5 September–October 1978
Volume 43 : Number 5

Coden: JFDAZ 43(5) 1359–1636 (1978)
ISSN: 0022–1147

JOURNAL of FOOD SCIENCE

BASIC SCIENCE

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IFT *A PUBLICATION OF THE INSTITUTE OF FOOD TECHNOLOGISTS*

APPLIED SCIENCE AND ENGINEERING

RESEARCH NOTES

JOURNAL OF FOOD SCIENCE
September—October 1978
Volume 43: Number 5

AUTHOR INDEX

Letters

Iron bioavailability assay—Methodology challenged

In the November-December, 1977, issue of *J. Food Sci.*, the paper by Farmer et al. reported on measurements of iron bioavailability from hand-deboned and mechanically deboned meats. They employed an animal model and used ferrous sulfate as the reference standard. I am writing this letter because of my concern with the appropriateness of the methodology.

There are many methods available for assessing iron bioavailability and an excellent discussion of the relative merits of each has been presented by Monsen (1974). I do not wish to elaborate here.

Hemoglobin iron is poorly absorbed by the rat (Fritz et al., 1970; Weintraub et al., 1965) whereas in the human, it is usually highly available (Turnbull et al., 1962). Within each species, there are marked differences not only in the rates of heme iron absorption but also differences relating to influencing factors. Enhanced absorption of hemoglobin iron is experienced by iron deficient human subjects, but no similar enhancement is observed in rats. Thus, caution must be taken in interpreting the results of experiments with rats for heme iron absorption in relation to human situations. Moreover, it may have been more meaningful for Farmer et al. to use heme iron as their reference standard rather than ferrous sulfate which is absorbed so differently.

The authors also reported a significantly lower liver iron content of animals fed mechanically deboned meat (MDM) compared to those fed hand-deboned meat (HDM). The liver iron of MDM fed animals did not appear to be significantly lower than HDM fed animals as the data were presented (25.8 and 26.0 μg/g vs 35.0 and 34.6 μg/g, respectively, L.S.D. at 5% level of probability = 18.8).

I would hope that you would bring my comments to the attention of the authors and to readers of the Journal.

Thank you for your consideration.

– *Mary Frances Picciano*, Asst. Professor, Nutrition, University of Illinois, College of Agriculture, School of Human Resources and Family Studies, 260 Bevier Hall, Urbana, IL 61801.

Thank you for allowing us to comment on Dr. Picciano's letter. Very correctly, an error was made in the manuscript concerning statistically significant differences in liver iron content among the treatments (Farmer et al., 1977). In Table 2, the LSD values for $P < 0.05$ and $P < 0.01$ should have been 8.2 and 11.0 μg Fe/g liver. We remain with the conclusion that rats fed mechanically deboned meat diets had lower liver iron stores than those fed hand-deboned meat diets.

We did not use hemoglobin as a standard. Ferrous sulfate was selected because of its known purity and because mammalian (and avain) species consistently respond in a predictable manner when consuming it. The absorption of hemoglobin iron is highly variable (Amine and Hegsted, 1971) and, therefore, is not a good reference source of iron. On the other hand, the efficiency of converting dietary ferrous sulfate iron into hemoglobin by the rat is quite similar among experiments and among laboratories (Mahoney et al., 1974; Mahoney and Hendricks, 1976).

The rat and the human being seem to utilize iron from hemoglobin and ferrous sulfate with similar efficiencies. Iron deficient human beings utilize 15–20% of the hemoglobin iron and about 50% of ferrous sulfate iron (Amine and Hegsted, 1971; Mahoney et al., 1974). This suggests that the rat models the human being quite well when both are of similar nutritional status.

The iron deficient rat responds as the iron deficient human being does, by enhancing its efficiency of iron absorption and retention when fed food iron, hemoglobin iron or ferrous ammonium sulfate (Monsen, 1974; Amine and Hegsted, 1971; Sørensen, 1965).

Philosophically, our approach to nutrient bioavailability assay is to determine the total amount of nutrient that the organism is able to extract from the food. Hence, when assaying the bioavailability of food iron, the iron deficient model is used and, iron is provided as the limiting nutrient in the diets. Knowing the efficiency of converting the food iron into hemoglobin and the total amount of iron in the food, we can determine the total amount of metabolizable iron present in that food.

– *Arthur W. Mahoney*, Professor of Nutrition, and *Deloy G. Hendricks*, Assoc. Professor of Nutrition, Dept. of Nutrition & Food Sciences UMC 87, College of Agriculture, Utah State University, Logan, UT 84322.

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Analysis of data criticized

The paper "Effect of bird type, growers and season on the incidence of salmonellae in turkeys" by McBride et al. (1978) is an interesting paper on an important subject. I realize a lot of good work has been done and I hope the authors are able to continue their investigations in the future.

However, since information of this type is hard to get, it should be effectively exploited (i.e. efficiently analysed). Because of the widespread interest which may be shown in this paper and the economic importance of the subject, I feel obliged to criticize certain aspects of the design, analysis and interpretation of the data, and to suggest an alternative analysis.

(1) No details of the design of the sample are given. Imbalance in the data may have been unavoidable but analysing and interpreting unbalanced data is always difficult and often

—Continued on page iv

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JOURNAL of FOOD SCIENCE

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inefficient. Also, the scope of the experiment (comparing single versus multiple growers, hens versus toms versus broilers, summer versus winter etc.) is too wide for the sample size used. Because of these problems it is perhaps not surprising, that few positive statements are made, based on these data, about flocks and growers in general. The last sentence in the abstract follows from previous work, and the last sentence in the top paragraph on the right of page 324 is more a factual statement than an inferential statement "substantiated" by analyses (their next sentence).

(2) No details of the calculations involved in the analyses of variance are given. For me, this produced two problems: (a) I did not know which flock was omitted from the analyses nor why it was omitted (it turns out to be G's 72% flock), and (b) I could not reproduce the numbers in Table 3 (there appears to be an error and perhaps the authors might like to submit a correction note.

(3) The interpretation of the analysis of variance is incorrect. The 23 degrees of freedom represent *among flock* variation, whereas the other 1176 represent *within flock* variation and should not be used for testing any subset mean square of the *among flock* sum of squares. The objective of the survey was presumably to investigate patterns among, and differences between, such "treatments" as growers, season, bird type etc., and for this purpose a residual variance estimate is needed which reflects natural variability within a well-defined group of flocks such as: all flocks from the same grower, or all flocks of the same type from the same grower. Whichever group is chosen, a within group error variance estimate should be obtained from the entire set of flocks. For example, if all flocks from the same grower were regarded as containing the sort of base variability with which to compare other differences, the "among flocks/growers of multiple flocks" mean square is an appropriate error to use. For these, or similar reasons, Tables 4 and 5 are incorrect. In Table 6, on the other hand, the authors appear to be at least partially aware of the problems mentioned above, but inconsistent interpretation of similar analyses of variance is not fair to the reader.

(4) For binomial data, a simple analysis of variance of the raw data is generally inappropriate. Sometimes a prior transformation of the data is all that is needed, but there are often instances where a more powerful analysis can be made. This is the situation here. A pattern begins to emerge, if the data for growers of multiple flocks are displayed in multiway tables. For example, for grower G:

Grower	Bird type	Season	Number negative	Number positive
G	T	S	32	18
G	H,T	S	14	36
G	H	W	40	10
G	T	W	46	4
			132	68

Firstly, there is an obvious increase in variability between flocks for "worse" growers, and clear indications of interactions between grower and season. For example, ignoring bird type, the approximate χ^2 test for 2 X 2 tables increases from a negligible size for grower A through 3.1(E), 7.5*(I), 24.9*(C) to 35.6*(G including all 4 flocks), the significant values indicating higher summer incidence. This kind of analysis of the data seems to me to be much more informative. The data are too scanty to make general statements about bird type effects.

It is disappointing that the authors, in trying to use statistical techniques to support perfectly reasonable statements, failed to use them correctly. It is more disappointing that their hard won data were analysed inefficiently, when there exist simple statistical methods by which much more information could be obtained.

– *M. R. Binns*, Statistical Research Section, Engineering & Statistical Research Institute, Agriculture Canada, Ottawa Canada.

In response to Dr. Binns' letter concerning the paper by McBride et al. (1978), the following should be considered.

As noted by Binns, we inadvertently omitted stating that flock 13 (hens and toms) in Table 1 was not included in the statistical analysis. This flock was omitted since one of our objectives was to determine whether there was any relationship between bird type and the incidence of salmonellae. Dr. Binns is correct in that there are several minor errors in Table 3. The revised table is reproduced herewith with the corrected values underlined.

	df	MS	F
All flocks	23	1.2574	21.1**
Growers of single flocks/ growers of multiple flocks	1	0.3041	5.09*
Among growers of single flocks	9	1.7431	29.2**
Among growers of multiple flocks	4	1.5501	25.9**
Among flocks/growers of multiple flocks	9	0.7474	12.5**
Samples	1176		
Total	1199		

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

Dr. Binns has recognized the difficulties in analyzing and interpreting unbalanced data. With regard to the interpretation of the analysis of variance outlined in Table 3, it is our opinion that the within flock variation or sample variation is appropriate for testing among flock variation. As Binns has suggested, among growers might indeed be better tested with among flocks within growers. However, this is not possible for growers with single flocks. For these growers, the among grower variation is not distinguishable from among flock variation, and is also tested by sample variation.

Although Tables 4 and 5 appear similar to Binns, they are, in fact, quite different. Table 4 is possibly oversimplified by the pooling of among flock and error variances, but it cannot be called incorrect. Larger F values could be obtained in this table only if the among flock variances were very small. In Table 5 it is appropriate to test each among flock variance against its corresponding within flock variance.

Dr. Binns might like to consult Li (1964) in which the analysis of binomial data by χ^2 analysis and analysis of variance are extensively discussed. The "more powerful" χ^2 analysis as suggested by Binns for Table 6, does not consider among flock variance. In Table 4, the conclusions cannot be changed if Binns' approach is followed. However, in Table 6, the conclusions differ according to the choice of F – denominator. When among flock variance is not considered, the season effect does appear to be significant. However, when this was considered by the authors in ANOV II, Table 6, the season effect was not significant at $P > 0.05$.

In conclusion, we feel that the data have been analysed in the most efficient manner possible due to the reasons stated above.

– *G. B. McBride* and *B. J. Skura*, Dept. Food Science, University of British Columbia, Vancouver, B.C., Canada, V6T 1W5.
– *B. Brown*, B.C. Ministry of Agriculture, 10344 E. Whalley Ring Road, Surrey, B.C., Canada, V3T 4H4.

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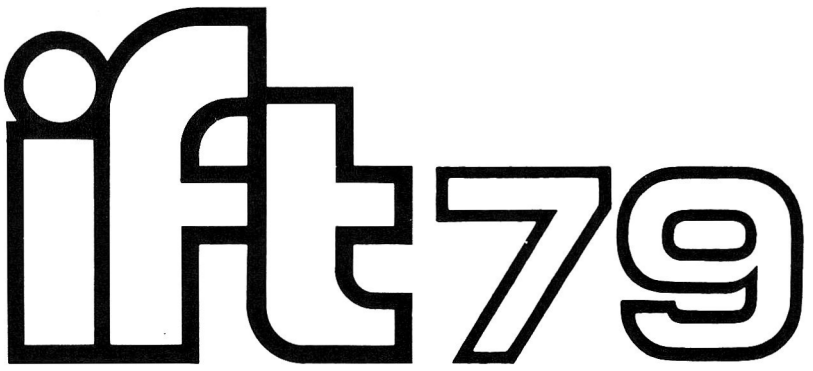

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A relatively simple and efficient method has been developed and is described for the preparation of lysozyme from food or biological tissues prior to quantitation. Deaminated chitin has a high specificity and capacity for the enzyme (squid chitin more so than crab chitin), along with good stability and fast flow rates. The enzyme-substrate reaction is effective at pH 7.0-8.0; all other proteins pass through the column. Elution is carried out under acid conditions. Studies have shown the method to be excellent for tissues and foods.

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PREDICTING PROTEIN EFFICIENCY RATIO BY THE CHEMICAL DETERMINATION OF CONNECTIVE TISSUE CONTENT IN MEAT

YU BANG LEE, JAMES G. ELLIOTT, DAVID A. RICKANSRUD and ELROY C. HAGBERG

ABSTRACT

The influence of connective tissue content on amino acid composition and rat PER was studied in an attempt to develop a regression equation for predicting PERs of meat from the simple chemical analysis of collagen. Collagen content was highly correlated to essential amino acid content and rat PER with correlation coefficients of −0.99 and −0.98, respectively. The developed regression equation, PER = −0.02290 (collagen content) + 3.1528, effectively predicted rat PER within ± 0.2 units when tested on various meat ingredients. These results indicated that the chemical determination of collagen content can be employed to provide a rapid, inexpensive and easily adaptable assay for the estimation of protein quality of meat. Mechanically deboned red meat had a reasonably good PER (2.65) and only a moderate level of collagen (19%); whereas, partially defatted chopped beef showed wide variation in PER as well as in collagen content with different processors. Both raw and cooked mechanically deboned chicken also showed good PERs, 3.0 and 2.6, respectively.

INTRODUCTION

NEW DEVELOPMENTS in processing technology have generated new meat ingredients such as mechanically deboned meat and high or low temperature rendered meats which can be utilized in formulated meat products. Inexpensive cuts of meat can also be more efficiently utilized to produce higher value products by restructuring (Mandigo, 1975; Farrington, 1975). These meat ingredients have wide variation in composition and nutritional value with different processors (Happich et al., 1975) and the USDA in 1976 proposed interim regulations on the nutritional quality of such products. The proposed regulation specified a minimum PER of 2.5 and a minimum essential amino acids content of 32% for most products. Since the PER test, a biological assay of protein quality described by the AOAC, is both time consuming and costly, many investigators have worked to develop methodology for a faster and cheaper way to determine PERs. USDA researchers (Alsmeyer et al., 1974) attempted to predict PERs with regression equations derived from amino acid analysis, while Satterlee et al. (1977) tested a *Tetrahymena* bioassay and in vitro digestibility.

Since the early work of Mitchell and Carman (1926), it has generally been recognized that inexpensive cuts of meat and meat by-products high in connective tissue have relatively poor biological value. Bender and Zia (1976) recently reported that low quality meat with 23.6% collagen had a net protein utilization (NPU) of 69 and high quality meat with 2.5% collagen had a NPU of 82. However, no extensive study has been reported that establishes a definite linear relationship between the connective tissue content and PER.

The objective of the present study was to determine the influence of a wide range of connective tissue contents on amino acid composition and PER, and furthermore, to establish a regression equation for predicting PER of meats from the chemical analysis of connective tissue content.

All authors are affiliated with the Campbell Institute for Food Research, Campbell Place, Camden, NJ 08101.

0022-1147/78/0005-1359$02.25/0

Table 1—Combination of beef round and partially defatted chopped beef for experimental beef mixes

Beef mix	Beef round %	Partially defatted chopped beef, %
Experiment 1		
1	100	0
2	70	30
3	55	45
4	37.5	62.5
5	22.5	77.5
6	7.3	92.7
Experiment 2		
7	100	0
8	86	14
9	65	35
10	52	48
11	38	62
12	24	76
13	14	86
14	0	100

Tetrahymena bioassay was also tested to determine its applicability to meat systems.

EXPERIMENTAL

Preparation of beef mixes

Two experiments were conducted with meat ingredients for each experiment from different production dates. Cow beef rounds were obtained from a local meat packer and closely trimmed to remove excess fat covering and connective tissue. Frozen partially defatted chopped beef (PDCB) was also obtained from a commercial processor. These two meat ingredients were separately ground through a plate with 0.63 cm orifices using a Hobart meat grinder and blended in a silent cutter in different proportions to vary collagen content from 5–45% of total protein (Table 1). After thorough blending, samples were taken for proximate analysis, amino acid analysis, collagen determination, *Tetrahymena* bioassay and rat bioassay.

Chemical analyses

Moisture, protein and ash were determined according to AOAC methods (1975). Percent protein was calculated from Kjeldahl nitrogen times the factor 6.25. Fat was determined by the Bligh and Dyer method (1959). Amino acids were analyzed in duplicate using a Hitachi KLA-5 amino acid analyzer on samples hydrolyzed with 6N HCl at 121°C for 12 hr.

Collagen content

Beef mix samples were frozen in liquid nitrogen and pulverized to a homogeneous fine powder according to the procedure described by Borchert and Briskey (1965). Duplicate 5-g samples were hydrolyzed with 250 ml 6N HCl at 121°C for 9 hr. Hydroxyproline content was determined in triplicate on acid-hydrolyzed samples by the method of Switzer and Summer (1971). Collagen was calculated from the hydroxyproline content by multiplying by a conversion factor of 7.25.

Bioassays of protein quality

Rat Protein Efficiency Ratio (PER) was determined with the diet protein level adjusted to 10% according to AOAC procedures (1975). Net Protein Ratio (NPR) was also determined in Experiment 2 on the 10th day of the feeding test according to the method of Bender and Doell (1957). NPR was calculated by dividing the sum of the weight gain of a test group fed a diet containing protein and the weight loss of a control group receiving a protein-free diet by the amount of protein

consumed by the test group. *Tetrahymena* Relative Nutritive Value (RNV) was determined as described by Evancho et al. (1977).

Statistical analyses

Data were analyzed using regression analysis (Steel and Torrie, 1960).

RESULTS & DISCUSSION

PROXIMATE COMPOSITION and collagen content of the experimental beef mixes are shown in Table 2. Protein content decreased slightly and fat content increased as the proportion of partially defatted chopped beef (PDCB) increased. All beef mixes possessed reasonably similar protein content, ranging from 18.3–22.4%. Collagen content linearly increased from 4–45.8% of total protein as the mixing proportion of PDCB increased from 0–100%.

Assuming that the collagen-to-elastin ratio is 3:1 (Wilson et al., 1954), the total connective tissue content would be 33% higher than the collagen value reported in Table 2.

Results of amino acid analyses are shown in Table 3. Since rats require arginine and histidine, these amino acids are also included as essential amino acids. As expected, the increase of connective tissue resulted in linear decrease of total essential amino acid content from 51% in lean beef to 36% in PDCB. Among the essential amino acids, arginine and valine showed little change, which would be explained by the fact that collagen contains a high level of arginine; whereas, elastin contains an exceptionally high level of valine. Among the nonessential amino acids, a gradual increase in nonpolar and a gradual decrease in polar amino acids were observed, the net result being the increase of total nonessential amino acids with the increase of connective tissue.

Table 2—Proximate analyses and collagen content of experimental beef mixes

Beef mixes	Proximate composition				Collagen content	
	Solid %	Protein %	Fat %	Ash %	mg/g sample	% of total protein
Experiment 1						
1	30.1	22.4	6.7	1.0	8.9	4.0
2	30.1	21.2	7.9	1.0	29.5	13.9
3	30.5	20.3	9.2	1.0	42.2	20.8
4	31.1	20.1	9.9	1.1	53.3	26.5
5	32.3	19.8	11.5	1.0	65.0	32.8
6	33.1	19.9	12.2	1.0	79.6	40.0
Experiment 2						
7	25.1	20.2	4.0	1.0	13.7	6.8
8	25.8	20.0	4.5	1.0	22.4	11.2
9	25.8	19.7	5.6	1.0	36.4	18.5
10	26.1	19.5	5.6	0.9	53.1	27.2
11	25.4	18.9	6.2	0.8	59.6	31.5
12	25.6	18.8	6.2	0.8	64.0	34.0
13	26.1	18.3	6.9	0.8	72.9	39.8
14	26.6	18.6	7.2	0.7	85.2	45.8

Protein quality determined by rat and *Tetrahymena* bioassays is summarized in Table 4. Rat PERs ranged from 3.09 in beef mix 1 (lean beef) to 2.08 in beef mix 14 (PDCB). The relationship between the collagen content and the rat PER is illustrated by a linear regression curve shown in Figure 1. The regression equation was defined as: $Y = -0.02290\ X + 3.1528$, where Y = rat PER and X = collagen content. The correlation coefficient between these parameters was highly significant ($r = -0.98$, $p < 0.001$), and this means that 97% of the total variation in rat PER was accounted for by collagen content. These results strongly suggest that rat PER can be effectively predicted by chemical determination of collagen content in meat samples. In the regression curve, a PER of 2.5 corresponds to 28.5% collagen. This means that collagen content should be less than 28.5% of total protein to give a PER of 2.50 or higher.

Other regression equations are also shown in Table 5. Essential amino acids accounted for 95% of the total variation in rat PER, indicating that amino acid analyses would also effectively predict PER of meat samples, as previously reported by Alsmeyer et al. (1974) and Happich et al. (1975). Advantages of using collagen determinations over amino acid analysis to predict PER of meat samples are: (1) no sophisticated instrument such as an amino acid analyzer is needed, (2) it is simpler and less expensive, and (3) small processors can easily perform this analysis in their quality control laboratory.

Since tryptophan cannot be determined by routine amino acid analysis, and dietary requirements of arginine and histidine for human adult have not been established, the con-

Table 3—Amino acid analyses of beef mixes[a]

Amino acid	Experimental beef mixes													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Essential														
Arg	7.2	6.7	7.1	7.0	7.4	7.4	7.3	7.3	6.9	6.7	6.9	6.7	6.3	6.0
His	4.3	3.7	3.3	2.8	2.5	2.0	3.7	3.6	3.3	2.8	2.7	2.3	2.0	1.7
Ileu	5.2	4.5	4.0	3.9	3.5	3.3	5.1	4.7	4.6	4.3	4.0	3.7	3.5	3.3
Leu	8.6	7.7	7.4	7.2	6.8	6.5	8.6	8.5	7.9	7.4	7.2	6.9	6.9	6.5
Lys	9.1	7.6	7.2	6.7	6.1	5.7	8.7	8.1	7.7	7.1	6.6	6.2	5.7	5.2
Met	2.4	2.1	1.7	1.7	1.6	1.4	2.9	2.5	2.2	2.1	1.8	1.8	1.6	1.5
Phe	4.3	3.9	3.8	3.7	3.5	3.4	4.1	4.0	4.0	3.8	3.6	3.6	3.5	3.4
Thr	4.2	3.8	3.6	3.4	3.2	3.0	3.6	3.4	3.3	2.9	2.8	2.5	2.4	2.3
Tryp[b]	–	–	–	–	–	–	0.5	0.5	0.3	0.4	–	0.3	–	–
Val	5.7	6.0	6.1	6.1	5.5	5.6	5.8	5.8	5.6	5.7	5.8	5.8	5.8	6.0
Total	51.0	46.0	44.2	42.5	40.1	38.3	50.3	48.4	45.8	43.2	41.4	39.8	37.7	35.9
Nonessential														
Ala	6.1	6.6	6.5	6.4	7.1	7.4	6.1	6.5	6.8	7.2	7.3	7.2	7.5	8.4
Asp	8.6	7.8	7.5	7.1	7.3	6.8	9.2	8.6	8.2	7.8	7.6	7.4	7.2	6.9
Cys	1.1	0.8	0.9	0.9	1.0	1.0	1.3	1.0	1.1	1.1	0.9	1.1	1.0	1.0
Glu	14.6	13.3	12.7	12.3	11.6	11.2	14.5	14.7	13.5	12.9	12.5	12.2	11.8	11.3
Gly	4.9	7.6	9.7	10.9	11.8	13.2	5.5	7.0	8.1	9.5	10.9	11.8	13.2	13.5
Hypro	1.8	5.5	5.4	6.5	7.1	7.7	1.0	1.6	3.8	5.3	6.2	7.3	8.1	8.8
Pro	5.0	6.3	7.0	7.2	8.2	9.0	4.7	5.5	6.3	6.8	7.4	7.6	8.2	9.2
Ser	3.6	3.6	3.5	3.5	3.5	3.5	4.0	3.7	3.5	3.5	3.4	3.4	3.4	3.3
Tyr	3.3	2.7	2.6	2.7	2.3	1.9	3.4	3.0	2.9	2.7	2.4	2.2	1.9	1.7
Total	49.0	54.0	55.8	57.5	59.9	61.7	49.7	51.6	54.2	56.8	58.6	60.2	62.3	64.1

[a] Expressed as grams of amino acid residue per 100g of total amino acid residues

[b] Tryptophan was not analyzed separately

tent of the remaining seven essential amino acids was calculated and related to PER (Table 5). The same correlation coefficient (r = 0.97) was observed regardless of whether ten or seven essential amino acids were considered. The quantity of essential amino acids to give a PER of 2.50 was 42% and 32.2% for the ten and seven amino acid groups, respectively. This result agrees with the interim regulations proposed by the USDA (1976) which stated that the minimum content of the seven essential amino acids should be 32% and the minimum PER should be 2.5 for the new meat ingredients identified.

The correlation coefficient between collagen and essential amino acids content was −0.99 and the regression equations were given as either Y = −0.3575 X + 52.1949 or Y = −0.2745 X + 40.1456, depending on the number of amino acids considered. This result clearly indicates that the amount of essential amino acids can be accurately predicted by the determination of connective tissue content and vice versa.

Rat NPRs determined on eight beef samples are shown in Table 4. NPR was highly correlated (r = 0.97) to rat PER, suggesting that the NPR test may be equivalent to the PER test. One advantage of the NPR test is that it is a faster (10–14 days) and consequently a less expensive method than PER. However, more extensive data must be accumulated to confirm the reliability of the NPR test.

PER estimated from *Tetrahymena pyriformis* W bioassay was not highly correlated to rat PER, accounting for only 28% of the total variation (Table 5). This result does not agree with the recent study of Evancho et al. (1977) who reported that the *Tetrahymena* bioassay could be effective for estimation of protein quality of commercially prepared foods. Further investigation is needed to determine whether *Tetrahymena* bioassay can be adapted for meat ingredients.

The effectiveness of regression equation 1, obtained from the collagen content data and the rat PER assay, was tested for various red and poultry meat samples and the results are presented in Table 6. The results clearly indicated that the proposed equation successfully predicted the PER of all the meat samples tested within ± 0.2 PER. Since it is generally assumed that the standard error among duplicate PER determinations in the rat assay is 0.2 PER, a prediction equation which can predict PER within ± 0.2, such as equation 1, should be regarded as a reliable and effective estimator of PER. It should not be construed, however, that the proposed method of PER estimation can also be applied to formulated meat products containing extenders such as soy proteins. This developed prediction equation would have application to meat products that are composed primarily of animal tissues.

The data presented in Table 6 also showed that there was a significant difference between the two partially defatted

Table 4—Protein quality of experimental beef mixes

Beef mixes	Corrected rat PER	Rat NPR[a]	Tetrahymena RNV	Calculated[b] tetrahymena PER
Experiment 1				
1	3.09	–	99.0	2.46
2	2.85	–	107.6	2.65
3	2.69	–	105.4	2.60
4	2.51	–	104.4	2.58
5	2.45	–	94.1	2.36
6	2.26	–	99.2	2.47
Experiment 2				
7	3.01	5.13	115.6	2.83
8	2.85	4.82	115.2	2.82
9	2.66	4.32	106.4	2.63
10	2.61	4.55	115.3	2.82
11	2.36	3.98	110.1	2.71
12	2.42	4.36	100.7	2.50
13	2.22	3.63	96.2	2.40
14	2.08	3.45	97.4	2.43

[a] NPR was determined only in experiment 2.
[b] PER = 0.286 + 0.022 (RNV)

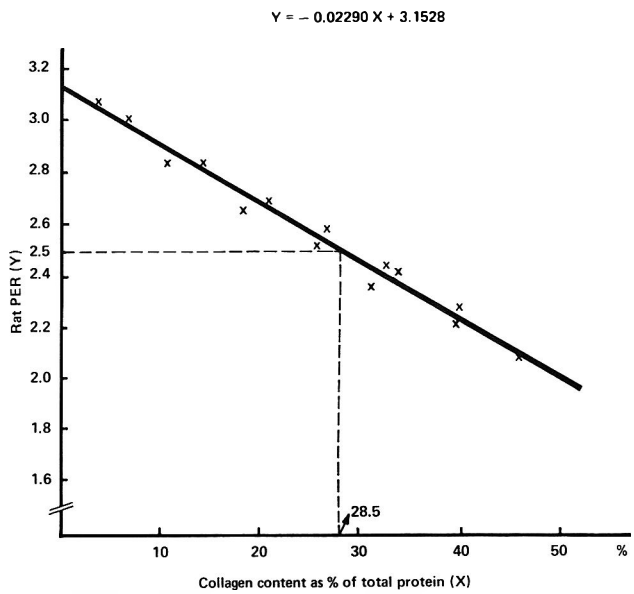

Fig. 1—Relationship between rat and PER and collagen content of beef mixes.

Table 5—Linear regression analyses

Regression equations		r^2	X value corresponding to Y = 2.5
1. Y = −0.02290 X + 3.1528;	Y = rat PER X = collagen content[a]	0.97	28.5%
2. Y = 0.06320 X − 0.1539;	Y = rat PER X = 10 essential amino acids (%)	0.95	42.0%
3. Y = 0.08084 X − 0.1094;	Y = rat PER X = 7 essential amino acids (%)[b]	0.95	32.2%
4. Y = 0.5333 X + 0.2435;	Y = rat PER X = rat NPR	0.94	4.2
5. Y = 0.9840 X + 0.027 9;	Y = rat PER X = calculated tetrahymena PER	0.28	
6. Y = −0.3575 X + 52.1949;	Y = 10 essential amino acids (%) X = collagen content[a]	0.98	
7. Y = −0.2745 X + 40.1456;	Y = 7 essential amino acids (%) X = collagen content[a]	0.98	

[a] Collagen content expressed as % of total protein
[b] lLeu, Leu, Lys, Met, Phe, Thr and Val

Table 6—Effectiveness of predicting PER from collagen content in various meat ingredients

Meat Products	Collagen content, % of total protein	Observed PER	Estimated PER[a]	Difference in PER (Obs est)
85% beef trim	11.1	2.96	2.90	+0.06
Partially defatted chopped beef 1	29.8	2.45	2.47	–0.02
Partially defatted chopped beef 2	46.8	1.92	2.08	–0.16
Partially defatted beef fatty tissue	57.0	1.70	1.85	–0.15
Mechanically deboned red meat 1	17.1	2.83	2.76	+0.07
Mechanically deboned red meat 2	18.8	2.60	2.72	–0.12
Mechanically deboned red meat 3	21.3	2.54	2.66	–0.12
Raw hand deboned chicken meat	4.0	3.13	3.06	+0.07
Raw mechanically deboned chicken	7.9	3.01	2.97	+0.04
Cooked hand deboned chicken	5.0	3.00	3.04	–0.04
Cooked mechanically deboned chicken	25.2	2.61	2.58	+0.03

[a] Y = –0.02290 X + 3.1528; Y = estimated rat PER, X = collagen content

chopped beef samples in collagen content and PER values. This indicates that this product can vary in nutritive quality with different processors. Mechanically deboned red meat showed a moderate level of connective tissue and an average PER of 2.65, which is better than that of casein (2.5).

In chicken meat, there was little difference in PER between raw hand deboned chicken meat (RHDC) and raw mechanically deboned chicken (RMDC). Collagen content was very low for both products. In contrast, cooked mechanically deboned chicken (CMDC) showed a significantly higher collagen content and lower PER than cooked hand deboned chicken (CHDC). Yet, the PER of CMDC (2.61) was better than that of casein. The high collagen content in CMDC was due to the fact that chicken skin was gelatinized during cooking and extruded with the meat during mechanical deboning.

In general, mechanically deboned meat (both red and chicken meat) possessed good protein quality and this product could be successfully utilized in formulated meat products. However, processors should continuously check the nutritional quality of such meat ingredients by estimating PER by the chemical determination of collagen content.

It was concluded from the foregoing results that the rat PER of meat ingredients can be effectively predicted by chemical determination of collagen content which is simple, fast, less expensive and easily adaptable by processors as compared to the rat bioassay or amino acid analysis. It was further suggested that a collaborative test by AOAC should be conducted to confirm the efficacy of the proposed method for predicting PER of meats.

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Ms received 11/27/77; revised 2/18/78; accepted 2/25/78.

The authors express their appreciation to Dr. C.W. Bretzloff of Campbell Institute for Agricultural Research and Mr. D.A. Hoben of Campbell Institute for Food Research for the analyses of amino acids and Tetrahymena bioassay.

A STUDY OF "SWEET" FLAVOR IN LAMB PRODUCED BY FEEDING PROTECTED SUNFLOWER SEED

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ABSTRACT

Cis γ-dodec-6-enolactone [synonym 4-hydroxydodec-cis-6-enoic acid lactone, indexed in Chem. Abs. as dihydro-5-(2-[Z]-octenyl)-2(3H)-furanone (18679-18-0) was found in the subcutaneous fat of lambs fed protected sunflower seed-casein. Larger amounts of this lactone were recovered after heating the fat. By extraction and chromatography of the polar lipids, presumptive evidence was obtained that the lactone was generated from a monohydroxydodecenoic acid triglyceride ester. Reducing the degree of protection of the supplement by including an equal amount of unprotected sunflower seed had no significant effect on the resulting lactone flavor in the cooked meat. The feeding of an undried, protected sunflower seed preparation brought about a highly significant reduction in yield of the lactone and in related flavor properties of the meat, compared to the flash-dried preparation. This implicates the drying process in the development of the sweet flavor defect.

INTRODUCTION

THE LINOLEIC ACID content of the subcutaneous fat of ruminant animals can be increased tenfold over normal values by supplementing their diet with protected formaldehyde–treated vegetable oil or oilseed–protein preparations (Scott et al., 1971).

Ford et al. (1975) found that the flavor of meat from lambs fed protected supplement was markedly different from that of conventionally fed lambs. When lambs were given a protected safflower oil-casein dietary supplement (Park et al., 1976) the meat possessed a characteristic "oily" flavor and odor, attributed to enhanced levels of *trans, trans* deca-2,4-dienal and other carbonyl compounds in the lipid portions of the cooked meat. These carbonyl compounds appear as normal products when foodstuffs containing lipids rich in linoleic acid are heated (Patton et al., 1959). However, when a protected sunflower seed-casein supplement was fed to lambs, the cooked meat possessed the "oily" flavor together with a characteristically "sweet" odor and flavor (Ford et al., 1975). This "sweet" flavor was attributed to the presence of enhanced levels of the lactone γ-*cis*, dodec-6-enolactone, also isolated from the lipid portions (Park et al., 1974). Although the "oily" flavor was not considered likely to be found objectionable by consumers, except at high concentrations, the presence of the lactone at detectable levels (about 1 ppm in the fat) was found quite objectionable by a majority of taste panel members (Park et al., 1975).

The origin of enhanced levels of γ-*cis* dodec-6-enolactone could not be readily explained. However, by analogy with the occurrence of this and other lactones in butter (Dimick et al., 1969) we considered it likely that the lactone would originate from decomposition of a 4-hydroxy-*cis*, dodec-6-enoic acid glyceride in the subcutaneous lipids of the meat. In this report we examine the unheated subcutaneous lipids from lambs which had consumed the protected sunflower seed-casein supplement for the presence of such lactone-generating glycerides.

No detectable quantities of such glycerides have been found in the lipids of sunflower seed or protected sunflower seed (Park, unpublished work; Stark and Urbach, 1974). The differing results obtained from feeding protected sunflower seed-casein or protected safflower oil-casein to lambs remains unexplained. Two hypotheses were apparent: (1) the lower degree of protection of lipids in the former supplement (determined by both "in vivo" and "in vitro" tests, Cook, 1975) may allow a biochemical conversion of a suitable precursor in the lipid prior to hydrolysis and absorption in the abomasum; or (2) chemical oxidation or reaction may occur during the drying of the supplement. Our observation in previous experiments that the quantity of lactone recovered from lamb meat lipids was inversely related to the degree of protection of the sunflower seed-casein supplement fed to the lambs supports the first hypothesis. In this report we have tested these hypotheses by feeding lambs protected, partially protected, unprotected or undried sunflower seed-casein supplements, and examing the meat by both taste panel and chemical analyses.

EXPERIMENTAL

Lactone precursor studies

Lactone-generating glycerides. Approximately 500g of subcutaneous fat tissue were removed from the rib eye area of frozen lamb carcasses possessing a linoleic acid content of 19.8 ± 1.5% of the total fatty acids. To achieve this level the lambs had been fed protected sunflower seed-casein supplement for 55 days before slaughter (Ford et al., 1975). Lipids were extracted from the tissue by mechanical stirring in 1L redistilled chloroform at 35–40°C. The lipid solution was filtered twice, once through paper (Whatman No. 1) and then through a layer of anhydrous sodium sulfate. The solvent was removed by evaporation in a rotary evaporator at low pressure (to 1 torr). The extracted lipids (ca 300g) were degassed in an all-glass vacuum distillation apparatus on a water bath at 35–40°C as described previously (Park et al., 1975). The pressure was reduced to 1×10^{-3} torr, the cold finger filled with liquid nitrogen, and distillation was continued until the pressure reached 1×10^{-5} torr. The distilled material was then removed from the cold finger and traps by extraction with boiling diethyl ether and concentrated to a volume of less than 1 ml (Fraction A). The remaining extracted lipid material was heated in air at 160°C for 90 min and re-distilled as above to a pressure of 1×10^{-5} torr. Distilled material was extracted and concentrated as before (Fraction B). These resulting fractions were examined by gas chromatography on a 100′ Carbowax 20M-coated stainless steel SCOT column as described previously for examination of cold finger fractions (Park et al., 1975). Identification of eluting components was by comparison of retention characteristics and olfactory qualities with those of components of similar distillate fractions previously identified by spectroscopic and chemical means (Murray et al., 1977).

Isolation of lactone precursor-rich fraction from subcutaneous lipids. Subcutaneous fat portions (1.4–1.5 kg) from the carcass portions described above, or from those described later in this text were ground through 3 mm holes and transferred to a 3L flask. The lipids were extracted by occasional stirring with acetone-light petroleum (b.p. 65–70°C) (1:1) on a water bath at 35–40°C. The resulting extract was filtered through a layer of glass wool and the acetone removed on a rotary evaporator "in vacuo," at 35–40°C. Portions of this light petro-

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0022-1147/78/0005-1363$02.25/0

leum extract (containing ca 200g lipid) were diluted with further light petroleum (1L) and then extracted twice in a 2L separating funnel with acetonitrile (400 and 300 ml) by vigorous shaking. The combined acetonitrile layers were washed twice with light petroleum (250 ml portions) and then dried over anhydrous sodium sulfate. The solvent was removed on a rotary evaporator at 35–40°C, leaving a straw-colored oily liquid (3.8g) (Fraction C). The light petroleum phase was likewise dried and the solvent removed (Fraction D). The lactone-generating fractions were identified in these and subsequent fractions through high-vacuum distillation, heating at 160°C and re-distillation of an aliquot as described above, followed by GC examination for the presence of *cis* γ-dodec-6-enolactone.

In a typical experiment a 1-g portion of the acetonitrile extract (Fraction C) was dissolved in 5 ml light petroleum (b.p. 60–75°C) and applied to a column (2.5 × 25 cm) of Silicic acid (Mallinckrodt TLC7) (30g) which had been previously heated overnight at 110–114°C and packed in light petroleum (b.p. < 50°C). The column was developed with further light petroleum (200 ml), 10% diethyl ether in light petroleum (300 ml), diethyl ether (200 ml), and finally methanol (100 ml), and 8 fractions (each 100 ml) were collected (Fractions E1–E8). These operations were carried out in a room at 3–7°C. Solvent was almost completely removed from the fractions on a rotary evaporator at 30–35°C.

Fractions E1–E8 were subsequently examined by TLC on 20 × 20 cm glass plates coated with 250 μm Silica Gel GF 254 (Merck). Developing solvents were either iso-octane-diethyl ether (3:2) (Jurriens and Oele, 1965) or, to limit streaking of the more polar components, iso-octane-diethyl ether-acetic acid (60:40:2 v/v), (Mangold, 1965). Spots were visualized either under light of 254 nm wavelength or by spraying with 5% Phosphomolybdic acid in ethanol and heating 5 min at 95–100°C. Reference lipids were tri-, di- and mono-glyceride fractions recovered from rendered sheep subcutaneous lipids.

Fraction E6 was re-chromatographed on another column of silica gel (30g) packed in 10% diethyl ether in light petroleum (b.p. < 50°C). This column was developed at 3–7°C with sequential 200 ml portions of 10%, 20%, 30% and 50% of diethyl ether in light petroleum and finally pure diethyl ether, 100 ml fractions being collected. These fractions (F1–F10) were evaporated and examined by TLC as before with the same reference glycerides and using the iso-octane, diethyl ether, acetic acid solvent mixture for developing.

Fractions F7 and F8 were combined and spotted on 1 mm thick plates of silica gel GF 254 and developed with the iso-octane, diethyl ether, acetic acid solvent system. After visualizing the plates under 254 nm ultraviolet light the main bands were removed by scraping the silica gel from the plate and washing off the lipids with cold diethyl ether (20 ml).

Each of the bands (G1–G4) was spotted on a 250 μm silica gel plate, developed and analyzed as previously. The main fractions (G1–G4) obtained from the 1 mm plates of R_f value 0.06–0.09, 0.20–0.23, 0.28–0.32, and 0.39–0.45 were dissolved in carbon disulfide (re-distilled) and examined by infrared spectroscopy in 0.1 mm pathlength KBr cells, using a Perkin Elmer 457 instrument.

The above fractions were recovered by removal of solvent and half of these chromatographed on a 45 × 2.5 cm column of porous styrenedivinylbenzene copolymer beads (Biobeads S-x8, Molecular weight exclusion 1,000, Bio-Rad Laboratories, California) packed in tetrahydrofuran (Parliment et al., 1966). 10 ml fractions were collected by fraction collector and monitored with a 254 nm photometric detector. The unused portions of the fractions G1–G4 were transferred to borosilicate glass tubes 40 × 6 mm, 25 μl of water added, the tubes sealed and then heated at 160°C for 3 hr. The tubes were opened next day, 50 μl diethyl ether added and 0.5 μl of the ether solution injected into the gas chromatograph using the Carbowax 20M-coated stainless steel SCOT column as described earlier.

Animal feeding studies

Animals and feed. In Experiment 1, 46 Border Leicester × Merino wether lambs approx. 20 weeks old, mean liveweight 27.7 ± 0.30 (S.E.) kg were divided into four groups. Three groups contained 12 lambs of mean liveweight 25.4 ± 0.29 kg selected on a stratified random basis. Each of these three groups was assigned to one of three dietary regimes and held outdoors in pens at the CSIRO Meat Research Laboratory. A basal ration of chopped alfalfa and cracked barley (50:50 w/w) was included in all three diets. The diets used were (1) basal ration plus fully portected sunflower seed-casein (70:30 w/w), (2) basal ration plus cracked sunflower seed (unprotected sunflower seed) (80:20 w/w) and (3) basal ration plus protected sunflower seed-casein and cracked sunflower seed (75:15:10 w/w) (partially protected sunflower seed). The amount of feed offered daily increased from 0.4 kg to 1.3 kg/head. A fourth group of 10 lambs (mean liveweight 33.05 ± 0.25 kg) were assigned to an all-grass forage diet (4). This last group was expected to have a higher subcutaneous fat cover than the other groups and therefore be more likely to match the others, which were consuming higher energy diets, at the end of the experimental feeding period. Six weeks after feeding commenced all lambs were slaughtered at a nearby abattoir. The carcasses were chilled, stored at −30°C and thawed when required as previously described (Park et al., 1976).

In Experiment 2, 36 Dorset Horn wether lambs, approx 6 months old of mean liveweight 23.3 ± 0.43 kg were divided into four groups of nine and assigned to one of four diets;

(1) basal ration as above plus dried sunflower seed-casein (70:30 w/w) (dried supplement);
(2) basal ration plus undried protected sunflower seed-casein (50:50 w/w) comprising ground sunflower seed, water, formaldehyde, casein, alkali and polyvalent cations ($CaCl_2$ and $MgCl_2$, Cook, 1974) (wet supplement);
(3) basal ration only;
(4) an all grass forage diet.

The lambs for this last group were selected from the heaviest lambs for the reasons discussed in Experiment 1 and had a mean initial liveweight of 26.5 ± 0.36 kg. The lambs in diets (1)–(3) had mean initial liveweights of 22.2 ± 0.56 kg, were selected on a stratified random basis and fed outdoors in pens. The amount of feed offered to the animals was increased gradually from 0.4 to 1.3 kg/head/day. Eight weeks after feeding commenced the animals were slaughtered and the carcasses treated as in Experiment 1.

Laboratory taste panel evaluations. Taste panel evaluations were carried out within 8 wk of slaughter. Twenty trained panelists, selected as described by Ford et al. (1975) scored the meat flavor properties as previously with one exception. In experiment 1 intensity ratings of "oily" and "sweet" flavors were included in place of "different" flavor on the same 9 point scale from "none" to "very strong" as other flavor properties. Panelists were instructed to score any aromas or flavors which they would not normally expect in lamb as "different." In both experiments the meat was prepared from rear legs roasted in stainless steel trays in a pre-heated forced-draught convection oven at 150°C, with a cooking time of 6 min for each 100g. Samples were tasted under red light and order of tasting was randomized through the panel. In Experiment 1 three samples were presented, hot, to the panel at each session. Weight rank-matched lambs were used, one from each of the dietary treatments (1), (3), and (4). The meat from the lambs given unprotected supplement was not included in the meats evaluated by the flavor panel since it was considered that the meat would not possess any unusual odor and flavor properties.

In Experiment 2 the meat was roasted uncovered, stored overnight at 5°C in polythene bags and served cold. One sample from each of the four dietary treatments was served at each session from weight rank matched lambs. The data were entered on computer cards and analyzed for differences between dietary treatments, for each flavor property evaluated.

Chemical examinations. Subcutaneous fat tissue was excised from the tail portion of all lambs after chilling the carcasses, and the percentage of individual fatty acids was determined by gas liquid chromatography of the methyl esters, as described previously (Park et al., 1975).

The steam-volatile components were obtained from the cooked, ground meat and the volatile components were recovered from the cold finger fraction obtained by high-vacuum degassing of the fat from the cooked meat as described by Park et al., (1975). These volatiles were examined by gas chromatographic techniques as described before. The columns employed were 50 meter glass SCOT columns coated with either Carbowax 20M or SF 96 operated under the conditions employed previously (Park et al., 1977). Components of interest had been identified previously (Murray et al., 1977) by gas chromatography-mass spectrometry techniques and by olfactory assessment of the compounds eluting from the gas chromatograph.

RESULTS & DISCUSSION

Lactone precursor studies

Gas chromatographic examination of the cold finger distillate (A) showed only 2 components present in significant quantities. These components were *cis* γ-dodec-6-enolactone

Table 1—Experiment 1—Mean daily liveweight gain, percentage linoleic acid in subcutaneous lipids and mean taste panel ratings for aroma and flavor properties of meat of lambs from experimental diets

Dietary treatment	Liveweight gain[a]	Linoleic acid[b]	Flavor property[a]					
			Meat aroma	Meat flavor	Different aroma	Oily flavor	Sweet flavor	Acceptability of flavor
Grass	–	2.4	4.24	4.31	1.05	0.70	0.65	4.77
Fully Protected	137	16.2	3.85	3.88	1.45	1.31	0.72	4.63
Part Protected	140	12.1	3.92	3.83	1.52	1.43	0.67	4.52
Unprotected	111	3.9	–	–	–	–	–	–
Significance of treatment			***	***	*	***	N.S.	N.S.
5% least significant differences			0.16	0.21	0.32	0.33	0.13	0.25

[a] Liveweight gain in g/day of treatment period

[b] Expressed as a percentage of the total fatty acids

[c] Aroma and flavor intensity ratings from 0 (zero) through 4 (moderate) to 8 (very strong) and acceptability on a hedonic scale from 0 (very poor) through 4 (moderate) to 8 (very good)

***, $p \leq 0.001$; *, $p \leq 0.05$; N.S., not significant.

and γ-dodecalactone, present in the ratio of 6.7:1, respectively. The unheated subcutaneous tissue then must contain free lactone, unless these were generated during the extraction and/or distillation. Further evidence supporting the presence of these lactones in the unheated subcutaneous fat tissue was found when several laboratory personnel with previous relevant flavor panel experience agreed that the subcutaneous fat tissue used here possessed the characteristic sweet odor, after warming to room temperature. The presence of these lactones in the unheated subcutaneous lipids parallels the occurrence of similar lactones in unheated milk fat (Boldingh and Taylor, 1962).

The degassed lipids, after heating at 160°C for 90 min and further degassing, produced a cold finger distillate (B) much more complex in composition than that obtained prior to heating. The principal components found from gas chromatographic analysis were *trans, trans* deca-2,4-dienal and *cis* γ-dodec-6-enolactone. The qualitative and quantitative composition were very similar to that of the corresponding cold finger distillate obtained from the cooked meat from similar sheep. The yield of *cis,* γ-dodec-6-enolactone was two to three times greater than from the unheated fat. Thus the quantity of lactone is increased considerably by heating. This again parallels the experience with milk fat after heating to similar temperatures (Boldingh and Taylor, 1962) and supports the hypothesis that these lactones obtained originate from a 4-hydroxy *cis*,-dodec-enoic acid triglyceride ester.

The methods employed in seeking presumptive evidence for the presence of a 4-hydroxy dodec-6-enoic acid triglyceride ester in the unheated fat from sunflower seed-casein fed lamb meat were similar to those employed by Jurriens and Oele (1965) and Parliment et al., (1966) in butterfat studies. A fraction rich in the lactone precursor was obtained by partitioning the lipid extracted from subcutaneous fat tissue between hexane and acetonitrile and recovering the polar components in the acetonitrile phase. The hexane phase (fraction D) did not produce any lactone on heating at 160°C for 2 hrs, whereas an aliquot of the acetonitrile extract (C) produced both the *cis,* γ-dodec-6-enolactone and its saturated counterpart, as found by gas chromatographic analysis. Liquid chromatography of the acetonitrile extract on silica gel further separated the components. TLC examination of the resultant fractions E1–E8 showed that fraction E6 (eluted with 100% ether) contained main components with Rf values close to that of simple 1,2 or 1,3 diglycerides, while fraction E5 had a strong odour of free lactone. Heating of an aliquot of the fraction E6 resulted in the production of the lactones. Rechromatography of Fraction E6 on another column of silica gel as described in methods section provided further separation of the components. Fractions F7 and F8, eluted with 50% diethyl ether in light petroleum were found by TLC to produce 4 main spots. After preparative TLC and extraction with diethyl ether, each fraction was substantially homogeneous as found by analytical TLC. On infrared spectroscopic examination of these fractions, Fraction G2 (R_f 0.2–0.23 cf. diglycerides 0.2–0.35) revealed hydroxyl absorption at 3460 and 1100 cm^{-1} and ester bands at 1740 and 1160 cm^{-1}.

When further chromatographed on Biobeads, TLC examination of fractions from G2 showed that it contained very little diglyceride material. The main component(s) had an elution volume intermediate in value between tri- and di-glycerides. When the other fractions (G1, 3, 4) were passed through the polystyrene gel they exhibited slightly higher elution volumes and consequently lower molecular weights.

Finally, equal aliquots of all the fractions obtained by preparative TLC separation on silica gel (*G1–4*) were heated with a little water in sealed tubes at 160°C and these examined by GC, to determine which contained the lactone generating glycerides. Fractions 1, 2 and 3 produced lactones, the amount in 2 being some five times greater than in 1 and ten times that in 3. Therefore Fraction 2, of R_f value 0.22 in the iso-octane-ether-acetic acid system had the highest amount of lactone precursor and possessed molecular weight, infrared absorption, TLC and column chromatographic properties consistent with a monohydroxyacyl triglyceride being a major component. While this is presumptive evidence only, it is considered adequate to be reasonably certain that the precursor of *cis,* ±-dodec-6-enolactone in the subcutaneous lipid from lambs consuming protected sunflower seed-casein is a triglyceride containing a 4-hydroxy dodec-*cis*, 6-enoic acid acyl group.

Animal feeding studies

Details of liveweight gain of the lambs on the various dietary treatments in Experiment 1, the percentage linoliec acid in the subcutaneous fat and the mean taste panel ratings from the comparison of the resultant meats are summarized in Table 1. The lambs on the grass forage diet showed no increase in liveweight during the experimental feeding period but had final liveweight and carcass weights very close to those lambs fed in the pens e.g. mean carcass weight of grass fed lambs was 13.5 ± 0.2 (S.E.) kg and those on fully protected supplement was 13.9 ± 0.3 kg. The linoleic acid contents of the subcutaneous (tail) fat for the various treatment groups were as expected, although that of the fully protected supplement group were a little less than desired. This was possibly due to an unusually poor palatability of the supplement to the lambs observed during the experiment.

The flavor panel results for the most part were similar to those obtained in previous experiments (Ford et al., 1975, Park et al., 1975), although the absence of a significant dif-

ference in both acceptability of flavor and "sweet" flavor scores was somewhat unexpected. The significantly lower "oily" flavor score obtained for grass-fed lamb meat was as expected, in view of the higher linoleic acid content of the other meats. The absence of any difference in sweet flavor intensity scores demonstrated clearly that the degree of protection of the sunflower seed lipids in the dietary supplement "per se" was not likely to be a significant factor in determining the intensity of "sweet" flavor in lamb meat.

The relative yields of the more significant volatile components from the cooked meat found in either the steam volatiles or in the cold finger fraction from high vacuum degassing of the lipids are shown in Table 2. These yields are expressed relative to that of one of the more innocuous components of the respective fractions as judged by lack of odor. These data complement that of the taste panel; particularly in relation to the "oily flavor" and "sweet flavor" intensity scores. The greatly increased yields of *t,t*-deca-2, 4-dienal obtained by feed fully or partially protected sunflower seed-casein supplements to the lambs compared to both grass fed and unprotected supplement-fed lambs are consistent with the increase in "oily flavor" scores in Table 1. Likewise the absence of any startling increase in corresponding *cis*, γ-dodec-6-enolactone levels confirms the taste panel responses and re-affirms the conclusion that the degree of protection of the supplement "per se" is not a significant factor in contributing to the lactone odor and flavor characteristic of this meat.

The data on daily liveweight gain, mean percentage of linoleic acid in subcutaneous tissue lipids and mean taste panel responses to the meat served cold from Experiment 2 are all summarized in Table 3. As in Experiment 1, the lambs consuming only a grass forage diet showed no increase in liveweight during the experimental feeding period but had final liveweight and carcass weights close to those of the penned animals. The linoleic acid content of subcutaneous lipid fatty acids was satisfactory, although those from the wet supplement treatment were lower than desired, due probably to an underestimation of the water content of the supplement. Palatability of the wet supplement was very good, although it had to be stored at 0°C to inhibit mold growth.

The flavor panel results demonstrate a significant treatment difference between diets for all flavor properties except meat flavor intensity and the panel significantly preferred meat from lambs fed the basal diet. The dry and wet supplement treatments both showed a highly significant greater intensity of "different" flavor than basal and grass diets. On examination of taste panel members' comments we found that the "oily" flavor was apparently the main contributor and the "sweet" flavor secondary. The dry supplement treatment elicited nearly twice as many comments on the presence of a "sweet" odor than did the wet supplement when tasted cold, which is when the assessment has been found to be most sensitive and reliable.

The relative yields of the more significant volatiles obtained from the cooked meat from animals in Experiment 2 and found by GC examination are shown in Table 4. These data support our assessment of the taste panel responses in relation both to the "sweet" odor and "oily" odor or flavor in wet and dry supplement treatments. The yield of *cis*, γ-dodec-6-enolactone from the wet supplement treatment group was less than half that from the dry supplement and this was statistically highly significant ($P < 0.01$). The standard error of the difference between the two treatment groups was 1.7.

The yield of *t,t*-deca-2, 4-dienal was also lower from the wet supplement treatment, but not significantly so.

We consider that these two sets of data offer evidence that the wet supplement treatment produces a lower amount of prescursor of the *cis*, γ-dodec-6-enolactone in the subcutaneous lipids than the dried supplement. The slightly lower amount of linoleic acid in the tissue lipids of the sheep from the wet supplement could have contributed to the lower lactone precursor level, but not sufficiently to account for such differences in our experience (Park et al., 1975). These observations support our second hypothesis, as stated in our introductory remarks that some chemical changes could occur in the supplement during the drying process, leading to the production of an intermediate capable of metabolism to the lactone precursor. The conditions during drying of this supplement in a Raymond flash-drier are quite severe (Scott and Cook, 1973), as the powdered supplement is dried by a hot air (ca 200°C) flash drier. Numerous reactions are possible including oxidative and additive reactions, considering the presence of unsaturated lipids, formaldehyde and hot air. It is of interest to note that we have examined some lamb from New Zealand which contained well over 20% linoleic acid and was produced by feeding a protected sunflower seed preparation which had been dried in another (Ring) drier. The cooked meat contained only a low level of the *cis* γ-dodec-6-enolactone, similar to that from feeding protected safflower oil-casein (Park et al., 1976) and not sufficient to impart any sweet flavor characteristics to the meat.

Table 2—Experiment 1—Relative[a] yield[b] of selected volatiles from lamb meat from experimental diets

Volatile component	Source	Experimental diets: Grass	Unprotected sunflower seed	Part-protected sunflower seed	Fully protected sunflower seed
n-hexanal	aqueous	0.7	11	15	23
n-pentanol	aqueous	0.4	14	17	20
t,t deca-2, 4-dienal	lipid	<0.1	1.2	30	30
γ-dodecalactone	lipid	2	1.4	1.0	0.7
γ,*cis*-dodec-6-eno lactone	lipid	<0.1	1.2	3	2

[a] Mean yield of components from aqueous distillates relative to that of the component of arithmetic retention index 1360 or of lipid cold finger distillates relative to that of n-octadecane.

[b] Yield calculated from peak heights as obtained by GC examination on Carbowax 20M or SF96– coated glass SCOT columns.

Table 3—Experiment 2—Mean daily liveweight gain, percentage linoleic acid in subcutaneous fat and mean taste panel ratings of cold roast meat from lambs comsuming experimental diets

Dietary treatment	Liveweight gain[a]	Linoleic acid[b]	Flavor property[c]: Meat aroma	Meat flavor	Different aroma	Different flavor	Acceptability of flavor
Basal	139	2.5	3.94	3.85	1.15	1.61	4.29
Grass	–	2.7	3.53	3.74	1.14	1.68	4.04
Dry supplement	186	18.8	3.81	3.63	1.83	2.49	3.74
Wet supplement	141	16.7	3.81	3.61	1.66	2.38	3.72
Significance of treatment[d]			*	N.S.	***	***	*
5% least significant difference between treatments			0.27	0.27	0.32	0.45	0.39

[a,b,c,d] See Table 1 footnotes

Table 4—Experiment 2—Relative yield[a] of selected volatiles from lamb meat from experimental diets

Volatile component	Source	Experimental diets			
		Grass	Basal	Wet supp	Dry supp
n-hexanal	aqueous	13	72	115	110
n-pentanol	aqueous	8	18	14	10
t,t-deca-2, 4-dienal	lipid	< 0.1	20	50	85
γ-dodecalactone	lipid	3	2	1.4	6.5
γ, *cis*-dodec-6-enolactone	lipid	< 0.1	2	4.5	10.5

[a] Calculated as for Table 2

The first of our introductory hypotheses that lower degrees of protection of the supplement lead to higher lactone precursor production was, however, not supported by our experimental observations. This was proposed to explain how less efficiently protected sunflower seed-casein gave higher lactone levels in lamb than the better protected safflower oil-casein. It then is of interest to note that the safflower oil-casein preparations were dried in Rogers or Niro spray driers (Scott et al., 1971) and these provide much milder conditions than the Raymond flash-drier. This further implicates the latter drier as a likely source for production of lactone precursors and supports our second hypothesis.

CONCLUSIONS

IN CONCLUSION we have shown:

(1) the precursor of *cis* γ-dodec-6-enolactone in the subcutaneous lipids of sheep consuming protected sunflower seed-casein is probably a 4-hydroxy-dodec-*cis*, 6-enoic acid, diacyl triglyceride;

(2) the degree of protection of the sunflower seed "per se" is not a significant factor governing the production of elevated levels of the lactone precursor in the subcutaneous lipids and

(3) feeding of undried protected sunflower seed-casein supplement to lambs significantly lowers the production of the lactone in the cooked meat.

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Ms received 6/27/77; revised 11/21/77; accepted 12/1/77.

These experiments were funded in part by grants from Alta Lipids (Aust.) Pty. Ltd. and from the Australian Meat Research Committee. Some of the animals used were obtained from the flocks of the CSIRO Division of Animal Production, Armidale, New South Wales. The protected sunflower seed-casein supplement was provided either by Dr. L.J. Cook of the CSIRO Division of Animal Production, Prospect, New South Wales or by Alta Lipid Pty. Ltd., Windsor, New South Wales. The technical skills of Phillipa Hancock, Lynn Ussher, I. Griffiths and T. Steginga and the participation of laboratory staff as taste panel members are gratefully acknowledged.

