset of sporulation could increase the health hazard of foods contaminated with *C. perfringens*. With an inoculum of log_{10} 4.51 vegetative cells/g, detectable spores (>log_{10} 1.00/g) of strain PS44 at 37°C first occurred in chicken thigh meat at the 8-hr sampling period (Table 3). Spores of this strain were not detected in chicken + 30% SPC until 14 hr. At 16 hr, spore counts were one log or more greater in chicken than in chicken-SPC. In beef with 30% ISP, strain PS17 inoculated at log_{10} 4.38 vegetative cells/g first formed detectable spores at 12 hr compared to 16 hr for the beef control. Sporulation at 16 hr was log 2.81 higher in beef-ISP than in beef.

Schroder and Busta (1971) found that soy protein concentrate, soy protein isolate, textured soy flour and soy grits did not affect growth rates of *C. perfringens* in beef. Kokoczka and Stevenson (1976) found that soya powder and textured soy product did not affect growth rates of *C. perfringens* in beef or chicken. In our preliminary experiments SPC did not change the growth rate of *C. perfringens* in chicken. However, our results (Table 2) show that of three soy protein products, TSP and SPC decreased sporulation in beef and chicken, and ISP increased sporulation in

Table 3—Sporulation of Clostridium perfringens in chicken, chicken + 30% SPC, beef, and beef + 30% ISP incubated anaerobically at 37° C.

Meat-soy combination	Strain[a]	Log_{10} spores/g[b] 6 hr	8 hr	10 hr	12 hr	14 hr	16 hr
Chicken	PS44	<1.00	1.17	3.15	4.58	5.35	5.30
Chicken + SPC	PS44	<1.00	<1.00	<1.00	<1.00	2.70	4.05
Beef	PS17	<1.00	<1.00	<1.00	<1.00	<1.00	2.14
Beef + ISP	PS17	<1.00	<1.00	<1.00	2.16	4.05	4.95

[a] Inocula (log_{10} vegetative cells/g): PS44, 4.51; PS17, 4.38.
[b] Each value is the mean of four samples from two replicate trials.

beef. Sporulation in a meat product apparently is more susceptible than vegetative cell growth to changes that are associated with soy protein additions.

The reduced sporulation in meat samples containing SPC and TSP could not be attributed to changes in water activity (a_w). The a_w of all cooked meat and meat-soy products were essentially the same (a_w = 0.99).

Sporulation of *C. perfringens* is dependent upon the pH in liquid media (Torrey et al., 1930; Labbe and Duncan, 1974), and in foods (Kim et al., 1967; Mead, 1969). In our study, sporulation of *C. perfringens* was lower in chicken and beef with than without SPC and higher in beef with than without ISP (Table 2). The initial pH of beef-SPC was 0.4 pH unit lower, chicken-SPC 0.9 pH unit lower and beef-ISP 0.2 pH unit higher than the pH of their controls (Table 4). When the pH of hydrated SPC or hydrated ISP was adjusted, before its addition to chicken or beef, so that the initial pH of the meat and meat-soy mixtures were similar, sporulation was not significantly different between these products (Table 5). TSP also inhibited sporulation when added to beef and chicken (Table 2), even though the initial pH was similar for the meat-TSP mixtures and their respective controls. However, after 24 hr the pH of chicken-TSP and beef-TSP dropped to levels that were lower than those of the respective controls by 0.3–0.9 of a pH unit (Table 4). The TSP used in this study contained 32% carbohydrate (dry weight) compared to 15% for SPC and less than 10% for ISP. Sporulation of *C. perfringens* is inhibited in the presence of a metabolizable carbohydrate

Table 4—Hydrogen-ion concentration of cooked meats and meat-soy mixtures inoculated with Clostridium perfringens and incubated anaerobically at 37° C

Meat-soy combination	Initial pH[a]	pH after 24 hr, Strain 7947	PS17	PS44
Beef	6.0[b]	ND[c]	6.0	6.2
Beef + ISP	6.2		6.2	6.3
Beef + SPC	5.6		5.5	5.5
Beef + TSP	6.1		5.7	5.7
Chicken	6.8	6.6	ND	6.7
Chicken + ISP	6.7	6.4		6.7
Chicken + SPC	5.9	5.7		6.3
Chicken + TSP	6.6	5.7		6.3
Pork sausage	6.4	ND	6.3	6.2
Pork + ISP	6.7		6.3	6.4
Pork + SPC	5.8		5.5	5.7
Pork + TSP	6.3		5.3	6.2

[a] pH as measured after 42–44 hr at 4° C prior to inoculation.
[b] All values are the mean of 2 replicate trials, 2 samples per trial, 3 measurements per sample.
[c] Not determined.

because acids produced during growth lower the pH (Torrey et al., 1930). *C. perfringens* did not sporulate when 1% glucose was added to the medium (Nishida et al., 1969). Perhaps the TSP contained some carbohydrates that were metabolized by *C. perfringens* during growth, reducing the pH in the meat-TSP mixtures.

The soy products were added at a protein concentration of 1.5% to Duncan-Strong sporulation medium (DS), DS without proteose peptone (DSB) and DS without proteose peptone and yeast extract (DSC), and the pH was adjusted to 7.5. For *C. perfringens* strains PS17 and PS44, sporulation in liquid media containing each of the three soy products (TSP, SPC, ISP) as an additional protein source (DS + soys), the primary protein source (DSB + soys), or the only protein source (DSC + soys) was equal to or greater than ($p < 0.05$) sporulation in DS medium without soy proteins (Table 6).

—Continued on page 762

Table 5—Sporulation of Clostridium perfringens after anaerobic incubation for 24 hr at 37° C in cooked meats supplemented with 30% hydrated soy proteins (pH adjusted)

Meat-soy combination	Initial pH	Log_{10} spores/g[a] Strain[b] PS44	PS17
Chicken	6.5	7.04a[c]	ND[d]
Chicken + SPC	5.8	5.85b	
Chicken + adjusted SPC	6.6	7.26a	
Beef	6.0	ND	4.55a
Beef + SPC	5.6		<1.00b
Beef + adjusted SPC	6.0		4.43a
Beef[e]	6.0	ND	3.16b
Beef[e] + ISP	6.2		5.93a
Beef[e] + adjusted ISP	6.0		3.74b

[a] Values represent the mean of 6 samples from 3 replicate trials for beef and beef-soy and 4 samples from 2 replicate trials for chicken and chicken-soy.
[b] Inocula (log_{10} vegtative cells/g): PS44, 4.51; PS17, 4.38.
[c] For each meat-soy type, values within a column with the same letter are not significantly different.
[d] Not determined.
[e] These samples prepared from a different batch of beef.

Table 6—Effect of soy proteins on sporulation of Clostridium perfringens in Duncan-Strong sporulation medium after 24 hr at 37° C

Medium	Strain PS17[a] pH (24 hr at 37° C)[b]	Log_{10} spores/ml	Strain PS44[a] pH (24 hr at 37° C)[b]	Log_{10} spores/ml
DS[c]	5.6	7.41de[f]	6.0	5.97b
DS+ISP	6.1	7.74abc	6.1	7.39a
DS+SPC	6.1	7.52cd	6.0	7.22a
DS+TSP	5.7	7.53cd	5.9	7.34a
DSB[d]	6.1	5.09f	6.2	3.50c
DSB+ISP	6.1	7.59bcd	6.0	6.91a
DSB+SPC	6.0	7.20e	6.0	7.35a
DSB+TSP	5.5	7.84ab	5.6	7.36a
DSC[e]	7.5	0g	7.4	0d
DSC+ISP	6.9	7.20e	6.6	5.90b
DSC+SPC	6.6	7.87ab	6.8	7.01a
DSC+TSP	5.8	7.96a	5.6	7.29a

[a] Inocula (log_{10} vegetative cells/ml): PS17, 6.38; PS44, 6.51.
[b] Initial pH of all samples adjusted to 7.5.
[c] Duncan-Strong sporulation medium.
[d] DS medium without proteose peptone.
[e] DS medium without proteose peptone and yeast extract.
[f] Values in a column with the same letter are not significantly different.

GAS CHROMATOGRAPHIC CHARACTERIZATION OF SAGES OF VARIOUS GEOGRAPHIC ORIGINS

HAN Y. RHYU

ABSTRACT

Many studies published to date do not include a comprehensive comparison among the various types of sage available in commerce. Sages procured from Yugoslavia, Albania, Greece, Turkey and Italy during 1971–1977 were ground and steam-distilled in the laboratory. Gas chromatographic profiles obtained on the resulting volatile oils suggest that the Greek, Turkish and Italian are strikingly similar and are believed to be of *Salvia triloba* L., and that though both the Yugoslavian and Albanian are of *S. officinalis* L., they display substantial differences. It is also shown by the use of linear discriminant function that the Yugoslavian sage may be subclassified into those grown in the Dalmatia region and those grown elsewhere in Yugoslavia.

INTRODUCTION

SAGE, the dried leaves of a perennial shrub of the genus *Salvia*, is native to the Mediterranean region (Parry, 1969) and is reported to include more than 500 species of which the best known for culninary use is *S. officinalis* L. (Rosengarten, 1969). According to the U.S. Department of Agriculture (Anonymous, 1975), more than 3 million pounds a year are imported into the U.S. mostly from Albania and Yugoslavia but some from Greece, Turkey and Italy as well. Traditionally, the sage from Yugoslavia, particularly the one grown in the Dalmatia region, has been highly esteemed and economically more valuable than the rest.

Though there have been a number of studies on the composition of sage oil, such as those by Brieskorn and Wenger (1960), Rasmussen et al. (1971), Dalferth (1963) on *S. triloba* L. from Greece and more recently Buil et al. (1977) on *S. triloba* L. of a Turkish origin, no comprehensive work on the gas chromatographic characterization of sages of various geographic origins has been published. In this paper, therefore, we describe the use of gas chromatograph for obtaining the compositional profiles and their use for comparing the sages from Yugoslavia, Albania, Greece, Turkey and Italy.

EXPERIMENTAL

A number of lots of sage (lot sizes usually varying 100–200 bales at 110 lb each) imported during 1971–1977 from various areas of the world through the normal commercial channels were sampled by the ASTA schedule (ASTA, 1968) and ground to pass a screen with 1 mm openings using a Wiley laboratory mill, Model 4. A few selected lots were also ground in the production scale mill, Jacobson hammer mill to pass a screen with 0.29 mm openings. The ground samples were steam-distilled (ASTA, 1968) within a day after being ground. The volatile oils thus obtained were saved in glass vials with Teflon-lined caps away from light until analyses by GC, usually within 0–6 months.

A Varian GC Model 2704 with flame ionization detector was fitted with a 0.01 in. × 200 ft stainless steel capillary tubing (Applied Science Laboratories, Inc., State College, PA), coated with Carbowax 20M by the method of Teranishi (1971). The operating conditions were, column at 90°C for 4 min, linear temperature programmed at 4°/min to 180° and held; injector and detector at 250°; nitrogen flow 1.9 ml/min, hydrogen 25 ml/min, air 300 ml/min and make-up gas 17 ml/min; split ratio 1:74; volume injected 0.1 μl. The peaks were recorded with a Varian Model A-25 recorder at 1 mV span, 0.25 in/min and integrated with a Varian Data System CDS 101. Some of the major peaks were identified by peak enrichment method (addition of either pure substances or volatile oil from different spices with previously identified peaks).

RESULTS & DISCUSSION

ALL LOTS analyzed are listed in Table 1 where only those peaks present in more than 0.1% (16 peaks) are included. Each of the columns in this table adds up to about 90%, or, 10% of the peaks areas are taken by the minor peaks numbering over 100. Classification into the type headings is by the original markings at the time of procurement with the exceptions indicated in the footnote. Figure 1 (a–d) shows the typical chromatograms of the Dalmation, Yugoslavian, Albanian and Turkish sages. While it may not be too clear in these figures, all four types displayed an identical set of peaks as judged by the retention times of the integrator printout. This was also true of all the lots of each type. The identity of the peak sets was further confirmed by peak enrichment method, either with pure substances or cross-blending. It should be recognized that this method does not provide sufficient evidence for identification but the degree of certainty may still be acceptable in profile comparison which is the concern of this study. What varied among lots or types then was of a quantitative nature.

Since it was occasionally up to 6 months after steam distillation before GC analysis was carried out, we were concerned about the possible compositional changes of the volatile oil during storage; however, periodic analyses of a stored oil indicated, as shown in Table 2, no significant compositional changes occurred during storage. Or, the changes are not much greater than the precision of the GC analysis as shown by the triplicate runs at t = 0 mo. The same data suggest additionally that the precision of the GC analysis as used in this study was sufficiently good for profile comparisons of the various types.

A cursory inspection of the data in Table 1 shows that the between-lot variations are much smaller than the between-type variations. This suggests comparison of the averages of each type as in Table 3 would be more useful for detecting possible between-type variations. In Table 1 we note that though only one lot each of the Cretian, Italian, Greek and Turkish was analyzed, they together display striking similarities, apart from the rest. Only the Greek was identified by the supplier as *S. triloba* L. Possibly, all four belong to it as Buil et al. (1977) reported; hence, the heading of "Greek" in Table 3.

Continuing with Table 3, we see that the Greek is characterized by its higher β-pinene, 3.5% against 1.1% and 0.5% for the Yugoslavian and Albanian, respectively; higher myrcene, 2.1% against about 0.6% for the rest; much higher cineole, 42.5% against 10% for the Dalmatian and Yugoslavian and 6.9% for the Albanian; much lower thujone, 2.6%

–Text continued on page 760

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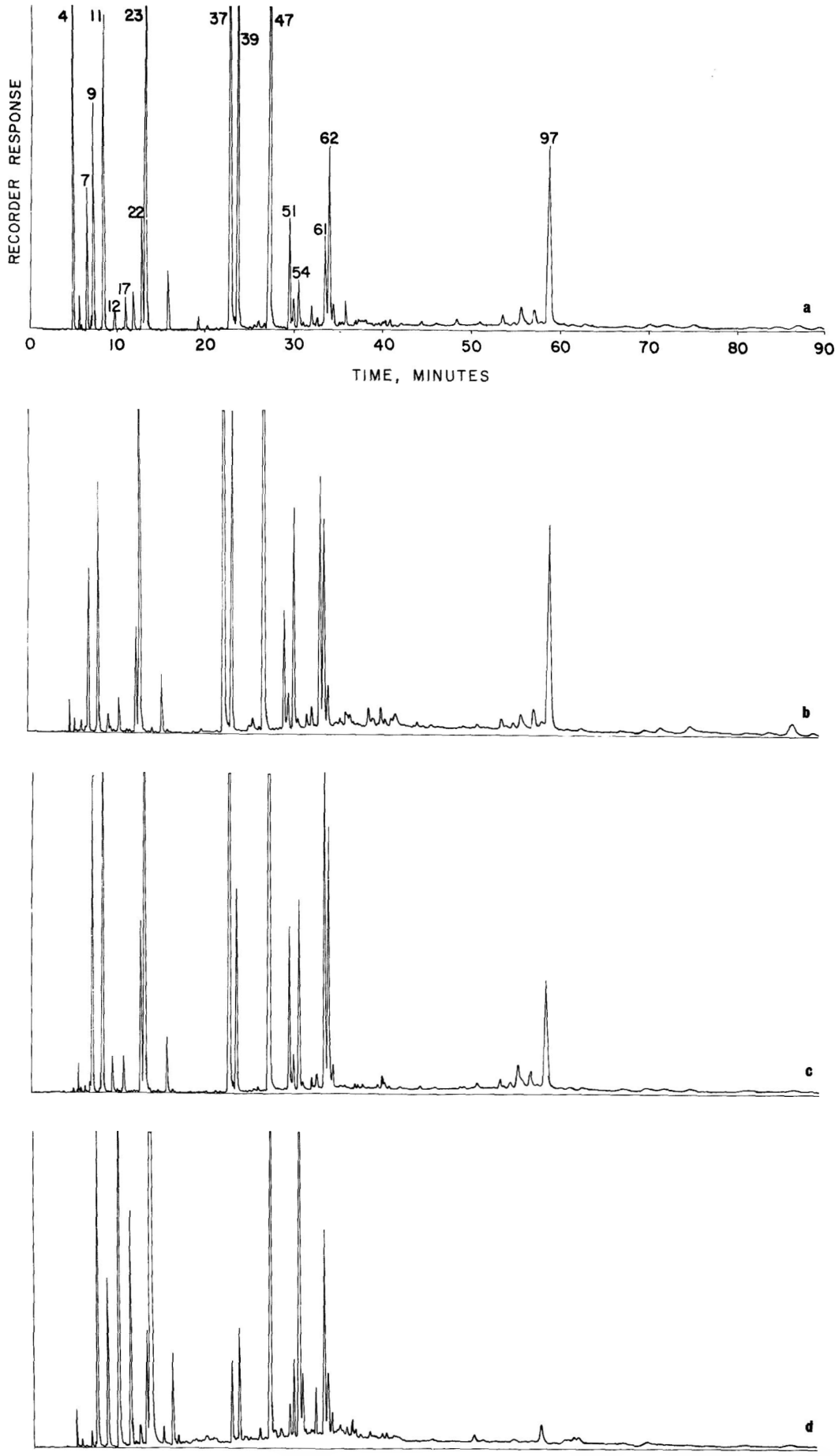

Fig. 1—Typical chromatograms of sage: (a) Dalmatian, Lot 18—76; (b) Yugoslavian, Lot 01—76; (c) Albanian, Lot 51—76; and (d) Turkish, Lot 55—71.

Table 1—Composition, % by area, of volatile oil of sages from various sources

		4	7	α-pinene 9	camphene 11	β-pinene 12	myrcene 17	d-limonene 22	1,8-cineole 23	thujone 37	d-camphor 39	d-linalool 47	51	β-caryophyllene 54	α-terpineol 61	62	97
Dalmatian[a]																	
15-76		1.5	0.7	4.0	4.0	0.6	1.0	1.5	10.2	27.0	14.1	14.3	1.1	0.8	1.8	3.0	4.6
82-75		13.3	6.9	4.3	5.1	0.3	0.4	1.0	9.3	19.4	8.1	14.7	1.3	0.9	1.6	2.1	4.5
11-75		+	+	3.0	4.3	0.4	0.3	1.2	12.9	32.9	12.8	16.4	1.8	0.1	0.6	3.6	1.8
42-75		+	+	2.7	4.0	0.4	0.3	1.1	11.7	31.2	11.8	25.7	1.2	+	0.8	3.6	1.4
17-76		2.8	1.4	3.8	3.1	0.3	0.5	1.0	9.9	25.8	12.8	18.9	0.9	0.6	2.1	2.2	8.1
18-76		3.8	2.2	3.3	5.4	0.3	0.6	1.9	9.3	23.3	8.9	19.2	1.9	1.0	1.5	3.6	6.6
90-75		+	+	3.9	4.7	0.5	0.9	1.9	11.1	29.7	12.3	11.9	2.0	0.5	0.1	6.2	5.2
Yugoslavian[b]																	
01-76		0.3	0.2	2.0	3.7	0.3	0.6	1.6	9.7	24.2	5.9	26.2	1.9	3.7	4.0	3.3	6.4
95-75		0.2	0.1	5.2	9.1	3.3	+	1.2	9.3	19.7	4.4	29.5	2.9	0.3	1.0	3.9	1.6
78-75		+	1.2	4.6	7.5	0.8	0.9	2.7	10.0	24.6	6.3	24.9	2.3	1.7	5.7	2.1	4.3
84-75		+	+	4.2	7.7	0.9	1.0	2.5	13.2	27.0	6.5	26.3	3.0	1.1	5.9	1.9	3.6
09-75		4.0	2.3	2.8	5.3	0.9	0.6	2.8	9.7	18.0	2.3	22.3	2.1	3.0	3.2	3.6	4.3
42-75		6.4	5.4	4.0	7.0	0.5	1.0	2.7	9.2	17.7	1.2	25.0	2.0	5.5	4.3	2.0	0.1
40-73		7.9	4.9	2.7	4.6	0.7	0.4	1.4	7.3	20.1	3.3	23.8	1.4	3.5	4.0	2.4	4.7
Albanian																	
77-77		5.3	2.6	1.3	3.4	0.7	0.7	1.8	5.6	17.3	2.7	28.5	2.5	4.1	6.8	5.7	3.7
57-77		0.2	0.2	1.4	3.6	0.3	0.4	1.6	6.8	15.0	3.1	33.7	2.9	4.0	6.8	5.7	5.6
46-77		+	+	2.1	3.9	0.4	0.6	2.4	9.5	12.7	1.9	21.5	1.6	3.0	5.3	3.5	3.0
51-76		+	+	4.0	6.8	0.6	0.6	2.7	10.1	18.3	3.3	31.1	2.4	2.9	5.6	3.9	2.9
81-77		+	3.6	1.5	3.8	0.6	0.6	1.6	5.5	15.8	2.0	26.1	2.2	4.2	6.2	5.2	4.2
91-75		3.5	7.8	0.6	1.2	0.6	0.5	3.7	5.5	15.2	2.7	35.0	3.6	2.5	9.1	1.4	1.5
79-75		0.6	10.9	0.3	3.8	0.7	0.5	4.3	6.4	15.6	3.2	27.2	3.0	3.6	6.9	3.4	2.2
04-72		+	2.4	1.7	4.4	0.2	0.8	1.5	5.9	18.2	3.3	23.5	2.2	3.4	6.5	4.9	6.7
Cretian	10-72	0.7	+	4.1	4.0	2.3	1.5	2.3	40.7	1.0	1.6	16.8	4.2	5.8	2.0	4.6	0.3
Italian	00-78	13.8	8.5	2.7	1.7	2.5	1.5	1.0	27.6	5.4	3.6	7.4	0.8	7.1	2.1	1.3	1.6
Greek[c]	34-73	1.5	+	6.0	2.7	3.8	2.4	1.7	48.0	2.8	2.6	9.0	1.7	5.0	1.4	7.6	1.3
Turkish	55-71	0.3	0.2	4.6	2.2	5.3	3.0	1.5	54.1	1.1	1.5	7.0	0.5	7.6	2.5	0.8	0.4

[a] Lot identification. The last 2 digits refer to the year of procurement.
[b] Lots 01, 95 and 84 received as Dalmatian.
[c] Additionally, identified as *S. triloba* L.

against over 20% for the Dalmatian and Yugoslavian and 16% for the Albanian; and similary much lower in linalool and peak 97 than the rest.

The Albanian, *S. officinalis* L. like the Dalmatian, is, as expected, much closer to the Dalmatian than the Greek is, but we note that it is lower in cineole, thujone, camphor and higher in linalool, caryophyllene, and α-terpineol than the Dalmatian.

Sage from Yugoslavia was marked, when received, either as "Dalmatian Sage, Produce of Yugoslavia" or as simply "Yugoslavian Sage, Produce of Yugoslavia." We do not know if any significance can be attached to the different markings, but we have often observed that the one marked as Dalmatian is generally fluffier, woollier, providing the quality of "rubbed" sage more acceptable to the consumer. Because the lots 01, 95 and 84, though received as the Dalmatian, appeared more similar to the Yugoslavian, they are grouped under Yugoslavian in Table 1.

Returning to Table 3, we find that the Dalmatian and Yugoslavian are more similar to each other than to either the Albanian or "Greek," but a closer examination shows minor differences in camphene, limonene, peak 51 and unmistakable differences in thujone, especially in camphor and linalool, caryophyllene and terpineol. In view of the lot-to-lot variations as shown in Table 1, questions may be raised as to the justification of this classification; therefore, these two types were tested by linear discriminant function as shown in Table 4. Though a linear combination of all peaks would be more desirable, we considered, instead, several 3-dimensional sets because their computations can be easily managed without a computer, using an ordinary desk-top programmable calculator. In the five triplets con-

—Text continued on page 762

Table 2—Composition, % by area, of volatile oil, lot 51, stored for various length of time before analysis

peak no	0 mo run #1	0 mo run #2	0 mo run#3	9 mo	17 mo
4	+	+	+	+	+
7	+	+	+	+	+
9	3.9	4.0	4.0	4.1	4.0
11	7.0	6.9	6.8	7.1	7.0
12	0.6	0.7	0.6	0.2	0.8
17	0.6	0.6	0.6	0.5	0.8
22	2.7	2.9	2.7	2.7	3.0
23	10.3	10.1	10.1	10.4	10.6
37	18.0	17.9	18.3	17.8	17.8
39	3.3	3.3	3.3	2.9	3.3
47	29.8	29.8	31.1	31.1	29.6
51	2.5	2.5	2.4	2.6	2.6
54	3.1	3.3	2.9	3.5	3.2
61	5.5	5.6	5.6	7.0	5.3
62	3.9	3.9	3.9	5.6	3.4
97	2.9	3.0	2.9	3.0	3.0

Table 3—Average composition, % by area, of volatile oil of sages from various sources[a]

peak no	compound	Dalmatian n = 7 m	Dalmatian n = 7 s	Yugoslavian n = 7 m	Yugoslavian n = 7 s	Albanian n = 8 m	Albanian n = 8 s	"Greek" n = 4 m	"Greek" n = 4 s
4		3.10	4.73	2.71	3.37	1.25	2.01	4.08	6.50
7		1.64	2.45	2.03	2.28	3.46	3.95	2.23	4.18
9	α-pinene	3.57	0.58	3.64	1.16	1.61	1.12	4.35	1.36
11	camphene	4.37	0.77	6.41	1.93	3.86	1.53	2.65	0.99
12	β-pinene	0.40	0.12	1.06	1.01	0.51	0.19	3.48	1.39
17	myrcene	0.57	0.28	0.66	0.34	0.59	0.12	2.10	0.73
22	d-limonene	1.37	0.40	2.13	0.70	2.45	1.06	1.63	0.54
23	1,8-cineole	10.63	1.34	9.77	1.75	6.91	1.85	42.60	11.40
37	thujone	27.04	4.70	21.61	3.63	16.01	1.87	2.58	2.06
39	d-camphor	11.54	2.21	4.27	2.08	2.78	0.56	2.33	0.98
47	d-linalool	17.30	4.52	25.43	2.08	28.33	4.74	10.05	4.58
51		1.46	0.44	2.23	0.56	2.55	0.61	1.80	1.68
54	β-caryophyllene	0.57	0.36	2.69	1.77	3.46	0.62	6.38	1.19
61	α-terpineol	1.21	0.72	4.01	1.64	6.65	1.15	2.00	0.45
62		3.47	1.37	2.74	0.83	4.21	1.47	3.58	3.17
97		4.60	2.40	3.57	2.09	3.73	1.74	0.90	0.65

[a] m = mean, s = standard deviation, n = number of lots averaged.

Table 4—Values of z for linear discriminant function (Dalmatian vs Yugoslavian)

(x_1,x_2,x_3)[a]	λ_1 / λ_2 / λ_3	Dalmatian 15	82	11	42	17	18	90	Yugoslavian 01	95	78	84	09	42	40
(37,39,47)	0.066 / −0.242 / 0.047	−0.96	0.01	−0.16	0.41	−0.51	0.29	−0.46	1.41	1.63	1.27	1.45	1.69	2.06	1.65
(51,54,61)	0.709 / 0.242 / 0.158	1.26	1.39	1.40	1.00	1.11	1.83	1.55	2.87	2.29	2.94	3.32	2.72	3.43	2.47
(23,37,39)	0.021 / −0.092 / 0.270	1.53	0.59	0.69	0.56	1.28	0.45	0.82	−0.43	−0.43	−0.36	−0.45	−0.83	−1.11	−0.81
(39,47,61)	−0.169 / 0.071 / 0.228	−0.95	0.04	−0.86	0.01	−0.34	0.20	−1.21	1.77	1.57	2.00	2.11	1.92	2.55	2.04
(17,22,23)	0.469 / −0.430 / 0.052	0.35	0.24	0.29	0.27	0.32	−0.05	0.18	0.10	0.01	−0.22	0.08	−0.42	−0.22	−0.04

[a] Denotes that the variables considered are the areas of the three peaks listed. Thus, for the data of the first line, $z = 0.066x_1 - 0.242x_2 + 0.047x_3$.

Table 5—Composition, % by area, of volatile oil distilled from low- and high-heat generating mill ground products

Peak no.	lot #51 low heat	lot #51 high heat	lot #01 low heat	lot #01 high heat
4	+	6.5	0.3	+
7	+	3.6	0.2	+
9	4.0	3.1	2.0	0.9
11	6.8	5.1	3.7	1.9
12	0.6	0.4	0.3	0.1
17	0.6	0.3	0.6	0.3
22	2.7	1.4	1.6	0.9
23	10.1	10.7	9.7	6.4
37	18.3	13.2	24.2	19.5
39	3.3	2.4	5.9	4.9
47	31.1	26.0	26.2	25.1
51	2.4	1.6	1.9	1.9
54	2.9	3.6	3.7	5.1
61	5.6	9.6	4.0	6.3
62	3.9	3.3	3.3	4.4
97	2.9	7.0	6.4	12.5

Table 6—Comparison of S. triloba L., lot 55 oil with other published data

	Dalferth (1963)	Buil (1977)	Rhyu (1978)
α-pinene	3.2	6.3	4.6
camphene	2.4	5.5	2.2
β-pinene	7.9	7.4	5.3
sabinene		0.6	+
myrcene		5.6	3.0
γ-terpinene		0.3	0.3
limonene			1.5
1,8-cineole	64.0	42.4[a]	54.1
α-terpinene		0.3	0.2
p-cymene		0.8	1.2
terpinolene		0.2	0.1
thujone	2.3[b]	1.0	1.1
d-camphor	8.2	9.1	1.5
d-linalool	1.4		7.0
β-carylphyllene	2.0	8.2	7.6
α-terpineo			2.5

[a] Limonene and cineole combined

[b] As α-thujone, 2.8% as β-thujone also reported.

sidered we see few overlaps in the z values.

Preliminary investigation with sage and other spices (Rhyu, 1978) indicated that the heat generated in grinding the spices for steam distillation not only reduced the quantitative yield of the oil but also significantly affected its composition. The loss in low-boiling compounds and "gain" in high-boiling compounds occur as a result of a quantitative redistribution which is influenced by the loss of the low-boiling compounds. An example is shown in Table 5. Further evidence may be the greater between-lot variations in the early peak areas, especially those of peaks 4 and 7 as shown in Table 1 in individual values and in Table 3 in standard deviation units, reflecting the varying degrees of loss on grinding and also on distillation because of incomplete condensation in cold traps.

A comparison in Table 6 of the present data on the Turkish sage with other published data (Dalferth, 1963; Buil, 1977) shows reasonably good agreements. It should be pointed out that this table lists only those compounds identified in the present work and that the earlier work cited includes a number of other compounds not identified in the present work.

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SPORULATION OF C. PERFRINGENS IN MEAT . . . From page 757

When the initial pH differences were eliminated, the soy materials evidently were good nutrient sources for sporulation. Perhaps the increased sporulation observed with addition of soy products to a liquid medium is due to absorption of compounds, that are inhibitory to sporulation, by the insoluble constituents of soy products. Such an explanation was proposed when *C. perfringens* sporulation increased in media with added activated carbon (Duncan and Strong, 1968) and with added anion exchange resin (Clifford and Anellis, 1971).

In summary, addition of some commercial soy materials to meats inhibited and addition of others stimulated sporulation of *C. perfringens*. Our data indicated that sporulation was significantly affected when soy proteins that changed the pH of the meat-soy mixtures were used.

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EVALUATION OF A FLUOROMETRIC TECHNIQUE FOR QUANTITATIVE DETERMINATION OF SOY FLOUR IN MEAT-SOY BLENDS

A. C. ELDRIDGE and LEO G. HOLMES

ABSTRACT

Textured soy flour containing 50% protein has been added to fresh ground beef at different levels. Samples were analyzed utilizing fluorescence spectrophotometry. Knowing only what product was added, it was possible to measure the amount of soy flour addition at the 30% level within ±2.4%.

INTRODUCTION

UTILIZATION of textured vegetable proteins in various meat products has increased markedly over the past decade. The National School Lunch Program (FNS, 1971) provides for addition of up to 30% hydrated textured vegetable protein.

Because of government regulations that limit quantities of soy products in some foods (Rakosky, 1970), it is essential to have an analytical procedure that will measure amounts added. Many analytical procedures have been suggested and have been reviewed by P. R. Smith in 1974. Formo et al. (1974) proposed magnesium and fiber analysis for measuring soy flour in meat-soy blends. Lee et al. (1975, 1976) developed an electrophoretic procedure to quantitate and differentiate various vegetable proteins. In 1977, Holmes was issued a patent on a method of determining soy material in foods based on fluorescence measurements. The procedure of Holmes has been studied further and this paper describes the development of a quantitative procedure to measure soy in fresh meat-soy flour blends.

MATERIALS

TEN DIFFERENT textured soy flours (TSF) containing 50% protein were obtained from various manufacturers. Samples contained soy flour only, colorings, vitamins, minerals, or combinations of all of these additives.

Four different lots of fresh ground beef were used in this study. All were obtained from local supermarkets and consisted of ground chuck and/or ground round steak.

Ultra pure quanidine hydrochloride is necessary to provide a low fluorescence background in the ultraviolet region of the spectra. Material from either Schwarz/Mann (136 Liberty St., New York, NY 10006) or Pierce Chemical (Box 117, Rockford, IL 61105) has proven satisfactory.

METHODS

A STANDARD CURVE is prepared for the soy material added to the meat. The dry soy materials are first ground in a Wiley Mill (60 mesh screen). Samples of the ground material (10–50 mg) are weighed accurately and dispersed in 20.0 ml 6M guanidine hydrochloride for 2 min at room temperature (water bath) in a Lourdes Homogenizer (Model MM) at maximum speed. The homogenates are centrifuged, and the extracts are filtered through Whatman 2 V paper and read in a fluorometer (Turner Model 110) with a 7-60 primary filter followed by 2A and 47B filters. This arrangement of filters gives an excitation of 365 nm and a fluorescence emission at 440 nm.

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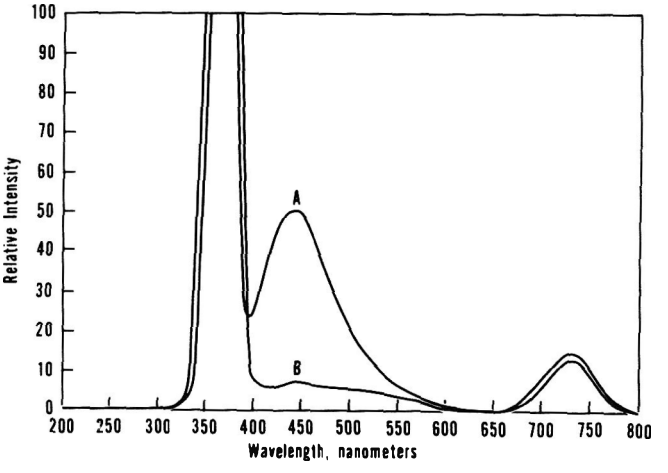

Fig. 1—Fluorescence spectra for extracts of soy flour (2.27 mg/ml) (A) and fresh ground beef (9.79 mg/ml) (B). Excitation is at 360 nm and maximum fluorscence is at 440 nm.

The blend of ground beef-textured soy flour must be ground to very fine particle size to ensure uniform sampling. This was accomplished by passing the blend through a Hobart food grinder (Model 4-B) equipped with a 5/64 in. plate for grinding. The mixture was passed through the grinder five times until the blend had the consistency of a frankfurter emulsion.

A blank was prepared to contain an amount of beef equivalent to the amount of beef in the blend. The amount of blend needed for optimum fluorescence is 1.4g. If one assumes an addition of 30% TSF, then 1.0g or 70% of 1.4g of fresh ground beef is weighed. This is transferred to the homogenizer cup and 20.0 ml of 6M guanidine hydrochloride is added. Homogenize for 2 min at maximum speed (place cup in room temperature water bath to avoid heating). Decant slurry into centrifuge cup, centrifuge 5 min at 8,000 × G, and filter supernatant through Whatman 2 V paper. Dilute the filtrate five times with guanidine hydrochloride. This is the blank and is used to zero the instrument. Unknown samples are read against this blank, thus a net reading is obtained.

Weigh 1.40g of the blend and proceed with extraction and dilution as described above. Read and record the deflections and calculate amount of soy from standard curve.

RESULTS & DISCUSSION

THE FLUORESCENCE SPECTRUM of an extract of defatted soy flour is shown in Figure 1. The extract was prepared from Amsoy variety of soybeans after dehulling, defatting and then extracting with 6M guanidine hydrochloride. The curve shown in Figure 1 was observed on an Aminco-Bowman spectrophoto-fluorometer. All soy protein products (i.e., flours, concentrates and isolates) examined exhibit the same general spectra in the recording fluorometer. Also included in Figure 1 is the spectrum of a 6M guanidine hydrochloride extract of fresh ground beef purchased in a supermarket.

A possible source of error in the analysis could arise from beef that had been stored for long periods. Large fluorescence peaks were observed in the 440-nm region in extracts of a laboratory sample of beef that had been freeze dried, defatted, ground in a Wiley mill and stored at room

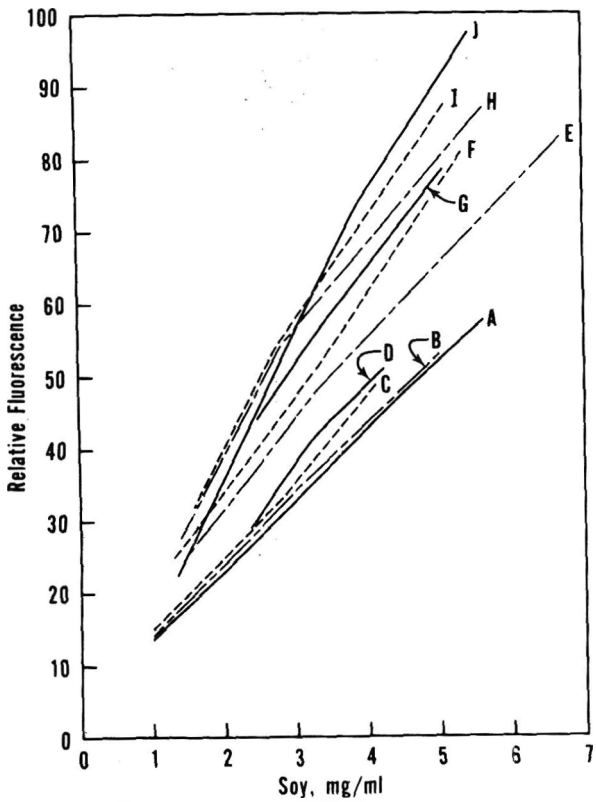

Fig. 2—Relative fluorescence for extracts of 10 texturized soy flour products.

Table 1—Summary of prediction estimates based on various groupings of the data

Basis of calculation	s[a]	Slope	s(b)[b]	Factor 95% limits on predicting P[c]	RSD[d] for P (%)
A single series ignoring samples	0.0891	0.8489	0.1050	1.62	27
Two series					
E,F,G,H,I,J	0.0428	0.8295	0.0516	1.27	13
A,B,C,D	0.0224	0.8425	0.0266	1.13	6.3
Single sample	0.0156	0.8405	0.0186	1.089	4.4

[a] Standard deviation based on scatter about fitted line or lines
[b] Standard error of slope
[c] Percent soy added
[d] Relative standard deviation

Table 2—Mean percent soy based on n analyses of nine preparations

n	Meat-TSF samples	Mean % soy		Mean difference	95% limits factor
		Observed	Actual	Obs-act.	for dif
8	A	22.33	17.53	4.80*	1.51
9	B	30.27	29.08	1.19	1.43
3	C	22.80	20.00	2.80*	2.47
3	D	22.83	20.00	2.83*	2.47
5	E	10.06	14.40	−4.34*	1.92
3	C_1	17.73	20.00	−2.27	2.47
3	D_1	18.93	20.00	−1.07	2.47
5	W	32.14	31.50	0.64	1.92
5	X	25.88	27.00	−1.12	1.92
2	Y	31.15	29.80	1.35	3.03
2	Z	28.70	29.30	−0.60	3.03

* Significant at 0.05 level

temperature for about 2 yr. The reason for this fluorescence is probably because of browning reaction product formation in meat, which has been studied by Dillard and Tappel (1976). Results indicated that the effects of storage of meat-soy flour blends must be evaluated.

Figure 2 shows standard curves obtained for 10 different texturized soy flours. Samples A, B, C and D were uncolored, and these four products tend to give one family of curves. Samples E, F, G, H, I and J are samples containing caramel coloring, and they yielded a second family of curves. Carbohydrate-amine, browning, and caramelization are known to cause fluorescence (Chio and Tappel, 1969).

Each manufacturer of TSF probably adds different quantities of caramel color from various sources to their products, which accounts for the differences noted in the curves in Figure 2.

Data presented in Figure 2 were analyzed statistically, and the results are given in Table 1. As seen, if one knows what product was added, a relative standard deviation of about 4% could be expected when a sample is examined.

After these initial studies, nine different meat-textured soy flour blends were prepared from four different lots of ground beef and from five different TSF's. Samples were prepared and presented to four colleagues in a completely randomized design. The blends contained different amounts of TSF and were analyzed as unknown samples (i.e., the analyst did not know the TSF content). Some samples were examined twice, others nine times. When all the data were examined (48 values), a standard deviation of 2.42 per analysis was obtained. If the data from a heterogenous sample, Sample A (improper grinding), is omitted, the standard deviation is lowered to 2.10.

Table 2 shows a summary of the means of observed and actual values for the various preparations. Also shown is the mean difference. Whenever the mean difference exceeds the 95% limits factor, the difference is significantly greater than zero. This was the result for four preparations (A, C, D, and E). Samples C and D were tested a second time (C_1 and D_1) and showed a reversal of the difference.

The fluorescence method o Holmes (1977) has been studied further and a quantitative procedure developed. Textured soy flour containing 50% protein was added to ground beef at different levels. Knowing only what product was added, it was possible to measure the amount of soy flour addition at the 30% level within ±2.4%.

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SALT CONCENTRATION, pH, AND FLOUR CONCENTRATION EFFECTS ON NITROGEN SOLUBILITY AND EMULSIFYING PROPERTIES OF PEANUT FLOUR

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ABSTRACT

Nitrogen solubility in 2% dispersions and emulsifying properties in 8% dispersions of unheated peanut flour (50.9% protein) in water, 0.1M NaCl (low salt), and 1.0M NaCl (high salt) were measured over a pH range 2.0–10.0. Flour concentration effects were measured over the same range in water only at 2, 4, 6 and 8% flour levels (1, 2, 3, 4% protein). Both nitrogen solubility and emulsion-forming and thickening properties of the water and low-salt suspensions were lower at pH 4.0 than at levels below and above 4.0, increases in solubility being quite substantial below pH 3.0 and above pH 6.0. In the high-salt suspensions, nitrogen solubility was low at pH 3.0 and below, high at pH 6.0 and above, with near linear increase between 3.0 and 6.0; no emulsions formed at pH 2.0–4.0, with generally low-viscosity emulsions at pH 5.0–10.0. A flour concentration of 2% (1% protein) in water could support emulsion formation only at pH levels of 2, 3, 8, 9 and 10, and the quality of these emulsions was extremely poor (low viscosity); at higher flour concentrations (4, 6, 8%), emulsion capacities and viscosities were improved substantially by adjusting the pH to levels below or above 4.0, with more viscous emulsions produced below pH 4.0 than above. Multiple regression analyses confirmed that deviations of pH from 4.0 were better predictors of emulsifying properties of peanut flour than simple pH alone. Altering the electrovalent properties, ionic environment, or flour concentration strongly influenced these functional characteristics.

INTRODUCTION

PEANUTS are an important oilseed grown throughout the world as a source of oil and protein. Interest in their protein component as a nutritious and functional ingredient in foods has increased in recent years since several commercial peanut protein products are now available to food processors (Anon., 1976; Ayres and Davenport, 1977). The bland flavor and low concentration of certain anti-nutritional factors of peanuts make them more desirable as food supplements than some other plant proteins in use today (Conkerton and Ory, 1976; Calloway et al., 1971).

Early research with peanut proteins investigated hydration properties (Burnett, 1945), viscosity patterns (Burnett et al., 1945), and solubility profiles (Burnett and Fontaine, 1944; Fontaine and Burnett, 1944). More recently, fiber spinning (Fletcher and Ahmed, 1977; Ahmed and Fletcher, 1977), gelation (Schmidt et al., 1978) and emulsification and foaming properties (McWatters and Cherry, 1977; McWatters et al., 1976; Ramanatham et al., 1978) have been investigated. Various processing factors such as heat (Fontaine et al., 1944; McWatters and Cherry, 1975; Cherry et al., 1975), extraction techniques (Rhee et al., 1972), pH (Rhee et al., 1973), and enzymatic and chemical hydrolysis (Beuchat, 1977) have been shown to alter functional properties of peanut proteins.

One of the important functions specialized proteins perform in certain food systems is emulsification. Acting as surface-active agents, these types of proteins have the capacity to lower the interfacial tension between immiscible liquids, such as oil and water, thereby enabling a mixture of the two. Many factors influence emulsification capacities including equipment design, rate of oil addition, temperature, pH, protein type, solubility and concentration, kind of oil used, salt (type and concentration), sugars, and water content (Saffle, 1968; Kinsella, 1976).

Previous studies conducted in our laboratory revealed that peanut protein's emulsion capacity and solubility were significantly influenced by pH and salt concentration (McWatters et al., 1976). Since the initial study involved a limited number of pH treatments, the present investigation was designed to characterize pH effects on protein solubility and emulsifying properties over a wider pH range, using three dispersion media to test ionic environment effects. Variations in flour (protein) concentration were also investigated.

MATERIALS & METHODS

Flour preparation

Peanuts (Florunner variety, 1976 crop) were heated in a forced draft oven at 100°C for 15 min to facilitate skin removal. Blanched peanuts (skins removed) were coarsely ground in a Hobart cutter (model 84142), partially defatted by extraction with hexane, air dried, reground in a pilot-scale stone mill (Morehouse-Cowles, Los Angeles, CA) set at a stone clearance of 0.5 mm, then re-extracted with hexane, and air dried again. Following screening, the material which rode a 60-mesh screen was reground in the Morehouse mill set at a stone clearance of 0.25 mm, screened again, and the flour stored in glass jars at 1°C until used. The proximate composition of the flour was 6.8% moisture, 50.9% protein (N × 5.46), 1.5% oil, 4.5% ash, and 36.3% carbohydrate (by difference).

Nitrogen solubility

Levels of soluble nitrogen at pH values ranging from 2.0–10.0 were determined on 2% suspensions (w/v) of peanut flour dispersed in water, 0.1M NaCl, and 1.0M NaCl. Solutions for nitrogen assay were obtained by adding 6N HCl to flour suspensions adjusted to pH 2.0–5.0 and either 1N HCl or 1N NaOH for all other pH adjustments. Solutions were stirred gently for 1 hr at 23°C, then centrifuged at 13,000 × G for 10 min to remove undissolved flour residues; 25-ml samples of the pooled supernate were analyzed for nitrogen content by the Kjeldahl procedure (AOAC, 1975). The percentage of nitrogen was calculated and plotted against corresponding pH values.

Emulsion capacity

In order to determine the effects of flour concentration, ionic environment, and pH on emulsion properties, 2, 4 and 6% flour suspensions in water and 8% suspensions in water, 0.1M NaCl, and 1.0M NaCl were each adjusted to pH values ranging from 2.0–10.0. Emulsion capacities, expressed as ml of oil per mg of protein, were determined by adding refined peanut oil from a buret (0.5 ml/sec) to 25 ml portions of these suspensions, while blending at low speed in an Osterizer blender, to the point at which the emulsion separated into distinct oil and water phases.

Emulsion viscosity

Emulsions for viscosity measurements were prepared as described above, terminating the additions of oil at 90% of the amount which resulted in phase separation. Viscosities were determined at 23°C using a Brookfield Model RVT Viscometer and Model C Helipath Stand with a T-B spindle turning at 2.5 rpm. Results were

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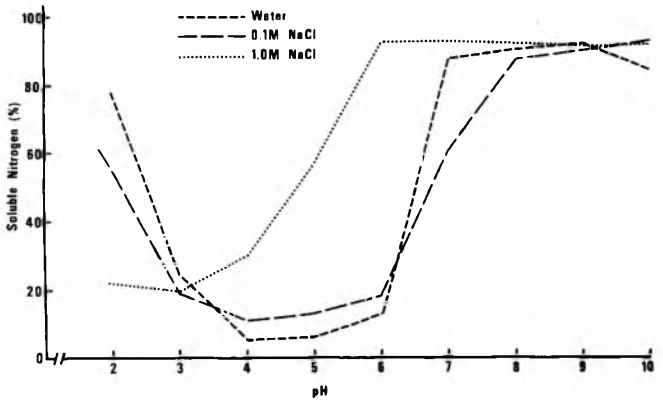

Fig. 1—Nitrogen solubility of peanut flour suspended in distilled water, 0.1M NaCl, and 1.0M NaCl (2% suspensions, w/v) in the pH range 2.0–10.0.

expressed in centipoises (cps) as the averages of the three or four readings obtained per sample.

Complete details of procedures employed in the preparation and examination of samples for measurements of emulsion properties, as well as for determinations of nitrogen solubilities as noted above, were described in a related study of soy flour (McWatters and Holmes, 1979).

Statistical analysis

Multiple regression analysis was used to quantify the relationships between nitrogen solubility and emulsifying properties of peanut flour dispersed in water and salt solutions and various independent variables. A step-down computer program was used in the selection of equations or models of best fit as described by Cherry et al. (1975), McWatters and Cherry (1975), and McWatters et al. (1976). For each of the models, a number of combinations of independent variables was used in combination with step-down elimination. Relative importance of the variables included was determined by comparison of corresponding regression coefficients and of β-values of the variables.

Independent variables used in multiple regression models included pH, absolute deviations of pH above 4.0 (set equal to zero for pH of 4.0 or less), absolute deviations of pH below 4.0 (set equal to zero for pH of 4.0 or above), salt level (0, 0.1 and 1.0M NaCl), interaction of deviations of pH from 4.0 with M NaCl, flour concentration (2, 4, 6 and 8%), quadratic and cubic powers of these variables, and the log (natural) of pH. Dependent variables included percentage soluble nitrogen, emulsion capacity, and emulsion viscosity. Since similar responses were noted for the water and low-salt treatments, two separate sets of models were developed, one for the water and low-salt treatments combined and another for the high-salt treatment.

RESULTS & DISCUSSION

Nitrogen solubility

Nitrogen solubility profiles of peanut flour dispersed in water, 0.1M NaCl, and 1.0M NaCl solutions are shown in Figure 1. As indicated, nitrogen solubility response to changing levels of pH in the water and low-salt suspensions was similar and substantially lower than that of the high-salt suspensions in the pH range of 4.0 to 6.0. In the high-salt samples (1.0M NaCl), the percentages of soluble nitrogen were lowest at pH 2.0 and 3.0 but increased steadily as the pH was increased from 3.0 to 6.0 (Fig. 1). Maximum solubility for the high-salt treatment was noted at pH 6.0, with no increases noted as the pH was adjusted to higher levels. Of the three dispersion media investigated, water solubilized the greatest percentage of nitrogen at pH 2.0 and 3.0 and the least at pH 4.0–6.0. The high-salt solution (1.0M NaCl) solubilized the greatest percentage of nitrogen between pH 4.0 and 7.0, but at higher pH levels, little difference was noted in levels of soluble nitrogen among the three dispersion media. Similar trends were reported by Rhee et al. (1972).

Table 1—Influence of pH and salt concentration on viscosities (cps) of peanut flour emulsions[a]

pH	Water	0.1M NaCl	1.0M NaCl
2	133,280	99,680	no emulsion
3	96,800	29,440	no emulsion
4	1,920	no emulsion	no emulsion
5	6,400	3,200	2,400
6	2,400	2,080	4,960
7	8,320	5,760	6,080
8	24,320	10,080	4,960
9	31,680	8,640	2,400
10	26,400	4,800	1,600

[a] 8% flour suspensions (w/v); for comparison, a commercial mayonnaise had a viscosity of 44,640 cps and two commercial salad dressings, 6,400 and 16,800 cps.

Emulsion capacity and viscosity

pH and salt concentration effects. Emulsion capacities of peanut flour dispersed in water, 0.1M NaCl, and 1.0M NaCl solutions (8% suspensions, w/v) are shown in Figure 2. Suspensions of peanut flour in water had higher emulsion capacities than either of the salt suspensions at pH levels of 2.0–4.0 and 8.0–10.0. No emulsions were formed by the high-salt samples at pH 2.0–4.0 and by the low-salt suspensions at pH 4.0. Emulsion-forming properties of water and low-salt suspensions were improved by adjusting the pH to levels below or above pH 4.0. All suspensions above pH 4.0 increased in emulsifying capacity although high-salt samples peaked at pH 6.0, then declined gradually as pH levels were increased to 10.0.

Emulsion viscosities of peanut flour dispersed in water, 0.1M NaCl, and 1.0M NaCl are shown in Table 1. In most instances, water dispersions produced more viscous emulsions than either of the salt suspensions. At all pH levels except 6.0 and 7.0, emulsion viscosities decreased as salt concentration increased. The poorest emulsifying properties in terms of both emulsion formation and thickening were demonstrated by the high-salt suspensions. By contrast, thick, mayonnaise-like emulsions with viscosities ranging from 24,320–133,280 cps were produced by the water dispersions at pH 2.0, 3.0, 8.0, 9.0 and 10.0 and by the low-salt suspensions at pH 2.0 and 3.0 (99,680 and 29,440 cps, respectively). Pourable, salad dressing-like emulsions were produced by the water suspensions at pH 4.0–7.0 and by both salt suspensions at pH 5.0 and above.

These data indicate that altering the electrovalent properties of peanut protein by shifts in pH as well as changing the ionic environment of the protein strongly influenced the emulsifying properties of peanut flour. Emulsion capacity was poorest at pH 4.0, a level which is near the isoelectric point of the predominate native peanut protein (arachin); at this point proteins have a net electrical charge of zero, are at a point of minimum solubility and reactivity, and thus are easy to precipitate. Shifting the pH away from the isoelectric point apparently improved emulsion capacity of peanut flour suspended in water by giving the protein an electrical charge and possibly by increasing the protein's solubility. Changing the ionic environment by increasing the salt concentration adversely affected emulsifying properties of peanut protein; emulsion capacity was in fact totally inhibited at some pH levels by the presence of salt.

pH and flour concentration effects. Emulsion capacities and viscosities of peanut flour dispersed in water at 2, 4, 6 and 8% flour concentrations (or 1, 2, 3 and 4% protein

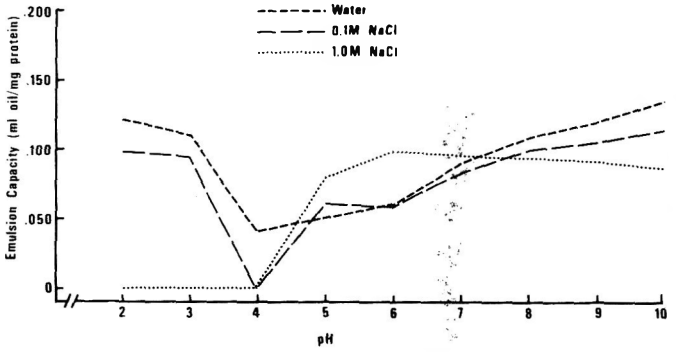

Fig. 2—Emulsion capacity (ml oil per mg total protein) of 25 ml of peanut flour suspended in distilled water, 0.1M NaCl, and 1.0M NaCl (8% suspensions, w/v) in the pH range 2.0—10.0.

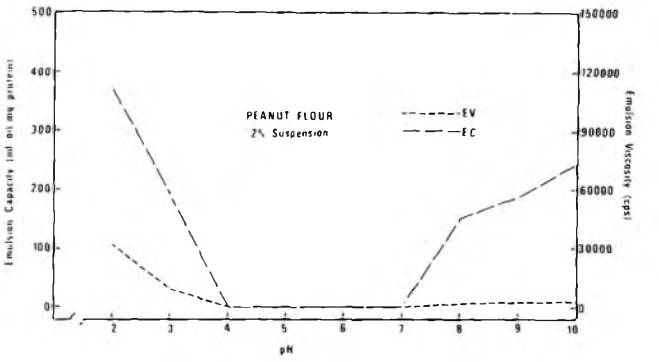

Fig. 3—Emulsion capacity (ml oil per mg total protein) and viscosity of emulsions prepared from 25 ml of peanut flour suspended in water (2% suspensions, w/v) in the pH range 2.0—10.0.

concentrations) are shown in Figures 3—6. Emulsion capacity of 2% suspensions (Fig. 3) was highest at pH 2.0 but was reduced as the pH was raised from 2.0 to 4.0. Between pH 4.0 and 7.0, no emulsions were formed; above pH 7.0, emulsion capacity increased as pH level increased, though not to the extent exhibited at pH 2.0.

The quality of emulsions formed, as measured by viscosity, was extremely poor at the 2% flour concentration (Fig. 3). The most viscous emulsions were produced at pH 2.0 (32,160 cps), but emulsion consistencies became less viscous as the pH was increased from 2.0 to 4.0. Emulsion viscosities were quite low at pH 8.0, 9.0 and 10.0 where only a slight thickening occurred. This flour concentration, therefore, was too low to promote emulsion formation at several pH levels; it also exhibited poor thickening properties at those pH levels where emulsions were formed.

Emulsion capacity of 4% suspensions (Fig. 4) was highest at pH 2.0 and, like the 2% suspension, was reduced as the pH was increased from 2.0 to 4.0. No emulsions were formed at pH 4.0 and 5.0, but above these levels, emulsion formation did occur with emulsifying capacity increasing as pH increased from 6.0 to 10.0.

Though the quantity of oil emulsified by 4% suspensions at pH 10.0 approached that of comparable suspensions at pH 2.0, the viscosities of the 4% emulsions were decidedly different (Fig. 4). Whereas emulsions formed at pH 2.0 or 3.0 were thick and mayonnaise-like in appearance and consistency (73,120 and 46,720 cps, respectively), those formed at pH 10.0 were thin and salad dressing-like (14,400 cps). The least viscous of the emulsions formed at this flour concentration were produced by suspensions adjusted to pH 6.0 and 7.0. Increasing the pH to 8.0, 9.0 or 10.0 increased emulsion thickness slightly.

Emulsion capacity of 6% suspensions (Fig. 5) was greatest at the two extreme pH levels (2.0 and 10.0) and least at pH 4.0 where no emulsions were formed. Adjusting the pH to levels either below or above pH 4.0 markedly improved emulsifying capacity of peanut flour at this concentration.

The consistencies of emulsions formed by 6% peanut flour suspensions at pH levels below 4.0 differed substantially from those formed above 4.0 (Fig. 5). At pH 2.0 and 3.0, extremely viscous, mayonnaise-like emulsions were produced (108, 480 and 76,800 cps, respectively) whereas thin, salad dressing-like emulsions were formed at pH 5.0, 6.0 and 7.0 (2,400—5,120 cps). Thickness of emulsions was increased somewhat by raising the pH to higher levels (8.0, 9.0 or 10.0) where viscosities ranged from 14,400—23,200 cps.

Like the 6% flour suspensions, emulsion capacity of 8% suspensions (Fig. 6) was greatest at the two extreme pH levels (2.0 and 10.0) and least at pH 4.0. This flour concen-

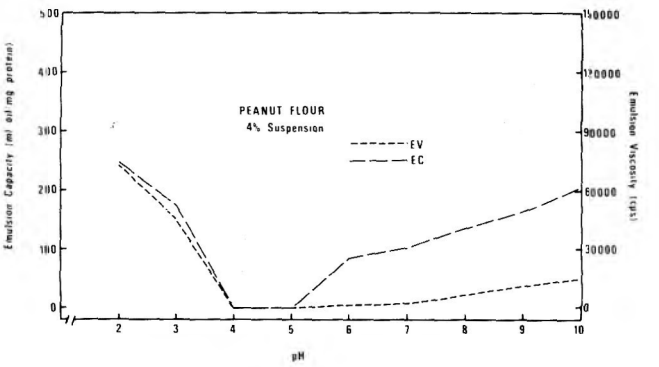

Fig. 4—Emulsion capacity (ml oil per mg total protein) and viscosity of emulsions prepared from 25 ml of peanut flour suspended in water (4% suspensions, w/v) in the pH range 2.0—10.0.

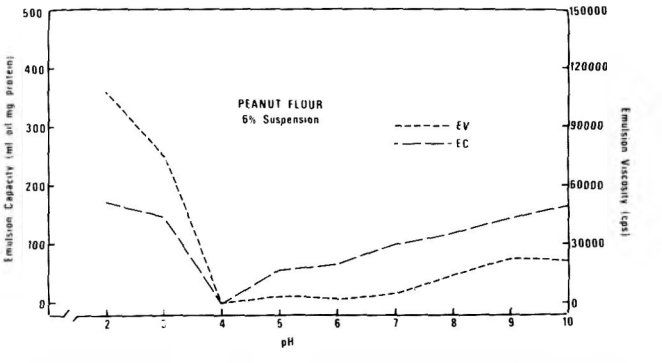

Fig. 5—Emulsion capacity (ml oil per mg total protein) and viscosity of emulsions prepared from 25 ml of peanut flour suspended in water (6% suspensions, w/v) in the pH range 2.0—10.0.

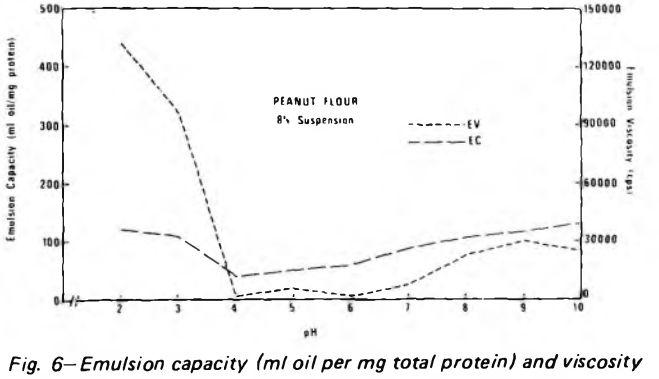

Fig. 6—Emulsion capacity (ml oil per mg total protein) and viscosity of emulsions prepared from 25 ml of peanut flour suspended in water (8% suspensions, w/v) in the pH range 2.0—10.0.

Table 2—Multiple regression models for prediction of nitrogen solubility (2% suspensions) and emulsifying properties (8% suspensions) of peanut flour as influenced by pH and salt concentration.

Dependent variable	Salt concentration	Variable description	Regression coefficient	Beta value	t-value[a]
% Soluble nitrogen (R^2 = 0.9130; adjusted R^2 = 0.9048; standard error of estimate = 11.04906)	0.0 and 0.1M NaCl	Constant	1.96429		
		$(pH\text{-}4.0)^2$	10.90121	3.78734	11.366**
		$(pH\text{-}4.0)^3$	−1.42321	−2.86733	−8.801**
		$(4.0\text{-}pH)^2$	16.32256	0.58101	9.746**
% Soluble nitrogen (R^2 = 0.9816; adjusted R^2 = 0.9776; standard error of estimate = 4.81500)	1.0M NaCl	Constant	23.22283		
		pH-4.0[b]	51.45546	3.55315	11.993**
		$(pH\text{-}4.0)^2$	−12.00318	−4.70766	−6.234**
		$(pH\text{-}4.0)^3$	0.88571	2.01443	4.068**
Emulsion capacity (R^2 = 0.9383; adjusted R^2 = 0.9280; standard error of estimate = 0.00908)	0.0 and 0.1M NaCl	Constant	0.03012		
		pH-4.0[b]	0.02552	1.65175	8.245**
		4.0-pH[c]	0.11532	2.30364	10.397**
		M NaCl	−0.14167	−0.21224	−4.679**
		$(pH\text{-}4.0)^2$	−0.00148	−0.54490	−2.991**
		$(4.0\text{-}pH)^2$	−0.03611	−1.36004	−6.624**
Emulsion capacity (R^2 = 0.9875; adjusted R^2 = 0.9849; standard error of estimate = 0.00544)	1.0M NaCl	Constant	0.00122		
		pH-4.0[b]	0.09237	4.64544	19.062**
		$(pH\text{-}4.0)^2$	−0.02604	−7.43661	−11.972**
		$(pH\text{-}4.0)^3$	0.00218	3.60913	8.861**
Emulsion viscosity (R^2 = 0.9556; adjusted R^2 = 0.9499; standard error of estimate = 8,896.60459)	0.0 and 0.1M NaCl	Constant	692.81046		
		pH-4.0[b]	4,854.62616	0.26769	5.168**
		4.0-pH[c]	73,424.31373	1.24945	22.198**
		(pH-4.0)(M NaCl)	−35,138.46154	−0.17240	−3.768**
		(4.0-pH)(M NaCl)	−280,800.00000	−0.35837	−7.058**
Emulsion viscosity (R^2 = 0.9452; adjusted R^2 = 0.9379; standard error of estimate = 564.16676)	1.0M NaCl	Constant	−15.09434		
		pH-4.0[b]	3,405.98383	15.76061	15.761**
		$(pH\text{-}4.0)^2$	−540.10782	−14.19509	−14.195**

[a] Double asterisk denotes significance at 1% confidence level.
[b] The variable described as "pH-4.0" is given a value of zero unless the pH is greater than 4.0.
[c] The variable described as "4.0-pH.. is given a value of zero unless the pH is less than 4.0.

tration was the only one of the four evaluated which produced an emulsion at pH 4.0. Adjusting the pH to levels either below or above 4.0 improved emulsifying capacity of peanut flour at this concentration.

Though the quantity of oil emulsified by the 8% flour suspensions at pH 2.0 and 3.0 was similar to that emulsified at pH 8.0, 9.0 and 10.0, the quality of the resulting emulsions as measured by viscosity was vastly different (Fig. 6). Emulsions formed at pH 2.0 and 3.0 were among the most viscous produced in the study (133,280 and 96,800 cps, respectively); those produced at pH 8.0, 9.0 and 10.0, on the other hand, had much lower viscosities, ranging from 24,320–31,680 cps. As noted in emulsion viscosity measurements of lower flour concentrations at pH 5.0, 6.0 or 7.0, emulsions produced by the 8% suspension at these same pH levels were likewise quite thin and salad dressing-like in consistency, having viscosities ranging from 2,400–8,320 cps.

In comparing emulsion capacities among the various flour concentrations, quantities of oil emulsified increased as flour concentration increased, though not proportionately. When expressed on a unit protein basis, however, emulsion capacity actually decreased with increasing flour concentration. Emulsion viscosities, on the other hand, increased as the concentration of flour increased.

Multiple regression analysis

The best fitting models for prediction of nitrogen solubility and emulsifying properties of peanut flour are shown in Table 2 (pH and salt concentration effects) and 3 (pH and flour concentration effects). In these models, the statistical significance of cubic and quadratic powers of independent variables indicates that as the value of an independent variable is increased, the marginal effects of that variable decline, i.e., the response of the dependent variable to increasing levels of the independent variable may be depicted as a curvilinear graph rather than a straight line.

In most of the models presented, absolute deviations of pH from 4.0, rather than pH itself, are used as independent variables with these deviations being separated into two variables: one for pH values above 4.0 and one for those below 4.0. This simply indicates that the effects of pH on the dependent variable, whether linear or curvilinear, depend on whether pH is above or below 4.0.

R^2 values indicate the percentage of variation in a dependent variable accounted for by its regression on the independent variables; the higher the value, the greater the accountability. All the models for each dependent variable produced very high R^2 values (>0.90) and in some instances accounted for as much as 98% of the variation. The β-values and t-values in the models suggest that equations including absolute deviations of pH from 4.0 would yield reliable estimates of nitrogen solubility and emulsification properties of peanut flour, given the flour (or protein) concentration and salt concentration.

Results of this study are in agreement with the findings of McWatters and Cherry (1975, 1977), McWatters et al. (1976), Ramanatham et al. (1978), and McWatters and Holmes (1979), who reported that emulsifying capacity of peanut protein is pH- and protein concentration-dependent, is adversely affected by high salt concentration at most pH levels, is less than that of soy flour, and cannot be predicted solely on the basis of protein solubility level; solubility appears to contribute more to the quality of emulsions formed than to quantities of oil emulsified. Thus, emulsifying capacity of peanut flour is influenced by complex interactions involving pH, ionic environment, protein concentration, level and character of soluble proteins, particle size,

Table 3—Multiple regression models for prediction of emulsifying properties of peanut flour suspended in water as influenced by pH and flour concentration

Dependent variable	% Flour concentration	Variable description	Regression coefficient	Beta value	t-value[a]
Emulsion capacity	2	Constant	−0.01393		
(R^2 = 0.9572; adjusted R^2 = 0.9515;		4.0-pH[b]	0.19556	17.34891	17.349**
standard error of estimate = 0.02905)		$(pH-4.0)^2$	0.00766	12.50002	12.500**
Emulsion capacity	4	Constant	−0.00768		
(R^2 = 0.9781; adjusted R^2 = 0.9734;		pH-4.0[c]	0.03511	0.94359	19.470**
standard error of estimate = 0.01349)		4.0-pH[b]	0.24402	2.02402	11.115**
		$(4.0-pH)^2$	−0.05884	−0.92027	−5.276**
Emulsion capacity	6	Constant	0.01630		
(R^2 = 0.9775; adjusted R^2 = 0.9727;		pH-4.0[c]	0.02602	1.03756	21.139**
standard error of estimate = 0.00921)		4.0-pH[b]	0.18954	2.33270	13.648**
		$(4.0-pH)^2$	−0.05635	−1.30762	−7.402**
Emulsion capacity	8	Constant	0.03609		
(R^2 = 0.9831; adjusted R^2 = 0.9795;		pH-4.0[c]	0.01673	1.11263	26.135**
standard error of estimate = 0.00479		4.0-pH[b]	0.10787	2.21354	13.838**
		$(4.0-pH)^2$	−0.03246	−1.25587	−8.197**
Emulsion viscosity	2	Constant	97,283.17460		
(R^2 = 0.9697; adjusted R^2 = 0.9632;		pH	−45,818.53776	−12.21420	−12.828**
standard error estimate = 1,912.56359)		$(pH)^2$	6,731.77489	21.91496	10.368**
		$(pH)^3$	−309.42761	−10.44652	−8.639**
Emulsion viscosity	4	Constant	−860.65359		
(R^2 = 0.9844; adjusted R^2 = 0.9823;		pH-4.0[c]	2,236.41457	5.05069	5.051**
standard error of estimate = 3,415.54737)		4.0-pH[b]	40,580.39216	28.28263	28.283**
Emulsion viscostiy	6	Constant	1,576.20915		
(R^2 = 0.9657; adjusted R^2 = 0.9612;		pH-4.0[c]	3,239.77591	3.36011	3.360**
standard error estimate = 7,437.37746)		4.0-pH[b]	59,006.27451	18.88610	18.886**
Emulsion viscosity	8	Constant	3,626.14379		
(R^2 = 0.9540; adjusted R^2 = 0.9478;		pH-4.0[c]	4,177.70308	3.07387	3.074**
standard error of estimate = 10,483.59176)		4.0-pH[b]	71,664.31373	16.27259	16.273**

[a] Double asterisk denotes significance at 1% confidence level.

[b] The variable described as "4.0-pH" is given a value of zero unless the pH is less than 4.0.

[c] The variable described as "pH-4.0" is given a value of zero unless the pH is greater than 4.0.

processing treatments, and nonprotein components occurring naturally in the seed. These factors would be important considerations to food processors anticipating the use of peanut proteins in certain food systems such as meat and bakery products, whipped toppings, or coffee whiteners where emulsifying properties were specifically required.

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INFLUENCE OF pH AND SALT CONCENTRATION ON NITROGEN SOLUBILITY AND EMULSIFICATION PROPERTIES OF SOY FLOUR

KAY H. McWATTERS and MAC R. HOLMES

ABSTRACT

Nitrogen solubility and emulsifying properties of soy flour were measured over a pH range of 2.0–10.0 and in three dispersion media including water, 0.1M NaCl (low salt) and 1.0M NaCl (high salt). Nitrogen solubility and emulsion capacity of water and low-salt suspensions were similar and were quite low at pH 4.0 but improved significantly at pH levels below or above 4.0. In high-salt suspensions, nitrogen solubility and emulsion capacity generally increased from pH 2.0 to pH 10.0. Whereas no emulsions were formed by water and low-salt suspensions at pH 4.0, thick mayonnaise-like emulsions were formed by the high salt treatment at this pH. Except for the water and low-salt suspensions at pH 4.0 and the low-salt suspension at pH 5.0, all emulsions formed were mayonnaise-like in consistency and compared favorably to a commercial mayonnaise. Multiple regression analyses showed that pH was the primary determinant of nitrogen solubility and emulsifying properties of soy flour. The influence of soluble nitrogen on emulsion capacity and viscosity was difficult to discern since it too was strongly influenced and confounded by pH effects. Changing the electrovalent properties of the protein, either by shifts in pH or by modifications in salt concentration, appeared to be the most significant factor influencing these functional characteristics.

INTRODUCTION

ONE of the important functions which proteins perform in a variety of food applications is emulsification. By possessing the capacity to lower the interfacial tension between hydrophobic and hydrophilic components in foods, proteins are effective surface-active agents. Functions performed in this role include emulsion and foam formation.

Various factors and conditions influence emulsifying capacities of proteins and have been reviewed by Saffle (1968). These include equipment design, rate of oil addition, temperature, pH, protein source, solubility and concentration, kind of oil used, salt (type and concentration), sugars, and water content. Therefore, emulsifying capacity is not solely a property of the protein under test but rather is a property of the emulsion system, the equipment, and method used to produce the emulsion (Tornberg and Hermansson, 1977; Pearce and Kinsella, 1978). Mattil (1971) has appropriately suggested that an investigation of the functional properties of any protein can be made more efficient if a systematic study is first made of the solubility properties of that protein in a variety of ionic environments. The manner in which proteins behave appears to be related to the solubility characteristics of the protein, though correlations between the two have been crude (Wolf and Cowan, 1971).

A preliminary study conducted in our laboratory using defatted soybean, peanut, field pea, and pecan flours revealed that each seed exhibited unique functional characteristics and varying responses to pH changes (McWatters and Cherry, 1977). Since the initial study employed only four pH treatments and one dispersion medium, the present investigation was expanded to include nine pH levels and three dispersion media to define more clearly how pH and salt concentration influence emulsion formation. Soy flour was selected because of its wide use by the food industry.

Though no standard methods are available for quantifying emulsifying properties of proteins, various model systems have been used effectively by a number of workers to measure these properties under specific test conditions and have been summarized by Kinsella (1976). A model system was employed in this study also to characterize emulsion formation properties of soy flour.

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EXPERIMENTAL

Flour preparation

Soybeans (Bragg variety, stored at 1°C, 1974 crop) were cracked by passing them through a stone mill set at a clearance of 0.5 mm and dehulled by screen separation. The coarsely ground material was partially defatted with hexane, air dried, reground, reextracted with hexane, air dried again, sifted through a 60-mesh screen, and stored in glass jars at 1°C until used. The proximate composition of the flour was 8.9% moisture, 46.0% protein (N × 5.71), 1.1% oil, 5.7% ash, and 38.3% carbohydrate (by difference). Analytical procedures previously described (McWatters, 1978) were used for proximate composition analyses.

Protein solution preparation, salt level, and pH adjustment

Soy flour (2% suspensions, w/v) was dispersed in either distilled water, 0.1M NaCl (low salt), or 1.0M NaCl (high salt) solutions by blending for 1 min in an Osterizer blender operated at low speed. Adjustments in pH were made over a range 2.0–10.0 in 1-unit increments by adding either 1N HCl or 1N NaOH to flour suspensions maintained at 23°C over a 1-hr period with constant, gentle stirring.

Nitrogen solubility

Flour suspensions for nitrogen solubility tests were prepared as described above except that 6N HCl was used for samples adjusted to pH 2.0–5.0. The pH-adjusted suspensions were centrifuged at 13,000 × G for 10 min; 25-ml samples of the pooled supernate were analyzed for nitrogen content by the Kjeldahl procedure (AOAC, 1975). The percentage of soluble nitrogen was calculated and plotted against corresponding pH values.

Emulsion capacity

Oil-in-water emulsions were prepared in triplicate at room temperature by pipetting 25 ml of the pH-adjusted flour suspensions into a partially covered Osterizer blender jar. Refined peanut oil (Dothan Oil Mill Co., Dothan, AL) was added in 10-ml portions from a buret at an average rate of 0.5 ml/sec while the flour mixture was blending at low speed. Blending was interrupted periodically (after each 10-ml addition of oil) so that the sides and bottom of the blender jar could be scraped and to prevent a rise in emulsion temperature. Oil was added until a sudden drop in emulsion viscosity occurred due to separation of oil and water into two phases. The breakpoint at which phase inversion occurred was considered to be the emulsion capacity of the flour suspension. Emulsion capacity is expressed as ml of oil emulsified per mg of protein in 25 ml of the flour/water suspension.

Emulsion viscosity

Emulsions for viscosity measurements were prepared by the procedure described above for emulsion capacity; the addition of oil was terminated when the emulsion was at maximum viscosity (just before inversion), at an oil level of 10% less than the breakpoint. Each emulsion was transferred to a straight-wall, 50-mm diam glass

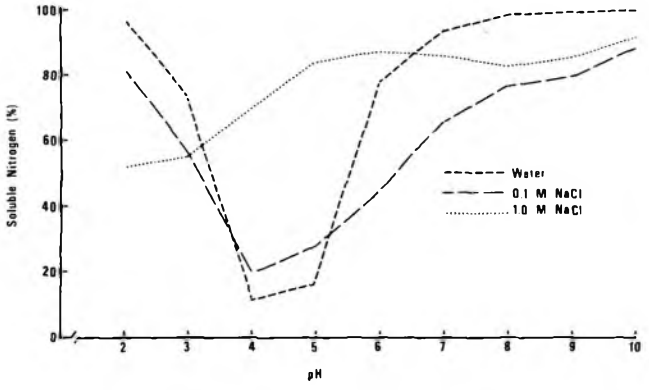

Fig. 1—Nitrogen solubility of soy flour suspended in distilled water, 0.1M NaCl, and 1.0M NaCl (2% suspensions, w/v) in the pH range 2.0–10.0.

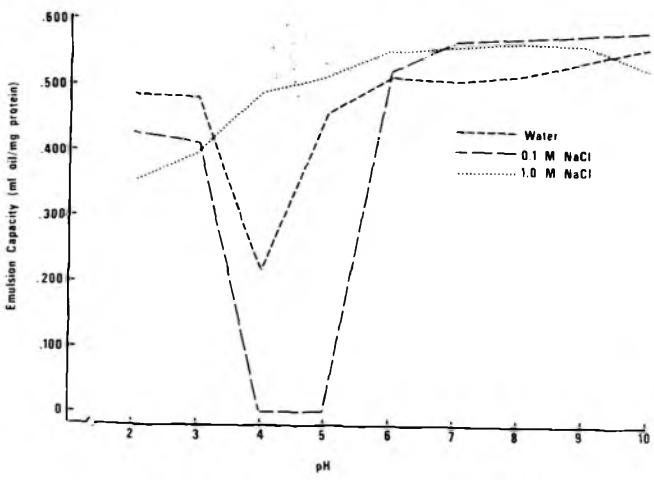

Fig. 2—Emulsion capacity (ml oil per mg total protein in 25 ml of 2% suspension) of soy flour suspended in distilled water, 0.1M NaCl, and 1.0M NaCl.

jar (4 oz) for viscosity evaluation. All measurements were made at 23°C using a Brookfield Viscometer (Model RVT) and Helipath Stand (Model C) equipped with a T-B spindle and operated at 2.5 rpm. Results were reported in centipoises (cps) as the average of the total number of readings obtained per sample; generally this was three to four, depending upon the volume of the sample.

Statistical analysis

Multiple regression analysis was used to quantify the relationships between nitrogen solubility and emulsifying properties of soy flour dispersed in water and salt solutions and various independent variables. A step-down computer program was used in the selection of equations or models of best fit as described by Cherry et al. (1975), McWatters and Cherry (1975), and McWatters et al. (1976). For each of the models, a number of combinations of independent variables was used in combination with step-down elimination. Relative importance of the variables included was determined by comparison of corresponding regression coefficients and of β-values of the variables.

Variables equated in multiple regression models included pH, absolute deviations of pH above 4.0 (set equal to zero for pH of 4.0 or less), absolute deviations of pH below 4.0 (set equal to zero for pH of 4.0 or above), salt level (0, 0.1 and 1.0M NaCl), percentage soluble nitrogen, and quadratic and cubic powers of these variables. Dependent variables included percentage soluble nitrogen, emulsion capacity, and emulsion viscosity. Since similar responses were noted for the water and low-salt treatments, which differed significantly from the high-salt treatments, two sets of models were developed, one for the water and low-salt treatments combined and another for the high-salt treatment.

RESULTS & DISCUSSION

Nitrogen solubility

Nitrogen solubility profiles of soy flour dispersed in water, 0.1M NaCl, and 1.0M NaCl solutions are shown in Figure 1. As the graph indicates, nitrogen solubility response to changing levels of pH in the water and low-salt suspensions differed from that of the high-salt suspensions. Between pH 4.0 and 5.0, pH levels which encompass the isoelectric point of soy proteins, nitrogen solubility of water and low-salt samples was significantly lower (11.4 and 19.9%, respectively) than that of the high-salt suspensions (70.1%). As pH was reduced to levels below or increased to levels above 4.0, nitrogen solubility of water and low-salt suspensions increased substantially, while a much different response occurred in similarly treated high-salt suspensions up to pH 8.0.

In the high-salt samples, the percentage of soluble nitrogen increased as pH was increased from the low-point at pH 2.0–6.0; a slight decrease was noted between pH 6.0–8.0, followed by a gradual increase from pH 8.0–10.0. Higher levels of soluble nitrogen were consistently noted in water suspensions than in low-salt suspensions except at pH 4.0 and 5.0. Of the three dispersion media investigated, water solubilized the greatest percentage of nitrogen at pH 3.0 or less and at pH 7.0 or above. At pH 10.0, nitrogen was almost totally soluble (99.9%) in suspensions prepared with water. These data confirm those of pH and salt concentration effects on soybean proteins reported in the pioneering work of Smith and Circle (1938); one exception is the higher salt concentration (1.0M NaCl) used in the present study.

Emulsion capacity

Emulsion capacities of soy flour dispersed in water, 0.1M NaCl, and 1.0M NaCl solutions are shown in Figure 2. The shape of the curves for emulsion capacity generally resemble those of the nitrogen solubility profiles in Figure 1. Similar findings were also reported by Franzen and Kinsella (1976). Response of emulsion capacity to pH changes was similar for water and low-salt suspensions which differed markedly from that of high-salt suspensions. In the low-salt treatment, no emulsions were formed at pH 4.0 and 5.0, but shifting the pH to levels below 4.0 and above 5.0 dramatically improved emulsifying properties. Likewise, emulsion capacity was poor for water suspensions at pH 4.0 (0.220 ml oil/mg protein) but was substantially improved when the pH was shifted to levels below or above pH 4.0.

By contrast, emulsion capacity of the high-salt suspensions increased as pH was increased from pH 2.0 to a level of 9.0 where values peaked then declined slightly beyond this point. At pH 4.0, emulsion capacity of the high-salt suspensions was significantly higher than either the water or low-salt suspensions. The emulsifying behavior of proteins at pH levels of 3.0–4.0 is of particular importance in preparation of salad dressing and mayonnaise products since the pH of these products fall within this range.

At pH 6.0 and above, all three dispersion media demonstrated high emulsifying activity. At these pH levels, the role which soluble protein plays in emulsion formation is not completely clear since the highest levels of soluble nitrogen were not necessarily associated with the greatest emulsifying capacity of soy protein. At pH 7.0 and above, the water suspensions had the highest levels of soluble nitrogen yet emulsified smaller quantities of oil than either of the salt suspensions. Near the isoelectric point (ca pH 4.0), however, the level of soluble protein may have been of greater significance since the high-salt suspension which

contained the highest level of soluble nitrogen at this point also emulsified the largest amount of oil.

Thus, the effects of salt and changes in ionic environment become readily apparent. Collidal particles (e.g., proteins) carry an electrical charge; this charge promotes stability in the colloid itself as well as in emulsions formed by it by causing particles of similar charge to repel each other, thereby preventing precipitation (Paul and Palmer, 1972). Neutralization of this charge, which occurs at a protein's isoelectric point, causes colloidal particles to become both unstable and less soluble. This effect of electrical charge was demonstrated in our study at pH 4.0 where a dramatic change in the electrovalent properties of soy protein, accomplished by increasing the salt level to 1.0M NaCl, counteracted the effects of pH at this point.

Wolf and Cowan (1971) reported that the two major soybean proteins, the 7S and 11S globulins, are sensitive to changes in their ionic environment in aqueous systems. Each protein undergoes association-dissociation reactions when ionic strength is changed, but their rate and extent of response to such changes are quite different. This difference in response may have accounted for the differences in emulsifying properties of soy proteins observed at pH 4.0 in the presence of salt at the high concentration (1.0M NaCl). Further comparative studies to determine the contribution of specific components of soy proteins to emulsifying properties are clearly warranted.

Table 1—Influence of pH and salt concentration on viscosities (cps) of soy flour emulsions

pH	Water	0.1M NaCl	1.0M NaCl
2	51,680	47,520	53,600
3	70,240	71,520	55,320
4	2,880	no emulsion	39,360
5	36,640	no emulsion	43,680
6	50,880	44,000	41,280
7	29,760	43,200	40,960
8	28,320	39,520	44,640
9	41,440	39,840	42,720
10	42,240	40,960	23,520

Emulsion viscosity

The viscosities of emulsions prepared from soy flour dispersed in water, 0.1M NaCl, and 1.0M NaCl and adjusted to pH levels ranging from 2.0–10.0 are shown in Table 1. Though these emulsions contained different quantities of oil (all contained 10% less than their respective breakpoints), some comparisons can nevertheless be made.

At pH levels of 2, 3, 6, 7, 8, 9 and 10, all emulsions formed were mayonnaise-like and spoonable in consistency, regardless of salt concentration. At 4.0, however, poor emulsifying properties were demonstrated by the water suspension which formed a thin, pourable emulsion (2,880 cps) and by the low-salt suspension which formed no emulsion. Poor emulsifying properties were also demonstrated by the low-salt suspension at pH 5.0; by contrast, both the water and high-salt suspensions produced thick, spoonable emulsions at this same pH. Most of the emulsions formed compared favorably with a commercial mayonnaise which had a viscosity of 44,640 cps.

Multiple regression analysis

Multiple regression models for prediction of pH and salt

Table 2—Multiple regression models for prediction of nitrogen solubility and emulsifying properties of soy flour as influenced by pH and salt concentration

Dependent variable	Salt concentration	Variable description	Regression coefficient	Beta value	t-value[a]
% Soluble N_2	0.0 and 0.1M NaCl	Constant	15.93522		
(R^2 = 0.9032; adjusted R^2 = 0.8871;		pH-4.0[b]	29.71500	2.16478	8.630**
standard error of estimate = 10.10708)		4.0-pH[c]	72.74009	1.63537	5.894**
		Salt level	−140.47222	−0.23686	−4.170**
		$(4.0\text{-pH})^2$	−16.30420	−0.69119	−2.688*
		$(\text{pH-4.0})^2$	−2.59732	−1.07471	−4.711**
% Soluble N_2	1.0M NaCl	Constant	22.28343		
(R^2 = 0.9053; adjusted R^2 = 0.8927;		pH	15.65848	6.82997	6.830**
standard error of estimate = 4.65944)		pH^2	−0.91726	−4.88596	−4.886**
Emulsion capacity	0.0 and 0.1M NaCl	Constant	0.22125		
(R^2 = 0.8385; adjusted R^2 = 0.7981;		pH-4.0[b]	0.18278	2.22597	6.409**
standard error of estimate = 0.08084)		4.0-pH[c]	0.45081	1.69426	4.438**
		Salt level	−2.41853	−0.68171	−4.590**
		$(4.0\text{-pH})^2$	−0.16459	−1.16638	−3.393**
		$(\text{pH-4.0})^2$	−0.02292	−1.58531	−5.197**
		(Salt level)(pH-4.0)	0.59235	0.64185	3.997**
		(Salt level)(4.0-pH)	1.11412	0.31403	2.320*
Emulsion capacity	1.0M NaCl	Constant	0.15479		
(R^2 = 0.9830; adjusted R^2 = 0.9807;		pH	0.10792	21.02003	21.020**
standard error of estimate = 0.01043)		pH^2	−0.00708	−16.83727	−16.837**
Emulsion viscosity	0.0 and 1.0M NaCl	Constant	4,701.90476		
(R^2 = 0.7846; adjusted R^2 = 0.7568;		pH-4.0[b]	18,031.42857	1.90794	5.183**
standard error of estimate = 10,210.91732)		4.0-pH[c]	117,147.14286	3.82536	9.395**
		$(4.0\text{-pH})^2$	−47,409.04762	−2.91915	−7.737**
		$(\text{pH-4.0})^2$	−2,127.61905	−1.27866	−3.819**
Emulsion viscosity	1.0M NaCl	Constant	98,207.61905		
(R^2 = 0.7955; adjusted R^2 = 0.7517;		pH	−28,811.39971	−8.58810	−3.473**
standard error of estimate = 4,441.52041)		pH^2	4,945.71429	18.00317	3.280**
		pH^3	−277.17172	−10.46334	−3.332**

[a] *Significant at 5% confidence level; **significant at the 1% level.
[b] The variable described as "pH-4.0" is given a value of zero unless the pH is greater than 4.0.
[c] The variable described as "4.0-pH" is given a value of zero unless the pH is less than 4.0.

Table 3—Simple correlation coefficients for selected pairs of variables for soybean flour dispersed in 0.0 and 0.1M NaCl solutions[a]

Variable	pH	(pH-4.0)[b]	(4.0-pH)[c]	% Soluble nitrogen	Emulsion viscosity	Emulsion capacity	Salt level	$(4.0\text{-pH})^2$	$(pH\text{-}4.0)^2$	Salt level X (pH-4.0)	Salt level X (4.0-pH)
pH	1.00	0.98**	−0.71**	0.43**	−0.12	0.49**	0.00	−0.65**	0.91**	0.55**	−0.47**
(pH-4.0)[b]		1.00	−0.54**	0.58**	0.01	0.60**	0.00	−0.48**	0.96**	0.56**	−0.36*
(4.0-pH)[c]			1.00	0.24	0.48**	0.03	0.00	0.97**	−0.41*	−0.30	0.67**
% Soluble nitrogen				1.00	0.51**	0.72**	−0.24	0.26	0.58**	0.13	0.07
Emulsion viscosity					1.00	0.68**	−0.09	0.36*	0.04	0.02	0.30
Emulsion capacity						1.00	−0.19	0.03	0.53**	0.36*	−0.04
Salt level							1.00	0.00	0.00	0.61**	0.33*
$(4.0\text{-pH})^2$								1.00	−0.36*	−0.27	0.65**
$(pH\text{-}4.0)^2$									1.00	0.54**	−0.27
(Salt level)(pH-4.0)										1.00	−0.20
(Salt level)(4.0-pH)											1.00

[a] *Significant at the 5% confidence level; **significant at the 1% level.
[b] The value of this variable was set to zero if the pH was less than or equal to 4.0.
[c] The value of this variable was set to zero if the pH was greater than or equal to 4.0.

concentration effects on nitrogen solubility and emulsifying properties are presented in Table 2; relevant simple correlation coefficients for some of the variables used in the analysis are shown in Table 3 (0.0 and 0.1M NaCl) and 4 (1.0M NaCl).

The β-values and t-values in all the models suggest that pH was the primary determinant of percentage soluble nitrogen, emulsion capacity, and emulsion viscosity, given the salt concentration. The models also indicate that pH had a curvilinear effect on these properties; their marginal response to pH declined as pH was increased for the high-salt treatment or declined as the absolute difference between the pH and a pH of 4.0 was increased for the water and low-salt treatments.

Multiple R^2 values indicate the percentage of variation in a dependent variable accounted for by its regression on the independent variables; the higher the value, the greater the accountability. The multiple regression models in Table 2 produced the highest percentage R^2 values of 90.32% and 83.85% for percentage soluble nitrogen and emulsion capacity, respectively, for the water and low-salt suspensions; corresponding values for the same variables in the high-salt suspensions were 90.53% and 98.30%, respectively.

Two other models which had higher R^2 values than those indicated in Table 2 exist for prediction of variation in emulsion viscosity. However, these models included percentage soluble nitrogen as one of the independent variables. Since the role that percentage soluble nitrogen played in emulsifying properties was not completely clear because it too was strongly influenced by pH, these models were not considered to be the most reliable predictors of emulsion viscosity. The equation for prediction of emulsion viscosity of water and low-salt suspensions which included percentage soluble nitrogen had a multiple R^2 value of 84.88% whereas the corresponding value of the equation excluding soluble nitrogen was 78.46%. For the high-salt suspensions, the equation which included percentage soluble nitrogen had a multiple R^2 value of 88.57% whereas the corresponding value of the equation excluding soluble nitrogen was 79.55%.

The effects of soluble nitrogen were evidently confounded with the effects of pH, a phenomenon which made its appropriate contribution to emulsification difficult to discern. The results of this research do indicate, however, very profound effects from changes in salt concentration. Further study based on additional salt levels might be profitable, perhaps yielding more precise estimates of the effects of both pH and salt concentration over a wider range.

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—Continued on page 781

Table 4—Simple correlation coefficients for selected pairs of variables for soybean flour dispersed in 1.0M NaCl solution[a]

Variable	pH	(pH-4.0)[b]	(4.0-pH)[c]	% Soluble nitrogen	Emulsion viscosity	Emulsion capacity	pH^2	pH^3
pH	1.00	0.98**	−0.71**	0.87**	−0.80**	0.81**	0.98**	0.95**
(pH-4.0)[b]		1.00	−0.54*	0.77**	−0.74**	0.69**	0.99**	0.98**
(4.0-pH)[c]			1.00	−0.88**	0.69**	−0.93**	−0.59**	−0.49*
% Soluble nitrogen				1.00	−0.82**	0.94**	0.78**	0.70**
Emulsion viscosity					1.00	−0.66**	−0.79**	−0.78**
Emulsion capacity						1.00	0.69**	0.59**
pH^2							1.00	0.99**
pH^3								1.00

[a] *Significant at the 5% confidence level; **significant at the 1% level.
[b] The value of this variable was set to zero if the pH was less than or equal to 4.0.
[c] The value of this variable was set to zero if the pH was greater than or equal to 4.0.

INFLUENCE OF MOIST HEAT ON SOLUBILITY AND EMULSIFICATION PROPERTIES OF SOY AND PEANUT FLOURS

KAY H. McWATTERS and MAC R. HOLMES

ABSTRACT

Nitrogen solubility and emulsifying properties of unheated and heated (live steam at 100°C for 10, 20, and 30 min) soy and peanut flours were determined. Results showed that each of these functional properties was reduced as heating time increased. Nitrogen solubility of soy flour was more sensitive to extension of heating time than peanut flour. However, emulsion-forming and thickening properties of soy flour were not as adversely affected by moist heat treatment as peanut flour. Viscous, mayonnaise-like emulsions (27,840–41,760 cps) were produced by unheated soy and peanut flours and soy flour heated for 10 or 20 min. Thick salad dressing-like emulsions (9,520–14,960 cps) were produced by soy flour heated for 30 min and peanut flour heated for 10 or 20 min. Poor emulsifying properties were exhibited by peanut flour steamed for 30 min where a thin, pourable emulsion (2,400 cps) was formed. High levels of nitrogen solubility were not necessarily associated with maximum emulsifying capacity. Multiple regression analyses confirmed that heating time was the primary determinant in reduction of functionality of both flours and that flours derived from peanuts and soybeans responded differently to moist heat treatment.

INTRODUCTION

IN THE PROCESS of preparing soy protein products for human consumption, moist heat is employed for a dual purpose: to inactivate antinutritional factors and to improve flavor quality. Generally, live steam treatment at 100°C for 20–30 min provides an adequate margin of safety to ensure maximal nutritional benefit. A combination of hexane:ethanol extraction and steaming has also been shown to improve flavor as well as nutritive value of defatted soy flakes (Rackis et al., 1975).

Nitrogen solubility levels which are frequently used as indicators of protein functionality and potential end use (Johnson, 1970) are reduced as extent of steaming increases (Rackis et al., 1975). In a previous study with moist-heated peanuts (McWatters and Cherry, 1975), levels of soluble protein were reduced as temperature and time of heating increased yet emulsion and foam-forming properties were retained. The amount of soluble protein in moist-heated peanuts was shown to play a secondary role in terms of functionality to the conditions of heating to which the nuts were exposed.

Oilseed-derived protein products have both current and expanding applications in meat (Drake et al., 1977; Terrell and Staniec, 1975; McWatters, 1977) and bakery products (Kissell and Yamazaki, 1975; French, 1977; Tsen and Hoover, 1973; McWatters, 1978). Since most protein products intended for use in human food would require exposure to rather severe heating conditions (live steam), the effects of such processing conditions on functionality merit investigation. The present study was designed to measure the influence of selected conditions of moist heating on solubility and emulsification properties of soy and peanut flours.

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EXPERIMENTAL

Flour preparation

Flours (60-mesh) were prepared from 1974 crop, Bragg soybeans and from 1976 Florunner peanuts by procedures described in a related study (McWatters and Holmes, 1979a). Proximate composition of the soy flour was 8.9% moisture, 46.0% protein (N × 5.71), 1.1% oil, 5.7% ash, and 38.3% carbohydrate (by difference). Proximate composition of the peanut flour was 6.8% moisture, 50.9% protein (N × 5.46), 1.5% oil, 4.5% ash, and 36.3% carbohydrate (by difference).

Moist heat treatment

Flours were spread in thin layers (1.5 cm) in aluminum pans, placed in a temperature-controlled retort, and steamed for 10, 20, and 30 min. Following heat treatment, the pans containing the heated samples and an unheated control were transferred to a forced draft oven at 25°C and dried overnight. The flours were pulverized the following day and stored in glass jars at 1°C until used.

Protein solution preparation and pH adjustment

Soy flour (2% suspensions, w/v) and peanut flour (8% suspensions, w/v) were dispersed in distilled water and blended for 1 min in an Osterizer blender operated at low speed. The pH of each flour/water suspension was adjusted to 8.0 by adding 1N NaOH over a 1-hr period with constant, gentle stirring. Related studies with soy flour (McWatters and Holmes, 1979a) and with peanut flour (McWatters et al., 1976; McWatters and Holmes, 1979b) had shown that these flour concentrations at this pH level promoted emulsion formation and produced emulsions with comparable viscosities.

Nitrogen solubility

The pH-adjusted suspensions were centrifuged at 13,000 × G for 10 min; 25-ml samples of the pooled supernate were analyzed for nitrogen content by the Kjeldahl procedure (AOAC, 1975). The percentage of nitrogen in samples which was soluble in water was then determined.

Emulsion capacity and viscosity

Oil-in-water emulsions for breakpoint and viscosity measurements were prepared in triplicate as described by McWatters and Holmes (1979a). Emulsion capacity is expressed as ml of oil emulsified per mg of protein in 25 ml of the flour/water suspension. Emulsion viscosity is reported in centipoises (cps) as the average of four readings per sample (Brookfield Viscometer–Model RVT and Helipath Stand–Model C, T-B spindle, 2.5 rpm, 23°C).

Statistical analysis

Multiple regression analysis was used to quantify the relationships between nitrogen solubility and emulsifying properties of moist-heated soy and peanut flours and various independent variables. A step-down computer program was used in the selection of equations or models of best fit as described by McWatters and Cherry (1975) and McWatters et al. (1976). For each of the models, several combinations of independent variables were used in conjunction with step-down elimination. Relative importance of the variables included was determined by comparison of corresponding regression coefficients and of β-values of the variables. Variables equated in multiple regression models included heating time, heating time squared, square root of heating time, and the log (natural) of percent soluble nitrogen. Dependent variables included percent soluble nitrogen, emulsion capacity, and emulsion viscosity.

RESULTS & DISCUSSION

Nitrogen solubility

Nitrogen solubility levels of both soy and peanut flours were reduced by the application of moist heat, as shown in Figure 1, though soy flour was far more sensitive to heat treatment than peanut flour. For both flours, the greatest reduction in solubility occurred during the first 10 min of heating; during this time, nitrogen solubility was reduced from 98 to 57% for soy flour and from 91 to 83% for

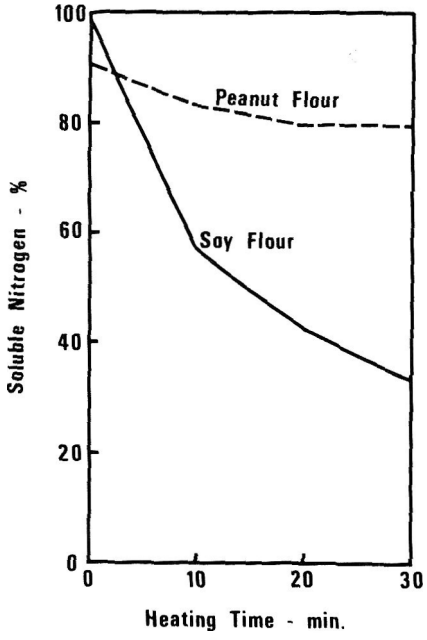

Fig. 1—Percentage of soluble nitrogen in unheated and heated (live steam at 100° C for 10, 20, and 30 min) soy and peanut flours; flours were dispersed in water (soy — 2% suspensions, w/v; peanut — 8% suspensions, w/v) and adjusted to pH 8.0.

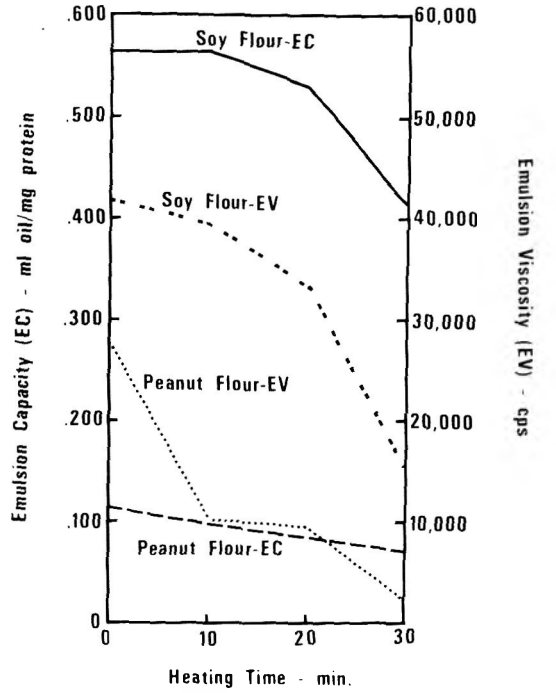

Fig. 2—Emulsion capacity (EC) and emulsion viscosity (EV) of unheated and heated soy and peanut flours. Soy and peanut flour emulsions contained 229.95 and 1017.8 mg protein, respectively.

peanut flour. After 20 min of heating, levels of soluble nitrogen were further reduced to 80% for peanut flour and to 43% for soy flour. Negligible reductions in nitrogen solubility levels of peanut flour occurred when heating time was extended to 30 min; in soy flour, however, levels were further reduced to 34%.

Emulsion capacity and viscosity

Emulsion capacities and viscosities of unheated and steam-heated soy and peanut flours are shown in Figure 2. Emulsion capacity of unheated soy flour was about five times that of unheated peanut flour, even though peanut flour emulsions contained about 4.4 times as much protein (1017.8 mg) as soy (229.95 mg). Obviously the unique protein quality of each of the flours differed dramatically in ability to form oil-in-water emulsions.

Emulsion capacity response to moist heat was similar to nitrogen solubility response in that both were reduced as heating time increased. In emulsion capacity response, however, peanut flour was more sensitive to moist heat treatment than soy flour. No reduction in emulsion capacity occurred in soy flour during the first 10 min of steaming whereas a 14% reduction occurred in peanut flour. Between 10 and 20 min of steam-heat treatment, emulsion capacity of soy flour was reduced by 6% while a 14% reduction occurred in similarly-treated peanut flour. Only between 20 and 30 min of heating did the percentage of reduction in emulsion capacity of soy flour exceed that of peanut flour (22 and 17%, respectively). At each heating time, however, the emulsifying capacity (quantity of oil emulsified per unit of protein) of soy flour was five to six times greater than that of peanut flour. Emulsion capacity reductions were more gradual for peanut flour (almost a straight-line effect) than for soy.

The viscosities of emulsions produced by both flours decreased as length of heating time increased. In emulsion viscosity response, peanut flour was more sensitive to moist heat treatment than soy flour. Thickness of peanut flour emulsions was reduced by 63% during the first 10 min of heating while that of soy flour emulsions was reduced by only about 6% during the same period. Between 10 and 20 min, however, a reverse response occurred; greater reduction (15%) in thickness occurred in soy flour emulsions than in those prepared from peanut flour (7%). Between 20 and 30 min heating, sharp declines in consistencies of both peanut and soy flour emulsions occurred. Whereas thick, spoonable mayonnaise-like products were produced by soy flour receiving no heat (41,760 cps) or heated for 10 or 20 min (39,360 and 33,280 cps, respectively), a pourable salad dressing-type product was produced by the 30 min sample (14,960 cps.)

Soy flour emulsions were consistently more viscous than emulsions prepared from similarly-treated peanut flour. The consistency of emulsions prepared from unheated peanut flour was thick and mayonnaise-like (27,840 cps) while that of emulsions prepared from peanut flour heated for 10 or 20 min resembled thick salad dressings (10,240 and 9,520 cps, respectively). The 30 min heat treatment for peanut flour produced a very poor emulsion which was quite thin and pourable in consistency (2,400 cps).

As demonstrated in a related study (McWatters and Holmes, 1979a), high levels of soluble nitrogen were not necessarily associated with maximum emulsifying capacities. The heated peanut flours consistently contained higher levels of soluble nitrogen than heated soy flours yet emulsified smaller quantities of oil and produced thinner emulsions than their soy counterparts. Thus, defatted flours derived from peanuts and soybeans are inherently different and respond differently to moist heat treatment.

Multiple regression analysis

Multiple regression models for prediction of nitrogen solubility and emulsifying properties of moist-heated soy and peanut flours are presented in Table 1. Examination of the coefficients of determination (R^2) and t-values in all the models indicates that heating time was largely responsible for the decreases in nitrogen solubility and in emulsifying properties. All the regression models include nonlinear transformations of heating time because the marginal effects (negative) of heating time on soluble nitrogen, emulsion capacity and viscosity of both soy and peanut flours begin to increase at some point as heating time is increased

Table 1—Multiple regression models for prediction of nitrogen solubility and emulsifying properties of soy and peanut flours as influenced by moist heat

Flour	Dependent variable	Variable description	Regression coefficient	Beta value	t-value[a]
Soy	% Soluble N_2[b]	Constant	97.19396		
		Square root of heating time	−12.00149	0.28800	−41.671**
	Emulsion capacity[c]	Constant	0.56220		
		Heating time	0.00394	5.09568	5.096**
		(Heating time)^2	−0.00031	−12.35910	−12.359**
	Emulsion viscosity[d]	Constant	42,118.09524		
		(Heating time)^2	−29.02313	1.67726	−17.304**
Peanut	% Soluble N_2[e]	Constant	90.53242		
		Square root of heating time	−2.22345	0.11382	−19.535**
	Emulsion capacity[f]	Constant	0.11317		
		Heating t me	−0.00143	0.00004	−40.273**
	Emulsion viscosity[g]	Constant	26,394.66667		
		Heating t me	−1,573.86667	−5.40254	−5.403**
		(Heating time)^2	27.46667	2.95154	2.952*

[a] *Significant at the 5% confidence level; **significant at the 1% level.
[b] R^2 = 0.9943; adjusted R^2 = 0.9937; standard error of estimate = 2.05804.
[c] R^2 = 0.9875; adjusted R^2 = 0.9847; standard error of estimate = 0.00855.
[d] R^2 = 0.9677; adjusted R^2 = 0.9645; standard error of estimate = 2033.56863.
[e] R^2 = 0.9745; adjusted R^2 = 0.9719; standard error of estimate = 0.81333.
[f] R^2 = 0.9939; adjusted R^2 = 0.9933; standard error of estimate = 0.00138.
[g] R^2 = 0.9090; adjusted R^2 = 0.8887; standard error of estimate = 3223.64597.

(or the rate of change in these characteristics increases as heating time is increased).

For prediction of levels of soluble nitrogen in both soy and peanut flours, the equations of best fit included the square root of heating time as the independent variable. R^2 values indicate the percentage of variation in a dependent variable accounted for by its regression on the independent variables; the higher the value, the greater the accountability. Results showed that 99.43% of the variation in soluble nitrogen of soy flour and 97.45% of that in peanut flour could be accounted for by the square root of heating time.

In emulsion capacity of soy flour, response was due to the independent variables of heating time and heating time squared whereas in peanut flour, variation was due to heating time only. R^2 values for this functional property were 98.75% for soy flour and 99.39% for peanut flour. Models for prediction of viscosity of soy flour emulsions included the independent variable of heating time squared while models for peanut flour included both heating time and heating time squared. R^2 values for emulsion viscosity equations were 96.77% for soy flour and 90.90% for peanut flour. Since these data show very profound effects from changes in heating time, the precision of models developed for prediction of functional properties of these particular flours could be increased in future studies by inclusion of additional heating times (e.g., 1-min rather than 10-min intervals).

Under the conditions employed in this study, solubility and emulsification properties of soy and peanut flour were reduced by the application of moist heat. Soy flour was more sensitive to increases in heating time than peanut flour in terms of nitrogen solubility but consistently emulsified larger quantities of oil and produced more viscous emulsions. Particularly noteworthy was soy flour's capacity to form emulsions even after the severe heat treatment of steaming for 30 min. Though peanut flour's emulsion-forming properties were more adversely affected by moist heat than soy flour, less heat may in fact be required for satisfactory processing of this flour for human consumption. Certain inherent qualities of peanuts, viz., bland flavor and low concentration of anti-nutritional factors and flatulence-producing carbohydrates, make them more desirable for food supplements than some of the plant proteins currently in use (Conkerton and Ory, 1976).

Since the present study was limited to an assessment of the effects of moist heat on soy and peanut flours adjusted to only one pH level (8.0), it would be difficult to speculate on effects which might result with the same heat treatments over a wide range of pH levels. More extensive studies to characterize the interacting effects of moist heat and pH on solubility and emulsifying properties of oilseed flours are needed to provide definitive information for food processors anticipating the use of these materials for these specific functional properties.

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ORANGE PYRUVATE DECARBOXYLASE: ISOLATION AND MECHANISTIC STUDIES

W. R. RAYMOND, J. B. HOSTETTLER, K. ASSAR and C. VARSEL

ABSTRACT

Pyruvate decarboxylase, isolated from orange vesicle tissue, was purified 66-fold by Carbowax®-4000 and ammonium sulfate precipitations, and carboxymethylcellulose chromatography. The enzyme was characterized for optimum pH, stability, and divalent cation and inhibitor effects. Kinetic studies indicated that the orange enzyme is mechanistically similar to yeast pyruvate decarboxylase, having separate binding sites for Mg^{++} and thiamine pyrophosphate. The enzyme differs from its yeast counterpart, in having only one active site. It is specific for pyruvate and 2-ketobutyrate, and is competitively inhibited by 2-ketononanoate. The enzyme apparently exists in the fruit, primarily as an inactive cyclizable ternary complex of *apo*-enzyme, Mg^{++}, and thiamine pyrophosphate. Cyclization to the active *holo*-enzyme is apparently controlled by the available pyruvate concentration.

INTRODUCTION

ALIPHATIC ALDEHYDES are reputed to be important constituents of the volatile profile of the orange, not only from a compositional standpoint (Shaw, 1977), but also in terms of the contributions they make to the character of orange flavor (Ahmed et al., 1973).

One of the predominant aldehydes in orange juice is acetaldehyde (Kirchner and Miller, 1957), which exerts a significant positive influence on orange flavor (Ahmed et al., 1973), and which is present in orange juice at a concentration more than eight times its taste threshold in water (Ahmed et al., 1978).

The formation of acetaldehyde in both climacteric (Dilley, 1970) and nonclimacteric fruits (Hawker, 1969) is mediated by pyruvate decarboxylase (E.C.4.1.1.1.). Decarboxylase activity has also been reported in freshly reamed orange juice and in neutralized fresh orange juice extracts (Roe and Bruemmer, 1974). The extracts catalyzed oxidative decarboxylation not only of pyruvate but also of longer-chain 2-keto acids through C-6.

The reported contribution of acetaldehyde to orange flavor, plus the limited information available about plant pyruvate decarboxylases, have prompted an investigation of the mechanism of orange pyruvate decarboxylase in vitro. The enzyme was isolated from orange vesicle tissue, free from other enzymes which might contribute to biosynthesis or degradation of aldehydes, and certain of its mechanistic properties were investigated and compared to those of its well-studied counterpart from brewers' yeast (*Saccharomyces carlsbergensis*). This paper reports the results of that investigation.

EXPERIMENTAL

Materials

Freshly harvested ripe Valencia oranges were obtained from a local grove. Yeast alcohol dehydrogenase (YADH), reduced nicotinamide adenine dinucleotide (NADH), thiamine pyrophosphate (TPP), and all keto acids used as substrates, were obtained from Sigma Chemical Company, St. Louis, MO. Cleland's reagent (dithiothreitol) was obtained from P-L Biochemicals, Inc., Milwaukee, WI. Carbowax (PEG)-4000, laboratory grade, was obtained from Fisher Scientific Co., Fair Lawn, NJ.

All UV studies were conducted with a Perkin Elmer 402 UV/visible spectrophotometer equipped with an automatic multi-sampler, and a Thomas Model 68 concentration readout and Model 80 printer (Arthur H. Thomas Co., Philadelphia, PA). Titrimetric studies were done with a Fisher Model 36 automatic titrimeter. Gas chromatographic analyses were performed on a Hewlett-Packard 5840 gas chromatograph equipped with dual flame ionization detectors. A 6' × 1/8" glass column packed with Tenax G.C., used for G.C. analyses, was obtained from Applied Science Laboratories, Inc., State College, PA.

Isolation and purification of orange pyruvate decarboxylase (PDC)

Oranges free of blemishes and bruises were carefully peeled. After removal of all traces of adhering albedo, the fruit were washed and the sections were separated carefully to prevent rupture of the juice vesicles, and carefully deseeded. The sections were chilled to 3°C.

A blending medium was prepared containing 133 ml of 1M Tris base, 10 mM each of $MgCl_2$ and thiamine pyrophosphate (TPP), 1g of cysteine-HCl, 200 mg of dithiothreitol (DTT), and 50g of Carbowax (PEG)-4000, in a total volume of 500 ml. One hundred milliliters of this mixture, chilled to 3°C, and 20g of polyvinylpolypyrollidone (PVPP) were added to 100g of chopped ice in a chilled Waring Blendor jar.

Two hundred grams of orange sections were added to the mixture, one section at a time, while blending at medium speed. Blending was continued until the sections were thoroughly macerated. The mixture was centrifuged for 15 min at 30,000 × G and the pellet was discarded. All further operations were performed at 3°C.

To the supernatant solution were added 10 g/100 ml of PEG-4000 with slow stirring to avoid foaming. The solution was centrifuged as above and the pellet was discarded. To the supernatant solution were added 20 g/100 ml of PEG-4000 with stirring and the mixture was held 30 min at 3°C. After centrifugation as above the supernatant solution was discarded.

An elution mixture, pH 6.5, was prepared containing 0.2 mg/ml DTT, 1 mg/ml cysteine·HCl, 10 mM each of TPP and $MgCl_2$, and 20 mM phosphate buffer.

The PEG-4000 pellet was dissolved in 20 ml of the elution mixture and centrifuged 10 min at 8000 × G. The supernatant solution was desalted on a 2.5 × 80 cm column of Sephadex G-25 equilibrated to the elution mixture. The fractions containing PDC activity were pooled and brought to 40% saturation with solid $(NH_4)_2SO_4$.

After standing 12 hr at 3°C, the solution was centrifuged 30 min at 30,000 × G. The enzyme was contained in a waxy epiphase. The aqueous phase was removed by insertion of a pipette through the epiphase.

The epiphase was dissolved in 5 ml of elution mixture and desalted as before. The activity-containing fractions were pooled and loaded onto a 2.5 × 40 cm column of carboxymethylcellulose equilibrated to 20 mM acetate buffer, pH 5.5. The column was washed with the same buffer and fractions of 25 ml were collected. Pyruvate decarboxylase activity was eluted by the buffer while the bulk of the protein, including orange alcohol:NAD oxidoreductase, remained on the column. The active fractions were pooled and lyophilized.

Preparation of apo-orange PDC

To remove TPP and Mg^{++} from the protein component, the enzyme, after CM-cellulose treatment, was brought to 10% saturation with solid $(NH_4)_2SO_4$ in an ice bath and the pH was adjusted

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to 9.1 with dilute NH_4OH. The solution was brought to 40% saturation by addition of small increments of $(NH_4)_2SO_4$ while maintaining a pH of 9.1 with NH_4OH, and then centrifuged at 7800 × G for 10 min. The pellet was washed first with one volume of 40% $(NH_4)_2SO_4$ solution, pH 9.1, and then with 40% $(NH_4)_2SO_4$, recentrifuging each time as above. The washings were added to the original supernatant solution and the pellet was discarded.

The combined solution was adjusted to pH 8.5 with dilute HCl and then brought to 80% saturation with solid $(NH_4)_2SO_4$, again added in small increments while maintaining the pH at 8.5 with dilute NH_4OH. The 80% solution was stirred gently for 15 min and then centrifuged as above. The supernatant solution was discarded and the pellet washed first with 80% $(NH_4)_2SO_4$, pH 8.5, and then 80% $(NH_4)_2SO_4$, centrifuging each time as above. The pellet was dissolved in 1 volume of 30 mM phosphate buffer, pH 6.8, and used immediately.

Measurement of enzyme activities

Orange pyruvate decarboxylase was routinely assayed by a spectrophotometric method similar to that of Bergmeyer et al. (1974). The assay mixture contained 20 mM phosphate buffer (pH 6.0), 10 mM sodium pyruvate, 0.1 mM TPP, 1 mM $MgSO_4 \cdot 7H_2O$, 0.16 mM NADH, 0.1 ml YADH (308 units/ml), and 0.01 to 0.1 ml PDC in a total volume of 3 ml. Oxidation of NADH was followed at 340 nm and 25°C. A blank solution was prepared as above using heat-inactivated PDC. One unit of PDC is defined as that activity corresponding to the oxidation of 1 nmole of NADH per min under the above conditions.

Peroxidase was assayed by the method of Pütter (1974), lipoxygenase by the method of Bergmeyer et al. (1974), and orange alcohol:NAD oxidoreductase by the method of Bruemmer and Roe (1971). Protein was measured by the method of Lowry et al. (1951).

Characterization of orange PDC

The pH optimum of orange PDC was determined directly using the pH-statt assay method of Schellenberger et al. (1963), titrating with 1 mM HCl.

The effects of 1 mM divalent cations (Ca^{++}, Co^{++}, Mg^{++}, Mn^{++}, Ni^{++}, and Zn^{++}) were determined by the spectrophotometric method, or by direct assay of acetaldehyde formed by a modified (Raymond and Hostettler, 1978) 6-mercapto-3(substituted)-*s*-triazolo[4,3-*b*] tetrazine (MTT) method (Dickenson and Jacobsen, 1970). The enzyme was first dialyzed against the previously described elution mixture but without $MgCl_2$.

Known noncompetitive inhibitors were tested by the spectrophotometric method, both by direct addition of the inhibitor (1 mM) to the blank and assay mixtures, and by assay of the enzyme after dialysis first against 20 mM phosphate buffer (pH 6) containing 1 mM inhibitor, and then against buffer alone. Enzyme used as a control was dialyzed against buffer only.

For competitive inhibition studies, the activity of orange PDC on sodium pyruvate at 2–30 mM was assayed alone and in the presence of 1 mM 2-ketocaproic, 3-ketovaleric, or 2-ketononanoic acid, by the spectrophotometric method (pH 7.0).

Substrate specificity of the enzyme was tested with various 2-keto acids (1 mM) by direct aldehyde assay using the MTT method against controls containing heat-inactivated enzyme. Possible activity with 3-ketovaleric acid was tested by gas chromatographic analysis for 2-butanone.

Active site studies

Evidence for the number of active sites was obtained by assaying orange PDC over a wide range of pyruvate concentrations, including very low levels (0.05–50 mM), and obtaining a Hill plot from the experimental data.

To determine whether a sulfhydryl group is involved in substrate binding to the enzyme protein, two aliquots of the enzyme were first dialyzed for 3 hr, one against 5000 volumes of 20 mM phosphate buffer (pH 6.5), 1 mM in TPP, 3 mM in Mg^{++}, and 10 mM in pyruvate, and the second against the same mixture containing no pyruvate. The enzyme from each treatment was further divided into smaller aliquots which were dialyzed against the same respective solutions containing 0.01 mM $HgCl_2$ for 15, 45, and 90 min, after which the aliquots were redialyzed against fresh volumes of the original solutions (6 hr total dialysis). Controls were dialyzed against the initial solutions for the same total time period. The enzyme samples so treated were assayed by the spectrophotometric procedure.

The kinetics of recombination of *apo*-PDC with Mg^{++} and TPP were determined at pH 6.1, 6.6, 6.8, and 7.0 by the method of Schellenberger and Hübner (1967). Thiamine pyrophosphate recombination (0.1–16 mM TPP) was assayed in the presence of 1 mM and 8 mM Mg^{++}. Recombination of Mg^{++} (2–20 mM) was assayed in the presence of 0.6 mM and 1 mM TPP.

Table 1—Purification of orange pyruvate decarboxylase

Treatment	Total volume (ml)	Total protein (mg)	Sp. act. (units/mg protein)	Recovery %	Relative activity
Crude tissue extract	200	985	65	100	1
Desalted PEG-4000 precipitate	33	70.6	710	78	10.9
Desalted $(NH_4)_2SO_4$ precipitate	49	31.4	995	49	15.3
CM-cellulose column	113	4.1	4278	27	65.8

RESULTS

Purification and general properties of the enzyme

Purification of orange PDC is summarized in Table 1. Use of PEG-4000 in the initial extraction and precipitation steps resulted in a 10-fold greater PDC activity than was obtained by maceration without PEG-4000 followed by $(NH_4)_2SO_4$ precipitation. Precipitation of PDC with PEG-4000 also resulted in irreversible precipitation of considerable intensely yellow-pigmented material, assumed to be flavoprotein, which could not be separated from PDC by gel filtration either on Sephadex G-100 or G-200. The $(NH_4)_2SO_4$ precipitate was free of peroxidase and lipoxygenase activities but retained considerable alcohol dehydrogenase activity (E.C.1.1.1.1). This activity was quantitatively removed from PDC by CM-cellulose column chromatography.

The $(NH_4)_2SO_4$ precipitate was stable, retaining ca. 90% of its original activity after 14 months at −25°C. After CM-cellulose chromatography, the purified enzyme lost its activity within a week at the same temperature. The *apo*-enzyme was very unstable, having a half-life of about 12 hr at −25°C. The enzyme was totally inactivated within 1 min at 100°C.

Throughout the purification sequence, a combination of 2 mM Cleland's reagent (DTT) and 6 mM cysteine was found necessary to prevent loss of activity. Removal of either or both compounds resulted in a 10% loss in activity per hr as compared to a control containing both. Removal of Mg^{++} and TPP, as expected, resulted in a 90% loss of activity over a 4 hr period. Up to 60% of the lost activity was regenerated in the presence of 1 mM Mg^{++} and 0.1 mM TPP at pH 6.0.