ANTIBOTULINAL ROLE OF ISOASCORBATE IN CURED MEAT

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ABSTRACT

The effect of antioxidants, reducing agents, and a chelating agent were tested in perishable canned cured meat. Isoascorbate, ascorbate, and cysteine enhance the antibotulinal effect of nitrite in perishable canned cured meat. It was determined that this effect was not due to the antioxidant or reducing properties which these compounds possess. The data indicate that they enhance the effect of nitrite by sequestering a metal ion(s) in the meat. It is suggested that nitrite (nitric oxide) reacts with a cation dependent material within the germinated botulinal cell and blocks a metabolic step which is essential for outgrowth. Enhancement of nitrite by isoascorbate, and similar compounds, may be due to preventing repair of damaged material or formation of new cation dependent material.

INTRODUCTION

TWO REPORTS show that reducing agents enhance the antimicrobial effect of nitrite in shelf-stable canned cured meat. Johnston and Loynes (1971) found that adding ascorbate or cysteine to an under-processed meat slurry greatly reduced the amount of nitrite required to inhibit botulinal spore outgrowth. Thioglycollate was less effective. Ashworth and Spencer (1972) obtained the same responses in ground pork heated at 115°C for 20 min. Two additional reductants, sodium formaldehyde sulfoxylate and sodium formaldehyde bisulfite, did not show this effect. Tompkin et al. (1978a, b, c) demonstrated isoascorbate to enhance the antibotulinal effect of nitrite in perishable canned cured meat. The addition of isoascorbate was considered a major reason for the differences in the magnitude of botulinal inhibition by nitrite as reported by different laboratories. Understanding the role of isoascorbate might elucidate the mechanism by which nitrite inhibits botulinal outgrowth in perishable canned cured meat.

The function of nitrite in meat curing is fourfold: (a) to stabilize the color of the lean tissues, (b) to contribute to the characteristic flavor of cured meat, (c) to inhibit growth of a number of food poisoning and spoilage microorganisms, and (d) to retard development of rancidity (Kramlich et al., 1973).

Borenstein (1965) listed the functions of isoascorbate in cured meat as (1) act as an oxygen scavenger, (2) shift the redox potential of the system to a reducing range, and/or (3) reduce undesirable oxidation products.

Others have listed the functions of ascorbate and its derivatives as: (a) to participate in the reduction of metmyoglobin to myoglobin, thereby accelerating the rate of curing; (b) react chemically with nitrite to increase the yield of nitric oxide from nitrous acid; (c) in excess they act as an antioxidant, thereby stabilizing both color and flavor (Kramlich et al., 1973).

Ascorbic acid is known as a synergist for preventing rancidity of fats and oils. This function has been attributed to chelation of powerful pro-oxidants (e.g., copper and iron) which are commonly present in food fats (Lundberg, 1962; Chipault, 1962). Ethylenediaminetetraacetic acid (EDTA) is another synergist with antioxidants which functions in a like manner (Furia, 1964).

EDTA in sufficient concentration prevents outgrowth of *C. botulinum* type E in haddock homogenate (Segner et al., 1966) and type A in white fish chub homogenate (Winarno et al., 1971). EDTA and other chelating agents have been reported to stimulate germination as well as inhibit outgrowth (Riemann, 1963). Schroeder and Busta (1974) found EDTA to enhance glucose inhibition of *C. perfringens* in a sugar and amino acid medium.

Borenstein and Smith (1968) patented a combination of EDTA, nitrite, and ascorbate to accelerate the formation of cured meat pigment. Borenstein (1976) reported EDTA to accelerate the disappearance of ascorbate in the presence of nitrite plus myoglobin. He suggested that the catalytic, ascorbate potentiator effect of EDTA in cured meat pigment synthesis is related to pigment reduction rather than nitric oxide production.

The research described herein was done to learn how isoascorbate enhances the antibotulinal effect of nitrite. The foregoing suggests several possible mechanisms by which this may occur. Tests are described wherein antioxidant, reducing, and chelating agents were compared with isoascorbate in a perishable canned cured meat.

EXPERIMENTAL

Inoculum

The *C. botulinum* inoculum consisted of a mixture of 5 type A (33A, 36A, 52A, 77A, and 12885A) and 5 type B (ATCC 7949, 41B, 53B, 213B and Lamanna B) strains prepared as previously described (Christiansen et al., 1973). The mixed spore suspension was heat-shocked at 80°C for 15 min and added to the meat during formulation using a target level of 100 spores/g of product.

Formulation and processing

Perishable canned comminuted cured pork was formulated with salt, water, and sugar; inoculated; processed; and chilled as described earlier (Christiansen et al., 1973). Pork hearts or beef round were used in place of fresh pork ham in some of the tests as indicated.

The effect of antioxidants was tested in the first experiment by substituting butylated hydroxyanisole (BHA) and tertiary butylhydroquinone (TBHQ) from Eastman Chemical Prod., Inc. (Kingsport, TN) for sodium isoascorbate. Fresh pork ham was used with 50 μg/g sodium nitrite added (w/w) on the basis of the meat portion of the formulation. Controls included one variable with no antioxidant and another with 0.02% sodium isoascorbate.

The reducing agents, sodium sulfide ($Na_2S \cdot 9H_2O$ – A.S. LaPine & Co., Chicago) and L-cysteine hydrochloride hydrate (Sigma Chemical Co., St. Louis) were added at levels of 0.071% and 0.1% (w/w) on the basis of the meat portion of the formulation. Beef was used in the product containing cysteine. Sodium ascorbate was tested in both beef and pork at a level of 0.02% on the basis of the weight of the meat in the formulation. All variables were formulated with 156 μg/g sodium nitrite and without sodium isoascorbate.

The tetrasodium salt of ethylenediaminetetraacetic acid (Na_4EDTA $\cdot 2H_2O$; Sigma Chemical Co., St. Louis) was used to determine the effect of a chelating agent having no reducing properties. The product for this test consisted of pork hearts formulated with 156 μg/g sodium nitrite and 0.02% sodium isoascorbate. Product was prepared with and without EDTA (500 μg/g) added on the basis of the total formulation.

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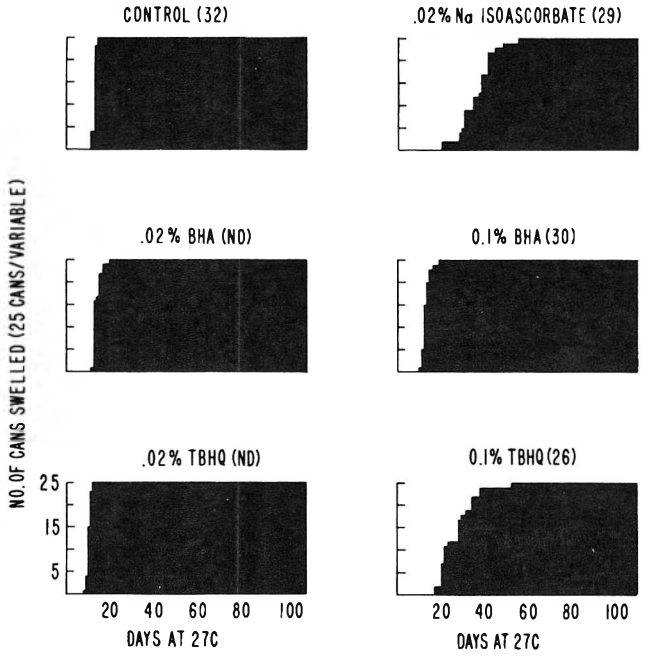

Fig. 1—Effect of antioxidants on botulinal outgrowth in perishable canned cured pork formulated with 50 μg/g sodium nitrite and placed at 27° C after processing. Values in parentheses (i.e., 32, 29, 30, 26) are for residual nitrite after cook. ND = not done.

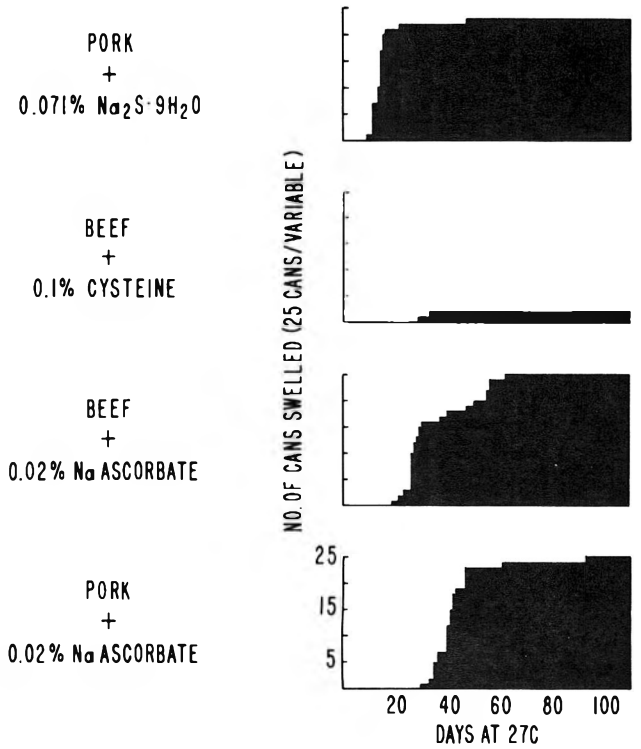

Fig. 2—Effect of reducing agents on botulinal outgrowth in perishable canned cured pork or beef formulated with 156 μg/g sodium nitrite and placed at 27° C after processing.

Holding conditions

Twenty-five cans of inoculated product per test variable were placed at 27°C for up to 110 days. Cans were removed from incubation as they swelled.

Microbiological and chemical analyses

Spore levels, toxin assays, and chemical analyses were determined as previously described (Christiansen et al., 1973). The first 5 cans to swell from each test variable were tested for toxin.

RESULTS

FIFTY-SEVEN CANS representing the first 5 cans to swell from each test variable were tested for botulinal toxin. Fifty-four of the cans contained botulinal toxin. This high correlation between swelled cans and toxic product supports the opinion that the swell patterns in the accompanying figures represent botulinal outgrowth in the product. The three non-toxic swells are believed due to botulinal outgrowth with sublethal toxin titers since these samples were also putrid and proteolysed.

The data presented in Figure 1 show that BHA at levels as high as 0.1% does not enhance the effect of nitrite. TBHQ at 0.02% had no effect. TBHQ at a level approximating 5 times the level of isoascorbate did cause a delay in botulinal outgrowth. The data indicate that the synergistic effect of isoascorbate is not due to its antioxidant properties.

The results presented in Figure 2 show that sodium sulfide had no effect on the efficacy of nitrite. The inhibition observed in the product containing sodium sulfide was of the same magnitude as occurs with 156 μg/g sodium nitrite in the absence of isoascorbate (Tompkin et al., 1978a). Cysteine enhanced the effect of nitrite. These results show that of the two reducing agents cysteine, but not sodium sulfide, shares a common property with isoascorbate.

The addition of EDTA, a strong chelating agent, caused a substantial delay of botulinal outgrowth in pork heart meat (Fig. 3) in the presence of nitrite and isoascorbate. The control product without EDTA showed no botulinal inhibition.

LEVEL OF EDTA ADDED (μg/g)	RESIDUAL $NaNO_2$ AFTER COOK (μg/g)
0	97
500	96

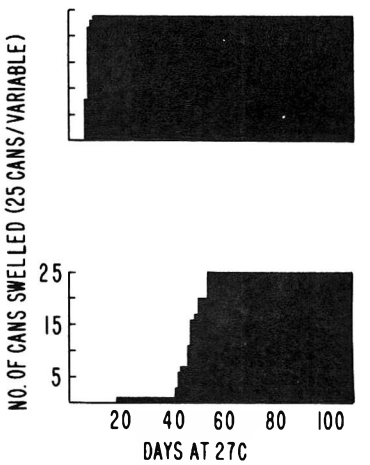

Fig. 3—Effect of EDTA on botulinal outgrowth in perishable canned cured pork hearts formulated with 156 μg/g sodium nitrite and 0.02% sodium isoascorbate and placed at 27°C after processing.

DISCUSSION

THE MECHANISM by which isoascorbate enhances the antibotulinal effect of nitrite does not appear to be due to either its antioxidant or reducing properties. The antioxidants, BHA and TBHQ, did not show the same effect as isoascorbate at the 0.02% level. All three compounds have similar molecular weights (180, 166, 198). Thus, differences in molar concentration did not influence the results. The reason for the increased

inhibition with TBHQ at the 0.1% level is as yet not clear. This material may have reactive properties aside from its antioxidant capability.

Sodium sulfide, a reducing agent having neither antioxidant nor sequestering properties, did not alter the efficacy of nitrite when added at the level tested. Sodium sulfide has been used as a reducing agent in media for rumen bacteria (Bryant and Robinson, 1961) and rumen protozoa (Clarke, 1963). It seems incongruous that adding reducing agents to this meat product should delay the outgrowth of *C. botulinum*, a strict anaerobe. The fact that botulinal outgrowth readily occurs without the addition of a reducing agent indicates some other function for isoascorbate in the inhibitory system. It does not appear that a more reduced environment is necessary for nitrite to become more inhibitory.

Isoascorbate, ascorbate, and cysteine are capable of sequestering cations. The greater inhibition of cysteine compared with isoascorbate and ascorbate is probably due to the fact that a higher molar concentration of cysteine was used. Cysteine was used at about five times the molar concentration of the other two compounds.

It is significant that EDTA, a strong chelating agent which is not considered a reducing agent, enhances the antibotulinal effect of nitrite as does isoascorbate, ascorbate, and cysteine. Figure 3 shows that 0.02% sodium isoascorbate is inadequate for botulinal inhibition in heart meat even though formulated with 156 μg/g sodium nitrite. The loss of inhibition when pork hearts are substituted for pork ham is reproducible (Tompkin et al., 1978c). Figure 3 indicates that some of the inhibition is regained when sodium isoascorbate is supplemented with EDTA. In a separate test (data not shown) all 25 cans swelled by the ninth day when 500 μg/g EDTA was added to fresh pork ham without sodium nitrite or isoascorbate. Neither isoascorbate nor EDTA, alone, can delay botulinal outgrowth in perishable canned meat. However, they share a common property of being able to act synergistically with nitrite to delay botulinal outgrowth.

We conclude from these results that isoascorbate, ascorbate, cysteine, and EDTA tie up one, or more, cations which are essential for botulinal outgrowth. The fact that, in the absence of added sodium nitrite, EDTA and isoascorbate are not inhibitory suggests that EDTA and isoascorbate retard repair of nitrite-injured cells by withholding an essential cation. Outgrowth does eventually occur. The time at which outgrowth occurs in this product is dependent on the inoculum level and amount of added nitrite (Christiansen et al., 1973). At a given inoculum level, outgrowth corresponds with the decreasing level of residual nitrite in the product (Christiansen et al., 1978; Tompkin et al., 1978b).

It appears that an essential metabolic step involving a cation is blocked by the reaction of nitric oxide within the vegetative cell. Eventual outgrowth could be dependent upon depletion of nitrite (nitric oxide) to nontoxic levels, repair of nitric oxide damaged material within the vegetative cell, and/or synthesis of new cation dependent material by the cell.

It is not unreasonable to assume that the complex formed by the reaction of nitric oxide and the cation dependent material within the vegetative cell is reversible. This complex would be formed in most instances after thermal processing as the spores germinate during storage. Subsequent heat is not applied which might cause such a reaction to be irreversible as is the case when cooked cured meat pigment (nitrosyl hemochrome) is formed.

Another possibility which has not been addressed is a direct reaction of nitric oxide with the cation. If such a reaction is possible, it would have to have the effect of detoxifying nitric oxide to agree with the data.

The sequestering function of isoascorbate is apparent. An additional function of isoascorbate may be important early in the incubation period when high residual nitrite levels still exist in the product. It is known that isoascorbate and cysteine form nitroso-reductant intermediates which break down to release nitric oxide (Fox and Ackerman, 1968). The commercial value of this reaction is an increased rate of cure color development. The possibility exists that an increased generation of nitric oxide, a bactericidal agent, helps to destroy germinated botulinal cells. This function of ascorbate and its derivatives was applied in the patented process of Schack and Taylor (1966). It is a valid question at this time whether the increased stability of product noted by Schack and Taylor was primarily due to the generation of nitric oxide or the sequestering of an essential cation by ascorbate.

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Ms received 12/16/77; revised 3/28/78; accepted 4/5/78.

FACTORS AFFECTING INHIBITION OF *Clostridium botulinum* IN CURED MEATS

S. H. LEE, R. G. CASSENS and H. SUGIYAMA

ABSTRACT

Ground pork trim was formulated with various levels of nitrite and one of two NaCl concentrations, canned and heated at 90, 100 or 110°C. Cured meats were similarly prepared with meat whose sulfhydryls had been reacted with silver ions. Antibotulinum activity in these cured products was tested by inoculating *Clostridium botulinum* spores directly into canned meats (96 spores/150g) or into homogenates (1:1, w/v) adjusted to 30 ppm $NaNO_2$ (48 spores/16.5 ml). Antibotulinum activity was detected by both challenges. It was greater in meats cured with higher nitrite concentrations, was lower if processing was at high temperature (110 vs 90°C) and decreased during storage of the cured meats. Inhibitory activity was found in cured products made of silver-treated meats.

INTRODUCTION

THE NITRITE used to cure meat produces products with desirable appearance, specific organoleptic characteristics, and microbiological stability. Reduction in the amount of nitrite for curing meats has been proposed since small amounts of carcinogenic nitrosamines have been detected in some products (Krol and Tinbergen, 1974). Drastically reducing the ingoing level of nitrite would be a simple solution to the potential carcinogenic problem, but such action is likely to result in products having unacceptable microbiological hazards.

Most commercially cured meats are not heated sufficiently to kill the heat resistant bacterial spores, such as those of *Clostridium botulinum*, but they have had an excellent safety record. Since curing of meats includes processing with heat, the suggestion has been made that the microbiological safety of these meats may be due to the "Perigo factor," an inhibitor of bacteria that is formed when nitrite is heated in certain bacteriological culture media (Perigo et al., 1967; Johnson et al., 1969). The inhibitor is formed by the reaction of nitrite with sulfhydryls and iron (Asan and Solberg, 1976; Huhtanen and Wasserman, 1975; Moran et al., 1975), and may be due to a mixture of several sulfhydryl complexes including nitrosocysteine, Roussins-salt and cysteine-NO-Fe complex (Incze et al., 1974; van Roon, 1974).

However, more recent observations indicate the inhibitor in cured meats is likely different from the Perigo factor. The Perigo factor is formed at 105°C or higher (Perigo et al., 1967), a temperature range not used in processing cured meats (Ashworth et al., 1973; Greenberg, 1973). Additionally, the antibacterial activity is neutralized by meat particles (Johnston et al., 1969; Moran et al., 1975).

We report here the presence of antibacterial activity in cured meats and its stability during storage of the products. Requirements of sulfhydryls for the formation of the inhibitor in cured meats was studied. The findings with meat are compared to results obtained with the Perigo factor.

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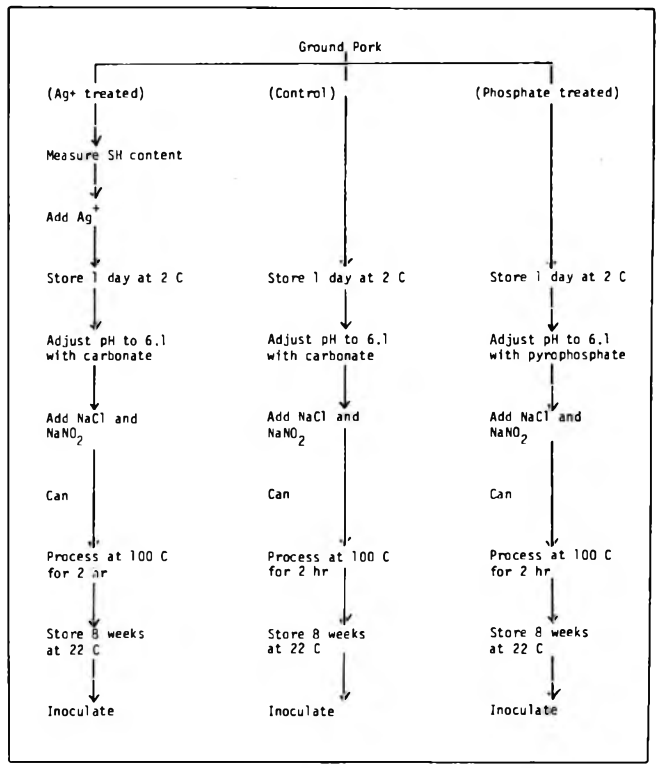

Fig. 1—Flow diagram for preparing cured meat samples of ground meat lot #1.

MATERIALS & METHODS

Meat preparations

Lean pork meat was obtained by deboning the whole carcass of animals which had been slaughtered and chilled at 2°C for 24 hr in the Meat Laboratory of the University of Wisconsin. The muscles were ground through a plate with 3/8 in. holes. Except where noted, sodium carbonate was used to adjust meats to pH 6.1. Sodium carbonate required was determined with 50g ground meat and the amount needed for 1 kg was added as a solution in 100 ml of water. Final pH was always confirmed.

Dry salts were mixed in ground meats with a Hobart mixer to obtain batches having 2% NaCl and 0, 50, 100, 200 or 300 ppm $NaNO_2$. Portions of 150g were placed in 300 × 200 cans, sealed under vacuum and heated. Approximate final composition was 63% H_2O, 22% fat and 12% protein.

Steps in preparing lot 1 meat are shown in Figure 1. These cans were all heated at 100°C for 2 hr. Meat of lot 2 (Fig. 2) differed in that pH was adjusted to 6.0 instead of 6.1 and NaCl was 2.5 instead of 2.0%. Cans in this lot were heated at 90°C for 1.5 hr, 100°C for 2 hr or 110°C for 2.5 hr.

To test the hypothesis that sulfhydryl groups are required to form the inhibitor, these groups were blocked with silver ions before adding nitrite. Silver lactate (15 mmoles in 80 ml of water) was mixed with each kg of meat which, by assay (see analytical procedures) contained 15 mmoles of SH. After overnight storage of 2°C, SH was again assayed and more silver lactate added when titrated SH was above 1 mM. Care was exercised not to use excess silver. The treated meat was adjusted to the desired pH with sodium carbonate in 20 ml water. Further steps to prepare the SH-blocked meat samples for canning paralleled those used for the controls (Fig. 1 and 2).

—Text continued on page 1372

The pH adjustment of one group of lot 1 samples was accomplished by adding 100 ml of 3.5% sodium pyrophosphate solution per kg of meat (Phosphate-treated meat). A portion of lot 2 ground pork was cured with 100 ml of 5% glucose added.

Samples of lot 2 were challenged with *C. botulinum* spores after the processed meat, held in the cans for 1 day or 6 wk at 22°C, were made into homogenates (Fig. 2). The contents of two cans (300g total) were blended in a quart Mason jar with 300 ml of buffer (0.1M phosphate, pH 6.1 containing 3.0% NaCl). Residual nitrite in the different formulations was determined. The homogenates were dispensed in 16.5 ml portions into 13 × 150 mm screw-capped culture tubes with 20 replicates for each meat formulation. The homogenates were de-aerated by holding the tubes in flowing steam (100°C) for 6 min. These homogenates were inoculated with spores after all were made to 30 ppm of $NaNO_2$ (based on homogenate volume) by addition of appropriate volumes of 0.5% $NaNO_2$. This procedure simplified the interpretation of results by avoiding the potential difficulties that arise from different residual nitrite concentrations at the time of inoculation. Aseptic precautions were used throughout the handling of the canned meats.

Inoculation

Inocula were suspensions of *C. botulinum* strain 62A spores in distilled water. Spore counts were based on the average of triplicate, 5-tube most probabie number analyses (Am. Public Health Assoc., 1967) made in Bacto Cooked meat medium (Difco Laboratories). The spore suspension to be inoculated was heated 20 min in an 80°C water bath to destroy residual toxin and activate the spores for germination.

In the experiments with meat of lot 1, the spores were inoculated into the cans in which meat had been heated and stored for 8 wk at 22°C. Can lids, sterilized by burning off alcohol, were punctured near the center with a sterilized ice pick. The inoculum of 0.5 ml containing 96 spores was deposited in about six different parts of the meat mass via a 3 1/2 in. length, 19 gauge hypodermic needle that was inserted through the hole. Cans were resealed with 10 mm square, sterile metal sheets placed over the hole and fixed to the lid with epoxy cement (Chang and Akhtar, 1973).

The tubes of homogenates (lot 2 preparations) were each inoculated with 0.5 ml containing 48 spores; this inoculation was done immediately after equalizing nitrite concentration in all the tubes. After the additions, the tubes were inverted three times to distribute the nitrite and spores evenly in the homogenates. A sterile mixture of 80% corn oil and 20% paraffin was layered on the homogenates so that O_2 absorption would be retarded and gases produced by growth of the inoculum would be trapped in the homogenate. Tubes were closed tightly with the caps and examined for gas production during incubation at 30°C.

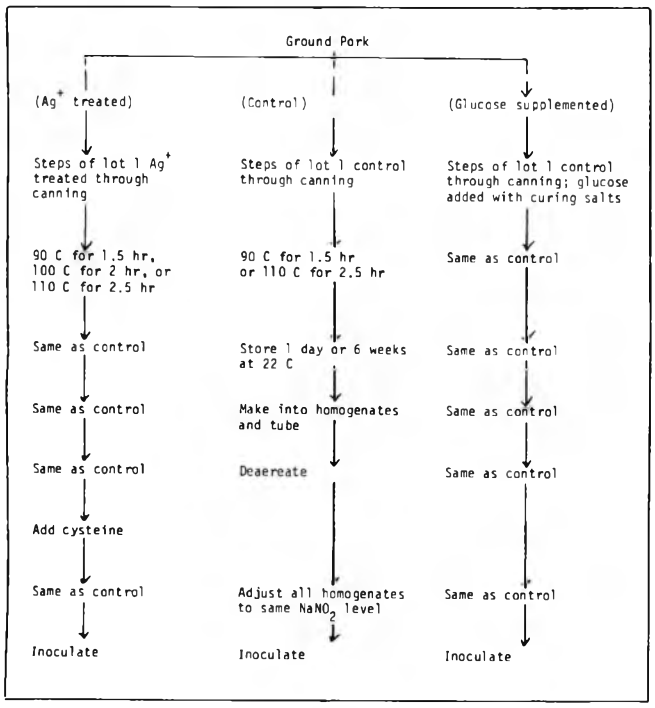

Fig. 2—Flow diagram for preparing cured meat samples of ground meat lot #2.

Analytical procedures

Nitrite was determined by a modification (Lee et al., 1978) of the AOAC (1970) method.

A modification of the Ellman (1959) procedure was used to assay SH. Buffer was 0.1M, pH 8.0, sodium phosphate with 8M urea and 2 mM EDTA. Buffer was added to 10g of minced meat to a final 100 ml volume and the meat solubilized by stirring the suspension with a magnetic bar at 3°C for 6 hr in a N_2 atmosphere. The resulting solution was diluted 1:39 (v/v) with buffer and 7.8 ml of the dilution reacted with 0.2 ml of 0.01M 2- nitrobenzoic acid (DTNB) in 0.1M sodium phosphate buffer, pH 7.0. Color, completely developed within 20 min, was read at 412 nm. The standard was reduced glutathione. Correction for the intrinsic color of the samples was with blanks made by substituting distilled H_2O for DTNB.

To detect botulinum toxin in the incubated meat homogenate, the slurry was centrifuged (18,000 × G, 15 min, 4°C), and the clear part of the supernatant saved. A mouse (20–24g weight) was injected intraperitoneally with 0.5 ml and observed for 4 days. Survival indicated absence of botulinum toxin. When the animal died, the sample was retested in paired mice. One was injected intraperitoneally with 0.5 ml of type A antitoxin containing one international unit of activity and then both were challenged with the sample. Death of the animal not receiving antitoxin and survival of the one given antitoxin showed the presence of type A botulinum toxin.

RESULTS & DISCUSSION

THE PROCESSED MEATS of lot 1 were stored for 8 wk before being inoculated with spores. Following storage, the originally added nitrite had been virtually depleted so that even those formulated with 300 ppm had three or less ppm of $NaNO_2$. This residual nitrite level should have no or negligible effect on the growth of the inoculum (Johnston et al., 1969). However, even with residual nitrite near zero, growth of the inoculum was adversely affected (Fig. 3). Among the several meat formulations, the first swells occurred in those heated with no or lesser nitrite concentrations and the least numbers of swells occurred in those given the higher nitrite cures. Similar observations have been made by others (Chang et al., 1974). The results confirm that an antibacterial activity is produced when meats are heated with nitrite.

The usual explanation of this type of observations is that an inhibitor of bacteria is formed by nitrite reacting with a meat constituent(s). We use this precedent and discuss the antibacterial activity as being due to an inhibitor, although serious consideration has not yet been given to nitrite changing an essential nutrient(s) of the inoculated bacteria so that the meat no longer supports growth of the organism.

Use of sodium pyrophosphate to adjust the pH of ground pork was expected to give cured products having a different level of inhibition than those treated with sodium carbonate. However, under the conditions of our experiment, the substitution had little effect since the antibacterial activities were similar to those observed in Figure 3.

Silver lactate was used in place of silver nitrate which has the disadvantage of its NO_3^- being reduced to NO_2^- in certain meat products (Kueper and Trealease, 1974; Polenske, 1891; van Roon, 1974). It, however, should react similarly to silver nitrate which combines with all SH groups in meat, including those buried in the folded protein molecules (Hamm and Hofmann, 1965).

Among the cured preparations made with SH-blocked meat, there was a trend toward greater inhibition of *C. botulinum* in meats cured with larger amounts of nitrite (Fig. 4). Since the spores were inoculated into meats with essentially no residual nitrite, the results indicate the inhibitor is not nitrite itself but an agent(s) whose production does not require participation of SH groups. Contrasting with the inhibitor in cured meats, the Perigo factor is not formed in a culture medium unless SH and iron are present (Asan and

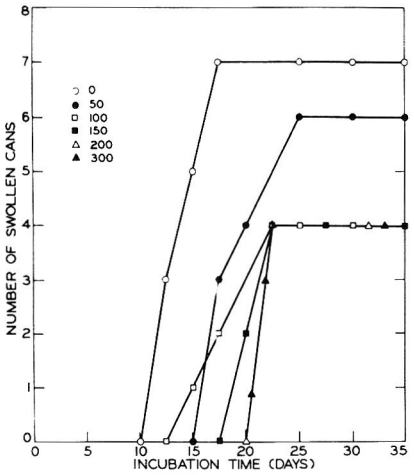

Fig. 3—Inhibition of C. botulinum in ground pork cured with different levels of nitrite. Meats heated in cans at 100°C for 2 hr, stored 8 wk at 22°C, inoculated with spores and incubated at 22°C. Seven cans per nitrite concentration.

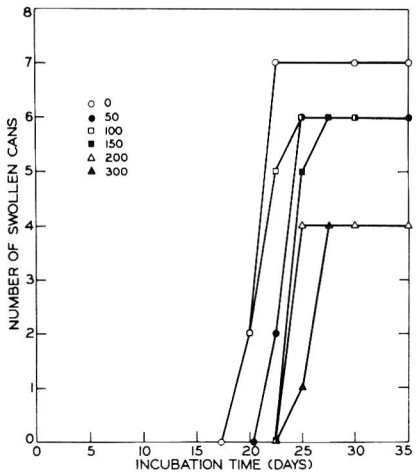

Fig. 4—Production of inhibitor in cured products made of meat previously treated to block sulfhydryls. Experiment otherwise same as in Fig. 3.

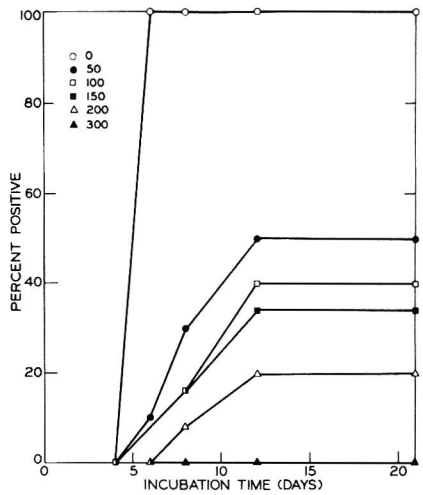

Fig. 5—Inhibitory activity in homogenates prepared of meats cured with different levels of $NaNO_2$. Meats processed at 100°C for 2 hr, stored 1 day at 22°C, and made into homogenates by 1:1 (v/w) dilution with buffer. All homogenates have 30 ppm nitrite at time of inoculation with C. botulinum spores. 20 replicates of homogenate for each nitrite concentration used for curing. Incubation at 30°C for growth of inoculum.

Solberg, 1976; Huhtanen, 1975; Moran et al., 1975; Polenske, 1891).

The final numbers of swells among the meats cured with the same amount of nitrite were not greatly different, but the time to first detectable swelling was delayed in the SH blocked meats that had received no nitrite before heat processing. Incubation of 22 days was needed before growth was detected. The slow growth was probably due to lack of sulfhydryl groups in SH-blocked meat rather than to toxicity of residual silver, since the blocking treatment avoided excess silver, and, if any did remain, it would have been tied up as AgCl when the 2.0% NaCl was added later. Addition of the NaCl did not increase titratable SH groups. In any case, all cans of both groups did develop into swells.

Table 1 shows residual nitrite in cured meats 24 hr after they had been prepared by heating 2 hr at 100°C. Tests for inhibitor in these preparations were made after their conversion into homogenates containing equal amounts of assayable $NaNO_2$. Of the nitrite added for this equalization, more might react and disappear in homogenates that are derived from meat not processed with nitrite. However, this possibility seems unlikely. When nitrite levels were made the same in homogenates of a set comparable to that used in our test, the assayable $NaNO_2$ was essentially the same in the different homogenates (Johnston et al., 1969).

Figure 5 shows that all 20 replicate tubes of the control meat (heated without nitrite) showed gas within 6 days of inoculation with the spores. Growth did not occur during the 3-wk observation period in those tubes containing meat cured with 300 ppm $NaNO_2$. The maximum percentages of replicates showing growth were related to the amounts of nitrite used for curing.

Homogenates of cured meats made with SH-blocked meat were supplemented to contain 15 mM cysteine before being inoculated with spores (Fig. 2). These homogenates had inhibitory activities not notably different from those of meats which had not been treated with silver lactate (Fig. 5). These results further indicate that formation of inhibitor in cured meats does not require the presence of reactive SH groups. Furthermore, the inhibitory activity of the cured meats is probably not because of deficiency in SH compounds that the organism needs for growth.

The cured products made of meat supplemented with 0.5% glucose were generally less inhibitory than the controls without added sugar (Fig. 6 *vs* Fig. 5). These results are different from the observations on the Perigo factor which showed greater inhibitory activity when nitrite is heated in a culture medium containing 0.2% glucose (Huhtanen, 1975).

Table 1—Residual nitrite levels in meats mixed with different concentrations of $NaNO_2$ and heated 2 hr at 100°C. Residual nitrite determined 1 day after heat processing

Sodium Nitrite, ppm	
Added	Residual
0	0
50	20
100	35
150	45
200	51
300	58

The stability of the inhibitor in cured meats was studied. Cured meats were stored in their original can containers for 6 wk at 22°C before they were made into the test homogenates. Results are plotted in Figure 7 for normal cured meats; similar results were obtained with SH-blocked cured products. Growth of *C. botulinum* in products aged 6 wk was considerably better than in the comparable preparations aged 1 day. These observations agree with a previous study (Chang and Akhtar, 1974) that the inhibitor in cured meats is not completely stable and becomes less potent during storage.

A study was done on the effect of processing temperatures on formation of inhibitor. The antibotulinum activity in meats processed at 110°C for 2.5 hr was less than in comparable formulations heated at 90°C for 1.5 hr (Fig. 8). Products heated 100°C for 3 hr seemed to have slightly less inhibitory activity than those heated 1.5 hr at 90°C.

Gas production during incubation with *C. botulinum* spores was used as evidence for absence of antibotulinum

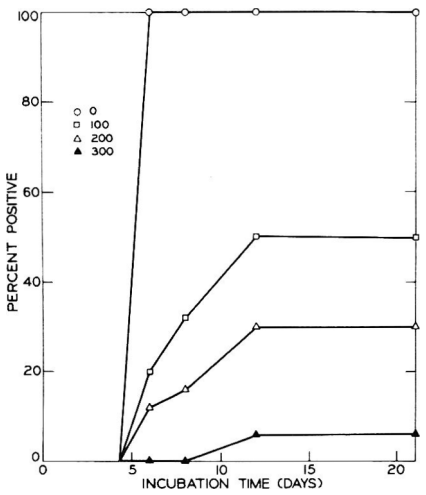

Fig. 6—Effect of glucose on inhibitory activities in meats cured with different levels of nitrite. Same experiment as in Fig. 5 except cured products are made with meat containing 0.5% added glucose.

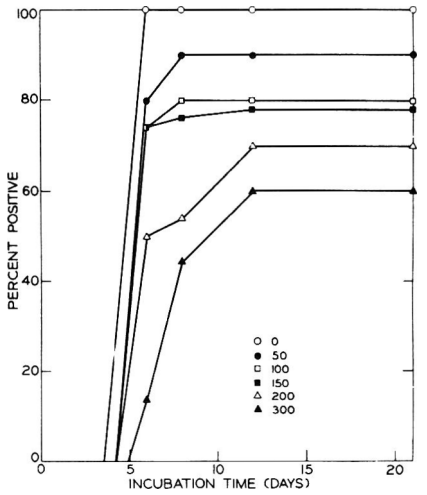

Fig. 7—Effect of aging on activity of inhibitor in cured meats. Experiment same as in Fig. 5 except that canned products stored 6 wk at 22° C before being tested in homogenate form.

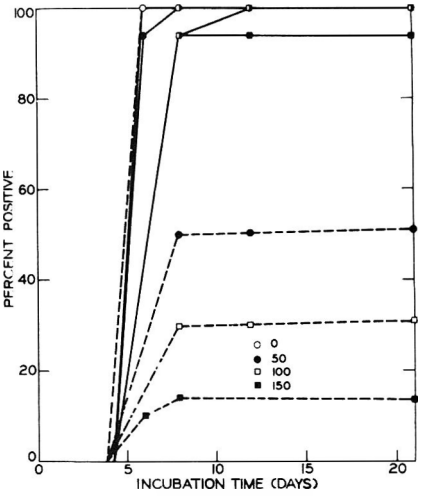

Fig. 8—Effect of processing temperature in developing inhibitory activities in meats cured with different nitrite levels. Solid lines are results from samples heated at 110° C for 2.5 hr and broken lines are results from samples heated at 90° C for 1.5 hr.

factor and, conversely, absence of gas was taken as failure of *C. botulinum* to grow.

To prove that the observed gas is due to growth of *C. botulinum* and not a contaminant, representative samples were tested for botulinum toxin. The tested homogenates included 30 not showing gas in the data of Figure 8; 15 were those cured with 150 ppm $NaNO_2$, 10 with 100 and 5 with 50 ppm. Botulinum toxin was found in only one of the thirty. The ten homogenates which had gas production were tested for toxin. Six were formulated without nitrite and four were cured with 50 ppm $NaNO_2$; botulinum toxin was present in all of these samples.

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Ms received 8/16/77; revised 4/15/78; accepted 4/20/78.

Research supported by the College of Agricultural and Life Sciences, University of Wisconsin-Madison, by the Food Research Institute and by the American Meat Institute. Muscle Biology Manuscript No. 117.

Appreciation is expressed to R.B. Tompkin and L. Christiansen of Swift and Co. Research Lab. for their willing advice and discussion.

LIPID OXIDATION IN MECHANICALLY DEBONED RED MEAT

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ABSTRACT

Mechanically deboned meat (MDM) from flat bones and neck bones of cattle was tested against ground beef for differences in rate of lipid oxidation. To measure the oxidative changes in MDM and ground beef, the fatty acid disappearance from the polar and nonpolar lipids and the production of monocarbonyls during storage were monitored. The meat was tested under two separate temperatures; 2–3°C and analyzed at intervals on days 0, 2, 4, 6, 9 and 13; –35°C and analyzed at intervals on days 0, 30 and 90. Two additional flat bone samples were stored (aged) for 5–6 days and then deboned and analyzed. Differences in the slopes of regression lines for oxidation of each polyunsaturated fatty acid showed no major differences between the disappearance of the fatty acids in MDM when compared to ground beef. Little change occurred in the polar and nonpolar fatty acids during storage at 2–3°C or at –35°C. Monocarbonyls were a better indicator of oxidation rate than total carbonyls. Increases in monocarbonyls during storage reached a peak on the sixth day of storage at 2–3°C and then declined. No significant differences were found in monocarbonyl content of MDM when compared to monocarbonyl content of ground beef. Polyunsaturated fatty acid/hemoprotein molar ratios of all samples analyzed were in the area where hemoproteins act as antioxidants. Overall, lipids in MDM from beef bones (aged or fresh) oxidized at about the same rate as lipids in ground beef.

INTRODUCTION

LIPID OXIDATION is a primary consideration in the storage stability of meat and meat products (Tappel, 1962). Lipid oxidation may be especially acute in mechanically deboned meat (MDM) since some increases in meat temperature as well as air incorporation is inherent in the deboning process. Several reports have already indicated that a problem of oxidative stability exists in MDM from poultry (Maxon and Marion, 1970; Dimick et al., 1972; Froning et al., 1971; Moerck and Ball, 1974). Suggestions as to the cause of this oxidation have centered on the increased concentrations of polyunsaturated fatty acids of the phospholipids (Moerck and Ball, 1974) and increased quantities of heme pigments from bone marrow (Lee et al., 1975). The phospholipids of MDM from beef, pork and lamb have previously been reported to contain relatively high concentrations of polyunsaturated fatty acids (Kunsman and Field, 1976). The heme pigment concentration in MDM from red meats is also higher than in hand boned meat (Field, 1976). Thus with adequate substrate available (polyunsaturated fatty acids of the phospholipids) and increased levels of known pro-oxidants present (heme pigments), as well as the finely divided physical nature of the product, MDM from red meats might be a product susceptible to lipid oxidation.

The purpose of this investigation was to identify the extent of lipid oxidation in MDM from beef stored at typical cooler or freezer temperatures. Differences in rate of oxidation in MDM from bones mechanically deboned immediately after removal from the carcass (fresh) and bones held after removal from the carcass (aged) were also studied.

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0022-1147/78/0005-1375$02.25/0

EXPERIMENTAL

Preparation of MDM

Flat bones (bones from the spinal column plus the ribs) from the right sides of mature cow carcasses and neck bones (cervical vertebrae) from 2-yr-old steers were obtained from the University of Wyoming Meat Laboratory and from commercial sources. The bones with the normal amount of lean attached after hand boning were ground through a bone cutter and mechanically deboned utilizing a Beehive mechanical deboner with 0.46 mm diameter holes in the cylinder head within 24 hr after they were removed from the carcasses. The product obtained is referred to as MDM from fresh flat bones or MDM from fresh neck bones throughout this paper. Immediately after deboning the MDM was cooled to 0°C. Samples of MDM from each lot were stored at 2°C and analyzed at intervals: days 0, 2, 4, 6, 9 and 13. Samples of MDM from these same lots were stored frozen at –35°C and analyzed after 0, 30 and 90 days storage. Flat bones from the left sides of the cow carcasses were stored for 6 days at 2°C before they were mechanically deboned. MDM from these bones was then referred to as MDM from aged bones. Two batches of each MDM prepared from different lots of steer or cow bones were analyzed in duplicate. Ground beef produced in the University of Wyoming Meat Laboratory from the hand boned steer meat was used as the control for these experiments.

Carbonyl analysis. Dinitrophenylhydrazine derivatives of carbonyls were prepared and extracted in duplicate according to the method of Keller and Kinsella (1973). Hexane used in the extraction was rendered carbonyl free by the method of Schwartz et al. (1963). The carbonyl derivatives were separated from unreacted lipid using a Celite-Sea Sorb (1:1) column (Schwartz et al., 1963). The unreacted lipid was discarded and the carbonyl derivatives fractionated into monocarbonyl and dicarbonyl fractions using an alumina column essentially as described by Schwartz and Parks (1961). Alumina (80–200 mesh) was adjusted to a pH of 4.7 and 6g sifted into a 0.9 cm by 30 cm glass column containing hexane. The sample containing the carbonyl derivatives eluted using 100 ml of methylene chloride. This elution procedure was checked using authentic mono- and dicarbonyl standards (Supelco Inc., Bellefonte, PA). Absorbance of the monocarbonyls was read spectrophotometrically at a wave length of 347 nm. The values were converted to μmoles/g of meat using E = 22,500.

Fatty acid analysis. The lipids of the MDM were extracted and separated into nonpolar, polar and glycolipid fractions as previously described by Kunsman and Field (1976). Methyl esters of the fatty acids from the nonpolar and polar lipid fractions were prepared by the procedure of Metcalfe et al. (1966). Gas chromatography was performed with a Perkin Elmer model 881 gas chromatograph equipped with glass columns (1.8m X 3.17 mm) containing 10% SP 2340 or 10% SP 2330 on 100/120 chromosorb W (Supelco, Inc., Bellefonte, PA). The conditions were: column temperature 195°C: injection temperature 240°C; detector temperature 220°C; carrier gas (nitrogen) 20 ml/min.

Heme pigment analysis. Total heme pigment analysis as well as hemoglobin, myoglobin analysis was carried out using the Sephadex gel filtration method of Franke (1973).

Fat, moisture, calcium and iron analysis. Standard AOAC (1970) methods were used for fat and moisture determinations. Concentrations of Ca and Fe were found using procedures and known standards of the Perkin Elmer Corp., (1964). All glassware used for Ca and Fe analysis by atomic absorption spectroscopy was washed and rinsed in demineralized water. Funnels and filtering apparatus were stored in a 1% EDTA solution.

Statistical analysis. Regression analysis (Steel and Torrie, 1960) was performed to determine the relationship between time and amount of oxidation. The slopes of the lines were compared using t-tests (Dixon

and Massey, 1969) to find confidence intervals of the differences between the slopes enabling determination of differences in lipid oxidation rate between MDM and ground beef during storage.

RESULTS & DISCUSSION

TABLE 1 lists the average yield of MDM from each of two lots along with the fat, moisture, calcium, iron and heme pigment concentration. The yield values, approximate analysis and mineral content of the MDM is typical of that previously reported for MDM from our laboratory (Field, 1976). Proportionally higher amounts of iron in MDM (0.005–0.006%) than in ground beef (0.001%) when compared to total heme pigment of 6.4 mg/g for MDM and 3.7 mg/g for ground beef is partially due to the red bone marrow present in the MDM. Red bone marrow contains high amounts of nonheme iron in the form of hemosiderin and ferritin which raises the figure for total iron in MDM but does not raise the figure for total heme pigment (Blum and Zuber, 1975). Heme pigment values for MDM are almost twice as high as the corresponding values for ground beef. Hemoglobin is the major pigment present in MDM in contrast to ground beef where myoglobin always prevails (Franke, 1973). These figures are in good agreement with unpublished data from our laboratory which suggests that MDM has a heme pigment concentration between 6 and 12 mg/g, 50–85% of which is hemoglobin. The lower values for hemoglobin found in the MDM from neck bones when compared to the values for MDM from flat bones, reflects the fact that more myoglobin-rich meat was left on the neck bones after hand boning than was left on the flat bones. Therefore, MDM from neck bones contained more muscle which is reflected by the higher values for myoglobin. The value of 28% hemoglobin in the ground beef is also in agreement with unpublished data from our lab as well as others (Hankin, 1965; Warriss and Rhodes, 1977).

Monocarbonyls were chosen as a measure of oxidation rather than total carbonyls (Fig. 1). As previously reported by other workers (Dimick et al., 1972) total carbonyl values change in an erratic manner over time. Monocarbonyl values give a steady, more predictable value over a time period. Figure 2 contains a comparison of monocarbonyl concentrations over time in MDM from both batches of fresh bones, aged flat bones and ground beef. Confidence intervals for the differences in slopes of the regression lines for the monocarbonyls in MDM when compared to ground beef are listed in Table 2. No significant differences ($P < 0.05$) in the slopes of the lines for monocarbonyl concentration existed when MDM and ground beef were compared at storage temperatures of 2–3°C or of −35°C. Therefore, when one looks at the analysis of the specific products of lipid oxidation there is very little difference in lipid oxidation between MDM and ground beef stored under similar conditions. In addition, MDM from flat bones aged 6 days at 2–3°C prior to mechanical deboning was similar in monocarbonyl content to ground beef as well as MDM from fresh flat bones (Fig. 2).

The slight decline in monocarbonyl content in all samples after 6 days (Fig. 2) is difficult to explain. It has been known for some time that a wide spectrum of bacteria can metabolize carbonyls (Smith and Alford. 1968). By the end of these experiments with the refrigerated meat samples (13 days) we noted an off aroma and slight discoloration in all samples. This suggests that microbial growth in these samples was present and that some carbonyl destruction could have occurred after 6 days of storage.

Lipid extracted from MDM of flat bones was 97% nonpolar

Table 1—Percent yield, calcium, iron and approximate analysis of mechanically deboned meat and ground beef[a]

Item	MDM from flat bones		MDM from neck bones	Ground beef
	Fresh	Aged	Fresh	
Yield,[b] %	28	29	32	
Fat, %	33	34	20	23
Moisture, %	49	48	50	53
Calcium, %	0.80	0.85	0.55	0.017
Iron, %	0.006	0.006	0.005	0.001
Heme pigment mg/g	6.4	–	6.4	3.7
Hemoglobin (% of total heme)	85	–	54	28
Myoglobin (% of total heme)	15	–	46	72

[a] Each mean represents the average for two different lots of MDM or ground beef.
[b] Yield = weight of MDM obtained ÷ total weight of bones used.

Table 2—Confidence intervals for the differences in the slope of the lines for total monocarbonyls in MDM when compared to total monocarbonyls in ground beef[a]

Days storage	Storage temp	Monocarbonyls in MDM from		
		Fresh flat bones	Aged flat bones	Fresh neck bones
0–13	2–3° C	−0.052 ± 0.390	0.057 ± 0.170	−0.035 ± 0.350
0–90	−35° C	−0.004 ± 0.028		−0.009 ± 0.026

[a] Differences in slopes of the regression lines for MDM when compared to slopes of the regression lines for ground beef were not different ($P < 0.05$).

Table 3—Means for percentage of the major fatty acids of the polar and nonpolar lipid fractions of MDM from flat bones (fresh and aged), neck bones (fresh) and ground beef at 0 days storage

Source of meat	Lipid class	% of lipid class[a]									
		C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	C20:4	C20:5	C22:4	C22:5
MDM (fresh flat bones)	Polar	13.7	1.3	16.1	31.2	19.1	3.1	10.2	.45	2.0	2.0
MDM (fresh flat bones)	Nonpolar	24.3	3.4	21.7	41.6	2.2	.8	–	–	–	–
MDM (aged flat bones)	Polar	10.8	1.7	15.8	32.8	17.5	2.4	11.3	1.7	1.5	4.0
MDM (aged flat bones)	Nonpolar	25.9	3.7	21.2	44.8	2.2	.9	–	–	–	–
MDM (fresh neck bones)	Polar	14.5	1.5	14.5	23.9	31.0	.4	8.5	.5	2.1	2.5
MDM (fresh neck bones)	Nonpolar	23.5	2.7	17.5	76.5	6.3	1.0	–	–	–	–
Ground beef (fresh)	Polar	12.4	1.9	16.0	23.9	30.1	.4	10.7	.6	.7	1.4
Ground beef (fresh)	Nonpolar	22.8	3.8	16.1	48.4	2.8	.5	–	–	–	–

[a] Expressed as percent of polar or nonpolar lipid class

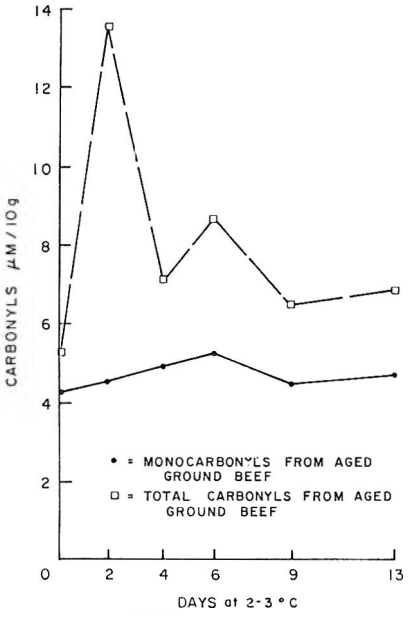

Fig. 1—Total carbonyls and monocarbonyls from aged ground beef.

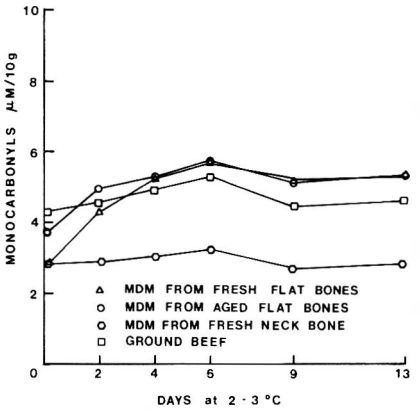

Fig. 2—Monocarbonyl content of mechanically deboned meat and ground beef.

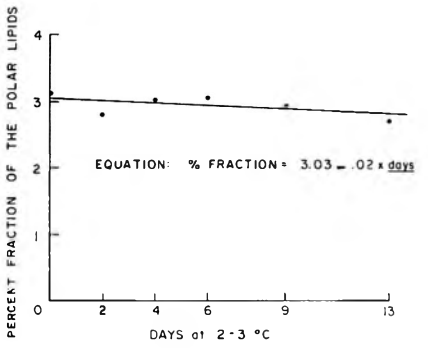

Fig. 3—Scatter diagram and linear regression of percent of C18:3 from polar lipid fraction from fresh flat bones stored at 2–3°C.

and 3% polar whereas lipids extracted from MDM of neck bones was 95% nonpolar and 4% polar. The lipid from ground beef which acted as the control was 96% nonpolar and 4% polar. The polar lipid of all samples analyzed contained small amounts of glycolipid (0.15–0.5%). Table 3 lists the major fatty acids found in the MDM and ground beef. These data agree with those of Kunsman and Field (1976).

The percentage change during storage of each fatty acid was plotted against days of storage as shown in Figure 3 for 18:3 from the polar lipid. The constant in the equation shows the percentage of the fatty acid in the lipid fraction at day 0. The slope of the regression line shows that little change occurred during storage. Tables 4 and 5 show the confidence intervals of the differences in the slopes of the regression lines for polyunsaturated fatty acids and whether or not the slopes were significantly different ($P < 0.05$) from the slopes for ground beef. The confidence intervals indicate that very little change occurred in the polar and nonpolar lipids during the 13-day storage period at 2–3°C. Differences between slopes of the lines for MDM and ground beef were small and in most cases not significant ($P < 0.05$).

Lee et al. (1975) studied the effect of added hemoprotein concentrations on the oxygen uptake rate catalyzed by mechanically deboned chicken meat homogenates. They found that at high molar ratios of linoleate to hemoprotein of 500:1, oxygen uptake was maximum. This high oxidation rate was applicable also at ratios of 350:1. However, as the ratio decreased below these levels the oxidation rate declined. At a ratio of 180:1, the oxygen uptake was only one third of the maximum level. As the ratio approached 90:1, the oxygen uptake fell to one-thirtieth of the maximum level. From these data the authors suggested that at high linoleate/hemoprotein ratios the hemoproteins act as prooxidants but as the ratio falls below 350 they become increasingly antioxidant. In fact, at low ratios of 90:1 the hemoprotein seem to exert strong antioxidant activity.

In Table 6 is listed the nonpolar and polar lipid content of the meat samples. Using this data as well as the polyunsaturated fatty acid content (those acids with 2 or more double bonds) of the meat samples (Table 3) one can calculate the μmoles of polyunsaturated fatty acids present in 1g of meat sample (Lee et al., 1975). Using MDM from flat bones as

Table 4—Confidence intervals for the difference in the slope of the lines for polyunsaturated fatty acids of the polar and nonpolar lipids in MDM when compared to the polyunsaturated fatty acids in ground beef after 0–13 days storage at 2–3° C

	MDM from		
Fatty acid	Fresh flat bones	Aged flat bones	Fresh neck bones
Polar lipids			
C18:2	0.028 ± 0.330	0.005 ± 0.283	0.134 ± 0.325
C18:3	−0.017 ± 0.027	−0.023 ± 0.101	0.006 ± 0.004
C20:4	0.036 ± 0.254	0.089 ± 0.257	0.105 ± 0.256
C20:5	0.008 ± 0.022	−0.47 ± 1.260	−0.368 ± 0.370*
C22:4	0.038 ± 0.033	−0.011 ± 0.028	−0.101 ± 0.081*
C22:5	−0.08 ± 0.072*	−0.096 ± 0.076*	−0.096 ± 0.080*
Nonpolar lipids			
C18:2	0.099 ± 1.91	0.034 ± 0.059	−0.130 ± 0.270
C18:3	0.024 ± 0.018	0.032 ± 0.016	−0.019 ± 0.031

* Slope is significantly different from slope for ground beef ($P < 0.05$).

Table 5—Confidence intervals for the difference in the slope of the lines for polyunsaturated fatty acids of the polar and the nonpolar lipids in MDM when compared to the polyunsaturated fatty acids in ground beef after 0–90 days storage at −35° C

	MDM from	
Fatty acid	Fresh neck bones	Fresh flat bones
Polar lipids		
C18:2	0.028 ± 0.038	0.103 ± 0.335
C18:3	−0.002 ± 0.001*	−0.001 ± 0.001
C20:4	−0.016 ± 0.017	−0.090 ± 0.135
C20:5	0.001 ± 0.001*	−0.044 ± 0.090
C22:4	−0.001 ± 0.011	−0.022 ± 0.026
C22:5	−0.0004 ± 0.002	−0.022 ± 0.041
Nonpolar lipids		
C18:2	−0.014 ± 0.029	−0.026 ± 0.063
C18:3	0.004 ± 0.002*	−0.001 ± 0.009

* Slope is significantly different from slope for ground beef ($P < 0.05$)

Table 6—Means for the nonpolar and polar lipid content of MDM and ground beef[a]

Meat source	g lipid per 1g sample	
	Nonpolar	Polar
MDM (fresh flat bones)	0.322	0.008
MDM (fresh neck bones)	0.191	0.009
Ground beef	0.222	0.008

[a] Each mean represents the average for two different lots of MDM or ground beef.

Table 7—The molar ratios of polyunsaturated to hemoprotein of MDM and ground beef

Meat source	Molar ratio
	Total PUFA[a]/hemoprotein
MDM (fresh flat bones)	107/1
MDM (fresh neck bones)	152/1
Ground beef	168/1

[a] Polyunsaturated fatty acids (2 or more double bonds)

an example one can calculate that 0.322g of nonpolar lipid per g of sample (Table 6) multiplied by 0.9 (assuming fatty acids make up 90% of the weight of the lipid) yields 0.299g of fatty acids. Since 3.0% of these acids are polyunsaturated, this yields 8.69×10^{-3}g of polyunsaturated fatty acids. The major polyunsaturated fatty acid is linoleate, therefore, one can use its molecular weight (280) as representative and thus convert the grams of polyunsaturated fatty acids to moles ($8.69 \times 10^{-3} \div 280 = 3.1 \times 10^{-5}$ moles) or 31 μmoles. If one does the same calculation for the polar lipid of the MDM (fresh flat bones) one gets another 9.7 μmoles. Thus the total μmoles of polyunsaturated fatty acid present in this 1g MDM sample can be estimated as 40.7. Using again the method of Lee et al. (1975) one can calculate the μmoles of the hemoprotein present by multiplying the g of heme pigment present by its iron content (0.335%) and dividing by the molecular weight of iron (55.85). Thus there are 0.38 μmoles of hemoprotein in 1g of MDM (fresh flat bones) ($0.0064 \times 0.00335 \div 55.85$). This yields a polyunsaturated fatty acid to hemoprotein molar ratio of 107/1 (Table 7) for the MDM (fresh flat bones).

One can note from Table 7 that the polyunsaturated fatty acids to hemoprotein molar ratios are all within the area where the hemoproteins increasingly act as antioxidants.

It is apparent from the analysis of the precursors of oxidation (unsaturated fatty acids) and the products of oxidation (monocarbonyls) that MDM from flat bones has almost the same oxidation rate as ground beef. MDM from neck bones appears to have better stability than ground beef since oxidation takes place at a slightly slower rate. MDM from aged flat bones had almost the same oxidation rate as MDM from fresh flat bones. The oxidative stability of all samples was better at -35°C than at $2-3^{\circ}$C storage temperatures. Overall, MDM from beef bones (aged or fresh) oxidizes at about the same rate as ground beef.