The activity vs pH of the enzyme is shown in Figure 1. The enzyme activity peaked sharply between pH 4.7 and 4.8. Above pH 5.2, a skewed activity curve was obtained with an apparent shoulder at ca. pH 6.0. The enzyme retained about 8–10% of its maximum activity at pH 7.0. The molecular weight of the enzyme was not precisely determined but the activity was excluded on Sephadex G-100 indicating a molecular weight of at least 150,000.

Dialysis against sodium azide or EDTA had no more effect upon orange PDC than dialysis against a buffered control solution. Exhaustive dialysis against buffer consistently failed to decrease PDC activity by more than 85–90%. The requirement for sulfhydryl protective reagents was confirmed by rapid and complete loss of activity in the presence of Hg^{++} or *p*-hydroxymercuribenzoate.

Magnesium, manganese, and calcium caused the activity of orange PDC to increase, relative to the control enzyme

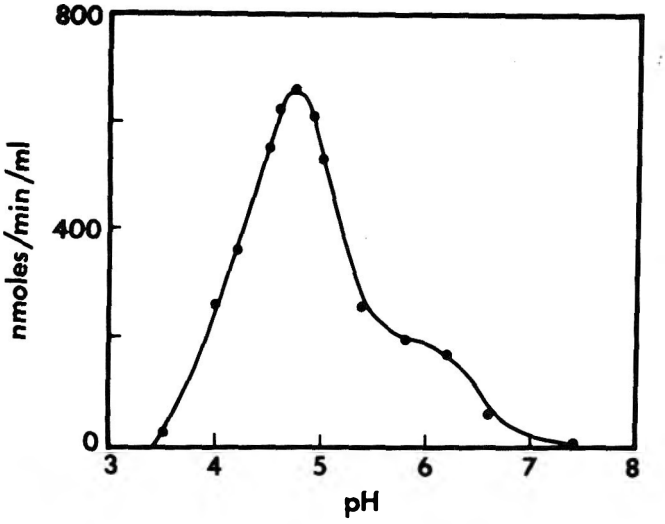

Fig. 1—Effect of pH on the activity of orange pyruvate decarboxylase.

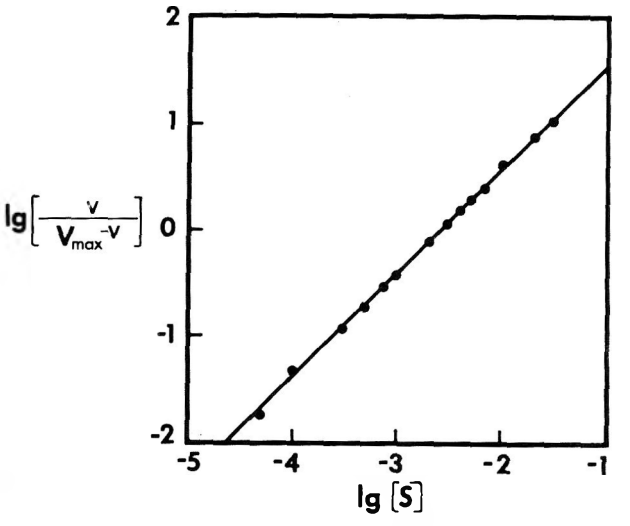

Fig. 2—Hill plot of orange pyruvate decarboxylase with pyruvate (0.05–50 mM) as substrate. The reaction mixtures contained, in addition to substrate (S), 20 mM phosphate buffer (pH 6.0), 0.1 mM TPP, 1 mM Mg $SO_4 \cdot 7H_2O$, 0.16 mM NADH, 31 E.U. of YADH, and 37 units of orange pyruvate decarboxylase in a total volume of 3 ml. Velocity (v) determined as rate of absorbance loss at 340 nm.

(no cation), by factors of 15%, 10%, and 9%, respectively. Zinc and cobalt were moderately inhibitory, causing decreases in activity of 15% and 31%, respectively, relative to the control, and 0.1 mM Hg^{++} caused a 93% loss of activity within 15 min. The effects of Ni^{++} and Cu^{++} could not be satisfactorily determined because both were inhibitory to yeast ADH and both interfered with the MTT aldehyde assay.

The enzyme was very specific in its substrate requirements exhibiting rates with 2-ketobutyrate, 2-ketovalerate, and 2-ketocaproate, of only 33.3%, 1.1%, and 0.6%, respectively, relative to the rate observed with pyruvate. No measurable activity was observed with C-8 and C-9 acids or branched-chain acids, and no 2-butanone was detected by gas chromatography in reaction mixtures containing levulinic acid, indicating that the enzyme requires a keto group adjacent to the carboxyl group for activity.

Since little or no activity was evidenced with 2-keto acids C-6 and longer, the C-6 and C-9 acids as well as levulinic acid were tested with pyruvate for competitive inhibition. The K_m and V_{max} data (Table 2) revealed little change in K_m in the presence of 2-ketocaproic and levulinic acids indicating little or no affinity of these compounds for the enzyme. However, 2-ketononanoate was a strong competitive inhibitor, having a relative affinity for the enzyme (K_i = 0.5 mM) greater than that of pyruvate (K_m = 3.2 mM).

Table 2—Kinetics of inhibition of orange pyruvate decarboxylase

Substrate	K_m(mM)	K_i(mM)	V_{max} (nmole/min)
Pyruvate	3.2	—	440
Pyruvate + 1 mM 2-Ketocaproate	3.4[a]	19	440
Pyruvate + 1 mM 3-Ketovalerate	3.6[a]	11	440
Pyruvate + 1 mM 2-Ketononanoate	9.5[a]	0.5	440

[a] $K_{m_{app}}$

Table 3—Kinetics of recombination of apo-orange pyruvate decarboxylase with Mg^{++} and TPP

Mg^{++}	
TPP(mM)	$K_{Mg}{}^{++}$(mM)
0.6	1.8
1.0	1.8
TPP	
Mg^{++}(mM)	K_{TPP}(mM)
1.0	0.26
8.0	0.27

Active site studies

The K_m of orange PDC determined at pH 6.0 was 0.8 mM, somewhat lower than the value of 3.2 mM determined at pH 7.0. A Hill plot (Fig. 2) over a pyruvate concentration range of 0.05 to 50 mM yielded a straight, unbroken line having a slope of 0.99. The ratio of $[S]_{90}/[S]_{10}$ (i.e. those substrate concentrations yielding rates, relative to V_{max}, of 90% and 10%, respectively) was calculated to be 79.9 compared to the theoretical value, $[S]_{90}/[S]_{10} = 81$, for enzymes having single or multiple non-cooperative substrate binding sites (Segal, 1975).

In the presence of 0.01 mM Hg^{++}, orange PDC saturated with pyruvate was inactivated at the same rate relative to its control as was enzyme from which pyruvate was removed, indicating that a sulfhydryl group is not involved in binding substrate to the enzyme.

The kinetics of recombination of TPP and Mg^{++} with the *apo*-enzyme are summarized in Table 3. The equilibrium constant, K_{TPP}, was independent of the Mg^{++} concentration; likewise, K_{Mg} was independent of TPP concentration. The results indicate that Mg^{++} and TPP are bound to the *apo*-enzyme at separate sites by independent binary equilibria. The enzyme exhibited a sharp recombination optimum at pH 6.8 with both Mg^{++} and TPP, the recombination constants at pH 6.6 and 7.0 increasing by an order of magnitude over those determined at pH 6.8.

DISCUSSION

THE LOWER of the two apparant pH optima determined for orange PDC (ca. 4.8) is considerably below the optima exhibited by most cytoplasmic enzymes. This may indicate

that the enzyme in vivo is exposed to a more acidic environment than that of the cytoplasm. Between pH 4.0 and 7.5, the initial enzyme rates remained constant with time indicating that the enzyme is stable in this pH range. Below pH 4.0, the initial rates began to decrease with time, approaching zero more rapidly as the pH approached 3.0. This is consistent with the results of Roe and Bruemmer (1974) who reported that PDC activity in freshly reamed orange juice (pH 3.4) sustained an 80% loss in activity within 1 hr at 30°C. The second pH optimum of orange PDC (ca. pH 6.0) compares more favorably with the optimum of yeast PDC (6.0–6.2) reported by Ullrich (1974). The apparant dual pH optima of orange PDC may reflect different functional aspects of the enzyme, e.g. binding of substrate, etc., and catalysis of decarboxylation.

The effects of divalent cations upon orange PDC resemble, somewhat, those observed with its yeast counterpart. Orange pyruvate decarboxylase, like the yeast enzyme (Schellenberger and Hübner, 1967) is relatively nonspecific in its cofactor requirements, being activated by Mn^{++} and Ca^{++} as well as Mg^{++}. Unlike yeast PDC, however, orange PDC is moderately inhibited by Co^{++} and Zn^{++}

Like yeast PDC, the orange enzyme is dependent upon sulfhydryl groups for activity; however, unlike yeast PDC (Schellenberger and Hübner, 1968), it is not protected by its substrate from attack by sulfhydryl reagents. Hence, a sulfhydryl group is apparently not active in substrate binding. In this regard, the enzyme resembles the PDC present in the pyruvate dehydrogenase complex of *Escherichia coli* described by Schwartz and Reed (1970).

The Hill plot, obtained over a pyruvate concentration range of 5 orders of magnitude, is typical of a mono-catalytic site enzyme, having a slope approaching 1, as opposed to yeast PDC which yields Hill plot slopes of 1.7–1.8 below 3 mM pyruvate, indicating the presence of two active sites (Ullrich and Donner, 1970).

The K_m values obtained for orange PDC (0.8 mM at pH 6.0; 3.2 mM at pH 7.0) are comparable to values of 1.13 mM (Ullrich, et al., 1970) and 3 mM (Ullrich and Donner, 1970) reported for yeast PDC. The increase in affinity of the orange enzyme for pyruvate at pH 6.0 is consistent with the activity vs pH data (Fig. 1). However, further work is needed to determine the true effect of pH upon the ionization of the prototropic groups of the free enzyme.

Studies of the recombination of TPP and Mg^{++} with *apo*-orange PDC have shown that, like its counterpart in yeast (Schellenberger and Hübner, 1967), both coenzyme and metal are bound to the *apo*-enzyme via independent equilibria at separate sites, both enzymes exhibiting the same pH optimum for recombination. The dissociation constants for the *apo*-PDC-Mg^{++} and *apo*-PDC-TPP complexes ($K_{Mg^{++}}$ and K_{TPP}, respectively) were on the same order as the corresponding constants (1.13 mM and 0.65 mM, respectively) for yeast PDC.

Schellenberger and Hübner (1967) have demonstrated that following the independent loose binding of Mg^{++} and TPP to the protein, the protein undergoes a conformational change with the binding of TPP to Mg^{++} to form a cyclic ternary complex (*holo*-PDC). Cyclization occurs at a slow but measurable rate, and the rate of cyclization is accelerated in the presence of pyruvate ($K_{Pyruvate}$ = ca. 17 mM). The *holo*-enzyme, once formed, is stable below pH 8. Above pH 8, the enzyme is cleaved into its components within a few minutes.

The formation of such a cyclic ternary complex appears to be true also of orange PDC. The orange enzyme, after exhaustive dialysis or Sephadex G-25 gel filtration in the absence of Mg^{++} and TPP, consistently retained 10–15% of its original activity. The activity present after these treatments was stable and no further activity loss occurred in the presence of either sodium azide or EDTA. Hence, it is believed that this remaining activity corresponds to the cyclic ternary *holo*-enzyme. Quantitative elimination of this activity was achieved only by ammonium sulfate precipitation of the protein at pH 8.5–9.0. The enzyme, both before and after precipitation, exhibited the same degree of activity regeneration in the presence of Mg^{++} (1 mM) and TPP (0.1 mM).

Holzer and Beaucamp (1961) have reported direct proof that decarboxylation of pyruvate by yeast PDC proceeds via a condensation of pyruvate with TPP at C-2 of the thiazolium ring, followed by elimination of CO_2 and subsequent release of acetaldehyde by exchange with another pyruvate molecule. Thus TPP is responsible for the actual decarboxylation reaction, the enzyme protein acting as a template to bring pyruvate and TPP into the proper proximity and orientation, as well as providing a favorable microenvironment for the reaction to occur. Since the mechanism of thiamine catalysis is unlikely to change, and since orange and yeast PDC exhibit similar recombination kinetics with Mg^{++} and TPP, the mechanisms of the two enzymes must be similar, except for the obvious difference in their modes of substrate binding.

If the *holo*-enzyme represents 10–15% of the total orange PDC activity as suggested above, then the predominant form of the enzyme in vivo must be either the *apo*-enzyme or the inactive, uncyclized ternary complex. The instability of the former argues in favor of the latter. Cyclization of this ternary complex to the active *holo*-enzyme in response to increases in the concentration of available pyruvate, as occurs with yeast PDC, offers a mechanism for the pyruvate-stimulated activity increase of PDC in fresh orange juice observed by Roe and Bruemmer (1974). The rate of aldehyde accumulation in fresh juice containing no added pyruvate was only ca. 20% of the maximum rate observed in the presence of 10 mM pyruvate. This indicates in turn that only about 20% of the total decarboxylase present in the juice was in the active form, which agrees relatively well with the 10–15% *holo*-enzyme content indicated by the present study.

Based on the present K_m/V_{max} data, the rate of PDC-catalyzed decarboxylation should be almost negligible at the level of free native pyruvate (0.04 mM) in orange juice (Roe and Bruemmer, 1974), and the proportion of active *holo*-enzyme would be expected to be considerably lower than 10–20% of the total enzyme present. Hence, the physiological concentration of pyruvate available to PDC in the intact juice sacs must be considerably higher, and is probably better reflected by the K_m (0.8 mM) of the enzyme (Segal, 1975).

Pyruvate-stimulated cyclization of an inactive ternary PDC complex may also explain the results of Davis et al. (1973) who observed little change in PDC levels of waxed Valencia oranges during controlled atmosphere storage, yet found substantial increases in concentrations of both acetaldehyde and ethanol with decreasing O_2 concentration in the storage atmosphere. Anaerobiosis in the fruit would result in a shift of available pyruvate from the tricarboxylic acid cycle to the PDC pathway thus activating "uncyclized" PDC already present.

Of the substrates tested with purified orange PDC, only pyruvate and 2-ketobutyrate were decarboxylated at significant rates, in contrast to the relatively nonspecific decarboxylase activity reported by Roe and Bruemmer (1974) in neutralized orange juice extracts. The relative reactivities of the purified enzyme with pyruvate and 2-ketobutyrate were identical to those reported for the extract activity. These results suggest the presence of more than one 2-ketoacid decarboxylase in orange vesicle tissue, one (PDC) which is specific for C-3 and C-4 acids, and the other(s) which at-

tacks longer straight- and branched-chain homologs.

The minimal observed rates of the purified enzyme preparation with 2-ketovalerate and 2-ketocaproate may or may not be due to PDC activity. Either the presence of C-3 or C-4 acids as contaminants in the C-5 and C-6 substrates, or traces of other 2-ketoacid decarboxylase activity in the PDC preparation, could also account for the observed rates. Since 2-ketocaproate (1 mM) did not act as a competitive inhibitor of PDC with pyruvate, it is concluded that PDC would probably not catalyze decarboxylation of the C-5 and C-6 acids at their physiological concentrations, having virtually no affinity for them.

Of interest, in regard to competitive inhibition, is the strong affinity of the enzyme for 2-ketononanoate (K_i = 0.5 mM). This indicates that steric factors are not responsible for the inability of the enzyme to decarboxylate C-5–C-9 acids. Ullrich et al. (1970) stated that due to the greater lipophilicity of the larger alkyl groups of long-chain 2-keto acids, they are bound better to the lipophilic binding sites of yeast PDC. This may explain the strong binding of 2-ketononanoate to orange PDC without release of product, but does not account for the behavior of the enzyme toward 2-ketocaproate which should also be quite lipophilic. Since the substrate binding site of orange PDC appears to be different from that of its yeast counterpart, the explanation of these results must await further investigation of the nature of the orange PDC binding site.

The results of this investigation indicate that the direct contribution of orange PDC to the orange volatile profile is limited to acetaldehyde and possibly propionaldehyde, both of which are known constituents of aqueous orange essence (Shaw, 1977). Since aldehydes enter into other equilibrium reactions in orange, particularly alcohol formation (Bruemmer and Roe, 1971), the influence of PDC upon the composition of the orange volatile profile may be reflected indirectly by other volatile constituents which contain ethyl and propyl residues. Evidence of a second 2-keto-acid decarboxylase in the orange has been presented but its contribution, if any, to the orange volatile fraction, awaits further investigation.

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SOY FLOUR SOLUBILITY AND EMULSION PROPERTIES . . . From page 773

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FACTORS AFFECTING THE THRESHOLD OF METHYL AND ETHYL OLEATE EMULSIONS IN RAISINS AND RAISIN PASTE

D. G. GUADAGNI and A. E. STAFFORD

ABSTRACT

Raisins prepared by dipping in methyl or ethyl oleate emulsions and dried in a tunnel dehydrator at 65.5°C for 12 hr had significantly lower off-flavor thresholds than those in which oleate was added after drying. This indicates that time-temperature combinations of this magnitude caused oxidative changes or interactions between constituents in the emulsions and raisins yielding a mixture of increased off-flavor potency. Whole raisins had an apparent lower oleate threshold than the same raisins ground into paste prior to tasting because of the nonuniform distribution of oleates in whole raisins. Consequently, conditions improving the uniform distribution of oleates in whole raisins would reduce off-flavor detection in oleate-treated fruit. Emulsifiers used in commercial oleate preparations can be the most significant source of off-flavor contribution to the dried raisins.

INTRODUCTION

THE PERTINENT REFERENCES dealing with the history of the use of fatty acid esters to facilitate drying of grapes into raisins, and the problems involved in the time-honored natural sun drying process were listed in a previous report (Guadagni et al., 1975). The authors pointed out the problem of possible off-flavors arising from the use of fatty acid esters in raisins and gave thresholds of a number of fatty acid esters in raisin paste and whole raisins. Thresholds of different compound preparations varied within a wide range and even those from the same compound preparation obtained from different sources varied widely. Part of the variation was attributed to known impurities, part to variation in percentage of the principal compound and part to unknown factors. This paper is concerned with threshold effects of methyl and ethyl oleate preparations on the surface of whole raisins as contrasted to a uniform concentration throughout the raisin mass as in paste, and to the possible effect that the heat applied during drying might have on fatty acid ester threshold. The effect of emulsifier in the commercial preparation of methyl oleate used in these experiments is also considered.

MATERIALS & METHODS

THE METHYL and ethyl oleate oils used for the dipping treatments were made from high oleaic safflower oil, and both were obtained from the same commercial supplier. Total ester content of the ethyl and methyl oleate oils was 70.0 and 92.5% respectively. For the ethyl oleate oil, 75.6% of the total esters was ethyl oleate, 18.8% ethyl linoleate and 5.6% ethyl palmitate. For the methyl oleate oil, 76.4% of the total esters was methyl oleate, 16.6% methyl linoleate and 7.0% methyl palmitate. The ester composition of the dipping oils and treated raisins was determined by the GLC method described by Stafford et al. (1974). The methyl and ethyl oleate oil emulsions were formed by blending the oil in water in a large Waring Blendor for 60 sec and mixing with additional water to give the desired concentration of oil.

Thompson seedless grapes were obtained from Fresno, Ca, transported to the laboratory and held at 1.1°C overnight. The berries were stripped from the bunches by hand, and the individual berries were hand dipped in emulsions containing from 2–16% methyl oleate, or 1–8% ethyl oleate for 1 min, drained 1 min and placed on stainless steel wire mesh trays. Controls for the oleate-treated berries were dipped in plain water. The dipped berries were placed in a tunnel dryer and dried at 65.5°C for 12 hr. All samples were then finish dried at 43.3°C until the moisture content reached 12–14%. The raisins were allowed to equilibrate at room temperature for 1 wk and the moisture of all samples was adjusted to 16% by dipping in 37°C water for 30 sec and equilibrating. The raisins were then sealed in gallon glass jars and held at 1.1°C until the sensory experiments were begun.

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Sensory evaluation

Detection thresholds for the dipping oils retained on the raisins were determined exactly as described previously (Guadagni et al., 1975). Briefly the procedure was as follows: Samples dipped in oleates were paired with water-dipped samples and presented to a panel of 20 judges trained to detect off-flavors in oleate-treated raisins. The task was to determine the sample with the off-flavor. Each pair was replicated at least three times giving 60 judgements per treatment. All samples were coded with two-digit numbers, and order of presentation was completely randomized. Because of the persistency of fatty acid ester flavors, only one pair was presented per session held in mid-morning and mid-afternoon. Thresholds were determined by plotting the results from four or five concentrations from a given treatment on log-probability paper and determining the concentration where 50% of the judgements detected an off-flavor. These values and the 95% confidence intervals are equivalent to the familiar LD_{50} used in dose-response experiments (Litchfield and Wilcoxon, 1949).

To evaluate the effect of surface concentration and heat during drying, three sets of threshold measurements were made. First, oleate thresholds were determined on whole raisins (RW) by comparing the samples dipped in varying concentrations of oleate emulsion with similar samples dipped in water alone. Secondly, the same oleate and water-dipped raisins described above were ground to a paste (RP), and thresholds were determined on the uniformly mixed paste, using the paste from the water-dipped raisins as control. This procedure equalizes the oleate concentration throughout the paste whereas in whole raisins it may be concentrated on the surface. Thirdly, only the water-dipped raisins were ground to a paste (RPU), and thresholds of the oleate emulsion were determined by adding varying amounts directly to the ground paste. Any difference between the second and third (RP vs RPU) set of measurements would indicate the effect of heat on the oleates during drying.

RESULTS & DISCUSSION

Application of oleates

The relation between oleate concentration in the dip solution and in dried raisins is shown in Figure 1. For methyl oleate emulsion, the relationship between percent oleate in the dip solution and ppm on the whole raisins was linear with a correlation coefficient of 0.999 and standard error of estimate of 18 ppm. The linear relation for ethyl oleate emulsion had a correlation coefficient of 0.994 and standard error of estimate of 26 ppm. These statistical parameters indicate that oleate concentration on the dried raisins could be estimated fairly accurately for the conditions employed in these experiments.

Methyl oleate emulsion

The threshold data for methyl oleate on whole raisins (RW), ground whole raisins (RP) and in raisin paste (RPU) prepared from plain water dipped raisins from the same lot of fresh grape berries is shown in Figure 2. The slopes of

Table 1—Taste thresholds of methyl and ethyl oleate emulsions in raisins and raisin paste

	Threshold and 95% confidence limits (ppm)		
	Whole raisins (RW)	Oil-dipped raisin paste (RP)	Water-dipped raisin paste (RPU)
Emulsion			
Methyl oleate	735 (609–886)	850 (675–1070)	1310 (1100–1558)
Ethyl oleate	235 (209–265)	470 (418–529)	730 (651–818)
Total esters			
Methyl oleate	680 (563–820)	786 (624–990)	1212 (1018–1441)
Ethyl oleate	179 (159–201)	357 (318–402)	555 (495–622)
Individual esters			
Methyl oleate	515 (426–620)	595 (473–749)	917 (770–1091)
Ethyl oleate	125 (111–140)	249 (221–230)	387 (345–433)

the lines relating methyl oleate in the raisins or raisin paste and the sensory data corrected for chance selection indicate some differences in panel sensitivity depending on treatment of the product being tasted, (Rpu > Rw > Rp) but the differences in slope function for the three different products were not significant at $P \leq 0.05$. The thresholds varied from 735–1310 ppm methyl oleate emulsion with 95% confidence intervals ranging from 609–886 to 1100–1558 ppm (Table 1). For this particular lot of methyl oleate emulsion, oleate threshold was lowest in RW and highest in RPU. Although not significant at $P \leq 0.05$, threshold of RP was greater than that of RW and both RP and RW gave significantly ($P \leq 0.05$) lower threshold values than RPU.

In general this confirms the previous contention that detection of oleates on whole raisins occurred at a lower oleate concentration than in raisin paste because of unequal distribution of the oleates in whole raisins compared to the uniform mixture in paste. Perhaps the long moisture equilibration procedure used in these studies reduced the concentration gradient between surface and interior of the whole raisins and thus reduced the magnitude of the threshold differences between RW and RP. However, with the relatively concentrated oleate dip solutions and rapid drying procedures used in this study, it is reasonable to expect that oleate concentration on whole raisins would be higher on the surface area than in the central region. The perception of oleate flavor would then probably occur as a result of surface contact with the taste buds before chewing and mastication makes a reasonably uniform mixture between oleates and raisins. Initial oleate perception would be derived from surface concentration rather than the concentration based on total weight of the raisin sample. This indicates that conditions which allow equilibration of oleate throughout the raisins would reduce the oleate flavor perception of a given amount of oleate picked up during dipping.

The significant ($P \leq 0.05$) flavor threshold difference between RPU and RP suggests that the heat treatment applied during air drying either intensified the flavor of the oleate emulsion itself through oxidation to lower threshold compounds or caused interactions between oleate and raisin constituents or resulted from a combination of these possibilities. Another possible reason for the lower threshold of RP compared to RPU could be an interaction between the non-oleate emulsifier and raisin constituents in the presence of heat. The importance of the emulsifier in off-flavor detection in raisins will be discussed later.

Ethyl oleate emulsion

The threshold results for ethyl oleate emulsions are shown in Figure 3. The dose-response lines for the three

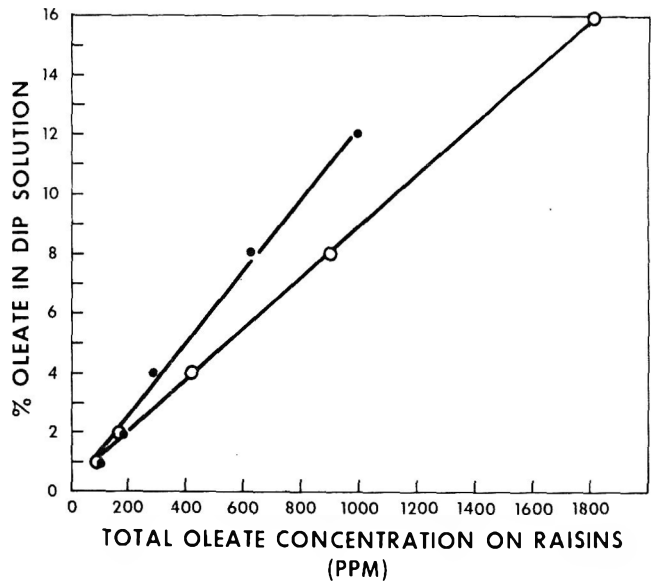

Fig. 1—Relationship between oleate concentration in dip solution and oleate content of dried raisins: ○—○ methyl oleate, ppm in raisins = 115.45 X % in dip solution − 37.4; ●—● ethyl oleate, ppm in raisins = 80.72 X % in dip solution + 5.1. (ppm in dip solution = % X 10^4.)

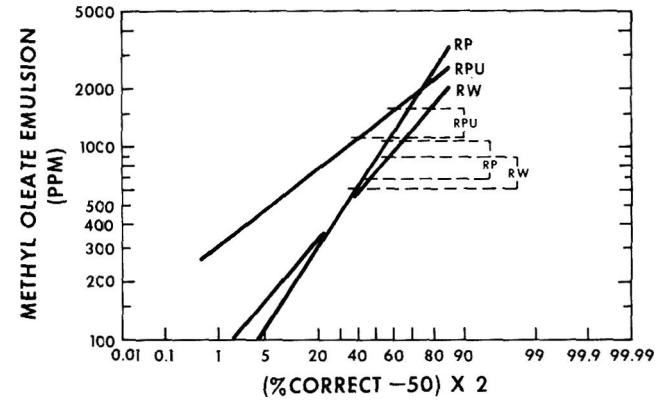

Fig. 2—Relation between concentration of methyl oleate emulsion in raisins and raisin paste and sensory detection of off-flavor: RPU—paste from water-dipped raisins, r = 0.94; RP—paste from oleate-dipped raisins, r = 0.95; RW—oleate-dipped whole raisins, r = 0.95. Dotted lines represent 95% confidence interval.

different raisin products are essentially parallel indicating no significant differences in sensitivity of the panel to detection of the ethyl oleate emulsion in these products. While the threshold values of ethyl oleate follow the same increasing order in RW, RP and RPU observed with methyl oleate, the actual values are much lower, (Table 1) and the differences among raisin samples are all highly significant and much more distinct than those observed for methyl oleate. The same reasons for the differences observed in the samples dipped in methyl oleate emulsions apply to the ethyl oleate dipped material. Namely, the lower threshold in RW as compared to RP is probably due to surface concentration effects, and the difference between RP and RPU is undoubtedly due to the heat effect obtained during drying the grapes in the presence of oleates and or emulsifiers. The large difference between RW and RPU represents the combination of both surface and heat-oleate interaction effects. Since the surface and heat effects observed for methyl and ethyl oleate are qualitatively similar and detection of the ethyl oleate preparations occur at about 1/3–1/2 the concentration of methyl oleate preparations, it is clear that methyl oleate would be preferable from an off-flavor standpoint.

Effect of emulsifier

Because the threshold of the methyl oleate emulsion used in these studies was considerably lower than that observed for pure methyl oleate, (Guadagni et al., 1975) the emulsion was vacuum distilled thus separating the nonvolatile emulsifiers from the volatile fatty acid esters. Table 2 shows thresholds of the methyl oleate emulsion with and without emulsifier in three different lots of raisin paste prepared from untreated raisins. In all cases, it is clear that the emulsifier greatly reduced threshold values in the paste, causing off-flavor detection at oleate levels 25–27% of those without emulsifier.

From the composition of the distilled oil and threshold values of pure methyl oleate, methyl linoleate and methyl palmitate (Guadagni et al., 1975), it was found that each of these constituents behaved additively in contributing to threshold of the fatty acid mixture. Assuming that the non-oleate impurities would also behave additively with the oleates in raisin paste, the threshold values for the impurities were calculated for the three raisin paste samples in Table 2. Since the composition of original methyl oleate emulsion was: methyl oleate, 70.6%; methyl linoleate, 15.4%; methyl palmitate, 6.5%; and emulsifier and other impurities, 7.5%; the equation for calculating threshold of the impurities took the form: $70.6/1.05 + 15.4/0.88 + 6.5/2.45 + 7.5/X = 100/Y$. In this equation the numerator represents percentage of the constituent in raisin paste; the denominator represents threshold (%) of the pure constituent; X represents threshold of the combined impurities, and Y is the threshold of the entire mixture. According to this concept, the calculated impurity thresholds for samples 1, 2 and 3 in Table 2 are 266, 111 and 152 ppm respectively. The ratio of the thresholds of the impurities to that of the distilled oleate mixture varied from 37.6–45.9 for the three raisin paste samples. This indicates that the impurities in this particular sample of methyl oleate emulsion were about 37–46 times more potent in terms of off-flavor contribution than the distilled methyl oleate mixture. Therefore, it would seem to be especially important to control the amount and nature of the emulsifiers used in oleate mixtures. These results emphasize the fact that additives other than fatty acid esters are also very important in determining acceptability of raisins treated with drying aids.

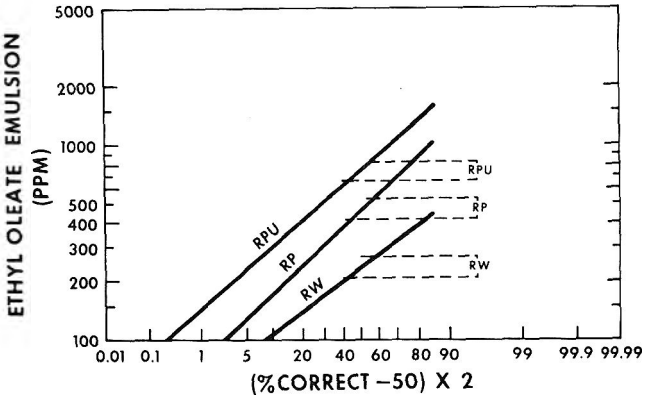

Fig. 3—Relation between concentration of ethyl oleate emulsion in raisins and raisin paste and sensory detection of off-flavor. RPU—paste from water-dipped raisins, r = 0.98; RP—paste from oleate-dipped raisins, r = 0.95; RW—oleate-dipped whole raisins, r = 0.99. Dotted lines represent 95% confidence interval.

Table 2—Effect of emulsifier on threshold of methyl oleate emulsion in raisin paste

Raisin paste sample	Threshold and 95% confidence limits (ppm)	
	With emulsifier	Without emulsifier
1	2700 (1995–3727)	10,000 (8607–11,617)
2	1310 (1100–1558)	5100 (4350–5979)
3	1722 (1608–1872)	6700 (5980–7506)

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Reference to a company and/or product named by the Department is only for purposes of information and does not imply approval or recommendation of the product to the exclusion of others which may also be suitable.

HEAT INACTIVATION OF PEROXIDASE IN CORN-ON-THE-COB

Y. C. LEE and J. K. HAMMES

ABSTRACT

Sweet corn was blanched in steam to investigate heat inactivation of peroxidase in corn-on-the-cob and the relationship between the residual peroxidase activity and flavor of stored frozen corn-on-the-cob. Heat inactivation of peroxidase in the kernel section was quickest and that in the center cob section was slowest. Heat penetration studies supported the fact that slow heat inactivation in outer cob and center cob sections was due to slow heat penetration in these sections. Temperature dependence of heat inactivation of peroxidase was accounted for by the Arrhenius equation and Ea for inactivation of peroxidase was estimated to be 7.4 ± 0.4 Kcal/mole. A computer model was developed to predict the residual peroxidase activity in blanched corn-on-the-cob and the prediction was reasonably accurate. Corn-on-the-cob blanched less than 15 min at 99°C developed varying degrees of off-flavor as determined by panel members. Peroxidase activities in the outer cob and the kernel sections of corn-on-the-cob had significant correlations with off-flavor development in corn-on-the-cob.

INTRODUCTION

WHEN RAW or underblanched vegetables are held in frozen storage for extended periods of time, a progressive deterioration in quality, such as the development of off-flavors and color destruction, occurs. These changes in quality are believed to be the result of enzyme action.

Peroxidase appears to be one of the most heat stable enzymes present in many fruits and vegetables. Therefore, it has been used as a prime index of blanching vegetables prior to canning and freezing. Yamamoto (1958) reported that peroxidase in whole kernel sweet corn had a heat labile fraction and a heat stable fraction which represented 5% of total peroxidase activity. Guyer and Holmquist (1954), Labbee and Esselen (1954) and Nebesky et al. (1951) reported that the peroxidase activity in processed vegetables was closely associated with off-flavor development in products during storage. The objectives of this research were to study heat inactivation of peroxidase in corn-on-the-cob (C-O-C) under various blanching conditions and to determine the relationship between the residual peroxidase activity and flavor of stored frozen C-O-C.

MATERIALS & METHODS

Blanching sweet corn

A hybrid developed by Green Giant Company (Code 48), harvested at 75 ± 1% of moisture content, was used for the blanching study. The ears with a maximum diameter of 5 ± 0.3 cm were trimmed to 14 cm in length. The trimmed ears were steam blanched at 82, 88, 93 and 99°C for 4, 7, 10 and 15 min, cooled in cold water and frozen by submersion in "Freon" for 9 min. Frozen C-O-C was stored in −18°C until used for enzyme analysis. Eight ears per treatment with four replications were used for the blanching study.

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Table 1—Distribution of peroxidase in nonblanched C-O-C of 11 hybrids

	Kernel	Outer Cob	Center Cob
Avg peroxidase activity ($\bar{X} \pm \sigma$)	9.0 ± 2.8	22.2 ± 6.9	5.3 ± 2.1
Percent of total activity	24.7	60.8	14.5

Heat penetration study

Thermocouple probes were positioned at 0.8, 1.7 and 2.5 cm from the surface of the middle section of the ear to monitor heat penetration at 82, 88, 93 and 99°C. Six ears per blanching temperature with three replications were used for the study.

Enzyme study

The frozen C-O-C was divided into kernel (diameter ≥3.4 cm), outer cob (1.6 <d <3.4 cm) and center cob (d ≤1.6 cm) sections for enzyme analysis. The distribution of peroxidase activity in raw C-O-C was determined by a random sample of eleven Green Giant experimental hybrids (six ears per hybrid) harvested on the same day at a similar maturity.

Peroxidase activity was determined by the o-phenylenediamine method (Vetter, et al., 1958). One peroxidase activity unit was defined as changes in one optical density per five minutes per gram of sample.

Flavor evaluation of frozen C-O-C

Flavor evaluation was confined to C-O-C blanched at 99°C for 4, 7, 10 and 15 min. Twelve panel members, who were prescreened on their ability to consistently identify flavor characteristics of nonblanched versus blanched C-O-C, were employed for the study. C-O-C stored at −18°C for 9 months was heated in boiling water for 16 min and tasted for flavor without addition of butter or salt.

RESULTS & DISCUSSION

Distribution of peroxidase in fresh C-O-C

Peroxidase activity in kernel, outer cob (including germ) and center cob sections of 11 sweet corn hybrids is summarized in Table 1. High peroxidase activity was found in the outer cob section, which had 60.8% of total peroxidase activity. The peroxidase activity in kernel and center cob sections was 24.7% and 14.5% of total peroxidase activity, respectively.

Heat penetration into C-O-C during blanching

Although the time required for heat to penetrate C-O-C was dependent on blanching temperature and time, the pattern of heat penetration at all temperatures was similar. The heat penetration during blanching at 99°C is shown in Figure 1. The heat penetration at 0.8 cm from the surface of C-O-C was rapid for the first 4 min and then leveled off. The heat penetration at 1.7 cm and 2.5 cm from the surface was similar, except the latter had 2–3 min of initial lag time and was slower. Temperature at 1.7 cm from the surface reached 66 and 80°C after 10 and 15 min blanching, respectively.

Heat inactivation of peroxidase in C-O-C

Heat inactivation of peroxidase was quickest in the kernel section and slowest in the center cob section (Fig. 2). The slow heat inactivation of peroxidase in outer cob and

center cob sections was due to the slow heat penetration into these sections (Fig. 1).

The heat inactivation of peroxidase in the kernel section was fast for the first 7 min of blanching and then slow (Fig. 2). The plots of blanching time versus the logarithm of percent residual activity showed broken curves. This could mean that corn kernels contained two fractions of peroxidase which had different heat resistance. Yamamoto (1958) reported that sweet corn kernels had a heat sensitive peroxidase fraction and a heat resistant peroxidase fraction. The heat resistant fraction represented 5% of total peroxidase activity and was primarily located in the pericarp. The kernel section of C-O-C blanched at 99°C had 3% residual peroxidase activity after 7 min blanching and 1% after 15 min blanching.

The plots of logarithms of percent residual peroxidase in outer cob and center cob sections versus blanching time showed straight lines (Fig. 2). Percent residual peroxidase activity in outer cob and center cob sections of C-O-C, blanched at 99°C for 15 min, was 13 and 45%, respectively.

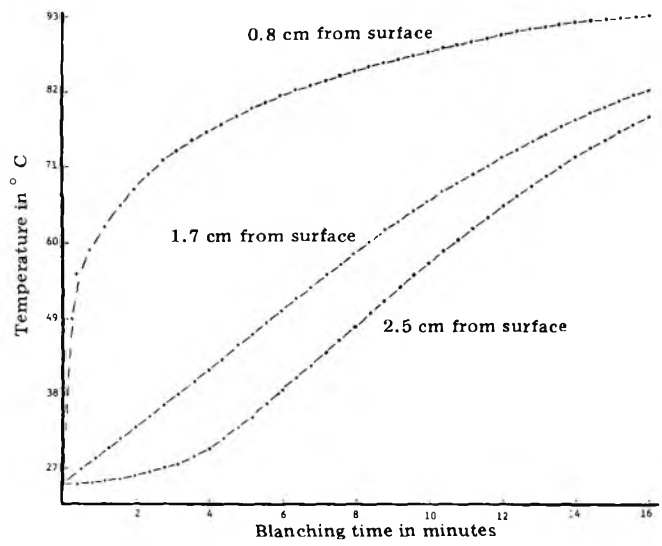

Fig. 1—Heat penetration into C-O-C during blanching at 99°C.

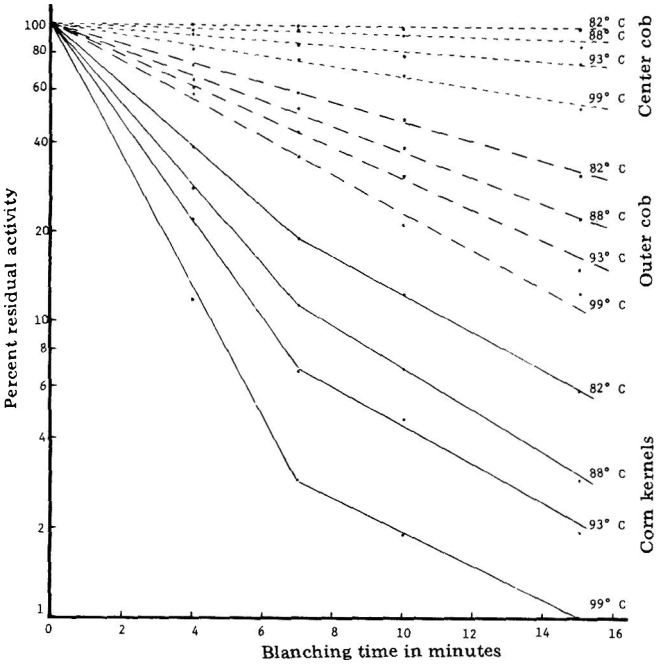

Fig. 2—Residual peroxidase activities in three sections of C-O-C blanched at 82, 88, 93 and 99°C

Effect of temperature on heat inactivation of peroxidase

The Arrhenius equation is one of the commonly used methods to account for temperature dependence of reactions:

$$\ln K = \ln A - \frac{Ea}{R}\frac{1}{T} \qquad (1)$$

where K = rate constant of a reaction; A = Arrhenius constant; Ea = energy of activation (for inactivation of peroxidase in this case); R = gas constant (1.987 cal/°K-mole); T = temperature (°K).

Heat inactivation of peroxidase in the sweet corn kernel was studied at 82, 88, 93 and 99°C of steam and the rate constant of heat inactivation of peroxidase at each temperature was calculated by plotting logarithm of percent residual activity versus blanching time.

A plot of the rate constant versus the reciprocal of the blanching temperature is shown in Figure 3. An Ea for inactivation of peroxidase in the sweet corn kernel was calculated from the Arrhenius plot, which was 7.4 ± 0.4 Kcal/mole. Svensson and Ericksson (1974) reported an Ea of 8.2 Kcal/mole for heat inactivation of peroxidase in peas.

Prediction of residual peroxidase activity in C-O-C

Based on the kinetics of heat inactivation of peroxidase in corn kernels, a computer program was developed to predict residual peroxidase activities in outer cob and center cob sections of blanched C-O-C. Residual peroxidase activity in the outer cob section of blanched C-O-C was of particular interest, because the peroxidase activity in the outer cob sections was high and it was assumed that off-flavor of frozen C-O-C during storage could be developed in the outer cob section.

Assumptions made to develop the prediction model were:

1. Heat penetration into three sections of C-O-C, monitored by using thermocouple probes, represented the mass average temperature of each section.
2. Heat inactivation of peroxidase in C-O-C was an accumulative effect of temperature which changed with blanching time.
3. Outer cob and center cob sections of C-O-C did not have a noticeable amount of heat resistant peroxidase, and heat inactivation of peroxidase followed a first-order reaction (Fig. 2).

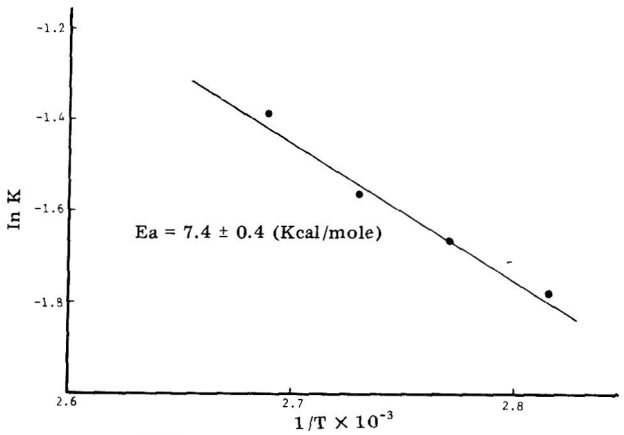

Fig. 3—An Arrhenius plot of peroxidase inactivation in sweet corn kernels

Table 2—Subjective flavor evaluation of frozen C-O-C stored at 0° F[a]

	Blanching time at 99°C							
	4 Min		7 Min		10 Min		15 Min	
Storage	Flavor acceptable	Off-flavor	Flavor acceptable	Off-flavor	Flavor acceptable	Off-flavor	Flavor acceptable	Off-flavor
9 Months	3/12	9/12	8/12	4/12	11/12	1/12	12/12	0/12

[a] "Acceptable flavor" ranges from excellent to fair flavor without off-flavor development. The data show number of panel members indicating the designated flavor. Example: 8/12 under "Acceptable flavor" = Eight members out of 12 judged C-O-C, blanched for 7 min and stored for 9 months, did not detect any off-flavor.

4. The cooling cycle did not considerably affect the heat inactivation of peroxidase.

The mathematical model used to develop the computer program was:

1. Heat penetration of C-O-C as a function of blanching time: used the Fourier Series of which coefficients were calculated based on the experimentally determined heat penetration data.
2. The heat inactivation rate of peroxidase (K) as a function of blanching temperature: used the Arrhenius Eq (1).
3. The residual peroxidase activity: The residual peroxidase activity as a function of blanching time was calculated by using the first-order equation.

$$\text{First order equation: } \ln C/Co = -Kt \qquad (2)$$

where K = rate constant of heat inactivation of peroxidase; C = residual peroxidase activity at blanching time t; Co = initial peroxidase activity; t = blanching time in minutes.

The information required to predict the residual peroxidase activity using this computer program was heat penetration data, Ea for heat inactivation of peroxidase and initial peroxidase activity. The program would define the blanching time and blanching temperature which would be used to estimate temperature at the section of C-O-C using the Fourier Series. The estimated temperature was then used to calculate the heat inactivation rate of peroxidase from the Arrhenius equation. The heat inactivation rate was then employed to calculate the residual peroxidase activity at the defined blanching time, and the results were printed out.

A few examples of the predicted residual peroxidase activity in blanched C-O-C were compared with those experimentally determined (Fig. 4). The overall results showed that the computer program could predict the residual peroxidase in C-O-C blanched under various conditions with reasonable accuracy.

Relationship between enzyme activities and flavor scores

The selected panel members were asked to evaluate flavor of C-O-C blanched at 99°C for 4, 7, 10 and 15 min and stored at −18°C for 9 months. The results are summarized in Table 2.

C-O-C blanched for 15 min at 99°C did not develop off-flavor in a 9-months storage period. C-O-C blanched less than 15 min developed varying degrees of off-flavor. Incidences of off-flavor detected increased with a decrease in blanching time. A separate flavor preference test on 9-months old C-O-C showed that 11 out of 12 panel members preferred 10 min blanched C-O-C to that blanched for 7 min.

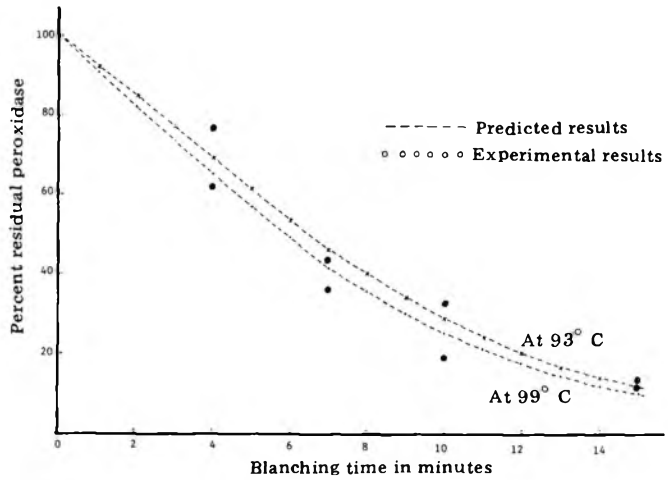

Fig. 4—Comparison of predicted peroxidase activities with experimentally determined peroxidase in outer cob section

The relationship between the residual peroxidase activity and off-flavor score of frozen C-O-C stored for 9 months was studied by the regression analysis. There was significant correlation between off-flavor score and the residual peroxidase activity in the outer cob section (r = 0.999) and off-flavor score and the residual peroxidase activity in the kernel section (r = 0.96).

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WATER-SOLUBLE PRODUCTS FROM PATULIN DURING ALCOHOLIC FERMENTATION OF APPLE JUICE

E. E. STINSON, S. F. OSMAN and D. D. BILLS

ABSTRACT

During the course of alcoholic fermentation, 50 ppm of ^{14}C-patulin added to apple juice was converted to other materials. The products were largely nonvolatile and water soluble. Little, if any, patulin was metabolized to CO_2. Comparison of the thin-layer chromatographic (TLC) migration of the major patulin products from fermentation with the products of the reaction of patulin with cysteine indicated that at least 58% of the added patulin was converted to substances other than adducts of cysteine, peptides and proteins. In addition to a major TLC-immobile component (21%), at least six TLC-mobile patulin products were observed.

INTRODUCTION

PATULIN is a mycotoxin reported to produce tumors in rats at the point of injection when administered subcutaneously (Dickens and Jones, 1961). Patulin is produced in high yields by a number of food-contaminating fungi (Enomoto and Saito, 1972). One of these fungi, *Penicillium expansum*, is an important cause of storage rot of apples, and patulin has been isolated from infected apples and commercial apple juice (Harwig et al., 1973a; Stoloff, 1975). The fate of patulin during alcoholic fermentation has not been resolved. French workers have reported the presence of residual patulin following fermentation of apple juice (Drilleau and Bohuon, 1973), but others have reported that patulin "disappears" or is substantially reduced during fermentation of apple juice (Burroughs, 1977; Harwig et al., 1973b; Stinson et al., 1978).

This study was undertaken to determine whether patulin per se persists following alcoholic fermentation of apple juice and to determine the nature of other products that may arise from patulin during fermentation.

EXPERIMENTAL

Materials

Uniformly labeled ^{14}C-patulin was prepared by an unpublished modification of the fermentation method of Norstadt and McCalla (1969) that we developed. Dr. Norstadt supplied a freshly isolated strain of *Penicillium urticae*. The fungus produced the highest yields of patulin when the medium (potato dextrose broth) was autoclaved long enough to give a golden yellow color (121°C for 30-45 min). Uniformly labeled ^{14}C-glucose was added after autoclaving to serve as the radioactive precursor. Monitoring the fermentation by pH measurement rather than elapsed time was crucial for favorable yields. Two successive physiological phases of *P. urticae* growth control patulin biosynthesis (Bu'Lock et al., 1965). When this medium is used, the pH increases slowly from 5.2 to 6.7 during the first phase and dips slightly to 6.3 during the second. Maximum patulin concentration was attained at the end of the second phase, which was detected by the dip in the pH. It was important to harvest patulin at this point as autolysis immediately ensued (marked by increasing pH) and rapidly destroyed patulin. Peak patulin concentration was variable, but 1000 mg/liter after 10 days was representative, falling to 600 mg/liter at 12 days. When the pH started to rise after reaching a minimum, the mycelial growth was removed by filtering through cheesecloth. The fermentation broth was concentrated in vacuo and extracted with ethyl acetate. The extract was dried over $MgSO_4$, concentrated to a thick sirup, and placed on a silicic acid column, and the patulin was eluted with benzene/chloroform, 3:1. The solvent was removed under vacuum, and the patulin was purified by successive recrystallizations from benzene and ether. Minimal heating was used to avoid the decomposition during recrystallization that has been reported (Katzman et al., 1944). Identity and purity of the labeled patulin were established by comparing its IR spectrum and migration on thin layer chromatographic (TLC) plates with those of authentic patulin supplied by R. D. Stubblefield (NRRC, USDA, Peoria, IL) and by running a mixed melting point with authentic patulin. The specific activity of the purified material was 0.00355 mCi/mmol as measured by the procedure described later. All of the ^{14}C activity was associated with the patulin TLC spot. Other procedures for preparing ^{14}C-patulin have been reported (Lovett, 1972; Dailey et al., 1977; Nip and Chu, 1977).

Patulin-cysteine adduct was prepared by reacting patulin with cysteine in aqueous solution in 1:1 ratio at 0.0065M concentrations. Base (0.1N NaOH) was added as required to maintain the pH at 3.5, the approximate pH of apple juice. After the solution had stood for 2 wk at room temperature, the characteristic UV absorbtion peak for patulin at 275 nm diminished to approximately 50%. No new UV peaks were observed. Patulin, but not cysteine, could be detected by TLC. The solution became yellow and a small quantity of yellow precipitate developed, corresponding to the precipitate described by Ciegler et al. (1976). Patulin-cysteine adduct solution was concentrated by evaporation under nitrogen prior to application to TLC plates.

Fermentation procedure

Standard fermentation practices were followed. A commercial "Champagne" yeast, *Saccharomyces cerevisiae* v. *ellipsoides* (Universal Foods, Red Star Yeast Division, Milwaukee, WI; originally from Institute Pasteur, Paris, France) was obtained as a lyophilized powder in an aluminum foil package labeled as sufficient for 5 gal. This was suspended in 100 ml water, and 0.5 ml was used for inoculation. A stock solution was prepared from whole, pasteurized apple juice by adding sugar to 22°B. Other components were 50 ppm ^{14}C-patulin, 0.08% $(NH_4)_2SO_4$, and 20 ppm Dowex Anti-Foam A (conveniently added after being absorbed on sugar). A 125-ml aliquot of this solution was placed in a 125 ml Erlenmeyer flask (actual capacity approx 140 ml) and inoculated with the yeast suspension. The flask was covered with a one hole rubber stopper in which a glass tube had been inserted. The gas evolved by the fermentation was bubbled successively through three solutions: the standard fermentation gas lock in which the gas is bubbled through water to insure anaerobic conditions, then two solutions of aqueous KOH to absorb the CO_2 formed by the fermentation. As evolution of gas slowed in the latter part of the fermentation, it was necessary to raise the inlet tube of the second flask above the liquid level to prevent backup of liquid into the fermentation gas lock.

The fermentation was allowed to proceed 28 days at 21°C, until gentle swirling of the flask produced no further gas evolution, and then allowed to stand two additional days to permit the yeast to form a compact sediment.

A control experiment consisted of adding ^{14}C-patulin to unfermented, pasteurized apple juice containing 20% added ethanol to prevent microbial growth. This solution was analyzed for patulin and ^{14}C-patulin products after standing for 30 days at 21°C.

Detection of radioactivity

Radioactive events were detected by a Nuclear-Chicago Mark IV liquid scintillation counter (G.D. Searle & Co., Des Plains, IL), and Aquasol, a liquid scintillation solvent containing a thixotropic material (Nuclear-Chicago). This formed a stiff gel upon addition of sufficient water. Liquid materials could be analyzed when added to the

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solvent. Solid materials were analyzed after being suspended in stiff thixotropic gels.

TLC procedures

Uniplate silica gel GF 250 μ plates (Analtech) and preabsorbant LQF 250 μ plates (Kontes) were used. The organic phase of a mixture of n-butanol/acetic acid/water (10:1:3) was used as the developing solvent. Zones on the TLC plates were detected by UV quenching, ninhydrin, I_2 vapor, and ^{14}C counting of material scraped from the plates.

Patulin analysis

Patulin was quantitatively analyzed by the method of Stinson et al. (1977).

RESULTS & DISCUSSION

THE ALCOHOLIC FERMENTATION yielded a fermented cider with a typical, pleasant aroma. The pH of the initial apple juice was 3.5, and that of the fermented cider was 4.0. The final Brix was -0.5°. Analysis of the fermented cider for patulin gave negative results by the Stinson et al. (1977) procedure.

The opalescent, fermented cider was clarified to a brilliant clear liquid by centrifugation for 40 min at 20,000g in a refrigerated centrifuge. The sediment from this centrifugation was combined with the original sediment for ^{14}C analysis. The solutions from the fermentation and the sediment were analyzed for ^{14}C (Table 1). The original juice before fermentation contained 2,540 cpm/ml.

Table 1—Distribution of ^{14}C after fermentation of apple juice containing ^{14}C - Patulin

Fraction	Total cpm	% of original
Clarified fermented cider (118 ml)	289,400	91.6
Sediment from settling and centrifuging 1.6g, ~2 ml	9,200	2.9
Fermentation lock (H_2O; 130 ml)	12,800	4.1
First CO_2 trap (10% KOH; 300 ml)	6,200	2.0
Second CO_2 trap (10% KOH; 15 ml)	165	0.05
	317,765	100.65

The fractions of ^{14}C labeled material consisted of 91.6% water-soluble material that remained in the fermentation flask, 2.9% in the sediment, and 2.0% in the CO_2 traps. The occurrence of the sizeable ^{14}C fraction, 4.1%, in the fermentation lock was not expected. There was no reason to assume that foaming had occurred, since no tendency was observed during the laboratory working day. A possible explanation could be mechanical entrapment of microscopic droplets during effervescence of the CO_2 bubbles, as approximately 6.5 liters of CO_2 resulted from the fermentation. The finding that the CO_2 traps contained only 2.0% of the ^{14}C indicated that little, if any, patulin was metabolized to yield CO_2.

Attempts to cleanly separate by TLC the soluble, ^{14}C-containing materials in clarified, fermented cider were unsuccessful. After the plates were developed and dried, the disposition of ^{14}C was 21% at or near the origin from R_f 0.00–0.08, 16% from R_f 0.08–0.66, 11% from R_f 0.66–0.75, and 47% from R_f 0.75–1.00. Five percent of the applied activity was not recovered from dried TLC plates and was assumed lost in the form of volatile compounds; 95% was present in relatively nonvolatile compounds which persisted on TLC plates even after prolonged drying. Components on TLC plates at R_f's 0.00–0.08 and 0.66–0.75 were visualized by either UV light (254 nm) or treatment with iodine vapor; ninhydrin-positive components were observed only at R_f 0.00–0.08. Since a fermented apple juice control (unspiked with ^{14}C-patulin and containing no detectable patulin prior to fermentation) also yielded similar components that could be visualized with UV light, iodine vapor, or ninhydrin, the actual ^{14}C-containing products may not be the visible components.

In the unfermented control, over 98% of the 50 ppm added ^{14}C-patulin was recovered unchanged at the end of 30 days; only 0.6 ppm was converted to nonvolatile substances that were insoluble in ethyl acetate. This agrees with published data (Scott and Somers, 1968). Preliminary work indicated that the products arising from patulin in unfermented apple juice consisted of at least six components; however, these components existed at such comparatively low concentrations in fermented juice that they could not be detected in the presence of the other products resulting from patulin. Thus, the reaction of patulin with material initially present in unfermented apple juice could account for only 1.2% of the patulin converted to other substances during fermentation.

The reaction of patulin with sulfhydryl-containing amino acids and proteins to form uncharacterized adducts has been advanced to explain the disappearance of patulin in many foods, including apple juice (Ciegler et al., 1976). The sulfhydryl content of apple juice has been reported as less than 0.003 mmole per 100 ml (Scott and Somers, 1968). In a bimolecular reaction, this could account, at most, for the loss of only 4.6 ppm patulin, whereas fermentation resulted in the loss of 50 ppm patulin. To determine whether the formation of adducts with compounds containing sulfhydryl groups could account for the disappearance of patulin, we subjected the patulin-cysteine adduct preparation to TLC analysis under the same conditions as the fermentation product. The most intense UV quenching band occurred at the origin. A number of mobile bands extended up as far as R_f = 0.40, with the most prominent bands at R_f 0.14–0.19 and 0.32–0.40. The lack of mobility of the adduct in comparison to the bulk of the ^{14}C-containing material from the fermentation indicated that much of the patulin was converted to substances other than adducts of cysteine under the circumstances of this experiment. Adducts of patulin and peptides, such as glutathione, or proteins containing sulfhydryl groups would exhibit even less TLC mobility.

It has been reported that yeast does not produce extracellular materials that can react with patulin (Harwig et al., 1973b). If this is the case, the formation of new ^{14}C-containing materials from ^{14}C-patulin during fermentation must result from the metabolic activity of yeast cells on apple juice substrates and/or patulin, itself.

Further work is required to determine the chemical composition and toxicologic properties of the products that arise from patulin during alcoholic fermentation. Thus far, efforts here and elsewhere (Ciegler et al., 1976) to chemically characterize products of patulin degradation or reaction have been unsuccessful because of the apparent instability of the compounds. Ciegler et al. (1976) demonstrated that patulin-cysteine adducts retain teratogenic activity in chick embryo tests. It is possible that other products that may arise during alcoholic fermentation also are biologically active, and it would be unwise to assume that the "disappearance" of patulin per se in any food system nullifies the potential health hazard posed by its initial presence.

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EFFECT OF STORAGE TIME AND CONDITIONS ON THE HARD-TO-COOK DEFECT IN COWPEAS (Vigna unguiculata)

S. SEFA-DEDEH, D. W. STANLEY and PETER W. VOISEY

ABSTRACT

Storage under unfavorable conditions is known to adversely affect the cooking quality of legumes by leading to the "hard-to-cook" defect. This phenomenon was studied by examining the texture and microstructure of Ghanainan cowpeas, variety "Adua ayera," following storage at 0°C, 80% RH; 21°C, 35% RH and 29°C, 85% RH for up to 12 months. Scanning electron microscopy revealed no changes in raw beans after 12 months storage but soaking in water produced a loss of some protein bodies in seeds stored at 29°C. Texture measurements indicated that the rate of cooking of the beans decreased with increasing storage temperature and that storage at 29°C introduced the formation of the "hard-to-cook" defect. The microstructure of the defective beans showed an incomplete breakdown of the middle lamella which may partially explain this defect.

INTRODUCTION

COWPEAS (*Vigna unguiculata*), also known as blackeye peas, are an important source of protein in developing countries. It is now established that after prolonged storage under unfavorable conditions cowpeas and other legume seeds may develop the "hard-to-cook" defect, i.e. an increase in cooking time required for tenderness. Cook time for legumes is a function of their moisture content and storage temperature, time and relative humidity (Weston and Morris, 1954; Morris and Wood, 1956; Burr et al., 1968; Kon, 1968; Burr, 1973). Reports on methods to minimize the "hard-to-cook" defect (Burr et al., 1968; Kon, 1968; de Ruiloba, 1973; Molina et al., 1976) indicate that this phenomenon may be the result of chemical or enzymatic changes in the beans.

Softening of beans during cooking was found to be accompanied by structural changes such as breakdown of the middle lamella in the cotyledons (Rockland and Jones, 1974; Sefa-Dedeh et al., 1978). This softening followed first order kinetics (Sefa-Dedeh et al., 1978). It is possible that the "hard-to-cook" defect may also be reflected in the microstructure of the stored beans. The objective of this study was to use the SEM to monitor structural changes in cowpeas during storage as well as water absorption and textural measurements in an attempt to explain the "hard-to-cook" defect.

MATERIALS & METHODS

Cowpea samples

The cowpea (*Vigna unguiculata*) variety "Adua ayera," grown in Ghana, was used throughout this work.

Storage

Studies were conducted using three storage conditions, 0°C, 80% RH; 21°C, 35% RH and 29°C, 85% RH, with cowpea samples in 10 kg lots packed in cotton flour bags. Half of the sample stored at 29°C, 85% RH was also placed in cardboard cartons to provide additional insulation. Changes in moisture, microstructure, texture and cooking properties were evaluated after 3, 7, 9 and 12 months storage. Unstored cowpea samples are referred to as "standard."

Moisture determination

Moisture in the samples was determined using the American Association of Cereal Chemists (AACC) Method 44-15 (AACC Approved Methods, 1969) of oven drying at 130°C for 1 hr.

Scanning electron microscopy

The stored cowpea samples were fixed for 48 hr in 2% glutaraldehyde (in 0.025M phosphate buffer, pH 6.8) at 4°C. The fixed samples were transferred to the phosphate buffer (4°C) which was changed five times over a 1-hr period to remove the glutaraldehyde. The samples were sectioned with a razor blade and dehydrated in five 20-min changes of 10, 20, 40, 60 and 80% ethanol and three 20-min changes in 100% ethanol. The dehydrated samples were dried using the critical point drying technique (Anderson, 1951), mounted on specimen stubs with colloidal silver and coated with a 300 nm layer of gold using a Hummer II sputter coater with gold/palladium (60:40) alloy (Technics, Inc., Alexandria, VA). The coated samples were viewed and photographed in an ETEC Autoscan Scanning Electron Microscope (ETEC Corp., Hayward, CA) at an accelerating voltage of 20 kv. Cooked cowpeas were prepared by first fixing in 2% glutaraldehyde, then post-fixing in 2% buffered osmium tetroxide for 1 hr and dehydrated through a graded series of ethanol as described above.

Texture measurements

A 40-g sample of raw cowpeas soaked in water for 1, 3, 6, 12, 18 or 24 hr was tested to determine their hardness using the wedge method described by Sefa-Dedeh et al. (1978). Twenty measurements were performed at each soaking time.

A 500-g cowpea sample was soaked in water at 25°C for 24 hr and then cooked by simmering in 1200 ml of water on a hot plate for 2 hr. The texture of the cooked beans was evaluated using an Ottawa Texture Measuring System (OTMS) test cell (10 cm^2) with an eight-bar wire extrusion grid (Canada Machinery Ltd., Simcoe, Ontario). The cell was operated in an Instron testing machine using a crosshead speed of 10 cm/min. A 48-g sample of cooked beans was used for each determination and the measurements were done in triplicate.

Water absorption

Water absorption properties of cowpeas were determined by soaking 10-g samples in water at 25°C for 1, 3, 6, 12, or 18 or 24 hr. After soaking the water was drained and the cowpeas blotted to remove surface water. The sample was then weighed and the increase in weight taken as the amount of water absorbed. Triplicate determinations were performed.

RESULTS & DISCUSSION

General effects

Cowpeas stored under different humidities and temperatures showed changing moisture contents with storage time (Fig. 1). The changes were affected by the relative humidity of the storage chamber with samples stored at higher humidity showing a higher moisture

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content. Other changes observed in storage included darkening of the beans. The samples stored at 29°C (85% RH) showing a more pronounced change in color after 9 months than those kept at 0°C (80% RH) or 21°C (35% RH). Some of the seeds stored at 29°C (85% RH) show a slight mould growth (estimated at 10% incidence) after 12 months.

Effect of storage on water absorption

Hydration properties of the cowpeas stored for up to 9 months showed little change. The rate of water absorption was similar after 7 and 9 months in storage for all storage conditions (Fig. 2). After 12 months a relatively high rate of water absorption at short soaking times was observed for cowpeas stored at high humidity irrespective of storage temperature. This higher rate of water absorption may be due to the greater amounts of imbibed water during storage. The seed coat, the first barrier to absorption, had had time to equilibrate with the high humidity environments, thus allowing easy penetration of water during soaking. Conversely, cowpeas stored under low humidity tended to have a slower absorption rate, possibly due to their lower moisture content.

Effect of storage on soaked cowpea texture

All the cowpea samples showed softening of the seeds with soaking time as reflected in the wedge cutting forces (Fig. 3). Storing cowpeas at 0°C (80% RH) for periods up to 9 months caused a relative increase in the hardness of the cowpeas at short soaking times when compared to the

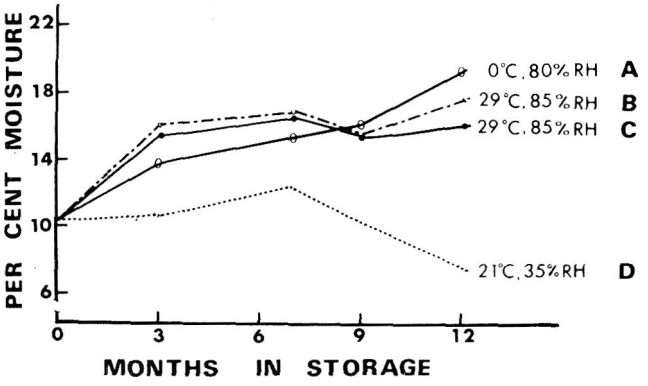

Fig. 1—Effect of storage conditions on moisture content of cowpeas.

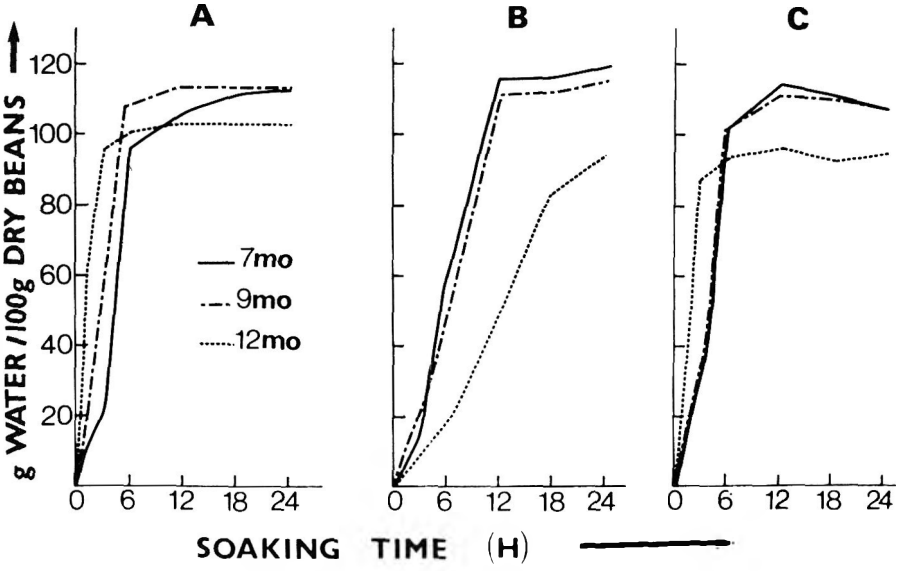

Fig. 2—Effect of soaking time on the hydration of cowpeas (Vigna unguiculata) stored under different conditions. A—0° C, 80% RH; B—21° C, 35% RH; C—29° C, 85% RH.

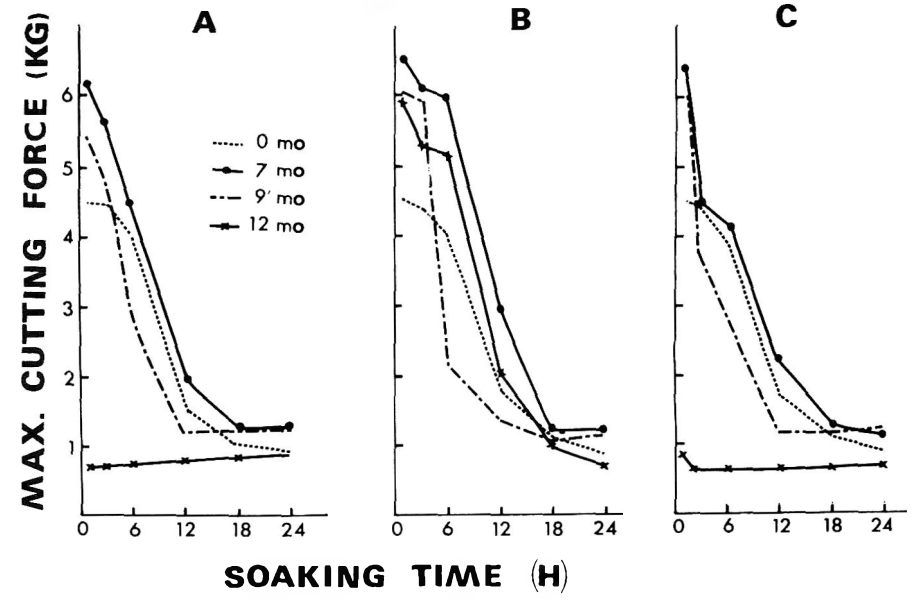

Fig. 3—Effect of soaking time on hardness of cowpeas (Vigna unguiculata) stored under different conditions as indicated by the maximum cutting force: A—0° C, 80% RH; B—21° C, 35% RH; C—29° C, 85% RH.

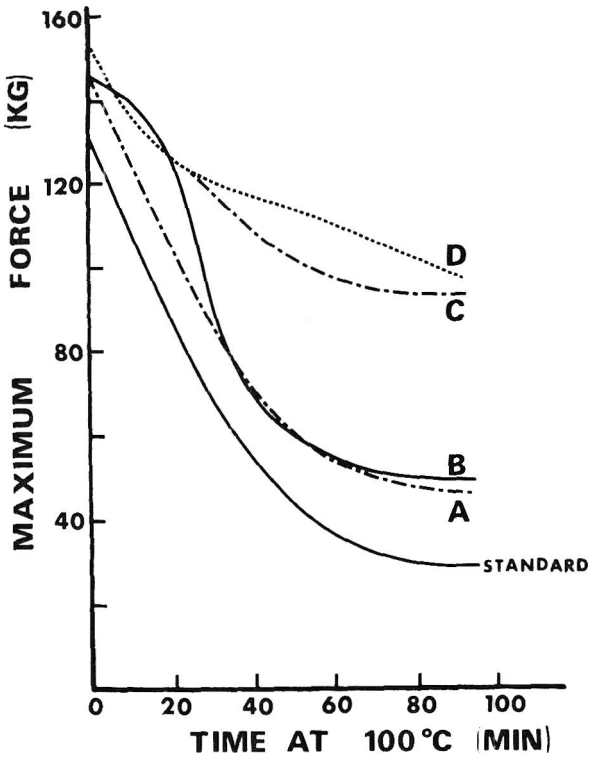

Fig. 4—Effect of heating at 100° C on texture of cowpeas stored for 12 months as indicated by the maximum force recorded in the OTMS wire extrusion cell: A—0° C, 80% RH; B—21° C, 35% RH; C—29° C, 85% RH (In bag inside a box); D—29° C, 85% RH (In bag alone). Standard = unstored cowpeas.

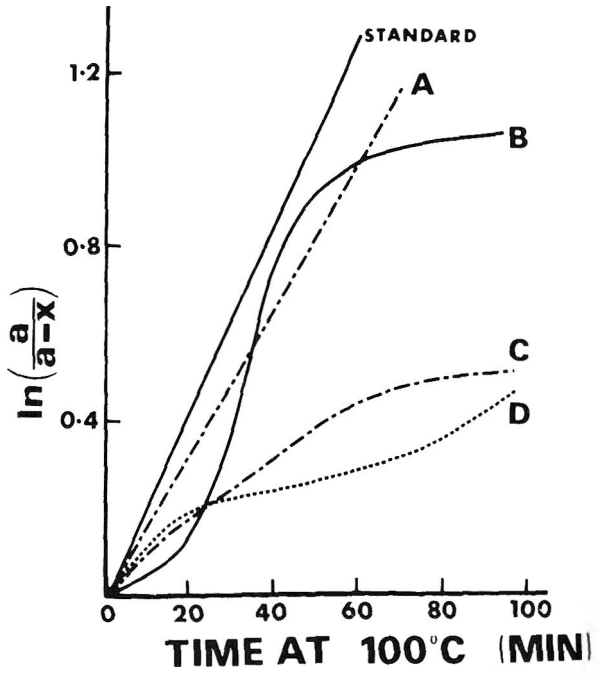

Fig. 5—The thermal softening of cowpeas (Vigna unguiculata) stored for 12 months under different conditions, plotted according to the equation: $\ln[a/(a-x)] = k_1 t$ where a = maximum force on cowpeas heated to 100° C; a−x = maximum force on cowpeas heated at 100° C for t min; k_1 = first order reaction rate constant; and t = time of heating at 100° C (min). A—0° C, 80% RH; B—21° C, 35% RH; C—29° C, 85% RH (In bag inside a box); D—29° C, 85% RH (In bag alone). Standard = unstored cowpeas.

control (Fig. 3A). However, after 12 months storage, the soaked cowpeas possessed a soft texture at all soaking times. The same phenomena were found for cowpeas stored at 29°C (85% RH). It has been previously reported (Sefa-Dedeh et al., 1978) that the water absorption characteristics of cowpeas show a high negative correlation with soaked cowpea hardness. A similar relationship is found in Figures 2 and 3; the cowpea samples stored at 0°C (80% RH) and 29°C (85% RH) for 12 months exhibited a high rate of water absorption at short soaking times and consequently a soft texture. Storage in a high humidity environment seemed to influence moisture uptake and consequently the hardness of the soaked stored cowpeas. Storage at 21°C in a low humidity environment (35% RH) consistently showed harder beans at the short soaking times. This is due to slow water uptake by these samples.

Effect of storage on cooked cowpea texture

After cooking for 90 min at 100°C, all the 12 month cowpea samples showed greater hardness when compared to the standard (Fig. 4) by an amount that was dependent on storage conditions. Samples stored at 29°C (85% RH) for 12 months exhibited the greatest increase in hardness. Cowpea samples stored at 0°C (80% RH) and 21°C (35% RH) showed a lower but similar hardness at the same cooking conditions.

Heating standard cowpeas at 100°C for 90 min resulted in an exponential reduction in the maximum force needed to compress, shear and extrude the cowpeas in the wire extrusion cell (Fig. 4). Stored cowpeas showed a similar pattern with the exception of those kept at 29°C (85% RH) which exhibited a harder texture. It was observed earlier (Sefa-Dedeh et al., 1978) that the rate of softening of standard cowpeas follows first order kinetics, the softening being related to the breakdown of the middle lamella. Applying these equations to the rate of softening of the stored cowpeas, it was found that only the sample stored at 0°C (80% RH) followed this type of kinetics (Fig. 5) whilst others showed anomalous behavior. Since the softening of cowpeas during cooking at 100°C is mainly dependent upon the breakdown of the middle lamella (Sefa-Dedeh et al., 1978), it may be inferred from Figure 5 that storage at high temperature and humidity for 12 months produced a failure of this process.

Effect of storage on microstructure

Microstructural changes were not apparent after 3 or 7 months storage for the three storage conditions (Fig. 6). The cotyledon cells showed starch granules embedded in a protein matrix with protein bodies. Similar structures were observed for cowpeas stored for 9 months (Fig. 7 A, B, C) and 12 months (Fig. 7 D, E, F). It seems that storage had no effect on the microstructure of raw, untreated cowpeas. This is of interest since prolonged storage under unfavorable conditions can lead to hardening of the seed with a concomitant increase in cooking time (Molina et al., 1976).

Scanning electron microscopy showed that after soaking for 24 hr at 25°C, the cowpea samples stored for 12 months at 0°C (80% RH) and 21°C (35% RH) had a structure similar to that of standard cowpeas (Fig. 8 A, B). The cowpea sample stored for 12 months at 29°C (85% RH) however, showed a loss of some protein bodies (Fig. 8C and 9). In contrast, similar samples examined under the SEM without a prior soaking treatment showed no loss of protein bodies (Fig. 7F). This suggests that the loss occurred during the soaking process, possibly due to the action of proteases that might have been stimulated during

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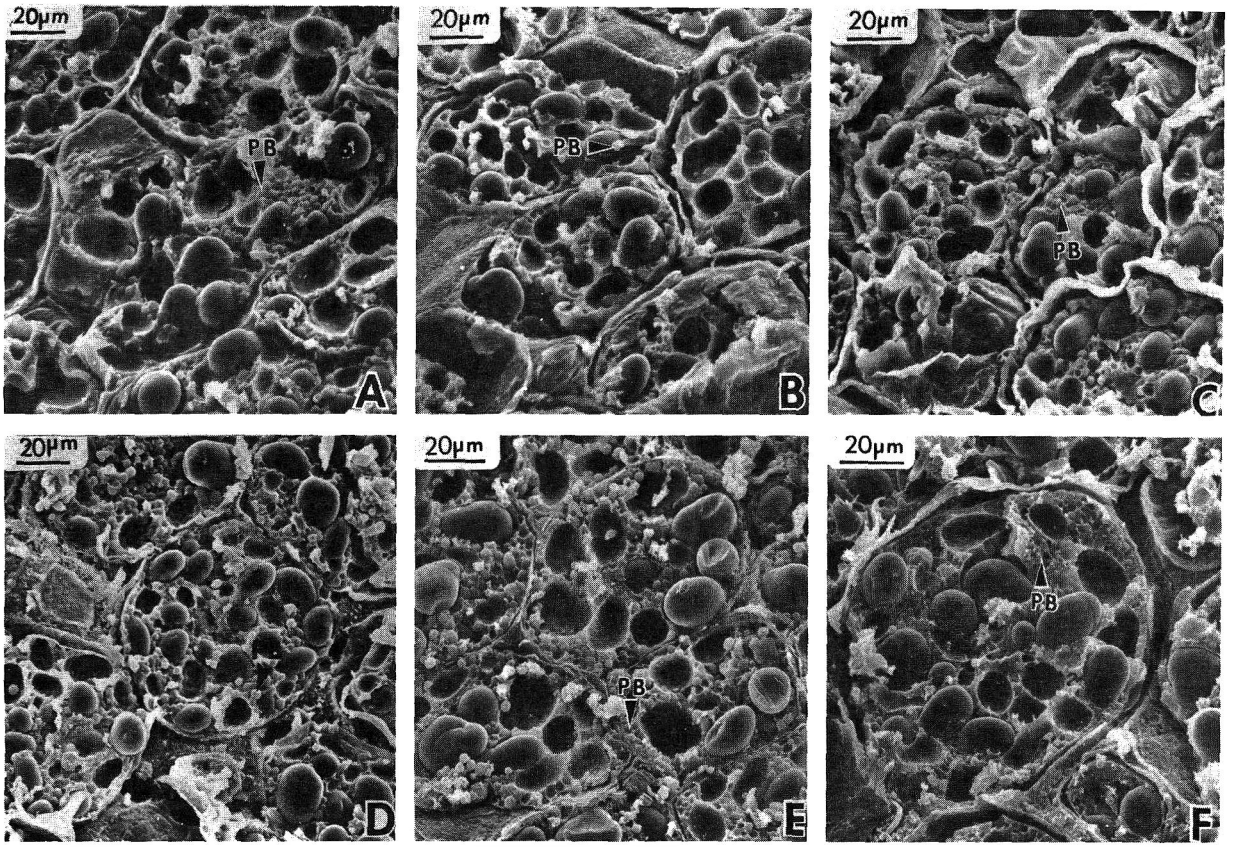

Fig. 6—Cross-sections of cotyledons of cowpea (Vigna unguiculata) stored for 3 months (A, B, C) and 7 months (D, E, F) at differe temperatures: A, D—0° C, 80% RH; B, E—21° C, 35% RH; C, F—29° C, 85% RH. S = Starch granule; PB = Protein body.

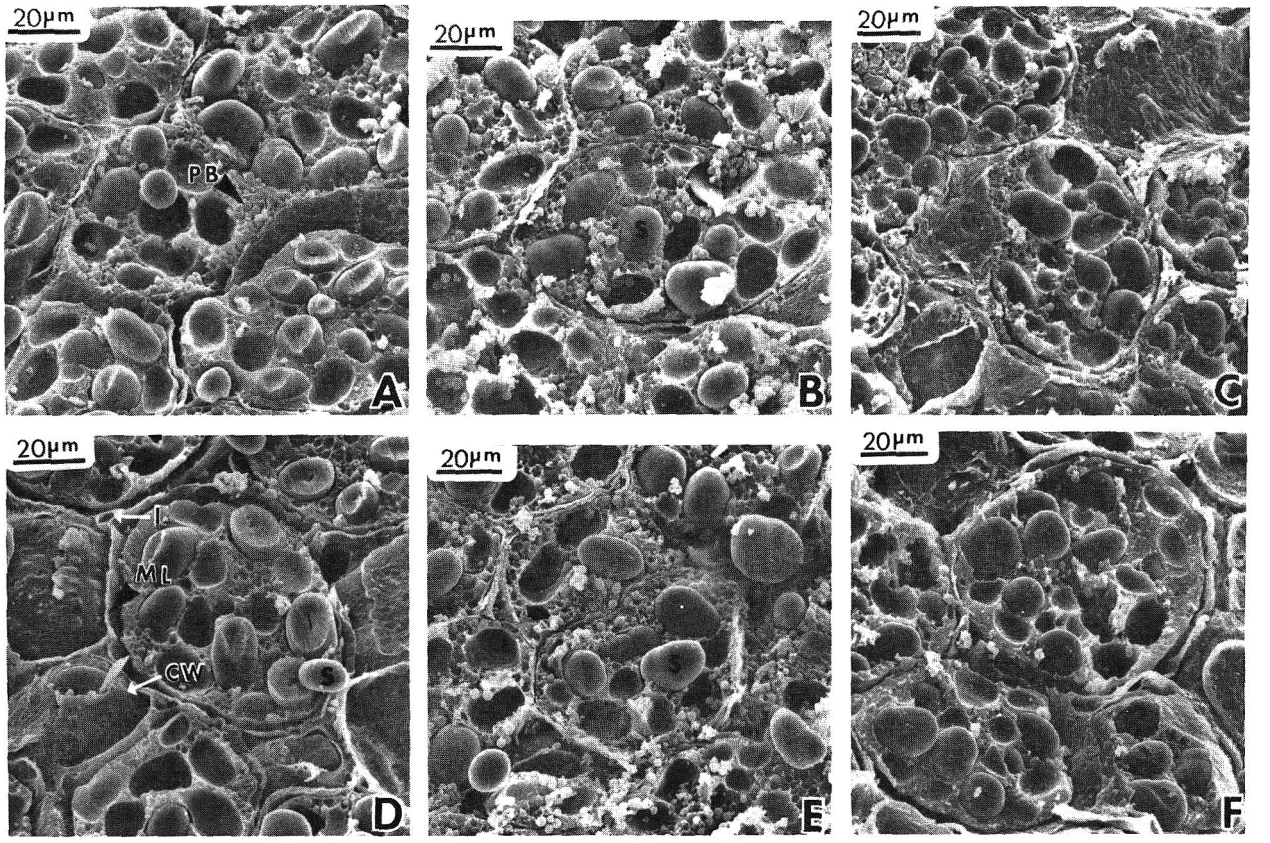

Fig. 7—Cross-sections of cotyledons of cowpeas (Vigna unguiculata) stored for 9 months (A, B, C) and 12 months (D, E, F) at different temperatures: A, D—0° C, 80% RH; B, E—21° C, 35% RH; C, F—29° C, 85% RH. I = Intercellular space; CW = Cell Wall; ML = Middle lamella; S = Starch granule; PB = Protein body.

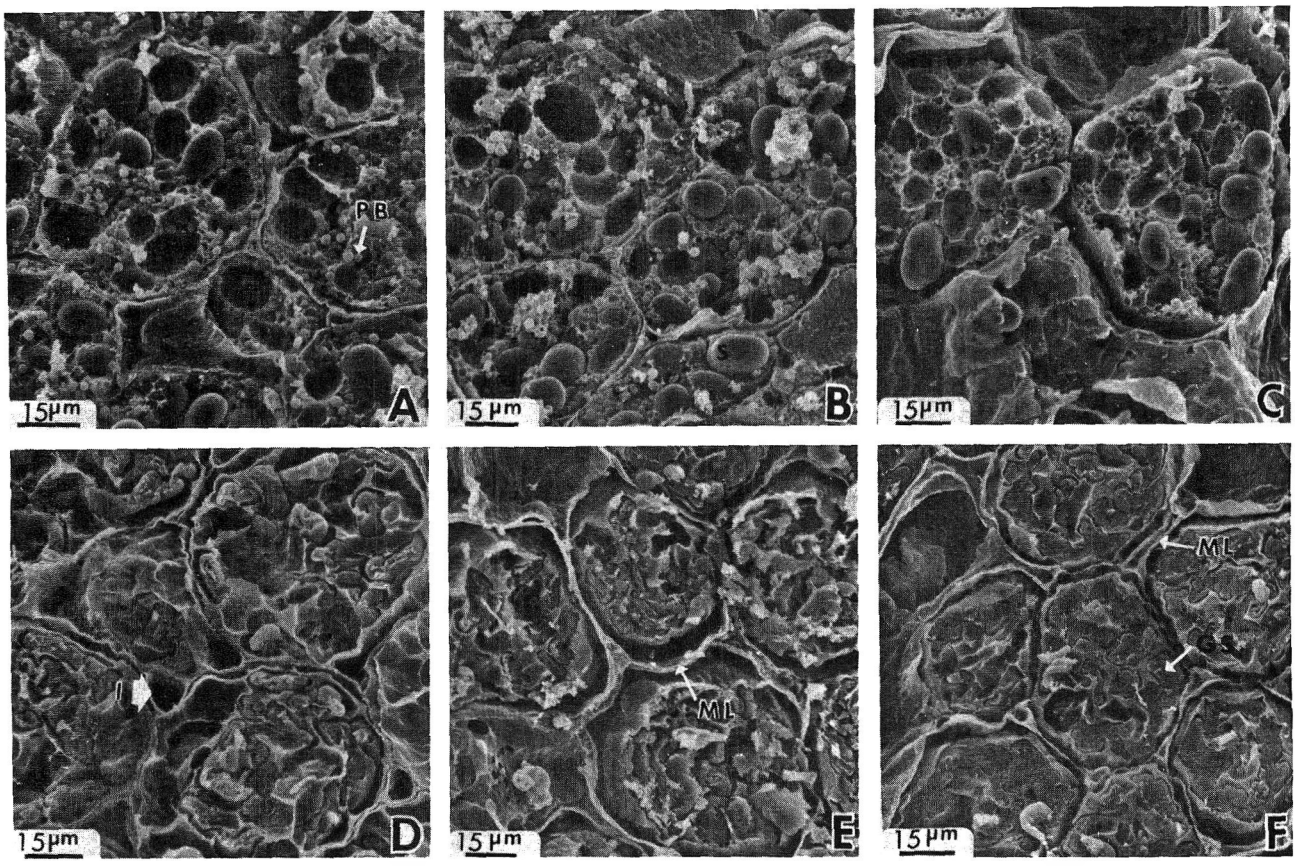

Fig. 8—Cross-sections of cotyledons of 12 month stored cowpeas (Vigna unguiculata) soaked for 24 hr at 25° C (A—C) and heated to 90° C (D—F): A, D—0° C, 80% RH; B, E—21° C, 35% RH; C, F—29° C 85% RH. PB = Protein body; ML = Middle lamella; GS = Gelatinized starch; I = Intercellular space; S = Starch granule.

storage. This is an observation which may explain some of the nutritional and functional changes that have been reported to occur during storage (Molina et al., 1975).

When heated to 90°C all the stored cowpea samples showed the beginning of gelatinization of starch (Fig. 8 D, E, F) in the form of deformed starch granules. A gradual change in microstructure was observed for all samples heated for 1 min and 5 min at 100°C (Fig. 10). At this stage of cooking the middle lamella exhibited a loss in structure which is probably the beginning of the softening process. Continued heating at 100°C caused an increased breakdown of the middle lamella for samples stored at 0°C (80% RH) and 21°C (35% RH) (Fig. 11 A, B, D, E). Cowpea samples stored at 29°C, however, showed little breakdown in the middle lamella (Fig. 11 C, F) even after 90 min. The increased hardness detected in these cowpeas may be due to the presence of intact middle lamella. This suggests that storage at high temperature and humidity might have caused changes in the middle lamella or cotyledons making subsequent thermal degradation difficult resulting in the "hard-to-cook" defect.

CONCLUSIONS

THESE STORAGE STUDIES confirmed what has been reported for other legumes, i.e. high temperature and high humidity adversely affect the cooking quality of the beans. This was evidenced by discoloration, some mould growth and the occurrence of the "hard-to-cook" defect. The water absorption properties and the hardness of the soaked cowpea changed during storage. This was more pronounced after 12 months of storage for all the different storage conditions used. In general, increasing soaking time decreased the hardness of the cowpeas. The soaked cowpea

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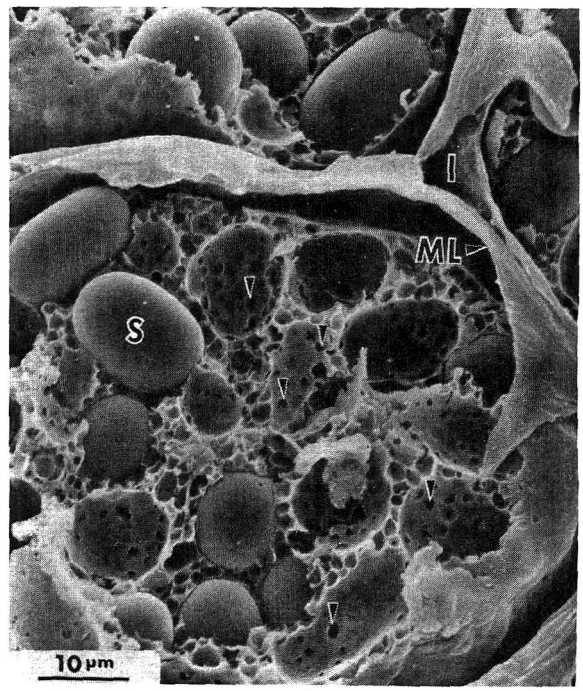

Fig. 9—Cross-section of cotyledon of cowpeas (Vigna unguiculata) stored at 29° C, 85% RH for 12 months and soaked at 25° C for 24 hr: ML = Middle lamella; I = Intercellular space; S = Starch granule. Arrows indicate points of protein body loss.

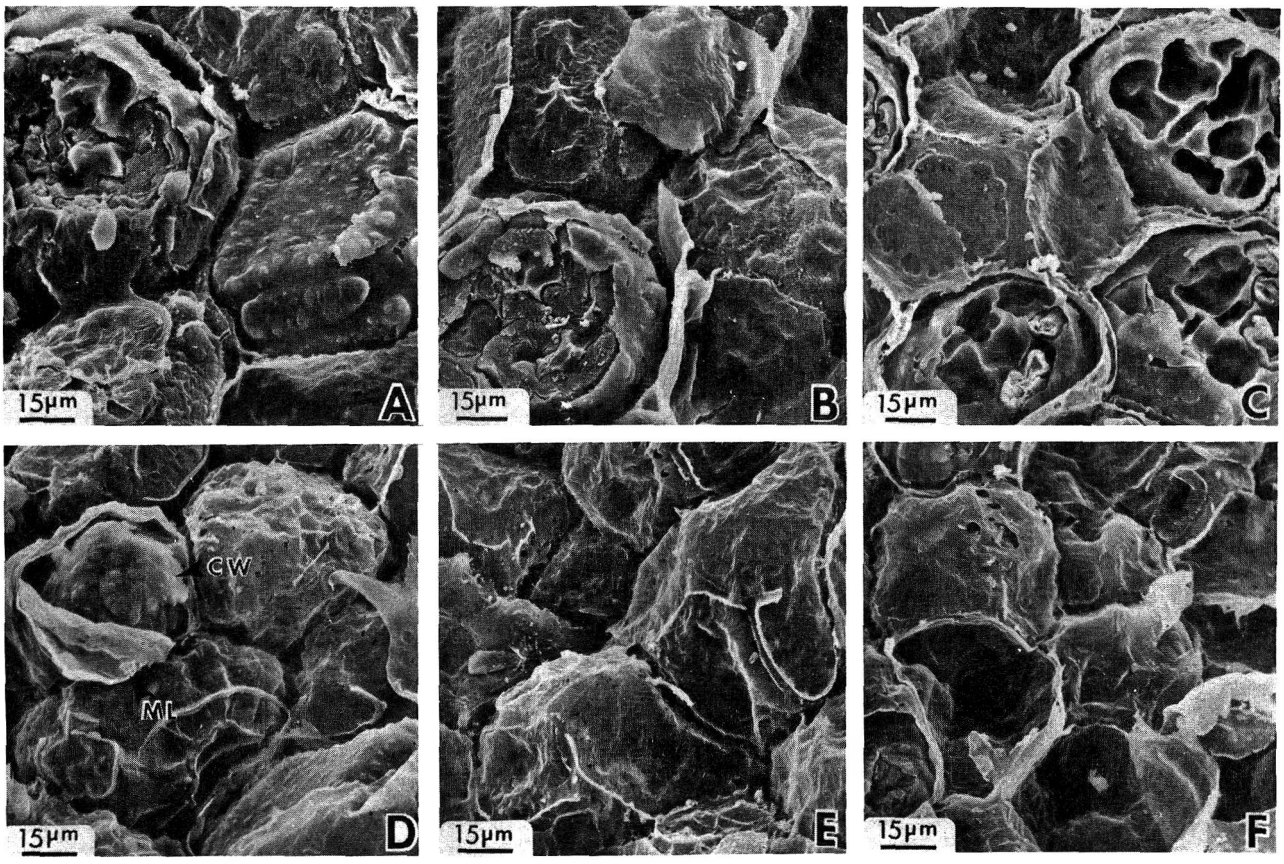

Fig. 10—Cross-section of cowpeas (Vigna unguiculata) stored for 12 months, soaked at 25° C for 24 hr and heated to 100° C for 1 min (A, B, C) and 15 min (D, E, F): A, D—0° C, 80% RH; B, E—21° C, 35% RH; C, F—29° C, 85% RH. CW = Cell wall; ML = Middle lamella.

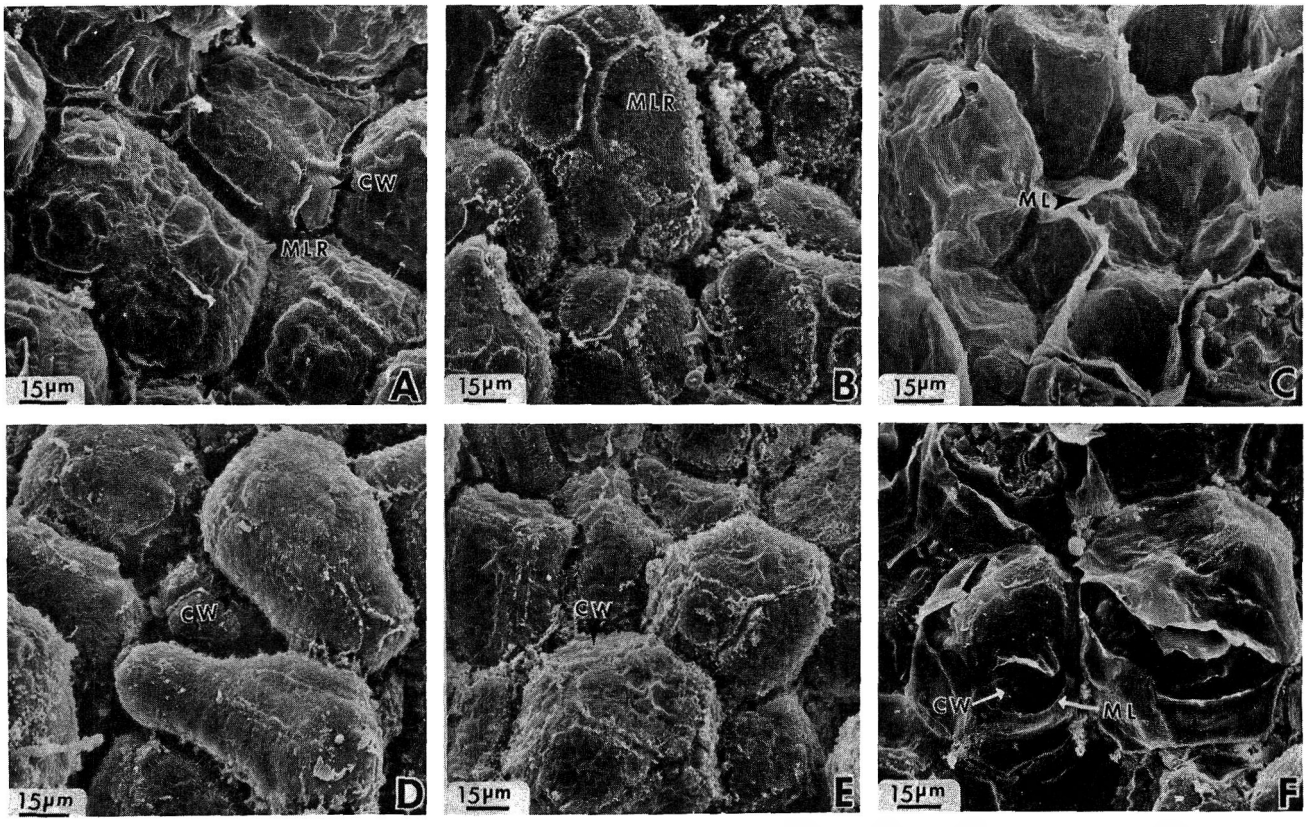

Fig. 11—Cross-section of cowpeas (Vigna unguiculata) stored at 12 months, soaked at 25° C for 24 hr and heated at 100° C for 45 min (A, B, C) and 90 min (D, E, F): A, D—0° C, 80% RH; B, E—21° C, 35% RH; C, F—29° C, 85% RH. CW = Cell wall; ML = Middle lamella; MLR = Middle lamella remnants.

hardness, however, changed with soaking time depending on the temperature and time at which the beans were stored and the storage time.

After 12 months in storage the cowpeas stored at 0°C (80% RH) and 29°C (85% RH) exhibited high moisture levels, high rates of water absorption and the corresponding soaked beans were soft. However, upon cooking the samples stored at 29°C (85% RH) for 12 months showed a difference in hardness. It is possible that changes had taken place in the samples stored at high temperature and humidity which did not occur in the samples stored at low temperature for 12 months.

The softening of cowpeas stored at 29°C (85% RH) and 21°C (35% RH) did not follow first order kinetics as found for the standard unstored cowpeas. Since this softening was found to be accompanied by the breakdown of the middle lamella, these observations suggest that some changes associated with storage had affected the course of this breakdown. Microstructural evidence revealed that the middle lamella in the cowpea samples stored at 29°C (85% RH) did not break down during cooking at 100°C. This may partly explain the difference in hardness observed for the cowpeas stored at high temperature and humidity. It was also observed that cotyledon cells of cowpea seeds soaked for 24 hr after a 12-month storage in high temperature and humidity lost some protein bodies.

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PATULIN PRODUCTS/FERMENTATION . . . From page 789

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PASTING BEHAVIOR OF RICE: A NEW METHOD OF VISCOGRAPHY

K. R. BHATTACHARYA and C. M. SOWBHAGYA

ABSTRACT

Brabender viscograms of 45 rice varieties, belonging to eight distinct property profiles, were determined at several slurry concentrations each. The conventional criteria, viz. peak viscosity (*P*), breakdown and setback, obtained at an arbitrarily fixed slurry concentration, did not provide useful information. For each rice variety there was a *P* value below which the breakdown was zero but rose progressively above it, as well as another *P* value at which the setback was zero but became positive below and negative above it. These two critical *P* values were fairly characteristic of the rice type: e.g. the zero-setback *P* value rose from about 350 B.U. for rice varieties of Group VIII (waxy) to 3500 B.U. for those of Group I (some dwarf *indica*). But the most useful test criterion was the 'relative breakdown,' i.e. breakdown/(breakdown + setback). Its value, at a *P* value of 1000 B.U., rose from about 3% for Group I to 300% for Group VIII. The three mutual ratios of the peak, hot-paste and cold-paste viscosities provided other useful distinguishing criteria. These viscogram indices were closely related to the total and insoluble amylose contents of the samples.

INTRODUCTION

ALTHOUGH STUDY of the pasting behavior of rice flour by the Brabender viscograph has been routinely applied in rice quality research for two decades, the results have not provided much useful information. This aspect was examined in a previous paper (Bhattacharya and Sowbhagya, 1978), and it was concluded that the failure arose from the following fallacy in the current methodology of viscography: Brabender viscograms are usually determined at a fixed slurry concentration (usually 8–10% for rice flour) and the resulting peak viscosity, breakdown and setback values are used as criteria for characterizing the samples. However, as a critical review of some of the literature on viscography showed, the magnitudes of breakdown and setback varied enormously with the peak viscosity, which in turn varied exponentially with the slurry concentration. In fact preliminary viscograms of a few rice samples showed that no rice as such was inherently characterized by a high or low breakdown or a positive or negative setback; any variety could show any or all of these properties depending on the slurry concentration chosen. These conventional criteria were thus largely meaningless. To be meaningful, comparison of viscogram parameters had to be made at a fixed *peak viscosity* and not at a fixed *slurry concentration*. The validity of this approach was demonstrated with literature data and a tentative set of test criteria was also proposed.

Results of a study of 45 rice varieties of different quality types by applying this new methodology are presented in this paper. The aim was, firstly, to finalize the new method of viscography, and secondly, to throw light on varietal differences in rice quality.

EXPERIMENTAL

Rice

The samples used in the study were taken from a larger lot of about 180 rice varieties, comprising a broad cross section of Indian and other rice, and collected during the summer of 1973 from various agricultural experiment stations in the country. As stored after fumigation in the laboratory, their moisture contents lay in the range of 11–13% (wet basis).

The 180 samples were used during 1974 for a detailed study of the physicochemical properties of rice (results to be published). It was observed that the conventional viscograph parameters of these samples shed very little light on their quality; similar paradoxes were revealed at this time in another study on the aging of rice, already reported (Indudhara Swamy et al., 1978). Therefore, exploratory studies on viscography with different slurry concentrations, on the reasonings discussed earlier (Bhattacharya and Sowbhagya, 1978), were carried out with a few samples during parts of 1974 and 1975. As these results showed good possibilities, 45 rice varieties belonging to different prototypes were selected from the large lot and were subjected to the present detailed studies during 1975. [By this time the samples had attained an age of about 2.5 yr (reckoned from the time of harvest) and certainly their viscograms were not what their normal viscograms would be, say a year after harvest. However, as the results below would show, this fact by no means detracted from the central relations obtained. Besides, one of the premises of the study was to reveal some fundamental basis of interpreting pasting behavior that would be unaffected by aging or by other treatment.]

The 45 varieties could be grouped into eight groups depending on their various characteristics as shown in summary in Table 1. These data, determined by methods cited in a previous work (Bhattacharya et al., 1978), are taken from the larger study mentioned above (unpublished). The main basis of classification was the total and insoluble amylose contents whose importance has been discussed elsewhere (Manohar Kumar et al., 1976; Bhattacharya et al., 1978). As can be seen, the groups also differed markedly in other characteristics. It was therefore of interest to see how far the proposed viscographic test could categorize the varieties into the above or comparable groups or prototypes.

Methods

Rice was milled in the laboratory with a McGill or a Satake sheller and a McGill miller No. 3 by standard methods. Milled rice was ground in a Buhler disc grinder followed by in a Raymond hammer mill to obtain a roughly 65-mesh powder. Coarser flour (40–45 mesh), generally used so far for viscograph studies of rice, was quite unsatisfactory.

Viscography was carried out by the general method of Halick and Kelly (1959), but at several slurry concentrations each in the range of 5–16% on dry weight basis. The total slurry weight was always maintained at 500g. The heating was up to 95°C, 'cooking' was for 20 min, and cooling was down to 50°C.

It was observed that appreciable quantities of slurry (5–30 ml) overflowed from the viscograph bowl during the stage of cooking and/or cooling whenever the slurry concentration was very high (over 12%). Reducing the quantity of slurry to 450g or 400g reduced but did not prevent the overflow. This problem was therefore ignored, for it did not seem to seriously affect the relations observed, as proved by the waxy and low-amylose rices which also gave precisely the same pattern of results but at much lower concentrations at which no overflow occurred.

The resulting *P*, *H* and *C* (see Definitions) values for each sample were plotted against the slurry concentration in a log-log graph, as explained earlier (Bhattacharya and Sowbhagya, 1978). For this purpose a special log-log graph sheet with one log cycle of 200 mm in the abscissa and two log cycles of 100 mm each in the ordinate was got printed. Various viscosity ratios were then calculated for each sample at specified *P* values. Peak viscosity values of 350, 500, 700, 1000 and 1400 B.U. were chosen for this purpose (being nearly equidistant from each other in the log scale).

Definitions

The following symbols and parameters, explained in detail earlier (Bhattacharya and Sowbhagya, 1978), are briefly defined here for convenience:

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Table 1—Classification and general properties of the rice varieties

Group No.	Group Description	No. of varieties	Amylose, % d.b. Total	Amylose, % d.b. Insoluble	EMC-S[a] % w.b.	Alkali degradation type	Cooked rice Stickiness[b]	Cooked rice Consistency[b]
I	High amylose A	3	27–30	15–18	29–30	B, A	Very low	Very high
II	High amylose B	9	–do–	13–15	27–28.5	A,B_1,(B)	↓	↑
III	High amylose C	15	–do–	11–13	27–28.5	A,B_1,(B)	↓	↑
IV	Scented	7	23–26	8–10	28–29	A(C),B_1 (C)	↓	↑
V	Medium amylose	4	24–26	8–10	29.5–30.5	AC	↓	↑
VI	*Bulu*	2	23–25	8–9	28.5–29.5	C(A)	↓	↑
VII	Low amylose	3	19–21	7–9	31–32	C	↓	↑
VIII	Waxy	2	5–6	4–5	34–36	D[c]	Very high	Very low

[a] Equilibrium moisture content attained by rice upon soaking in water
[b] In general the stickiness of cooked rice increased from Group I to VIII and consistency in the reverse order.
[c] A new type not observed earlier (to be published)

P = Peak viscosity. Whenever the viscosity shows no clear peak but continues to rise until the end of 95°C heating, the H value is taken as the P value also.
H = Hot-paste viscosity, i.e. viscosity after heating at 95°C for 20 min.
C = Cold-paste viscosity, i.e. viscosity after cooling to 50°C.
BD = Breakdown = $P - H$
SB = Setback = $C - P$
SB_t = Total setback = $C - H$ = BD + SB. Japanese scientists designate this parameter as consistency (Chikubu, 1975). However, consistency has an entirely different connotation to texture scientists and therefore it is better to use the term total setback.
H/P = Breakdown ratio
C/P = Setback ratio
C/H = Total setback ratio
BD_r = Relative breakdown = $BD/SB_t = (P - H)/(C - H)$
P.C intersection point = Zero-SB point = Viscosity value at which P equals C
$P^{max}_{BD=0}$ = Highest P value at which BD is zero, i.e. at which P is identical with H
c_2P/c_P = Concentration ratio, i.e. the factor by which the slurry concentration has to be raised for the P to be doubled (say from 500 to 1000 B.U.)

RESULTS

THE VISCOSITIES given by the samples when plotted against the slurry concentration gave a characteristic pattern common to all the rices. The curves of one representative variety from each rice group are shown in Figure 1 for illustration. The broad features of these curves have already been pointed out in an earlier paper (Bhattacharya and Sowbhagya, 1978).

The range of values, within each rice group, of the major indices computed from the above curves is shown in Table 2 and the group means are shown in Figures 2 and 3.

The peak viscosities of the different samples at any one slurry concentration varied rather widely, e.g. from about 500 to 1500 B.U. for 10% (dry basis) slurry (Table 2). But it showed no apparent correlation either with the rice groups or any other property of the samples.

The $P \cdot C$ intersection point showed a dramatic pattern among the groups (Fig. 1). The viscosity value at this point was fairly constant within each rice group but rose from about 350 B.U. for Group VIII to about 3500 B.U. for Group I (Table 2). However, it was not a practical test index; for, apart from some scatter in its values, its determination would need the laborious determination of the entire curve complex for each sample.

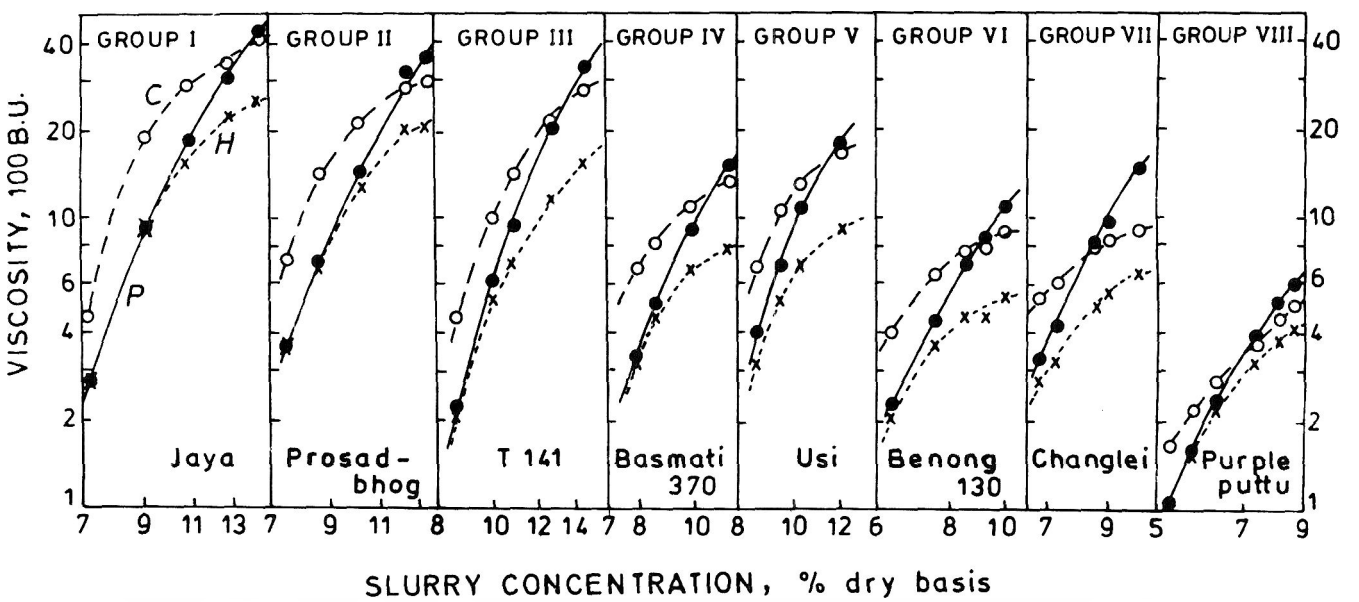

Fig. 1—Brabender viscograms of one representative rice variety from eight groups: ●——● P = peak; X– –X H = hot-paste; ○– –○ C = cold-paste viscosities.

Table 2—Range of viscograph indices within each rice group

Group	No. of varieties	Range of P values, B.U., at 10%(d.b.) slurry	$P = C$	$P^{max}_{BD=0}$	Range of values, at P = 1000 B.U., of H/P	C/P	C/H	BD_r
I	3	820–1390	3100–3820	650–950	0.96–1.00	1.74–2.05	1.81–2.07	0.00–0.05
II	9	765–1470	2040–3550	300–500	0.78–0.91	1.44–1.74	1.77–1.91	0.16–0.27
III	13	505–1030	2050–2530	<100–<200	0.56–0.78	1.35–1.75	1.78–2.80	0.31–0.55
	P_{tb} 9[a]	1230	–	<200	0.79	1.63	2.06	0.25
	S 317[a]	860	1670	<100	0.60	1.16	1.95	0.71
IV	6	890–1090	1130–1550	150–200	0.64–0.72	1.08–1.22	1.60–1.91	0.56–0.81
	Badshabhog[a]	740	2110	250	0.79	1.50	1.90	0.30
V	3	950–1150	1200–1950	<150–<200	0.60–0.70	1.11–1.29	1.84–1.92	0.56–0.78
	Co 25[a]	800	2140	200	0.75	1.43	1.91	0.37
VI	2	1060–1090	720–840	<200	0.53	0.83–0.88	1.56–1.66	1.34–1.57
VII	3	1025–1330	750–940	<200	0.54–0.60	0.84–0.96	1.56–1.59	1.11–1.53
VIII	2	820–870	355–405	130–200	0.56–0.60	0.72–0.74	1.19–1.31	2.52–3.33

[a] These varieties were exceptional for their respective groups; their values were excluded while showing the group ranges.

The $P^{max}_{BD=0}$ value was similar. It also showed reasonably good group specificity (Table 2), but was not of much practical value due to the same reasons as above.

The slope of the P lines decreased slightly as the P increased. Calculated at any given P value, the slope decreased progressively from Group I to Group VIII (see Fig. 1). However, on examination it was found that this difference in the slope of the P line among the different groups was largely but a reflection of the difference in their $P \cdot C$ intersection points, for the slope was nearly identical for all samples at the respective intersection points; its rate of fall with rising P was also identical among the samples when reckoned from that point. In other words, the various P lines, although looking slightly different from each other as drawn, would fully overlap if positioned precisely at the $P \cdot C$ intersection points. These facts can be illustrated by quoting some values of the concentration ratio C_{2P}/C_P, the inverse of the slope. The value of this ratio was approximately 1.25 at the $P \cdot C$ intersection point for each variety, regardless of the viscosity value at that point; for each variety again, it decreased to about 1.12 at one log cycle of P below the intersection and rose to about 1.5 at one log cycle of P above.

The various ratios H/P, C/P and C/H, computed at specified P values, showed a fair degree of group specificity (Table 2, Fig. 2), though not enough for unequivocal identification or classification. It should be noted that the ratio C/H showed the least variation among varieties and over a wide range of P values—the only exception being the waxy class, Group VIII, which gave a very low value (Fig. 2, right). That this ratio is reasonably constant for different rice varieties and shows a characteristic value for different starch species has already been noted earlier (Bhattacharya and Sowbhagya, 1978).

Finally, the relative breakdown (BD_r)—whose significance as a viscogram parameter has been discussed earlier (Bhattacharya and Sowbhagya, 1978)—of the samples showed a very characteristic pattern (Table 2), and the locus of this ratio through a range of P values neatly distinguished the rice groups from each other (Fig. 3). No doubt its values too were rather close for a few groups at some regions. But the other ratios above could be used as subsidiary discriminatory indices at these points.

DISCUSSION

THE PEAK VISCOSITY (at a given slurry concentration) is obviously an important characteristic of any starch or flour. This value differed appreciably among the rice varieties, but the variation was quite random. It cannot therefore be correlated with any known characteristic of rice at this time, which must await further research.

It appears from the present results that the primary viscogram character differentiating cultivars within a species

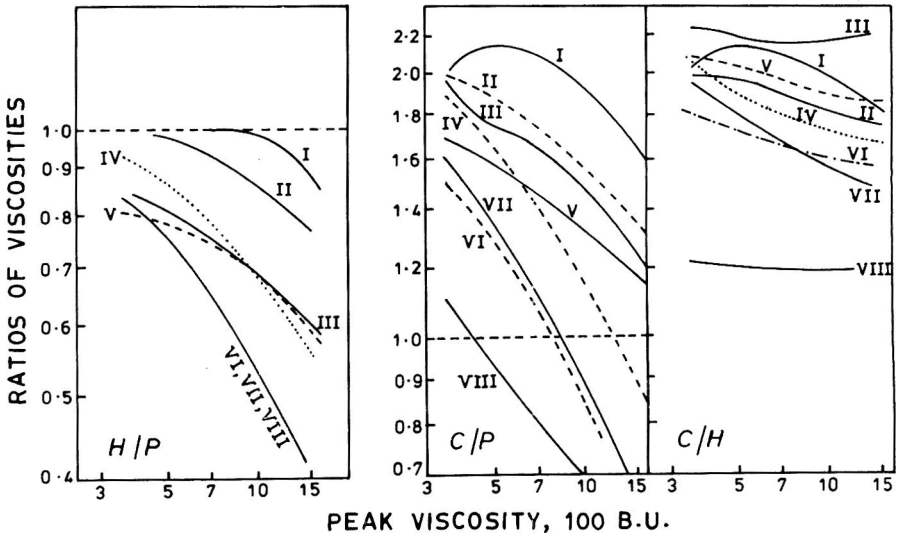

Fig. 2—Ratios of key viscosities of rice of different groups at various peak viscosities. The horizontal lines at ratio 1.0 in the left and center figures represent zero-breakdown and zero-setback points, respectively.

(at least rice) is not the setback but the breakdown—notwithstanding the importance currently attached to the former (for instance it is said that a high setback is a distinguishing characteristic of a high-amylose rice, and vice versa). This is evident from a glance at the curves of Figure 1, i.e. if the breakdown and setback parameters are considered as ratios, as they should (Bhattacharya and Sowbhagya, 1978). The total rise in viscosity upon cooling, i.e. the total setback ratio C/H, is reasonably constant among the varieties. The magnitude of the observed setback ratio, C/P, of any variety at any point is then but largely an inverse reflection of the corresponding breakdown ratio, H/P. Of course, it should be remembered that for interspecies comparison the setback also is a relevant index, for the value of C/H differs widely among starch species (Bhattacharya and Sowbhagya, 1978).

The breakdown, it appears, is best expressed as 'relative breakdown' for maximum sensitivity. That this latter parameter may be a fundamental property is shown by the fact that it distinguished the different starches also the most effectively (Bhattacharya and Sowbhagya, 1978).

The breakdown is presumably an indication of the ease with which the swollen starch granule can be disintegrated, i.e. of the degree of its organization. It is therefore significant that the BD_r data bore an excellent inverse relation to the amylose contents of the sample (cf. Tables 1 and 2). An especially significant point in this context is the observed relation with the insoluble amylose. It has been shown earlier (Manohar Kumar et al., 1976; Bhattacharya et al., 1978) that the insoluble amylose content is as important as, if not more than, the total amylose as a determinant of rice quality. And indeed the BD_r data too bore this out well as shown particularly by the varieties of the first three groups (Table 2, Fig. 3), all of which had a uniformly high total amylose content but markedly different insoluble amylose (Table 1). Again the varieties Mahsuri (26%) and GEB 24 (27%) were rather intermediate in total amylose; yet they gave type I and II viscograms, respectively, which was consistent with their high insoluble amylose contents (14% and 13% respectively).

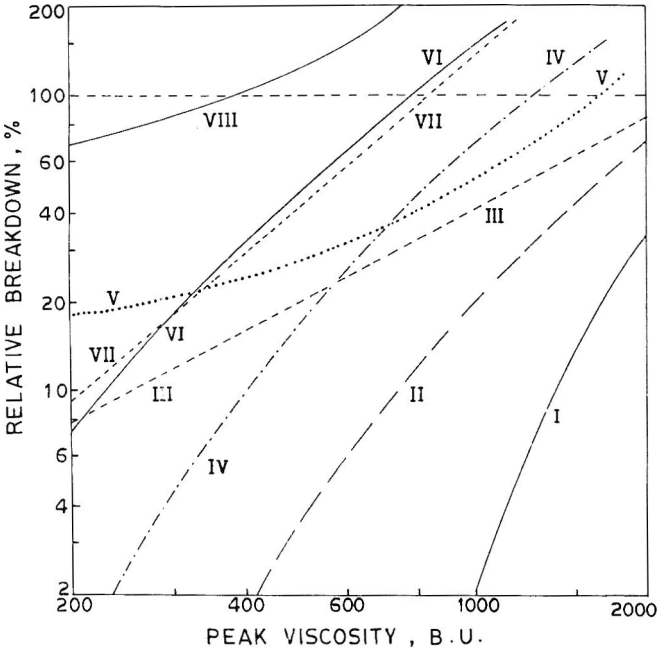

Fig. 3—Relative breakdown of rice of different groups at various peak viscosities. The horizontal line at 100% BD_r represents the zero-setback line.

The excellent correlation with the total and insoluble amylose contents, and the fact that the BD_r and the other values fell into several sharply different categories, simulating the classification based on other properties of the varieties (Table 1), is eloquent proof of the validity of the proposed new viscogram criteria. A very interesting case in point is that of Group IV. Initially this group was arbitarily constituted with scented rice varieties. Surprisingly, although collected from different parts of India, all the properties of these diverse varieties fell within a narrow range as if they all belonged to a single prototype (Table 1). And indeed they also gave a unique viscogram type here (Fig. 2 and 3)—distinctly different even from that of Group V, although both these groups showed similar amylose contents. The only instance where the proposed viscogram indices failed to discriminate between two otherwise distinct groups of rice was the pair Groups VI and VII, the reason for which is not known at this time.

The proposed new indices also showed up other peculiarities not revealed by other conventional criteria of rice quality. Firstly there were a few interesting exceptions within the individual groups. Ptb 9 (Group III), Badshabhog (IV) and Co 25 (V), and S 317 (III) gave viscograms which were more like those of types II, III and V respectively (Table 2). Significantly, their stickiness (cooked rice) results were also atypical (not shown). Secondly some of the BD_r data were very interesting. For example Group III samples gave an unusually high BD_r at low P values (Fig. 3) despite their very high total and moderately high insoluble amylose contents; accordingly their $P^{max}_{BD=0}$ values were one of the lowest among all groups (Table 2). At high P values, however, their relative breakdown was only moderately high as to be expected. Varieties of Group IV on the other hand, despite their rather low amylose contents, gave a surprisingly low BD_r at low P values, and accordingly a rather high $P^{max}_{BD=0}$ (Table 2), but an expectedly high BD_r at high P values. The significance of these behaviors is not known at this time. But clearly they reveal other unknown areas to be clarified for a thorough understanding of rice quality, and give further evidence of the usefulness of the new viscographic technique as a research tool.

Finally it should be recalled that the samples used in this study were about 2.5 yr in age (after harvest). Since aging has a profound influence on the viscogram of rice (Barber, 1972; Indudhara Swamy et al., 1978), it is very likely that the magnitude of the suggested criteria too would change with age: to determine in what manner is the subject of a current study.

Test methodology

It is clear from the above results that to use viscography as a tool for research or quality control, one should discard the traditional peak viscosity-breakdown-setback (at a fixed slurry concentration) approach and resort instead to comparison of viscosity ratios at a given peak viscosity. This seems true not only of rice but of cereals and starches in general.

For a thorough study of a rice sample, it should preferably be viscographed over a range of slurry concentrations as above and determine the relation of its various viscosity ratios to P. By comparing the results with the standard curves in Figures 2 and 3, one can then confidently place the rice sample in one group or another, and thus predict its behavior. Besides, the process will also reveal any unusual characteristics (exceptions noted above), thereby giving more insight into the sample quality and perhaps enabling new relations to be discovered.

For routine tests such an elaborate procedure would not be necessary. It would be sufficient to determine the viscogram preferably at two concentrations (at a fair distance from each other, say 6% and 11%, dry basis), or, for a

—Continued on page 804

SONICATION EFFECT ON POTATO STARCH AND SWEET POTATO POWDER

A. AZHAR and M. K. HAMDY

ABSTRACT

Maximum degradation of potato starch was detected after 30 min sonication at 1–2°C. Degradation, evidenced by decrease in viscosity, may occur to starch granules rather than the individual polymers. This hypothesis was supported by the fact that no difference in rate of enzymatic hydrolysis was detected between sonicated and nonsonicated potato starch when either was used as substrate for β-amylase. Neither the specific activity of pure preparation of β-amylase, nor its electrophoretic behavior was altered due to cavitation action of sonication. Ultrasonic waves also did not alter activities of β-amylase intrinsic of sweet potato.

INTRODUCTION

THE EFFECT of ultrasonic radiation on macromolecules has been examined for many years. Richards and Loomis (1927) were the first to report that ultrasonic waves are able to produce a great diversity of physico-chemical changes such as accelerating the rate of hydrolysis of dimethyl sulfate in basic solution. Szent-Gyorgyi (1933) showed that ultrasonic radiation decomposed cane sugar into monosaccharides and that highly polymerized compounds such as starch, gum arabica, and gelatin were depolymerized by such radiation energy, as evidenced by the rapid decrease in their viscosity. Morehead (1950), through electron microscopic examinations, noted that native cellulose fibers were broken into thin fibrills upon exposure to ultrasonic waves. Coakley et al. (1973), studying the effect of ultrasonic waves on the catalytic action of several enzymes, reported that while catalase was slightly affected, both alcohol dehydrogenase and lysozyme were inactivated at an exponential rate. These authors also suggested that the mechanism of inactivation was chemical rather than physical.

Because of the rapid increase in demand for grains, both as food and feed, there is an urgent need for substitution of grain starch with other substrates for alcohol as well as for various other industrial fermentations. Therefore, it was decided to examine the effect of ultrasonic radiation on the sweet potato (*Ipomoea batatas*) and evaluate the possibility of utilizing the end product formed for industrial fermentation. Sweet potato is considered to be a suitable substitute for grain in this respect, since it has a higher starch yield, per unit land cultivated compared to grains (corn). It also contains a considerable amount of highly active β-amylase which is the primary saccharifying enzyme in sweet potato (Schwimmer, 1947; Balls et al., 1948), responsible for conversion of starch into maltose units during baking at elevated temperatures (60–80°C).

MATERIALS AND METHODS

Preparation of freeze-dried sweet potato

Fresh (uncured) Georgia jet sweet potatoes were sliced to a thickness of 0.4 cm and freeze-dried for 18 hr under a reduced pressure of 50–20μ at a shelf temperature of 0–4°C. The moisture content of the freeze-dried sweet potato, determined by drying samples in a vacuum oven at 80°C to a constant weight (AOAC, 1975), was found to be 8%. The freeze-dried sweet potato was ground in a Wiley mill and the resulting powder was allowed to pass through a No. 40 wire mesh screen and stored at 1–2°C until used.

Sonication

Powder of both potato starch (Baker Chemical Co.) and the freeze-dried sweet potato were used and the desired concentration of the former was boiled for 5 min in sodium acetate buffer (pH 4.8, 0.01€M), whereas a slurry of the latter was made and each preparation was then subjected to sonication at 1–2°C. This temperature was selected to limit the effect of heat generated within the sample due to molecular friction and cavitation bubble collapse. A sonic dismembrator (ARTEK #300) with an energy input of 300 watts, operated at a frequency of 20 ± 0.4 KHz and designed to transfer 98% of the input energy into usable acoustic energy was used. The sonicator is equipped with a titanium microtip (14.2 cm long, 1.90 cm diam) and when placed in solution reaches a depth of 1.27 cm. The rate of potato starch degradation was followed by relative viscosity measurements performed at 25 ± 0.5°C in a water bath using a Fenske-modified Ostwald viscosimeter and sodium acetate buffer as solvent.

Sugar analysis

The presence of reducing sugars, formed during ultrasonic degradation of potato starch, was determined based on their ability to reduce 3,5-dinitrosalicyclic acid (Bernfeld, 1955). In the case of sweet potato, reducing sugars were extracted following the procedure reported by McDonald and Newson (1970) and then quantitatively determined using the method of Furuholmen et al. (1964) and utilizing glucose as a standard.

Hydrolysis of starch by β-amylase

The ultrasonically degraded potato starch was also subjected to crystalline (3X) preparation of β-amylase (α-1,4-glucan maltohydrolase EC.3.2.1.2.). This enzyme was obtained from United States Biochemical Corp. and the specific activities herein are reported as μM maltose per min per mg protein. The latter was determined by Folin phenol reagent (Lowry et al., 1951) and using bovine albumin as standard. The activity of the β-amylase present in sweet potato (intrinsic enzyme) was also examined by incubating the ultrasonically degraded sweet potato slurry for 20 min at 55°C. This temperature was established by Giri (1934) to be optimal for the intrinsic β-amylase activity of the sweet potato.

Polyacrylamide-gel electrophoresis

Disc electrophoresis, on polyacrylamide gel (6.5 cm long × 5 mm diam), was carried out in a manner similar to that reported by Davis (1964). The monomer mixture contained the following components: acrylamide, 0.9g; potassium persulfate, 10 mg; N,N′-methylenebisacrylamide (Bis), 0.03g; 0.38M (pH 8.9) tris buffer up to 12 ml. To this mixture 5 λ of N,N,N′,N′-tetramethylethylenediamine (TEMED) was added as a catalyst to induce the formation of the gels. The buffer reservoir contained tris-glycine solution. The latter consisted of trishydroxymethylamino methane (tris), 0.6g; glycine, 2.9g per liter of distilled water and adjusted to pH 8.3. A sodium acetate buffer-enzyme mixture (pH 4.8, 0.016M) containing 28 μg β-amylase per ml was exposed to sonication at 1–2°C for 30 min. Aliquots (10μl) of the sonicated enzyme preparation were placed gently on top of the gel which was then subjected to a current of 4m ampere per gel tube using the upper electrode as the cathode. The electrophoresis was allowed to continue until the tracking dye (0.005% bromophenol blue) reached the bottom of the gel. The gel rods were then stained for two hours using Buffalo black (1g dye per 100 ml of aqueous 7% acetic acid) and the excess dye removed with 7% acetic acid.

—Text continued on page 802

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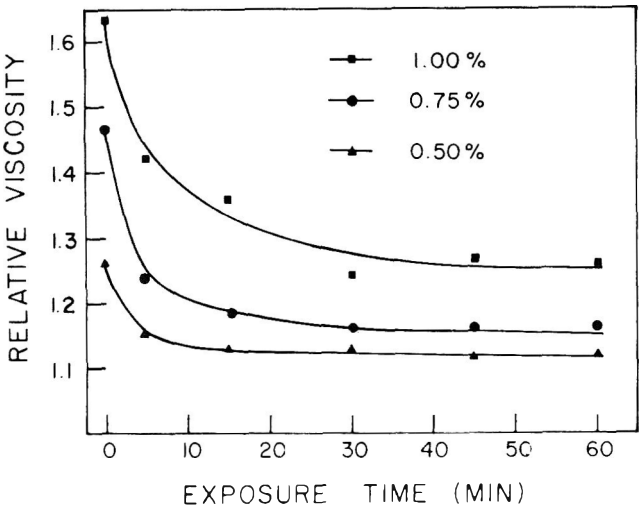

Fig. 1—Effects of sonication on relative viscosity of potato starch. Various concentrations of the polymer were prepared in Na acetate buffer (pH 4.8, 0.016M) and subjected to sonication at 1–2° C.

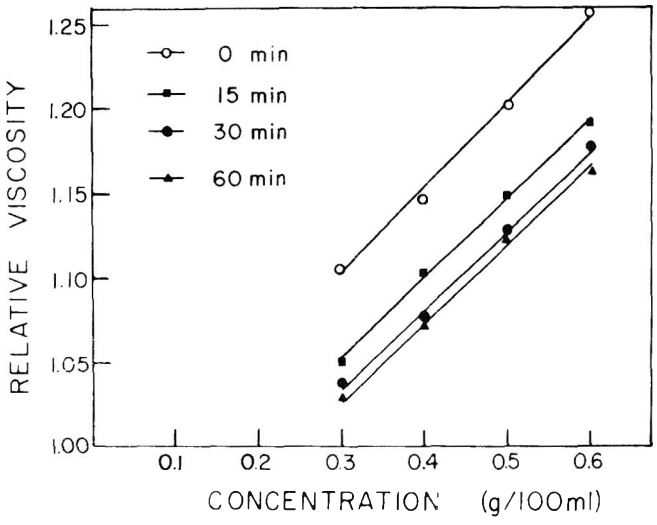

Fig. 2—Relationship between concentration and relative viscosity of potato starch solution subjected to sonication for different time intervals.

RESULTS AND DISCUSSION

Effect of sonication on potato starch

Figure 1 depicts the data obtained for the effect of sonicating different concentrations of potato starch polymer and indicates the rapid decrease in relative viscosity for these solutions, particularly during the first 5 min exposure. The relative viscosities for solutions containing 1.0, 0.75, and 0.50% starch were 13, 16, and 9% of the initial values after 5 min, respectively. However, for the following 10 min exposure, the rate of change in relative viscosity was much lower with very little changes thereafter.

When a 1% starch solution, exposed to sonication for various time intervals, was diluted to lower concentrations ranging from 0.3–0.6 g/100 ml and viscosity measurements performed (Fig. 2), a linear relationship existed between relative viscosity and level of starch in solution at any given exposure time. It was also noted that the slope of these lines was the same (0.47), indicating that the change in relative viscosity of the different levels of starch was identical. No reducing sugars were also detected at any time during the entire experiment.

Early studies (Szalay, 1933; Szent-Gyorgyi, 1933) showed that sonication of starch solution resulted in a decrease in viscosity. Szalay (1933) reported that during 20 min sonication of starch solution at a frequency of 720 KHz, the following substances, as qualitatively determined by iodine reaction, appeared in succession; amylodextrins stained blue, erythrodextrins that gave a red color, and achrodextrins with no reaction. Thieme (1938) observed a reduction in the viscosity of gelatin, agar agar, and gum arabica solutions due to ultrasound and that this effect was dependent on the concentration of the polymer. Stacey (1951) and Hamdy (1953) showed that ultrasonic waves decreased the viscosity of dextran solutions. Lockwood et al. (1951) and Hamdy (1953) failed to detect any reducing sugars during the depolymerization of dextran by sonication. Mostafa (1958a,b) demonstrated that high polymer molecules could be degraded by ultrasonic waves alone in the absence of cavitation, provided that kinetic energy of vibration was sufficient to break a C-C bond. Again, the author reported that for a constant ultrasonic frequency, the kinetic energy of vibration increased with the chain length of the macromolecule and that when the chain length reached a limiting value, the kinetic energy of vibration would be sufficient to break a C-C bond and rupture would be effective.

Radley (1954) established that the viscosity of colloidal solutions was influenced by the number and shape of the colloidal particles and that high specific viscosities are characteristic of colloidal solutions having thread-like micelles or macromolecules present. This investigator also stated that these macromolecules could form an irregular, ever-shifting network, and the effective length of the micelles and their molecular weights determine the viscosity. It is believed that the starch molecules have an intermediate shape between the thread-like molecules of the cellulose and the spherical ones of glycogen and that in solutions containing thread-like molecules, the intrinsic viscosity is directly proportional to the molecular weight (Radley, 1954). Thus, an increase in reducing power, which is dependent on the terminal aldehydic group of the starch molecule, will occur upon hydrolysis resulting in a decreased molecular weight. Based on the data obtained in the present investigation, the possibility of rupturing any glycosidic bond in starch molecules under the condition examined was excluded. During gelatinization the swollen starch granules become increasingly susceptible to disintegration by mechanical force (Radley, 1954). This type of force, such as those occurring during sonication of the higher molecular weight polymers, may lead to configurational modification of the granular structure rather than a true alteration of the individual polymer. Again the data obtained on the effect of β-amylase on sonicated starch (Table 1) is in support of this hypothesis. It was noted that sonication of potato starch for 60 min did not significantly alter the individual polymer and the rate of enzymatic hydrolysis was no different from that of the nonsonicated. Thus it appears that even though sonication modified the potato granules, as indicated by the decrease in relative viscosity, yet such a configurational modification did not result in the formation of reducing groups nor did it affect the rate of enzymatic hydrolysis of starch granules by β-amylase. This configurational modification, as affected by sonication, could be a diffuse erosion and pitting of the starch grains as observed by Degrois et al. (1974). By means of scanning electron microscopy, these authors were able to show that the erosion of the starch grains depended on the nature of the dissolved gas and that the pit size increased inversely to the solubility of the gas. For example, marked overall erosion

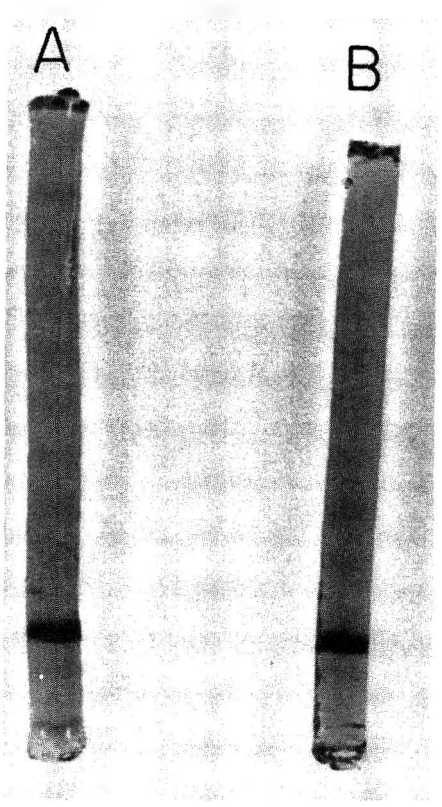

Fig. 3—Polyacrylamide gel electrophoretogram of β-amylase before (A) and after (B) sonication for 30 min at 1–2°C.

and pitting were detected in air as compared to hydrogen, oxygen or carbon dioxide.

Effect of sonication on freeze-dried sweet potato

Table 2 shows the comparative effect of sonication on sweet potato slurry before and after sonication. When freeze-dried sweet potato slurry, exposed to sonication for 30 min, was incubated for 20 min at 55°C (action of intrinsic α- and β-amylases), no appreciable change in the amount of sugar liberated was observed. Again, this indicated that ultrasonic waves apparently did not alter the individual starch polymer as indicated from the action of intrinsic amylases.

Since sweet potato contains appreciable amounts of highly active amylases responsible for converting starch to reducing sugars upon incubation at elevated temperatures (Hoover and Harmon, 1967; Deobald et al., 1969), it was decided to examine the effect of sonication on one of these enzymes, namely β-amylase. In this experiment, sodium acetate buffer-enzyme (14.15 μg protein/ml) was subjected to sonication for 30 min. The results (Table 3) revealed that the specific activity of β-amylase was not appreciably altered by sonication.

The effect of ultrasonic waves on enzymes has been studied by several investigators. Coakley et al. (1973) examined the behavior of several enzymes after exposure to sonication with a frequency of 20 KHz for varying lengths of time and showed that catalase was least affected whereas both alcohol dehydrogenase and lysozyme were inactivated at an exponential rate. The rate of enzyme inactivation decreased with increasing protein concentration. Stefanovic et al. (1960) reported a 73% decrease in the diastase enzymatic activity as the result of exposure to 3 MHz ultrasound for 30 min. The nature of changes in enzyme in an ultrasound field mainly depends not only on the structure of these proteins, but also on the nature of the dissolved gas present and energy of the ultrasonic waves applied. The

Table 1—Effects of time of sonication (at 1–2°C) on starch polymer; the starch was then used as substrate for β-amylase[a]

Sonication (min)	Specific activities[b] μmole maltose/min/mg protein
0	208.5 ± 3.4
15	209.6 ± 2.1
30	209.9 ± 2.3
45	206.2 ± 3.0
60	208.2 ± 1.5

[a] Reaction mixture: 1 ml Na-acetate buffer contained 5.0 mg starch and 0.06 mg β-amylase, incubated at 25°C for 5 min and assayed.
[b] Expressed as mean of three samples ± 1 standard deviation.

Table 2—Effects of sonication (at 1–2°C) on sweet potato determined by measuring reducing sugar liberated as the result of intrinsic β-amylase action[a]

Sweet potato slurry	Reducing sugars formed[b]
Before sonication	
(a) At zero time	2.38 ± 0.07
(b) After 20 min	15.97 ± 0.24
After sonication	
(a) At zero time	2.38 ± 0.07
(b) After 20 min	15.77 ± 0.62

[a] Reaction mixture: 10 ml Na-acetate buffer (pH 4.8, 0.016M) contained 100 mg freeze-dried sweet potato powder (Containing both substrate and intrinsic enzyme), incubated at 55°C for 20 min.
[b] mg reducing sugar (glucose)/100 mg freeze-dried sweet potato. Expressed as mean of three samples ±1 standard deviation.

Table 3—Effects of sonication (at 1–2°C) on activity of β-amylase[a]

Enzyme solution	Specific activity[b]
Before sonication	110.0 ± 1.6
After sonication	111.5 ± 3.8

[a] Reaction mixture: 1 ml acetate buffer contained 5.0 mg starch, 7.0 μg β-amylase and incubated at 25°C for 5 min.
[b] μmole maltose/min/mg protein. Expressed as mean of three samples ±1 standard deviation.

inactivating effect of ultrasonic waves is usually revealed when enzymes are irradiated in the presence of oxygen. El'piner (1964) noticed that while the biocatalytic activity of trypsin is reduced by 75–80% after exposure to ultrasound in the presence of oxygen, it is quite unaffected by sonication in the presence of hydrogen. On the basis of results obtained, it is concluded that sweet potato β-amylase is resistant to the action of sonication under the conditions examined.

Effect of sonication on the electrophoretic characteristics of β-amylase

Figure 3 depicts the electrophoretogram of the gel patterns for the pure preparation of β-amylase before (A) and after (B) sonication for 30 min at 1–2°C. A single narrow

band with the same apparent mobility was detected in each gel indicating that sonication under the conditions examined did not alter the molecular configuration of the enzyme as detected by this technique. It should be pointed out that the same concentration of the enzyme (0.28 μg) was added to each gel column. However, ultrasonic waves with energy higher than that used in this study (294 watts) could possibly disrupt the three dimensional structure of the enzyme and thus alter to some degree its electrophoretic behavior. It is also possible that such high energy could cause slight denaturation of the enzyme and decrease its activity. Several investigators have reported that ultrasonic waves change the activities of some enzymes such as trypsin, diatase, catalase, alcohol dehydrogenase and lysozyme (Coakley et al., 1973; Stefanovic et al., 1970; El'piner, 1964). However, search in the available literature so far revealed no data on the effect of sonication on the electrophoretic behavior of these enzymes.

CONCLUSIONS

THIS STUDY has investigated several areas pertaining to the use of ultrasonic, with an energy input of 300 watts, for the degradation of potato starch and sweet potato powder. Specifically, maximum degradation of potato starch was observed after 30 min sonication at 1–2°C as evidenced by the decrease in relative viscosity. This degradation may occur to the starch granules rather than the individual polymers. Sonication of sweet potato slurry for 30 min did not alter the activities of β-amylase intrinsic of the sweet potato.

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PASTING BEHAVIOR OF RICE . . . From page 800

slightly less accurate test, just at one concentration (say 8%). The various viscosity ratios should now be calculated and then compared, against the observed *P* values, with the standard curves. This should enable one to determine with a reasonable degree of assurance the group to which the sample belongs, and hence to predict its behavior.

The same approach should hold for viscograph studies of starches and cereals in general. It will be best if standard curves such as in Figures 2 and 3 are first prepared after working with as many cultivars and at as many slurry concentrations as possible.

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COMPOSITON OF RAW AND COOKED POTATO PEEL AND FLESH: PROXIMATE AND VITAMIN COMPOSITION

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ABSTRACT

A comparative analysis of raw and cooked tuber peel and flesh was performed. Whether raw or cooked, and regardless of the cooking methods used tuber peel contained significantly higher amounts of ash, crude fiber, protein and riboflavin but less thiamine than the corresponding flesh. Raw peel contained less ascorbic acid and niacin, more folic acid than and about the same amount of vitamin B_6 as the corresponding tuber flesh. However, the relative amounts of these four vitamins changed depending on the cooking method used.

INTRODUCTION

FOLKLORE has often made claims that most of the nutrients of the potato tuber are contained in its peel. In the past years the authors received numerous inquiries regarding the nutritional value of potato peel. Outside of nitrogenous compounds (Neuberger and Sanger, 1942) and some nonnutritive constituents such as alkaloids (Lampitt et al. 1943; Gull and Isenberg, 1960), there appears to be little information on the compositional value of peel or of the peel relative to the corresponding tuber flesh. Because of apparent public interest in this subject, a study was undertaken to document the nutritional value of raw and cooked potato peel. This report covers the findings on some constituents of the proximate class as well as water soluble vitamins.

MATERIALS & METHODS

Cultivars

Field grown potato samples were obtained during two seasons from the following cultivars and locations: Russet Burbank (Idaho), Katahdin (Maine) and Norchip (North Dakota). The cultural practices used in growing these potatoes were what is considered "normal" for each particular cultivar and location. Following arrival at the laboratory, all samples were stored at 7°C and 90% relative humidity for approximately 90 days prior to further processing and analysis.

Cooking experiments

Triplicate sublots of 16 tubers of each cultivar were chosen for each cooking test. The same sublot size was used for the raw, uncooked tuber material. The tubers were cooked in boiling water (30 min), in a conventional household oven at 218°C (425°F, 60 min), and in a microwave oven (20 min). Immediately following cooking the samples were hand-peeled with a conventional household knife. The raw uncooked samples were peeled using a regular household peeling knife. Subsamples for each treatment were obtained for dry matter and ascorbic acid determinations. The remainder of each sample was frozen by immersion in liquid nitrogen, freeze-dried, ground through a Wiley Mill using a 40-mesh screen, placed into sealed containers and stored at −20°C until further analysis. Fresh samples were handled as outlined by Toma et al. (1978).

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Analytical

The samples were analyzed for dry matter, total ash, crude fiber, protein, ascorbic acid, thiamine, riboflavin, niacin, total folic acid and total vitamin B_6. The analytical methods used were those outlined in the AOAC (1975). Ascorbic acid was determined by indophenol titration, thiamine and riboflavin by fluorometry, and niacin by colorimetry. Folic acid and vitamin B_6 were determined microbiologically. Prior to analysis, bound folic acid was converted to its free form by treatment with purified hog kidney conjugase. The latter was extracted from hog kidneys according to Eigen and Shockman (1963) and purified according to Shin et al. (1972).

RESULTS & DISCUSSION

TABLE 1 summarized the average comparative nutrient analysis of raw and cooked potato flesh and peel. The data show that the raw peel area contained significantly higher amounts of total ash, crude fiber, protein and riboflavin and folic acid than the corresponding flesh. However, tuber peel was significantly lower in thiamine and niacin than the tuber flesh. These differences, with the exception of folic acid and niacin, persisted through cooking regardless of the cooking method. Tuber peel originating from potatoes which were cooked in boiling water contained less folic acid than the corresponding flesh; oven-baked and microwave-cooked peel contained higher amounts of niacin than the corresponding flesh. This is because the moisture content of peel relative to flesh decreases during cooking, and because niacin is very heat stable. A similar trend was observed with Vitamin B_6. Differences in the dry matter content between raw and cooked tubers as well as between tuber flesh and peel make it difficult to truly assess nutrient retention in the peel during cooking. A more equitable way of evaluating these changes, would be a comparison of the data on a dry weight or equal moisture basis. However, in this particular instance, because of differences in the morphological composition of the raw and the difference in cooked peel material such a comparison would be meaningless. If changes did occur in the nutrient composition between raw and cooked potato peel, it is impossible to determine which part of the change was due to the effects of the cooking method and which due to the differences in the thickness of the peel.

When one considers the fact that the peel fraction in terms of the entire tuber material varied between 6, 2, 10 and 4% for raw, boiled, oven-baked and microwave-cooked tubers, respectively, it becomes obvious that peel thickness can have quite an effect on its composition. Initial attempts to separate the periderm from the cortex were not successful, because not only was this procedure so extremely tedious and time consuming that it could affect nutrient retention; but more so, the completeness of such a separation left so much to be desired that it introduced a considerable margin of error in terms of the fractions of cortex tissue present, and it was almost impossible to totally remove all cortex tissue from the peel. For this reason, it was decided to separate the peel from the flesh in a manner in which the homemaker or the consumer conventionally does it in the kitchen or on the dinner table. This, of course, made it impossible to truly assess nutrient retention during home preparation.

–Text continued on page 806

Table 1—Proximate and vitamin composition of the flesh (F) and peel (P) of raw and cooked potatoes (100g WB)[a]

	Raw		Boiled-unpeeled		Oven-baked		Microwave-cooked	
	F	P	F	P	F	P	F	P
Dry matter, %	21.6 b	17.0 a	22.4 c	21.0 b	24.9 d	48.1	25.4 d	35.8 f
Ash, %	0.86 a	1.67 c	0.90 a	1.50 c	1.03 ab	1.90 d	1.00 b	2.00 d
Crude fiber, %	0.37 a	1.83 b	0.38 a	3.00 d	0.35 a	1.90 b	0.40 a	2.50 c
Protein, %	1.81 a	2.67 d	1.92 a	2.95 e	2.03 a	3.90 f	2.16 c	4.30 g
Ascorbic acid, mg	14.4 d	11.8 bc	12.2 c	6.7 a	11.7 bc	12.3 b	12.6 b	10.7 b
Thiamine, mg	0.10 c	0.03 a	0.10 bc	0.03 a	0.10 bc	0.08 b	0.11 c	0.09 bc
Riboflavin, mg	0.036 b	0.058 d	0.031 a	0.046 c	0.027 a	0.103 e	0.034 ab	0.057 d
Niacin, mg	1.57 bc	1.05 a	1.61 c	1.41 b	1.79 d	2.84 e	1.64 c	3.55 f
Folic acid, ug	12.8 bc	17.3 e	11.6 b	8.9 a	12.3 bc	19.7 d	11.7 b	17.3 e
Vitamin B_6, mg	0.22 b	0.21 ab	0.24 b	0.18 a	0.25 b	0.42 c	0.26 b	0.40 c

[a] Within each analytical group, values followed by the same letter are not significantly different ($P < 0.05$).

TABLE 2 shows the peel/flesh ratio of the raw and cooked potatoes on a dry weight basis. The data show ash, crude fiber, protein (as determined by the Kjeldahl method), riboflavin and folic acid to be present in higher concentrations in the raw peel than in the corresponding flesh. However, these ratios change somewhat depending on the cooking methods involved, and consequently because of the results of heat, dehydration and leaching as well as a result of differences in the peel thickness associated with these methods.

As indicated above, peel amounts to a small fraction of the entire tuber, whether raw or cooked. Therefore, even a relatively high P/F ratio may not mean very much nutritionally when one consumes an entire tuber. Just how much it means for the nutrients in question is shown in Table 3. Any figure in this table which exceeds the percentage value of peel listed at the top of each column signifies that the tuber contains a disproportionally higher amount of that particular nutrient. This is the case of protein, crude fiber and ash which includes minerals regardless of the cooking methods used. This also holds true for riboflavin. As for the other vitamins, raw and cooked tuber peel contains a disproportionately low amount of thiamine. It contains roughly the same amount of ascorbic acid, higher amounts of folic acid in raw peel, and higher amounts of niacin, folic acid and vitamin B_6 with dry cooked tuber peel. Thus while tuber peel contains many of the nutrients in disproportionately higher amounts in peel than in flesh, these values with the exception of crude fiber and riboflavin do not justify claims, that most of the nutrients are contained in the peel.

Table 2—Peel-flesh ratio of nutrients in raw and cooked potatoes (DWB)

	Raw	Boiled in water	Oven-baked	Microwave-cooked
Ash	2.5	1.8	1.0	1.4
Crude Fiber	6.3	8.4	2.8	4.5
Protein	1.9	1.6	1.0	1.4
Ascorbic acid	1.0	0.6	0.5	0.6
Thiamine	0.4	0.3	0.4	0.6
Riboflavin	2.0	1.6	1.9	1.2
Niacin	0.9	0.9	0.8	1.5
Folic acid	1.7	0.8	0.8	1.1
Vitamin B_6	1.2	0.8	0.9	1.1

Table 3—Nutrient content of tuber peel as percent of the nutrient content of the whole tuber

	Raw	Boiled with peel	Oven-baked	Microwave-cooked
% Peel/tuber	6	2	10	4
Dry matter	4.7	1.9	17.7	5.5
Protein	8.3	2.9	16.7	7.6
Crude fiber	34.4	14.9	37.3	20.8
Ash	15.9	3.3	17.0	7.7
Ascorbic	5.0	1.1	10.5	3.4
Thiamine	1.7	0.6	7.8	3.1
Riboflavin	9.4	2.9	28.6	6.6
Niacin	4.1	1.8	15.0	8.3
Folic acid	8.0	1.6	15.2	5.8
Vitamin B_6	5.6	1.7	15.6	6.0

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CHANGES IN THE NUTRIENT COMPOSITION DURING COMMERCIAL PROCESSING OF FROZEN POTATO PRODUCTS

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ABSTRACT

Unit operations in several commercial processing operations of frozen potato products were evaluated regarding their effects on the protein and water-soluble vitamin composition of the products. The investigation included processing lines for French fries and preformed patties. Overall retention values ranged from a low of 53% for ascorbic acid in preformed patties to a high of 90% for vitamin B_6, also in preformed patties. With the exception of the case of ascorbic acid in preformed patties, water blanching was the chief contributor toward the reduction of nutrients. The effects of the other unit operations were statistically nonsignificant. Retention values with small cut French fries were lower than those of the large size cuts. Retention values were significantly greater with steam blanching than with water blanching.

INTRODUCTION

INFORMATION regarding the effects of commercial processing on the nutritional value of potatoes has been published repeatedly (Hendel, 1960; Olson and Harrington, 1955; Bring et al., 1963; Bring and Raab, 1964; and Watt and Merrill, 1963). However, these studies were limited to laboratory or pilot plant scale operations. If done on a commercial scale, they were limited to comparative nutrient evaluations of the raw versus the finished product, and failed to investigate specific effects of the individual unit operations, e.g. blanching, deep-fat frying, mashing, dehydration, etc. Furthermore, nutrient coverage in general involved one or two vitamins. There also appears to be a total lack of information on nutrient changes during commercial processing of frozen potato products.

The purpose of this investigation was to determine the nutritional effects of the various unit operations in commercial potato processing plants. This report deals with frozen potato products. Changes in the nutrient contents in potatoes during commercial dehydration have been published earlier (Augustin et al., 1979).

MATERIALS & METHODS

SEVERAL commercial frozen potato product lines were sampled in southern Idaho as well as in south central Washington. Product lines investigated were large sized (½ in.) and small sized (¼ in.) French fries and preformed patties.

Flow sheets for each product are shown in Figure 1. These flow sequences are patterned after those outlined by Blackstock and Skiver (1974). It should be borne in mind that processing steps vary depending on the processing plant and/or company. Furthermore, one should also realize that some processing parameters, e.g. time and temperature, may vary considerably depending on the company involved as well as on the quality of the raw material.

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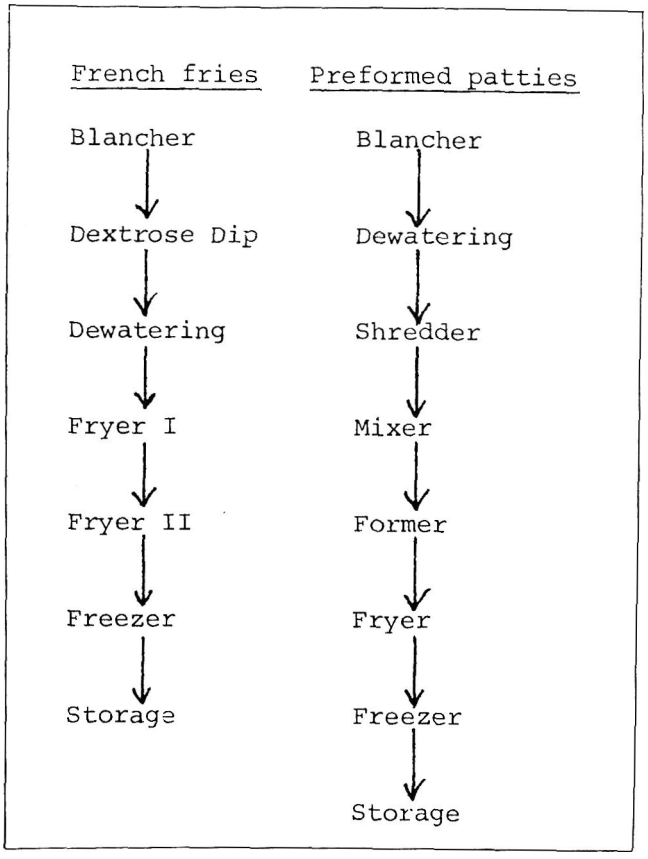

Fig. 1—Unit operations in frozen potato production lines.

Each product line was sampled twice with three replicate samples of approximately 500g each for every sampling point. The basis of selection of sampling points within each line were exposure of the product to water and/or high temperature. The sampling schedule for each line followed the same time sequence as the product flow through that line.

Following removal from the line, each sample was immersed separately into liquid nitrogen, packaged in Whirl-Pak pouches, stored at freezing temperatures, and then transported to the laboratory in insulated boxes using dry ice and stored at −20°C. Within 1 wk or less after sampling, part of each frozen sample was used for dry matter determination and ascorbic acid analysis. The remaining part of each sample was freeze-dried, using ambient plate temperatures, then ground through a Wiley Mill using a 40 mesh screen, and stored in a nitrogen atmosphere at −20°C until further analysis. Each of the replicate samples was analyzed separately.

Analytical

Total nitrogen was determined by the micro-Kjeldahl method. Analysis of the vitamins was done by the following methods: ascorbic acid by the indophenol titration method, thiamine and riboflavin by fluorometry, and niacin by colorimetry; folic acid and vitamin B_6 were determined microbiologically. The methods used in general were those outlined in the AOAC (1975) with the following modifications:

Total folic acid instead of free folic acid was determined. Bound folic acid was converted to its free form by treatment with purified hog kidney conjugase. This was prepared from

fresh, defatted hog kidneys according to Eigen and Shockman (1963), and purified, i.e., separated from conjugated folates by gel chromatography according to Shin et al. (1972) using Sephadex G-25. The Dextran Blue fraction which contained the purified conjugase was frozen and stored until used. Sample incubation with conjugase was 4 hr at 38°C. Total vitamin B_6 rather than the individual three forms were determined with pyridoxine as the standard.

RESULTS & DISCUSSION

TABLE 1 summarizes the compositional ranges encountered with the raw material, i.e., the fresh potatoes, throughout this study. These values, when recomputed onto a 150g basis, generally fall within the compositional ranges published by Augustin et al. (1978) which were based on a nationwide investigation encompassing freshly harvested as well as potatoes stored up to 9 months. The data shown in Table 1 are limited to potatoes which were stored between 2 and 6 months. As a rule, the potato processing season in the Pacific Northwest lasts over a period of 8–10 months. Since storage has been reported to affect notably ascorbic acid, folic acid and vitamin B_6 (Augustin et al., 1978, 1979; Augustin, 1975; Yamaguchi et al., 1960) values for these vitamins in the unprocessed potatoes can amount to as high as 36 mg and 21 μg, and as low as 0.13 mg per 100g raw tuber material respectively in the early part of the season, and as low as 7 mg and 8 μg and as high as 0.40 mg respectively at the end of the storage period.

Table 1—Composition ranges of 100g raw potatoes

Protein, g	1.5 – 2.3
Ascorbic acid, mg	10.7 – 18.3
Thiamine, mg	0.08 – 0.13
Riboflavin, mg	0.03 – 0.05
Niacin, mg	1.32 – 2.03
Vitamin B_6, mg	0.18 – 0.31
Folic acid, mg	5.9 – 17.3

Table 2—Composition of 100g frozen potato products

	½ in. French fries	¼ in. French fries	Preformed patties
Protein, g	2.1*(19.3)**	2.1 (13.9)	2.4 (33.1)
Ascorbic acid, mg	12.3 (12.0)	11.1 (25.1)	13.7 (56.8)
Thiamine, mg	0.12 (34.4)	0.12 (20.4)	0.12 (18.7)
Niacin, mg	1.81 (26.9)	1.53 (15.8)	1.32 (22.9)
Vitamin B_6, mg	0.25 (29.1)	0.24 (24.8)	0.27 (16.1)
Folic acid, μg	10.6 (24.1)	8.8 (39.6)	11.2 (58.6)

* Mean
** Coefficient of variation

Table 2 summarizes the mean protein and vitamin composition of the finished products. The values are somewhat higher than those published by Watt and Merrill (1963); However, this is mainly because of differences in the moisture content of the products. When computed on an equal moisture basis the data between the two groups are in good agreement.

Because of differences in the moisture and fat content between the raw and the finished product, changes in the nutrient composition cannot be properly assessed in this manner. Instead they have to be computed on an equal moisture of dry weight and fat-free basis. Consequently, the data listed in the following tables were computed on such a basis.

Table 3 summarizes the protein and vitamin retention values obtained in this investigation. It includes both the total retention, i.e., the retention of nutrients in the finished product as compared to the corresponding raw material as well as the nutrient retention during water blanching. In general, the total retention values were highest with protein, thiamine and niacin, and the lowest with ascorbic and folic acids. Differences between total retention and retention during water blanching in general were insignificant. An analysis of variance revealed that these differences were statistically nonsignificant except in one instance, i.e., in the case of ascorbic acid with preformed patties. In this case additional reductions in retention values occurred during the deep-fat frying operation. The data clearly show water blanching to be the main point of nutrient loss during commercial frozen potato product operation.

With the exception of thiamine and folic acid, retention values were significantly lower ($P \leq 0.05$) with the smaller ¼ in. fries than with the large ½ in. fries. This was probably due to differences in leaching losses of some nutrients as a result of different volume-to-surface area ratios of the two size cuts. It would appear logical to extrapolate this reasoning to the preformed patties which were blanched prior to shaping and which during blanching consist of bits and pieces, smaller in diameter than that of the smallest size French size cut; one would expect that retention values of this product during blanching to be significantly less than that of the small French fry cut. However, the data in Table 3 show the opposite to be true. One likely explanation is the fact that these bits and pieces which are used in the preformed pattie operation are extensively exposed to water prior to blanching, both in flumes and in holding tanks. It is conceivable that during this prolonged exposure to water prior to blanching could result in extensive leach losses. However, since the inception point of sampling started at the blanching inlet, and since product flow and handling prior to blanching was such as to make it physically impossible to obtain representative samples, this theory has not been substantiated by experimental evidence.

Table 3—Percent retention of nutrients during commercial processing

	Large sized French fries (5)[a]		Small sized French fries (4)[a]		Preformed patties (3)[a]	
	Total	Water blanching	Total	Water blanching	Total	Water blanching
Protein	85[b] (12.0)[c]	89 (6.2)	81 (8.5)	81 (12.7)	90 (4.4)	97 (7.0)
Ascorbic acid	69 (14.4)	75 (10.2)	61 (20.3)	69 (22.0)	53 (26.6)	80 (6.3)
Thiamine	80 (11.4)	80 (9.3)	81 (16.8)	88 (5.9)	88 (9.9)	96 (5.6)
Niacin	84 (1.25)	85 (8.0)	74 (13.6)	78 (18.4)	90 (18.2)	93 (7.9)
Vitamin B_6	78 (12.5)	79 (12.0)	74 (11.9)	77 (19.0)	91 (12.8)	92 (9.6)
Folic acid	66 (19.4)	69 (19.5)	65 (23.3)	66 (21.1)	73 (16.2)	81 (13.2)

[a] Number of plants sampled
[b] % retention
[c] Coefficient of variation

The data in Table 3 clearly show water blanching to be the major source of nutrient reduction during commercial frozen French fry production. This method of blanching is predominant in the industry. On a few occasions steam blanching is used. The results of a comparative evaluation of the two blanching methods which are shown in Table 4, clearly demonstrate that all nutrients were retained better during steam blanching as compared to water blanching. Based on the results obtained in this study, from a nutritional standpoint at least, steam blanching is preferable to water blanching.

Table 4—Comparative percent retention of nutrients in potatoes during water and steam blanching

	Water blanching	Steam blanching
Protein	81	94
Ascorbic acid	69	89
Thiamine	88	90
Niacin	78	93
Vitamin B_6	77	97
Folic acid	66	93

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SENSORY PROPERTIES OF HIGH FRUCTOSE CORN SYRUP ICE CREAM FORMULATIONS

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ABSTRACT

Ice cream formulations containing varying levels of high fructose corn syrup (HFCS) and corn sweeteners of differing dextrose equivalents (DE) were compared on preference and three sensory components with a sucrose control by 160 untrained panelists. The sensory components studied were sweetness, mouthfeel and gumminess. A significant improvement in preference was found with the HFCS ice cream formulations relative to the sucrose control with increasing concentration of corn sweeteners up to about 33% of total sweetener at 28–31 DE corn sweetener. A very high correlation between preference and mouthfeel was found yielding a multiple R^2 of 0.98 of which over 96% was due to mouthfeel in the multiple regression model relating preference to sweetness, mouthfeel and gumminess.

INTRODUCTION

IMPROVED TECHNOLOGY in the processing of corn sweeteners has resulted in the development of 90% fructose syrups. With the increasing availability of a variety of corn sweeteners, the possibility of complete replacement of sucrose with corn sweeteners in frozen desserts exists. Arbuckle (1972) investigated the use of 17% and 42% fructose syrups. Long (1978) revealed that considerable study is now being given by the frozen dessert industry to the use of a blend of 55% fructose syrup and 36 DE conventional corn syrup as the total sweetener. The recent appearance of 90% fructose syrups opens up new and exciting possibilities in this regard.

It is realised that the inclusion of corn sweeteners in ice cream will result in changes in sweetness, freezing point and in textural characteristics, including body, compared with formulations in which sucrose is used as a sweetener. A primary objective of this study was, therefore, to assess the impact of high fructose formulations on sensory components before adjusting mix composition to compensate for changes in physical parameters.

With respect to these changes, it was of the utmost importance to determine the extent to which they affected the sensory characteristics perceived by untrained panelists when they taste ice cream. The problem was shaped by two questions: (a) did the new formulations differ in preference ratings?; and (b) if so, which sensory component was most likely to be responsible for the difference in preference? In order to answer the latter of these two questions, an extremely simple discriminative test involving perceived sweetness, mouthfeel, and gumminess was conducted.

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Table 1–Sweetener combinations consisting of 90% high fructose corn syrup (HFCS) and corn sweeteners of differing dextrose equivalents (DE)

Sweetener	Combination %
Sucrose	100.0
HFCS	70.7
9–12 DE corn sweetener	29.3
HFCS	69.3
13–17 DE corn sweetener	30.7
HFCS	68.0
19–23 DE corn sweetener	32.0
HFCS	66.7
28–31 DE corn sweetener	33.3
HFCS	59.4
41–44 DE corn sweetener	40.6

MATERIALS & METHODS

Ice cream formulations

Fifteen pound batches of ice cream mix were prepared according to the following basic formulation: 12% fat, 11% serum solids, 15% sweetener (dry basis) and 0.2% stabilizer-emulsifier. A list of the sweetener combinations used are shown in Table 1. The mixes were pasteurized (74°C for 10 min) and homogenized in the usual manner. After a 24-hr aging period they were frozen in a small batch freezer to 80% overrun and dispersed in 40 ml paper cups with lids.

The sensory scale

The analysis of sensory rating scales has been the topic of considerable debate in recent years and several important papers have been published on this subject (Lasztity and Orsi, 1975, 1976; Howard, 1972, 1976; Chapman and Wigfield, 1970; Levitt, 1974; Herrmann, 1976). Cloninger et al. (1976) studied 5-, 7-, 9-, and 15-point scales measuring flavor, aroma and juiciness for equal spacing and normalization. The 5-point scale demonstrated the least central tendancy, since extremes on the 9- and 15-point scales were avoided. Magnitude estimates have been used successfully in sensory research (Mc Burney, 1966; Stevens, 1962; Moskowitz, 1974) and are highly reliable for statistical analysis. Stone et al. (1974) described the background to the development of a word-anchored scaling technique in which panelists marked a continuous line to indicate the degree of a sensory characteristic relative to extreme anchor terms. This method is easily understood and the data can be subjected to parametric statistical analysis in general, without violating the assumptions inherent in the analysis of variance. A slightly modified version of this scale was used in the present study in which the line length was complete at 10 cm rather than 5 in. with extending ½ in. segments as described by Stone et al. (1974).

Experimental design

Experimental designs for sensory research have been reviewed by Sidel and Stone (1976). Some attention has been focused on the use of incomplete blocks in sensory studies (Gacula and Kubala, 1972) and composite complete-incomplete block designs have been developed (Cornell and Knapp, 1972, 1974). In this study an incomplete block design was used, plan 11.5, Type 1 from Cochran and Cox (1957) where t (number of treatments) = 6, k (number of treatments per block) = 3, r (number of replications) = 10, b (number of blocks or panelists) = 20, λ (number of times two treatments appear together in the same block) = 4. This basic plan of 20 blocks was repeated 8 times and the order of presentation of the samples to panelists was randomized.

Table 2–Analysis of variance table for preference

Source of variation	Degrees of freedom	Sums of squares	Mean squares
Replications	79	614.6	
Treatments (unadjusted)	5	343.1	
Blocks within Reps. (adj)	80	373.4	4.7 E_b
Intra-block error	315	1145.7	3.6 E_e
Total	479	2476.8	

Efficiency (relative to randomised blocks):	1.01
Effective error variance per unit:	3.80
Adjusted treatments mean square:	64.00
F-ratio:	16.8 ($P < 0.001$)

Table 3–Analysis of variance table for sweetness

Source of variation	Degrees of freedom	Sums of squares	Mean squares
Replications	79	312.3	
Treatments (unadjusted)	5	195.4	
Blocks within Reps. (adj)	80	336.6	4.2 E_b
Intra-block error	315	1090.1	3.5 E_e
Total	479	1934.4	

Efficiency (relative to randomised blocks):	1.01
Effective error variance per unit:	3.60
Adjusted treatments mean square:	40.10
F-ratio:	11.20 ($P < 0.001$)

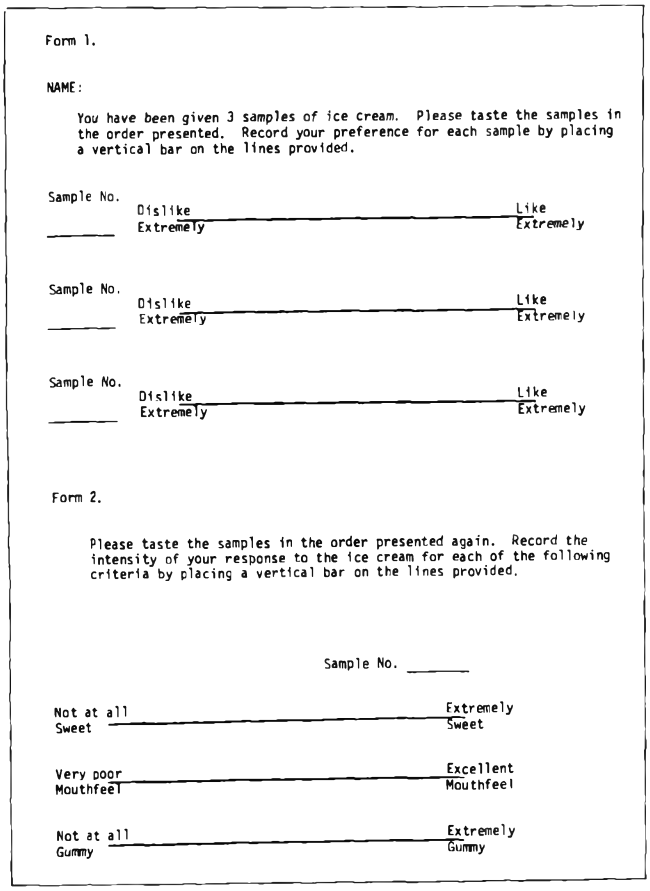

Fig. 1–Sensory evaluation forms used to assess the sensory properties of ice cream formulations.

Sensory evaluation

The ice cream formulations were evaluated by 160 panelists according to the design already described. The panelists were randomly drawn from the University of Guelph student population of 10,000 by contacting every fifth student registered for the Winter Semester, 1978 by mail. These panelists were untrained and can be considered to represent their age group and socio-economic class reliably. Once a sensory roster was established, each panelist was invited to participate once at a specific time between 10:00 am to 12:00 noon or 2:00 pm to 4:00 pm on Tuesdays, Wednesdays or Thursdays, which cover optimum sensory testing times. The samples were tasted in individual booths under white lights from 40 ml cups using disposable plastic spoons. The panelists had been informed in advance that the sensory study concerned ice cream formulations so that individuals who dislike ice cream could elect not to participate.

Panelists were requested to evaluate three samples of ice cream for preference and then, on subsequent forms, to score each sample separately for perceived sweetness, mouthfeel and gumminess. Thus, panelists were not influenced in scoring for preference by these sensory terms since they did not see them until the preference scores were given. Scoring was accomplished by placing a vertical bar or line on a work-anchored horizontal scale of 10 cm in length. For example, the preference anchor terms were "dislike extremely" and "like extremely" so that when the line is measured from the left anchor term end to the vertical bar, a score of from 0.0 cm to 10.0 cm can be obtained depending on the panelist's response to the sample. Figure 1 shows the two types of forms used. The choice of the terms "sweetness," "mouthfeel" and "gumminess" was based on the expected changes in sensory characteristics and an attempt to group these changes under terms which would be simple enough to be understood by untrained panelists. More informative, but more complex, textural terms were avoided because of the danger of confusion. Analysis of the data resulting from sensory testing involved analysis of variance (using the IMSL subroutine ABIBAN) and multiple regression (using BMD 02R).

RESULTS

TABLES 2, 3 and 4 are the analysis of variance tables for preference, sweetness and mouthfeel. The gumminess characteristic did not exhibit a significant F-ratio at the 95% level. These tables show that efficiency of the incomplete block design was between 1.00 and 1.05 relative to randomised blocks. The F-ratios were very highly significant (>99% confidence).

Table 5 gives the means (adjusted for block effects) for the six ice cream formulations separated using Duncan's Multiple Range test at the 95% level. It can be seen that preference scores generally increase as the DE of the sweetener increases to a maximum at the 19-23 DE and 28-31 DE formulations and thereafter (41-44 DE formulation) scores drop drastically. The simple correlation coefficient between preference and sweetness is low (0.47), which can be seen in Table 6. This table gives the simple correlation coefficient matrix and it can be seen that the most important sensory criterion influencing preference within the experimental region is mouthfeel (or body). The simple correlation coefficient, r, between preference and mouthfeel is

Table 4–Analysis of variance table for mouthfeel

Source of variation	Degrees of freedom	Sums of squares	Mean squares
Replications	79	526.1	
Treatments (unadjusted)	5	228.7	
Blocks within reps (adj)	80	575.1	7.2 E_b
Intra-block error	315	1333.7	4.2 E_e
Total	479	2663.7	

Efficiency (relative to randomised blocks):	1.05
Effective error variance per unit:	4.60
Adjusted treatments mean square:	44.60
F-ratio:	9.70 ($P < 0.001$)

Table 5—Adjusted means of six ice cream formulations for preference, sweetness and mouthfeel[a]

Formulation	Preference	Sweetness	Mouthfeel
Sucrose	5.4 b	6.6 a	5.2 b
HFCS plus 9–12 DE sweetener	5.8 ab	5.3 b	5.0 a
HFCS plus 13–17 DE sweetener	6.0 ab	5.1 b	5.7 ab
HFCS plus 19–23 DE sweetener	6.4 a	5.6 b	5.2 a
HFCS plus 28–31 sweetener	6.3 a	5.5 b	5.4 a
HFCS plus 41–44 sweetener	3.9	4.4 c	4.3

[a] Means within columns followed by the same letter are not significantly different from each other at the 95% confidence level using Duncan's Multiple Range Test.

Table 6—Simple correlation coefficient matrix for the sensory characteristics of ice cream formulations

Sensory characteristic	Sensory characteristic			
	Preference	Sweetness	Mouthfeel	Gumminess
Preference	1.00			
Sweetness	0.47	1.00		
Mouthfeel	0.97	0.33	1.00	
Gumminess	−0.69	−0.45	−0.59	1.00

Table 7—Relative importance of the sensory characteristics, sweetness, mouthfeel and gumminess in influencing preference

Sensory characteristic	% of Coefficient of determination (R^2)
Mouthfeel	96.76
Sweetness	2.37
Gumminess	0.87

0.97. This relationship is clearly evident in Table 5 also where the sucrose control scored highest for sweetness and numerically second lowest for mouthfeel and preference.

Multiple regression analysis of preference as a function of sweetness, mouthfeel and gumminess established the following relationship:

$$P = 0.98 + 0.17S + 1.03M - 0.67G$$

where P = preference, S = sweetness, M = mouthfeel, and G = gumminess.

The coefficient of determination, R^2, was 0.98 for this relationship and the analysis of regression was significant at > 99%. Table 7 shows that mouthfeel constitutes 96.76% of the explainable variation.

DISCUSSION & CONCLUSIONS

THE USE OF CORN sweetners plus HFCS in ice cream had profound effects on preference and a closely related sensory component, mouthfeel, in sensory studies with untrained panelists. The replacement of sucrose with HFCS plus other corn sweeteners at levels up to about 33% of total sweetener as 28-31 DE corn sweetener improved preference scores for ice cream significantly. This improvement was due almost entirely to a change in the sensory components suggested to panelists under the term "mouthfeel." Although significant differences in sweetness were found, sweetness was shown to have very little bearing on preference *within the experimental region*. This means that although there were noticable differences between formulations in sweetness, the differences were not sufficient to influence preference when "mouthfeel" differences were so glaringly obvious to panelists. Panelists could not perceive differences in the ice cream samples on the basis of gumminess. This is not surprising considering the low level of stabilizer-emulsifer used and the minimum impact on gumminess expected from the HFCS or the corn sweeteners.

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SWEETNESS OF LACTULOSE RELATIVE TO SUCROSE

F. W. PARRISH, F. B. TALLEY, K. D. ROSS, J. CLARK AND J. G. PHILLIPS

ABSTRACT

The sweetness of lactulose over the range of concentration 5–35% (W/V), measured by a trained panel using paired comparison with standard reference solutions of sucrose of varying concentrations, is 48% to 62% of that of sucrose. In addition, sensitivity thresholds and recognition thresholds for sweetness of lactulose and sucrose were determined by a rating-scale method. The sweetness of a mixture containing 10% (W/V) lactulose and 5% (W/V) sucrose showed a synergistic effect of 22%, and a mixture of 5% (W/V) lactulose and 2.5% (W/V) sucrose showed 12% synergism. Partial hydrolysis of lactulose to give a mixture containing 5% (W/V) lactulose, 2.5% (W/V) galactose, and 2.5% (W/V) fructose caused a 6% synergistic effect on sweetness.

INTRODUCTION

THE ANNUAL WHEY production in the United States has been estimated at 36 billion pounds (2.4 billion pounds of solids) (Lough, 1974), approximately half of which is discarded (Johnson et al., 1976). Economic considerations have led Drews (1975) to advocate utilization of whey protein concentrate and lactose derived from whey rather than yeast protein. Isolation of whey protein concentrate by ultrafiltration has been described (McDonough et al., 1971). Deionization of the ultrafiltrate yields α-lactose monohydrate (Parrish et al., 1979) or β-lactose (Kavanagh, 1975), both of which have been used as partial replacements for sucrose in bakery goods (Ash, 1976; Goldman and Short, 1977). However, Ash (1976) found that lactose is not useful when sweetness or high solubility of the sugar components is required.

We thought that lactulose [4-O-β-D-galactopyranosyl-β-D-fructofuranose] (Isbell and Pigman, 1938; Perlin et al., 1973) might possess sweetness and solubility properties which would make it useful in baking and confectionery applications. Isomerization of lactose to lactulose was first accomplished by Montgomery and Hudson (1930) who recognized that lactulose is sweeter than lactose, but not as sweet as sucrose; in addition, these workers found lactulose to be very soluble in water, and that crystallization to produce anhydrous lactulose was difficult. In tests with finely powdered crystalline lactulose, Lee and Birch (1976) confirmed the sweetness observation of Montgomery and Hudson (1930) and found that lactulose was devoid of bitter taste. The work described here presents our findings on the sweetness of lactulose, derived from lactose by isomerization, compared to that of sucrose, together with determinations of sensitivity thresholds and recognition thresholds for sweetness of these sugars.

EXPERIMENTAL

Materials

α-Lactose monohydrate was obtained from Sigma Chemical Co., and sucrose from Fisher Scientific Co. Lactulose [4-O-β-D-galactopyranosyl-β-D-fructofuranose] was prepared from α-lactose monohydrate by isomerization with calcium hydroxide by the procedure of Corbett and Kenner (1954), seed crystals of lactulose being obtained from Aldrich Chemical Co., Inc. Partially hydrolyzed lactulose was obtained by treating a 10% (W/V) lactulose solution (1000 ml) with food-grade β-galactosidase (1g; 40,000 ONPG units) (Enzyme Development Corporation) from *Saccharomyces lactis* for 4 hr at 30°C and pH 6.7, after which time the solution was heated to 70°C for 5 min to inactivate the enzyme. The solution was decolorized with charcoal (Eastman Kodak Company), and spectrophotometric analyses (see later) showed the extent of hydrolysis of lactulose to be 70%. Untreated lactulose was added to give a mixture containing 5% (W/V) lactulose, 2.5% (W/V) galactose, and 2.5% (W/V) fructose. Distilled water for taste testing was obtained from Lehigh Valley Cooperative Farmers.

Analytical methods

Purity of the sugar materials was determined from optical rotation measurements on 2% (W/V) aqueous solutions in a 1 dm tube with a Perkin Elmer Model 141 polarimeter; specific optical rotation data used in the calculation of purity were those reported by Buma and van der Veen (1974) for α-lactose monohydrate, Isbell and Pigman (1938) for lactulose, and Bates (1942) for sucrose. Moisture content was determined on 50–500 mg samples by use of a Photovolt Aquatest II instrument, and ash content was measured by the AOAC method I (1975).

The presence of other sugars as impurities in the test sugars was tested by thin-layer chromatography on cellulose plates with ethyl acetate/pyridine/water (10/4/3, by volume) used as solvent (Aspinall and Ferrier, 1957), and visualization of the spots was made with orcinol reagent (Klevstrand and Nordal, 1950) for ketose sugars, with aniline oxalate (Horrocks and Manning, 1949) for reducing sugars and sucrose, or with alkaline silver nitrate (Trevelyan et al., 1950) for all classes of sugars. In addition, gas-liquid chromatography of trimethylsilyl derivatives of the sugars (Sweeley et al., 1963) was performed on a 4 ft × 1/8 in. column of 3% SP-2100 on Supelcoport (100–120 mesh) with temperature programming from 150–210°C and a flame ionization detector. Other parameters were helium flow-rate 30 ml/min, injector temperature 215°C, and detector temperature 220°C.

Spectrophotometric analyses were made with a copper reagent to determine monosaccharides in the presence of disaccharides (Tauber and Kleiner, 1932), with 3,5-dinitrosalicylic acid to measure total reducing sugars (Miller, 1959), and with thiobarbituric acid to determine ketose sugars (Percheron, 1963) to evaluate the purity of the sugars.

Method used for sensory evaluation

Sensory evaluations were made by a forced choice paired comparison method (Pangborn, 1963) in which the judges were asked to determine the sweeter member of each pair comprising a fixed concentration of lactulose or lactose and one of three sucrose solutions of differing concentrations. The choice of method is based on the observation of Stone and Oliver (1969) that paired-comparison is the most exact method because responses are the result of direct comparison between two stimuli.

Panel selection and composition

The panel consisted of in-house personnel with previous experience in sweetness evaluation of sugar sirups. Selection was based on their ability to determine relative sweetness of samples in paired comparison tests with different levels of sucrose; two series of sucrose solutions were presented and each judge was asked to find the samples of equal sweetness. In another test two series of lactose and sucrose solutions were presented to determine the concentrations for equal sweetness; the findings were compared with literature values (Amerine et al., 1965).

The 31 panel members were scientists, engineers, technicians,

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and secretaries. There were 21 males and 10 females ranging in age from 20 to 62. In each session 20 judges were used.

Material evaluation

All tests were performed in the morning. Solutions were prepared 16 hr before being tested to allow mutarotational equilibrium of lactose and lactulose to be established (Pangborn and Gee, 1961). Serving order was randomized within and between pairs. Samples were coded and served at 22°C in 30 ml amounts in odorless plastic cups. Three pairs of samples were tested in each session, one of lactulose or lactose for comparison with each of three concentrations of sucrose. All tests were replicated twice.

Environmental conditions

A separate taste panel room with partitioned booths was used. The room was maintained at 20°C under positive pressure. Air, brought in from outside, was filtered through charcoal. The room was equipped with white fluorescent lights and lights with a choice of colored filters. The white fluorescent lights were used in most of the tests except that when a discernable color difference occurred green lights were used.

Data analysis

The equal sweetness point was found by plotting the percentage of judges who selected the lactulose or lactose solution as sweeter than the sucrose solution versus the sucrose concentration (Pangborn, 1963). The concentration of sucrose was calculated for which 50% of the judges selected the lactulose or lactose solution, and this concentration of sucrose is the equal sweetness point (Fig. 1). This calculation was made for each concentration of lactulose or lactose tested.

Sensitivity threshold and recognition threshold for sweetness for lactulose and sucrose were made by a modification of the method of Gregson (1962) by limiting the choice of responses to the following:

1. Definitely the same as water.
2. A perceptible taste, cannot say what.
3. A barely recognizable sweet taste.
4. A definitely recognizable sweet taste.

An ascending series was tested for sucrose comprising 0, 0.125, 0.25, 0.50, 1.00 and 2.00% (W/V) sucrose. For lactulose the concentrations were 0, 0.25, 0.50, 1.00, 2.00 and 4.00% (W/V). The percentage of judges selecting responses 1 and 2 was plotted against the solution concentration. The sugar concentration was interpolated for the 50% response level, and this is the sensitivity threshold. Similarly, the 50% response level for selection of responses 1, 2, and 3 is the recognition threshold.

Synergism between lactulose and sucrose was examined by comparing the sweetness of a mixture containing 10% (W/V) lactulose and 5% (W/V) sucrose with standard solutions containing 10, 11, 12 and 13% (W/V) sucrose. The 50% response level was used to calculate the equal sweetness point. Similarly, a mixture of 5% (W/V) lactulose and 2.5% (W/V) sucrose was evaluated, using 4.5, 5.0, 5.5, 6.0 and 6.5 (W/V) sucrose.

The effect on sweetness of partial hydrolysis of lactulose was studied by comparing a mixture containing 5% (W/V) lactulose, 2.5% (W/V) galactose, and 2.5% (W/V) fructose with standard solutions of 6.5, 7.0, 7.5, 8.0, and 8.5% (W/V) sucrose. The 50% response level was used to calculate the equal sweetness point.

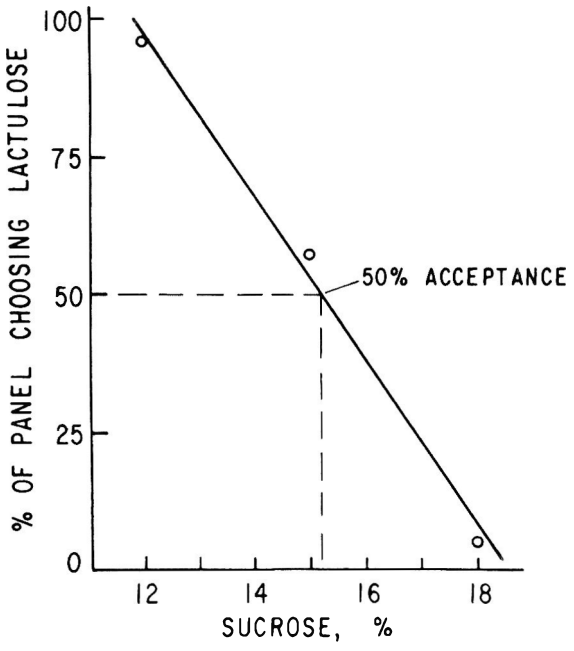

Fig. 1—Concentration of sucrose having sweetness equal to that of lactulose or lactose test solution. Example shown is for 25% (w/v) lactulose.

Table 1—Analyses for α-lactose monohydrate, β-lactulose and sucrose

	α-Lactose monohydrate	β-Lactulose	Sucrose
Specific optical rotation[a] (589 nm)	+52.62°	−50.63°	+66.49°
Purity %[b]	99.82	99.87	99.94
Moisture %	5.12[c]	0.015	0.0048
Ash %	0.091	0.034	0.001

[a] Equilibrium value at 20°C
[b] Based on specific optical rotation
[c] Includes water of hydration

RESULTS & DISCUSSION

THE PURITY (based on specific optical rotation data at equilibrium), moisture content, and ash content of the sugars used in the sweetness studies are shown in Table 1. No other sugars were detected as impurities by thin-layer chromatography on cellulose. Gas-liquid chromatography of trimethylsilyl derivaties of the crystalline sugars (Sweeley et al., 1963) showed a single peak for all three sugars. The absence of monosaccharides in lactulose, lactose, and sucrose, whether examined in the crystalline state or from equilibrated, aqueous solution, was shown by application of the spectrophotometric procedure using copper reagent (Tauber and Kleiner, 1932) and by gas-liquid chromatography of the trimethylsilyl derivatives (Sweeley et al., 1963). Two other spectrophotometric analyses were found to be of value in following the transformation of lactose to lactulose and for examining the purity of the crystalline lactulose. With 3,5-dinitrosalicylic acid reagent (Miller, 1959) lactose and lactulose showed identical absorbances at 540 nm, whereas sucrose gave no reaction. With thiobarbituric acid reagent (Percheron, 1963) lactulose and sucrose showed identical absorbances at 432 nm., whereas lactose gave only 3.8% of the absorbance of lactulose or sucrose. The results of all of these analytical procedures provided assurance of the high purity of the sugar materials used in our sweetness studies.

In our sweetness measurements we used aqueous solutions of sugars which had been allowed to stand 16 hr in order to achieve mutarotational equilibrium (Pangborn and Gee, 1961) of lactulose and lactose, whereas other workers (Montgomery and Hudson, 1930; Lee and Birch, 1976) used crystalline sugars applied to the tongue. Since in food applications sugars are used in an aqueous environment, we thought it was more practical to examine the equilibrated, aqueous sugar solutions rather than crystalline or freshly prepared materials.

The concentrations of the three sucrose solutions used for measuring the sweetness of each lactulose solution are shown in Table 2. The sweetness of equilibrated lactulose over the range of concentration 5–35% (W/V) was found to be 48% to 62% of that of sucrose (Table 3).

In the determination of threshold response levels (Greg-

son, 1962), we measured sensitivity thresholds and recognition (for sweetness) thresholds (Amerine et al., 1965) for lactulose and sucrose (Table 4); the latter values agree with the sweetness relationships shown in Figure 2. Our values of 0.36% for sensitivity threshold and of 0.53% for recognition threshold for sucrose (Table 4) are similar to the median values of 0.342% and 0.582%, respectively, reported by Pfaffmann (1959).

Sweetness comparison of lactose (1.5–16%) and sucrose was made in a study which was curtailed when it was found that the data were closely similar to those of Pangborn (1963), whose results are interpolated in Table 3 to allow comparison of the concentrations for equal sweetness of lactulose and lactose. This comparison shows clearly that lactulose is sweeter than lactose over the range of concentration we examined. Synergistic effects on sweetness for mixtures of sugars are well known (Amerine et al., 1965). We observe a synergistic effect in a mixture containing 10% (W/V) lactulose and 5% (W/V) sucrose. On the basis of additivity of sweetness contributions, this combination would have a sweetness equivalent to 10.07% (W/V) sucrose, whereas by sensory evaluation we find sweetness equivalent to 12.2% (W/V) sucrose, i.e., 22% synergism. Similarly, a mixture containing 5% (W/V) lactulose and 2.5% (W/V) sucrose was expected to have a sweetness equivalent to 4.91% (W/V) sucrose, whereas the value obtained by sensory evaluation was 5.5% (W/V) sucrose, i.e., 12% synergism.

We have kept 70% (W/V) aqueous solutions of lactulose at 4°C for 2 yr, and they have shown no formation of crystalline material or color development.

Partial hydrolysis of 10% (W/V) lactulose was performed with food-grade β-galactosidase to give a mixture containing 5% (W/V) lactulose, 2.5% (W/V) galactose, and 2.5% (W/V) fructose. The mixture was equivalent in sweetness by sensory evaluation to 7.44% (W/V) sucrose, whereas the arithmetic sum of the sweetness contributions of the individual sugars gives an expected value of 7.02% (W/V) sucrose based on our value for lactulose (Table 3) and those of Amerine et al. (1965) for galactose and fructose. These values of 7.02 and 7.44% (W/V) differ significantly ($p < 0.05$) and indicate a synergistic effect of 6%.

The greater sweetness and solubility of lactulose compared to lactose make it of interest to study lactulose as a partial replacement for sucrose in food applications, as Ash (1976) has done for lactose. This work is in progress in our laboratories.

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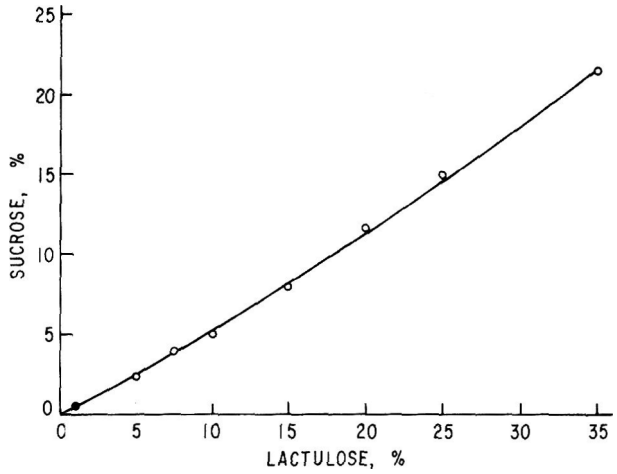

Fig. 2—Concentration of sucrose having sweetness equal to that of lactulose test solution.

Table 2—Concentrations of sucrose solutions used in paired-comparison test of sweetness of lactulose solutions

Concentration % (W/V)			
Lactulose	Sucrose		
5.00	1.0	2.0	3.0
7.50	3.0	4.0	5.0
10.0	4.0	5.0	6.0
15.0	8.0	9.0	10.0
20.0	9.0	11.0	13.0
25.0	12.0	15.0	18.0
35.0	18.0	21.0	24.0

Table 3—Equal sweetness concentration values for lactose, lactulose, and sucrose

Concentration % for equal sweetness		
Lactulose	Sucrose[a]	Lactose[b]
5.00	2.41(0.39)	7.48
7.50	4.01(0.31)	11.12
10.0	5.07(0.13)	13.53
15.0	8.04(1.15)	20.27
20.0	11.79(0.99)	28.79
25.0	15.16(0.60)	36.45
35.0	21.84(2.67)	51.63

[a] Value in parentheses is standard error for duplicate analyses using 20 judges.

[b] Calculated from Pangborn (1963) data.

Table 4—Sensitivity threshold and recognition threshold (for sweetness) of lactulose and sucrose

	Concentration % (W/V)	
Sugar	Sensitivity threshold[a]	Recognition threshold[a]
Lactulose	0.53 (0.30–0.94)	1.22 (0.64–2.34)
Sucrose	0.36 (0.24–0.52)	0.53 (0.41–0.67)

[a] Values in parentheses are 95% confidence intervals for duplicate analyses using 20 judges.

PRODUCTION OF PRE-COOKED CORN FLOURS FOR AREPA MAKING USING AN EXTRUSION COOKER

O. SMITH, T. S. de BUCKLE, A. M. de SANDOVAL and G. E. GONZÁLEZ

ABSTRACT

A commercially available extrusion cooker was successfully used to produce pre-cooked corn flours for use in making arepas, a thick unsalted unleavened corn cake worked by hand into small patties and then roasted. Sixteen trials were run and eight flour samples were obtained that formed a consistent dough that was easy to shape into arepas. The arepas were roasted and observed from 1–20 hr afterwards and no change was noted in sensory or textural properties. This extrusion cooker can be used for elaborating pre-cooked corn flour for arepa production.

INTRODUCTION

IN COLOMBIA and neighboring South American Countries there is a great demand for pre-cooked corn flour, used for making a widely consummed product called the arepa. The arepa is a thick unleavened corn cake that is worked by hand into small patties and then roasted. Until recently, the only process that has been commercially exploited for pre-cooked corn flour for arepa production was steam cooking in a column-roller (IIT, 1973). Colombian industry is interested in rapidly expanding the pre-cooked corn flour market, because the arepa is an integral part of the typical Colombian diet. There are several extrusion cookers already installed in the country. In order to develop a new product line for the unused installed capacity, this Institute, in collaboration with an equipment manufacturer, decided to attempt to produce an extrusion-cooked corn flour suitable for arepa production. Generally, cooker extruders produce high starch modification because their principal application has been in the field of expanded product and instant-type food products that require high temperature and low moisture operating conditions (Smith, 1974). Studies carried out at IIT for several years have shown that pre-cooked flours for arepa making should have an intermediate degree of starch modification which, when measured as dextrose index (described under methods), should give a value between 50 and 70 and a slightly modified amylogram. Dough prepared from these flours must be soft, cohesive, homogenous and nonsticky. The increase in hardness in the roasted arepa when stored should be minimum. These properties can be indirectly measured by compression and puncture tests (Sandoval, 1977). The purpose of this study was to explore the possibilities of producing adequate pre-cooked flours for arepa making by properly adjusting working conditions of a commercial cooker extruder.

An explanation of the type of starch modification that takes place under different extrusion conditions is being carried out.

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MATERIALS & METHODS

Materials

U.S. yellow corn grits were used (7%–60 mesh; 8%–120 mesh, U.S. standard).

Dough and arepa preparation

Arepas were made in the traditional way and by using the flours under study. In the traditional method, degermed hard field corn was soaked overnight in water at room temperature, simmered for about 3 hr, cooled, drained and ground to a moist dough in a kitchen grinder. Similar dough was made by mixing pre-cooked corn flour and water (2.5% salt was added in all cases to the dough) which was then rolled out and cut into flat discs, (arepas) 7.5 cm in diameter and 10 mm thick. Arepas were placed on a metal plate heated to 340°C for 1½ min each side. The arepa was then ready for eating (Bourne et al., 1975).

Equipment

A Wenger X-20 extruder was used with an auger configuration composed of five screw sections (68327-1, 68327-1, 68327-1, 68326-1, 68321-1) and five heating heads. A 1.27 cm diam die was used. The extruder speed and feeding rate were kept constant (400 rpm, 157 lb/hr).

Variables used during extrusion (a) steam fed to the mixing cylinder, 30, 40 and 60 lb/hr which provided moisture contents of the grits at the inlet of 26, 30 and 37%. No steam or water was supplied to the extruder barrel. (b) Temperatures in the 4th (38–149°C) and 5th (79–135°C) heating heads. Other heads were kept under refrigeration. The extrudate was dried at 55°C and milled in a Fitz mill through a 0.95 cm square opening screen. The flour was then sieved through an 80 U.S. standard mesh.

Analytical methods

Chemical. Chemical analysis of the grits was: 1.3% fat, 6.7% protein (N × 6.25) 0.5% ash; 0.7% fiber; and 11% moisture as analyzed by AOAC methods (1975).

The degree of starch modification was measured using the dextrose index method (Buckle and Pardo) (1973). The dextrose index method is a variation of other methods for the determination of the degree of starch modification in wheat flours and related materials (Nielsen, 1969; Toyama et al., 1966; Farrand, 1964; Campbell et al., 1950).

Reagents

Extracting solution. 20.0g of NaCl and 0.2g Ca-acetate are diluted to 1 liter with demineralized water.

Buffer solution (pH: 4.6–4.8). 4.1g anhydrous Na-acetate and 3 ml glacial acetic acid are diluted to 1 liter with demineralized water.

α and β-amylase solution. 0.25g α and 0.25g β-amylases (Nutritional Biochemicals Corp.) are diluted to 50 ml in demineralized water.

Sulphuric acid, 10% (V/V).

Sodium tungstate, 12% (W/V).

Hydrochloric acid, 7.78N.

Sodium hydroxide, 20% (W/V).

Other reagents as specified in AOAC method No. 14.023 (AOAC, 1975).

Procedure

One-gram sample is place into a 125 ml Erlenmeyer and is suspended into 45 ml of a 1:1 mixture in a water bath at 30°C ± 0.5. After 10 min, 10 ml α- and β-amylase solution is added and the mixture is incubated for exactly 1.0 hr with constant stirring. At the end of the prescribed time, 2 ml sodium tungstate and 2 ml sulphuric acid are added and the flasks are taken out of the water bath.

The contents of the Erlenmeyer flasks are then filtered through Whatman No. 4 paper and repeatedly washed with distilled water until a total volume of 100 ml is obtained. A 50-ml aliquot of the

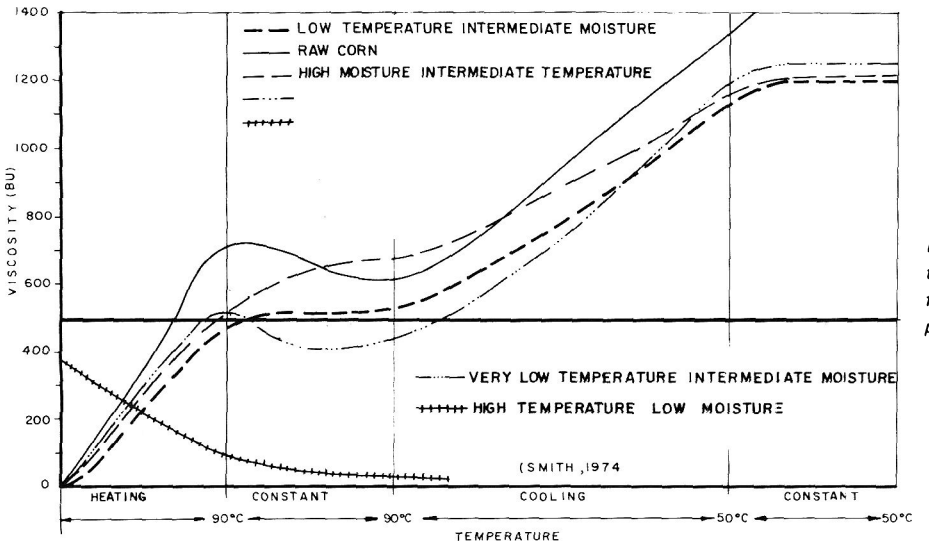

Fig. 1—Effect of operating conditions on the Amilograph viscosity behavior of corn flours. Column-roller and extrusion-cooking process.

filtrate is put into a 250 ml boiling flask. 5.0 ml hydrochloric acid are added and the mixture is then refluxed during 1 hr. At the end, the mixture is cooled and neutralized with sodium hydroxide using phenolphtalein as indicator. The final solution is then filtered through Whatman No. 4 paper into a 100 ml volumetric flask and is washed with distilled water up to the mark.

The reducing power of the solution is evaluated by AOAC method 14.023 (AOAC, 1975) and expressed as glucose. A 0.25% glucose solution in water is used as a standard.

Calculations

Dextrose index is calculated as the weight percentage of the total sample (dry basis) that is converted to glucose.

Physical

Physical testing was done on flours, doughs and arepas using specific methodologies for each case.

Amylography of flours

Evaluation of flour properties was done through amylography (10% aqueous suspension on a dry basis, Brabender visco amylograph, model VS-6, 350 cmg torsion head). When interpreting the amylographs, careful attention was given to the shape of the curve during the 20-min holding period at constant temperature (90°C).

Table 1—Effect of operating conditions on the properties of corn flour for arepa making extrusion-cooking process

Moisture at the inlet (%)	Temperature °C last heating heads 4 TH	5 TH	Amylograph section (20 min at 90°C)		Dough Characteristics	Run N°
26	38	79		500 BU	Very sticky[a]	1
26	38	93		500 BU	Very sticky[a]	2
26	38	107		500 BU	Very sticky[a]	3
30	121	121		500 BU	Good	4
30	135	135		500 BU	Good	5
30	135	135		500 BU	Good	6
30	121	121		500 BU	Good	7
30	93	93		500 BU	Sticky	8
30	38	93		500 BU	Sticky	9
30	38	79		500 BU	Sticky	10
30	38	79		500 BU	Sl. sticky	11
37 (c)	38	93		500 BU	Good	12
37	38	107		500 BU	Good	13
37	121	121		500 BU	Good	14
37	135	121		500 BU	Good	15
37	149	121		500 BU	Very good	16

[a] Unable to form arepas

Water absorption

Water absorption of the pre-cooked corn flour samples was estimated by measuring the precise amount of water needed to obtain a soft, cohesive, homogeneous and smooth dough.

Hardness of dough and arepa

Dough hardness was evaluated using a compression test in the Instron Universal testing machine (Model 1132) provided with a 50 kg cell and a plunger (3.6 cm diam). Cylindrical dough samples having 5.5 cm diam and 5.5 cm height were used. Crosshead speed: 6 cm/min; chart: 12/cm/min. The reported results correspond to a single determination of the maximum force needed for a 71% compression.

Arepa hardness was measured by a puncture test similar to the one described for beans by Bourne (1972) in the Instron machine already mentioned. A 5 mm diam flat-faced metal punch was driven through the arepa at a speed of 200 mm/min. A total of 15 punches was made in each of three arepas for each test. The maximum force readings were grouped into 50g force ranges and a frequency histogram was constructed. The most frequent range was taken as the hardness reading.

Sensory evaluation

The arepas were presented to a trained sensory texture profile panel who had previously developed a comparative texture profile ballot sheet for arepas (Bourne et al., 1975).

Arepas made by both the traditional method and from precooked flours were presented to the panel immediately after roasting.

Storage tests

Storage tests were performed on arepas which were wrapped in chemical pulp Kraft paper and kept at 20°C and 60% R.H. for a maximum of 20 hr.

RESULTS & DISCUSSION

THE PARTICLE SIZE distribution of the flours tested was the following: 30% + 60 U.S.; 22% −60 + 80; 21% −80 + 150; 27% −150. Figure 1 shows the amylograph viscosity behavior of precooked corn flours produced during this work under different extrusion cooking conditions (curves 2 to 4). These are compared with one obtained by Smith (1974) during a regular extrusion cooking process for corn grits run under low moisture-high temperature conditions (curve 5) and with a precooked corn flour produced by the conventional column-roller process (curve 1). Curves 2, 3 and 4 show that it is possible to adjust extrusion processing conditions to produce flours with similar amylographic behavior to that from the conventional column-roller process.

Table 1 shows the relationship between extruder operating conditions, a section of the amylograph, and dough characteristics. Interesting relationships are shown between

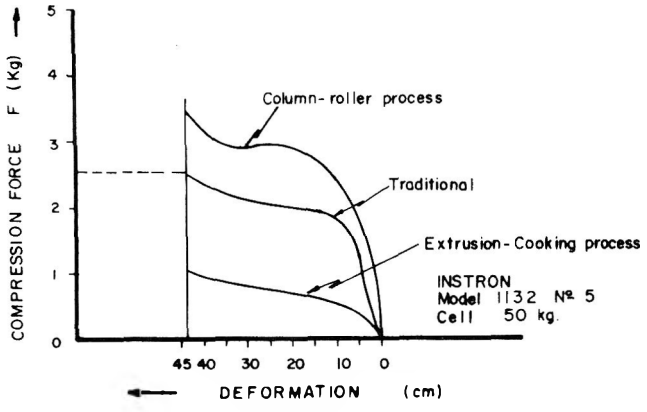

Fig. 2—Compression test-behavior of traditional dough and of doughs produced from pre-cooked corn flours from two different processes.

Table 2—Properties of pre-cooked corn flours for arepa making extrusion cooking process

Run No.	Dextrose index	Water absorption (g H_2O/g flour)	Instron hardness reading during storage (g)[a]	
			1 hr	20 hr
1	41	1.1	–	–
2	47	1.4	–	–
3	51	1.1	–	–
4	58	1.4	1.000	2.500
5	58	1.2	1.000	3.000
6	58	1.4	1.000	2.000
7	54	1.4	1.000	2.500
8	54	1.4	1.000	2.000
9	52	1.4	1.500	3.000
10	50	1.4	1.000	2.000
11	48	1.5	1.000	3.000
12	53	1.5	1.000	3.000
13	48	1.6	1.000	3.000
14	52	1.8	1.000	2.000
15	49	1.4	1.500	2.600
16	54	1.7	1.500	2.000

[a] Instron 50g cell – 20°C, 60% RH

the shape of this section of the amylograph and the properties of the dough obtained with the flours. Good dough properties were observed with flours having a constant or an increasing viscosity during this period, with values equal or greater than 500 BU. The best doughs were obtained when extrusion was carried out in the presence of high moisture (37% at the inlet). Under this condition the temperature of the heating heads was not critical for dough quality (runs 12 to 16, Table 1). At intermediate moisture levels (26 and 30%) adequate amylographs and good doughs were obtained only with temperatures between 121 and 135°C. (runs 4 to 7, Table 1). Unstable pasting behavior (a sagging viscosity curve) is related to a very sticky dough that is unable to produce arepas, extreme cases were runs 2 and 3, Table 1.

The foregoing implies that high moisture (37.%) intermediate temperatures (90–121°C) are adequate extruding conditions for producing corn flours for arepa making. At intermediate moisture (26–30%) it will be necessary to work with high temperature in the last two heating heads (121–135°C).

Table 2 shows the dextrose index, the water absorption capacity of the flours, and the hardness reading for roasted arepas fresh and under storage. (These latter values read from graphs like the one shown in Fig. 3).

Water absorption of the flours shows a tendency to increase with increasing moisture levels during processing. Dextrose indexes ranged from 41 to 58 but clear relationships were not found between this value, the amylographic behavior and the dough characteristics. Flours with equal susceptibility to α- and β-amylases (dextrose index) gave different amylographs and very different dough properties. This is the case of run 2 and 13 and 8 and 16. This implies that the final properties of the flours depend not only on the degree of starch modification (measured by enzymatic methods) but on the type of modification suffered by the starch under different extruding conditions of moisture and temperature.

No significant differences were found in the Instron hardness readings for arepas (fresh and stored) made with 14 extrusion precooked flours (Table 2).

The compression behavior of three doughs is compared in Figure 2. Run No. 15 from the extrusion cooking process gives the lowest of the three curves which implies that this flour produces a dough softer than the ones obtained by either the traditional corn cooking method or the con-

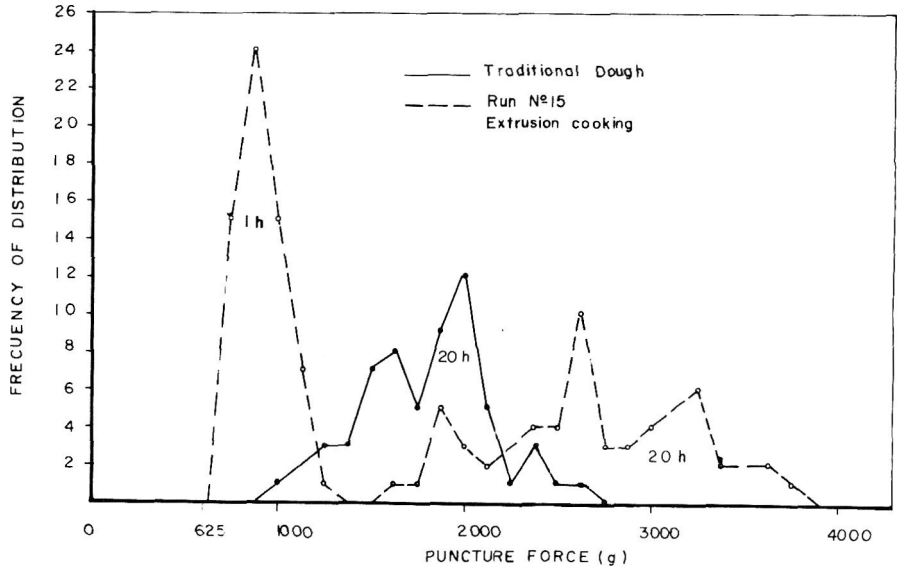

Fig. 3—Corn arepa-puncture test after 20 hr of storage. Arepas produced with the traditional dough and with flours from two different precooking processes.

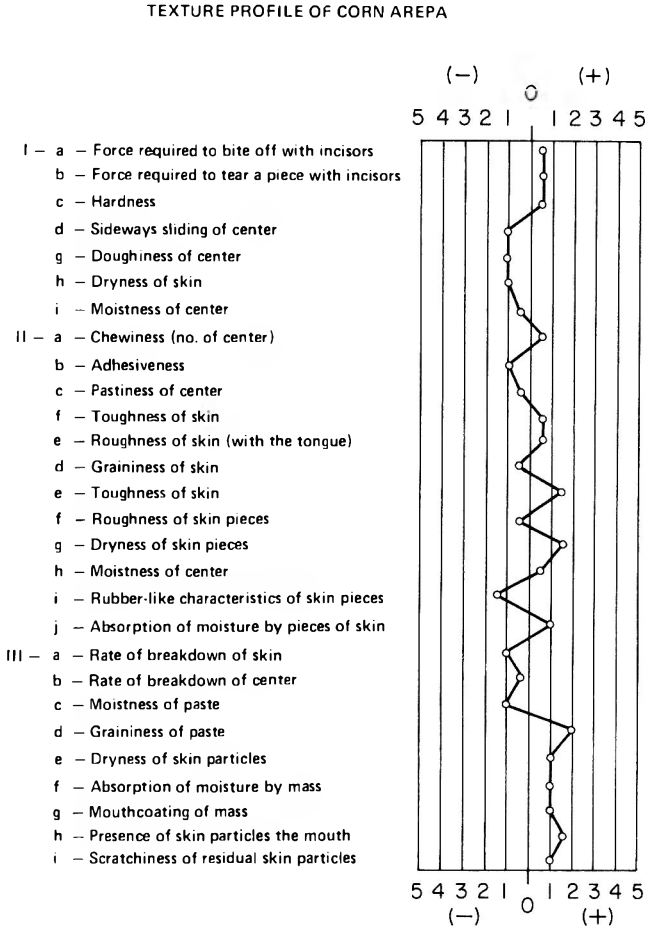

Fig. 4—Texture profile of corn arepa Run No. 15 vs Arepa made from traditional corn dough.

ventional commercially established column-roller process.

Figure 3 shows the hardness of stored arepas (20 hours) prepared with the doughs of Figure 2. The least hardness reading after 20 hr storage is shown by the arepas made from the traditional dough. The closest behavior to this one is shown by the arepas produced from the extruded flour Run No. 15. The column-roller process produces the greatest hardness reading.

Figure 4 shows the texture profile of arepas obtained with the flour from run 15. In this type of test only deviations from the central line which corresponds to the standard greater than ± 1 represent a significant difference: hence it could be said that the texture of the arepas is very acceptable. Other commercial flours generally present a greater number of deviations and of greater magnitude (± 2–4) (Sandoval, 1977).

CONCLUSIONS

OUT OF 16 extrusion cooking trials, 9 pre-cooked flours were found to be suitable for preparing arepa dough. The behavior of doughs and of fresh and stored arepas of these flours was found equal or better than flours from the conventional column-roller process as measured by instrumental and sensory techniques. Most appropriate extrusion conditions were found to be high moisture (37%) and intermediate temperature (93–121°C). It can be concluded that extrusion cooking is a suitable process for producing precooked corn flour for arepa making if conditions are properly adjusted. The process might be used as a good alternative to column-roller pre-cooking.

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PEANUT-FORTIFIED FOOD BLENDS

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ABSTRACT

Defatted and partially defatted peanut flours were tested in fortified food blends utilized in food aid programs. Peanut flours were compared with soy flours as components of blends containing partially or fully gelatinized corn meal, nonfat dry milk, soybean oil, vitamins and minerals. Changes in flavor, peroxides, free fatty acids, available and added L-lysine HCl after storage for 2, 6 and 12 months were evaluated and found satisfactory. Protein efficiency ratios (PER) were about 0.7 units lower in blends containing peanut rather than soy protein. Increased PER values were obtained by the addition of 0.3–0.5% L-lysine HCl or by combining peanut with soy proteins. No further improvement in PER values was effected in peanut formulations by the addition of DL-methionine in combination with L-lyine HCl.

INTRODUCTION

IN THE Foods for Peace Program, formulated food products meeting established guidelines are provided by the United States to needy people abroad to alleviate malnutrition (Senti, 1972). The formulated blends are composed of various cereals fortified with concentrated protein sources such as defatted and full-fat soy flour, nonfat dry milk, dry whey, dry buttermilk, processed corn germ and wheat concentrate. Small amounts of vitamins, minerals and oils are added to provide complementary nutrients and improve caloric density.

Peanut protein is also recognized as a valuable concentrated protein source (Milner, 1962) and is being studied for possible inclusion in the donation program. Recent work was carried out with blends of degermed, uncooked corn meal fortified with 15% defatted peanut flour (Bookwalter et al., 1978). It was shown that foods with improved nutritional value, satisfactory quality and adequate storage stability can be achieved with degermed corn meal fortified with peanut flour. This type of product fortified with soy flour is utilized in feeding programs for older children and adults where there is a need for additional protein in the diet (USDS, 1978).

Another major thrust of the Food for Peace Program is directed toward supplying special food supplements for weanling infants, young children and adult health cases. Food blends such as corn-soy-milk (CSM), instant CSM, instant CSM (sweetened) and corn-soy (CS) blend (Bookwalter, 1977) are utilized to provide supplementary calories, protein, vitamins and minerals.

The objectives of the experiments reported here were to evaluate edible peanut flours as a concentrated protein source in these types of special food supplements. Side-by-side comparisons were made with peanut and soy flours to evaluate their effect on nutritional characteristics and storage stability in food systems containing processed corn meal, nonfat dry milk, sweetener, soybean oil and enrichment materials.

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MATERIALS & METHODS

Formulations and ingredients

The experimental food blends utilized in this study are shown in Table 1. The blends referred to as "regular" contain partially gelatinized corn meal, whereas the "instant" types contain completely gelatinized corn meal. These blends were prepared according to USDA specifications. Defatted or partially defatted peanut flour was substituted for all or part of the soy flour in the comparative formulations. The standardized formulas in Table 1 are: regular CS (USDA, 1973), regular CSM (USDA, 1975a), instant CSM, sweetened (USDA, 1975b) and instant CSM (USDA, 1975c). Substitution of peanut flour in these formulations is noted by the letter "P" (defatted) or "Px" (partially defatted). All of the blends contain soybean oil and all but the regular CS or corn-peanut (CP) contain nonfat dry milk. All formulations contain antioxidants, vitamins and minerals; some formulations contain added L-lysine HCl and DL-methionine as shown in Table 1.

Commercial and laboratory-prepared ingredients were utilized in the preparation of the experimental blends. They are listed with their analyses in Table 2. The partially gelatinized corn meal was prepared to meet specifications of Announcement CSM-7 (USDA, 1975a). The instant (completely gelatinized) corn meal and toasted defatted soy flour were prepared to meet specifications of Announcement CSM-6 (USDA, 1975b). The hexane-defatted peanut flour was prepared from selected peanuts according to Ayres et al. (1974). The partially defatted peanut flour was prepared from the peanut meal residue, which was partially defatted by a screw press. Other ingredients used in this study complied with commercial standards of identity. Table 2 shows the analytical profiles of the various food blend combinations of Table 1.

Analytical tests

Peroxide values, free fatty acids of the pentane-hexane extracted fat and other fat assays were determined by standard procedures (AOCS, 1970). Available lysine was measured by a method based on reaction with dinitrofluorobenzene (Rao et al., 1963). Added free L-lysine HCl was measured after storage according to the method of Finley et al. (1972). Moisture analyses were made on a Brabender moisture tester (120°C, 60 min). Other chemical analyses were determined by standard procedures (AACC, 1962). Protein efficiency ratio (PER) was determined by the method outlined in AOAC (1975), with the diets standardized to 10% protein and compared to an Animal Nutrition Research Council casein reference standard adjusted to 2.5 PER. Digestibility of the diet was determined by: (feed intake-fecal weight)/feed intake × 100. Nitrogen digestibility was determined by: [(nitrogen intake − fecal nitrogen)/nitrogen intake] × 100.

Storage stability

Stability tests were made on the food blends (Table 1) containing peanut or soy flours, with or without the inclusion of 0.28–0.53% L-lysine HCl. The mixtures were packaged in glass containers with foil-lined screw closures. Such containers maintained moisture at constant levels in samples stored at 37 and 49°C and at approximately 25°C room temperature. Controls were held at −18°C. Withdrawals from storage were made at 49°C after 0, 1 and 2 months; at 37°C after 3 and 6 months; and at 25°C after 6 and 12 months. The samples were evaluated for changes in peroxide values, free fatty acids, available lysine, added L-lysine HCl and flavor.

The samples were made into gruels (10% solids) for flavor tests. The "regular" formulations containing partially gelatinized corn meal were stirred into boiling water and cooked for 2 min. The "instant" products containing fully gelatinized corn meal were stirred into boiling water. Flavor changes over the period of 12 months of storage were evaluated by a trained, experienced 14-member taste panel. The product combinations, stored at −18°C and at an elevated temperature, were randomized and compared for

Table 1—Formulations[a] of peanut- and soy-fortified food blends

Ingredients	Regular[b] CS[c]	Regular CP	Regular CSM[d]	Regular CPM	Regular C $\frac{S}{2}\frac{P}{2}$ M	Regular C $\frac{S}{4}\frac{3P}{4}$ M	Instant[b] CSM[e] (sweetened)	Instant CPM (sweetened)	Instant CSM[f]	Instant CPM	Instant CP_xM
Corn meals											
Partially gelatinized	71.0	71.0	63.8	63.8	63.8	63.8	–	–	–	–	–
Completely gelatinized	–	–	–	–	–	–	52.35	52.35	63.8	63.8	63.8
Peanut flours											
Defatted (P)	–	22.0	–	24.2	12.1	18.2	–	27.5	–	23.7	–
Partially defatted (P_x)	–	–	–	–	–	–	–	–	–	–	25.8
Soy flour											
Defatted, toasted	22.0	–	24.2	–	12.1	6.0	27.5	–	23.7	–	–
Nonfat dry milk	–	–	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Soybean oil	5.0	5.0	5.0	5.0	5.0	5.0	5.5	5.5	5.5	5.5	3.4
Sucrose	–	–	–	–	–	–	7.5	7.5	–	–	–
Flavoring	–	–	–	–	–	–	0.15	0.15	–	–	–

[a] L-lysine HCl (0.28–1.59%) and DL-methionine (0.1–0.2%) were added to some formulations. The blends contain combinations of 2.5 mg butylated hydroxyanisole (BHA) and 2.5 mg butylated hydroxy toluene (BHT) or 2.5–5 mg tertiary butyl hydroquinone (TBH) per 100g of final formulated product. All blends contain 1.9% mineral premix and 0.1% vitamin premix (USDA, 1975a).

[b] Regular C = partially gelatinized corn meal, Instant C = fully gelatinized corn meal, P = defatted peanut flour, P_x = partially defatted peanut flour, S = defatted, toasted soy flour, M = nonfat dry milk.

[c] USDA, 1973

[d] USDA, 1975a

[e] USDA, 1975b

[f] USDA, 1975c

flavor at each time interval. Samples were rated for flavor according to the 10-point scoresheet described by Bookwalter et al. (1971a). Scores of 6 and above were considered acceptable. Samples with objectionable flavors were assigned scores of 5 and below. Statistical evaluations were made on the experimental data after completion of each taste panel series. Analysis of variance, regression analysis (Snedecor and Cochran, 1968) and Duncan's multiple range test (Duncan, 1955) were used to assess results.

RESULTS & DISCUSSION

Nutritional aspects

Higher protein contents were achieved with peanut flour substituted for soy flour in standard USDA formulations (Tables 1, 2). The protein quality of corn meal is substantially improved by fortification with peanut flour (Bookwalter et al., 1978). However, the protein quality was poorer with peanut than with the respective soy blends as evidenced by PER determinations. Table 3 shows the PER results and digestibility of regular corn-soy (CS) and regular corn-peanut (CP) formulations as well as those of regular corn-soy-milk (CSM) and regular corn-peanut milk (CPM). In formulations without nonfat dry milk, the addition of L-lysine HCl was associated with higher nitrogen digestibility values. Other digestibility data were similar. PER values for CP and CPM mixtures containing peanut flour are significantly lower than the respective CS and CSM mixtures containing soy flour. The nutritive quality of peanut protein is limited primarily by its content of lysine and methionine (Milner, 1962). Soy protein is also deficient in methionine (Bookwalter et al., 1975). Total sulfur amino acid levels of peanut and soy proteins are approximately 2.0% of protein. Soy protein is high in lysine, the first limiting amino acid in corn meal (Kato and Muramatsu, 1971). Available lysine values for the corn, peanut and soy products were 2.3, 3.2 and 6.1% of protein, respectively. Available lysine of the CS and CP formulations were 4.2 and 2.7% of protein, respectively. Available lysine of the CSM and CPM formulations were 4.8 and 3.4% of protein, respectively. The lower available lysine values of the CP and CPM formulations could contribute to their lower PER values as shown in Table 3. We found threonine levels of 2.5% and 4.2% for peanut and soy proteins, respectively, which could also contribute to the lower PER values for the CP and CPM formulations.

Table 2—Analyses[a] of ingredients and their formulations

Base materials and blends	Moisture (%)	Protein[b] (%)	Fat (%)	Ash (%)	Crude fiber (%)
Base materials					
Corn meal, partially gelatinized	9.1	8.5	0.5	0.4	0.5
Corn meal, completely gelatinized	9.0	8.5	0.5	0.5	0.6
Peanut flour, defatted	8.6	66.8	0.6	5.3	3.8
Peanut flour, partially defatted	6.2	55.6	9.3	4.4	4.9
Soy flour, defatted, toasted	7.2	52.5	1.4	7.0	3.3
Nonfat dry milk	3.0	36.0	0.1	8.4	0
Blends					
Regular CP[c]	6.3	20.5	8.6	4.2	2.1
Regular CS	6.3	18.1	8.7	4.5	2.0
Regular CPM	8.1	25.3	8.0	4.3	2.3
Regular CSM	9.0	22.9	8.0	4.6	2.1
Regular C $\frac{S}{2}\frac{P}{2}$ M	8.4	23.9	7.9	4.4	2.4
Regular C $\frac{S}{4}\frac{3P}{4}$ M	8.2	24.9	8.0	4.3	2.4
Instant CPM (sweetened)	7.5	24.4	6.5	3.7	1.4
Instant CSM (sweetened)	6.6	21.2	6.6	4.1	1.3
Instant CPM	7.6	22.6	7.8	3.5	1.5
Instant CP_xM	7.2	21.8	7.6	3.6	1.4
Instant CSM	7.5	19.9	8.0	3.9	1.3

[a] Dry matter basis

[b] Nitrogen X 6.25

[c] See footnotes, Table 1

The effect of adding L-lysine HCl to CP and CPM formulations is shown in Table 3. Addition of 0.53% and 0.55% L-lysine HCl to the CP and CPM formulations, respectively, increased their available lysine levels to those of the CS and CSM formulations. A significantly higher PER value was achieved by the addition of 0.53% L-lysine HCl to the CP formulation. However, the PER values showed no further increase when as much as 1.59% L-lysine HCl was added to this formulation. A significantly higher PER value was attained by the addition of 0.28% or 0.55% L-lysine HCl to

Table 3—PER[a] values and digestibility of food blends containing peanut and/or soy flours, with and without L-lysine HCl

Formulations[b]	Added L-lysine HCl (% of blend)	PER	Digestibility (%)[c] Diet	Nitrogen
Regular CS	–	2.31 A	91	81
Regular CP	–	1.56 C	91	84
Regular CP	0.53	1.81 B	92	88
Regular CP	1.06	1.87 B	92	87
Regular CP	1.59	1.79 B	92	89
Regular CSM	–	2.43 K	93	85
Regular CPM	–	1.74 N	93	87
Regular CPM	0.28	2.06 L	93	88
Regular CPM	0.55	1.99 LM	93	85
Regular CPM	1.10	1.83 MN	93	87
Regular CSM	–	2.26 W	92	85
Regular CPM	–	1.69 Z	92	84
Regular C $\frac{S}{2}\frac{P}{2}$ M	–	2.08 WX	91	84
Regular C $\frac{S}{4}\frac{3P}{4}$ M	–	1.88 Y	92	84

[a] PER (protein efficiency ratio) = gram weight gain per gram protein intake. Animal Nutrition Research Council casein = 2.50. Two PER values with no common letter, within A-C, K-N and W-Z, differ significantly (5% level).
[b] See Table 1 footnotes.
[c] Digestibility was determined from the 7th through 14th day of PER tests.

the CPM formulation. However, PER decreased with the addition of 1.10% L-lysine HCl to this formulation. At this higher addition level, the PER was not significantly different from the CPM with no added L-lysine HCl. Although significant increases in PER values were achieved by the addition of L-lysine HCl to the CP and CPM formulations, the values were significantly below those of the CS and CSM formulations, respectively. PER tests were made with combinations of CP containing 0.53% L-lysine HCl with and without 0.15% and 0.30% DL-methionine. Although all PER values were significantly higher than CP with no amino acid enrichment, there were no significant changes in PER values that were associated with added DL-methionine.

Table 4—PER values and digestibility of instantized food blends containing peanut and soy flours, with and without L-lysine HCl

Formulations[a]	Added L-lysine HCl (% of blend)	PER[b]	Digestibility (%)[b] Diet	Nitrogen
Instant CSM (sweetened)	–	2.47 A	93	86
Instant CPM (sweetened)	–	1.63 C	93	86
Instant CPM (sweetened)	0.50	1.96 B	94	89
Instant CPM (sweetened)	1.00	1.82 B	94	90
Instant CSM	–	2.34 W	93	85
Instant CPM	–	1.63 Y	93	87
Instant CP_xM	–	1.75 Y	93	87
Instant CP_xM	0.28	2.07 X	93	89
Instant CP_xM	0.55	2.14 X	93	89
Instant CP_xM	1.10	2.02 X	93	84

[a] See Table 1 footnotes.
[b] See Table 3 footnotes.

PER tests were carried out (Table 3) on CSM and CPM formulations and blends with combinations of peanut and soy flours (Table 1). When soy was substituted for one-half of the peanut flour, the PER value was not significantly different from the CSM formulation. However, when soy was substituted for one-fourth of the peanut flour, the PER value was significantly higher than the CPM blend but significantly lower than the CSM blend.

Table 4 shows the PER results and disgestibility of instant corn-soy-milk, sweetened [I-CSM(S)] and instant corn-peanut-milk, sweetened [I-CPM(S)] as well as instant corn-soy-milk (I-CSM), instant corn-peanut-milk (I-CPM) and instant corn-partially defatted peanut-milk (I-CP_xM). Formulations for these mixtures are shown in Table 1. No marked differences were noted in digestibility results. PER values for I-CPM(S) and I-CPM or I-CP_xM mixtures containing peanut flour were significantly lower than the respective I-CSM(S) and I-CSM mixtures containing soy flour. Available lysine values for the I-CSM(S) and I-CPM(S) formulations were 4.4 and 2.9% of protein, respectively. The avail-

Table 5—mean flavor scores[a] of stored food blends containing peanut and/or soy flours, with different antioxidants,[b] with and without L-lysine HCl

Formulations[c]	Added L-lysine (% of blend)	Temperature (months) −18°C	49°C		37°C		25°C	
		0	1	2	3	6	6	12
Regular CP	–	7.6	7.5	6.8	7.2	6.6	7.4	7.2
Regular CS	–	7.5	6.8	6.9	7.3	6.6	7.7	7.2
Regular CP	0.53	7.3	7.8	6.9	7.6	6.5B	7.9	7.1B
Regular CS	0.53	7.5	7.4	7.2	7.4	5.8A	7.7	6.3A
Regular CPM	–	7.5	7.1	7.2	7.0	7.0	7.6	7.4
Regular CPM (TBH)	–	7.5	7.4	7.4	7.6	7.3	7.3	7.4
Regular CPM $(\frac{TBH}{2})$	–	7.5	7.1	6.8	7.6	7.3	7.5	7.4
Regular CSM	–	7.5	6.8AB	7.2	6.9	7.2	7.2	6.9
Regular C $\frac{S}{2}\frac{P}{2}$ M	–	7.5	7.3B	7.1	6.8	7.1	7.7	7.0
Regular C $\frac{S}{2}\frac{P}{2}$ M $(\frac{TBH}{2})$	–	7.6	6.5A	7.3	7.3	7.4	7.4	6.9
Regular CPM	0.28	7.5		7.1		7.2		7.4
Regular CPM (TBH)	0.28	7.5		7.0		7.2		7.4
Regular CP_2M	0.28	7.4		7.4		7.0		7.4
Regular CSM	0.28	7.3		6.7		7.1		7.6
Regular C $\frac{S}{2}\frac{P}{2}$ M	0.28	7.9		7.0		7.0		7.7

[a] Each mean score based on scores from 14 panelists. Coded means (A,B) with no letter in common differ significantly (5% level).
[b] All formulations contain either combinations of BHA and BHT or TBH (footnote "a", Table 1).
[c] See Table 1, footnotes "a" and "b" (P and P_2 are defatted peanut flours produced at different times.

able lysine values for the I-CSM, I-CPM and I-CP_xM formulations were 4.4, 3.3 and 3.3% of protein, respectively. The lower available lysine values of the I-CPM(S), I-CPM and I-CP_xM formulations could contribute to their lower PER values.

A significantly higher PER value (Table 4) was achieved by the addition of 0.50% L-lysine HCl to the I-CPM(S) formulation. However, the PER values showed no further increase when as much as 1.00% L-lysine HCl was added to this formulation. The PER values of the I-CPM(S) formulations with added L-lysine HCl were significantly lower than those of the I-CSM(S) blend.

PER values of the I-CPM and I-CP_xM mixtures were not significantly different (Table 4). This indicates that defatted or partially defatted peanut flours are nutritionally similar. PER values of the I-CPM and I-CP_xM formulations were significantly lower than those of the I-CSM containing soy flour. The addition of 0.28% L-lysine HCl to the I-CP_xM formulation resulted in a significantly higher PER value. However, the PER values showed no further increase when as much as 1.10% L-lysine HCl was added to this formulation. The PER values of the I-CP_xM formulations with added L-lysine HCl were significantly lower than those of I-CSM.

Storage stability

Mean flavor scores of stored blends of various combinations of regular (partially gelatinized) corn meal, peanut flour, soy flour, nonfat dry milk and two different antioxidant systems, with and without added L-lysine HCl, are shown in Table 5. The mean flavor scores are shown for each time-temperature condition and were compared with a control sample stored at −18°C. Initial flavor scores of the formulations stored at −18°C were in the highly satisfactory range (7.3 or above). There were no significant differences in the initial flavor scores associated with the presence of peanut or soy in the blends. All scores were in the satisfactory range (5.8 or above) during storage at these conditions. In the regular CP and regular CS without added L-lysine HCl, no significant changes were noted in flavor scores during storage at these conditions. In the CP and CS formulations with added L-lysine HCl, no flavor changes occurred during storage at 49°C. However, after storage at 37°C for 6 months and 25°C for 12 months, the CS samples with added L-lysine HCl received significantly lower flavor scores. This is of no practical importance since the CS formulation is not lysine deficient.

No significant flavor differences were found between stored samples of regular CPM formulated with different antioxidant systems (Table 5). CPM received similar flavor scores when formulated with a combination of butylated

Table 6—Mean flavor scores[a] of stored instantized food blends containing peanut or soy flours, with and without L-lysine HCl

Formulations[b]	Added L-lysine (% of blend)	Temperature (months) −18°C	49°C		37°C		25°C	
		0	1	2	3	6	6	12
Instant CPM (sweetened)	0.51	7.6	6.6	6.3	6.5	6.6	6.6	6.6
Instant CSM (sweetened)	0.51	7.4	7.0	6.4	6.6	6.3	6.8	6.9
Instant CPM (sweetened)	–	7.4	7.0	6.8	6.4	6.8	6.8	6.5
Instant CSM (sweetened)	–	7.7	6.4	6.8	6.6	6.6	6.8	6.8
Instant CPM	–	7.5	8.0	6.3	7.0	7.7	7.6B	7.1
Instant CP_xM	–	7.4	7.8	6.9	7.7	7.2	7.6B	7.1
Instant CSM	–	7.6	7.4	7.2	7.7	7.0	6.7A	7.1

[a] See Table 5, footnote "a".
[b] See Table 1, footnote "b" (P and P_x are defatted and partially defatted peanut flours, respectively).

Table 7—Analyses of stored food blends containing peanut and/or soy flours, with different antioxidants,[a] and 0.28% added L-lysine HCl

Formulations[b]	Storage Months	Storage Temp (°C)	Peroxide value (meq/kg fat)	Free fatty acids (% oleic)	Total available lysine[c] (% of protein)	Recovered free L-lysine HCl (% of blend)
Regular CPM		−18	1.6	0.9	4.6	0.29
Regular CPM (TBH)		−18	3.2	0.8	4.7	0.30
Regular CP_2M		−18	2.0	0.8	4.5	0.28
Regular CSM		−18	2.0	0.7	5.9	0.28
Regular C$\frac{S}{2}\frac{P}{2}$M		−18	2.8	1.0	5.3	–
Regular CPM	2	49	4.3	5.4	4.2	0.29
Regular CPM (TBH)	2	49	4.7	5.4	4.3	0.28
Regular CP_2M	2	49	6.7	5.1	4.4	0.30
Regular CSM	2	49	2.4	4.7	5.1	0.27
Regular C$\frac{S}{2}\frac{P}{2}$M	2	49	3.5	5.5	4.7	–
Regular CPM	6	37	17.0	7.4	4.4	0.32
Regular CPM (TBH)	6	37	16.0	7.7	4.4	0.33
Regular CP_2M	6	37	20.0	6.4	4.4	0.33
Regular CSM	6	37	14.0	6.4	5.5	0.31
Regular C$\frac{S}{2}\frac{P}{2}$M	6	37	15.6	7.3	5.0	–
Regular CPM	12	25	5.1	6.4	4.5	0.30
Regular CPM (TBH)	12	25	5.5	6.7	4.5	0.29
Regular CP_2M	12	25	5.1	6.2	4.5	0.28
Regular CSM	12	25	3.1	5.1	6.0	0.32
Regular C$\frac{S}{2}\frac{P}{2}$M	12	25	2.3	6.2	5.3	–

[a] All formulations contain either combinations of BHA and BHT or TBH (footnote "a" Table 1).
[b] See Table 1, footnotes "a" and "b," (P and P_2 are defatted peanut flours produced at different times).
[c] Total available lysine (available plus added L-lysine HCl)

hydroxy anisole (BHA) and butylated hydroxy toluene (BHT), the same level (5 mg/100g mix) of tertiary butyl hydroquinone (TBH) or one-half of this level of TBH. With one exception, there were no significant flavor differences between stored samples of regular CSM or the blends of corn and milk with one-half the soy replaced with peanut flour and containing either BHA or BHT or one-half the level of TBH as shown in Table 5. No significant flavor differences were noted in stored, L-lysine HCl fortified combinations of CPM formulated with two different peanut flours and containing either BHA and BHT or TBH, or CSM with and without one-half the soy flour replaced with peanut flour.

Mean flavor scores of stored blends of various combinations of instant (completely gelatinized) corn meal, peanut flour, partially defatted peanut flour, soy flour and sugar, with and without added L-lysine HCl, are shown in Table 6. Mean flavor scores are shown for each time-temperature condition and were compared with a control sample stored at −18°C. Initial flavor scores of the blends stored at −18°C were in the highly satisfactory range (7.4 or above). No significant flavor changes occurred during storage that could be associated with product differences shown in Table 6. There were no flavor differences in stored samples of I-CPM(S) or I-CSM(S), with or without added L-lysine HCl. No differences in flavor were noted in stored samples of I-CSM and formulations with the soy flour replaced with standard defatted peanut flour or partially defatted peanut flour.

All blend combinations shown in Tables 5 and 6 were tested for changes in peroxide values, free fatty acids, available lysine, and retention of added L-lysine HCl during storage. Two different antioxidant systems were used, because peroxide values were slightly higher in initial tests of blends containing peanut flour than those of control blends containing soy flour. BHA and BHT, as specified (USDA, 1973; 1975a,b,c), were included along with TBH. Previous studies (Bookwalter et al., 1971b) had shown TBH to be more effective in resisting peroxide development than the BHA and BHT combination.

Analytical data indicated adequate stability for the blend combinations shown in Tables 5 and 6. The added L-lysine HCl was retained by all combinations during these storage conditions. The blends containing peanut flour were generally slightly higher in peroxide values than control blends containing soy flours. However, the peroxide values were well below the levels that could be associated with the development of "off flavors" as demonstrated by the mean flavor scores shown in Tables 5 and 6. Analytical results of some of the peanut blend combinations, along with their soy blend controls, are demonstrated in Tables 7, 8 and 9.

Table 7 shows the comparison of regular CPM containing two different defatted peanut flour samples and the antioxidant combinations of BHA and BHT or TBH. In addition, a control sample of regular CSM is included along with a sample of regular CPM with one-half the peanut flour replaced with soy flour. All combinations contained 0.28% added L-lysine HCl, which was found to be stable under these storage conditions. Satisfactory stability is indicated by these levels of peroxide values, free fatty acids and total available lysine. All peroxide values were low except after storage at 37°C for 6 months. There were no differences in peroxide values of CPM containing the combination of BHA and BHT or TBH. Peroxide values were lowest in the regular CSM samples, followed by the blend of regular corn-1/2 peanut flour-1/2 soy flour-nonfat dry milk. Free fatty acids were relatively low when these results are compared to those of other food blend combinations (Bookwalter et al., 1971a).

Analytical results of instant CPM containing defatted peanut flour (P) and a partially defatted peanut flour (P_x), as well as those of an instant CSM control blend, are shown in Table 8. Peroxide values of the I-CP_xM were much lower

Table 9—Analyses of stored food blends containing peanut and/or soy flour, with different antioxidants[a]

Formulations[b]	Storage: Months	Storage: Temp (°C)	Peroxide value (meq/kg fat)	Free fatty acids (% oleic)	Available lysine (% of protein)
Regular CPM		−18	0.4	1.1	3.4
Regular CPM (TBH)		−18	2.2	1.1	3.5
Regular CPM(TBH/2)		−18	1.0	1.0	3.7
Regular CSM		−18	0.6	1.1	4.8
Regular C S/2 P/2 M		−18	0.6	1.1	4.1
Regular C S/2 P/2 M(TBH/2)		−18	0.8	0.9	3.9
Regular CPM	2	49	2.8	1.2	3.0
Regular CPM (TBH)	2	49	2.8	1.3	3.1
Regular CPM(TBH/2)	2	49	2.4	1.3	3.0
Regular CSM	2	49	2.4	1.4	4.0
Regular C S/2 P/2 M	2	49	1.8	1.3	3.5
Regular C S/2 P/2 M(TBH/2)	2	49	2.2	1.3	3.5
Regular CPM	6	37	4.5	1.4	3.0
Regular CPM (TBH)	6	37	2.9	1.5	3.1
Regular CPM(TBH/2)	6	37	3.9	1.4	3.0
Regular CSM	6	37	3.9	1.6	4.2
Regular C S/2 P/2 M	6	37	7.8	1.7	3.6
Regular C S/2 P/2 M(TBH/2)	6	37	3.3	1.4	3.9
Regular CPM	12	25	8.2	1.3	3.0
Regular CPM (TBH)	12	25	7.4	1.3	3.0
Regular CPM(TBH/2)	12	25	7.4	1.3	2.7
Regular CSM	12	25	12.0	1.6	4.2
Regular C S/2 P/2 M	12	25	5.5	1.4	3.2
Regular C S/2 P/2 M(TBH/2)	12	25	5.8	1.4	3.8

[a] All formulations contain either combinations of BHA and BHT or TBH (see Taole 1, footnote "a").
[b] See Table 1, footnote "b".

Table 8—Analyses of instantized food blends containing peanut or soy flours

Formulations[a]	Storage: Months	Storage: Temp (°C)	Peroxide value (meq/kg fat)	Free fatty acids (% oleic)	Available lysine (% of protein)
Instant CPM		−18	7.8	0.8	3.3
Instant CP_xM		−18	8.8	0.9	3.3
Instant CSM		−18	5.9	0.8	4.4
Instant CPM	2	49	10.8	1.1	2.6
Instant CP_xM	2	49	3.3	1.2	2.6
Instant CSM	2	49	6.9	1.2	3.7
Instant CPM	6	37	37.0	1.0	2.5
Instant CP_xM	6	37	5.9	0.8	2.6
Instant CSM	6	37	4.3	0.9	3.7
Instant CPM	12	25	9.9	0.9	2.9
Instant CP_xM	12	25	3.0	0.6	3.1
Instant CSM	12	25	2.0	1.2	3.9

[a] See Table 1, footnotes "a" and "b" (P and Px are defatted and partially defatted peanut flours, respectively).

than those of the I-CPM during storage at these conditions. These results indicate better oxidative stability for samples containing P_x than P. Available lysine values for both peanut formulations were similar, although they were substantially lower than those of the I-CSM control blend. However, values for both available lysine and free fatty acids indicate adequate stability.