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Ms received 2/16/78; revised 4/3/78; accepted 4/10/78.

Journal Paper 934 of the Wyoming Agricultural Experiment Station.

LIPID–SOLUBLE CARBONYL COMPONENTS OF OVINE ADIPOSE TISSUE

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ABSTRACT

Three Suffolk (*Ovis aries*) castrate male animals (wethers) were used in this study. Following exsanguination, chilling and primal/subprimal processing, samples were taken from the subcutaneous adipose tissue (*Panniculus adiposus*) covering the thoraco-lumbar area, and randomly allotted to three low temperature (−29°C) storage treatments (0, 5 and 10 months). During the analyses, each sample was further separated and subjected to three heat/preparation treatments: cooked (163°C) tissue (CT), cooked (163°C) fat (CF), melted (50°C) fat (MF). Carbonyl analyses of each sample were conducted on hexane extracts passed over Celite impregnated with 2,4-DNPH to convert the carbonyls to their 2,4-DNPH derivatives. The monocarbonyl derivatives were eluted from the total carbonyls and were further fractionated into classes. The results indicated that after 5 months of storage the amount of total carbonyls was reduced by 52%, and after 10 months the amount present was only 21%. However, most of the monocarbonyls were present in greater amounts at 5 months than initially or after storage for 10 months. Generally, cooking was observed to increase the amount of all carbonyls present. In fact, alk-2,4-dienals were only detected in the cooked samples. Of the monocarbonyls usually detected in the various samples, the alk-2-ones and alkanals were the predominant classes.

INTRODUCTION

DURING THE PAST several decades, the world's consumption of sheep meat (lamb/mutton) has declined steadily while that of other species has increased (USDA, 1976). The low consumer acceptance of lamb and mutton in many countries has been attributed to the objectionable cooking smell and to the flavor of the cooked meat (Ziegler and Daly, 1968; Batcher et al., 1969; Weidenhamer et al., 1969). Noting the importance of fat or adipose tissue in the generation of the characteristic sheep meat flavor (Hornstein and Crowe, 1963; Sink and Caporaso, 1977), we became interested in identifying those compounds responsible for the distinctive lamb/mutton flavor.

Although the fatty acids, especially the branched chain and unsaturated C_{8-10} acids, have been identified as flavor constituents (Wong et al., 1975), the carbonyls are the most numerous, and perhaps the most important, of all the various classes of compounds identified in meat flavor studies (Herz and Chang, 1970; Caporaso et al., 1977). Although there are both water- and fat-soluble carbonyls, Sanderson et al. (1966) indicated those involved in meat flavor are primarily lipid-soluble. Since the importance of lipid compounds in flavor (Sink, 1973) and in storage (Huston et al., 1965) changes have been demonstrated, the carbonyls can be implicated. Thus, the focus of this study was to identify the types and amounts of lipid-soluble carbonyls important in lamb/mutton flavor.

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0022-1147/78/0005-1379$02.25/0

MATERIALS & METHODS

Experimental design

Three Suffolk (*Ovis aries*) castrate male animals (wethers) were exsanguinated and then chilled (3°C) according to standard industry practices. Samples (ca 300g each) were taken from the subcutaneous adipose tissue (*Panniculus adiposus*) covering the thoraco-lumbar area and randomly allotted to three low temperature (−29°C) storage treatments: 0, 5 and 10 months. Then, the samples were removed from the freezer, thawed and ground through a 3 mm plate. Each sample was split into two 150-g portions.

One portion of the ground adipose tissue was cooked in a standard commercial oven at 163°C for 45 min as recommended for the usual preparation of lamb/mutton roasts. This sample (CT) was then filtered through several layers of cheesecloth and 10g of the lipid filtrate dissolved in 250 ml of carbonyl-free hexane (Schwartz and Parks, 1961) for subsequent analysis.

The other portion of the ground adipose tissue was gently melted at 50°C and filtered through several layers of cheesecloth. From the lipid filtrate two samples were taken and treated as follows: one (10g) sample (MF) of this melted fat was dissolved in 250 ml of carbonyl-free hexane; the other sample (CF) was cooked at 163°C for 45 min, as described above, and then (10g) dissolved in 250 ml of carbonyl-free hexane.

Carbonyl analyses

Analyses were conducted in duplicate on the hexane extracts which were passed over a Celite column impregnated with 2,4-dinitrophenylhydrazine (Schwartz et al., 1963) to convert the carbonyls to their DNPH derivatives. The total concentration of these carbonyl derivatives was determined using a Beckman Model DB Spectrophotometer set at a wavelength of 350 nm.

Additional lipids were removed from the DNP-hydrazones by passing the mixture over a 20g Celite 545-Sea Sorb 43 column (1:1, w/w), followed by 200 ml of hexane. The absorbed monocarbonyl derivatives were eluted from this column with 140 ml of chloroform-nitromethane (3:1, v/v). Ketoglyceride derivatives were separated from the monocarbonyl DNP-hydrazones on an alumina column. Neutral alumina (40g of 80–100 mesh), activated by heating 24 hr at 150°C, partially deactivated by addition of 6% (w/w) distilled water and allowed to equilibrate 16–20 hr, was found necessary for proper column preparation. The monocarbonyl fraction was eluted with benzene:hexane (1:1, v:v). The resulting monocarbonyl DNP-hydrazones were evaporated to dryness, dissolved in a known volume of chloroform and the concentration determined spectrophotometrically at 365 nm.

The DNP-hydrazone classes were isolated from the monocarbonyl fraction by an adaptation of the procedure of Schwartz et al. (1963). A 10-g column was prepared using activated (150°C for 24 hr) Celite 545-Sea Sorb 43 (1:1, w/w) and separation of the DNP-hydrazone classes accomplished by elution with the following sequence of solvent solutions: 100 ml each of 20% and 40% chloroform in hexane; 150 ml of 80% chloroform in hexane; 150 ml of chloroform; 100 ml each of 1%, 2%, 5% and 10% methanol in chloroform; and 125 ml of nitromethane in chloroform. A Buchler Automatic Fraction Collector equipped with a UV source to monitor the eluant from the column aided in determining the separation. The DNP-hydrazone derivatives were evaporated to dryness, pooled according to class, dissolved in a known volume of chloroform, and their concentration determined spectrophotometrically. Classes were established on the basis of the following absorption maxima: alkanals, 355 nm; alk-2-ones, 365 nm; alk-2-enals, 373 nm; and alk-2,4-dienals, 390 nm. The carbonyls of each class were then regenerated from their DNP-hydrazones and subjected to olfactory evaluation (Bassette and Day, 1960). These evaluations were performed individually by three experienced flavor researchers.

–Text continued on page 1380

RESULTS & DISCUSSION

THE RESULTS of the carbonyl analyses from this study are presented in Table 1, and that from previous reports in Table 2. In Table 3, the results of the olfactory evaluation of the regenerated carbonyls are recorded.

Although total carbonyl values between the cooked fat (CF) and cooked tissue (CT) samples were not different from each other, both were higher than that found in the melted fat (MF) sample. A treatment effect was noted in that the amount of total carbonyls decreased with storage time. However, sample differences among treatments were not consistent. More carbonyls were present in CF and CT than in MF samples at 0 and 10 months of frozen storage whereas the converse was observed in samples stored for 5 months. Comparisons of these values with those in the literature are not possible since none have been reported.

The total monocarbonyl levels found in this study were in relative agreement with those reported previously. The CF sample was noted to contain a greater amount of total monocarbonyls than the MF sample, with the CT sample intermediate between the two. Thus, the increased temperature associated with the cooking (CF and CT) treatments did increase the total monocarbonyl fraction, most likely via increased oxidation (Lea, 1962). In the storage experiment, the greatest amount of monocarbonyls was observed at the end of 5 months and the least amount in the fresh, nonstored, tissue.

The alk-2-ones, or methylketones, generally accounted for more than 45% of the total carbonyl fraction. Although the amount of these constituents was not different between the two cooked samples (CF and CT), it was higher than that observed in the MF sample. Further, it can be noted that there was an increase in the alk-2-one content of ovine fat samples with storage time. However, neither Ellis et al. (1961) nor Hornstein and Crowe (1963) reported finding any alk-2-ones, and Riley et al. (1971) reported being unable to detect this fraction. The widespread occurrence of alk-2-ones in food products (Forss, 1972), notably the volatiles of lamb

Table 1—Carbonyl values for ovine fat (μmoles/10g)

	Carbonyl classes					
Treatment[a]/Sample[b]	Total carbonyls	Total monocarbonyls	Alk-2-ones	Alkanals	Alk-2-enals	Alk-2,4-dienals
0 Months						
MF	86.1 ± 15.8	0.95 ± 0.17	0.19 ± 0.04	0.42 ± 0.09	0.08 ± 0.01	— —
CF	93.2 ± 2.5	3.92 ± 0.02	2.03 ± 0.01	0.49 ± 0.03	0.29 ± 0.03	0.04 ± 0.02
CT	97.2 ± 3.7	2.01 ± 0.03	0.55 ± 0.11	0.96 ± 0.01	0.07 ± 0.01	— —
5 Months						
MF	53.9 ± 2.9	1.76 ± 0.43	0.75 ± 0.01	0.64 ± 0.02	0.08 ± 0.01	— —
CF	38.8 ± 1.3	6.89 ± 0.04	0.96 ± 0.33	1.01 ± 0.31	2.13 ± 0.18	0.57 ± 0.03
CT	40.8 ± 0.1	2.02 ± 0.43	1.27 ± 0.17	0.67 ± 0.12	0.04 ± 0.00	0.04 ± 0.02
10 Months						
MF	13.3 ± 2.5	2.07 ± 0.84	1.32 ± 0.78	0.30 ± 0.04	0.09 ± 0.04	— —
CF	24.8 ± 0.9	3.25 ± 0.07	0.40 ± 0.12	0.64 ± 0.04	1.02 ± 0.01	0.33 ± 0.03
CT	19.8 ± 0.9	3.64 ± 1.13	1.74 ± 0.73	0.90 ± 0.29	0.27 ± 0.01	— —
All Samples						
MF	33.2 ± 7.1	1.59 ± 0.48	0.75 ± 0.28	0.45 ± 0.05	0.08 ± 0.02	— —
CF	52.3 ± 1.6	4.67 ± 0.04	1.13 ± 0.15	0.71 ± 0.16	1.15 ± 0.07	0.31 ± 0.03
CT	52.6 ± 1.6	2.57 ± 0.53	1.19 ± 0.34	0.84 ± 0.14	0.13 ± 0.01	0.01 ± 0.01
All Treatments						
0 Months	92.2 ± 7.3	2.29 ± 2.07	0.92 ± 0.02	0.62 ± 0.08	0.15 ± 0.02	0.01 ± 0.01
5 Months	44.5 ± 1.4	3.56 ± 0.30	0.99 ± 0.17	0.77 ± 0.15	0.75 ± 0.06	0.20 ± 0.02
10 Months	19.3 ± 1.4	2.99 ± 0.68	1.15 ± 0.54	0.61 ± 0.12	0.46 ± 0.02	0.11 ± 0.01

[a] Months stored at −29°C

[b] MF = melted fat; CF = cooked fat; CT = cooked tissue.

Table 2—Reported carbonyl values for ovine fat (μmoles/10g)

	Carbonyl classes					
Treatment/Sample	Total carbonyls	Total monocarbonyls	Alk-2-ones	Alkanals	Alk-2-enals	Alk-2,4-dienals
Rendered in Air[a]						
Unheated	N.R.[d]	4.76	N.R.	2.56	1.97	0.23
Heated	N.R.	10.31	N.R.	5.31	4.09	0.91
Rendered in Nitrogen[b]						
Unheated	N.R.	0.94	N.R.	0.94	0.00	0.00
Heated in Air	N.R.	1.08	N.R.	1.03	0.05	0.00
Heated in Nitrogen	N.R.	2.23	N.R.	2.23	0.00	0.00
Volatiles	N.R.	0.00	N.R.	0.00	0.00	0.00
Chloroform Extract[c]						
Unheated, fresh	N.R.	8.50	0.00	5.60	2.90	0.00
Unheated, aged	N.R.	4.20	0.00	1.80	1.70	0.70
Cooked, aged	N.R.	4.00	0.00	1.90	1.70	0.40
Drippings	N.R.	7.40	0.00	2.50	4.20	0.70

[a] Ellis et al. (1961)

[b] Hornstein and Crowe (1963)

[c] Riley et al. (1971)

[d] Not reported

(Jacobson and Koehler, 1963) and other meat products (Herz and Chang, 1970; Wilson and Katz, 1972), make the detection of this fraction in ovine fat quite reasonable.

As can be noted from the data presented in Table 1, the amount of alkanals was greater in the cooked tissue (CT) than in the cooked fat (CF) sample, and both cooked samples had more alkanals than the MF sample. In all previous studies, except that of Riley et al. (1971), alkanal concentration was increased by heating. This increase probably reflects an increase in lipid oxidation. During storage, the alkanal fraction was largest at 5 months whereas the amount in the 10-month sample was not different from the fresh, nonstored sample.

The alk-2-enal fraction was greater than the 2,4-dienal fraction. This is consistent with the literature although some researchers have failed to detect any 2,4-dienals in ovine fat samples. In comparing the melted (MF) and cooked (CF and CT) samples of these fractions, only a small amount of alk-2-enals, and no 2,4-dienals, were detected in MF samples. This illustrates the influence of heat in accelerating lipid oxidation. However, the cooked tissue (CT) samples generally showed a decrease upon heating. This result is not easily explained but may be due to tissue binding of these materials. As noted in the other carbonyl classes, the greatest amounts of alk-2-enals and alk-2,4-dienals were observed in fat samples stored for 5 months. The least amount of these compounds was present in fresh, nonstored samples, and the amounts of these constituents found in samples stored for 10 months was intermediate between the fresh, nonstored samples and those stored for 5 months.

Generally, these studies have indicated that cooking increased the amount of all carbonyls present. Although the amount of total carbonyls decreases continuously during frozen storage, the amount of total monocarbonyls as well as that of the individual classes of monocarbonyls appears to increase up to 5 months and then decrease thereafter to 10 months.

In regenerating the carbonyls from their DNP-hydrazone derivatives, the alkanal fraction appeared to retain more of the "typical" lamb/mutton aroma than was observed in the total carbonyl fraction. However, the distinctive "sulfur" note was noticeably absent (Sink and Caporaso, 1977). This would be expected since sulfur-containing compounds are generally not found among the many alkanals identified in ovine fat (Hornstein and Crowe, 1963; Jacobson and Koehler, 1963). The alk-2-enals appear to contribute a "cucumber" note to the overall aroma of ovine fat. This may be due to *trans*-2 C_9 and/or C_{10} components (Badings, 1970). These odor analyses must be evaluated carefully considering the various sources of error associated with the hydrazone regeneration technique.

Table 3—Olfactory evaluation of regenerated carbonyls

Carbonyl class	Panelists		
	1	2	3
Total carbonyls	Musty, Sulfur	Sulfur, Rotten eggs	Sulfur, Animal-like
Alk-2-ones	Sweet, Fruity	Fruity	Waxy, Oily
Alkanals	Painty, Musty	Weak Oxidized	Waxy, Oxidized, Animal-like
Alk-2-enals	Strong Cucumber	Cucumber, Vegetable, Oxidized	Cucumber, Waxy, Animal-like
Alk-2,4-dienals	Smoky	?	Fresh Laundry-like

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Ms received 9/28/77; revised 3/4/78; accepted 3/8/78.

Paper No. 5241 in the journal series of the Pennsylvania Agricultural Experiment Station.

The authors express their appreciation for technical assistance to Elaine Austin, and to G.W. Smeal and D.E. Butts for assistance in procurement of the ovine adipose tissue samples.

PROTEIN EXTRACTION FROM MEAT AND THE POSTMORTEM PERMEABILITY OF MUSCLE FIBERS

E. G. CERRELLA and H. A. MASSALDI

ABSTRACT

The extractability of intracellular proteins of bovine meat in a Ringer solution was determined as a function of the transfer area of regularly cut samples. With the aid of theoretical estimations of the extracted fraction–by application of diffusion theory–it is shown that proteins migrate uniformly in all directions and do not proceed exclusively from the fibers ruptured by the cuts. This result is discussed in connection with the postmortem permeability of tissue and its relevance in the models of freezing injury of meat.

INTRODUCTION

THE INCREASING DEMAND of frozen meat in the world market has caused concern for gaining more insight into the freezing process and its relationship with the quality of the final product, intended for direct consumption.

One of the characteristics of frozen meat is the exudation of fluid, or drip, that takes place upon thawing. Dissolved in the fluid can be found, typically, sarcoplasmic proteins, DNA and salts (Grau, 1965). Whereas the loss of liquid is normally considered a consequence of the distribution of ice in the frozen state and partial reabsorption of water during thawing, the presence of such intracellular components in the fluid deserves additional explanation.

In living tissue, the cellular membrane – sarcolemma – retains selectively the intracellular components, and regulates the absorption and removal of water in the cell by means of osmotic changes. In other words, it exerts the properties of semipermeability. The hypotheses given in the literature to explain drip from thawed tissue are based on a specific injury to the cell membrane, either by mechanical action of growing ice crystals (Meryman, 1960) or chemical damage (Lovelock, 1953) caused by the exposition of the membrane proteins to highly concentrated salt solutions, left by the crystallization of water. In the first case the membrane would "leak" the N_2-rich compounds together with the intracellular water. In the latter case, the semipermeability of the membrane would be lost by denaturation of membrane proteins, and the intracellular components could leave the cell more readily.

Even in the case that either, or both mechanisms take place to some extent during freezing, there exists a third possibility that can account for the presence of intracellular components in the exudated fluid. Preliminary experiments in this laboratory showed that proteins, and probably other intracellular components could be lost from fresh meat prior to freezing and thawing, and appeared in the drip with a concentration similar to that found in frozen-thawed tissue. Several works from the literature seem to confirm this fact. Zierler (1958a, b) and Zierler et al. (1953) discussed the membrane properties of rat muscle in connection with the observed efflux of different enzymes after incubation for an hour of dissected muscle. Dawson (1966), measured the efflux rate of seven sarcoplasmic enzymes from rat muscle. Protein diffusion from chicken intact muscle in relation to pH of a Ringer solution was studied by Osner (1966). This author also tested this effect in connection with the problem of muscle passing rigor mortis attached or not attached to the bone. On this basis, the present work was undertaken to elucidate whether sarcoplasmic proteins could be lost from post-rigor, nonfrozen meat under conditions that insured no internal damage to the tissue, namely, by extraction of meat pieces in a Ringer solution at moderate temperature. Due to the necessity of cutting meat samples, however, it was also necessary to determine what incidence the cut surfaces had on the total amount of protein found in the extract.

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0022-1147/78/0005-1382$02.25/0

EXPERIMENTAL

Materials

Samples of semitendinosus bovine muscle, provided by Frigorífico CAP La Negra, and aged at 1°C during 72 hr after slaughter, were used. A Ringer phosphate solution, pH–7.4, was used (Rappaport, 1956) with composition: NaCl, 8g; KCl, 0.2g; KH_2PO_4 0.2g; Na_2HPO_4, 1.15g; excipient: distilled water, 1L.

Analytical method

The method of Lowry et al. (1951) was applied to determine soluble proteins in the extract. Absorbance readings were taken at λ = 750 nm in an Arolab Spectrophotometer and were converted to concentration values by calibration of the apparatus with solutions of bovine serum albumin of varying concentrations. The reproducibility of determinations was found to be within 5%.

Experimental procedure

The samples to be tested were obtained by proper cutting of meat in the form of regular parallelepipeds of similar dimensions: 1 cm × 1 cm × 4 cm (referred to below as original geometry). Some of these were then subdivided in a prescribed manner and all were classified according to the following list (see Fig. 1):

I: Original geometry, longitudinal cut (parallel to the fibers).
II: Type I, divided into two pieces by a longitudinal cut.
III: Type I, divided in three pieces by two longitudinal cuts.
IV: Type I, with the top and bottom sides sealed with paraffin (not shown).
V: Original geometry, transverse cut (perpendicular to the fibers).
VI: Type V, divided into two pieces by a tranverse cut.
VII: Type V, divided into 10 pieces, as shown.
VIII: Type V, divided into 20 pieces, as shown.

At least two replicated runs with each geometry were carried out by submerging the pieces in 110 ml of saline buffered solution for 16 hr. Moderate, but gentle stirring was achieved by placing the flasks in a thermostated shaker at 12°C. To avoid maceration, the pieces were hung from the flask cover by means of a wire tied to the piece so that contact with the walls was minimal. Two additional runs were carried out with the Type VIII geometry for a longer time (approx 24 hr) in order to obtain a measure of the maximum extractable protein by this method, as determined by the constancy of concentrations in two succesive measures. Another two runs were carried out with Na_3N added to the solution in order to prevent the possible development of microorganisms.

Since most of the experiments with each geometry were conducted separately on different days, the samples sometimes came from different animals (always the same breed) and sometimes from the same animal but with a different aging time. For this reason, and to make the

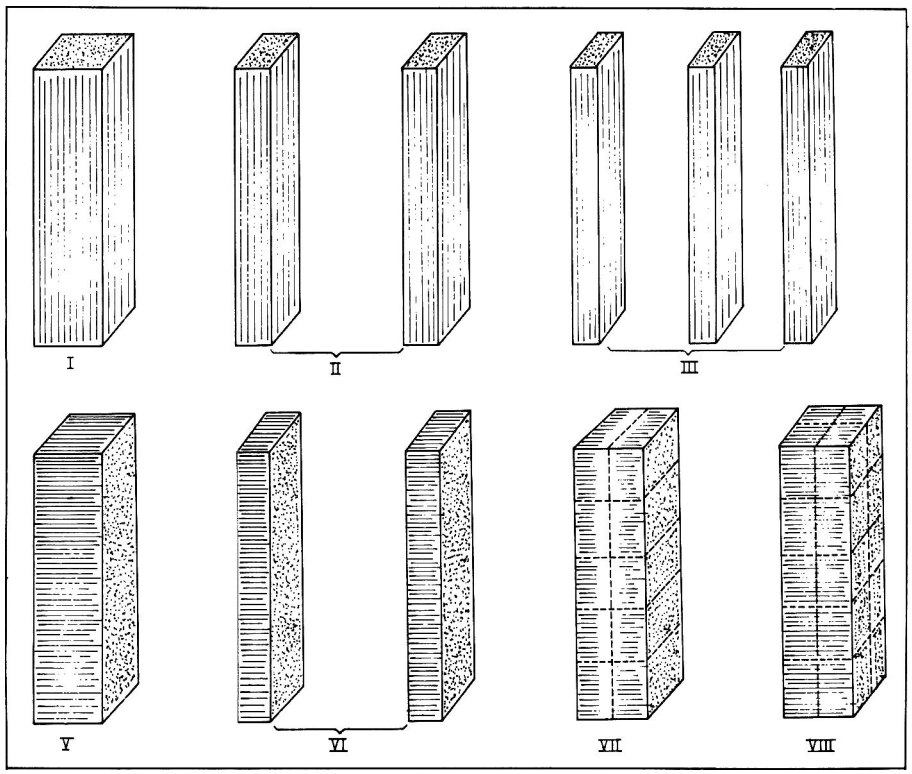

Fig. 1—Geometry of the samples used in the determination of protein extraction. Classification according to type number is indicated in the text.

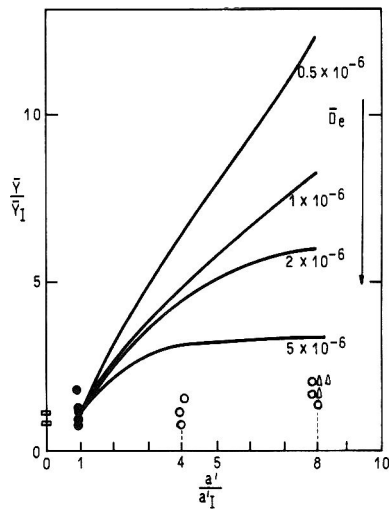

Fig. 2—Protein fraction extracted as a function of tranverse cut area. Full lines are theoretical estimations. The mean effective diffusivity is the parameter. Y_I ranges from 30—70%. w is 5—6% g protein/g meat.*

results from different geometries comparable, a simultaneous run with the type I geometry was conducted in every case, and the result was used as a reference value in plotting the data, so that all other results were normalized with respect to this geometry. In this way, biological variations from different animals, or differences in the aging time of meat were absorbed. Also other possible sourses of errors, as mentioned below, were minimized by this procedure.

The solution was separated from the flask and centrifuged in a refrigerated centrifuge (2500 × G) for 15 min. Two aliquots were taken from the supernatant and were diluted, when necessary, for analysis.

RESULTS

FOR EACH RUN, the amount of protein extracted, w, expressed as % grams of protein in solution per gram of meat, was calculated from the sample weight, the solution volume and the spectrophotometer reading. The maximum extractable protein by this method, w*, was found to be, w* = 5—6% g protein/g of meat. The fraction of protein extracted is $\bar{Y}$ = w/w*. The normalized ratio $\bar{Y}/\bar{Y}_I$, however, is independent of w*, and was calculated for every run as $\bar{Y}/\bar{Y}_I = w/w_1$.

The same experimental results and the theoretical estimations of protein extracted as a function of transfer area are presented in Figures 2 and 3 in the form of $\bar{Y}/\bar{Y}_I$ vs a'/a'_I and a/a_I respectively. a', in Figure 2, is just the area produced by the cut transverse to the fiber, so that only the direction leading to the cut surfaces was considered to be effective for extraction. In Figure 3, a is the total exposed area of the piece and so the three dimensions were assumed to be equally effective for extraction.

The experimental data are shown in Figures 2 and 3 by the circles, triangles and squares. The full lines in Figures 2 and 3 represent theoretical estimations of $\bar{Y}$, corresponding to changes of transfer area in one direction only. The segments in Figure 3 represent the theoretical estimation for Type VII and VIII geometries, where the change in transfer area is due to changes in two dimensions simultaneously and so the intermediate values cannot be represented by a unique line. Table I

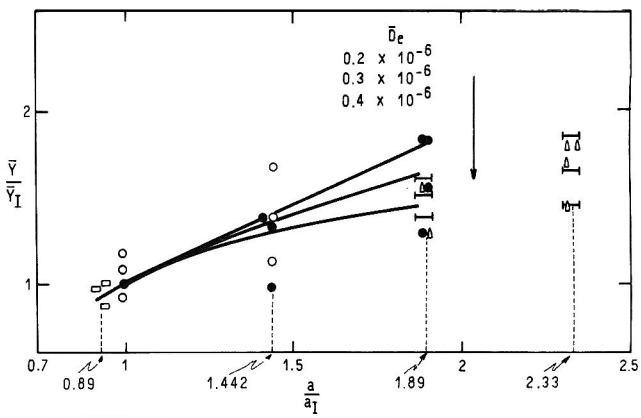

Fig. 3—Protein fraction extracted as a function of total exposed area. Full lines and segments are theoretical estimations. The mean effective diffusivity is the parameter. Y_I ranges from 30—70%. w is 5—6% g protein/g meat.*

Table 1—Meaning of symbols and corresponding transfer area

Geometrical type	Cut type	Symbol	a'/a'_I	a/a_I
I	Longitudinal	●	2/2 = 1	18:18 = 1.0
II	Longitudinal	●	2/2 = 1	26/18 = 1.44
III	Longitudinal	●	2/2 = 1	34/18 = 1.89
IV	Type I, paraffin sealed extremes	□	0/2 = 0	16/18 = 0.89
V	Transverse	○	8/2 = 4	18/18 = 1.0
VI	Transverse	○	16/2 = 8	26/18 = 1.44
VII	Longitudinal and Tranverse	Δ	16/2 = 8	34/18 = 1.89
VIII	Longitudinal and Tranverse	Δ	16/2 = 8	42/18 = 2.33

shows the meaning of the symbols and the value of the abscissa for the data in each Figure. Accordingly, the triangles are to be compared with the segments in Figure 3. The solid-circle in the origin of the curves ($\bar{Y}/\bar{Y}_I = 1$), corresponds to the data with the type I geometry. Other solid circles at $a'/a'_1 = 1$ in Figure 2 correspond to type II and III geometries. The open circles at $a/a_1 = 1$ in Figure 3 represent data for the type V geometry.

Several data points are coincident in Figure 2, and some of them are shown slightly shifted even though they correspond to the same value of a'/a'_1, as indicated in the abscissas to the figures. The data show more scatter when plotted in Figure 3 because of the stretching of scales. Also the data from geometrical types VI, VII and VIII, that have the same value, $a'/a'_1 = 8$ in Figure 2, split up to the values shown at $a/a_1 = 1.44$ (Type VI), 1.89 (Type VII) and 2.33 (Type VIII), concurrently with Type II geometry at $a/a_1 = 1.44$ and Type III at $a/a_1 = 1.89$. Results for the runs with Na_3N-added solution didn't show extraction values out of the average and so were not identified in the figures.

The theoretical curves and segments were calculated by assuming that proteins migrate by ordinary diffusion within the meat. The parameter for these curves is the mean effective diffusivity of proteins within the meat, and the values chosen are the possible ones that can render curves comparable with the experimental data.

The extraction of protein from the piece of meat was considered to be governed by the equation,

$$\frac{\partial c}{\partial t} = D_e \nabla^2 C \qquad (1)$$

with initial and boundary conditions,

$$C = C_o;\ t = 0;\ \text{every } x, y, z \qquad (2)$$

$$C = 0;\ t > 0;\ x = \pm x_o,\ \ y = \pm y_o,\ \ z = \pm z_o \qquad (3)$$

$$\frac{\partial c}{\partial x} \cdot \frac{\partial c}{\partial y} \cdot \frac{\partial c}{\partial z} = 0;\ t \geq 0;\ x = y = z = 0 \qquad (4)$$

Boundary condition (3) implies two assumptions: first, that protein concentration is zero in the solution at all times, which is a reasonable approximation considering the relatively large volume of solution used. Secondly, that the external mass transfer resistance is negligible. This was assumed to be easily achieved—even considering the gentle stirring used—because of the low expected values of the mean effective diffusivity ($\bar{D}_e < 10^{-6}$ cm^2/sec).

The solution to Eq (1), subject to conditions (2), (3), and (4) has been presented by Newman (1931), as the product of the solutions corresponding to each dimension, assuming infinite slab geometry. The result, in terms of the protein fraction remaining, for one dimension, $\bar{Y}_i$, is:

$$\bar{Y}'_i = \sum_{n=1}^{\infty} \frac{8}{\pi^2 (2n+1)^2} \exp\left[\frac{-\bar{D}_e (2n+1)^2 \cdot \pi^2 \cdot t}{4.1_i^2}\right] \qquad (5)$$

The total fraction remaining is,

$$\bar{Y}'_T = \prod \bar{Y}'_i;\ \ i = 1, 2, 3 \qquad (6)$$

and the total fraction extracted is,

$$\bar{Y} = 1 - \bar{Y}'_T \qquad (7)$$

The total fraction extracted, $\bar{Y}$, is calculated from Eq (7), with $\bar{Y}'_T$ evaluated either from plots (Chorny and Krasuk, 1966) or from Eq (6), where $\bar{Y}'_i$ can be obtained from the Table given by Sherwood et al. (1975).

DISCUSSION

A FIRST POINT to be observed is the substantial amount of protein extracted from the samples, as indicated by the values of $\bar{Y}_I$ and w*, included in the legends to Figures 2 and 3.

It may be argued that proteolysis is the cause of the extensive presence of proteins in the solution. It is possible that some degree of proteolysis had taken place during the experiments. Also, it is felt that the values of $\bar{Y}$ are strongly dependent on the aging time of the meat used. However, the normalization of results for every run with respect to a fixed geometry (Type I) eliminates the uncertainty that this would introduce on the absolute values of protein extracted from different runs. A second order effect, such as increasing proteolysis at a higher degree of subdivision (higher a/a_I), would render more confidence to the findings drawn from this work, as is discussed below.

However, the question can be raised as to what extent the proteins migrate from the inside of the piece with equal probability in all directions, or they proceed just from the cells ruptured by the cut.

In both cases, it seems reasonable to assume that the proteins migrate by an ordinary diffusional mechanism, going through the intracellular space in the first case, or going through the intracellular, the membrane, and the extracellular phases in the second, before getting to the extractive solution.

In order to discriminate between those alternatives, we can consider Figures 2 and 3. In Figure 2, the curves represent the extracted fraction function that should be expected if the proteins proceed just from the transverse cut surfaces. It is apparent that the experimental data do not follow the trend of the theoretical curves shown, e.g., they slightly increase with an augment in the effective transfer area. It can be observed that a higher value of $\bar{D}_e$, of the order of 1×10^{-5} or greater, would render a curve that might fit the data. However, this value results too high when reported values of protein diffusivity in water solutions, are considered [myoglobin: $D = 1.03 \times 10^{-6}$, phosphoglucomutase, $D = 4.83 \times 10^{-7}$; (Sober, 1973)]. This, together with the fact that the diffusion medium is restricted by the presence of membranes, connective tissue, etc., led us to consider just the values of $\bar{D}_e$ shown. Furthermore, higher values of $\bar{D}_e$ would result in a fraction $\bar{Y}_I$ of order 95% whereas the observed values of $\bar{Y}_I$ range from 30–70%.

Now, we turn to consider Figure 3, which shows the extracted fraction function corresponding to a total surface transfer area. The curves and segments in this figure correspond to lower values of $\bar{D}_e$, which are also the more probably ones. It is apparent that the experimental data are reasonably well fitted by the theoretical curves, considering the reduced range of $\bar{D}_e$ shown.

Although the scatter of the data at $a/a_1 = 1.89$ and 2.33 is considerable, the overall behavior of results in both figures is different enough to allow stating that it is the whole exposed surface of the piece that is effective for protein transfer. Even considering the geometries separately, such as geometries Type I and V and geometries Type II and VI, that show about the same extraction value in Figure 2, the above statement seems correct. This is further supported by the results with the paraffin-sealed piece—the squares in Figures 2 and 3—that show a non zero extraction value in Figure 2 and fit fairly well the theoretical level in Figure 3.

This whole picture can be logically interpreted if the cell membranes are considered to be permeable to intracellular solutes. Voyle (1974) suggested a continuous increase in the membrane permeability of meat to proteins during rigor

mortis, together with a decrease in ATP content and pH. Also Fenichel and Horowitz (1965) found a substantial increase in protein efflux from the sartorius muscle of frog after induction of rigor mortis with 1-propanol. The phenomenon is frequently mentioned in the literature—though somewhat laterally—in connection with the postmortem changes of meat. In this sense, the present work provides an experimental support to those hypotheses.

Fiber dimensions and the protein extraction process

During the experimental work, the question was raised as to what effect the fiber dimensions may have on the behavior of results. Clearly, the fiber thickness was not considered to be important since it is several orders of magnitude less than the smallest dimension of the piece. The fiber length, however, could be thought to represent a new parameter in the analysis of results, since it can have a value similar to, or greater than, that of the piece.

Figure 4 shows a schematic representation of the two limiting situations: (a) short and (b) long fibers. Table 2 summarizes the possibilities.

A short analysis of Figure 4 [(a) and (b)] indicates that, if only the cut fibers were responsible for the loss of protein, the functions $\bar{Y}/\bar{Y}_I$ vs a'/a'_I would be different for short or long fibers. In the first case it would be a 45 degree line in Figure 2, since a fixed increase in effective area would roughly give the same increase in protein loss. For long fibers, a unidirectional diffusion model should be postulated, as in fact was assumed in the previous discussion. As was discussed above, it was found that this model does not represent the data, nor the 45 degree line does.

On the other hand, the multidirectional diffusion model—that was found to fit the data—is insensitive to the length of the fibers, since, apparently, the proteins can go through the sarcolemma with a probability similar to that through the intracellular medium.

CONCLUSIONS

PROTEIN LOSS from post rigor, nonfrozen meat can be found to be significant when a proper—but not destructive—extraction solution is used. It was shown that this is not the consequence of cells having been ruptured by the cuts necessary for handling. Instead, it seems that the cell membrane of fibers (sarcolemma) has lost, at least to a great extent, its functionality in fresh, post rigor meat. On this basis, it is not necessary to postulate a specific membrane damage undergone during the freezing step to allow for the presence of intracellular components in the drip. More simply, it can be proposed that the proteins and other intracellular dissolved substances are carried along convectively with the water that migrates out of the cell (as occurs in moderate or slow freezing) and accumulate—and/or precipitate—between the crystals and the dehydrated fibers. It should be noted, however, that the external evidence of damage—loss of nutrients and weight as drip—depends also on the characteristics of the thawing step, where partial reabsorption of the fluid into the fibers can take place.

NOMENCLATURE

- a Total exposed area of the sample, cm^2
- a' Transverse cut area of the sample, cm^2
- C Protein concentration in solution, g/L
- C_o Initial protein concentration in solution, g/L
- $\bar{D}_e$ Mean effective diffusivity of protein within meat, cm^2/sec
- l_i Length of dimension i, cm (= x_o, y_o, z_o)
- t Time, sec
- w Amount of protein extracted, % g protein/g meat
- w^* Maximum amount of protein extracted, % g protein/g meat

Table 2—Extraction model and permeability of cells

Cells	Short	Long
Permeable	Follow multi-directional diffusion	Follow multi-directional diffusion
Not permeable	Do not follow diffusion. Follow 45 degree line	Follow unidirectional diffusion

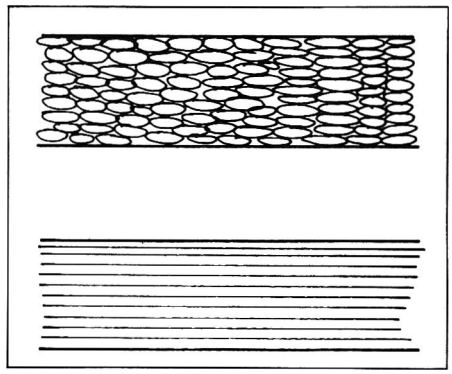

Fig. 4—Schematic representation of short and long fibers.

- x_o Length of dimension x, cm
- y_o Length of dimension y, cm
- $\bar{Y}$ Fraction (with respect to maximum) of total protein extracted, = w/w*
- $\bar{Y}'_i$ Fraction of protein remaining for dimension i, Eq (5)
- $\bar{Y}'_I$ Fraction of total protein remaining, Eq (6)
- z_o Length of dimension z, cm

Subscripts

- I Type I geometry

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Ms received 6/13/77; revised 11/17/77; accepted 11/23/77.

EFFECT OF SELECTED SOLUTES ON GROWTH AND RECOVERY OF A RADIATION–RESISTANT Moraxella SP.

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ABSTRACT

A highly radiation-resistant *Moraxella* sp. from beef was more resistant to gamma radiation in frozen beef than *Clostridium botulinum* 33A spores. Even though the *Moraxella* sp. was extremely radiation-resistant, its recovery after irradiation was markedly influenced by the plating medium. Fewer colony-forming units were recovered in Tryptic Soy Agar (TSA) than in Plate Count Agar (PCA), and differences in recovery became more pronounced with increasing radiation dose. Growth studies of the nonirradiated *Moraxella* sp. suggested the presence of dialyzable inhibitory factor(s) in Trypticase Soy Broth (TSB) and TSA. The low (0.5%) concentration of NaCl in TSA was shown to be mainly responsible for the slow growth and reduced recovery after irradiation. Reduced recovery was also obtained by plating the *Moraxella* sp. in PCA plus 0.5% NaCl or PCA plus 6% glucose after irradiation. It was noted that 2 other highly radiation-resistant isolates identified as *Moraxella* sp. gave similar results. Sensitivity to low solute concentrations, therefore, appeared to be a general phenomenon for this group.

INTRODUCTION

Moraxella sp. and *Acinetobacter* sp. are gram-negative to gram-variable nonsporeforming bacteria that have frequently been found in irradiated meats (Tiwari and Maxcy, 1972; Ito and Sato, 1973). These closely related bacteria have been designated *Moraxella-Acinetobacter* (M-A). M-A isolated from beef in a study by Welch and Maxcy (1975) exhibit a wide range of resistance to gamma radiation. Three of the M-A from beef (Isolates 4, 7, and 13) had radiation resistance that appeared to be greater than that of bacterial spores.

For many years it has been known that another nonsporeformer, *Micrococcus radiodurans*, is more resistant to radiation than are spores (Duggan et al., 1963; Thornley, 1963). Other highly resistant micrococci (Davis et al., 1963; Lewis, 1971) have also been isolated from irradiated foods. The fact that certain micrococci and M-A exhibit exceptionally high resistances to radiation is of fundamental and applied interest in anticipating irradiation of food as an acceptable commercial process.

Radiation-resistant asporogenous bacteria may constitute a significant percentage of the microflora of a wide variety of food products. For example, radiation-resistant micrococci and M-A have been reported to comprise as much as 0.05–0.1% (Anderson et al., 1956) and 27% (Tiwari and Maxcy, 1972), respectively, of the total microflora of fresh ground beef. While the residual microflora in beef treated with low radiation doses (200–500 Krad) may be more heterogeneous, the highly resistant micrococci and M-A would appear to comprise the main part of the residual microflora in beef given higher doses (1000 Krad). Therefore, the proportion of these highly radiation-resistant bacteria in relation to the total number of survivors in an irradiated food product would increase with increasing doses of gamma radiation.

The significance of these residual bacterial populations in irradiated foods needs to be determined. *M. radiodurans* and the more recently isolated *Micrococcus radiophilus* sp. nov. have no presently recognized public health significance (Lewis, 1971; Thornley, 1963). M-A have been found in a variety of food products (Koburger, 1964; Shewan, 1971; Snodgrass and Koburger, 1967) without being associated with spoilage, except in marine fish and shellfish (Herbert et al., 1971). However, M-A have been associated with both human and animal infections (Henriksen, 1973). Determination of chemical and physical factors that control the growth of the residual flora of irradiated meat is important, because these bacteria become the potential spoilage flora.

The purpose of this work was to investigate the effects of certain nutrient media on growth and recovery of highly radiation-resistant M-A.

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0022-1147/78/0005-1386$02.25/0

MATERIALS & METHODS

Cultures and inocula

Preliminary experiments showed that growth responses in various media were similar for Isolates 4, 7, and 13. Isolate 4 was selected as a representative of the group for further study. Welch and Maxcy (1975) classified Isolate 4 as a member of Group M-5 of the bacteria that resemble *Moraxella* sp. Cultures of Isolate 4 were propagated on Plate Count Agar (PCA; Difco) slants by incubating for 24 hr at 32°C. Between transfers cultures were stored at 2°C. *Clostridium botulinum* 33A spores were supplied by A. Anellis of the U.S. Army Natick Research & Development Command. The spore suspensions were diluted tenfold in M/15 phosphate buffer (pH 7.2) and heat shocked at 80°C for 10 min before inoculation into beef prior to irradiation.

Cultures of Isolate 4 to be irradiated were grown in m Plate Count Broth (PCB; Difco) at 32°C in a rotary shaker bath for either 24 or 48 hr. Inocula for growth studies were obtained from 24-hr cultures grown in Trypticase Soy Broth (TSB; BBL) at 32°C. PCB (pH 7.0) consists of 10g tryptone, 5g yeast extract, and 2g dextrose per liter, whereas TSB (pH 7.3) consists of 17g Trypticase, 3g phytone, 5g NaCl, 2.5g K_2HPO_4, and 2.5g dextrose per liter of distilled water.

Plate counts and special media

Dilution and plate counts of Isolate 4 were performed according to procedures outlined by Sharf (1966). Survival of *C. botulinum* 33A spores after various radiation doses was determined by counting colonies after 48 hr in roll tubes containing Thiotone-Yeast Extract-Trypticase Medium (TYT, pH 7.2) and $NaHCO_3$ (Anellis et al., 1975). NaCl, glucose, and sucrose tolerances of Isolate 4 were determined in a nutrient medium (TP broth, pH 7.0), which consisted of 17g Trypticase (BBL) and 3g Bacto-Peptone (Difco) per liter of distilled water.

Radiation inactivation curves in beef

Ground beef obtained from a local commissary was treated at ambient temperature with 2 Mrad gamma radiation from a Cobalt-60 source providing 7 Krad per min (Tiwari and Maxcy, 1972) and then chilled at 2°C. Plate counts of uninoculated meat yielded less than ten bacteria per gram. After inoculation with either Isolate 4 or *C. botulinum* 33A spores, the product was aseptically ground 5 times.

Patties of approximately 30g of inoculated beef were placed in corners cut from plastic sandwich bags, dropped into Cryovac pouches (Standard gauge, Type P 850; W.R. Grace Co.), and flattened to a thickness of 8 mm. The pouches were sealed with a vacuum of approximately 125 mm Hg.

Patties were held at −25°C overnight before irradiation. The patties were held next to dry ice during irradiation so that the temperature was maintained at −30 ± 10°C as determined with a thermocouple. After irradiation, patties were thawed in a 32°C water bath and sampled for plate counts by transferring 11g meat to a Waring Blendor with 99 ml phosphate buffer and blending for 1 min.

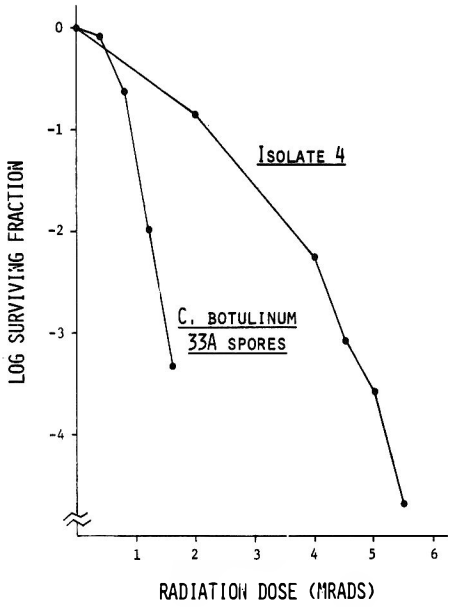

Fig. 1—Comparative radiation resistance of Isolate 4 and C. botulinum 33A spores in ground beef at −30° C. Initial concentrations were: Isolate 4, 1.4 X 10^7 CFU/g, and C. botulinum spores, 1.6 X 10^6 CFU/g.

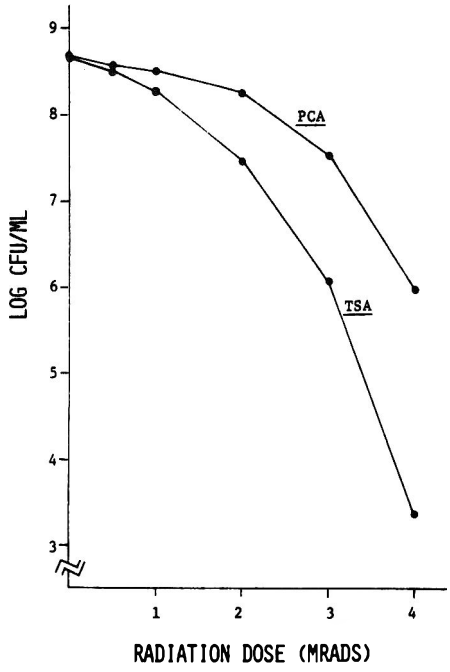

Fig. 2—Comparative recovery of Isolate 4 at 32° C in TSA and PCA after irradiation in PCB at −30° C.

Comparative recovery of radiation-injured Isolate 4

Samples from a 24-hr PCB culture of Isolate 4 were quick-frozen in a dry ice-acetone bath and irradiated in test tubes next to dry ice. Irradiated samples were thawed at 32°C, and plate counts were made with PCA and TSA. Later studies used PCA with added NaCl or filter-sterilized glucose. Plates were incubated until there was no further increase in the number of colonies on the plates (up to 7 days incubation for samples receiving radiation doses above 3 Mrad).

Growth of nonirradiated Isolate 4 in nutrient media

Population densities of Isolate 4 growing out from equivalent inocula were determined after 48 hr in PCB, TSB, and TSB to which 5g yeast extract (YE) per liter had been added. Growth rates of Isolate 4 in PCB, TSB and dilute TSB (1:4) were observed by plating after 24 and 48 hr. Broths were inoculated with cells previously grown in TSB.

Concentrated TSB was dialyzed against distilled water at 2°C. Enough distilled water was then added to the dialyzable and nondialyzable fractions to make each fraction to the appropriate volume for converting the concentrated TSB into standard TSB. Growth of Isolate 4 in these fractions, which were sterilized by autoclaving, was compared to growth in standard TSB and TP.

Salt and sugar tolerance levels of nonirradiated Isolate 4

NaCl, glucose, and sucrose tolerance levels were determined by inoculating TP broths containing NaCl or filter-sterilized sugar with enough cells to obtain an initial concentration of 10^3 cells/ml. Flasks were examined visually for turbidity up to 72 hr and positive cultures were streaked on PCA to check for contamination.

Salts found in TSB were added to PCB at equivalent concentrations. Growth rates of Isolate 4 were compared in PCB containing 0.25% K_2HPO_4 (0.014M) and in PCB containing 0.5% NaCl (0.085M) plus 0.25% K_2HPO_4. The pH of both media was 7.3.

The following results for all experiments represent averaged values from duplicate trials.

RESULTS

Radiation inactivation curves in beef

Results of radiation inactivation curves in beef at −30°C are shown in Figure 1. The radiation inactivation curve for *C. botulinum* 33A spores was in agreement with the one obtained by Grecz et al. (1965) in frozen canned ground beef. The radiation inactivation curve for Isolate 4 had a more extensive shoulder than did the curve for *C. botulinum* 33A spores.

Effect of plating medium on recovery of radiation-injured Isolate 4

There was very little difference between TSA and PCA in the numbers of recovered nonirradiated bacteria. Colony size of Isolate 4, however, was smaller in TSA than in PCA. The percentage of bacteria recovered after irradiation in TSA, when compared to PCA, decreased with increasing radiation dose. At 4 Mrad only approximately 0.1% of the bacteria recoverable on PCA yielded colonies on TSA. Comparative recovery curves are shown in Figure 2.

Growth of nonirradiated Isolate 4 in PCB and TSB

Isolate 4 multiplied more slowly in TSB than in PCB. Table 1 shows 48-hr population densities of Isolate 4 cultures in PCB, TSB, and TSB with 5 g/l YE. These results suggested that the primary cause of the differences in population in TSB and PCB was attributable to an inhibitory rather than a nutritional factor. This was demonstrated by the enhanced growth response observed in a 1:4 dilution of TSB (Fig. 3).

Inhibitory factor(s) in TSB were removed by dialysis as was indicated by results in Figure 4. The nondialyzable fraction supported growth comparable to growth in TP, which had the same peptone concentration in TSB without NaCl. Growth in the dialyzable fraction was comparable to growth in TSB. These data suggested that salt concentration was a key factor in the inhibition of growth. NaCl, at a concentration of 0.5% is the primary contributor of ions in TSB. The effects of added salts on growth of Isolate 4 in PCB are shown in Figure 5. K_2HPO_4, at a concentration of 0.25%, had little effect on growth rate, while further addition of 0.5% NaCl to PCB slowed growth significantly.

Sensitivity to osmotic concentration

The possibility that growth of Isolate 4 could be suppressed by nonspecific increases in solute concentration was explored. Tolerance levels of NaCl, glucose, and sucrose were determined as the highest solute concentrations that would allow enough

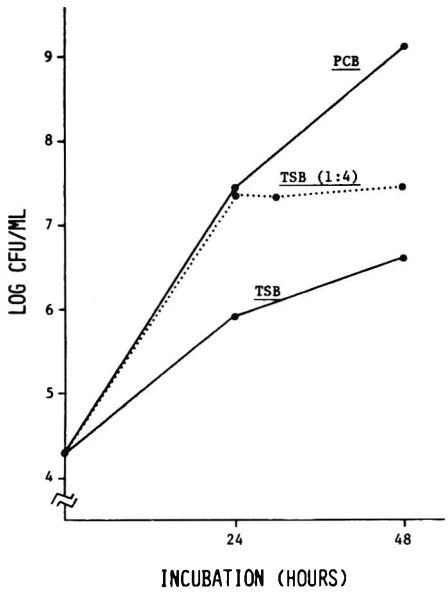

Fig. 3—Growth of Isolate 4 in PCB, TSB, and TSB diluted 1:4 at 32° C.

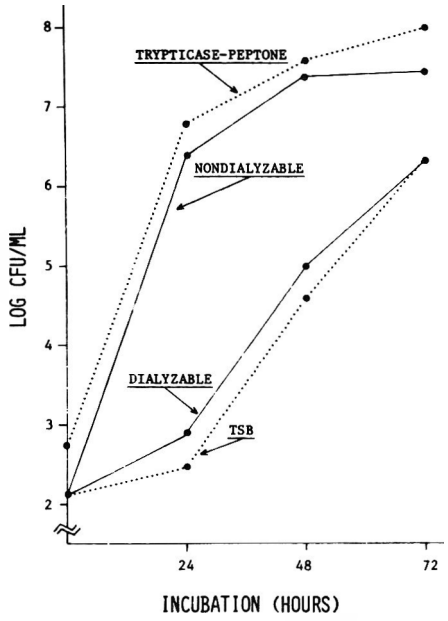

Fig. 4—Growth of Isolate 4 in dialyzable and nondialyzable fractions of TSB at 32° C.

outgrowth from an initial concentration of 10^3 cells/ml to produce visible turbidity in TP broth within 72 hr. The solute concentrations tolerated by Isolate 4 in TP broth were 0.75% NaCl, 7% glucose, and 11% sucrose. Isolate 4 appeared to be somewhat more sugar-tolerant than salt-tolerant, because the osmolar concentration of 0.75% NaCl corresponds to 4.6% glucose, assuming complete dissociation of NaCl.

Effect of NaCl and glucose on radiation recovery in PCA

Colony counts of Isolate 4 before and after irradiation with 2.5 Mrad were made on TSA and PCA plus 0.5% NaCl (Table 2). (The data are presented as percent recoveries, with 100% recovery based on colony counts obtained with PCA.) After 2.5 Mrad colony counts were still approximately four times greater on PCA plus 0.5% NaCl than on TSA. This difference may have been due to peptone type and content or to the presence of yeast extract in PCA.

Radiation inactivation curves for Isolate 4 plated on PCA and PCA plus 6% glucose are shown in Figure 6. The percentage of cells recovered on PCA plus 6% glucose declined rapidly with increasing radiation dose.

The low concentrations of solutes in TSA, PCA with 0.5% NaCl, and PCA plus 6% glucose were all effective in reducing yields after irradiation, even though colony counts before irradiation were not significantly affected.

Table 1—Population density of Isolate 4 in broth at 32° C after 48 hr

Broth	CFU/ml
PCB	1.4×10^9
TSB	1.9×10^7
TSB + 5 g/1 YE	2.1×10^7

Table 2—Effect of plating medium on recovery of Isolate 4[a] before and after irradiation with 2.5 Mrad at −30° C in PCB

	Nonirradiated		Irradiated	
Medium	CFU/ml	% recovery[b]	CFU/ml	% recovery[b]
PCA	6.6×10^8	100	1.3×10^8	100
TSA	6.0×10^8	91	9.2×10^6	7
PCA + 0.5% NaCl	5.9×10^8	89	4.1×10^7	32

[a] 24-hr cultures
[b] 100% recovery based on PCA plate counts

DISCUSSION

RADIATION INACTIVATION CURVES in frozen beef show that Isolate 4 is more resistant to gamma radiation than are *C. botulinum* spores of one of the most resistant types (Anellis and Koch, 1962). The M-A isolated from ground beef in this laboratory have exhibited a wide range of radiation resistance. Among them, Isolates 7 and 13 (Welch and Maxcy, 1975) have resistance similar to that of Isolate 4. The fact that some asporogenous bacteria are more resistant to radiation than are spores must be considered in radiation processing schemes.

An understanding of the significance of radiation-resistant M-A in irradiated meats should involve determining the effects of any inhibitory factors on their growth. For nonirradiated Isolate 4 colony development is slower, and colony size is smaller in TSA than in PCA. There is very little difference in plate counts of Isolate 4 in TSA and PCA before irradiation. After irradiation, however, colony counts are reduced in TSA when compared to PCA, and the reduction in colony yield is greater with increasing radiation dose. The slower growth of Isolate 4 in TSA and TSB and its reduced recovery in TSA after irradiation appear to be mainly due to the 0.5% NaCl concentration in these media. While 0.5% NaCl is commonly added to some media to enhance the growth of some fastidious microorganisms, this concentration is actually inhibitory for Isolate 4.

Sensitivity to low solute concentrations was not limited to Isolate 4. Further experiments showed that the salt and sugar concentrations that inhibited Isolate 4 were also inhibitory for Isolates 7 and 13, which indicated a more generalized phenomenon. Sensitivity of the highly radiation-resistant M-A to these solute concentrations was greater after irradiation. One reason for the sensitivity of highly radiation-resistant M-A to nonspecific increases in solute concentration may be a very high water activity requirement for growth (Snyder, 1977). Therefore, while radiation-resistant M-A may be able to survive high radiation doses, their outgrowth in some foods, particularly cured meats, may be restricted by lower water activities. In studies by Krabbenhoft et al. (1964) *C. botulinum* spores were also shown to be more sensitive to radiation in meat to which NaCl and $NaNO_3$ had been added.

It is interesting to note that the highly radiation-resistant

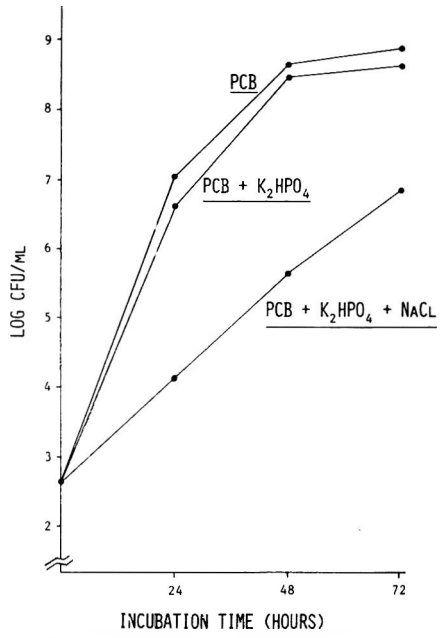

Fig. 5—Growth of Isolate 4 in PCB without salts, PCB plus 0.25% K_2HPO_4, and PCB plus 0.25% K_2HPO_4 and 0.5% NaCl.

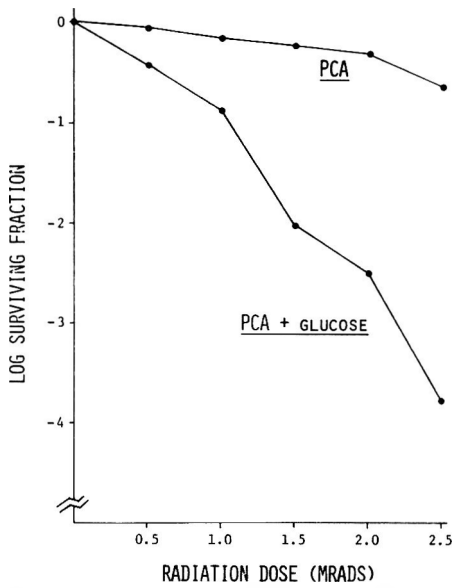

Fig. 6—Comparative recovery of Isolate 4 at 32° C in PCA and PCA plus 6% glucose after irradiation in PCB at −30° C. Initial concentration was 2 X 10^8 CFU/ml.

M. radiodurans is also markedly inhibited by 1% NaCl (Anderson et al., 1956). In addition, a highly radiation-resistant chromogenic bacterium (*Pseudomonas radiora*) isolated from irradiated rice was reported to tolerate up to only 0.5% NaCl in a potato dextrose medium (Ito and Iizuka, 1971). Further studies on the different radiation-resistant asporogenous bacteria that have highly effective systems for recovery from radiation may detect a common factor that could be used for their control in irradiated foods.

In this work the plating medium had a marked effect on the recovery of radiation-resistant M-A after irradiation. Further experiments provided strong evidence for the role of NaCl in reducing recovery. Nonirradiated M-A were highly sensitive to low levels of either NaCl or glucose in a growth medium and their sensitivity was increased after irradiation.

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Ms received 12/9/77; revised 1/20/78; accepted 1/30/78.

This work was supported in part by Contract No. DAAG17-76-G0008 from the United States Army Natick Research & Development Command.

The technical assistance of Kenneth Ma is greatly appreciated.

Published as Paper No. 5340, Journal Series, Nebraska Agricultural Experiment Station. Research reported under Project No. 16-023.