Table 9 shows stability results for regular CPM formulated with BHA and BHT, TBH and one-half level of TBH, as well as regular CSM containing BHA and BHT. Two other blends of regular CPM with one-half of the peanut flour replaced with soy flour are also included, one with BHA and BHT and the other with one-half level of TBH (Table 1). As shown by peroxide values, there were no substantial differences in the two antioxidant systems or with the lower level of TBH. Adequate stability is indicated by the absence of substantial changes in peroxide values, free fatty acids and available lysine during storage at these conditions.

CONCLUSIONS

FOOD BLENDS with improved nutritional value, satisfactory organoleptic quality and storage stability can be prepared with blends of either partially or fully gelatinized corn meal fortified with defatted or partially defatted peanut flour, nonfat dry milk, fat and enrichment materials.

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PRODUCTION OF TEXTURED VEGETABLE PROTEIN FROM COTTONSEED FLOURS

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ABSTRACT

Texturized cottonseed products with good nutritional and functional properties were obtained by an extrusion cooking process, using cottonseed flours as raw materials. Free gossypol levels in the extruded products were low (0.009–0.025%). Available lysine (3.6 g/100g protein) and biological values (55–57 vs 100 for egg) were measured. Texture profile and ultrastructure were found similar to soybean extrudates. Best results were obtained with water, pH 7.0–8.5, using flours with lower fiber content (5.3%). The texturized product represents an interesting alternative for tropical countries where cottonseed flours abound.

INTRODUCTION

COTTONSEED CAKE is a good protein source, available in large quantities in tropical countries. Its use as human food and even as a feed for poultry or swine is usually restricted due to gossypol, a pigment present in the seeds. Great research efforts have been devoted to devise practical methods for reducing the cake's gossypol content. In 1974, IIT started a study on the possible use of extrusion cooking for improving both the textural and the nutritional characteristics of cottonseed flours to be used as human food.

It is well known that extrusion of defatted soybean flour produces important modifications on the physical properties of the material. Properties such as volume, elasticity, chewiness and hardness, similar to those found in meat, are generated. It is also known that the properties of texturized soy protein can change according to variables such as extruder configuration, exit die dimensions, temperature distribution, moisture and the feed water pH. (Smith, 1974a).

In 1974 preliminary studies conducted at IIT showed that cottonseed flours could be texturized in a similar way to soybean flour and that free gossypol contents were simultaneously reduced to safe levels (Buckle et al., 1975).

The objective of this work was to study the influence of raw materials and processing conditions on the physical and textural properties of the extrudates prepared from cottonseed flours, to verify the consistent reduction of free gossypol contents and to evaluate some nutritional properties of the product.

EXPERIMENTAL

Raw materials

Three cottonseed flours from commercial oil extraction were used as starting materials. They were ground so that at least 95% passed through a 100 mesh U.S. standard sieve.

Chemical analysis

Moisture, fat, crude fiber and protein were measured according to AOAC (1975).

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Free gossypol was measured following the AOCS standard method (1975) that has been applied even to chemically treated cottonseed meals (King et al., 1962).

Available lysine was determined by the Conkerton and Frampton method (1959).

Physical analysis

Bulk density of the extrudates was evaluated by using a calibrated beaker, without spout, 600–700 ml capacity. Extruded material was cut to a length of 1 cm. This material was then fed by gravity from a height of 20 cm through a powder funnel into the beaker until an excess was present. The surface of the material was leveled without compressing the material, by using a flat surface that slides over the rim of the beaker. The beaker plus content was then weighed. Bulk density is easily calculated if the weights of the empty beaker and of the beaker completely filled with distilled water are known.

Integrity index of the textured materials was measured according to known procedures (USDA, 1974). Water absorption index (WAI) was measured by the following procedure: exactly 50.0g of textured material was added to 1L of water maintained at 30°C. The mix was gently and constantly stirred for 10 min. The material was then drained on a sieve for 10 min and weighed. WAI was calculated by the relationship:

$$\text{WAI} = \frac{\text{Wt hydrated material} - \text{Wt dry material}}{\text{Wt dry material}} \times 100$$

Expansion index was calculated as the ratio of the extruded product diameter to the exit die diameter.

Fine structure of the products was observed by scanning electron microscopy. A Cambridge Stereoscan S-4-10 was used. For examination, pieces of the extruded material were attached to standard stubs and coated with gold in a vacuum evaporator.

Texture profiles were constructed according to IIT methods (Bourne et al., 1975). Ground meat was used as a standard (Sandoval, 1977).

Biological values

Biological values of cottonseed and soy extrudates and whole hen's egg were measured using the slope ratio technique as developed by Hegsted and Chang (1965).

Sample extrusion at laboratory scale

Samples C and D were extruded on a laboratory scale by means of a Wenger X-5 extruder cooker. Operating conditions are shown in Table 1.

The extrusion variables under consideration were: die hole diameter, die temperature, die type, flour-to-water ratio and water pH. Although the initial intention was to vary simultaneously only one of the parameters in order to ascertain its influence on the quality of the product, it was soon realized that it was impossible, if a steady state was to be maintained in the machine operation. So, die temperature and flour-to-water ratio were two variables that were simultaneously adjusted and because of that it is difficult to ascertain their separate influence on the end product.

The exit dies used are sketched in Figure 1. Plate die is commonly used for testing cereals, cereal flours or cereal-protein mixtures. The die opening is variable and coaxial with the extruder screw. TSP die is mainly used when the raw material has a high protein content, such as soy flours or concentrates. TSP die openings are situated at right angles to the axis of the extruder screw. In this case, pressure and shear forces higher than those developed by the plate die, are originated.

Sample extrusion at industrial level

Samples C and G were extruded through a Wenger X-25 CF extruder cooker. Processing conditions were as follows: 5 screw sections separated by steamlocks (initial two heating heads: spiral

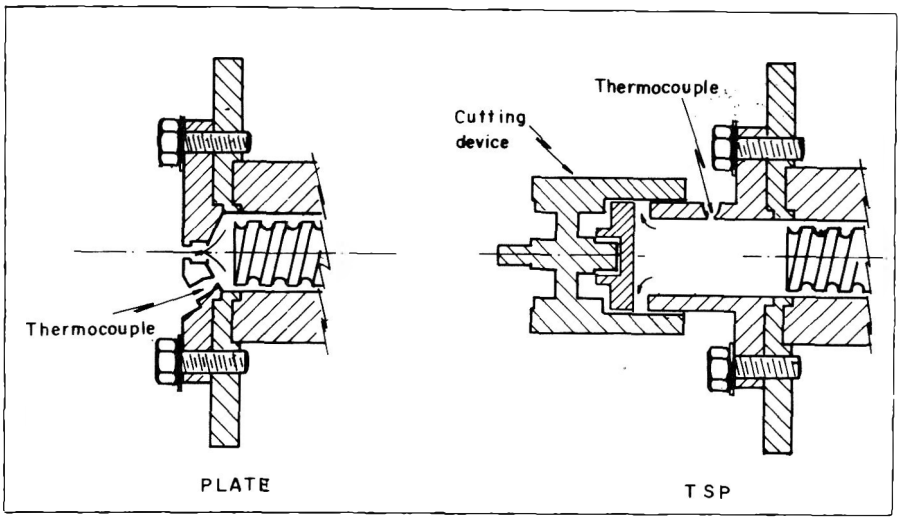

Fig. 1—X-5 extruder—exit dies.

rib; third and fourth heads: straight rib; last head: spiral-conic); screw speed: 300–350 rpm. Temperature at the exit die varied between 103 (sample C) and 135°C (sample G). Product moisture at the exit die varied from 12.0% (sample C) to 16.0% (sample G). Extruded samples were dried in warm air at 40–55°C.

RESULTS & DISCUSSION

Raw material characteristics

Chemical analysis of the three cottonseed flours used as raw materials are shown in Table 2. One of the samples (C) is remarkably lower in fiber content than the other two, although fat and alkali soluble protein levels are similar in all three. Free gossypol content of all three samples is similar.

Table 1—Cottonseed flour extrusion conditions (X-5 laboratory extruder)

Run[a]	Die hole diam (in.)	Die temp (°C)	Flour-to-water mass ratio[b]
C – 6	3/16	90–100	2.65 (1)
C – 8	13/64	100–102	7.14 (1)
C – 9	13/64	105–115	7.88 (1)
C –10	3/16	88–92	8.41 (1)
C –11	3/16	88–92	8.76 (1)
C –12	3/16	93–97	8.07 (2)
C –13	3/16	94–98	7.88 (3)
D –17	1/8	75–90	4.12 (1)
D –18	1/8	90–100	5.44 (1)
D –19	5/32	90–95	4.12 (1)
D –22	3/16	75–85	4.07 (1)
D –24	3/16	98–102	9.52 (4)
D –26	13/64	102–107	8.30 (1)

[a] Samples C-8, C-9 and D-26 were run by using a TSP die. A plate-type die was used for all other runs.
[b] Water pH as follows: (1) 6.3; (2) 7.0; (3) 8.5; (4) 10.0

Table 2—Chemical analysis of cottonseed flours[a] (moisture free basis)

Sample	Fat (%)	Crude Fiber (%)	Protein (N X 6.25) (%)	Free gossypol (%)	Available lysine g/100g protein
C	0.73	5.27	57.6	0.077	3.8
D	0.59	13.98	47.5	0.082	3.8
G	0.75	12.85	46.3	0.073	–

[a] Alkali soluble protein (NaOH, 0.02N) was 70.5–71.0 for all three samples

Nutritional characteristics of the extruded products

Results of biological value determinations for two cottonseed textured products extruded at two different temperatures are included in Table 3. Values for soy textured protein were measured for comparison with egg as a reference and are also included in Table 3. Available lysine (g/100g of protein) values for the four products and free gossypol of the two cottonseed textured flours are also shown in the same Table.

It is interesting to note that nutritional values of cottonseed textured proteins are similar to those of soy textured protein. Available lysine was not reduced greatly as the levels in textured products are comparable to those in commercial cottonseed flours for human consumption (Bressani, 1965). The free gossypol content has been reduced to safe levels (under 0.055%) (USDA, 1960) as can be observed in Table 4. If it is supposed that one mole of free gossypol combines with 2.8 moles of available lysine (Cater et al., 1969), a reduction of free gossypol from 0.077% to 0.01% will theoretically reduce the available lysine in the final product from 3.80 to 3.71 g/100g protein. The values of available lysine registered in the extruded product agree with this figure and with the acceptable results obtained for the biological value.

Scanning electron microscopy of extruded materials

Photomicrographs (Fig. 2a,b) show some fine structural components of both soy and cottonseed textured proteins. Both samples show intact and ruptured protein bodies, rivu-

Table 3—Biological value, available lysine and free gossypol of some textured products[a]

Product	Biological value	Available lysine (g/100g protein)	Free gossypol (%)
Hen's egg (whole)	100	6.9	–
Cottonseed textured protein[b]	57	3.6	0.014
Cottonseed textured protein[c]	55	3.6	0.016
Soy textured protein	56	6.3	–

[a] Using cottonseed flour C (Table 2)
[b] Extrusion temperature: 150°C
[c] Extrusion temperature: 175°C

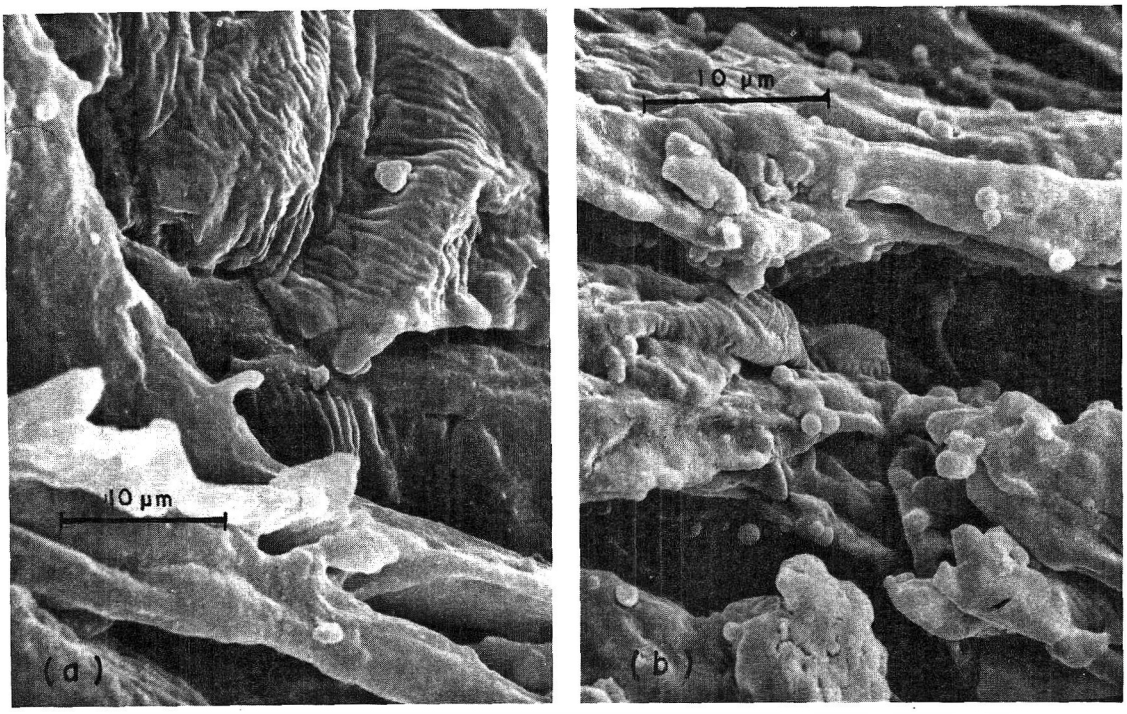

Fig. 2—Scanning electron micrographs of textured products. Cross fractures of soybean (a) and cottonseed (b) extrudates.

lets of protein, protein strands and their systematic layering. These structural characteristics correlate with the textural profiles found by sensory evaluation, as discussed later.

Photomicrographs (Fig. 3a,b,c) show some protein bodies that remain unmodified after extrusion both in cottonseed (a) and in soy textured protein (b). The higher number of unmodified protein bodies in cottonseed extrudates compared to soybean could be explained by the lower water solubility of the former.

Strand formation from ruptured protein bodies is illustrated in Figure 2c. One of the protein bodies is just being elongated and another is fully elongated and connects two layers of orientated protein bodies.

Photomicrographs (Fig. 4a,b) show the fiber orientation observed both in cottonseed texturized protein (high fiber content) (a) and in soybean texturized protein (b). In the former, two dimensional layering which resembles that of bovine muscle tissue is observed (Varriano-Marston et al., 1976). In the latter the fibers (layers) show a more complex interweaving.

Processing conditions and chemical and physical characteristics of the products

Some of the physical and chemical characteristics of the extrudates from cottonseed flours are included in Table 4. High integrity index seems to correspond to high water absorption index, low bulk density and high expansion index. This means that the highly expanded product absorbs and retains more water and withstands more mechanical work and cooking than the less expanded material.

No relationship was found between water pH and free gossypol reduction, or between die diameter and expansion index. No significant differences were found in expansion indexes for the extruded material under various processing conditions. This index does not seem to be a critical variable for cottonseed textured proteins as it is for cereal based extruded products.

Extrusion conditions for cottonseed flours are more stable and give products with better properties, when a plate-type exit die is used instead of the TSP die. The latter seems to produce excessive heat treatment and make expansion difficult, thus giving products with a high bulk density, low expansion and low water absorption index (See samples C-8, C-9 and D-26, Table 4).

Extrusion with water at a pH between 7.0 and 8.5 gives products with better properties. This result is in agreement with Smith (1974b) who states that alkaline treatment will promote enhancement of properties such as chewiness and high water absorption in soybean textured proteins.

Table 4—Physical and chemical characteristics of cottonseed textured proteins

Sample	Free gossypol[a] (%)	Integrity index	WAI[b] (g/100g)	Bulk density (g/ml)	Expansion index[c]
C – 6	0.016	27	192	0.209	1.15
C – 8	–	–	40	0.460	1.11
C – 9	–	–	40	0.430	1.22
C – 10	0.012	45	140	0.200	1.29
C – 11	0.011	65	160	0.190	1.49
C – 12	0.009	53	240	0.140	1.41
C – 13	0.012	57	267	0.150	1.47
D – 17	0.015	16	141	0.226	1.45
D – 18	0.013	17	181	0.175	1.15
D – 19	0.015	20	181	0.182	1.26
D – 22	0.025	17	134	0.297	1.04
D – 24	0.022	26	180	0.210	1.01
D – 26	–	–	60	0.420	0.79
C –X25	0.018	44	248	0.262	n.d.
G –X25	0.014	46	225	0.333	n.d.
C_1 –X25	0.016	47	160	0.200	n.d.

[a] Original free gossypol content: Sample C 0.077%, D 0.082, G 0.073

[b] Water Absorption Index

[c] n.d.: Not determined

Sensory evaluation of texture of the products

Sensory evaluation by the texture profile method (Bourne et al., 1975) of cottonseed extrudates showed that

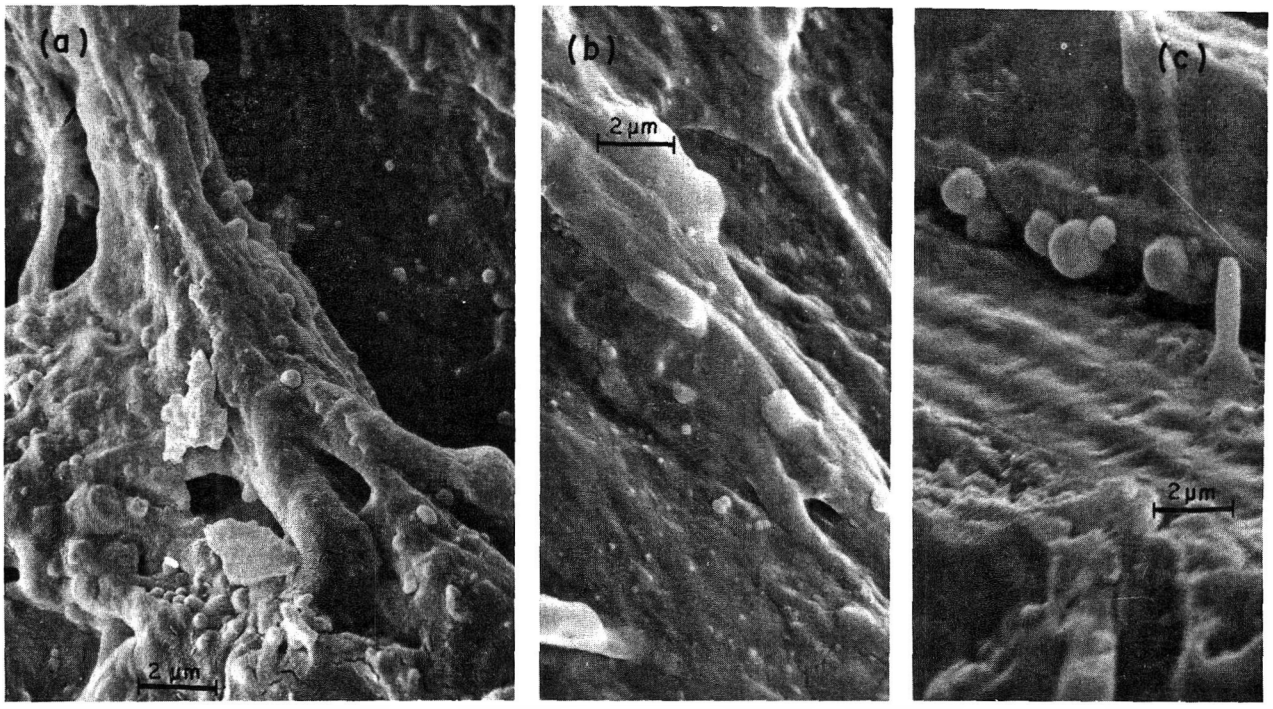

Fig. 3—Scanning electron micrographs of cross fractures of extruded products showing transformation degrees to textured forms: (a) and (c) cottonseed; (b) soybean.

during initial masticatory and residual sensations, the outstanding mechanical parameter was roughness. As the sample breaks down moisture liberation, oiliness and geometrical properties become evident. It is interesting to note that when texturized soy protein is compared to ground beef, roughness is not important.

Textured products prepared from high-fiber cottonseed flour tend to show greater differences when compared with the standard, than the product obtained from low-fiber flours. The latter is very similar to the standard, and shows just a little more roughness.

Integrity index

This property is closely related to the functional characteristics of the products, because it is a measure of the resistence of the material to the different procedures followed during food preparation.

USDA (1974) regulations state that textured products having an integrity index higher than 35 can be classified in group 1. Using this criterion, most of the low-fiber samples and products from commercial extrusion belong to this group. It is remarkable that none of the textured products

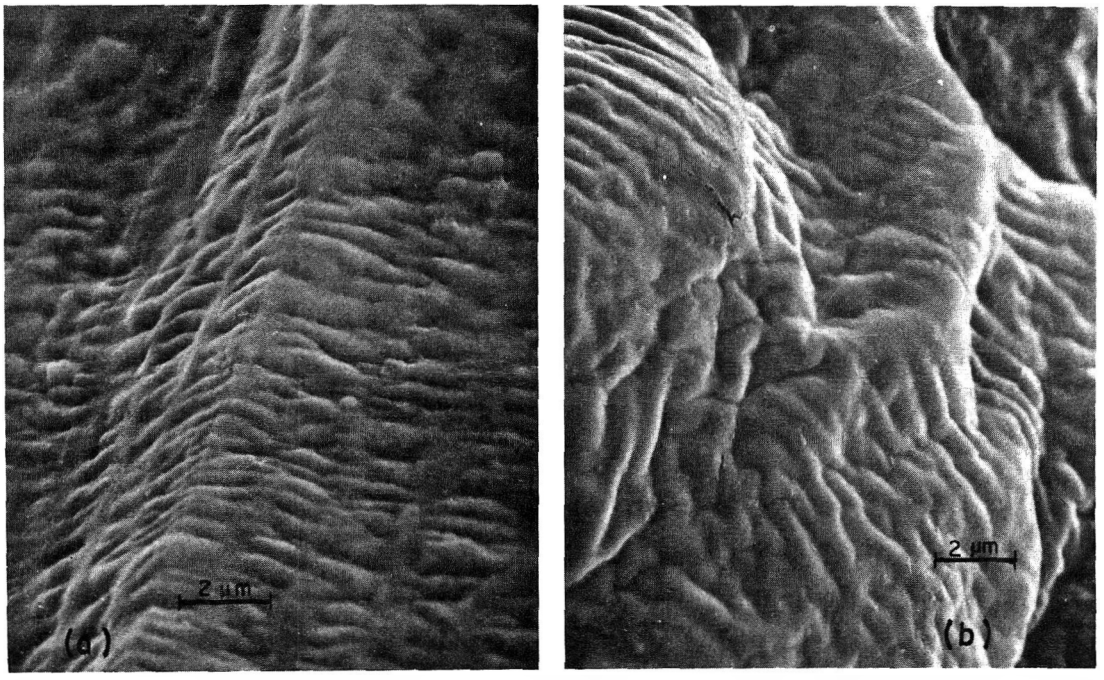

Fig. 4—Scanning electron micrographs of longitudinal fractures of fibrillar arrangements of extruded products: (a) cottonseed; (b) soybean.

obtained from high-fiber cottonseed flour (D type) can be classified into this group. This can be related to the higher differences in texture for those samples. The use of a commercial extruder (X-25) also helps to produce a higher integrity index in the textured proteins both for the low and high fiber cottonseed flours, than a laboratory extruder (X-5), as can be observed in Table 4.

Cottonseed extrudates produced in an X-25 extruder have a lower integrity index (44–67) than soybean extrudates processed in the same equipment (86–95). This is probably due to the more complex three-dimensional interweaving observed in soy textured proteins, as compared to the two-dimensional layering present in cottonseed textured proteins (Fig. 4a,b).

CONCLUSIONS

From the foregoing it may be concluded that:

1. It is possible to produce textured cottonseed protein from commercially available defatted cottonseed flours. The products obtained both on laboratory and industrial scales offer similar properties to those of ground beef, although in texturized cottonseed, lysine in maintained above minimum acceptable values for cottonseed proteins (3.6 g/100g protein). Nutritive value of the products is similar to that of textured soybean proteins.
2. Better product properties are obtained when water pH is between 7.0–8.5 and when low-fiber cottonseed flours are used. Extrusion in industrial equipment greatly improves the functional properties of the product.
3. Scanning electron microscopy reveals that the basic structural components of textured proteins (strands, rivulets, layers) are present in cottonseed textured products. More intact protein bodies are apparent than in soy textured protein. A two-dimensional structure is observed in high-fiber products as compared to low-fiber or soy textured proteins, and this probably causes low mechanical resistence as measured by integrity index in the high fiber cottonseed products.

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HEATING CHARACTERISTICS OF CREAM-STYLE CORN PROCESSED IN A STERITORT: EFFECTS OF HEADSPACE, REEL SPEED AND CONSISTENCY

MAURICE R. BERRY, JR., ROBERT A. SAVAGE and IRVING J. PFLUG

ABSTRACT

The heat penetration factors (f_h and j) and sterilization value (F_o) for cans of cream-style corn heated in an FMC Steritort were determined from time-temperature data as a function of container headspace, reel speed, and product consistency. Four commercial instruments were used to measure product consistency. For commercially prepared cream-style corn in 303 × 406 cans, headspace (or fill weight) is the most critical of these parameters. Sterilization values that ranged from 39 to 78 min for 1/4 in. gross headspace were reduced to less than 1 min when the headspace bubble was eliminated. Positive headspacing devices or net-weight sensors are recommended for control of product lines. Other parameters that significantly influence the degree of agitation induced within the can are rotational speed of the cooker and product consistency. At low reel speeds or high product consistencies, sterilization values decrease dramatically.

INTRODUCTION

AS EARLY AS 1939, agitating cans of food during heating was shown to be effective as a means of increasing the rate of heat penetration (Adam and Stanworth, 1939). For viscous products such as cream-style corn (CSC), agitation induces convection within the can that allows short-time, high-temperature processing that improves appearance, flavor, and texture.

The Sterilmatic cooker (FMC Corp., Canning Machinery Div., San Jose, CA), recommended in 1949 for processing of CSC, is today the most widely used continuous agitating processing machine in the United States. Cans enter the retort and are indexed into a revolving reel; the cans move through the machine in a spiral pattern. Agitation is provided by allowing the cans to roll freely across the bottom of the retort.

The objective of this investigation was to determine the significance of the several critical parameters that affect the rate at which heat is transferred to cans of CSC heated in a Steritort (FMC Corp.) and, in turn, the sterilization value received by the product. The results of these studies using a Steritort would be applicable to commercial CSC processed in the FMC Sterilmatic processing machines.

MATERIALS & METHODS

Experimantal plan

This study to determine the effect of headspace, product consistency, and Steritort rotational speed on the heating rate and lag factor of cream-style corn was carried out at the pilot plant facility of the Green Giant Company, Le Sueur, MN. The CSC was obtained from the production line of an adjacent CSC canning plant.

The cream for CSC was prepared from corn, starch, sugar, salt, and water. The cream and slit corn kernels were combined and passed through a DeZurik consistency controller (DeZurik Corp., Sartell, MN). The torque required to rotate a paddle in the CSC is measured and small amounts of hot water are added by the unit to maintain a constant torque, which is empirically related to product consistency. The product then flows directly to the filling machines.

Since commercial CSC varies within rather broad manufacturing limits, it was recognized at the onset of this project that we would be working with a variable product. It was decided that two sequential tests of six cans each would be carried out from each batch of CSC. The CSC for the duplicate test would be held under controlled temperature conditions while the first test was carried out. In this way each product test condition was evaluated twice.

Cream-style corn physical characteristics

CSC at or above 180°F was collected immediately before the filler bowl in the canning plant. Approximately 10 gal of corn was diverted into a 15-gal, stainless steel stock-pot.

In this report, we use the term "consistency" to describe the product characteristics. Consistency measurements were made on the product before heating and afterward on the fastest and slowest heating cans. Four commercial instruments were used to measure this quantity: the FMC consistometer (paddle A, C.W. Brabender Instr., Inc., South Hackensack, NJ), the Brookfield viscometer (Model HBT, Spindle 2, Brookfield Engineering Lab., Stoughton, MA), the Adams consistometer (Cefaly Experimental Co., Brentwood, MD), and the Stormer viscometer (A.H. Thomas Co., Philadelphia, PA).

Both the Brockfield viscometer and the FMC consistometer were used to measure total consistency. Each of these instruments measures the torque required to rotate a paddle in the product. The Adams consistometer measures the spread of a premeasured amount of CSC on a calibrated flat surface in 20 sec. The Stormer viscometer is used to measure the characteristics of the cream portion of the product. The CSC was strained through a No. 8 sieve, the cream portion was placed in a cup, and the time required for a constant amount of energy to be dissipated in rotating a cylinder in the cream was determined. The Stormer viscometer was used to monitor consistency in the plant, where product values were normally between 6.0 and 9.0 sec.

Washed, drained residue (WDR) is the percent of solids remaining on a No. 8 sieve after washing the product with water and draining. The WDR of the product in each test was measured. The WDR of the product from the plant varied from 25% to 40%.

In some cases, it was desired to evaluate a range of product conditions by modifying the product as obtained from the canning plant. Two techniques were used:

1. To vary the cream consistency and maintain the same WDR, extra cream was collected in the canning plant before the cream and slit corn kernels were mixed. The CSC sample was then split in the pilot plant and equal amounts of cream and water were added. The addition of about 1 qt of cream or water to 5 gal of CSC resulted in a difference of about 4 sec in the Stormer consistency readings.
2. To obtain a range of WDR's for the same cream consistency, a CSC sample was divided into two equal parts, and the kernels were strained from one part and added to the other.

Filling and sealing cans

The test cans were hand filled. Four filling conditions were used: fill weights of 480g, 488g, and 493g (all ± 0.2g) and full to overflowing (approx 510g). A fill of 488g in a 303 × 406 can results in a gross headspace of approximately 0.25 in. and was considered normal fill. After filling, the gross headspace was measured and then the cans were closed.

Heat penetration measurement

All heating tests were carried out using 303 × 406 cans. An Ecklund (Ecklund Custom Thermocouples, Cape Coral, FL) locking-type receptacle was located in the center of the manufacturer's end of the can. Ecklund CNS copper-constantan thermocouples,

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2–3/16 in. long and 0.067 in. in diameter, were installed along the center line of the can at the geometric center.

An Ecklund thermocouple lead wire harness (rotary contactor) was used. The device included a slip ring unit that attached to the locking receptacle on the can and lead wire that connected to the thermocouple junction unit on the reel hub. The thermocouple lead wires were brought out of the Steritort through an external commutator unit at the end of the reel shaft.

The slip ring unit that attached to each can was inspected before and after each test to be sure that there was free movement between parts. The results of some heating tests had to be discarded either because drag developed in the slip ring unit or the slip rings stopped rotating altogether.

The temperature in six instrumented cans was measured during each heating test. Two thermocouples were located in the Steritort near the mercury-in-glass thermometer to obtain the heating medium temperature.

The thermocouple outputs, which were recorded using a strip-chart, temperature-recording potentiometer, were also measured, recorded, and punched on paper tape by a Doric Digitrend 220 data acquisition system (Doric Scientific Crop., San Diego, CA). Both systems were connected in parallel to all the thermocouples. Temperatures were measured at 30-sec intervals.

Steritort operation

Four Steritort reel rotational speeds were evaluated – 5.0, 6.5, 8.0 and 10.6 rpm. Normally, within each run at a given reel speed, two sets of three cans each had different product-container parameters. The difference was usually fill weight, since consistency and WDR varied routinely for the commercial product. But to better understand the effects of consistency and WDR, for some runs the commercial product was modified as previously described. A variation of these characteristics could thus be obtained within the same Steritort test.

The six thermocouple-equipped cans plus two additional cans for consistency testing were loaded into the Steritort. Thermocouple leads were connected, reel rotation was started, and the movement of cans was observed to be sure that all cans were rolling freely. The Steritort door was then closed and sealed.

The automatic temperature control was set for an operating temperature of 270°F, and the steam by-pass valve was opened. At time zero, the main steam valve was opened. The vents were closed at the end of 1 min of heating, and the by-pass valve was closed as the temperature approached 270°F.

The heating time (measured from steam-on to steam-off) was 22 min for tests at 5 rpm and 16 min for all other tests. At the end of the heating period, the steam valve was closed, and the 4-in. drain valve was opened along with the cooling water spray valve.

When cooling was completed (at a product temperature of about 100°F), the door was opened and the rotation of reel and cans observed to be sure that all cans were still rolling freely. The reel was then stopped.

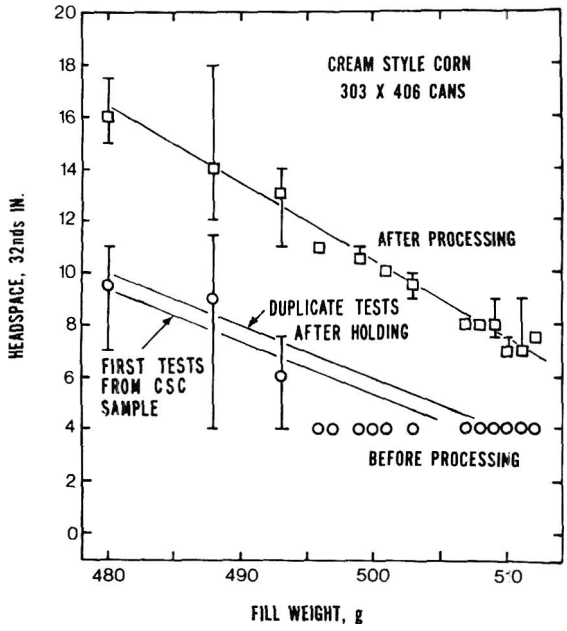

Fig. 1—Headspace correlation for cream-style corn in 303 X 406 cans.

Post-heating evaluation

The cans were removed from the Steritort and the vacuum measured to make certain the thermocouple packing glands had not leaked. The cans were opened and the headspace measured. The consistency of the CSC in the fastest and slowest heating cans was determined. The WDR of the CSC in two cans was also determined.

Data analysis

At the end of each day of testing, the heat penetration data on the punched paper tapes record was transferred to the University of Minnesota computer where, using appropriate software, sterilization values (F_o) were calculated by the general method and heating rate parameters (f_h, f_2 and j) were calculated. This rapid reduction of data produced results that were used to decide which subsequent tests were needed to best accomplish the objective in the limited time available.

Sterilization values were also calculated from manually determined heating rate parameters from the computer generated heat penetration graphs. The Ball method was used and lethalities were calculated using the actual initial temperature (T_o) for each can and an assumed initial temperature of 170°F.

RESULTS

Results for each of the variables evaluated (headspace, reel rotational speed, and product consistency) will be presented separately even though they interact.

Headspace

Agitation of the product in a can heated in a Steritort is facilitated by the presence and movement of the headspace bubble. The size of the bubble, the rotational speed of the can, and the consistency of the product together determine the rate of heat penetration.

Gross headspace before and after processing as a function of fill weight is shown in Figure 1. Both the mean (data symbols) and range (vertical lines) of headspaces at each fill weight are shown. The curves shown in Figure 1 represent linear regression analysis of the data. The two curves labeled "before processing" are for the first and duplicate tests made from a given sample of CSC. The difference in measured headspaces before processing for the two tests is probably due to deaeration of the CSC. The vapor was partially removed and the CSC cooled as it stood for about 45 min between tests. This effect was not significant for the after-processing data, and thus only one curve is shown. Headspace increased approximately 6/32 in. during processing. Gross headspaces before processing for cans with no headspace bubble are shown as 4/32 in. (the approximate counter-sink depth of the can). These cans were filled to overflow before seaming but headspaces were not actually measured. We believe that the variation in fill weight (496–512g) for the cans with no headspace is primarily due to variations in entrapped gases in the CSC.

In this study, we varied the fill weight of the cans to produce variations in headspace. The minimum sterilization value (calculated with T_o of 170°F) for nonmodified product for each fill weight for the various reel speeds is shown in Figure 2. The minimum F_o data shown are for cans with a high consistency; however, we have excluded the data from tests where we purposely changed the product (i.e., where WDR or consistency was modified). Changes in fill weight of as little as 5g were easily detected by the heat penetration data and subsequent sterilization value calculations. The most obvious effect is the significant reduction in sterilization value as the ingoing headspace bubble is reduced to zero.

The effect of fill weight on the heating rate parameter

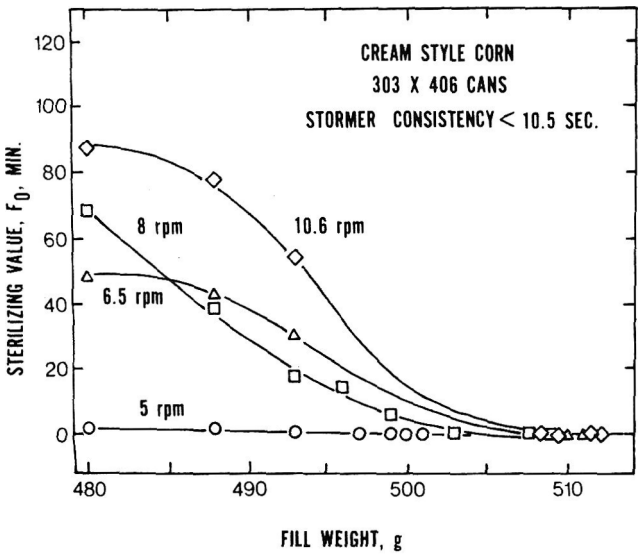

Fig. 2—Effect of fill weight on sterilization value.

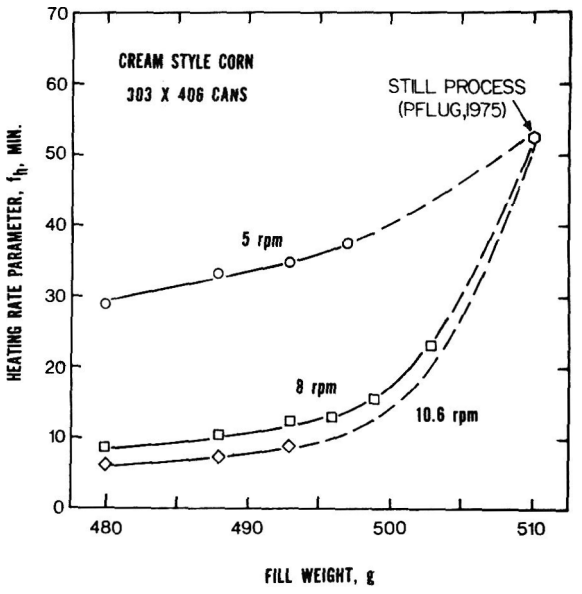

Fig. 3—Effect of fill weight on the heating rate parameter.

(f_h, or minutes required for the heat penetration curve to traverse one log cycle) is shown in Figure 3. The data represent the measured heating rate parameter for the slowest heating cans. No data are shown for the 6.5 rpm tests because broken-heating curves were obtained and a single value of the heating rate could not be computed. The heating rate parameter increases uniformily with increasing fill weight at all reel speeds. The data are shown approaching an f_h value typical of a still cook for CSC in 303 x 406 cans at the brimful condition (Pflug, 1975). Above about 500g, no data are shown since the process time was not long enough to fully develop the straight-line portion of the heating curve.

Reel speed

The influence of reel speed on the sterilization values and heating rate parameters as a function of fill weight and consistency are shown in Figures 2 and 3, and 4 and 5, respectively. The results indicate that the higher the reel speed, the more effective the agitation and the larger the sterilization value for the same process time. Although sterilization values were slightly larger at 8 rpm, there was little difference between the 6.5 and 8.0 rpm runs. The most interesting difference was the broken-heating phenomenon that consistently occurred at 6.5 rpm. After 4–8 min, the heating rate parameter increased by 50–100%. Straight-line heating occurred only with high consistencies or zero headspace.

Consistency

In Figure 6 are shown the FMC consistometer readings as a function of WDR. The FMC consistometer measures in Brabender units (B.U.), and the measure is influenced by the two components of CSC; cream and kernels. To show the role of the cream portion of the CSC, the data have been keyed to the relative Stormer readings: low/5.6–7.5, medium/7.5–9.0, and high/9.0–16.2 sec as represented by the different symbols.

Lines have been drawn approximating Stormer values of 7.5 and 9.0. In general, FMC B.U. values can be the result of thick cream and a low percentage of kernels, or thin cream and a high percentage of kernels. Either a Stormer consistency of 9.0 sec and a WDR of 26%, or a Stormer of 7.5 sec and a WDR of 34% have an FMC consistency of 70

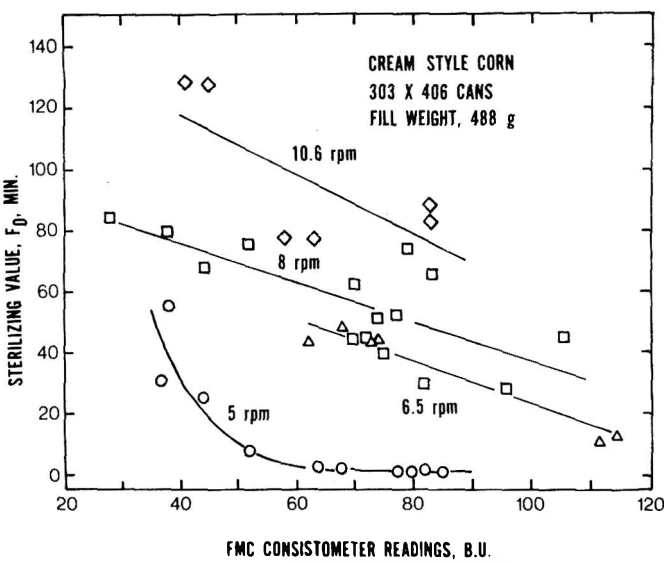

Fig. 4—Effect of consistency on sterilization value.

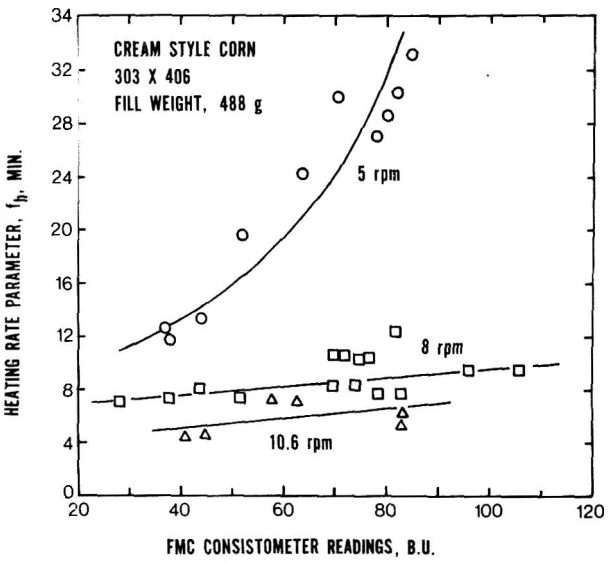

Fig. 5—Effect of consistency on the heating rate parameter.

B.U. (dashed lines, Fig. 6). A similar correlation was found for the Brookfield consistency data.

Consistency is a measure of a product's internal resistance to flow when subjected to an external stimulus. The higher the consistency, the thicker the product, or the more resistant it would be to movement such as agitation in a Sterilmatic cooker. For a product like CSC, consistency is influenced both by the thickness of the cream and the amount of particulate matter (WDR) in the mixture (Fig. 6). Consistency measurement is further complicated by the product temperature and rheological effects. Consistency comparisons must be made at the same temperature (normally 170 or 180°F for CSC), with the same viscometer. Even then the unknown rheological effects of holding and mixing may influence the results.

Control of product temperature for the consistency measurements was not possible. Some insight as to the effect of temperature on consistency can be obtained by comparisons of the same sample of corn at different temperatures. For most combinations of processing parameters, an initial run was followed immediately by a duplicate run. The average product temperature was 168°F for the ingoing FMC consistency measurements for the initial runs. For the duplicate runs, the temperature was 148°F. The combined result of the holding time and the decrease in product temperature was to increase the average FMC consistency from 70 to 81 B.U. Based on the close agreement between the sterilization values (corrected to an initial temperature of 170°F) in the first and second tests of each sample of CSC, we conclude that the difference in measured consistencies are primarily the result of the temperature differences and only to a much lesser extent to changes in rheological properties. If only a temperature effect is assumed, a correction factor of about −0.5 B.U./°F appears reasonable. For the Brookfield and Stormer viscometers, the factors are −25 centipoise/°F and −0.1 sec/°F, respectively.

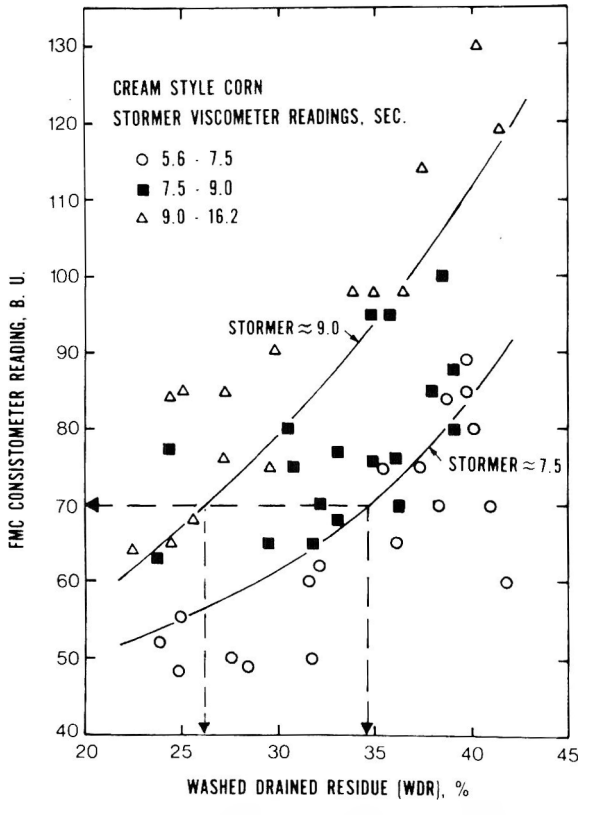

Fig. 6—Components of total consistency for cream-style corn.

Both the FMC consistometer and the Brookfield viscometer measure total consistency, whereas the Stormer viscometer characterizes the cream portion only. FMC and Brookfield consistencies were in agreement, probably because the two instruments are similar and both measurements were taken from one sample and at the same temperature. But the FMC consistometer was more sensitive to WDR, probably because of the difference in the shape of the shearing devices. The Brookfield spindle was T-shaped and made of small-diameter rods, whereas the FMC paddle was a flat, rectangular plate. For the same Brookfield consistency, CSC with higher WDR yielded higher FMC consistencies.

The effect of product consistency on the sterilization value is shown in Figure 4 at reel speeds of 5, 6.5, 8, and 10.6 rpm. Data points shown are for a fill weight of 488g and represent the slowest-heating can of each run at a given consistency. FMC consistometer (paddle A) readings are for the ingoing product and have been corrected to a temperature of 170°F. Readings greater than 100 have been extrapolated from actual data taken with the B paddle. The data indicate a definite decrease in sterilization value with increasing consistency at all reel speeds. All runs conducted at 5 rpm were processed for 22 min, 6 min longer than at other reel speeds. Even with the additional processing time, sterilization values for the tests conducted at 5 rpm were minimal at all FMC consistencies over 65 B.U., which represents about half the CSC tested. Heat penetration was greatly reduced at 5 rpm.

The effect of the reduced agitation on the measured heating rate parameter at 5 rpm is shown in Figure 5. At 10.6 and 8 rpm, the change in heating rate parameter over the range of consistency readings is relatively small. When the reel speed is 5 rpm, a dramatic change occurs in the heating rate parameter with increasing consistency. At low FMC consistometer readings, the heating rate parameter is about 12. But it increases to more than 30 when the consistency reaches 80 B.U. A single-valued heating rate parameter could not be shown in Figure 5 for the 6.5 rpm tests since broken-heating occurred.

DISCUSSION

THE OBJECTIVE of this investigation was to determine the effects of variation in processing parameters on the heating parameters and sterilization values of commercially formulated CSC heated in a Steritort. The study required that a large number of heat penetration tests be carried out during the 2-wk CSC pack. Since commercial CSC was used we did not have precise control of product formulation and true replicate or repeat tests could not be made.

For most processes, an initial run from a sample of CSC was followed immediately with a duplicate run. At the start of processing, initial temperatures of the CSC varied from 120–175°F. The average initial temperature of the first run was 158°F. This temperature had dropped to 150°F for the duplicate runs because of the delay before filling the duplicate cans, even though filled cans were placed in a 170°F holding bath to await processing. The effect on the average sterilization value (general method) was a reduction of 1.5 min between initial and duplicate runs. When the Ball formula method (T_o = 170°F) was used, the difference was 0.5 min. This result indicated good agreement between duplicate runs, considering the possible rheological effects due to holding the product at elevated temperatures. Consistency readings for the ingoing product were corrected to a temperature of 170°F. F_o value comparisons are based on initial-temperature-corrected data. The data from minimum F_o value (slowest heating) cans are used in the discussion and figures.

Product agitation in a Sterilmatic cooker is a consequence of the motion of the headspace bubble within the can. For fill weights above 505g sterilization values are dramatically reduced (Fig. 2), even though gross headspace measured after processing is 0.25 in. (Fig. 1). Consequently, a positive indication of headspace after processing does not insure an adequate headspace during processing.

Within limits, the data show that the agitation and the rate of heat transfer increase with the reel speed. For a fixed-capacity cooker, however, an increase in reel speed is accompanied by a reduced retention (process) time. Whether this reduction is compensated by the increased heat penetration rate depends on the characteristics of the product being heated.

The effectiveness of the induced convection for CSC depends on accurate filling machines to control headspace, precise formulation and preparation to control product consistency, and avoidance of delays between filler and retort that tend to increase product consistency and lower initial temperatures (Kueneman, 1953; Wilson, 1953). Until recently, however, very few additional data on continuous agitating retorts have been published (Houtzer and Hill, 1977).

CONCLUSIONS

THE RESULTS of this investigation warrant the following conclusions regarding the processing of CSC in 303 × 406 cans in a Steritort:

1. Headspace is the most critical processing parameter. Regardless of consistency or reel speed, an inadequate headspace resulted in a low sterilization value. The critical nature of headspace suggests the use of positive headspacing devices or net-weight sensors for control on production lines.

2. Agitation of the product in the container is determined by reel speed. At reel speeds of 8 and 10.6 rpm, the effect of a change in consistency on heating rate was small. But at 5 rpm, the f_h value increased dramatically with increasing product consistency.

3. Consistency of CSC is influenced by both cream consistency and WDR, and both measurements are necessary to characterize the product completely. Devices that measure total product consistency such as the FMC consistometer and the Brookfield viscometer were helpful in establishing the effect of consistency on the sterilization value and can be used to establish consistency limits for effective processing.

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LACTULOSE VS SUCROSE SWEETNESS . . . From page 815

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SWEET CORN: VARIETAL AND DEVELOPMENTAL DIFFERENCES IN AMINO ACID CONTENT AND COMPOSITION OF GRAIN

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ABSTRACT

Grain of three varieties of hybrid sweet corn was harvested and analyzed at approximately 15, 30, 45 and 60 days after pollination to establish variation in composition and amino acid content among varieties and during course of grain maturation. In all three varieties, percent protein content on a dry basis dropped markedly after 15 days post-pollination but remained fairly constant at 12–13% during later development. Fat increased steadily from 3 to 7 or 8% (dry basis) in all varieties. Lysine content decreased after 15 days, but sulfur amino acids increased to a maximum at 60 days. At 28–30 days, when sweet corn is generally harvested for canning, lysine content of the grain ranged from 3.8–4.3g per 100g of protein.

INTRODUCTION

SWEET CORN (*Zea mays L.* ssp. *saccharata*) is one of the largest vegetable crops grown in the United States; in 1975 over 3 million tons were produced (USDA, 1976). Despite this large production and wide consumption as fresh, canned and frozen vegetable, until recently little research has been devoted to evaluation of sweet corn as a source of dietary protein. Primary interest has been directed to carbohydrates, since in the milky stage, when the grain is harvested for food use, carbohydrates determine flavor and texture (Huelsen, 1954). Nordstrom and Meade (1969) reported that one variety of sweet corn they examined as mature grain contained 49% more lysine and 79% more sulfur amino acids than normal dent field corn. Luk'yanenko (1974) and Pukrushpan et al. (1977) demonstrated that the protein composition in terms of solubility fractions changes with maturity of the grain. In kernels analyzed shortly after pollination, free amino acids and salt-soluble proteins predominate; whereas in mature kernels zein and glutelin are the major proteins. At the milky stage used for food, an intermediate composition occurs. These changes in composition during grain maturation were reflected in decreased available lysine content in the older grain. Also Luk'yanenko (1974) and Pukrushpan et al. (1977) reported that protein composition at each stage of development differed among varieties.

To obtain additional information on the nutrient value of sweet corn, we have conducted proximate and amino acid analyses on three different varieties of sweet corn commonly grown for canning, which were harvested at four different stages of grain development. The data obtained supports the idea that sweet corn protein quality and content differ with variety and time of harvest, and that sweet corn can serve as a good source of nutritious protein with proper selection of variety and harvest time.

EXPERIMENTAL

Preparation of corn samples

Three hybrid sweet corn varieties were planted in the area of Princeville, IL, by the Joan of Arc Canning Company in the spring of 1974. Wondergold is an early maturing hybrid produced by Northrup King Co., Minneapolis, whereas Midway and Commander, which mature later, were grown from seeds from Asgrow Seed Co., Kalamazoo, MI. The Wondergold was grown in a field with 66 lb of N per acre applied as anhydrous ammonia while the Midway and Commander received 102 lb N per acre. The sweet corn was taken from a row in the center of each field. Approximately 25 ears of each variety were harvested at or near 15, 30, 45 and 60 days after pollination (more exact dates and intervals of harvest are given in Table 1).

Husks were removed from ears after picking, and the ears were then frozen in dry ice. The frozen ears of corn were placed in sealed plastic bags and stored at −29°C. Four ears of each variety and stage of maturity were selected at random. The 15-day frozen kernels were cut from the cobs in a cryostat, and the older kernels were removed from the frozen cobs manually. Weights of the kernels were determined and the samples were lyophilized and reweighed. The freeze-dried samples were ground to 40 mesh with a Wiley mill. Moistures on the lyophilized samples were determined by drying portions in an oven at 105°C for 1 hr.

Proximate analyses

Analyses for fat were conducted by the standard butt extraction method (30–25) of the AACC (1961). Portions of the defatted samples were used for fiber and amino acid analyses. Nitrogen was determined by a semimicro Kjeldahl method and protein calculated by multiplying the percent nitrogen by 6.25. Acid-detergent fiber was determined by Method 7.055 of the AOAC (1975). Total ash was determined on the ground meals by Method 08–01 of the AACC (1961). Nitrogen-free extract was calculated as the weight of material remaining after subtraction of protein, lipid, ash, fiber and moisture weights from the total kernel weight; it consists primarily of noncellulosic carbohydrates.

Amino acid analyses

Duplicate samples of defatted ground sweet corn containing 1 mg N were refluxed in constant-boiling HCl (2 ml per mg sample) for 24 hr. The acid was evaporated from the samples under vacuum. Water was added and evaporated twice from the sample to remove residual acid. Internal standards were introduced and the hydrolyzates were again evaporated to dryness. They were then diluted to 10 ml with pH 2.2 citrate buffer. Amino acids were determined on aliquots of hydrolyzates with a Beckman Model 121 amino acid analyzer. Results were integrated on an Infotronics Model CRS-210 integrator and calculated by computer according to a program of Cavins and Friedman (1968).

RESULTS & DISCUSSION

THE PROXIMATE ANALYSES of the three varieties of sweet corn at different stages of maturity, based on percent of the whole grain, are summarized in Table 1. Trends in compositional changes among the varieties during maturation are very similar. Moisture content dropped from 85–87% at 15 days to 23% or less at 60 days post-pollination. At 28–30 days the moisture content of 72–75% is optimum for harvesting for whole kernel canned corn; cream style canned corn is generally prepared from 68–70% moisture grain (Huelsen, 1954). Protein in the grain on a dry basis dropped from 18–21% at 15 days to 12–13% at 28–30 days and stabilized at that value. Ash declined from 4.2–5.5% to 1.6–2.7% of dry weight as the grain matured. Fiber was high in the early 15-day stage due to inclusion of glumes from the poorly developed cobs. After dropping in percent composition in the 30-day grains, the fiber fraction increased slightly as the kernel matured. Fat increased steadily to 45 days, probably due to increase in size and fat content of the germ. Nitrogen-free extract

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Table 1—Varietal and developmental differences in sweet corn grain. Proximate analyses, percent weight basis

Variety	Development: Harvest date	Development: Approx days after pollination	Percent: Water in whole kernel	Percent: Fat (dry basis)	Percent: Ash (dry basis)	Percent: Protein (dry basis)	Percent: Acid-detergent fiber (dry basis)	Percent: Nitrogen-free extract (dry basis)
Wondergold	7–26	15	87.7	3.3	5.5	20.7	6.9	63.6
	8–10	30	73.7	4.2	2.7	11.8	2.8	78.5
	8–30	50	33.8	6.3	1.6	13.7	3.7	74.7
	9–9	60	14.9	7.1	2.0	13.1	3.2	74.6
Midway	8–12	15	88.3	1.7	4.2	17.6	11.7	64.8
	8–25	28	74.7	4.3	2.4	12.5	2.2	78.6
	9–10	45	54.7	6.9	1.9	12.6	3.2	75.4
	9–25	60	42.3	7.1	1.6	11.7	3.1	76.5
Commander	8–26	15	87.9	3.2	4.4	20.9	7.1	64.4
	9–10	30	76.2	4.2	2.8	13.3	2.5	77.2
	9–23	43	68.8	6.0	2.6	11.8	2.3	77.3
	10–10	60	28.0	8.4	2.7	13.0	3.1	72.8

increased from 15–30 days post-pollination but remained a fairly constant fraction of the kernel after that.

Since kernel size and polysaccharide and sugar contents increase rapidly during development of the kernel, analyses of amounts of components per 100 kernels (Table 2) gives a better insight into compositional changes than does percent composition. On a 100-kernel basis, most constituents except fiber and ash showed several-fold increases during the period 15–30 days after pollination; ash and fiber content was almost doubled. Moisture declined markedly after 28 days. Ash content leveled off at 45 days. Nitrogen-free extract, which is primarily sugars, starch, phytoglycogen, and hemicellulose, continued to increase at a vigorous pace until 45 days when it generally stabilized. Fat continued to be deposited in the kernel until 60 days. Protein and fiber content also rose after 30 days but at a reduced rate. These changes explain why the percent protein of the grain is near maximum at 28–30 days, whereas the percent fat is greatest in the mature kernels.

At 28 days, the Midway variety had the highest dry weight per 100 kernels; Wondergold was heaviest at maturity (Table 2). These variations may be due to different moistures and temperatures during the growth season, since the varieties developed grain at different times. The high dry weight of mature Wondergold grain is primarily due to a large carbohydrate content on a weight-per-100-seed basis. Thus, although Midway at the milky or canning stage (28–30 days) has more protein on a weight-per-100-kernel basis (Table 2), it contains less protein than Commander on a percent basis (Table 1). Of the three sweet corn varieties, Wondergold was lowest in protein at 28 days but was highest at maturity (Tables 1 and 2). Some of these differences may be due to the lower application of nitrogen on the field in which Wondergold was grown compared to the fields in which the other sweet corns were produced.

All three varieties of sweet corn show changes in amino acid analyses during maturation (Table 3) consistent with the changes in protein fractions observed by Pukrushpan et al. (1977) and Luk'yanenko (1974). Lysine content was high in Midway and Commander at 15 days post-pollination, 5.3 and 4.9g per 100g protein, respectively. Their lysine contents decreased sharply in the 15- to 30-day period. Although the 15-day stage of Midway was highest in lysine, its lysine content decreased the most in the next 15 days. Lysine content of Commander remained relatively high even at 60 days after pollination. Content of alanine and aspartic acid also declined in all three varieties during maturation. Proline, leucine and cystine increased in all varieties between 15 and 60 days. Proline and leucine are major components of zein. There was a greater difference in amino acid composition between varieties at 15 days than at 60 days, possibly because more rapid changes occurring at the earlier period make it more difficult to obtain samples at precisely identical stages. However, at 30 days the

Table 2—Varietal and developmental differences in sweet corn proximate analysis, 100-kernel basis

Variety	Approx days after pollination	g per 100 kernels: Weight	Water	Dry weight	Fat	Ash	Protein	Acid-detergent fiber	Nitrogen-free extract
Wondergold	15	14.07	12.34	1.73	0.06	0.10	0.36	0.12	1.10
	30	29.70	21.89	8.01	0.33	0.21	0.92	0.22	6.33
	50	26.65	9.01	17.64	1.11	0.28	2.42	0.65	13.18
	60	19.33	2.88	16.45	1.17	0.33	2.15	0.53	12.30
Midway	15	9.15	8.08	1.07	0.02	0.05	0.19	0.13	0.69
	28	39.45	29.47	9.98	0.43	0.24	1.25	0.22	7.84
	45	24.72	13.52	11.20	0.77	0.21	1.41	0.36	8.45
	60	23.47	9.93	13.54	0.96	0.21	1.58	0.42	10.37
Commander	15	11.57	10.17	1.40	0.05	0.06	0.29	0.10	0.90
	30	31.18	23.76	7.42	0.31	0.21	0.99	0.19	5.72
	43	41.99	28.89	13.10	0.79	0.34	1.55	0.30	10.12
	60	18.65	5.22	13.43	1.13	0.36	1.75	0.42	9.77

Table 3—Amino acid composition of sweet corn of three varieties at different stages of development[a] (g per 100g protein)

Amino acid	Variety											
	Wondergold				Midway				Commander			
	Days after pollination											
	15	30	50	60	15	28	45	60	15	30	43	60
LYS	4.3	3.8	3.6	3.7	5.3	3.8	3.3	3.0	4.9	4.3	4.1	3.9
HIS	2.0	2.7	2.7	3.3	1.9	2.5	2.8	2.8	2.0	2.3	2.6	2.9
NH_3	3.0	2.4	2.2	2.4	3.8	2.5	2.3	2.2	2.9	3.0	2.3	2.5
ARG	4.8	4.9	5.7	5.4	4.6	4.0	4.9	4.7	4.4	4.6	5.0	5.6
ASP	10.1	7.8	7.0	6.7	9.7	6.7	7.9	6.8	8.4	8.2	7.4	8.0
THR	3.8	3.9	3.9	3.8	3.5	3.6	3.9	3.9	3.3	3.6	3.9	3.8
SER	4.7	4.9	4.8	4.9	4.2	4.4	5.0	5.1	4.2	5.0	5.0	4.8
GLU	17.6	19.0	17.1	18.7	23.4	20.0	13.4	20.6	18.3	20.7	19.9	18.6
PRO	8.6	8.7	8.4	9.1	5.8	10.7	9.6	10.3	8.1	8.1	9.4	9.2
GLY	4.9	4.6	4.9	4.5	4.0	3.9	4.0	4.1	3.9	3.8	4.0	4.2
ALA	12.5	9.7	7.8	8.2	11.1	11.7	3.5	8.6	17.7	9.3	9.9	7.9
½ CYS	0.22	0.74	0.97	0.81	0.45	0.88	1.6	1.3	0.62	0.86	0.97	1.2
VAL	5.5	5.9	5.8	5.8	4.7	5.6	5.5	5.5	4.9	5.3	5.5	5.5
MET	2.0	2.9	2.1	2.1	1.6	2.2	2.3	2.1	1.8	1.9	2.1	2.1
ILE	3.4	3.9	3.6	3.7	3.2	3.6	3.9	3.9	3.0	3.7	3.8	3.7
LEU	7.0	11.1	11.2	12.8	5.6	11.7	13.5	14.0	5.7	10.8	11.8	12.1
TYR	2.5	3.9	4.3	4.4	3.0	4.6	5.0	4.8	2.9	4.1	4.4	4.5
PHE	3.2	4.5	4.8	5.1	3.2	4.4	5.2	5.3	3.1	4.6	4.5	4.7

[a] Average of duplicate analyses

amino acid composition of Commander appeared to be somewhat superior nutritionally over those of Midway or Wondergold.

These changes in amino acid content of sweet corn during kernel development are similar in direction to those observed in normal field corn by Moureaux and Landry (1972) except that the decrease in lysine and increase in leucine and proline are not as great in sweet corn. Consistent with the observations of Pukrushpan et al. (1977) and Luk'yanenko (1974), the amino acid data indicate that zein synthesis is not as rapid in the later stages of development of sweet corn kernels as it is during maturation of normal dent field corn. The lysine content in the protein of Commander sweet corn at 30 days is similar to that of W64A *opaque*-2 high-lysine corn at a similar stage of development, although the high-lysine corn retains a higher lysine content upon maturity, 4.6g lysine per 100g protein (Dalby and Tsai, 1975). The sweet corn appears to be similar to inbred field corn containing the *su* gene in its content of lysine and protein fractions (Dalby and Tsai, 1975).

The claim for high sulfur amino acids, methionine and cystine in a sweet corn variety (Nordstrom and Meade, 1969) is not consistent with values determined in varieties investigated in this study. The sulfur amino acid content in both 28-day and 60-day harvested sweet corn are nutritionally adequate but do not exceed values for normal dent field corn (Paulis et al., 1978). Also the sulfur amino acid analyses in the 28-day milky stage, when sweet corn is usually consumed, is less than in the mature kernel.

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BAKING CHARACTERISTICS AND PROTEIN QUALITY OF SOY-WHOLE EGG SOY-EGG YOLK AND SOY-EGG WHITE SUPPLEMENTED BREADS

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ABSTRACT

Full-fat soy (S), and other full-fat soy-whole egg (SE), soy-egg yolk (SEY) and soy-egg white (SEW) flours were produced and utilized as 12% supplements for patent wheat flour in breads. Addition of 0.5% sodium stearoyl-2-lactylate (SSL) improved loaf volumes of all bread types except SEY. Generally, 30 or 45 ppm bromate supplementation also resulted in increased loaf volumes. Organoleptic evaluations of bread types indicate that 100% wheat flour and SEY supplemented loaves scored well in most quality characteristics. Protein efficiency ratio analyses showed that all S and soy-egg fortified breads produced better growth than 100% wheat flour bread. The results indicate that supplementation of breads with SEY flour produces breads of good quality without the need for SSL or bromate.

INTRODUCTION

THE NUTRITIONAL VALUE of full-fat soy and other full-fat soy-whole egg flours as well as the effects of their supplementation upon the baking quality in breads have been previously reported (Erdman et al., 1977). A 50% sponge baking process was utilized to produce 12% supplemented soy and soy-whole egg breads, with and without 0.5% sodium stearoyl-2-lactylate (SSL). Soy supplementation, as noted by Tsen et al. (1971), adversely affected loaf size and quality. SSL addition, as predicted by Tsen and Hoover (1973), increased loaf volumes and acceptability of soy loaves. The soy-egg supplemented loaves, in contrast, produced loaf volumes and organoleptic scores comparable to 100% wheat flours, without the need for SSL. Protein efficiency ratio (PER) and net protein ratio (NPR) tests showed soy-egg flours to have significantly improved protein quality as compared to soy flour. Howarter and Klein (1978) subsequently reported that quick breads of acceptable organoleptic qualities could be made with up to 30% substitution of soy or soy-whole egg flours for wheat flour.

The primary purpose of the present investigation was to evaluate 12% full-fat soy, and other full-fat soy-whole egg, soy-egg white, and soy-egg yolk flour breads baked by the K-State Process (Tsen and Tang, 1971). This short time, high protein baking process which is fully applicable for baking 12% soy flour breads, was developed by the Department of Grain Science at Kansas State University. The second objective was to determine the protein quality of bread baked with 100% patent wheat flour or supplemented with selected soy-egg flours.

EXPERIMENTAL

DRUM-DRIED FLOURS were produced as described previously (Erdman et al. 1977). The flours produced included, soy (S), 75, 80, 85 parts soy to 25, 20, or 15 parts whole egg (dry basis) (75, 80, 85SE, respectively), 80 parts soy to 20 parts egg white (SEW), and 80 parts soy to 20 parts egg yolk (SEY). Fresh grade A eggs were used whole or as separate components after division with separator cups. Protein, fat, ash and moisture were determined for all flours according to standard AOAC (1975) methodology.

Enriched patent wheat flour (compliments of Purity Sunbeam Baking, Co., Champaign, IL: 11.5% protein, 0.45% ash, farinograph absorption 60.5%, moisture 13.8%) was supplemented with 12% of one of the soy or soy-egg flours. Breads were baked according to the K-State Process (Tsen and Tang, 1971) with slight modifications. The bread formula is found in Table 1.

Table 1—Bread formula

Ingredient[a]	Amount (g)	% of Patent flour[b]
Patent flour (14% H_2O basis)	700	100
Substituted flour (d.b.)	84	12
Yeast	21	3
Salt	14	2
Sugar	35	5
Shortening[c]	variable	variable
Water[d]	variable	variable
SSL	3.5	0.5
Bromate[e]	variable	variable

[a] Patent flour was a gift from Sunbeam Purity Bakers, Champaign, IL. Fleischmann's Compressed Yeast, SSL (Sodium stearoyl-2-lactylate) compliments of Patco Co., Kansas City, MO. Food Grade $KBrO_3$, Fischer Scientific Company.

[b] By weight

[c] Amount of shortening added varied according to the fat content of the substituted flour. In most studies where soy-egg yolk loaves were produced, fat content of all other loaves had to be raised to 4% with shortening to equal the high fat content of the soy-egg yolk breads.

[d] Amount of water used varied with the type of flour used and the presence or lack of SSL. Enough water was added to each to produce the proper texture during kneading.

[e] Bromate was added in amounts equal to 0, 15, 30, 45, 60 or 75 ppm of patent flour weight.

Compressed yeast was dissolved in warm water (40°C). Potassium bromate (if appropriate) was dissolved in water and both of these solutions were used to dissolve the sugar. The white flour, substituted flour, salt and SSL (if used) were thoroughly mixed and added to the previous solution alternately with more water to achieve the proper consistency. Shortening was added after the first flour addition. The dough was kneaded at low speed for 1 min in a Hobart Kitchen Aid Mixer, Model K5-A (Troy, OH) followed by medium speed until optimum development was reached (about four minutes). Doughs, scaled to 517g were allowed to ferment for 40 min at 30°C and 80% relative humidity in a fermentation chamber (National Mfg, Co., Lincoln, NB). The doughs were then shaped, panned and proofed under the same conditions until the top of the dough was 1.5 cm above the edge of the pan. Loaves were baked in a rotary oven (National Mfg. Co., Lincoln, NB) at 205°C for 40–50 min depending upon loaf type. (Soy supplemented loaves required longer heating times to be cooked to doneness).

Ten minutes after baking, specific loaf volumes were measured by the rape-seed displacement method (Cathcart and Cole, 1938). Subjective organoleptic evaluations of breads were performed on selected bread types by 10 membered panels 25 hr after baking. Each panel consisted of the same persons. A score sheet was developed using the criteria set forth by Rose and Solle (1968).

Protein efficiency ratios were determined to evaluate the protein quality of 12% substituted breads containing no SSL or bromate.

—Text continued on page 840

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All breads for PER studies were baked at 205°C for 50 min, sliced, crumbled, allowed to dry overnight, freeze-dried and milled. The resultant flours were formulated into 10% protein diets. Diets conformed to recommendations for Protein Efficiency Ratio Studies (AOAC, 1975). The PER study was carried out as described previously (Erdman et al., 1977).

The organoleptic and PER data were subjected to standard analysis of variance and least significant difference tests while the specific loaf volume data was analyzed with a 6 × 6 × 2 fractional design (Steel and Torrie, 1960).

Table 2—Composition of selected flours[a,b]

	S	80SE	SEW	SEY
Fat	24.8	31.2	19.5	37.0
Protein	51.2	48.0	57.0	46.2
Ash	3.8	3.8	3.8	3.5
Carbohydrate & fiber[c]	20.2	17.0	19.7	13.3

[a] Values are averages of duplicat samples taken from representative batches of flour and are expressed on a dry basis.
[b] S = full fat soy flour, 80SE = 80 parts soy to 20 parts whole egg (d.b.), SEW = 80 parts soy to 20 parts egg white, and SEY = 80 parts soy to 20 parts egg yolk.
[c] Carbohydrate and fiber are calculated by difference.

Table 3—Specific loaf volumes (cc/g) of soy & soy-egg supplemented breads at various bromate levels[a]

Flour type[b]	0.5%SSL	Bromate levels (ppm) 0	15	30	45	60	75
W	–	4.31	4.03	4.65	4.23	3.80	3.60
W	+	4.21	4.52	4.91	4.68	4.35	4.08
S	–	3.92	2.68	2.69	2.78	2.78	2.74
S	+	3.47	4.03	3.85	4.39	3.74	3.82
85SE	–	4.14	---[d]	3.56	3.88	3.81	3.31
85SE	+	4.10	---	4.07	4.17	4.40	4.13
75SE	–	3.19	3.05	3.47	3.46	3.43	3.45
75SE	+	3.87	3.87	3.98	3.97	3.94	4.77
SEY	–	4.34	4.06	4.15	4.31	4.27	---
SEY	+	4.33	4.33	4.35	4.50	4.26	---
SEW	–	2.62	---	2.68	2.96	2.74	---
SEW	+	3.88	---	4.23	4.07	4.09	---

Source of variation	Analysis of variance[c] df	ss	ms	f
A Flour type	5	16.82	3.36	238.2 **
B Bromate level	5	0.56	0.11	7.9 **
C SSL	1	12.61	12.61	893.9 **
AB	25	3.91	0.16	11.1 **
AC	5	4.47	0.89	63.4 **
BC	5	1.56	0.31	22.1 **
ABC	25	2.48	0.10	7.0 **
Error	56	0.79	0.014	---
Total	127	43.20	---	---

[a] Values represent the average of two loaves.
[b] W = 100% patent wheat, S = 12% supplemented soy flour, 85SE = 12% supplemented 85SE flour (85SE flour = 15 parts whole egg plus 85 parts soy flour), 75SE = 12% supplemented 75SE flour (75SE = 25 parts egg plus 75 parts soy flour) SEY = 12% supplemented SEY flour (SEY flour = 20 parts egg yolk plus 80 parts soy flour), SEW = 12% supplemented SEW flour (SEW flour = 20 parts egg white plus 80 parts soy flour), SSL = sodium stearoyl-2-lactylate.
[c] A 6 X 6 X 2 factorial design was used for analysis; **P < 0.01.
[d] Dashes (---) indicate that no breads were baked utilizing these specifications.

RESULTS & DISCUSSION

THE PROXIMATE COMPOSITION of selected test flours appears in Table 2. The fat content varied with the part and amount of egg added; increasing with yolk addition and decreasing with egg white addition. Incorporation of whole egg or egg yolk decreased the protein content while egg white increased protein content. The ash content ranged between 3.5 and 3.8% on a dry basis. The calculated content of carbohydrate (digestible plus indigestible) decreased with egg addition as expected.

The specific loaf volumes of six flour types with and without 0.5% SSL, and containing 0, 15, 30, 45, 60 or 75 ppm of bromate are found in Table 3. In general, SEY and 85 SE supplemented loaves approximated all-wheat flour loaves while S and SEW loaves were smaller than the 100% wheat flour loaves. Twelve percent soy supplementation is known to adversely affect loaf volume (Tsen et al., 1971 and Erdman et al., 1977). The volumes of bromate-free loaves produced in this study are increased (about 1 cc/g) compared to the previous 50% sponge procedure (Erdman et al., 1977) but 100% wheat flour and 12% soy flour-supplemented loaf volumes are not as large as produced by Tsen and Tang (1971). In the 50% sponge study, SSL improved specific loaf volumes of the 100% wheat flour and 12% soy-supplemented breads but not the soy-egg supplemented breads. This differential effect was attributed to the natural emulsifiers in egg that were thought to provide the same surface active properties as SSL.

In the present study and under the specified conditions, SSL improved loaf volumes of most bread types; the greatest increase in volume occurred in soy and soy-egg white supplemented loaves. Soy-egg yolk breads, which contain the greatest quantities of egg emulsifiers, were affected the least by SSL at the various bromate levels. Bromate addition affected loaf volumes of most loaf types. However, it is apparent that each of the flours react differently to variation of $KBrO_3$ level. In general, the medium supplementation levels, 30 or 45 ppm, resulted in the highest loaf volumes. Statistical analyses revealed significant interaction ($P < 0.01$) of SSL, flour types and bromate levels; the addition of bromate improving the action of SSL in most cases.

Based upon the results of loaf volume analysis, 45 ppm $KBrO_3$ was chosen as a good supplementation level for organoleptic evaluation. Additional breads were baked to contain (a) neither SSL or $KBrO_3$; (b) 0.5% SSL without $KBrO_3$; or (c) 0.5% SSL plus 45 ppm $KBrO_3$. The results of the taste panels are found in Table 4. Each quality tested was significantly ($P < 0.01$) affected by both flour type and treatment. The loaves containing neither SSL nor $KBrO_3$ were generally scored the lowest in each category. In the case of the SEY breads, significant differences due to treatment were only found in one of the nine categories (crust), indicated that SEY is least improved by additional emulsification or oxidant. One hundred percent wheat loaves scored near the top in each category, while S, 75 SE and SEW loaves were commonly rated inferior to the others. The poor quality of S and SEW loaves is predictable from the loaf volume data in Table 3 and from previous results (Erdman et al., 1977), while 85SE ratings were lower than expected. Figure 1 shows representative loaves of experimental breads whose evaluation appears in Table 4.

The results of the PER studies of ground bread diets appear in Table 5. Since 100% wheat diets are severely deficient in lysine, and since bread loses additional lysine during staling (Rosenburg and Rohdenburg, 1951), it is not surprising that the wheat bread-based diets resulted in the poorest growth response. The corrected PER value of 0.38 for white bread is considerably lower than that obtained by other researchers (0.79 by Tsen et al., 1977 and approxi-

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Table 4—Organoleptic evaluation of breads[a]

Flour type[b]	Treatment SSL	Treatment $KBrO_3$	Shape	Crust	Volume	Texture	Grain	Crumb color	Flavor	Aroma	General desirability
W	0	0	6.5	5.5	5.9	6.8	5.4	7.3	7.2	7.2	6.6
	0.5%	0	8.8	7.3	8.7	7.6	8.1	8.5	8.4	7.5	8.1
	0.5%	45ppm	6.8	7.6	7.5	8.0	6.8	8.3	7.7	7.5	7.7
S	0	0	4.0	4.2	2.8	5.1	4.0	5.2	5.2	5.2	5.3
	0.5%	0	7.2	7.1	6.5	7.6	7.1	7.2	7.5	6.0	7.4
	0.5%	45ppm	6.5	7.5	6.5	8.0	6.1	6.9	7.7	7.2	7.2
85SE	0	0	4.8	4.3	5.2	5.7	4.5	5.5	5.7	5.7	5.6
	0.5%	0	6.2	6.5	7.4	6.8	6.4	7.0	6.3	6.4	6.3
	0.5%	45ppm	6.0	6.1	6.4	7.2	4.1	6.6	6.8	6.6	6.5
SEW	0	0	3.9	4.2	2.9	5.0	4.1	5.1	5.2	5.3	4.9
	0.5%	0	5.5	5.8	5.4	6.6	4.8	7.0	6.7	7.0	5.9
	0.5%	45ppm	6.8	7.3	5.8	7.2	7.3	7.1	7.3	7.1	6.7
SEY	0	0	7.1	5.8	6.8	6.6	7.0	7.3	6.2	5.3	6.3
	0.5%	0	6.5	5.1	7.3	7.5	6.2	6.5	6.5	5.4	6.6
	0.5%	45ppm	7.3	6.9	7.3	7.1	6.2	6.5	6.5	6.2	6.8
lsd ($P < 0.01$)[c]			1.4	1.5	1.3	1.4	1.3	1.1	1.6	1.3	1.4

[a] Values represent average of 10 responses where 1 = very poor, 9 = very good.
[b] W = 100% patent wheat flour; S = 12% supplemented soy flour; 85SE = 12% supplemented 85SE flour; SEW = 12% supplemented SEW flour; SEY = 12% supplemented SEY flour; SSL = Sodium stearoyl-2-lactylate.
[c] lsd refers to least significant difference test. Values can be used for comparisons within an individual bread quality.

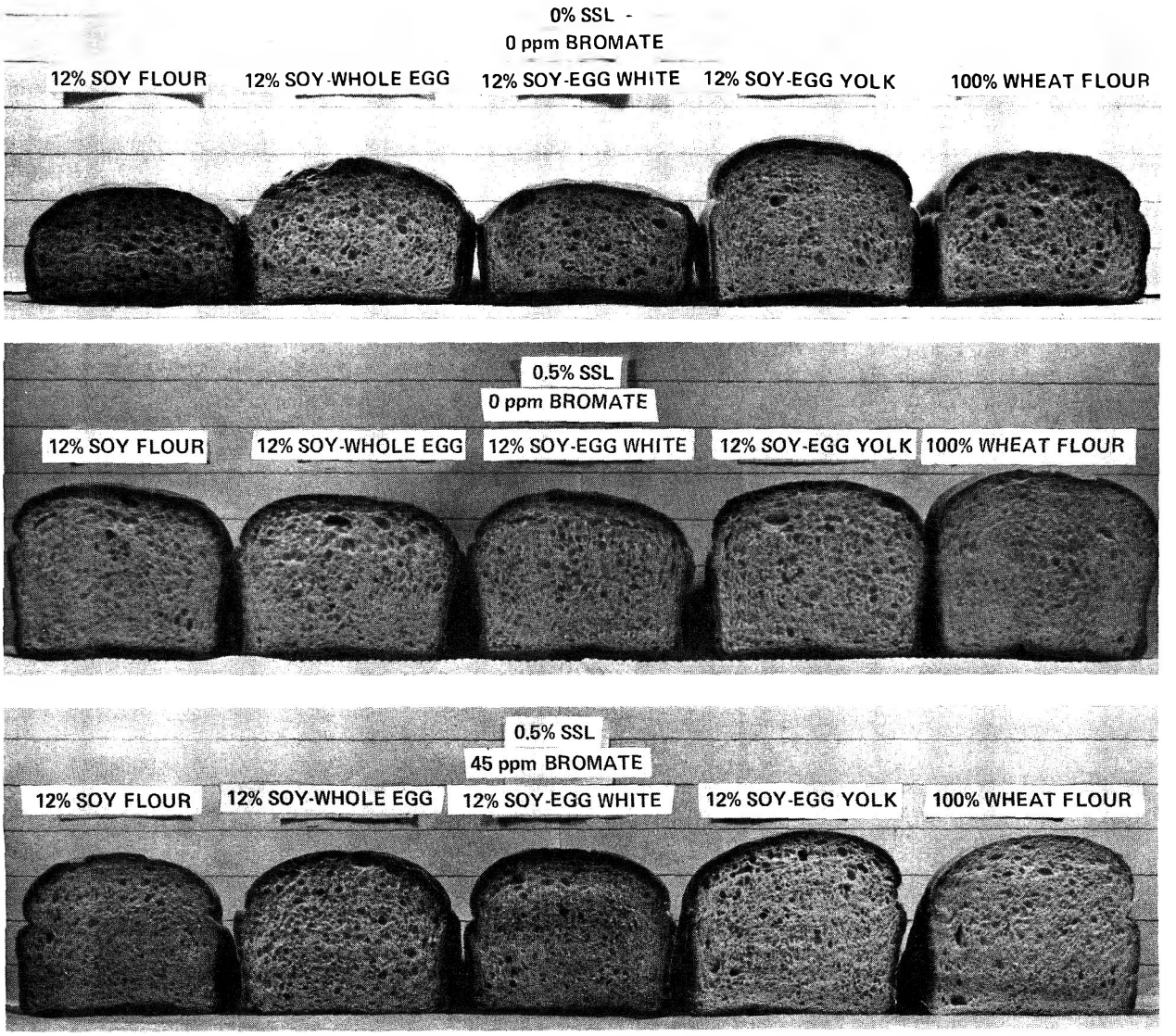

Fig. 1—Typical breads prepared with 100% patent wheat flour, wheat flour plus 12% supplementation of S, 80SE, 80SEW, or 80SEY, with or without SSL and/or Bromates.

Table 5—Protein efficiency ratios of breads[a]

Protein source[b]	Food consumed (g)	Weight gain (g)	Uncorrected PER[c]	Corrected PER[d]
100% Wheat	177.1	7.7	0.43	0.38w
88% Wheat 12% 80SEY	205.2	22.3	1.04	0.92x
88% Wheat 12% S	189.7	20.4	1.07	0.95xy
88% Wheat 12% 80SE	203.7	24.3	1.16	1.03xy
88% Wheat 12% 80SEW	194.2	23.0	1.19	1.06y
Casein	258.0	72.7	2.82	2.50z

[a] Data represent average of 10 animals.
[b] All diets contain 10% protein. 80SEY = 80 parts soy plus 20 parts egg yolk; S = full fat soy flour; 80SE = 80 parts soy plus 20 parts whole egg; 80SEW = 80 parts soy plus 20 parts egg white.
[c] PER = Protein Efficiency Ratio (wt gain/protein consumed) calculated after 28 days.
[d] Vertical means with unlike letter differ at $P < 0.01$.

mately 1.0 by Hoover, 1974). This is probably due to the longer baking time employed in the current study. In order to ensure equal heat treatment for all bread types, all loaves were baked at 205°C for 50 min. This rather long baking time was required to bake soy and soy-egg supplemented loaves to doneness. The 100% wheat flour loaves were probably overbaked as their optimal cooking time was about 40 min. Therefore, the resulting protein quality of these loaves was reduced. Jansen and coworkers (1964) have shown that available lysine, as well as breads' growth-supporting potential decreases with baking time.

All soy and soy-egg supplemented breads resulted in significantly increased PER's compared to the all-wheat breads. The PER of soy loaves is comparable to that reported by Turro and Sipos (1968) but is less than reported by Tsen et al. (1977) and Hoover (1974). Whole egg, egg white or egg yolk supplementation did not result in further improvement in PER compared to soy loaves.

The PER of SEW bread is significantly higher than that of SEY bread. Due to the differences in protein concentration (dry basis) between egg yolk (32%) and egg white (87%) flours, the proportion of total protein in the bread from egg varies from about 11% for the SEW to 4% for the SEY loaves. Since egg protein is high in methionine, the limiting amino acid of soy, and lysine, the limiting amino acid of wheat, the increased egg protein supplementation should improve the overall amino acid profile of the bread by supplying more of these amino acids.

Although the effects of flour type, SSL and/or bromates upon various handling, fermentation and baking variables were not investigated in the present study, it seems clear under the conditions set forth that soy-egg yolk flour utilization in breads results in breads with good loaf volume and organoleptic acceptability. Soy-whole egg breads also had acceptable quality, while soy and soy-egg white loaves were less acceptable, even when fortified. All soy and soy-egg fortified breads had greater protein quality than the 100% wheat breads. Therefore, drum drying soy with egg yolk and to some extent, whole egg resulted in a reduction of the inferior functional properties of soy-supplemented breads baked by the K-State procedure. Horn (1977) has theorized that co-processing of egg and soy creates beneficial lipid/lipoprotein structures which allow for a reduction of egg use in some food systems. The positive nature of the functional characteristics of SEY flour in breads suggests its use as a nutritional supplement in baked goods.

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EFFECTS OF STORAGE CONDITIONS ON FIRMNESS OF BRINED CUCUMBERS

R. L. THOMPSON, H. P. FLEMING and R. J. MONROE

ABSTRACT

Brined, fermented cucumbers were stored up to 1 yr under various conditions of pH (3.3, 3.8), NaCl concentration (5.5, 11.4%), temperature (4.4, 15.5, 26.6°C), storage time (3, 6, 9, 12 months), and $CaCl_2$ addition (0.1%). Polygalacturonase (PG) activity in the brine was negligible. Firmness of the cucumbers was determined with a Magness-Taylor fruit pressure tester. Temperature had the greatest influence upon retention of firmness, but effects of pH, % NaCl, and storage time also were significant ($P \leq 0.01$). Rates of firmness loss ranged from <0.05 to ca 0.5 lb/month, depending upon pH, % NaCl and temperature. Firmness retention was greatest at 4.4–15.5°C, pH 3.8 and 11.4% NaCl. A prediction equation for the rate of firmness loss was developed. Addition of 0.1% $CaCl_2$ to the brine resulted in firmer cucumbers, particularly those stored at pH 3.3 ($P < 0.01$). Results indicated that firmness of cucumbers can be retained at lower brine strengths than currently used, provided the cucumbers are washed prior to brining to remove softening enzymes as in the controlled fermentation process, and temperature of the brine stock is maintained at ca 15.5°C or lower.

INTRODUCTION

FIRMNESS and crispness are primary traits affecting consumer acceptance of pickled cucumbers. Variations in firmness among cucumber cultivars may account for differences in quality of the pickled product (Jones et al., 1954; Breene et al., 1973; Sneed and Bowers, 1970). Processing methods undoubtedly play a major role in firmness. The firmness of fresh pack cucumbers is affected by variables such as pasteurization time and temperature, pH and acidity, and storage time and temperature (Monroe et al., 1969, Fellers and Pflug, 1965; Pangborn et al., 1959).

Firmness of brined cucumbers depends on the vagaries of microbial activities as well as the storage conditions of the cucumbers. Microbial polygalacturonases, whether introduced into the brining tank by attachment to the fruit or flower as shown by Etchells et al. (1958) or produced in the brining tank, can greatly reduce the firmness of the brined cucumbers. The initial load of such enzymes–particularly on small fruits, which retain a greater percentage of polygalacturonase-laden flowers–could be effectively reduced if the brining tank is drained after ca 36 hr (Etchells et al., 1955). Microbial polygalacturonases, active in softening brined cucumbers, are inhibited by high levels of NaCl (Bell and Etchells, 1961); and briners have used high levels of salt to retard enzymatic softening. Increasing difficulties with salt disposal have caused briners to seek means of reducing the level of salt used.

The controlled fermentation process of Etchells et al. (1973) provides a possible means of storing brined cucumbers at low levels of salt. The process includes a washing step to remove soil, enzymes and unwanted microorganisms. Thus, softening of cucumbers by microbial polygalacturonases should be low. Under these conditions the major factors affecting the firmness of brined cucumbers would include temperature, pH, salt concentration, and storage time.

The purpose of this study was to determine the relative importance of the above factors on the firmness of cucumbers fermented by the controlled fermentation process. Also, effects of calcium chloride addition to the storage brine on firmness were evaluated.

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MATERIALS & METHODS

THE CUCUMBERS used in these studies were brined according to the controlled fermentation process of Etchells et al. (1973). In Experiment I, cucumbers (size no. 3, 3.8–5.1 cm diam) free from disease and mechanical injury were carefully hand washed and brined under laboratory conditions in four, 44-gal, pilot-scale brining tanks described earlier (Fleming et al., 1977). One month after brining, the fully fermented, essentially bloater-free, brine-stock cucumbers were used for storage studies. The brine analyses of these fermented cucumbers before further adjustments were: pH 3.5, 1.18% acid (as lactic) and 5.4% NaCl.

In Experiment II, cucumbers (size no. 2, 2.5–3.8 cm diam) were brined in a 1,000-bu commercial tank. The cucumbers were tanked by fluming in ca 7% NaCl; they were then covered with brine, drained, and re-covered with brine. The cucumbers received no other washing treatment. Brine analyses of the fully fermented cucumbers, ca 1 month after brining, were: pH 3.5, 0.58% acid (as lactic), and 6.1% NaCl.

Salt-stock cucumbers, prepared as described above for Experiments I and II and free of visible injury and of uniform size, were thoroughly mixed and separated into four lots. Two lots of cucumbers with sufficient brine to cover them were adjusted to 11.4% w/v NaCl and two lots were adjusted to 5.5% NaCl. Also, lots of cucumbers from each salt concentration were adjusted with NaOH or lactic acid to pH 4.00 or 3.5 ± 0.05 in Experiment I, and pH 3.8 or 3.3 ± 0.05 in Experiment II. The design of Experiment II is illustrated in Fig. 1. Experiment I was designed similarly, but only two incubation temperatures were used.

The cucumbers were packed into 1-gal jars, 20 per jar in Experiment I and 25 per jar in Experiment II (pack-out ratio cucumbers:brine, ca 60:40 in each experiment), and placed into incubators at 15.5 and 26.6°C in Experiment I and 4.4, 15.5, and 26.6°C in Experiment II. At 3-month intervals for 12 months, duplicate jars from each treatment were removed, equilibrated to room temperature (24 ± 2°C) for 48 hr, and analyzed for firmness by use of a Magness-Taylor fruit pressure tester (FPT) equipped with a 0.79-cm tip, according to Bell and Etchells (1961). All FPT readings were made by the same individual. Adjective ratings corresponding to FPT readings (Etchells et al., 1973) were: for size no. 2 cucumbers, very firm (18 lb and above), firm (15–18 lb) and inferior (11–14 lb); for size no. 3 cucumbers, very firm (20 lb and above), firm (16–19 lb) and inferior (11–15 lb).

Brines were analyzed for polygalacturonase activity by the method of Bell et al. (1955).

A split-plot design was utilized in the layout of the experiments. Due to the lack of replicates for the pH and salt adjustment treatments, a pseudo-error structure was developed for the purpose of statistical analyses. Four replications, each consisting of five (Experiment I) or six (Experiment II) cucumbers, randomly selected from all jar-treatment combinations, were used to create the error structure for the appropriate test of main-plot and sub-plot effects. The resulting analyses yielded a mean square error (MSE) for sub-plots essentially the same as the residual MSE computed from the difference among individual cucumbers. The residual MSE was considered appropriate for the statistical tests.

Calcium chloride, to equilibrate at 0.1%, w/v, was added to the

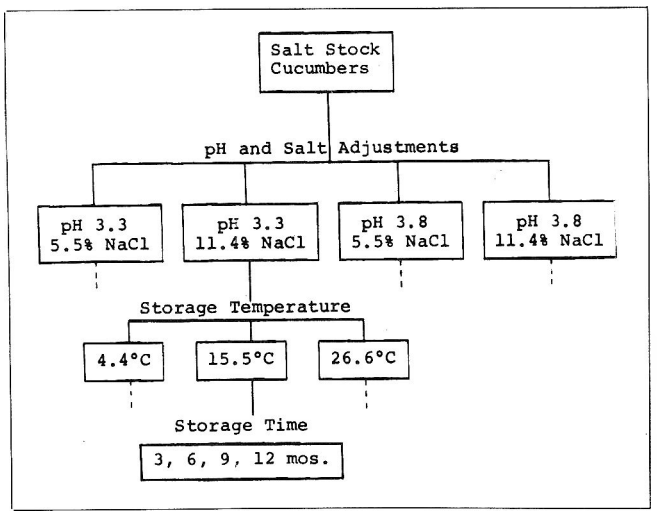

Fig. 1—Experimental design for storage study of salt-stock cucumbers.

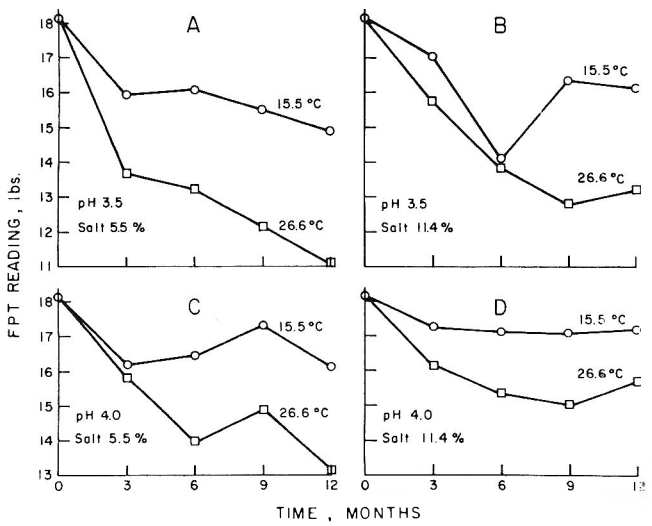

Fig. 2—Changes in firmness of size no. 3 salt-stock cucumbers during storage. See Experiment I.

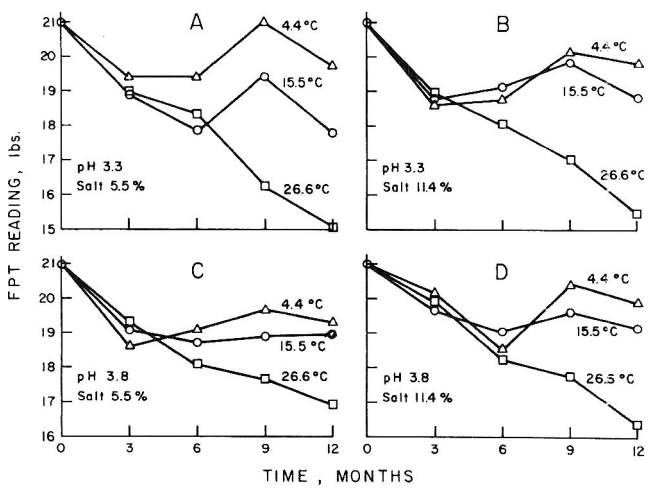

Fig. 3—Changes in firmness of size no. 2 salt-stock cucumbers during storage. See Experiment II.

cover brines of 16 additional 1-gal jars of cucumbers used in Experiment II. Duplicate jars of cucumbers with and without added calcium chloride were stored at 15.5 and 26.6°C at the pH and salt concentrations indicated in Figure 1. Pressure tests were made after 6 months' storage.

RESULTS

BRINES from Experiments I and II were "negative to weak" (ranging from 0–3 units) in polygalacturonase activity, when analyzed according to Bell et al. (1955), which we consider to be negligible in this study. Effects of four factors (pH, NaCl concentration, temperature, and time) on the firmness of brined cucumbers are shown in Figures 2 and 3. Generally, the factors similarly affected the cucumbers fermented under laboratory (Fig. 2) and commercial conditions (Fig. 3). Firmness retention was greatest at low temperature, high salt concentration, and high pH. By the end of the 12 months, the loss in FPT reading for cucumbers stored at 4.4°C was less than 2 lb, regardless of pH and salt concentration (Fig. 3). Firmness retention was slightly lower at 15.5°C, than at 4.4°C, but strikingly less at 26.6°C after 9 months' storage (Fig. 2 and 3). At 4.4 and 15.5°C, FPT readings became nearly constant after 3 months' storage; but readings steadily declined at 26.6°C (Fig. 3).

Effects of individual factors on firmness of the brined cucumbers when averaged over all other factors, and statistically significant interactions, are given in Table 1. Again, temperature was indicated to be the most important factor influencing firmness ($P \leq 0.01$, Table 2). The overall reduction in FPT readings due to storage at high (26.6°C) as compared to low (15.5°C) temperatures was 2.2 lb in Experiment I and 1.4 lb in Experiment II. Firmness loss was greater at low than at high pH values in both experiments ($P \leq 0.01$, Table 2). The overall reduction in FPT readings due to low pH was greater in Experiment I (1.5 lb) than in Experiment II (0.3 lb) (Table 1). Firmness loss was greater at 5.5 than 11.4% NaCl ($P \leq 0.01$ and 0.05 for Experiments I and II, respectively, Table 2). The overall reduction in FPT readings due to low salt concentration was 0.8 lb in Experiment I and only 0.2 lb in Experiment II.

Data from Experiment II were summarized to show the

Table 1—Effects of pH, salt, temperature, and time on the firmness of brined cucumbers[a]

Variable	FPT (lb) Exp I	Exp II
pH		
3.3	–	18.5
3.5	14.4	–
3.8	–	18.8
4.0	15.9	–
Salt, %		
5.5	14.8	18.6
11.4	15.6	18.8
Temperature, °C		
4.4	–	19.5
15.5	16.3	19.0
26.6	14.1	17.6
Time, months		
0	18.2	20.9
3	16.0	19.2
6	15.0	18.6
9	15.1	19.0
12	14.7	18.1

[a] FPT readings for pH, salt, and temperature are averaged over the 3–12 month readings. Readings for 3, 6, 9 and 12 months, are averaged over pH, salt and temperature. Zero time readings were determined before any adjustments.

effect of temperature on the rate at which firmness was lost during storage (Fig. 4).

Addition of 0.1% calcium chloride to storage brines of cucumbers resulted in increased FPT readings after 6 months' storage at all combinations of temperatures, salt concentration, and pH tested (Table 3). The greatest increases in FPT readings due to calcium chloride addition occurred in cucumbers stored at pH 3.3 ($P \leq 0.01$ for three of four treatment combinations). At pH 3.8, only one treatment combination resulted in a significant increase in FPT reading due to calcium chloride addition (5.5% salt, 26.6°C, Table 3).

DISCUSSION

ALTHOUGH polygalacturonase activity in storage brines in this study was considered to be negligible, low levels of enzyme activity could contribute to the slow softening of cucumbers under prolonged storage. Also, the pectinolytic enzyme activity of the cucumber fruit might promote softening, particularly at low salt concentration and high storage temperature. Cucumbers contain a polygalacturonase (Pressey and Avants, 1975).

These storage studies indicated that the firmness of brined cucumbers can be maintained at 5.5% NaCl (20° salometer), provided the storage temperature is 15.5°C or lower and the polygalacturonase activity is negligible or within the limits used in these experiments. Thus, if proper conditions are maintained, it may be unnecessary to increase brine strength to between 11.4 and 17.6% NaCl (40–60° salometer) after the fermentation period, as has been the practice for many years. In geographic locations where freezing is not a problem, it may be possible to maintain brines below 8.4% (30° salometer) during storage. Further studies are needed to confirm these conclusions.

Storage temperature was the most important factor influencing firmness of brined cucumbers in this study. Storage temperature also has been shown to be a highly important factor influencing firmness of fresh pack cucumbers, the rate of firmness loss being particularly noticeable above ca 26.6°C (Pangborn et al., 1959; Nicholas and Pflug, 1960; Fellers and Pflug, 1965). At 1.1°C, the firmness of fresh pack pickles increased upon storage (Pangborn et al., 1959), which is consistent with our observation that firmness of brined cucumbers may increase at some point during storage (see e.g. Fig. 3, 4.4°C, the higher FPT readings at 9 and 12 months than at 3 and 6 months).

Firmness retention might be improved if low pH brine (e.g. pH 3.3) after fermentation is adjusted to ca pH 3.8. This adjustment can be made with NaOH, but partial adjustment with lime [$Ca(OH)_2$] up to about 0.07% (calcium equivalent of 0.1% $CaCl_2$), would improve firmness of the brine stock. Such adjustments [neutralization of lactic acid with NaOH or $Ca(OH)_2$] should be made carefully to avoid exceeding pH 4.0. It would be unwise to partially neutralize the fermentation acids in brines contained in tanks which leak excessively. Brine is customarily added to compensate for losses, but the added brine usually is not acidified; hence, acidity may be reduced below the level needed for preservation.

Design and type of materials used in the construction of brining tanks may influence the environmental conditions under which brine stock are held and, thus, the quality of the cucumbers. For example, underground storage tanks should maintain brined cucumbers at a fairly uniform temperature, reducing seasonal extremes due to ambient temperatures. Thus, cooler brine temperatures in summer would benefit product firmness while warmer temperatures in winter would allow the use of lower brine strengths without freezing damage. Although wooden tanks have been the

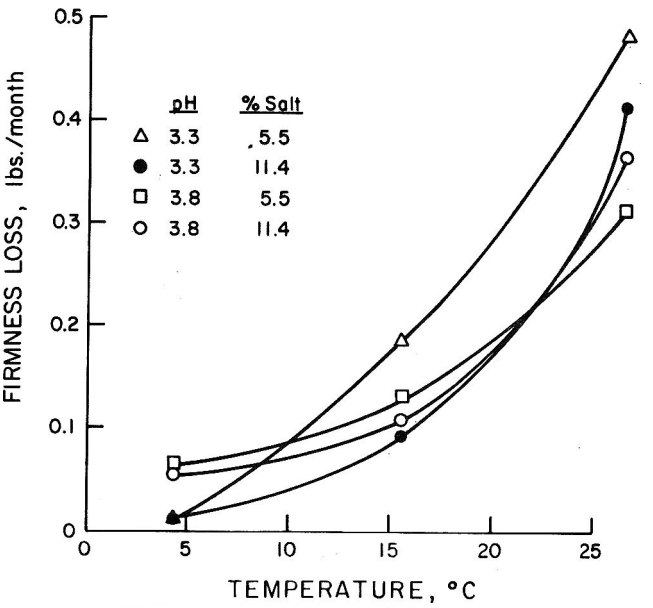

Fig. 4—Effect of temperature on firmness loss of salt-stock cucumbers during storage. Data based on Experiment II.

Table 2—Analysis of variance for factors affecting firmness of brined cucumbers

Source of variation	Degrees of freedom	Mean square
Experiment I		
pH	1	488.15**
Salt	1	282.04**
Temperature	1	1353.35**
Time	3	59.90**
pH X temperature	1	28.23*
Temperature X salt	1	42.05*
Time X salt	3	39.62*
Time X temperature	3	43.44*
Error	1118	7.048
Experiment II		
pH	1	56.93**
Salt	1	36.38*
Temperature	2	732.91**
Time	3	136.40**
pH X temperature	2	41.94**
Time X temperature	6	181.18**
Error	2254	6.51

* Denotes $P \leq 0.05$.
** Denotes $P \leq 0.01$.

Table 3—Effect of $CaCl_2$ addition on firmness of brined cucumbers[a]

	Average increase in FPT readings due to added $CaCl_2$			
	pH 3.3		pH 3.8	
	Salt, %		Salt, %	
Temp (°C)	5.5	11.4	5.5	11.4
15.5	4.1**	1.9**	1.0	.75
26.6	2.4**	1.0	1.5*	.52

[a] Used size no. 2 cucumbers, as described for Experiment II. Asterisks denote levels of significance for the increase in FPT readings of brined cucumbers to which $CaCl_2$ was added as compared to cucumbers without added $CaCl_2$; $P < 0.01$ (**), $P < 0.05$ (*).

primary storage vessel for brined cucumbers, some companies are testing and using coated concrete and fiberglass tanks to some extent. Both of these latter type tanks could be placed underground. In southern California, Spanish-type green olives are presently being fermented and stored in underground fiberglass tanks (Webster, 1978).

We attempted to predict the effects of the variables we studied on firmness of brined cucumbers. By response surface analysis, an equation for predicting firmness loss of brined cucumbers was developed, based on data obtained in Experiment II:

$$\frac{\text{FPT lb loss}}{\text{month}} = -0.47 + 0.042\,(^{\circ}\text{C}) + 0.00071\,(^{\circ}\text{C})^2 + 0.16\,(\text{pH}) - 0.0039\,(\%\,\text{NaCl}) - 0.014\,(^{\circ}\text{C} \times \text{pH})$$

The rate of firmness loss for each pH, salt, and temperature treatment (Fig. 4) was used as the dependent variable in a multiple linear regression analysis. Salt, pH, temperature, and selected interactions were the independent variables. Correlation coefficients between measured and predicted FPT readings were 0.81 and 0.86 for Experiments I and II, respectively. The above equation would be relevant only when polygalacturonase activity in the brine is negligible.

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SENSORY QUALITY OF CUCUMBERS BEFORE AND AFTER BRINING

DANIEL M. ENNIS and J. O'SULLIVAN

ABSTRACT

Cucumbers were evaluated on sensory characteristics by panels of 5–6 judges before and after brining. Multiple regression analysis of the data resulting from word-anchored sensory scales revealed that only three characteristics, color, shape and seed size, influenced overall quality before brining. These characteristics separated out on a 60:27:13 basis using a 100-point scale. Texture differences between the 24 cultivars tested were not sufficient to include texture in the model before brining. Twenty cultivar-treatments were evaluated after brining on nine sensory characteristics of which color, seed size, crispness and firmness were significant. The relative importance of these characteristics was 38:27:21:14 on a 100-point scale.

INTRODUCTION

IN A RECENT REVIEW on cucumber quality (Ennis and O'Sullivan, 1979), we pointed out the need for quantitative justification for the selection of particular scores for quality characteristics in numerical rating systems for cucumbers. The problem is further exacerbated by the fact that sensory rating of cucumbers before brining may differ from rating after brining so that care should be taken in interpreting fresh cucumber quality characteristics in relation to brined quality.

The purpose of the work reported here was to examine the quality components of cucumbers before and after brining to determine what, if any, differences exist in sensory quality due to processing and to assign numerical scores to the statistically important components using multiple regression.

The selection of a reliable sensory rating scale for parametric statistical use is not an easy task. Since Peryam and Pilgrim (1957) introduced the hedonic rating scale, there has been considerable research activity on word category, numerical, semi-structured and ratio scales (Chapman and Wigfield, 1970; Gridgeman, 1960; Howard, 1972; Moskowitz, 1975; Stone et al., 1974; Cloninger et al., 1976). For the purpose of this study, the word-anchored scale was chosen because of published evidence supporting equal spacing on the scale.

MATERIALS & METHODS

Fresh quality

Twenty-four cultivars of cucumbers in the 2–3 grade size ranges (2.50–4.13 cm diam) were evaluated by six judges drawn from pickle processing companies in Ontario and the Ontario Horticultural Experiment Station at Simcoe. All of these individuals had extensive experience in judging cucumbers for commercial use as brined stock and preliminary sessions were held to define and agree on the meaning of the sensory terms used. The cucumbers were assessed on a word-anchored scale of 12.7 cm (5 in.) for shape, ridges and spines, color, cavity (placenta) size, seed size, and overall quality.

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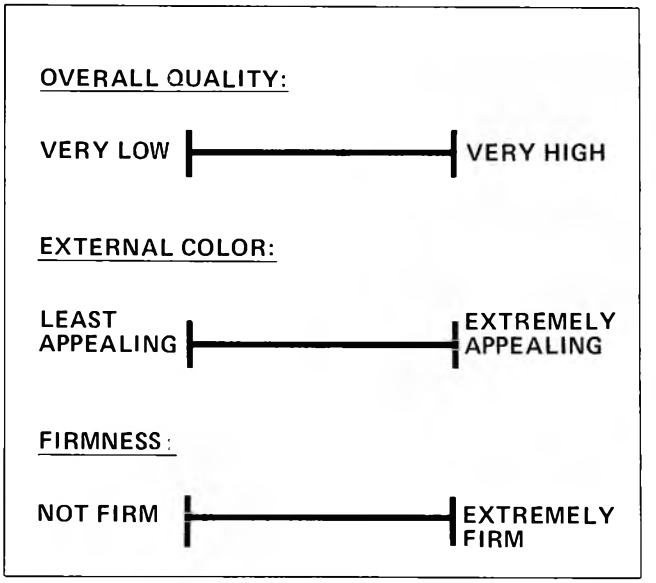

Fig. 1—Examples of word-anchored scale lines. The panelist scored the characteristic by placing a vertical bar on the line according to the intensity of the response required.

Brining conditions

The cucumbers used in the brining phase of the study were in two groups differing in length of time in brine. The first group were harvested in August 1977 and held in an 8-ton wooden brining tank 8 ft in height. The cucumbers were placed 5 ft from the top and the cover brine was increased from 20° salometer to 45° salometer during the 90-day period. The cucumbers were stored under ambient conditions, and when removed from the tank were desalted to 7–8° salometer before tasting. The second group was brined under identical conditions but was harvested in August 1976 and brined for 15 months before assessment. Two of the four cultivars in this group had been treated with ethephon during the growing season.

Brined quality

Twenty cultivars were evaluated by 5 panelists in a similar manner to the fresh cucumbers for shape, ridges and spines, external color, internal color uniformity, crispness, firmness, placenta size, seed size and overall quality. The length of the word-anchored scale line was reduced to 10 cm for ease of interpretation of the sensory means.

Sensory testing conditions

The sessions were conducted in individual isolation booths under white lights and all of the cultivars (either fresh or brined, but not both) were assessed in one session. Descriptive terms were used in each case to anchor the line on which panelists marked the extent of the sensory response for each criterion. Examples of the scale are given in Figure 1. The length: diameter ratio (L:D ratio) was also recorded and included in the multiple regression model as an independent variable for fresh cucumbers. A randomized block design was used and the samples were coded with 3 digit numbers taken from a random number table.

Scale establishment

In order to establish a 100-point scale for cucumber quality

assessment, the relative importance of each criterion was calculated from the partial regression coefficients as a fraction of the coefficient of determination (Kramer and Twigg, 1970) as follows:

$$\text{Color: Score} = R^2 \left(\frac{100\, b_A}{(b_A + b_B + b_C)} \right)$$

$$\text{Shape: Score} = R^2 \left(\frac{100\, b_B}{(b_A + b_B + b_C)} \right)$$

$$\text{Seed size: Score} = R^2 \left(\frac{100\, b_C}{(b_A + b_B + b_C)} \right)$$

Table 1—Mean comparisons for 24 cultivars of fresh cucumber with respect to sensory scores (12.7 best and 0.0 poorest for shape, color and overall quality; 0.0 smallest and 12.7 largest for seed cavity and seed size)[a]

Cultivar	Score				
	Shape	Color	Cavity size	Seed size	Overall quality
C589	7.0 abcd	3.2 ghi	5.1 cd	4.3 gh	4.0 cdef
XP 76	7.5 abc	5.6 bcdef	7.4 ab	7.8 abc	5.6 abc
XP 1149	6.8 abcd	6.6 abc	6.2 abcd	5.2 efgh	5.7 abc
FX 4164	4.8 cde	6.5 abcd	7.1 abc	8.7 a	5.1 bc
EXP 823	6.2 abcde	5.5 bcdefg	5.9 abcd	5.3 efgh	7.1 ab
La Salle	4.6 de	6.5 abcd	5.9 abcd	5.5 efgh	4.7 cde
NCX 5003	6.0 abcde	8.5 a	6.1 abcd	6.4 bcdef	7.1 ab
Multipick	4.7 cde	5.1 bcdefghi	6.5 abcd	6.2 bcdefg	4.6 cdef
Calypso	5.8 abcde	3.0 hi	5.3 bcd	3.6 h	4.8 cde
Pioneer	5.1 cde	8.6 a	6.1 abcd	5.2 efgh	7.2 a
Premier	6.8 abcd	7.1 ab	6.2 abcd	5.9 cdefg	5.5 abc
Trispear	6.3 abcde	2.8 j	6.7 abc	5.8 defg	2.9 ef
H-1077	7.0 abcd	7.3 ab	7.0 abc	7.9 ab	5.9 abc
CP 183	6.8 abcd	3.6 efghi	5.8 abcd	5.7 efg	4.3 cdef
Green Beauty	3.6 e	5.9 bcde	7.7 a	6.0 bcdefg	2.6 f
Earlypick 14	6.3 abcde	3.4 fghi	6.9 abc	5.1 efgh	4.0 cdef
Tally	8.1 a	7.0 ab	5.4 bcd	5.2 efgh	7.1 ab
Pennant	4.7 de	4.5 cdefghi	4.4 d	4.2 gh	3.4 def
Bounty	7.9 ab	8.2 a	6.4 abcd	7.0 abcde	7.4 a
Liberty Bell	5.4 abcde	5.0 bcdefghi	5.9 abcd	5.4 efgh	4.5 cdef
Femcap	4.9 cde	4.4 cdefghi	6.2 abcd	6.0 bcdefg	4.3 cdef
Green Star	6.1 abcde	4.2 defgh	5.3 bcd	7.7 abcd	5.7 abc
Capir	4.8 cde	4.1 efgh	4.4 d	6.2 gh	3.9 cdef
Caroline	5.3 bcde	5.2 bcdefgh	5.3 bcd	4.7 fgh	4.9 cde

[a] Means within columns followed by the same letter are not significantly different at the 5% level using Duncan's Multiple Range test

Table 2—Multiple regression parameters for fresh cucumber quality

Variable entered	Multiple R	Multiple R^2	F-to-enter[a]
Color	0.78	0.61	217.28
Shape	0.82	0.68	26.33
Seed size	0.83	0.69	5.62

Multiple Regression Equation:

$$Q = 1.50 + 0.59(A) + 0.22(B) - 0.11(C)$$

Q = Overall Quality
A = Color
B = Shape
C = Seed Size

[a] All F-ratios are significant at >95% confidence

where, b_A = partial regression coefficient for color; b_B = partial regression coefficient for shape; and b_C = partial regression coefficient for seed size.

RESULTS

Fresh quality

An analysis of variance for each sensory response before brining showed that the F-test was significant at the 95% level for shape, color, cavity size, seed size and overall quality. The mean scores for the 24 cultivars were separated using Duncan's Multiple Range test and are shown in Table 1. The cultivars yielding significantly the highest scores for overall quality included Bounty, Tally and Pioneer. The cultivars yielding significantly the lowest scores for overall quality included Pennant, Green Beauty and Trispear. The relationship between color and overall quality is evident from this table.

Table 2 gives the multiple correlation coefficients (R) and coefficients of determination (R^2) for characteristics which demonstrated a significant F-to-enter (F-test for the contribution to the sums of squares for regression for a variable) at the 95% level. This table also gives the multiple regression model for the relationship between overall quality, color, shape and seed size. From Table 2, one would expect that the order of importance for sensory components influencing overall quality should be color, shape and seed size since this is the order in which they were chosen to enter the equation and the F-ratios attest to their importance at a particular stage in the development of the model. Table 3 bears out this observation with a rating assigned to color of 60, shape of 27, and seed size of 13, calculated as described earlier. This means that when panelists rate fresh cucumbers for overall quality they will, on the average, be most influenced by these characteristics with the relative intensities of 60:27:13.

Brined quality

Of the 9 sensory criteria measured, the analysis of variance indicated a significant F-ratio for shape, external color, crispness, firmness, placenta size, seed size, and overall quality and Table 4 shows the means (separated) for the 20 cultivar-treatments. Calypso, Tally and Pioneer scored in the highest group of non significantly different means, while Earlypik and Pennant were in the lowest group of non significantly different means. The importance of firmness and crispness in influencing cucumber preference can be seen in this table in contrast to sensory assessment before brining when textural differences were found to be non significant.

Table 5 shows the same multiple regression statistics as were reported for fresh quality and it can now be seen that the relevant sensory criteria used after brining, within the range for each characteristic, differ from those for fresh cucumbers with respect to textural quality.

Table 6 shows that the score for external color for brined cucumbers, although still the most important criterion in influencing overall quality, is lower than for fresh quality (38 vs 60). Crispness and firmness take up 35 points (21 and 14) for brined cucumbers. Seed size, at 27 points, completes the 100 point total.

Table 3—Scores for fresh cucumber quality on the basis of sensory criteria

Sensory criterion	Score
Color	60
Shape	27
Seed size	13
Total	100

Table 4—Mean comparisons for 20 cultivar-treatments of cucumber after brining (10 = best or greatest intensity of characteristic, 0 = worst or lowest intensity of characteristic; for seed and placenta size, score decreased with increasing size)[a]

Cultivar	Sensory characteristic						
	Shape	External color	Crispness	Firmness	Placenta size	Seed size	Overall quality
Tally (Ethrel)[b]	4.4 def	4.3 bcde	5.9 abcde	6.7 a	7.3 a	7.6 a	5.3 abcd
Pioneer (No Ethrel)	4.5 cdef	3.6 e	4.8 e	5.7 abcde	6.2 abcde	6.5 ab	4.2 cd
County Fair	4.9 bcdef	5.0 abcde	5.5 bcde	4.7 cdef	5.5 bcdef	6.6 ab	4.9 bcd
Tally (No Ethrel)	6.9 a	4.7 abcde	5.8 abcde	6.8 a	7.5 a	7.7 a	5.8 abc
Pioneer (Ethrel)	6.2 ab	4.2 cde	4.9 de	6.4 abc	6.6 abc	7.4 a	5.0 bcd
Pioneer	6.9 a	6.1 a	6.6 abc	6.6 ab	6.1 abcde	6.4 ab	6.2 ab
XP 76	6.1 abc	5.7 abc	5.5 cde	4.9 cdef	5.3 cdef	5.8 bc	5.0 bcd
FX 4164	4.4 cdef	4.9 abcde	5.5 cde	4.9 bcdef	5.2 def	5.6 bc	4.8 bcd
CP 183	4.3 def	5.7 abc	5.4 cde	6.1 abcd	6.7 ab	6.8 ab	5.4 abcd
C 589	5.7 abcde	4.1 cde	6.2 abcd	5.8 abcde	6.9 ab	7.0 ab	5.7 abc
Pennant	3.7 e	3.8 e	5.4 cde	4.1 ef	4.5 f	3.4 d	4.0 d
Triple Mech	5.5 abcde	3.9 de	6.4 abc	5.8 abcde	6.1 abcde	5.7 bc	4.7 bcd
H 1077	4.2 ef	5.7 abcd	5.4 cde	6.2 abc	6.1 abcde	7.0 ab	5.6 abcd
XP 1149	6.4 ab	5.1 abcde	6.9 ab	6.0 abcd	6.8 ab	6.4 ab	6.2 ab
XP 823	5.9 abcd	5.2 abcde	5.9 abcde	4.4 def	6.5 abcd	7.5 a	6.1 ab
Tally	5.7 abcde	6.0 ab	6.6 abc	5.5 abcdef	6.8 ab	7.6 a	6.6 a
Bounty	6.7 a	6.0 ab	6.2 abcd	6.1 abcd	5.0 ef	5.6 bc	5.6 abcd
Earlypik	6.4 ab	5.0 abcde	6.0 abcde	3.9 f	4.4 f	4.5 cd	4.2 cd
Multipik	5.9 abcd	5.3 abcde	6.4 abc	6.4 abc	6.2 abcde	5.8 bc	5.4 abcd
Calypso	5.3 abcde	5.7 abc	7.2 a	6.7 a	5.8 bcdef	6.5 ab	6.7 a

[a] Means within columns followed by the same letter are not significantly different at the 5% level using Duncan's Multiple Range test
[b] Ethrel and No Ethrel treatments were brined for 15 months

DISCUSSION & CONCLUSIONS

AT THE PRESENT TIME, it is not possible to assess brining cucumber quality with reference to fresh characteristics. In fact, the misuse of scores on fresh cucumber quality can lead to erroneous conclusions about the brined stock. In this sense, scoring fresh cucumbers for textural characteristics and including this score in the total score for assessment of cucumber cultivars for brining may be somewhat artificial. It is apparent from the work reported here that the sensory perception of texture among cultivars before brining may not indicate any differences, but that textural characteristics such as crispness and firmness become of paramount importance after brining. This is not to say that textural differences cannot be found instrumentally before brining, in fact, there is voluminous literature to the contrary. In order to assess potentially important cultivars adequately during or immediately after the growing season for brining it is now apparent that a significant amount of work needs to be done on instrumental methods for texture evaluation (example, the Instron texture profile analysis) and its relationship to the sensory perception of crispness and firmness after brining. Although considerable research has been done on cucumber texture in recent years, both instrumental and sensory, there is insufficient reliable numerical information available to construct a scoring system which would predict cucumber quality after brining on the basis of fresh cucumber characteristics.