EFFECT OF HISTAMINE, PUTRESCINE AND OF CANNED SPOILED TUNA ON GROWTH IN YOUNG JAPANESE QUAIL

EDWARD R. BLONZ and HAROLD S. OLCOTT

ABSTRACT

Newly hatched quail were fed for 9 days with chick starter meal, or diets based upon 60% freeze-dried spoiled or nonspoiled tuna. The diets were fed alone, or with added histamine or putrescine. Histamine in the chick starter meal depressed weight gain while the putrescine had no apparent effect at the level used. The diets based upon 60% freeze-dried spoiled tuna significantly depressed weight gain when compared to the nonspoiled tuna diets. No significant effect was observed when histamine was added to nonspoiled tuna diets at levels equaling or exceeding that in the spoiled fish. Quail might be useful bioassay animals for the isolation and identification of toxic factors in canned spoiled tuna fish.

INTRODUCTION

THE SCOMBROIDAE FAMILY of fish includes skipjack tuna (*Katsuwonus pelamis*), bonito, mackerel, yellowfin, bluefin, kingfish and albacore. Such fish characteristically contain large amounts of free histidine in the muscle tissue which can undergo bacterial decarboxylation to histamine during spoilage. Consumption of such spoiled fish may cause "scombroid poisoning," the symptoms of which may include flushing about the head and neck with accompanying sensations of heat, epigastric burning, thirst and burning about the mouth, headache, and gastrointestinal distress. Reviews of the subject are available (Arnold and Brown, 1978; Sapin-Jaloustre, 1957; Halsted, 1967).

The symptoms of "scombroid poisoning" resemble those of a histamine intoxication, yet when taken orally, histamine has been reported to be relatively harmless (Weiss et al., 1932; Douglas, 1970), with few exceptions (Sjaastad, 1966). The part histamine plays in the toxic reaction, as well as identification of any possible cofactors, synergists, etc., has been extensively discussed (Geiger, 1955; Aiso et al., 1958; Olcott and Lukton, 1961; Kawabata et al., 1955a, b, c). A bioassay technique is needed to detect toxic substances. With such a test, identification of the critical factors could be achieved by fractioning the spoiled tuna and feeding various combinations of the fractions until a toxic reaction reappears.

Possible synergists or potentiators under consideration are the diamines putrescine, cadaverine, and spermine (Parrot and Nicot, 1965; Ienistea, 1973). In the present work, newly hatched Japanese quail (*Coturnix coturnix japonica*) were fed diets containing histamine, putrescine, and/or tuna, to investigate their effect on growth of these laboratory animals.

EXPERIMENTAL

IN EACH OF TWO experiments, Japanese quail (*Coturnix coturnix japonica*) were taken 6 hr after hatching and were randomly assigned to the experimental groups. At the same hour for eight successive days, the birds were weighed, after which fresh feed and water were supplied. Those birds which had died were removed prior to the daily weighings.

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0022-1147/78/0005-1390$02.25/0

Table 1—Composition of diets fed to quail

Ingredient	g/kg Exp 1	Exp 2
Soybean protein[a]	284.0	–
Freeze-dried tuna[b]	–	600.0
DL-Methionine	4.5	–
Soybean oil	37.5	–
Cornstarch	521.0	244.0
Cellulose[c]	50.0	60.0
Microcell[d]	30.0	30.0
$CaCO_3$	10.0	19.6
$CaHPO_4 \cdot 2H_2O$	30.0	13.4
NaCl	9.9	9.0
Vitamin premix[e]	10.0	10.0
Mineral premix[e]	13.1	13.2
Protein (N X 6.25)	271.0	306.0
Met. energy[f]	3250 Kcal	4675 Kcal
Pro:Kcal	0.083g/Kcal	0.065g/kcal

[a] RP-100 Purina Assay Protein (Ralston Purina Company, St. Louis, MO)
[b] Average of 57% weight reduction upon drying
[c] Solka-Floc (Brown Company, Berlin, NH)
[d] Microcell S (calcium silicate) was added to the diet to prevent caking. (Johns-Manville, New York, NY)
[e] Vitamin & Mineral premix (Vohra, 1972)
[f] Agricultural Handbook 8, 1975

Table 2—Group treatments[a] in 1st Experiment

Experimental group	Concentration (g/kg diet) Hm[a]	Pt[b]
Control	–	–
1	–	2.2
2	1.2	–
3	3.6	–
4	3.6	2.2
5	9.6	–

[a] All treatments dissolved in 100 ml phosphate buffer; pH = 7.0
[b] Putrescine and histamine were used as the dihydrogen chlorides.

Total weight per group per day was recorded along with the number of quail present.

In the first experiment, histamine and/or putrescine were added to a standard chick-starter diet (Table 1). There was one cage for each of six experimental groups, with 20 birds per cage. Histamine and/or putrescine dietary treatments are shown in Table 2.

In the second experiment there were five experimental groups, with 10 quail per cage, and two cages per group. The feed was based on commercially canned tuna which was freeze-dried and used as 60% of the diet (Table 1). Two separate lots of tuna were used. The first was *spoiled* tuna which had been recalled by the packer and saved for research purposes. It had an elevated histamine content (200 mg/100g) as measured by thin-layer chromatography (Lin et al., 1977). The second lot of tuna was purchased at a local supermarket and was of good quality with no detectable histamine (by the same method). The quail received various diets containing tuna, either alone, or with added histamine (Table 3).

In both experiments, comparison of growth (weight gain) between groups was handled by subtracting one equation for the linear growth curve ($y = mx + b$) from the other, and plotting this new equation. If in the duration of the experiment the "y" value of the new equation

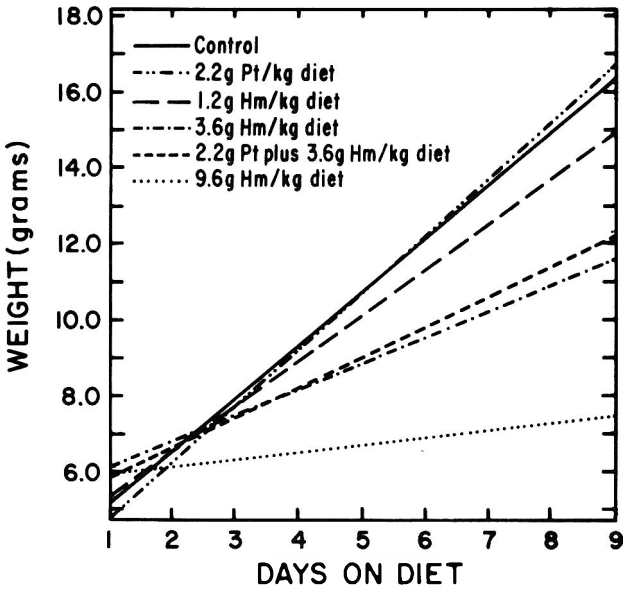

Fig. 1—Average weight per bird on chick starter diets.

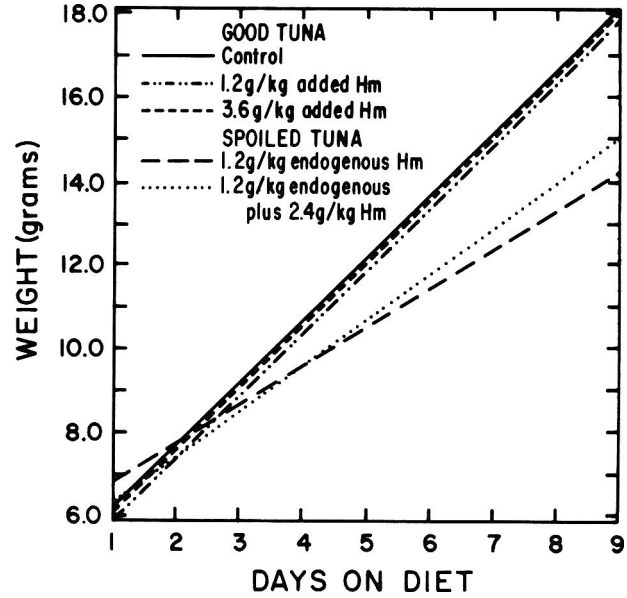

Fig. 2—Average weight per bird on 60% freeze dried tuna based diets.

Table 3—Description of freeze-dried tuna and treatments used in second experiment

Experimental group	Diet	Added[a] histamine g/kg	Total histamine g/kg
Control	60% Unspoiled tuna	–	–
1	60% Unspoiled tuna	1.2	1.2
2	60% Unspoiled tuna	3.6	3.6
3	60% spoiled tuna	–	1.2
4	60% spoiled tuna	2.4	3.6

[a] Histamine was added as the dihydrogen chloride

equaled or exceeded twice the square root of the sum of the squares of the standard errors of estimate for the two original equations ($\hat{s}_\alpha$, $\hat{s}_\beta$) then the difference in growth between the two groups was defined as significant ($p < 0.05$) (Alder and Roessler, 1960).

$$y \geq 2\left(\sqrt{(\hat{s}_\alpha)^2 + (\hat{s}_\beta)^2}\right)$$

RESULTS & DISCUSSION

ANALYSIS OF DATA in the first experiment revealed three levels of treatment effect on growth as illustrated in Figure 1. The first level included Group 1 (2.2g putrescine/kg diet) and Group 2 (1.2g histamine/kg diet), both of which failed to show any difference from control. The second level did show a significant depression [all significance reported at the 0.05 level] and included Group 3 (3.6g histamine/kg diet) and Group 4 (3.6g histamine, and 2.2g putrescine/kg diet). The third level of effect consisted of Group 5 (9.6g histamine/kg diet) whose daily average weight was significantly lower than that of all other groups including control. The addition of putrescine had no apparent effect at the level used, when added alone, or with histamine.

In the second experiment, those groups whose diets were based on 60% *spoiled* tuna, i.e., Group 3 (1.2g endogenous histamine/kg diet) and Group 4 (1.2g endogenous PLUS 2.4g histamine/kg diet), both showed significant depression when compared with groups having diets based on 60% *unspoiled* tuna (Fig. 2). It is to be noted that a significant depression in weight gain was found in the quaii fed the untreated *spoiled* tuna (Group 3) when compared with those fed the *unspoiled* tuna plus histamine in Group 2, even though Group 2 received more histamine. No difference was found between the two groups receiving the *spoiled* tuna, and likewise no significant difference was found between the control and two experimental on the *unspoiled* tuna diets.

In a preliminary experiment it was found that there was no significant difference in growth during the first 6 days, between quail receiving a diet based upon chick starter meal (26% protein) and one based upon *spoiled* tuna (31% protein). The present work is of longer duration, but there were similar findings if only the first 6 day's data were used. In the total 9-day treatment period, *spoiled* tuna-based diets did show a significant depression in weight gain when compared to the chick starter meal. Comparisons between the present two experiments were approached cautiously, there being different values for dietary protein (27.1% vs 30.6%) and metabolizable energy (3250 vs 4675 Kcal/kg diet). However, it should be noted that at the conclusion of the respective experiments, the *unspoiled* tuna-based diet containing 3.6g histamine/kg diet (Experiment 2, Group 2) showed significantly *greater* growth than the control group receiving the untreated chick starter diet (Experiment 1, Control).

In these experiments, feed consumption was not recorded. It is unknown whether the reported rates of gain were a direct reflection of feed consumption, or a reaction to the presence or absence of histamine and/or unidentified toxins, OR an interaction of both.

Chick starter meal plus histamine caused weight depression. Putrescine had no apparent effect at the level used. In the freeze-dried tuna-based diets, levels of histamine were not useful in predicting depression of weight gain—the rate of growth was depressed by unknown substances.

Finding growth depressed in *spoiled* vs *unspoiled* tuna based diets—which was unmodified by changing levels of histamine—would indicate that young Japanese quail might be utilized in the identification of toxic substances. Variance in protein and energy content of experimental diets fed to quail have to be considered if growth is the parameter to be measured.

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—Continued on page 1399

EFFECT OF LOW VOLTAGE STIMULATION OF BEEF CARCASSES ON MUSCLE TENDERNESS AND pH

P. E. BOUTON, A. L. FORD, P. V. HARRIS and F. D. SHAW

ABSTRACT

The tenderness of muscles from sides of beef which had received one of three different electrical stimulation treatments was compared with that of muscles from unstimulated control sides. Comparisons were also made between muscles from stimulated and unstimulated sides suspended by the Achilles tendon or by the sacro-sciatic ligament. The maximum stimulation voltage applied was 110v d.c. Stimulation caused a marked increase in the tenderness of muscles which were removed from the carcasses 22–24 hr after slaughter. Muscles from stimulated sides had significantly lower pH values at 1, 4 and 24 hr after slaughter than muscles from control sides.

INTRODUCTION

A MAJOR CAUSE of meat toughness is postmortem shortening of muscle (Newbold and Harris 1972). This shortening can be minimized or prevented by suspending the carcasses, pre-rigor, from the pelvis. This technique to prevent contraction has not so far been widely adopted, probably because it requires changes in subsequent 'boning-out' procedures.

Other methods for preventing cold-shortening include conditioning the carcasses pre-rigor at 10–20°C (Locker and Hagyard, 1963; Smith et al., 1971; Bouton et al., 1973) and electrical stimulation of the carcass pre-rigor (Chrystall and Hagyard, 1975, 1976; Grusby et al., 1976; Gilbert and Davey, 1976; Davey et al., 1976; Smith et al., 1977; Savell et al., 1977). Electrical stimulation has become of increasing interest to meat processors because (a) it requires little change in normal abattoir practice and (b) the removal of meat from the carcass pre-rigor, (hot-boning) could become a practical possibility.

Voltages as high as 700v (Bendall et al., 1976) or even 1600v (Davey et al., 1976) have been used for the stimulation of beef carcasses. High voltages present major safety problems, particularly in an abattoir environment where strict safety precautions are essential. Stimulation of beef carcasses with low voltages (<120v) increases the rate of pH fall (Shaw and Walker, 1977) and improves tenderness (Savell et al., 1977; Walker et al., 1977).

Three experiments are described in this paper. The first compares the effect of two different methods of application of low voltages to carcasses on muscle tenderness and pH. The second and third experiments compare the properties of muscles from stimulated and unstimulated sides of beef suspended by the Achilles tendon or by the sacro-sciatic ligament (pelvic hung).

EXPERIMENTAL

Animals and stimulation treatments

Experiment 1. Eight Hereford steers, 2–4 years old, of carcass weight 150–180 kg were used. The animals were divided equally into two groups, one group was stimulated via a rectal probe (Fig. 1) immediately after stunning and bleeding, the other group received no stimulation at this time. After dressing and splitting of the carcasses, stimulation was applied via multi-point electrodes (Fig. 2) to one side of each carcass. The 16 sides were therefore divided equally into four treatments.

Group I
Treatment 1– No stimulation–control sides
Treatment 2– Stimulation of side after dressing of carcass completed (side stimulation)

Group II
Treatment 3– Stimulation of carcass via rectal probe but no further stimulation of side (rectal stimulation)
Treatment 4– Stimulation of carcass via rectal probe and further stimulation of one side after dressing of carcass completed (rectal + side).

The rectal probe was of the type used for electro-ejaculation of bulls. Rectal stimulation was applied after bleeding was completed (3–5 min after stunning), when the animal was hoisted and shackled by one hind leg.

Stimulation of sides, hung from the Achilles tendon, was carried out after the dressed carcass was split in two (32–38 min after stunning). One of the multi-point electrodes was inserted at the distal end of the junction of the biceps femoris muscle with the semitendinosus muscle. The other was inserted into the brachiocephalicus muscle.

Pulsed direct current (40 pulses per second, pulse width 2 msec) was used for rectal and side stimulation. Preliminary experiments with rectal stimulation had shown that when the current was initially applied the free hind leg rapidly extended posteriorly to assume a near vertical position. This rapid extension sometimes caused a jerking of the entire carcass giving rise to the possibility that the carcass could be dislodged from the supporting rail. It was therefore decided to apply a low voltage initially and to increase the voltage in a series of steps to 110v. Stimulation was applied for 4 min with the voltage being increased as follows: 0–30 sec 10v, 30–60 sec 50v, 60–120 sec 75v and 120–240 sec 110v. The same voltage program was used for rectal and side stimulation. The muscles usually contracted with each increase in voltage and began to relax about 30 sec later. With rectal stimulation the free hind leg tended to extend with each change in voltage. With side stimulation the foreleg extended and with the higher voltages a lateral twisting of the forequarter occurred. In one case the rectal electrode appeared to initially make intermittent contact–this resulted in alternate extension and relaxation of the free hind leg and vibration of the carcass for 15 seconds.

Experiment 2. Six Hereford steers 2–3 years old of carcass weight 230–250 kg were used. Paired sides were allocated to a treatment group in accordance with the balanced incomplete block design (plan 11.1) of Cochran and Cox (1957). The treatments were:

(i) Control – Achilles hung
(ii) Stimulated – Achilles hung
(iii) Control – pelvic hung
(iv) Stimulated – pelvic hung

Stimulation commenced immediately after the dressed carcass was split into sides (30–40 min after stunning) and was applied for 1½ min with the voltage being varied as follows: 0–10 sec 10v, 10–30 sec 50v, 30–60 sec 75v and 60–90 sec 110v. The pulse characteristics were the same as in Experiment 1. Sides were suspended from the Achilles tendon during stimulation and completion of dressing. They were then retained in a holding area (air temperature 10°C, air velocity 0.1 $msec^{-1}$) until 2 hr after stunning when they were then transferred to a chiller (air temperature 1°C, air velocity 0.5 $msec^{-1}$). While in the

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0022-1147/78/0005-1392$02.25/0

holding area and in the chillers, three stimulated and three control sides were suspended by a hook through the sacro-sciatic ligament (pelvic hung) while the remaining six sides were conventionally hung from the Achilles tendon. All sides were assigned to the hanging treatments according to the experimental balanced incomplete block design.

Experiment 3. The experimental design and method were identical to that of Experiment 2. Six Brahman cross heifers, aged about 6 months and of carcass weight 90–130 kg, were used.

Removal and treatment of muscles

Experiment 1. Stimulated and control sides were quartered at the 10th rib and transported to the holding area approximately 45 min after the animals were stunned. One hour after stunning the semitendinosus (ST) and deep pectoral (DP) muscles were removed from the carcass. Two hours after the animals were stunned the triceps brachii (TB) and semimembranosus (SM) muscles were removed. Each muscle was placed in a plastic bag and immersed in ice water until 24 hr after slaughter. The hindquarters were transferred from the holding area to the chiller two hours after stunning. They were stored in the chiller and hung by the Achilles tendon for 22 hr. Four muscles [biceps femoris (BF), gluteus medius (GM), vastus lateralis (VL) and longissimus dorsi (LD)] were then removed.

Experiments 2 and 3. The sides were stored in the chiller for 22 hr. Seven muscles (SM, BF, VL, LD, GM, ST and TB) were then removed.

Temperatures and carcass chilling rates

The temperatures of two muscles on several different carcasses were measured during chilling. Copper constantan thermocouples were inserted deep (10 cm) into the VL and about 0.5 cm into the LD.

Aging and pressure-heat treatments

In Experiment 3 each of the SM and BF muscles (i.e. the part remaining after removal of a sample for objective measurements) was further divided into two sub-samples which weighed about 300–400g. One of each pair of muscle sub-samples was vacuum sealed in a Cryovac, gas impermeable, bag and aged for 4 wk at 5–6°C while the other sample was used immediately (i.e. 0 wk aging). Each sample (at both 0 and 4 wk aging) was further subdivided into two samples each weighing approximately 80–100g–one was the control and the other was subjected to a pressure-heat treatment (pre-heat at 45°C and atmospheric pressure for 30 min followed by 30 min at 150 MNm^{-2} and 60°C). This pressure-heat treatment is believed to primarily affect the myofibrillar structural component (Bouton et al., 1977; Ratcliff et al., 1977). All samples were then cooked at 80°C for 90 min and cooled as described below. After overnight storage at 0–1°C the samples were prepared for WB shear force measurements.

Measurement of pH

In Experiment 1 the pH of three muscles (LD, BF and VL) was measured at 1, 4 and 24 hr after stunning. In Experiments 2 and 3 the pH of five muscles (SM, BF, VL, LD and TB) was measured at 1, 4 and 24 hr after stunning. Measurements were made with a portable pH meter (Townson meat pH meter) which had a Phillips probe-type combined electrode (C64/1). The probe electrode was inserted into a shallow cut made into the muscle. pH values for individual muscles were taken as the mean of readings from three different sites.

Cooking methods

Samples taken from each muscle for objective assessment weighed about 200g and were cooked for 90 min in polyethylene bags totally immersed in water maintained at 80°C (± 0.5°C). The cooked samples were then cooled in cold running water for 30 min. Excess surface moisture was removed with an absorbent paper towel. The samples were then rewrapped in polyethylene and stored at 0–1°C overnight.

Subjective assessments

In Experiments 2 and 3 samples for taste panel assessment were taken from the LD and GM muscles. The samples, which weighed about 400 and 350g respectively (Experiment 2) and 350 and 250g respectively (Experiment 3), were cooked under the same conditions as used for the samples which were required for objective assessments. After cooking the samples were held overnight at 1°C and served to the taste panel the following day. The meat was cut into ½-in. cubes and served at room temperature under green lights. Twelve tasters assessed four samples of the same muscle type (i.e. either LD or GM), one from each treatment at each of 6 sessions. The order of tasting was randomized through the panel to cancel out any sequential effect. Tasters were asked to rate the samples on 25-point unstructured scales for tenderness and juiciness, with end points defined as extremely tender or juicy and extremely tough or dry.

Table 1–Mean pH values obtained at 1, 4 and 24 hr post slaughter for the BF, VL and LD muscles from beef sides subjected to different electrical stimulation treatments pre-rigor (Experiment 1)

Time after slaughter	Muscle	Group I		Group II	
		Control	Side stimulation	Rectal stimulation	Rectal and side stimulation
1 hr	BF	7.18	6.55	6.28	6.15
	VL	7.18	6.68	6.22	6.15
	LD	7.25	6.40	6.20	6.13
	MEAN	7.20	6.54	6.23	6.14
4 hr	BF	6.80	6.05	5.85	5.83
	VL	6.78	6.20	5.88	5.88
	LD	7.00	6.20	5.93	5.90
	MEAN	6.86	6.15	5.89	5.87
24 hr	BF	5.80	5.62	5.58	5.60
	VL	5.78	5.65	5.60	5.63
	LD	5.88	5.65	5.55	5.58
	MEAN	5.82	5.64	5.58	5.60
LSD[a] between 2 treatment means in same group					
	1 hr	0.22			
	4 hr	0.07			
	24 hr	0.10			
LSD[a] between 2 treatment means in different groups					
	1 hr	0.24			
	4 hr	0.29			
	24 hr	0.15			

[a] Least significant difference at $P < 0.05$ level

Measurement of sarcomere length, Instron compression and shear measurements

Sarcomere lengths of raw and cooked meat samples were measured using a light diffraction method (Bouton et al., 1973). The Instron compression (IC) and Warner-Bratzler (WB) shear force measurements were carried out as previously described (Bouton and Harris, 1972).

Statistical treatment of results

Analyses of variance for a split plot design (Experiment 1) and a balanced incomplete block design (Experiments 2 and 3) were used to test for significance of treatment effects and to calculate appropriate standard errors.

RESULTS & DISCUSSION

THE RATE OF CHILLING used throughout the three experiments was sufficient to reduce the temperature of the LD muscle to about 8°C within 5 hr of slaughter and that of the VL to 8°C within 15 hr of slaughter. Davey et al. (1976) recorded loin temperatures of 8°C within 8 hr of slaughter for beef sides which were rapidly chilled and, in some cases, the deep leg temperature had not reached 8°C 24 hr after slaughter.

Rate of fall of pH

The pH values obtained at 1, 4 and 24 hr after slaughter for the selected muscles from Experiments 1, 2 and 3 are shown in Tables 1 and 2 respectively. In all three experiments stimulation had reduced pH values substantially at 1 and 4 hr after slaughter. In Experiment 1, side stimulation alone reduced pH by nearly 0.7 within 1 hr and by 1.0 within 4 hr. Rectal stimulation (both with and without additional side stimulation) appeared to be more effective in reducing pH than side stimulation alone. As pH was not measured before stimulation it is not possible to say whether rectal stimulation produced a real increase in rate of fall of pH after stimulation or whether there had been a greater fall in pH during rectal stimulation than during side stimulation. In any event electrical stimulation at or near the bleeding area could be inconvenient at abattoirs, particularly in those with high rates of slaughter

Table 2—Mean pH values obtained for several muscles (SM, BF, VL, LD and TB) from Achilles tendon and pelvic (sacro-sciatic ligament) animals both electrically stimulated and unstimulated — measured at 1, 4 or 24 hr after slaughter (Experiments 2 and 3)

Time after slaughter	Muscle	Treatment				
		Control	Stimulated control	Pelvic	Stimulated pelvic	LSD[a]
1 hr	SM	6.81	6.21	6.60	6.18	
	BF	6.81	6.12	6.75	6.17	
	VL	6.91	6.27	6.85	6.25	0.04
	LD	6.73	6.24	6.63	6.22	
	TB	7.05	6.41	6.98	6.40	
	MEAN	6.86	6.25	6.76	6.24	
4 hr	SM	6.51	5.96	6.50	5.91	
	BF	6.46	5.97	6.35	5.98	
	VL	6.51	6.04	6.65	6.04	0.18
	LD	6.50	5.99	6.45	5.94	
	TB	6.93	6.21	6.72	6.14	
	MEAN	6.58	6.03	6.54	6.00	
24 hr	SM	5.66	5.58	5.71	5.55	
	BF	5.70	5.61	5.79	5.59	
	VL	5.78	5.64	5.82	5.59	0.08
	LD	5.75	5.61	5.69	5.60	
	TB	5.88	5.69	5.89	5.67	
	MEAN	5.75	5.63	5.78	5.60	

[a] Least significant difference at $P < 0.05$ level

Table 3—Mean Warner-Bratzler shear values (kg) obtained for muscles removed at various times post slaughter from 4 beef sides subjected to different forms of electrical stimulation pre-rigor. Muscles cooked at 80° for 90 min

Time removed post slaughter	Muscle	Group I		Group II	
		Control	Side stimulation	Rectal stimulation	Rectal + side
1 hr	ST	18.5	17.4	11.4	12.9
	DP	13.6	19.1	17.4	16.1
	MEAN	16.0	18.2	14.4	14.5
2 hr	SM	22.7	9.8	6.5	5.6
	TB	17.9	14.4	18.6	12.4
	MEAN	20.3	12.1	12.5	9.0
24 hr	BF	12.3	6.5	7.0	6.3
	VL	15.0	9.5	7.5	8.0
	LD	15.8	12.7	10.1	10.3
	GM	10.5	5.8	5.1	4.5
	MEAN	13.4	8.6	7.4	7.3

LSD[a] between 2 treatment means in same group

1 hr	4.4
2 hr	2.5
24 hr	1.5

LSD[a] between 2 treatment means in different groups

1 hr	3.8
2 hr	3.1
24 hr	1.6

[a] Least significant difference at $P < 0.05$ level

(Smith et al., 1977). Subsequent work in Experiments 2 and 3 therefore involved side stimulation only.

The results obtained for Experiments 2 and 3 (Table 2) confirmed those obtained in Experiment 1 as stimulation had reduced pH values in the five selected muscles to about 6.2 in 1 hr and 6.0 in 4 hr. Analyses of variance of these pH results showed highly significant differences due to stimulation between experiments (i.e. muscles from the young animals in Experiment 3 had significantly higher pH values than muscles from the older animals in Experiment 2) and between muscles (i.e. the TB muscle – see Table 2 – tended to have higher pH values than the other muscles at 1 and 4 hr). There was, however, no significant interaction between stimulation, age and muscle. The results obtained for the LD muscle were different from those obtained by other workers. Bendall et al. (1976) found that, when a beef carcass was stimulated with 100v, this muscle took 8 hr to reach a pH of 6.0. In the case of the TB muscle the results of the present work agree with those reported for lambs by Bendall (1976) who found a slower rate of pH fall for the TB than for the LD or BF muscles. Bendall (1976) suggested that electrode positioning could affect the amount of stimulation which individual muscles receive via the nervous system. For example, with the electrodes positioned at the hind legs and the neck, the full number of stimuli may not reach the posterior part of the brachial motor-nerve plexus which innervates the TB muscles.

Mechanical measurements

Experiment 1. The shear force measurements obtained for the cooked muscles from Experiment 1 are shown in Table 3. For the muscles excised at 1 hr, side and rectal stimulation had little effect on the DP and only rectal stimulation had any effect on the ST muscle. For the muscles excised at 2 hr the TB muscle was not greatly affected by stimulation although stimulation produced a large decrease in the shear force values obtained for the SM muscle. The fact that the forequarter muscles (DP and TB) showed less effect due to stimulation than did the hindquarter muscles (SM and ST) could be due either to fundamental differences in the response of the muscle per se to stimulation or to differences in current distribution through the carcass or side.

At 24 hr after slaughter the results (Table 3) show that stimulation significantly ($P < 0.001$) reduced shear force values for all four muscles examined. Low voltage stimulation of carcasses, therefore, appears to reduce the increased toughening normally associated with rapid chilling. The LD muscle showed least effect, but this muscle generally responds well to aging (Bouton et al., 1973) which would reduce shear force values to a more acceptable 5–7 kg. The results in Table 3 therefore indicate that rectal stimulation (and, to a lesser extent, side stimulation) will successfully reduce the deleterious effects of cold shortening in some of the important back and hindquarter muscles (the SM, BF, VL, LD and GM muscles).

Experiments 2 and 3. The results for shear force, sarcomere length, Instron compression and 24 hr pH for the SM, BF, VL, LD, GM and TB muscles from both these experiments are shown in Table 4. Stimulation significantly reduced shear force values and the samples obtained from sides which were stimulated then hung by Achilles tendon gave shear values comparable with those not stimulated hung by the pelvis. Sarcomere length values (for the raw samples) showed that, while samples from stimulated sides tended to have longer sarcomeres than those from unstimulated controls, these differences were not always significant. The Instron compression (IC) results showed very little effect due to either stimulation or hanging method but they did show that muscles from the older animals (Experiment 2) gave significantly larger values than those from the young animals (Experiment 3). The pH values, measured at 24 hr, indicated that the stimulated sides had significantly lower values than the controls. The control sides from the young animals yielded muscle with significantly higher values than did those from the older animals but no significant difference existed for the stimulated sides. Buchter (1974) has reported that, particularly for young animals, some toughening due to myofibrillar contraction could occur when muscles were removed from the restraint of the skeletal framework even as late as 24 hr post slaughter

Table 4—Shear values (kg), sarcomere lengths (μm) Instron compression values (kg) and 24 hr pH values obtained for muscles from stimulated and control sides hung by the Achilles tendon or by the sacro-sciatic ligament (pelvic-hung)[a]

	Exp no.	Achilles hung control	Achilles hung stimulated	Pelvic hung control	Pelvic hung stimulated	LSD[b] A	LSD[b] B
Shear values	2	9.2	6.6	6.4	5.0	1.2	1.1
	3	12.9	8.7	8.3	6.7		
Sarcomere length	2	1.86	1.94	2.54	2.60	0.21	0.14
	3	1.73	1.89	2.35	2.62		
Instron compression	2	2.13	2.11	1.95	1.86	0.23	0.17
	3	1.56	1.57	1.51	1.48		
pH values at 24 hr	2	5.69	5.59	5.70	5.57	0.10	0.12
	3	5.81	5.66	5.86	5.63		

[a] Values are means for six muscles (SM, BF, VL, LD, GM, TB) six sides per treatment.
[b] LSD—A is least significant difference at ($P < 0.05$) for comparing any two means except for the same treatment levels where B should be used.

when the muscles would be close to their ultimate pH. The sarcomere lengths obtained for the muscles from the control sides of the younger animals were shorter than those from the control sides of the older animals (Table 4). In addition shear force values were higher for the muscles from the younger animals than for those from the older ones.

Muscles restrained from shortening (Bouton et al., 1973) on the pelvic hung carcasses (i.e. SM, BF, VL, LD, GM) all gave decreased shear force values when the carcasses were stimulated. Whether stimulation per se was responsible for this decrease or whether the muscles from the unstimulated sides shortened when taken off the carcass at 24 hr post slaughter was not established.

Taste panel results

The taste panel results for the LD and GM muscles from Experiments 2 and 3 are shown in Table 5. Pelvic hanging had a much larger effect on tenderness than did stimulation but stimulation improved the tenderness of the muscles from the sides hung by the Achilles tendon. The taste panel found no animal age effect which supports the objective results where, although the younger animals had higher shear force values, this was compensated by lower IC values. The higher shear force values would indicate increased toughness in the myofibrillar component of the meat structure, whereas the lower IC values suggest a decrease in connective tissue toughness (Bouton and Harris, 1972).

Pressure-heat treatment and aging effects

The BF and SM muscles from Experiment 3 were subjected to pressure-heat and aging treatments—both treatments believed to primarily affect the myofibrillar structures (Bouton et al., 1975, 1977; Ratcliff et al., 1977). Pressure-heat treatment eliminated the differences in shear force values due either to different hanging method, electrical stimulation or aging (Table 6). Aging reduced shear force values for all four treatments but the main effect was to eliminate differences between the shear force values obtained for muscles for sides which were pelvic hung, pelvic hung plus stimulated and Achilles tendon hung plus stimulated. The shear force values obtained for the Achilles tendon hung controls were significantly greater than the others both before and after aging.

The sarcomere length values obtained for both the raw and cooked meat samples indicated that stimulation decreased myofibrillar shortening although the effect was not always significant.

Table 5—Taste panel tenderness and juiciness scores for muscles from stimulated and control sides hung by the Achilles tendon or by the sacro-sciatic ligament (pelvic-hung) Experiments 2 and 3)[a]

	Achilles hung control	Achilles hung stimulated	Pelvic hung control	Pelvic hung stimulated	LSD[b]
			L. dorsi		
Tenderness	18.1	15.7	8.9	10.3	3.1
Juiciness	11.8	10.7	9.5	8.3	1.8
			G. medius		
Tenderness	13.3	10.4	7.6	6.9	2.5
Juiciness	9.6	9.3	8.9	7.6	1.6

[a] Values are mean scores for eight tasters, six sides per treatment. Subjective scale 1 = extremely tender or juicy; 25 = extremely tough or dry.
[b] Least significant difference at $P < 0.05$ level.

—Text continued on page 1396

Table 6—Shear force and sarcomere length values obtained for samples of fresh and aged (5–6° C for 4 wk) muscles (BF and SM results combined) from stimulated and control sides hung by the Achilles tendon or by the sacro-sciatic ligament (pelvic hung) and cooked at 80° C for 90 min after pressure-heat treatment (30 min at 150 MNm^{-2} and 60° C after pre-heat at 45° C for 30 min)—P/H—or control (i.e. no pressure-heat treatment)—N

Parameter measured	Aging period (wk)	Treatment	Achilles hung control	Achilles hung stimulated	Pelvic hung control	Pelvic hung stimulated	LSD[a]
Peak shear force values (kg)	0	N	14.0	9.1	10.0	5.9	2.0
		P/H	2.6	2.5	2.7	3.0	
	4	N	9.0	4.4	4.7	4.3	
		P/H	3.6	2.9	3.1	3.8	
Sarcomere length (raw) in μm	0	—	1.57	1.88	2.58	2.98	0.37
Sarcomere length values of cooked meat (μm)	0	N	1.27	1.64	1.88	2.24	0.34
		P/H	1.19	1.69	1.85	2.41	
	4	N	1.23	1.55	1.65	2.31	
		P/H	1.25	1.59	1.57	2.40	

[a] Least significant difference at $P < 0.05$ level

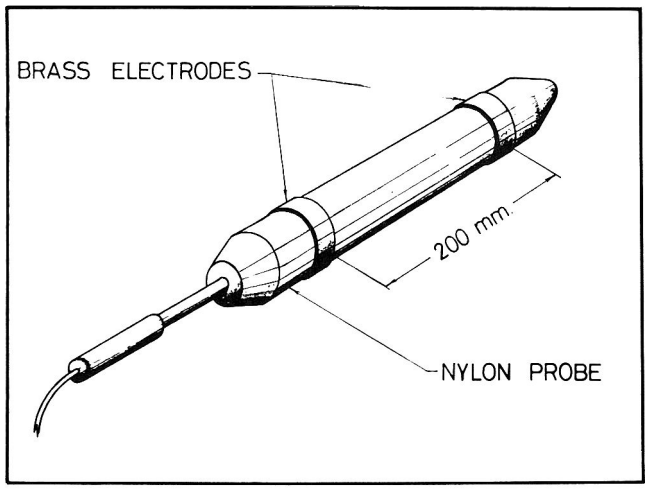

Fig. 1—Rectal probe.

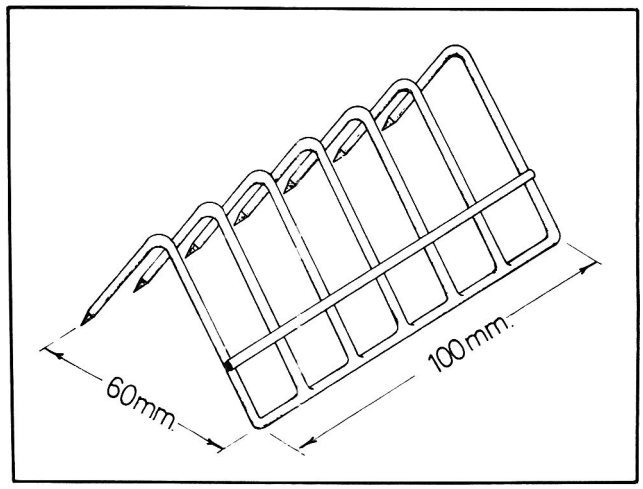

Fig. 2—Multi-point electrode probe.

CONCLUSIONS

EARLIER EXPERIMENTS (Shaw and Walker, 1977) showed that low voltage electrical stimulation increased the rate of pH fall in beef carcasses. The experiments reported in this paper have shown that this low voltage stimulation reduces the shear force values obtained for cooked samples of muscles removed from the back and hindquarter 24 hr after slaughter. Stimulation was less effective in preventing cold shortening and toughening of muscles, particularly forequarter muscles (DP and TB), removed within 1–2 hr of slaughter and cooled rapidly by direct immersion in ice water—a treatment representing far more drastic cooling than would be found in normal commercial practice. The rate of pH fall indicated by the results in Tables 1 and 2 suggest that boning out might be feasible 2–4 hr after slaughter for at least the commercially important muscles of the back and leg.

While the method of stimulation used has obviously reduced the toughening effects normally associated with rapid chilling it is not possible to state without equivocation that the reduction was due to changes in myofibrillar contraction state. The sarcomere length data does not always show that stimulation results in a significantly greater sarcomere length. However, a number of muscles (e.g. SM, BF, GM, LD, VL) from rapidly chilled conventionally processed carcasses, hung from the Achilles tendon, contract to give sarcomere lengths of $<1.8–2.0$ μm, where quite small differences can produce disproportionately large differences in shear force values (Bouton et al., 1973).

If electrical stimulation has not beneficially altered myofibrillar contraction state then it is possible that it either disrupts the myofibrillar structure in such a way as to weaken it or that it has affected the connective tissue structure. The latter seems unlikely because the IC results (Table 4) should have shown a much larger effect due to stimulation. This method of measurement is known to be relatively sensitive to connective tissue (Bouton and Harris, 1972). When the myofibrillar contribution to the mechanical shear strength of the cooked meat is reduced by means of the pressure-heat treatment any apparent effect attributable to stimulation is eliminated. If stimulation had affected the connective tissue structure then differences should have become apparent once the myofibrillar contribution was removed. Muscles normally restrained from shortening by the skeletal framework also show some reduction in shear force values when stimulated albeit not always significantly. If these muscles are restrained and thus have identical myofibrillar contraction states then it could be argued that stimulation has an effect over and above that on myofibrillar contraction state alone.

Evidence presented in this paper shows that relatively low voltages can reduce the deleterious effects normally associated with rapid chilling of either the whole carcass or hot boned cuts. Aging of meat from stimulated carcasses results in additional tenderizing which makes it more tender than aged meat from unstimulated carcasses.

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Ms received 12/21/77; accepted 2/25/77.

This work was supported in part by the Australian Meat Research Committee. The assistance of Mr. P.N. Jones, CSIRO, Division of Mathematics and Statistics in analyzing the data is gratefully acknowledged, as is the willing co-operation of taste panel members. Mrs. Robin Efimov, Mrs. Lynn Ussher and Mr. S. Penna provided skilled technical assistance.

RELATIONSHIP OF THYROID ACTIVITY TO DISTRIBUTION OF FAT IN RABBIT CARCASSES

T. M. McBRIDE and D. A. CRAMER

ABSTRACT

The effects of exogenous administration of T_4 and a combination of T_4 and Synovex (12.5 mg progesterone and 1.25 mg estradiol) on the distribution of fat in rabbit carcasses have been studied. The results demonstrate that increasing thyroid activity will cause production of leaner rabbits that contain adequate amounts of intramuscular fat. Further, implantation of estrogenic hormones tend to stimulate growth and counteract the depressed weight gains in treated rabbits. A possible T_4-Synovex interaction is indicated. Rabbits receiving both hormones had the lowest amount of waste fat and the highest amount of intramuscular fat of all treatment groups.

INTRODUCTION

ONE OF THE MAJOR problems confronting the cattle industry is the production of beef with desirable amounts of properly distributed lipids. With the high cost of feed grains and increased emphasis on energy conservation, feeding animals to a high degree of finish has become wasteful. In addition, consumer aversion to animal fats is encouraging the production of leaner beef. However, certain minimum amounts of intramuscular fat or marbling are required for beef carcasses to have a quality grade of USDA choice which is important in marketing of beef.

Exogenous administration of hormones that are related to thyroid activity offer the potential of producing beef that is adequately marbled without excessive amounts of waste fat. McBride et al. (1976) reported that thyrotropin releasing hormone appeared to cause slight reductions of waste fat deposits in rabbits without concurrent loss of muscle fats. In addition, the treated rabbits continued to gain weight. Thyroid stimulating hormone (TSH) has been shown by several authors (Payne, 1949; Hart and McKenzie, 1971; White and Engel, 1959; Rudman, 1961) to be effective in inducing lipid mobilization. In a recent study, Fredericks and Cramer (1975) reported that injections of TSH in rabbits reduced fatty depots without loss of intramuscular fat.

The success of the TSH experiment prompted further studies with the thyroid hormones, triiodothyronine (T_3) and tetraiodothyronine (T_4), to determine if the lipolytic effects of TSH were a direct action or if they resulted through mediation of the thyroid. Several investigators have demonstrated that thyroid hormones can be effective in inducing lipolysis in both in vitro and in vivo studies. La Chance and Page (1953) reported that thyroxine (T_4) is effective in reducing perirenal fatty depots in rats. Ellefson and Mason (1962) noted that rats fed T_4 had elevated plasma levels of nonesterified fatty acids. Krishna et al. (1968) showed that rats injected with thyroxine had an increased rate of lipolysis. This agreed with Debons and Schwartz (1961), who reported that both T_3 and T_4 enhanced the lipolytic effect of catecholamines. Deykin and Vaughan (1963) demonstrated that rat epididymal fat pad treated with T_3 released more free fatty acids (FFA) into the medium and contained more FFA after one hour of incubation than did control tissues.

In two separate trials, Fredericks et al. (1975) reported that both T_3 and T_4 could be effective in reducing waste fat deposits in rabbits although the dose levels (1.0 mg per day and 0.5 mg per day) were too high as the treated rabbits had reductions in muscle fat and body weight. The overall results indicated that T_4 appeared to have an advantage over T_3 in reducing fatty depots with less deleterious effects. Furthermore, the lower dose levels (0.5 mg per day) were effective in reducing fatty depots with less weight loss in the treated animals. In a more recent study, McBride et al. (1976) reduced the daily administration of T_4 tenfold (100 μg and 50 μg) and was still successful in reducing waste fat deposits in rabbits. In addition, these levels did not adversely affect the percent fat in the primal cuts in males and female rabbits showed an increase in primal cut fat. Although loss of weight was considerably reduced as compared to the previous studies, the treated rabbits continued to lose weight, particularly in the 100 μg dose level.

Estrogens have been shown to exert a protein anabolic effect in a variety of animals (Guyton, 1971). In addition to stimulating growth, estrogens (Gassner et al., 1948) and progesterone (Werner, 1957) have been reported to stimulate thyroid activity. Synovex, which contains estradiol benzoate and progesterone, has been shown by several researchers to increase growth rates and efficiency of feed utilization in cattle fed finishing rations (Woods and Tolman, 1967; Preston and Cahill, 1971).

The present studies were designed to determine if Synovex implants used in conjunction with T_4 injections in rabbits could counteract the weight losses incurred with use of T_4 without losing the lipolytic activity. Also, since the results reported by McBride et al. (1976) showed that following T_4 treatment there was an increase in intramuscular fat in T_4-treated females as compared to control females, another objective was to determine if similar results could be obtained by injecting T_4 into castrated males implanted with female hormones.

EXPERIMENTAL

TWO EXPERIMENTS were conducted with New Zealand White rabbits. In Experiment I, twenty-two 14-wk old rabbits were randomly divided into four groups: Control, T_4, T_4 + Synovex, and Synovex. Each group consisted of three males and three females with the exception of the Synovex group which consisted of two males and two females. The T_4 and T_4 + Synovex treated rabbits each received a 50 μg intramuscular injection of T_4 every 12 hr for 2 wk. Rabbits with placebo saline injections had been compared to noninjected controls in previous experiments. No differences were observed so the use of placebo injections was discontinued. All rabbits receiving Synovex were implanted subcutaneously in the neck 7 wk prior to the beginning of the trial with a tablet containing 10 mg progesterone and 1 mg estradiol benzoate. The Synovex was implanted at this time in order to establish growth curves of control and Synovex implanted rabbits before starting the thyroid hormone injections.

In Experiment II, forty-eight 14-wk old rabbits were randomly divided into the same four groups as in the previous experiment with each group consisting of six females and six castrated males. The rabbits receiving Synovex were implanted 3 wk prior to the beginning of the trial with tablets containing 12.5 mg progesterone and 1.25 mg estradiol benzoate. All male rabbits were castrated at the same time. At

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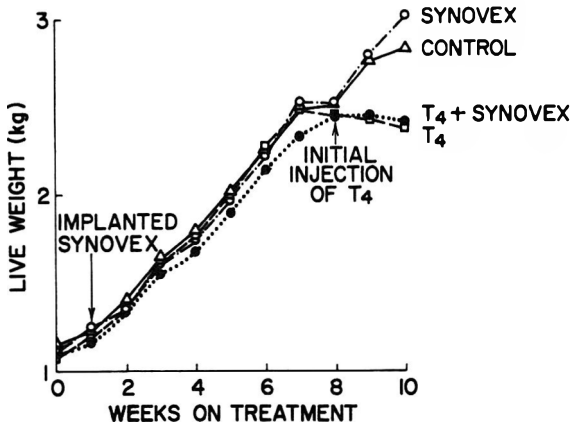

Fig. 1—Rate of growth during treatment period, Experiment I.

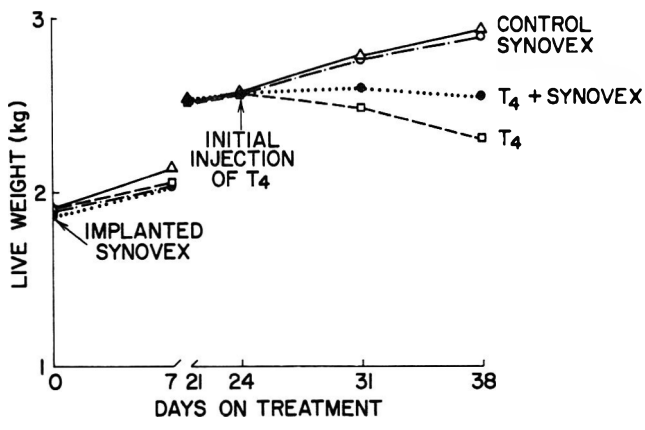

Fig. 2—Rate of growth during treatment period, Experiment II.

time of slaughter, subcutaneous fat, perinephric fat and mesenteric fat depots were weighed and stored at −20°C for further analysis.

In Experiment I, the left side of each carcass was boned out and all the soft tissues were mixed and ground. A sample was then chemically analyzed (AOAC, 1965) from this mixture to obtain the proximate composition of carcass soft tissues not including subcutaneous or perinephric fat. In Experiment II, intramuscular fat was determined by dissecting a major muscle from the leg, loin and shoulder of each rabbit and preparing it for proximate analysis. This included trimming off all external fat and connective tissue. The major muscles were biceps femoris, longissimus and supraspinatus.

Statistical analysis of the data consisted of the least squares analysis of variance described by Harvey (1960). Mean values for treatments, sexes and percentage of fatty tissues were further analyzed for statistical differences by the least significant difference test (Snedecor and Cochran, 1967).

RESULTS & DISCUSSION

IT WAS POSTULATED that weight losses in rabbits (Fredericks et al., 1975; McBride et al., 1976) and rats (Hsieh, 1962; Ellefson and Mason, 1962; Grossie and Turner, 1961) caused by administration of T_3 and T_4 could be counteracted by the anabolic effect of Synovex. However, in Experiment I, implantation of Synovex in rabbits treated with T_4 did not improve weight gains over rabbits injected with T_4 alone (Fig. 1).

Even though the implant size was extrapolated by body weight to the ¾ power according to Brody (1945) from the amount given to cattle, the optimum pellet weight was likely underestimated. In Experiment II, the pellet size was increased and the time from implant to initiation of T_4 injections was reduced. In this case the weight gains of the rabbits treated with both T_4 and Synovex were intermediate between those treated only with T_4 and the controls (Fig. 2).

In Experiment I, the rabbits receiving T_4 injections had significant depletions in percent subcutaneous, perinephric and total separable fats. The T_4 + Synovex group was intermediate between the T_4 group and the Synovex group in fat deposits (Table 1).

Although the percent carcass fat was slightly lower in both the T_4 and the T_4 + Synovex groups as compared to the control and Synovex groups the differences were nonsignificant. The females had lower mean values for body fat than the males, particularly in the T_4 + Synovex group, but the differences were not significant ($P > 0.05$).

The male rabbits ir. Experiment II were castrated since the treated females in Experiment I had superior weight gains and less waste fat than the males. Similar sex differences in cattle implanted with Synovex have been reported in the literature. Implanted steers and heifers gained significantly more than controls (Preston and Cahill, 1971; Woods and Tolman, 1967) but bulls did not (Preston et al., 1971).

The results of Experiment II show that there were significant reductions of the fatty tissues in all three treatment groups without loss of intramuscular fat (Table 2). There were no significant differences between females and castrated males

Table 1—Effects of T_4 and Synovex on the distribution of fat in rabbits, Experiment I

Tissue	Control	T_4	T_4 + Synovex	Synovex
No. of animals	6	6	6	4
Total sep. fat[c], % HCW[d]	8.75	5.64[a]	6.72[a]	8.48
Subcutaneous fat, % HCW	3.38	2.04[b]	2.12[b]	2.77
Perinephric fat, % HCW	2.82	1.72[a]	1.95	2.59
Mesenteric fat, % HCW	2.55	1.88	2.65	2.98
Carcass fat, %[e]	8.59	7.23	6.63	9.18

[a] Difference between treatment and control, sexes pooled ($P < 0.05$).
[b] Difference between treatment and control, sexes pooled ($P < 0.01$).
[c] Total separable fat = subcutaneous, perinephric and mesenteric fat.
[d] HCW = hot carcass weight including the separable fats.
[e] Carcass fat is percent of boneless carcass after removal of subcutaneous, perinephric and mesenteric fat.

Table 2—Effects of T_4 and Synovex on the distribution of fat in rabbits, Experiment II[c]

Tissue	Control	T_4	T_4 + Synovex	Synovex
No. of animals	12	12	12	12
Total sep. fat, % HCW	10.09	7.20[b]	6.12[b]	7.57[b]
Perinephric fat, % HCW	3.49	2.62[b]	1.40[b]	2.15[b]
Subcutaneous fat, % HCW	4 16	2.66[b]	2.39[b]	2.92[b]
Mesenteric fat, % HCW	2.45	1.92[a]	2.32	2.51
Intramuscular fat, %	1.16	1.06	2.01[a]	1.28

[a] Difference between treatment and control, sexes pooled ($P < 0.05$).
[b] Difference between treatment and control, sexes pooled ($P < 0.01$).
[c] There were no significant differences between females and castrated males ($P > 0.05$).

for any of the fat depots. There appeared to be a synergistic interaction between Synovex and T_4 as the T_4 + Synovex rabbits had the greatest reduction of body fats and still had the largest amount of intramuscular fat.

In summary, it may be concluded that intramuscular injections of T_4 will decrease the amount of separable fat in rabbits without excessive depletion of intramuscular fat. Subcutaneous implantation of Synovex tends to counteract the weight losses incurred from use of T_4, particularly in castrated males and females. There appeared to be an interaction between T_4 and Synovex as rabbits treated with both had the smallest amounts of separable fats and the largest amount of intramuscular fat.

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Ms received 6/1/77; revised 2/24/78; accepted 2/28/78.

Presented at the 37th Annual Meeting of the Institute of Food Technologists, Philadelphia, June 5-8, 1977.

Scientific Series No. 2255 of the Colorado State University Experiment Station, Fort Collins, CO. From the MS thesis of T.M. McBride, Colorado State University, Fort Collins, CO.

This study was conducted in cooperation with Regional Project NCR-97.

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Ms received 11/27/77; revised 2/16/78; accepted 2/25/78.

This work is a result of research sponsored in part by the Tuna Research Foundation and by the NOAA Office of Sea Grant, Department of Commerce, under Grant # 04-7-158-44121.

AMMONIA-PRODUCING ENZYMES IN WHITE SHRIMP TAILS

CHIA-PING S. YEH, RANZELL NICKELSON II and GUNNAR FINNE

ABSTRACT

Of a number of potential ammonia-producing enzymes tested, only adenosine deaminase and AMP deaminase could be detected in significant levels in white shrimp tails. In addition to these two enzymes, arginase was also shown to be present and formation of ammonia from arginine can thus potentially take place through urea and bacterial urease. At every temperature tested, enzymatic ammonia production from shrimp tissue during initial postmortem storage accounted for more than half of total ammonia production. Optimum temperature for the enzymatic ammonia formation was shown to be 37°C, while the pH profile showed two distinct peaks: one near 6.0 and the other around 8.4. The results of this study show the important part native shrimp tissue enzymes play during spoilage of iced shrimp.

INTRODUCTION

UP TO NOW, most of the studies on the causes of the quality deterioration of iced shrimp have centered around microbial activity. Several reports on microbial concentrations and variation in microbial populations during iced shrimp storage are available (Campbell and Williams, 1952; Carroll et al., 1968; Vanderzant et al., 1970; Cobb et al., 1976). Specific interest has centered around the nitrogenous constituents in shrimp because of their contribution to the flavor and off-flavor and thus their direct affiliation with organoleptic characteristics. The sweet flavor of freshly caught shrimp is believed to be due to free amino acids (Hashimoto, 1965; Rajendranathan Nair and Bose, 1965), while volatile nitrogen compounds normally reported as total volatile nitrogen (TVN), represents part of the off-flavors associated with spoilage. Increase in total volatile nitrogen in shrimp tails during the postmortem storage period on ice has been reported by several investigators (Iyengar et al., 1960; Cobb and Vanderzant, 1971; Cobb et al., 1973b, 1976). The primary component included in the total volatile nitrogen fraction was shown by Vanderzant et al. (1973) to be ammonia.

Although a large number of investigators have examined factors affecting shrimp quality, few have attempted to differentiate effects caused by natural shrimp enzymes from those caused by postmortem bacterial activity. Cobb and Vanderzant (1971) did, however, note a progressive increase in total volatile nitrogen in sterile shrimp extracts, indicating that endogenous enzymes may contribute to the TVN production.

This study reports on postmortem ammonia-producing enzymes in tails of white shrimp from the Gulf of Mexico.

MATERIALS & METHODS

Shrimp

White shrimp (*Penaeus setiferus*) were obtained directly from fishing boats in Galveston Bay and immediately packed in ice and shipped to the laboratory. The shrimp were deheaded, shelled, deviened, thoroughly washed in distilled water and analyzed immediatley or held at −24°C until used (maximum of 3–4 days for repeat analysis).

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0022-1147/0005- 140002.25/0

Chemical analysis

Protein determination was done according to Kjeldahl nitrogen content × 6.25 (AOAC, 1975) or the Buiret procedure (AOAC, 1965). Ammonia was determined by the microdiffusion method using Na_3PO_4 as releasing agent as described by Cobb et al. (1973a) and by the colorimetric method described by McCullough (1967).

Enzyme assays

Arginase. Shrimp muscle was homogenized with 3 volumes of 0.1% hexadecyltrimethylammonium bromide (CTB, Eastman Chemicals) in a blender for 30 sec (Brown and Cohen, 1959). The homogenate was centrifuged at 16,500 × G for 20 min and the supernatant was used for the determination of arginase activity.

Arginase activity was determined by a modification of the method of Andrews and Reid (1972) using L-(+)-arginine as a substrate and pure urease for the conversion of urea to ammonia. The assay mixture contained 0.5 ml of 0.24M L-arginine, 0.5 ml of 3.6 mM magnesium sulfate, 1.0 ml of urease solution (1g urease, 15g NaCl dissolved to 50 ml with distilled water, stirred for 30 min and allowed to stand at room temperature for 3 hr before being filtered through a Whatman no. 41 filter paper and adjusted to pH 7.0), 2.5 ml of shrimp extract and 0.2 ml of 0.01M glycine buffer, pH 9.6. This was also the pH of the final mixture. After incubation for 30 min the reaction was terminated by the addition of 0.5 ml of 20% trichloroacetic acid (TCA). The ammonia liberated was determined by the microdiffusion method described by Cobb et al. (1973a).

Urease. For the determination of urease activity the same tissue extraction was used as for arginase. Urease activity was measured by determining ammonia released from shrimp extracts with added urea. To 5.0 ml of crude enzyme extract were added: 4.0 ml of 0.02M phosphate buffer (pH 7.0) and 0.05 ml Procaine penicillin G in dihydrostreptomycinsulfate solution (Diamond). The assay system was incubated in room temperature and terminated by the addition of 1.0 ml of 20% TCA solution. Ammonia released from the mixture was determined by the microdiffusion method.

Adenase, adenosine deaminase, AMP deaminase and guanase. For the determination of nuceotide deaminase reactions shrimp were homogenized with 11 volumes of prechilled distilled water in a blender for 30 sec. The homogenates were stirred at 1°C for 1 hr, and then centrifuged at 14,000 × G for 25 min.

Enzyme activities were measured by a modification of the method of Stone (1970). The assay system consisted of 3.6 ml of substrate and 0.4 ml of shrimp muscle extract. The substrates were:

(a) 9.0 mM adenine in 0.05M phosphate buffer, pH 7.0
(b) 9.0 mM adenosine in 0.1M barbital buffer, pH 8.6
(c) 9.0 nM AMP in 0.1M citrate buffer, pH 6.5
(d) 10.0 nM guanine in 0.1M tris buffer, pH 8.0

The reactions were carried out at 37°C for 30 min. Adenase, adenosine deaminase and AMP deaminase activities were measured by the decrease in absorbance at 265 nm (Stone, 1970). Guanase activity was measured by the decrease in absorbance at 290 nm (Shuster, 1955). Ammonia formed was determined by the colorimetric method of McCullough (1976) and by the microdiffusion method of Cobb et al. (1973a).

Amino acid oxidase activity. Shrimp were homogenized with 1.0 volume of prechilled distilled water in blender for 30 sec. The homogenate was centrifuged at 4500 × G for 30 min. One ml of Procaine penicillin G in dihydrostreptomycin solution was added to 100 ml of supernatant and this mixture was used for amino acid oxidase determinations.

Amino acid oxidases were assayed by a modification of the method of Curti et al. (1968). To 4.0 ml of shrimp extract were added: 1.0 ml of 0.2M tris buffer (pH 7.8) containing 2.5 μg/ml catalase and 1.0 ml of 0.05M substrate in 0.2M tris buffer, pH 8.0. Substrates included arginine, proline, glycine, alanine and serine. Reaction time was varied from

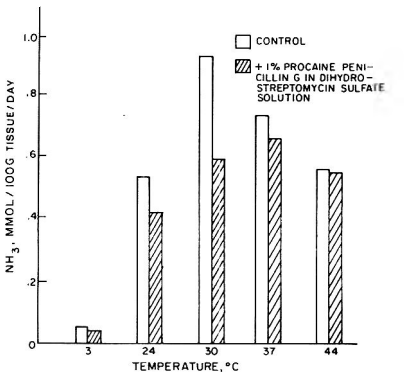

Fig. 1—Ammonia production in shrimp extracts at different temperatures.

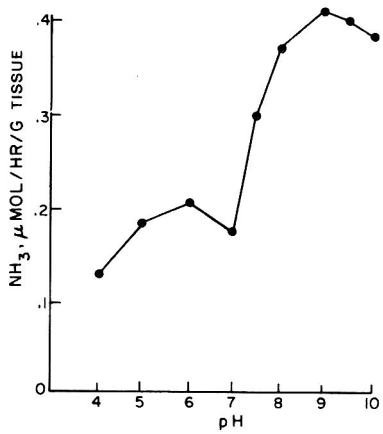

Fig. 2—Effect of pH on the ammonia-producing enzymes from shrimp muscle tissue at 37° C.

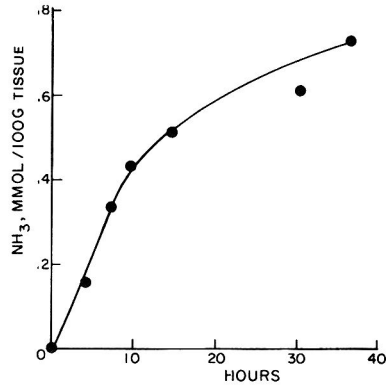

Fig. 3—Rate of ammonia production in antibiotic treated shrimp muscle extract at 24° C.

1–24 hr. The ammonia content was determined in the sample at the beginning and end of the reaction and in a control (no substrate added).

RESULTS & DISCUSSION

Production of ammonia in shrimp muscle extracts during storage

To investigate the ammonia produced by bacteria and by tissue enzymes, shrimp extracts with and without antibiotics were incubated at different temperatures for one day, after which the ammonia levels were determined. Ammonia production in these shrimp muscle extracts are shown in Figure 1. Although bacterial enzymes may be present in low levels, the ammonia produced in antibiotic treated samples should indicate the activity of tissue enzymes for the most part. The ammonia produced in the control samples would be contributed by bacterial as well as tissue enzymes. As indicated in Figure 1, the difference in ammonia production between the control and antibiotic treated extracts was markedly affected by incubation temperature. The large differences noted at 30°C is probably due to a more accelerated bacterial growth at this temperature. At 37°C bacterial ammonia production is lowered because this temperature is above the maximum growth temperature of many of the organisms normally associated with shrimp spoilage. At all the temperatures tested, the ammonia production by tissue enzymes far exceeded that of bacterial production, indicating the importance of tissue enzymes during postmortem quality deterioration.

pH effect on ammonia production in sterile shrimp extracts

To determine the optimal pH for ammonia production by tissue enzymes, 1.0 ml of antibiotic treated shrimp muscle extract was mixed with 4.0 ml of different buffer solutions (0.1M) and incubated for 3 hr at 37°C. Citrate-phosphate buffer was used for pH values between 4.0–8.4, and Sorensen's glycine buffer was used for the pH range 8.4–9.4. Effect of pH on the ammonia production in sterile shrimp muscle extracts are shown in Figure 2. According to this figure, it appears that two enzyme systems are responsible for the ammonia production: one with a pH optimum of around 6.0, and another with a pH optimum in the region of 8.4.

Rate of ammonia production in sterile shrimp muscle extracts

The rate of ammonia production in antibiotic treated shrimp muscle extracts at pH 9.6 and 24°C is shown in Figure 3. Ammonia production follows first order kinetics for at least 9 hr at a rate of 0.045 mmol/hr/100g. The decrease in ammonia production after nine hours of incubation could be due to either enzyme instability or depletion of the substrate pool.

Testing of the individual enzymes

Of all the potential ammonia-producing enzymes tested, only arginase, adenosine deaminase and AMP deaminase were found to be present in significant levels in antibiotic treated shrimp muscle extracts. This is in close agreement with earlier works. Crustaceans, like insects, appear to be devoid of all enzyme activities associated with the urea cycle, except arginase (Chefurka, 1965). Hanlon (1975) examined five species of crab and showed that all had low to undetectable digestive gland urease activities. Amino acid oxidase activities have also been shown to be low or absent in a variety of crustaceans. Thus Sisini (1963) stated that serin oxidase is the only amino acid oxidase that has been found in crustaceans. The lack of adenase and guanase in shrimp extracts is in agreement with Stone (1970) who failed to detect adenase in salmon, crab and weathervane scallops, and with Tarr and Comer (1964) who reported that lingcod muscle possessed a very weak adenase activity and indeed suggested that the deamination of the added substrate might be caused by the presence of adenosine deaminase.

SUMMARY & CONCLUSIONS

THIS STUDY has shown the importance of ammonia-producing tissue enzymes during postmortem storage of deheaded shrimp. The ammonia produced by tissue enzymes in white shrimp tails increased with increasing temperature, reaching a maximum at 37°C. At this temperature, more than 85% of the total ammonia production in shrimp muscle extracts after 1 day incubation could be attributed to shrimp tissue enzymes. The percentage will vary, of course, with initial bacterial load and types. Ammonia production by shrimp tissue enzymes was also shown to be relatively high during the early stages of ice storage.

The ammonia production by tissue enzymes was shown to be pH dependent with two optimal pH values of 6.0 and 8.5.

The rate of ammonia production at pH 9.6 followed first order reaction kinetics with a rate of 0.045 mmol/hr/100g tissue.

Of a large number of ammonia-producing enzymes tested, only adenosine deaminase and AMP deaminase were found to be present in significant levels. In addition to these two enzymes, arginase was also found to be present. Although ammonia is not formed directly by arginase, the urea produced by arginase during postmortem storage could be hydrolyzed by bacterial ureases.

—Continued on page 1404

ISOLATION AND CHARACTERIZATION OF POLYPHENOLIC COMPOUNDS IN CRANBERRIES

P. L. WANG, C. T. DU and F. J. FRANCIS

ABSTRACT

Methanolic extracts of cranberries were evaporated and reextracted with ethyl acetate. Residues from the dried ethyl acetate extract were isolated by thin-layer, column, and paper chromatography. A large diffuse area indicated the presence of high molecular weight polymeric polyphenols. In addition, seven discrete spots were identified as: (a) catechin; (b) epicatechin; (c) a dimeric epicatechin with a C_4-C_8 linkage; (d) an unknown polymer which degraded on acid hydrolysis to three compounds, of which one was cyanidin; (e) an unknown polymer which degraded to at least four compounds of which one was cyanidin and one was the compound described above as (d). Compounds (f) and (g) were present in very small amounts. These compounds may contribute to the astringency of cranberries.

INTRODUCTION

POLYPHENOLIC POLYMERS, especially leucoanthocyanins, have been investigated in many foods (Bate-Smith, 1954a, b; Forsyth, 1955; Forsyth and Roberts, 1964; Goldstein, 1964; Hsia et al., 1964; Saburo and Joslyn, 1965; Geissman and Dittmar, 1965; Van Buren et al., 1966; Bhatia et al., 1968; Schmidt and Neukom, 1969; Weinges et al., 1968, 1969). In many cases, they contribute to the flavor and appearance of food.

Products made from cranberries (*Vaccinium Macrocarpon* Ait.) owe their appeal mainly to their attractive color and flavor. The taste is astringent with a little bitterness. The astringency may logically be attributed in part to monomeric flavan-3,4-diols and flavan-3-ols or polymeric flavan-3-ols and flavan-3,4-diols (Bate-Smith, 1954a, b; Weinges et al., 1969b). The bitterness may be caused in part by flavan-3-ol compounds, mainly catechin (Nakabayashi et al., 1967). It is obvious from the taste that cranberries must contain polymeric polyphenols but none has been reported in the literature.

This work was undertaken to characterize some of the polyphenolic compounds which may contribute to the flavor of cranberries.

EXPERIMENTAL

Materials

Cranberries (*Vaccinium Macrocarpon* Ait.) obtained from Ocean Spray Cranberries, Inc., Middleboro, MA, were stored at −20°C for these studies.

Extraction

One kg of cranberries was macerated in a Waring Blendor with 2 liters of methanol, and allowed to stand overnight in the refrigerator. After filtration, extracts were concentrated under reduced pressure to about 300 ml, and then extracted with ethyl acetate three times (500, 300 and 200 ml). Dehydrated ethyl acetate extracts were evaporated to near dryness and dissolved in ethyl alcohol for isolation.

At the time the work reported in this paper was done, all authors were with the Dept. of Food Science & Nutrition, University of Massachusetts, Amherst, MA 01003. Author Wang is now deceased. Author Du is now affiliated with Stokely Van Camp, Inc., Indianapolis, IN.

0022-1147/78/0005-1402$02.25/0

Isolation

Paper chromatography. The ethyl acetate extract was streaked on Whatman #3 paper. The papers were developed in a descending direction with 2% acetic acid for 8 hr. Bands were detected by vanillin-HCl reagent. This approach is similar to that used by Luh et al. (1967) for peaches.

Column chromatography. Polyamide powder (Woelm) was pretreated with 95% ethanol and stirred occasionally over a 2- or 3-day period. The alcohol was filtered off, and the polyamide was washed twice with 95% alcohol. It was then suspended in 95% ethanol and poured into the column. Samples were applied as ethanolic solutions to top of the column. Ethanol was used as the eluting solvent. Each 250 ml eluate fraction was collected and evaporated to dryness. Compounds were detected using paper chromatography and spraying with vanillin-HCl solution.

Identification

Detection reagents (Swain and Hillis, 1959). A freshly prepared vanillin reagent was made by mixing a 1% ethanolic solution of vanillin with an equal volume of concentrated HCl. $FeCl_3$-$K_3Fe(CN)_6$ reagent was made of equal volumes of 0.5% $FeCl_3$ and 0.5% $K_3Fe(CN)_6$ (Keppler, 1957). Toluene-p-sulphonic acid (3%) in ethanol was made by heating at 90°C for 5 min (Roux, 1957). The 0.1N HCl was used as suggested by Seikel (1962). Confirmation of leucoanthocyanin identification was obtained by heating the material on paper for 5 min with 0.1N acid at 100°C. Leucoanthocyanin appears as red spot. The ferric chloride solution was 0.2% in ethanol.

Two dimension paper chromatography. Extracts soluble in ethyl acetate were chromatographed on Whatman #1 paper, with development first in butanol:acetic acid:water (BAW, 4:1:2.2) and second, in 2% acetic acid. The spots were detected by vanillin-HCl.

Thin-layer chromatography. Extracts soluble in ethyl acetate were spotted on cellulose thin layer plates and run first in BAW (6:1:2.2), and second, in 5% n-butanol. They were detected by vanillin-HCl.

Controlled hydrolysis. Compounds were hydrolyzed by suspending in 0.1N HCl and holding in a water bath at 100°C for 5 min. Another procedure was to suspend in leucoanthocyanidin reagent (BuOH-HCl) in a water bath at 97°C for 40 min (Swain and Hillis, 1959).

Conversion of leucoanthocyanin to anthocyanidin (Seikel, 1962; Swain and Hillis, 1959). Leucoanthocyanin reagent (concentrated hydrochloric acid diluted (1:10) with n-butanol) was added to a solution of leucoanthocyanins or proanthocyanins, and then placed in a water bath at 97°C for 40 min. After cooling, the pigments were measured by spectral methods or identified by paper chromatography along with authenic compounds.

Identification of anthocyanidins and sugars. Anthocyanidins were identified through a combination of R_f values in three solvent systems and spectral properties. Sugars were identified by conventional paper chromatography and TLC (Harborne, 1958; Francis, 1967; Wang and Francis, 1972).

Chemical methods: Alkali degradation. Samples were treated with 25% NaOH solution, refluxed for 30 min under a stream of nitrogen, cooled and acidified. The reaction mixture was extracted several times with ether, evaporated to dryness, spotted on TLC plates and run with known standards for comparison. Acetylation: One ml pyridine and 1 ml of acetic anhydride were added to the sample which was then allowed to stand at room temperature for one week. The mixture was poured into ice water. The precipitate was filtered off, washed with a small amount of ice water, and dissolved in methanol for TLC.

RESULTS & DISCUSSION

BOTH TWO dimensional paper chromatography and thin-layer chromatography of ethyl acetate crude extracts of cranberry revealed a diffuse area and five major spots. The diffuse area indicated the presence of high polymeric polyphenols. In addition, two other less intense spots also existed, but their quan-

Table 1—Color reactions of cranberry polyphenols with diagnostic reagents

Diagnostic reagents	Spot 1	Spot 2	Spot 3	Spot 4	Spot 5	Spot 6
Vanillin-HCl	Red-brown	Red-brown	Brown	Brown	Pink	Red
Toluene-sulfonic acid	Brown	Brown	Brown	Brown	Pink	–
0.1N HCl, 100°C, 10 min	Brown	Brown	Brown	Brown	Pink	Brown
$FeCl_3$-$K_3Fe(CN)_6$	Blue	Blue	Blue	Blue	Blue	Blue
$FeCl_3$	Green	Green	Green	Green	Green	Green
Air (1 month)	Brown	Red-brown	Brown	Brown	Pink	Red-brown

tity were too small to be investigated. This paper described the isolation and identification of five major isolated compounds (Fig. 1).

The color reactions of these five major flavan spots on paper chromatograms with diagnostic reagents are presented in Table 1. None of these flavan spots showed a blue color reaction with $FeCl_3$, indicating the absence of free gallocatechin or gallates.

On the basis of color reactions and co-chromatography with authentic compounds, spots 3 and 4 were identified as catechin and epicatechin respectively.

Two dimensional paper chromatography gave good separation of spots 1 and 2, whereas column chromatography using polyamide gave poor resolution of these compounds. Column chromatography did give good separations of spot 5.

Spot 1, when acid hydrolyzed with 0.1N HCl at 100°C for 10 min, yielded (−)-epicatechin. On more prolonged hydrolysis, at 15 min with 2N HCl, it gave cyanidin which was confirmed by exhibiting identical R_f values with authentic cyanidin and typical cyanidin spectra with a positive aluminum chloride shift. Thus spct 1 was identified as a dimeric epicatechin. The identity of this compound was further confirmed by exhibiting identical chromatographic mobilities with one spot in Red Delicious apples extracted in the same manner. The compound in apples was previously identified as a dimeric epicatechin (Saburo and Joslyn, 1965; Schmidt and Neukom, 1969) which also gave cyanidin and epicatechin on acid hydrolysis. Upon acetylation of spot 1 obtained by polyamide column chromatography (fraction 14), it showed identical R_f values with the decaacetate of dimeric 1-epicatechin with a C_4 and C_8 linkage. No sugar was detected with this dimer.

The end products of acid hydrolysis of spot 2 and spot 5 are presented in Table 2. Further identification of the end products is presented in Table 3. B_2 from spot 5 had an E_{max} of 438 nm and gave a positive aluminum shift. It also had identical chromatographic mobilities with authentic cyanidin, similar alkali degradation products and thus was identified as cyanidin. B_1 had an E_{max} of 436 nm and positive aluminum

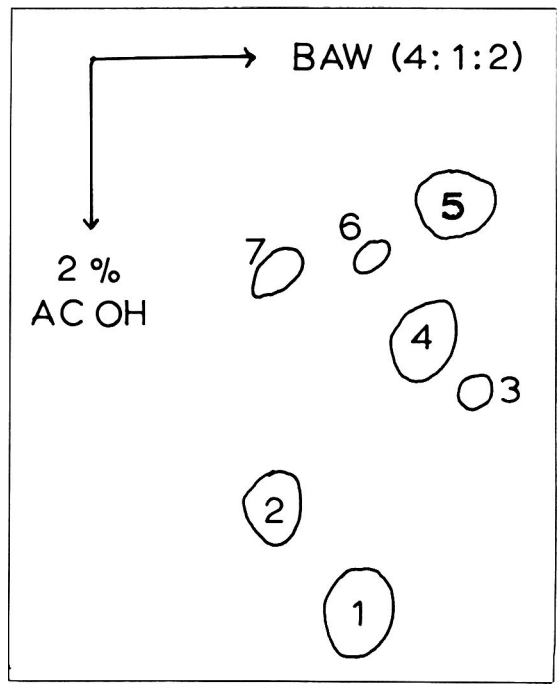

Fig. 1—Two dimensional paper chromatograph showing the separation of polyphenols from ethyl acetate extract of cranberry.

chloride shift, but it had a lower R_f value than that of delphinidin in Formic and Forestal solvent systems. Alkali degradation of B_1 indicated that it was not delphinidin due to the absence of gallic acid. Comparison with 6-hydroxycyanidin made synthetically from quercetagetin-7-glucoside revealed that they were different in color characteristics on paper chromatograms. B_1 is closer in color to cyanidin.

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Table 2—Acid hydrolysis of Spots 2 and 5

Spot no.	Conditions of acid hydrolysis	Acid hydrolysis products
Spot 2	0.1N HCl, 10 min, 100°C	Original spot 2 (−)-epicatechin Spot 5
	2N HCl, 15 min, 100°C	3 pigments (same as spot 5)
Spot 5	0.1N HCl, 10 min, 100°C	Original Spot 5 3 pigments:
	2N HCl, 15 min, 100°C	B_1 (unknown) B_2 (cyanidin) B_3 (unknown)
	5N HCl, 5 min, 100°C	2 pigments: Original spot 5 Unknown spot

Table 3—Alkali degradation of the acid hydrolyzed products from Spot 5

Degraded samples	Proto-catechuic acid	Gallic acid	Vanillic acid	Phloro-glucinol
Standards				
Delphinidin		(+++)[a]		(+)
Cyanidin	(+++)			(++)
Peonidin			(+++)	(++)
6-hydroxycyanidin	(+++)			(–)
Samples				
B_1	(+++)			(+)
B_2	(+++)			(++)
B_3	(+++)			(?)

[a] The number of plus marks indicates the color intensity of spots.

Both spot 1 and 5 caused precipitation of gelatin solutions and might contribute to the astringency of cranberry juice.

CONCLUSIONS

PAPER and thin-layer chromatography of extracts from cranberries revealed a complex pattern. A large diffuse area indicative of large polymers was evident together with five major and two minor spots. Spot 1 was a dimeric epicatechin with a C_4-C_8 linkage. Spot 2 was an unknown compound which hydrolyzes into at least four components. Two of these are (−)-epicatechin and cyanidin. Spot 3 was catechin. Spot 4 was epicatechin. Spot 5 is an unknown which hydrolyzes to at least 3 components, one of which is cyanidin. Spot 5 can be produced from 2 by acid hydrolysis. Spots 6 and 7 were present in quantities too small for chemical analysis.

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Ms received 12/22/77; revised 3/16/78; accepted 3/20/78.

Contribution No. 2186 from the Univ. Mass. Agricultural Experiment Station, Amherst, MA. Appreciation is expressed to the Glass Packaging Inst., Washington, DC for partial financial support.

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Ms received 2/21/78; revised 5/1/78; accepted 5/10/78.

We are deeply grateful to the late Dr. Dryant F. Cobb III who did most of the experimental design for this study. This work was partially supported through Institutional Grant 04-7-158-44105 to Texas A&M University by the National Oceanic and Atmospheric Administration's Office of Sea Grants, Department of Commerce.

SCANNING ELECTRON MICROSCOPY STUDIES ON STARCH GRANULES OF RED KIDNEY BEANS AND BEAN SPROUTS

HUGO C. SILVA and B. S. LUH

ABSTRACT

Scanning electron microscopic examinations of cotyledons from red kidney beans (*Phaseolus vulgaris*, L.) revealed changes on the surface starch of granules during germination of the seeds. There were distinct transformations of the surface of the granules after the eighth day of germination. After germination for 10 days the granules appeared to be very fragile and broken into small, irregular fragments. Observations were also made on starch granules of cooked dry beans and in bean cotyledons germinated for 4 days followed by heating in edible oil at 95°C for 10 min.

INTRODUCTION

SCANNING ELECTRON MICROSCOPE (SEM) has been used to show the surface characteristics of starch granules from tender white, pinto and lima beans (Hall and Sayre, 1971). In a SEM investigation of the cotyledon interior of water-soaked soybeans, protein bodies of 1–10μ in diameter exhibited a covering spongy network (Wolf and Baker, 1972). Changes on cell wall and cell structure of cotyledons from large lima beans were revealed by Rockland and Jones (1974) who used SEM to study the effects of cooking on water-soaked and salt water-soaked beans. Cotyledons from fababean seed showed the presence of starch granules and protein bodies ranging from 1–5μ in diameter (McEwen et al., 1974). Cell structure in the seed coat of cooked soybeans was observed by transmission electron microscopy and SEM (Saio et al., 1973). Hahn et al. (1977) used light and scanning electron microscopy to characterize intracellular configurational changes of starch granules during gelatinization of standard and quick-cooking Lima bean cotyledons. Intracellular gelatinization of starch was initiated at about 76°C for water-soaked and at 85°C for quick-cooking beans. Protein bodies and spherosomes of soybean cotyledons were observed in SEM studies of commercial soy flours, protein concentrates, and protein isolates (Wolf and Baker, 1975).

Little is known about starch granules in sprouted bean seeds. This paper reports SEM studies on starch granules in cotyledons of germinated red kidney beans (*Phaseolus vulgaris*, L.). Starch and free sugars were determined in the beans during germination to relate SEM with chemical changes. The changes on starch granules in cooked red kidney beans, and in beans germinated for 4 days followed by heating for 10 min in an edible oil at 95°C were studied.

EXPERIMENTAL

Dry red kidney beans

Dry red kidney beans (*Phaseolus vulgaris*, L.), 1976 crop, were supplied by the seed laboratory of the University of California, Davis. The moisture content of the beans was 7.7%. They were kept at 20°C in tightly covered cans for 4 wk prior to experiments.

Germination of beans

Red kidney dry beans were rehydrated by soaking in 0.02% calcium hypochlorite (30–35% available chlorine) solution for 24 hr at 20°C. After washing, the beans were placed on a spongy cloth in a screen basket. The basket was placed in a covered ceramic pot. The beans were germinated for a total of 10 days, and during this period were rinsed with tap water every 4 hr during the day.

Preparation of samples for SEM

Soaked samples. At the end of the soaking period, a sample was taken, and the seed coat removed from the seed. The cotyledons were placed in a 3% glutaraldehyde–0.2M phosphate buffer (pH 7.1) and later dehydrated in increasing concentrations of ethanol (25, 50, 75, 90 and 100%). The cotyledons were than dried by the critical point method (Anderson, 1951) and fractured to provide a cross-section near the embryo.

Sprouted samples. For studies of sprouted specimens, germinating beans were harvested at 2-day intervals for a total of 10 days. The hypocotyl and seed coat were removed and the cotyledons treated in the same way as the soaked samples.

Cooked samples. Dry beans were cooked in boiling tap water for 1 hr. After cooking the hypocotyl and seed coat were removed. The cotyledons were submitted to the same procedure used for soaked samples.

Beans germinated for 4 days were heated in edible oil at 95°C for 10 min. After cooking, the hypocotyl and seed coat were removed. The cotyledons were treated in the same way as the soaked samples.

Examination of specimens by SEM

The samples of soaked, sprouted and cooked beans as well as fractured cotyledons from dry beans were mounted on aluminum stubs with silver paint, sputtered with gold to a thickness of about 40 nm and examined in a Cambridge Mark II A microscope at an accelerating potential of 10 kv.

Starch determination

Samples from germinating beans were taken at 2-day intervals and weighed. After extraction of the sugars the starch in the free-sugar residue was hydrolyzed following the method for direct acid hydrolysis of starch described by McCready (1970). An aliquot of the final filtrate was used to determine glucose by the phenol-sulfuric acid method (Dubois et al., 1956). The determined amount of glucose × 0.9 = the amount of starch.

Powdered (20 mesh) whole dry bean samples were submitted to the same procedure.

Determination of free sugars in bean sprouts as glucose

During germination, 20g of bean sprouts were taken at 2-day intervals and placed in 100 ml of 80% ethanol. After blending the mixture was refluxed for 1 hr and filtered. The residue was washed 3 times with 100-ml portions of hot 80% ethanol. The extracts and washings were combined and concentrated to 10 ml under vacuum at 48°C. The concentrated sugar extract was clarified with 1 ml of saturated lead acetate solution, and the supernatant was separated after centrifugation. Powdered whole dry beans (5g) were extracted for sugars in the same way as the germinated samples. An aliquot of the final sugar extract was used to determine glucose by the phenol-sulfuric acid method (Dubois et al., 1956).

Moisture

Samples from germinating beans were weighed and blended with an equal weight of absolute ethanol. The moisture content was determined by drying 5g of the blend in an oven at 70°C for 16 hr under a vacuum of 40 Torr. Powdered dry bean samples (5g) were dried directly in the vacuum oven under the same conditions.

All analyses were made in duplicate.

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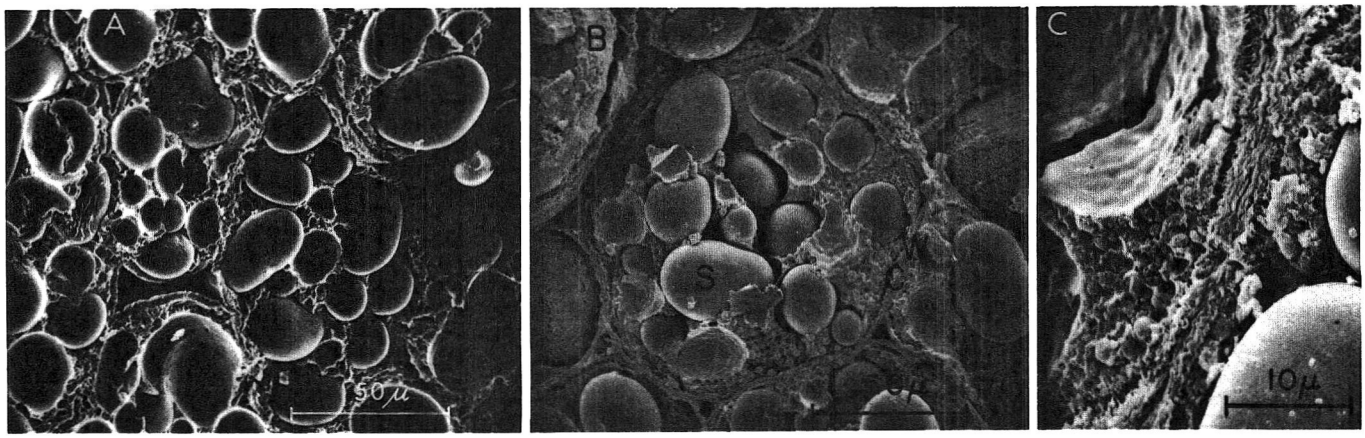

Fig. 1—Cross-section of bean cotyledons: A—starchy cells in dry beans; B—cell in soaked bean: (s) starch granule, (cw) wall; C—cell wall and granules on higher magnification in soaked bean.

RESULTS & DISCUSSION

Starch content and free sugars

During the germination of red kidney beans there was a decrease in starch and an appreciable increase in free sugars expressed as glucose (Table 1). The results suggest that after germination for 10 days, the free sugar content decreased. The phenomenon may be explained by the less availability of starch at that stage. The starch contents obtained here are in good agreement with the results obtained in cotyledons of germinating seeds by Young and Varner (1959) who stated that during germination, the activities of amylase and phosphatase increased manyfold. The amylase activity increased in cotyledons germinated for 6 days and was still high in seeds germinated for 8 days. The amylase activity caused the decrease in starch content during germination.

Table 1—Starch and free sugars during germination of red kidney beans

Period of germination (day)	Moisture (%)	Starch (dry basis, %)	Free sugars as glucose (dry basis, %)
0	55.7	59.1	1.3
2	58.8	46.0	2.2
4	69.2	37.0	3.2
6	90.0	25.6	11.3
8	92.0	10.9	14.1
10	92.0	7.2	11.4

SEM observations on germinated beans

When the samples were fractured the cell walls were ruptured, thus exposing the cell content for SEM study. Figure 1A and 1B show respectively the cotyledon cells from dry and soaked beans. The cotyledon cells were somewhat angular in shape with diameters of about 50–100μ in length and from 10–25μ in width. They did not show furrows or grooves under SEM, but had smooth surfaces with no breaks. On the other hand furrows could be found on granules from dry beans, similar to those seen in air-dried purified Lima beans starch (Hall and Sayre, 1971). Cell walls of adjacent cells in the soaked bean seeds were observed in Figure 1C, measuring about 1.5μ in thickness.

Figure 2A shows the cell contents of a sample on the second day of germination. Fracture of the cotyledons resulted in loss of some material in which the granules were embedded. After germination for 4 days, the loss of the cytoplasmic matrix in the fractured cotyledon is more clearly seen (Fig. 2B) in contrast to the unbroken and smoothly surfaced starch granules which appear to be covered with a film or membrane-like material (Fig. 2C). Probably the membrane-like materials are proteinaceous in nature (McEwen et al., 1974). There were no alterations visible by SEM on the starch granules from seeds germinated for 6 days. Some modifications appeared on the surface of the starch granules after germination for 8 days (Fig. 3A). At this stage, the cell contents were

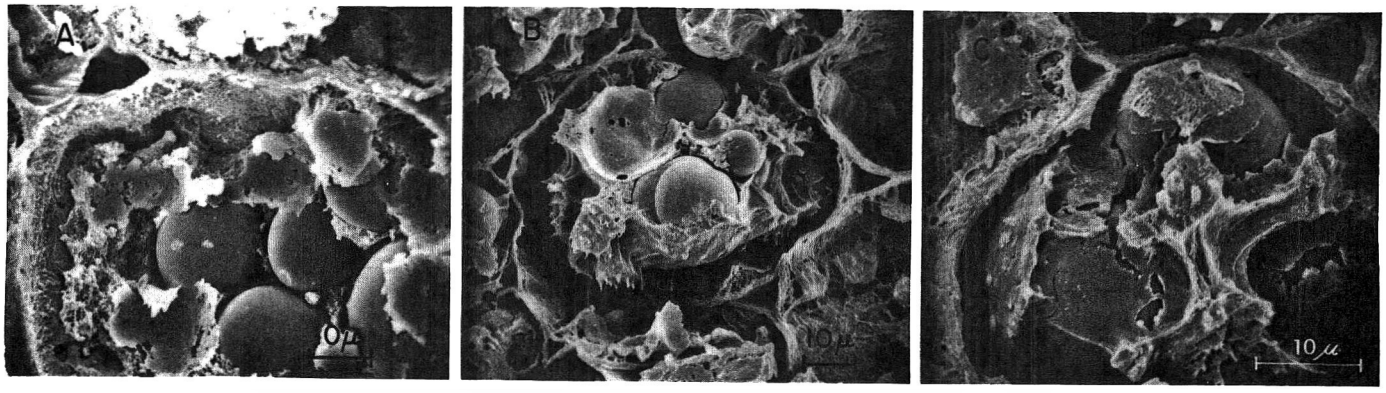

Fig. 2—Bean cotyledons during germination: A—starch granules in beans germinated for 2 days; B-starch granules in beans germinated for 4 days; C—enlarged view of the granules.

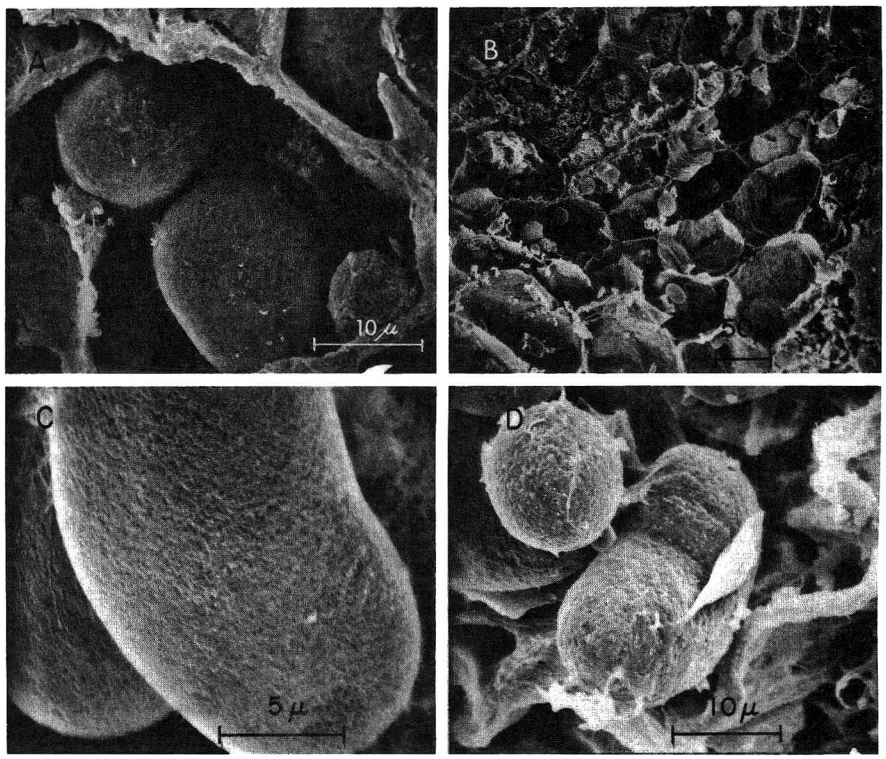

Fig. 3—Bean cotyledons germinated for 8 days: A—modified surface of starch granules; B—the cell contents; C—enlarged view of granule surface. D—alterations on small and large granules.

loosely attached, and were easily detached during fracture of the cotyledons (Fig. 3B). A SEM picture of the surface of the starch granules is presented in Figure 3C. The entire surface of both small and large granules (Fig. 3D) appeared rough and porous when compared to the smooth surface of granules from cotyledons of earlier stages. This observation is quite different from that reported on the enzymatic erosion of starch granules in wheat after germination for 8 days (Dronzek et al., 1972). These authors reported that the small and large granules were differentially attacked at specific sites on the surface.

After germination for 10 days, the starch granules were very porous (Fig. 4A and Fig. 4B). They appeared to be so fragile that irregular fragments were frequently seen (Fig. 4C). Cotyledons germinated for 10 days and cut under liquid nitrogen (Fig. 4D and Fig. 4E) showed the same effects as those which were fractured after critical-point drying. The surfaces of the granules cut under liquid nitrogen showed that alterations occured throughout the entire starch granule, and were

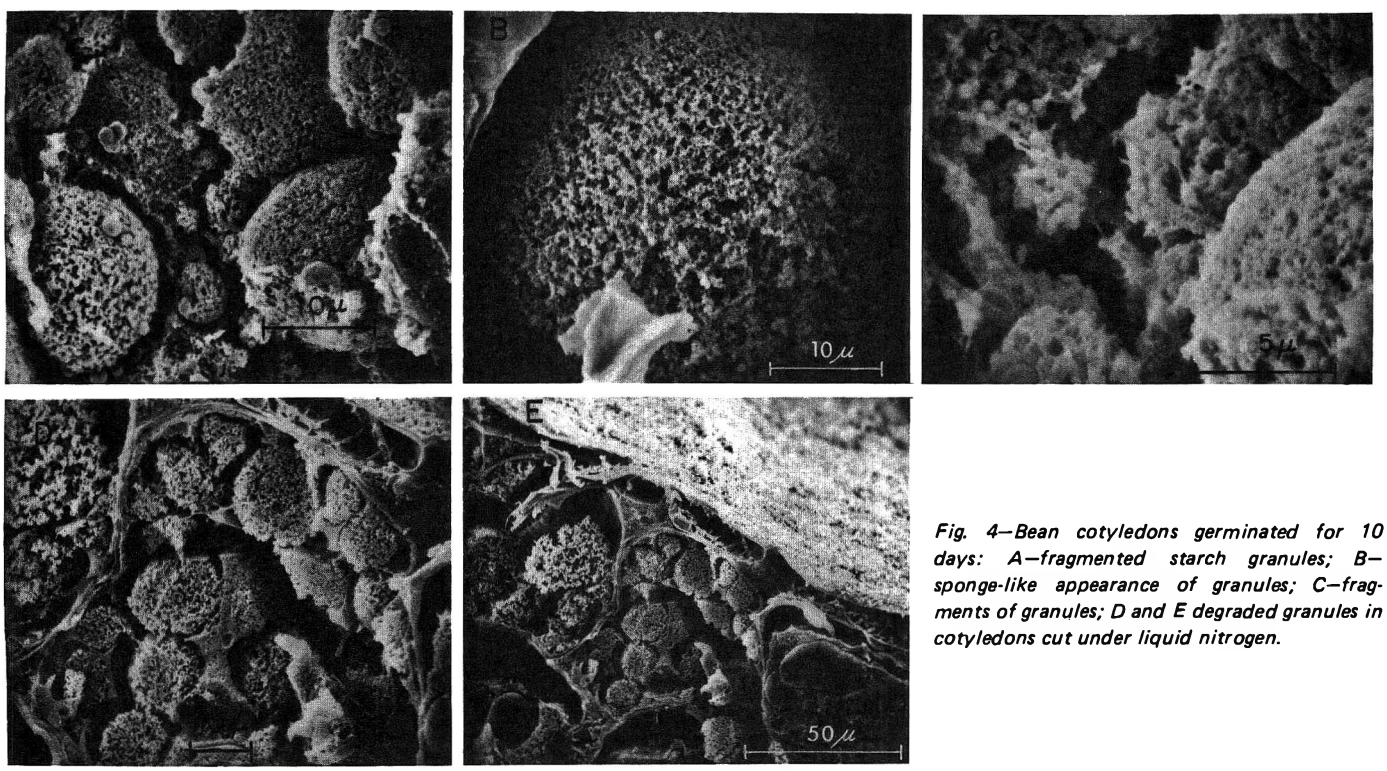

Fig. 4—Bean cotyledons germinated for 10 days: A—fragmented starch granules; B—sponge-like appearance of granules; C—fragments of granules; D and E degraded granules in cotyledons cut under liquid nitrogen.

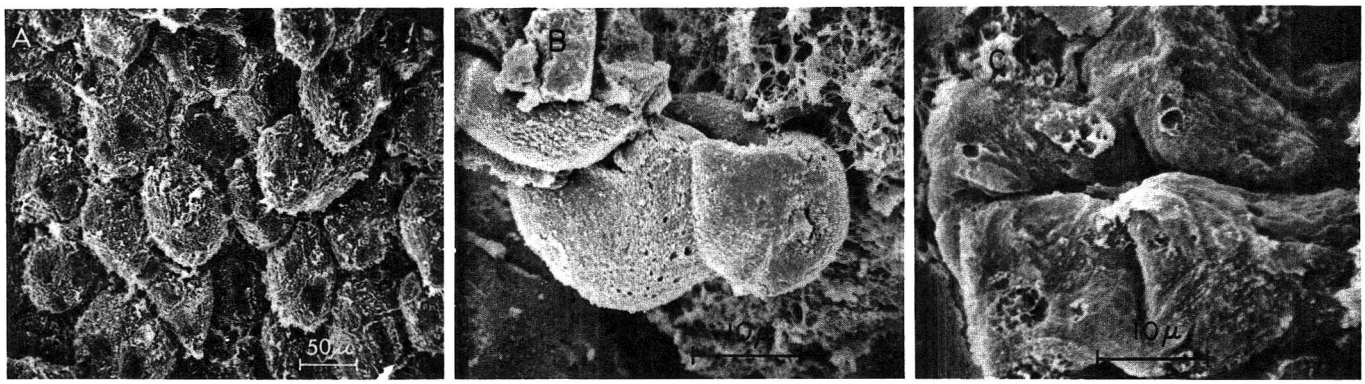

Fig. 5—Cooked bean cotyledons: A—unruptured cells in cooked dry beans; B—modified starch granules in cooked dry beans; C—shapeless granules in beans germinated for 4 days followed by oil-heating.

not confined to the surface. Both methods revealed a few starch granules which had not been attacked.

SEM of cooked beans

Cells from dry beans cooked in boiling water remained intact after fracturing the critical-point-dried cotyledons (Fig. 5A). Occasionally, isolated starch granules were observed which had some porous and flattened surfaces (Fig. 5B). The resistance of cooked bean cells to fracturing was also reported by Rockland and Jones (1974) who worked on air-dried, cooked lima beans.

The appearance of the cells from beans germinated for 4 days, followed by heating in oil at 95°C for 10 min, was the same as those in water-cooked dry beans. The cells from oil-heated beans also kept their integrity, and resisted fracturing. However, occasionally the cells were ruptured, revealing starch granules which had completely lost their basic spherical shape and were agglomerated into an amorphous mass (Fig. 5C).

In summary, starch granules in the cotyledons of red kidney beans showed visible signs of deterioration after germination for 8 days. By the tenth day, the granules were spongy and began to fragment. It is not clear why this starch degradation differed from that in wheat (Dronzek et al., 1972). In wheat, starch degradation started along specific equatorial sites on the larger granules and was followed by degradation of the smaller granules. Our observations have shown no differential effects which would lead us to speculate that the structures of starch granules in red kidney beans and wheat differ. The cooked dry beans, as compared with those germinated, differ not only in the changes noted on starch granules, but also by their resistance to fracture.

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Ms received 9/19/77; revised 4/24/78; accepted 5/5/78

The senior author thanks CAPES, Institution of the Ministry of Education and Culture, Brazil, for a fellowship which made this study possible. The authors also thank Dr. Jack Pangborn of the Facilities for Advanced Instrumentation for his help and advice on this work.

PROTEIN COMPOSITION OF SEEDS OF Lupinus albus

PAOLO CERLETTI, ALESSANDRO FUMAGALLI and DANIELA VENTURIN

ABSTRACT

Proteins were extracted from dehulled white lupine seeds. The extraction yields compare favorably with those from other legumes. Globulins were separated from albumins and were further analyzed by ion exchange chromatography, gel filtration and cellulose acetate electrophoresis. Eight fractions were resolved, all with acidic character. Their mol wt and subunit composition were established.

INTRODUCTION

AT THE PRESENT STATE of agricultural and technological development, leguminous seeds are one of the most conspicuous alternative protein sources for human and animal nutrition. Soya bean is by far the most important among them. Several countries depend upon import for their supply of soya, and this has stimulated lively studies to find a substitute for it.

The high protein content and the adaptability to climatic and soil conditions qualify lupine for this purpose. The original varieties contain some toxic and anti-nutritional factors which restrict their application, but they were significantly improved by breeding, and varieties now available compare well in many aspects, with soya beans (Hudson et al., 1976).

Some of the studies done were aimed at characterizing the seed proteins of lupine. Joubert (1955a, b; 1956) evidenced two globulins in seed extracts from *Lupinus luteus* (yellow lupine) and *L. albus* (white lupine) and three in those from *Lupinus angustifolius* (blue lupine), and determined their electrophoretic and sedimentation behavior. Gerritsen (1956) determined the amino acid composition of the globulins of *L. angustifolius* and *L. luteus*. Gillespie and Blagrove (1975a) separated three globulins, conglutine α, β and γ in *L. angustifolius* as well, and found that the first two are poor in sulfurated amino acids, but the third one, which is the smallest component, has a fair content of it. By comparing different lupine species, these authors (1975b) found that *L. albus* is one of the richest in conglutine γ.

These results indicate that breeding programs for lupine may aim also at improving the content of sulfurated amino acids, which, as known, are limiting in most leguminous seeds. A detailed knowledge of protein composition in lupine species would appear necessary for economic and processing proposals. Work is being done to this end in our laboratory, and the results so far obtained are reported in this paper.

EXPERIMENTAL

ALL CHEMICALS were reagent grade. Biogel A 0.5m, A 1.5m and P 10 were from BioRad; Ultrogel AcA 22, AcA 34 and AcA 54 were from LKB; and Sephadex G 25 from Pharmacia. Standard proteins for molecular weight determinations were obtained from Sigma, namely beef tyroglobulin, Jack Beans urease, beef fibrinogen, beef liver catalase, rabbit muscle aldolase, yeast alcohol dehydrogenase, human transferrin, beef serum albumin, egg albumin, α-chymotrypsinogen, horse heart myoglobin and cytochrome C.

L. albus seeds were of commercial origin.

Total nitrogen was estimated by the AOAC micro-Kjeldahl procedure (1970), soluble proteins by a biuret and a micro-biuret method (Gornall et al., 1949; Itzaki and Gill, 1964).

Cellulose acetate electrophoresis was performed as described by Chin (1970) in phosphate buffer pH 7.0 I = 0.1 or tris barbiturate buffer pH 8.6, I = 0.1, with an applied current of 0.75 mA cm^{-1}. In some cases the continuous electric field was substituted with a pulsing field (Pulsephor electrophoresis, Italian patent Bosi N. 833364), which gives less diffuse bands, and veronal buffer was used as specified in Figure 1. Proteins were stained with Amido Black. Sodium dodecyl sulphate (SDS) electrophoresis on acrylamide gel was according to Shapiro et al. (1967). At the end of the run peptides were stained with Coomassie blue, the gel was scanned at 550 nm and the separated bands were quantitated as per Hsieh and Anderson (1975); their molecular weight was determined according to Osborn and Weber (1969).

Gel filtration columns (2 × 140 cm for Ultrogels, for Sephadex G 25, 5 × 20 cm) were equilibrated and eluted with 0.2M NaCl in 0.05M phosphate buffer pH 7.0. Elution was followed at 280 nm and the eluate was collected in 3 ml fractions. Molecular weight determinations were performed as indicated by Andrews (1965).

Analysis of variance was done according to Lison (1961).

Extraction of proteins

Dehulled lupine seeds (moisture content 8.3%) were blended with grinder mod. 320 of Moulinex, the flour was extracted with pentane for 3 hr in a Soxhlet apparatus, and it was then suspended in distilled water (1:20 w/v) and stirred overnight at 4°C. The suspension was then centrifuged 30 min at 20,000 × G and the supernatant was filtered through gauze and separated. The pellet was resuspended in 1M NaCl in 0.05M phosphate buffer pH 7.0 (1:10 w/v) and stirred 1 hr at 4°C. The suspension was centrifuged as described and the supernatant was collected. When necessary, the precipitate was extracted again twice as described. All suspensions were homogenized with a Virtis homogenizer.

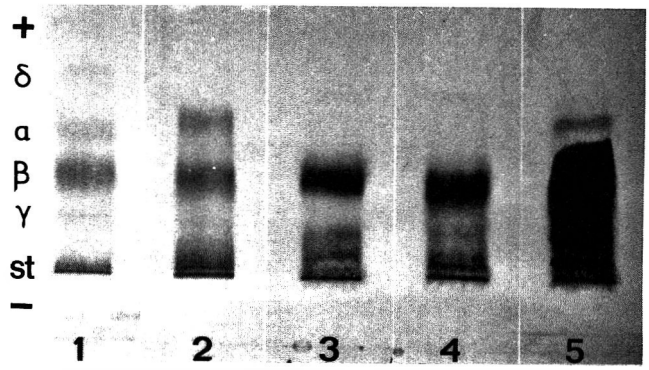

Fig. 1—Pulse electrophoresis on cellulose acetate of saline extracts of leguminous seeds: 1 white lupine, 2 lentils, 3 navy beans, 4 soya beans, 5 chick pea. St = start. Sodium veronal buffer pH 8.6; I = 0.2, 30 min, 20° C.

RESULTS

Protein solubility

The water extract of lupine flour contained 12.8 ± 1.0% of total proteins estimated by total nitrogen determination, and the pH was 5.0. An additional 77.4 ± 1.0% of the total protein was extracted from the sediment with 1M NaCl whereas 7.9% ± 0.7% remained unextracted. If the pH during water extrac-

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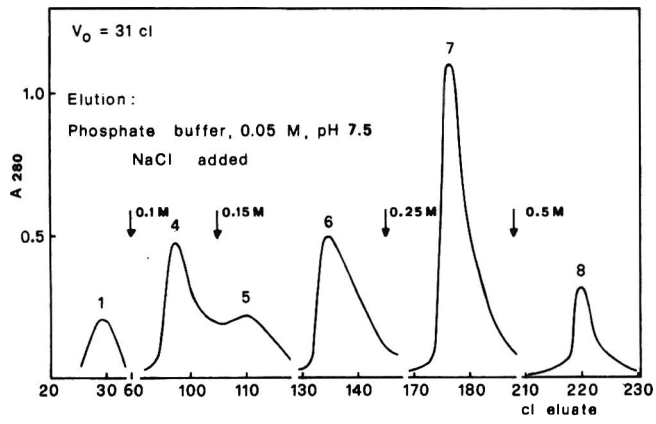

Fig. 2—Separation of the desalted globulin extract of white lupine seeds on Whatman DE 52 cellulose. The column, equilibrated with 50 mM phosphate buffer pH 7.5, was eluted at 20° C with the buffer with stepwise additions of NaCl. Flow rate 100 ml hr^{-1}.

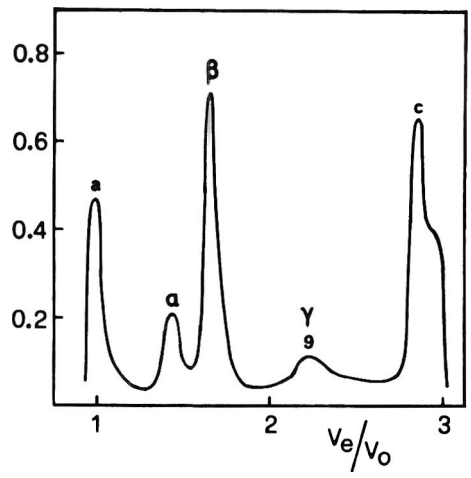

Fig. 3—Separation of the globulin extract of white lupine seeds on a column of Ultrogel AcA 34 equilibrated and eluted with 0.2M NaCl in 50 mM phosphate buffer pH 7.5 at 20° C. Vo = 60 ml. Flow rate 4.8 ml (cm^2)$^{-1}$ hr^{-1}.

tion was maintained at 7.0 by adding 0.1N NaOH, 93.3 ± 1.0% of total proteins were solubilized, and 5.8 ± 0.8% remained undissolved. The solution was not stable and after 18 hr at 4°C only 38.7% ± 1.6% of total was still dissolved. The medium had an ionic strength, calculated by conductance measurements, of about 0.01.

If NaCl were in the initial extraction, it solubilized 94.7 ± 1.1% of the proteins and 4.8 ± 0.6% remained in the residue. The solution was stable at 4°C. Dialysis for 48 hr against distilled water precipitated 83.5 ± 1.0% of the original proteins, 7.2 ± 1.2% being left dissolved. (All were averages on 6 samples). The ionic strength of the dialysate was 10^{-4}.

The albumin fraction soluble after dialysis and proteins extracted at pH 5 showed similar electrophoretic behavior: five bands were separated on cellulose acetate at pH 7.0 and 18 components with molecular weights ranging from 117,000 to 6,000 appeared after SDS electrophoresis. It is likely that not all molecular species are detected in the conditions used.

The globulin proteins in the precipitate after dialysis, the water extract at pH 7, and the saline extract are mutually similar and differ from the albumins. On cellulose acetate electrophoresis they gave four bands, δ, α, β and γ with decreasing anodic mobility. The subunit composition is discussed more in detail in connection with the separated fractions.

For routine work albumins were first separated with water at pH 5.0, then globulins were extracted with buffered 1M NaCl or with water at pH 7.0.

Low ionic strength denatured some of the globulins. Indeed total protein solubilized with water at pH 7 followed by 1M NaCl, was slightly less than when buffered saline was used as first and only extractant: the electrophoretic band γ and DEAE cellulose fraction 1, (see later) remained practically unextracted. Some denaturation also occurred during dialysis and during storage of low ionic strength extracts.

Flours from various leguminous seeds which had not been defatted were extracted once with saline solution as described above. The percent of solubilized proteins and, in parentheses, grams of protein obtained from 100g flour were as follows: *Lupinus albus*, 60% (22g); *Cicer arietinum*, 72% (14g); *Lens culinaris*, 50% (11g); *Glycine max*, 50% (20g); *Phaseolus vulgaris*, 40% (14g). Figure 1 shows the separation of the extracts by cellulose acetate pulse electrophoresis.

Fractionation of the globulins

Ion exchange. The globulin extract was desalted on a column of Sephadex G-25 equilibrated with 0.05M phosphate buffer pH 7.0. This removed also a yellow non amino-acidic compound, mol wt less than 1000, which interferes with the micro-biuret reading: it corresponds to 9.8% of the biuret absorbancy of the extract. The extract was then fractionated on Whatman DE 52 DEAE cellulose. A typical separation is shown in Figure 2.

One component (peak 1) was not retained on the column. A second one (peak 4) was delayed, but was still eluted with the buffer. Three more globulin peaks were separated by increasing salt concentration stepwise, a procedure that in this case gave better results than continuous gradients. Fractions 4 and 5 could not be completely resolved from each other. Fractions 1, 4, 5, 6 and 7 represent respectively 6.4 ± 1.1%, 31.5 ± 3.4%, 5.4 ± 1.2%, 16.1 ± 3.8% and 41.7 ± 1.9% of the eluted proteins (values from 4 determinations). Fraction 8 was not of protein nature. Total recovery ranged between 90 and 95% of the applied extract.

Albumins, when present in the sample applied were eluted with fraction 1.

On cellulose acetate electrophoresis fraction 1 corresponds to band γ and fraction 7 to band α, whereas fractions 4, 5 and 6 all move as band β.

The molecular size distribution within each fraction was determined using appropriate gel filtration media. Before gel filtration the fractions were dialyzed against 0.05M phosphate buffer pH 7.0 and freeze dried. Each yielded a single sharp peak except for fraction 7 which was partially resolved into two components 7_1 and 7_2.

The subunit composition was assayed by SDS electrophoresis for the central tubes of the fractions eluted from the ion exchanger and on the peaks recovered after gel filtration. The mobility and relative amount of the components observed coincided in samples before and after gel filtration except for fraction 7 where the subunits found in peaks 7_1 and 7_2 in part do not correspond either quantitatively or qualitatively to those evidenced in fraction 7. Table 1 summarizes the results of all the determinations mentioned.

Gel filtration. The total globulin extract was also fractionated by gel filtration chromatography. The separation obtained on Ultrogel AcA 34 is shown in Figure 3.

The proteins in some of the peaks show electrophoretic mobilities which correspond to those of bands separated by cellulose acetate electrophoresis of the total globulin extract. These peaks are named after the electrophoretic band which characterizes them. Their elution volumes agree with the molecular weights of the DEAE cellulose fractions that have similar electrophoretic behavior. The small amounts of each component available, the insufficient resolution of the gel filtra-

Table 1—Seed globulins of Lupinus albus

Fraction[a]	Electrophoretic component	Mol wt	Subunit composition: Mol wt and ratio[b]							
1	γ	300,000						37,000$_4$	23,000$_6$	
9	γ	26,500								13,500$_2$
4	β	187,000		63,000			44,500	35,500	24,500	17,000
5	β	225,000		70,000	52,000		44,300	34,500	23,000	
6	β	260,000	73,000		53,000	48,000		40,000	27,000	19,800
7	α		76,000	63,500	56,000	50,000	45,000	39,000	28,700	
								36,500	27,200	
7_1	α	440,000	77,000$_1$	70,000$_1$	58,000$_2$		45,000$_2$		22,800$_4$	
7_2	α	330,000	77,000$_1$	70,000$_1$	59,000$_1$	50,500$_1$	45,500$_1$		22,800$_2$	
	δ									

[a] Fractions 1, 4 and 5 are the gel filtrate of peaks separated by DEAE cellulose chromatography. Fraction 7 is before gel filtration, 7_1 and 7_2 separate from it after gel filtration. Fraction 9 appears only in the gel filtrate of the total globulin extract. Samples analyzed were the central tubes of each peak. Component δ was evidenced only on cellulose acetate electrophoresis and none of the fractions mentioned corresponded to it.

[b] Subscripts give the ratios between subunits, calculated as detailed in the text. Where they do not appear, a 1:1 ratio was found.

tion medium in the range of molecular weights where most components of the total extract fall, and the number of bands therefore evidenced on SDS electrophoresis in each peak, made subunit analysis difficult.

Peak 9, which moved in electrophoresis as band γ, did not correspond to any of the fractions isolated on DEAE cellulose. It was preparatively isolated on Ultrogel AcA 54. Its mol wt and subunit composition are shown in Table 1. It represents 4.7% of the applied proteins.

Peaks a and c do not contain proteins: nucleic acids are probably present in the peak a and compounds with molecular weight less than 1,000 in peak c.

DISCUSSION

ONE SINGLE TREATMENT with saline solution extracted a higher percentage of seed protein from lupine than from other leguminous seeds assayed, except chick pea.

In lupine, albumins form only a minor part of proteins extracted. They appear to contain at least five different species, while Joubert (1955a) evidenced in blue lupine seed only one albumin component.

Globulins are the major component of the extract. Water adjusted to pH 7.0 is a good extractant, but proteins extracted under these conditions gradually precipitate upon storage. In practice, however, this may represent a useful result since it allows the easy recovery of extracted proteins.

Globulins appear to contain at least seven distinct molecular species, which differ in molecular weight, subunit size and distribution as well as in ionization behavior as seen upon electrophoresis and ion exchange chromatography. These characteristics are kept in conditions as different as ion exchange and the subsequent gel filtration; therefore, the fractions isolated likely are real associations existing in the seed, and not occasional aggregates formed during isolation. They are not all recognized on gel filtration of the total extract since in the range of mol wt from 200,000 to 300,000 where five of them fall, the medium has too limited separating capacity to resolve so many species, which make up more than 70% of total proteins.

The behavior in electrophoresis and on the ion exchanger indicated that all components have acidic character, which is weakest in fractions 1 and 9, and which increases in the order of fractions 4, 5, 6, 7_1 and 7_2 and δ.

The electrophoretic bands α, β and γ behave respectively as the conglutins α, β and γ described by Gillespie and Blagrove (1975a) in *Lupinus angustifolius*. Neither of them is however homogeneous: they all contain two or more species, which differ in size, subunit composition and ionic behavior.

When the subunit distribution in each fraction isolated by ion exchange and then gel filtration was assayed, we assumed that these fractions, because of their analytical homogeneity, represented each a single molecular association; therefore, an integer ratio between subunits was expected.

The molar fraction of each subunit was calculated from the ratio between relative amount measured by densitometry of the gel and molecular size indicated by mobility on SDS electrophoresis. In Table 1, subscripts give ratios between molar fractions when they differ from 1:1. For some subunits the calculated ratios were not easily reduced to integers. It is known that the staining characteristics of various proteins and peptides depend upon their binding to Coomassie blue dye (Hsieh and Anderson, 1975); therefore, some scattering of values is not surprising. However, extreme cases, e.g. the high molecular weight subunits of fractions 7_1 and 7_2, developed much weaker color than that expected for a peptide of given molecular size in unitary molar ratio to other subunits. An integer ratio to the other components of the association was nevertheless assumed; the scarce reactivity with Coomassie blue suggests the presence of a non amino-acidic component, which from preliminary assays we suspect to be a carbohydrate. Glycoprotein subunits have been isolated from the globulins of soya bean by Lee et al. (1976) and of navy bean by Ishino et al. (1975).

The data available show that fractions 1, 9, 7_1 and 7_2 are oligomeric associations with repeating subunits of a given molecular weight. The molecular weights calculated for each entire fraction from these association patterns agree well with the values determined directly by gel filtration. In fractions 4, 5 and 6 the sum of molecular weights of the subunits is about equal to the value determined directly on the fraction; therefore, the association cannot contain more than one subunit of each type.

Protein fractions that behave as a unique aggregate and contain a high number of different peptides, several of which have quite similar molecular weights, are not unusual among legumes; vicilin of *Vicia faba*, e.g., it not a uniform protein but a collection of different, although highly similar, molecules (Manteuffel and Scholz, 1975). This may be the case for fraction 7.

Three subunits in fraction 4 have molecular weights identical or differing within the range of the standard error to the corresponding subunits of fraction 5. The apparent fact of three subunits in common in fractions 4 and 5 might be the basis of their very similar ionic behavior. Except for the subunit of mol wt 50,500 in 7_2, all other components in fractions 7_1 and 7_2 have identical molecular weights. Gel filtration only partially resolves fractions 7_1 and 7_2 and their relative proportion vary from run to run. All this suggests that these two fractions may rise from shifts in the aggregation pattern of a same pool of components.

Our findings show that globulins of lupine seed have a more complex composition than had previously been reported.

—Continued on page 1414

SACCHARIDES IN PROSO AND FOXTAIL MILLETS

ROBERT BECKER and KLAUS LORENZ

ABSTRACT

The saccharide composition of 8 samples of proso and foxtail millets was determined. Sucrose was the major saccharide followed by raffinose. Glucose, fructose, and galactose were present only in trace amounts. Myo-inositol was found in small amounts in some of the samples. Maltose and maltotriose were not detected. Autolysis after 16 hr at pH 6.5 and 5.0, respectively, changed the saccharide composition. Sucrose and raffinose values decreased. Maltose values increased with autolysis at pH 6.5, but were much lower with incubation of the samples in a pH 5.0 acetate buffer. The amounts of myo-inositol were higher after autolysis at the lower pH presumably due to hydrolysis of phytic acid by phytase.

INTRODUCTION

MILLETS constitute one important group of food plants in many parts of the world, but especially in regions of rather primitive agricultural practices and high population density. With the exception of the species *Panicum miliaceum*, millet is a relatively minor cereal crop and of little economic importance in the U.S. *Panicum miliaceum* is also called proso millet, hershey, broom corn or hog millet. The species is extensively grown in Russia and central Asia and is probably the ancient millet of the Chinese, perhaps antedating wheat in cultivation (Schery, 1963).