Table 5—Multiple regression parameters for brined cucumber quality

Variable entered	Multiple R	Multiple R^2	F-to-enter[a]
External color	0.78	0.61	154.35
Seed size	0.87	0.76	56.14
Crispness	0.89	0.79	16.60
Firmness	0.90	0.81	5.73

Multiple Regression Equation:

$$Q = -0.75 + 0.40(A) + 0.30(B) + 0.24(C) + 0.16(D)$$

Q = Overall Quality
A = External Color
B = Seed S ze
C = Crispness
D = Firmness

[a] All F-ratios are significant at >95% confidence

Table 6—Scores for brined cucumber quality on the basis of sensory criteria

Sensory criterion	Score
External color	38
Seed size	27
Crispness	21
Firmness	14
Total	100

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PREMIUMS AND COUPONS AS A POTENTIAL SOURCE OF OBJECTIONABLE FLAVOR IN CEREAL PRODUCTS

M. G. HEYDANEK, JR., G. WOOLFORD and L. C. BAUGH

ABSTRACT

Current practice in the cereal industry is the use of multi-colored graphics on plastic-foil constructions and various coupons and premiums as packaging inserts. The volatiles in these inserts, if not controlled, can impart undesirable flavor to packaged foods. Volatiles isolated from various printed coupons and premiums exhibit a wide range of component concentration and odor. Chemical and organoleptic methods for measuring simulated volatile transmission from coupons and premiums through overwrap films have shown classical materials to be unsuitable in many cases. Instrumental measurements showed a polyester/PVDC/polyester film to be an almost absolute barrier to premium and coupon volatiles at the concentrations normally encountered.

INTRODUCTION

IN TODAY'S ready-to-eat cereal market, a common promotional tool is the insertion of a premium or coupon inside the cereal box. The fact that these inserts are in direct product contact presents the possibility of flavor alteration. Common practice is the use of an overwrap film to prevent direct contact and contamination of the product. Use of conventional polyethylene, polypropylene, or PVDC coated cellophane as overwrap films was found to be unreliable in preventing flavor alterations from occurring when various toys and coupons were inserted in RTE cereals. The present study is an investigation designed to identify volatiles present in commonly used premiums and coupon constructions and measure the effectiveness of selected overwrap films as flavor transfer barriers.

EXPERIMENTAL

Identification of premium and coupon volatiles

Several representative premiums of varied composition were analyzed. All had molded polyethylene bodies with color and standard industry additives present. One had about 40% by weight clear polystyrene as one part. Coupons were standard printed materials which used both flexographic and rotagravure printing processes and their associated ink-solvent systems. Representative samples of unwrapped inserts, coupons, or printing inks were placed in glass Mason jars, fitted with stainless steel lids which contained a septum sealed port for withdrawing headspace samples. After heating the jars at 125°C for 15 min, 10 ml of headspace gas were withdrawn via a syringe and injected into a Hewlett-Packard 5700 gas chromatograph coupled (through a silicone membrane separator) to a Hewlett-Packard 5930A dodecapole mass spectrometer. The sample was chromatographed on 10% Carbowax 20M on Gas-Chrom Q 10 ft × 1/8 in., programmed from 85°C to 160°C at 8°/min.

Measurement of transmission rates

Selection of test film overwraps. Overwraps were chosen (a) to conform with required cost constraints and (b) for potentially promising barrier properties. Polypropylene was selected as the control system for comparison purposes. PVDC-coated cellophane, another commonly used overwrap was found to contain toluene and would therefore interfere with transmission measurements. The following materials were selected for study:

0.9	mil	Polypropylene
1.25	mil	Polypropylene
1.25	mil	HDPE/Tie Layer/Nylon/EVA Blend
1.5	mil	LDPE/PVDC/LDPE
2.5	mil	LDPE/PVDC/LDPE
0.5	mil	PVDC/Polyester/PVDC
0.75	mil	PVDC/Polyester/PVDC

Test odorants. Styrene, toluene and n-pentanal were Aldrich 99%. Hexane was Mallinckrodt Nanograde and 2-butanone was supplied by Eastman. (See Results & Discussion for choice of these odorants.)

Preparation of overwrap materials for transmission rate measurement. Each overwrap was cut into 4 in. × 7 in. strips, then folded and sealed on two sides using an impulse sealer. Initial experiments used fin-type seals, but these were found to leak at the T-junction, after 100% transmission was noted in several cases after a short storage time. Thereafter, three side/sealed pouches were used.

The amount of each of the chemicals used in the experiments was determined by assuming a "worst case" level. This was estimated to be approximately 100 ppm of each chemical based on the weight of a premium. The average weight of premiums currently used is 10g, so that 1 mg of each chemical placed in the sealed overwrap would represent the required level. To represent a premium, copper wire was coiled into a short (4 cm) length and a small strip of folded filter paper placed in the coil. 5 μl of a mixture of each component were injected by syringe onto the filter paper, the liquid being prevented from contacting the overwrap by the copper coil. Immediately after injection of the mixture, the coil was placed in the 2-side sealed pouch, and the third side then sealed. Each pouch and coil was stored in glass quart Mason jars, which represented cereal packages. The Mason jar lids were stainless steel assemblies containing a septum and fitted with a rubber gasket to facilitate good sealing between the jar and lid (R.J. Harvey Instrument Corporation). Preliminary experiments showed that the volatile components that had diffused through the overwrap were absorbed into the rubber gaskets so that the levels of measured volatiles were lower than they should have been. This was overcome by placing a sheet of aluminum foil, exactly cut to fit, between the top of the jar and the rubber gasket. A separate jar was needed for each sampling time of a particular film because the syringe needle pierced the aluminum foil during sampling.

Two different temperatures were used in the studies. In one series of experiments, all samples were kept at ambient, (approximately 20°C), to simulate storage of cereal in warehouse and retail stores. Another set of samples was kept at 100°C to represent extreme abuse, and also to give an early indication of the relative effectiveness of the films under test. All data points represent replicated sets of samples.

Gas chromatography of headspace samples. For each sampling time, 10 ml of the headspace from a Mason jar was injected into a Hewlett-Packard 7620A Gas Chromatograph, equipped with a 10 ft × 1/8 in. 10% Carbowax 20M stainless steel column, programmed 75°C to 190°C at 10°/min after an initial hold period of 8 min. The F.I.D. response was integrated electronically and the area obtained compared to a GC trace of a standard mixture of the five contaminants, prepared daily. Injection port septa were replaced after each injection to minimize the possibility of leaks.

Taste evaluation of premium contaminants

Method of contamination. Ready-to-eat cereal (RTE) finished product, unexposed to packaging material, was used as the test medium. Gallon sized jars were filled with 400g of cereal. To the lids of the jars (except those containing control sample) a paper clip was

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attached and a small piece of filter paper impregnated with a known amount of the test chemical hung from the clip. The control and experimental jars were sealed tightly and stored for 7 days in total darkness at ambient temperature.

Taste panel evaluation. Product exposed to the test chemicals at various concentrations (1-200 ppm) were graded in preliminary blind tests by trained judges familiar with the flavor character of the product. Their evaluations were used to select levels at which the flavor of each test chemical 1) could not be perceived, 2) barely perceived (threshold), 3) definite off-flavor.

Using the levels determined above, more rigorous panel evaluations were made. For each evaluation, judges were given a reference sample of control cereal to taste. They were then served consecutively two coded samples of cereal, one of which was the contaminated sample. In each case, they were asked if the coded sample was "like" the reference sample or "different." If "different," they were asked to say how "different" using an unstructured line, and to identify the difference. Values for the degree of over-all difference (0–60) were determined after grading, and were submitted to analysis by the Wilcoxon Signed Rank Test (Marascuilo and McSweeny, 1977). Product was served in odor-free glass dishes and was removed from the glass jars just prior to serving each sample. Samples were served in a randomized balanced presentation. The Wilcoxon Signed Rank Test for nonparametic differences was used to analyze the responses.

RESULTS & DISCUSSION

Identification of premium and coupon volatiles

In order to understand the odor barrier properties of overwrap films, one must know the odorants involved. To insure identification of as many commonly encountered materials as possible, a large variety of premiums and coupons were subjected to headspace GC-MS analysis.

The results of the GC-MS analyses are summarized in Table 1. These analyses represent combined information from many different types of materials. Not all of these materials would likely be present in any one given system. Due to the large number of diverse chemicals with the potential of being present, a test mixture of five representative chemical classes was selected. Toluene, styrene and hexane are obvious candidates for inclusion in the transmission study, because they are present in the premiums. Two oxygenated compounds, pentanal and 2-butanone were also included; the former because this potent odorant has been recognized in lacquer overcoats and 2-butanone for its typical use as an industrial solvent in many printing and adhesive applications and to provide information on this class of polar compounds.

Table 1—Volatile components identified in premiums and coupons

			Premium		Coupon	
Compound	GC	MS	Poly-ethylene	Poly-ethylene/ Poly-styrene	Flexo-Print	Roto-Print
Decane	+[a]	+[b]	+	+	+	+
Decene		+			+	+
Dodecane	+	+	+	+		
Hexane		+	+	+	+	+
Hexene		+	+		+	+
Heptane	+	+			+	+
Heptene		+			+	
Nonane	+	+			+	
Nonene		+			+	+
Octane	+	+			+	+
Octene	+	+			+	+
Undecane		+			+	+
Undecene		+			+	+
Toluene	+	+	+	+	+	+
Xylene	+	+		+		
Ethyl benzene	+	+		+		
Propyl benzene	+	+		+		
Styrene	+	+		+		
Acetone		+			+	+
Ethanol	+	+			+	+
Ethyl acetate	+	+			+	+
Ethyl cellusolve	+	+			+	
Hexanal	+	+			+	
Methanol		+			+	+
Methyl cellusolve	+	+			+	+
Methyl ethyl ketone	+	+			+	+
Methyl-iso-butyl ketone	+	+				+
1-Nitropropane	+	+				+
2-Nitropropane	+	+				+
Pentanal	+	+				
n-Propyl acetate	+	+			+	+
i-Propyl acetate	+	+			+	+
l-Propanol	+	+			+	+
2-Propanol	+	+			+	+

[a] (+) matches authentic compound RT
[b] (+) matches authentic compound mass spectra or published spectra

Measured transmission rates

The transfer of chemicals through a polymer barrier is complex. Each component, when considered singly, has a particular interaction with the overwrap film. Generally the principle of "like dissolves like" operates and thus, more polar compounds will transfer more rapidly through polar films and more slowly through apolar film. Another factor affecting transmission rates is the easier a vapor is to condense, the faster the transmission rate. Additionally, there is likely to be interaction between the chemicals as each chemical affects the transmission rate of the others. This will occur as each chemical dissolves in the film and causes the film to undergo stress and deformation. In this study we have chosen the worst possible case; that is, for each chemical to be present at what we consider to be the maximum practical limit. The rates we have measured are thus likely to be faster than those which would be measured for each chemical singly.

The classical theory of diffusion of gases is based on Fick's laws. (Stannet et al., 1962). Diffusion in homogenous substances is based upon the assumption that the rate of transfer, R, of a gas passing perpendicularly through unit area of a section is proportional to the concentration gradient through the section. This is expressed as

$$R = -D\frac{dc}{dx} \quad (1)$$

where D is the diffusion coefficient, c is the concentration of diffusant and x is the thickness of the material. Here, D is assumed to be independent of concentration, a close approximation for gases such as oxygen and nitrogen through polymers at 1 atm.

The concentration of diffusant inside a polymer is related to the gas pressure, P, outside the membrane

$$c = SP \quad (2)$$

where S is the solubility coefficient of the gas in the film (Henry's Law).

Integration of Eq (1) and combination with Eq (2) allows the formation of an expression relating the quantity of gas permeating a membrane to the diffusion and solubility coefficients.

$$q = \frac{DSA\,(p_1 - p_2)t}{L} \quad (3)$$

where A is the cross sectional area: p_1 and p_2 are the

pressures across the membrane, t is time and L is thickness. At unit A, $(p_1 - p_2)$, t and L, q is the permeability coefficient, p, and

$$p = DS \quad (4)$$

p is a constant for a given penetrant-membrane system at a given temperature.

Henry's law, however, does not hold for diffusion of organic vapors and liquids through polymers, where concentrations can be high, and swelling of the membrane matrix can take place.

It is also known that D, the diffusion coefficient, is concentration dependent for liquids and easily condensable vapors in certain polymers, i.e.

$$Dc = Do[1 + f(c)] \quad (5)$$

where Do is the diffusion at zero concentration and f(c) is a function of concentration dependent on the particular system studies. In the system, the polymer swells, and some of the bonds rupture, so that a time dependence is also a factor. Thus, in the present system studied, with several (5) diffusants as organic vapors through rigid polymers, a likely expression of the change in concentration of diffusant across the polymer is

$$\frac{dc}{dx} = \frac{d}{dx}\left(D\frac{dc}{dx}\right) \text{ where } \begin{matrix} D = f(t,c) \text{ and is unknown} \\ C = f^1(p) \end{matrix} \quad (6)$$

Thus, true rate constants could not be measured and explains why transmission rates were compared instead.

Table 2—Molar transmission rates at ambient[a] temperature

	Hexane	2-butanone	Pentanal	Toluene	Styrene
Propylene (1.25 mil)	91	250	250	240	190
HDPE/TieLayer/Nylon/EVA	0.51	42	550	7.7	9.8
LDPE/PVDC/LDPE (1.5 mil)	0.6	11	0.63	5.4	5.7
LDPE/PVDC/LDPE (2.5 mil)	0.37	3	b	1.1	2.1
PVDC/Polyester/PVDC (0.5 mil)	b	0.41	b	b	0.74

[a] Expressed as cm^{-2} day^{-1} $\times 10^5$
[b] No transmission at 24 day

Table 3—Molar transmission rates at 100° C[a]

	Hexane	2-butanone	Pentanal	Toluene	Styrene
Polypropylene (1.25 mil)	69	69	69	69	69
LDPE/PVDC/LDPE (1.5 mil)	5.8	15	8.6	8.6	34
LDPE/PVDC/LDPE (2.5 mil)	1.9	8.6	8.6	8.4	4.1
HDPE/Tie Layer/Nylon/EVA	0.46	6.9	5.1	5.3	5.2
PVDC/Polyester/PVDC (0.5 mil)	0.039	0.51	0.15	0.87	2.4
PVDC/Polyester/PVDC (0.75 mil)	b	0.3	0.075	0 58	0.84

[a] Expressed as cm^{-2} $day^{-1} \times 10^2$
[b] No transmission at 5 hr.

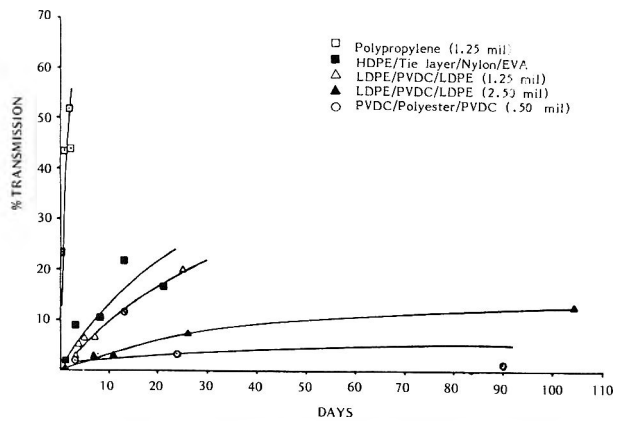

Fig. 1—% Transmission of styrene with time through various films at ambient temperature

With this qualification in mind, the data obtained for both the ambient and high temperature experiments enables a clear ranking of the overwrap films, from the standpoint of ability to prevent transmission of the premium contaminants. The ranking of overwraps based on their performance at ambient temperature is:

1. PVDC/Polyester/PVDC (0.5 mil)
2. LDPE/PVDC/LDPE (2.5 mil)
3. LDPE/PVDC/LDPE (1.5 mil)
4. HDPE/Tie Layer/Nylon/EVA (1.25 mil)
5. Polypropylene (1.25 mil)

The ranking of overwraps based on their performance at 100°C is:

1. PVDC/Polyester/PVDC (0.75 mil)
2. PVDC/Polyester/PVDC (0.5 mil)
3. HDPE/Tie Layer/Nylon/EVA (1.25 mil)
4. LDPE/PVDC/LDPE (2.5 mil)
5. LDPE/PVDC/LDPE (1.5 mil)
6. Polypropylene (.9 mil)

The production of these rankings is based solely on the transmission data of the type graphically presented in Figures 1—4. Transmission data for the two most odorous test chemicals, pentanal and styrene, at ambient and 100°C are presented. Similar curves are obtained for the other test chemicals. Several conclusions can be drawn.:

1. A conventional overwrap, 1.25 mil polypropylene film, performed by far the worst as a barrier for the test chemicals. With the exception of methyl ethyl ketone, polypropylene allowed the transmission of all other chemicals at least 20 and up to 180 times faster than the next worse film, at ambient temperatures.

2. PVDC/Polyester/PVDC (0.75 and 0.5 mil) was easily the best film. After three months storage, transmission of three of the chemicals, hexane, pentanal and toluene, was too small to be measured, and transmission of 2-butanone and styrene was significantly lower than in the next best film.

3. In the higher temperature study, all the transmission rates were faster; there was also a change in order of the middle ranked films, with HDPE/Tie Layer/Nylon/EVA

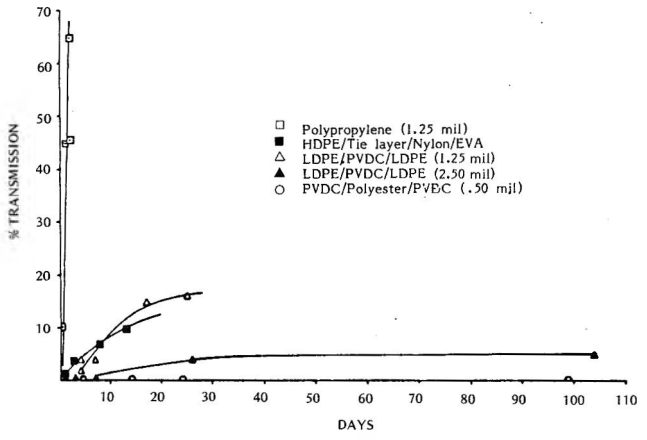

Fig. 2—% Transmission of pentanal with time through various films at ambient temperature

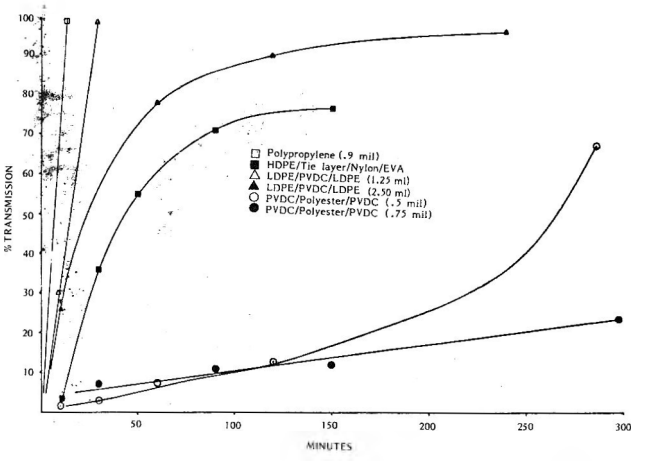

Fig. 3—% Transmission of styrene with time through various films at 100° C.

showing less thermal deformation than LDPE/PVDC/LDPE films and consequently, lower transmission rates. PVDC/Polyester/PVDC still remained the film of choice, with no measurable hexane diffusion, even after 5 hr at 100°C through the 0.75 mil film. Polypropylene was again the film that allowed the fastest transmission, all chemicals being completely through in less than 15 min.

The taste panel data were now needed to determine how effective these barriers are to the test chemicals in terms of normal product shelf-lives. The transmission rate at the worst case concentration of 100 ppm for each test chemical present in a premium or coupon is now known, but not their flavor effect level in a typical RTE cereal. To develop this information, the cereal was exposed to known concentrations of each individual test chemical and taste paneled as described in the methods section. The concentrations at which an objectionable off-flavor was noted in the RTE cereal were:

Styrene	4 ppm
2-butanone	25 ppm
Hexane	200 ppm
Pentanal	5 ppm
Toluene	100 ppm

Having the objectionable flavor concentration and transmission rates available, it is possible to calculate the time required for an odorant to diffuse through the films and reach an objectionable flavor level.

Table 4 shows values for the time taken to reach a threshold of off-flavor for each chemical and film, assuming sufficient contaminant in the premium and assuming the chemical continues to diffuse at the rate measured in this

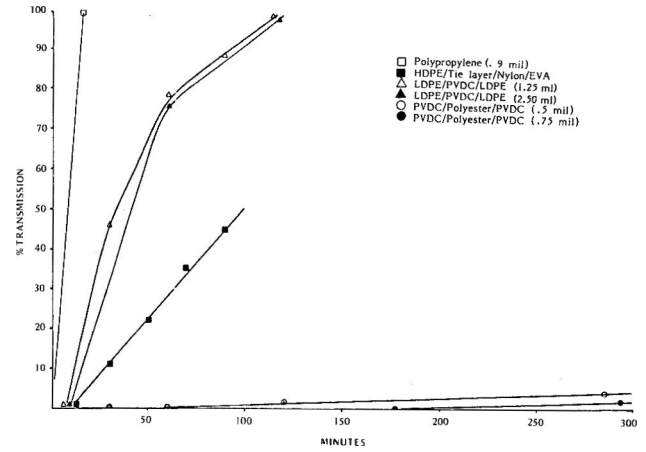

Fig. 4—% Transmission of pentanal with time through various films at 100° C.

study. From the threshold levels found for these compounds, styrene was one of the most potent contaminants. It can be seen that the PVDC/Polyester/PVDC and LDPE/PVDC/LDPE (2.5 mil) films should provide sufficient resistance to an off-flavor problem from this chemical.

The time for the first two films, polypropylene and HDPE/Tie layer/Nylon/EVA, to reach unacceptable levels of pentanal and styrene is less than 200 days, certainly within the normal shelf-life of a RTE cereal and therefore

—Continued on page 856

Table 4—Predicted days for contaminants to reach threshold off-flavor levels at ambient[a] temperature

	Hexane	2-butanone	Pentanal	Toluene	Styrene
Polypropylene (1.25 mil)	585	22	5	84	4
HDPE/Tie Layer/ Nylon/EVA	585	132	200	2.6×10^3	79
LDPE/PVDC/LDPE (1.5 mil)	585	516	176	3.8×10^3	138
LDPE/PVDC/LDPE (2.5 mil)	585	1.9×10^3	1×10^5	1.9×10^4	383
PVDC/Polyester/PVDC (0.5 mil)	585	1.3×10^4	1×10^5	2×10^6	1150

[a] Assuming required amount of contaminant present

PREPARATION OF A SHELF-STABLE, INTERMEDIATE MOISTURE FOOD PRODUCT FROM MUSCADINE GRAPE SKINS

L. F. FLORA, L. R. BEUCHAT and V. N. M. RAO

ABSTRACT

A study was designed to produce a shelf stable food ingredient from muscadine grape press cake using enzyme modification and intermediate moisture technology. Two pectinases and a cellulase were screened for their capacity to tenderize the grape skins; sucrose syrup and grape concentrate were tested as osmotic soaking solutions. Treatment of deseeded press cake in an aqueous enzyme solution of 0.05% Rohament P, followed by soaking in grape concentrate and drying at 60°C, yielded a chewy, shelf-stable food ingredient with a tart grape flavor. Sensory and instrumental evaluation confirmed the texture modification. Baked and snack foods containing the grape skin product in place of raisins or dates were highly acceptable based on sensory panel scores.

INTRODUCTION

GRAPES are commonly processed by pressing out juice for wines, juices, and jellies, leaving as residue the pulp, skins, and seeds. Except for the relatively small amount of pulp and skins used in jams and preserves, most of the residue is discarded. This waste problem is particularly pronounced with the southern muscadine grape, which has a thick, tough skin that can account for almost one-fourth of its fresh weight.

Grape skins or deseeded press cakes require tenderizing, usually by heating, before incorporating into preserves, jams, or other products. The desirable texture of heat-tenderized skins is difficult to achieve; skins often remain tough or too mushy or soft. Moreover, heat-softened skins are not shelf stable and have limited applications. Enzymes, on the other hand, have been successfully used to alter texture of muscadine grape skins for use in preserves (Rizley et al., 1977).

Kitson and Britton (1978) described the preparation of low and intermediate moisture combination dried fruit snacks/ingredients from drum-dried fruit sauce flakes and juice concentrate. The purpose of our research was to apply enzyme and intermediate moisture technology to produce, from deseeded muscadine grape press cakes or skins, a shelf-stable food or food ingredient with good texture and flavor. The development of such a product could not only provide an additional use for the fruit but could also provide an alternative for material presently considered waste.

EXPERIMENTAL

Ripe fruit from two cultivars of muscadine grapes were mechanically deseeded and cold-pressed (Flora, 1977), and the press cakes were frozen until treated. A thin-skinned cultivar, Creek, and a thick-skinned cultivar, Jumbo, were used to give an indication of the feasibility of the process.

In preliminary trials, three enzymes were screened for their capacity to tenderize the grape skins: Pectinol 59-L (Rohm and Haas Co., Philadelphia, PA), Rohament P (Rohm Enzyme, Darmstadt, Germany), and Cellzyme (Wallerstein Co., Deerfield, IL). The first two are pectinases; the third is a cellulase. Each enzyme was dispersed in 200 ml of water which was then combined with 100g of grape skins. Pectinol 59-L was tested at concentrations of 0.005, 0.05 and 0.1% (v/v); Rohament P was tested at concentrations of 0.05, 0.5 and 1.0% (w/v). These concentrations covered and exceeded the levels recommended by the manufacturers. Cellzyme was tested at concentrations of 0.05, 0.5 and 1.0% (v/v).

Grape skins were submerged in the enzyme solutions for 1 hr at 35°C, with occasional agitation. The skins were drained, submerged in a sucrose syrup containing 70g sucrose and 100 ml water (approx 41° Brix) for 14–16 hr at 22°C, drained again and soaked for a second 14–16 hr period. The treated skins were then dried in a forced air oven at 60°C for 24–28 hr. In later experiments, a 65° Brix red muscadine grape juice concentrate replaced the sucrose soak solution, and drying time was reduced to 14 hr. Upon assessment of the grape skins treated with the three enzymes at various concentrations, Rohament P (a pectin glycosidase) was tested further at concentrations of 0.05, 0.25, 0.50, 0.75 and 1.0% (w/v). Based on these results, a concentration of 0.05% (w/v) Rohament P was used in the production of grape skins for product formulations.

Samples of the treated grape skins were equilibrated at 21°C overnight, and water activity (a_w) was measured using a Sina-scope hygrometer (Sina Ltd., Zurich, Switzerland).

To objectively assess textural changes, skins were stored in a desiccator containing a saturated NaCl solution, brought to an equilibrium relative humidity of 75.5% (a_w 0.755) at 21°C, and puncture tests were performed as described by Hankinson et al. (1977) using the Universal Testing Machine (Instron Model 1130). Tests were run using a crosshead speed of 5 cm/min and were replicated at least four times per sample. Prior to testing, the thickness of each skin was determined using a micrometer. Failure force, as recorded from the charts, and skin thickness were analyzed using one way analysis of variance and least significant difference.

A 10-member sensory panel evaluated texture and flavor of the treated grape skins using a 9-point scale (1 = extremely poor, 5 = borderline, 9 = excellent). A maximum of three samples was presented to panel members at any given evaluation. Treatments were compared using rank totals (Kramer, 1963).

To evaluate the feasibility of using the treated grape skins as a dried fruit, they were chopped and incorporated into several food recipes in place of raisins and dates. Products evaluated included oatmeal-raisin cookies, date-nut bread, and a cereal based snack food containing dates. Products containing treated grape skins were then compared to those containing traditional dried fruits using an 18-member sensory panel as described above. Appearance, color, aroma, texture and flavor were evaluated. Comparisons were made using rank totals (Kramer, 1963).

Shelf life was assessed by holding the treated skins in closed polyethylene bags at room temperature (~21°C) for 3 months. After this period they were evaluated for microbial growth or degradation and color, texture and flavor changes. Skins from a hot-pressed juice operation (Flora, 1977) were similarly treated and evaluated for quality.

RESULTS & DISCUSSION

Evaluation of enzymes and soak solutions

Naturally dried or dehydrated grape skins typically are leathery or brittle in texture in addition to possessing little flavor. Skins treated with Pectinol 59-L and Cellzyme exhibited little change in texture over the range of concentrations used. Treatment with Rohament P, however, resulted in marked softening of the skins at all test concentrations,

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Table 1—Texture and flavor scores[a] of treated muscadine grape skins

Cultivar	Tenderization treatment	Soak treatment	Mean sensory score[b] Texture	Flavor
Jumbo	Enzyme	Sucrose	5.1 ab	5.6 a
		Grape conc	6.8 c	7.4 b
	Control	Sucrose	4.1 a	4.9 a
		Grape conc	5.9 bc	7.2 b
Creek	Enzyme	Sucrose	6.4 b	5.2 b
		Grape conc	7.6 c	7.3 c
	Control	Sucrose	4.2 a	4.1 a
		Grape conc	5.9 b	7.3 c

[a] Values in the same column, for each cultivar, not followed by the same letter are significantly different ($p \leq 0.05$).
[b] 1 = Extremely poor, 5 = borderline, 9 = excellent

Table 2—Thickness and failure force of treated muscadine grape skins

Cultivar	Tenderization treatment	Soak treatment	Mean Thickness[a] (mm)	Mean Failure Force[a] (Newtons)
Jumbo	Enzyme	Sucrose	.47 d	6.99 d
		Grape conc	.46 d	3.60 b
	Control	Sucrose	.51 e	8.59 e
		Grape conc	.52 e	9.23 e
Creek	Enzyme	Sucrose	.24 a	2.31 a
		Grape conc	.30 c	2.49 a
	Control	Sucrose	.25 ab	5.92 c
		Grape conc	.29 bc	6.48 cd

[a] Values in the same column not followed by the same letter are significantly different ($p \leq 0.05$).

with undesirable gumminess occurring at high levels. Since some retention of skin characteristics was desirable, a level of 0.05% Rohament P in the tenderizing solution was chosen as the most appropriate level for these studies. The pH of the enzyme solutions after addition of the grape skins was near that of the grapes, between 3.4 and 3.5. The optimum pH for the three enzymes is about 4.0, just slightly above the pH of the grape skin/enzyme/water mixtures.

There was some indication of off-flavors in skins treated at concentrations of Rohament P above 0.25%. Although there appeared to be a progressive increase in softening and stickiness as the enzyme concentration was increased, a 10-member sensory panel asked to evaluate grape skins after treatment with Rohament P at concentrations of 0.05, 0.5 and 1.0% (w/v) indicated no detectable differences in texture or flavor among the three concentrations.

Skins soaked in the sucrose syrup were drier and more brittle after oven drying than skins soaked in grape concentrate. The concentrate-soaked skins were more chewy and moist. The effects of the enzyme and soak treatments on panel scores are shown in Table 1. Enzyme-treated skins generally scored better in texture than did skins that were not enzyme-modified. Skins soaked in grape concentrate consistently scored better in texture and flavor than did sucrose-soaked skins. The concentrate-soaked skins had a tart, grape-like flavor while the sucrose-soaked skins were woody or grainy in flavor.

There was little difference in sensory scores between cultivars, except for texture scores of the enzyme-treated skins which were higher for the thinner Creek skins. Though not substantiated in the sensory scores, preliminary evaluations of the two cultivars indicated that treated Jumbo skins might provide a chewier product and be more suitable for incorporation in foods.

The color of the sucrose-soaked skins was lighter than that of the grape concentrate-soaked skins, which varied from brown to purple depending upon the concentrate quality. The process ultimately used for preparation of the grape skins is shown in Figure 1. Though it would be advantageous to combine the enzyme treatment and soaking steps, the high solids and low pH of the concentrate would likely inhibit the enzyme action. An artificial osmotic soaking solution containing appropriate amounts of sugar, acid, and artificial or natural colors and flavors could be used in place of the concentrate.

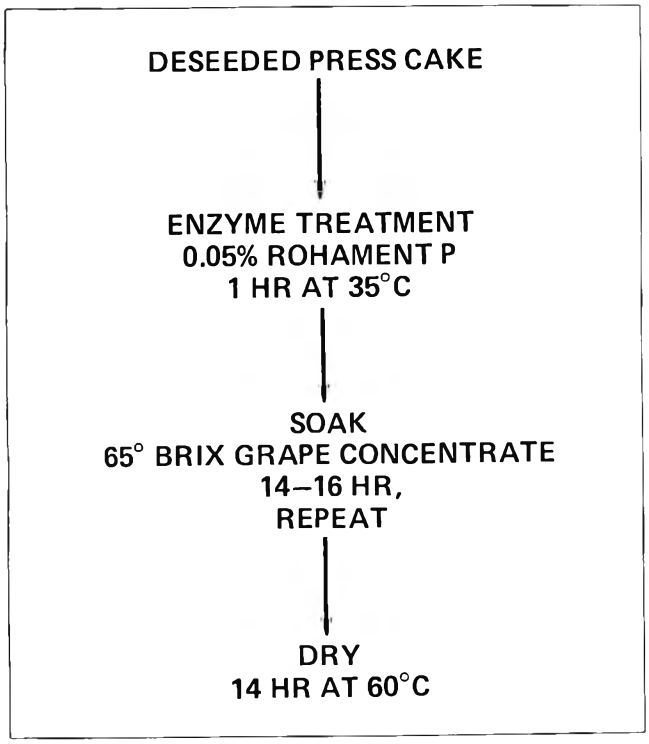

Fig. 1—Optimum process for preparing texturized muscadine grape skins.

Objective texture measurements of skins

Skins of Jumbo grapes were approximately twice as thick as the Creek (Table 2). Enzyme treatments evidently caused some sloughing of the cells from the Jumbo skins, thus reducing their thickness. Soaking in grape concentrate apparently caused a greater swelling of the skins of the Creek cultivar than did the sucrose solution soak.

Enzyme treatment significantly lowered the failure force of the grape skins (Table 2). These results correspond to the improved texture scores for the enzyme-treated skins shown in Table 1; skins were less tough and more chewy than those not treated with enzyme. The difference between cultivars is also evident for failure force; the thicker Jumbo skins were more difficult to puncture than the thinner Creek skins. Seemingly opposed to the findings of Hankinson et al. (1977) on raw grape skins, skin thickness did affect resistance to puncture for treated grape skins.

Evaluation of products made with treated skins

Products containing grape skins were usually darker in color than the original recipe products containing raisins or dates. Panel scores of the grape skin-substituted products were similar to those assigned to original recipe products

containing raisins or dates (Table 3). Substitution of grape skins for raisins and dates in the three products did not significantly alter panel scores. The grape-skin cookies were somewhat harder than those containing raisins. Appearance and aroma were scored slightly higher for the cereal-based snack containing grape skins, and aroma and flavor were scored slightly higher for the nut bread containing grape skins. Overall, scores of sensory characteristics for the grape-skin substituted products corresponded to ratings of good to very good.

Table 3—Sensory scores of original recipe vs grape skin-substituted products

Product	Fruit	Mean sensory score[a]				
		Appearance	Color	Aroma	Texture	Flavor
Oatmeal cookies	Raisins	8.2	8.2	7.7	7.4	7.9
	Grape skins	7.6	7.5	7.4	6.8	7.3
Cereal-based snack	Dates	7.3	7.5	7.2	7.7	7.8
	Grape skins	7.6	7.5	7.6	7.3	7.1
Nut bread	Dates	7.6	7.9	6.8	7.3	6.6
	Grape skins	7.4	7.5	7.2	7.1	7.1

[a] 1 = Extremely poor, 5 = borderline, 9 = excellent.

Evaluation of shelf life and hot-press skins

The a_w of the skins dried for 24–28 hr was 0.41–0.45, which would prevent microbial growth. After drying treated skins for only 14 hr at 60°C, sufficient moisture was removed to reduce the a_w from 0.82 to 0.70. These skins had a more chewy texture and were judged superior to those dried to an a_w of 0.41–0.45.

After 3 months of storage in a closed container at room temperature, there were no visible signs of microbial growth or degradation of the skins treated with enzyme and concentrate as shown in Figure 1. Little if any change was noted in aroma and flavor. Skins having a purple color at the onset of storage experienced a gradual browning. However, this brown color was not objectionable in the products tested. Texture was not noticeably affected during storage.

Skins from hot-press juicing operations processed by the procedure shown in Figure 1 proved as acceptable as cold-press skins.

CONCLUSIONS

DESEEDED muscadine grape waste, consisting mostly of skins, can be converted to a usable food ingredient. Enzyme level would be determined by the degree of tenderization required for a given application. Other pectinase preparations may also be suitable for tenderizing grape skins when used in appropriate concentrations and under suitable conditions.

Maintenance of red or purple color would not be critical in the baked and snack products tested in this study. The product developed in this study might also be successfully incorporated into products such as candy and ice cream. Extrusion or further form manipulation of the treated skins may improve their acceptance as a dried fruit product. It is likely that the above process could be successfully applied to waste skins of grapes other than muscadines.

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PREMIUM-CEREAL OFF-FLAVOR TRANSFER . . . From page 853

unacceptable as a barrier film for these odorants. The calculated time of the other films, PVDC/Polyester/PVDC and LDPE/PVDC/LDPE, to reach objectionable flavor transfer is 1 year or more and therefore, should be useful as barriers in this type of application. On the basis of data obtained, the PVDC/Polyester/PVDC film should be, from a practical viewpoint, an absolute barrier to most normally encountered volatiles originating from premiums and coupons. Premiums and coupons can contain a large number of potentially odorous components. To ensure the minimum transmission of these odorants to a food product that will readily absorb them, we have found that a copolymer overwrap construction of PVDC/polyester/PVDC is a suitable barrier.

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PREVENTION OF TOUGHENING OF CHICKEN BREAST MUSCLE CUT IMMEDIATELY AFTER SLAUGHTER BY CONTROL OF pH DECLINE

D. W. PETERSON and A. L. LILYBLADE

ABSTRACT

The influence of slowing the rate of pH decline on the toughening effect of cutting chicken pectoralis muscle shortly after slaughter was studied by injection of $NaHCO_3/Na_2CO_3$ buffer into the muscle immediately after evisceration. Buffer injected to a calculated level of 0.48% solids resulted in cut muscles with an ultimate mean pH of 6.5, which were completely tender after 24 hr aging. Buffer injected at a calculated level of 0.72% solids resulted in muscles which were adequately tender after 5 hr aging and extremely tender (ultimate pH 6.7) after 24 hr aging. Untreated cut control muscles were relatively tough after 24 hr aging (ultimate pH 5.75–5.78).

INTRODUCTION

CUTTING of chicken muscle shortly after slaughter prevents the muscle from achieving the same degree of tenderness after aging that is obtained with intact muscle aged for the same period of time (Lowe, 1948, Koonz et al., 1954; Pool et al. 1959; de Fremery and Pool, 1960; Klose et al., 1971, 1972; Webb and Brunson, 1972).

Peterson (1977) found that if sodium polyphosphates were injected into chicken pectoralis muscle within 20 min postmortem the toughening effect of cutting the muscle off the carcass 1 hr postmortem was prevented. It was suggested that this effect might be produced by slowing down the rate of pH decline since the ultimate pH after aging was considerably higher in the polyphosphate treated muscles.

The pH of pectoralis muscles in chickens immediately at slaughter is 7.2. In the first few minutes after slaughter there is a rise in pH followed by a rapid decline (Dodge and Peters, 1960). This decline is the result of the increase in lactic acid from glycolysis. The rate of decline of pH is the factor which appears to be most directly related to the degree of toughness of the muscle (de Fremery and Pool, 1960, 1963; Khan and Nakamura, 1970). Grey et al. (1974) stated that rigor commences between pH 6.13 and 6.25 assuming from the data of de Fremery and Pool that rigor occurs when the ATP level has declined to 30% of its initial value. Khan (1971) stated that if the postmortem pH drops to 6.3 when the body temperature is still high rigor develops rapidly with resulting toughening of the muscle. When rigor is allowed to occur under conditions where lactic acid formation is minimal the muscle is relatively tender without aging and the ultimate pH after aging will be relatively high. This has been shown by injection of adrenaline pre-slaughter, allowing sufficient time for the lactic acid formed from the resultant pre-mortem glycolysis to be removed from the muscle tissue (de Fremery and Pool 1963, Khan and Nakamura, 1970). de Fremery and Pool (1963) were also able to obtain muscle which was tender without aging by injection of iodoacetate which inhibits lactic acid formation. Injection of iodoacetate resulted in pectoralis muscle with a mean pH of 6.76. On the other hand Khan and Nakamura (1970) showed that injection of adrenaline 2 hr pre-slaughter which caused pre-mortem glycolysis resulted in tough muscle of low pH because insufficient time was allowed for removal of lactic acid from the tissues prior to slaughter.

Khan (1974) found that (isometric) tension began to develop in strips of chicken breast muscle between pH 6.1 and 6.3 and that ultimate shear force after 20 hr aging was related to the maximum tension developed during rigor and not to the rate of rigor decline. It would appear that muscle cut shortly after slaughter would be able to undergo a greater degree of rigor contraction since it is not restrained by complete attachment to the bones. This may be in part an explanation of toughness in cut muscle.

The purpose of this study was to determine to what extent the toughening induced by cutting the muscle shortly after slaughter could be prevented by injection into the muscle of an alkaline buffer (Na_2CO_3-$NaHCO_3$) to slow down the rate of pH decline.

EXPERIMENTAL

MALE WHITE CHICKENS 8–10 wk old from a commercial broiler strain were used in all of these experiments. Each bird was individually slaughtered in the conventional manner, scalded at 57°C for 70 sec, defeathered using a small laboratory size picker and eviscerated.

Immediately after evisceration the pectoralis muscle from one side was injected with the test buffer solution using a hypodermic needle and syringe. Injection was done at numerous locations to distribute the solution as evenly as possible throughout the muscle. Both the treated muscle and the untreated muscle from the opposite side, which served as the control, were cut off the carcass and placed in heat sealed, water-impermeable plastic bags. The entire procedure was accomplished within 10–20 min. The muscles were then placed in a water-ice bath at 4°C until cooked, or if they were to be cooked after 24 hr aging they were placed in a refrigerator at 4°C after 6 hr in the water bath.

Just before cooking the pH of each muscle was determined, using a Corning pH meter and a KCl glass electrode inserted into slits in the muscle at two different locations. Muscles were then weighed and cooked in boiling water for 20 min between aluminum plates spaced 0.64 cm apart as described by Pool et al. (1959). The treated muscle and the untreated control muscle from the opposite side were cooked between the same plate to make conditions as uniform as possible. The plates were then cooled in cold tap water. A circular sample 2-1/8 in. in diameter was cut from the center of the cooked muscle and maximum shear force was measured on each sample with a L.E.E. Kramer apparatus using the special head previously described by Peterson and Lilyblade (1969). Since this device employs 25 steel cylinders which are forced simultaneously through the sample over its entire area, the shear force readings are higher than those determined by conventional single blade devices. Thus a very tender sample will give a reading in the neighborhood of 40 lb.

The buffer solution used in these experiments consisted of equal parts of 4.84% $NaHCO_3$ and 2.88% Na_2CO_3 (in water) which were mixed just prior to injection. The pH of this solution was 9.6. In the first experiment this buffer was injected at the level of 12.5 ml per 100g of muscle assuming the muscle weight to be 7% of the eviscerated weight. This would result in the injected solids content being 0.48% of the muscle weight assuming that all material injected was retained which it obviously was not.

In the second experiment, the buffer was injected at the rate of 18.75 ml per 100g of muscle which would have resulted in the injected solids content being 0.72% of the muscle weight again mak-

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Table 1—Results of injecting $NaHCO_3/Na_2CO_3$ buffer at 12.5 ml/100g muscle[a]

Time post-mortem cooked	No. of birds	Mean pH		Shear force lb		P Level
		Inj.	Control	Inj.	Control	
2 Hr	5	6.84	6.11	86.6 ± 7.0	101.1 ± 9.5	n.s.
3 Hr	7	6.83	5.78	57.6 ± 6.7	67.3 ± 8.2	n.s.
4 Hr	9	6.72	5.76	62.1 ± 3.3	82.3 ± 5.7	<.0'
5 Hr	14	6.80	5.91	56.5 ± 4.2	68.8 ± 4.9	<.1
6 Hr	9	6.54	5.77	59.4 ± 2.0	68.2 ± 4.1	<.05
24 Hr	24	6.50	5.75	38.5 ± 2.2	52.6 ± 2.0	<.001
24 Hr[b] (control)	12	—	5.77	—	41.2 ± 1.0	—

[a] Buffer contained 2.42g $NaHCO_3$ and 1.44g Na_2CO_3 per 100 ml solution

[b] Muscles not cut, aged on the carcass for 24 hr

ing the improbable assumption that all injected material was retained.

In experiment 1, treated and control samples which had been aged at 4°C were cooked at 2, 3, 4, 5, 6 and 24 hr postmortem.

In experiment 2, paired samples aged at 4°C were cooked at 2, 3, 4, 5 and 24 hr postmortem.

RESULTS & DISCUSSION

THE DATA presented in Table 1 show that pectoralis muscles cut off the carcass which had been injected with $NaHCO_3$-Na_2CO_3 buffer to a level of about 0.5% solids content were significantly more tender after aging for 24 hr than the untreated control muscles cut at the same time and aged for the same period. The resulting tenderness of the treated muscles was equivalent to that of pectoralis muscles aged on the carcass for 24 hr. It could not be concluded from this experiment what the minimum time would be for aging muscle treated with $NaHCO_3$-Na_2CO_3 since there was sufficient variability in tenderization of individual muscles that highly significant differences in shear values were not yet obtained after 6 hr aging, even though the mean values for all periods of aging were lower than those of the untreated cut muscles. The main factor responsible for the high degree of ultimate tenderization of the treated muscles was apparently the decrease in the rate of fall of pH and the ultimate high pH (mean 6.5) of these muscles. Although it has been stated by Grey et al. (1974) and Khan (1974) that rigor begins when the pH of the muscle is somewhat under pH 6.3 it is obvious that these treated muscles went through rigor and subsequent tenderization even though the mean pH values were at all times above pH 6.5. High shear values were obtained at 2 hr postmortem but after 24 hr aging the shear values had declined to normal levels expected in uncut adequately aged muscle. The statements of Grey et al. and Khan would only appear to be valid in normal situations where pH values are correlated with ATP levels.

The untreated cut control muscles were tougher after 24 hr than muscles aged for the same time on the carcass even though they did undergo a considerable degree of tenderization. This result is in agreement with those of previous workers as indicated in the introduction.

When the level of injection of the $NaHCO_3$-Na_2CO_3 buffer was increased by 1½ times (Table 2) all periods of aging from 2–24 hr resulted in significantly more tender meat than was obtained with the untreated cut controls. In this treatment muscles which had been cooked after 5 hr aging would probably be considered adequately tender. After 24 hr aging the cut untreated controls were twice as tough as the treated muscles. Treated muscles were very tender. In this experiment there was no further fall in the mean pH values after 2 hr postmortem. At this higher level of injection the treated samples appeared to be more uniformly tender than those in the first experiment (Table 1) even though in some cases the mean pH value was not higher. This may possibly be the result of a more uniform distribution of the buffer throughout the muscle. There was still, however, an appreciable tenderization from 5–24 hr of aging. Control samples as expected were tough after 24 hr of aging.

Table 2—Results of injecting $NaHCO_3/Na_2CO_3$ buffer at 18.75 ml/100g muscle[a]

Time post-mortem cooked	No. of birds	Mean pH		Shear force lb		P Level
		Inj.	Control	Inj.	Control	
2 Hr	12	6.74	5.89	49.4 ± 3.1	88.2 ± 5.2	<.001
3 Hr	10	6.84	5.76	48.8 ± 3.6	77.6 ± 5.2	<.01
4 Hr	10	6.91	5.78	48.7 ± 2.5	67.8 ± 6.2	<.01
5 Hr	10	7.12	5.88	43.8 ± 3.7	69.1 ± 7.8	<.01
24 Hr	12	6.70	5.78	31.3 ± 1.7	60.9 ± 4.2	<.001

[a] See footnote Table 1.

These experiments show that tenderization in cut muscles can be increased by retarding the rate of pH decline.

A small scale laboratory taste panel was conducted on several samples of the treated and control muscle. At the lower level of injection of buffer the panel was unable to distinguish a flavor difference between the treated and control samples. At the higher level of injection, however, a difference was found but tasters showed a preference for the injected samples. This unexpected result may possibly be explained on the basis of a salt effect.

The method described here for tenderization of cut muscle could possibly be used to advantage in the preparation of products using "hot boned" poultry meat where it is desired to obtain a product with maximum tenderness. Use of the procedure would, of course, require approval by the appropriate regulatory agencies.

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CONSUMER ACCEPTABILITY AND RETAIL YIELD IN BEEF AFTER PELVIC SUSPENSION AND DELAYED CHILLING

E. D. ABERLE and M. D. JUDGE

ABSTRACT

The effect of pelvic versus Achilles' tendon suspension, combined with reduced rate of beef carcass chilling during the first 24 hr postmortem, on tenderness and consumer acceptability of round and loin steaks and on retail yield from the hindquarter were determined in this study. Chilling temperature (−2.2, 3.3 or 8.9°C) did not affect retail yield. Pelvic suspended hindquarters yielded less lean trim ($P < 0.001$) but had higher yields of round steak and rump roast. Total retail yield was not affected by method of carcass suspension. Pelvic suspension reduced the dimensions of round steaks and produced a more round shape in short loin steaks as compared to normal suspension. Loin steaks from pelvic suspended carcasses were rated more tender ($P < 0.001$) by a laboratory panel, had longer sarcomeres ($P < 0.001$) and slightly greater myofibril fragmentation; however, chilling temperature did not significantly affect these traits. 614 consumer evaluations of overall palatability in loin and round steaks were received. Consumers ranked steaks from pelvic suspended carcasses higher ($P < 0.01$) than those from normally suspended carcasses. Consumers who indicated they normally purchased Choice grade beef perceived less difference between suspension methods and ranked all steaks lower than consumers who normally purchased Good grade or "whatever was sold in the supermarket." Chilling temperature did not affect consumer rankings of palatability. It is believed that the fat cover on the carcasses was sufficient to prevent muscle temperature from dropping to levels at which cold shortening occurs, even with the lowest chill temperatures used in this study.

INTRODUCTION

MUSCLE CONTRACTION or shortening before and during the onset or rigor mortis decreases meat tenderness (Locker, 1960). Meat tenderness is improved if shortening is prevented (Herring et al., 1965a; Marsh and Leet, 1966). While the amount of muscle shortening is somewhat limited by the tendinous attachment of muscles to the skeleton, the positioning of the limbs and back, either extended or flexed, also influences the degree to which a muscle may shorten. For example, the common practice of hanging carcasses from the Achilles' tendon at the hock allows many muscles of the back and hind limb to shorten (Locker, 1960; Herring et al., 1965b). Herring et al. (1965b) demonstrated that round and loin muscles of beef carcass sides positioned horizontally, with the limbs perpendicular to the back during rigor mortis development, had longer sarcomeres and lower shear values compared to sides suspended vertically from the hock. Reduced muscle shortening and improved meat tenderness can be obtained by suspending beef carcass sides from the pelvic bone (hip suspension) as opposed to Achilles' tendon suspension (Hostetler et al., 1970, 1972, 1973).

Locker and Hagyard (1963) described the cold shortening phenomenon in prerigor excised beef muscle exposed to temperatures near 0°C. Toughening due to cold shortening is maximum at 40% shortening in beef neck muscles (Marsh and Leet, 1966) with less toughening at either lesser or greater amounts of shortening. Rapid post-slaughter chilling of either lamb or beef carcasses can cause cold shortening and toughening of muscle (McCrae et al., 1971; Smith et al., 1971), even though the muscles remain attached to the skeleton. Slower chilling treatments or delayed chilling of carcasses has improved meat tenderness.

Hostetler et al. (1975) investigated the individual and combined effects of method of carcass suspension and chilling temperature on beef tenderness as measured by taste panel and instrument methods. This study was conducted to determine the effect of pelvic suspension combined with reduced rate of carcass chilling post-slaughter on consumer acceptability of round and loin steaks and on yield of retail cuts from the hindquarter.

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EXPERIMENTAL

FORTY-TWO Hereford steers, that had been fed a corn grain and corn silage based ration, were slaughtered in the university abattoir as they reached 454 kg live weight. Carcasses were alloted to chilling treatments of either −2.2, 3.3 or 8.9°C for the first 24 hr postmortem. One side of each carcass was hung vertically from the Achilles' tendon (normal suspension) while the opposite side was hung vertically from the pelvis (Hostetler et al., 1970). The suspension treatments were alternated between right and left carcass sides within each chilling treatment. At 24-hr postmortem, carcasses that had been chilled at either −2.2 or 8.9°C were transferred to the 3.3°C cooler. Data for estimation of USDA Yield and Quality grades were obtained from the sides suspended by the Achilles' tendon at 48 hr post-slaughter. All carcasses were aged for 168 hr before retail cuts were prepared.

Since pelvic suspension alters the shape of the hindquarter but has little affect of the forequarter, retail cut yields were determined on only the hindquarters. For sides hung by the Achilles' tendon, the procedure described by Orts (1962) was followed in separating the hindquarter into wholesale cuts. The tip (knuckle) was removed from the round and from the sirloin portion of the loin before retail cuts were prepared. Skeletal reference points for dividing pelvic suspended hindquarters into wholesale cuts were identical to those for normally suspended quarters. The flank and round tip were removed first by a cut beginning at the 13th rib and extending posteriorly, meeting the femur opposite the hip joint and then proceeding distally along the femur. The round was removed by cutting across the femur immediately ventral and parallel to the long axis of the aitch bone. The rump was then removed from the loin by a cut beginning at the junction of the 4th and 5th sacral vertebrae and extending immediately in front of the hip joint. All retail cuts were trimmed to approximately 0.6 cm fat thickness and were bone-in cuts except for the rump and round tip.

Samples for sarcomere length determination were obtained with a small cork borer from the center of the longissimus muscle opposite the 11th rib at 24 hr postmortem. Sarcomere length was determined as described by Locker (1960) and Herring et al. (1965b) and was expressed as the mean of 25 myofibrils. Samples for measurement of myofibril fragmentation were obtained at 48 and 168 hr postmortem from the longissimus muscle opposite the 12th rib and were frozen for later analyses. Myofibril fragmentation was determined by homogenizing 0.25g of muscle in 30 ml of 0.06M KCl, 0.05M potassium phosphate, pH 7.0 for 60 sec in a Lourdes blender at full speed. The homogenate was centrifuged at 2000 × G for 20 min, the supernatant discarded and the sedimented myofibrils resuspended in 30 ml of the original buffer. Several one-drop aliquots of this suspension were examined by phase contrast microscopy at

1000 × magnification and the number of sarcomeres in 100 randomly selected myofibril fragments was determined. Myofibril fragmentation was expressed as sarcomeres/myofibril fragment with higher values indicating less fragmentation.

The short loin was cut into 2.54 cm thick steaks beginning at the anterior end of the loin. The first steak removed was used for Warner-Bratzler shear value determination while the second and third steaks were evaluated for palatability by a taste panel. Both shear and taste panel steaks were oven broiled to an internal temperature of 66°C. Steaks for Warner-Bratzler shear were cooled to room temperature before 1.27 cm cores were cut for shearing. Hot samples were served to the taste panel immediately after cooking and panel members were asked to evaluate tenderness, juiciness and flavor on a nine-point scale.

Steaks from the short loin and the round (cut 1.9 cm thick) were evaluated for acceptability by a group of consumers who had agreed to participate in the study. Most consumer panelists were members of Purdue University academic, professional or service staffs and were believed to be representative of the general consumer who purchases steak for home consumption. Each consumer purchased 12 loin steaks (two steaks from each suspension by chilling temperature treatement) and 6 round steaks (one steak from each suspension by chilling temperature treatment). The steaks were packaged separately by treatment, frozen (−40°C) and stored (−23°C) for a maximum of 30 days before being distributed to consumers. Eighty-four consumers participated in this study. They were asked to prepare the steaks by whatever method they normally used for home preparation and to return a score sheet (Table 1) for each pair of loin steaks and for each round steak, comparing overall palatability of the sample to that of steaks usually purchased for home consumption. In addition, they were asked to indicate what grade or quality of steak they usually purchased.

Data were subjected to analysis of variance for a 3 × 2 factorial design and individual treatment means were computed using the Statistical Package for the Social Sciences (Nie et al., 1970).

Table 1—Consumer score card

Sample number_____Round or Loin Steak. (circle one)

The overall palatability (tenderness, juiciness, flavor) of this sample is described as (check one):

( ) Equal to the steaks (round or loin) I usually buy for home preparation.

( ) Inferior to the steaks (round or loin) I usually buy for home preparation. If so, in what way?_____

_____.

( ) Superior to the steaks (round or loin) I usually buy for home preparation. If so, in what way?_____

_____.

The steaks I usually buy for home preparation are (check one):

( ) Choice grade
( ) Good grade
( ) Whatever is sold in supermarkets
( ) Other_____

Table 2—Means for carcass traits and quality and yield grades

Item	Mean[a]	S.D.
Carcass weight, kg	278.0	5.0
Fat thickenss, 12th rib, cm	1.57	1.38
Rib eye area, cm^2	66.9	5.5
Kidney, heart and pelvic fat, %	3.7	0.7
USDA Yield grade	3.8	0.6
Marbling score[b]	5.9	1.3
Quality grade[c]	12.8	1.4

[a] n = 42
[b] 5 = small, 6 = modest
[c] 11 = high Good, 12 = low Choice, 13 = average Choice

Table 3—Yield of retail cuts from normal and pelvic suspended beef hindquarters[a]

Retail cut	Normal suspension	Pelvic suspension
Hind quarter	67.03	66.66
Loin steak	7.14	6.84
Flank steak	0.65	0.63
Round tip	3.97	4.19
Sirloin steak	5.69	5.66
Round steak	8.96	9.52
Rump roast	3.09	3.31
Lean trim	13.16***	12.23***

[a] All values are expressed in kg and are the mean of 28 observations.
*** $P < 0.001$.

RESULTS & DISCUSSION

SINCE CARCASSES were randomly allotted to one of the chilling treatments and one side of each carcass was allotted to one of the two suspension treatments, means for carcass traits are presented for the experimental population (Table 2) but individual treatment means are not presented. The mean quality grade was nearly average Choice and carcasses were slightly fatter than the average U.S. beef carcass, with a mean fat thickness of more than 1.5 cm and mean yield grade of 3.8.

Pelvic suspension alters the shape of the hindquarter and might be expected to change the proportional yields of the respective retail cuts. For this reason, yields of retail cuts were measured in contralateral sides subjected to the two suspension procedures (Table 3). Since the range of carcass weights was very small (cattle slaughtered at constant weight), and the treatments were imposed on opposite sides of the same carcass, data are presented in kg of each retail cut rather than percentage yields. Pelvic suspended hindquarters yielded significantly less lean trim and tended to yield slightly less loin steak. However, yields of round tip, round steak and rump roast were generally higher in pelvic hung quarters. Differences in round steak and rump roast approached significance ($P < 0.05$).

The physical shape of both round and loin steaks was affected by suspension method. Steaks from the short loin of pelvic suspended sides were more round in appearance while those from normally suspended sides tended to be more elongated with a straight or even a dished outline along the fat cover surface (Fig. 1). Round steaks from pelvic hung sides were smaller compared to those from normally hung sides. This difference might be expected since pelvic suspension stretches muscles in the round. As a muscle is stretched, its cross-sectional size decreases. Even though round steaks were smaller in the pelvic hung sides, these sides yielded one or two more round steaks and almost the same weight of this retail cut as the normally suspended sides.

As has been shown by other workers (Hostetler et al., 1970, 1972, 1973), pelvic suspension of beef carcasses increased sarcomere length of the longissimus muscle compared to normal suspension. The laboratory taste panel ranked steaks from these sides higher in tenderness but did not find differences in flavor or juiciness (Table 4). Warner-Bratzler shear values were slightly but not significantly lower in the pelvic hung sides. Pelvic suspension was also associated with a tendency for greater myofibril fragmentation in the longissimus and the difference approached significance at 168 hr postmortem.

Slower carcass chilling, either 3.3 or 8.9°C compared to −2.2°C, during the first 24 hr postmortem did not significantly affect sarcomere length or myofibril fragmentation and did not improve tenderness as measured by shear value or as perceived by the taste panel (Table 4). Chill tempera-

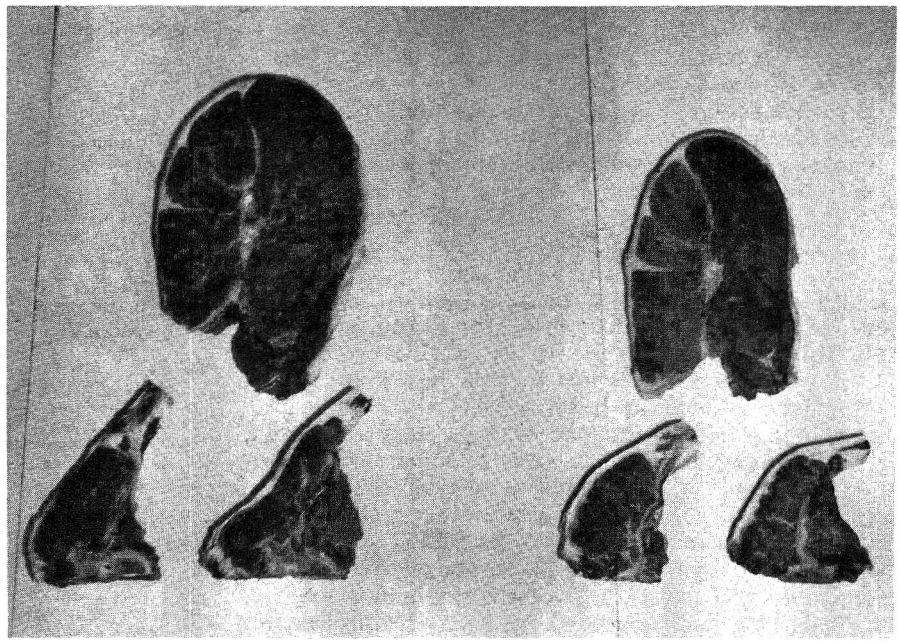

Fig. 1—Round (top) and loin (bottom) steaks from contralateral sides of a beef carcass suspended by the Achilles' tendon (left) and the pelvis (right).

ture by suspension method interactions were not significant for any of the variables. Parrish et al. (1969) were unable to detect significantly improved tenderness in beef longissimus muscles of carcasses chilled at 7–15°C for the first 24–48 hr postmortem followed by 2°C, as compared to chilling at 2°C for the entire postmortem period. However, others demonstrated that chilling temperatures ranging from 14–19°C for 16–24 hr postmortem significantly improved tenderness (Smith et al., 1971; Parrish et al., 1973; Fields et al., 1976) as opposed to chilling immediately at 0–2°C. Hostetler et al. (1975) suggested that pelvic suspension produced greater improvements in tenderness than did chilling at 16°C for 20 hr post-slaughter and that use of a combination of pelvic suspension and elevated temperature produced the most tender beef when the processes were compared.

In the consumer acceptability segment of this study, 614 replies were received out of 1008 replies possible, a return rate of 60.9%. For data analyses, numerical values were assigned as follows: 1 = inferior to-, 3 = equal to-, 5 = superior to palatability of steaks usually purchased for home preparation. Round steaks from pelvic suspended carcasses were ranked higher ($P < 0.01$) in overall palatability but there was no significant preference for loin steaks from pelvic suspended sides over those from sides suspended by the Achilles' tendon (Table 5). Palatability scores for loin steaks were generally higher than for round steaks but round steaks from pelvic hung sides received rankings nearly equal to rankings for loin steaks from either suspension method. Space was provided for panelists to indicate in what way steaks were inferior or superior in palatability to steaks usually purchased for home preparation (Table 1). Every reply did not contain comments and no analyses were conducted of those comments offered. The most com-

Table 4—Sarcomere length, myofibril fragmentation, Warner-Bratzler shear, and laboratory taste panel scores

Item	Chilling temp, °C[a]			Suspension method[a]	
	−2.2	3.3	8.9	Normal	Pelvic
Sarcomere length, μm	2.25	2.18	2.19	2.04***	2.37***
Myofibril fragmentation					
48 hr[b]	9.10	10.40	10.39	10.30	9.62
168 hr[b]	6.80	7.63	7.31	7.60	6.88
W-B shear value, kg	2.23	2.34	2.36	2.34	2.28
Tenderness score[c]	6.95	7.03	6.96	6.72***	7.24***
Juiciness score[c]	7.40	7.49	7.38	7.38	7.46
Flavor score[c]	7.50	7.44	7.49	7.45	7.57

a Means for chilling temperature include both suspension methods and means for suspension method include three chilling temperatures.
b Sarcomeres/myofibril fragment
c 1 = extremely tough, extremely dry, or extremely bland flavor; 9 = extremely tender, extremely juicy or extremely strong flavor.
*** $P < 0.001$.

Table 5—Consumer rankings of round and loin steaks

Item	n	Chilling temp, °C[a]			Suspension method[a]	
		−2.2	3.3	8.9	Normal	Pelvic
All consumers						
Round steaks	281	3.34[b]	3.65	3.46	3.22**	3.76**
Loin steaks	333	3.93	3.81	3.94	3.83	3.96
Combined round & loin steaks	614	3.65	3.73	3.73	3.54**	3.87**
Choice grade purchasers[c]	370	3.55	3.54	3.58	3.42*	3.71*
Good grade purchasers[c]	75	3.90	3.95	3.71	3.78	3.88
Supermarket beef purchasers[c]	150	3.90	4.11	4.26	3.77**	4.39**
Other purchasers[c]	19	2.67	3.00	3.50	3.22	3.00

a Means for chilling temperature include both suspension methods and means for suspension method include three chilling temperatures.
b Consumer replies were coded as follows: 1 = inferior to-, 3 = equal to-, 5 = superior to steaks usually purchased for home preparation. See Table 1.
c Values are for round and loin steaks combined.
* $P < 0.05$
** $P < 0.01$

mon reason given for ranking steaks superior to those usually purchased was that they were more tender. Consumers detected no difference in acceptability among steaks from carcasses subjected to different rates of postmortem chilling. There were no significant chill temperature by suspension method interactions.

Consumer replies were analyzed according to purchasing habits indicated on the score sheets. Approximately 60% of the consumers indicated they normally purchased Choice grade steaks, about 25% said they purchased whatever was sold in the supermarket and 12% bought Good grade (Table 5). All three groups of consumers ranked steaks from pelvic suspended sides higher than those from normally hung sides, although the difference was not significant for purchasers of Good grade. Consumers who normally purchased Choice grade did not rank steaks as highly as those persons who bought either Supermarket or Good grade beef. This probably indicates that Choice grade consumers are accustomed to a higher quality product and thus are more demanding in their expectations of acceptability in steak.

This study demonstrated that prevention of muscle shortening by hanging beef sides from the pelvic bone will improve meat tenderness, as had been shown by other research cited previously, and that this is reflected in greater consumer acceptability of the product. Pelvic suspension does not markedly affect the yield of the respective retail cuts from the hindquarter but may improve the appearance of steaks from the short loin, largely due to the shape of the cuts.

Delayed chilling treatments did not affect tenderness or acceptability. Cold shortening and its effects on tenderness have been conclusively demonstrated in beef (Locker and Hagyard, 1963; Marsh and Leet, 1966). Bendall (1972) indicated that the critical muscle temperature for onset of cold shortening is 11°C and that a muscle temperature of 3°C causes the most severe cold shortening. Longissimus muscle temperature at the 12th and 13th rib junction might decrease to the critical temperature for cold shortening before rigor onset when chilling carcasses at −2°C but this is less likely at a 3°C cooler temperature (Glover et al., 1977). The subcutaneous adipose tissue is a very effective insulator in preventing cold shortening and maintaining tenderness in the beef longissimus muscle (Meyer et al., 1977). Since the carcasses in the present study were slightly fatter than those in the report by Glover et al. (1977), it is unlikely that muscle temperature reached the critical level for development of cold shortening before rigor developed. In that case, one would not expect the chilling treatments to affect tenderness. On the other hand, a slower rate of chilling postmortem would probably be effective in improving tenderness of those carcasses with very little backfat over the longissimus muscle.

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SHELF-LIFE AND QUALITY CHARACTERISTICS OF POULTRY PARTS DIPPED IN POTASSIUM SORBATE

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ABSTRACT

Fresh, broiler parts were dipped 30 or 60 sec in solutions of potassium sorbate. Drumsticks were stored at refrigerator temperature and evaluated daily for off-odor development and total counts. Thighs and breasts were baked to internal breast temperature of 85°C, cooled, then evaluated for shear press values, moisture retention, and sensory properties. Drumsticks dipped in potassium sorbate developed off-odors later than the controls. Those parts dipped in 10% potassium sorbate had a 20-day shelf-life at 4°C, based on off-odor development and time for total counts to reach 10^7. Parts dipped in potassium sorbate for 30 sec, drained, then baked had similar Shear Press (tenderness) and Carver Press (moisture retention) values regardless of the sorbate concentration used. Taste panel members could not distinguish between those parts dipped in 5 or 10% potassium sorbate and those parts dipped in distilled water. Sensory evaluations were significantly lower for those parts dipped in 15% potassium sorbate solutions for 1 min. Parts dipped in 5 or 10% solutions for 1 min were indistinguishable from the controls.

INTRODUCTION

THE RELATIVELY SHORT shelf-life of fresh, unfrozen poultry has been a major problem for the poultry industry. The initial bacterial load greatly influences the shelf-life of broilers (Arafa and Chen, 1977). In retail outlets, fresh broilers can be expected to have an initial concentration of 10^4-10^5 microorganisms/cm^2 (Brune and Cunningham, 1971; Walker and Ayres, 1956; Woodburn, 1964).

Sorbic acid has been reported to be an effective antimicrobial agent when applied to poultry as a 7.5% solution in a 70:20:10 propylene glycol, water and glycerine mixture at 60°C (Perry et al., 1964).

Sorbic acid has also been reported to inactivate *S. typhimurium* in media, milk, and cheese (Park et al., 1970; Park and Marth, 1972). Robach and Ivey (1978) and Robach (1978) reported that a 5% potassium sorbate dip significantly reduced the total plate count of chicken. They also observed that a solution of 5% potassium sorbate reduced the growth of salmonellae inoculated onto the surface of the chicken.

This report concerns the shelf-life of fresh poultry parts dipped in potassium sorbate solutions and the sensory properties of cooked chicken parts after sorbate treatment.

METHODS & MATERIALS

Meat

Fresh poultry (cut-up broilers) was purchased from a commercial processor.

Sorbate dip

Chicken parts were dipped 30 or 60 sec in 5-liter solutions of either distilled water, 5.0% (W/V), 10.0%, or 15% potassium sorbate, drained for 10 min, and packaged in sterile, polyethylene plastic bags.

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Table 1—Shelf-life of drumsticks (at 4°C) when dipped 30 sec in potassium sorbate

% Potassium sorbate	Day off-odor detected	Day total count reached 10^7/cm^2
0	10 ± 0.5	11 ± 0.5
2.5	13 ± 0.5	13 ± 0.5
5.0	14 ± 0.5	15 ± 0.5
7.5	16 ± 0.5	17 ± 0.5
10.0	20 ± 0.5	20 ± 0.5

Microbiological examination

All samples were stored at 4°C. Six parts were analyzed per variable.

Samples were enumerated by adding 100 ml of the sterile 0.005M phosphate buffer (pH 7.2) to the packaged chicken, shaking for 1 min, and diluting further before plating with standard plate count agar (Difco). Total plate counts were determined after 48 hr of incubation at 29°C. All counts are reported on a per cm^2 basis according to the method of Goresline and Haugh (1958).

Cooking method

The treated poultry parts, without batter or breading, were baked (on racks) in a commercial rotary oven to an internal temperature of 85°C. The oven was set at 204°C.

Carver Lab Press

After baking and cooling, 500 mg squares were cut from the muscle of each poultry part, placed on 9 mm filter paper (Watman #1), and pressed in the Carver press between two Plexiglas plates. The samples were pressed for 5 min at 10,000 psi. The expressed moisture was measured by determining the area of spread with a compensating polar planimeter.

Kramer Shear Press

For shear press determinations, cooked muscles were cut into approximately 2 × 5 × 1 cm and trimmed to a weight of exactly 10g. Using a 15-sec downstroke, we attached a 1,500 kg ring to the hydraulic ram of the Kramer shear press, and with the instrument set in the 100 kg range, the total force required to shear each sample was recorded electronically.

Sensory evaluations

The taste panel was composed of six adults previously used on poultry meat sensory panels. They were asked to score samples for flavor, tenderness, juiciness, and overall acceptability using a nine-point hedonic scale ranging from like extremely (9) to dislike extremely (1). Each sample consisted of one uncoated drumstick, thigh, or breast with skin intact. Sensory evaluation was carried out over a 6-month period, resulting in 32 observations per treatment. An analysis of variance was conducted on each treatment and panel scores. The Newman-Keuls test (Anderson and McLean, 1974) was done on the means when statistical differences existed.

In addition, triangular taste tests (Boggs and Hanson, 1949) were used to determine if potassium sorbate dips affected flavor. Significance of these tests was determined from standard tables (Roessler et al., 1948).

RESULTS & DISCUSSION

SHELF-LIFE of fresh broiler drumsticks at refrigerator temperature after being dipped in potassium sorbate solutions for 30 sec is shown in Table 1. Untreated drumsticks

remained acceptable (no off-odor) for 10 days. As determined by off-odor development and total counts reaching $10^7/cm^2$, drumsticks dipped in 5% potassium sorbate had about a 4-day extension of shelf-life. Those dipped in 10% solutions took 20 days to spoil by these criteria. These data agree with earlier work that sorbates effectively control the growth of spoilage organisms associated with fresh poultry (Horwitz and Gangarosa, 1976; Park and Marth, 1972).

Properties of baked chicken as affected by dipping for 30 sec in potassium sorbate are shown in Table 2. Those parts dipped in either 0, 5, 10, or 15% solutions had similar Shear Press and Carver Press values, indicating no difference in tenderness and moisture retention. Likewise, taste panel data indicated no difference in flavor, juiciness, or tenderness of parts dipped in 0, 5, 10 or 15% solutions. At the 15% dip level, however, taste panel scores were slightly lower, but not significantly so, for over-all acceptability.

To test thoroughly the sensory properties of fresh poultry parts dipped in 5% potassium sorbate, we conducted triangular taste tests on cooked poultry parts after a 30-sec dip treatment (Table 3). From a total of 51 judgments, only 17 were correct. By chance alone, 1/3 of the responses should have been correct. Panel members could not detect cooked poultry parts dipped for 30 sec in 5% potassium sorbate.

Sensory evaluation of poultry parts after a 1-min dip in potassium sorbate is shown in Table 4. Panel members could not distinguish between control pieces and those dipped in 5 or 10% solutions. As indicated in Table 2, however, panel members scored those parts which had been dipped in 15% solutions (1-min) lower in flavor, tenderness, juiciness, and over-all acceptability. By analysis of variance, those differences were significant at the 1% level of probability.

We found that using potassium sorbate on fresh poultry extends the shelf-life of the product, and that 5 or 10% potassium sorbate used as a dip for 30 or 60 sec does not affect sensory characteristics of cooked poultry.

Table 2—Properties of baked poultry parts as affected by dipping 30 sec in potassium sorbate

	Potassium sorbate (%)			
	0	5	10	15
Shear Press values	4.5	4.7	4.4	4.5
Carver Press values	0.52	0.50	0.49	0.51
Taste panel means				
Flavor	5.9	6.0	5.8	5.8
Juiciness	5.2	5.2	5.1	5.1
Tenderness	6.0	6.0	5.9	5.9
Acceptability	5.8	5.8	5.8	5.6

Table 3—Triangular taste test of cooked poultry parts as affected by dipping 30 sec in 5% potassium sorbate

Control	Dipped	TJ[a]	CJ[b]
1	2	26	9 n.s.
2	1	25	8 n.s.

[a] TJ = total judgments
[b] CJ = correct judgments; n.s. = not significant

Table 4—Means of sensory evaluation scores of baked chicken as affected by dipping 1 min in potassium sorbate[a]

	Flavor	Tenderness	Juiciness	Acceptability
Control (no dip)	6.21a	6.10a	6.25a	6.10a
Treatment				
5% dip	6.20a	6.15a	6.20a	6.15a
10% dip	6.10a	6.10a	6.15a	6.10a
15% dip	5.00b	5.22b	5.18b	5.21b

[a] Means with different letters in the same column differ significantly at $P < 0.01$.

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EFFECTS OF OILSEED PROTEINS, AT TWO REPLACEMENT LEVELS, ON CHEMICAL, SENSORY AND PHYSICAL PROPERTIES OF FRANKFURTERS

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ABSTRACT

Frankfurters (all meat) and frankfurter-like products (oilseed proteins replaced 10% or 30% of the meat) were prepared. Oilseed proteins were hydrated to a protein content of 13% during batter preparation. There were no significant differences among finished products in protein content; however, frankfurter-like products containing 10% oilseed proteins had ($P < 0.05$) higher moisture, fat and ash percentages than did frankfurters or frankfurter-like products containing 30% oilseed proteins. Among frankfurter-like products, bioyield values from Instron force-deformation curves were greater for products containing 10% oilseed protein than for those containing 30% oilseed protein. Although not significantly different in all comparisons at the 10% and 30% replacement levels, frankfurter-like products containing soy concentrate, soy isolate and textured soy flour had greater bioyield values than did those products containing cottonseed proteins. Sensory panel ratings for overall satisfaction of frankfurters and frankfurter-like products containing 10% oilseed proteins revealed a significant difference ($P < 0.05$) between the all-meat frankfurters and all frankfurter-like products except those containing soy concentrate and textured soy flour. Differences in sensory panel ratings for overall satisfaction and texture desirability were more apparent among frankfurter-like products when 30%, rather than 10%, of the meat was replaced with oilseed proteins. None of the frankfurter-like products containing 30% oilseed proteins was as desirable in overall satisfaction or texture desirability as the all-meat frankfurter.

INTRODUCTION

INCREASING COSTS of conventional protein foods (meat, milk, eggs) have encouraged the sausage manufacturer to study alternative protein sources (Wilding, 1974). Terrell and Staniec (1975) described the apparent economic basis for using vegetable proteins in comminuted meats and suggested that a nutritional-cost concept was of major consumer concern. Impetus for this concept was provided by the FNS-219 regulation (USDA, 1972) for type-A menus in the School Lunch Program which allowed up to 30% of the meat in the finished product to be replaced with a nutritionally equivalent vegetable protein hydrate. Frankfurters exceeding 3½% added proteins on a cooked product basis are nonspecified products with no USDA standard of identity and must be labeled with a qualifying phrase contiguous to the products fanciful name (USDA, 1977).

Functional properties of various proteins in processed meats have been studied (Sofos and Allen, 1977; Skurray et al., 1976; Lin et al., 1975; Terrell and Staniec, 1975; Smith et al., 1973). Generally, these properties include improving yields, retaining moisture, reducing fat, reducing manufacturing cost or improving fat binding in sausage products. However, the use of high levels of vegetable proteins in frankfurters with protein, fat and moisture contents equivalent to those of an all-meat product may cause problems in emulsion stability, texture, flavor and color (Cassens et al., 1975, 1977). The objective of the present study was to determine the effects of eight different types of oilseed proteins at two meat replacement levels on the chemical, physical and sensory properties of vacuum packaged frankfurters.

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EXPERIMENTAL

Manufacturing

Two replications of frankfurter treatments involving replacement of 10% or 30% of the raw meat with eight different oilseed proteins were prepared. Two batches of an all-meat control formula were made for comparative purposes. Included in the eighteen 4.5 kg batches, where appropriate, were the following proteins (identified by description, percentage of protein, brand name–if applicable–and source): glandless cottonseed flour (GLCF), 65% protein, TAMU; soy flour (SF), 50% protein, Soyafluff 200W, Central Soya Co.; liquid cyclone processed glandless cottonseed flour (LCP), 70% protein, Promasoy, Central Soya Co.; cottonseed storage protein isolate (IC), 90% protein, Cenpro M, Central Soya Co.; textured glandless cottonseed flour (TC), 65% protein, TAMU; textured soy flour (TS), 50% protein, Archer-Daniels Midland Co. To maintain a constant fat content (estimated 28%), in each formulation, the meat block for use with 10% replacement studies was formulated with 34% beef and 66% pork (Table 1). Added proteins were calculated to contain an equivalent content of 13% protein (Table 1). Added protein was hydrated in the 3-blade bowl chopper with meat during the 1 hr time lag before heat processing (Ziemba, 1974). Proteins were added to batters after all meats, other dry ingredients and 2/3 of the total water were chopped 4–6 min (1/3 total water was added simultaneously when proteins were added). Lean meats, salt, cure and 1/3 total water were first chopped 1–2 min before addition of other ingredients. Batters were chopped to 20°C (8–10 min), discharged directly to the stuffer (pneumatic piston), stuffed into 23 mm clear cellulose casings and linked (14 cm). A 1.5 hr conventional cook and smoke cycle (internal frankfurter temperature of 72°C) followed by a 3 min cold water shower and overnight chilling to 3°C was completed prior to peeling and vacuum packaging (nylon/saran/PVC film).

Analytical

Vacuum packaged samples were frozen (–34°C) for 2 wk and subsequently analyzed for moisture, fat, protein and ash according to AOAC (1975) procedures. Just prior to sensory panel and Instron analysis, frankfurters were steeped in boiling water for 7 min. An eight-member untrained sensory panel evaluated samples for juiciness, texture desirability, and overall satisfaction using a nine-point hedonic scale (9 = like extremely; 1 = dislike extremely). Instron shear force-deformation analysis using a L.E.E.-Kramer shear cell was performed according to the procedure of Bourne et al. (1966). Analysis of variance (Steel and Torrie, 1960) and mean separation (Duncan, 1955) were performed on the data.

RESULTS & DISCUSSION

MEAN CHEMICAL COMPOSITIONS according to replacement levels are presented in Table 2. There were no significant ($P > 0.05$) differences in protein contents of any of the batches of frankfurters. However, the 10% level frankfurter-like products had higher ($P < 0.05$) percentages of

Table 1—Formulations for control frankfurters and frankfurter-like products

Replacement level (%)[a]	Protein added[b]	Beef (%)	Pork (%)	Dry ingredients[c] (%)	Dry protein (%)	Added water (%)		Total (%)
						For meat	For dry protein	
0	None	56.0	19.1	5.18	0.0	20.7	0.0	100
10	GLCF	54.5	12.1	5.18	1.6	20.7	5.8	100
10	SF	54.5	12.1	5.18	1.9	20.7	5.5	100
10	LCP	54.5	12.1	5.18	1.5	20.7	5.9	100
10	SC	54.5	12.1	5.18	1.4	20.7	6.0	100
10	IC	54.5	12.1	5.18	1.1	20.7	6.3	100
10	IS	54.5	12.1	5.18	1.1	20.7	6.3	100
10	TC	54.5	12.1	5.18	1.6	20.7	5.8	100
10	TS	54.5	12.1	5.18	1.9	20.7	5.5	100
0	None	54.5	20.2	5.18	0.0	20.7	0.0	100
30	GLCF	17.8	34.1	5.18	4.8	20.7	17.4	100
30	SF	17.8	34.1	5.18	5.8	20.7	16.4	100
30	LCP	17.8	34.1	5.18	4.4	20.7	17.8	100
30	SC	17.8	34.1	5.18	4.2	20.7	18.0	100
30	IC	17.8	34.1	5.18	3.2	20.7	19.0	100
30	IS	17.8	34.1	5.18	3.2	20.7	19.0	100
30	TC	17.8	34.1	5.18	4.8	20.7	17.4	100
30	TS	17.8	34.1	5.18	5.8	20.7	16.4	100

[a] Meat replacement based on raw meat (green meat formula weight)
[b] GLCF = glandless cottonseed flour; SF = soy flour; LCP = liquid cyclone processed cottonseed flour; SC = soy concentrate; IC = cottonseed isolate; IS = soy isolate; TC = textured cottonseed flour; TS = textured soy flour
[c] Commercial blend: Salt, corn syrup solids, dextrose, mustard, hydrolyzed plant protein, paprika and other spices, natural smoke flavor, spice extractives, onion and garlic powder, sodium erythorbate, sodium nitrite

moisture and lower percentages of fat and ash than did the control or 30% level products; these differences were probably due to understuffing of the control and 30% level products. Understuffing tends to increase shrinkage and in particular, loss of moisture, which would increase the fat content. Another possible explanation for higher moisture contents in the 10% level frankfurter-like products as opposed to those with 0% or 30% replacement is that added water for meat (Table 1) was not simultaneously reduced by 10% and 30%, respectively, as meat was replaced. In developing protein-added formulations where meat is replaced at different levels, statistical comparisons of products between replacement levels is invalid unless the fat, moisture and protein contents are equivalent (Sofos and Allen, 1977). However, from a commercial standpoint, it is more difficult to produce acceptable products of equal fat and moisture contents when 10% versus 30% of the meat is replaced with oilseed proteins. What actually is encountered in the former case is that fatter meats are used at the 30% replacement level in order to approximate fat contents equivalent to those for products formulated at the 10% replacement level. Therefore, more of the collagen-type protein is automatically included in the 30% replacement as opposed to the 10% replacement frankfurter-like formulation; hence a softer, generally less desirable product is produced (Randall et al., 1976). For acceptable commercial products, the volume dilution of fat, with added proteins and skeletal meats would be more preferable (i.e., finished fat content of 20–22%; Terrell, 1975).

Sensory panel scores for juiciness, texture desirability and overall satisfaction for all-meat product and product with 10% of the meat replaced are presented in Table 3. LCP frankfurter-like products had significantly ($P < 0.05$) higher juiciness scores than TS products but neither were different from any of the other products including the all-meat frankfurters. There was no difference ($P > 0.05$) in texture desirability between control and TS frankfurter-like products; however, all-meat frankfurters had higher ($P < 0.05$) texture desirability scores than all of the other frankfurter-like products. All-meat frankfurters had higher overall satisfaction ratings than all of the frankfurter-like products except those containing TS or SC. Terrell (1975), Cassens et al. (1975) and Roberts (1974) reported that at low meat replacement levels (i.e., 10% and less) in sausage products containing fat contents of 20–30%, a variety of oilseed

Table 3—Mean ratings for juiciness, texture desirability and overall satisfaction for control frankfurters and for frankfurter-like products made at the 10% meat replacement level

Protein additive[a]	Juiciness rating[b]	Texture desirability rating[b]	Overall satisfaction rating[b]
None	4.9cd	5.7c	5.4c
GLCF	4.8cd	4.5d	4.6d
SF	4.8cd	4.9d	4.8d
LCP	5.6c	4.5d	4.8d
SC	4.8cd	5.0d	5.0cd
IC	4.8cd	4.7d	4.8d
IS	4.7cd	4.0d	4.6d
TC	5.0cd	4.8d	4.7d
TS	4.3d	4.9d	4.9cd

[a] GLCF = glandless cottonseed flour; SF = soy flour; LCP = liquid cyclone processed cottonseed flour; SC = soy concentrate; IC = cottonseed isolate; IS = soy isolate; TC = textured cottonseed flour; TS = textured soy flour
[b] Like extremely = 9; dislike extremely = 1. Means in the same column followed by a common letter do not differ ($P > 0.05$)

Table 2—Percentages of chemical components for control frankfurters and frankfurter-like products[a]

Chemical component	Meat replacement level		
	0%	10%	30%
Protein	13.78a	13.78a	13.41a
Moisture	54.35a	56.98b	54.41a
Fat	26.50a	23.58b	26.24a
Ash	3.62a	3.07b	4.04a

[a] Means in the same row followed by a common letter do not differ ($P > 0.05$)

Table 4—Mean ratings for juiciness, texture desirability and overall satisfaction for control frankfurters and for frankfurter-like products made at the 30% meat replacement level

Protein additive[a]	Juiciness rating[b]	Texture desirability rating[b]	Overall satisfaction rating[b]
None	5.1cd	5.8c	5.7c
GLCF	5.3cd	2.2f	2.4ef
SF	5.0cd	2.9ef	2.9def
LCP	5.7c	2.1f	2.2f
SC	4.5d	3.6de	3.4de
IC	5.2cd	2.7ef	2.9def
IS	5.1cd	3.3de	3.4def
TC	4.8cd	4.0de	3.0def
TS	4.6d	4.2d	3.9d

[a] GLCF = glandless cottonseed flour; SF = soy flour; LCP = liquid cyclone processed cottonseed flour; SC = soy concentrate; IC = cottonseed isolate; IS = soy isolate; TC = textured cottonseed flour; TS = textured soy flour

[b] Like extremely = 9; dislike extremely. Means in the same column followed by a common letter do not differ ($P > 0.05$)

proteins alone (extruded flours, nonextruded flours and concentrates) may produce acceptable products; however, when higher replacement levels are used, a combination of protein types may be required in order to produce acceptable products. Sensory panel evaluation is an important indicator of potential consumer preferences (juiciness, texture, overall satisfaction); likewise visual product appearance is also quite important. It was observed that frankfurter-like products containing cottonseed proteins had a more brownish-greenish color than any of the other products. Regardless of functional properties, the cottonseed proteins used in this study would not likely be the protein additive of current choice for incorporation into cured sausage products, because of visual color problems.

Performance of the various oilseed proteins with respect to juiciness, texture desirability and overall satisfaction at the 30% meat replacement level are presented in Table 4. The LCP frankfurter-like products had higher ($P < 0.05$) juiciness ratings than TS and SC frankfurter-like products but none of the frankfurter-like products differed in juiciness from all-meat frankfurters. All-meat frankfurters had higher ($P < 0.05$) texture desirability and overall satisfaction ratings than all other products. Generally, the GLCF and LCP frankfurter-like products had higher juiciness ratings and lower texture desirability and overall satisfaction ratings than did frankfurter-like products made with soy proteins. Apparently, use of cottonseed proteins at the 30% level resulted in greater moisture retention which may have decreased texture desirability and overall satisfaction ratings. Comparison of data in Tables 3 and 4 for texture desirability and overall satisfaction indicates that frankfurter-like products containing 30% oilseed protein were assigned lower ratings than were products containing 10% oilseed protein. These data suggest that an upper limit on meat replacement in frankfurter formulations may exist which is not necessarily in accordance with the maximum level suggested in FNS-219 (USDA, 1972). Adjustments in seasoning (spice formulation) are made commercially to compensate for loss of flavor with increasing meat replacement levels and to improve overall desirability. Sofos and Allen (1977) reviewed the importance of relationships among skeletal meat, fat and replacement levels with textured soy protein in formulating emulsion-type sausage products.

Objective texture measurements (shear force-deformation curves) for frankfurter-like products containing 10% or 30% of oilseed proteins, using an Instron Universal Testing

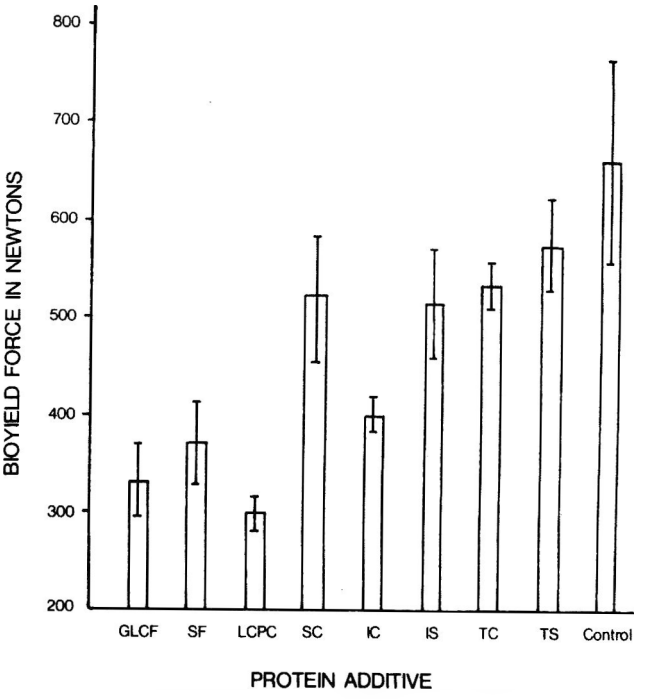

Fig. 1—Instron bioyield force (L.E.E.-Kramer cell) for control frankfurters and for frankfurter-like products made at the 10% meat replacement level. Means with overlapping bars are not different ($P > 0.05$). GLCF = glandless cottonseed flour; SF = soy flour; LCP = liquid cyclone processed cottonseed flour; SC = soy concentrate; IC = cottonseed isolate; IS = soy isolate; TC = textured cottonseed flour; TS = textured soy flour.

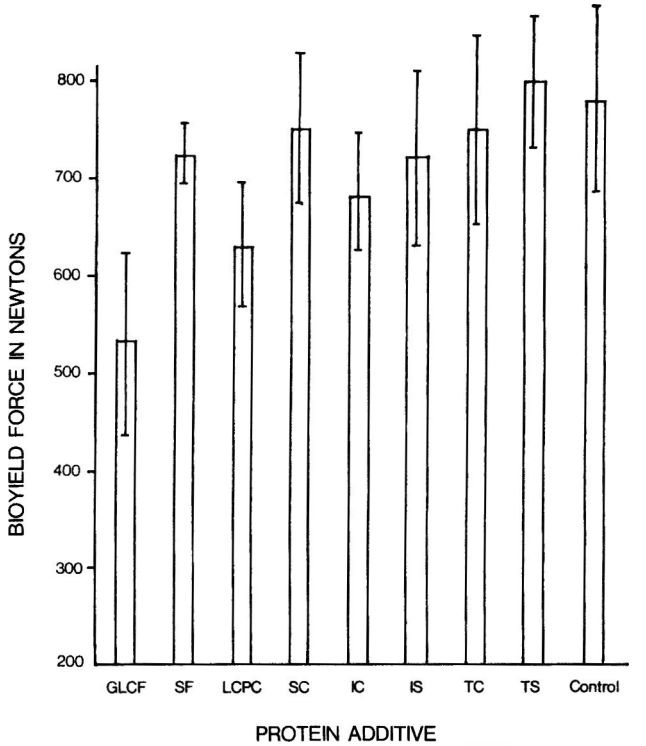

Fig. 2—Instron bioyield force (L.E.E.-Kramer cell) for control frankfurters and for frankfurter-like products made at the 30% meat replacement level. Means with overlapping bars are not different ($P > 0.05$). GLCF = glandless cottonseed flour; SF = soy flour; LCP = liquid cyclone processed cottonseed flour; SC = soy concentrate; IC = cottonseed isolate; IS = soy isolate; TC = textured cottonseed flour; TS = textured soy flour.

machine with a L.E.E.-Kramer cell, are presented in Figures 1 and 2. Bioyield determinations from the shear force deformation curve of frankfurters have been described by Terrell et al. (1979). Bioyield forces (Newtons per cm^2) were generally greater for the frankfurter-like products with 10%, as compared to 30%, oilseed protein. These results confirm sensory panel results with regard to effects of meat replacement on texture desirability and overall satisfaction (Tables 3 and 4).

Frankfurter-like products containing 10% or 30% texturized soy protein or 10% or 30% texturized cottonseed protein had bioyield forces which did not differ from those of all-meat frankfurters (Fig. 1 and 2). Apparently, extruded proteins contribute to textural properties of frankfurters as measured by the Instron bioyield force (Terrell, 1975). Differences in protein functionality as measured by the bioyield force are quite dramatic at the 30% replacement level (Fig. 2). The GLCF, SF, LCP and IC frankfurter-like products had lower ($P < 0.05$) bioyield forces than any of the other products. These protein additives alone contributed little to improvement of textural properties of frankfurter-like products when 30% of the meat was replaced. On the other hand, textured soy (TS), textured cottonseed (TC), soy concentrate (SC) and soy isolate (IS) improved the bioyield force when used to replace meat at the 30% level.

These data suggest that in order to improve textural properties of frankfurter-like products at relatively high meat replacement levels, different combinations of oilseed proteins should be considered. Terrell (1973) reported that a combinations of soy concentrate and textured soy flour improved the textural properties (Instron bioyield force) of beef patties at higher meat replacement levels to a greater extent than was possible with either protein alone. Research by Terrell and Staniec (1975) also suggested that combinations of oilseed proteins improved the textural properties of batter-type sausage products.

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EFFECTS OF PRECOOKING BEEF PATTIES ON PALATABILITY, COOKING PROPERTIES AND STORAGE STABILITY

H. R. CROSS, D. MUSE and E. C. GREEN

ABSTRACT

Precooked and raw frozen patties varying in level of soy protein and spices were prepared at two commercial locations. The patties were cooked or reheated by either roasting, broiling, or microwave. Precooked, reheated patties were rated tougher and drier by the trained panel. Panel ratings for precooked, reheated patties were low enough to be considered of practical importance. The addition of soy protein to the mixture significantly increased the panel ratings for tenderness.

INTRODUCTION

THERE HAS BEEN increased interest in the meat industry to produce precooked meat items for use by the institutional trade. Several elementary and secondary schools in the U.S. are using precooked, frozen beef patties rather than frozen, raw beef patties. Some advantages cited in favor of precooking are reduced cooking time prior to serving and fewer cooking losses. There are almost no published research data to support these statements. Soy added to meat may reduce the stale or warmed-over flavor in reheated meat because certain vegetable extracts are effective antioxidants. Sato et al. (1973) found that adding one of several cereal protein products to ground-meat loaves prevented rancidity. Bowers and Engler (1975) added soy protein to raw patties that were subsequently either cooked or precooked, frozen, and reheated and found the reheated patties to be less juicy regardless of the amount of soy in the formulation. The soy-containing patties that were not precooked also had less meaty aroma and flavor than the all beef controls (Bowers and Engler, 1975).

To our knowledge, no one has evaluated the palatability of patties precooked with commercial equipment. We, therefore, undertook to evaluate the effects of precooking under commercial conditions on palatability and cooking properties of patties containing various levels of soy protein. Different spice and soy levels were included in the design to simulate current school lunch/industry practices and provide a product that could vary in cooking and palatability traits.

EXPERIMENTAL

SEVEN ground beef formulations were prepared at two locations as outlined in Table 1. All raw materials were selected on the same days at each location. U.S. Utility square-cut chucks were used as the primary lean source; fat from U.S. Good and Choice plates was used to adjust the fat content. Minimum batch size of each formulation was 45 kg, and two batches of each formulation were prepared at both locations. Each batch was prepared from different chuck and plates.

Comminution

Location A. All material was chopped in a commercial chopper for 1 min and sampled for fat analysis. After a 1-min mix, 10 grab samples (0.60 kg each) were analyzed for fat with the Anyl-ray instrument. Fat content ranged between 28.5–29.7% for two batches, and the formulation was mixed for 1 min. Hydrated soy flour (1.5 water to 1 soy) and soy protein concentrate (see Table 1) were added to the formulation, and the batch was mixed again for 1 min. The product was passed through a 0.32-cm plate for the final grind. The ground beef mixture was formed into 75g patties with a formax patty machine. The patties were chilled with CO_2 to an internal temperature of about 0–2°C. They were then either frozen or precooked.

Table 1–Patty formulation

Formulation	Beef %	Salt %	Black pepper %	Spices %	Soy flour %	Soy conc %	Water %
1	98.55	1.00	0.05	–	–	–	–
2	89.25	–	–	0.75	4.00		6.00
3	78.50	–	–	1.50	8.00		12.00
4	68.00	–	–	2.00	12.00		18.00
5	87.75	–	–	2.25	2.00	1.40	6.60
6	77.00	–	–	3.00	5.00	2.15	12.85
7	67.50	–	–	2.00	7.00	3.00	20.50

Location B. All raw material was passed through a 1.90-cm breaker plate, mixed for 1 min and sampled for fat analysis. Fat content was determined with a Honeywell fat analyzer. Fat content was 28.5–29.5%, respectively, for the two batches. The mixtures were formulated and the patties prepared as described for location A.

Precooking methods

Location A. Patties were broiled in a custom-made gas broiler to an internal temperature of approximately 50–55°C. The design of the cooking apparatus was such that the patty moved along a belt and was heated from both sides. The time from beginning of cooking until the product reached the freezer was approximately 4 min. Patties then passed through a liquid nitrogen tunnel for 5–6 min and were chilled to an internal temperature of about −5°C.

Location B. Patties were broiled in a custom-made gas broiler to an internal temperature of approximately 50–55°C. The heat source was from both directions as in location A. The oven was 13m long and the time from beginning of cooking until the product reached the freezer was approximately 4 min. Patties were frozen in a NH_3 carousel to an internal temperature of approximately −10°C.

Frozen patties from each location (raw and precooked) were stored at −10°C for 2 days until shipment via air (−10°C) to the Meat Science Research Laboratory at Beltsville, MD, where they were stored at −20°C for analysis.

Cooking losses

Fifty raw patties from each batch/formulation/location combination were randomly selected for weighing before and after the precooking/freezing treatment. Patties were weighed to the nearest 28.0g. The weights of 15 raw, frozen patties and 15 precooked, frozen patties were determined; each was reweighed after cooking at the Meat Science Research Laboratory, and total cooking losses were determined.

Cooking procedures

Frozen raw and frozen precooked patties were cooked in an electric oven preheated for 30 min at 225°C. Patties were placed four to a pan, two pans per oven, and roasted at 200°C for 13 min. The patties were not turned during cooking. Preliminary cooking trials revealed that frozen patties whether precooked or raw, required approximately the same amount of time to reach an internal

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temperature of 55°C. Internal temperature was measured with iron/constantan thermocouples connected to a Honeywell recorder.

Frozen, precooked patties were also cooked in a General Electric model 500 microwave oven. Four patties were placed on a glass plate and cooked for 2 min. Then the plate was rotated one-quarter turn and the patties were cooked 2 more min.

Sample preparation and presentation to panel

Samples were served warm to the panelists (within 2 min after removing from the oven). Each patty was quartered, and two pieces from each sample randomly allotted to each panelist. Samples were placed in nonwaxed paper cups and served to the panelist one cup at a time. To prevent cooling during serving, the paper cups were placed on heated sand for no more than 2–3 min. Panelists were served distilled water at room temperature so that they could rinse their mouths between samples.

Trained descriptive attribute panel

An eight-member panel, trained according to Cross et al. (1978a), evaluated samples from each of the three cooking treatments in a total of 24 sessions; and six samples were evaluated at each session. The trained panel evaluated each sample for tenderness, fragmentation, juiciness, detectable connective tissue, and flavor intensity on the basis that 8 = extremely tender, juicy, no connective tissue, and intense flavor; and 1 = extremely tough, dry, an abundant amount of connective tissue, and bland flavor. The panel also subjectively evaluated the samples for flavor acceptability in order to detect those samples that were intense but unacceptable in flavor from those with intense acceptable flavor. Only four of the seven formulations (Table 1) were presented to the sensory panel.

Instron shear

Ten patties from each treatment were sheared with a single blade device attached to the Instron as described by Cross et al. (1978b). Each patty was allowed to cool 2 hr and sectioned into four 2.54-cm squares. Each square was sheared once.

Oxidative rancidity

Five patties from each treatment were randomly selected, frozen in liquid nitrogen, and powdered in a high-speed blender. Rancidity, as TBA values, was determined by the procedures of Tarladgis et al. (1964).

Statistical analysis

The experimental design was a 4 × 3 × 2 factorial with incomplete blocks (formulation; cooking method and location) and each contrast (comparison) was estimatable in at least two of the six replications. Mean separation was performed according to Scheffe (1959). In designing the study, we were concerned that the precooking location interactions might confound the reheating methods location interactions. If location is found to have significant effects, all location interactions will vary accordingly; however, cooking method and formulation can still be studied if they are averaged over location. Consequently, when an interaction involving location was significant at the $P < 0.05$ level, we constructed a second ANOVA table (not shown) such that each sum of squares involving location was pooled with the error sum of squares into a residual sum of squares term. This enabled us to test the remaining mean squares and to gain information about main effects averaged over location. The estimate of pure error from the first ANOVA table (not shown) was used in each means analysis only when all interactions were nonsignificant ($P < 0.05$); otherwise, the pooled residual mean square from the second ANOVA table was used.

Table 2—Mean panel tenderness ratings for each level of formulation, formulation by cooking method, and formulation by location

Formulation[b]	Formulation avg	Tenderness rating[a] cooking method: Precook & roast reheat	Precook & microwave reheat	Raw and roast	Location: 1	2
1	4.64[e]	4.26	4.33	5.32	4.88	4.39
2	5.07[d]	4.70	4.42	6.10	5.32	4.82
3	5.57[c]	5.31	4.99	6.40	5.82	5.31
4	5.80[c]	5.49	5.29	6.62	5.90	5.69
Avg	5.27	4.94[g]	4.76[g]	6.11[f]	5.49	5.05

[a] 8 = extremely tender and 1 = extremely tough.
[b] Refer to Table 1 for composition of each formulation.
[c,d,e] Means in the same column with different superscripts differ significantly ($P < 0.05$).
[f,g] Means in the same row with different superscripts differ significantly ($P < 0.05$).

Table 3—Instron maximum shear force means (kg) by formulation, formulation cooking method, and formulation location

Formulation[a]	Formulation avg	Maximum shear force Cooking method: Precook & roast reheat	Precook & microwave reheat	Raw and roast	Location: 1	2
1	5.11[bc]	5.46	5.62	4.26	4.00	6.22
2	5.78[b]	6.32	5.61	5.50	4.75	6.66
3	5.00[bc]	5.63	4.96	4.29	3.97	5.90
4	4.60[c]	5.06	4.64	4.11	4.72	4.49
5	4.85[c]	4.66	4.78	4.11	4.13	5.58
6	4.51[c]	4.96	4.70	3.87	4.15	4.87
7	4.40[c]	4.50	4.72	3.97	4.20	4.59
Average	4.89	5.24[e]	5.15[e]	4.34[f]	4.28	5.51

[a] Refer to Table 1 for composition of each formulation.
[b,c,d] Means in the same column with different superscripts differ significantly ($P < 0.05$).
[e,f] Means in the same row with different superscripts differ significantly ($P < 0.05$).

RESULTS & DISCUSSION

MEAN SQUARES associated with the main effects of formulation (except for juiciness) and cooking method were highly significant and quite large compared to each significant interaction mean square (data not shown). Consequently, real differences in formulation and/or cooking methods could be expected. This was confirmed with the second ANOVA (data not shown), which also showed no significant interactions between formulation and cooking method.

Sensory panel and Instron maximum shear force results are presented in Tables 2 through 8. Mean separation was calculated across all formulations and the three cooking methods. Mean values for tenderness are presented in Table 2. The addition of soy to the formulation significantly increased the panel ratings for tenderness. The all-beef formulation (no soy proteins) was rated "slightly tough" by the panel. Experience indicates that a rating of less than 5 (8-point scale) by a trained panel usually indicates that the product would be rated as "unacceptable" by a consumer panel. Cross et al. (1976b), Huffman and Powell (1970) and Twigg et al. (1975) have also reported increased tenderness with increasing levels of soy. U.S. Utility chuck is a common raw material for use in ground beef – including that accepted by U.S.D.A. school lunch purchase programs. Cross et al. (1976a) reported that ground meat produced from U.S. Utility or lower grade meat was unacceptably tough, due primarily to large amounts of "tough" connective tissue. Likely, the addition of soy protein diluted the connective tissue in the final product. Although panel tenderness ratings for patties prepared at location A were higher, formulations were ranked in the same order at each location.

Patties that were cooked from the frozen, raw state were significantly more tender than patties that were precooked and then reheated. The differences were large enough to be important. Differences between cooking methods were also significant within each formulation.

Table 4—Mean panel ease of fragmentation ratings by formulation, formulation cooking method, and formulation location

Formulation[b]	Formulation avg	Fragmentation rating[a] Cooking method: Precook & roast reheat	Precook & microwave reheat	Raw and roast	Location: 1	2
1	4.95[e]	4.71	4.71	5.42	5.20	4.69
2	5.19[de]	4.96	4.67	5.96	5.40	4.99
3	5.44[cd]	5.24	4.82	6.26	5.64	5.24
4	5.65[c]	5.37	5.18	6.39	5.78	5.51
Average	5.31	5.07[g]	4.84[h]	6.01[f]	5.50	5.11

[a] 8 = extremely easy and 1 = extremely diff cult.
[b] Refer to Table 1 for composition of each formulation.
[c,d,e] Means in the same column with different superscripts differ significantly ($P < 0.05$).
[f,g,h] Means in the same row with different superscripts differ significantly ($P < 0.05$).

Table 5—Mean panel connective tissue ratings by formulation, formulation cooking method, and formulation location

Formulation[b]	Formulation avg	Connective tissue rating[a] Cooking method: Precook & roast reheat	Precook & microwave reheat	Raw and roast	Location: 1	2
1	4.52[f]	4.40	4.33	4.83	4.76	4.28
2	4.85[e]	4.57	4.50	5.47	4.98	4.72
3	5.27[d]	5.06	5.00	5.74	5.37	5.16
4	5.57[c]	5.38	5.35	5.97	5.67	5.46
Average	5.05	4.85[h]	4.80[h]	5.50[g]	5.20	4.91

[a] 8 = no detectable connective tissue and 1 = abundant.
[b] Refer to Table 1 for composition of each formulation.
[c,d,e] Means in the same column with different superscripts differ significantly ($P < 0.05$).
[g,h] Means in the same row with different superscripts differ significantly ($P < 0.05$).

Mean values for Instron maximum shear force by formulation, cooking method, and location are presented in Table 3. There was a significant formulation/cooking method interaction but in view of the highly significant effect of cooking method, we concluded that differences exist between cooking methods. Panel and maximum shear force evaluations of tenderness agreed closely. All beef (no soy protein) patties were significantly ($P < 0.05$) tougher than patties containing high levels of soy protein. Precooked patties were significantly tougher than patties cooked from the raw state.

Panel ratings for ease of fragmentation (Table 4) followed trends similar to panel evaluations of tenderness (Table 2) and maximum shear force (Table 3). As it did for tenderness, the addition of soy protein increased the ratings for ease of fragmentation. Precooking with subsequent reheating adversely affected texture. Patties that were reheated with the microwave oven were rated significantly lower than were those cooked by the other two methods.

Panel ratings for connective tissue are presented in Table 5. The addition of soy protein to the formulation tended to offset the adverse effects of connective tissue. Cross et al. (1977) reported that the addition of mechanically deboned beef had a similar effect on ground beef produced from U.S. Utility grade meat. Cooking method significantly affected panel ratings for connective tissue. Patties cooked from the raw, frozen state had less panel-detectable connective tissue than reheated patties.

The addition of soy protein to the formulation did not significantly affect panel ratings for juiciness (Table 6) but cooking method had a pronounced effect. Precooked, reheated patties were rated significantly lower than patties cooked from the raw state, and the differences were large enough to be of practical concern. There were differences between locations; but, as with previous palatability attributes (Tables 2–5), the rankings were identical between locations.

Tables 7 and 8 present data for panel ratings of flavor intensity and flavor acceptability. Acceptability ratings are usually used only with untrained consumer panels but were used in this study to "flag" possible off-flavors. In general, the panel objected to the level of pepper and spices in the formulations. Many panelists commented that the flavor was not necessarily undesirable but just not that of ground beef. This comment was reflected in the intensity ratings which were all above 6.0 (Table 7). No trends could be established with regard to soy, spice level, or formulation. An exception was the all beef formulation (No. 1); it received the highest ratings for intensity and the lowest for acceptability (Tables 7 and 8). Formulation 1 was the only one containing salt and black pepper. Huffman et al. (1978) reported that low levels of salt usually enhanced the flavor

Table 6—Mean panel juiciness ratings by formulation, formulation cooking method, and formulation location

Formulation[b]	Formulation avg	Juiciness ratings[a] Cooking method: Precook & roast reheat	Precook & microwave reheat	Raw and roast	Location: 1	2
1	5.32[c]	4.86	5.05	6.05	5.78	4.86
2	5.45[c]	5.05	5.05	5.96	5.57	5.33
3	5.52[c]	5.14	4.98	6.44	5.80	5.23
4	5.39[c]	5.07	4.84	6.26	5.42	5.36
Average	5.42	5.03[e]	4.98[e]	6.25[d]	5.64	5.20

[a] 8 = extremely juicy and 1 = extremely dry.
[b] Refer to Table 1 for composition of each formulation.
[c] Means among different formulations were not significantly different ($P < 0.05$).
[d,e] Means in the same row with different superscripts are significantly different.

Table 7—Mean panel flavor intensity ratings by formulation, formulation cooking method, and formulation location

Formulation[b]	Formulation avg	Flavor intensity[a] Cooking method: Precook & roast reheat	Precook & microwave reheat	Raw and roast	Location: 1	2
1	6.71[c]	6.79	6.82	6.52	6.56	6.86
2	6.12[d]	6.14	6.40	5.82	6.02	6.22
3	6.23[de]	6.20	6.38	6.12	6.27	6.19
4	6.41[e]	6.34	6.55	6.34	6.45	6.37
Average	6.37	6.37[g]	6.54[f]	6.20[h]	6.32	6.41

[a] 8 = extremely intense and 1 = extremely bland.
[b] Refer to table 1 for composition of each formulation.
[c,d,e] Means in the same column with different superscripts differ significantly ($P < 0.05$).
[f,g,h] Means in the same row with different superscripts differ significantly ($P < 0.05$).

Table 8—Mean panel flavor acceptability ratings by formulation, formulation cooking method, and formulation location

Formulation[b]	Flavor acceptability[a] Formulation avg	Cooking method: Precook & roast reheat	Cooking method: Precook & microwave reheat	Cooking method: Raw and roast	Location 1	Location 2
1	3.26[d]	3.23	3.18	3.38	2.93	3.60
2	4.21[c]	4.29	4.04	4.29	4.25	4.16
3	4.08[c]	4.23	3.75	4.27	4.12	4.05
4	3.90[c]	4.03	3.61	4.06	3.98	3.82
Average	3.86	3.95[e]	3.65[e]	4.00[e]	3.82	3.91

[a] 8 = extremely acceptable and 1 = extremely unacceptable.
[b] Refer to Table 1 for composition of each formulation.
[c,d] Means in the same column with different superscripts differ significantly ($P < 0.05$).
[e] Means among cooking method not significantly different ($P < 0.05$).

Table 9—Mean degree of doneness ratings by formulation, formulation cooking method, and formulation location

Formulation[b]	Degree of doneness[a] Formulation avg	Cooking method: Precook & roast heat	Cooking method: Precook & microwave reheat	Cooking method: Raw and roast	Location 1	Location 2
1	1.8	1.6	1.4	2.6	2.3	1.3
2	1.9	1.5	1.4	2.6	1.9	1.9
3	1.9	1.7	1.8	2.3	2.1	1.8
4	1.8	1.8	1.6	2.1	1.8	1.9
5	2.0	2.0	1.8	2.3	2.3	1.8
6	2.1	2.1	1.9	2.4	2.3	2.0
7	2.2	1.9	2.1	2.5	2.4	2.0
Average	2.0	1.8	1.7	2.4	2.2	1.8

[a] 8 = rare and 1 = well done (color photographs).
[b] Refer to Table 1 for composition for each formulation.

of ground beef. Likely, the black pepper was the main contributor to the unfavorable ratings; but to be certain, additional research will be necessary. Cooking method significantly affected ratings for flavor intensity but not for flavor acceptability (Tables 7 and 8). As mentioned, the spice level was likely so high that any differences due to cooking method were masked.

Mean ratings for degree of doneness are presented in Table 9. The formulation × location × cooking method interaction was highly significant ($P < 0.005$). After adjustment for location, the formulation × cooking method interaction (data not shown) was also highly significant ($P < 0.005$). Results for formulation and cooking method were significant ($P < 0.05$); however, we do not know whether the significance was due to the formulation × cooking method interaction or real differences in formulation and/or cooking method. Patties that were not precooked tended to be rated less well done than patties that were precooked and reheated. Reheating by roasting or with microwave seemed to have little effect on degree of doneness.

Mean TBA values by cooking method are presented in Table 10. The formulation × location interaction was significant (data not shown). We then determined whether the formulations differed significantly in TBA values given the significant interaction. The F value for formulation was significant ($P < 0.002$) and indicated that formulation 1 generally had higher TBA values than the remaining formulations. Cooking method had no significant effect on TBA values. By the end of 2 months storage, most patties had reached a level of oxidative rancidity that could be normally detected by a trained panel.

One of the often cited advantages for precooking and reheating frozen patties is reduced cooking loss as compared to that of nonprecooked patties. Cooking loss can best be evaluated on the basis of the total loss from raw patty to the final cooked patty. Total loss can be determined from Table 11, which presents the stepwise losses. Cooking losses for patties that were precooked and reheated were from 10–15 percentage points greater than those for the nonprecooked patties. As expected, cooking loss was the highest for the all-beef patty.

CONCLUSIONS

THE LEVEL of soy protein in the formulation and cooking method significantly affected palatability. The primary objective of this study was to evaluate the effects of precooking beef patties on ultimate palatability. Various levels of soy protein in the formulation were included in the experimental design only so that we could evaluate the effects of cooking methods on patties widely different in palatability. Patties that were precooked, frozen, and reheated were tougher and drier, and lost more weight during processing than those not precooked. The all-beef patty had unacceptably high TBA values regardless of location or cooking method. Our study indicates that there is little advantage to precooking beef patties.

—Continued on page 877

Table 10—Mean TBA values by cooking method and storage item

Formulation[a]	TBA values: Precooked Initial	Precooked 2 months	Precooked 4 months	Raw Initial	Raw 2 months	Raw 4 months
1	3.26	6.97	4.48	2.38	5.32	11.56
2	0.91	1.83	2.87	1.05	5.39	5.54
3	0.72	1.37	2.01	0.56	1.04	1.64
4	0.69	0.91	0.88	0.57	1.16	0.79
5	1.13	2.08	4.05	0.90	1.72	2.11
6	0.91	1.68	3.01	0.82	1.88	2.38
7	1.11	1.86	2.84	0.66	1.48	1.72
Average	1.25	2.38	2.88	0.99	2.57	3.68

[a] Refer to Table 1 for composition for each formulation.

Table 11—Cooking losses for each formulation by cooking method

Formulation[a]	Cooking loss %: Raw to precooked A	Precook to roast reheat B	Precook to microwave reheat C	Raw to roast D
1	21.74	17.18	18.78	28.22
2	22.95	14.99	16.95	26.96
3	14.80	13.46	13.96	15.38
4	13.00	11.10	12.63	13.11
5	18.19	16.90	16.69	17.65
6	13.43	13.62	14.23	16.07
7	13.45	16.01	14.98	14.50
Average	16.79	14.71	15.46	19.11

[a] Refer to Table 1 for composition for each formulation.

VITAMIN B_{12} AND ASCORBIC ACID IN KIMCHI INOCULATED WITH *Propionibacterium freudenreichii* ss. *shermanii*

S. L. RO, M. WOODBURN and W. E. SANDINE

ABSTRACT

Inoculation of kimchi, a Korean style fermented Chinese cabbage, with *Propionibacterium freudenreichii* ss. *shermanii* (ATCC No. 13673) increased vitamin B_{12} production to a maximum of 102 ng/100g from 47 ng/100g in the control at 1 wk of fermentation at 4°C. Soy flour (0.5%) or beef extract (0.05%), added as protein sources to the inoculated kimchi, further increased the vitamin activity to 197 and 203 ng/100g, respectively, at 1 wk. The fresh, unfermented kimchi contained 16.8 mg of ascorbic acid per 100g and the vitamin decreased at a constant rate during the first 5 wk of fermentation.

INTRODUCTION

VITAMIN B_{12} is essential in the human diet as evidenced by the development of pernicious anemia in humans unable to absorb the vitamin. Deficiency levels of serum vitamin B_{12} (45–193 pg/ml with an average value of 111 pg/ml as compared to usual values of 200–320 pg/ml) have been found in adults who have subsisted on vegetarian diets for extended periods (Wokes et al., 1955).

Vitamin B_{12} is unique among the vitamins in being produced exclusively by microorganisms. Wherever the vitamin is found in nature, its origin can be traced back to microorganisms growing in soil or water or in the rumen or intestine of an animal (Smith, 1965).

Fermented soybean and fish products have been found to contain vitamin B_{12} (Lee et al., 1958). Nutritionally significant amounts of vitamin B_{12} were also found in the Indonesian fermented products, ontjom and tempeh (Liem et al., 1977). The microbial production of vitamin B_{12} in kimchi, Korean fermented vegetables, including cabbage, has been reported (Lee et al., 1958; Kim et al., 1960). The strain producing the vitamin during the fermentation was identified as *Bacillus megaterium*.

Inoculation of fermented foods with strains known to produce vitamin B_{12} has been evaluated as a vitamin enrichment method. Soybean paste inoculated with *Bacillus megaterium* and fermented was found to contain increased vitamin levels (Choe et al., 1963; Ke et al., 1963). *Propionibacterium* species, widely used in the industrial production of vitamin B_{12} (Wuest and Perlman, 1968), have been recommended for vitamin fortification of some dairy products. Karlin (1961) fortified kefir with vitamin B_{12} by the addition of *Propionibacterium* to the kefir grains. Kruglova (1963) prepared vitamin-enriched curds from pasteurized cow's milk by fermentation with equal parts of cultures of lactic acid and propionic acid bacteria (2.5% each). The curds had approximately 10 times more vitamin B_{12} than when produced in the usual way with only lactobacilli.

In this study, an attempt was made to increase the vitamin B_{12} production in kimchi. Changes in the ascorbic acid content during the kimchi fermentation were also followed.

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Table 1—Proportions of kimchi ingredients

Ingredients	Distribution % by weight
Korean cabbage (Chinese cabbage)	81
Radish roots (white)	9
Salt (pickling salt)	3.5
Green onion	2.7
Salted shrimp (imported from Korea)	1.8
Red pepper powder (dried)	0.8
Garlic (fresh)	0.7
Ginger (fresh)	0.5

METHODS

Preparation of kimchi samples

A typical winter kimchi was studied, as it is the major and most popular type among Koreans. The ingredients and their proportions are shown in Table 1.

Washed Chinese cabbages were cut into 4–6 pieces longitudinally and mixed with three-quarters of the total salt. No additional water was used and the cabbages were held overnight in a cool room. Radish roots were sliced into thin pieces and mixed with all other minced or sliced ingredients and spices, including the remaining quarter of salt. Salted shrimp were omitted in part of the kimchi for canning trials. Soy flour (0.5%) (hammer milled, Star flour, Eugene, OR) or beef extract (0.05%) (BBL, Division of BioQuest, Cockeysville, MD) was added in some inoculation experiments. This mixture was then sandwiched between the salted cabbage leaves. The stuffed cabbages were packed tightly into a 2-gal glass jar and pressed with a weight (a bottle of water) to exclude air. The cabbages were covered with the juice without additional water. Fifteen pounds of kimchi were made for each sample. The jar was covered and kept in a refrigerator at 4°C, for a 10-wk fermentation period. Three replications were done.

Vitamin B_{12} was assayed at 0 day; 4 days; 1, 2, 3, 4, 5, and 10 wk of fermentation. The 10-wk sample was also cooked for 13 min and the vitamin B_{12} content again determined. Ascorbic acid, pH and total acidity, and microbial counts were determined at the same time as vitamin B_{12}.