Foxtail millet belongs to the species *Setaria italica*, which also includes varieties such as German millet, Hungarian grass, White Wonder and Siberian millet. At one time these were named varieties, but due to the lack of pure seed sources, these terms now often refer only to seed or plant type, color or shape (Hinze, 1972).

White foxtail millet (*Setaria italica*) is usually grown for hay and forage, proso millet (*Panicum miliaceum*) is used in feeding rations, as birdseeds, and also as human food. Food applications of dehulled proso millet in the U.S. include consumption as a puffed cereal or cooked as a hot breakfast cereal. Pearl millet flour can partially replace wheat flour in certain baked product formulations and other household recipes, imparting a distinct nutlike flavor (Awadalla, 1974; Badi et al., 1976).

Both proso and foxtail millets have been reported to be higher in protein than rice, sorghum, corn or oats (Matz, 1959). They are higher in ash and fiber compared to other cereals used for human consumption (Hinze, 1972; Lorenz and Hinze, 1976). There are few reports in the scientific literature on the nature and composition of the polysaccharides of millets (Casey and Lorenz, 1977). The mono- and oligosaccharides of millets have not been reported.

It was the purpose of this investigation to examine the seeds of different varieties of proso- and foxtail millets for mono- and oligosaccharides.

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MATERIALS & METHODS

Sample identification

Samples selected for this study included six varieties of proso millet: Abarr and Leonard are named varieties grown in Colorado; experimental lines are identified as Black Russian (P.I. 346937), Big Red (P.I. 346946), Akron Proso 73-21-1 (a selection of Common White), and Akron Proso (73-1055), a white type of unknown origin. The foxtail millets used were: Golden German, a variety grown in Colorado, and P.I. 391638, a Chinese line. All varieties were grown at the Central Great Plains Field Station of the Colorado State University Agricultural Experiment Station at Akron, CO.

Chemical analyses

Moisture, protein, ash, fat and phosphorus were determined using AACC (1969) approved methods.

Sugar analysis

Kernels of proso and foxtail millets were milled 30–45 sec in a stainless steel ball mill. Weighed amounts of the powders were mixed with 70% ethanol (v/v) in screw cap vials to give 100 mg/ml, heated at 70–75°C for 1 hr, cooled, and centrifuged. Aliquots of the supernatant were evaporated to dryness under nitrogen at 60°C and silylated overnight at room temperature with shaking using Tri-Sil reagent. The saccharides were separated on a Hewlett Packard 5830A gas chromatograph with dual flame ionization detectors. The unit was equipped with dual 1/8 in. × 6 ft stainless steel columns packed with 3% OV-1 on Chromosorb W (HP 80-100). It was temperature programmed from 180°C to 330°C at 15°C/min (Becker et al., 1977). Sucrose, maltose, maltotriose, and raffinose were identified by their retention times and were quantified by comparison of their peak areas with standard curves constructed using known amounts of sugar. The myo-inositol peak was quantified using known amounts of myo-inositol. The reported results are averages of two or more separate determinations and agreed within 10%.

The GLC separations showed only trace amounts of the monosaccharides glucose, fructose and galactose eluted before sucrose in a complicated series of overlapping peaks; the monosaccharides were not quantified.

Autolysis

Weighed amounts of ball-milled millet powders were mixed with deionized water or 0.1N acetate buffer (pH = 5.0) in screw cap vials to make 100 mg/ml, 2 drops of toluene were added and the vials incubated at 37°C for 16 hr. The water slurries were initially near pH 6.4, but dropped to about pH 5.9 after the 16 hr of incubation. All samples were cooled to room temperature, made to 70% ethanol, centrifuged, and aliquots evaporated to dryness under nitrogen at 60°C. The samples were assayed for their sugar content by GLC as described above. The results are averages of two or more separate determinations.

RESULTS & DISCUSSION

Proximate analysis

The proximate analysis of the millet samples are shown in Table 1. Contents of fat and ash were higher than normally found in several other cereal grains, which agrees with values in the literature (Matz, 1959; Hinze, 1972; Casey and Lorenz, 1977). Protein contents, expressed on a 14% moisture basis, ranged from 9.1% for the foxtail millet Golden German to 12.9% for the experimental line Big Red.

Saccharide composition of ethanol extracts

The monosaccharides glucose, fructose and galactose were present only in trace amounts in all samples of proso- and

foxtail millets. This general absence of free monosaccharides in millets is typical of mature cereal grains (Täufel et al., 1959). Sucrose was by far the main free sugar in both species of millet, as shown in Table 2, occurring in 8 to 10 times greater amounts than the next abundant sugar raffinose. The amounts of sucrose are comparable to amounts found in other grains (MacLecd and Preece, 1954; Becker et al., 1977). Raffinose, however, occurred at only about 1/10 the amounts reported in mature kernels of wheat, rye, and triticale (Becker et al., 1977). Maltose and maltotriose were not detected in any of the samples of proso- and foxtail millet. There was little free myo-inositol. Myo-inositol and inorganic phosphate are the hydrolysis products of phytic acid, a common constituent of cereal grains. Glass and Geddes (1960) isolated myo-inositol from wheat stored at 30°C under nitrogen for 24 wk. The saccharide is not normally found in freshly harvested kernels of other cereal grains.

Table 1—Proximate analysis of millet samples

Species and Variety	Moisture (%)	Fat[a] (%)	Protein[a] (%)	Ash[a] (%)
Proso millets:				
Abarr	7.9	3.7	11.6	2.61
Leonard	7.3	3.1	10.7	3.39
Big Red (P.I. 346946)	6.9	4.0	12.9	3.79
Black Russian (P.I. 346937)	7.7	4.7	12.9	3.07
Akron Proso (73-21-1)	8.2	3.4	9.8	2.76
Akron Proso (73-1055)	9.1	4.7	12.2	3.26
Foxtail millets:				
Golden German	7.0	3.6	9.1	2.67
Chinese (P.I. 391638)	7.4	3.8	11.4	2.97

[a] 14% moisture basis; Protein = (N X 6.25)

Saccharide composition after autolysis

Methods involving extracts with water yielded different and appreciably higher saccharide results in spite of precautions designed to prevent amylase action during extraction of samples of wheat for saccharide determinations (D'Appolonia et al., 1971). Such methods provide an indication of indigenous saccharidase activity of a grain sample and were applied to the millet samples. The free carbohydrate composition of millets was markedly altered by incubation in the presence of water (initial pH 6.5) and acetate buffer (pH 5.0), respectively, as shown in Table 3. Incubation with water caused the indigenous enzymes to hydrolyze starch to maltose and maltotriose, and sucrose to glucose and fructose. Because of the higher amounts of sucrose and the lower amounts of maltose noted in proso millets, it is apparent that amylase and invertase activity was markedly less in the proso millets than in the foxtail millets. The possible presence of enzyme inhibitors in the proso millets would be an alternate explanation. After autolysis, sucrose was completely destroyed in the foxtail millets, but only partially hydrolyzed in the proso millets. The decreases in raffinose content after autolysis were presumably also due to invertase action.

Autolysis at pH 6.5 produced higher amounts of myo-inositol compared to extraction with ethanol. The largest amounts were produced at pH 5.0, however, the pH optimum for phytase (Table 3). Invertase activity was enhanced at pH 5.0 and hydrolyzed most of the sucrose and much of the raffinose. The production of di- and trisaccharides by amylase was reduced at that pH compared to autolysis at pH 6.5.

—Text continued on page 1414

Table 2—Saccharides in millets: Percent, dry weight basis[a] (Extraction with 70% ethanol)

Species	Variety	Sucrose	Maltose	Maltotriose	Raffinose	Myo-inositol
Proso Millets —	Abarr	0.66	0	0	0.10	0.01
	Black Russian	0.48	0	0	0.06	0
	Leonard	0.58	0	0	0.12	0.01
	Akron Proso (73-21-1)	0.60	0	0	0.10	0
	Akron Proso (73-1055)	0.72	0	0	0.08	0.01
	Big Red	0.90	0	0	0.04	0
Foxtail Millets —	Golden German	1.12	0	0	0.08	0.01
	Chinese	0.90	0	0	0.12	0.01

[a] Averages of two or more separate determinations.

Table 3—Saccharides in Millets: Percent, dry weight basis (After autolysis at pH 6.5 and 5.0, respectively)[a]

Species	Variety	Autolysis pH	Sucrose	Maltose	Maltotriose	Raffinose	Myo-inositol
Proso —	Abarr	6.5	0.14	0.37	0.04	0.05	0.09
		5.0	0.01	0	0	0	0.20
	Black Russian	6.5	0.33	1.39	0.20	0.07	0.05
		5.0	0.01	0	0	0.03	0.25
	Leonard	6.5	0.24	0.31	0.04	0.08	0.06
		5.0	0.04	0.03	0	0	0.24
	Akron Proso (73-21-1)	6.5	0.26	0.17	<0.01	0.05	0.08
		5.0	0.02	0	0	0	0.24
	Akron Proso (73-1055)	6.5	0.30	0.32	0.03	0.07	0.13
		5.0	0.02	0	0	0	0.22
	Big Red	6.5	0.38	1.25	0.13	0.03	0.02
		5.0	0.01	0.01	0	0	0.22
Foxtail —	Golden German	6.5	0	2.06	0.24	0.05	0.21
		5.0	0	1.13	0	0	0.24
	Chinese	6.5	0	2.09	0.44	0.08	0.16
		5.0	0	0.38	0	0	0.27

[a] Extraction with water (pH 6.5 and with 0.1N acetate buffer (pH 5.0), respectively. Data are averages of two or more separate determinations.

Table 4—Phytic acid in Millets[a]

Species	Variety	Calc from Phosphorus Detms ($P_{Total} - P_{inorg.}$)	Calc from Myo-Inositol Values (after pH 5 Autolysis)
Proso —	Abarr	1.45	1.21
	Black Russian	1.75	1.50
	Leonard	1.61	1.44
	Akron Proso (73-21-1)	1.72	1.45
	Akron Proso (73-1055)	1.56	1.34
	Big Red	1.45	1.31
Foxtail —	Golden German	1.88	1.62
	Chinese	1.86	1.43

[a] Expressed in mmoles.

There was general agreement on the amount of phytic acid (Table 4) as calculated from phosphorus determinations ($P_{total} - P_{inorg.}$) or from the myo-inositol values after pH 5 autolysis. The results are comparable to the amount of phytic acid found in other cereals.

CONCLUSIONS

GLUCOSE, fructose, and galactose were present in only trace amounts in 70% ethanol extracts of mature kernels of millets. Sucrose was the major sugar followed by raffinose. Maltose and maltotriose were not detected. Myo-inositol was present in small amounts in some of the millet samples.

Autolysis at pH 6.5 and 5.0, respectively, changed the saccharide composition. Sucrose and raffinose values decreased. Maltose values increased with autolysis in water, but decreased again with autolysis of the samples in the pH 5.0 acetate buffer. The amounts of myo-inositol increased with decreasing pH of the autolysis solution. Phytic acid was present in amounts typical of cereal grains.

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Ms received 1/20/78; revised 4/21/78; accepted 4/25/78.

The authors thank G. Hinze, Department of Agronomy, Colorado State University, Fort Collins, CO, for supplying samples of millets, and from WRRC Ok-Koo K. Grosjean for technical assistance in conducting some of the experiments and G.E. Secor for phosphorus determinations.

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PROTEIN COMPOSITION OF L. albus SEEDS . . . From page 1411

Their fractionation allowed further interesting insights; namely that fair amounts of sulfurated amino acids were found (Unpublished data) not only in fraction 1, i.e. in conglutine γ, which corresponds to less than 6% of total protein, but also in fraction 7, which contains more than 41% of total protein, a result which reveals stimulating nutritional possibilities.

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Ms received 8/19/77; revised 2/21/78; accepted 2/28/78.

This research was supported in part by contracts 76.00085.76.115.-0950 and 77.00085.76.115.0950 of the Italian National Research Council (C.N.R.) program: New Protein Foods.

DIFFERENCE IN POLYGALACTURONASE COMPOSITION OF CLINGSTONE AND FREESTONE PEACHES

R. PRESSEY and J. K. AVANTS

ABSTRACT

The composition of polygalacturonase activity and the solubility of pectin were compared in clingstone and freestone peaches. At the unripe stage, all varieties of both types of peaches had low levels of water-soluble pectin and virtually no polygalacturonase activity. Ripe clingstone peaches had exopolygalacturonase activity and essentially insoluble pectin. Ripe freestone peaches had exopolygalacturonase and endopolygalacturonase activity and high levels of water-soluble pectin. These differences in composition of polygalacturonases may account for the markedly different textural characteristics of the two types of peaches.

INTRODUCTION

RIPENING CLINGSTONE PEACHES soften considerably less than do freestone varieties. This difference in texture has been explained in terms of pectin composition (Nightingale et al., 1930). Ripening in peaches is accompanied by conversion of protopectin to soluble pectin, but pectin solubilization is much less pronounced in clingstone than in freestone fruit (Postlmayer et al., 1956; Sterling and Kalb, 1959; Shewfelt, 1965). If the greater retention of firmness of clingstone peaches is due to less degradation of protopectin, the two types of peaches may differ in the nature and quantity of their pectic enzymes.

Because pectolytic enzymes in higher plants are specific for deesterified polygalacturonans (Pressey, 1977), the first step in pectin degradation probably involves pectinesterase. Sterling and Kalb (1959) found that, during ripening, there was a continual decrease in the content of methyl esters in the pectin of Elberta peaches. Shewfelt et al. (1971) fractionated the pectin from four varieties of freestone peaches and showed that deesterification with advancing ripeness was maximum in the protopectin. But pectinesterase is present in peaches at all stages of development and ripeness, and the amount does not differ greatly between clingstone and freestone varieties (Shewfelt, 1965). The level of this enzyme, therefore, does not appear to be the controlling factor in pectin solubilization.

Deesterified blocks of galacturonic acid residues in the pectin chain produced by pectinesterase render the chain susceptible to degradation by polygalacturonase. Pressey et al. (1971) detected polygalacturonase activity in ripe peaches. The activity in freestone peaches is due to a combination of endopolygalacturonase and exopolygalacturonase (Pressey and Avants, 1973a). Endopolygalacturonase cleaves the pectate chain in a random fashion and effectively reduces its molecular size. In contrast, exopolygalacturonase removes monomer units from the nonreducing end of the substrate chain with a minimal effect on the macromolecule. To help understand the difference in pectin solubility of clingstone and freestone peaches, we have examined a number of varieties of each type of peach to compare the levels of endo- and exopolygalacturonase.

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MATERIALS & METHODS

Sources of fruit

Peaches were obtained from the University of Georgia Horticulture Farm, Watkinsville, and the Southeastern Fruit and Tree Nut Research Station, Byron, GA, in 1976. Peaches from each lot were tested for firmness, and the remainder was frozen and then stored in polyethylene bags at −23°C.

Determination of firmness

Two Magness-Taylor pressure testers for the pressure ranges of 0–10 and 0–30 pounds and equipped with 5/16 inch tips were used to measure peach firmness. Six peaches were randomly taken from each lot, and readings were recorded for both cheeks of each peach after the skin was removed with a sharp knife. The mean of the 12 readings represented the average firmness of each lot.

Substrates

For purification of sodium pectate (Sigma Chemical Co.), a 1% aqueous solution was precipitated with three volumes of 95% ethanol. The precipitate was collected on Miracloth, squeezed dry, and redissolved in water. The pectate was precipitated with ethanol two more times, washed with 95% ethanol and then acetone, and dried in vacuo over Drierite.

To prepare polygalacturonate, we treated the purified pectate with fungal pectinase (Sigma Chemical Co.). 20 mg of pectinase dissolved in 10 ml of 0.2M NaCl were added to 2 L of 1% pectate at pH 5 and 35°C. The solution was immediately placed on a hot plate and heated to the boiling point (approximately 15 min). It was cooled and adjusted to pH 2 by addition of HCl. The precipitated polygalacturonate was collected by centrifugation and dried by washing with ethanol and acetone (Pressey and Avants, 1971). The degree of polymerization of this substrate was 79.

Assay for polygalacturonase in water-washed cell-wall residues of peaches

Six peaches from each lot were thawed, peeled, and sliced; 100g of slices in about equal amount from each peach was added to 100 ml of cold water containing 12% Carbowax 4000 and 0.2% sodium bisulfite (pH 5). The suspension was blended for 2 min in a VirTis homogenizer, then centrifuged at 8000 × G for 20 min. The supernatant solution was discarded, and the residue was washed twice with 200 ml cold water by homogenizing and centrifuging each time. The insoluble fraction was finally suspended in 100 ml of cold 0.5M NaCl.

The reaction mixture for endopolygalacturonase consisted of 0.4 ml of 0.2M acetate (pH 4), 1 ml of peach residue suspension, and 1 ml of 1% polygalacturonate (pH 4) in a 17 × 100 mm Corex round-bottom centrifuge tube. The reaction for exopolygalacturonase consisted of 0.4 ml of 0.2M acetate (pH 5.5), 1 ml of peach residue suspension, 0.1 ml of 0.02M $CaCl_2$, and 1 ml of 1% polygalacturonate (pH 5.5). The substrate solutions were prepared by dissolving polygalacturonate in water and adjusting the pH with dilute HCl or NaOH. We prepared blanks for both assays by heating reaction mixtures in boiling water for 3 min before adding the substrate. Chloramphenicol and Acti-dione (Sigma Chemical Co., 100 μg of each dissolved in 0.1 ml water) were added to prevent microbial growth. The samples were incubated at 30°C and shaken to keep the residues in suspension. After 16 hr, the samples were heated for 3 min, cooled, and centrifuged. The supernatants were analyzed to measure the number of reducing groups by the arsenomolybdate method (Nelson, 1944). A unit of polygalacturonase activity is defined as that amount which catalyzes the formation of 1 μmole of reducing groups in 16 hr.

Assay for polygalacturonase in ultrafiltered extracts of peaches

All of the steps of this procedure were conducted at low temperature with cold solutions. One kilogram of peach slices was added to 1

liter of water containing 0.2% bisulfite (pH 5). The tissue was homogenized with a Polytron (Brinkmann Instrument, Inc.) set at high speed for 2 min. The homogenate was centrifuged at 8000 × G for 20 min, and the supernatant was discarded. The residue was washed twice by homogenizing and centrifuging; 2 liters of water containing 0.2% sodium bisulfite were used each time. The washed residue was suspended in 2 liters of 0.2M NaCl, and the suspension was adjusted to pH 5 by addition of 1N NaOH and stirred for 3 hr. The suspension was centrifuged at 8000 × G for 20 min, and the supernatant was concentrated to about 60 ml by ultrafiltration by use of an Amicon Corp. model 2000 UF cell with a PM-10 membrane and 20 psi pressure. The concentrated extract was dialyzed overnight against 0.2M NaCl.

The reaction mixture for endopolygalacturonase consisted of 0.5 ml of 0.2M acetate buffer (pH 4) 0.5 ml peach concentrate and 1 ml 1% polygalacturonate (pH 4). The reaction mixture for exopolygalacturonase also included 0.1 ml 0.02M $CaCl_2$, and the buffer and substrate solutions were at pH 5.5. We prepared blanks by heating duplicate samples in boiling water for 3 min before adding the substrate. After 1 hr at 37°C, the samples were heated for 3 min and analyzed for reducing groups by the arsenomolybdate method. A unit of polygalacturonase activity is defined as that amount which catalyzes the formation of 1 μmole of reducing groups in 1 hr.

In the viscometric method for measurement of polygalacturonase, 5 ml of 1% pectate (pH 4) and 4 ml of 0.15M NaCl were mixed with 1 ml of concentrated peach extract. After 1 hr at 37°C, 5 ml of the solution was transferred to an Ostwald viscometer immersed in a water bath at 37°C and the flow time was measured. The results are expressed as relative viscosity, with the flow time for the reaction mixture without enzyme equal to 1.0 and the flow time for water equal to 0.

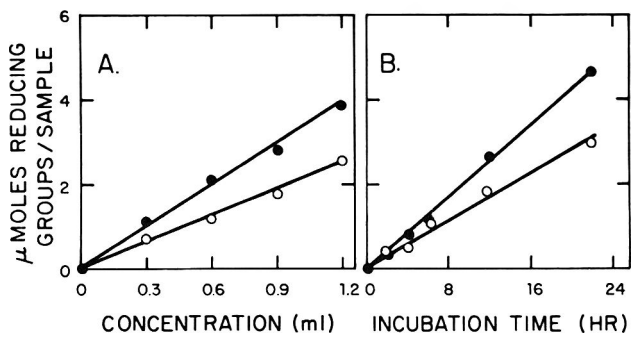

Fig. 1—Effects of enzyme concentration (A) and incubation time (B) on the rate of reducing group formation by peach endopolygalacturonase (●——●) and exopolygalacturonase (○——○). The enzyme was a suspension of water-washed cell-wall residue from ripe Red Globe peaches. In graph A, the incubation time was 16 hr. In graph B, the amount of cell-wall suspension was 1 ml per sample. All other assay conditions were standard.

Table 1—Polygalacturonase activity in suspensions of water-washed cell-wall residues from peaches

Variety	Ripeness	Stone Freeness	Firmness (lb)	Polygalacturonase Endo (units/ml)	Polygalacturonase Exo (units/ml)	Pectin Soluble (% AGA)	Pectin Total (% AGA)
Baby Gold 6	unripe	cling	12	0.1	0	5.8	30
Baby Gold 6	ripe	cling	2.7	0.2	3.8	8.8	34
Baby Gold 7	ripe	cling	5.0	0.5	4.6	6.7	32
Baby Gold 8	ripe	cling	4.3	0.3	3.2	7.7	27
Piedmont Gold	ripe	cling	6.6	0.2	3.7	6.5	32
Mountain Gold	unripe	cling	15	0	0.1	5.8	33
Mountain Gold	ripe	cling	2.5	0.6	3.5	7.4	30
Suncling	unripe	cling	17	0	0	6.6	32
Suncling	ripe	cling	3.0	0.2	4.7	8.1	28
Red Globe	ripe	free	0.7	4.0	3.2	18.3	29
Summer Gold	unripe	free	12	0	0.4	6.3	31
Summer Gold	ripe	free	0.4	4.7	3.7	18.0	28
Fay Elberta	unripe	free	17	0.1	0	5.3	32
Fay Elberta	ripe	free	0.8	3.7	3.3	17.2	28
Sullivan Elb.	ripe	free	0.6	3.3	2.4	17.0	33
Cornet	ripe	free	0.6	4.5	2.2	15.5	24
Blake	unripe	free	20	0	0.1	3.5	27
Blake	ripe	free	0.5	6.2	2.8	13.9	25

Pectin analyses

Alcohol-insoluble solids were prepared by adding 100g of peach tissue (approximately equal amounts from 6 peaches) to 430 ml of boiling 95% ethanol. After being refluxed for 20 min, the sample was cooled, homogenized in a VirTis blender, and filtered. The residue was extracted with 250 ml of 80% ethanol by 20 min of refluxing and then filtered. It was washed with 95% ethanol and acetone and dried under vacuum.

The amount of water-soluble pectin was determined by extracting 0.1g of the alcohol-insoluble solids with 50 ml of water at 25°C for 2 hr. The insoluble material was removed by centrifugation and then washed with water. The anhydrogalacturonic acid (AGA) content of the combined supernatants was measured by the carbazole method (Rouse and Atkins, 1955) with galacturonic acid (CalBiochem) as the standard.

Total pectin content was determined by the method of McCready and McComb (1952). Alcohol-insoluble solids (0.1g) were suspended in 50 ml of 0.5% EDTA. The pH was adjusted to 11.5 and held constant for 30 min at 25°C. The mixture was then acidified with 2N HCl to pH 5 and treated with 10 mg fungal pectinase dissolved in 2 ml 0.1M NaCl. After 1 hr at 25°C, the sample was centrifuged and analyzed for AGA as above.

RESULTS & DISCUSSION

THE LEVEL of pectolytic activity in peaches is relatively low (Pressey et al., 1971; Pressey and Avants, 1973a); in comparison, the activity in ripe tomatoes is approximately 50 times as high (Pressey and Avants, 1973b). In addition to the low pectolytic activity, the high concentration of pectin that accompanies the pectic enzymes in peaches presents a problem. These factors preclude the use of conventional methods for extracting and assaying peach enzymes. Therefore, we used the two previously developed methods, which are based on the inextractability of pectic enzymes in water and allow tissue homogenates to be washed with cold water to remove some of the pectin along with other soluble components. The activity in the water-washed cell-wall residues can then be assayed by adding suspensions of the residue to reaction mixtures and incubating for long periods (16 hr). An alternative method is to solubilize the enzyme with 0.2M NaCl and then concentrate it by ultrafiltration.

The objective of this study was to measure not the total pectolytic activity but the levels of endopolygalacturonase and exopolygalacturonase. Two important differences between the enzymes make it possible to measure them individually without resorting to physical separation. First, the pH optima for the endo- and exoenzymes are 4.0 and 5.5, respectively. Second, divalent cations do not affect the activity of the endoenzyme whereas Ca^{2+} is necessary for the activity of the exoenzyme (Pressey and Avants, 1973a). By adjusting the pH and the Ca^{2+} concentration, the two enzymes can be measured with minimal interference from each other, and the assays can be conducted with either peach cell-wall residues or ultrafiltered extracts.

In water-washed cell-wall residues, the rates of reaction for both endopolygalacturonase and exopolygalacturonase were linear with respect to enzyme level and reaction time (Fig. 1). We used the 16 hr incubation period for this assay so that absorbance readings with the arsenomolybdate method for measuring reducing groups would be reasonably high despite the low enzyme activity in peaches. Overnight incubation of the samples enabled us to begin analyses of peach extracts in the afternoon and complete them the next morning.

Ripe fruit of each of 6 varieties of clingstone and 6 varieties of freestone peaches was assayed for the polygalacturonases according to the cell-wall residue method and analyzed to determine the amounts of water-soluble pectin and of total pectin (Table 1). Some of the varieties were also analyzed at the mature but unripe stage. The proportion of pectin in the

water-soluble form was low in unripe fruit of both types of peaches. On ripening, the freestone peaches softened considerably more than did the clingstone varieties. The soluble fraction of pectin increased sharply in freestone peaches but remained low in clingstones. The results of the changes in pectin content confirm published findings (Postlmayer et al., 1956; Sterling and Kalb, 1959; Shewfelt, 1965). Very low polygalacturonase activity was detected in unripe fruit of either clingstone or freestone peaches. Enzymatic activity was present in ripe fruit of all varieties studied, but the two types of peaches were clearly differentiated by their polygalacturonase compositions. All freestone varieties contained both endopolygalacturonase and exopolygalacturonase, generally with higher levels of the former; the clingstone varieties had much lower levels of endopolygalacturonase activity and high levels of exopolygalacturonase activity.

The difference in polygalacturonase composition of clingstone and freestone peaches was confirmed by studying the enzyme activity in ultrafiltered extracts of representative varieties (Table 2). Extracts of clingstone peaches contained exopolygalacturonase but very low amounts of endoenzyme. The ultrafiltered extracts of freestone peaches contained both enzymes, as did cell-wall residues. The ratio of exoenzyme to endoenzyme, however, was higher in the extracts than in the residues. This difference might be explained by the high amount of pectin in the ultrafiltered extracts. The prewashing of peach cell-wall fragments with cold water before the extraction of the polygalacturonases removes some of the pectin, and the 0.2M NaCl used to extract the enzymes solubilizes additional pectin. The pectin contents of ultrafiltered extracts of Suncling and Fay Elberta peaches determined as anhydrogalacturonic acid, were 3.7 and 3.2 mg/ml, respectively. The high pectin content of the extracts was also evident from their high viscosities (data not shown). The effect of peach pectin on the enzymes was not determined, but such high levels of pectin might inhibit one enzyme more than the other.

Ultrafiltered extracts of Fay Elberta and Suncling peaches were also analyzed by gel filtration (Fig. 2). Polygalacturonase activity in the fractions was measured by the reductometric method at pH 4 and pH 5.5 (in the presence of Ca^{2+}) and the viscometric method at pH 4. Two peaks of polygalacturonase activity, as measured by the reductometric method, were found in the fractions from the Fay Elberta extract. Only the second peak was detected viscometrically, indicating the presence of both endopolygalacturonase and exopolygalacturonase in this extract. Assay of the fractions from the Suncling extract showed the presence of a single peak of activity corresponding to exopolygalacturonase. Some activity was evident when the fractions were analyzed at pH 4; likely, however, the activity was due not to endopolygalacturonase but to the exopolygalacturonase, which was weakly active at that pH. This also suggests that the low polygalacturonase activity measured in water-washed cell-wall residues of clingstone peaches may be due to exopolygalacturonase at pH 4 rather than endopolygalacturonase.

An explanation for the textural difference between clingstone and freestone peaches has long been sought. Addoms et al. (1930) proposed that ripe freestone peaches are distinguished by having thinner cell walls, weaker cellular coherence in the hypodermal tissue, and more extensive cell-wall breakdown than do ripe clingstone peaches. Reeve (1959) confirmed that the loss of cellular coherence is more pronounced in freestone than in clingstone varieties but disagreed on the other two features. He found that cell-wall thickness decreased to the same extent in both types of peaches but could not find evidence for rupturing of parenchyma cell walls in freestone peaches, even in overly ripe fruit that contained extremely thin cell walls.

In addition to the structural changes, peach ripening is accompanied by conversion of protopectin to soluble pectin, a

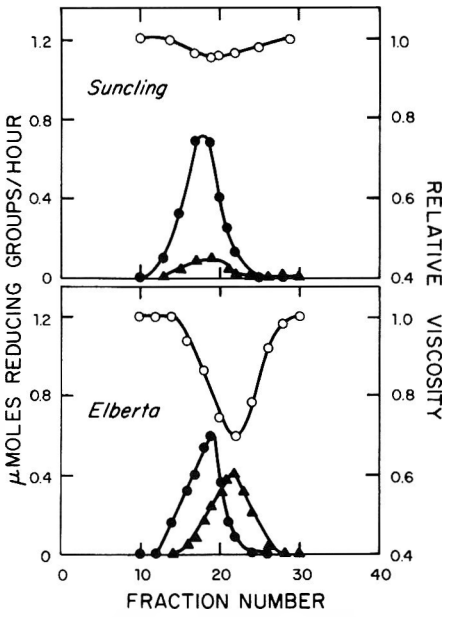

Fig. 2—Chromatography on Sephadex G-100 of ultrafiltered extracts from ripe Suncling and Fay Elberta peaches; 10-ml aliquots of the extracts were applied to a 2.5 X 90 cm column of the gel equilibrated with 0.15M NaCl. Elution was conducted with 0.15M NaCl and 10 ml fractions were collected. ○—○, viscometric assay for polygalacturonase at pH 4; ●—●, reductometric assay at pH 5.5 in the presence of 0.5 mM $CaCl_2$; ▲—▲, reductometric assay at pH 4.

Table 2—Polygalacturonase activity in ultrafiltered extracts of peaches

Variety	Stone freeness	Polygalacturonase Endo units/ml	Exo
Baby Gold 6	cling	0.2	3.0
Mountain Gold	cling	0.2	2.6
Suncling	cling	0.2	4.2
Fay Elberta	free	4.6	4.8
Sullivan Elberta	free	4.0	3.8

transformation that is more extensive in freestone than in clingstone peaches and probably involves the release of soluble fragments by the action of polygalacturonase. Evidence supporting a role for polygalacturonase in peach softening is the absence of activity in unripe peaches and its appearance during ripening (Pressey et al., 1971).

The results of this study indicate that the difference in pectin solubilization between clingstone and freestone peaches is not necessarily due to differing levels of total polygalacturonase activity but rather to the enzyme composition. Freestone peaches contain both endo- and expolygalacturonase. Because endopolygalacturonase attacks the linear polygalactironase chains of proptopectin in a random fashion, it can degrade the macromolecular structure very effectively. Pectin is a major component in peach cell walls and middle lamellae, and its destruction should lead to the weakening of the cell walls and separation of cells observed in freestone peaches. Presumably, the endopolygalacturonase functioning alone could effect pectin solubilization in freestone peaches, but the exoenzyme may also be involved.

Clingstone peaches, in contrast, contain high levels of exopolygalacturonase and very low levels or no endopolygalacturonase. The apparent absence of the endoenzyme is consistent with the high retention of protopectin in clingstone peaches.

—Continued on page 1423

VITAMIN ASSAY BY MICROBIAL AND PROTOZOAN ORGANISMS: RESPONSE TO VITAMIN CONCENTRATION, INCUBATION TIME AND ASSAY VESSEL SIZE

M. N. VOIGT, R. R. EITENMILLER and G. O. WARE

ABSTRACT

Standard vitamin calibration curves for the microbiological determination of thiamine, riboflavin, vitamin B_6, vitamin B_{12}, biotin, niacin and pantothenic acid were prepared to determine the minimal and maximal vitamin concentrations that may be assayed by various microorganisms and protozoa, and to determine the incubation periods required for the growth response of the microbial vitamin assays to stabilize. The effect of assay vessel size on growth of *Tetrahymena pyriformis* was also determined. Vitamin assays using *Tetrahymena pyriformis* were shown to have wider concentration limits than those of bacteria and yeast. Analyses based on *Ochromonas danica* and *Ochromonas malhamensis* possessed approximately equal vitamin concentration limits when compared to corresponding bacterial assay methods. Accepted vitamin assay methodology using microorganisms terminate vitamin assays when the growth response has stabilized. This study indicated that some test organisms such as *Lactobacillus viridescens*, (thiamine), *Lactobacillus casei* (riboflavin) and *Saccharomyces uvarum* (vitamin B_6) do not achieve a point of stable response, thus requiring a defined incubation period. *Tetrahymena pyriformis* growth was depressed by use of small diameter assay vessels. Optimum response of this organism requires the use of flasks in which assay media, when dispensed, will have a large surface to volume ratio.

INTRODUCTION

MICROBIOLOGICAL METHODS for quantitating vitamins are based on the observation that a given microorganism can reproduce only in the presence of a specific vitamin. When an aliquot of the sample containing the vitamin being assayed is added to the initially clear assay medium followed by inoculation with the test organism, the organism grows in proportion to the vitamin content. Growth response can be measured photometrically or the accumulation of metabolic products can be measured (acids or CO_2). Over a certain concentration range, the measured response will be directly proportional to the amount of vitamin present, and, within this range, the sample and reference solutions can be compared accurately. As a group, the lactobacilli are most widely employed as test organisms. Although yeasts, molds and protozoa are used, their growth characteristics are often less suitable.

A quantitative vitamin assay depends on the relationship between the vitamin dosage and the test organism response. As the dose is increased from that which gives no effect to one giving a maximal effect, the response increases continuously in a sigmoid dosage-response curve. No statistical techniques have been developed for handling a sigmoid curve directly that are as simple as those for a straight line. As a result, development of computer programs to evaluate data from vitamin analyses requires the redefining of the dosage and response units to transform the sigmoid curves into straight lines. The equation describing the transformed dosage-response curve is known as the regression equation and its slope and intercept are known as the regression coefficients. The sampling error is computed from the variation in the assay response. A computer program has been developed to conduct these calculations (Voigt, 1977).

Analyses of the B-vitamins using protozoa yield data that accurately represent the B-vitamin activities relative to mammalian metabolism (Baker and Frank, 1968). This is due to the more mammalian-like nutrient requirements of the protozoa vs bacteria and yeast. If there are significant differences in the vitamin contents indicated by the protozoan and the conventionally used methods of vitamin analyses, the accuracy of data in current food composition tables and on product nutrition panels (percentages of U.S. RDAs) would be in question. Vitamin analyses methods utilizing protozoa have not been used routinely for quantitating food vitamin levels, and optimal assay conditions have not been established. The objective of this study was to evaluate several assay parameters related to the application of protozoa to the analyses of vitamin levels in foods. Parameters studied included vitamin concentration, incubation period and assay vessel size. The data provided by this study can be used to formulate specifications for vitamin analyses procedures. Vitamin levels in foods cannot be accurately quantitated when less than optimal methods of vitamin analyses are employed.

Table 1—B-vitamin assay conditions

Vitamin	Assay Culture[a]	Vitamin levels[b] (ng/ml)	References
Thiamine	*L. viridescens* (12706)	0.2 – 10	Difco (1968)
	O. danica (30004)	0.1 – 100	Baker and Frank (1968)
Riboflavin	*L. casei* (7469)	5 – 200	Difco (1968)
	T. pyriformis (30008)	1 – 200	Baker and Frank (1968)
B_6[c]	*S. uvarum* (9080)	0.1 – 8	Difco (1968)
	T. pyriformis (30008)	0.1 – 100	Baker and Frank (1968)
B_{12}	*L. leichmannii* (7830)	0.005 – 0.2	Difco (1968)
	O. malhamensis (11532)	0.0003 – 1	Baker and Frank (1968)
Biotin	*L. plantarum* (8014)	0.003 – 0.4	Difco (1968)
	O. danica (30004)	0.003 – 3	Baker and Frank (1968)
Niacin[d]	*L. plantarum* (8014)	1 – 400	Difco (1968)
	T. pyriformis (30008)	1 – 3000	Baker and Frank (1968)
Pantothenate	*L. plantarum* (8014)	2 – 30	Difco (1968)
	T. pyriformis (30008)	3 – 3000	Baker and Frank (1968)

[a] ATCC strain number is in parenthesis.
[b] Specific vitamin levels used are listed in Table 2.
[c] *S. uvarum* (pyridoxine), *T. pyriformis* (pyridoxine, -al, -amine; equal molar amounts of each isomer).
[d] Nicotinic acid + Nicotinamide; equal molar amounts of each isomer.

MATERIALS & METHODS

STANDARD VITAMIN calibration curves for the microbiological determination of thiamine, riboflavin, vitamin B_6, vitamin B_{12}, biotin, niacin and pantothenic acid were prepared to examine the effects of vitamin concentration and incubation time on the growth of various microbes (Table 1). The effect of assay vessel size (18- and 25-mm test tubes compared to 25 ml Erlenmeyer flasks) on growth of *Tetrahymena*

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0022-1147/78/0005-1418$02.25/0

Table 2—B-vitamin levels in assay media used for calibration curves[a]

Tube no.	Thiamine		Riboflavin		B_6[b]		B_{12}		Biotin		Niacin[c]		Pantothenate	
	B[d]	P[d]	B	P	B	P	B	P	B	P	B	P	B	P
1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	0.2	0.1	5	1	0.1	0.1	5	0.3	3	3	1	1	2	3
3	0.5	0.3	10	2	0.2	0.3	10	1	5	10	3	3	4	10
4	1	1	15	10	0.4	1	15	3	10	30	10	10	6	30
5	1.5	3	20	20	0.8	2	20	10	20	100	20	30	10	30
6	2	5	30	30	1.2	3	25	30	40	300	40	100	14	50
7	3	10	40	50	1.6	5	30	100	60	1000	60	300	16	100
8	4	30	60	100	2	10	50	300	100	2000	100	1000	18	200
9	5	100	100	200	4	30	100	1000	200	3000	200	3000	20	1000
10	10		200		8	100	200		400		400		30	3000

[a] ng/ml, except B_{12} and biotin (pg/ml). Assay volumes were 5 ml.
[b] B(pyridoxol), P (pyridoxol, -al, -amine; equal proportion each isomer).
[c] Nicotinic acid + nictoinamide; equal proportions each isomer.
[d] B = bacterial or yeast assay, P = protozoan assay, corresponding cultures listed in Table 1.

pyriformis was also determined. Vitamin assays using bacteria and yeast as the test organisms are conducted in test tubes, it would simplify routine analysis if the assays using *Tetrahymena* could be conducted in test tubes rather than flasks. Assays utilizing bacteria or yeast were completed following procedures outlined by Difco (1968). Protozoan assays were performed according to procedures of Baker and Frank (1968). Table 1 shows the test organisms used for each vitamin and the concentration ranges commonly used for standard curve preparation for each assay. Table 2 shows the standard curve concentrations used with each assay method.

The growth responses of the bacteria and yeast were measured daily for 4 days, while the protozoan assays were monitored for 7 days. All assays were conducted in triplicate. Growth response was measured as absorbance at 620 nm. The sigmoid dosage-response curves obtained from these measurements were transformed by the logistic function into linear curves. The transformations had minimal correlation coefficients of 0.87 ($P \leq 0.0001$). The transformation is illustrated in Figure 1. Slope and intercept coefficients were evaluated to determine the length of incubation required to attain maximal growth; that is, the time required for the slopes and intercepts of the dosage-response curves to stabilize. Thus, the null hypothesis evaluated the length of time required for the differences in the slope and intercept coefficients of dosage-response curves from successive incubation intervals to equal zero.

The regression equations describing the transformed dosage-response curves corresponding to minimal incubation times required for growth stability were then evaluated to determine the assayable vitamin limits. The thresholds for the lower and upper limits were evaluated by considering both the rate of slope change of the dosage-response curve and the technical limitations encountered in measuring the absorbance of the growth response; that is, the inability of the Spectronic 20 to accurately measure absorbances greater than 1.0. Particle counters (e.g. Coulter ZB1, Hialeah, FL) can be used to measure growth response, since this avoids many of the problems inherent in absorbancy measures; however, this procedure is cumbersome for routine analysis of vitamins. The mathematical formula employed in calculating the doses corresponding to any given rate of slope change assumes that the sigmoid curve is symmetrical. This requirement is not met when the absorbance of maximal growth is greater than 1.0 or when metabolic by-products change the shape of the sigmoid at the upper limit. Thus, the same rate of slope change used to determine the limit at the lower threshold cannot always be used to determine the limit at the upper threshold (Table 3). The slope of the dosage-response curves indicates the ability of the assay to resolve small changes in dose. The greater the slope, the better the resolution. However, this is at the expense of obtaining a narrower sensitivity range, since a greater slope will pass through the measurable response region over a narrower dose range.

In this study, slope and intercept coefficients from the dosage-response curves for each day were compared to those from the last day of incubation; that is, to the fourth day in the bacterial and yeast assays and to the seventh day in the protozoan assays. The Student t-test was employed at the 95% level to evaluate the null hypothesis. Differences in both the intercept and the slope coefficients from the succeeding days had to be insignificantly different for the growth response to be considered stabilized. The slope and intercept coefficients for any given day were entered into the following formulas to determine the sensitivity limits at any given rate of change in slope:

$$D = (b - 4bx^2)/4$$

$$A = 0.5 \pm \sqrt{(b - 4D)/4b}$$

$$\text{dose} = \sqrt[b]{(A/(1-A)e^a}$$

where: x = estimated A − 0.5; b = slope; a = intercept; D = rate of slope change; A = absorbance at the calculated limits; and dose = concentration at the calculated limits. A computer program was developed to evaluate these calculations.

RESULTS & DISCUSSION

TYPICAL GROWTH RESPONSES of *L. viridescens* and *O. danica* to thiamine at increasing times of incubation are shown in Figure 2. The *L. viridescens* assay must be read at the end of 16–18 hr incubation, since longer incubation periods allow lysolytic enzymes time to digest the bacterial cells, which results in a decrease in absorbance. The upper sensitivity limit of the *O. danica* assay is defined by the technical inability to accurately measure absorbances greater than 1.0. *O. danica* required 5 days incubation to reach stabilized maximal growth. Under these conditions, the thiamine sensitivity limits of the two assay methods were about the same; that is, from about 0.2–10 ng per ml (Table 3). The *O. danica* assay had a slightly lower initial threshold, 0.2 vs 0.3 ng per ml which is due to its lower slope value, 1.3 vs 1.5. The *O. danica* assay has the advantage of reaching a stabilized growth response; however, it requires four more days incubation than does *L. viridescens*.

Studies on vitamin B_{12} analyses utilizing *L. leichmannii* and *O. malhamensis* revealed that the assay using *L. leichmannii* required 2 days to stabilize; whereas growth of *O. malhamensis* did not stabilize within 7 days. In practice, 16–20 hr incubations are used for the assay using *L. leichmannii*. The shorter incubation time introduces an opportunity for large deviations in growth response to occur due to inhibition or stimulation from components of the sample extract. Such response variations may be largely eliminated by allowing the growth response to stabilize. Incubation periods longer than 7 days for *O. malhamensis* are not practical. Sensitivity limits of vitamin B_{12} assays with both *L. leichmannii* and *O. malhamensis* were 1–100 pg/ml (Table 3). The upper limit for the assay of vitamin B_{12} by *O. malhamensis* was determined by the technical absorbance measurement limit of 1.0.

Results of the biotin assays with *L. plantarum* and *O. danica* showed that *L. plantarum* required 2 days incubation for growth to stabilize and *O. danica* required 5 days. The assay limits were 3–100 pg/ml for both organisms (Table 3).

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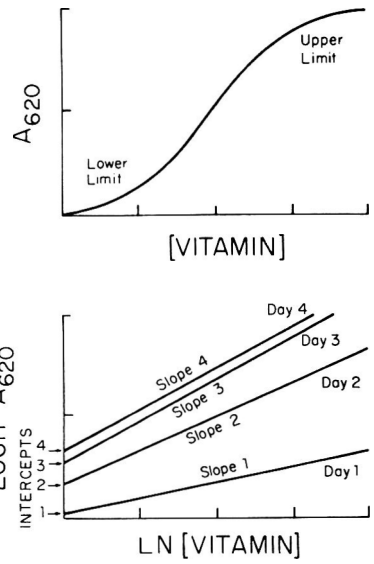

Fig. 1—Top—The lower and upper limits of the sigmoid dose-response curve. Bottom—The intercepts and slopes of four transformed dose-response curves. The four linear transformations correspond to four daily monitorings of a vitamin assay which is attaining stability in the measured growth response.

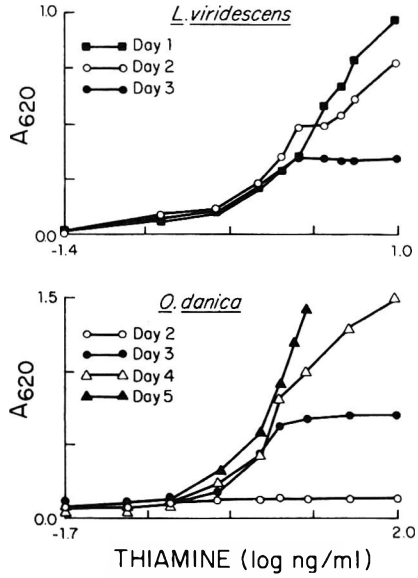

Fig. 2—Effect of time on the growth of two thiamine assay cultures as measured by the absorbance (A_{620}) of the liquid culture.

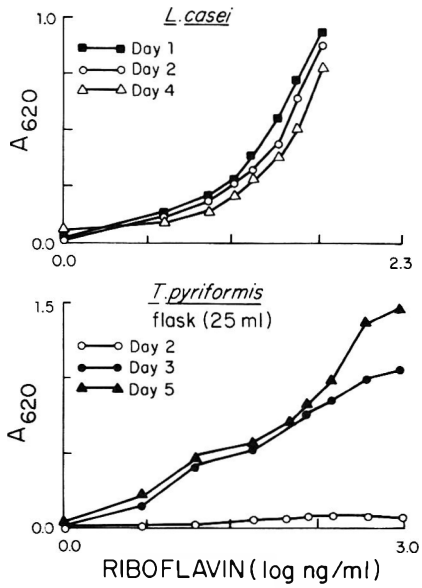

Fig. 3—Effect of time on the growth of two riboflavin assay cultures as measured by the absorbance (A_{620}) of the liquid culture.

Table 3—Length of incubation period to reach stable microbial growth responses and the corresponding vitamin assay sensitivity limits

Vitamin assay	Days incubated[a]	Sensitivity limits (ng/ml)		Slope	% Δ Slope at limits	
		Lower	Upper		Lower	Upper
Thiamine						
L. viridescens	1[b]	0.3	10	1.5	0.08	0.12
O. danica	5	0.2	10	1.3	0.08	0.08
Riboflavin						
L. casei	1[b]	4	60	1.8	0.08	0.19
T. pyriformis – 18 mm tube	5	0.2	70	0.4	0.05	0.09
T. pyriformis – 25 mm tube	4	0.6	40	1.0	0.10	0.10
T. pyriformis – flask	4	0.3	90	1.0	0.05	0.05
Vitamin B_6						
S. uvarum	1[b]	0.1	4	1.2	0.1	0.1
T. pyriformis – 18 mm tube	4	0.1	2	0.5	0.04	ND[c]
T. pyriformis – 25 mm tube	5	0.1	4	0.9	0.05	0.2
T. pyriformis – flask	4	0.1	5	0.8	0.06	0.18
Vitamin B_{12}						
L. leichmannii	1[d]	0.001	0.2	0.8	0.04	0.14
L. leichmannii	2	0.001	0.1	1.6	0.05	0.05
O. malhamensis	6[b]	0.001	0.1	1.0	0.09	0.09
Biotin						
L. plantarum	1[d]	0.003	0.2	1.0	0.03	0.21
L. plantarum	2	0.003	0.1	1.0	0.07	0.19
O. danica	5	0.003	0.1	0.8	0.07	0.19
Niacin						
L. plantarum	0.67[d]	0.5	100	1.3	0.02	0.08
L. plantarum	1	0.5	20	1.0	0.05	0.10
T. pyriformis – 18 mm tube	6	1	100	0.9	0.03	0.21
T. pyriformis – 25 mm tube	5	1	100	1	0.04	0.18
T. pyriformis – flask	5	1	100	0.9	0.03	0.19
Pantothenate						
L. plantarum	0.67[d]	1	30	2.5	0.03	0.1
L. plantarum	1	1	15	2.8	0.07	0.15
T. pyriformis – 18 mm tube	5	3	200	0.8	0.06	0.17
T. pyriformis – 25 mm tube	5	2	100	1.7	0.05	0.5
T. pyriformis – flask	5	2	100	1.2	0.11	0.11

[a] Day 1 signifies 16–18 hr unless data are given for both day 1 and day 0.67, then the former signifies 24 hr and the latter 16–18 hr.
[b] Growth response does not stabilize.
[c] "ND" not determined.
[d] Incubation period employed in practice. The growth response has not stabilized.

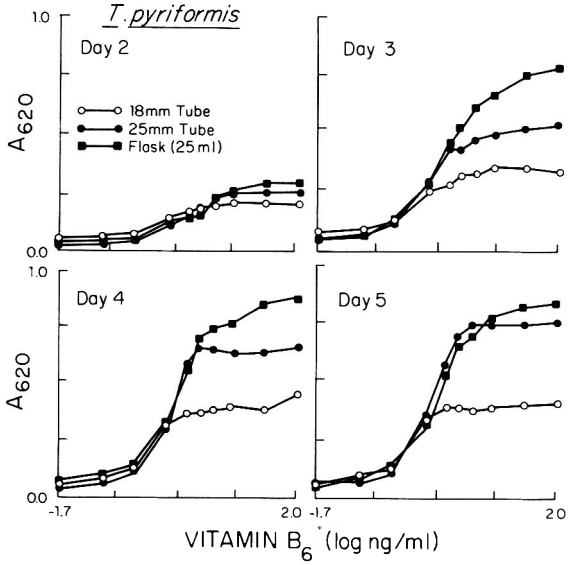

Fig. 4—Effect of time (5 days) and assay vessel size (18 mm tube, 25 mm tube, and 25 ml flask) on the growth of T. pyriformis in response to vitamin B_6 as determined by the absorbance (A_{620}) of the liquid culture.

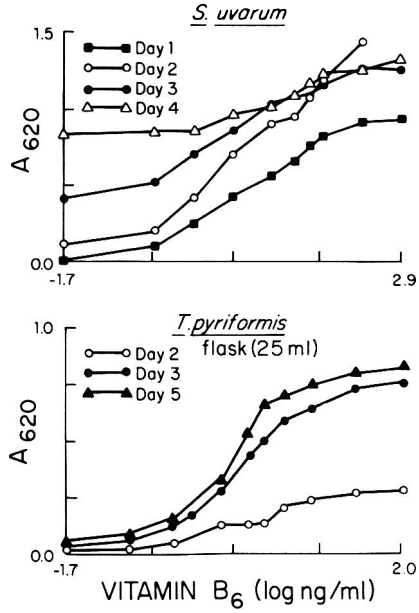

Fig. 5—Effect of time on the growth of two vitamin B_6 assay cultures as measured by the absorbance (A_{620}) of the liquid culture.

Again, the upper limit for the assay using *Ochromonas* was set by the technical limit of measuring response absorbances less than 1.0.

The results of the riboflavin assays using *L. casei* and *T. pyriformis* are given in Figure 3. *L. casei* assays for riboflavin must be read after 16–18 hr, since longer incubations allow lysolytic enzymes to digest bacterial cells. *T. pyriformis* required 4–5 days to reach a stable growth response. The growth of *T. pyriformis* was depressed by the use of the assay vessels with smaller surface to volume ratios. This is evidenced by the lower slope of the assays performed in 18 mm tubes compared to growth in flasks, 0.4 vs 1.0, and by the lower maximal growth attained in assays for vitamin B_6 using 18 and 25 mm tubes (Fig. 4). The assay for riboflavin using *L. casei* had narrower concentration limits than did the assay using *Tetrahymena* in flasks, 4–60 vs 0.3–90 ng per ml. The wider limits again were due to the smaller slope of the latter, 1.0 vs 1.8.

The effects of incubation time on assays for vitamin B_6 using *S. uvarum* and *T. pyriformis* are shown in Figure 5. The assay by *S. uvarum* must be read at the end of 16–18 hr incubation. With time, the biomass of yeast cells continues to increase at the lower doses of vitamin B_6. This indicates that cells of *S. uvarum* do not quantitatively tie up vitamin B_6 in proteins and cellular complexes, since the calibration curves obtained were not stable. The assays using *T. pyriformis* required 4–5 days to reach stable growth and growth was depressed by the use of the assay vessels with smaller surface to volume ratios. However, the effect was overcome in the assays conducted in 25 mm tubes by allowing an additional day of incubation. The assay with *S. uvarum* for analysis of vitamin B_6 had slightly narrower assay limits than did the assays using *T. pyriformis*, conducted in flasks, 0.1–4 vs 0.1–5 ng per ml. Again, the latter had a smaller slope, 0.8 vs 1.2 (Table 3). The *T. pyriformis* assay had the advantage of reaching a stable growth response, while the differences in sensitivities were of no practical value.

The responses of the *L. plantarum* and *T. pyriformis* in assaying for niacin and pantothenic acid were characteristic of the growth responses noted for the other microbiological assays. The dosage-response curve of *L. plantarum* in the niacin assay was stabilized after 24 hr. Recommended procedures call for 16–18 hr for this assay. The assay for niacin by *T. pyriformis* required 5–6 days to stabilize and the use of small diameter assay vessels depressed the growth of the organism in a manner which was not overcome by increasing incubation time. The niacin assay using *L. plantarum* had a more sensitive lower limit but a lower upper limit than did the *T. pyriformis* assay; that is, 0.5–20 vs 1–100 ng/ml (Table 3). The lower limit difference would be of no practical value.

The pantothenic acid assay using *L. plantarum* required 24 hr to stabilize, which is longer than the 16–18 hr incubation time conventionally used. The *T. pyriformis* assay for pantothenic acid required five days to stabilize. The growth of *T. pyriformis* was depressed when small diameter assay vessels were used; however, the effect was overcome in 25 mm tubes after 5 days of incubation. As with the niacin assay using *L. plantarum*, the *L. plantarum* assay for pantothenate had a slightly more sensitive lower assay limit but a smaller upper limit than did the corresponding vitamin assays which utilized *Tetrahymena*. The corresponding sensitivity limits were determined to be 1–15 and 2–100 ng per ml for *L. plantarum* and *T. pyriformis*, respectively.

Table 4 compares the vitamin assay sensitivity limits reported in the literature to those determined in this study. Most of the limits given in the literature were not defined as the assayable sensitivity limits. Strohecker and Henning (1966) and Baker and Frank (1968) indicated that the lower limits they cited are minimal sensitivity levels. Generally, the limits determined in this study agree with those given in the literature except for the upper limits given for the protozoan vitamin assays by Baker and Frank (1968). Their limits appear to be from 3–30 times higher. Higher concentrations were used in this study, but they either caused no increase in growth response or the measured response occurred above an absorbance of 1.0; thus, they could not be accurately measured. Also, little change occurred in the responses recorded at the higher doses. The *Ochromonas* assays for thiamine, biotin and vitamin B_{12} had the same practical assay limits as did the bacterial assay methods. However, the *O. danica* assay reached a stable growth response while the *L. viridescens* did not. The vitamin assays with *T. pyriformis* had wider sensitivity limits than did the bacterial and yeast assays. This advantage was slight for vitamin B_6, but of practical

Table 4—Comparison of literature and experimental minimal and maximal concentrations of vitamins that may be assayed microbiologically

Vitamin Assay	Literature (ng/ml)[a]					Experimental[b]
	AOAC (1)	Difco (2)	Strohecker (3)[c]	Baker (4)[c]	AVC (5)	
Thiamine						
L. viridescens	NR[d]	0.1 – 2.5	NR	NR	NR	0.3 – 10
O. danica	NR	NR	0.1 – 10	0.1 – 100	NR	0.2 – 11
thiochrome	10000 – 25000	NR	1000 – 50000	NR	10000 – 30000	NR
Riboflavin						
L. casei	5 – 100	2.5 – 30	0.5 – 10	NR	5 – 25	4 – 60
T. pyriformis[e]	NR	NR	NR	1 – 1000	NR	0.3 – 90
Vitamin B_6						
S. uvarum	0.1 – 0.5	0.2 – 1	0.5 – 10	NR	0.5 – 3	0.1 – 4
T. pyriformis[e]	NR	NR	NR	0.1 – 100	NR	0.1 – 5
Vitamin B_{12}						
L. leichmannii[f]	0.001 – 0.02	0.0025 – 0.025	0.001 – 0.1	NR	0.001 – 0.015	0.001 – 0.1
O. malhamensis	NR	0.005 – 0.08	0.001 – 0.1	0.001 – 1	NR	0.001 – 0.1
Biotin						
L. plantarum	NR	0.0025 – 0.1	0.05 – 1	NR	0.01 – 0.08	0.003 – 0.1
O. danica	NR	NR	NR	0.003 – 3	NR	0.003 – 0.1
Niacin						
L. plantarum[g]	10 – 200	5 – 50	5 – 100	3 – 30	5 – 40	0.5 – 20
T. pyriformis	NR	NR	NR	1 – 300	NR	1 – 200
Pantothenate						
L. plantarum[g]	0.5 – 10	1 – 10	1 – 10	NR	2.5 – 20	1 – 15
T. pyriformis[e]	NR	NR	NR	10 – 300	NR	2 – 100

[a] Literature limits were not always defined as the usable sensitivity limits. References: (1) AOAC, 1975; (2) Difco, 1968; (3) Strohecker and Henning, 1966; (4) Baker and Frank, 1968; (5) Association of Vitamin Chemists, 1966.
[b] Experimental limits are the usable sensitivity limits determined in this study.
[c] Lower limits are the minimum sensitivity levels.
[d] "NR" not reported.
[e] *T. pyriformis* assays in 25 ml Erlenmeyer flasks.
[f] Incubation length 48 hr vs the conventional 16–18 hr.
[g] Incubation length 24 hr vs the conventional 16–18 hr.

significance for riboflavin, niacin and pantothenate. Table 5 summarizes the optimal vitamin concentration ranges determined in this study for the various vitamin assay methods.

The AOAC (1975) recommends terminating vitamin assays when the turbidity change after two hours additional incubation results in an insignificant change in the growth of the assay organism in the standard tubes containing the highest level of vitamin. The AOAC estimated the required incubation time to be about 16–24 hr for bacterial and yeast analyses, but an "insignificant change in growth" was not defined. This study showed that the *L. viridescens* and *S. uvarum* vitamin assays should be read at 16–18 hr, the *L. plantarum* niacin and pantothenate assays required a minimum of 24 hr incubation, the *L. plantarum* biotin and *L. leichmannii* vitamin B_{12} assays required a minimum of 48 hr incubation, the *Ochromonas* vitamin assays required 5–6 days, and the *T. pyriformis* vitamin assays required 4–5 days.

The employment of assay vessels of smaller diameters, and hence less surface to volume area, had a depressive effect on the *Tetrahymena* vitamin assays. This effect has a biochemical explanation. The smaller surface area of the assay medium lowers the oxygen tension and increases the carbon dioxide tension (Hutner, 1964). This activates the Crabtree effect, the inhibition of respiration by glucose. In *T. pyriformis*, this effect is dependent upon pH and carbon dioxide and implies that, under appropriate conditions the cell will utilize more glucose through the glycolytic pathway for production of ATP, and will use the respiratory system (TCA) less than glycolysis. Thus, glycolytic products accumulate when the oxygen tension is low. Under anaerobic conditions in the

Table 5—Recommended B-vitamin levels of calibration curves for future studies[a]

Tube no.	Thiamine		Riboflavin		B_6[b]		B_{12}		Biotin		Niacin[c]		Pantothenate	
	B[d]	P[d]	B	P	B	P	B	P	B	P	B	P	B	P
1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	0.2	0.1	5	1	0.1	0.1	5	0.3	3	3	0.1	1	2	3
3	0.5	0.3	10	1.5	0.2	0.3	7	1	5	10	2	2	4	5
4	1	0.5	15	2	0.4	0.5	10	3	7	15	3	3	6	10
5	1.5	1	20	5	0.6	1	15	10	10	20	5	5	8	15
6	2	2	30	10	0.8	1.5	20	20	15	30	10	10	10	20
7	3	3	40	15	1.2	2	25	30	20	40	15	15	12	25
8	4	4	50	20	1.6	2.5	30	50	30	60	20	30	14	30
9	5	5	60	30	2	3	70	70	40	100	30	50	16	50
10	10	10	75	90	4	5	100	100	100	250	40	100	20	100

[a] ng/ml, except B_{12} and biotin (pg/ml). Assay volumes at 5 ml.
[b] B (pyridoxol), P (pyridoxol, -al, -amine; equal proportion each isomer).
[c] Nicotinic acid + nicotinamide; equal proportions each isomer.
[d] B = bacterial or yeast assay, P = protozoan assay; corresponding cultures listed in Table 1.

presence of carbon dioxide, glycogen is converted to succinate, acetate and lactate; and carbon dioxide is fixed. In the absence of carbon dioxide, glycogen is converted to lactic acid. Under aerobic conditions, glucose is converted to carbon dioxide and water (Hutner, 1964). Oxygen is required for growth of *Tetrahymena* but cells can survive for several days in static conditions under anaerobic conditions. When high cell concentrations are present, oxygen is depleted and carbon dioxide can accumulate, resulting in the inhibition of growth and respiration (Hill, 1972). This would be expected to occur in the *Tetrahymena* vitamin assays conducted in the 18 mm tubes, and to a smaller extent in the assays employing 25 mm tubes, and to be negligible in the assays using flasks. The opposite of the Crabtree effect, the inhibition of glycolysis by oxygen, is called the Pasteur effect. This response is strong in *T. pyriformis.* Respiration (TCA) is not normally at the expense of the intracellular glycogen reserves; glycogen actually increases aerobically in the absence of nutrients, indicating a reversal of glycolysis.

CONCLUSION

VITAMIN ASSAYS using *T. pyriformis* as the test organism had wider concentration limits than the corresponding assay methods which used bacteria and yeast. The procedures using *Tetrahymena* would be an advantage to the food analyst, since he would have more latitude in estimating the sample dilution necessary to remain within the concentration limits of an assay method. Analysis procedures using *Ochromonas* possessed approximately equal vitamin concentration limits compared to their corresponding bacterial methods. Optimal growth of *T. pyriformis* required the use of flasks rather than test tubes. Test tubes would be more convenient for the food analyst. The protozoan method of vitamin analyses require incubation periods that are 3–5 times longer than the conventionally used bacterial and yeast methods. Incubation periods given in standard methods that employ bacteria and yeast as test organisms are not always consistent with the incubation times required to reach a stabilized growth response, this is complicated by the fact that some of the test organisms do not always reach a sustained stabilized growth response. Inaccurate data can be expected from analyses employing organisms that do not reach a stabilized response, since the presence of interfering agents (especially inhibitors) in sample extracts could not be overcome by an extended incubation period. This deduction should be evaluated in future research. Research needs to be completed to determine the optimal methods of preparing vitamin extracts from various types of food samples to be analyzed by the protozoa for vitamin content. This research is needed to determine the accuracy of vitamin contents reported in current food composition tables.

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But clingstone peaches do soften somewhat, and some of the protopectin is solubilized during ripening, indicating that the exoenzyme may be involved in these processes. The mere removal of terminal galacturonic acid residues from long polygalacturonase chains would not have a large effect on pectin solubility or cell-wall structure. This is assuming that the terminal monomer units are not linked to other cell-wall components. But if such linkages do exist (and plant cell-wall structure is not now adequately understood to exclude them), exopolygalacturonase could have an important function in cell-wall degradation.

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We thank Dr. Gary A. Couvillon, Dept. of Horticulture, University of Georgia, Athens, and Mr. V.E. Prince, Southeastern Fruit and Tree Nut Research Station, Byron, GA, for the samples of peaches. Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture, and does not imply its approval to the exclusion of other products that may also be suitable.

PHYTOSTEROL BIOSYNTHESIS IN RIPENING TOMATOES

EDWARD T. S. CHOW and JOSEPH J. JEN

ABSTRACT

Phytosterols from mature green tomatoes ripened under red light treatment or in the dark were extracted and quantitated by GLC at 3-day intervals. Tomatoes ripened under light showed 8 phytosterols while those ripened in the dark showed 5 phytosterols. The 4 phytosterols identified and quantitated were cholesterol, campesterol, stigmasterol and β-sitosterol. This is the first time cholesterol was identified in tomato pericarp tissues. Red light treatment advanced and slightly enhanced the biosynthesis of tomato phytosterols in comparison with tomatoes ripened in the dark. Approximately a 10% increase in total phytosterols was noted and the increase was attributed mostly to campesterol and stigmasterol. In mature green tomatoes, the phytosterol content was 37 mg/100g dry wt of which two-thirds was β-sitosterol. As the fruit matured, stigmasterol became the dominant phytosterol with β-sitosterol closely behind. Peak phytosterol content was 106 mg/100g dry tissue at the ripe stage of maturation and the content declined as tomatoes became senescent. The results support the fact that the effect of red light on terpenoids biosynthesis was a general one and was not specific to carotenoids biosynthesis in ripening tomatoes.

INTRODUCTION

PHYTOSTEROLS are the terminal metabolites of a branched metabolic pathway in the mevalonic acid-terpenoid biosynthetic pathways. As carotenoids and other terpenoids, the phytosterols were considered as secondary waste products of plant tissues in the past, but as pointed out by Bean (1973), more and more functions of phytosterols were uncovered in recent years. It was suggested that they were membrane components; they acted as hormones; they could be used as steroid precursors (Heftmann, 1971); and they could influence the plant cell membrane permeability and the rate of tissue maturation (Bean, 1973).

An obvious increase in carotenoid contents in ripening tomatoes induced by red light was observed by Jen (1974) and Thomas and Jen (1975b). The phenomenon was suggested to be mediated by phytochrome (Thomas and Jen, 1975a) which was found to decline in ripening tomatoes (Jen et al., 1977). Red light also was shown to advance respiration and ethylene evolution in ripening tomatoes (Jen and Watada, 1977). Since the biosyntheses of carotenoids and phytosterols share the early parts of their biosynthetic pathways (Goodwin and Mercer, 1972; Yamamoto and Mackinney, 1967), the comparison of phytosterol biosynthesis in tomatoes ripened under the influence of light versus those ripened in the dark could provide information on the overall effect of red light on the biosynthesis of terpenoids.

EXPERIMENTAL

TOMATOES (*Lycopersicon esculentum* Mill cv. Walters) were grown on the Clemson Experimental farm and harvested at the mature green stage. After transportation to the laboratory, the tomatoes were sorted for uniform color, size, weight (between 150–200g) and specific gravity (between 30–40% aqueous ethanol solution) (Jen, 1974). The fruit was washed and soaked in a 0.1% sodium hypochlorite solution for 5 min and air dried before being placed in environators for ripening. For far-red light experiment, greenhouse grown Tropic tomatoes of between 145–155g and specific gravity between 15–30% aqueous solution were used.

Light treatments

Seven groups of tomatoes with six in each were placed in each of two identical environators. The red light treatment and dark control were given as described previously (Thomas and Jen, 1975a) except the temperature was at 20 ± 0.5°C instead of at 26 ± 0.5°C. The red light intensity was measured by an ISCO spectroradiometer to be 540 mW/cm² provided by 8 standard Gro-Lux fluorescent lamps. One group of tomatoes was removed at 3 day intervals from each environator for the analysis of phytosterols. For far-red light experiment, the light intensity was 320 mW/cm². Tomatoes were ripened for 8 days before analysis.

Phytosterol extraction

After removing the seeds and locular tissues, 400g of tomato pericarp tissues were blended with 200 ml of acetone for 3 min before filtering through No. 1 Whatman filter paper under suction. The residue was re-extracted 4 times, each with 200 ml of acetone, and the filtrates were combined. Phytosterols in the acetone extract were transferred to petroleum ether by partitioning in separatory funnels with 4 × 100 ml of petroleum ether, which had been purified through silica gel (Jen and Mackinney, 1970). The combined petroleum ether layers were washed with distilled water twice and dried over anhydrous Na_2SO_4. The extracts were then filtered and taken to dryness in a flash evaporator under reduced pressure. The dried extracts were dissolved in 40 ml of 95% ethanol containing 0.67%(v/v) conc sulfuric acid and refluxed for 15 hr. Then, 25 ml of 95% ethanol containing 10%(w/v) KOH was added and the contents were refluxed for an additional hour to complete the hydrolytic cleavage of glucosides and esters (Foote and Jones, 1974). After adequate distilled water was added, the free phytosterols were transferred to petroleum ether by partitioning as described above using 4 × 50 ml of petroleum ether. The free phytosterols were precipitated with digitonin, collected on sintered-glass funnels and re-obtained by cleavage with hot Dimethyl sulfoxide as described by Foote and Jones (1974). The free phytosterols were then transferred to petroleum ether and taken to dryness in a flash evaporator. The residues were stored in a vacuum desiccator and resuspended in 1 ml of CS_2 just before GLC analysis.

GLC quantitation of phytosterols

The tomato phytosterols were separated and quantitated with the Bendix model 2600 GLC and integrator system described previously (Dixon and Jen, 1977). Dual stainless steel columns (6 ft × ¼ in. i.d.) packed with 3.8% SE-30 on 80/100 mesh VAR-A-PORT #30 were used. The following conditions gave good chromatograms:

Sample size	10μl in CS_2
Column temp.	isotherm at 250°C
Inlet temp.	260°C
Detector temp.	290°C
Carrier (He) flow	80 ml/min

Quantitation was achieved by preparing standard calibration curves with high purity cholesterol, campesterol, stigmasterol, β-sitosterol and internal standard, cholestane (Applied Science Laboratories, Inc., College Park, PA). The weight ratios for each authentic sterol and internal standard were plotted against the resultant peak area ratios. The retention times and relative weight ratios (RWR) are listed in Table 1, where RWRs were defined as the weight of authentic sample over the weight of internal standard, to give equal peak areas. To each tomato sample, 1 mg of cholestane was added as an internal standard. Duplicate injec-

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0022-1147/78/0005-1424$02.25/0

tions of each tomato phytosterol sample were performed and the standard deviation was less than 1% in all cases.

The whole experiment was repeated once by using separate batches of tomatoes from the same source but harvested 1 wk apart from the previous batch.

RESULTS & DISCUSSION

FIGURE 1 shows a typical GLC chromatogram of phytosterols from ripe tomatoes 12 days after harvest ripened under red light treatment and in the dark. There were 8 peaks in red-light treated tomatoes, but only 5 were observed in dark control. The four main peaks had the same retention time and co-chromatographed with authentic cholesterol, campesterol, stigmasterol and β-sitosterol standards. Yamamoto and Mackinney (1967) mentioned the existence of stigmasterol, β-sitosterol and possibly campesterol in tomato fruits and seeds. The existence of cholesterol in higher plants has been well documented by Heftmann and his associates (Johnson et al., 1963; Bennett et al., 1963), Knights (1965) and other workers. However, this is the first time that existence of cholesterol was reported in tomato fruits. Unknown peaks I, II, and III appeared only in the red-light treated tomatoes and their contents decreased before they reached the senescence stage of maturation. After the tomatoes reached the senescence stage, these unknowns appeared more irregularly in both red-light treated tomatoes and dark controls. Unknown IV was observed earlier in red-light treated tomatoes than it was in dark controls. The total contribution of the four unknowns was in the range 0–20% of the total peak area depending on the stage of maturation.

Figure 2 shows that the total phytosterol content in red-light treated tomatoes was slightly higher than that in the dark controls throughout the ripening period. The effect of red light was significant during the early stages of ripening. Red light seemed to have advanced the rate as well as the total tomato phytosterol biosyntheses. After reaching full ripeness, the phytosterol content decreased somewhat in senescent tomatoes. The result corresponded well with the enhanced carotenoid biosyntheses (Jen, 1974), respiration rate, and ethylene evolution (Jen and Watada, 1977) in ripening tomatoes. This seems to suggest the whole terpenoid biosynthetic pathway was turned on by the red light treatment before the precursor of both phytosterols and carotenoids, farnesyl pyrophosphate, was formed. The increase in phytosterols in red-light treated tomatoes, however, was much less in magnitude than that of carotenoids in the same fruit (Jen, 1974).

Table 1—Retention time (RT) and relative weight ratio (RWR) of tomato phytosterols

Sterols	RT[a]	RWR	SD[b]
Cholestane	1.00	1.00	–
Cholesterol	1.76	1.75	0.024
Campesterol	2.24	1.05	0.047
Stigmasterol	2.43	1.45	0.045
β-sitosterol	2.82	1.25	0.032

[a] Cholestane as internal standard has a retention time of 12.3 min = 1.00 RT.
[b] Standard deviation

To check the effect of far-red light, Tropic tomatoes were treated with far-red irradiation and compared with those ripened in the dark. Table 2 shows that in pericarp tissues, tomatoes treated with far-red light had 10% less total phytosterols than those kept in the dark. The major difference lies in the content of stigmasterol in the two groups. In tomato seeds, on the other hand, a drastic reduction of phytosterols in the far-red light treated sample was seen in comparison with those in the dark control. It was therefore evident from all the data in this study that phytosterol biosynthesis in ripening tomatoes was partially mediated by phytochrome as the carotenoid biosynthesis.

In mature green tomatoes, β-sitosterol was the main phytosterol and constituted nearly two thirds of the total phytosterols. As the fruit ripened, stigmasterol took over as the dominant phytosterol, but the β-sitosterol content remained high (Fig. 3). Cholesterol remained as a minor component and did not change much throughout the ripening period. The dehydrogenation of β-sitosterol to stigmasterol in tomato fruits was reported by Bennett et al. (1961). Our results supported the proposal by Heftmann (1971) that β-sitosterol functions as a reserve supply from which plants can produce other phytosterols including stigmasterol and possibly cholesterol. An interesting observation on campesterol was that the amount of campesterol increased more than five-fold in red-light treated tomatoes while it only doubled in dark controls during the ripening period (Fig. 2). On a percentage of the total phytosterol basis, the campesterol contributed a rather constant 5% in all dark controls but continued to increase from 5% to 12% in red-light treated tomatoes (Fig. 3). This difference can account for the differences in total phytosterol contents in senescent tomatoes between the two groups. Campesterol is

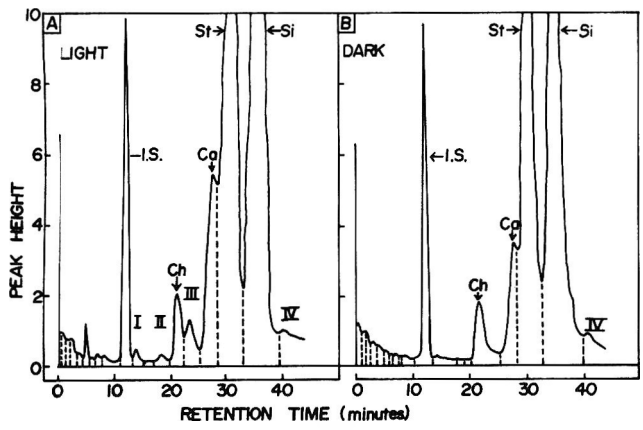

Fig. 1—GLC chromatograms of tomato phytosterols. (A) Tomatoes ripened with red light treatment for 12 days; (B) Tomatoes ripened in the dark for 12 days; I.S. = Internal standard, cholestane; Ch = Cholesterol; Ca = Campesterol; St = Stigmasterol; Si = β-sitosterol. I, II, III, IV are unknown peaks.

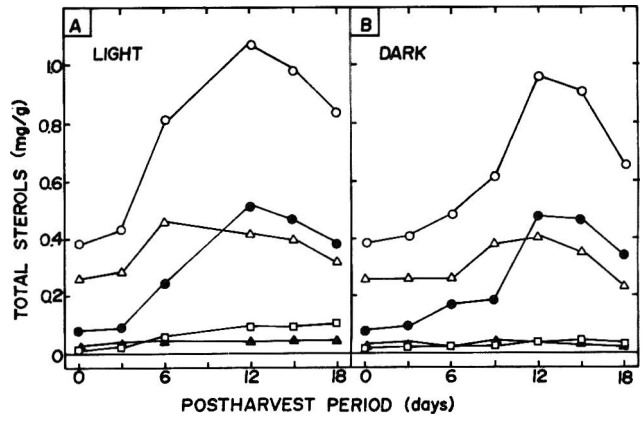

Fig. 2—Phytosterol contents of mature green tomatoes held under red light treatment (A) or in the dark (B). ○—○, total phytosterols; △—△, β-sitosterol, ●—●, stigmasterol; □—□, campesterol; ▲—▲, cholesterol. Sterols were expressed as mg sterols per g dry wt tomato pericarp tissues.

Table 2—Effect of far-red light on phytosterol contents of tomato pericarp tissues and seeds

	Pericarp tissues			Seeds	
		8 Days		8 Days	
Sterols[a]	0 Day	Dark	Far-red	Dark	Far-red
Cholesterol	2.3	2.1	1.7	47.4	10.1
Campesterol	1.6	2.4	1.8	26.0	6.2
Stigmasterol	6.1	20.1	16.7	33.2	5.9
β-sitosterol	18.1	12.6	13.9	214.7	20.1
Total	28.1	37.2	34.1	321.3	42.3

[a] Sterol concentration expressed as mg/100g dry wt.

another terminal metabolite just as stigmasterol in phytosterol metabolism (Grunwald, 1975). Red light enhanced campesterol formation and caused its accumulation in the pericarp tissues. Since the maximum contents of both β-sitosterol and stigmasterol were higher in red-light treated tomatoes than tomatoes kept in the dark (Fig. 2), the effect of red light was probably a general one on the biosynthesis of all terpenoids.

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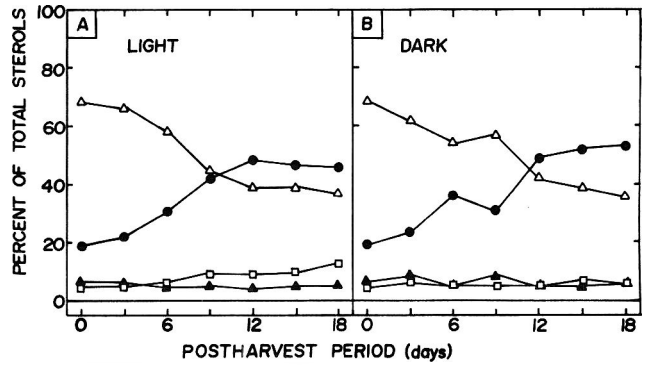

Fig. 3—Weight percentage of individual phytosterols in tomatoes ripened under red light treatment (A) or in the dark (B); symbols are the same as Fig. 2.

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Ms received 2/18/78; revised 4/18/78; accepted 4/25/78.

Technical contribution No. 1561 of South Carolina Agricultural Experiment Station, Clemson, SC 29631.

PATULIN PRODUCTION DURING STORAGE OF BLACKCURRANT, BLUEBERRY AND STRAWBERRY JAMS INOCULATED WITH Penicillium expansum MOULD

S. LINDROTH, A. NISKANEN and O. PENSALA

ABSTRACT

The effects of storage temperature, water activity (a_w) and a CO_2 rich atmosphere on patulin production by *Penicillium expansum* in blackcurrant, blueberry and strawberry jams were studied over a storage period of 6 months. Reduction of storage temperature from 22 to 4°C resulted in decreases both in hyphal growth and patulin production. An atmosphere containing 10% CO_2 had the effect of reducing patulin synthesis but did not significantly affect fungal growth. When the water activity of stored jam was reduced by the addition of 20 and 44% sugar, toxin production in the jams was reduced to a level of 1/10–1/1250 of the maximum occurring in unsweetened jams, despite the fact that the addition of sugar stimulated hyphal growth. Maximum concentrations of patulin were usually observed after 1–2 months of storage, after which the toxin level fell significantly.

INTRODUCTION

PATULIN is a toxic metabolite produced mainly by species of *Penicillium* and *Aspergillus*. The toxin has been shown to be produced readily in fruits (Harwig et al., 1973; Buchanan et al., 1974; Frank et al., 1977) and vegetables (Frank et al., 1977). Some work has previously been published in which patulin was identified in naturally contaminated blueberries (Åkerstrand et al., 1976) and strawberry juice (Percebois et al., 1975).

The aim of this research was to examine the capability of a model strain, *P. expansum* HPB 231170-2, to produce patulin in berry products often employed in both domestic and industrial fruit processing. The fruits chosen were blackcurrant (*Rubus nigrum L*), blueberry (*Vaccinium myrtillus L*) and strawberry (*Fragaria × ananassa Duch*). Particular note was made of the effect of alteration of easily-controlled external storage conditions, such as temperature, water activity (a_w) and ambient CO_2 concentration, on the growth of the *P. expansum* strain and on the production of patulin in the berry products.

MATERIALS & METHODS

Preparation of jams

Three lots of unsweetened and two of sweetened jams were prepared from blackcurrants, blueberries and strawberries. Tap water (10% v/v) was added to the blackcurrant and blueberry lots and the sweetened jams all received 20 or 44% (w/v) sucrose. The jams were boiled for 20 min with occasional stirring, divided into 100 ml portions in 150 ml autoclaved glass jam jars (i.d. 5.5 cm) and sealed.

Inoculation of jams

Patulin-producing *Penicillium expansum* strain HPB 231170-2 (obtained from Dr. J. Harwig, Health Protection Branch, Health and Welfare Canada, Ottawa), was grown on potato dextrose (PD, Difco) plates for 5 days at 30°C. The plates were rinsed with 0.05% Tween-80 (GT Gurr) in 0.9% saline and diluted further in saline to a suspension containing 10^6 CFU/ml as measured by plate counting on PD. Inocula of 1.0 ml of this *P. expansum* suspension were introduced to experimental jars of the cooled jams, while control jars (not inoculated) provided a standard of normal shelf-life.

Stability of patulin in jams

In order to take into account the possible degeneration of patulin in the jam, some noninoculated jars received an amount of 300 μg of patulin standard in 2 ml chloroform (2 duplicates for each type of jam in each set of storage conditions). Residual patulin levels were assayed after 6 months' storage.

Storage of jams

Sweetened jams were stored at room temperature, while unsweetened jams were subjected to three different storage regimes: room temperature (22°C), 4°C and at 22°C in an atmosphere containing 10% CO_2 (Forma Scientific un-i-trol CO_2 incubator model 329). The lids of the jars were kept slightly open during storage to facilitate respiration of the fungi and therefore accelerate mycelial growth and toxin production.

Determination of water activity (a_w)

The a_w values of the jams were measured at the beginning of the experiment using the apparatus of Hygrodynamics Inc. (model 15-3001).

Determination of mycelial dry weight

The mycelial layer was skimmed off the mouldy samples, washed with distilled water, dried at 60°C for 24 hr and weighed. This determination was carried out on samples stored for 0.5, 1, 2 and 6 months.

Measurement of patulin

After removal of the mould layer, the jam was mixed well and a 50-g sample was taken into a flask to which were added 200 ml ethyl acetate. The flask was then shaken for 30 min on a Griffin flask shaker, after which the liquid was filtered through a glass filter (G 1) coated with 10g Kieselgur (E. Merck). The first 150 ml of filtrate were taken for patulin determination by the method of Scott and Kennedy (1973). Thin-layer chromatography was carried out using E. Merck precoated silica gel 60 plates. Patulin determinations were carried out on jams stored 0.5, 1, 2 and 6 months.

RESULTS

Mould growth and patulin production

P. expansum strain HPB 231170-2 was found to produce patulin in blackcurrant, blueberry and strawberry jams in all the conditions examined. Toxin production was in general greatest in strawberry and least in blueberry jam (Fig. 1). The highest toxin concentrations were in unsweetened jams stored at room temperature. In blackcurrant jams stored at 22°C, the patulin concentrations in lots containing 0, 20 and 44% added sugar were 738.4, 7.3 and 2.2 mg/kg respectively. For blueberry the corresponding values were 375.7, 4.5 and 0.3 mg/kg and for strawberry 477.8, 41.2 and 6.9 mg/kg. The highest levels of patulin were in most cases found after 1 or 2 months storage, after which a decline in the level of toxin was observed. The decrease of patulin levels from their maximum value to the value observed after 6 months varied from 0.26–437.1 mg/kg, representing a decrease of over 90% in 12 cases out of 15. In blackcurrant jams stored at 22°C the patulin level had apparently not reached its maximum during the experiment and therefore no decrease in patulin level could be observed in these jams. The disappearance of patulin standard from the noninoculated jars over the 6 months storage period was on average 38% in unsweetened and 85% in sucrose containing jams.

–Text continued on page 1428

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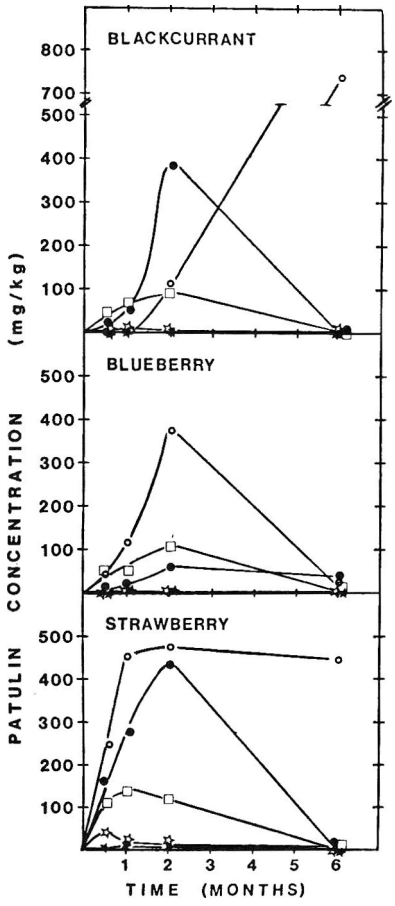

Fig. 1—Patulin concentration in blackcurrant, blueberry and strawberry jams inoculated with P. expansum strain HPB 231170-2 during storage in different conditions. Symbols: ○ 22°C; ● 4°C; □ 10% CO_2, 22°C; ☆ 20% sucrose, 22°C; ★ 44% sucrose, 22°C.

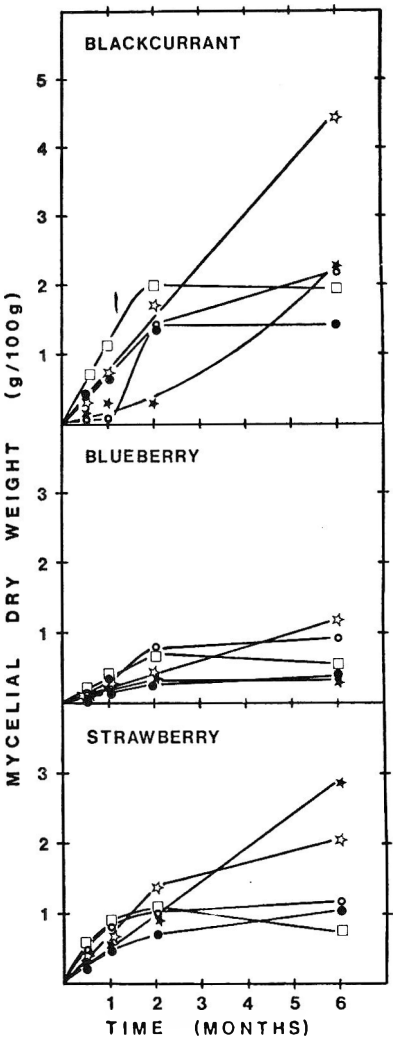

Fig. 2—Mycelial dry weight of P. expansum strain HPB 231170-2 grown in different storage conditions in blackcurrant, blueberry and strawberry jams. Symbols: ○ 22°C; ● 4°C; □ 10% CO_2, 22°C; ☆ 20% sucrose, 22°C; ★ 44% sucrose, 22°C.

Fungal growth was most vigorous in the blackcurrant and least vigorous in the blueberry jams (Fig. 2). The greatest amount of mycelium after 6 months storage was, in the case of blackcurrant and blueberry jams, in those lots containing 20% added sugar, while in the case of the strawberry jams in those lots with 44% added sugar. In general, the amount of mycelium increased continuously throughout the period of storage.

Patulin production per unit mass of mycelium, as a function of storage time, is presented in Table 1. Relative production was observed to be greatest in strawberry and least in blackcurrants. Highest levels of production of patulin per unit mass of mycelium were found in unsweetened blackcurrant and strawberry jams stored at 4°C and in blueberry jams stored at 22°C. Patulin production per unit mycelial weight, like total patulin production was considerably less in sweetened than in unsweetened jams.

Effect of temperature on patulin production

The effect of temperature on patulin production by *P. expansum* strain HPB 231170-2 growing in berry jams was examined by comparing toxin production in unsweetened jams stored at room temperature (22°C) or in cold storage (4°C). In blackcurrant and blueberry jams patulin formation at 4°C was considerably less than at 22°C (Fig. 1). Toxin production in blackcurrant jams at the lower temperature was about half that found in the lots stored at 22°C, in which patulin levels were still increasing after 6 months. In blueberry jam stored at 4°C the highest measured patulin concentration was only 15% of the corresponding concentration in jam stored at 22°C. In the case of the strawberry jams storage temperature appeared to have only a slight effect on maximum patulin levels, in the range tested, with maxima of 437.2 and 477.8 mg/kg in the 4°C and 22°C lots respectively. However, cold storage must still on the basis of these results be considered advantageous also in the case of strawberry jam, as after the full 6 months of storage the toxin levels had fallen in the 4°C lot to 0.1 and in the 22°C only to 445.0 mg/kg.

Effect of water activity on patulin production

Sugar was added (20 or 44%) to the berries in order to reduce their water activity (a_w). In the case of blackcurrants, sugar additions of 20 and 44% caused reductions in a_w-value from the original 0.98 to 0.94 and 0.90 respectively (Table 2). With blueberries the corresponding reductions were from 0.98 to 0.95 and 0.92 and with strawberries from 0.98 to 0.96 and 0.94. Reduction in water activity was in all cases highly correlated with reduction in patulin production. In blackcurrants with 20 and 44% added sugar, toxin production in the jam was at a level of only 1:100 and 1:340, respectively, compared to that in unsweetened jam. The corresponding values for blueberries were 1:80 and 1:1250 and for strawberries 1:10 and 1:70.

Effect of high-CO_2 atmosphere on patulin production

The effect of a modified atmosphere on the formation of patulin in jams was investigated by storing some of the inoculated jams at 22°C in an incubator with a constant CO_2 level of 10%. This atmospheric modification was found to inhibit the production of patulin in comparison with production in jams stored in a normal atmosphere (0.3% CO_2). The highest toxin concentration observed in blackcurrant jam stored in the high-CO_2 atmosphere at 22°C was 88.2 mg/kg, whereas in a normal atmosphere the highest level was 738.4 mg/kg (Fig. 1). For blueberries the corresponding levels were 108.7 and 375.7 mg/kg and for strawberries 139.6 and 477.8 mg/kg. The

inhibiting effect of high-CO_2 atmosphere on patulin production was greatest in the case of blackcurrant jam.

pH and patulin levels of jams

pH was not used as a variable in this work, and changes occurring in pH during storage were a function of fungal growth. As patulin is known to be unstable in alkaline conditions (Chain et al., 1942) pH was measured in all jam samples. The general tendency was towards an increase in pH with longer storage times (Table 3). No correlation was observed between pH and patulin formation in or disappearance from the jams.

DISCUSSION

THE RESULTS reported here indicate that all the berry products examined (blackcurrant, blueberry and strawberry) are potentially high-risk products, as patulin production was possible under all the storage conditions examined. Previous work has been published concerning the occurrence of patulin in naturally contaminated blueberries (Åkerstrand et al., 1976) and strawberry juice (Percebois et al., 1975). Contamination of berry jams and juices by fungal spores is usually more easily prevented in the case of industrially processed foods than in domestic processing, and germination of spores in the commercial product is usually impossible because of the high concentrations of sugar and preservatives employed. Domestic berry products, on the other hand, often become mouldy during storage. As it is quite common practice in such cases that the mouldy layer is removed and the underlying jam eaten, the consumer is exposed to a very real risk of patulin intake.

Although the sucrose additions used in this work (20 and 44%) reduced the production of patulin, fungal growth was in fact stimulated by the presence of the added sugar. The synthesis of patulin has been shown to be prevented by a_w values of 0.82 or less (Frank, 1974), but the growth of potential patulin-producing fungi is possible at a_w values down to 0.77. These values are too low to be achieved reliably by sugar addition alone, even if some of the sucrose is replaced by monosaccharides. The use of chemical preservatives for the storage of berry jams is therefore essential if it is not possible in the production technology to prevent contamination of the product after the heat treatment.

As the ripening of berries usually occurs during a rather short period, an intense picking season is necessarily followed by a long period of storage. Storage in an incubator with a CO_2 concentration of 10% was found to considerably reduce the production of patulin but not to prevent it completely. Lovett et al. (1975) and Orth (1976) made a similar finding in the case of controlled atmospheres having high carbon dioxide and low oxygen partial pressures compared to normal air. In the present work the CO_2-atmosphere storage took place at 22°C, while Lovett et al. (1975) and Orth (1976) used 0–3 and 25°C respectively. The results indicate that it is possible to delay both fungal growth and patulin production in contaminated jams using a controlled atmosphere.

Reduction of temperature from 22 to 4°C caused a significant drop in patulin level, although patulin production per unit mass of fungal growth was in fact greater at 4°C than at 22°C in the case of blackcurrant and strawberry jams. Total amounts of patulin in samples stored at 4°C were considerably lower than those in similar samples stored at 22°C. As reduction in a_w and in temperature of storage both caused reduction in patulin production, the combination of these two factors may be employed to good effect, but total prevention is not attainable under conditions of the present study.

An interesting results was that patulin levels in the stored jams except blackcurrant stored at 22°C first reached a maximum level and then showed a marked decrease with continued storage. This decrease in toxin concentration varied from 26

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Table 1—Patulin production per unit mass of mycelium during storage of blackcurrant, blueberry and strawberry jams inoculated with P. expansum strain HPB 231170-2

Growth condition	Patulin/mycelial dry weight (mg/g)			
	2 wk	1 month	2 months	6 months
Blackcurrant				
22°C	0.5	0.2	7.9	33.7
4°C	7.4	8.4	28.2	0.1
10% CO_2, 22°C	7.2	2.9	4.4	<0.1
20% sucrose, 22°C	<0.1	1.1	<0.1	<0.1
44% sucrose, 22°C	<0.1	0.8	<0.1	<0.1
Blueberry				
22°C	30.4	38.5	48.7	1.6
4°C	5.0	16.7	21.2	10.4
10% CO_2, 22°C	26.6	12.0	16.3	0.6
20% sucrose, 22°C	3.6	0.4	0.2	<0.1
44% sucrose, 22°C	<0.1	0.2	<0.1	<0.1
Strawberry				
22°C	34.5	52.8	46.0	36.9
4°C	101.0	54.0	62.6	<0.1
10% CO_2, 22°C	21.0	16.4	11.4	<0.1
20% sucrose, 22°C	10.7	3.0	0.8	0.3
44% sucrose, 22°C	0.7	1.3	0.4	<0.1

Table 2—Initial water activities (a_w) and maximum patulin concentrations observed in blackcurrant, blueberry and strawberry jams inoculated with P. expansum strain HPB 231170-2

Jam	a_w	Max patulin conc (mg/kg)
Blackcurrant	0.98	738.4
Blackcurrant, 20% sucrose	0.94	7.3
Blackcurrant, 44% sucrose	0.90	2.2
Blueberry	0.98	375.7
Blueberry, 20% sucrose	0.95	4.5
Blueberry, 44% sucrose	0.92	0.3
Strawberry	0.98	477.8
Strawberry, 20% sucrose	0.96	41.2
Strawberry, 44% sucrose	0.94	6.9

Table 3—Variations in pH during storage of blackcurrant, blueberry and strawberry jams inoculated with P. expansum strain HPB 231170-2

Growth condition	pH				
	Initial	2 wk	1 month	2 months	6 months
Blackcurrant					
22°C	3.2	3.4	3.5	3.8	3.8
4°C	3.2	3.4	3.4	3.5	3.5
10% CO_2, 22°C	3.2	3.4	3.5	4.0	6.6
20% sucrose, 22°	3.2	3.3	3.4	3.4	3.4
44% sucrose, 22°	3.1	3.3	3.4	3.4	3.3
Blueberry					
22°C	3.2	3.4	3.3	3.7	5.8
4°C	3.2	3.4	3.3	3.3	3.4
10% CO_2, 22°C	3.2	3.4	3.4	3.5	5.2
20% sucrose, 22°	3.1	3.3	3.4	3.3	3.3
44% sucrose, 22°	3.1	3.2	3.2	3.3	3.5
Strawberry					
22°C	3.6	3.5	3.9	4.0	3.9
4°C	3.6	3.6	3.7	3.6	6.4
10% CO_2, 22°C	3.6	3.7	3.9	4.6	7.1
20% sucrose, 22°	3.5	3.4	3.5	3.5	5.5
44% sucrose, 22°	3.5	3.4	3.4	3.4	3.5

COMPRESSION CREEP BEHAVIOR AND SYNERESIS WATER OF AGAR–AGAR AND ACTOMYOSIN GELS

T. NAKAYAMA, M. KAWASAKI, E. NIWA and I. HAMADA

ABSTRACT

Typical hydrogels of food material were characterized on creep behavior and syneresis water. An autographic creep meter, consisting of a balance and two parallel plates, was designed for agar-agar and actomyosin gels. In the case of agar-agar gel, instantaneous modulus was independent of applied stress level up to 19000 dyne/cm², but from retardation modulus the critical boundary of linear viscoelastic region was estimated to be 7600 dyne/cm² in stress and 0.020 in strain. A large amount of syneresis water came out during the creep run. In the case of actomyosin gel, the critical boundary of linear viscoelastic region was 5700 dyne/cm² in stress and 0.107 in strain. And even over the boundary, syneresis water was negligible during the creep run. Much syneresis water from the agar-agar gel was due to both the smaller linear viscoelastic region and the easiness of water movement in the gel. Namely, the line width of the proton NMR spectrum of agar-agar gel was narrower than that of actomyosin gel.

INTRODUCTION

THE VISCOELASTIC PROPERTIES of gel-type foods are of considerable practical importance for industrial texture control and home cookery. The static measurements of their properties require times as long as possible. However, most ingredients of food are not in a state of equilibrium but are continuously changing, little by little. Therefore, the interval of time of measurement is inevitably limited.

Nakayama and Sato (1971) reported on the stress relaxation responses of hen actomyosin gels. The gels were held at constant tensile strain of 0.05, because both instantaneous and relaxation moduli were almost independent of applied strain up to 0.10.

Agar-agar gel is a prototype of hydrogels, and its critical boundary of linear viscoelastic region has been estimated to be 0.13 in strain, only from the measurement of instantaneous modulus (e.g.: Arakawa, 1961; Isozaki et al., 1976). However, the gel shows considerable syneresis when aged (e.g.: Yamazaki and Kato, 1957; Kojima et al., 1961). As the result, a gradual decrease in the gel volume might occur, and the critical boundary of linear viscoelastic region must be retreated when retardation modulus is measured. Therefore, compression creep behaviors of agar-agar and carp actomyosin gels were compared in relation to syneresis water.

EXPERIMENTAL

Gel preparation

Agar powder was purchased from Wako Pure Chemical Industries, Ltd. The powder (0.75 gr) was mixed with distilled water (50 ml) in an Erlenmeyer flask with rubber stopper, and preheated on a water bath at 65°C for 1 hr. Then the mixture was heated on an oil bath at 103°C for 30 min with a reflux condenser, cooled to 50°C at room temperature, poured into a box-type mould with a lid and a frame for easy removal, and annealed at room temperature for 1 hr. The agar-agar gel was removed from the mould and aged at 5°C for 18 hr in a beaker filled with liquid paraffin in order to avoid the evaporation of water. The dimension of the gel was about 3 cm in height and 2 cm in both length and breadth. Its pH was 6.8 ± 0.1.

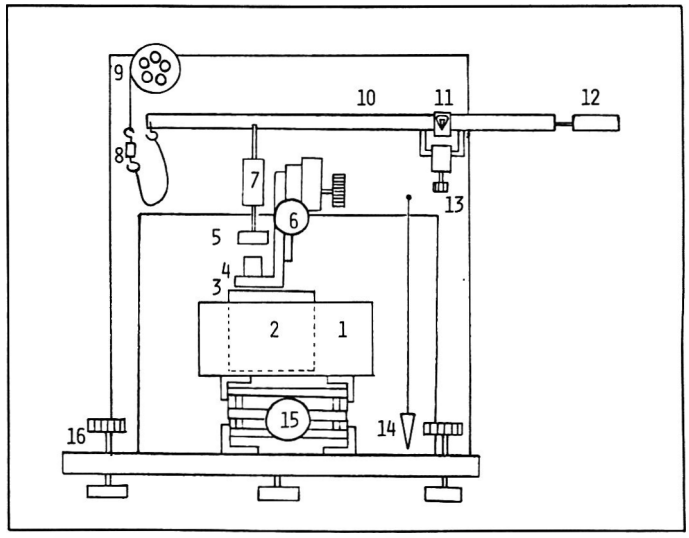

Fig. 1—Schematic diagram of creep meter. (1) water bath. (2) liquid paraffin; (3) bottom plate; (4) specimen gel; (5) top plate; (6) gap setting knob; (7) differential transformer; (8) weight; (9) pulley; (10) balance beam; (11) knife edge; (12) balancer. (13) beam release knob; (14) level; (15) jack; and (16) leveling screw.

Carp actomyosin was prepared by Takashi's procedure (Takashi et al., 1970), except that NaCl was used instead of KCl, and concentrated by Nakayama's method as follows (Nakayama and Sato, 1971). The actomyosin solution was diluted with 10 volumes of cold water, centrifuged at 6000 × G for 10 min, and recentrifuged at the higher gravitational field of 22000 × G for 1 hr. Then three parts of NaCl were added to 100 parts of the precipitate, and the actomyosin sol was centrifuged once more at 22000 × G for 1 hr to remove the foams produced. The sol was packed in a box-type mould with a lid and unfixed bottom, wrapped in polyvinylidene chloride film, heated in a water bath at 90°C for 30 min, and cooled by tap water. Then the actomyosin gel was extruded from the mould by a square rod and aged at 5°C overnight in a beaker filled with liquid paraffin. The dimension of the gel was about 2 cm in height and 2 cm in both length and breadth. Its pH was 6.3 ± 0.1.

Measurement of compression creep and syneresis water

An autographic creep meter was designed as shown in Figure 1. The specimen gel (4) was placed on the bottom plate (3) (Fig. 1 shows this step), which was carefully raised by the gap setting knob (6) until the top of the gel just touched the top plate (5). This was indicated by a flicker on the recorder. Immediately the surface of liquid paraffin (2) was set by the jack (15) higher than the top plate. The balance beam (10) was set to swing freely by the beam release knob (13). All additions of weights (8) were done through a pulley (9) fixed above an end of the beam to speed the weighing process. The movement of the top plate was detected by the differential transformer (7).

The interval of time of measurement in each creep run was limited to 160 min or less. The amount of syneresis water was defined as the difference between the weights of gel before and after the creep run. To weigh a gel, liquid paraffin was wiped off by rolling the gel along a sheet of parchment paper.

Agar-agar or actomyosin sol was injected into a standard NMR sam-

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ple tube (5 mm i.d.) by means of a syringe and silicone rubber tube. Agar-agar sol was annealed at room temperature for 1 hr and actomyosin sol heated at 90°C for 30 min, to make them gel. The gels were aged at 5°C for 18 hr and their proton NMR spectra were obtained using a high-resolution NMR spectrometer (Model R 22, Hitachi Manufacturing Co., Ltd.).

RESULTS & DISCUSSION

Creep behavior of gels

The shape of creep curve is written as:

$$\gamma(t) = \sigma \left\{ \frac{1}{E_o} + \sum_i \frac{1}{E_i}(1 - e^{-t/\tau_i}) + \frac{t}{\eta_{st}} \right\} \quad [1]$$

where $\gamma(t)$ is the strain at time t, σ is applied stress, E_o and E_i are instantaneous and retardation moduli respectively, τ's are the retardation times, and η_{st} is the viscosity in the steady flow region. If the creep data of a gel are in the linear viscoelastic region, the parameters, E_o, E_i, τ_i and η_{st}, are constants characteristic of the gel. At any time t, Eq [1] can be written with the constant C as:

$$\gamma(t) = C\,\sigma\,(t) \quad [2]$$

In the case of actomyosin gel, both instantaneous and retardation moduli were almost constant with applied stress up to 5700 dyne/cm^2. By 5700 dyne/cm^2, the gel was deformed to the strain of 0.107 during 160 min. When applied stress was increased to 7600 dyne/cm^2, the strain responses deviated from linear viscoelastic region defined by Eq [2] (Fig. 2).

In the case of agar-agar gel, a different aspect appeared. Instantaneous modulus was constant with applied stress up to 19000 dyne/cm^2, but retardation modulus was constant only with applied stress up to 7600 dyne/cm^2. By 7600 dyne/cm^2, the gel was deformed to the strain of 0.020 during 160 min (Fig. 2).

Mitchell and Blanshard determined the creep response in shear at 24°C using 1.39% pectate (1976a) and 1.46% alginate (1976b) gels and reported that both instantaneous and retardation moduli were constant with applied stress up to 7400 dyne/cm^2. By 7400 dyne/cm^2, the pectate and alginate gels were deformed to the strains of 0.074 and 0.180 during 60 min, respectively. They referred to the question of syneresis, but did not actually determine the syneresis water.

Strain change and amount of syneresis water

In the creep response of gels, the magnitude of the strain is important rather than that of the stress. Therefore, the relationship between the strain and the amount of syneresis water was determined along each creep run (Fig. 3). The relationships in agar-agar gels almost superimposed without any shift. Up to the strain 0.036 the syneresis water of about 2.2% was detected irrespective of the strain change, and over that value the amount of syneresis water increased up to 10.8% with the strain up to 0.139. The strain of 0.036, over which the increase of syneresis water began, roughly coincided with the strain of 0.020, which was the strain in the critical boundary of linear viscoelastic region. Therefore, the increase of syneresis water could be due to the deviation of creep response from linear viscoelastic region. But the perfect coincidence was not demonstrated.

In the case of actomyosin gels, the amount of syneresis water was much less than in agar-agar gels, and remained around 1% irrespective of the strain change up to 0.170 (Fig. 3).

Okada et al. (1952) reported that the higher the pressure, the more the dehydration was achieved in 1.0 or 2.0% agar-agar gel. The pressure they applied covered the range from 98000–980000 dyne/cm^2. A similar tendency appeared in our agar-agar gels in the stress range from 11400–19000 dyne/cm^2, and disappeared when the stress was still decreased. Okada (1963) reported that the higher the pressure, the more the amount of expressible water was in Kamaboko (Fish Meat Jelly produced in Japan). The pressure he applied covered the range 4900000–34300000 dyne/cm^2. This tendency was not found in our carp actomyosin gels in the stress range 1900–7600 dyne/cm^2.

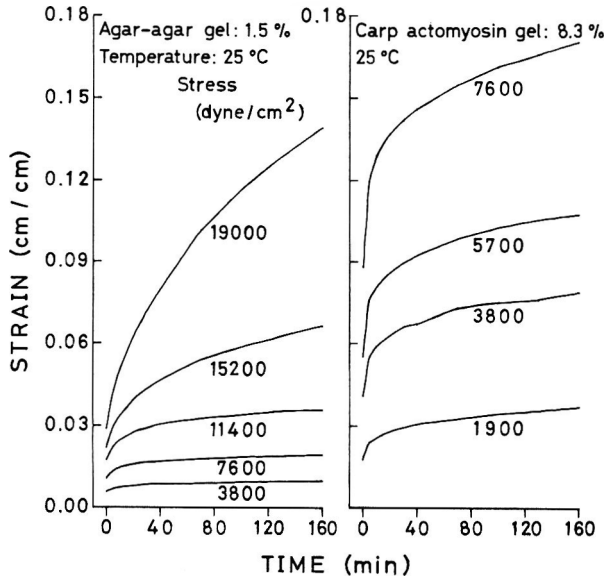

Fig. 2—Effect of stress on the shape of creep curve. Each line for agar-agar and actomyosin gels represents the arithmetic average of five and two determinations, respectively.

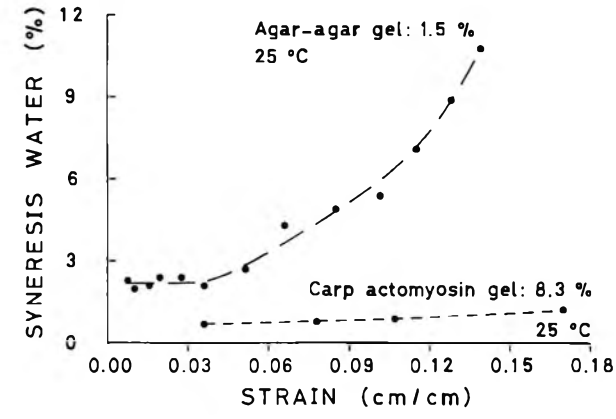

Fig. 3—Change of the amount of syneresis water on the increasing strain. Each point for agar-agar and actomyosin gels represents the arithmetic average of five and two determinations, respectively.

Water in agar-agar and actomyosin gels

The proton NMR spectra for agar-agar and actomyosin gels are illustrated in Figure 4. The line width at half of the maximum peak height was wider in actomyosin gels (0.23 ppm) than in agar-agar gels (0.13 ppm). This result corresponded to the fact that the amount of syneresis water was less in actomyosin gels than in agar-agar gels, when the stress of 3800 or 7600 dyne/cm^2 was applied to both gels (Fig. 2 and 3). Such a relation between the line width and the amount of syneresis water was found in Kamaboko as well (Suzuki, 1973).

The linear viscoelastic region was smaller in agar-agar gel

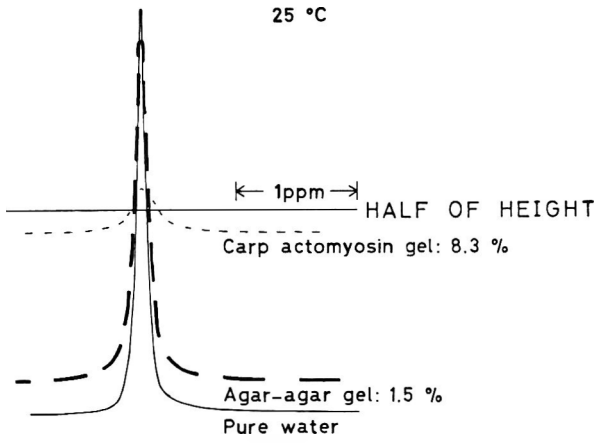

Fig. 4—Proton NMR spectra of agar-agar and actomyosin gels. Each line represents the arithmetic average of two determinations.

than in actomyosin gel, from the measurement of retardation strain response (Fig. 2). And the line width of the proton NMR spectrum of agar-agar gel was narrower than that of actomyosin gel (Fig. 4). Furthermore, much syneresis water came out from agar-agar gel alone. Therefore we conclude that much of the syneresis water from agar-agar gel was due to both the smaller linear viscoelastic region and the easiness of water movement in the gel.

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Ms received 12/31/77; revised 4/11/78; accepted 4/20/78.

We thank H. Isozaki of Showa Women's College for her expert technical advices and suggestions from the start of our work, and Prof. F. Hamada of Mie University for her valuable guidance.
We also thank Prof. Z. Kumazawa of Mie University for the NMR spectral determinations.

PATULIN PRODUCTION IN JAMS WITH P. expansum . . . From page 1429

μg to 44 mg per jar, being in several samples over 90%. Sommer et al. (1974) and Ciegler et al. (1977) found a similar surprising fall in patulin levels during patulin production tests in cultivation broth, using *P. expansum* as the test organism. Sommer et al. (1974) postulated that the disappearance was due to metabolic destruction and/or the lability of patulin in aqueous solutions, while Ciegler et al. (1977) were of the opinion that the explanation lay in adduct formation with other compounds in the menstruum. In the present work reduction in patulin levels was observed in both mouldy and noninoculated jams, indicating that patulin is unstable in blackcurrant, blueberry and strawberry jams. The primary reason for this was probably the high a_w-value of the jams, as patulin is labile in aqueous solution. As the disappearance of patulin appeared to be more marked in the mouldy jams, than in the noninoculated jams, it is possible that the disappearance of patulin from mouldy jams was in part a result of fungal metabolism. This possible metabolic breakdown of patulin, as well as a detailed study of the stability of patulin in berries and berry products, is at present under investigation.

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Ms received 1/3/78; revised 4/6/78; accepted 4/10/78.

We are indebted to J. Harwig of the Health Protection Branch, Health and Welfare Canada, Ottawa, for P. expansum strain HPB 231170-2. Excellent technical assistance was afforded by L. Aalto, A. Murrola and P. Salovaara.

CO_2 EMISSION FROM AN ORANGE JUICE POWDER: INDICATION OF AN OXIDATIVE ORIGIN

D. PAPANICOLAOU, F. SAUVAGEOT and D. SIMATOS

ABSTRACT

To gain knowledge of the circumstances associated with the release of CO_2 and better understand the mechanisms of deterioration of orange juice quality, the quantity of CO_2 emitted by freeze-dried orange juice powder was measured. At the same time, quality attributes (flavor, color, ascorbic acid) were evaluated as a function of time, temperature, water content and oxygen content of the atmosphere. Results of these experiments are reported.

INTRODUCTION

WHEN AN ORANGE JUICE POWDER with a water content ranging from 3–40% is stored at a temperature between 20–70°C, alterations in organoleptic properties are observed (browning, appearance of off-flavors). These alterations are of great practical significance, since these deleterious conditions can occur during storage of the dry product, or during the final step of certain drying processes.

From the numerous research works which have dealt with this problem (Gee et al., 1969; Karel and Nickerson, 1964; Tatum et al., 1967; Shaw et al., 1970; Foda et al., 1970) it can be concluded that nonenzymatic browning reactions are responsible for the above mentioned alterations in color and flavor.

The increase in optical density of an aqueous extract has often been used as an index of product alteration. We could observe, however (Papanicolaou, 1975), that at rather high temperatures, the formation of off flavors anticipates the detection of browning reaction.

Flavor characteristics are not easily usable for routinely evaluating the change of product quality. A physical or chemical parameter, simply and quickly measured, would be useful, if it undergoes a change preceding the alteration in flavor. Carbon dioxide emission appeared to be such a test. It is known that CO_2 can be produced in the course of nonenzymatic browning reactions by several mechanisms:

(a) Decarboxylation of amino-acids through Strecker-degradation (Hodge, 1953);
(b) Decarboxylation of ascorbic acid (Curl, 1949) or citric acid (Lalikainen et al., 1958).

Moreover, a knowledge of the circumstances associated with the release of CO_2 can help understand the mechanisms of the deterioration of product quality. We have measured therefore the quantity of CO_2 emitted by a freeze-dried orange juice powder. At the same time quality attributes (flavor–color–ascorbic acid) were evaluted, as a function of time, temperature, water content and oxygen content of the atmosphere.

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0022-1147/78/0005-1433$02.25/0

EXPERIMENTAL

Processing of product

Freeze-dried granules of orange juice (3.5% residual moisture) were distributed in vials (25 ml "penecillin" vials, about 3g of product in each one) and accurately weighed. Water was added with a microsyringe to obtain the desired water content in each sample. The homogenous distribution of moisture in the products was obtained by stirring with a spatula. Vials were closed with special rubber stoppers and metallic caps.

Different atmospheres inside the vials were obtained through 2 injection needles, one being connected to a vacuum line, the other permitting the introduction of nitrogen when vacuum was obtained. Four types of atmospheres were studied: air (760 Torr); air at low pressure (about 1 Torr); vacuum (less than 5.10^{-2} Torr); nitrogen (760 Torr).

The thermal treatment of the product was performed by immersing the vials in water-baths in which the water was well stirred. When the desired heating time was over, the vials were quickly cooled in water at 20°C and the CO_2 content in the vials measured.

Measurement of CO_2

A sample of the gaseous atmosphere inside the vial was taken and injected in the column of the gas chromatograph, through a special sampling system: the sampling valve (volume ÷ 5 ml) under vacuum, was connected with the vial atmosphere through an injection needle.

The sample passed through 2 columns successively, the first one (silicagel, 30–60 mesh, 150°C) separated CO_2 from the mixture O_2/N_2, the second one (molecular sieve, 30–60 mesh, 20°C) separated oxygen from nitrogen. The chromatograph (thermal conductivity detector 250 mA, 250°C) was a Becker 406 (Packard). The carrier gas was hydrogen, with a flow of 16.5 ml · mn^{-1}. The system (gas chromatograph + injection system) can detect 2.4 · 10^{-6} g of CO_2.

Detection of free radicals

The number of free radicals existing in the samples after processing was measured by electron spin resonance (ESR; Varian E9 Spectrometer). Freeze-dried orange juice was introduced in ESR quartz tubes, which were then closed with butyl stoppers.

RESULTS

Quantity of CO_2 emitted as a function of time, temperature and moisture content

Figure 1 shows the emission of CO_2 as a function of duration of the treatment for different temperature and moisture content conditions. After a rapid increase of the quantity of emitted CO_2, the emission rate became very low and then increased again. This behavior strongly suggests that CO_2 emission is the result of two different mechanisms which take place successively in the product. As the object of these studies was to use CO_2 emission as a test for an early change in the material, only the first phase was investigated.

At temperatures higher than 50°C, it was not possible to study accurately the first phase of CO_2 emission in samples of high moisture content, because the CO_2 production was too rapid to test. Samples with moisture contents of 3.5, 4.5 and 7% have thus been stored at 20°C. As shown in Figure 2, the CO_2 emission after a given length of time increased with an increase in temperature or moisture content.

When the CO_2 content had been measured, after this first storage period, the vials were opened and flushed with air for a few seconds. Following this, the vials were closed again and placed in the water-bath at 61°C for 15 min. After cooling to 20°C, CO_2 content was measured.

Figure 3 shows the combination of the two successive treat-

ments on the same samples: 1st (continued lines) production of CO_2 as a function of the time of storage at 20°C (black symbols) or at 4°C (open symbols). 2nd (discontinued lines) production of CO_2, from the samples after storage at 20°C or 4°C for various time periods, after 15 min at 61°C. The total amount of emitted CO_2 for each sample is denoted as Σ. It appeared to be constant and independent of moisture content.

It is concluded from this experiment that the first phase of CO_2 production is finished with the exhaustion of a substrate, or substrates in the sample. Substances which are present in large amount in the orange juice, such as citric acid and sugars, may be substrates for the second phase of CO_2 production, but not for the first one. Moreover, in solutions of sugars and amino acids (Cole, 1967), CO_2 production does not exhibit a course analogous to the first phase reported in the present studies.

It is possible that CO_2 can be produced in the first phase through oxidation of lipids, which are present in small amounts in orange juice (less than 0.1%) but contain a large proportion of unsaturated fatty acids (Askar et al., 1973). It is known that unsaturated fatty acids can be oxidized at moderate temperatures with production of CO_2 (Loury, 1961). Oxidation of lipids is enhanced in freeze-dried juices by their dispersion on carbohydrates (Labuza, 1971). This hypothesis has been tested in two ways:

(1) A study of the influence of O_2 in the conditioning atmosphere on the amount of CO_2 produced; and (2) A study of the free radicals decay.

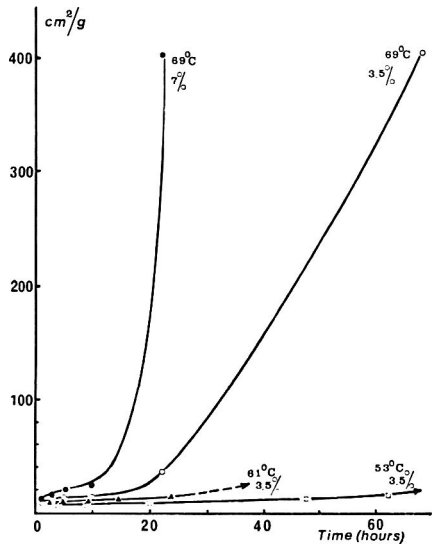

Fig. 1—Production of CO_2 as a function of time, for different temperature and humidity conditions (conditioning atmosphere: air 760 Torr) (ordinates: area of gas-chromatograph peak corrected for the sample weight).