Kimchi at the end of 1 wk of fermentation was canned and stored at room temperature for 4 wk, then compared with the product fermented for 5 wk. Canning was done in pint glass jars using the method recommended by the USDA for sauerkraut (USDA, 1970). This involved a hot pack and 10-min processing in a boiling water bath. Before canning, the kimchi juice was adjusted to pH 4.5 with white vinegar.

Inoculation with vitamin B_{12}-producing organism

A lyophilized culture of *P. freudenreichii* ss. *shermanii (P. shermanii*, ATCC No. 13673), was dispersed in 10 ml of sodium lactate broth (Vedamuthu and Reinbold, 1967). The broth culture was incubated at 30°C for 48 hr and transferred (Moore and Holdeman, 1974) into sodium lactate agar stabs for stock cultures grown at the same temperature. For inoculation into kimchi samples, the stab culture was again transferred into broth and grown at 30°C for 48 hr. At the time the kimchi was made, it was inoculated with 10 ml of a standardized suspension of the organism per 600g kimchi. This gave an additional 10^6 cells/g.

Determination of vitamin B_{12}

Vitamin B_{12} produced during the kimchi fermentation was determined by a microbiological assay method with *Lactobacillus*

leichmannii as the test organism. The assay procedure followed the *Official Methods of Analysis* of AOAC (1975, p. 839) except for the buffer substitution described below.

Stock cultures of *Lactobacillus leichmannii* (ATCC No. 7830) in agar culture medium (Difco Lactobacilli broth plus 1.5% agar) were stored at 7°C. Before using in an assay, successive daily transfers of the stab culture were made over a 2-wk period at 37°C with incubation for 18–20 hr. Better growth as determined visually was obtained by daily rather than every-other-day transfer.

Metabisulfite-buffer solution was recommended as the vitamin B_{12}-extracting solution in the AOAC method. However, preliminary studies showed that kimchi samples extracted with water were higher in vitamin B_{12} than samples extracted with the metabisulfite-buffer solution (Table 2). Cyanide-buffer solutions have been recommended to convert the less stable noncyanocobalamins to the more stable cyanocobalamin (Volcani et al., 1961; Rosenthal, 1968). Cyanide solution is also active in releasing vitamin B_{12} from cells (Wuest and Perlman, 1968). Therefore, a cyanide-buffer (pH 6.0) solution was evaluated and found to yield higher values of vitamin B_{12} as compared to those obtained from samples extracted with metabisulfite-buffer or water (Table 2). The cyanide-buffer solution contained 3.25g Na_2HPO_4, 1.5g citric acid · H_2O, and 10 mg KCN per 250 ml. Assay samples were prepared with kimchi juice and the extracting solution. The recovery of added vitamin B_{12} (1 ng/ml) averaged 107%.

A hydrolytic method was used to test for the presence of non-B_{12} compounds such as those identified by Ford et al. (1953) and Hoffman et al. (1948) in certain foods as having no biological activity for animals but stimulating growth in microbial assays.

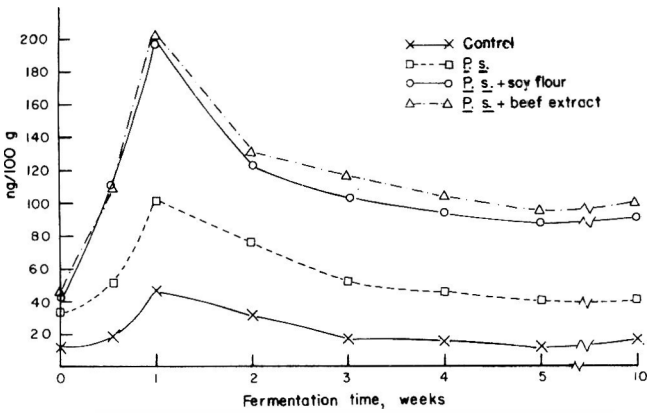

Fig. 1—Content of vitamin B_{12} in control and inoculated kimchi, with or without soy flour or beef extract, and fermented at 4° C. (P.s. Propionibacterium freudenreichii ss. shermanii).

Table 2—Effects of buffer solutions and heat treatments on vitamin B_{12} extraction from kimchi

		Replication				
		1	2	3	3	3
Solution	Heat treatment	Total ng/g	Total ng/g	Total ng/g	B_{12} analog ng/g	B_{12} ng/g
H_2O	15 min steaming	2.45	3.68	3.66	3.44	0.22
S_2O_5-soln	15 min steaming	2.80	3.10	3.21	3.03	0.18
S_2O_5-soln	10 min autoclaving[a]	2.35	2.96	2.54	2.49	0.05
CN-soln	15 min steaming	2.39	3.61	NT[b]	NT[b]	NT[b]
CN-soln	10 min autoclaving[a]	2.43	3.83	3.78	3.34	0.44

[a] Autoclaving was 121°C.
[b] Not tested

These preliminary studies revealed the presence of large quantities of nonvitamin B_{12} activity in kimchi (representative data in Table 2). Therefore, to provide a correction factor for this activity, sample extracts were hydrolyzed at pH 12.0 for 30 min at 121°C to destroy vitamin B_{12} (Shenoy and Ramasarma, 1955). The difference in the results before and after the alkali treatment was reported as the amount of vitamin B_{12} present in the sample. The addition of vitamin B_{12} before hydrolysis did not result in increased microbial growth, demonstrating the effectiveness of the procedure.

Determination of ascorbic acid

During the kimchi fermentation, ascorbic acid and dehydroascorbic acid were determined by the microfluorometric method (AOAC, 1975, p 829). Sampling was done on 50g of kimchi juice and 150g of the solids portion, blended 5 min. The fluorescence was read on an Aminco-Bowman Spectrophotofluorometer (American Instrument Company, Inc., Silver Spring, MD). Recovery of added ascorbic acid averaged 99%.

Microbial counts

During the fermentation of kimchi, numbers of anaerobic microorganisms, lactic acid bacteria, and propionibacteria were determined. The total number of anaerobes was determined on samples plated in lactic agar (Ellker et al., 1956) incubated anaerobically (GasPak Anaerobic Jar, BBL) at 37°C for 48 hr. Total counts of lactic acid organisms were made on plated samples incubated anaerobically at 37°C for 48 hr in LBS agar (Rogosa et al., 1951). Similarly, numbers of propionibacteria were determined from plated samples incubated anaerobically at 30°C for 6 days in sodium lactate agar (Vedamuthu and Reinbold, 1967).

Identification of propionibacteria

Selected colonies from the sodium lactate agar plates were studied to identify the individual *Propionibacterium* species. After incubation for 24 hr at 30°C in sodium lactate broth in anaerobic jars, gram stains, colonial observations, and the following tests were done: hydrolysis of gelatin and esculin; acid formation from mannitol, lactose, trehalose, maltose and ribose; reduction of nitrate; and production of indole. These identification criteria were assessed for speciation by reference to Moore and Holdeman (1974). *Propionibacterium freudenreichii* ss. *shermanii* ATCC No. 13673 was used as the reference organism.

pH and total acidity measurements

The pH of each sample was measured as well as total acidity by titration to pH 8.3 with 0.0990N NaOH solution; results were expressed as percent lactic acid. A Fisher Titrimeter, Model 36 (Fisher Scientific, Chicago, IL) was used.

Sensory evaluation

The difference in flavor between the control and inoculated kimchi samples with added soy flour or beef extract was judged by 9 Koreans (6 women and 3 men), who lived in Corvallis, OR. All were graduate students attending Oregon State University except for two student wives. A triangle test (Amerine et al., 1965) was used. Panel members were trained with several different kimchi samples. Although the panel was too small to give other than a preliminary assessment, members were also asked to judge the acceptability. Two different fermentation stages, 1 wk (under) and 3 wk (optimum), were evaluated for each replication. Data were analyzed using a significance table for triangular tests (Roessler et al., 1948).

RESULTS & DISCUSSION

THE OPTIMUM FERMENTATION of kimchi as judged by a pH of 4.5–4.8 was reached by 3 wk of fermentation. The panel members also agreed that the kimchi was at the optimum stage. In Figure 1, levels of vitamin B_{12} production were compared for each sample. Maximum production of vitamin B_{12} was achieved at 1 wk of fermentation in all samples. At that time, the vitamin B_{12} content was 47 ng/100g in control kimchi, which had not been inoculated with *P. freudenreichii* ss. *shermanii*. Inoculation of kimchi samples with the *Propionibacterium* increased the vitamin production to 102 ng/100g. The addition of soy flour (0.5%) or beef extract (0.05%) in inoculated kimchi further increased the vitamin content to 197 and 203 ng/100g, respectively.

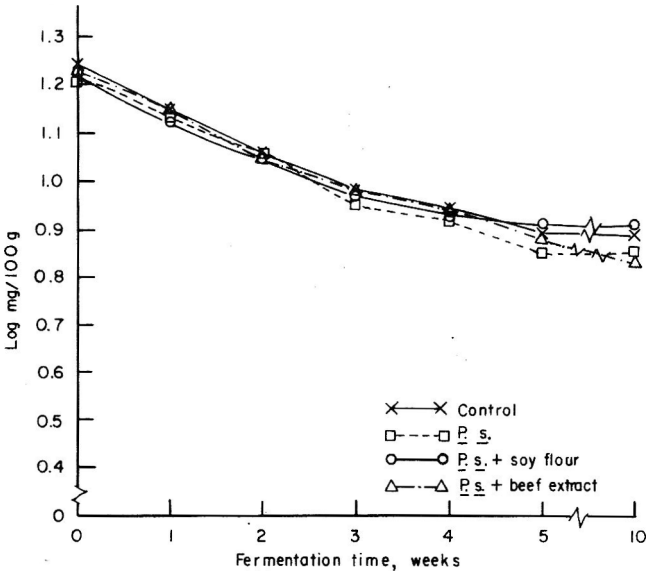

Fig. 2–Ascorbic acid content of control and inoculated kimchi, with or without soy flour or beef extract, and fermented at 4°C. (P.s. Propionibacterium freudenreichii ss. shermanii).

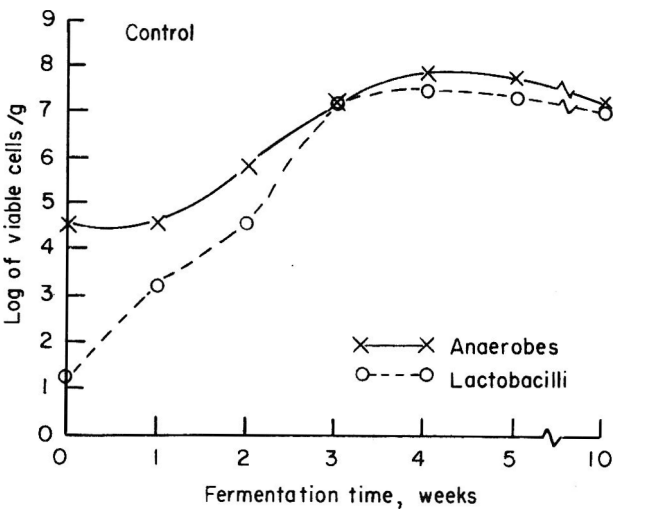

Fig. 3–Total anaerobic microorganisms and lactobacilli per gram of control kimchi, fermented at 4°C.

The level of vitamin B_{12} was reduced markedly by 2 and 3 wk of fermentation. After 3 wk, the vitamin decreased gradually to 5 weeks of fermentation; no further decrease was seen at 10 wk of fermentation. Cooking of the kimchi for 13 min after 10 wk of fermentation lowered the vitamin B_{12} in all samples. Kimchi is usually cooked if it is overfermented.

The exact process by which the vitamin B_{12} decreased is not known. Vitamin B_{12} is known to be relatively stable at pH 4.0–7.0. Hartley et al. (1950) reported that the vitamin is unstable in the presence of oxidizing or reducing substances. Destruction of vitamin B_{12} by ascorbic acid has been reported (Gakenheimer and Feller, 1949; Herbert and Jacob, 1974). The vitamin may be metabolized by the microorganisms involved in the kimchi fermentation.

The F values from the analysis of variance (Table 3) for the effects of fermentation time and inoculation with *P. freudenreichii* ss. *shermanii* were significant at the 1% level. The difference between the effects of added soy flour or beef extract was not significant at the 5% level.

In a preliminary study, addition of 1 ppm of Co^{++} ($Co(NO_3)_2 \cdot 6H_2O$, Baker Chemical Co., Phillipsburg, NJ) did not increase the vitamin B_{12} production in kimchi. Hendlin and Ruger (1950) had found that supplementation of the medium with as little as 1–2 ppm of cobalt increased the vitamin B_{12} activity in fermentation by various microorganisms.

The per capita consumption of kimchi in Korea ranges from 200–300g (about 1–1½ cups) per day in the winter season (Kwon, 1972). Three hundred grams of the inoculated kimchi with added soy flour or beef extract would provide about 0.6 μg of vitamin B_{12} at 1 wk of fermentation, or 30% of the daily requirement established by FAO-WHO (1970). The same amount of the inoculated kimchi would provide 0.3 μg, while that of the control kimchi would give 0.14 μg.

The ascorbic acid content did not differ significantly between the control and inoculated kimchi with or without soy flour or beef extract. The average content of the vitamin in fresh, unfermented samples was 16.8 mg/100g. The content of ascorbic acid gradually decreased during kimchi fermentation (Fig. 2). After 5 wk, ascorbic acid content was 7.7 mg/100g. No further decrease was observed between 5 and 10 wk of fermentation. Lee et al. (1960) reported that the ascorbic acid content of kimchi fermented at 2–7°C decreased during the first week and then slightly increased by the end of 2 wk and again decreased gradually as the fermentation continued. A temporary increase was not found in this study. A linear relationship between the ascorbic acid content and the fermentation time to 5 wk existed; the negative correlation coefficient, r, was significant at the 1% level. A regression equation, Y = 1.207 mg – 0.069 X, was derived; Y is the content of ascorbic acid in log mg/100g and X is the fermentation time in weeks.

Table 3–ANOVA table for vitamin B_{12} production in kimchi

Source of variation	d.f.	Mean square	F value
Total	31		
Fermentation time	7	3,656	12.6**
Inoculation	3	15,079	52.1**
Control vs Inoculation	1	29,400	101.7**
Among inoculation types	2	7,918	27.4**
Error	21	289	

** Indicates significance at the 1% level.

Kimchi is regarded as an important source of ascorbic acid. Three hundred grams of fresh, unfermented kimchi provide about 50 mg of ascorbic acid. The same amount of kimchi at the stage of optimum (3 wk) and excessive (10 wk) fermentation would provide 28 and 23 mg, respectively. The daily requirement established by FAO-WHO (1970) for the adult is 30 mg.

Total anaerobic counts of microorganisms and lactobacilli are presented in Figure 3 for the control. No differences in the total counts were observed between the control and inoculated kimchi with or without soy flour or beef extract. By 4 wk of fermentation, the total number of lactobacilli had increased sharply from an average of 1.8×10 per g in the fresh, unfermented kimchi to a maximum of 2.3×10^8 per g. Almost all of the anaerobic microorganisms at 3 wk of fermentation were lactobacilli. Kim and Chung (1962) identified some of the facultative anaerobic bacteria from kimchi: *Lactobacillus plantarum, L. brevis, Leuconostoc mesenteroides,* and *Pediococcus cerevisiae.*

The number of propionibacteria in the control kimchi increased from 6.7 per g at 0 day to 5.0×10^2 per g at 3 wk, after which there was a slight decrease (Fig. 4). The

species identified were *P. freudenreichii* ss. *globosum*, *P. granulosum*, *P. avidum*, *P. freudenreichii* ss. *freudenreichii*, and *P. acidi-propionici*. Of these, only *P. freudenreichii* ss. *freudenreichii* has been reported to be a vitamin B_{12}-producing organism (Leviton and Hargrove, 1952). *P. freudenreichii* ss. *shermanii* was not found in the control kimchi.

P. freudenreichii ss. *shermanii*, which was inoculated into kimchi, did not increase in numbers; however, there was survival after 10 wk (Fig. 4). The survival pattern of the *Propionibacterium* was similar in all of the inoculated samples. The addition of soy flour or beef extract apparently caused increased production of vitamin B_{12} rather than increased growth.

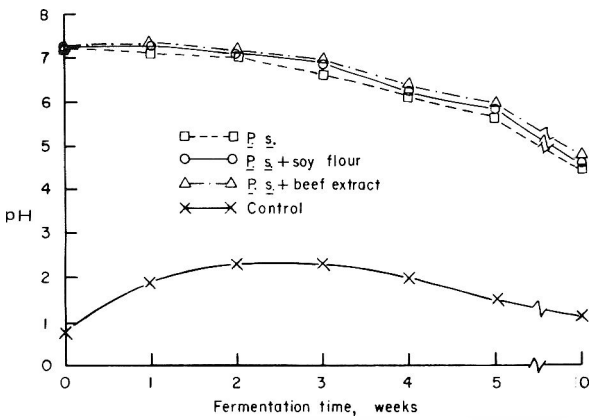

Fig. 4—Total number of propionibacteria per gram of control and inoculated kimchi, with or without soy flour or beef extract, and fermented at 4°C. (P.s. Propionibacterium freudenreichii ss. shermanii).

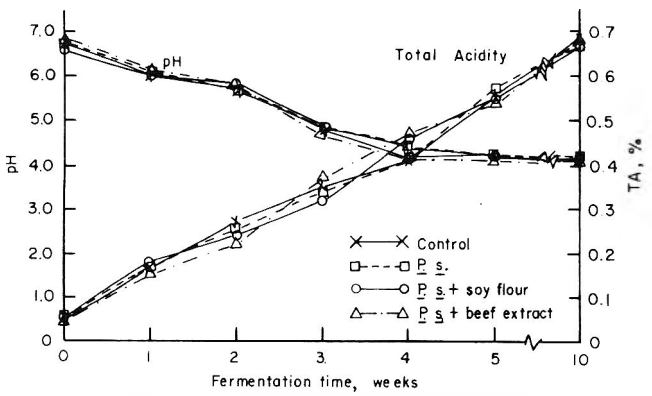

Fig. 5—pH and total acidity of control and inoculated kimchi, with or without soy flour or beef extract, and fermented at 4°C. (P.s. Propionibacterium freudenreichii ss. shermanii).

Table 4—Results of sensory evaluation for control and inoculated kimchi with added soy flour or beef extract and fermented at 4°C

	Fermentation time (wk)	Total judgments	Samples correctly identified
Reference[b] vs			
P.s. plus soy flour	1	26	11[a]
	3	26	9[a]
Reference[b] vs			
P.s. plus beef extract	1	26	2[a]
	3	26	9[a]

[a] Not significant at the 5% level
[b] Reference was control kimchi which had not been inoculated with *P. freudenreichii* ss. *shermanii* or had soy flour or beef extract.

The pH of all samples decreased during the fermentation; control and inoculated kimchi with or without soy flour or beef extract showed no significant differences in values (Fig. 5). The average pH value of the fresh, unfermented kimchi was 6.7 and that after 3 wk of fermentation, 4.8. The average value for total acidity of the fresh, unfermented kimchi was 0.05% expressed as lactic acid. At the optimum point, 3 wk of fermentation, the average value for total acidity was 0.35%. No significant difference was observed between control and treated samples. During the 5–10 wk of fermentation, the pH dropped slightly, from 4.2 to 4.1, while the total acidity increased from 0.56% to 0.68%.

Results of the triangle tests showed no significant difference (5% level) in flavor between the control and kimchi inoculated with *P. freudenreichii* ss. *shermanii* and with added soy flour or beef extract (Table 4). The panels described all of the samples, including the control, as good kimchi.

Control kimchi and inoculated kimchi with added soy flour were canned after 1 wk of fermentation to determine if the vitamin B_{12} content could be stabilized (Table 5). Although there was a small loss of vitamin B_{12} in the canning process and a decrease during storage, the content in both types of kimchi was greater in the canned products than in those fermented for the additional time. The ascorbic acid content was significantly decreased both in control and treated samples by the canning process. Storage of canned products at room temperature for 4 wk resulted in further loss.

A continuous loss of ascorbic acid in canned food throughout the storage life was reported by Feaster et al. (1949), in canned orange juice by Nagy and Smoot (1977), and in tomato juice by Lee et al. (1977). Anaerobic destruction of ascorbic acid in citrate-phosphate buffer at pH 3–4 was observed by Huelin (1953). The destruction was accelerated by fructose, fructose-6-phosphate, and fructose-1, 6-diphosphate, the effect increasing with lower pH. Lee et al. (1977) found a linear relationship between the copper concentration and the rate of anaerobic ascorbic acid destruction.

To provide a practical procedure for the inoculation of *P. freudenreichii* ss. *shermanii*, cultures of the organism could be combined with soy flour, dried, and packaged so

Table 5—Vitamin B_{12} and ascorbic acid in fresh and canned kimchi[a]

Sample	Control		Inoculated plus soy flour	
	Fresh	Canned	Fresh	Canned
Vitamin B_{12}	(ng/100g)	(ng/100g)	(ng/100g)	(ng/100g)
1 wk				
With salted shrimp	59	54	186	180
Without salted shrimp	17	16	82	81
5 wk				
With salted shrimp	15	35[b]	92	129[b]
Ascorbic acid	(mg/100g)	(mg/100g)	(mg/100g)	(mg/100g)
1 wk				
With salted shrimp	12.0	9.0	13.4	8.9
Without salted shrimp	14.4	8.8	14.3	9.3
5 wk				
With salted shrimp	8.7	7.7[b]	8.6	7.5[b]

[a] Data are averages of 2 replications.
[b] Canning was done at 1 wk of fermentation and jars were stored at room temperature for 4 wk to compare with unprocessed product fermented for 5 wk.

that homemakers could easily buy and use them. Inoculation of the organism into kimchi has the advantage of increasing vitamin B_{12} production without any effect on ascorbic acid content. Exploration to stimulate the growth of inoculated *P. freudenreichii* ss. *shermanii* might be worthwhile for further increase in the vitamin B_{12} production.

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Mention of brand names does not imply endorsement by the U.S. Government.

DISCRIMINATION OF FISH AND SEAFOOD QUALITY BY CONSUMER POPULATIONS

J. B. WESSON, R. C. LINDSAY and D. A. STUIBER

ABSTRACT

The quality of selected commercial fish and seafood products was first characterized with an experienced descriptive sensory analysis panel, and then the samples were evaluated by consumer preference panels. Consumer panelists readily discriminated and preferred fillet, portion and pattie fish products without distinct off-flavors over those exhibiting marked off-flavors, particularly oxidized flavors. Although flavor characteristics were major determinants of preference when distinctively oxidized flavors were present, texture was an extremely influential determinant of preference when samples exhibited moderate to low intensities of fishy and/or oxidized flavors.

INTRODUCTION

FRESHLY-CAUGHT FISH and seafoods are well-known for their distinct, but mild and delicate flavors which are highly-prized by consumers where products of this quality are readily available. Adaptation of frozen food technology to fish and seafoods has extended distribution of many products to all of the developed countries of the world, including the inland areas of the United States, where substantial markets have been developed (Gallup Organization Inc., 1971; National Economic Research Service, 1973). However, many of the characteristics of freshly caught fish are altered during current processing and distribution practices for frozen products (Dyer et al., 1957; Lane, 1966; Connell and Howgate, 1968; 1969). Notable are the development of fishy and oxidized flavors, and tough or chewy textures. As a result, many inland populations are unfamiliar with the sensory properties of freshly-caught fish, and therefore assume that all fish are characterized by distinct and often undesirable fishy flavors and tough textures.

Additionally, recent heavy demands on the world's fishery stocks have resulted in the depletion of some high-value species. This has led to the exploitation of traditionally less-sought-after fish stocks, and to the development of technology for recovery of boneless, minced fish flesh. Availability and usage of alternate species and minced fish products by consumers has been limited and has depended largely upon pricing strategies. Emphasis on marketing these products has been minimal because many food purveyers have been reluctant to handle unfamiliar items for which quality and consumer acceptance data are not available.

Technology for preserving much of the quality of freshly-caught fish is available and includes rapid freezing and storage temperatures below −20°C (Lane, 1964; Young, 1950), polyphosphate dipping to reduce freeze-thaw drip loss (Boyd and Southcott, 1965) and vacuum packaging to retard dehydration and lipid oxidation (Lindsay, 1977). Each of these practices increases costs of production, and the seafood industry has been reluctant to extensively implement them because information demonstrating that consumers are able to discriminate fish flavor quality adequately to justify added costs is lacking. A recent market study (Wesson et al., 1977) clearly indicated that greater amounts of fish would be purchased if consistent, consumer-acceptable products were available through dependable marketing channels. Since data on the ability of consumers to discriminate fish quality attributes have not been reported, this study was undertaken to investigate the degree to which an inland population was able to recognize fish quality and to determine factors which influenced their perception of quality.

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MATERIALS & METHODS

Samples

Fish were purchased from wholesale and retail distributors either in Madison, Milwaukee, and Port Washington, WI, Chicago, IL, or Astoria, OR. These included fresh and frozen whitefish fillets (*Coregonus clupeaformis*); fresh lake or yellow perch fillets (*Perca flavascens*); frozen perch fillets (*Perca fluviatalus*; imported from Holland); frozen cod fillets (*Gadus sp.*); frozen, dressed channel catfish (*Ictalurus punctalus*); and a variety of battered and breaded frozen fish portions.

Fresh unfrozen fish samples were held at ca 5°C between acquisition and utilization, and were prepared for sensory analyses within 24 hr of acquisition. Commercially prepared frozen samples were retained in original packaging materials and held at −24°C, for use within 2 wk of purchase.

Frozen, battered and breaded portions included 2-oz wedges (fish 'n' chip style) of haddock (*Melanogrammus aeglefinus*) and pollack (*Pollachius vireno*) fillets (Icelandic®, Coldwater Seafood Institute, Scarsdale, NY) and 3½-oz rectangular sandwich portions prepared from haddock fillets (Icelandic®) or minced cod (Sea Star® Coldwater Seafood Institute, Scarsdale, NY). Also obtained were 4-oz minced shrimp (*Pandalus jordani*) -minced rockfish (*Sebastes melanops*) patties (50-50 shrimp-fish; Shrim-Bo's®, Pacific Shrimp Co., Astoria, OR).

Frozen whitefish and frozen dressed catfish (wholesale, Chicago, IL) were obtained in individually quick frozen (IQF) form.

Frozen IQF catfish were thawed, filleted and prepared into 5-lb blocks and refrozen at −24°C. Frozen cod fillets were obtained in 1-lb blocks. Individual portions (ca 4 × 4 × 1 cm) of cod and catfish were sawed from frozen blocks with a band saw, and were battered and breaded in the laboratory while frozen (Golden Dipt® Batter and Golden Dipt Gourmet®, Breading, Golden Dipt. Co., Milstadt, IL). Portions were brought to −24°C before loosely packaging in heat-sealed polyethylene pouches (2 mil), and held at −24°C.

Sample preparations for evaluations

Deep-fat frying. Fish portions which were prepared by deep-fat frying were cooked in corn oil (Fry-wel®, Milwaukee Cheese Co., Milwaukee, WI) at 183 or 191°C according to label instructions or experimentally determined times to achieve the desired doneness. Commercially prepared portions had been pre-cooked, and therefore the frying was completed in a single short frying operation (4 min for fish 'n' chip style wedges and shrimp-fish patties, and 3 min for sandwich portions). Laboratory-prepared portions were pre-fried for 1 min, returned to a blast freezer (−24°C) until frozen, and then were finally fried for 2 min at the designated cooking temperature.

For serving to panelists, 1-oz samples of either deep-fried fish 'n' chip style wedge portions (approx 1/2 portion), or laboratory-prepared cod or catfish portions were placed in 5 oz odor-free paper sample cups coded with three-digit random numbers. Cooked haddock and cod sandwich portions were cut in half, and placed in

one-half commercial sandwich buns (Gardner's Bakery, Madison, WI). Shrimp-fish patties were cut into thirds, and each 1/3 patty was placed in a half-sized bun. Each half sandwich was then placed on a 15 cm diameter paper plate coded with a three-digit random number. All deep-fried samples were held under an infrared warming light (ca 45°C) after frying, and samples were presented to panelists within 15 min of preparation.

Oven baking. Fresh or thawed whitefish fillets were placed skin side down in aluminum pans (30 × 24 × 7 cm) with 1/4 cup butter per lb of fillets. The pans were covered tightly with aluminum foil, and baked for 25–30 min at 177°C in a household oven (General Electric). Cooked whitefish fillets were coarsely flaked, and the flesh from each lot was pooled. Approximately 2 oz of flaked samples were portioned (ca 2 oz each) into coded paper sample cups (3¼ oz). Portioned samples were covered tightly with aluminum foil, and held in a steam table (Wells portable) at ca 62°C for a maximum of 30 min after baking before presentation to panelists.

Oven frying. Fresh and thawed perch (Lake and Holland, respectively) were dipped in fresh homogenized whole milk, and then were rolled in all-purpose flour. Single layers of perch fillets were placed skin side down in aluminum pans (30 × 24 × 7 cm) which had been sprayed with commercial lecithin (Pam®, Boyle-Midway, Inc., NY). Melted butter (1/4 cup/lb) was added to the pans, and the samples were oven-fried in a household oven (General Electric) at 233°C. After 15 min, the fillets were turned, and oven-fried for 5 more min. Fillets were cut into 2-½ × 5 cm portions which were placed in 4 oz paper sample cups coded with three-digit random numbers. Portioned samples were held under an infrared warming light (ca 45°C) for a maximum of 30 min before presentation to panelists.

In-home panel product preparation

For the in-home consumer panel, freshly processed lake perch fillets were frozen for 5 days at −24°C before preparation and distribution to panelists. Both lake and Holland perch were thawed at 5°C, and were prepared for cooking as described for oven-frying. A meal-size portion of perch fillets (ca 2 lb) were then placed in each aluminum pan (30 × 24 × 7 cm) which was sprayed with commercial lecithin (Pam®, Boyle-Midway, Inc., NY), and tightly closed with an aluminum cover. Holland perch samples were refrozen at −24°C before distribution, but lake perch samples were prepared and held at ca 5°C on the day of distribution to encourage immediate usage.

Instructions for both oven- and pan-frying were provided to consumer panelists. The method for oven-frying was consistent with that described in this paper. For the pan-frying method, panelists were instructed to fry the fish pieces in 1/8–1/4 inch of oil in a skillet over medium heat. Initially, fillets were to be fried for ca 7 min with the skin-side of the fillets down, then turned and continue frying until done (ca 5 min).

Sensory analyses

Conditions for sensory testing were consistent with those recommended by ASTM (1968). For store-setting consumer panels, from 175–200 volunteer panelists were recruited for each panel from the usual clientele of the University of Wisconsin Dairy Products Salesroom. The panelists were seated in individual booths in the salesroom, and were each presented with coded or identified samples, eating utensils, condiments (tartar sauce and/or salt) for ad libitum seasoning when appropriate, and a ballot.

For the in-home consumer evaluation, samples were distributed to 85 customers of the University of Wisconsin Dairy Products Salesroom. Each panelist was given a bag containing a pan of thawed lake perch fillets, a pan of frozen Holland perch fillets, two additional aluminum pans, butter for use in home preparation, suggested instructions for home preparation, a ballot, and an addressed return envelope. Seventy-two panelists evaluated the samples and returned ballots.

For consumer preference evaluations, ballots with traditional seven-point hedonic scales were used. The ballot for in-home consumer evaluation of lake and Holland perch also contained questions for demographic information, specific preparation methods chosen, and potential product use.

Each lot of fish evaluated by consumer panels was also characterized by descriptive panels using semi-structured Quantitative Descriptive Analysis (QDA) (Stone et al., 1974) scales for selected attributes. Individual observations were obtained for each sample from each of 20–30 panel members who were graduate students, faculty, and staff of the Dept. of Food Science, University of Wisconsin-Madison. Panelists who participated had extensive and broad experience in the sensory evaluation of food products. These panels were conducted in a room equipped with individual booths, running water, and indoor fluorescent lighting (ca 78 ft-c). Each panelist received a tray of samples coded with random, three-digit numbers, a plastic eating utensil when appropriate, and a ballot.

Statistical analysis of data

QDA and hedonic scales were coded on a seven-point basis. Coded values were analyzed using a program for one-way analysis of variance for a completely random design and the University of Wisconsin Univac 1110 computer. Individual analyses were performed for each quality attribute of samples evaluated in each panel session, and mean scores for each sample attribute, and least significant difference (LSD) for attributes of each pair of samples were computed (Steel and Torrie, 1960).

RESULTS & DISCUSSION

Fresh and frozen fillet products

Whitefish. This regionally popular freshwater species contains an intermediate fat content (2–5%; Ackman, 1967), but is prone to rapid flavor and texture deterioration. Because of its availability and economic importance to the Great Lakes area, whitefish was selected for determination and demonstration of quality extremes in commercial fresh and frozen fish products. Initial efforts to identify and obtain samples exhibiting extreme quality contrast employing descriptive sensory analyses (Table 1) showed that both fresh and frozen whitefish obtained through usual marketing channels were extremely variable in quality. After identifying fresh and frozen samples exhibiting extremes in quality, both descriptive and consumer evaluations were conducted, and the results are presented in Table 2. The fresh whitefish sample was characterized by the descriptive panel as significantly less oxidized in flavor and dry and tough in texture than the frozen whitefish sample. The overall preference scores from the descriptive analysis indicated that the small group of experienced panelists was more critical of both samples than the consumer panel, but expressed a statistically significant preference for the fresh product. The laboratory consumer preference data showed, however, that the fresh whitefish was distinctly preferred over the frozen sample. The magnitude (5.3 on a 7-point scale) of the preference score for the selected fresh sample indicated a favorable acceptance of this product when it exhibits desirable characteristics. These results supported the initial hypothesis that consumers could readily recognize and discriminate the flavor and texture quality ex-

Table 1—Mean scores for the descriptive sensory analyses of fresh and frozen whitefish prepared by oven baking

Whitefish sample	Oxidized flavor[a]	Texture[b]
Frozen	3.4[x]	3.9[x]
Fresh	3.1[x]	5.1[y]
Frozen	2.9[x]	4.2[x]
Fresh	2.9[x]	5.3[y]
Frozen	4.9[x]	4.1[x]
Fresh	1.9[y]	5.4[y]
Frozen	3.7[x]	4.7[x]
Fresh	2.8[y]	4.7[x]
Frozen	3.4[x]	4.5[x]
Fresh	3.0[x]	4.3[x]
Frozen	3.9[x]	3.4[x]
Fresh	2.9[y]	4.6[y]

[a] Scale: 1 = None; 7 = Extreme.
[b] Scale: 1 = Dry, Tough; 7 = Moist, Tender.
[x,y] Scores with the same superscript within the same column are not different from each other at the 5% level of significance. Number of panelists = 20–30.

Table 2—Mean scores for descriptive and consumer preference sensory analyses of fresh and frozen whitefish prepared by oven baking

Samples	Descriptive panel			Laboratory consumer preference[c]
	Oxidized flavor[a]	Texture[b]	Preference[c]	
	Mean values			
Fresh	2.5[x]	5.6[x]	4.8[x]	5.3[x]
Frozen	5.1[y]	3.4[y]	2.5[y]	4.3[y]

[a] Scale: 1 = None; 7 = Extreme.
[b] Scale: 1 = Tough, Dry; 7 = Tender, Moist.
[c] 1 = Dislike Extremely; 7 = Like Extremely.
[x,y] Scores with the same superscript within the same column are not different from each other at the 5% level of significance. Number of panelists: Descriptive = 21; Consumer = 208.

tremes in fish prepared by a procedure yielding a relatively bland product.

Freshwater perch. Lake or yellow perch (*P. flavescens*) is a low fat species (0.8% fat; Kinsella et al., 1977) of substantial economic importance in the Great Lakes area. Since there was a high demand for locally harvested perch, difficulties were encountered in identifying low quality stored products. However, a nearly identical species (*P. fluviatalus*) imported from Holland was found to have contrasting sensory qualities, and was used in the test design. The descriptive panel documentation of quality (Table 3) revealed that the fresh lake perch sample was distinctly less oxidized in flavor, and markedly less dry and tough in texture than the frozen Holland perch product. The in-home consumer preference evaluation allowed the panelists some choice in the method of preparation, and an opportunity to experience fish odors during preparation. Gillespie and Houston (1975) have found that inexperience in preparation of fish and unpleasant odors during preparation of fish are the basis for many consumer complaints about home-prepared fish. The in-home consumer preference data presented in Table 3 show a significant preference for the fresh sample, again demonstrating the ability of the consumers to discriminate flavor and quality extremes in fish. Data based on the perceived quality of the samples by the consumer respondents indicated that 85% reported a willingness to buy the fresh lake perch compared to 50% reporting a willingness to buy the frozen Holland perch.

Species-related quality differences

Fish'n'Chip style portions. Surveys and opinion polls have shown the importance of the food service and restaurant trade for marketing fish products (Wesson et al., 1977; Gallup Organization, Inc., 1971; 1976). Typical food service-type fish'n'chip-style portions were selected for evaluation because these battered fish products have been estimated to account for about 1/3 of the national frozen fish market (Anonymous, 1978). The haddock and pollock portions chosen were identical to those served in many fast food and fish specialty operations, and the comparison included product quality attributes which are considered influential in acceptance and pricing. Descriptive analysis (Table 4) showed that the pollock flesh was significantly darker in color than the haddock fillet portions. The dark outer layer of fatty flesh had been retained in pollock portions, and accounted for the contrasting colors of flesh between the samples. The pollock sample was also significantly more soft and mushy than the haddock sample. However, the descriptive analysis did not reveal an expected difference between samples in intensity of fish flavors.

Overall, the documented quality differences were considered moderate, and more a result of species characteristics rather than abuse in distribution. Results from the consumer preference analysis (Table 4) showed a significant preference for the haddock portions over the pollock product which was interpreted as consumer perception of moderate quality differences based principally on color and texture. Although flavor is usually considered a major determinant in acceptance of fish products (Raseth et al., 1970; Connell and Howgate, 1968), these data clearly show that color and texture are also influential when fishy or oxidized flavors are equivalent and not pronounced.

Table 3—Mean scores for descriptive and consumer preference sensory analyses of fresh lake perch and frozen Holland perch prepared by oven- or pan-frying

	Descriptive panel			In-home consumer preference[d]
	Oxidized flavor[a]	Texture[b]	Preference[c]	
	Mean values			
Fresh lake perch	2.4[x]	4.5[x]	4.9[x]	5.8[x]
Frozen Holland perch	4.6[y]	3.7[y]	3.1[y]	4.1[y]

[a] Scale: 1 = None; 7 = Extreme.
[b] Scale: 1 = Dry, Tough; 7 = Moist, Tender.
[c] Scale: 1 = Dislike Very Much; 7 = Like Extremely.
[d] Scale: 1 = Dislike Very Much; 7 = Like Extremely.
[x,y] Scores with the same superscript within the same column are not different from each other at the 5% level of significance. Number of panelists: Descriptive = 24; Consumer = 72.

Table 4—Mean scores for descriptive and consumer preference sensory analyses of fish-and-chip style haddock and pollock portions

Samples	Descriptive panel			Laboratory consumer preference[d]
	Color[a]	Texture[b]	Fishy flavor[c]	
	Mean values			
Haddock fillets	5.6[x]	4.0[x]	3.1[x]	5.3[x]
Pollock fillets	2.8[y]	3.3[y]	3.2[x]	4.6[y]

[a] Scale: 1 = Greyish Brown; 7 = Snow White.
[b] Scale: 1 = Soft, Mushy; 4 = Flaky & Tender; 7 = Tough, Chewy.
[c] Scale: 1 = None; 7 = Pronounced.
[d] Scale: 1 = Dislike Very Much; 7 = Like Extremely.
[x,y] Scores with the same superscript within the same column are not different at the 5% level of significance. Number of panelists: Descriptive = 30; Consumer = 183.

Catfish and cod. In the determination of consumer acceptance of fish based on species identity, it is essential to consider the perceived image of the species involved. Channel catfish was chosen as an appropriate species to include because the image of this species is not high in the Great Lakes area. Preliminary evaluations, however, had shown that the characteristics of catfish in deep-fried fillet form were similar to those of regionally popular walleye pike (*Stizostedion vitreum*) and lake perch. Since maintenance of sample anonymity was an objective in a portion of this particular experiment, both cod and catfish were prepared and evaluated as battered-and-breaded deep-fat fried portions.

Descriptive analysis of the samples (Table 5) showed that the catfish sample was more brown in color than the cod sample. Although the experienced panel easily detected the color difference between the species, the mode of preparation de-emphasized the contrasting colors of beefy-red, raw catfish fillets and white, raw cod fillets. The descriptive panel did not note significant differences in texture. However, they did find the catfish sample to be distinctly more fishy in flavor than the cod sample, but voluntary com-

Table 5—Mean scores for descriptive and consumer preference sensory analyses of cod and catfish in breaded deep-fat fried portion form

	Descriptive panel			Laboratory consumer preference		
Samples	Color[a]	Texture[b]	Fishy flavor[c]	Species image only[d]	Coded[d]	Identified[d]
Cod	5.4[x]	3.7[x]	2.9[x]	5.6[x]	5.5[x]	5.7[x]
Catfish	3.2[y]	3.3[x]	4.6[y]	4.3[y]	5.3[x]	5.6[x]

[a] Scale: 1 = Greyish Brown; 7 = Snow White.
[b] Scale: 1 = Soft, Mushy; 7 = Tough, Chewy.
[c] Scale: 1 = None; 7 = Pronounced.
[d] Scale: 1 = Dislike Very Much; 7 = Like Extremely.
[x,y] Scores with the same superscript within the sample column are not different at the 5% level of significance. Number of panelists: Descriptive = 30; Consumer = 175–200.

Table 6—Mean scores for descriptive and consumer preference sensory analyses of haddock fillet and minced cod breaded sandwich portions

	Descriptive panel			Laboratory consumer preference[d]
Samples	Color[a]	Texture[b]	Fishy flavor[c]	
	Mean Values			
Haddock fillets	4.8[x]	4.2[x]	3.8[x]	5.3[x]
Minced cod	3.6[y]	3.4[y]	3.1[y]	4.7[y]

[a] Scale: 1 = Greyish Brown; 7 = Snow White.
[b] Scale: 1 = Soft, Mushy; 7 = Tough, Chewy.
[c] Scale: 1 = None; 7 = Pronounced.
[d] Scale: 1 = Dislike Very Much; 7 = Like Extremely.
[x,y] Scores with the same superscript within the same column are not different at the 5% level of significance. Number of panelists: Descriptive = 30 Consumer = 193.

ments indicated that this flavor was not strongly perceived as typical oxidized, fish flavor.

The test design for consumer reactions to these products included three sequential testing sessions (Table 5). For the initial test, samples were not provided and consumers were asked to rate the desirability of "Southern Catfish" and "Ocean Cod" on the sole basis of their perceived image of the two species. Data from this session showed a distinctly lower image of catfish compared to cod. This was anticipated because of regional unfamiliarity with catfish. Subsequent consumer tests involved sequential evaluation of actual samples in coded and in identified situations. However, in both of these cases cod and catfish were similarly preferred. Considering the earlier data on perceived quality, the results of these evaluations were somewhat unexpected, especially in the case of the identified samples. However, the data indicate that species prejudices can be overcome when consumers are introduced to products with acceptable quality and in a popular form. The preference data also suggest a tolerance of noticeable fishy flavors, particularly if it is not strongly oxidized and is blended with deep-fat fried flavors. However, the textural quality must be acceptable, and the results support the contention that texture is an important determinant of consumer preference of fish.

Processing and compositional quality influences

Minced cod and haddock fillet sandwich portions. Although minced fish flesh recovery technology increases recovery of boneless product and lowers overall costs, the mincing process disrupts cellular structures and creates opportunities for accelerated quality deterioration and changes in physical characteristics of the flesh (Babbitt, 1972; Lee and Toledo, 1977; Silberstein and Lillard, 1978). One of the major considerations for inclusion of minced fish flesh into prepared portions is the resulting texture, and the adequacy of the minced flesh for satisfying consumer expectations of texture. Top-line commercially used sandwich portions containing either minced cod or haddock fillets were selected as models to assess influences on consumer reactions which principally result from processing technologies.

Descriptive analysis data (Table 6) showed that the minced cod portions were darker in flesh color and softer in texture than the haddock fillet portions. The haddock fillet portions were significantly more fishy flavored than the minced portions, but the data did not indicate a marked difference in the intensity of fishy flavor between the samples. In consumer preference analysis, the haddock fillet portions were significantly preferred over the minced cod portions. Consumers appeared to unfavorably discriminate against the darker color and softer texture of the minced cod, but did not object to the slightly more fishy flavor of the haddock fillet portions. As with the pollock vs haddock and catfish vs cod comparisons, textural and possibly color characteristics were determining factors in consumer preference decisions. Other investigators (Raseth et al., 1970; Connell and Howgate, 1968) have reported that flavor is the most important determinant of acceptance of fish products. The current data suggest that texture may be the major determinant of preference when the products exhibit reasonably low intensities of fishy flavors. However, acceptance reactions of consumer populations to fishy flavor intensities are probably somewhat dependent upon geographical locations and resultant experiences with seafood products. On the other hand, when fishy flavors are strong and distinctly oxidized, textural considerations probably assume a secondary role in fishery product acceptances.

It is also important to note that much of the current data were developed with battered and breaded, deep-fat fried portions. With this type of preparation, low levels of fishy flavors may be less apparent to consumers than under home preparation conditions where broiling, baking, or moist heat cooking may be employed. Since consumption of deep-fried fish products is paralleling the increasing consumer tendency to eat out, textural characteristics of these fish products should receive appropriate attention in the future.

Minced shrimp-fish and haddock fillet sandwich portions. Incorporation of minced shrimp (*P. jordani*) into a shrimp-fish portion has been reported to enhance the acceptability of mechanically deboned minced fish muscle (Babbitt et al., 1974). Additionally, the shrimp component has been found to exert an antioxidant effect during frozen storage of minced fish flesh (Babbitt et al., 1976). Minced shrimp-fish (50-50) portions similar to those described by Babbitt et al. (1974; 1976) were evaluated comparatively with the top-line haddock fillet portions used previously in the comparisons with minced cod portions.

Results of the descriptive analyses (Table 7) indicated that the minced shrimp-fish patties were significantly more pink in color, firmer, and more crumbly in texture than the haddock fillet portions. The minced shrimp-fish portion was also characterized as markedly more flavorful than the haddock portions. The consumer preference data show that the haddock fillet and minced shrimp-fish portions were similarly preferred, demonstrating the favorable image, flavor, and texture contributions of minced shrimp to a minced fish (*S. melanops*) portion. These results contrast with those for minced cod and haddock fillet portion comparisons where color and textural characteristics of the minced cod product resulted in unfavorable consumer pref-

erences. The favorable consumer reaction to a new high quality shrimp-fish portion should provide encouragement for the development of other products utilizing shrimp flavor and texture of lower-grade shrimp pieces, and other traditionally less sought-after fish species similar to the *S. melanops* included in this study.

SUMMARY

THE RESULTS of this study clearly demonstrate the ability of an inland consumer population to recognize moderate and extreme quality differences in fish products which are due to fishy and oxidized flavors, darker colors from inclusion of high-heme tissues, and tough textures resulting from protein denaturaions. These findings reemphasize the need for application of improved preservation technology and distribution systens for commercial fishery products. Further, recent evidence that malonaldehyde, which is a product of lipid oxidaion in foods, may be carcinogenic (Shamberger et al., 1974) and mutagenic (Mukai and Goldstein, 1976) provides a more pressing need for improved preservation practices for fish to maintain and improve the safety of commercial products. The widespread occurrence of relatively high concentrations of malonaldehyde in commercial fishery products (Siu and Draper, 1978; Sinnhuber et al., 1962) lends support to this concern.

Discrimination of quality characteristics based on species differences and product form was also evident from this study. Substitutions of species with differing characteristics in typical fast-food portions could significantly affect consumer acceptance of products. In the continuing development of minced fish product technology, textural aspects should receive attention equivalent to flavor considerations. An understanding of consumer quality discrimination abilities and expectations is essential for providing direction to fish product development and marketing strategies.

Table 7—Mean scores for descriptive and consumer preference sensory analyses of haddock fillet and minced shrimp-fish breaded sandwich portions

	Descriptive panel				Laboratory
Samples	Color[a]	Textural firmness[b]	Structural character[c]	Flavor intensity[d]	consumer preference[e]
Haddock fillet portions	5.8[x]	4.8[x]	4.7[x]	2.85[x]	5.0[x]
Minced shrimp-fish portions	2.2[y]	3.8[y]	3.5[y]	4.45[y]	5.1[x]

[a] Scale: 1 = Pinkish, White; 7 = Snow White.
[b] Scale: 1 = Firm, Rubbery; 7 = Flaky, Tender.
[c] Scale: 1 = Crumbly, Discontinuous; 7 = Fibrous, Continuous.
[d] Scale: 1 = None, Bland; 7 = Strong, Pronounced.
[e] Scale: 1 = Dislike Extremely; 7 = Like Extremely.
[x,y] Scores with the same superscript within the same column are not different from each other at the 5% level of significance. Number of panelists: Descriptive = 28; Consumer = 183.

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STABILITY OF REFRIGERATED MULLET SAUSAGE

L. H. DALEY, J. C. DENG and J. L. OBLINGER

ABSTRACT

A storage study including microbiological assay, chemical determination of rancidity, and sensory evaluation was performed on a sausage-type product developed from minced mullet. Eight treatments were tested to determine the effect of a liquid smoke flavoring, a textured soy flour, and two methods of flesh recovery (hand filleted or mechanically deboned flesh). Results indicated a shelf-life of approximately 2 wk at 2°C with no preservatives other than the liquid smoke flavoring (0.5%) and sodium nitrite (100 ppm). The limiting factor was the development of off-flavors which correlated to total bacterial counts of approximately 4.8×10^5 APC/g.

INTRODUCTION

A SAUSAGE-TYPE PRODUCT was developed in an attempt to increase the acceptability and marketability of mullet as described in two previous studies (Daley and Deng, 1978; Daley et al., 1978). Mullet is plentiful but underutilized•primarily because it has a strong fishy flavor and is susceptible to oxidative rancidity (Deng et al., 1977) which limits long-term frozen storage. Since fish proteins are of high biological value (Finch, 1970) mullet could provide an inexpensive protein source if it can be incorporated into acceptable products.

The sausage product was designed to overcome the limitations of the mullet species and to be acceptable to the American population. The distinctive flavor characteristics of mullet can be masked by the relatively high spice levels often used in sausages. Mullet has a low meat yield, but a sausage product allows for the use of mechanically deboned fish. The dark flesh of mullet is not detrimental to sensory acceptability because visible particles are typical to coarse-ground sausages.

An added bonus of a sausage product is consumer convenience, an important marketing factor in American society. No fat is added to the fish sausages, so they contain substantially fewer calories than similar products currently marketed. The lipids of fish are highly unsaturated and contain little cholesterol, an important consideration for many Americans on cholesterol-restricted diets. The protein content of fish sausage should be equal to or greater than that of meat sausages, since the fat is replaced by textured soy protein. Fish sausages should also be less expensive than their meat counterparts.

A storage study was designed to compare the effect of hand-filleted (F) or mechanically deboned (DB) fish flesh, soy (S) or no soy (NS), and a liquid smoke flavoring, Charsol (C), or no Charsol (NC). The effect of deboned flesh was studied because the recent development of fish deboning machines can substantially increase the flesh yield (Crawford et al., 1972). The use of this deboned flesh could reduce manufacturing costs, if it becomes commercially available in the future. Soy was studied because it is currently less expensive than fish (Liener, 1972) and an even greater price difference is expected in the future between proteins of animal and vegetable origin (John, 1974). Liquid smoke flavoring (Charsol C-6) was studied because it can inhibit rancidity development, improve sensory acceptability, and inhibit microbial growth (Kramlich et al., 1973).

The purpose of this study was to determine the approximate shelf-life of the product to evaluate the possibility of mass production and distribution.

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EXPERIMENTAL

Materials

Materials were obtained as described by Daley and Deng (1978) with the exception of the fish purchase. Mullet was purchased in one 200-lb lot from Placida, FL, in late March (1977) and transported on ice to Gainesville. One hundred pounds of the fish were filleted, skinned, and washed by hand; the remaining fish was halved and gutted by hand, then mechanically deboned using a Baader 694 (Nordischer Machinenbau, Lubeck, Germany) separator with 5mm diameter holes. All flesh was stored in a 2°C cooler before sausage manufacture.

Methods

Sausage preparation. Sausages were prepared the day following fish purchase as described by Daley and Deng (1978) using the same constant levels of salt (1.6%), sugar (0.9%), spices (1.1%) and sodium nitrite (100 ppm). The formulations for the eight samples used were chosen from optimum combinations predicted by response surface analyses previously reported (Daley et al., 1978), although the sodium tripolyphosphate and water levels were each adjusted to a constant value (0.25% and 9.7%, respectively) to allow for comparison of other factors. Four samples were prepared using the following combinations of ingredients: 0% soy with 0% Charsol, 19.5% soy with 0% Charsol, 0% soy with 0.5% Charsol, and 19.5% soy with 0.5% Charsol. These same four samples were made with both mechanically deboned flesh and hand-filleted flesh to give a total of eight samples. In all sample formulations, the total raw weight was held constant by adjusting the flesh content. All samples were stored at 2°C in a barrier bag (Cryovac®) to prevent moisture loss.

Sensory evaluation. Sensory panels were held on the second, tenth, and seventeenth days following sample preparation. The sausages were evaluated by the same nine judges using the same method and scoring system described by Daley et al. (1978). The reference sample was always fresh, prepared the day before evaluation.

Analytical methods. Thiobarbituric acid (TBA) number measures malonaldehyde (a decomposition product) as an estimate of rancidity. The samples were flushed with nitrogen, vacuum packaged, and frozen at −34.5°C prior to analysis. The TBA analysis was performed according to the method of Deng (1978), using frozen sausage samples (approximately 50g) blended with two volumes of cold deionized water. Although nitrite, in general, interferes with the TBA reaction, nitrite at 100 ppm level according to Houghan and Watts (1958) did not interfere in the Turner modification of the TBA test. Thus, the modified method of Zipser and Watts (1962) was not used in the analysis. Duplicate absorption readings were made at 535 nm and the averaged values were reported as mg malonaldehyde per 1000-g sample.

Both standard plate counts and yeasts and mold counts were enumerated using accepted procedures (Thatcher and Clark, 1968). For all microbiological evaluations, analyses were done in duplicate and in replicate, and each reported count is an average of the four samples. All media (Difco Laboratories, Detroit), glassware, and diluent (Butterfield's phosphate buffer, pH 7.2) were autoclaved at

121°C for 15 min. Media were tempered to 45 ± 1°C in a water bath before use. Twenty-five-gram samples were aseptically removed from sausages stored at 2°C and homogenized for 2 min with 225g of diluent in blender jars at approximately 8000 rpm. Serial dilutions were made and 1 ml aliquots of each dilution were used for all assays.

The standard plate count was obtained using plate count agar incubated at 35°C for 48 ± 2 hr. Counts were made every 3 days and data were averaged and expressed as aerobic plate count per gram at 35°C (APC/g).

Yeasts and molds were enumerated using plate count agar with antibiotics added at a level of 100 mg/L for both cholorampheniçol and chlortetracycline HCl after tempering (Koburger, 1970). Plates were incubated at 25°C for 5 days before counting. The assay was performed every 3 days and averaged data were expressed as colony forming units per gram at 25°C (CFU/g).

Statistical design. The statistical design used was a 2 × 2 × 2 factorial over time comparing the effects of filleted vs deboned flesh, soy vs no soy, and Charsol vs no Charsol. Analysis of variance was calculated by computer using the Statistical Analysis System of Barr et al. (1976).

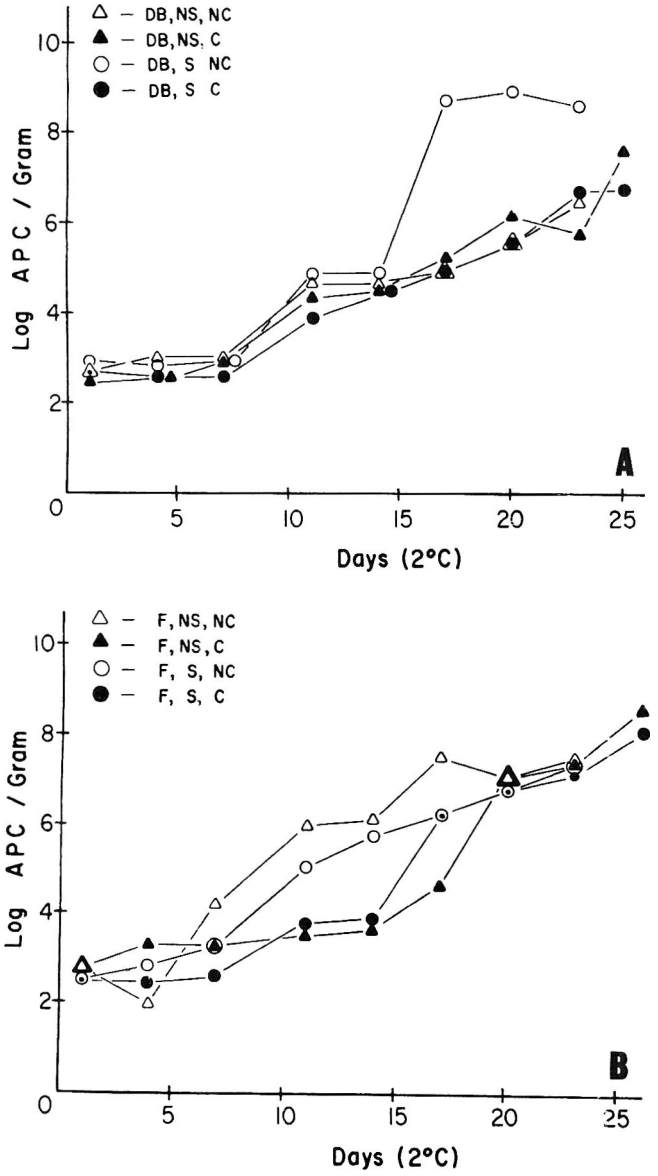

Fig. 1—Effects of storage time (2° C) and treatments on log bacterial count (APC/g) in mullet sausage.

RESULTS & DISCUSSION

Microbiological assays

The initial bacteria counts for the sausage raw materials and processing steps are shown in Table 1. The counts for both bacteria and fungi were very low for the textured soy flour, probably because of the heat treatment during the extrusion processing of textured soy protein. In contrast, the spice mix showed typical high counts for both bacteria and fungi (Frazier, 1967). Surprisingly, the counts for halved mullet were not much higher than the counts for filleted mullet. Higher counts were expected for the halved fish because the halving process requires gutting (which permits contamination of the flesh by intestinal microorganisms), while skinless filleting does not. Both grinding and deboning of mullet increased the corresponding bacterial and fungal counts, but deboned mullet showed bacteria counts only slightly greater than ground fillets for a relatively small load of fish (approximately 100 lb). After cooking at 185°C for 50 min, the bacterial counts were extremely low for all sausages with little variation between the treatments on the first day as shown in Figure 1.

An analysis of variance for the bacterial counts in Figure 1 shows that the day effect was highly significant (α = 0.0001). Of the three factors considered, only the Charsol level showed a significant inhibitory effect (α = 0.0001). Neither the type of flesh nor the soy level alone affected the bacterial counts. Soy was not expected to affect bacterial counts because of low bacterial numbers for raw soy, and results of other authors (Judge et al., 1974) who reported no significant differences in bacteria counts for ground beef or beef-soy blends. The insignificant effect of deboned fish confirmed the results of the raw material analysis and demonstrated that the small differences in counts for ground fillets and deboned fish were equalized by the heat treatment.

Neither the soy nor flesh type alone had a significant effect on bacterial numbers, but the interaction of soy and flesh type was significant. Figure 1 shows the effect of soy and Charsol on bacteria counts in sausages made from deboned flesh (A) and filleted flesh (B). Initially (day 1), the Charsol had no effect on bacterial counts, but a slight inhibitory effect was observed in certain samples (particularly dramatic with deboned flesh) later in the storage period. With both deboned and filleted flesh, slightly higher counts were observed for samples with no Charsol. This small bacteriostatic effect of Charsol agrees with literature results for liquid smoke flavorings (Erdman et al., 1954). No clear trends were observed for the flesh type or the soy effect.

Between days 20 and 23, obvious spoilage (slime forma-

Table 1—Initial microbial counts (day 0) for raw materials and processing steps used in mullet sausage manufacture

Sample	APC/g[a]	CFU/g[b]
Spice mix	1.0×10^6	3.3×10^3 [c]
Soy (Centex 400, caramel)	1.3×10^2	<10
Filleted mullet	8.8×10^3	2.1×10^2
Ground mullet fillets	1.8×10^4	8.1×10^2
Halved mullet	1.3×10^4	1.6×10^3
Deboned mullet	1.4×10^5	3.1×10^3
F,S,C (raw)	1.2×10^5 *	5.6×10^2
F,S,C (cooked)	4.2×10^2	<10
DE,NS,NC (raw)	1.2×10^4	9.5×10^1
DE,NS,NC (cooked)	4.9×10^2	<10

[a] Aerobic Plate Count, incubation 35° C for 24 hr
[b] Yeast and mold counts, incubation at 20° C for 5 days
[c] Estimated value

tion at approximately 1.0×10^8 APC/g and odor at approximately 1.0×10^7 APC/g) had occurred in the non-Charsol samples. Similar spoilage was found in the Charsol samples between days 25 and 28. The spoilage again corresponded to bacterial numbers of approximately 10^7 APC/g. The mullet sausages therefore had a shelf life of approximately 3 wk (from the bacterial viewpoint) which compares well with kamaboka (2 wk) or frankfurters (4 wk vacuum packaged) (Hing et al., 1972).

Fungal counts are shown in Table 2. No fungi were found for any of the samples before day 11 when two samples showed low levels present. Two additional samples showed growth on day 14, and by day 23 all but two samples showed fungal growth although all counts were still low. There was no discernible trend concerning fungal growth in samples.

TBA analysis

The average values of duplicate TBA numbers (always expressed as mg malonaldehyde per 1000-g sample) are shown in Figure 2 for both deboned (A) and filleted flesh (B).

Statistical analyses for the TBA values showed significant effects for both flesh type and soy level, but factor interactions prevent pooling of data to simplify the trends. A comparison of individual curves in Figures 2-A and 2-B show, in most cases, a slightly higher TBA value for the deboned fish, perhaps because a greater surface area is exposed by the deboning process which may promote oxidative rancidity.

For both filleted and deboned flesh, the sample with 0% soy and 0.5% Charsol showed the lowest TBA value (Fig. 2-A and 2-B). The trend for soy effect is not consistent, but shows a slight prooxidant effect, in disagreement with a report of antioxidant properties of soy in beef-soy blends (Kotula et al., 1976). However, Williams and Zabic (1975) reported no significant effect of soy on TBA values, but the soy blends had initially higher TBA values. The reason for the prooxidant effect of soy observed in the present study is unknown, but may be a result of the particular soy brand used (Kotula et al., 1976), possibly a difference in lipid content.

The insignificant effect of the Charsol level (Fig. 2) was a surprise because of literature reports and results from a preliminary study in November which are shown in Figure 3. In the November study, the addition of 0.5% Charsol substantially decreased the TBA values until day 25 but Charsol had no significant antioxidant effect in the March study. The TBA values for the November study were much higher (4–23) than for the March storage study (2–7). This agrees with a report by Mendenhall (1972) who also found lower TBA values in the Spring, particularly for May mullet. The increase in dark flesh (Deng et al., 1977) and lipid content during the spawning season of November is responsible for the relative higher TBA number since dark flesh

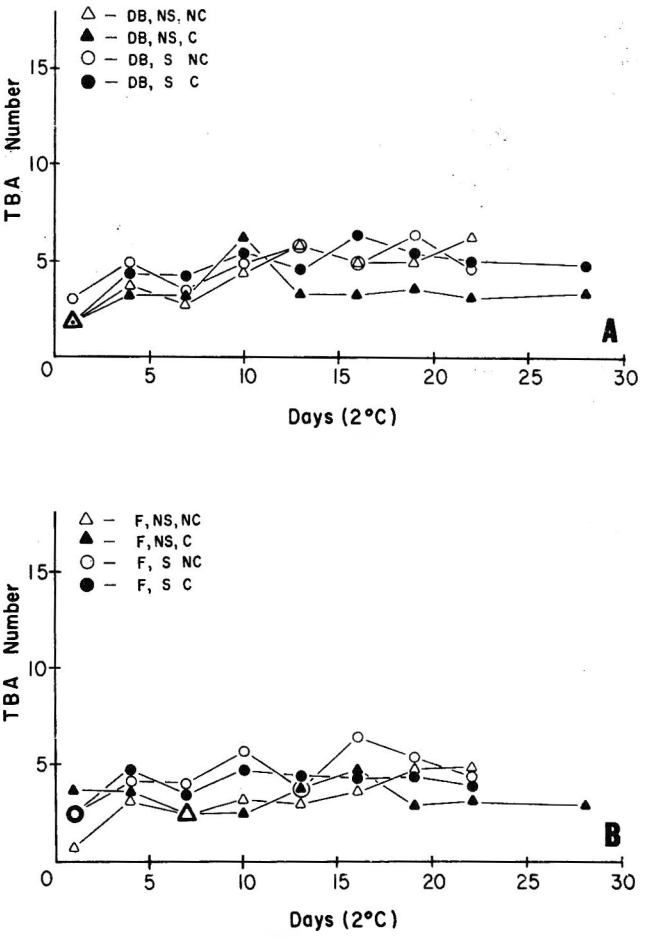

Fig. 2–Effect of storage time (2° C) and treatments on TBA values in mullet sausage (March).

contains components capable of catalyzing oxidative rancidity (Fischer and Deng, 1977). An assay for lipids showed a total lipid content in November mullet which was twice that of March mullet. In any case, rancidity was not a problem in the sausages used for the March storage study, and the corresponding TBA values were consistently low. A future storage study is required to determine if these formulations are stable to rancidity development during the spawning season. Vacuum packaging has been reported to inhibit rancidity development in beef (Yu et al., 1973) and could possibly allow for frozen storage of the sausages if it inhibits its rancidity in mullet flesh also. Antioxidant compounds could also be included in the formulations to retard rancidity.

–Text continued on page 886

Table 2–Effect of storage time (2° C) and treatments on fungal counts (CFU/g) in mullet sausages

	Day								
Treatments	1	4	7	11	14	17	20	23	26
F,NS,NC	<10	<10	<10	9.0×10^1	1.8×10^2	5.4×10^2	5.3×10^2	1.9×10^3	
F,NS,C	<10	<10	<10	<10	1.0×10^1	1.0×10^1	3.6×10^2	1.5×10^3	4.2×10^3
F,S,NC	<10	<10	<10	<10	1.0×10^1	<10	<10	<10	
F,S,C	<10	<10	<10	<10	<10	<10	<10	<10	1.2×10^2
DB,NS,NC	<10	<10	<10	<10	<10	<10	<10	1.0×10^1	
DB,NS,C	<10	<10	<10	<10	<10	<10	1.9×10^2	7.1×10^3	1.8×10^4
DB,S,NC	<10	<10	<10	1.0×10^1	<10	1.8×10^2	2.6×10^3	3.0×10^2	
DB,S,C	<10	<10	<10	<10	5.0×10^1	4.0×10^1	9.1×10^2	4.6×10^3	1.7×10^3

Table 3—Effect of storage time (2° C) and treatments on sensory panel scores (using a fresh reference) for mullet sausages

Sample	Flavor[a]			Texture[a]			Overall Acceptability[a]		
	Day 2	Day 10	Day 17	Day 2	Day 10	Day 17	Day 2	Day 10	Day 17
DB,S,C	4.89	4.38	3.00	4.78	4.75	4.71	4.78	4.34	3.43
DB,NS,C	4.33	4.25	3.00	4.44	4.13	4.00	4.33	4.34	3.00
F,S,C	4.56	3.63	2.29	4.78	4.50	4.43	4.67	3.75	2.43
F,NS,C	4.22	4.00	3.43	4.33	4.50	4.29	4.33	4.34	3.71
Average	4.50	4.07	2.93	4.58	4.47	4.36	4.53	4.20	3.14

[a] Scoring system: 1 = extremely poorer than reference; 4 = same as reference, 7 = extremely better than reference.

Table 4—Effect of storage time (2° C) and treatments on bacterial counts and TBA number for mullet sausage

Sample	Bacterial Counts[a]			TBA number		
	Day 1	Day 11	Day 17	Day 1	Day 10	Day 16
DB,S,C	5.4×10^2	7.7×10^3	9.7×10^4	1.61	5.23	6.43
DB,NS,C	3.2×10^2	2.2×10^4	1.6×10^5	1.89	6.23	3.37
F,S,C	4.2×10^2	6.1×10^3	1.6×10^5	2.49	4.71	4.40
F,NS,C	6.0×10^2	3.2×10^3	4.5×10^4	3.67	2.47	4.60
Average	3.9×10^2	9.7×10^3	4.8×10^5	2.42	4.68	4.70

[a] Aerobic Plate Counts, incubation at 35° C for 48 hr

Sensory evaluation

The samples without Charsol were not evaluated by panelists because of the flavor contribution of liquid smoke flavoring studied in previous work (Daley and Deng, 1978), so Table 3 shows sensory scores only for samples with Charsol. Also shown in Table 4 are the corresponding bacterial counts and TBA numbers. Flavor panel scores decreased with time as expected, dropping about 0.5 points from day 2 to day 10, but over a full point from day 10 to day 17. Neither the flesh type nor the soy level significantly affected the flavor, although flavor scores for soy samples degraded at a faster rate than samples without, regardless of flesh type. The panelists could detect off-flavors on day 17, reducing the shelf life to approximately 2 wk from the flavor viewpoint. A significant (0.05 level) correlation coefficient of −0.695 was calculated for the flavor scores (Table 3) and the bacterial counts of approximately 4.8×10^5 APC/g corresponded to a distinct off-flavor. A correlation between the flavor scores and the TBA values (−0.430) was not significant. Moledina et al. (1977) also found no correlation between flavor panel scores and the TBA number along with a quadratic TBA trend and a linear decrease in flavor panel scores with frozen flounder fillets.

The texture panel scores (Table 3) decreased only slightly with time. Statistical analysis showed significantly improved texture ($\alpha = 0.01$) in the soy sample but the flesh type had no effect on texture. However, there was a significant interaction between flesh type and soy level.

The overall acceptability scores (Table 3) again decreased with time. Although neither flesh type nor soy level was a significant factor, their interaction was significant. The overall acceptability scores correlated significantly (at the 0.01 level) to the bacterial count, but not to the TBA number. This confirmed that the sensory acceptability of the product was primarily affected by off-flavors resulting from bacterial growth.

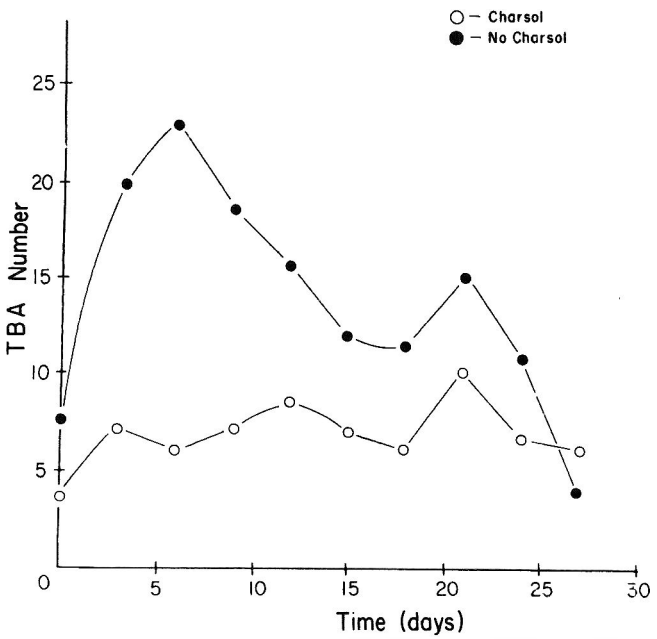

Fig. 3—Effect of storage time (2° C) and treatments on TBA values in mullet sausage (November).

CONCLUSIONS

THE STORAGE STUDY showed an estimated shelf life of 2 wk for mullet sausage stored at 2° C under aerobic conditions. The limiting factor was off-flavors which correlated to bacterial numbers of approximately 4.8×10^5 APC/g. Bacterial counts could possibly be reduced by vacuum packaging, by using impermeable casings, or by adding other preservatives. The fungal counts were very low, so these organisms were not important factors in the product's storage stability. Oxidative rancidity (TBA number) was not a problem during 26 days of storage. If the shelf life could be extended for 1 or 2 more wk, the product would be commercially marketable from a stability basis.

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—Continued on page 891

ESSENTIAL ELEMENTS IN FRESH AND IN CANNED ONIONS

ANTHONY LOPEZ and H. L. WILLIAMS

ABSTRACT

The content of 15 essential elements was determined in fresh and canned onions by atomic absorption spectrophotometry. The elemental content of fresh onions was compared with that of canned onions. All data were analyzed statistically. There were significantly higher amounts of chloride, cobalt, copper, iron, nickel, sodium, and tin in all the canned onions than in the fresh onions; higher amounts of magnesium, phosphorus and potassium in the fresh onions than in the canned onions, and no significant differences in manganese and zinc between the fresh and the canned samples. The addition of calcium chloride to seven canned samples produced a significantly higher content of calcium in the processed samples while there was no significant difference in the calcium content of fresh and processed onions in the nine samples that did not have calcium chloride added during processing. Fresh and canned onions supply small percentages of the essential elements included in the research for which RDA's have been established or estimated. Element retention in the canned onions was at least 65% and in several instances over 100%.

INTRODUCTION

PREVIOUS WORK done on essential element content of onions (*Allium cepa*) is either out-dated or incomplete. Most of the data reported concern only fresh or processed onions. Calcium, copper, iron, magnesium, phosphorus, and potassium content have most often been determined in fresh onions (Ajakaiye and Greig, 1976; Furr et al., 1976a, b; Koivistoinen et al., 1974). Data on calcium, iron, phosphorus, potassium and sodium content in fresh and cooked onions have been compiled by Heinz International Research Center (1962) and Watt and Merrill (1963). No data are available on the effects of canning on onions.

Much of the data reported have been the result of atomic absorption spectrophotometric (AAS) analyses (Ajakaiye and Greig, 1976; Koivistoinen et al., 1974; Haddad et al., 1973; Thomas et al., 1974). Furr et al. (1976a, b) determined copper, chromium, and nickel by AAS and made determinations on six other elements using neutron activation analysis (NAA).

The objectives of this research were (1) to determine and compare the content of 15 essential elements in fresh and in canned onions, and (2) to determine the effect of canning operations on the amount of these elements.

EXPERIMENTAL

Sampling

Samples of fresh and canned open pollinated onions of the pungent cooking type, Downing Yellow Globe variety grown in New York State, were obtained from a commercial plant located in the eastern United States. The onions were harvested in New York State in the winter of 1977 and commercially canned within a few days of harvesting.

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Truckloads of onions were received at the processing plant. They were size graded for #303 cans and placed on a cutting machine to trim the ends. The trimmed onions were washed in a rotary washer and peeled on a carborundum roller peeler with water sprays. The onions were placed in a continuous, submerged reel lye peeler that contained a 5–10% lye solution at a temperature of 98–99°C to complete the peeling operation. After leaving the lye peeler, the onions were washed with high pressure water sprays and inspected before being placed in #303 "C" enameled cans. Containers were semi-mechanically filled leaving 3/4 in. headspace and a 2% citric acid-sodium chloride solution was added before the cans were sealed. Seven of the canned samples had calcium chloride, a firming agent, added to the brine before the process. A processing time of 25 min at 100°C was used. Product pH after processing was 4.0–4.2.

Fresh onion samples were taken at the beginning of the processing line. The canned samples were taken 50 min later, the time it takes onions to go through the complete process.

Samples were taken twice a day on 8 days over a period of 5 wk, making a total of 16 samples each of fresh and canned onions. In-processing samples of water, lye and brine were taken daily.

Statistical design and analysis

Twenty fresh onions were randomly selected for each fresh onion sample. Two onions were taken from each of 10 cans of onions for each canned sample. Twenty ml of canned brine was pipetted from each can of onions for the canned brine samples.

The fresh and canned onions were peeled, sliced and dried at 70°C under 26 in. vacuum overnight. The dried samples were ground with a mortar and pestle and stored in small tightly closed polyethylene bottles in a desiccator until ashed.

The data were analyzed using analysis of variance and paired "t" tests (Sokal and Rohlf, 1969) to compare the element content of the fresh onions with that of the canned onions. Comparisons of the element content of fresh and canned brines were also made.

Materials

Hydrochloric acid, reagent A.C.S. grade, 37.5%, was obtained from Fisher Scientific Company, Fair Lawn, NJ (Fisher). Atomic absorption standards were obtained from Harleco, Philadelphia, PA. Lanthanum oxide, 98% pure, obtained from J.T. Baker Chemical Company, Phillipsburg, NJ (Baker), was dissolved in water and hydrochloric acid, for a 5% (w/v) solution. Cesium chloride was obtained from Fisher and dissolved in water for a 0.5% (w/v) solution.

The following materials were used for the phosphorus determination: ACS certified potassium dihydrogen phosphate (Fisher) was used to prepare a 500 ppm standard; ammonium molybdate, ACS certified (Fisher) was used to prepare a 10% solution; 53.5g ammonium chloride, Fisher certified, was dissolved in water, 70 ml of 30% ammonium hydroxide, ACS grade (Fisher), was added and the solution was diluted to 1L; 1-pentanol and diethyl ether, both reagent grade, were obtained from Baker and mixed 1:5.

Used for the chloride determination were ACS certified silver nitrate (Fisher) to prepare a 1000 ppm standard, and ACS grade nitric acid, 70% (Baker).

Sample preparation

A dry ashing procedure was used for atomic absorption spectrophotometric (AAS) analyses of all elements except chloride. Triplicate 1.00 ± 0.05g dried samples were weighed into clean, dry platinum crucibles. Each sample was pre-ashed and ashing completed by placing the sample in a Lindberg electric muffle furnace at 500°C for 4 hr. The ash was dissolved in 5 ml of 20% HCl. The solutions were warmed to effect complete solution and filtered through acid washed filter paper (Whatman #42) into 100 ml volumetric flasks. After diluting to volume, the solutions were ready for analyses by AAS (Anon., 1971).

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Table 1—Content of essential elements in fresh and canned onions

Element	No $CaCl_2$ added			$CaCl_2$ added		
	Fresh (mg/100g)	Canned (mg/100g)	Difference[a]	Fresh (mg/100g)	Canned (mg/100g)	Difference
Calcium	11.5 ± 2.8[b]	9.39 ± 1.23	ns	9.06 ± 1.99	91.9 ± 3.4	**
Chloride	19.9 ± 6.4	142. ± 42	**	19.1 ± 3.9	460. ± 57	**
Chromium	<0.007[c]	<0.006[c]	—	<0.007[c]	<0.006[c]	—
Cobalt	<0.009[c]	0.07 ± 0.02	**	<0.010[c]	0.08 ± 0.003	**
Copper	0.04 ± 0.01	0.06 ± 0.01	**	0.03 ± 0.01	0.06 ± 0.01	**
Iron	0.14 ± 0.05	0.30 ± 0.08	**	0.12 ± 0.05	0.40 ± 0.13	**
Magnesium	7.61 ± 0.79	5.63 ± 0.53	**	7.27 ± 0.79	5.44 ± 0.30	**
Manganese	0.12 ± 0.03	0.10 ± 0.02	ns	0.12 ± 0.02	0.10 ± 0.01	ns
Molybdenum	<0.028[c]	<0.023[c]	—	<0.029[c]	<0.024[c]	—
Nickel	<0.009[c]	0.14 ± 0.04	**	<0.010[c]	0.16 ± 0.005	**
Phosphorus	38.8 ± 4.1	27.9 ± 5.6	**	34.0 ± 5.4	27.7 ± 3.8	*
Potassium	170. ± 12	111. ± 6.4	**	167. ± 16	110. ± 5.1	**
Sodium	0.85 ± 0.43	344. ± 44	**	0.51 ± 0.17	406. ± 14	**
Tin	<0.056[c]	4.90 ± 0.67	**	<0.059[c]	6.04 ± 0.46	**
Zinc	0.25 ± 0.04	0.28 ± 0.03	ns	0.23 ± 0.07	0.28 ± 0.04	ns

[a] Statistical difference in paired "t" test: ns = No significant difference at 1% and 5% level. * = Significant difference at 5% level. ** = Significant difference at 1% level.
[b] Standard deviation
[c] Element below the detection limit of the AAS instrument

In-processing samples of water, brine and canned brine were analyzed for all 15 elements, except phosphorus and chloride, by direct aspiration of the liquids. Lye solutions were diluted 1/1000 before being introduced into the AAS unit.

Analytical methods

All element analyses were performed with a Perkin-Elmer Model 403 atomic absorption spectrophotometer. A 4-in. burner head and standard air-acetylene flame were used for all element analyses except chromium and molybenum. A nitrous oxide-acetylene burner and flame were used for chromium and molybdenum analyses. Single element hollow cathode lamps were used for all elements. The instrument settings and other experimental conditions were in accordance with the manufacturer's specifications.

For some element determinations it was necessary to dilute the samples with deionized water in order to obtain readings in a suitable range. Appropriate dilutions were used to determine calcium, magnesium, potassium and sodium in all samples. Samples diluted for calcium and magnesium analyses contained 1% (w/v) lanthanum to overcome potential anionic interferences. Cesium chloride solution was added to the samples before dilution to give a final concentration of 2000 ppm cesium for sodium analysis and 1000 ppm cesium for potassium analysis.

Duplicate samples of the dry-ashed solutions were used for the indirect determination of phosphorus. Aliquots of 0.5 ml of the fresh onion solutions and 1.0 ml of the canned onion solutions were taken through the procedure described by Parker (1972). Phosphorus determinations were also made on duplicate aliquots of water (2 ml), brine (5 ml), lye (1 ml) and canned brine (1 ml).

Chloride was also determined indirectly (Parker, 1972). Duplicate dried samples of 0.5–1.5g fresh onions and 0.1–0.3g canned onions were analyzed for chloride, as were duplicate samples of 0.1 ml water, 0.1 ml brine, 10 ml lye and 0.05 ml canned brine.

RESULTS & DISCUSSION

TABLES 1, 2 AND 3 give the content of 15 essential elements of fresh and canned onions, of the brine before and after processing, of the lye used for peeling, and of the plant tap water. Percentage retention of the essential elements are given in Table 4 and percentages of recom-

Table 2—Content of essential elements in freshly prepared and processed[a] brines

Element	No $CaCl_2$ added			$CaCl_2$ added		
	Fresh (mg/100g)	Processed (mg/100g)	Difference[b]	Fresh (mg/100g)	Processed (mg/100g)	Difference
Calcium	3.76 ± 1.39[c]	7.28 ± 1.23	**	224. ± 6.1	106. ± 9.3	**
Chloride	2,600 ± 530	2,500 ± 990	ns	3,750 ± 580	3,680 ± 480	ns
Chromium	0.003 ± 0.001	0.005 ± 0.004	*	0.007 ± 0.001	0.008 ± 0.003	ns
Cobalt	0.03 ± 0.005	0.02 ± 0.004	**	0.07 ± 0.00	0.03 ± 0.003	**
Copper	0.01 ± 0.008	0.02 ± 0.01	*	0.01 ± 0.004	0.01 ± 0.004	ns
Iron	0.10 ± 0.10	0.28 ± 0.09	*	0.15 ± 0.07	0.39 ± 0.19	*
Magnesium	0.78 ± 0.06	5.29 ± 0.39	**	1.21 ± 0.14	5.46 ± 0.51	**
Manganese	0.01 ± 0.001	0.08 ± 0.01	**	0.01 ± 0.001	0.09 ± 0.01	**
Molybdenum	0.04 ± 0.009	0.02 ± 0.003	**	0.05 ± 0.005	0.03 ± 0.00	**
Nickel	0.09 ± 0.02	0.06 ± 0.02	**	0.19 ± 0.01	0.10 ± 0.004	**
Phosphorus	0.90 ± 0.07	15.1 ± 1.2	**	0.89 ± 0.11	16.5 ± 2.0	**
Potassium	0.91 ± 0.13	128 ± 7.7	**	0.89 ± 0.12	125 ± 5.3	**
Sodium	857. ± 74	369 ± 74	**	911. ± 56	406. ± 29	**
Tin	2.78 ± 0.12	1.89 ± 0.17	**	4.81 ± 0.13	2.86 ± 0.10	**
Zinc	0.01 ± 0.01	0.30 ± 0.04	**	0.005 ± 0.001	0.32 ± 0.05	**

[a] "Processed" were brine samples taken from containers of canned onions.
[b] Statistical difference in paired "t" test: ns = No significant difference at 1% and 5% level. * = Significant difference at 5% level. ** = Significant difference at 1% level.
[c] Standard deviation

Table 3—Content of essential elements in water and in the lye solution

Element	Water (mg/100g)	Lye solution (mg/100g)
Calcium	2.24 ± 0.24[a]	6.12 ± 2.17
Chloride	2,290 ± 670	36.8 ± 27.5
Chromium	<0.001[c]	0.005 ± 0.001
Cobalt	<0.001[c]	<1.0[c]
Copper	0.002 ± 0.001	<0.2[c]
Iron	0.06 ± 0.04	8.33 ± 9.22
Magnesium	0.79 ± 0.02	0.99 ± 0.42
Manganese	0.003 ± 0.000	<0.2[c]
Molybdenum	<0.003[b]	<3.0[c]
Nickel	<0.001[b]	<1.0[c]
Phosphorus	1.15 ± 0.23	2.33 ± 0.90
Potassium	0.77 ± 0.06	51.6 ± 36.4
Sodium	2.33 ± 0.14	3,140 ± 1,750
Tin	<0.006[c]	<6.0[c]
Zinc	0.003 ± 0.003	<0.2[c]

[a] Standard deviation
[b] Element below the detection limit of the AAS instrument

Table 4—Retention of essential elements in canned onions and processed brines

Element	Fresh canned onions		Fresh processed brine	
	No $CaCl_2$ Added Retention (%)	$CaCl_2$ Added Retention (%)	No $CaCl_2$ Added Retention (%)	$CaCl_2$ Added Retention (%)
Calcium	82	1,014	194	47
Chloride	715	2,406	96	98
Chromium	a	a	167	114
Cobalt	a	a	52	46
Copper	158	222	150	100
Iron	211	326	280	260
Magnesium	74	75	678	451
Manganese	85	83	790	636
Molybdenum	a	a	57	57
Nickel	a	a	66	54
Phosphorus	72	81	1,678	1,854
Potassium	65	66	14,077	14,000
Sodium	40,340	79,258	43	45
Tin	a	a	68	59
Zinc	115	121	5,000	6,400

[a] Element below detection limit of the AAS instrument.

mended dietary allowances per 100g of fresh onions, canned onions and processed brines are given in Table 5. Availability to the human body of the different elements discussed varies with valence, solubility, and chemical species in which each element is present in the food. The reported, calculated percentages of RDA's or of estimated requirements assume 100% availability of the element. This is done to be able to provide some measure of the contribution of elements in processed foods to the diet.

There were significantly higher amounts of chloride, cobalt, copper, iron, nickel, sodium and tin in the canned onions than in the fresh onions; significantly higher amounts of magnesium, phosphorus and potassium in the fresh onions than in the canned onions; and no significant differences in manganese and zinc in the fresh and canned onions. The seven canned onion samples that were processed with calcium chloride ($CaCl_2$) had significantly higher amounts of calcium in the canned onions than in the fresh onions, whereas the calcium content of the nine samples processed without $CaCl_2$ was not significantly different from the fresh onion samples. Chromium and molybdenum were below the detection limit of the AAS instrument (Table 1).

Table 2 shows that there were significantly higher amounts of iron, magnesium, manganese, phosphorus, potassium and zinc in the thermally processed brines than in the brines before processing; significantly higher amounts of cobalt, molybdenum, nickel, sodium and tin in the brines before processing than in the processed brines; and no significant differences in chloride in the brines before and after processing. The brine samples that had $CaCl_2$ added showed a significantly higher amount of calcium in the freshly prepared brine than in the processed brine. Those samples that did not have $CaCl_2$ added had higher amounts of calcium in the processed brine. Chromium and copper were present in significantly higher amounts in the processed brine samples that did not have $CaCl_2$ added. There was no significant difference in chromium and copper in the samples that had $CaCl_2$ added to the brine.

Fresh onions (calcium content: 11.5 and 9.06 mg/100g) and canned onions processed without $CaCl_2$ (9.39 mg/100g) are relatively poor sources of calcium, supplying approximately 1% of the RDA for calcium in a 100g serving. Canned onions, which had $CaCl_2$ added to the brine, supply 11.5% of the RDA, and the processed brine supplies 13.3% per 100g brine (Table 5). Calcium values of 19.2–32

Table 5—Percentages of recommended dietary allowances per 100g of fresh onions, canned onions and processed brines (100g onions ≃ one 2½ in. diameter onion or ½ cup chopped onions)

Element	No $CaCl_2$ added			$CaCl_2$ added		
	Fresh onions	Canned onions	Processed brine	Fresh onions	Canned onions	Processed brine
Calcium	1.4[a]	1.2	0.9	1.1	11.5	13.3
Chloride	b	b	b	b	b	b
Chromium	c	c	0.5[d]	c	c	0.8
Cobalt	c	7.0[d]	1.7	c	7.9	3.2
Copper	1.8[e]	2.8	0.9	1.4	3.0	0.7
Iron	1.0[a]	2.2	2.0	0.9	2.9	2.8
Magnesium	2.3[a]	1.7	1.6	2.2	1.7	1.7
Manganese	2.5[f]	2.1	1.7	2.5	2.1	1.9
Molybdenum	c	c	2.1[d]	c	c	3.0
Nickel	b	b	b	b	b	b
Phosphorus	4.8[a]	3.5	1.9	4.2	3.5	2.1
Potassium	8.5[g]	5.6	6.4	8.3	5.5	6.2
Sodium	0.09[h]	34.4	36.9	0.05	40.6	40.6
Tin	b	b	b	b	b	b
Zinc	2.0[a]	2.3	2.4	1.9	2.3	2.6

[a] Recommended Dietary Allowance as set by the Food & Nutrition Board, National Research Council (Anon., 1974). % RDA in 100g serving for adult male.
[b] RDA not established or estimated
[c] Element below detection limit of AAS instrument
[d] RDA has been estimated to be 1 mg (Sarett, 1974)
[e] RDA has been estimated to be 2 mg (Sarett, 1974)
[f] RDA has been estimated to be 4 mg (Sarett, 1974)
[g] RDA has been estimated to be approximately 2000 mg (Sarett, 1974)
[h] RDA has been estimated to be approximately 1000 mg (Sarett, 1974)

mg/100g have been reported in fresh onions by Furr et al. (1976b), Heinz International Research Center (1962), Koivistoinen et al. (1974), Watt and Merrill (1963), and White and Selvey (1974). Retention values show no significant increase or decrease (82%) in calcium in onion samples that have not been processes with $CaCl_2$ added to the brine and a significant increase (194%) in calcium in the canned brine of the same samples. When $CaCl_2$ is added to the brine, the calcium content of the canned onions increases significantly (1,014%) and the calcium content of the processed brine decreases significantly by 53%.

—Text continued on page 890

Chloride values of 19.9 and 19.1 mg/100g were found for fresh onions. These results compare to 12.6–21.0 mg/100g of chloride previously reported (Anon., 1941; Furr et al., 1976b). Canned onions without the addition of $CaCl_2$ contained 142 mg chloride/100g and canned onions with $CaCl_2$ contained 460 mg chloride/100g. A chloride retention of 715% shows the effect of adding brine without $CaCl_2$ to the canned onions and the additional effect of $CaCl_2$ in the brine is reflected by a retention value of 2,406%. Canned onions contain more chloride due to the brine used in processing.

Chromium values of < 0.007 mg/100g for fresh onions and <0.006 mg/100g for canned onions were below the detection limit of the AAS instrument. Thomas et al. (1974) and Furr et al. (1976b) reported chromium values in fresh onions of 0.019 mg/100g and 0.011 mg/100g, respectively. Chromium was detected in fresh and canned brines (0.003–0.008 mg/100g). Sarett (1974) estimated the RDA for chromium to be 1 mg/day.

Cobalt content in fresh onions were found to be below the detection limit of the AAS instrument (<0.009 and <0.010 mg/100g). These results compare with values of <0.001 mg/100g (Thomas et al., 1974) and 0.013 mg/100g (Heinz International Research Center, 1962). Sarett (1974) estimated the RDA for cobalt to be 1 mg. Canned onions contained 0.07 and 0.08 mg/100g of cobalt and supply 7.0 and 7.9% of the above mentioned estimated RDA in a 100g serving. This increase in cobalt during processing is probably due to the amount of cobalt present in the brines (0.03–0.07 mg/100g). Retention values of 52% and 46% show a significant decrease in the amount of cobalt in the brines after processing.

Copper content of fresh onions (0.04 and 0.03 mg/100g) compares with those cited by Ajakaiye and Greig (1976), Furr et al. (1976a, b) and Koivistoinen et al. (1974). Sarett (1974) estimated the RDA for copper to be 2 mg. Fresh onions supply 1.8% and 1.4% of the estimated RDA given for copper while canned onions supply 2.8% and 3.0%. Retention values (150–222%) show significant increases in copper due to processing in all samples except brine that contained added $CaCl_2$. The copper picked up may originate from a copper or copper-containing food contact surface in the plant.

Fresh onions had 0.14 and 0.12 mg/100g of iron, supplying 1% of the RDA in a 100g serving. Canned onions had 0.30 and 0.40 mg/100g of iron, which are 2.2% and 2.9% of the established RDA. These results compare with the fresh onion value of 0.26 mg/100g given by Koivistoinen et al. (1974). Retention values show a significant increase in iron in all products due to processing. This increase could possibly be due to the amount of iron (8.33 mg/100g) present in the lye solution (Table 3), or be due to contact with steel equipment contact surfaces.

Fresh onions supply 2.3 and 2.2% of the RDA established for magnesium with values of 7.61 and 7.27 mg/100g. Canned onions and processed brine supply 1.7% of the RDA for magnesium. Furr et al. (1976b) and Haddad et al. (1973) reported comparable results of 7.2 mg/100g and 7.3–8.7 mg/100g for magnesium in fresh onions, respectively. Retention values of 74% and 75% for onions show that a significant amount of magnesium diffuses to the brine during processing, as shown by a significant increase in magnesium in the processed brines.

Sarett (1974) estimated the RDA for manganese to be 4 mg/day. The manganese content of 0.12 mg/100g found for fresh onions and 0.10 mg/100g for canned onions supply 2.5% and 2.1% respectively of the RDA estimated by Sarett (1974). These results compare with values for fresh onions reported by Koivistoinen et al. (1974) and Haddad et al. (1973). There was no significant difference in the manganese content of onions due to processing although the retention values show a 15–17% loss. There was a significant increase in the amount of manganese in the brines after processing.

Molybdenum content of fresh and canned onions was found to be below the detection limit of the AAS instrument (<0.023–<0.029 mg/100g). The results compare to previously reported values of 0.0057 mg/100g (Lupea and Marin, 1973) and 0.007 mg/100g (Furr et al., 1976a, b). Freshly prepared brine contained 0.04 and 0.05 mg/100g of molybdenum. Processed brine contained 0.02 and 0.03 mg/100g of molybdenum, supplying 2.1 and 3.0% of the RDA which was estimated by Sarett (1974) to be 1 mg. Retention values of 57% show a significant decrease in molybdenum in the brines due to processing.

Fresh onion values of <0.009 and <0.010 mg/100g for nickel were below the detection limit of the AAS instrument. Thomas et al. (1974) and Furr et al. (1976a, b) reported nickel results for fresh onions of 0.009 mg/100g and 0.022 mg/100g, respectively. The nickel content of canned onions was significantly higher (0.14 and 0.16 mg/100g). This increase could have come from the nickel present in the freshly prepared brine (0.09 and 0.19 mg/100g). Retention values of (66% and 54%) show a significant decrease in nickel in the brines due to processing.

Fresh onions (38.8 and 34.0 mg/100g) supply 4.8 and 4.2% of the RDA established for phosphorus. Canned onions (27.9 and 27.7 mg/100g) supply 3.5% of the RDA. These values are comparable to those given by Heinz International Research Center (1962), Watt and Merrill (1963), White and Selvey (1974) and Koivistoinen et al. (1974). Fresh brines (0.90 and 0.89 mg/100g) and processed brines (15.1 and 16.5 mg/100g) with retention values of 1,678 and 1,854% reflect the significant increase in phosphorus due to processing. Retention values (72 and 81%) show significant decreases in the phosphorus content of onions due to processing.

Sarett (1974) and Anonymous (1974) respectively report 2,000 mg and 2,500 mg as RDA estimates for potassium. Fresh onions contain 170 and 167 mg/100g of potassium and supply 8.5 and 8.3% of the estimated RDA. Canned onions contain 111 and 110 mg/100g of potassium and supply 5.6 and 5.5% of the estimated RDA. Watt and Merrill (1963) reported 157 mg/100g of potassium in fresh onions and 110 mg/100g of potassium in cooked onions. Retention values show significant decreases in potassium in the onions and significant increases in the brines due to processing.

Sarett (1974) estimated the RDA for sodium to be approximately 1,000 mg/day. Fresh onions (0.85 and 0.51 mg/100g) supply less than 0.1% of that estimated RDA value for sodium, while canned onions (344 and 406 mg/100g) supply 34.4 and 40.6%. This increase in sodium is due mostly to the addition of brine containing 857 and 911 mg/100g of sodium. Retention values of 40,340% and 79,258% for onions and 43% and 45% for brines explain the significant increases and decreases in sodium content due to processing. The sodium content of the onions could also have increased due to lye peeling (Table 3).

The tin content of fresh onions was below the detection limit of the AAS instrument (<0.056 and <0.059 mg/100g). Canned onions contained 4.90 and 6.04 mg/100g of tin. This significant increase is probably due to the addition of brine. Fresh brine (2.78 and 4.81 mg/100g) and processed brine (1.89 and 2.86 mg/100g) showed a decrease in tin content due to processing. No RDA has been established or estimate given for tin.

Fresh onions (0.25 and 0.23 mg/100g) and canned onions (0.28 mg/100g) supply approximately 2.0% of the RDA established for zinc. Comparable results of 0.22

mg/100g (Ajakaiye and Greig, 1976), 0.40 mg/100g (Koivistoinen et al., 1974), and 0.18 mg/100g (Haddad et al., 1973) have been reported for fresh onions. The zinc content of the processed brines (0.30 and 0.32 mg/100g) increased significantly over the content found in the fresh brines (0.01 and 0.005 mg/100g), as reflected in the retention values (5,000% and 6,400%). This increase is probably due to the zinc oxide present in the "C" enameled cans used.

Percentages of the established and estimated RDA's for the essential elements determined in this study show that fresh onions are relatively poor sources of all the elements except phosphorus and potassium. Canned onions are relatively high in cobalt, phosphorus and potassium and high in sodium due to the addition of brine. When calcium chloride was added to the brine, the canned onions and brine were high in calcium.

The content of 11 of the 15 essential elements in onions significantly increased or decreased as a result of processing. Cobalt, nickel, sodium, and tin increased in the processed onions and decreased in the processed brines. Magnesium, phosphorus, and potassium decreased in the processed onions and increased in the processed brines. Iron increased in both the processed onions and brines. The amount of manganese and zinc in onions was not significantly affected by processing while processed brines showed significant increases in the two elements. Processing increased chloride in onions, but there was no significant change in the chloride content of processed brines. Copper increased in all the onion and brine samples that did not contain $CaCl_2$. Chromium and molybdenum in onions were below the detection limit of the AAS instrument; however, chromium in processed brine without $CaCl_2$ increased and molybdenum decreased in all brine samples. As expected, the addition of $CaCl_2$ to brine samples caused a significant, very large increase in calcium of canned onions. The differences in element content observed between the fresh and the processed samples cannot be explained on a mass balance basis.

Both canned and fresh onions are better sources of sodium, potassium, and phosphorus than of any of the other elements included in the study. Element retention in the canned onions was at least 65% and in several instances over 100%.

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STABILITY OF REFRIGERATED MULLET SAUSAGE . . . From page 886

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APPLICATION OF HPLC TO CHARACTERIZATION OF INDIVIDUAL CARBOHYDRATES IN FOODS

W. J. HURST, R. A. MARTIN JR. and B. L. ZOUMAS

ABSTRACT

High performance liquid chromatography (HPLC) has been applied to the characterization and quantitation of the simple sugars (glucose, fructose, sucrose, maltose and lactose) present in a broad variety of food items, both fresh and processed. Following one of two simple extraction procedures, HPLC analysis is complete in 15 min. Recovery studies indicate good recovery from a variety of food matrices (94–104%). Relative standard deviations indicate excellent precision. The method permits an update of current carbohydrate literature both qualitatively and quantitatively.

INTRODUCTION

TRADITIONALLY the carbohydrate composition of food has been determined by difference using the proximate analysis scheme with its attendant errors. The compilation of carbohydrates in food compiled by the U.S. Government in Handbook 8 exemplifies this use of proximate analysis. For individual sugar characterization, several time-consuming and sometimes difficult techniques have been used. These techniques might be enzymatic (BFMIRA, 1958a, 1967; Worthington Biochemicals, 1976) or spectrophotometric (Morell, 1941; Forsee, 1938; McCready et al., 1974; Miller, 1959). They might involve a GLC determination (Birch, 1973; Parris, 1976; Snyder and Kirkland, 1974) or the use of paper chromatography (Lee et al., 1970).

The advent of HPLC in recent years and its applicability to many fields of analytical chemistry now allows the rapid characterization of many nutritionally significant carbohydrates in food products. The HPLC analysis of five individual sugars (fructose, glucose, sucrose, maltose, lactose) can be completed in a short time using simple sample preparation. The technique has good precision and accuracy. Additionally, the use of an autoinjector with the HPLC allows analysis on a 24-hr basis. When a data unit is also used, sample analysis and data handling are optimized.

MATERIALS & METHODS

High Performance Liquid Chromatography (HPLC)

The HPLC used in this study was a Waters Associates' Model ALC/GPC 201. This unit contained a Model R401 Differential Refractometer, and an M6000A Solvent Delivery System. The injection system was the Waters Intelligent Sample Processor (WISP) Model 710 having a capacity of 48 sample vials with each vial individually programmed if so desired. The recorder was a variable input, variable speed Sargent Welch Model XKR. The Shimadzu E1A Data Processor was coupled with the HPLC and autoinjector and used for data processing. Carbohydrate separations were accomplished on a "μ Bondapak/Carbohydrate" column (4 mm i.d. × 30 cm long) from Waters Associates. A stainless steel precolumn (2 mm i.d. × 55 mm) packed with Bondapak C_{18}/Porasil from Waters Associates was used as a guard column.

The HPLC mobile phase consisted of a mixture of LC grade acetonitrile/H_2O (80/20 v/v). The mobile phase was filtered through a 0.45μ filter and degassed prior to use and was stirred continuously when the HPLC was operating.

Flow rate was 2.0 ml/min. The concentrations of sugar in the samples were obtained by comparison of peak areas of samples to those of standard sugar solutions of known concentration which were made from Sigma Chemical Company sugar standards (St. Louis, MO). These solutions were checked daily to insure no degradation of the standards had occurred. Detection limits and the linearity of the system have been previously established in earlier work (Hurst and Martin, 1977).

Solids data for liquid samples were obtained by using a Bausch and Lomb refractometer with constant temperature accessory at 25°C; otherwise, solids data were obtained using those procedures of the AOAC (1975).

Samples

All food samples were commercially available and had national distribution.

Sample preparation

Depending on the food matrix, sample preparation and extraction methodology varied. The foods were divided into different food groups and extraction methodologies evaluated for each group. Extraction schemes (Technicon, 1972; BFMIRA, 1968b; Cerbulis, 1954; Kroplein, 1974; Lee et al., 1970; Palmer and Brandes, 1974) were evaluated to obtain optimum sugar extraction conditions. The extraction methodology for chocolate candy developed earlier (Hurst and Martin, 1977) was also used in this study. The drinks were analyzed as received except for minor sample preparation steps. Carbonated drinks were decarbonated in an ultrasonic bath, filtered through 0.45μ filters and prepared for injection. Noncarbonated drinks (e.g., fruit punches) were filtered through 0.45μ filters and prepared for injection. For the remaining three categories (fresh fruits, canned fruits, and dried fruits), individual studies were carried out to arrive at optimum extraction conditions and optimum extraction times. Several commercial instruments were evaluated for efficient sugar extraction. Various blenders, a Virtis mixer and finally a Sorvall Omni-mixer (DuPont Instruments) were evaluated. The Sorvall Omni-mixer was the instrument of choice. The extraction solvent used was $CH_3OH/H_2O/HOAc$ (79/20/1). This solvent is very similar to that used by Lee et al. in 1970. Our variations included the addition of a small amount of acetic acid and the use of the Omni-mixer rather than hot alcohol solutions (requiring refluxing). In our samples, quantitative extraction was complete in 7–10 min.

Dairy products in our study included ice cream, ice milk, puddings and yogurt. These dairy products were extracted with H_2O at 70–80°C. Water was used to overcome the low solubility of lactose. Temperatures were chosen so as to facilitate quantitative extraction but not too high to allow possible inversion of added sucrose (Hoynak and Bollenback, 1966). Extraction conditions were an adaptation of published methods (Boehringer and Mannheim, 1977). A time of 25 min allowed optimum extraction of carbohydrates.

Snack items in our study included: cookies, cakes, and several snack bars. Due to the recent introduction of many of these items, little published information is available. The same extraction methods employed for dairy products were used and found to be adequate. Again temperatures were kept sufficiently low to allow sugar extraction but not allow possible inversion or hydrolysis of the starches.

In those cases where a heat extraction was performed, the pH of solutions was kept near neutral because the use of heat coupled with low pH would again allow inversion of sucrose (Hoynak and Bollenback, 1966).

–Text continued on page 895

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Table 1—Carbohydrate analysis data

Product	% Fructose	% Glucose	% Sucrose	% Maltose	% Lactose	Other sacch.	Total sugars	Total solids	% Sugar dry wt
Drinks									
Apple juice	7.53	3.18	1.29	–	–	–	12.0	12.2	98.4
Grape juice	8.30	6.80	<0.5	–	–	–	15.1	16.8	89.9
Grapefruit juice	3.08	2.97	1.26	<0.5	–	–	7.31	10.0	73.1
Tomato juice	2.09	1.47	<0.5	–	–	–	3.56	6.4	55.63
Gatorade	2.03	2.57	–	–	–	–	4.6	5.6	82.14
Hi-C cherry	4.14	5.22	0.76	<0.5	–	–	10.7	12.3	86.99
Hawaiian punch	4.35	3.73	3.21	–	–	–	11.29	11.4	99.03
V-8	1.90	1.40	<0.5	–	–	–	3.3	5.2	63.46
Iced tea	<0.5	<0.5	7.97	–	–	–	7.97	8.8	90.57
Orange juice	4.55	4.04	<0.5	–	–	–	8.59	10.9	78.81
Orange drink	<0.5	<0.5	7.20	–	–	–	7.20	12.0	60.0
Cola 1	5.05	4.79	<0.5	–	–	–	9.84	10.2	96.47
Cola 2	5.45	5.11	0.88	–	–	–	11.44	12.2	93.77
Cola 3	5.32	5.31	<0.5	–	–	–	10.63	11.2	94.91
Root beer	3.15	3.18	5.58	–	–	–	11.91	12.2	97.62
Fruits									
Pineapple									
Heavy syrup	7.2	7.5	2.2	–	–	–	16.9	19.1	88.48
Own juice	6.5	7.7	<0.5	–	–	–	14.2	18.2	78.02
Fruit cocktail	6.0	6.0	3.3	–	–	–	15.3	17.6	86.93
Pear halves	4.8	4.5	6.4	–	–	–	15.7	18.8	83.51
Peach halves	6.0	6.65	4.43	–	–	–	16.11	17.1	94.21
Apple sauce	7.5	4.3	4.7	–	–	–	16.5	17.8	92.69
Raisins, Golden	36.8	35.3	<0.5	–	–	–	72.1	84.8	85.02
Raisins 1	34.9	34.3	<0.5	–	–	–	73.7	82.1	89.77
Raisins 2	33.0	33.0	<0.5	–	–	–	66.0	70.8	93.22
Prunes 1	17.6	45.4	<0.5	–	–	–	63.0	67.4	93.47
Prunes 2	14.8	34.4	<0.5	–	–	–	49.2	70.8	69.49
Dates	29.5	34.2	11.6	–	–	–	75.3	91.6	82.20
Peaches, dried	15.6	15.8	13.2	–	–	–	44.6	73.6	60.59
Mixed fruit, dried	22.3	22.0	7.2	–	–	–	51.5	78.2	65.86
Apricots, dried 1	11.8	20.5	7.3	–	–	–	39.6	72.3	54.77
Apricots, dried 2	12.6	20.1	5.6	–	–	–	38.3	68.4	55.99
Chocolate									
Milk chocolate 1	–	–	46.70	–	7.05	–	53.75	98.5	54.57
Milk chocolate 2	–	–	33.73	–	7.38	–	41.11	98.3	41.82
Milk chocolate 3	–	–	48.46	–	8.11	–	56.57	98.4	57.49
Milk chocolate 4	–	–	45.49	–	5.66	–	51.15	98.5	51.93
Milk chocolate 5	–	–	55.09	–	7.64	–	62.73	98.2	63.88
Carob 1	8.05	4.89	24.7	–	–	–	37.67	N/A	N/A
Dairy items									
Ice cream	1.65	3.45	7.75	1.87	8.44	–	23.66	40.20	58.85
Ice milk	1.66	3.44	9.26	2.52	9.01	–	25.89	38.52	67.21
Yogurt, plain	–	–	–	–	4.18	–	4.18	12.84	32.55
Yogurt, blueberry	3.71	5.68	6.54	.57	4.25	–	20.75	25.52	81.83
Pudding, chocolate	–	–	14.22	–	2.11	–	16.33	34.3	47.61
Pudding, strawberry	5.91	6.76	2.73	.23	1.19	–	16.28	26.1	62.37
Sherbert	2.66	3.60	12.02	.80	3.26	–	22.34	33.93	65.84
Fresh fruit									
Strawberry	1.88	1.62	.88	–	–	olig.[a]	4.38	8.42	52.01
Banana	1.8	3.58	0.45	–	–	–	12.28	N/A	N/A
Pears	4.57	3.58	0.45	–	–	unid.[b] mono.	8.6	13.40	64.1
Snack foods									
Chocolate cookie	1.26	0.21	37.18	1.20	.62	unid. mono.	40.47	94.4	42.87
Breakfast bar	3.04	6.42	29.06	1.81	1.03	unid. mono.	41.36	82.1	50.37
Snack bar 1		6.7	12.01	2.59	3.98	–	25.28	84.3	29.98
Cake mix	–	2.7	42.24	–	–	–	–	–	46.71
Canned frosting	–	–	72.40	–	–	–	72.40	88.4	81.90
Miscellaneous									
Baby food dessert	7.61	5.21	–	–	–	unid. mono.	12.77	–	–

[a] olig. = oligosaccharide
[b] unid. mono. = unidentified monosaccharide

Table 2—Recovery of added carbohydrate to various food groups

Food type	Sucrose			Glucose			Fructose			Lactose		
	% Added	% Recovered	Net recovery %	% Added	% Recovered	Net recovery %	% Added	% Recovered	Net recovery %	% Added	% Recovered	Net recovery %
Milk chocolate	0	0	—	0	0	—	0	0	—	0	0	—
	5	4.84	96.8	2	1.82	91.0	2	1.94	97.0	2	2.10	105
	10	9.14	91.4	3	2.84	94.7	3	3.18	106.0	3	2.87	95.7
	15	15.58	103.9	4	4.16	104	4	4.15	103.85	4	3.72	93.0
		Avg	97.4	5	4.91	98.2	5	5.17	103.4	5	4.71	94.2
					Avg	97.0		Avg	102.5		Avg	97.0
Apple juice	1	1.12	112									
	2	1.74	87									
	5	4.73	94.6									
	10	9.47	9.47									
		Avg	97.1									
Pudding	1	0.89	89									
	2	1.87	93.5									
	5	4.63	92.5									
	10	9.78	97.8									
		Avg	93.3									
Dried fruit	1	1	100	1	0.90	90	1	1.05	105			
	2	1.83	91.5	2	1.82	91	2	1.89	94.5			
	5	5.06	101.2	5	4.32	86.4	5	5.05	101			
	10	11.1	111	10	9.65	9.65	10	9.92	99.2			
		Avg	100.9		Avg	90.9		Avg	99.9			
Chocolate cookies	1	0.93	93									
	5	4.83	96.6									
	10	9.44	94.4									
		Avg	94.7									

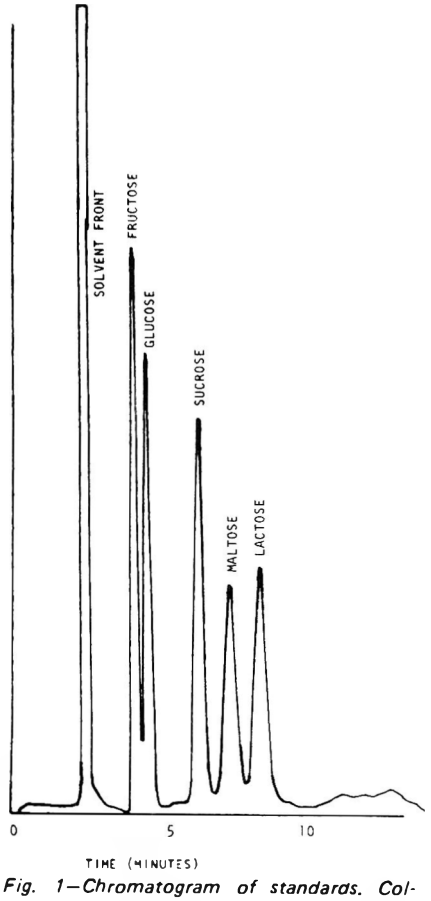

Fig. 1—Chromatogram of standards. Column: μ Bondapak/carbohydrate: Mobile phase: CH_3CN/H_2O (80/20, v/v); Flow rate: 2.0 ml/min.

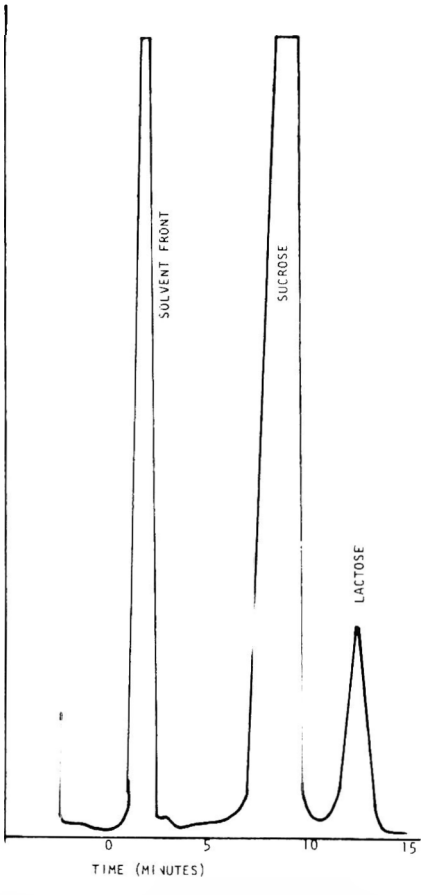

Fig. 2—Chromatogram of milk chocolate extract with no corn syrup solids. Column: μ Bondapak/carbohydrate; Mobile phase: CH_3CN/H_2O (80/20, v/v); Flow rate: 2 ml/min.

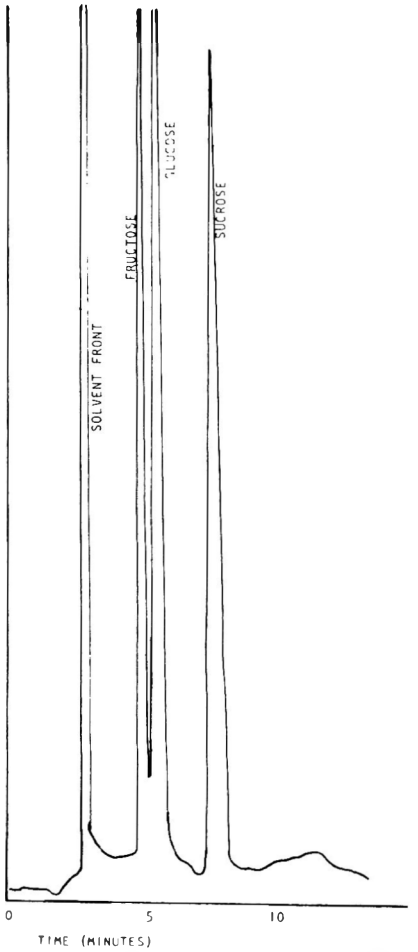

Fig. 3—Chromatogram of ginger ale. Column: μ Bondapak/carbohydrate; Mobile phase: CH_3CN/H_2O (80/20, v/v); Flow rate: 2.0 ml/min.

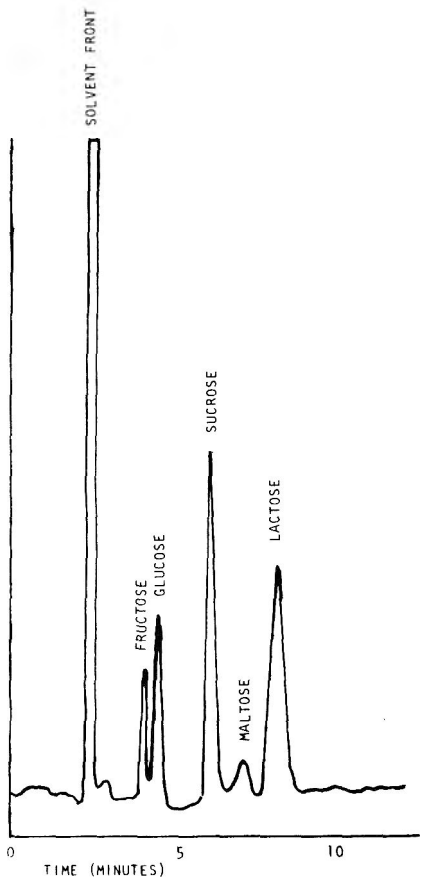

Fig. 4—Chromatogram of ice cream extract. Column: μ Bondapak/carbohydrate; Mobile phase: CH_3CN/H_2O (80/20, v/v); Flow rate: 2.0 ml/min.

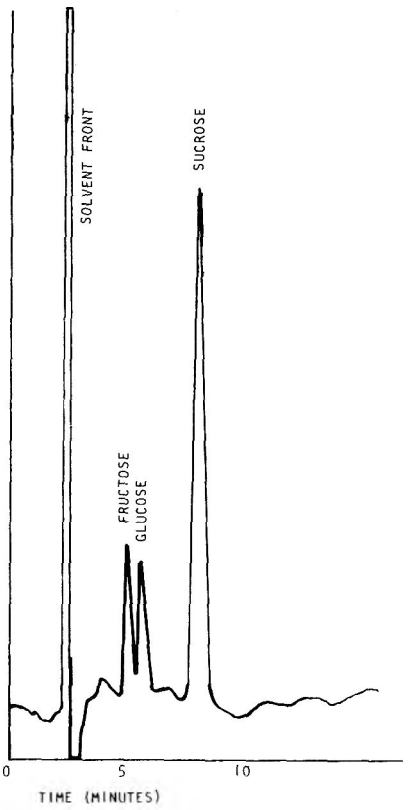

Fig. 5—Chromatogram of banana extract. Column: μ Bondapak/carbohydrate; Mobile phase: CH_3CN/H_2O (80/20, v/v); Flow rate: 2.0 ml/min.

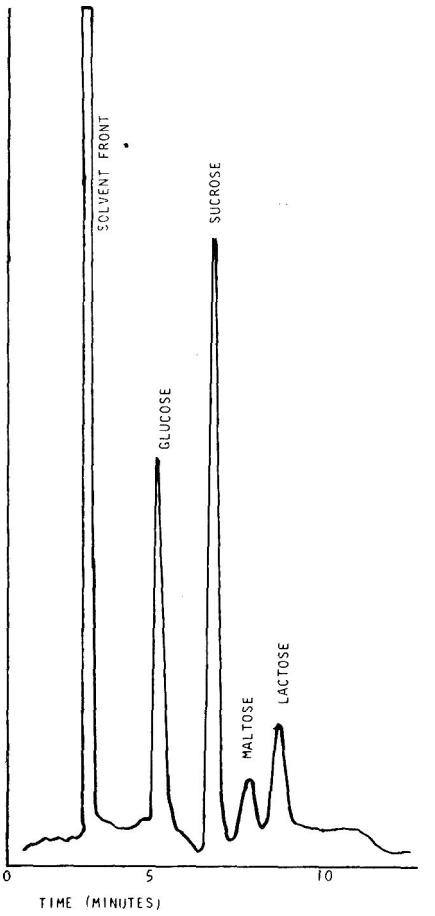

Fig. 6—Chromatogram of snack bar extract. Column: μ Bondapak/carbohydrate; Mobile phase: CH_3CN/H_2O (80/20, v/v); Flow rate: 2.0 ml/min.

RESULTS

DATA IN TABLE 1 reflect duplicate analysis of over 50 food items divided into five groups. Recovery studies were performed on each of the five food groups. These data are presented in Table 2 for candy, drinks, dairy, fruit, and snack items. Additionally, Figures 1, 2, 3, 4, 5, and 6 show chromatograms for sugar standards and samples representative of each of the groups.

DISCUSSION

MULTIPLE INJECTIONS were performed on several days to check the accuracy and reproducibility of the WISP and integrator unit. The coefficient of variation (CV) for the retention time was less than 2% with CV for area also being less than 2%.

Data generated in this study (Table 1) were compared with data available from other sources. The sources included U.S. Government publications, consumer publications, professional publications and industry publications (USDA, ARS, Handbook 8; USDA, ARS, Handbook 456; USDA, ARS, Handbook 8-1; PB262-668, FDA; Consumer Reports, Jan., 1978; March, 1978; Delmonte Nutrition Guide; Sweeney, 1977; Lee et al., 1970; Palmer and Brandes, 1974). The data in Table 1 could serve to expand and update carbohydrate literature in these sources.

The μ Bondapak/Carbohydrate column has a limited life span. The use of the guard column packed with μ Bondapak C_{18}/Porasil B will extend column life. With reasonable care, the column is useful for several thousand injections. Other columns are available for sugar analysis. The work in our laboratory has been limited to the use of the Waters μ Carbohydrate column.

The HPLC enables the characterization of these five saccharides in less than 15 minutes. Even without autoinjector capability and automatic data evaluation, the HPLC is a valuable addition to a laboratory with an interest in sugar analysis.

CONCLUSION

HPLC is now available as an analytical tool for characterization of many major and minor sugars of nutritional interest. To profile other monosaccharides such as xylose, ribose, raffinose, and mannose or to use it to characterize the higher oligosaccharides present, a simple adjustment of mobile phase and flow rate can be made. HPLC also has been successfully applied to other areas of carbohydrate research as diverse as enzyme studies on polysaccharides and analysis of the resulting fraction (Palmer, 1977). Sugar analysis by HPLC is a technique whose time has come and it now allows the food manufacturers a rapid quantitation with good accuracy and reproducibility.

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—Continued on page 904

OPTIMIZATION OF A LACTOSE HYDROLYSIS PROCESS

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ABSTRACT

A design equation was developed which represents the enzymatic hydrolysis of lactose in whey. Various processing schemes and conditions were studied to determine an optimal cost for the hydrolysis process. Consideration was given to catalyst costs, product value and reactant concentration.

INTRODUCTION

APPROXIMATELY 4 billion pounds of acid whey, a by-product of cottage cheese manufacturing, must be disposed of each year. In the past, and even currently in a large number of dairies, acid whey has been dumped directly into the municipal sewage system. Due to whey's high BOD (biological oxygen demand) many municipalities are now imposing stringent waste treatment controls on dairies and they have started charging additional fees for any whey treatment.

The best alternative to the disposal of whey is its conversion to a usable food additive. The main problem to be overcome is the low solubility and low sweetness value of lactose. Hydrolysis of lactose in whey to glucose and galactose can overcome these problems. Upon hydrolysis, the sugar mixture is sweeter and more soluble thus, lending itself to greater use in the food industry.

Lactose can be hydrolyzed by using ion exchange resins (Block, 1950), strong mineral acids (Coughlin and Nickerson, 1975), and enzymes (Shukla, 1975). Ion exchange resins and strong acids tend to destroy the functionality of the whey protein through irreversible denaturization. The use of hydrolyzing enzymes lowers the lactose content without adversely affecting the proteins and other components in whey. Through immobilization, enzymes can be repeatedly used for lower operating and separation costs.

Immobilization of enzymes

Excellent reviews of immobilized enzyme technology can be found in the literature (Stark, 1971; Zaborsky, 1973; Gutcho, 1974). Many different enzymes such as glucose isomerase, amylase, urease and lactase have been successfully immobilized. The immobilization of lactase has been of great interest because of the whey disposal problem. Lactase has been immobilized to many solid supports which include porous glass (Okos and Harper, 1974; Woychik and Wondolowski, 1972; Weetal et al., 1974; Wierzbicki and Kosikowski, 1973), phenol-formaldehyde resins (Olson and Stanley, 1973; Okos, 1975; Knopf, 1976; Hyrkas et al., 1976), stainless steel (Hasselberger et al., 1974), polyacrylmide (Bunting and Laidler, 1972), cellulose (Sharp and Lilly, 1969; Pastore et al., 1976), alumina (Paruchuri, 1975) and collagen (Giacin et al., 1974). While all these supports have successfully been used to immobilize lactase, not all are industrially applicable. The phenol-formaldehyde resin meets the criteria for an economical, stable, rigid, inert and regenerable support.

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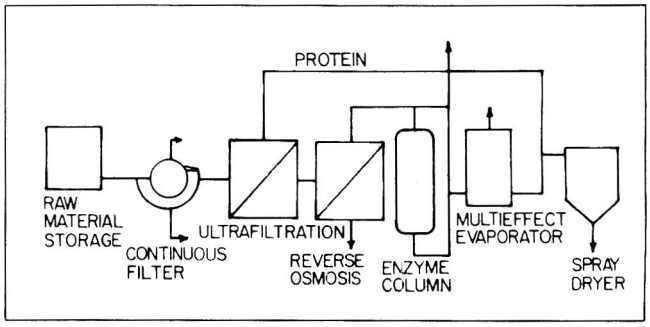

Fig. 1—Flow diagram of lactose hydrolysis.

Application of immobilized β-galactosidase

One of the most important criteria for an economical lactose hydrolysis plant is a long catalyst operational life. A long catalyst life reduces the costs associated with the enzyme, immobilization procedure and downtime while the reactor is being charged with new catalyst. Olson and Stanley (1973) reported that columns of phenol-formaldehyde bound *A. niger* β-galactosidase had no apparent loss in activity after 6 wk of operation when pure lactose was used. However, when using reconstituted nonfat dried milk, they lost 10% of the enzyme's activity after 17 hr of operation. For β-galactosidase bound to porous glass, the half-life at 40°C, 10% lactose solution, was 27.4 days (Weetal et al., 1974). Pastore et al. (1976) reported no loss in enzymatic activity when milk lactose was used in a column run 30 days (5°C) with β-galactosidase entrapped into cellulose triacetate fibers. Hasselberger et al. (1974) reported operating a column of *A. niger* β-galactosidase bound to stainless steel for 120 days (25°C) with little loss of activity, when pure lactose was used as feed. Okos et al. (1978) reported a column of β-galactosidase bound to phenol-formaldehyde resin retained nearly full activity for over 120 days (40°C) of continual hydrolysis of lactose in acid milk whey. From these examples it can be seen that a long operational life process with immobilized lactase is possible.

However, to date, little work has been done to consider the effects of lactose concentration on conversion or enzyme loading on processing costs. A design equation which accurately represents the immobilized enzyme column operation over a range of conditions is needed, and is presented in this paper.

The authors consider various processing schemes for whey concentration and lactose hydrolysis, and investigate the effects of various operating parameters on processing costs. As shown in Figure 1, the processing system uses ultrafiltration for protein removal, reverse osmosis for preconcentration, an immobilized enzyme column for hydrolysis of lactose to glucose and galactose, and evaporation and spray drying to form the final dried product. Enzyme costs

are optimized based on relative product sweetness, enzyme operating life, catalyst size, conversion attained in the reactor and the price of the enzyme and support.

PROCEDURE

THE KINETICS of the immobilized enzyme (lactase) hydrolysis of lactose are necessary in order to perform an economic optimization. A stable immobilized enzyme was produced by adsorbing β-galactosidase to a porous phenol-formaldehyde resin, Duolite ES-762 and then crosslinking with gluteraldehyde similar to the procedure of Okos et al. (1978). Only 0.65% of the initial enzyme activity was lost during immobilization using this procedure.

The kinetics of lactase were investigated using soluble, immobilized batch, and immobilized column reactors (Knopf, 1976). The batch and column hydrolysis data for enzymes immobilized on 50 mesh or smaller particles with a loading of 460 activity units/gram dry resin (0.071 gram enzyme/gram dry resin or 34g enzyme/liter resin) superimposed the soluble enzyme hydrolysis data. This indicated no appreciable effects due to diffusion (internal and external) or heat transfer for the catalyst. As loading increases, internal mass transfer becomes significant.

Grulke et al. (1978) considered the mass transfer and kinetic effects of enzymes immobilized to spherical particles. A finite difference model was used to solve the unsteady state equation for diffusion and reaction in spherical coordinates. This model was used to predict the mass transfer effects of higher enzyme loadings.

The kinetics of lactase were modeled using soluble, immobilized batch and immobilized column data. Inlet and operating conditions were varied to adequately describe most industrial situations. The enzyme concentration was varied a 250-fold span (from 0.04 mg enzyme/ml of 5% lactose to 10 mg/ml of 5% lactose). Initial lactose concentration was varied 12-fold (from 12 mg sugar/ml of solution to 250 mg/ml) and reaction data obtained. A reversible rate equation (Eq. 2) was found to fit the data with equilibrium obtained at 97% conversion.

The general mechanism consists of the enzyme (E) combining with the reactant (S) to produce a complex (AE) which then breaks down to give the product (P) and the original enzyme:

$$S + E \underset{k_2}{\overset{k_1}{\rightleftarrows}} AE \underset{k_4}{\overset{k_3}{\rightleftarrows}} E + P \qquad (1)$$

or specifically

$$\text{Lactose} + \beta\text{-Galactosidase} \underset{k_2}{\overset{k_1}{\rightleftarrows}} \text{Activated Complex} \underset{k_4}{\overset{k_3}{\rightleftarrows}} \text{Glucose} + \text{Galactose} + \beta\text{-Galactosidase} \qquad (2)$$

The total amount of enzyme in the system remains constant so by a material balance with E_o representing the initial concentration of enzyme in the solution

$$E_o = E + (AE) \qquad (3)$$

If the steady-state approximation is assumed for the rate of change of the enzyme (E) and enzyme complex (AE) the rate of product formation can be written as:

$$-\frac{dS}{dt} = \frac{dP}{dt} = \frac{(k_1 k_3 S - k_2 k_4 P)E_o}{(k_2 + k_3) + k_1 S + k_4 P} \qquad (4)$$

Eq. (4) can be integrated and solved for $E_o * t$ where E_o is the original enzyme concentration and t is the time of reaction in seconds. The result is shown in Eq. (5).

$$E = 1/t(C/A + B * D/A^2) + \text{Ln}(B - A * S_o)/(B/A * S)) + D/A * (S_o - S) \qquad (5)$$

where E = enzyme concentration, g/L; t = time of reaction, sec; S = S_o (1 − x); S_o = initial lactose concentration, moles/L; $A = k_1 k_3 + k_2 k_4$; $B = k_2 k_4 S_o + k_2 k_4 P_o$; $C = k_2 + k_3 + k_4 S_o + k_4 P_o$; $D = k_1 - k_4$; and P_o = initial galactose concentration, moles/L. If no product (galactose) is initially present, then $P_o = 0$.

RESULTS & DISCUSSION

USING the reaction data obtained from the soluble, immobilized batch and immobilized column, a Rosenbrock rotating coordinate search (Rosenbrock and Story, 1966) was used to minimize the sum of the squares of the percent difference between the experimental value of $E_o * t$ and the value calculated using various constants in the integrated, Eq. (5). Based on 22 experimental runs the values for k's were determined to be:

$k_1 = 0.1353 \times 10^{-2}$ liters/(sec-gram)
$k_2 = 0.8024 \times 10^{-7}$ moles/(sec-gram)
$k_3 = 0.5949 \times 10^{-3}$ moles/(sec-gram)
$k_4 = 0.2517$ liters/(sec-gram)

These values compare favorably with literature values (Okos et al., 1978; Woychik and Wondolowski, 1972). As shown in Figure 2, Eq. (4) with the k values listed above, represents the experimental data of various lactose concentrations.

Figure 3 shows little increase in efficiency (g glucose/g enzyme) is gained from increased lactose concentration.

—Text continued on page 898

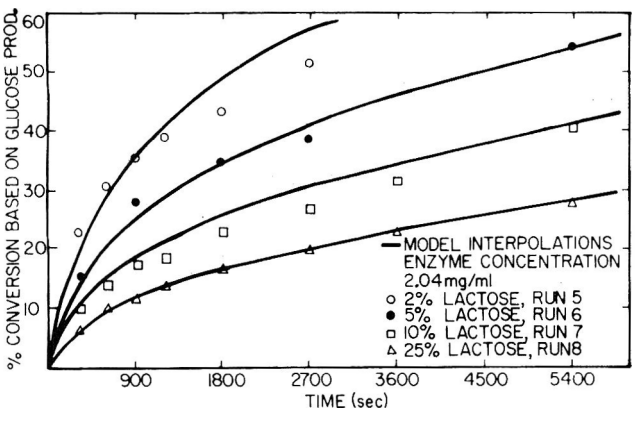

Fig. 2—Effect of lactose concentration on percent conversion vs time of reaction.

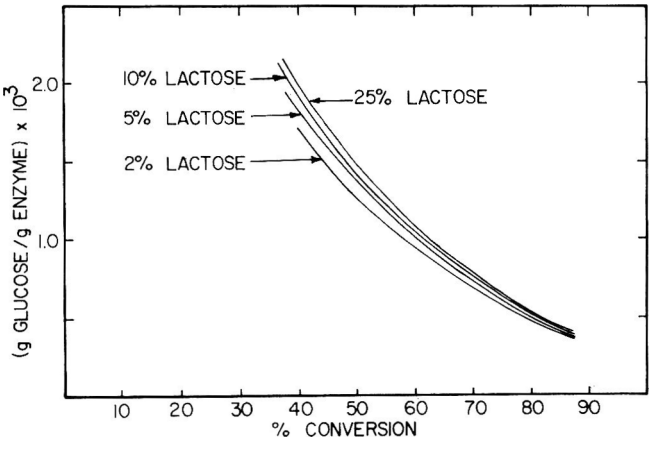

Fig. 3—Comparison of enzyme efficiency at various lactose concentrations.

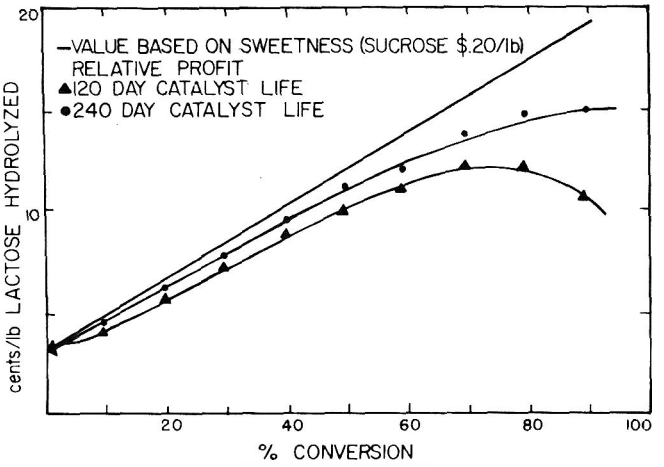

Fig. 4—Relative profit of lactose hydrolysis based on sweetness value.

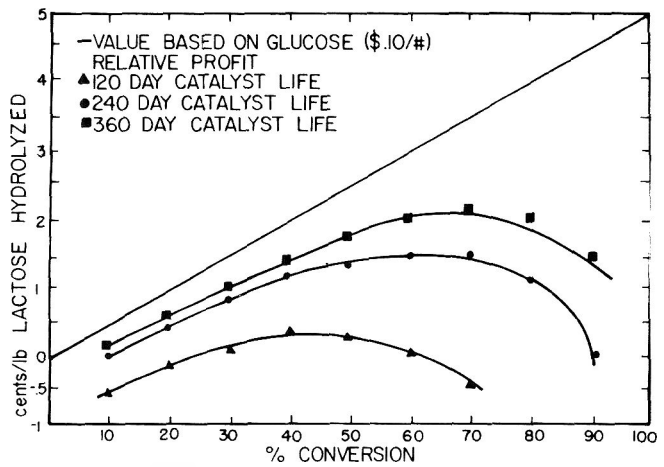

Fig. 5—Relative profit of lactose hydrolysis based on glucose.

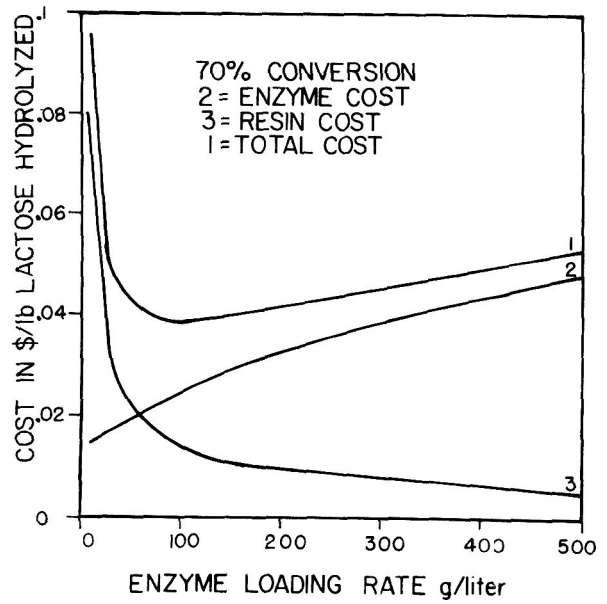

Fig. 6—Factors contributing to total catalyst cost.

Economic considerations

Before any process can be considered feasible, a preliminary cost estimate must be determined. The cost for the immobilized enzyme system was determined and an optimization program was written for the proposed system shown in Figure 1.

The cost estimate for the immobilized enzyme system consisted of three major components. The first of these components is the cost of the immobilized enzyme itself. This cost is comprised of the carrier, enzyme and enzyme loading.

The second component is the processing cost. This cost includes the labor for operating and maintaining the process, supervision costs, and the costs of utilities and supplies. The third component is the equipment cost, which appears as depreciation (10-yr straight line, 10% recovery value).

The degree of hydrolysis is also an economic consideration. Sweetness relative to sucrose can be used as an economic base. Although sweetness varies with concentration, temperature and possibly other variables, one might use as an example glucose 74%, galactose 32% and lactose 16%, of the sweetness of sucrose. With sucrose at 20¢/lb the value of the hydrolyzed whey is shown in Figure 4. Based on sweetness value and catalyst costs alone, 75% hydrolysis represents an optimum value for a catalyst life of 120 days.

If the catalyst were stable for 240 days, the optimum would be shifted to 90% conversion since the cost of the catalyst decreases relative to the value of the product. When a comparison is based on the comparative value of glucose at 10¢/lb the optimum percent hydrolysis becomes 40% as shown in Figure 5 at 120 catalyst life. At 120 day life the relative profit is very marginal. The relative profit was determined based on product value minus catalyst costs. If the catalyst life could be increased to 360 days the relative profit would increase 2¢/lb lactose hydrolyzed at 67% conversion. The optimum conversion will change with sugar price variation.

In order to determine the entire economic picture, the fixed and operating costs must also be considered. It is necessary to determine the effect of enzyme loading on catalyst costs. As the enzyme loading increases, the amount of support needed will obviously decrease. But due to mass transfer limitation, the amount of enzyme needed for a given amount of lactose hydrolysis will increase. Using the data of Figure 2 and assuming a catalyst life of 120 days at 70% conversion, a plot of cost of hydrolysis of 1 lb of lactose at various enzyme loadings is shown in Figure 6. The calculations were based on an enzyme cost of $1.00/g and a support cost of $53/L. Under the above conditions, a minimum catalyst cost is reached at an enzyme loading of 100g enzyme/L support. As shown in Figure 7 at 30, 50, 70 and 90% conversion of 5% lactose the optimum enzyme loadings are 95.3, 98.4, 103.1 and 118.7g enzyme preparation per liter of catalyst. These values correspond to 1.8, 2.6, 3.9 and 7.5¢/lb of lactose hydrolysed.

The optimum enzyme loading increases slightly with output conversion from the reactor. If low conversions are desired a lower enzyme loading is most economical. At lower conversions the rate of hydrolysis is faster than the rate of mass transfer; therefore, lower amounts of enzyme become optimal.

Particle size also affects the optimum enzyme loading. As shown in Figure 8, the minimum cost for a 0.05 cm diam support particle at 70% conversion is 102g enzyme/liter of support. If smaller particles are used, greater amounts of enzyme can be economically adsorbed to the support. Particles 0.03 cm diam have an optimal loading at 133g enzyme/liter support. As discussed above, with various conversions, smaller particles have lower mass transfer resistances; therefore, they can use more enzyme at a lower cost.

All of the above calculations were made for enzymes costing $1/g and support cost at $53.5/L. If the price for either the enzyme or the support changes, the minimum cost will vary significantly and can be easily determined for 70% conversion by changing the relative cost shown in Figure 6.

Catalyst costs are only one part of the economics. From the preliminary process flow diagram (Figure 1) and based on an amount of whey to be converted and the degree of conversion, equipment sizes and costs can be determined. The computer model simulates this entire process with any combination of units, utility costs and concentrations possible. Various case studies were performed with increasing sophistication and the costs were optimized.

Optimization of the hydrolysis process

A computer program was written to simulate the whey concentration process shown in Figure 1. Various case studies were performed to determine the effect of different combinations of unit operations and operating conditions on the selling price of glucose.

Although product quality and other intangibles may be important, optimizing a process and choosing operating parameters depend initially on economic considerations. Profitability determinations are difficult in this system because the value of the products (glucose and galactose) and reactant (whey) vary. For calculation purposes, the value of whey and galactose were assumed negligible. Using this assumption it was possible to calculate the price glucose must sell for (break-even) to pay for the catalyst (1-yr life), equipment (10-yr straight line depreciation, 10% recovery) and the operating costs. No time value for money, taxes or insurance were included.

An enzyme column with a loading of 100g of enzyme/L of resin, was used in combination with a single effect evaporator, double effect evaporator, spray drier and reverse osmosis unit. The various processing schemes studied are shown in Table 1. The concentration of lactose (solids)

Table 1—Processing schemes studied

Study I:	Reactor, single effect
Study II:	Reactor, double effect
Study III:	Reactor, double effect, spray drier
Study IV:	Reactor, double effect, reverse osmosis (10%)
Study V:	Reverse osmosis (30%), reactor, double effect

Table 2—Optimization program cost estimation

Capital		
Reverse osmosis		
tubes		$500/stage
pumps		$500/hp
	(Palmer, 1970)	
Reactor		
tube	($5.402/ft^3 * Volume in ft^3 + $567.0)(532.0/238.7)	
	(Regression: Page, 1963)	
resin	$53.0/liter *Volume in liters	
enzyme	depr. *loading*$1.0/g Enz.*Volume in liters	
	depr. = 10. for 300 day (1 yr) enzyme life, 10 yr straight line	
	loading = 100g Enz/L	
	(From Manufacturer's Data)	
Evaporator		
	$exp [0.88009 *ℓn (total area in ft^2/1000) + 10.775]	
	(Regression: E.R.D.A., 1977, p. 72 for forced circulation, integral heater)	
Drier		
($100 hr/#evaporation)*(Evaporation rate #/hr)		
	(Cook, 1966)	
Operating		
Steam $3/100C#		
Electricity 2¢/KWH		
Gas $1/1000 ft^3		
	(E.R.D.A., 1977, p. 5)	

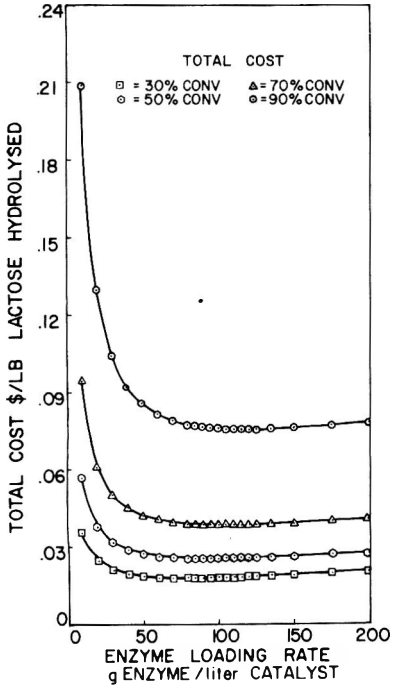

Fig. 7—Catalyst cost as affected by conversion.

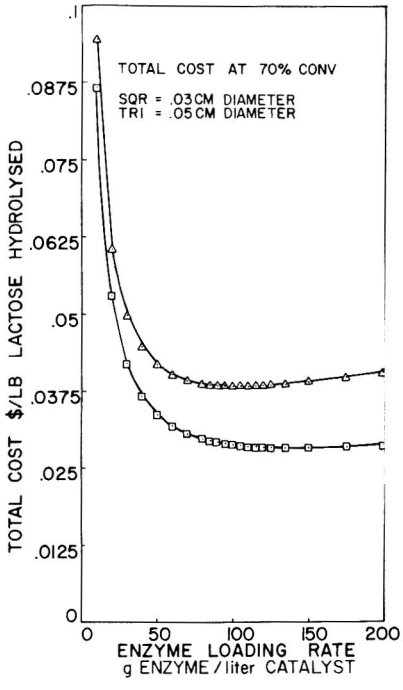

Fig. 8—Catalyst cost as affected by particle size.

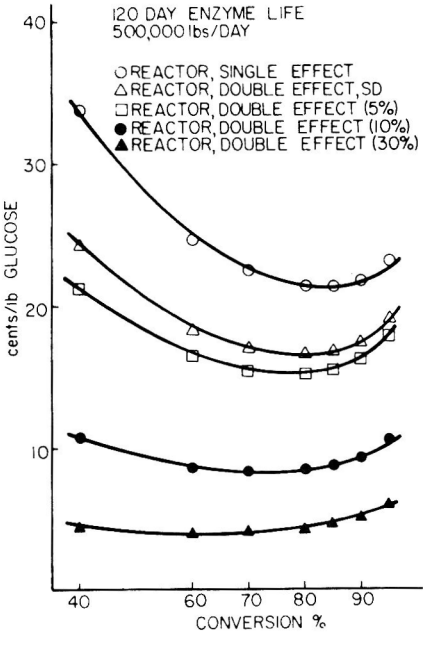

Fig. 9—Cost of lactose hydrolysis with various processing schemes for an enzyme life of 120 days.

leaving the reverse osmosis unit was evaluated at 10 and 30% to determine the effect of preconcentration (from 5% solids) on glucose selling price.

For programming purposes, the reactor was assumed in plug flow operation, and the evaporator(s) had an overall heat transfer coefficient of 500 BTU/hr-ft^2-°F. The concentration leaving the evaporators was fixed at 40% solids and the concentration leaving the spray drier was fixed at 96% solids.

Costs were calculated using manufacturer's data and the Estimator's Manual of Equipment and Installation Costs (Page, 1963). A Marshall-Stevens index of 532 was used to update capital costs. Table 2 lists costs.

As shown in Figure 9, the selling price of glucose is generally lowered with increasing design sophistication. Even more interesting is how increasing concentrations leaving the reverse osmosis unit lowers the optimum percent conversion in the reactor. As the percent solids (lactose) leaving the RO increases, the size of the evaporator necessary to reach 40% solids decreases. This reduction of evaporator costs more than offsets increasing reverse osmosis costs and thereby increases the significance of the catalyst costs.

The design equation as shown in Figure 3 is not significantly affected by lactose (solids) concentration. Thus, as catalyst efficiency or savings does not significantly increase with increasing lactose concentration, conversion will be lowered with decreasing evaporator costs.

To study the effect of enzyme life on costs, enzyme life was extended to 1 yr and the results plotted in Figure 10. As shown, the optimum conversion point in the reactor increases due to the decreased significance of catalyst cost.

From Figure 9, it would appear a process using reverse osmosis to concentrate whey to 30% lactose and an enzyme column operating at 60% conversion could sell glucose for 5¢/lb. A more sophisticated process producing a spray-dried product and operating at 80% conversion could produce glucose for 17¢/lb.

The profitability of this process is not the only consideration. Government regulations may soon establish that all

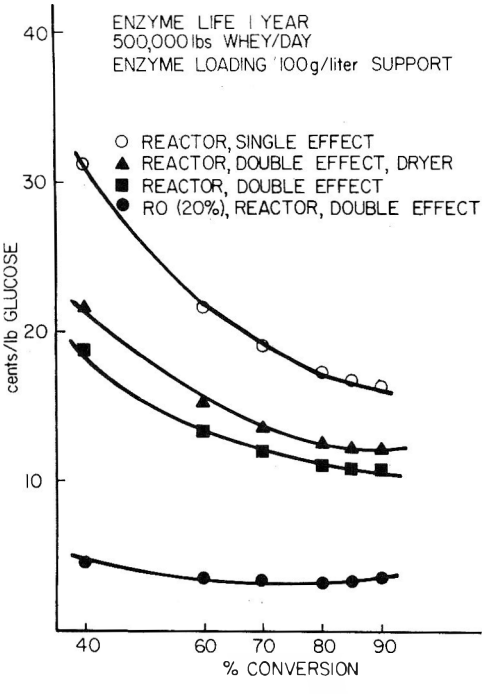

Fig. 10—Cost of lactose hydrolysis with various processing schemes for an enzyme life of 1 yr.

pollutants (including whey) must be treated with the best available technology and a zero pollutant level has been proposed for 1985.

CONCLUSIONS

A DESIGN equation which represents the effect of lactose concentration was determined from experimental results. The rate of lactose hydrolysis increased slightly with lactose concentration. It was also found the optimum conversion from the reactor was significantly affected by catalyst life and product value. The optimum enzyme loading was affected by particle size and conversion. When considering the total process the optimum conversion was found to be a function of capital investment and enzyme life.

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USE OF GRAPHIC LINEAR SCALES TO MEASURE RATES OF STALING IN BEER

DEBORAH J. SCHMITT and JOSEPH T. HOFF

ABSTRACT

Graphic linear scaling was used to estimate rates of staling for several American lager beers. Packaged, fresh beers were incubated at various elevated temperatures. Corresponding controls were incubated at 0°C. Air contents of the packages were also varied. Samples of the control and experimental beers were evaluated over time by a trained panel. Results showed that this method could accurately measure stale flavor as it increased with time. Regression analyses showed that acetaldehyde concentration, determined by a GLC headspace technique, correlated well with mean panel scores for stale flavor.

INTRODUCTION

LONG-TERM STORAGE has long been recognized in the brewing industry as detrimental to beer flavor. It is well known that stale flavors are due to higher aldehydes. The rate at which these aldehydes form is dependent upon composition of product, amount of oxygen present, and temperature at which the product is stored (Hashimoto and Eshima, 1977). The purpose of this investigation was to measure by sensory evaluation methods the rates of staling as affected by brand, temperature and air content, and to correlate the sensory data with some chemical changes.

Graphic linear scaling methods have been successfully used in the brewing industry in descriptively evaluating beer (Mecredy et al., 1974; Stone et al., 1974). These methods normally use analysis of variance to quantify differences between products. It was hypothesized that by using a panel of judges trained to rate stale flavor, rates of change in stale flavor could be determined from regression analyses of mean scores. At the same time headspace GLC was performed. Regression analysis could confirm a possible correlation between mean panel scores and acetaldehyde concentration as suggested by Hoff et al. (1978).

EXPERIMENTAL

Sample preparation

Several popular brands of American lager beer were obtained from commercial markets. Freshness or absence of stale flavor was validated by production code date and sensory analysis. Air contents were analyzed using a modified Zahm-Nagel procedure. Representative samples of fresh beer were then chosen based upon normal air values for each particular brand and the absence of stale flavor.

Three brands of American lagers containing approximately 1.1 ml air were used for Study IA. Two brands containing 0.5 ml air were used for Study IB. The samples for both studies were divided into two groups: one stored at 0°C (control) and one stored at 24°C. For each brand, samples were taken for sensory analysis every 3–4 days during the first 3 wk of storage and thereafter every 7 days to the end of storage.

For Study II, one brand of beer at two air levels (0.5 and 1.0 ml) was used. Beer at each air level was divided into four groups. The control group was held at 0°C and the other three groups were stored at 15.5°, 24° and 38°C, respectively. The sampling schedule for this study was identical to Studies IA and IB.

Sensory evaluation

Testing was conducted in a room used solely for sensory evaluation. Room temperature was held at 22 ± 1°C with 150 ft-c of illumination at testing surface. Partitioned booths equipped with water for rinsing and a sink for expectoration were used during testing. A positive air flow was incorporated to minimize odors. A cracker-type clearing agent was made available to panelists.

Prospective judges were selected from a group of employees who had proven ability to discriminate between beers with different sensory properties. The judges had qualified their abilities by triangular testing at the 95% significance level. After qualification, their abilities were monitored by Chi square and percent correct on significant triangulars during daily testing sessions. Judges had also been trained to consistently recognize and quantify various flavor notes, including stale flavor.

During each testing session, judges were presented with two samples of beer; a control and a sample taken from incubated storage. Each sample consisted of 3 oz of beer at a serving temperature of approximately 9°C contained in a 12 oz, smoke-colored beverage glass. Samples were coded with three-digit random numbers. Sample position was also randomized.

The ballot was composed of a 6-inch line with anchor marks 1/2 inch from each end. The anchors were labeled *not oxidized* at the left end and *extremely oxidized* at the right end. After testing, the line was divided into 24 quarter-inch segments. Judges' ratings were assigned a numerical score for each sample on this 24-point basis. The term "oxidized" was agreed on by the panel as being *the* term to describe staling. The judges were asked to rate the degree of oxidation for each sample.

Training samples for each brand of beer were prepared by incubating at 38°C. Each day, samples were taken from incubated storage and stored at 0°C. A full spectrum of oxidative change in each brand of beer was obtained in this manner. Three different samples, taken at random, were presented to each judge. The judge was instructed to rate the degree of staling in each sample. Each judge's ballot was checked immediately after completion of the training test, and he was shown how his scores deviated from proper rank order and group means as found in preliminary testing. Nine to twelve training sessions were necessary to standardize judges and enable them to consistently rate various degrees of stale flavor on a "day-to-day" basis. Approximately 1/3 of all prospective judges were unable to perceive or actually rate stale flavor as it increased during incubation and were not used in the actual study. The training samples were not available for use as reference during the actual testing. Judges relied totally on the preliminary training to rate degree of staling during actual testing.

A pool of 20–25 judges was used in each study. An average of 13 judges was used in each test. Judges were interchanged due to high absentee rates and company policy limiting the number of available judges. The 3–5 month duration of each study sometimes led to erratic scoring by a few judges on an infrequent basis. Due to these factors. Dixon's test for outliers at an α of 0.05 (Youden and Steiner, 1975) was applied before any other statistical analysis was performed.

GLC analyses

The following conditions were used with the Perkin-Elmer F-42 automatic headspace analyzer.

Headspace program:	(1)	Pressurization	= 30 sec
	(2)	Injection	= 5 sec
	(3)	Flush	= 15 sec
	(4)	Stabilization	= 2 min
Temperatures:	(1)	Detector	= 180°C
	(2)	Injector	= 150°C

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Table 1—Critical values for beers used in Study IA

Brand	k X 10^3 ($days^{-1}$)	t½(days)	r	n
A	62.9	11.0	0.89	15
B	17.8	38.9	0.98	16
C	12.8	54.1	0.95	24

Table 2—Critical values for beers used in Study IB

Brand	k X 10^3 ($days^{-1}$)	t½(days)	r	n
D	29.9	23.2	0.93	22
E	13.4	51.7	0.83	19

(3) Needle = 150°C
(4) H_2O bath = 25°C

Sensitivities: (1) FID Amp: range = 1; attenuator = 4
(2) Recorder = 5 mv

Gas Flows: (1) Air = 300 ml/min (3 atm)
(2) Hydrogen = 30 ml/min (2 atm)
(3) Nitrogen = 25 ml/min (1.4 atm)

Column: 2m × 2.8 mm i.d. glass packed with 0.2% Carbowax 1500 on 80/100 mesh Carbopack C

Temperature program: (1) Isothermal 2 min at 60°C
(2) 8°/min to 140°C
(3) 2°/min to 150°C
(4) Isothermal 32 min at 150°C

For sample preparation, 22 ml vials were purged with nitrogen. Five ml of beer, chilled to 0°C in an ice bath, were gently pipetted into the vials. Thirty µl of internal standard (2 ml n-hexanol diluted to 100 ml with absolute ethanol) was added and the sample placed in the water bath to equilibrate a minimum of 30 min.

The gas chromatograph was attached to a Spectrophysics System IV integrator and ppm acetaldehyde calculated. Each sample was analyzed in duplicate.

Statistical analyses

After applying Dixon's test for outliers to the sensory scores, mean values were calculated for each test sample. Using an iterative process, least squares estimates of the asymptotic regression (first order rate equation) coefficients were performed, and correlation coefficients were estimated with the aid of a computer (Snedecor and Cochran, 1967). The general equation was as follows:

$$Y = A-(A-I)e^{k-t}$$

where Y is the dependent variable "mean panel score;" A is the asymptote; I is the initial mean panel score; k is the rate constant; and t is the independent variable "time." Linear regression was performed for acetaldehyde concentration vs mean panel scores for two brands of beer to confirm possible correlations.

RESULTS & DISCUSSIONS

FIGURE 1 shows a typical curve for mean panel scores vs time obtained with the computer and the scatter of the 16 actual data points around the asymptotic regression line. The resulting rate equation was $Y = 18.3 - 14.6e^{-0.0178t}$.

A correlation coefficient of 0.98 ($p \leq 0.001$) and a residual variance about the asymptotic regression line of 0.734 show that the curvilinear fit is quite good.

Figure 2 shows the rate curves obtained for Study IA, and Table 1 shows the computed values for the rate constant, half-life, and correlation coefficient as well as sample number for each of the three brands. It can be seen that Brand A staled much more rapidly than either Brand B or Brand C, while Brand C staled less rapidly than Brand B.

Figure 3 shows the rate curves obtained for Study IB. The rate of staling for Brand D was approximately twice that for Brand E as evidenced by the data in Table 2.

The effect of packaged air content and temperature on rate of staling for Brand E was investigated in Study II. Since the computed asymptotes and initial ratings were similar for all cases, the general rate equation $Y = 16.8 - 12.6e^{-kt}$ was derived, and the rate constants estimated.

Figure 4 shows the computed rate curves obtained at the three elevated temperatures and an air content of 1.0 ml. Figure 5 shows the corresponding rate curves at an air content of 0.5 ml.

Table 3 shows the computed rate constant, half-life, correlation coefficient, and sample number for each curve. The data indicate that when the air content is reduced, the rate of staling at a particular temperature is reduced. In these

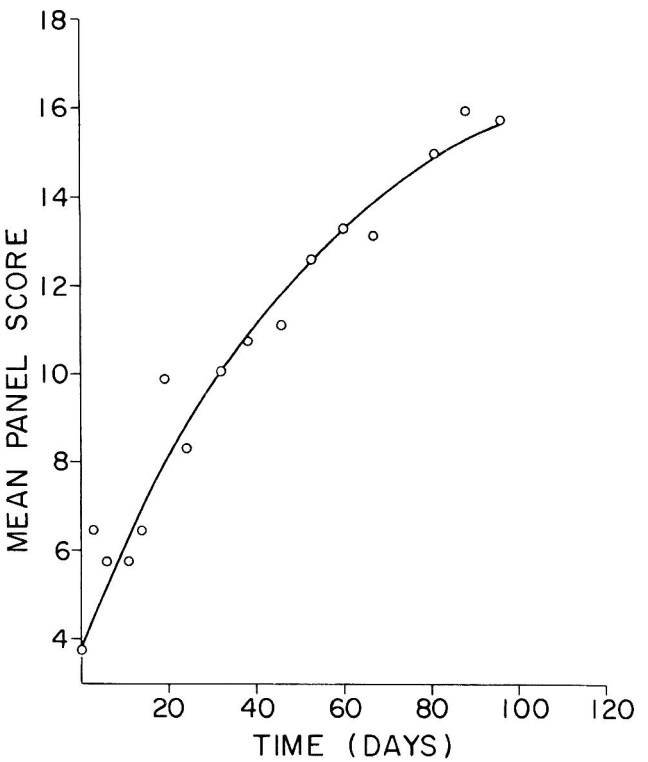

Fig. 1—Typical rate curve showing the scatter of 16 data points.

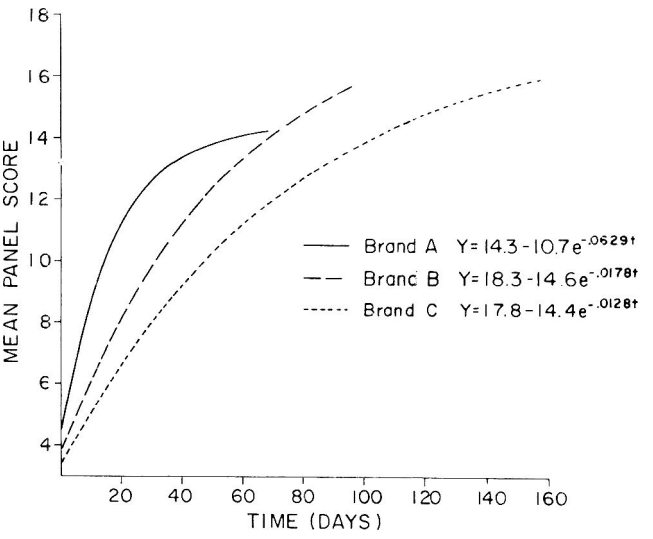

Fig. 2—Rate curves for Brands A, B and C containing 1.1 ml air.

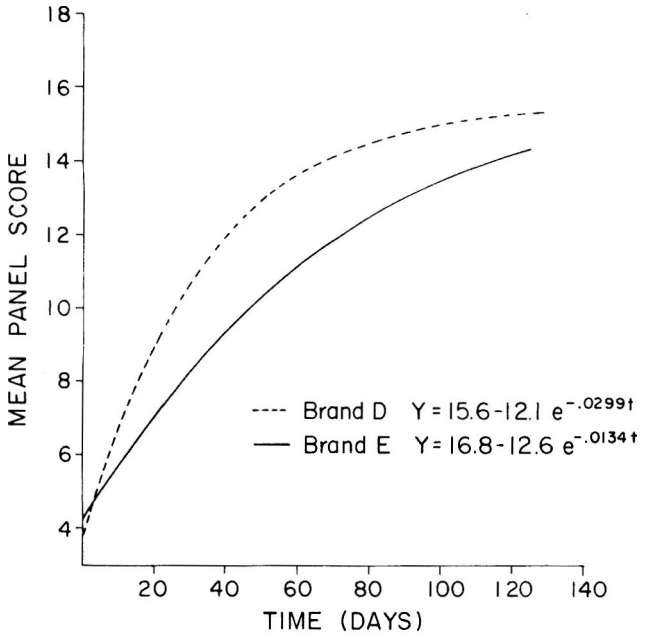

Fig. 3–Rate curves for Brands D and E containing 0.5 ml air.

particular cases, a halving of the air content halved the rate of staling; but we are not sure that this effect is linear over the normal range of air contents that can occur.

The Arrhenius plot (log k vs 1/T) was constructed for each air content. The linear relationships in Figure 6 show that, for the temperature range studied, the first order rate constants behave according to the Arrhenius equation where a 10°C increase in temperature approximately doubles the rate of staling.

Mean ppm acetaldehyde was calculated from headspace analyses done in duplicate on the same samples given the panel for Brand C and Brand D. Linear regression analyses were performed for the mean sensory scores vs acetaldehyde concentrations. The results are shown in Figure 7 for Brand C and in Figure 8 for Brand D. The high correlation coefficients ($p \leqslant 0.001$) indicate good linearity and are consistent with our earlier study (Hoff et al., 1978).

SUMMARY

THE BEER STALING PROCESS as measured in these studies was affected by such parameters as brand, air content, and temperature. The high correlation coefficients, 0.75–0.98 ($p \leqslant 0.001$) obtained when panel scores were fitted to the first-order rate curve, demonstrate that the use of graphic linear scales is a valid means of measuring stale flavor changes in beer. Although acetaldehyde does not contribute to stale flavor, the high degree of correlation between its concentration and sensory scores for stale flavor ($p \leqslant 0.001$) indicates its potential use as an index of beer staling.

–Text continued on page 904

Table 3–Critical values for brand E stored with varied air content and temperature

Air content	Temp (°C)	k X 10^3 ($days^{-1}$)	t½(days)	r	n
1.0 ml	38.0	119	5.82	0.97	14
	24.0	28.0	24.8	0.89	19
	15.5	12.5	55.4	0.93	19
0.5 ml	38.0	54.4	12.7	0.93	14
	24.0	13.4	51.7	0.83	19
	15.5	5.24	132	0.75	20

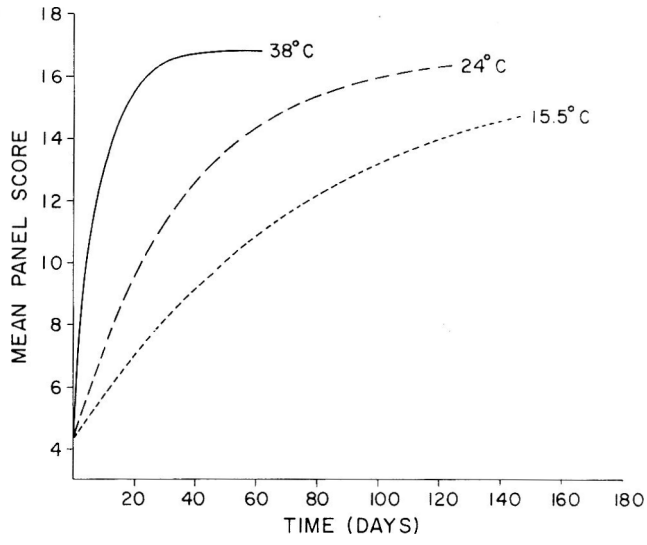

Fig. 4–Rate curves for Brand E containing 1.0 ml air at three temperatures.

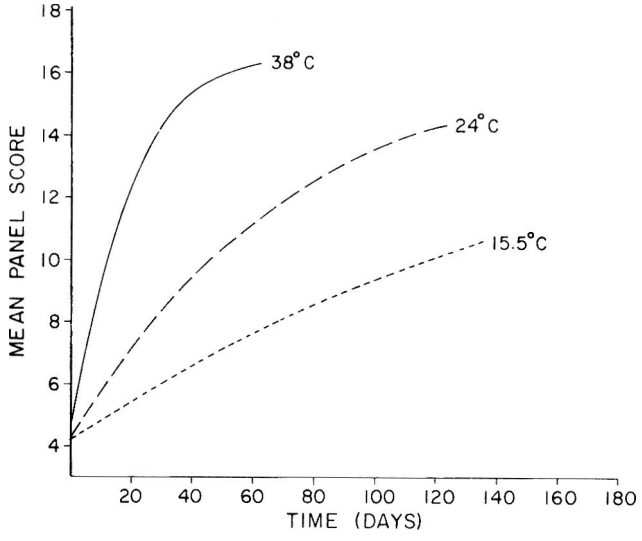

Fig. 5–Rate curves for Brand E containing 0.5 ml air at three temperatures.

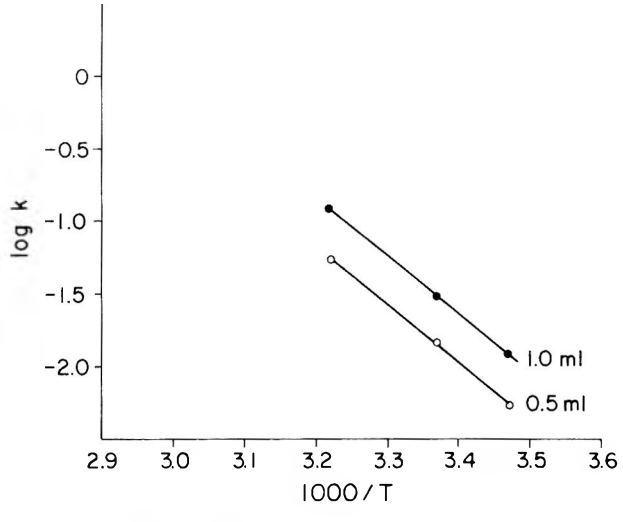

Fig. 6–Arrhenius plots for each air content.

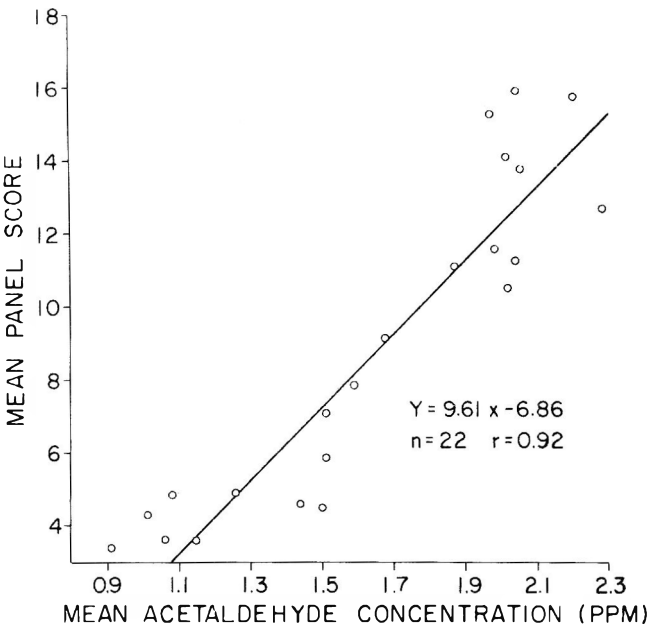

Fig. 7—Regression line of stale flavor vs acetaldehyde concentration for Brand C.

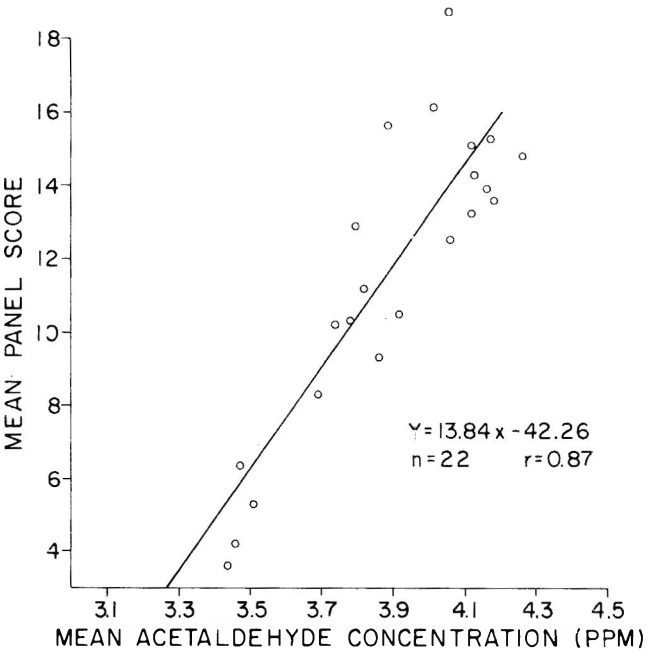

Fig. 8—Regression line of stale flavor vs acetaldehyde concentration for Brand D.

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HPLC CHARACTERIZATION OF FOOD CARBOHYDRATES . . . From page 895

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ANALYSIS OF CONTINUOUS STERILIZATION PROCESSES FOR BINGHAM PLASTIC FLUIDS IN LAMINAR FLOW

C. GUARIGUATA, J. A. BARREIRO and G. GUARIGUATA

ABSTRACT

A model was developed to represent the continuous sterilization of Bingham plastic fluid foods for the laminar, isoviscous and nonisoviscous flow, with applications to design. The length of the heating and cooling sections as well as nutrient retention were numerically calculated for a required sterilization value. Thiamine and *C. botulinum* were considered for this purpose. There was a considerable difference between solutions obtained for isoviscous and nonisoviscous flow. Therefore, it would not be advisable to overlook the temperature effect on rheological fluid properties. Optimal nutrient retention was shown to occur for the smallest tube diameter studied.

INTRODUCTION

CONTINUOUS STERILIZATION has proved to be more advantageous than batch methods. Properly designed, nutrient losses and operating costs are minimized.

The design of continuous sterilizers has been studied by several authors, such as Pfeifer and Vojnovich (1952), Whitmarsh (1954), and Deindoerfer and Humphrey (1959), which dealt only with turbulent flow applicable to sterilization of Newtonian fluid media. Later, Holdsworth (1969) took into account the fact that most of the food products subject to continuous sterilization are high apparent viscosity non-Newtonian fluids in laminar flow.

Among the sterilization analyses in laminar flow that should be mentioned are those of Aiba et al. (1965) and Charm (1966). They consider only isothermal conditions, with no variation of fluid properties with temperature.

The non-Newtonian fluids that have been subject to the most extensive studies are the pseudoplastics that follow the Ostwald-de Waele power law without yield stress. However, of all non-Newtonian fluids with yield shear stress, Bingham plastics show the simplest flow curve (Metzner, 1956; Skelland, 1967-a).

Unfortunately, there are very few data on the rheological behavior of fluid foods. According to Harper (1960), most liquid food materials show a yield stress, but its effect has been considered small and neglected. Nevertheless, Charm (1963) determined the value of this parameter for several foods, proving that values of other fluid characteristics are incorrect when the yield stress is neglected.

Heat transfer to Newtonian and non-Newtonian fluids in laminar flow through cylindrical tubes has been analyzed in many occasions. Graetz (1883) obtained analytical "isothermal" solutions to the theoretical relationships that describe heat transfer to Newtonian fluids through tubes. Brown (1960) presented similar solutions for tubes and flat plates.

The Graetz "isothermal" problem was extended by Wissler and Schechter (1959) to Bingham plastics allowing heat generation within the fluid. Hirai (1959) presented empirical solutions, but Schechter and Wissler (1959) proved his analysis led to error.

Isoviscous analyses for pseudoplastic fluids have been performed by Lyche and Bird (1956) for tube flow, and Tien (1962), for flat plate. Others have considered temperature effect on rheological properties; Christiansen and Craig (1962), Christiansen et al. (1966), Christiansen and Jensen (1969), and Kwant et al. (1973), studied nonisothermal pseudoplastic fluid flow through tubes.

Recently, Simpson and Williams (1974) developed a method for the design of continuous sterilizing equipment for pseudoplastic fluids in laminar and nonisoviscous flow.

The purpose of this research is to analyze and model continuous sterilizers for Bingham plastic fluids in laminar flow, allowing temperature effect on rheological properties, and maximizing nutrient retention while sterilizing the product.

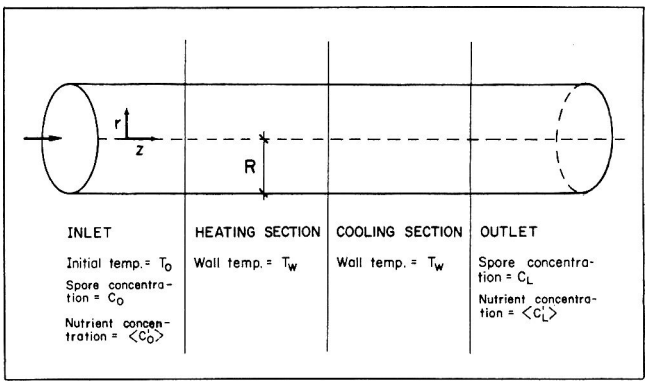

Fig. 1—Physical model of the sterilization process.

MODEL DEVELOPMENT & SOLUTION

THE STERILIZATION MODEL for Bingham plastics in laminar flow is developed under the following assumptions:

1. The flow is steady state and axisymmetric, and radial flow effects are negligible.
2. Velocity distribution is fully developed at the entrance to the heat transfer section.
3. The inlet temperature is uniform.
4. The fluid is heated and cooled at constant wall temperature.
5. Axial diffusion of energy is negligible.
6. The fluid thermal diffusivity, α, is independent of temperature.
7. Thermal energy generation in the fluid by viscous dissipation or any reaction is negligible.

Physical model

The physical model is similar to the one described by Simpson and Williams (1974) and is shown in Figure 1. It consists of a single tube exchanger into which the fluid is pumped at an inlet temperature T_0. The fluid enters first a heating section held at a constant wall temperature, T_W, and immediately followed by a cooling section also at constant wall temperature maintained by cooling water. A holding section is omitted in order to attain a "highest-temperature/shortest-time" process (Simpson and Williams, 1974).

Rheological model

The equation of the Bingham plastic flow curve is (Metzner, 1956; Skelland, 1967-a):

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$$\tau_{rz} - \tau_y = \frac{\eta}{g_c}\left(-\frac{du_z}{dr}\right) \quad (1)$$

For isoviscous flow (τ_y and η constant with temperature) the velocity profile derived from this flow model is (Skelland, 1967-b):

$$u_z(r) = \frac{g_c}{\eta}\left[\frac{\tau_w}{2R}(R^2 - r^2) - \tau_y(R-r)\right] \quad (2)$$

when $r_0 \leq r \leq R$, and

$$u_z(r) = \frac{\tau_w g_c R}{2\eta}\left(1 - \frac{\tau_y}{\tau_w}\right)^2 \quad (3)$$

when $r \leq r_0$, where:

$$r_0 = \frac{\tau_y}{\tau_w} R \quad (4)$$

and τ_w is related to the flow velocity V by means of Buckingham's equation:

$$\frac{8V}{D} = \frac{\tau_w g_c}{\eta}\left[1 - \frac{4\tau_y}{3\tau_w} + \frac{1}{3}\left(\frac{\tau_y}{\tau_w}\right)^4\right] \quad (5)$$

For nonisoviscous flow (τ_y and η dependent on temperature), the following variations of fluid properties with temperature are considered:

$$\eta = \eta_0 \exp\left(\frac{\Delta H_1}{R_G T(r,z)}\right) \quad (6)$$

and

$$\tau_y = \tau_{y0} \exp\left(\frac{\Delta H_2}{R_G T(r,z)}\right) \quad (7)$$

where ΔH_1 and ΔH_2 are activation energies for flow.

This type of powerlaw model has been used before to correlate the nonisoviscous data for food products and suspensions of similar consistency (Charm and Merrill, 1959; Christiansen and Craig, 1962; Christiansen et al., 1966).

The velocity profile derived here for nonisoviscous flow is of the form:

$$u_z(r,z) = \frac{g_c}{\eta_0}\left[\frac{\tau(R,z)}{R} I_1 - \tau_{y0} I_2\right] \quad (8)$$

when $r_0 \leq r \leq R$, and

$$u_z(r,z) = u(r_0,z) \quad (9)$$

when $r \leq r_0$, where:

$$I_1 = \int_r^R r \exp\left[-\Delta H_1 / R_G T(r,z)\, dr\right] \quad (10)$$

$$I_2 = \int_r^R \exp\left[(\Delta H_2 - \Delta H_1)/R_G T(r,z)\right] dr \quad (11)$$

Combining Eq (7) and Eq (4):

$$r_0 = \frac{\tau_{y0} \exp[\Delta H_2 / R_G T(r,z)]}{\tau(R,z)} \times R \quad (12)$$

The rheological fluid properties would be related in this case to wall shear stress by Rabinowitsch-Mooney's equation (Skelland, 1967-c):

$$\frac{2V}{D} = \frac{g_c}{\eta}\int_{\tau_y}^{\tau(R,z)} \frac{\tau_{rz}^2}{\tau(R,z)^3}(\tau_{rz} - \tau_y)\, d\tau_{rz} \quad (13)$$

Heat transfer

The basic differential equation that describes heat transfer to fluids in laminar flow through tubes is (Skelland, 1967-d):

$$\frac{\partial T(r,z)}{\partial z} = \frac{\alpha}{u(r,z)}\left[\frac{\partial^2 T(r,z)}{\partial r^2} + \frac{1}{r}\frac{\partial T(r,z)}{\partial r}\right] \quad (14)$$

The boundary conditions are:

$$T(r,0) = T_0 \quad (15)$$

$$T(R,z) = T_w \quad (16)$$

Eq (14), solved for the isoviscous and nonisoviscous velocity profiles, allows the calculation of the temperature profiles within the tube.

Thermal destruction of microorganisms and nutrients

For a first order kinetics of denaturation, it can be shown, from well known correlations (Stumbo, 1973), that:

$$-\ln\frac{C(r,z)}{C_0} = \frac{2.303}{D_0}\int_0^z \frac{1}{u_z(r,z)} \exp\left(2.303\,\frac{T(r,z) - 250}{Z_0}\right) dz \quad (17)$$

when T is in (°F).

This equation permits the evaluation of the remaining nutrient or spore fraction anywhere inside the tube from the velocity and temperature profiles, under the assumption that bacterial spores remain on streamlines throughout the process.

If product quality is interpreted in terms of fractional retention of nutrients, it is convenient to express the nutrient fraction as an average over the flow cross section (Simpson and Williams, 1974):

$$\left.\frac{\langle C'_L\rangle}{\langle C'_0\rangle} = \frac{\int_0^R \frac{C'(r)}{C'_0} u_z(r)\, 2\pi r\, dr}{\int_0^R u_z(r)\, 2\pi r\, dr}\right|_z \quad (18)$$

Approximations introduced to the sterilization model

As was mentioned before, the exact resolution of the model developed for the continuous sterilization process consists of solving successively the heat transfer differential equation for the isoviscous and nonisoviscous velocity profiles, determining the temperature distribution and spore and nutrient degradation along the heating and cooling sections. Therefore, in the nonisoviscous case, the relations (14), (15), (16), (8), (9), (10), (11), (12) and (13) must be solved simultaneously, which implies analytical and numerical complications, being necessary to use trial and error procedures for the evaluation of r_0 and the wall shear stress $\tau(R,z)$.

In order to simplify the cumbersome calculations mentioned above, the wall shear stress $\tau(R,z)$ is assumed here to vary with axial position according to:

$$\tau(R,z) = a + b\exp(-z) \quad (19)$$

The exponential relationship defined by Eq (19) was chosen among other simple functions such as linear and polynomial, to represent more accurately the variation of the wall shear stress with axial position through a small diameter tube, as it proved to simulate better the changes undergone by temperature and other rheological parameters near the wall, which are sharp at the beginning of the tube sections and asymptotic towards the outlet. Being a mathematical approximation, it should be subject to future research in order to evaluate the impact of the inherent error.

The constants "a" and "b" are calculated from the known extreme isothermal conditions ($T = T_0$ when $z = 0$ and $T = T_w$ when $z = \infty$).

Although analytic solutions to Eq (14) are unknown, numerical solutions were obtained through standard finite-difference techniques, frequently used to solve similar problems (Christiansen and Craig, 1962; Christiansen et al., 1966; Christiansen and Jensen, 1969). The tube diameter was divided into 20 equal parts of Δr each and the velocity and temperature distributions were computed for steps of length Δz, fixed at 0.69 ft for stability of the solutions.

Then, assuming that fluid at the centerline receives the least thermal treatment, the remaining *C. botulinum* spore fraction was evaluated along the sterilizer until complete sterilization at the centerline occurred, that is, 10^{-12} for the remaining spore fraction, as recommended by Stumbo (1973) for low-acid foods. The cooling section began immediately and was prolonged up to 150°F at the centerline, or 800 ft of total tube length, due to computational limitations. The nutrient average retention was then evaluated throughout both sections. Thiamine was selected as the model nutrient due to its first order kinetics of denaturation (Feliciotti and Esselen, 1957).

The sterilization model was solved by means of Fortran IV computer programming for the following conditions:

- Isoviscous case: tube radius of 0.0245, 0.0305, 0.0513 and 0.0722 ft; heating wall temperature of 730, 750, 770 and 790°R; and cooling wall temperature of 536°R.
- Nonisoviscous case: tube radius of 0.0305 ft; heating wall temperature of 750°R and cooling wall temperature of 536°R.

The fluid density, ρ, and thermal diffusivity, α, were arbitrarily fixed at 70 lbm/ft^3 and 2×10^{-6} ft^2/s respectively. The initial temperature utilized was 536°F, and the flow rate 0.28 lbm/s, in order to obtain laminar flow. For the rheological parameters, the figures chosen were $\eta = 0.06$ lbm/ft s, $\tau_y = 0.15$ lbf/ft^2, $\eta_0 = 2.47 \times 10^{-3}$ lbm/ft s, $\tau_{y_0} = 0.09$ lbf/ft^2, $\Delta H_1/R_G = 1710$°R and $\Delta H_2/R_G = 273$°R.

The calculated values of "a" and "b" in Eq (19) gave results of: $a = 0.3073$ and $b = 0.2277$ for the heating section, and $a = 0.5350$ and $b = -0.2277$ for the cooling section.

With regard to thiamine degradation, the values $D_0 = 157.2$ min and $Z_0 = 45$°F were utilized, as recommended by Barreiro et al. (1977) for phosphate buffer of pH = 6. $D_0 = 0.21$ min and $Z_0 = 18$°F were selected to represent *C. botulinum* spore denaturation.

RESULTS & DISCUSSION

THE RESULTS corresponding to the isoviscous case can be found in Figures 2, 3, 4, 5, 6, 7 and 10, and in Tables 1, 2, 3 and 4. Figures 2 and 3 show velocity distributions obtained for tube radii of 0.0305 and 0.0722 ft, and Figures 4, 5, 6 and 7 show temperature distributions for the different tube radii studied and a heating wall temperature of 750°R, at different radial distances ($r/R = 0$, $r/R = 0.4$ and $r/R = 0.8$). Similar results were obtained for the nonisoviscous case for a tube radius of 0.0305 ft and heating wall temperature of 750°R (Fig. 8 and 9).

In Figure 10, nutrient retention evaluated for the isoviscous case is plotted as a function of the different tube radii and heating wall temperatures studied.

Tables 1, 2, 3 and 4 show other results (heating and cooling lengths, and remaining spore fraction) computed for the isoviscous case.

Finally, Table 5 shows the heating and cooling lengths, percentage of nutrient retention and remaining spore fraction evaluated for the nonisoviscous case.

Isoviscous case

As shown in Figures 2 and 3, corresponding to isoviscous velocity distributions, an increase of the tube diameter implies a flattening of the profile and a decrease of the centerline fluid velocity. This should affect positively the product quality, because of a more uniform thermal treatment over the different fluid layers. Also, with a decrease in the centerline velocity, a smaller tube length should be sufficient to achieve the product sterilization. However, as observed in Figures 4 to 7 and Tables 1 to 4, larger heating and cooling sections are required for a same wall temperature

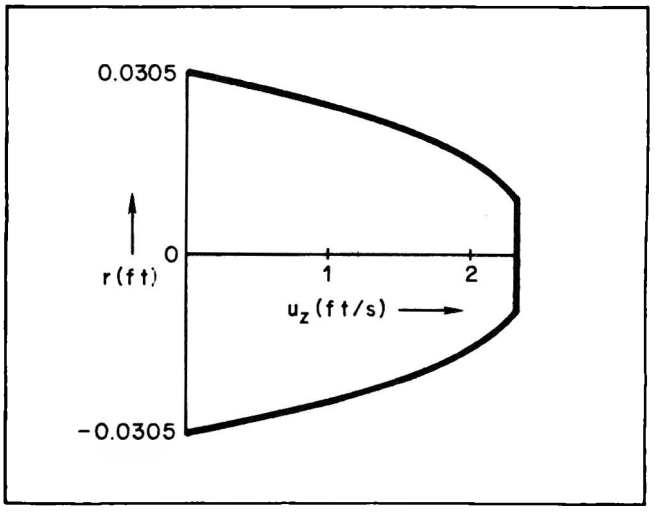

Fig. 2—Isoviscous case velocity distribution for a tube radius of 0.0305 ft.

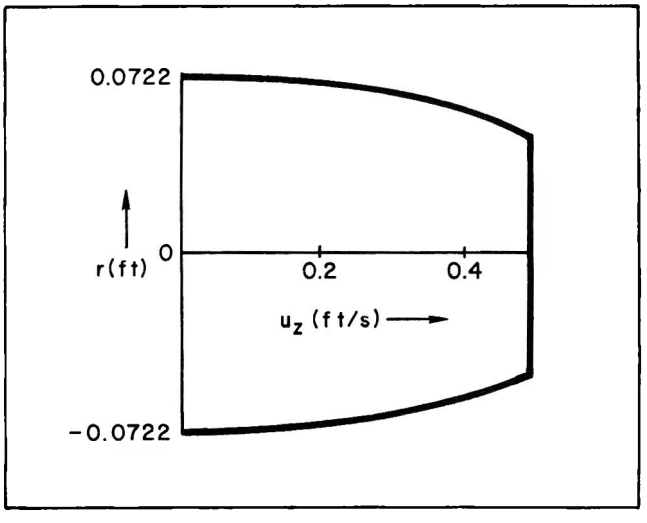

Fig. 3—Isoviscous case velocity distribution for a tube radius of 0.0722 ft.

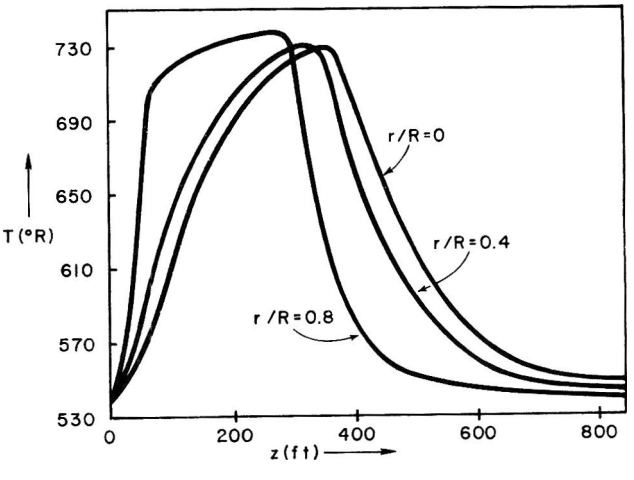

Fig. 4—Isoviscous case temperature distribution for a tube radius of 0.0245 ft, and heating wall temperature of 750° R.

Table 1—Results obtained for isoviscous flow, with heating wall temperature of 730° R

Tube radius (ft)	Heating length (ft)	Cooling length (ft)	Remain ng spore fraction (C_L/C_0)
0.0245	449	163	2.1×10^{-20}
0.0305	608	221	2.2×10^{-21}
0.0513	587	242	1.4×10^{-27}
0.0722	774	> 55	$<4.9 \times 10^{-23}$

Table 2—Results obtained for isoviscous flow, with heating wall temperature of 750° R

Tube radius (ft)	Heating length (ft)	Cooling length (ft)	Remaining spore fraction (C_L/C_0)
0.0245	332	170	3.4×10^{-34}
0.0305	463	249	3.2×10^{-36}
0.0513	470	276	4.6×10^{-47}
0.0722	636	>194	$<6.7 \times 10^{-56}$

Table 3—Results obtained for isoviscous flow, with heating wall temperature of 770° R

Tube radius (ft)	Heating length (ft)	Cooling length (ft)	Remaining spore fraction (C_L/C_0)
0.0245	276	176	1.1×10^{-67}
0.0305	387	257	1.2×10^{-65}
0.0513	400	285	$<1.0 \times 10^{-77}$
0.0722	546	>283	$<1.0 \times 10^{-77}$

Table 4—Results obtained for isoviscous flow, with heating wall temperature of 790° R

Tube radius (ft)	Heating length (ft)	Cooling length (ft)	Remaining spore fraction (C_L/C_0)
0.0245	235	180	$<1.0 \times 10^{-77}$
0.0305	339	264	$<1.0 \times 10^{-77}$
0.0513	352	292	$<1.0 \times 10^{-77}$
0.0722	484	>346	$<1.0 \times 10^{-77}$

Table 5—Results obtained for nonisoviscous flow, with heating wall temperature of 750° R

Tube radius (ft)	Heating length (ft)	Cooling length (ft)	Nutrient retention (%)	Remaining spore fraction (C_L/C_0)
0.0305	380	305	95.6	2.2×10^{-40}

when increasing the tube diameter, mainly because the centerline responds more slowly to heating and cooling effects proceeding from the wall.

The results of Tables 1, 2, 3 and 4 lead to the conclusion that at very high heating wall temperatures (770°R and 790°R), the remaining spore fraction at the centerline reaches values as low as 10^{-77}, which is not desirable, as it is the result of unnecessary overprocessing which affects negatively the nutritive and organoleptic quality of the product. This overprocessing must be even higher at locations closer to the wall; therefore it would be advisable to calculate spore degradation as an average value over the tube cross section.

Optimal nutrient retention, as seen in Figure 1C, occurred for a tube radius of 0.0245 ft, which was the smallest considered, and for a heating wall temperature of 750°R. This agrees qualitatively with the findings of Simpson and Williams (1974), which concluded that product quality requires the smallest tube diameter, the size reduction being limited by the physical tube blockage. Economic factors, such as operating and investment costs, would also have to be considered. In this respect, it should be mentioned that the investment costs are minimized with the most compact design. On the other hand, compactness increases pumping and maintenance costs. The wall temperature selection will also be affected by quality and economic factors. Overprocessing and undesirable reactions should be avoided, as should increased sterilization lengths. Also, for a complete analysis of the sterilization process, more than one important nutrient should be taken into account.

Nonisoviscous case

The nonisoviscous temperature profiles differ from the isoviscous results under the same conditions, as shown in Figures 5 and 9. This difference occurs as a consequence of

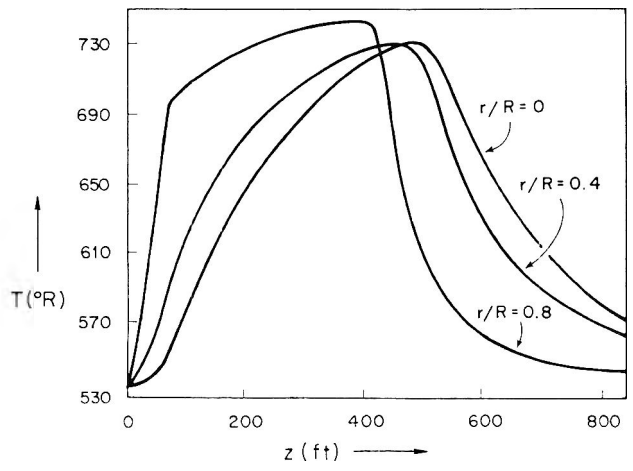

Fig. 5—Isoviscous case temperature distribution for a tube radius of 0.0305 ft, and heating wall temperature of 750° R.

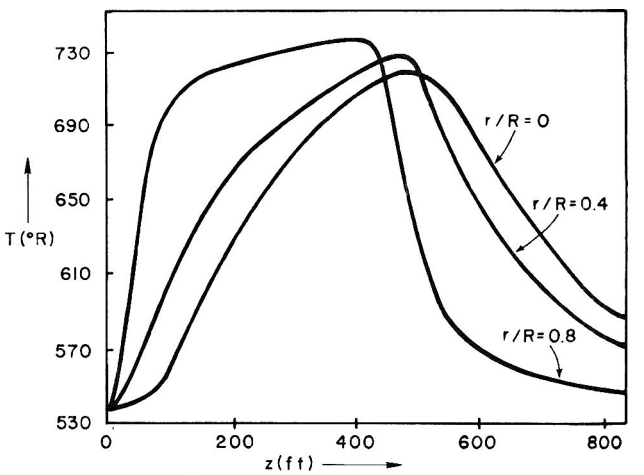

Fig. 6—Isoviscous case temperature distribution for a tube radius of 0.0513 ft, and heating wall temperature of 750° R.

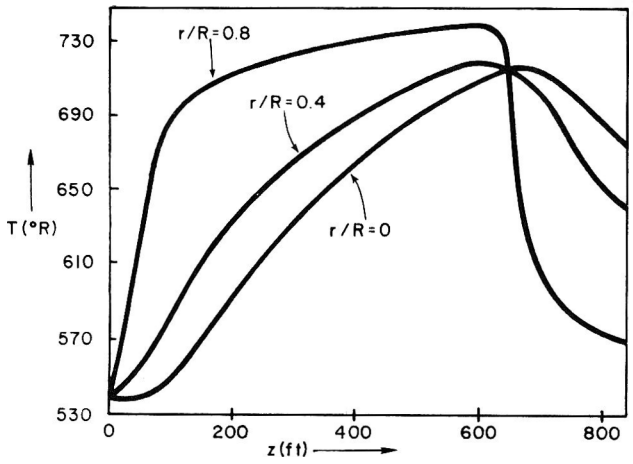

Fig. 7—Isoviscous case temperature distribution for a tube radius of 0.0722 ft, and heating wall temperature of 750° R.

the variation in the velocity distribution due to changes of rheological fluid properties with temperature (Fig. 8). Therefore, in the nonisoviscous case the temperature at the centerline increases more rapidly at the heating section, and for a same axial distance there will be more spore degradation than in the isoviscous case. The contrary happens at the cooling section.

As observed in Table 5, for the nonisoviscous case the calculated heating section length resulted of 380 ft, which is 22% less than the obtained in the isoviscous case (463 ft). Therefore, a substantial error is committed when neglecting rheological properties changes with temperature.

The cooling section length resulted of 305 ft (Table 5), due to the variations of the velocity distribution already mentioned. This value is 18% greater than the one determined for the isoviscous case (249 ft), which means that at the sterilizer outlet, when supposing rheological properties constant with temperature, the real average temperature will be greater than the calculated value, which can result in subsequent nutrient losses and other undesirable changes in the product. The real cooling section length must then be greater than the predicted by the isoviscous case.

The nutrient retention obtained for both isoviscous and nonisoviscous cases under the same conditions resulted by coincidence approximately the same (Fig. 10 and Table 5), but the value calculated for isoviscous flow cannot be reliable, as it depends on the velocity and temperature distributions. This can be stated also for spore degradation.

As a conclusion, it can be established that, in spite of the computational complications inherent to the resolution of the nonisoviscous case for the sterilization design, it is necessary to consider the effect of temperature over rheological fluid properties, in order to guarantee a product heating and cooling in agreement with the established patterns for the thermal processing of foods.

NOTATION

a, b	Constants in Eq (19)
C(r,z) (C′ (r,z))	Concentration of spores (nutrients), at any point in the tube (ft^{-3})
D	Tube diameter (ft)
D_0	Time to destroy 90% of spores or nutrients at a temperature of 250°F (s)
g_c	Conversion factor, 32.174 lb mass ft/lb force s^2
ΔH_1	Constant in Eq (6)
ΔH_2	Constant in Eq (7)

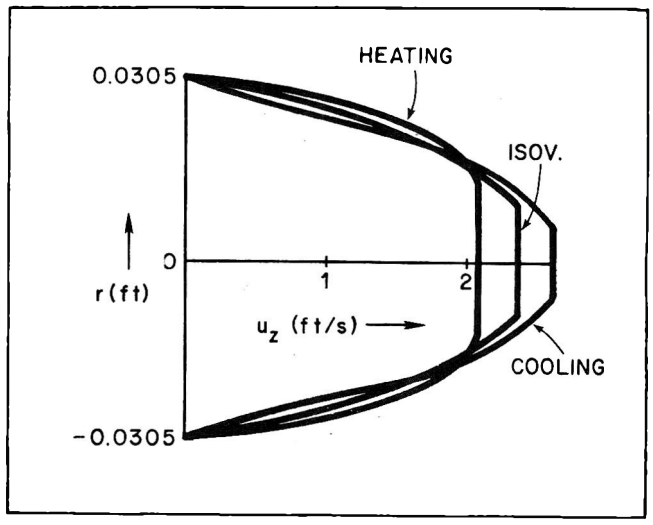

Fig. 8—Nonisoviscous case velocity distribution at the outlet of the heating and cooling sections vs isoviscous case, for a tube radius of 0.0305 ft, and heating wall temperature of 750° R.

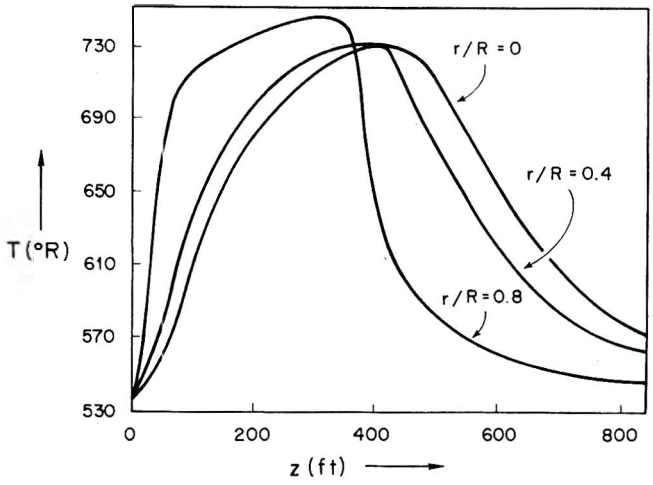

Fig. 9—Nonisoviscous case temperature distribution for a tube radius of 0.0305 ft, and heating wall temperature of 750° R.

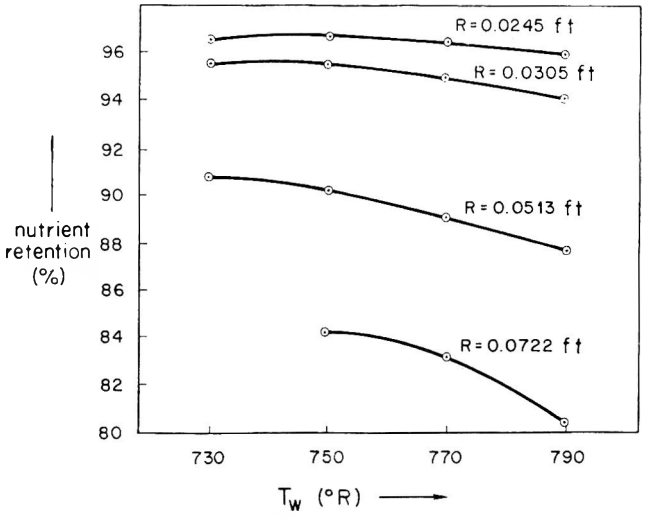

Fig. 10—Isoviscous case nutrient retention as a function of heating wall temperature and tube radius.

I_1	$= \int_r^R r \exp [-\Delta H_1 / R_G T(r,z)]\ dr$
I_2	$= \int_r^R \exp [(\Delta H_2 - \Delta H_1)/R_G T(r,z)]\ dr$
r	Radial distance (ft)
r_0	Radius of unsheared plug (ft)
R	Tube radius (ft)
R_G	Universal gas constant, 10.73 psia $ft^3/°R$ lb mole
T(r,z)	Temperature at any point (°R), (°F)
$u_z(r,z)$	Fluid velocity at any point (ft/s)
V	Average fluid velocity (ft/s)
z	Axial distance (ft)
Z_0	Number of Fahrenheit degrees required for the thermal destruction curve to traverse one log cycle (°F)

Greek symbols

α	Thermal diffusivity (ft^2/s)
η	Coefficient of rigidity or plastic viscosity (lb mass/ft s)
η_0	Constant in Eq (6) (lb mass/ft s)
ρ	Density (lb mass/ft^3)
τ_{rz}	Shear stress at any point (lb force/ft^2)
τ_y	Yield shear stress (lb force/ft^2)
τ_{y0}	Constant in Eq (7) (lb force/ft^2)
τ_w, $\tau(R,z)$	Shear stress at the wall (lb force/ft^2)

Subscripts

o	Inlet values
L	Outlet values
w	Wall values

Brackets

<C>	Average value of C over a flow cross section

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A Research Note

INFLUENCE OF ELECTRICAL STIMULATION ON CERTAIN CHARACTERISTICS OF HEAVY-WEIGHT BEEF CARCASSES

J. W. SAVELL, G. C. SMITH, Z. L. CARPENTER and F. C. PARRISH JR.

ABSTRACT

Eight heavy-weight, grain-fed cattle (475–550 kg steers) were slaughtered and the right sides were electrically stimulated (50 impulses, 440 volts, 5 amps, 60 cycles per sec) within 1 hr postmortem and before chilling. Measurements of pH were taken at 1, 6, 12 and 24 hr. Steaks were removed at 24 hr for palatability, fragmentation and sarcomere length determinations. Significant differences were observed for pH values at 1 hr ($P < 0.01$) and 6 hr ($P < 0.001$) postmortem for electrically stimulated vs control sides while no significant differences ($P > 0.05$) were observed for 12 hr or 24 hr pH values. Quality evaluations revealed that electrical stimulation resulted in improvements in lean color uniformity. Electrically stimulated beef also had less panel-detectable connective tissue amount and higher ratings for tenderness, flavor and overall palatability than unstimulated beef. Neither sarcomere length nor myofibril fragmentation index differed significantly ($P > 0.05$) between treatments. This study suggests that electrical stimulation is as effective in improving quality and palatability of heavy-weight, grain-fed beef as previous research has shown it to be in improving these characteristics in light-weight, short-fed or grass-fed beef.

INTRODUCTION

INDUSTRY RESPONSE to use of electrical stimulation of beef for quality and palatability improvement has been tremendous with commercial application of this method already in progress (Savell, 1978). Benefits of using electrical stimulation include improvements in tenderness (Davey et al., 1976; Grusby et al., 1976; Savell et al., 1977, 1978a, b, c; Smith et al., 1977); enhancement of lean color and lean maturity (Smith et al., 1977; Savell et al., 1978b, c); increased marbling (Savell et al., 1978b, c); prevention of "heat-ring" development (Smith et al., 1977; Savell et al., 1978b, c) and reduction in time necessary for aging (Savell et al., 1978c).

Numerous theories still exist regarding the mechanism by which tenderness is increased by use of electrical stimulation. These theories include prevention of cold shortening (Davey et al., 1976; Chrystall and Hagyard, 1976); increased proteolytic activity by natural enzymes (Dutson et al., 1979; Sorinmade et al., 1978; Parrish, 1977) and physical disruption of muscle fibers (Savell et al., 1978a).

Previous research on electrical stimulation of beef from this laboratory has been conducted with light-weight or grass-fed cattle. Since the quality and palatability characteristics of grain-fed cattle appear to be different than those of short-fed or grass-fed beef (Bowling et al., 1977), it was the purpose of this study to evaluate the influence of electrical stimulation on heavy-weight, grain-fed beef.

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EXPERIMENTAL

Design

Eight cattle (475–550 kg steers) were slaughtered, split longitudinally into sides and the right sides were electrically stimulated within 1 hr postmortem and before chilling. An "Electro-Sting" electrical stunner (Model #160, Type ESS) was used as the source of electrical stimulation. The stunner was modified for use in electrical stimulation by connecting two cables to the end of the lamb stunning attachment to facilitate extension of the stunning probes. A metal pin (approximately 0.6 × 20 cm) was attached to the end of each cable; one pin was placed in the muscles of the round near the achilles tendon and the other pin was inserted in the muscles between the scapula and the thoracic vertebrae. The machine setting was 100 volts, 5 amps and 60 cycles per sec; producing a 440 volt potential difference between electrodes. Sides were stimulated with 50 impulses of 1.0 sec duration; time between impulses was 0.5–1.0 sec.

pH determination

Measurements of pH were taken by inserting a combination electrode into small incisions made in the longissimus muscle of the forequarter region at an angle calculated to minimally damage the muscle fibers. These measurements were taken at 1, 6, 12 and 24 hr postmortem with at least 10 readings recorded at different depths in the muscle in order to obtain a pH measurement for each side at each time period.

Quality measurements

On the first day following slaughter (19–24 hr postmortem) each of the 16 beef sides was ribbed and quality characteristics were evaluated by TAMU personnel. Traits evaluated were skeletal, lean and overall maturity (USDA, 1975), lean firmness (8 = very firm, 1 = very soft), presence of "heat-ring" (Savell et al., 1978b) and lean color uniformity (8 = very uniform lean color, 1 = very uneven lean color).

Fragmentation

At 24 hr postmortem, one steak (2.5 cm in thickness) was removed from the 13th rib section of the longissimus muscle of each side, double-wrapped in polyethylene-coated paper, frozen and stored (−34°C) for 4 months. The steaks were removed from the freezer, boxed in plastic foam containers and shipped to the Iowa State University Meat Laboratory by air freight. Fragmentation of myofibrils from the steaks was conducted as described by Culler et al. (1978).

Palatability tests

One steak (2.5 cm in thickness) was removed from the 11–12th rib section of the longissimus muscle of each side at 24 hr postmortem, double-wrapped in polyethylene-coated paper, frozen and stored (−34°C) for 1 wk. Each steak was removed from the freezer, thawed (2°C) and broiled by use of a Farberware Open-Hearth broiler to an internal temperature of 70°C (monitored by the use of copper-constantan thermocouples and a recording thermometer). Samples (1 × 1 × 2.5 cm) were removed from each steak and evaluated by a 10-member sensory panel for juiciness, tenderness, amount of panel-detectable connective tissue, flavor and overall palatability (panelists were selected for their ability to highly correlate tenderness scores with Warner-Bratzler shear force values and for their repeatability in evaluating comparable samples during the same and different training sessions). Cores (1.27 cm diam) were also removed from each cooked steak for shear force determinations by use of the Warner-Bratzler Shear machine.

Sarcomere length

A 5-g sample of longissimus muscle was obtained at 24 hr postmortem from each side and frozen for 2 days. Each sample was placed in 35 ml of 0.25M sucrose and blended at low speed for 30

sec in a Virtis homogenizer. Sarcomere length was measured on the homogenate by use of a Zeiss WL phase-contrast microscope equipped with a Timbrell-Coulter Shearicon particle counter/size analyzer. The mean sarcomere length was determined by measuring 120 sarcomeres per sample (4 sarcomeres from each of 30 myofibrils).

Statistical analysis

The data were analyzed by use of paired-t distribution analysis (Steel and Torrie, 1960) to determine the significance of differences between control and electrically stimulated samples.

RESULTS & DISCUSSION

The beef carcasses used in this study had the following average characteristics: 529.6 kg liveweight; 334.0 kg carcass weight; 8.1 mm adjusted fat thickness, 12th rib measurement; 83.9 cm^2 longissimus muscle area; 2.0% kidney, pelvic and heart fat; and 2.3 yield grade. These carcass characteristics are more typical of the kind of beef sold by large chain supermarkets and hotels and restaurants than is the beef studied in research by Savell et al. (1977; 1978b, c); therefore, the present study is important from the standpoint of determining efficacy prior to industry-wide application of electrical stimulation to grain-fed, heavy-weight beef.

Postmortem pH decline in electrically stimulated and unstimulated sides of beef are reported in Table 1. Significant differences in pH between treated and control sides were found at 1 hr ($P < 0.01$) and 6 hr ($P < 0.001$) but pH did not differ at 12 and 24 hr postmortem. These results support the general observation that electrical stimulation accelerates postmortem glycolysis and thereby lowers pH initially but that it does not change the ultimate pH of the muscle.

Presented in Table 1 are mean values for certain quality-indicating characteristics of beef from electrically stimulated and control sides. As has been previously reported in other studies by Savell et al. (1978b, c), electrical stimulation improved the USDA lean and overall maturity scores of these sides when compared to the unstimulated control sides. Marbling scores increased as a result of electrical stimulation but final grade did not; differing widths of U.S. Choice (3 marbling scores) and U.S. Good (1 marbling score) grades cause average USDA quality grade to not necessarily correspond to average marbling scores. "Heat-ring" was completely alleviated by the use of electrical stimulation. The degree of "heat-ring" has been lessened or alleviated in previous research reported by Savell et al. (1978b, c). Although the firmness of the lean was not significantly affected by electrical stimulation, lean color uniformity was; the longissimus muscle was more uniform in color in electrically stimulated than in unstimulated (control) sides. The latter difference may have been due to the pH-temperature decline pattern from the time of stimulation to the time of muscle exposure by ribbing.

Mean values for palatability characteristics, sarcomere length and myofibril fragmentation index of beef from electrically stimulated and control sides are found in Table 2. Increases in sensory panel ratings for tenderness, connective tissue amount (indicative of less panel-detectable connective tissue), flavor and overall palatability as well as lowered shear force values occurred due to electrical stimulation. These differences in tenderness and panel-detectable connective tissue are well documented by Smith et al. (1977); Savell et al. (1977, 1978a, b, c); however, increases in flavor and overall palatability have been less consistently reported by researchers who have studied electrical stimulation. Flavor increases for electrically stimulated beef have been reported by Davey et al. (1976) and Savell et al. (1977, 1978a). Although the mechanisms by which flavor is increased by electrical stimulation have not been studied, merchandizable benefits which accrue from increased flavor may be as great as those realized from increased tenderness. Neither sarcomere length nor myofibril fragmentation index differed significantly between samples from electrically

Table 1—Mean values for postmortem pH and certain quality-indicating characteristics of beef from electrically stimulated and control sides

Trait	Treatment of paired sides: Electrically stimulated Mean	S.D.	Unstimulated (control) Mean	S.D.	Level of probability[a]
Postmortem pH					
1 hr	6.7	0.2	7.0	0.1	$P < 0.01$
6 hr	5.8	0.2	6.1	0.2	$P < 0.001$
12 hr	5.8	0.1	5.9	0.2	N.S.
24 hr	5.7	0.1	5.7	0.1	N.S.
Skeletal maturity[b]	A^{53}	10.3	A^{53}	10.3	N.S.
Lean maturity[b]	A^{47}	15.3	A^{76}	27.7	$P < 0.01$
Overall maturity[b]	A^{50}	11.5	A^{63}	13.8	$P < 0.01$
Marbling[b]	$Small^{17}$	76.2	$Small^{01}$	75.1	$P < 0.05$
USDA quality grade[b]	$Good^{88}$	46.9	$Good^{78}$	49.8	N.S.
"Heat-ring"[c]	5.0	0.0	3.8	0.9	$P < 0.01$
Lean firmness[d]	6.8	0.9	6.6	0.9	N.S.
Lean color uniformity[e]	7.4	0.7	5.1	1.6	$P < 0.01$

[a] The probability that the difference between treatments is statistically significant based on paired-t analysis (Steel and Torrie, 1960). $P > 0.05$ was reported as nonsignificant (N.S.).
[b] USDA (1975). For maturities, A^{00} = approximately 9 months of age; A^{100} = approximately 30 months of age.
[c] "Heat-ring" was evaluated using a 5-point scale (5 = none, 1 = extremely severe) as described by Savell et al. (1978b).
[d] 8 = extremely firm; 1 = extremely soft.
[e] 8 = very uniform lean color; 1 = very uneven lean color.

Table 2—Mean values for palatability characteristics and laboratory determinations of beef from electrically stimulated and control sides

Trait	Treatment of paired sides: Electrically stimulated Mean	S.D.	Unstimulated (control) Mean	S.D.	Level of probability[a]
Juiciness[b]	5.2	0.4	5.5	0.4	N.S.
Tenderness[c]	5.6	0.8	4.3	1.0	$P < 0.01$
Connective tissue amount[d]	6.0	0.5	5.4	0.7	$P < 0.05$
Flavor[e]	5.8	0.3	5.1	0.6	$P < 0.02$
Overall palatability[e]	5.4	0.6	4.4	0.8	$P < 0.02$
Warner-Bratzler shear force (kg)	5.5	1.7	7.4	2.1	$P < 0.02$
Sarcomere length (μm)	1.86	0.09	1.86	0.07	N.S.
Myofibril fragmentation index[f]	55.6	8.2	56.1	13.9	N.S.

[a] The probability that the difference between treatments is statistically significant based on paired-t analysis (Steel and Torrie, 1960). $P > 0.05$ was reported as nonsignificant (N.S.).
[b] 8 = extremely juicy, 1 = extremely dry
[c] 8 = extremely tender, 1 = extremely tough
[d] 8 = none, 1 = abundant; evaluated by sensory panel
[e] 8 = extremely desirable, 1 = extremely undesirable
[f] Determined by the procedures of Culler et al. (1978).

stimulated and control sides. Such results suggest that the mechanism by which tenderization is achieved via electrical stimulation cannot be elucidated by either of these measurements (myofibril fragmentation index, sarcomere length). Failure to associate sarcomere length to the enhanced tenderness attributed to electrical stimulation has now been reported by Savell et al. (1977, 1978a), Smith et al. (1977) and Bouton et al. (1978) which lend further credence to the natural enzyme activity and structural damage theories of the mechanisms associated with increased tenderness of electrically stimulated carcasses.

The possibility exists that concern may be registered by the USDA or others regarding improvements in quality-indicating factors in electrically stimulated beef. It should be noted that the magnitude of the improvement in palatability is much greater than is the improvement in the factors used in grading for predicting palatability. It is our contention that marbling is not increased by use of electrical stimulation but rather that it "sets up" more rapidly in such beef. It is widely believed in industry that marbling score increases as chilling time increases. The increase in marbling in this study and in other research (Savell et al., 1978b, c) associated with use of electrical stimulation is usually observed if carcasses are ribbed 19–24 hr postmortem but no research has been conducted in order to determine if carcasses that are ribbed 48–72 hr postmortem still exhibit these differences. It is our theory that if both sides of a carcass in which only one side has been electrically stimulated are held for 48 hr or more prior to ribbing, these differences between treatments may not exist. Increased palatability (especially tenderness) achieved by use of electrical stimulation should be the major impetus for industry utilization of this innovative processing technique.

The results of the present study indicate that grain-fed, heavy-weight beef carcasses that have been electrically stimulated will have higher USDA quality grade characteristics, will yield longissimus muscle steaks that are more tender, more flavorful and have greater overall palatability than steaks from unstimulated (control) sides. It appears that the improvements in quality and palatability characteristics that have been associated with use of electrical stimulation of light-weight and/or grass-fed beef, are also observed in heavy-weight, grain-fed beef thus assuring that electrical stimulation can effectively increase palatability of most of the beef presently sold in the United States.

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A Research Note

QUALITY OF BONELESS DRY-CURED HAM PRODUCED WITH OR WITHOUT NITRATE, NETTING OR POTASSIUM SORBATE

JAMES D. KEMP, B. E. LANGLOIS, M. B. SOLOMON and J. D. FOX

ABSTRACT

Two trials were conducted. In trial I, boneless hams were cured with salt, sugar, potassium nitrate and sodium nitrite or salt, sugar and nitrite only. Half of the hams in each group was tied with heavy twine and half was enclosed in elastic netting. There were no differences in any variable due to the presence or absence of nitrate. Hams that were netted had higher yields after curing and aging than tied hams. They also had a more desirable general appearance. There were no differences in composition, palatability scores or tenderness. In trial II one group of boneless hams was dipped in 2.5% potassium sorbate solution, placed in elastic netting, cured and aged while a similar group was not dipped. Treated hams were slightly more desirable in general appearance, slightly higher in moisture and lower in nitrite. Flavor ($P < 0.05$) and overall satisfaction scores ($P < 0.01$) favored the treated group. Saltiness scores also were lower ($P < 0.05$) in the treated group. Yeast and mold counts were lower ($P < 0.05$) for the treated groups after curing, after salt equalization and after aging 1 month, indicating that potassium sorbate could be used successfully as a yeast-and-mold-retardant in cured hams.

INTRODUCTION

DRY-CURED HAMS normally are cured without boning. However, some are being skinned (Montgomery et al., 1976), and others are being defatted and deboned (Kemp and Fox, 1977). Some advantages that have been noted for the boneless hams are ease of slicing and elimination of shank and butt slices. Disadvantages include lower yields owing to additional dehydration and development of mold in the seams where the bones have been removed (Kemp and Fox, 1977).

Nitrate has been used traditionally in dry curing. However, recent work (Kemp et al., 1974, 1975) has shown that intact hams cured either with nitrate, nitrite or a combination were similar in quality.

Retail markets generally use elastic netting rather than twine to hold boneless roasts in shape for display and sale. Netting saves time and often produces a neater product.

Potassium sorbate is approved as a mold retardant in some forms of dry sausage (USDA, 1973). Baldock and Frank (1978) showed that it was effective as a mold retardant also on cured unboned hams.

This study was designed to study the quality of boneless hams cured with or without nitrate, netting, or potassium sorbate.

EXPERIMENTAL

Experiment I

Twenty-eight hams of similar weight and quality were deboned and partially defatted. Fourteen hams were cured using a mixture composed of 96 parts salt, 16 parts white sugar, 3 parts potassium nitrate and 1 part sodium nitrite. The other 14 hams were cured with a similar mixture except that the nitrate was omitted. Fifty grams of the mixture were placed inside each ham. Half of each cure group was then tied with heavy meat twine at about 2.5 cm intervals and half was enclosed in a closely fitting elastic netting. Additional cure was applied on the outside at day 0 and day 5 so that ingredients equal to 5% of in-cure weights were applied.

After curing 21 days at 3°C, hams were removed from cure and hung for salt equalization at 13°C for 14 days. They were then smoked approximately 24 hr at 38°C and placed in an aging room at 24°C and aged 4 wk. Hams were weighed at each processing step. The hams were held at 24°C and were sliced within a week of weighing and evaluated for color, odor and general appearance. Three 1.27-cm slices were obtained. Two were used for palatability testing and one was trimmed of visible fat and subsequently analyzed for salt, moisture and nitrite (AOAC, 1975). One 2.54-cm slice was obtained for shear testing. The 1.27 cm slices were broiled in an electric oven. The slices were placed approximately 11 cm from the heat, cooked 5 min, turned and cooked 4 min. The cooked slices were divided into pieces approximately 2 cm^2, given code numbers and served in duplicate to a 7-member experienced palatability panel who rated them on a 9-point scale (Peryam and Giradot, 1952) for tenderness, flavor, saltiness and over-all satisfaction. Each 2.54 cm slice was fitted with a probe type steel thermometer, broiled to 54°C, turned, and removed at 77°C. The slices were cooled to room temperature. Cores of 2.54-cm diameter were removed from the semimembranosus and biceps femoris muscles and sheared on an Instron Universal testing device fitted with a Warner-Bratzler type shear.

Experiment II

Twenty hams were deboned and defatted. Ten were dipped in a 2.5% potassium sorbate solution, while the other 10 were not dipped. The same curing ingredients as used in Experiment I (minus nitrate) were used, and all hams were placed in elastic netting. The same curing, aging and evaluation procedures as in Experiment I were followed except 100g of cure was placed inside each ham and total cure equal to approximately 6% of weight was used. A standard Warner-Bratzler Shear was used, an additional muscle, the semitendinosus, was sheared and a 9-member palatability panel was used.

Five hams from each group were sampled for yeasts and molds after curing, after salt equalization and after aging one month. Sampling was done by swabbing six 12 cm^2 areas. Yeast and mold counts were made using Potato Dextrose Agar (Difco) acidified to pH 3.5, and incubating the plates at 26°C for 5 days. Counts are expressed as mean log counts per 12 cm^2.

Data were analyzed by Analysis of Variance using the Statistical Analysis System of Barr et al. (1976).

RESULTS & DISCUSSION

NO SIGNIFICANT DIFFERENCES were noted in any variable owing to the presence or absence of nitrate, showing that nitrite alone is capable of producing the desired effects in boneless dry-cured hams.

Table 1 shows the effect of netting on ham yields. At each weigh period the netted hams had higher yields ($P < 0.01$). Apparently the netting closed the seams more tightly, decreased the amount of exposed lean tissue and, thus, reduced evaporative and drip loss. Netted hams also were more desirable ($P < 0.05$) in general appearance. The outside was smoother and the inside color and overall appearance were more desirable as a few of the tied hams exhibited mold in the seams, while the netted hams were mostly free of mold. There were no significant differences in moisture, salt and nitrite levels. Average salt content by groups ranged from 3.1–3.6% which is below that specified for country ham by the USDA (1977). Nitrite levels were

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Table 1—Ham traits with significant differences – Experiment I

Group	Treatment	Yield by periods, %								General Appearance[c]	
		Cured	S.D.	Salt equal.	S.D.	Smoked	S.D.	Aged 1 Mo	S.D.	Mean	S.D.
1	Netted, NO_3 + NO_2	93.8[a]	0.8	85.0[a]	1.4	83.1[a]	1.3	73.7[a]	2.7	3.9[x]	.4
2	Netted, NO_2	93.7[a]	1.3	84.0[a]	1.4	82.3[a]	1.2	73.1[a]	1.6	4.0[x]	0
3	Tied, NO_3 + NO_2	91.0[b]	2.4	80.5[b]	2.0	78.3[b]	2.1	67.8[b]	2.3	3.3[y]	.5
4	Tied, NO_2	91.4[b]	1.0	80.2[b]	1.0	78.3[b]	0.9	67.5[b]	1.6	3.4[y]	.5

[ab,xy] Means in columns with different superscripts are different: [ab]$P < 0.01$; [xy]$P < 0.05$.
[c] 4 = excellent, 3 = good, 2 = fair.

Table 2—Ham traits with significant differences – Experiment II

Treatment	Palatability scores						Yeast and mold counts (Log_{10}/12 cm^2)					
	Flavor[c]		Saltiness[d]		Overall[c] satis.		After cure		After salt equalization		After aging 1 mo	
	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
Sorbate	6.8[x]	.2	5.6[a]	.2	6.6[a]	.3	3.45[x]	3.30	2.02[x]	2.10	4.10[x]	4.41
No sorbate	6.4[y]	.2	5.9[b]	.2	6.2[b]	.4	5.07[y]	5.11	4.26[y]	4.52	4.66[y]	4.84

[ab,xy] Means in columns with different superscripts are different: [ab]$P < 0.01$, [xy]$P < 0.05$.
[c] 9-point hedonic scale, 1 = dislike extremely, 9 = like extremely.
[d] 9-point scale, 1 = extremely devoid, 9 = extremely salty.

low with no differences due to treatment. Color in all hams was red so enough nitrite was present for proper color formation. Palatability and tenderness of the hams were acceptable with no differences due to treatment.

Ham yields by periods for Experiment II were similar to those in Experiment I (Table 1), with no differences attributed to sorbate. Color and general appearance scores were slightly more desirable for the sorbate hams even though, due to netting, very little internal mold was visible. Moisture, salt and nitrite values were similar for both groups. pH values were not affected even though sorbate was low, and the time between dipping and sampling was long the pH values were not affected.

Sorbate-treated hams were more desirable in flavor ($P < 0.05$) and overall satisfaction ($P < 0.01$) and were less salty ($P < 0.01$) (Table 2). Tenderness scores and shear values were similar for both groups. Therefore, it appears that potassium sorbate when used as a 2.5% dip is beneficial for improving acceptability of cured ham.

Table 2 also shows that potassium sorbate inhibited the growth of yeasts and molds. The untreated hams had higher counts at each testing period with the biggest difference occurring after salt equalization. The low counts after salt equalization apparently were due to the low relative humidity (about 58%) in the salt equalization room. Numbers increased again during aging probably because of a higher RH (about 75%). The percentage increase in numbers was greater for the treated than for the untreated group probably because the sorbate effect diminished during aging.

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A Research Note

ESTIMATION OF FRESH MUTTON COLOR BY REFLECTANCE SPECTROPHOTOMETRY

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ABSTRACT

Accuracy and suitability of various wavelengths employing reflectance spectrophotometry for measuring K/S ratios or RA values, was critically analysed for the estimation of fresh mutton color. The K/S ratio method using wavelengths 572 and 525 nm gave more consistent results than other wavelengths. The lower myoglobin content of mutton as compared to that of beef was, however, responsible for narrower ranges obtained for differentiation of fresh and oxidized mutton color. The commercial acceptability limit for fresh mutton color was 0.09 units less than the initial value of K/S 572/525 ratio.

INTRODUCTION

REFLECTANCE SPECTROPHOTOMETRY has been widely used (Naughton et al., 1958; Stewart et al., 1965; Snyder, 1965; Snyder and Armstrong, 1967; Eagerman et al., 1977; Eagerman et al., 1978) to estimate the color of fresh beef. Clydesdale and Francis (1971) and Govindarajan (1973) who reviewed the methods of estimation of meat color have pointed out the drawbacks of these methods. Since the use of reflectance spectrophotometry has not been reported for fresh mutton color, an attempt has been made to use this technique to estimate fresh mutton color and assess the applicability of various methods and their suitability for this purpose.

MATERIALS & METHODS

LONGISSIMUS MUSCLE of healthy male Bannur sheep (a local Indian breed) of about 1½ years of age was removed from the carcass immediately after slaughter, trimmed free of all visible fat and connective tissue and ground mechanically. The samples were analysed spectrophotometrically on a Bausch and Lomb spectrophotometer (Spectronie 20 colorimeter with reflectance attachment (Spec 20), Bausch and Lomb Optical Co., Rochester, NY).

Absorbance readings were obtained at various wavelengths using magnesium carbonate as reference standard. Limiting values for ratios were determined for mutton containing 0% methmyoglobin (MMb), after it was initially allowed to bloom fully in the refrigerator for 2 hr in a cellophane pouch (Snyder, 1965) and 100% MMb after it was mixed with potassium ferricyanide solution/the entire-chunk painted thrice with this solution at hourly intervals inside the refrigerator. Ten replicates of sample were taken for the analysis. K/S values were read from standard tables (Judd and Wyszecki 1963; Hood, 1973).

Visual color estimation on a 9-point hedonic scale was done on five replicates ot mutton samples kept at 0°C in a cellophane pouch for blooming for 2 hr and also after storing the same samples in the refrigerator for 10 days at 5°C. The stored samples after visual and spectrophotometric evaluations of color were oxidized with potassium ferricyanide and again evaluated for color as per Stewart et al. (1965).

RESULTS & DISCUSSION

VALUES obtained for K/S ratios at 572/525, 474/525, 507/573 and 473/597 nm and also the RA values at 632 nm and 'q' values i.e. A 580–630 nm have been presented in Table 1.

Ratio values of K/S 572/525 as shown in Table 1 were higher for MMb and lower for Mb/MbO_2, when compared to those reported for beef by Stewart et al. (1965). This can be attributed to lower myoglobin concentration (37%) in mutton than beef (62%) (Broumand et al., 1958). Absorbance values also did not exceed unity due to this lower myoglobin content in mutton and the use of bleached meat/grey card board was not necessary as in the case of beef (Stewart et al., 1965). Absorbance values were, therefore, directly obtained against white standard, the $MgCO_3$ block. Visually the beef color on oxidation with the same quantity of $K_3Fe(CN)_6$ looked duller and less acceptable than the corresponding oxidized mutton.

The K/S 474/525 nm values (Table 1) for beef were also higher than those obtained in the present investigation for fresh mutton again probably due to lower myoglobin content in mutton. The K/S 507/573 nm values were, however, comparatively of a much narrower range than those reported for beef, while K/S 473/597 nm values were quite low in comparison to the same values reported by Van den Oord and Wesdorp (1971) for beef (Table 1), possibly due to the differences in tissue fat, moisture content and other surface characteristics of mutton and beef apart from the difference in the myoglobin content.

The reflectivity value (q = (A 580–A 630) × 100) as described, by Van den Oord and Wesdorp (1971) decreased on successive oxidation of Mb/MbO_2 from 16.35 ± 2.55 and reached the value 6.27 ± 1.62 for 100% oxidised mutton samples. Limiting values of RA 632 according to the method of Franke and Solberg (1971) for fresh and oxidized mutton samples were 0.651 ± 0.056 and 0.450 ± 0.070 respectively. The difference in absorbance values of fresh and oxidized mutton samples at RA 632 was 0.175 ± 0.051. RA 632 values have not been reported so far for beef or mutton.

From the above, it is seen that the range of differentiation between Mb/MbO_2 and MMb in mutton is very small by all wavelength combinations described. All the wavelength combinations seem to be fairly good, yet no method is completely fool proof and reliable for ascertaining minor changes in fresh mutton color. Though K/S 473/597 ratio seems to give maximum range of differentiation as also 'q' value it may be mentioned here that the selection of wavelengths is the most important criterion for achieving the best results. It is observed that the wavelengths 525 and 572 are most sensitive and give most consistent results. Accordingly these wavelengths may be preferred for color estimation of fresh mutton by reflectance spectrophotometry measuring K/S ratios, according to Stewart et al. (1965).

Commercial acceptability limit

When the color of fresh mutton samples fell below the acceptability level on visual estimation, the difference was 0.089 ± 0.018 as shown in Table 2. Samples having visual color score of 6 or above were acceptable and corresponded to approximately 75 ± 2.8% Mb/MbO_2 or approximately 25% conversion of Mb/MbO_2 to MMb. This can be regarded as the commercial acceptability limit. This indicates that when the K/S 572/525 nm ratio changes from the original

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Table 1—Comparison of ratios of measured K/S values with those reported in literature and the results of values other than K/S ratios for mutton

Meat Pigment	Wave length d_1 nm / d_2 nm	Ratio of K/S values at d_1 and d_2 wavelengths: Stewart et al. (1965) (for beef) No. of samples	Avg K1/S1 / K2/S2	SD + −	Snyder and Armstrong (1967) (for beef) No. of samples	Avg K1/S1 / K2/S2	SD + −	Van den Oord and Wesdorp (1971) (for beef) No. of samples	Avg K1/S1 / K2/S2	SD + −	Present study (1978) for mutton No. of samples	Avg K1/S1 / K2/S2	SD + −	Range
Mb/	572/525 nm	20	1.40	0.16	35	1.327	0.071	12	1.49	0.04	10	1.186	0.091	1.06–1.35
MbO_2	474/525 nm				35	0.957	0.038	12	0.91	0.01	10	0.745	0.138	0.56–0.96
	507/573 nm							12	0.40	0.02	10	0.778	0,10	0.64–0.89
	473/597 nm							12	3.51	0.23	10	0.844	0.063	0.76–0.94
MMb	572/525 nm	20	0.56	0.04	35	0.608	0.038	4	0.55	0.03	10	0.805	0.093	0.62–0.92
	474/525 nm				35	0.962	0.02	4	1.06	0.02	10	0.853	0.112	0.66–0.98
	507/573 nm							4	2.13	0.13	10	1.177	0.123	0.98–1.46
	473/597 nm							4	2.26	0.13	10	1.331	0.113	1.18–1.46

Table 2—Commercial acceptability experiment—results[a]

Carcass no.	Ratio K/S $\frac{572}{525}^{nm}$ Value for fresh mutton samples	Visual color score for fresh mutton sample	K/S Ratio 572/525 nm value of stored mutton samples (after 10 days at 5°C)	Visual color score of stored mutton samples (after 10 days at 5°C)	Difference in ratio K/S $\frac{572}{525}^{nm}$ value	Average difference	Ratio of K/S $\frac{572}{525}^{nm}$ value of oxidised sample	Corresponding % Mb/MbO_2 present from values of column 4	Average % Mb/MbO_2 corresponding to column 7
1.	1.2108	9	1.1112	6	0.0996		0.8061	76	
2.	1.3001	9	1.1918	6	0.1083	0.0891*	0.8923	72	75*
3.	1.1251	8	1.0306	6	0.0945	±	0.7210	77	±
4.	1.1819	9	1.1208	6	0.0611	0.0182**	0.7923	72	2.83%**
5.	1.1186	8	1.0362	6	0.0024		0.7654	78	

[a] Difference in K/S 572/525 nm ratio values on spectrophotometric evaluation and visual color estimation of fresh and stored mutton samples

* Mean difference

** Standard deviation

value by about 0.09 units, the mutton is most likely to be found unacceptable by the consumers. It is, however, important that the initial K/S value should be known so that the difference is measured from the initial K/S 572/525 nm value. Hood (1974) found that a change in K/S 572/525 nm ratio of 0.15–0.17 is equivalent to 20% MbO_2 conversion to MMb, which was close to the limit of commercial acceptability for beef. The lower commercial acceptability limit obtained in the present investigation is again possibly due to a lower myoglobin content of mutton than of beef.

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A Research Note

CONNECTIVE TISSUE PROFILES OF VARIOUS RAW SAUSAGE MATERIALS

E. L. WILEY, J. O. REAGAN, J. A. CARPENTER and D. R. CAMPION

ABSTRACT

21 different sausage meats were chemically analyzed to determine the amounts of total collagen, insoluble collagen, and percentage soluble collagen present in each meat. A simple cookout test was also conducted to determine its usefulness in evaluating the bind characteristics of raw sausage materials. Values for total collagen, insoluble collagen, and percentage soluble collagen were quite variable among the meats sampled. Of the three collagen profile components studied, only percentage soluble collagen was observed to be highly related to the binding characteristics of meat. Values for milliliters of fat and water lost during cooking were not significantly related to bind values. This study indicates a need for additional research to investigate the role of collagen profile components in the formation of sausage batters.

INTRODUCTION

MEATS containing large quantities of collagen are frequently referred to in the sausage industry as high collagen meats. These meats have long been used by sausage processors as a means of reducing the manufacturing costs of processed meats. The accepted "rule of thumb" for using high collagen meats has been to limit their use to 15% of the total meat block. Most processors believe that if the 15% level is exceeded the probability of major defects occurring in the final product is greatly enhanced. Defects such as poor peelability, unstable batters, gel-pocket formation, and wrinkling of the outer skin have been associated with sausage products containing large quantities of high collagen meats (Saffle et al., 1964). Manufactured products containing large quantities of high collagen meats have been found to exhibit these undesirable characteristics when heated to smokehouse temperatures greater than 65°C (Kramlich, 1972; Saffle et al., 1964). However, Hamm (1972) reported that connective tissue proteins (collagen) utilize water-binding mechanisms similar to those used by myofibrillar proteins. Hamm (1972) further reported that gel-pocket formation in frankfurter-type sausages was minimal in the temperature range of 65–80°C. Although numerous researchers have studied the use of collagen meats in processed products, the exact role of collagen in sausage batters has not been determined. Prior to gaining a better understanding of the role of high collagen meats in sausage formulations, it is necessary to further characterize the raw materials used in sausage production. The primary objective of this study was to determine both the quantity and chemical state of collagen in 21 different types of meats which are commonly used in sausage production. In addition, a simple cookout test was evaluated to determine its usefulness in predicting the binding and waterholding properties of different types of meat.

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EXPERIMENTAL

TWENTY-ONE DIFFERENT meat samples were obtained from bovine and porcine animals slaughtered at the Meats Laboratory of the Food Science Department (University of Georgia) and at a nearby slaughter plant. Three samples of each type of meat were obtained in order to determine the amount of variation among carcasses. All samples were ground through a 4 mm plate, then powdered by freezing in liquid nitrogen and blending in a Waring Blendor. The powdered samples were placed in "Whirl pak" bags and stored at −20°C until chemically analyzed. Values for protein, fat, and moisture were determined according to AOAC procedures (1970). Hydroxyproline determinations were made by heating 4g of the powdered meat tissue in one-fourth strength Ringer's solution for 70 min in a 77°C water bath according to the procedures of Hill (1966). Values for hydroxyproline were determined for the soluble and insoluble fractions employing the procedures outlined by Woessner (1961). Values for total collagen (mg/g), soluble collagen, and insoluble collagen were obtained by multiplying the hydroxyproline content of the supernatant plus residue, supernatant alone, and residual alone fractions, respectively, by 7.25 (Goll et al., 1964).

Cookout determinations were used to estimate the binding characteristics of each type of meat. The cookout procedure involved placing 50g of the ground meat tissue in a Virtis jar containing 40 ml of distilled water. The meat tissue and water were then blended in a Virtis homogenizer at high speed for 30 sec. The meat slurry adhering to the sides of the jar and the blades of the homogenizer were scraped clean with a rubber policeman, then blended for an additional 60 sec. The sides of the jar and the blades were again scraped prior to covering the mouth of the jar with aluminum foil. The jars containing the meat slurry were then cooked for 1 hr in a 70°C hot water bath, after which the jars were inverted over a graduated cylinder in order to collect the fat and moisture fractions that were lost during the cooking process. The graduated cylinders were allowed to stand at room temperature for 1 hr to facilitate the separation of the fat and water layers. The amount of fat and water in the graduated cylinders were reported as milliliters of fat or water lost during cooking. Weights of the cooked meat tissue remaining in the jars after removing the fat and water mixture were also obtained. Bind values for sausage raw materials were determined by the method of Carpenter and Saffle (1964) and color values were determined by the method of Saffle (1973). Simple correlation coefficients (Snedecor and Cochran, 1967) were used to determine the relationship between bind values and collagen profile components.

RESULTS & DISCUSSION

MEAN VALUES for various chemical determinations and cookout losses for 21 different types of sausage materials are presented in Tables 1 and 2. All meats are listed by species in a descending order of their ability to bind fat (bind values) according to bind values which have been previously determined at this laboratory. Color values (Georgia) are also shown for each type of meat. Chemically determined values for total collagen, insoluble collagen, and percentage soluble collagen were quite variable among the 21 different types of meat evaluated. Total collagen (mg/g) ranged from 7.6 (skinned pork jowls) to 34.1 (pork snouts), insoluble collagen (mg/g) ranged from 4.0 (beef tongue) to 24.4 (pork snouts) while percentage soluble collagen ranged from 6.2 (cow meat) to 47.8 (pork blade meat) among the 21 different types of meat. No set trends were observed with respect to the relative amounts of total collagen, insoluble collagen, or soluble collagen associated with meats which are considered to be good, fair, or poor fat binders. For example, meats with bind values greater than 18 (good

Table 1–Mean values for protein, fat and moisture and values for bind and color for 21 different sausage raw materials

Meat	Protein (%)	$\overline{sx}$	Fat (%)	$\overline{sx}$	Moisture (%)	$\overline{sx}$	Bind (Ga)	Color (Ga)
Beef shank	22.7*	0.48	7.1	1.49	69.3	1.50	28.0	46.5
Cow meat	19.5	0.72	15.1	0.88	70.6	0.99	24.5	38.9
Beef trim 75/85	20.6	0.06	20.0	0.62	62.2	4.50	21.7	33.6
Beef neckbone trim	21.7	1.03	29.1	0.71	63.4	1.28	18.8	19.3
Beef plates	16.2	2.39	45.0	1.40	55.7	5.68	16.3	25.2
Beef cheek meat	23.5	1.56	14.2	0.44	67.9	2.03	13.9	47.9
Beef diaphragm	19.9	2.74	14.1	1.53	62.9	1.98	11.7	32.3
Beef tongue	16.4	0.26	24.2	1.52	64.6	2.77	8.3	35.9
Beef head meat	21.4	2.13	13.5	2.58	67.3	1.90	7.8	26.4
Beef heart	17.3	0.79	9.6	1.62	68.3	2.06	6.1	40.0
MDB chicken	12.8	1.41	15.9	1.09	65.3	2.16	16.3	19.9
Pork blade meat	21.4	0.58	9.2	1.50	72.6	1.61	23.7	20.0
Pork neckbone trim	19.6	2.06	19.7	5.94	56.2	2.60	21.0	30.0
Pork tongue trim	18.2	1.37	10.8	4.09	68.9	5.32	19.3	16.6
Pork diaphragm	22.1	2.63	17.7	3.74	61.3	2.38	15.9	18.1
Pork trim 50/50	11.1	1.17	25.5	6.05	41.9	3.15	11.7	9.0
Pork cheek trim	19.7	0.94	14.1	1.51	66.9	1.18	8.6	21.1
Pork head meat	20.0	0.82	25.0	3.90	62.6	3.24	7.5	15.6
Pork heart	18.7	0.75	6.9	0.66	73.6	2.89	6.1	32.3
Pork jowl, skinned	6.1	0.75	71.9	7.36	24.6	9.10	4.5	1.8
Pork snouts	21.0	2.58	58.6	1.40	31.9	1.07	2.5	0.5

* (n = 3)

binders) exhibited total collagen contents ranging from 8.2–24.3 mg/g.

Simple correlation coefficients (r) between bind values and the various collagen profiles were total collagen (r = 0.26), insoluble collagen (r = −0.07), and percentage soluble collagen (−0.45). In general, the observed relationships between collagen profile components and bind values were quite low, with the exception of percentage soluble collagen, which would indicate that the variables total and insoluble collagen are of little consequence in determining the ability of a raw material to bind adipose tissue. The significant negative relationship between percentage soluble collagen and bind values (r = −0.45) would suggest that with higher levels of soluble collagen, the possibility of gel-pocket formation may be increased and therefore limit the binding capacity of a meat. Additional studies are presently in progress to further investigate the role(s) of collagen profile components in the formation of sausage batters and to define maximum levels which high collagen meats may be used in sausage formulations without the occurrence of any detrimental effects.

Values for milliliters of fat and moisture lost during the cooking process indicate that there was little variation among the different types of meats for these variables (Table 1). Four of the 21 different types of meats bound portions of the added water while 15 meat samples lost a portion of the bound water in addition to the 40 ml of added water. Values for moisture loss during cooking were signifi-

–Continued on page 921

Table 2–Mean values for total collagen, insoluble collagen, soluble collagen, and water and fat lost on cooking for 21 different sausage raw materials

Meat	Total collagen (mg/g)	$\overline{sx}$	Insoluble collagen (mg/g)	$\overline{sx}$	Soluble collagen (mg/g)	$\overline{sx}$	Cookout (ml)	
							Water	Fat
Beef shank	23.5*	6.79	22.4	8.82	11.2	2.34	43.0	1.3
Cow meat	8.7	3.60	8.1	3.38	6.2	0.27	42.5	0
Beef trim 75/85	12.2	1.55	10.1	1.38	16.8	1.35	41.9	2.1
Beef neckbone trim	14.8	0.86	8.2	3.67	14.5	3.26	40.0	1.5
Beef plates	12.8	3.14	10.7	2.61	17.1	1.92	39.0	7.3
Beef cheek meat	25.3	4.63	19.1	4.64	23.4	5.34	45.6	1.7
Beef diaphragm	14.1	4.01	11.9	3.27	14.4	2.11	41.3	0.6
Beef tongue	16.1	2.62	4.0	2.21	13.2	2.08	46.3	5.8
Beef head meat	23.6	4.41	18.3	2.95	23.2	3.90	40.9	2.9
MDB Chicken	11.2	1.73	7.6	1.63	32.7	5.36	50.0	5.2
Pork blade meat	24.3	0.89	11.3	2.72	47.8	2.67	40.2	0.8
Pork neckbone trim	13.2	1.18	8.2	0.35	37.6	3.20	36.8	0.2
Pork tongue trim	8.1	1.66	6.7	1.01	28.1	4.13	44.3	0.1
Pork diaphragm	18.1	4.99	14.7	4.28	18.9	1.94	38.0	0.6
Pork trim 50/50	11.1	3.12	8.3	2.64	28.2	4.77	38.3	19.5
Pork cheek trim	24.3	4.95	13.5	3.45	47.8	4.61	43.5	0.7
Pork head meat	15.6	3.84	15.6	4.09	38.2	2.67	45.8	3.1
Pork heart	9.7	0.51	8.0	2.93	18.7	5.22	41.6	0
Pork jowl, skinned	7.6	1.79	7.6	2.13	35.8	7.71	40.0	16.4
Pork snouts	34.1	1.46	24.4	2.13	33.0	3.55	40.5	0.5

* (n = 3)

A Research Note

RELATIONSHIP BETWEEN METHYLMERCURY AND TOTAL MERCURY IN BLUE CRABS (Callinectes sapidus)

DONN R. WARD, RANZELL NICKELSON II and GUNNAR FINNE

ABSTRACT

Blue crabs were harvested from an area known to have a high level of mercury in the sediment. The edible body meat was extracted and analyzed for both total and methylmercury concentrations. A highly significant correlation was observed (0.957) with respect to the amount of total mercury present as methylmercury. Analysis indicated that approximately 35% of the total mercury was present as methylmercury.

INTRODUCTION

CONSIDERABLE INTEREST has been generated during the past several years with respect to mercury levels in the human diet. It is generally agreed that mercury enters our diet in a variety of ways, some is derived from natural origins and some resulting from man's activities. Furthermore, it is agreed that the form mercury enters our diet determines its potential toxicity, inorganic mercury being much less toxic than organic mercury. As a result of the variance in potential toxicity, disagreement has arisen over which form of mercury standards should be established. Total mercury, which the FDA limit of 1.0 ppm is based, is a measure of both inorganic and organic forms; whereas, France has a methylmercury standard of 0.5 ppm excluding Tuna and Swordfish which is 0.7 ppm (Hackley, 1978). While it is not the intent of this paper to discuss the advantages and disadvantages of either the organic or total mercury standard, it does point out that in some foods, such as crab meat, there may exist large differences in the percentage of methylmercury present in the total mercury concentration.

METHODS & MATERIALS

Crabs

Crabs were harvested from an area known to have a high concentration of mercury in the sediment due to an industrial chlor-alkali process. The crabs were frozen and shipped on dry-ice to the Food Quality and Safety Laboratory in College Station. Edible body meat was removed from the crabs in the following manner: carapaces and viscera were removed, the body rinsed in running tap water to remove bits of adhering viscera, and split in two. The meat from each half was pressed out into separate whirl-pak bags and frozen until required for analysis. One-half of the crab was analyzed for total mercury and the other half analyzed for methylmercury.

Total mercury

A Perkin-Elmer flameless atomic absorption spectrophotometer (Coleman 50) was used to analyze for total mercury content. The procedure of Hoover et al. (1971) was used with the following modifications: a 5:1 mixture of concentrated nitric and perchloric acid was used to digest crab samples in lieu of sulfuric acid.

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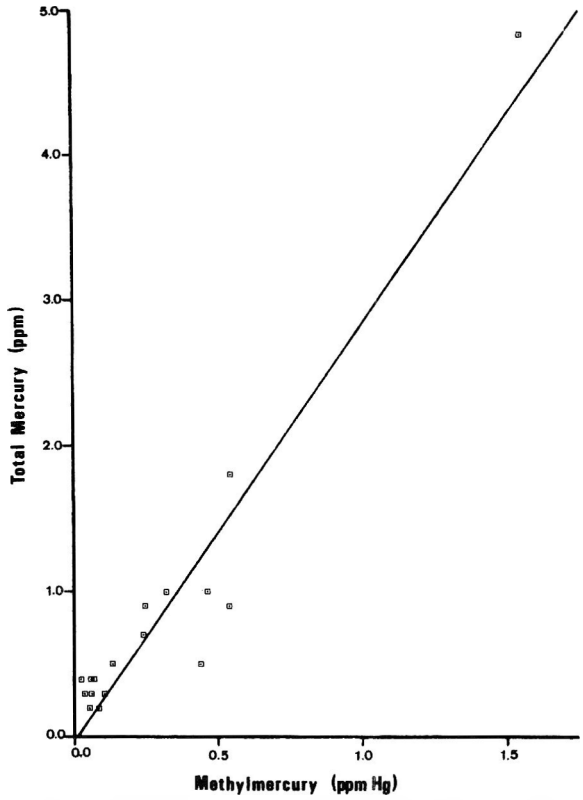

Fig. 1—Demonstrates the relationship between total mercury and methylmercury levels in the edible body meat of blue crabs.

Methylmercury

Methylmercury determinations were done according to Shin and Krenkel (1976). Five μl of the extract containing methylmercury was analyzed on a Tracor 560 gas chromatograph, using a 1/4 in. × 6 ft. glass column packed with a 1.5% OV-17 and 0.95% QF-1 mixture on 80/100 Chromosorb W-HP obtained from Altech Associates (Arlington Heights, IL). Chromatographic conditions were: column 160°C, detector (Ni 63) 350°C, carrier gas 5% methane 95% argon at a flow rate 45 cc/min.

Standard curve

A standard curve was prepared from methylmercury bromide (CH_3HgBr) obtained from Alfa Inorganics (Beverly, MA). Known concentrations of CH_3HgBr were analyzed by injecting a constant volume of 5 μl. The recorder response (area) was plotted as a function of injected concentration.

Extraction efficiency

Extraction efficiencies were determined in accordance with the procedure outlined by Shin and Krenkel (1976). Extraction efficiencies were determined for both the total and methylmercury analysis by spiking crab samples of known total and methylmercury values with a known concentration of inorganic mercury ($HgCl_2$) or methylmercury (CH_3HgBr) and determining the percent recovery. Extraction efficiencies for total and methylmercury determinations were 90-102% and 98-101%, respectively.

RESULTS & DISCUSSION

FIGURE 1 shows the relationship between methylmercury and total mercury in the edible meat of blue crabs. The

data indicate a highly significant correlation (r = 0.957) between the total mercury and methylmercury. Thirty-five percent of the total mercury is present as methylmercury. This information is significant since several studies have shown that the mercury present in fish and shellfish is almost entirely methylmercury (Westoo, 1966; Barke et al., 1972; Zetko et al., 1971). Blanton et al. (1972) found methylmercury to comprise from 17.24–100% of the total mercury in fish muscle. Their results indicated that fish which characteristically possess very high levels of tissue mercury (Blackfin Tuna, Bonita) were those which had methylmercury levels approaching 100% of the total mercury levels: whereas, those fish which characteristically possess low concentrations of tissue mercury (Dolphin, Mullet), methylmercury levels were less than 50% of the total mercury. Since blue crabs do not characteristically have high total mercury levels, Hall et al. (1978) indicated 0.105 ppm average for edible body meat of crabs harvested from the Gulf, it follows that the methylmercury content should be small in proportion to the total.

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SAUSAGE CONNECTIVE TISSUE PROFILES . . . From page 919

cantly related to percentage intramuscular fat (r = −0.29), percentage moisture (r = 0.37), milliliters of fat loss (r = −0.27), and color index (r = 0.31). Significant correlation coefficients observed for milliliters of fat lost during cooking included percentage protein (−0.55), percentage intramuscular fat (r = 0.50) and percentage moisture (r = −0.64). In general, the correlation coefficients for water lost during cooking were quite low which would indicate that water loss determined by this procedure was not indicative of binding characteristics of raw sausage materials. Values for fat losses determined by this cookout procedure also appear to be of little value for predicting bind characteristics of raw meats.

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A Research Note

AMINE CONTENT OF TOXIC AND WHOLESOME CANNED TUNA FISH

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ABSTRACT

Concentrations of cadaverine, putrescine, histamine, spermidine and spermine were determined in canned wholesome tuna and in canned tuna that had been implicated in an outbreak of scombroid poisoning in humans. The method employed is a modification of established extraction and thin-layer chromatographic techniques for analysis of amines. The average concentrations of amines in wholesome tuna were: putrescine, 0.35 mg%; cadaverine, 1.05 mg%; histamine, 2.74 mg%; spermidine, 3.26 mg%; and spermine, 1.23 mg%. Amine levels in toxic tuna ranged from 1.53 mg% and 1.16 mg% for putrescine and spermine, respectively, to 2.37 mg% for spermidine, 12.8 mg% for cadaverine, and 116 mg% for histamine.

INTRODUCTION

HISTAMINE appears to be an etiologic agent in scombroid fish poisoning (Arnold and Brown, 1978) which has occurred following consumption of scombroid fish (i.e., tuna). The fish contain relatively large amounts of histamine and the symptoms are generally those of histamine poisoning.

Histamine may not be the only causative agent of scombroid poisoning because histamine has a relatively low peroral toxicity in humans (Weiss et al., 1932). Several authors have suggested that substances in addition to histamine may be involved in poisoning (Geiger, 1955; Foo, 1976; Durand, 1973). Putrescine potentiates the peroral toxicity of subsequent doses of histamine in guinea pigs (Parrot and Nicot, 1966) and cadaverine causes a pronounced increase in the toxicity of histamine on simultaneous administration of the two compounds (Bjeldanes et al., 1978). Spermine has been reported to liberate endogenous histamine in intestinal fluids of the guinea pig (Parrot, 1948). Also, histamine taken with a meal (bread, milk and butter) has been reported to be absorbed to a greater extent than histamine consumed by itself (Mitchell and Code, 1954).

To determine if amines, in addition to histamine, may be involved in the etiology of scombroid poisoning, analyses of these substances in fish are required. Mietz and Karmas (1977) have described a high-performance liquid chromatography method to resolve tuna fish extracts. The method used for this report is a combination of the extraction technique of Spinelli et al. (1974) and the thin-layer chromatographic technique of Hammond and Herbst (1968). The method was used for the analysis of cadaverine, putrescine, histamine, spermidine and spermine in canned samples of wholesome tuna and of tuna which was implicated in an outbreak of human poisoning.

EXPERIMENTAL

Materials

The following chemicals were reagent grade unless otherwise noted: trichloroacetic acid (Merck & Co., Inc., Rahway, NJ), hydrochloric acid (B&A Allied Chemicals, Morristown, NJ), n-butyl alcohol and sodium hydroxide (Mallinckrodt Chemical Works, St. Louis, MO), anhydrous sodium sulfate, trisodium phosphate, and n-heptane 98% (J.T. Baker Chemical Co., Milwaukee, WI), ninhydrin (I.C.N. Pharmaceuticals Inc., Life Science Group, N.B.C. Division, Cleveland, OH).

Thin-layer chromatography was performed with two types of plates: one consisted of 20 × 20 cm glass plates coated with Whatman CC_{41} microgranular cellulose powder in a 0.25 mm layer with a Kensco #K4039 applicator and alignment tray (Kensington Scientific Corp., Emeryville, CA). The other plates were 20 × 20 cm commercially pre-coated with a 0.25 mm layer of cellulose MN_{300} (Macherey, Nagel Co., Düren, Germany; distributed by Brinkmann Instruments, Inc., Westbury, NY).

CC_{41} cellulose plates were prepared by a modification of the procedure described by Hammond and Herbst (1968). A mixture of Whatman CC_{41} microgranular cellulose powder (30g), water (90 ml), and EDTA (0.1 mg) was homogenized in a Waring Blendor (250 ml) for 2.5 min. This material was immediately applied to 10 glass plates (20 × 20 cm) in a 0.25 mm layer, allowed to air dry overnight, and stored in a desiccator.

Standard solutions of putrescine·2HCl, cadaverine·2HCl, spermidine·3HCl, and spermine·4HCl (Calbiochem, San Diego, CA) contained 2.5 mmol of amine salt and 0.1 mg EDTA in 1 liter of 0.1N HCl solution. The standard histamine (Aldrich Chemical Co., Inc., Milwaukee, WI) solution contained 12.5 mmol of amine salt and 0.1 mg EDTA in one liter of 0.1N HCl.

Spoiled tuna samples were 6.5 oz cans from lots D417 and D419 which have been implicated in outbreaks of human poisoning (Anonymous, 1973). Other tuna samples were 6.5 oz cans purchased from local supermarkets.

Methods

Amines were extracted from fish samples using a modification of the method of Spinelli et al. (1974). Samples were prepared by homogenizing 20g of ground fish in a Waring Blendor for 3 min with 80 ml of a solution containing 10% (w/w) trichloroacetic acid in 0.05N HCl. The mixture was allowed to stand overnight, or at least for 2–3 hr, at 4°C and then centrifuged. The residue was re-extracted with 20 ml of cold 5% (w/w) trichloroacetic acid and the combined supernatants (~ 100 ml) were adjusted to pH 12~13 with 20% NaOH. Thirty grams of a salt mixture containing 62.5g anhydrous sodium sulfate and 9g of trisodium phosphate was added. This solution was then extracted with three 200-ml portions of n-butyl alcohol. Each extraction included a 1-hr period of mechanical shaking. The combined n-butanol extracts were acidified to pH 1 with approximately 2 ml 1N HCl. An equal volume of n-heptane was added and the mixture was shaken for 1 min. The butanol fraction was re-extracted three times with 20 ml of 0.1N HCl. The total acidic extract was evaporated in vacuo at 50°C until a few milliliters remained. The volume was adjusted to 5 ml with 0.1N HCl, EDTA (0.1 mg) was added, and the solution stored at 4°C.

Amines were quantified by a modification of the method of Hammond and Herbst (1968). Fish extracts (5–10 μl) and standard solutions (5 μl) were spotted on the CC_{41} or MN_{300} cellulose plates. Chromatography was performed in two dimensions with ethylene glycol monomethylether (methylcellosolve)-propionic acid-water (70:15:15). Solvents were removed from the plates (2 hr in fume hood or 20 min in vacuo) and plates were heated to 65°C for 5 min. Cooled plates were sprayed with a ninhydrin solution (1%, w/w) and held at room temperature for 20 min in a fume hood. Plates were then heated to 65°C in vacuo (MN_{300} plates for 60 min, CC_{41} plates for 25 min).

By reference to standards, individual spots were located and identified. Spots were scraped from plates and extracted with ethanol (1 ml, 71%, w/w) by agitation (Vortex Jr. Mixer, model

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Table 1—TLC analysis of amines in commercial (nondecomposed) and decomposed canned tuna fish

Fish sample	n[a]		Amines (mg%) Cadaverine	Putrescine	Histamine	Spermidine	Spermine
Commercial canned tuna fish	34	Range	0 ∿ 3.70	0 ∿ 2.50	1.0 ∿ 17.0	0.47 ∿ 7.90	0.25 ∿ 2.90
		$\bar{X}$ ± SE	1.05 ± 0.60	0.35 ± 0.10	2.74 ± 0.71	3.26 ± 0.33	1.23 ± 0.09
		Median	0.80	0.12	1.97	2.5	1.15
Canned decomposed[b] tuna fish	15	Range	2.40 ∿ 21.0	0 ∿ 5.60	9.70 ∿ 200	0 ∿ 4.90	0 ∿ 2.2
		$\bar{X}$ ± SE	12.8 ± 1.66	1.53 ± 0.44	116 ± 6.24	2.37 ± 0.50	1.16 ± 0.15
		Median	10.8	1.25	118	2.63	1.30

[a] Total number of cans analyzed
[b] Lots D417 and D419 implicated in human poisoning

K-500-J, Scientific Industries Inc., Queens Village, NY). The mixture was centrifuged (5 min, 100 × g) and absorbances of aliquots of the supernatants were read at 575 nm. Concentrations of amines (mg/100g fish) were calculated from standard curves. Blank was prepared by extraction of an area on the TLC plate similar in size but remote from distinguishable spots.

Identities and purities of amines isolated from fish material were established by gas-liquid chromatographic analysis of silylated derivatives.

Rates of recovery of pure amines added to fish samples before extraction averaged 95 ± 2%. The relative standard deviations were 7% or less.

RESULTS & DISCUSSION

CADAVERINE (R_F = 0.47), putrescine (R_F = 0.40), histamine (R_F = 0.35), spermidine (R_F = 0.24), and spermine (R_F = 0.13) were clearly distinguishable with ninhydrin as separate violet spots on the MN_{300} and CC_{41} cellulose TLC plates. Other amines such as diethylamine, trimethylamine and dimethylamine present in marine fish (Gruger, 1972) have R_F's greater than 0.47 and were not individually resolved under the conditions used. Amino acids such as lysine, arginine and ornithine which have R_F's in the range 0.10–0.50 are extracted in only trace amounts by n-butanol and do not interfere with the assay. Histidine, which is present in relatively large amounts in tuna, was visible on ninhydrine-treated TLC plates as a yellow-brown spot (R_F = 0.29). It did not absorb light at 575 nm and hence did not interfere with the quantitation of histamine and spermidine.

The results of analyses of amines in canned tuna are summarized in Table 1.

Cadaverine, putrescine and histamine concentrations were lower in good tuna than in canned tuna implicated in human poisoning. The spermidine levels were decreased slightly in the latter tuna samples in comparison to good tuna whereas spermine levels were similar in both types of tuna. These trends are in general agreement with the results of Mietz and Karmas (1977). However, the concentrations reported by these workers are approximately one-tenth the present results for all amines assayed. The discrepancy between the present results and those of Mietz and Karmas (1977) is unresolved. The concentrations of histamine observed here are similar to values reported in several other studies (Nerrison, 1974; Anonymous, 1973).

The role in scombroid poisoning of amines which potentiate the toxicity of histamine is unclear. However, certain of these compounds such as cadaverine appear to occur at increased levels in toxic tuna and, therefore, might be significant in the etiology of toxicity.

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A Research Note

ENZYME NEUTRAL DETERGENT FIBER ANALYSIS OF SELECTED COMMERCIAL AND HOME-PREPARED FOODS

WEN-LI JOHN JWUANG and MARY E. ZABIK

ABSTRACT

To assess the potential dietary fiber contribution of selected commercial foods and home-prepared quick and yeast breads, over 40 commercially available breads, European crisp breads, breakfast cereals, crackers, cookies, and snack foods and 10 home-prepared breads were analyzed for their enzymatic neutral detergent fiber (ENDF) content. The commercial breads would provide from 1.5–3.9g ENDF per 1 oz serving while home-prepared breads had from 0.6–3.9g per ounce. European crisp bread contained from 4.7–9.7g per ounce ENDF. Snack foods were also good sources of dietary fiber providing from 3.1–9.3g ENDF per ounce while cookies and crackers analyzed ranged from 1–4g ENDF per ounce.

INTRODUCTION

RECENT REVIEWS have summarized the epidemiological evidence relating the lack of dietary fiber in Western diets to such diseases as diverticular disease, ischemic heart disease, colonic cancer, appendicitis, etc. (Burkitt and Trowell, 1975; Spiller and Amen, 1975, 1976; Eastwood, 1977). Dietary fiber consists of cellulose, lignin, hemicellulose, pectins, gums and mucilages. Since crude fiber values which are commonly tabulated in food composition tables only accounts for approximately 50–90% of the cellulose, 10–40% of the lignin and 20% of the hemicelluloses (Schaller, 1977), these values have very limited use in predicting the dietary fiber value of foods. The American Association of Cereal Chemists have adopted the neutral detergent fiber NDF method in combination with enzyme digestion with hog pancreas amylase as the official method for dietary fiber analyses (Schaller, 1977) but very few ENDF values have been published. Recently, Spiller et al. (1977) have advocated the establishment of a recommended daily dietary allowance for dietary fiber suggesting the level should be that quantity which produces a 36-hr intestinal transit time. Before a recommended allowance for dietary fiber can become very useful, the dietary fiber content of many more foods must be quantified. The purpose of this research was to determine the ENDF values of selected commercial products which could be significant sources of dietary fiber. In addition, five yeast-leavened and five quick breads were prepared and evaluated for their potential as sources of dietary fiber.

EXPERIMENTAL

THE COMMERCIAL PRODUCTS analyzed which are listed in Table 1 were obtained from local retail markets and included: five whole grain breads, fourteen breakfast cereals, seven European flat

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Table 1—Means and standard deviations of the mean for enzymatic neutral detergent fiber content in commercial whole grain breads, cereals, breads, cookies, and snacks.

Food items	% ENDF	g ENDF per 1-oz serving
Whole grain bread		
Schwazbrot	13.50 ± 0.02	3.85
Pumpernickle	11.33 ± 0.36	3.25
Stollen	7.84 ± 0.11	2.23
Arwrey Heart Health Wheat Bread	7.66 ± 0.20	2.18
Italian Rye Bread	5.21 ± 0.04	1.48
Cereals		
Kellogg's All Bran	34.06 ± 0.44	9.71
Kellogg's Bran Buds	33.84 ± 0.04	9.64
Kellogg's Raisin Bran Flakes	14.72 ± 0.15	4.20
Ralston's Wheat Chex	13.85 ± 0.13	3.95
Kellogg's Frosted Mini-Wheats	13.55 ± 0.19	3.86
General Mill's Wheaties	10.57 ± 0.55	3.01
Food Club Raisin Bran Flakes	8.80 ± 0.11	2.51
Grape-Nut Cereal	7.54 ± 0.45	2.15
Kellogg's Country Morning	6.34 ± 0.30	1.81
Nature Valley's Nature Cereals	5.87 ± 0.43	1.67
General Mill's Total	5.81 ± 0.19	1.66
Nature Valley Granola Cinnamon & Raisin Cereal	5.63 ± 0.25	1.60
C.W. Post Family Style Cereal	4.56 ± 0.28	1.30
Cap'n Crunch Crunch Berries	3.84 ± 0.18	1.09
Cookies and snacks		
Doritos Tortilla Chips	32.44 ± 0.46	9.25
Sesame and Bran Sticks	16.10 ± 0.12	4.59
Corn Chips	14.11 ± 0.04	4.02
Potato Chips	12.64 ± 0.28	3.60
Sesame Buds	10.85 ± 0.12	3.08
Nature Valley Granola Bar with coconut	8.09 ± 0.10	2.31
Old Fashioned Oatmeal Cookies	5.43 ± 0.16	1.55
Fig Bar	4.25 ± 0.16	1.21
Crackers and European crisp breads		
Siljan Swedish Rye Crisp Bread	36.27 ± 0.31	10.34
Ideal Flat Bread, Norwegian	28.23 ± 0.28	8.05
Rogga	25.40 ± 0.36	7.24
King's Crisp Bread	25.21 ± 0.22	7.24
Crisp Bread with Linseed	22.44 ± 0.09	6.40
Mors Flat Bread	17.77 ± 0.15	5.05
Crisp Bread with Sesame	16.44 ± 0.26	4.69
Triscuit Whole Wheat	14.67 ± 0.42	4.11
Wheat Square Crackers	5.41 ± 0.41	1.54
Honey Sorghum	4.76 ± 0.29	1.36
Hearty Wheat Snack Cracker	3.67 ± 0.38	1.05
Other food stuffs		
Red Wheat Bran	40.81 ± 0.12	
White Wheat Bran	39.77 ± 0.14	
Wheat Germ	24.26 ± 0.14	
Sesame Seeds	14.36 ± 0.10	
Jiffy Bran Muffin Mix	9.67 ± 0.08	
Whole Grain Natural Rice	5.97 ± 0.18	
Rolled Oat	5.63 ± 0.28	

Table 2—Means and standard deviations of the mean for enzymatic neutral detergent fiber content of home-prepared breads

Type of bread	% ENDF	g ENDF per 1-oz serving
Rye bread	13.75 ± 0.46	3.91
Fig bran bread	9.99 ± 0.09	2.85
Apple bread	6.32 ± 0.06	1.80
Bran bread	6.08 ± 0.19	1.73
Rolled oat-nut bread	6.06 ± 0.10	1.73
Raisin-nut oat bread	5.54 ± 0.13	1.58
Banana bran bread	5.49 ± 0.33	1.56
Raisin bread	4.48 ± 0.13	1.28
Carrot bread	3.44 ± 0.22	0.98
Zucchini bread	2.02 ± 0.05	0.58

breads, twelve cookie, crackers and snack foods and seven other food stuffs. In addition ten home made breads were prepared following formulas and procedures published in commercial cookbooks. The five chemically-leavened quick breads were banana bran bread, bran bread, carrot bread, fig bran bread and zucchini bread. The five yeast leavened breads were apple bread, raisin bread, raisin-nut oat bread, rolled oat nut bread and rye bread.

Duplicate samples of each of the three replications of each food were analyzed by the Enzymatic Neutral Detergent Fiber method described by Robertson and Van Soest (1977). The entire loaf of home prepared bread was ground before sampling. Foods high in fat were first defatted for 6 hr with diethyl ether using a Goldfish continuous extraction apparatus. Bacterial crude alpha amylase (Type VI-A) was used to remove the starch. All ENDF values are based on the original sample weight.

RESULTS & DISCUSSION

THE ENDF CONTENT (mean ± SD) of the commercial products and the grams of ENDF per 1-oz serving are in Table 1. The five commercial whole grain breads analyzed ranged from 5.2–13.5% with 1.5–3.8g of ENDF per 1-oz serving. Whole wheat bread had previously been reported to contain 14.9% NDF (Spiller and Amen, 1975). The cereals ranged from 3.8–34.0% ENDF providing from 1.0–9.7g ENDF per serving. The ENDF value for All Bran agrees closely with the NDF values reported by Spiller and Amen but the ENDF values for Wheat Chex and Wheaties were lower.

Fig bars and oatmeal cookies had 4.3 and 5.4% ENDF respectively while Granola Bars had 8.1%. Other snack foods evaluated ranged from 10.9 for Sesame Buds to 32.4% for Doritos Tortilla chips contributing from 3–9g ENDF per 1-oz serving. Doritos Tortilla chips are made from whole corn and tomato pulps. Whole corn kernels contain 13% NDF (Spiller and Shipley, 1976) while dry tomatoes contain 4.41% lignin, 6.72% cellulose, 4.15% pentoses and 4.34% uronic acids (Southgate, 1976). Corn chips had 14.11% ENDF while potato chips had 12.64% ENDF. Southgate (1976) reported the dietary fiber components of potatoes on a dry weight basis to be 6.24% lignin, 7.02% cellulose, and 6.24% hemicellulose.

European crispbreads are also good sources of dietary fiber with ENDF values ranging from 16.4–36.3%. The ENDF values of the crackers analyzed ranged from 3.7–14.7%; three of the crackers provided 1–1.5g ENDF per ounce while Triscuits which are made from whole wheat itself provide 4g of ENDF per ounce. The high percentage of ENDF found in bran and wheat germ account for the high ENDF values found in these whole wheat foods.

The ENDF values (means ± SD) of the home prepared breads are in Table 2. Rye bread had the highest ENDF value and would provide almost 4g dietary fiber per serving. This was slightly higher than the commercial breads. The quick breads tended to be slightly lower but all except for zucchini bread provide 1g or more of dietary fiber per serving.

Thus many commercial and home prepared foods do have significant ENDF values and can contribute to fulfilling a persons need for dietary fiber. As more and more foods are analyzed for their dietary fiber content, selection of foods to meet a recommended daily dietary allowance for dietary fiber becomes more feasible.

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A Research Note

HIGH-PRESSURE LIQUID CHROMATOGRAPHY OF CRANBERRY ANTHOCYANINS

A. L. CAMIRE and F. M. CLYDESDALE

ABSTRACT

The four major anthocyanins of cranberries were separated and purified by conventional paper chromatography. The purified individual anthocyanins were eluted from the paper by methanol-acetic acid-water (90:5:5) and concentrated on a rotary evaporator (<30°C). The individual anthocyanins were chromatographed separately and in a mixture by a reversed-phase high-pressure liquid chromatographic (HPLC) system. Complete separation of all four anthocyanins took less than 1 hr 40 min. A 50-ml sample of commercial Cranberry Juice Cocktail first purified by a GC-50 ion exchange column before injection into the HPLC showed the presence of two additional pigments when chromatographed.

INTRODUCTION

RECENT ADVANCES in high-pressure liquid chromatography (HPLC) together with the development of efficient microparticulate column packings has opened up new horizons in the field of analytical separation and identification of plant phenolics.

Manley and Shubiak (1975) were the first to investigate several HPLC systems potentially useful in rapidly separating mixtures of anthocyanins; however, their column packings were not the now "state of the art" microparticulates. It was necessary for these workers to couple together two 1/8 in. × 2 ft stainless steel columns packed with Pellidon to achieve a satisfactory separation of three monoglucosides of malvidin, petunidin, and peonidin using a chloroform-methanol (87:13) solvent system.

The HPLC of some anthocyanidins and flavonoids has been reported by Adamovics and Stermitz (1976). These workers coupled a C_{18} Corasil column to a μ Bondapak C_{18} column to achieve satisfactory separation of three flavonol glycosides with methanol-acetic acid-water (30:5:70) as eluent. The HPLC of anthocyanidins was more recently reported by Wilkinson et al. (1977). This group separated the six most common anthocyanidins by a reversed-phase HPLC system in less than thirty minutes with methanol-acetic acid-water (19:10:71) as eluent. Wulf and Nagel (1978) have reported the HPLC separation of the anthocyanin pigments of two varieties of Vitis vinifera grapes. These workers used solvent programming to generate a linear gradient which separated the pigments of Cabernet Sauvignon grapes into twenty discrete peaks. The separation was carried out using a reversed-phase octadecylsilyl (ODS) analytical column and a water-methanol eluant acidified to pH 1.5 with formic acid. Manufacturers of reversed-phase supports do not recommend using eluants with a pH less than 2.0 because the ODS groups may be hydrolyzed off of the silica; however, these workers have used this solvent system for several months with no apparent loss in column efficiency thus far.

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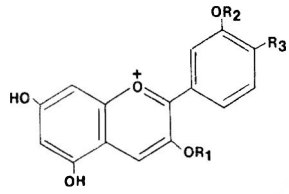
Table 1—Structures, retention times, capacity factors, and relative retentions of four cranberry anthocyanins



Peak No.	Compound	R_1	R_2	R_3	t_R (min)	k'	α
1	Cy-3-Ga	galactose	H	OH	64.43	2.96	1.15
2	Pn-3-Ga	galactose	CH_3	OH	72.04	3.41	1.13
3	Cy-3-Ar	arabinose	H	OH	79.19	3.86	1.20
4	Pn-3-Ar	arabinose	CH_3	OH	92.27	4.66	

Vincent and Scholz (1978) have recently reported an HPLC method for the separation and quantification of betacyanin and betaxanthin pigments in red beet extracts and liquid and powdered products. The method involves a reversed-phase column, a methanol-water mobile phase, and an ion pairing technique. The ion pairing approach was chosen in order to take advantage of the highly ionic character of the betacyanin pigments.

We would like to report a procedure using a modified version of the reversed-phase HPLC system developed by Wulf and Nagel (1976) for the separation of flavonoids and phenolic acids.

MATERIALS & METHODS

THE HPLC INSTRUMENT used in this work was assembled in this laboratory and consists of the following components: (a) Ultraviolet detector set at 280 nm with a flow cell of 8μl and an optical path length of 10 mm (Laboratory Data Control). (b) Milroyal®D Controlled volume pump, rated to a maximum discharge pressure of 1000 psi. (Milton Roy Company). (c) Stop-flow septum injector (Waters Associates). (d) 10 mv full scale strip chart recorder set at 6 in./hr (Leeds and Northrup Co.). (e) 400 ml capacity glass solvent reservoir (Buchler Instruments). (f) Reversed-phase octadecylsilyl (ODS) column packing (10μ) (Tridom Chemical, Inc.). A flow rate of approximately 0.3 ml/min was maintained at a pressure of 950 psi utilizing a mixture of methanol-acetic acid-water (37:10:53) by volume as the mobile phase. Two 4.6 mm i.d. × 25 cm stainless steel columns were coupled in series in order to achieve a satisfactory separation of the anthocyanin mixture.

Ion exchange purification of the cranberry juice cocktail to eliminate sugars and organic acids was carried out on an Amberlite GC-50, 100–200 mesh column (Mallinckrodt Chem. Works, St. Louis, MO) according to the method of Chiriboga (1969).

RESULTS & DISCUSSION

FIGURE 1a SHOWS a chromatogram of a standard mixture of the four major cranberry anthocyanins. The structures, retention times, capacity factors, and relative retentions of these anthocyanins are given in Table 1. Generally values of α greater than 1.15 are required in order to achieve a resolution of 1 (98% separation) between two compounds.

The flow rate of approximately 0.3 ml/min resulted in an elution time for this four-component mixture of 1 hr 40 min. The elution time could be reduced by using a higher flow rate; however, the pump used in this work was limited

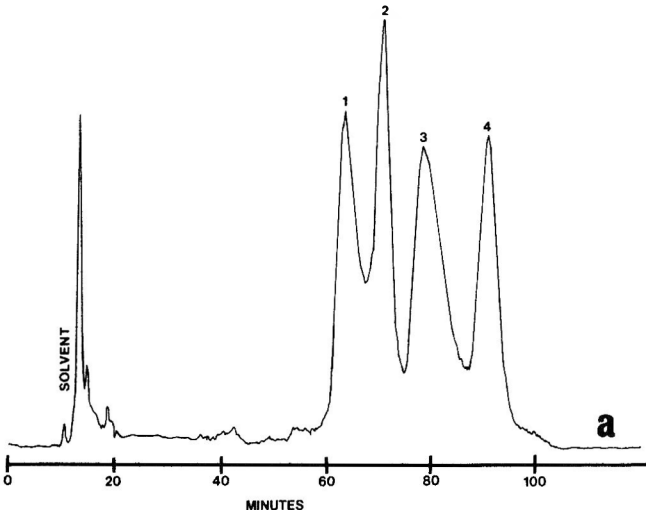


Fig. 1a—HPLC of the four purified major anthocyanins of cranberries. Peaks 1, 2, 3 and 4 represent Cy-3-Ga, Pn-3-Ga, Cy-3-Ar, and Pn-3-Ar respectively (Structures and separation parameters described in Table 1). Chromatogram obtained using a 10 µl injection on coupled reversed-phase ODS columns with water-acetic acid-methanol (37:10:53) by volume as the mobile phase. Detection was at 280 nm with attenuation 0.16 a.u.f.s. for 40 min and 0.01 a.u.f.s. from 40 min on.

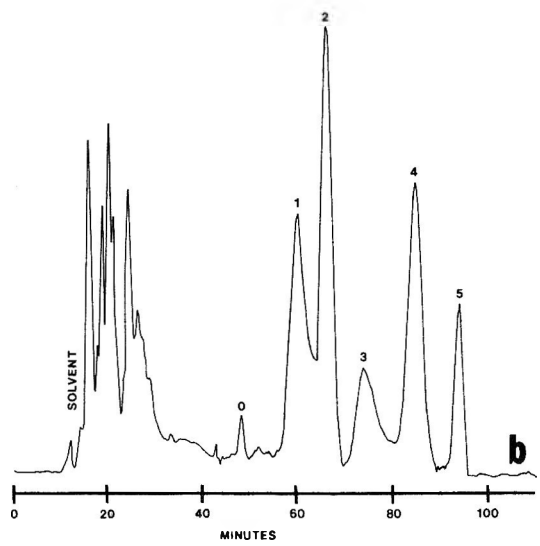


Fig. 1b—HPLC of anthocyanins from GC-50 purified cranberry juice cocktail. Peaks 1, 2, 3, 4 are described in Fig. 1a. Peaks 0 and 5 are tentatively identified as Cy-3-Gl and Pn-3-Gl. Separation conditions are the same as described in Fig. 1a except that a mobile phase of water-acetic acid-methanol (40:10:50) was used in order to speed elution and therefore shorten the total analysis time.

to a maximum discharge pressure of 1000 psi which would have to be exceeded in order to produce the higher flow rate.

A chromatogram of GC-50 ion exchange purified pigment from a sample of commercial cranberry juice cocktail is shown in Figure 1b. Four standard cranberry pigments, cyanidin-3-galactoside (Cy-3-Ga), peonidin-3-galactoside (Pn-3-Ga), cyanidin-3-arabinoside (Cy-3-Ar), and peonidin-3-arabinoside (Pn-3-Ar), identified previously on paper chromatography by Fuleki (1967) and numbered 1, 2, 3, 4 respectively may be seen in this Figure. Also two other peaks (0, 5 in Fig. 1b) are shown which the authors suspect represent two anthocyanin pigments previously identified on paper chromatography by Fuleki and Francis (1967) to be present in cranberries at less than 1% of the total. These minor pigments are cyanidin-3-monoglucoside and peonidin-3-monoglucoside. It is realized that it would be necessary to repeat the procedure of Fuleki and Francis (1967) to more positively confirm the peaks on the HPLC chromatogram but it is thought that the standards obtained in Figure 1a adequately confirmed the major peaks found in Figure 1b.

Although this procedure requires an ion exchange step for commercial beverages prior to analysis, it provides a rapid, quantitative technique which has potential application in both basic research and quality control.

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A Research Note

MICROBIOLOGICAL AND TOXICOLOGICAL EVALUATION OF FERMENTED COWPEAS (Vigna sinensis) AND CHICKPEAS (Cicer arietinum)

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ABSTRACT

Cowpeas and chickpeas were allowed to undergo natural fermentation for 4 days at room temperature (25°C). The average pH and titratable acidity (as percent lactic acid) attained for cowpeas were 4.12 and 1.32%, respectively, and for chickpeas, 3.92 and 1.02%, respectively. No toxic substance was detected in fermented cowpeas and chickpeas as determined by the in vivo tests in chicken embryos.

INTRODUCTION

IN A PREVIOUS PUBLICATION, it was shown that cowpeas and chickpeas after undergoing a natural fermentation at 25°C for 4 days increased significantly ($P < 0.05$) % relative nutritive value, in methionine, isoleucine and niacin. The thiamine content increased significantly ($P < 0.05$) in chickpeas but not in cowpeas whereas riboflavin increased significantly ($P < 0.05$) in cowpeas but not in chickpeas (Zamora and Fields, 1979). These findings are important since it is necessary to optimize the nutritional value of legumes in order to improve the diet and nutrition of people, especially those who utilize them to a great extent.

Since the protein content is high enough to give buffering action to the lactic acid produced by the microflora and possibly to allow growth food poisoning to grow, it was imperative that the safety of the fermented cowpeas and chickpeas be evaluated by testing for harmful microorganisms and by testing for toxicity of the product and its components.

MATERIALS & METHODS

Preparation and fermentation of beans

The procedure was described in a previous paper by Zamora and Fields (1979).

Microbiological analyses

Enumeration and tests for lactic acid bacteria. Serial dilutions of the fermenting samples taken from the initial to the last day of fermentation were prepared using 0.1% peptone water. One-tenth ml of the appropriate dilutions was pipetted on lactic and V-8 agar (APHA, 1976) plates and spread with a sterile bent glass rod. The plates were incubated at 37°C for 48 hr in an anaerobic jar. A lighted candle was placed inside the jar, and the lid was replaced. The expanding gases were allowed to escape. When the flame died, the lid was sealed securely. This provided the low oxygen tension necessary for the growth of the lactic organisms. This procedure was that of Lord (1959).

Gram positive, catalase-negative cocci or rods from lactic agar plates were considered as lactic acid bacteria. Colonies in V-8 agar which were green to black with yellow halo were counted as lactics.

Enumeration of coliforms. Purplish red colonies on violet red bile agar surrounded by a reddish zone of precipitated bile were counted as coliforms (APHA, 1976).

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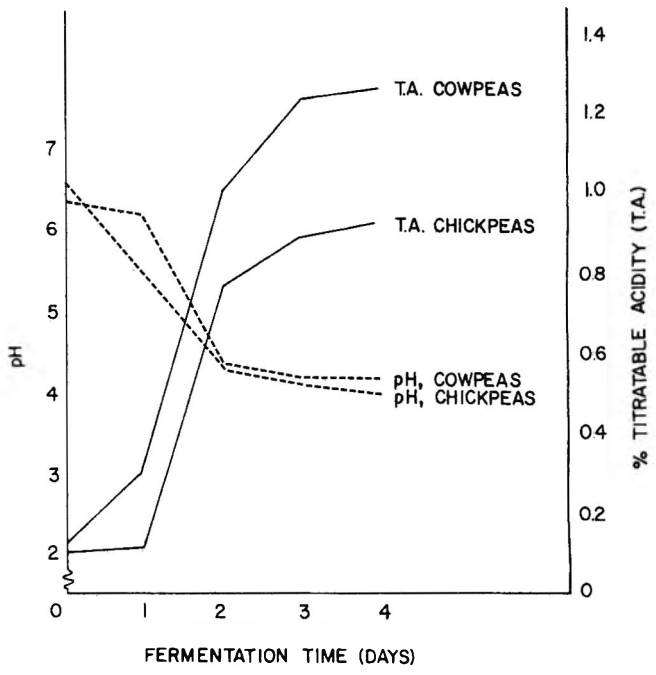

Fig. 1—pH and titratable acidity of fermenting cowpeas and chickpeas.

Toxicological evaluation of the fermented beans. Chicken embryo bioassay were carried out on the fermented samples. Fertile chicken eggs were obtained from the Poultry Department of the University of Missouri. The eggs were candled to eliminate eggs with cracks or with other shell imperfections, and to outline the location of air cells (AOAC, 1975). The eggs were incubated at 37–38°C at 65% relative humidity. After five days of incubation, the eggs were candled and only the ones with healthy embryos were selected. The eggs were surface-sterilized with mercurochrome. A hole was made with sterile pointed needle where the air cell was located (AOAC, 1975). Twenty-five eggs were used for each of the following treatments: (1) oil extract; (2) water extract; (3) sterile distilled water control; and (4) sterile oil control.

Inoculated eggs were incubated at 37–38°C for 4 days (Davis et al., 1975).

RESULTS & DISCUSSION

pH and titratable acidity

Figure 1 illustrates the trends in pH and titratable acidity of fermenting cowpeas and chickpeas. There was a sharp increase in titratable acidity from the first to the third day of fermentation, and it gradually leveled off from the third to the fourth day. With the abrupt increase in titratable acidity, there was the corresponding drop in pH followed by an almost constant pH from the third to the fourth day of fermentation. These results were observed for both cowpeas and chickpeas.

The pH of fermenting cowpeas was higher than that of chickpeas, but the titratable acidity was also higher for cowpeas than for chickpeas. This might be because the proteins in chickpeas were more soluble, so that the proteins buffered the acid produced. Therefore, the titratable acidity measured in fermenting chickpeas was lower than the actual amount present in the medium.

Enumeration of microorganisms present

Total lactics and coliforms were determined in the fermenting beans. The count of lactic organisms in cowpeas and chickpeas peaked on the third day of fermentation, after which counts leveled off in chickpeas and decreased gradually in cowpeas. The lactic counts were higher than one usually finds in lactic fermentations probably because of the added sugar. Coliform count was highest on the second day of fermentation, and there was a sudden decrease on the third and fourth days. No coliforms were detected on the fourth day of fermentation of chickpeas (Fig. 2).

Effect of fermented bean extracts on chicken embryos

Although no toxic substance was detected in the fermented cowpeas and chickpeas as determined by the in vivo tests in chicken embryos, one can theorize that since there is a rapid fermentation by lactic acid bacteria with a concomitant lowering of pH and production of lactic acid, there is no reason for food poisoning occurring. Zamora (1978) challenged similar fermentations and found that when pathogenic organisms were inoculated into the fermenting beans, the organisms survived after 24 hr. This was probably because it took about 24 hr for the acidity to develop to a level which suppressed the growth of the pathogenic organisms. After 24 hr, the respective *Bacillus cereus* and *Staphylococcus aureus* counts in fermenting cowpeas were 6×10^5 and 300 per g. For chickpeas, the respective *B. cereus* and *S. aureus* counts were 300 and 130 per g. The survival and growth of *B. cereus* and *S. aureus* might be dangerous because these organisms can produce toxins in the food. This means that contamination during fermentation should be avoided, and that it is necessary to use soil-free seeds. After four days fermentation, however, there was less than one per g sample of the pathogenic organisms.

The data show that in the development of high protein materials that are fermented, precaution should be taken to make sure that the foods will be safe and not go on hypotheses that are not tested for the particular fermentation being studied. The authors were particularly concerned about *B. cereus* in this research.

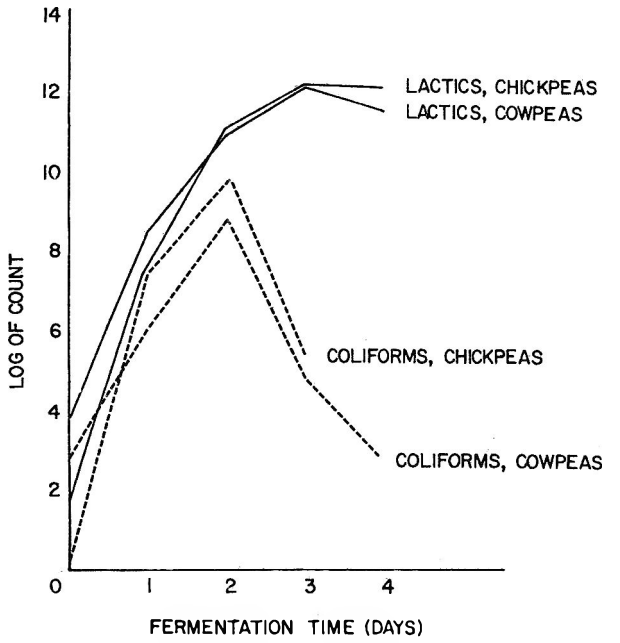

Fig. 2—Lactic and coliform counts on fermenting cowpeas and chickpeas.

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A Research Note

SENSORY EVALUATION AND NUTRITIVE VALUE OF SOUPS AND CHIPS MADE FROM FERMENTED AND NONFERMENTED BEANS

AGNES F. ZAMORA and MARION L. FIELDS

ABSTRACT

Soups prepared from the fermented samples with and without pH adjustment were in the "dislike" category, while the soup from nonfermented sample was in the "neither like nor dislike" category. Chickpea chips made from the fermented bean flours were in the "neither like nor dislike" category while those from the nonfermented flours were in the "like" category. Relative nutritive values (RNV) of soups and chips prepared from the fermented samples were significantly ($P < 0.05$) higher than those prepared from the nonfermented samples. Soups and chips made from fermented beans contained significantly ($P < 0.05$) greater amounts of limiting amino acids than products prepared from the nonfermented samples.

INTRODUCTION

BEANS are important dietary foodstuffs in many developing countries. However, people who rely on beans as a protein source do not get the high quality protein that animal proteins provide.

Zamora and Fields (1979) increased the relative nutritive value and availability of some amino acids by natural lactic fermentation of cowpeas (*Vigna sinensis*) and chickpeas (*Cicer arietinum*) since fermented foods are important components of the diet in many parts of the world such as Southeast Asia, the Near East and parts of Africa. This process offers a possible means of improving diets in these areas.

In this study, products were developed from the fermented cowpeas and chickpeas. Soups were prepared from cowpeas while chips were made from chickpeas. Hedonic rating and nutritional quality of the products were determined. The product must be acceptabe for human use, particularly in the target area for the food involved. Also, if it is to help overcome the nutritional gap in the less developed countries, significant improvement in the nutritive quality of the product must be demonstrated.

MATERIALS & METHODS

Preparation and fermentation of beans

The preparation and fermentation, the relative nutritive value (RNV), available methionine, isoleucine, and tryptophan and riboflavin procedures are described in a previous publication (Zamora and Fields, 1979).

Preparation of chips and soups

Bean chips were prepared from nonfermented (CC) and fermented chickpea flours (FC). The pH of the fermented chickpeas was adjusted to the original pH of the beans (6.4) with baking soda. Salt, water and oil were added to nonfermented and fermented beans and mixed thoroughly. The resulting dough was rolled thinly (less than 4/25 cm), cut into 3.8 × 3.8 cm pieces, and deep fried in corn oil at 177 ± 1°C. The chips made from nonfermented samples were fried for 45 sec while the chips made from fermented beans were fried for 15 sec. The ingredients for chickpea chips were bean flour (145g), salt (6g), baking soda for fermented bean flour (6g), oil (6g), and water (90g).

Bean soups were made from nonfermented (CS) and fermented cowpeas (FS). The pH of one of the soups made from fermented beans (FS) was 4.2, which was the pH of the cowpeas after fermentation, and the other one (MS) was adjusted to pH 5.5 with baking soda. Powdered onion and salt were added to the soup. The ingredients for cowpea soups were bean flour (79g), salt (6g), baking soda for adjusting pH to 5.5 (6g), powdered onion (6g) and water (1240g).

Sensory evaluation of chips and soups

One piece each of the two samples of chips (CC and FC) was served on one paper plate to 99 consumers. Each plate was coded with 3-digit random code numbers for each sample (Snedecor and Cochran, 1967). Fifteen ml of soups (CS,FS,MS) were served hot in coded souffle cups under red lights. Both soups and chips were served together with a score sheet. The products were scored on a 5-point hedonic scale.

Data were analyzed by analyses of variance and Duncan's New Multiple Range Test (Duncan, 1955).

Nutritional evaluation of bean chips and bean soups

The soup was dried at 50–55°C for 48 hr in an air circulating incubator (National Model, National Appliance Co., Portland, OR). The chips and the dried soups were ground in a Kitchen Aid Mill (Model K 5-A, Hobart Corp., Troy, OH). Chemical and microbiological procedures of the nutritional evaluation are described in Zamora and Fields (1979).

RESULTS & DISCUSSION

Consumer evaluation of bean soups and bean chips

The mean score (3) for CS was significantly ($P < 0.05$) higher than the means for FS (2.0) and MS (2.3). On a 5-point scale, CS would be in the "neither like nor dislike" category while FS and MS would be in the "dislike" category. Many of the consumers commented on the acid taste of FS which they found disagreeable. The lowest score for each of the soups was 1 while the highest was 5 for CS and 4 for FS and MS.

There was also a significant ($P < 0.05$) difference between CC and FC. On a 5-point scale, CC with a mean score of 4 was in the "like" category, while FC, with a mean score of 3.3 was in the "neither like nor dislike" category. The color might have had some influence on the scores given by the consumers. FC samples were darker than CC sample. CC resembled the color of some commercial corn chips. FC was darker than CC even before frying. The cooking process made them even darker, probably due to Maillard reaction and possibly also due to carmelization of the sugars in the fermented beans. The fermented beans were expected to have more sugars than the nonfermented samples, since Desikarchar et al., (1960), as cited by Rajalakshmi and Vanaja (1967), reported increases in free sugars during idli (fermented product from rice and black gram) fermentation. However, these researchers made no mention of the effect of the increased sugar content on the color of the product.

Nutritional evaluation of bean soups

The RNV of CS was significantly ($P < 0.05$) lower than the RNV's of FS and MS. It is also illustrated in Table 1 that the RNV of MS was significantly ($P < 0.05$) higher than the RNV of FS. The methionine content of CS was

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Table 1—Nutrient contents of nonfermented and fermented cowpeas

Factors analyzed	Cowpea flour		Cowpea soup		
	Raw control	Raw fermented	CS	MS	FS
RNV (%)	85.44[a]	92.52[b]	76.90[c]	86.60[a]	82.66[d]
Methionine (mg/g N)	2.26[a]	15.51[b]	3.94[a]	12.03[c]	13.72[c]
Isoleucine (mg/g N)	4.42[a]	35.40[b]	15.67[c]	37.08[d]	31.38[b]
Riboflavin (mg/g sample)	2.42[a]	2.86[b]	2.25[a]	2.84[b]	2.18[a]

[a,b,c,d] Where superscripts differ within a factor analyzed, means differ significantly ($P < 0.05$).

Table 2—Nutrient contents of nonfermented (control) and fermented chickpeas

Factors analyzed	Chickpea flour		Chickpea chips	
	Raw control	Raw fermented	CC	FC
RNV (%)	83.27[a]	91.65[b]	83.71[a]	91.74[b]
Methionine (mg/g N)	1.61[a]	10.41[b]	0.03[a]	8.42[c]
Isoleucine (mg/g N)	2.22[a]	18.83[b]	2.32[a]	16.22[b]
Tryptophan (mg/g N)	13.13[a]	16.07[b]	10.66[c]	13.29[a]

[a,b,c] Where superscripts differ within a factor analyzed, treatments differ significantly ($P < 0.05$).

lower than either FS or MS, but no significant difference was detected between the two soups prepared from fermented beans. Isoleucine was lower in CS compared with either FS or MS. FS, however, had a significantly ($P < 0.05$) lower isoleucine value than MS. This might have been reflected in the difference between the RNV's of FS and MS. The amino acid balance of MS was probably better than FS. CS and FS contained significantly ($P < 0.05$) less riboflavin than MS. This might have had an influence on RNV also, since MS had a higher RNV than CS or FS.

Nutritional evaluation of bean chips

For all nutrients evaluated (RNV, methionine, isoleucine and tryptophan), FC had significantly ($P < 0.05$) higher values than CC. It was mentioned earlier that FC was darker than CC probably due to Maillard reaction. This reaction did not have a detrimental effect on RNV. Table 2 shows that the RNV of FC did not vary significantly from the RNV of the uncooked, fermented product.

The methionine content of CC decreased considerably compared to the value of the uncooked control chickpeas. FC also had a lower methionine content than the uncooked fermented sample. This suggests that frying, which involved high temperature, had a deterimental effect on the methionine content.

The controls (bean flour and chips made from the nonfermented flour) did not differ from each other in available isoleucine. Also, no differences were detected in the fermented samples (fermented bean flour and chips prepared from the fermented beans). Thus, frying did not destroy the isoleucine.

Tryptophan content of FC was significantly ($P < 0.05$) higher than that of CC. Frying destroyed some of the tryptophan, as illustrated by the data in Table 2. The uncooked, control chickpeas had significantly ($P < 0.05$) higher tryptophan content than CC, and the uncooked, fermented samples also had a higher value than did FC.

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A Research Note

ASCORBIC ACID IN PEAS COOKED BY MICROWAVES

L. B. MABESA and R. E. BALDWIN

ABSTRACT

Market samples of frozen peas were cooked with and without water in a domestic microwave oven (115V, 550 watts cooking power) and in an institutional microwave oven (220V, 1150 watts cooking power). Total ascorbic acid, as determined by the 2,4-dinitrophenylhydrazine method, was higher in peas cooked without water regardless of cooking appliance. Microwave cooked peas tend to have greater retention of total ascorbic acid than those cooked conventionally.

INTRODUCTION

THE IMPORTANCE of nutritional contribution and special chemical activity of ascorbic acid has been recognized by food processors. Some losses of ascorbic acid may occur during harvesting and handling due to enzymatic action, particularly when tissues are damaged by cutting or bruising. Further losses of this vitamin can be expected during subsequent processing and storage. Due to its sensitivity to handling and storage conditions, retention of ascorbic acid in frozen peas is an appropriate objective test for quality. Conflicting results have been reported concerning the effects of conventional and microwave cooking on ascorbic acid retention in vegetables. Stevens and Fenton (1951), Kylen et al. (1961), Eheart and Gott (1964), and Bowman et al. (1975) reported no significant differences in ascorbic acid retention of various vegetables cooked by microwave and conventional methods. Greater retention in microwave than in conventionally cooked vegetables was indicated in the studies of Campbell et al. (1958), Gordon and Noble (1959), Chapman et al. (1960) and Eheart and Gott (1964). In 1965, the latter researchers found higher retentions of ascorbic acid with conventional than with microwave cooking when the water was limited and stir-fry was superior to microwave cooking for retention of this nutrient. Since this lack of agreement is evident, this study was conducted to investigate the effects of cooking in a domestic and an institutional microwave oven on the ascorbic acid content of peas.

MATERIALS & METHODS

Sampling and cooking procedure

Details of the sampling and cooking procedures were described by Mabesa and Baldwin (1977). Briefly, the procedure consisted of combining 48 market samples of frozen peas (680g each), portioning them into 400-g lots in freezer bags and storing (−18°C) them until needed (maximum 12 days). Peas (400g) were cooked by each of five methods as follows: control samples simmered with 100g water for 8 min on a surface burner (Thermador Model TMH35A, Thermador Norris Ind., Los Angeles, CA 90040); peas cooked 8.0 min with 100g water and 6.5 min without water in a domestic microwave oven (Litton Minutemaster 416, Litton Industries, Minneapolis, MN 55411; 115V, 550 watts cooking power, 2450 MHz) and 6.0 min with 100g water and 5.0 min without water in an institutional microwave oven (Litton Menumaster; 220V, 1150 watts cooking power, 2450 MHz). All samples were covered during cooking. The cooking methods will be referred to as Conv, MW-115V+, MW-115V, MW-220V+ and MW-220V, respectively.

Determination of ascorbic acid

The method outlined by the Association of Vitamin Chemists (1966) for total ascorbic acid determination was followed. This procedure is an adaptation of that described by Roe and Hall (1939). It involves the oxidation of ascorbic acid to dehydroascorbic acid followed by conversion to diketogulonic acid which couples readily with 2,4-dinitrophenylhydrazine derivative which is highly stable when treated with 85% sulfuric acid. Photometric measurement of the color completed the procedure. Values reported herein include any diketogulonic acid which may have been present in the vegetable. The major concern, however, is the effect of cooking method on ascorbic acid, not the amount of ascorbic acid which varies considerably from one vegetable lot to another. To facilitate conversion of percent retention of ascorbic acid on wet weight to dry weight basis, the raw and drained cooked peas were blended and dried in a vacuum oven at 70°C for 12 hr. Percent retention was calculated by dividing content of cooked peas by content of raw and multiplying by 100.

Statistical analysis

After six replications were completed, the data were subjected to analysis of variance (Snedecor and Cochran, 1967) to determine the effects of cooking on ascorbic acid. When significant differences occurred, Duncan's (1955) new multiple range test was applied to locate the significant ($P < 0.05$) differences among means.

RESULTS & DISCUSSION

COOKING PEAS without added water, as in the MW-115V and MW-220V methods, resulted in lower moisture content than raw peas and those cooked by other methods. The MW-220V process resulted in the lowest moisture content of all treatments.

Ascorbic acid content and retention of all samples of cooked peas were significantly lower than that of the raw sample except for peas cooked by the MW-220V method. With the exception of the MW-115V+ cooking procedure, peas cooked by microwaves tended to be significantly higher in ascorbic acid content than peas cooked by the Conv procedure (Table 1). This trend also was noted by Stevens and Fenton (1951), Campbell et al. (1958), Kylen et al. (1961) and Bowman et al. (1975) although no statistically significant differences were established in these studies.

Peas with added water cooked in both microwave ovens contained significantly less ascorbic acid than their counterparts cooked without water. Also, peas cooked by the Conv method, which required added water, contained significantly less ascorbic acid than peas cooked by microwave methods without water (Table 1). Bowman et al. (1975) reported the same trend, and attributed losses to the leaching of ascorbic acid into the cooking liquids. Campbell et al. (1958) found 25–30% of the original ascorbic acid in the cooking liquid.

Peas cooked by the Conv and MW-115V+ methods did not differ significantly in ascorbic acid content (Table 1). This particular comparison agreed with the conclusions of

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Table 1—Mean[a] moisture and total ascorbic acid contents and retention of peas cooked by microwaves and conventional methods

Treatment	Moisture (%)	Total ascorbic acid			
		Wet basis		Dry basis	
		Content (mg/100g)	Retention (%)	Content (mg/100g)	Retention (%)
Raw	78.81[b]	21.73[a]	100.00[a]	102.69[a]	100.00[a]
Conv, 8.0 min	80.54[a]	15.52[d]	71.40[d]	79.79[c]	77.68[c]
MW-115V + 100g water, 8.0 min	80.04[ab]	15.59[d]	72.19[d]	78.59[c]	76.71[c]
MW-115V, 6.5 min	76.85[c]	20.61[b]	95.61[b]	89.40[b]	87.21[b]
MW-220V + 100g water, 6.0 min	78.63[b]	17.02[c]	78.28[c]	79.89[c]	77.78[c]
MW-220V, 5.0 min	73.97[d]	21.94[a]	100.96[a]	84.28[bc]	82.19[bc]

[a] N = 6. Where exponent letters differ within a column, means differ significantly ($P < 0.05$).

Thomas et al. (1949) that the cooking time and the amount of water are the determinants of the retention of nutrients in the cooked vegetables. Equal amounts of water were added to peas cooked by the Conv, MW-115V+ and MW-220V+ methods. Total cooking times were the same for the Conv and MW-115V+ processes. However, 2 min less time were needed to cook peas by the higher power MW-220V+ method. Therefore, both the addition of water and the cooking time affected the ascorbic acid content of the cooked peas regardless of the cooking appliance. Griswold (1962) emphasized that volume of cooking water was important and stated that increasing the volume of water caused lower retention of ascorbic acid.

Content and retention of ascorbic acid on a dry weight basis was significantly higher in samples cooked by the MW-115V process than in the other cooked samples, except those cooked by the MW-220V method (Table 1). Addition of water to peas cooked in the domestic microwave oven significantly decreased the ascorbic acid retention, but this effect was not evident in the peas cooked in the institutional microwave oven.

Based on the above results, it appears that waterless cooking in domestic microwave ovens has potential for preventing losses of ascorbic acid in cooked peas. This is of particular importance since cooking water is rarely consumed. The lack of agreement in reports of previous research on losses of ascorbic acid, probably was due to the differences in cooking procedure.

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A Research Note

LOSS OF GREEN COLOR OF REFRIGERATED AND NONREFRIGERATED SOUTHERN PEAS

W. C. HURST and G. A. SCHULER

ABSTRACT

Southern pea quality is judged commercially according to USDA grading standards. The presence of green color determines USDA grades of Southern peas. Since USDA testing procedures are subjective in nature and the objective of this investigation was to provide commercial Southern pea processors a practical means of preserving pea color, it was determined that measurements should be made subjectively to give validity to the results. This study shows that Grade A peas can be maintained by hydrocooling and holding them under refrigeration subsequent to the harvesting and shelling operations.

INTRODUCTION

ALTHOUGH SOME WORK (Smittle and Keyes, 1976) has been done to assess quality deterioration of Southern peas during commercial operations, no practical solution has been given to the problem of maintaining pea color from the field to the commercial processor. Drastic losses in green color occur due to long exposures of shelled peas to high field temperatures. This problem is of such magnitude that Grade A peas (USDA Standards for Grades of Frozen Field Peas and Frozen Black-eye Peas, 1976) harvested and shelled in the field normally become a grade lower by the time they reach the processing plant. Loss of grade due to color is of great economic significance to food processors since the Southern pea is a major horticultural crop grown in the southeast.

Hydrocooling has proven a rapid and effective method of precooling (Ryall, 1966) and Stewart and Barger (1960) demonstrated that temperatures of packed English peas could be reduced 10–15°C in 20–30 min when hydrocooled in ice water maintained at 2°C.

This investigation was designed to monitor present and commercially feasible pea handling operations and to collect information on post-harvest changes in Southern pea quality relating to hydrocooling and refrigerated and nonrefrigerated storage.

EXPERIMENTAL

SOUTHERN PEAS were mechanically harvested and shelled in the field. Shelled peas were conveyed from combine storage bins into an open-bed, 18 ton truck for transport 15 miles to a cleaning plant. Approximately 16,200 lb of peas were field harvested and shelled over a 6-hr period.

A total of three trips were made to the cleaning plant. Each pea loading and in-transit operation took 1.5–3.0 hr. Pea temperatures at the end of each trip were 28°C, 31°C and 34°C, respectively. Following arrival at the cleaning plant, peas (8,230 lb) of the first 2 loading operations were treated as follows: First crate 1 was filled with uncleaned peas (1000 lb) and loaded into an 18-ton refrigerated truck. Secondly, uncleaned peas (2060 lb) were dried 5 hr at 45°C in a commercial seed dryer, distributed equally into crates 6 and 12, and loaded on the 18-ton refrigerated and open bed trucks respectively. Third, uncleaned peas (5170 lb) were distributed as bulk in the open-bed truck to simulate present commercial practices. The dimensions of all crates were 122 cm × 122 cm × 107 cm.

The cleaning operation was utilized to remove shells, skins, field debris, and accumulated heat from shelled peas (7970 lb) of the third load. Two thousand pounds of cleaned peas were discharged equally into crates 2 and 8 with each being placed respectively in the refrigerated and open-bed trucks. A representative sample of cleaned peas was drawn, given a 3-min blanch to set the color, and iced for later grading at the processing plant. Remaining cleaned peas (5970 lb) were distributed into 7 crates, loaded in the refrigerated truck and hauled 30 mi to a hydrocooler. On arrival crate No. 7 was immediately pre-cooled with crushed ice and loaded in the open-bed truck while the remaining 6 crates were placed in the hydrocooler. Sprays of water cooled with ice to 1°C passed through the crates cooling the peas in 45 min. After hydrocooling peas of crates 4 and 10 were treated with an aqueous 50 ppm Cl_2 solution, peas of crates 5 and 11 were treated with an aqueous 200 ppm SO_2 solution, and peas of crates 3 and 9 had no treatment other than precooling. Crates 3, 4, and 5 were loaded into the refrigerated truck while crates 9, 10 and 11 were loaded into the open-bed truck. Both trucks left the hydrocooler at the same time and proceeded to the processing plant located 125 mi distant.

Internal temperatures were recorded at 60 cm depths in the center of each crate 8 hr and 20 hr after leaving the hydrocooler. Composite 10-oz samples representing peas from five locations (taken at each corner and 60 cm depths in the middle of each crate) were drawn from the crates of each truck and checked for physical defects such as eyebleed, slime, and sprouting. A 3-min blanch was given all samples which were subsequently frozen and stored at −10°C until graded. Peas were graded according to USDA Standards for Grades of Frozen Field Peas and Frozen Black-eye Peas which require 10% distinct green color to meet Grade A standards. A 5-oz frozen portion was selected at random from each 10-oz sample, weighed, and placed in a shallow pan of water to thaw. After removing any trash an experienced grader segregated green peas by count from the colorless peas in each sample. All data are recorded in Table 1.

RESULTS & DISCUSSION

GREEN COLOR of Southern peas can best be preserved by hydrocooling cleaned peas with subsequent transport and storage under refrigerated conditions. Bulk pea temperatures of 30°C were reduced to 7°C in 45 min. Although there was a slight rise in temperature between the 8-hr and 20-hr sampling periods, the effects on color were negligible as indicated by the superior % green peas found in crates 3, 4 and 5 (Table 1). Differences in temperature readings of peas can be attributed in part to the fill of the crates. Since crates 3 and 5 were only half-filled with peas, it is assumed less heat was generated due to pea respiration. An important matter to reconcile is whether the refrigeration system in use on this truck could absorb the heat generated by 38 filled pea crates which constitutes a full load and still maintain the temperature at 13°C. Further research needs to be done in this area. Differences between hydrocooling peas and hydrocooling followed by treatment with a bacteriostatic agent were negligible, although peas treated with SO_2 had a slightly higher % green color. No visual defects were found on 20-hr old peas which had been cleaned, hydrocooled and stored under refrigeration. Results of grading the pea sample blanched immediately after cleaning showed an impressive 39.47% green color. It appears that blanching fixes green pea color, however, the use of field blanching to

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Table 1—Effects of hydrocooling, refrigerated and nonrefrigerated storage on Southern pea quality

Crate no.[a]	Treatment applied	Hydro-cool (°C)	8-hr samples[b] Temp (°C)	8-hr samples[b] Green pea[d] (%)	8-hr samples[b] Defects (visual)	20-hr samples[c] Temp (°C)	20-hr samples[c] Green pea[d] (%)	20-hr samples[c] Defects (visual)
1	no cleaning		50 (+)	14.63	none	50 (+)	4.67	eye bleed, slime
2	clean only		50 (+)	7.59	none	50 (+)	2.95	eye bleed, slime
3	clean, hydrocool	7	7	21.86	none	9	19.01	none
4	clean, hydrocool Cl_2 (50 ppm)	7	8	17.40	none	13	18.61	none
5	clean, hydrocool SO_2 (200 ppm)	7	9	20.09	none	9	21.19	none
6	dried, uncleaned		45	sample lost	none	40	10.26	mold
7	clean, iced	7	29	16.82	none	50 (+)	9.01	eye bleed, slime, sprout
8	clean only		30	16.94	none	50 (+)	9.63	eye bleed, slime, sprout
9	clean, hydrocool	7	29	20.04	none	50 (+)	6.96	eye bleed, slime, sprout
10	clean, hydrocool Cl_2 (50 ppm)	7	21	23.40	none	50 (+)	7.26	eye bleed, slime, sprout
11	clean, hydrocool SO_2 (200 ppm)	7	25	20.92	none	50 (+)	8.24	eye bleed, slime, sprout
12	dried, uncleaned		27	12.28	none	29	10.55	none
	uncleaned bulk control		29	16.38	none	50 (+)	4.97	eye bleed, slime, sprout

[a] Crates 1–6 refrigerated; crates 7–12 nonrefrigerated
[b] Ambient temp = 24° C; Refrigerated = 3° C
[c] Ambient temp = 35° C; Refrigerated = 4° C
[d] % Green pea = (green peas/no. of peas in sample) X 100.

retain color is not commercially feasible. Temperature readings taken of peas in crates 1, 2 and 6 which were not precooled indicate that hydrocooling is necessary to reduce temperature. Exposure of peas to prolonged high temperatures caused a significant decrease in the % green peas and the occurrence of eyebleed, slime and mold after 20-hr refrigerated storage. Apparently the difference between pea temperature and the refrigerated surrounding caused condensation to form on some peas of crate 6 enabling mold to grow easier. Temperature readings of peas held under nonrefrigerated conditions showed a positive increase with an increase in ambient temperature. Hydrocooling peas in crates 7, 9, 10 and 11 before loading on the open-bed truck arrested color loss for 8 hr, but was not sufficient for 20 hr. It should be noted that the 8-hr period before sampling represented dark hr and the same period in the sun most likely would not furnish the same results. After 20 hr under ambient conditions these peas exhibited eyebleed, slime and sprouting. Quality deterioration of cleaned peas (crate 8) and uncleaned peas (bulk control) not hydrocooled was manifested by a lower % green peas after 8 hr followed by eyebleed, slime, and sprouting after 20 hr storage under ambient conditions. Pea temperatures and color loss did not fluctuate greatly for dried peas in crate 12 for the two sampling periods.

The data in Table 1 demonstrate that Southern peas can meet Grade A standards if they are hydrocooled and refrigerated subsequent to field operations. Based on the obviously significant difference in using this treatment over any other in the investigation, it was decided that a statistical analysis was not needed to support the validity of the results. It is the authors recommendation that present commercial Southern pea handling operations be adapted to include hydrocooling and refrigerated storage to insure maximum pea color and quality.

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GERMINATION AND RELATIVE NUTRITIVE VALUE OF CORN MEAL AND CORN CHIPS

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ABSTRACT

Microbiological assay showed a significant increase in niacin ($P < 0.05$) and riboflavin ($P < 0.01$) in corn meal made from germinated corn as compared to the control corn meal. The thiamine content (microbiologically determined) remained the same as that in the control meal. The relative nutritive value (as determined by *Tetrahymena pyriformis W*) of the corn meal made from germinated corn was significantly ($P < 0.01$) higher (86%) than that of the control corn meal (69%). Processing the germinated corn meal into corn chips, decreased the levels of vitamins and %RNV but these values remained higher than the control chips. Bitterness and an earthy flavor were defects in the corn chips made from germinated corn.

INTRODUCTION

IN MANY developing countries, corn is the main staple of the diet of low income families. The protein quality of corn is low because the protein of corn lacks quantities of lysine and tryptophan (FAO, 1970). Economical ways to correct these deficiencies are highly desirable.

Wang and Fields (1978) reported that the highest relative nutritive value (RNV) occurred at 3–4 days at 30°C. In this experiment the % RNV increased from 66.8 to 99.5. The data of Wang and Fields suggested that germination of corn was a procedure that can be used at home to produce a more nutritious ingredient (with a better amino acid balance) from which to produce foods in developing countries or in the United States.

During the germination process, Fordham et al. (1975) found that ascorbic acid was higher in germinated beans and peas than in nongerminated dry seeds. The amounts of niacin, pyridoxine, pantothenic acid, inositol, tocopherol and vitamin K increased in some germinated legumes (Banerjee et al., 1955). The contents of ascorbic acid, riboflavin and niacin increased in germinated soybeans (Chen, 1970).

The nutritional value of corn is due to the amino acid balance, the vitamins, minerals and caloric contents. Since there was evidence that germination could improve both the amino acid balance and vitamin content, this research was instituted to determine the vitamin content and RNV after germination and to determine the acceptability of corn chips made from germinated corn.

MATERIALS & METHODS

Sample preparation

A flow chart showing the method of sample preparation is given in Figure 1.

Shelled, yellow field corn (*Zea mays*) was obtained from a local dealer in Columbia, MO. Broken and moldy seeds (visually) were removed by hand during and after screening the seeds. The seeds were washed three times in tap water, during which the floating seeds were discarded. The seeds were divided into two lots. One lot dried at 50°C (7–8 hr) in flowing air in a Freas Model 835 dryer were used as control. The other lot was soaked in four to five times the volume of the seeds in tap water at room temperature for 16–18 hr.

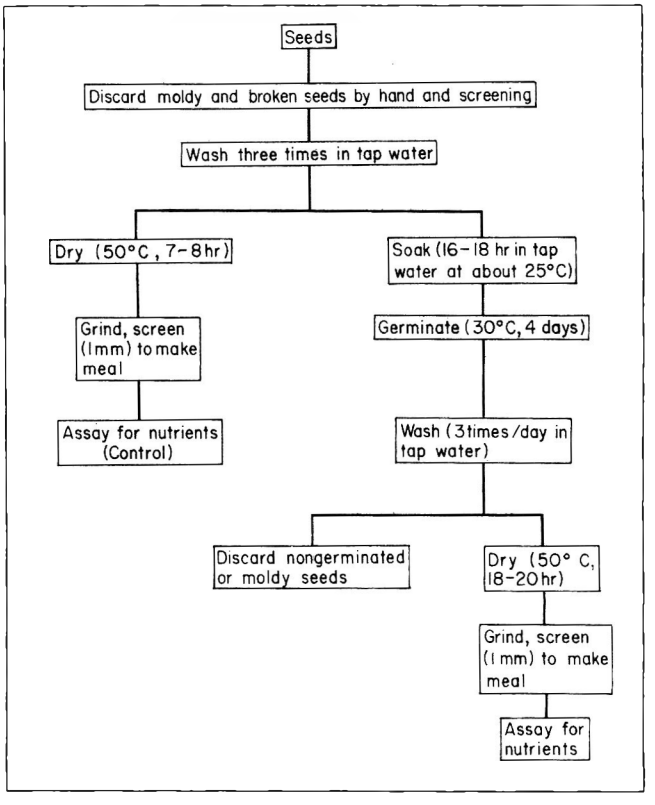

Fig. 1—Flow chart for germination of corn and preparation of sample.

The seeds were germinated in a wooden tray with a screened bottom measuring 19½ inches × 25 inches. The bottom was lined with wet paper towels. The soaked grains were washed in tap water and spread evenly about ½ inch in thickness. The seeds were covered with two layers of wet paper towels, and germinated in a 30°C air-circulating incubator for 4 days. The germinated seeds were washed three times each day with tap water during the germination period.

The germinated seeds were separated from the nongerminated and moldy seeds daily. The selected germinated seeds were washed, and then dried at 50°C for 18–20 hr.

The dried seeds were ground in a Thomas-Wiley Laboratory Mill (Model 4) using a 1-mm screen. The dried corn meal was sealed in glass jars and stored at 2°C until biological analyses were made and the corn chips were prepared (all analyses were made within 1 month).

Chemical and microbiological analyses

Moisture and micro-Kjeldahl were the methods of AOAC (1975). Relative nutritive value method is given in Wang and Fields (1978).

Assay for riboflavin, niacin and thiamine

Preparation of the samples was according to the Association of Vitamin Chemists (1966). Microbiological assays were the methods recommended by Difco (1977).

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Table 1–Means[a] on a dry-weight basis of nutritive value of corn meal and corn chips made from nongerminated and germinated corn.

	Corn meal				Corn chips			
Treatment	Riboflavin (mg/100g)	Niacin (mg/100g)	Thiamine (mg/100g)	RNV (%)	Riboflavin (mg/100/g)	Niacin (mg/100g)	Thiamine (mg/100g)	RNV (%)
Nongerminated	0.10a	2.19a	0.31a	69a	0.09a	1.97a	0.17a	62a
Germinated	0.27b	3.18b	0.33a	86b	0.21b	3.21b	0.20a	73b

[a] Mean of four replicates; means followed by the same letter are not significantly different ($P < 0.01$ for riboflavin and % RNV, $P < 0.05$ for niacin in corn meal; ($P < 0.01$) for all nutrients in corn chips).

Preparation, assay for nutrients, and sensory evaluation

The preparation, assay and sensory evaluation of the corn chips are described by Hashim (1977).

Statistical analyses

Data were analyzed by methods of Snedecor and Cochran (1967).

RESULTS & DISCUSSION

Corn meal

Results of the analyses on the corn meal made from nongerminated and germinated corn are given in Table 1. Germination increased the riboflavin from 0.10 mg/100g to 0.27 mg/100g and niacin from 2.19 mg/100g to 3.18 mg/100g significantly ($P < 0.01$ and $P < 0.05$, respectively) whereas the thiamine content remained about the same in both meals. The increase with germination in RNV was 17% ($P < 0.01$). The RNV data are in agreement with data of Wang and Fields (1978).

Corn chips

Data in Table 1 show that riboflavin and niacin contents were higher in chips made from germinated corn than in the control chips. The RNV of chips made from the fermented corn was significantly higher ($P < 0.01$) than chips made from the nonfermented corn.

Germination influenced the texture of the chips. Chips made from germinated corn (score of 3.4) were not as brittle as the control (score of 4.6). However, germination did not influence significantly the grainy character of the chips since the scores were 3.9 for the control and 3.4 for the germinated.

Flavor was influenced by germination even though the chips were made with only about 74% germinated corn meal. The following flavor characters were significantly different ($P < 0.05$). The control scores are listed first followed by scores of chips made from germinated corn meal: corn flavor (score of 3.8 vs 2.5), sweet (score of 2.0 vs 2.6), bitter (score of 2.0 vs 4.2) and earthy (score of 2.6 vs 4.0). There was no significance difference in the spicy flavor of the control chips and chips made from germinated corn meal (scores of 3.6 vs 3.2).

Panel members objected to the bitter-earthy flavor of corn chips containing germinated corn meal. If these two major defects can be corrected, more nutritious foods can be fabricated from germinated corn.

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A Research Note

INTENSIFICATION OF FRUIT-FLAVORS BY ASPARTAME

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ABSTRACT

Orange-, cherry-, and strawberry-flavored noncarbonated beverages containing 0.065% Aspartame were equivalent in sweetness to beverages containing 9.52% sucrose. The same flavors in gelatin desserts sweetened with 15% sucrose were equivalent to those sweetened with 0.070% Aspartame. Sensory scores indicated that both orange- and cherry-flavored beverages had a significantly higher intensity of fruit-flavor than their counterparts sweetened with sucrose. However, in the strawberry-flavored beverages or in the three flavored gelatins, no such differences in intensity of fruit-flavors were found between products sweetened with Aspartame or sucrose.

INTRODUCTION

PREVIOUS RESEARCH (Cloninger and Baldwin, 1970, 1974) suggested that Aspartame has considerable potential as a low-calorie sweetener. The relationship between sweetness of Aspartame and sucrose is almost linear when plotted on a log-log scale. Aspartame is 182 times sweeter than a 2% sucrose solution but only 43 times sweeter than a 30% sucrose solution.

The clean, full sweetness of Aspartame is similar to sucrose and complements other flavors. It has no bitterness and blends well with food flavorings (Beck, 1974; McCormick, 1975; Pangborn and Larson, 1975). Cloninger and Baldwin (1974) determined that orange-flavored beverage sweetened with 0.075% Aspartame or 2% sucrose did not differ in sweetness, sourness or acceptability. However, it was suggested by Beck (1974) and McCormick (1975) that Aspartame may have a potentiating effect on certain fruit-like flavors. Therefore, this study was undertaken to determine if Aspartame affected the intensity of orange-, cherry- or strawberry-flavors in a beverage or in gelatin.

EXPERIMENTAL

General procedures for sensory testing

Panelists were seated in individual booths in a sensory testing facility. Verbal instructions were given prior to each series of tests. Panelists were instructed to swallow neither the test samples nor the charcoal-filtered water provided for rinsing their mouths. They were requested to hold the sample in their mouths and to distribute it throughout their mouths so that an accurate response on sweetness or intensity of fruit-flavor could be determined.

Sweetness equivalents

Although 0.075% Aspartame was found by Cloninger and Baldwin (1974) to be comparable in sweetness to 10% sucrose in noncarbonated orange-flavored beverage, it was necessary to test the sweetening effect of Aspartame in cherry- and strawberry- as well as in orange-flavored beverage. For these determinations, a laboratory panel of staff (N = 8) was employed for a series of paired sensory tests on the fruit-flavored beverages sweetened either with Aspartame or with 9.5% sucrose, the level of sugar recommended by the manufacturer (Kool-Aid, General Foods Corp., White Plains, NY 10625). Beverage mix (0.37%) was dissolved in redistilled water prior to adding the sweetener. Concentrations of 0.095%, 0.075%, 0.070%, 0.065% and 0.060% Aspartame were prepared. All three flavors of beverage were tested with only one level of Aspartame at one panel session. Samples (15 ml, 4° C) were served in pairs, one of which was sweetened with sucrose and the other with Aspartame. Panelists were asked to indicate the sweeter sample within each pair.

Sweetness equivalents of Aspartame to sucrose in cherry-, orange- and strawberry-flavored gelatin were confirmed by paired sensory tests also. Concentrations of 0.15%, 0.12%, 0.10% and 0.07% Aspartame were paired in random order with gelatin sweetened with 15% sucrose, the usual level of sugar in gelatin-based desserts. Fifteen grams of unflavored gelatin were heated (boiling water bath) in approximately 300 ml of fruit-flavored beverage prepared as described previously. For the control samples, sucrose was dissolved in the hot solution which was then cooled to room temperature and brought to 1000 ml volume with fruit-flavored beverage. For products sweetened with Aspartame the sweetener was added after the gelatin mixture had cooled to room temperature and prior to bringing the solution to 1000 ml with fruit-flavored beverage. Although Aspartame is stable to mild heating (McCormick, 1975), this step was a precaution against possible heat-induced changes. Samples for the sensory panel were prepared by pouring approximately 20 ml of the gelatin mixture into 3-oz portion cups which were refrigerated (4 ± 1°C) 24 hr prior to evaluation. Since no previous information concerning sweetness equivalents of Aspartame to 15% sucrose in gelatin was available, responses of a relatively large number (50) of untrained judges (staff and students) were solicited.

Intensity of fruit-flavor

A design for evaluating intensity of flavor was selected which demonstrated minimal residual or carry-over flavor in a previous study (Korschgen et al., 1978). Twenty ml of fruit-flavored beverage and two 20-ml portions of charcoal filtered water for clearing the mouth were served to each panelist. Each of these was held in the mouth 10 sec before expectoration. Samples were served at 60-sec intervals. A similar procedure was followed for gelatin, but panelists spooned the product into their mouths.

One series of sensory tests was completed on fruit-flavored beverages and one series on fruit-flavored gelatins. The order of serving cherry-, orange-, or strawberry-flavored products was predetermined so that each flavor was served an equal number of times in the first, second or third position. The order of serving products sweetened with Aspartame or with sucrose was randomized. A total of 50 untrained judges (staff or students) scored the products on an intensity scale of 1, very weak fruit-flavor to 7, very strong fruit-flavor. A second series of tests (N = 50) was conducted in which a sample sweetened with Aspartame and one sweetened with sucrose were presented as a pair. Panelists were asked to indicate the sample within the pair which had the more intense fruit-flavor.

Statistical analyses

To determine the significant differences for the paired comparisons, the total number of judgments indicating greater sweetness or more intense flavor, depending on the test, for each sweetener was determined. This number was compared with the number required for a significant ($P < 0.05$) difference with a one-tailed paired test (Amerine et al., 1965). To analyze the scores for intensity of fruit-flavor, t-values were computed on data obtained for each flavor of beverage and of gelatin.

RESULTS

Sweetness equivalents

Since the beverage mix contained no carbohydrates, all sweetening effects were attributable to the respective sweet-

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Table 1—Mean scores[a] for intensity of fruit-flavor in beverages and in gelatins sweetened with sucrose or with Aspartame.

Flavor	Beverage			Gelatin		
	Sucrose (9.52%)	Aspartame (0.065%)	t-value	Sucrose (15%)	Aspartame (0.070%)	t-value
Orange	4.5 ± 0.19	5.4 ± 0.20	4.56*	4.6 ± 0.25	4.8 ± 0.21	0.59
Strawberry	4.8 ± 0.23	5.3 ± 0.16	1.20	3.5 ± 0.23	3.6 ± 0.22	0.30
Cherry	5.0 ± 0.19	5.8 ± 0.16	3.05*	4.4 ± 0.21	4.5 ± 0.23	0.30

[a] Means (N = 50) and standard errors of mean. Range of scores: 7, very strong; to 1, very weak.
* $P < 0.05$.

eners. A concentration of 0.065% Aspartame did not differ significantly in sweetness from 9.5% sucrose in non-carbonated beverage prepared with orange-, strawberry-, or cherry-flavored mix. In fruit-flavored gelatin, 0.070% Aspartame was equivalent in sweetness to 15% sucrose. Therefore, these concentrations of Aspartame and sucrose were used for the major part of this investigation which dealt with the intensity of fruit-flavors. At these concentrations, Aspartame was approximately 150 and 200 times sweeter than sucrose in the beverages and gelatin, respectively.

Intensity of fruit-flavor

When the intensity of fruit-flavor was scored by the sensory panel, both orange- and cherry-flavored beverage sweetened with Aspartame were significantly more intense in fruit-flavor than the counterpart sweetened with sucrose. However, no significant difference in intensity of strawberry-flavored beverage was found (Table 1). These results were confirmed by a paired comparison test (Table 2).

In gelatin, no significant differences in intensity of fruit-flavor were found between products sweetened with Aspartame and sucrose (Table 1). This also was confirmed by a paired comparison test (Table 2).

Discussion

In the two instances where intensification of flavor occurred, the magnitude on a 7-point scale was similar: 0.8 for cherry- and 0.9 for orange-flavored beverage. Since intensification was not found with strawberry-flavored beverage, it appears that specific flavor components must enter into the reaction with Aspartame. Gelatin, however, inhibited this reaction. In fact, gelatin appeared to depress the flavor intensity of products sweetened either with sucrose or with Aspartame. The strawberry-flavored gelatin sweetened with sucrose was an exception and was just as intense in flavor in the gelatin product as in the beverage (Table 1).

Although response to complex fruit flavors may differ from that observed with primary tastes, depression of primary taste intensity by gelatin has been well documented with sodium chloride, sucrose, caffeine and tartaric acid (Mackey and Valassi, 1956; Mackey, 1958). Moskowitz and Arabie (1970) found a power function with a negative slope that was generally applicable to the relation between taste intensity of glucose, citric acid or sodium chloride and viscosity of aqueous systems thickened with carboxymethylcellulose. However, as pointed out by Pangborn et al. (1973), both the nature of the thickening agent and of the taste substance may influence the response to taste intensity.

Table 2—Number of judgments indicating greater intensity of fruit-flavor within a pair[a] of samples

Flavor	Beverage		Gelatin	
	Sucrose (9.52%)	Aspartame (0.065%)	Sucrose (15%)	Aspartame (0.070%)
Orange	17	33*	23	27
Strawberry	19	31	27	23
Cherry	17	33*	30	20

[a] N = 50.
* $P < 0.05$.

Knowledge of the interrelationship of tactile and gustatory properties is of major importance to segments of the food industry concerned with flavor intensity and quality of products with various textural properties. Although flavor potentiation was not apparent with Aspartame in gelatin products, its selective flavor potentiating property may prove advantageous in certain aqueous product formulations.

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A Research Note

EXTRUDED CORN FLOUR AS AN ALTERNATIVE TO LIME–HEATED CORN FLOUR FOR TORTILLA PREPARATION

CARMEN D. BAZÚA, ROSAURA GUERRA and HANK STERNER

ABSTRACT

Experiments were conducted to produce an extruded corn dough (*masa*) and meal with the same organoleptic properties as lime heat-treated corn dough. Dry, raw corn kernels were ground in a cereal grinder. The resulting meal was combined with calcium hydroxide in a powder mixer and fed continuously through a Wenger X-5 Extruder along with a controlled flow of cold water. The dough and tortillas made in this way were compared with those made by traditional lime heat-treatment methods for organoleptic properties and total lysine, tryptophan and protein content. No appreciable differences were noted at comparable use levels of calcium hydroxide. Better color and smaller tryptophan losses were obtained with extruded products using 0.2% calcium hydroxide than with the lime heat-treatment method using 1.0% calcium hydroxide.

INTRODUCTION

FOR CENTURIES corn has been the staple food in Mexico and tortillas the main form of consumption. Dough (*masa*) for tortillas is made using a traditional cooking method called "nixtamalizacion," derived from the Nahuatl *nextli*: ashes or lime ashes, and *tamalli*: corn dough. "Nixtamalizacion," or lime heat-treatment, involves alkaline cooking by boiling corn grains in lime water. This method has been described and studied by several authors (Illescas, 1943; Bressani et al., 1958; Del Valle, 1972; Katz et al., 1974).

Research has now been conducted (Bazúa et al., 1976) for employing extrusion as an alternative method of producing the corn dough. The purpose of this preliminary study was to produce a dough and tortillas with the same organoleptic qualities as those resulting from lime heat-treatment.

MATERIALS & METHODS

COMMERCIAL VARIETIES of white crystalline corn were used for the experiments. One yellow crystalline variety was included for the purpose of color comparison. (Yellow corn products have generally low acceptability in Mexico). Extruded and lime heat-treated corn doughs and flours were obtained from these samples. Powdered lime [$Ca(OH)_2$] was used for the alkaline treatment in the laboratory. Commercial instant corn flour (*masa harina*) was employed for comparison with the products obtained in the laboratory.

The procedure for making the traditional dough was as follows: 100g corn grain were boiled 20 min in 300 ml of water containing 1g of $Ca(OH)_2$. This mixture was allowed to stand overnight (15 hr) and then the supernatant was decanted and the grain rinsed thoroughly with water. The alkaline-cooked corn was ground in a hand grinder. The resulting dough was then shaped into 30g balls and pressed with a hand tortilla maker to an approximate diameter of 10 cm. Tortillas were cooked on a griddle 1 min on each side.

The procedure for making the extruded dough was as follows: Corn grain was ground in an electric Superline S cereal grinder until the particle size was between 50 and 80 mesh, The raw cornmeal and $Ca(OH)_2$ were combined in a powder mixer, each batch with a different $Ca(OH)_2$ concentration, ranging from 0.1 to 1.0g $Ca(OH)_2$ to 100g cornmeal. A Wenger X-5 extruder without a die plate was employed for extruding the dry mix. Description of this equipment is given by Conway and Anderson (1973). The extruder was started with a water flow setting of 80 ml/min. Then, the dry mix (cornmeal-$Ca(OH)_2$) was fed through a hopper and conveyed to the extruder. Flour feed rate was 300g/min, and water rate was decreased gradually until a dough with the proper consistency was obtained. Water rate was at this point 60 ml/min. The extruder was shut off gradually by decreasing the solids flow rate and increasing the water flow rate until only water flowed out. Tortillas from extruded dough were made following the same procedure as for traditional dough.

Table 1–Color analyses for cornmeals

	L	a	b	ΔE
Calibration	+95.8	−1.4	+2.6	
Masa Harina (lime heat-treated)	+86.5	−3.4	+16.8	0
White corn (extruded, 0.2% $Ca(OH)_2$)	+86.3	−2.9	+15.5	1.4071
White corn (extruded, 1% $Ca(OH)_2$)	+80.8	−3.2	+26.5	11.2525
Yellow corn (extruded, 1% $Ca(OH)_2$)	+75.7	−3.8	+30.0	17.0599

Consistency in doughs, which gives an indirect measurement of viscosity, was measured with a consistometer (Haake, Germany). Operating procedure was as follows: A cylinder was filled with dough and maintained at a constant temperature of 30°C with a water bath. A piston was placed on cylinder's top, a known stress was applied on the piston, and the time elapsed in a given displacement of the piston was recorded. The viscosity of the dough in centipoises was calculated using the formula

$$\mu = \frac{G\,t\,K}{S}$$

where μ is the viscosity, in centipoises; G is the stress applied, in grams; t is the time, in sec, needed to displace the piston a distance S, in mm; and K is a constant for the cylinder used. Doughs made by the traditional process were found to have viscosities ranging from $6-8 \times 10^5$ cp. Extruded doughs with viscosities within this range were considered adequate for making tortillas.

Instant *masa* flours were made from doughs obtained by both methods. The doughs were dried with warm air in a tunnel drier (Aeroglide Model R-1-12-6-G) until a moisture content of approximately 10% was reached. A low drying temperature (60°C) was maintained to avoid color changes in the product. Other than temperature control, no attempt was made to determine optimum drying conditions.

The dried products were ground in the same cereal grinder to an average particle size of 60 mesh, and stored in 1 kg plastic bags, made of heavy 3 mil polyethylene film (Curtin Matheson Scientific Co.) at room temperature (approx 20°C). Stored flours were used within a week's period to make tortillas and present them to a panel of experts for organoleptic testing.

A panel of 10 trained people performed hedonic scalar tests for color, odor, flavor and texture to compare tortillas made from extruded and lime heat-treated flours and *masas*. Texture was tested as

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Table 2—Chemical analyses for corn kernels, meals and tortillas

	Whole raw corn kernel (Control)	Cornmeals			Tortillas	
		Lime heat treated (1% Ca^{++})	Extruded		Lime heat treated (1% Ca^{++})	Extruded (0.2% Ca^{++})
			0.2% Ca^{++}	1% Ca^{++}		
Total protein (g/100g sample)	10.43	10.40	9.60	9.90	10.20	9.80
Total lysine (g/100g protein)	2.76	2.57	2.41	2.32	2.54	2.39
Total tryptophan (g/100g protein)	0.765	0.505	0.781	0.585	0.570	0.704

olding and rolling qualities. Color tests were also conducted using a Neotec colormeter, following manual instructions.

The total protein content of the raw corn, flours and tortillas was determined by micro-Kjeldahl analysis (AOAC, 1970). Total lysine was determined by the Tsai method as modified by Villegas and Mertz (1971), and total tryptophan was measured using the Opienska-Blauth method as modified by Hernandez and Bates (1969).

RESULTS & DISCUSSION

IT WAS FOUND that $Ca(OH)_2$ concentrations affected the color of extruded *masas* as well as other properties. A level of 0.2%, based on raw cornmeal, was the optimum concentration for odor, color, flavor and texture (viscosity) in the extruded *masas*. Panelists could not detect differences in odor and textural properties (folding and rolling) of tortillas made from extruded *masa* and tortillas made from lime heat-treated *masa*.

In the lime heat-treatment, a considerable amount of calcium is removed during rinsing procedures. In the extrusion process, all the calcium added is preserved in the dough. Thus, a lesser amount is needed to achieve the same level of hydrolysis.

Table 1 presents the results of color testing with the Neotec colormeter of the flours obtained by the extrusion and lime heat-treatment methods (yellow corn flour was used only for comparison, given its low acceptability in Mexico). Glasser et al. (1958) developed equations to represent accurately colors in homogeneous colored solids. In these equations, L, a, and b, are color coordinates representing lightness, red-greenness and yellow-blueness, respectively. Total color difference, ΔE, in the cube-root coordinate system is expressed as follows:

$$\Delta E = (\Delta L^2 + \Delta a^2 + \Delta b^2)^{1/2}$$

The last column in Table 1 presents total color differences of extruded and dried samples with respect to the commercial lime heat-treated cornmeal (*masa harina*). The results agree with panelists' judgements that white corn extruded and dried meals processed with 0.2% $Ca(OH)_2$ gave a color almost equal to lime heat-treated corn products (typical dustrial tolerances for ΔE are around 1.0 to 1.5).

Table 2 presents the results of chemical analyses performed on raw white corn, meals, and tortillas. These data make clear the effect of $Ca(OH)_2$ concentration on tryptophan losses. In extruded products processed with 0.2% $Ca(OH)_2$, approximately 8% of the total tryptophan was lost a making tortillas while with 1% $Ca(OH)_2$ in lime heat-treated products over 25% of the total tryptophan was lost. These results corroborate previously reported effects of lime on tryptophan (Bressani et al., 1958).

With respect to total lysine, the differences between extruded and lime heat-treated products possibly slightly favored lime heat-treatment over extrusion. Bookwalter et al. (1974) presented results on available lysine changes in corn during extrusion, and no appreciable differences were found.

No appreciable difference was found for total protein in the extruded and lime heat-treated samples. The small differences noted (less than 4%) might be attributed to experimental error.

Further research is necessary to compare the nutritional value of the extruded products and traditional ones. Organoleptic results, however, indicate that it is possible to make culturally acceptable tortillas using extrusion as an alternative process to lime heat-treatment.

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A Research Note

A COMPARISON OF THE SUITABILITY OF THREE HYDROGEN DONORS IN THE DETERMINATION OF PEROXIDASE ACTIVITY

M. MARSHALL and G. W. CHISM

ABSTRACT

A plot of initial velocity versus increasing o-dianisidine concentration results in a nontypical Michaelis-Menten curve. Further analysis points out that the recommended o-dianisidine concentration for assaying peroxidase is below saturation and thus may give erroneous results. The data show that care should be taken when using o-dianisidine as a substrate for measuring total peroxidase activity. Guaiacol and pyrogallol are shown to be suitable substrates for peroxidase determination.

INTRODUCTION

PEROXIDASES' (E.C. 1.11.1.7) ubiquitous occurrence and stability has been studied in a number of food systems. Isoperoxidase and/or total peroxidase activity has been studied in relation to quality in orange juice (Bruemmer et al., 1976), blue crabmeat (Burnette and Flick, 1977), sweet potato (Buescher et al., 1975; Haard and Marshall, 1976), pear (Ranadive and Haard, 1972), banana (Nagle and Haard, 1975), green beans (Gkinis and Fennema, 1978) and blanched vegetables (Joslyn, 1970) to name a few.

Peroxidase assays use a wide variety of H-donors to determine enzyme activity. There are three hydrogen donors commonly used to assay peroxidase activity. These include o-dianisidine (Worthington, 1972; Shannon et al., 1971; Shinshi and Noguchi, 1975), guaiacol (Wise and Morrison, 1971; Gove and Hoyle, 1975, Birecka and Catalfamo, 1975) and pyrogallol (Srivastava and van Huystee, 1977).

The objective of this study was to examine, using simple kinetic parameters, the three substrates (o-dianisidine, guaiacol, pyrogallol) for their suitability as H-donors in determining total peroxidase activity.

MATERIALS & METHODS

HORSERADISH PEROXIDASE (HRP) (Type 1) was obtained from Sigma. Sweet potato, tomato, squash, egg plant and green beans were frozen in liquid nitrogen and 25g were extracted with 50 ml 0.1M potassium phosphate buffer, pH 6.0. The tissue was homogenized for 2 min in a Waring Blendor and filtered through a 10μ GAF filter. The extract was centrifuged at 50,000 × G for 15 min and the supernatant fraction was used for the analysis.

The assay mixture contained the H-donor (either o-dianisidine, pyrogallol or guaiacol), 0.5 mM phosphate buffer (pH 5.0) and 0.03% H_2O_2. Initial velocity measurements were determined by mixing 2.9 ml of the assay mixture and 0.1 ml of the enzyme preparation and placing this in a Coleman 124, Perkin-Elmer Double Beam Spectrophotometer. The temperature was constant during the assay procedure (25°C) and the change in absorbancy at 460 nm was continually measured with a recorder. The slope of the initial straight line portion of the curve was taken as the initial velocity (Vo).

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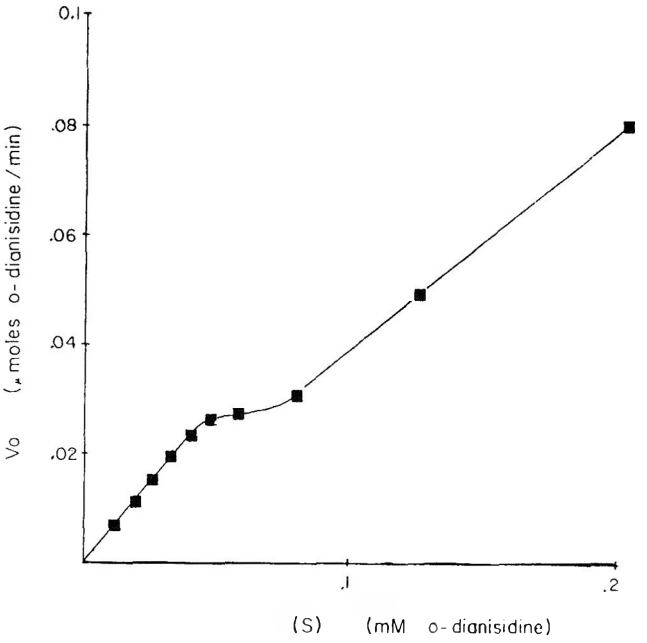

Fig. 1—Initial velocity versus o-dianisidine concentration of sweet potato peroxidase. Data are averages of duplicates and representative of three trials.

RESULTS & DISCUSSION

FIGURE 1 shows that o-dianisidine was not an adequate substrate for assaying peroxidase in sweet potato. The curve appeared to be reaching the maximal velocity near 0.1 mM but as o-dianisidine was increased, the reaction rate increased and failed to level off even at 1.0 mM. This is quite different from the typical Michaelis-Menten (M-M) kinetics. Recently, Lebedeva et al. (1977) demonstrated typical M-M kinetics using o-dianisidine and HRP. However, the maximum concentration that they used was in the area that follows M-M kinetics in Figure 1. They did not report results at any higher o-dianisidine concentrations.

Worthington (1972), Burnette and Flick (1977), Guilbault (1970), Fowler and Morgan (1972) and others, recommend 0.25 mM or less o-dianisidine to measure total peroxidase activity. Figure 2 shows that for HRP, which is representative of the effects seen with tomato, potato squash, egg plant, and green bean extracts, 0.25 mM was not high enough to give maximal velocity. If the enzyme is not at saturation, then total enzyme activity will be misleading, as shown in Figure 2a. At any enzyme dilution, the initial velocity is greater at the higher substrate concentrations. Also, saturation of the enzyme with substrate allows for small changes in substrate concentration with no effect on initial velocity. Figure 2a demonstrates a linear relationship between initial velocity and enzyme dilution for the different concentrations of o-dianisidine. It is possible to use 0.25 mM o-dianisidine and obtain and compare accurately enzyme activity. However, at a point below saturation (which 0.25 mM is shown to be), a small change in o-dianisidine concentration will have a dramatic effect on

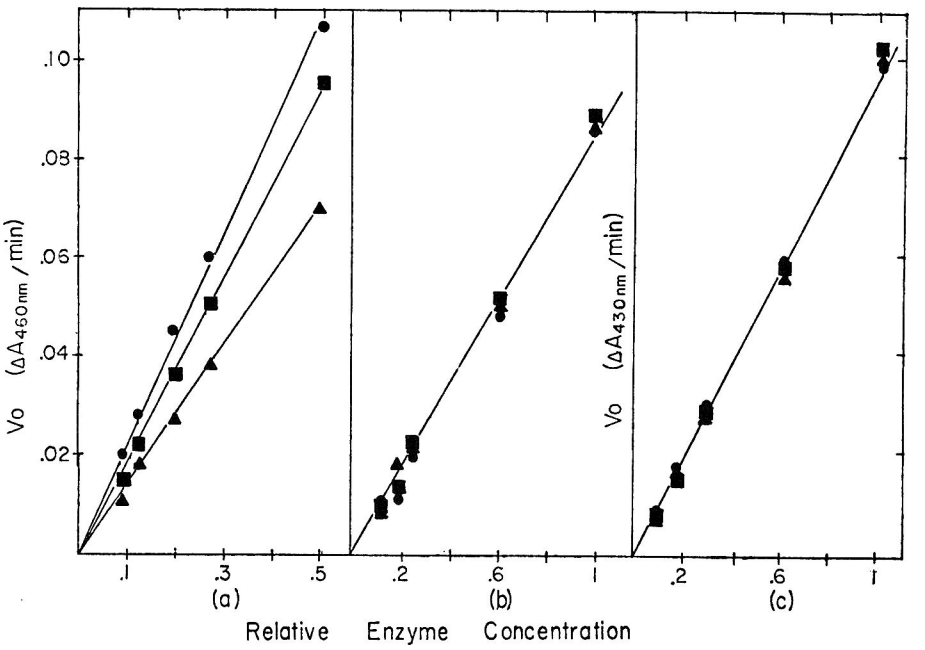

Fig. 2—Initial velocity versus relative enzyme concentration: (a) Substrate o-dianisidine at ●, 0.40 mM; ■, 0.3125 mM; ▲, 0.25 mM; (b) Substrate guaiacol at ●, 0.06M; ■, 0.049M; ▲, 0.043M; (c) Substrate pyrogallol at ▲, 0.043M; ■, 0.052M; ●, 0.06M. (Relative enzyme concentration is equal to ml of enzyme extract/ 4 ml PO_4 buffer.) Data are averages of duplicates and representative of three trials.

initial velocity. The instability of o-dianisidine increases this problem, because a stock solution of o-dianisidine in methanol will crystallize out over time, thereby changing the actual stock concentration. Thus, peroxidase assays done at 0.25 mM may or may not be misleading depending on the care the researcher takes in controlling the o-dianisidine concentration.

Figures 2b and c show that guaiacol and pyrogallol are more appropriate substrates for assaying peroxidase. Again, HRP was used and is representative of the effects seen with potato, tomato, squash, egg plant and green bean extracts. From the figures (2b, 2c) one can see that saturation (v_{max}) was obtained. The kinetics of these substrates follow the typical M-M kinetics.

CONCLUSIONS

FROM THE DATA presented, o-dianisidine should not be used for assaying total peroxidase activity, because enzyme activity is always dependent upon substrate concentration within a practical range. Thus, it is imperative that the substrate concentration be kept constant if any comparisons are to be made. If one must use o-dianisidine, extreme precaution should be taken to make sure complete solubility is achieved.

Guaiacol and pyrogallol appear to be much more suitable substrates for quantitative peroxidase determinations.

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A Research Note

EFFECTS OF PEANUT SHELL FLOUR ON FIRMNESS, COLOR AND ACCEPTANCE OF PEANUT BUTTER

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ABSTRACT

A flour was prepared from peanut shells and added to peanut butter at levels of 0–1%. The flour increased firmness, had only a slight effect on color, and produced no sensory preferences of the peanut butter. Results indicate that the flour may have usefulness as a stabilizer of peanut butter.

INTRODUCTION

QUALITY of peanut butter (PNB) encompasses many factors, but the separation of the oil phase and the solid particle phase has probably received the greatest consideration. According to Woodroof (1973), the earliest reference to the use of a stabilizer as a means of preventing the oil-solids separation was the incorporation of hydrogenated peanut (PN) oil in PNB. Since that time various substances and methods of preparation have been used in an attempt to prevent the oil-solids separation, to improve smoothness, spreadability, and consistency, and to eliminate stickiness.

In an effort to maximize functionality of the different ingredients during processing, storage and consumption of various products, the use of "additives" has been generous in many cases. Because of the vast, growing concern for human health, consumers have begun to display a great deal of anxiousness regarding the use of additives. Since the group of substances used to stabilize PNB may be labelled as "additive," it seems reasonable to investigate the use of a natural substance which may be used as a substitute for a portion, at least, of the stabilizers currently being used. Toward this end, a precursory study was made to determine the effect of a flour prepared from PN shells on firmness, color, and preference of PNB.

MATERIALS & METHODS

PEANUT SHELL FLOUR (PSF) was added as an ingredient to PNB. A local PNB processor provided all the ingredients except PSF (Benzinger, 1977).

The PSF was prepared from shells obtained from a PN processor (Zekert, 1977). The shells were separated from the debris, washed in a solution of sodium dodecyl sulfate (17 cc dry detergent per 38L water), and rinsed in three changes of fresh water. The shells were dried at 65°C, toasted at 149°C for 1 hr, and ground three times in a Wiley Mill to pass a 40-mesh sieve. After each grinding, material which passed a 140-mesh sieve was removed as PSF.

PNB was prepared according to a formulation suggested by the local processor. Eight treatments were prepared, four with 1.2% stabilizer and four, 1.4%. The amount of roasted PN (hearts and skin removed) and salt was held at 90.7 and 1.2%, respectively. At each level of stabilizer, four treatments were prepared to contain 0, 0.33, 0.67, or 1% PSF. Corn syrup solids were used to raise the amount to 100%. Samples of 150g were prepared per treatment in three replications. The sample with 1.4% stabilizer and 0% PSF was considered the reference for comparing with a commercial sample.

The ingredients were combined in a 250 ml jar and blended 2 min until liquified. The mixture was transferred to a 200 ml beaker, covered with plastic wrap, and allowed to stand 4 wk at 22°C before being tested.

Three 340g jars of commercial PNB processed by the local processor were purchased for comparative testing.

All samples were tested for firmness, color, and preference.

Firmness was determined by the Instron machine (model 1132) using a 5-kg load cell and a 7.94 mm diameter probe. Crosshead and recorder speeds were 10 cm per min. Testing consisted of forcing the probe 2 cm into the sample, with one measurement in each quadrant at the surface. The points tested were 90° apart. The four values (g force) were averaged for analysis.

Color values, L, "a" and "b" were obtained with the Hunter Meter (model D25D2M). Calibration was against a yellow tile (Hunter C2-138). Samples were placed in a cuvette and one reading was taken per sample.

A panel of 12 semi-trained people tested the samples (IFT, 1971). Cool white fluorescent light was used. Panelists considered color, mouthfeel, and flavor in determining acceptance. A 6-point hedonic scale (1 = dislike very much; 6 = like very much) was used. Three replications were tested, each on separate days.

The PSF was tested for aflatoxin; four, 2-g samples were used (Jones, 1977; Pons, 1968; Pons et al., 1966).

The data were analyzed as a factorial by analysis of variance; significance among means was determined by Duncan's Test (Li 1957).

Table 1—Firmness of peanut butter prepared with peanut shell flour (PSF) and by a commercial processor

PSF (%)	Stabilizer (%) 1.2	1.4	Mean PSF (%)
	grams force		
0.00	57.8[a]	58.8[a]	58.3[c]
0.33	61.2[a]	81.5[a]	71.4[bc]
0.67	64.8[a]	85.6[a]	75.2[b]
1.00	81.1[a]	89.3[a]	85.2[b]
Mean stabilizer (%)	66.2[e]	78.8[d]	
Commercial product[f]		58.0	

[a] Means are not different; N = 3.
[b,c] Means followed by same letter are not different ($P < 0.05$); N = 6.
[d,e] Means followed by same letter are not different ($P < 0.05$); N = 12.
[f] N = 3.

RESULTS & DISCUSSION

PSF affected firmness of PNB (Table 1). Mean firmness values increased with an increase in PSF, but only samples with 0.67 and 1% PSF were firmer than the control. Samples with 1.4% stabilizer were the firmer. The higher level of stabilizer was recommended by the PNB processor; the lower level was selected to compensate for the firming effect of PSF. Firmness values for the reference sample (58.8g force) and the commercial sample (58g force) were almost identical. Since PSF increased firmness, it may be possible to produce a product of appropriate firmness by reducing the level of stabilizer and adding PSF.

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All Hunter color values except "a" tended to decrease as the level of PSF was increased (Table 2). While the decrease was significant, the small difference in the L value (lightness) was discernable to the eye only when the samples were placed side by side.

The level of stabilizer had no effect on red and yellow coloration of the samples but had a slight effect on lightness.

Hunter values indicate that the commercial sample was slightly darker and less yellow, but more red than the experimental samples.

The taste panel showed no preference among samples with different levels of PSF. The mean score was 4.5, midway between *like slightly* and *like moderately*. This value was 0.6 units below that of the commercial sample.

Samples with 1.2% stabilizer (score = 4.6) were preferred over those with 1.4% (score = 4.3). This is most probably due to the less firm texture of samples with the lower level of stabilizer.

All samples were considered acceptable, thus, one may conclude that PSF can be added to PNB under certain conditions without producing important changes in appearance and taste attributes.

A few panelists complained that certain samples were either "dry," "oily," "sticky" or "grainy." These conditions should present no problems to the commercial processors since the methods used for blending the ingredients and reducing particle size of PN should eliminate most, if not all, of the objections. Obviously, the laboratory blender used had its limitations.

The yield of PSF from the shells was 19%. The PSF was soft to the touch, had a light tan color, and had a pleasant taste.

Aflatoxins were not detected.

Additional studies may be justified for determining the minimum amount of stabilizer necessary to yield PNB without undesirable changes in quality attributes with added PSF.

Table 2—Hunter color values of peanut butter prepared with peanut shell flour (PSF) and by a commercial processor

PSF (%)	Hunter values		
	L	"a"	"b"
0.00	48.6[a]	4.9[d]	22.1[e]
0.33	47.7[a]	4.7[d]	21.6[f]
0.67	46.8[b]	4.6[d]	21.4[f]
1.00	45.6[c]	4.7[d]	21.0[g]
Stabilizer (%)			
1.2	47.0[i]	4.7[j]	21.5[k]
1.4	47.3[h]	4.7[j]	21.6[k]
Commercial product[1]	44.1	6.1	20.9

[a-c] Means followed by same letter are not different ($P < 0.01$); N = 6.
[d] Means are not different; N = 6.
[e-g] Means followed by same letter are not different ($P < 0.01$), N = 6.
[h,i] Means are different ($P < 0.05$); N = 12.
[j] Means are not different; N = 12.
[k] Means are not different; N = 12.
[l] N = 3.

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A Research Note

STUDIES ON KINETICS OF INOSINE 5′-MONOPHOSPHATE INSTABILITY

JAE HEUNG LEE

ABSTRACT

The decomposition rate of inosine 5′-monophosphate was investigated at 100°C in a pH range 5.52–6.87, ionic strength 0.4. The rate was confirmed to be a pseudo first-order reaction and log rate-pH profile showed a maximum near pKa_2 of inosine 5′-monophosphate. The theoretical reaction model was proposed and the calculated rate constant was in good agreement with the results obtained from the experiments.

INTRODUCTION

HEAT DECOMPOSITION of inosine 5′-monophosphate (5′-IMP) was studied comprehensively by several investigators (Toda et al., 1965; Kuriyama et al., 1965; Fujita et al., 1965). The kinetics of the 5′-IMP decomposition were shown to be first-order with respect to 5′-IMP. Previous work (Davídek et al., 1972) demonstrated an increase in the rate of 5′-IMP loss with decreasing pH value at a constant temperature, and no influence of ionic strength was found. However, little information is available for the decomposition mechanism of 5′-IMP. The purpose of this study was to investigate the importance of the pK in decomposition kinetics, and propose the 5′-IMP decomposition mechanism based on the experimentally obtained results.

MATERIAL & METHODS

Material

The 5′-IMP produced from Cheil Sugar Co. was used to prepare this experiment. IR spectrum was taken and it was identical with that of authentic sample (Zorbach and Tipson, 1973). The purity was determined by using the Waters Associates Model 244 high-pressure liquid chromatograph (HPLC). It was found that this material was analytically pure grade. In order to determine the water of hydration of 5′-IMP, it was kept for a long time in a desiccator maintained by saturated NH_4Cl solution.

The Karl Fischer titration (Mitchell, 1951) was carried out on samples to determine the water of hydration. These studies revealed that the crystalline 5′-IMP exists as a stable octahydrate at 25°C.

Method

Experiments were made on 5′-IMP buffer solution (0.01 mole/L) in a pH range 5.52–6.87, ionic strength 0.4. Sterilized screwcap vials which were filled with 5′-IMP solutions were tightly sealed and then placed in a constant temperature oil bath (± 1°C). The vials were allowed to equilibrate thermally during appropriate time intervals at 100°C. The vials were removed from the bath and their contents analyzed by using the HPLC. The procedure was similar to that used by Bennett (1977) to measure nucleotide concentrations in orange juice. The carrier fluid was 0.01 mole/L buffer solution of $NH_4H_2PO_4$, and the flow rate was 2 ml/min. The HPLC column was a μ BONDAPAK C18.

Determination of pKa_2 of 5′-IMP

The pKa_2 of 5′-IMP was determined from the titration curve by measuring the pH using a Hitachi-Horiba pH meter. A solution containing 0.01 mole/L of 5′-IMP was prepared in distilled water and a calculated amount of NaCl was added to give ionic strength 0.4. The solution was titrated with 0.1N HCl at 60°C. The average of two potentiometric titrations yielded a pKa_2 = 5.9. The pKa_2 value reported for 5′-IMP was 6.0 (Dawson et al., 1974).

RESULTS & DISCUSSION

THE PRODUCT of 5′-IMP heat decomposition was inosine except trace amounts of hypoxanthine in the pH range 5.52–6.87. The results of 5′-IMP decomposition were analyzed by the first-order rate function (Fujita et al., 1965; Davídek et al., 1972);

$$\frac{dc}{dt} = -Kc$$

where c = concentration of 5′-IMP; t = time (hr) and K = first-order rate constant (hr^{-1}). The experimental results for the loss of 5′-IMP are presented in Figure 1, and the reaction rate constants were calculated. Fujita et al. (1965) reported an increase in the rate of 5′-IMP loss with decreasing pH value at a constant temperature. Examination of Figure 1 shows that the rate at pH 5.87, which is near pKa_2 of 5′-IMP, is larger than the rate at pH 5.52. In order to explain the experimental results, the following reaction mechanism was proposed.

$$IMP^{=} \xrightarrow{k_1} \text{INOSINE (Reaction 1)}$$

$$IMP^{=}\cdot IMP^{-} \xrightarrow{k_2} \text{INOSINE (Reaction 2)}$$

$$IMP^{-} \xrightarrow{k_3} \text{INOSINE (Reaction 3)}$$

In this reactions, IMP^- and $IMP^=$ are mono- and di-dissociated 5′-IMP, respectively, and $IMP^=\cdot IMP^-$ is a complex of them. The overall rate of 5′-IMP decomposition is a sum of the rates described above.

$$\frac{-d(IMPT)}{dt} = k_1 (IMP^{=}) + k_2 (IMP^{=}\cdot IMP^{-}) + k_3 (IMP^{-}) \quad (1)$$

$$(IMPT) = (IMP^{=}) + (IMP^{=}\cdot IMP^{-}) + (IMP^{-}) \quad (2)$$

If the complex has a formation constant, Kc,

$$Kc = \frac{(IMP^{=}\cdot IMP^{-})}{(IMP^{=})\ (IMP^{-})} \quad (3)$$

and if $k_2' = k_2 \cdot Kc$, then substituting this into Eq (1) give

$$\frac{-d(IMPT)}{dt} = k_1 (IMP^{=}) + k_2'(IMP^{=})\ (IMP^{-}) + k_3 (IMP^{-})$$

From the experiments, the overall reaction follows the first-order.

$$-\frac{d(IMPT)}{dt} = K\ (IMPT) \quad (5)$$

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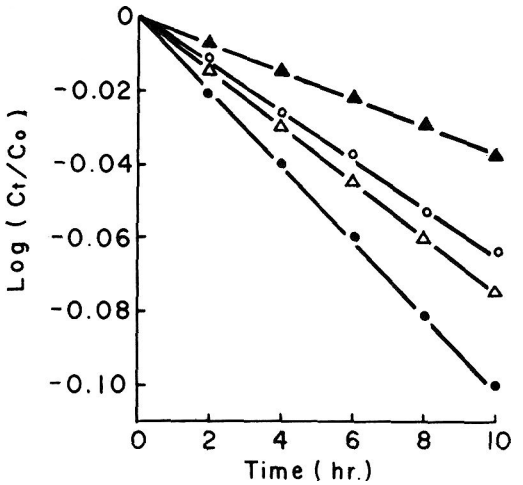

Fig. 1—Pseudo first-order plots for 5'-IMP decomposition at 100° C: ● pH 5.87; ◿ pH 6.25; ○ pH 5.52; ▲ pH 6.87.

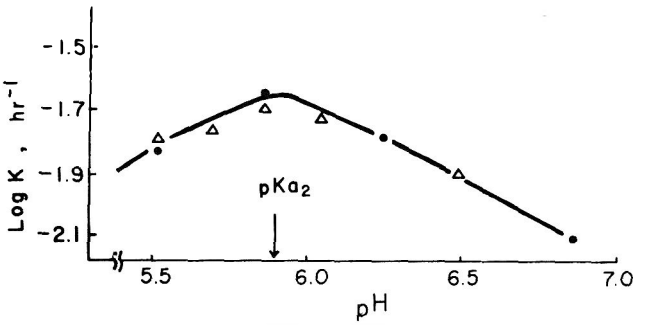

Fig. 2—LogK vs pH profile: ●; experimental rate constant; △; calculated rate constant.

Combining Eq. (5) and Ka_2 gives

$$K = \frac{k_1 Ka_2 + k_3 (H^+)}{Ka_2 + (H^+)} + \frac{k_2' Ka_2 (IMPT)(H^+)}{[Ka_2 + (H^+)]^2} \quad (6)$$

From Eq (6), the following equation can be derived:

$$(H^+) = \frac{Ka_2 (k_3 - k_1 + k_2' (IMPT)}{k_1 - k_3 + k_2' (IMPT)} \text{ for } \frac{dk}{d(H^+)} = 0 \quad (7)$$

If the 5'-IMP destruction is maximized at pH = pKa_2,

$$\frac{k_3 - k_1 + k_2' (IMPT)}{k_1 - k_3 + k_2' (IMPT)} = 1 \quad (8)$$

Therefore, $k_1 = k_3$. Substituting this into Eq (6) gives

$$K = k_1 \text{ (or } k_3) + \frac{k_2' Ka_2 (IMPT)(H^+)}{[Ka_2 + (H^+)]^2} \quad (9)$$

From Eq (9), the overall reaction rate constants can be calculated as a function of pH by using the value of 0.01 mole/L for (IMPT). The log K-pH profile is shown in Figure 2. The maximum rate of 5'-IMP decomposition was observed near pKa_2. At pH = pKa_2, the concentration of IMP^- is equal to $IMP^=$. Reaction 2 supposes the existence of a complex between IMP^- and $IMP^=$. It is shown that a reaction that presupposes the existence of a complex between the mono- and di-dissociated 5'-IMP helps to explain the observed pH-dependence of the decomposition. The relatively good agreement of the experimental data and the theoretical profile does not prove that the proposed reactions are the correct ones. However, the reasoning for them justifies their proposal.

The effect of temperature on the decomposition of 5'-IMP was studied in buffer solution. The loss of 5'-IMP in this study was temperature dependent and confirmed to the Arrhenius equation in the temperature region 100–120°C. The activation energy at pH 5.87 was found to be 26 Kcal/mole.

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ERRATUM NOTICE

J. Food Sci. 43(6): 1669–1674 (1978). Lipids of fish fillets: Changes following cooking by different methods by J. Mai, J. Shimp, J. Weihrauch and J.E. Kinsella. Page 1674, Table 10, the cholesterol content of Bluegill, the column under Dry should read as follows:

Raw	Cooked
244	169
130	59
241	198
204 ± 69	

On page 1671, second paragraph, under Lipid changes, the sentence beginning on line nine should read: "In general, baking resulted in a loss of lipids from the fillets of fat species."

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