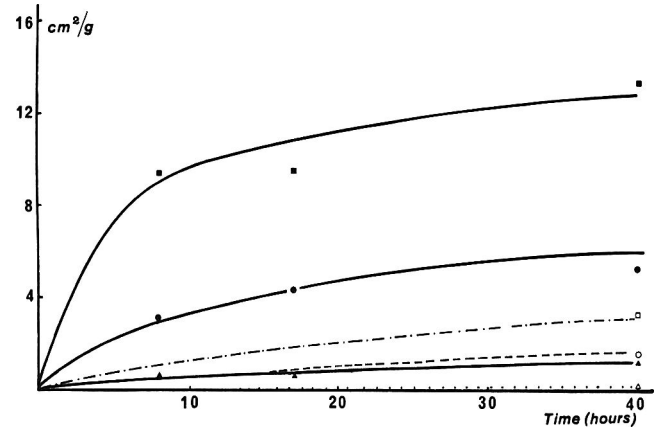

Fig. 2—Production of CO_2 as a function of time: (ordinates as in Fig. 1): water content, △ ▲ 3.5%; ○ ● 4.5%; □ ■ 7%. Conditioning temperature, black symbols, 20° C; open symbols, 4° C.

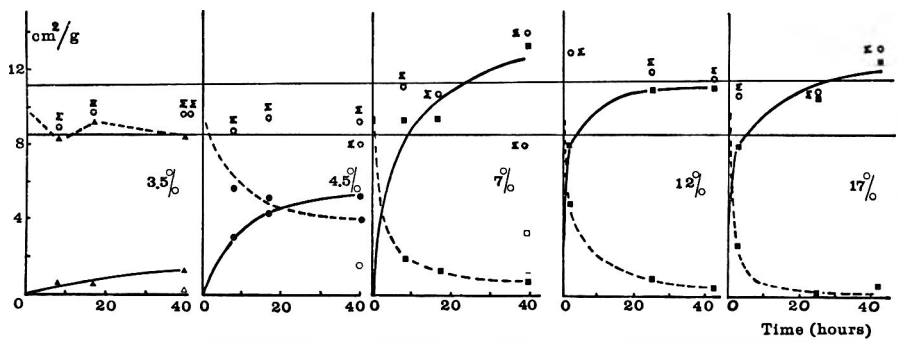

Fig. 3—Combination of two successive treatments on same sample. For details, see text.

Influence of the presence of oxygen in the conditioning atomsphere

The CO_2 content, measured after maintaining the sample at 61°C for 15 min, increased with the O_2 amount which was available in the vial at the beginning (Fig. 4). The emitted CO_2 (first period) is thus, at least in part, produced in reactions which use molecular O_2.

Change in free radicals content

Freeze-dried juice, stored at room temperature, and in the presence of air, exhibited an ESR signal, which was probably due to free radicals (g value = 2,002). The signal amplitude decreased when the sample was heated at 45°C for 30 or 60 min (Fig. 5) and completely disappeared after heating at 61°C for 15 min. The ESR signal amplitude and CO_2 amount showed symetrical changes during heating (Fig. 6). Addition of water to the sample produced the same effects: the ESR signal

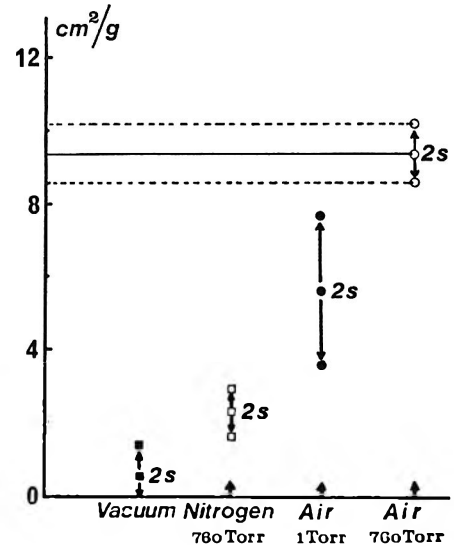

Fig. 4—Production of CO_2 by samples in presence of different conditioning atmospheres: (Conditioning, 61° C—15 min); S = standard deviation.

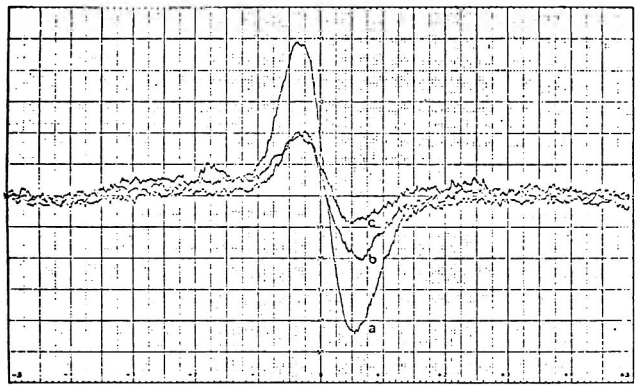

Fig. 5—ESR spectrum of dried orange juice (water content, 3.5%): (a) control; (b) after heating for 30 min at 45°C; (c) after heating for 60 min at 45°.

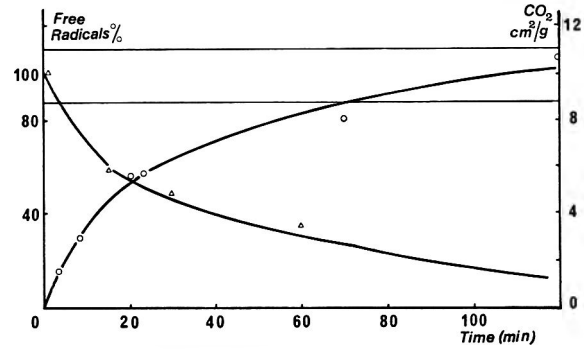

Fig. 6—ESR signal amplitude and CO_2 production as a function of time of heating at 45°C (water content of dried orange juice, 3.5%): ○ CO_2 production; △ ESR signal amplitude (% of control).

decreased and disappeared, the emitted CO_2 increased to the maximum value corresponding to the first emission phase. One can thus conclude that CO_2 is produced by the degradation of intermediate oxidation species, such as free radicals.

The presence of free radicals in freeze-dried products has been demonstrated by Munday et al. (1962) and Simatos (1966). Free radicals are detectable when the product undergoes contact with ambient O_2; free radicals content increases with the O_2 content in the atmosphere. Schaich and Karel (1975) showed that in the system, lysozyme + methyl-linoleate, free radicals species were probably formed from the fatty acid and then transferred to the protein.

It would be interesting to continue the investigation and confirm the nature of the oxidized substrate in dried orange juice. This could be done by adding to the product before drying the presumed substrates in a radioactive form and looking for radioactivity in emitted CO_2.

DISCUSSION & CONCLUSION

THE CO_2 PRODUCTION from an orange juice powder takes place in two successive phases. In the first one, the quantity of emitted CO_2 seems to be limited by the quantity of available substrate, if the sample is conditioned in the presence of a sufficient O_2 amount. During the first phase, CO_2 appears to be produced by the degradation of intermediate oxidation products.

In the second phase, the CO_2 production seems to be more affected by temperature, and probably results from non-enzymatic browning reactions.

Flavor alteration of the dried juice was always observed after the completion of the first phase of CO_2 production. It has not been possible, however, to find a simple relationship between the amount of emitted CO_2 and the flavor alteration.

In conclusion, the CO_2 production cannot be used as a test for early alteration of the orange juice powder. However, interesting information on the responsible reactions is suggested.

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Ms received 8/8/77; revised 4/10/78; accepted 4/20/78.

We acknowledge our gratitude to Professor M.J. Ashwood-Smith (Univ. of Victoria, B.C., Canada) who kindly reviewed the manuscript.

CHEMICAL AND GELATION CHARACTERISTICS OF AMMONIA–DEMETHYLATED SUNFLOWER PECTINS

W. J. KIM, F. SOSULSKI and S. C. K. LEE

ABSTRACT

Sunflower head and stalk pectins were demethylated by ammonia-ethanol treatments over a range of concentrations, duration and temperature conditions. The de-esterification treatments decreased the methoxyl and acetyl contents of the pectins and their molecular weights. The associated increases in proportions of acid amide and free carboxyl groups on the polygalacturonic acid molecules were greater in the modified pectins extracted from a head:stalk mixture (1:1 ratio) than in the modified head pectin samples. For all modified pectins there was a general increase in firmness and strength of the prepared gels as the percent esterification decreased from 32 to 14% in these samples. The modified head pectins were superior to those from the head-stalk mixture in pectin solubility, absence of pregelation, and gel smoothness, elasticity, uniformity and stability. The improved gel characteristics of the demethylated pectins were attributed to the increase in acid amide groups and greater randomization of free carboxyl groups in the pectin molecules. The optimum pH of 4.3 for gel formation in the unmodified and most demethylated samples was higher than that of a commercial low-ester pectin, suggesting a particular application in high pH dessert gels.

INTRODUCTION

THE POTENTIAL for commercial extraction of sunflower pectin as a byproduct of oilseed production has been investigated by several workers since Colin and Lemoyne (1940) first reported the presence of pectin-related substances in sunflower. Sunflower pectins are considered as naturally occurring low-ester pectins because of their low contents of methoxyl groups in the water-soluble head pectins (34.3–66.8%), oxalate-soluble head pectins (30.0–44.1%) and stalk pectins (15.9–21.4%) (Lin et al., 1975). Lin et al. (1975) reported that only one-quarter of the head pectins were water-soluble, the principal fraction being bound in the plant tissues and requiring a sequestrant such as ammonium oxalate or hexametaphosphate for extraction. The neck and stalk of the sunflower plant contained only bound pectins. The total pectin content in four sunflower cultivars varied between 15–24% in the heads and 4–7% in the stalks.

In the previous report on gelling properties of polyphosphate-soluble sunflower pectin extracted from a mixture of head and stalk, the gels showed marked sensitivity to pH and calcium concentration, which resulted in pectin insolubility, pregelation and gel brittleness (Kim et al., 1978c). These adverse gelation characteristics have been previously associated with a high proportion of free carboxyl groups and non-random distribution of methoxyl groups on the pectin molecule which is characteristic of enzymatically-demethylated citrus pectin.

At least four methods of hydrolysis (acid, alkali, enzyme and ammonia in alcohol) have been employed commercially for demethylation of citrus pectin. Demethylation using the ammonia system yielded a low-ester pectin which contained acid amide groups and ammonium salts as well as methoxyl and free carboxyl groups (Joseph et al., 1949). Kim et al. (1978a) reported that, as the temperature of treatment was decreased and concentration of NH_4OH increased, the depolymerization of pectin was minimized and the conversion of methoxyl groups to acid amide groups approached unity. The increase of amide groups in the low-ester pectin resulted in more elastic and stronger gels than was obtained with low-amide pectins.

In the present investigation, polyphosphate-soluble pectins from sunflower heads and stalks were modified by ammonia-ethanol treatments to add amide groups and randomize the distribution of methoxyl groups on the pectin molecules. The NH_4OH treatments involved a range of pH, pectin and alcohol concentrations, as well as time and temperature conditions. The effect of reaction time was investigated primarily in the head pectins while other variables were introduced for demethylation of the head-stalk pectin blend. The modified pectins were evaluated for their chemical composition, pregelation and gel properties in comparison with a commercial low-ester pectin.

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0022-1147/78/0005-1436$02.25/0

MATERIALS & METHODS

Sample preparation

Mature heads and stalks of Peredovik and Krasnodarets sunflowers were harvested from field plots at Saskatoon, Canada, air-dried and ground to 60-mesh (Tyler) after seed removal. Samples of the two cultivars were combined to provide samples of heads only and a 1:1 mixture of heads and stalks for pectin extraction. Prior to the extraction step, the two samples were washed with hot water (75°C) for 15 min at a solid:water ratio of 1:25 to remove soluble pectins, other carbohydrates and pigments. Insoluble pectins were extracted from the residue with 0.75% sodium hexametaphosphate at 75°C and pH 3.5 for 1 hr using a solid:liquid ratio of 1:20, followed by filtration through cheesecloth. The pectins were precipitated by acidification with concentrated HCl at pH 1.0 for 1 hr at 5°C, followed by pressing in a laboratory hydraulic press at 25–30 kg/cm² for 15 min (Lin et al., 1978). The pressed pectins were resuspended, washed and pressed, consecutively, with 0.25N HCl, 60% ethanol and 95% ethanol, and then freeze-dried.

Before treatment, the freeze-dried pectins were ground to pass a 60-mesh screen and 50-g samples were rehydrated in a series of ethanol solutions (0–60%) overnight at 4°C (Table 1). The zero ethanol treatment formed a viscous aqueous solution while the higher proportions of ethanol caused increased degrees of particle swelling and viscosity in these pectin-ethanol mixtures. The demethylation step was conducted by adjusting the solution or suspensions to 4° or 12°C and stirring with precooled NH_4OH in ethanol for 18–65 min of reaction time. The concentrations of NH_4OH and ethanol used for the demethylation reaction were varied to provide the same ethanol concentrations as were used for rehydration, but pectin concentrations varied from 1.0 to 5.0%. Demethylation was stopped by neutralizing the suspensions, which were about pH 12, to 4.0 with 6N HCl. The acid-precipitated pectins were washed several times with 60% ethanol until free of NH_4Cl, followed by final washings with 95% ethanol and acetone before air drying.

Pectin analysis and gel evaluation

Acid-washed samples of the unmodified and modified pectins were analyzed for galacturonic acid content, degree of esterification and molecular weight (Smit and Bryant, 1967), and acetyl content (Chang and Smit, 1973). The proportion of carboxyl groups which contained acid amide groups was determined on a micro-Kjeldahl distillaton apparatus using the formula (Kim et al., 1978a):

$$\%\ \text{acid amide groups} = \frac{\text{ml 0.1N HCl neutralized by } NH_4OH}{\text{g sample (ash- and moisture-free)}} \times \frac{16.024}{9.414}$$

The percentage of free carboxyl groups was calculated by subtracting % esterification and % acid amide groups from total galacturonic acid content. The compositional data are reported on an ash- and moisture-free basis.

The sunflower pectins were prepared into gels over a pH range 2.7–4.5 by the National Research Council (1972) procedure using 1% pectin, 30% sugar and 22 mg Ca^{++}/g pectin, which was found to be optimal for gel firmness and strength (Kim et al., 1978c), in all formulation. These results were compared to those of gels prepared with commercial low-methoxyl (LM) citrus pectin (Genu LM-15AB, The Copenhagen Pectin Factory Ltd., Denmark) using the same conditions as for sunflower pectin except that the recommended 25 mg Ca^{++}/g pectin (National Research Council, 1972) was used in the gel formulation. The firmness of the gels, expressed as % sag, was measured with the Sunkist Exchange Ridgelimeter and the strength, in g/cm^2 of breaking pressure, was determined on the Marine Colloids Gel Tester using a plunger downstroke rate of 5.0 cm/min. The firmness/strength (F/S) ratio was determined by dividing the breaking pressure value into the % sag and multiplying the value by 100. Low ratios were associated with sunflower pectins that were highly soluble, exhibiting little pregelation during preparation and forming firm, smooth, elastic gels (Kim et al., 1978c).

Table 1—Conditions used for rehydration and demethylation of pectins from sunflower head (H-series) and head-stalk mixtures (M-series)

Sample	Rehydration conditions (16 hr) Pectin (%)	Ethanol (%)	Reaction conditions Normality of NH_4OH in EtOH	Pectin (%)	Temp (°C)	Time (min)	$K^a \times 10^{-3}$
H-1	2.0	30	2.5	1.0	12	23	22.3
H-2	2.6	50	2.5	1.3	12	18	12.6
H-3	2.6	50	2.5	1.3	12	20	14.4
H-4	2.6	50	2.5	1.3	12	22	12.0
H-5	2.6	50	2.5	1.3	12	25	11.6
H-6	2.6	50	2.5	1.3	12	30	11.9
M-1	3.2	0	3.0	1.6	12	20	35.0
M-2	3.2	12	2.5	1.6	12	22	13.2
M-3	10.0	60	3.5	5.0	4	25	7.6
M-4	10.0	60	3.5	5.0	4	50	6.4
M-5	10.0	60	3.8	5.0	4	34	8.6
M-6	10.0	60	4.3	5.0	4	65	12.0

[a] K = specific reaction rate per min.

RESULTS & DISCUSSION

Characteristics of control samples

The chemical composition of the untreated head sample (H-0) and head-stalk mixture (M-0) is shown in Table 2. The head pectin-sample was found to contain 98.9% of galacturonic acid which had 40.4% esterification and 0.7% amidation of the carboxyl groups, the remainder (57.8%) being free carboxyl groups. The acetyl content of the head pectin was 1.9% and the molecular weight was 147,100. Carboxyl groups of the head-stalk pectins were 30.3% esterified and 0.7% amidated, while the acetyl content of the pectin was 1.4% and the pectin molecular weight was 122,200. Under standard gelation conditions at pH 4.3, the sag and breaking pressure were 12.7% and 76.7 g/cm^2 for the head pectin and 8.7% and 83.5 g/cm^2 for the pectin blend. Pregelation, brittleness and lack of elasticity were characteristic of gels from both unmodified pectins, especially in the gel from the head-stalk mixture.

The gelation characteristics of the sunflower head pectin were compared to those of a commercial low-ester citrus pectin over a pH range 2.7–4.5 with concentrations of pectin, sugar and calcium being kept constant at each pH (Fig. 1). The maximum breaking pressure and minimum sag of the head pectin were obtained at pH 4.3, as previously found for the head-stalk blend (Kim et al., 1978c). The least degree of sag for the citrus pectin occurred at a lower pH of 3.1–3.4 and maximum breaking pressure was also at pH 3.4. Thus the lowest F/S value for sunflower pectin occurred at pH 4.3 while citrus pectin showed this minimal ratio at pH 3.4, and the optimal range appeared to be broader in the commercial product. The citrus pectin had 28.5% methoxyl groups and 17.9% amide groups, the remainder (48.3%) being free carboxyl groups. The acetyl content of the pectin was 0.0% and the molecular weight was 100,260.

For the unmodified head pectin, increasing the gel pH from 3.4 to 4.3 improved gel strength, elasticity, smoothness and uniformity of texture (Fig. 2). At pH 3.4, the gels were soft, granular and lacked clarity while at pH 4.3 the pectins were more soluble and showed less tendency for pregelation during preparation. Even at its optimum pH, the sunflower gel (M-0) was rated as inferior in gel characteristics to the citrus LM pectin at pH 3.1–3.4. However, these results indicated that sunflower pectins may be superior for specific applications such as in high pH dessert gels.

These results differ slightly from those of Black and Smit (1972a) who obtained the maximum breaking pressure at pH 3.8 and minimum sag at pH 3.3–3.7. Their commercial citrus LM pectin contained 33.7% ester groups, 13% amide groups and 49.4% free carboxyl groups. Owens et al. (1949) measured gel strength as the shear modulus for an acid-demethylated LM pectin which contained 21.5% ester groups and no amide groups. They reported that the maximum gel strength

Table 2—Chemical composition and gel characteristics of untreated and demethylated sunflower pectins

Sample	Galacturonic acid (%)	Esterification (%)	Acid amide groups (%)	Free carboxyl groups (%)	Acetyl content (%)	Molecular weight X 10^3	Gel sag (%)	Breaking point (g/cm^2)	F/S X 100	Gel characteristics[a] Pregelation	Smoothness (+) Brittleness (–)	Elasticity
H-0	98.9	40.4	0.7	57.8	1.9	147.1	12.7	76.7	16.6	+	–	+
H-1	99.2	24.2	8.7	66.3	1.3	117.5	8.4	89.5	9.4	+	–	+
H-2	100.0	33.2	2.9	64.9	1.3	105.3	14.1	54.6	25.8	0	+++	+++
H-3	99.4	30.3	5.1	64.1	1.3	115.6	13.0	58.7	22.1	0	+++	+++
H-4	100.0	31.1	4.3	64.7	1.3	99.9	13.9	55.4	25.1	0	++	++
H-5	99.8	30.2	4.6	65.1	1.3	103.1	12.5	68.4	18.3	0	++	+++
H-6	100.0	28.3	3.6	68.1	1.3	103.9	10.5	72.8	14.4	0	–	+++
M-0	98.5	30.3	0.7	69.1	1.4	122.2	8.7	83.5	10.4	++	––	+
M-1	100.3	15.1	9.8	75.4	1.1	104.8	4.9	112.7	4.3	++	–––	+
M-2	99.3	22.7	6.3	70.3	1.0	87.3	9.1	78.9	11.5	+	––	+
M-3	98.8	25.1	5.2	68.5	1.3	113.2	7.6	75.0	10.1	+	––	+
M-4	98.6	22.0	7.0	69.7	1.1	110.0	6.7	90.2	7.4	+	––	+
M-5	99.5	22.6	7.4	69.5	1.1	111.5	6.3	101.2	6.2	+	––	+
M-6	100.0	13.9	9.3	76.8	0.6	114.0	3.6	212.5	1.7	+	–––	0

[a] + = high, 0 = none.

occurred at pH 2.7, with values decreasing on either side of this pH. It is generally accepted that gelation characteristics, to the extent that they are determined by hydrogen and calcium bonding, are associated with the proportions of methoxyl, amide and free carboxyl groups as well as their distribution on the polygalacturonic acid molecules.

Characteristics of the demethylated pectins

Since the sunflower pectins were sufficiently high in ester content to permit demethylation, experiments were conducted to randomize the distribution of free carboxyl groups by hydrolytic demethylation. Partial amidation of demethoxylated carboxyl groups would limit the increase in total free carboxyl groups and contribute to further randomization of free carboxyl groups. The net effect would be to produce a more uniform hydrogen and calcium bonding that should minimize pregelation and enhance the smoothness and strength of the sunflower gels. The NH_4OH treatments applied in the present study (Table 1) decreased the ester contents quite substantially in both the head (H-series) and head and stalk (M-series) samples (Table 2). Greater variation were observed in the M-series because of the wide range in treatments applied to these pectins, and some esterification levels were very low.

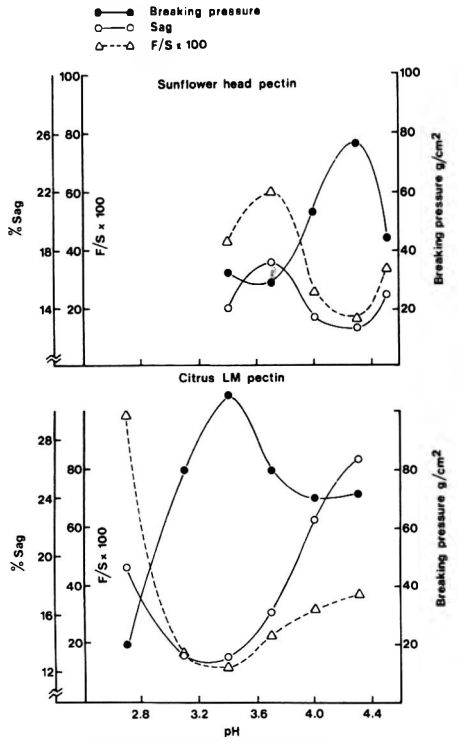

Fig. 1—Effects of pH on sag and breaking pressure of gels made from unmodified sunflower head pectin and commercial low-ester pectin.

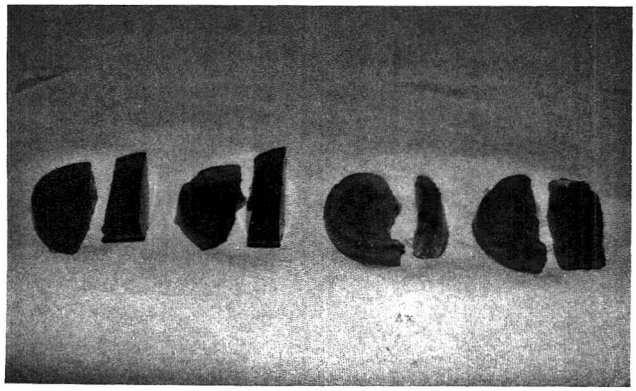

Fig. 2—Effects of pH on breaking characteristics of gel slices for unmodified head pectin. Left to right: pH 4.3, 4.0, 3.7 and 3.4.

Several workers have suggested a first order relationship between reaction time and the decrease of methoxyl groups during the demethylation process (Speiser et al., 1945; Black and Smit, 1972b; Kim et al., 1978a). Specific reaction rate constants were calculated using the formula: $K = (1/t)\ln(Co/C)$, where Co is the initial degree of esterification and C is degree of esterification at time t in min (Table 1). The K values obtained in this study (Table 1) were variable but within the range of values in other reports on NH_4OH demethylation, e.g. 25.1×10^{-3} at pH 11 and 5–7°C (Black and Smit, 1972b), 5×10^{-3} at pH 11 and 15°C (McCready et al., 1944) and $1.6–7.7 \times 10^{-3}$ at several concentrations of NH_4OH and temperatures (Kim et al., 1978a). In the present study, the highest rate of demethylation was achieved in the aqueous solution (M-1) and the rate decreased with increased ethanol concentration (Table 1). The specific rates of reactions, at constant ethanol concentration, were higher in the head (H series) than head-stalk (M series) mixtures, due possibly to the higher methoxyl content of the head sample.

The rates of ammonia demethylation were affected by temperature and concentrations of NH_4OH and pectin, but alcohol concentration appeared to be the most important variable in this study. Treatments which received higher aqueous:alcohol ratios (H-1, M-1, M-2) would permit greater hydration of pectin particles, more rapid penetration of ammonia and more uniform demethylation.

In addition to demethylation, the NH_4OH treatments decreased the acetyl contents, quite uniformly in the head samples, and increased the content of amide groups to 2.9–8.7% in head samples and 5.2–9.8% in mixed samples (Table 2). Decreases in molecular weights of the pectins due to the ammonia treatments were small in most mixed sunflower samples but the weights were reduced from 147,100 in the untreated head pectin to 99,900–117,500 in the treated pectins. It appeared that the low temperature of 4°C used for demethylation of several head-stalk pectins was effective in controlling depolymerization of the pectin while allowing greater exchange of acid amide groups for methoxyl groups. As is noted below, these improved chemical characteristics served to decrease gel sag and increase breaking strength, but gel brittleness and poor elasticity were still a problem in these gels.

Evaluation of the gel characteristics of the NH_4OH-treated pectins (Table 2) revealed hyperbolic relationships between degree of esterification and gel breaking strength or gel sag (Fig. 3). With greater de-esterification of the head and mixed head-stalk pectins, the sag decreased with an associated increase in breaking strength. These results support the earlier conclusions (Kim et al., 1978b) that the degree of esterification was the major factor influencing the gel strength and firmness as measured by the F/S ratio.

Although not directly correlated with degree of de-esterification, other gel characteristics were also improved by the NH_4OH treatments (Table 2). Generally, pectin solubility was increased and pregelation was less evident during gel preparation, especially in the treated head pectin samples. Smooth, clear and elastic gels were obtained with the H-2, H-3, H-4 and H-5 samples but the other gels still exhibited some brittleness and coarseness of texture, especially in the head-stalk pectin gels. These differences may be due to greater randomization of the methoxyl group distribution and less free carboxyl groups in demethylated head pectin molecules than occurred in the head-stalk mixtures.

Samples showing smooth and elastic gel properties such as

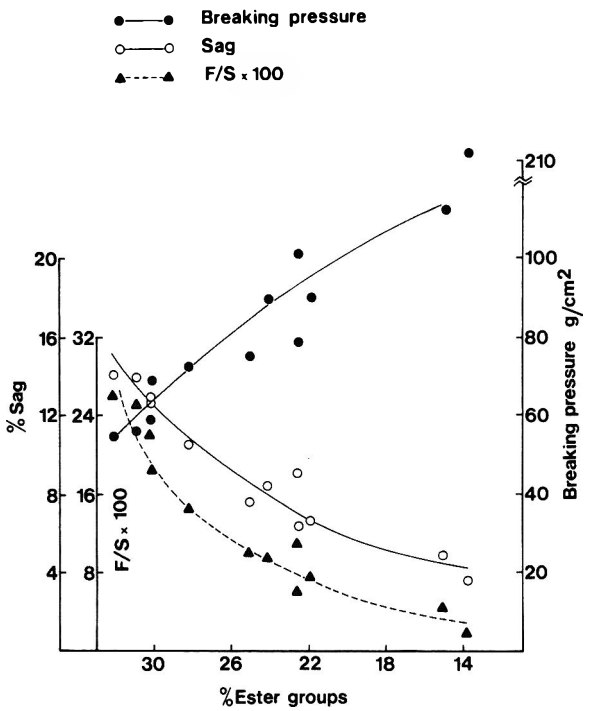

Fig. 3—Effects of degree of esterification on sag and breaking pressure of demethylated sunflower head and head-stalk pectin gels.

H-2 and H-3 are compared with the low-ester citrus gel and the more brittle M-2 gel in Figure 4. After standing at room temperature for several hours, gels made from the NH_4OH-treated sunflower samples retained their original shape and firmness to a greater degree than the gel made with commercial pectin. It appeared that ammonia modification was a practical procedure for improving the gel characteristics of sunflower pectins. Generally, gels from mixed head-stalk pectins were inferior to the modified head pectins, despite a wide range of demethylation treatments. The conditions of 1.3% pectin, 50% ethanol, 2.5N NH_4OH and 18–25 min reaction time were satisfactory for demethylation of head pectins. It appeared that 4°C was more desirable than 12°C to control depolymerization of the pectin.

Commercial low-ester pectins are reported to form gels over a wide range of pH from 2.5–6.5 (Anon., 1947). However, specific optimum pH levels for maximum breaking pressure and minimum sag can be determined for each type of pectin as shown in Figure 1. The modified sunflower pectins were also evaluated for gel characteristics over a range of pH and the lowest F/S ratios occurred at pH 4.3 in most samples, and none were below pH 4.0. The effect of pH on gel characteristics of the H-2 and H-5 samples are illustrated in Figure 5. Despite substantial differences between the samples in the pattern of breaking strength and sag, the lowest F/S ratio occurred at pH 4.3 in each sample. The pH stability of each pectin was much improved over that of the untreated head sample in Figure 1.

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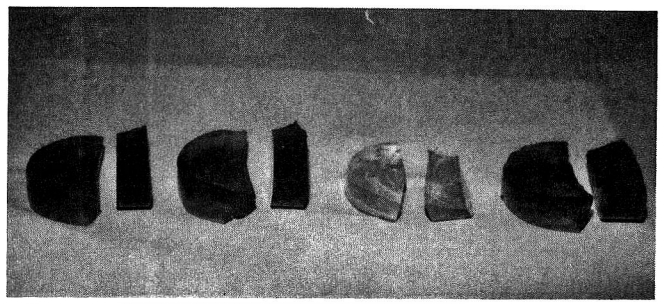

Fig. 4—Appearance of gels prepared from demethylated sunflower pectins at pH 4.3 and 22 mg Ca^{++}/g pectin and commercial low-ester pectin at pH 3.2 and 25 mg Ca^{++}/g pectin. Left to right H-2, H-3, citrus LM pectin and M-2.

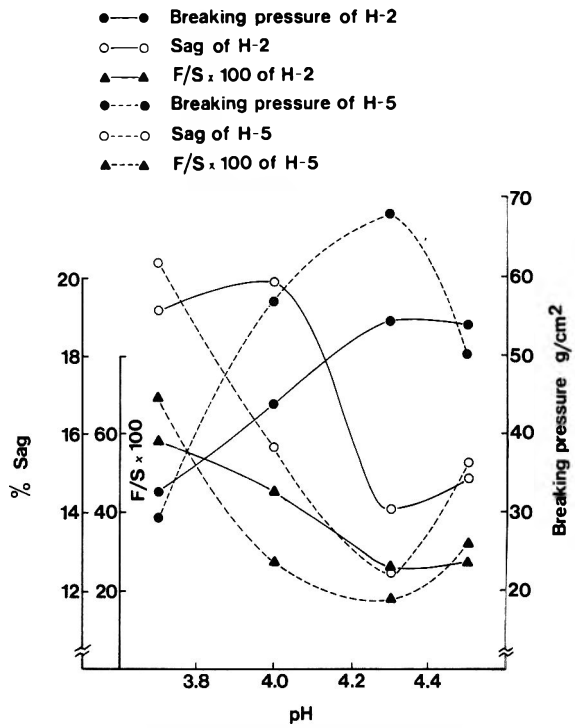

Fig. 5—Effects of pH on sag and breaking pressure of gels made from demethylated sunflower head pectins H-2 and H-5.

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This research was supported by grants from the National Research Council and the New Crop Development Fund.

DEGRADATION KINETICS OF ASCORBIC ACID AT HIGH TEMPERATURE AND WATER ACTIVITY

B. M. LAING, D. L. SCHLUETER and T. P. LABUZA

ABSTRACT

The kinetics of ascorbic acid degradation were studied in an intermediate moisture model food system as a function of water activity (0.69–0.90) and temperature (61–105°C). The disappearance of ascorbic acid in each case followed a zero order kinetic model. Rates of ascorbic acid degradation ranged from 1.5–10.5 mg/100g solids/min, while the activation energy was in the range of 14–17 Kcal/mole. It is suggested that dissolved oxygen concentration was limiting above 92°C, resulting in a rate decrease between 92 and 105°C. Rates of ascorbic acid degradation were found to increase with increasing a_w, except at 105°C, where the opposite was observed. An equation derived from the integrated zero order rate law was used to predict ascorbic acid losses during an unsteady state heating process approximating a linear temperature rise, with good results. The same equation was much less accurate (predictions were 2–4 times larger), when used to predict ascorbic acid losses during extrusion processing, most likely due to the difficulty in obtaining an accurate temperature history of the extruded product.

INTRODUCTION

THE LOSS OF nutritional quality during the processing and storage of foods has become an increasingly important problem with the introduction of nutrition labeling regulations. The loss of some nutrients, including ascorbic acid, may actually become the limiting factor in determining the shelf life of some products. There is an obvious need for methods which can be used to accurately predict the degree of nutrient loss occurring in specific food products. One such method is through the application of kinetic analysis to nutrient retention data. Labuza (1972), has discussed the application of chemical reaction kinetics to the study of deteriorative reactions occurring in dry and intermediate moisture foods.

The majority of data concerning the degradation of ascorbic acid in food is the result of endpoint analyses; that is, the measurement of ascorbic acid concentration prior to and at the conclusion of a given process or storage period. Nutrient retention data gathered in this manner are generally not subject to kinetic analysis.

Existing kinetic studies of ascorbic acid degradation in food are limited in one or more other respects. First, these studies have been performed at relatively low temperature, characteristic of product storage rather than processing. Secondly, predictions of nutrient stability based on kinetic analysis have been tested under conditions of constant temperature storage only, while temperatures most often vary during the processing and storage of foods. Finally, only several of the existing kinetic studies have considered the effect of water activity (a_w) on the kinetics of ascorbic acid degradation. Lee and Labuza (1975), have shown that rates of ascorbic acid loss in an intermediate moisture model food system are greatly affected by changes in a_w.

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0022-1147/78/0005-1440$02.25/0

Table 1—Model system composition

		%
Soy flour		40
Ground beef		25
Sucrose		20
Propylene glycol		10
Lard		5
Water		*
Ascorbic acid		∿400 mg/100g solids

* as per system

	a_w	% H_2O
System A	0.69	20
System B	0.80	26
System C	0.90	32

Lee et al. (1977), studied the kinetics of ascorbic acid degradation in canned tomato juice stored at temperatures ranging from 10–37.8°C. A computer simulation based on these results yielded accurate predictions of ascorbic acid stability in cans of tomato juice stored at constant temperatures. The use of a more complicated computer simulation technique to predict ascorbic acid losses during storage under varying temperature conditions was discussed. However, the feasibility of such a computer simulation was not verified experimentally. Recently Mizrahi and Karel (1977) have developed a kinetic-based computer simulation method which predicted accurately the loss of vitamin C during storage of dry tomato powder held isothermally under conditions of moisture gain.

In theory, the kinetics of ascorbic acid degradation for a given food system, in conjunction with the temperature history of the system, should provide sufficient information to make accurate predictions of the amount of ascorbic acid degradation occurring during a given nonisothermal process. Many food processes occur at high temperatures, for which little kinetic data exist; thus this theory has not been tested.

The objectives of this study were: (1) to determine the kinetics of ascorbic acid degradation in an intermediate moisture model food system at relatively high temperatures; (2) to determine the effects of a_w on the kinetics observed under these conditions; (3) to develop a mathematical model expressing the rate of degradation of ascorbic acid as a function of nonisothermal environmental conditions; and (4) to apply this model to the prediction of the amount of ascorbic acid degradation occurring during an unsteady state heating process.

MATERIALS & METHODS

Model system

The composition of the basic model food system used in this work is shown in Table 1. Water concentration was varied to achieve systems of three different a_w's within the intermediate moisture range (0.69, 0.80 and 0.90). The humectant propylene glycol was added to achieve an extrudable consistency at low water activities. The prepared systems were kept in frozen storage until used.

Moisture and a_w determinations

Water activity values were determined using the vapor pressure manometer according to the method of Labuza et al. (1976). Moisture analyses of the systems before and after extrusion were performed

using methanol-extraction gas-liquid chromatography (Tjhio et al., 1969).

Ascorbic acid determination

Ascorbic acid values were determined using a modification of the AOAC indophenol titration procedure (AOAC, 1975). Frozen samples of model system were blended with 99 ml of acetic acid-metaphosphoric acid extracting/stabilizing solution, centrifuged and filtered. The filtrate was titrated with indophenol dye to a visual end-point, yielding ascorbic acid concentration as mg ascorbic acid per 100g solids, thus accounting for any moisture changes in the model system during heating.

Determination of kinetic parameters for ascorbic acid degradation

Samples (about 100g) of each model system were preheated (for less than 60 sec) in a microwave oven and then placed into a Brabender Farinograph mixing bowl (300 cc capacity) equipped with a specially designed, tightly-fitting cover, based on the system used by Hsieh et al. (1976). Throughout heating, the sample was mixed at 100 rpm. A thermocouple mounted in the bowl cover and in actual contact with the model system provided temperature data. When a constant temperature was reached, the cover was raised and a 6–8g sample was removed, placed in a foil-laminated pouch, which was then dipped into liquid nitrogen to flash freeze the sample. The pouch was then vacuum sealed on a laboratory sealer and then the sample pouch was placed in frozen storage at −30°C until analyzed. The first such sample was designated time zero. Subsequent samples were obtained in an identical manner, at 5- or 8-min intervals, depending on the temperature under consideration. Since all samples were obtained at the same temperature, problems of come-up time were avoided. This procedure was repeated in duplicate for each model system at 61, 71, 79, 92, and 105°C. The rate of ascorbic acid degradation under each set of conditions was determined by linear regression analysis of concentration versus time. The resulting rate values (slope of above curve) were plotted versus the reciprocal of the absolute temperature to yield activation energies for the degradation of ascorbic acid in each model system.

Linear temperature rise

A heating profile which closely followed a linear temperature rise was achieved by placing a preheated sample of model system (100g) into the equilibrated (55°C) Brabender Farinograph mixing bowl, as described previously. Upon reaching a constant temperature, a sample was removed and immediately frozen. At this point, the temperature setting of the thermostat for the heating liquid circulating around the jacketed mixing bowl was increased to 120°C resulting in a gradual, approximately linear, temperature rise within the bowl. After a rise in sample temperature of 25–30°C, a final sample was obtained. This procedure was repeated in duplicate for each model system. All samples were subsequently analyzed for moisture, ascorbic acid and water activity.

Extrusion

A Brabender model 2003 3/4-inch extruder was specially modified for use in this work. Holes were drilled at five points along the length of the extruder barrel. A thermocouple was placed through each hole, into actual contact with the product flowing through the extruder. As shown in Figure 1, the protruding thermocouples were shielded from the rotating screw by the walls of a longitudinal groove along the interior surface of the extruder barrel. During extrusion, heating zones 1, 2 and 3 of the electrically heated barrel were set at 70, 80 and 83°C, respectively. Screw speed was set at 10 rpm. The temperature of the product as it passed along the extruder barrel was recorded continuously. Several minutes after the temperatures had all reached constant values, a sample of the product exiting at the die was collected and immediately frozen in liquid nitrogen. In addition, an unprocessed sample was also frozen. All samples were kept in frozen storage until analyzed for moisture, a_w, and ascorbic acid. Residence times for each model system were determined by measuring the transit time of a small rubber marker. Samples of each model system were extruded in triplicate.

RESULTS & DISCUSSION

FOR EACH SET of experimental conditions during the steady state experiments in the mixing bowl, a plot of change in ascorbic acid concentration ($A_o - A$) versus time yielded a straight line (example in Fig. 2). The disappearance of ascorbic acid in each case can thus be described by a zero order reaction model. This conclusion is contrary to the results reported by several workers including Waletzko and Labuza (1976) and Nagy and Smoot (1977). These authors described the loss of ascorbic acid using a first order kinetic mode. However, it must be remembered that previous studies have not generally considered the rate of degradation at high temperature. Further, the high initial concentrations of ascorbic acid necessary for high temperature studies may have resulted in an apparent zero order reaction, since the percent change in ascorbic acid concentration was relatively small. Even so, in several cases where degradation of ascorbic acid approached 50%, zero order character was still observed. Zero order rates of ascorbic acid loss for each system at each temperature as determined by linear regression analysis are presented in Table 2. For all values, a correlation coefficient of 0.97 or greater was obtained. As seen, the rate of ascorbic acid degradation in each system increases with increasing temperature from 61–92°C.

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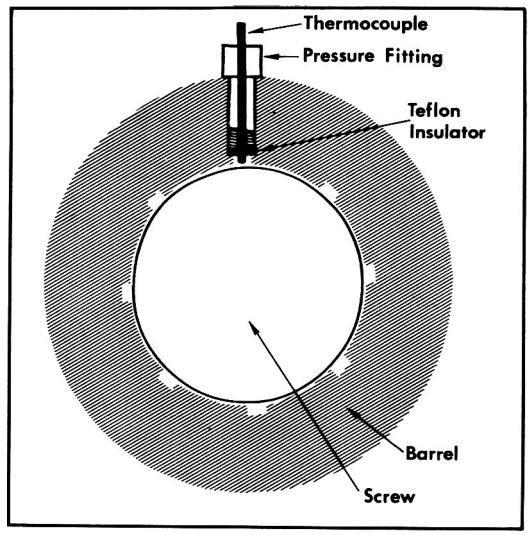

Fig. 1–Arrangement showing thermocouple bore in Brabender extruder.

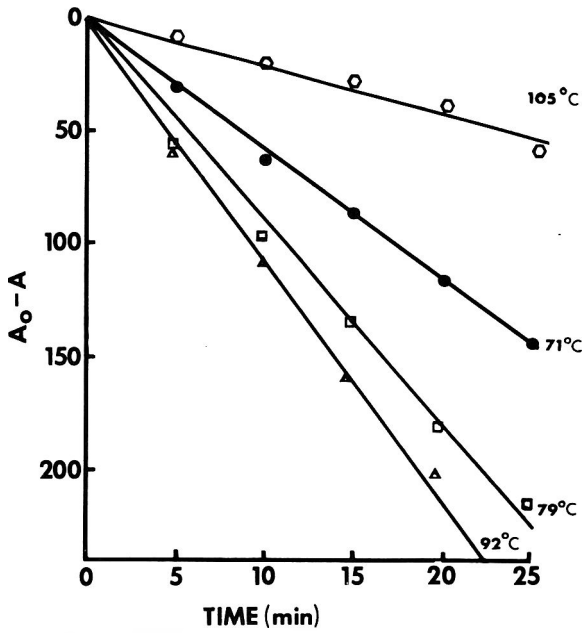

Fig. 2–Loss of ascorbic acid as a function of time in representative systems at a_w 0.9. Temperatures of heating shown on line. The Y axis is change in ascorbic acid content (mg per gram of system) from initial value of A_o.

In addition, within this temperature range, rates of ascorbic acid loss increase with increasing a_w. However, above 92°C, rates are seen to decrease with increasing temperature. Also, at 105°C, the ranking of the three model systems with respect to reaction rate is reversed; the system of lowest a_w showing the highest rate of degradation. The relationships among temperature, a_w and reaction rate can be seen more clearly in Figure 3, wherein rates of ascorbic acid degradation for each model system are plotted versus the reciprocal of the absolute temperature, yielding an Arrhenius plot for each model system. As seen, the effects of a_w and temperature on the rate of ascorbic acid loss appear to be interdependent and follow an unexpected pattern. The decline in reaction rate as temperature increased from 95–105°C suggests a negative activation energy for the degradation of ascorbic acid. A negative value for the activation energy of ascorbic acid degradation has previously been reported by Sizer and Maga (1976), studying extrusion of potato flakes. In both cases, these anomalous results can likely be explained by considering the effect of temperature on the solubility of oxygen in the aqueous phase of each system studied. The solubility of oxygen in pure water decreases with increasing temperature up to 100°C, where it approaches zero (Handbook of Physical Chemistry, 40th edition). According to Tannenbaum (1976) the oxidative and nonoxidative pathways for ascorbic acid degradation may operate simultaneously in the same system. The oxidative pathway, however, operates at a higher rate, and in the presence of O_2 will tend to predominate. In the present study, oxygen concentration within the aqueous phase of each model system may have become a limiting factor at temperatures approaching 100°C. Thus, the apparent negative activation energy observed is probably an artifact resulting from a shift in reaction mechanism from oxidative to nonoxidative. The same may be said of the negative value reported previously. As for the effect of a_w, increases in water content as a_w increases at the lower temperatures may have resulted in a rate increase simply because of a corresponding increase in the ratio of oxygen available to dissolve ascorbic acid. At the highest temperature studied, increases in a_w and thus water content would not have caused an increase in oxygen availability, since oxygen has little or no solubility at that temperature. Thus, at 105°C, increases in water content may only have served to dilute reactants and catalysts, resulting in the rate decreases seen. Kirk et al. (1977), in studying the effects of a_w on the kinetics of ascorbic acid degradation in a model cereal food system at normal storage temperatures, also found the ratio of ascorbic acid to soluble oxygen to have a significant effect on the rates of degradation observed.

Table 2—Rates of ascorbic acid loss (mg/100g solids/min)

System	a_w	Run	Temperature °C 61	71	79	92	105
A	0.69	1	1.58	3.34	6.09	7.79	4.95
		2	1.84	3.32	5.80	8.16	4.93
B	0.80	1	2.22	4.61	7.91	8.62	3.88
		2	2.21	4.61	7.89	8.50	3.83
C	0.90	1	3.11	5.66	8.30	10.57	2.34
		2	2.79	5.27	7.92	10.51	2.26

Table 3—Activation energies for the degradation of ascorbic acid

a_w	E_a (Kcal/mole)
0.69	14
0.80	17
0.90	16

Table 4—Comparison of actual and predicted losses of ascorbic acid for a linear temperature rise

System	a_w	Run	Concentration of ascorbic acid (mg/100g solids) Initial	Actual final	Predicted final
A	0.69	1	373.7	296.5	301.6
		2	377.3	284.4	295.2
B	0.80	1	384.2	285.9	289.0
		2	385.5	290.7	289.2
C	0.90	1	316.9	198.3	208.5
		2	357.3	253.2	250.3

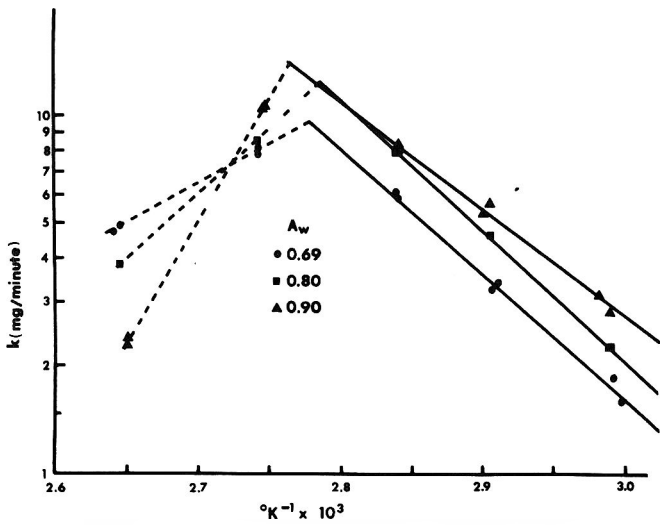

Fig. 3—Arrhenius plot of log rate of destruction (k mg/min) versus reciprocal absolute temperature.

Prediction of ascorbic acid losses during unsteady state heating processes

As seen in Figure 3, it is clear that the dependence of reaction rates on temperature between 61 and 92°C can be described well by use of the Arrhenius equation. The activation energy for the degradation of ascorbic acid in each model system, obtained by linear regression analysis, is presented in Table 3 and is not significantly different for the results up to 92°C. The data for 105°C cannot be included, since they would result in a negative value.

To predict the amount of ascorbic acid degradation occurring in each model system during a given unsteady state heating process, the following equation was derived from the integrated zero order rate law:

$$A_n = A_o - \sum_{i=o}^{i=n} (k_i \theta_i) \quad (1)$$

where, A_n = final concentration of ascorbic acid; A_o = initial concentration of ascorbic acid; k_i = zero order rate constant for ascorbic acid degradation; and θ_i = interval of time at each temperature T_i. To test the accuracy of Eq (1), samples of each model system were subjected to an unsteady state heating process approximating a linear temperature rise. A computer iteration procedure was used to solve Eq (1) for A_n. The value of θ_i was set at one second. The average temperature over each 1-sec interval was calculated from the slope of the heating curve. The value of k_i at each temperature was calculated using the Arrhenius equation and the Arrhenius parameters for each model system. The resulting predictions are presented in Table 4. As seen, agreement between the actual final concentrations of ascorbic acid as determined by measurement and the predicted final concentrations is good. Thus, Eq (1) appears to

yield accurate productions of ascorbic acid losses occurring during a single unsteady state heating process.

Extrusion is an example of a much more complicated unsteady state heating process. A typical temperature profile for extrusion of the model system, determined using the thermocouple arrangement described previously, is shown in Figure 4. Temperature gradients between thermocouples were assumed to be linear to simplify the solution of Eq (1). The time required for a product to reach each thermocouple was calculated from the residence time for each run, assuming a constant flow rate. A computer iteration procedure was again used to calculate the predicted values of A_n, based on the temperature profiles obtained for each run. Predicted final concentrations of ascorbic acid are compared with actual final concentrations as determined by measurement in Table 5. As shown, ascorbic acid losses during extrusion were slight, even at a screw speed of 10 rpm, where residence times were 4–6 min. Predicted final concentrations were much less than the actual final concentrations observed in every case. The difference between predicted and actual loss was about a factor of 2–4 times larger. This inaccuracy may be ascribed to several causes: first, despite the fact that each thermocouple was insulated, it may have been influenced by the temperature of the heated barrel, rather than the product alone; second, some stasis may have occurred near the tip of each thermocouple; third, a temperature gradient may have existed within the extruder barrel with higher temperatures being concentrated nearer to the heated barrel surface; and fourth, the temperature rise may not be linear between thermocouples. In the first three cases the thermocouples would have recorded an artificially high temperature, leading to an overestimate of the amount of ascorbic acid lost, as was observed. The last case could have partially compensated for this. In addition to these, it is possible that at the pressures involved in the extruder barrel (10 psig), oxygen solubility may not have been limiting. This however, would have resulted in a greater loss than actually found or calculated.

The results of this study indicate that the rates of ascorbic acid degradation in the model system studied are dependent upon oxygen availability, which is in turn dependent upon temperature and moisture content. The equation derived to predict the amount of ascorbic acid lost during an unsteady state heating process was successful when tested under conditions approximating a linear temperature rise. The same equation yielded less accurate predictions of ascorbic acid losses during extrusion, most likely due to the difficulty in obtaining an accurate temperature history of the extruded product.

Table 5—Comparison of actual and predicted losses of ascorbic acid during extrusion

System	a_w	Run	Residence time (min)	Concentration of ascorbic acid (mg/100g solids) Initial	Actual final	Predicted final
A	0.69	1	5.83	342.9	329.6	308.1
		2	6.26	342.9	332.9	305.6
		3	6.26	342.9	335.4	305.6
B	0.80	1	6.54	354.9	328.4	304.4
		2	5.75	354.9	331.7	310.2
		3	6.14	354.9	328.8	307.1
C	0.90	1	4.88	335.0	325.3	293.6
		2	4.80	335.0	324.2	293.4
		3	4.88	335.0	324.3	293.6

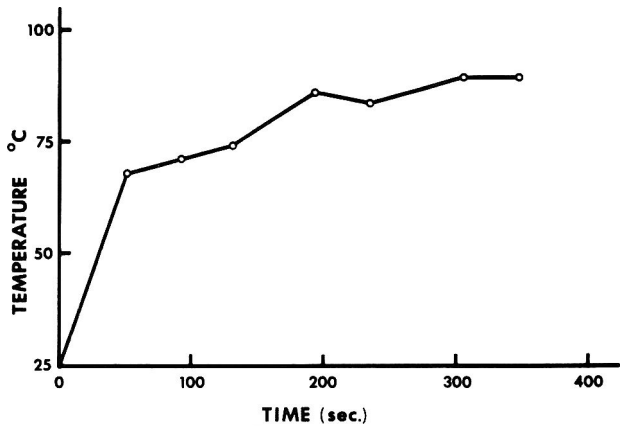

Fig. 4—Temperature profile in Brabender extruder during unsteady state process used to determine ascorbic acid losses.

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Ms received 11/3/77; revised 3/3/78; accepted 3/8/78.

This is paper no. 10,063 from the University of Minnesota Agric. Exp. Station. This study was supported in part by the University of Minnesota Agricultural Experiment Station Project 18-72, and 18-78. The authors also acknowledge the financial support received from the Pillsbury Foundation, and the U.S. Lyndon Johnson Manned Space Craft Center, Houston, TX, Contract NAS 9-12560.

RAPID DETECTION OF SALMONELLAE IN FOODS BY MEMBRANE FILTER–DISC IMMUNOIMMOBILIZATION TECHNIQUE

B. SWAMINATHAN, J. M. DENNER and J. C. AYRES

ABSTRACT

A membrane filter-disc immunoimmobilization technique was developed for rapid detection of salmonellae in foods. The method involved concentrating bacteria from the selective enrichment culture of a food sample by membrane filtration. The membrane filter, with the entrapped bacteria, was inverted and placed on the surface of a semi-solid selective medium contained in a 100 × 15 mm plastic Petri plate. A paper disc impregnated with *Salmonella* polyvalent flagellar antiserum was placed on the surface of the semi-solid agar approximately 2.5 cm from the nearest edge of the membrane filter. The plate was incubated at 37°C under high humidity. Motile salmonellae, if present in the sample, grew and migrated in the semi-solid medium. When the moving front of motile salmonellae came into contact with the diffusing flagellar antiserum, an antigen-antibody reaction occurred resulting in the immobilization of salmonellae. The formation of a line of immobilization indicated the presence of salmonellae in the sample. A semi-solid medium containing dulcitol, proteose peptone, brilliant green, and novobiochin as the major functional components was found to be more efficient than semi-solid modifications of *Salmonella-Shigella* agar and Hektoen enteric agar for the detection of salmonellae in foods by the membrane filter-disc immunoimmobilization procedure. The new method, when applied to the detection of salmonellae in raw meats and poultry, was found to give good correlation with the conventional cultural method.

INTRODUCTION

RECOGNITION of the presence of salmonellae in foods of animal origin has emphasized a need for rapid and simple detection methods that can be used in routine quality control. The fluorescent antibody technique (FA), which was granted Official First Action status by the Association of Official Analytical Chemists in 1975, requires the use of an expensive fluorescence microscope, costly fluorescein labeled antisera, and highly trained personnel to perform the test. The evaluation of fluorescence is subjective and there can be interferences due to nonspecific fluorescence and auto-fluorescence. For these reasons, it has not come into wide usage.

Several attempts have been made to devise other rapid and simple methods for the detection of salmonellae. Many of these methods involve selective motility media (Stuart and Pivnick, 1965; Harvey and Price, 1967; Banwart, 1968; Chau and Huang, 1974). The use of a semi-solid enrichment medium in a column or U-tube leads to the isolation of motile salmonellae from a mixed culture. The pure culture is then subjected to a few selected biochemical tests or O and H serology to confirm the presence or absence of salmonellae. Banwart et al. (1968) used such a method to screen and eliminate *Salmonella* negative samples of pasteurized dried whole egg. Fung and Kraft (1970), using a combination of motility medium and biochemical tests in a motility multi-layer flask could detect one *Salmonella* organism in 100 ml of culture medium in the presence of large numbers of contaminating bacteria in 17–36 hr. Chau and Huang (1974) reported the development of a 1-day selective migration procedure involving the passage of the organisms through a selective motility medium followed by the detection of H-antigens by slide agglutination. A higher detection efficiency was claimed using the selective migration procedure. The technique was further refined by making the motility medium more selective by the addition of magnesium chloride, brilliant green, and novobiocin (Chau and Huang, 1976).

Most of the selective motility procedures are primarily for the isolation of salmonellae from a mixed culture and require further confirmation through biochemical or serological tests. All the above methods also necessitate the use of a specially designed glassware apparatus. The present work purports to overcome these deficiencies and combines the isolation and reliable detection of motile salmonellae in one step. The method is based on the disc-immunoimmobilization technique suggested by Mohit et al. (1975) for the detection of salmonellae in feces in the presence of large numbers of contaminating bacteria.

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0022-1147/78/0005-1444$02.25/0

MATERIALS & METHODS

Cultures

Serotypes of salmonellae were obtained from Mrs. B.M. Thomason, Center for Disease Control, Atlanta, GA. Cultures of *Citrobacter freundii* and *Arizona hinshawii*, were provided by Dr. J.E. Williams, USDA, ARS, Southeast Poultry Research Laboratory, Athens, GA. Cultures of *Enterobacter aerogenes, Escherichia coli, Proteus vulgaris, Pseudomonas fluorescens, Serratia marcescens*, and *Shigella flexneri* were obtained from the culture collections at the Department of Food Science, University of Georgia.

Stock cultures of *Salmonella, Arizona*, and *Citrobacter* were maintained on semi-solid stab media (Thomason, 1974) at room temperature. Stock cultures of other bacteria were maintained on nutrient agar slants at 4°C. The stock cultures were activated, prior to use, by at least two passages through tryptic soy broth incubated overnight at 37°C.

Food samples

Samples of chicken parts were collected from a federally inspected poultry processing plant located in Athens, GA area. Samples of approximately 450g quantities of each specimen were collected in sterile plastic bags. Samples of raw meats and poultry in approximately 450g quantities, were purchased from five retail outlets in the Athens, GA area once every week over a 6-wk period.

Conventional cultural procedure

The examination of the meats and poultry samples by the conventional cultural method was carried out as recommended in the Bacteriological Analytical Manual for foods (1976). The samples were enriched in selenite-cystine and tetrathionate broths for 18–24 hr at 37°C. Brilliant green agar and Hektoen enteric agar were used as selective differential media for the isolation of pure colonies.

Selective motility media

Salmonella-Shigella motility medium (SSM) and Hektoen enteric motility medium (HEM) were prepared by reducing the concentration of agar to 0.4% but maintaining the recommended proportions of other ingredients (Difco Manual, 1953; King and Metzger, 1968). The dulcitol-brilliant green-novobiocin motility (DBN) medium had the following composition: dulcitol 4.0g; proteose peptone 12.0g; beef extract 3.0g; sodium chloride 5.0g; sodium thiosulfate 5.0g; bile salts 15.0g; ferric ammonium citrate 1.5g; sodium desoxycholate 2.0g; neutral red 0.025g; brilliant green 0.0004g; novobiocin 0.02g; agar 4.0g; and distilled water 1000 ml. The medium was heated to boiling and its

pH was adjusted to 7.0 ± 0.1. The medium was cooled to 45°C and dispensed in 12 ml amounts into plastic disposable Petri dishes (100 mm × 15 mm). After gelation of the medium, the Petri dishes were stored at 4°C and at high humidity until use.

Antiserum discs

Antiserum discs were prepared by impregnating 6.3 mm diameter sterile blank concentration discs (Difco Laboratories, Detroit, Mich.) with 0.02 ml of a 1 in 10 dilution of *Salmonella* polyvalent "H" antiserum in Tris-albumin buffer, pH 7.4 (Mohit, 1968). The antiserum discs could be stored in a freezer for up to one month without any apparent loss of activity.

Sensitivity of disc immunoimmobilization

For the determination of the sensitivity of the disc immunoimmobilization procedure, *Salmonella* cultures were grown in tryptic soy broth at 37°C for 18 hr, harvested by centrifugation and suspended in phosphate buffered saline (PBS). For the mixed culture, *P. vulgaris*, *P. fluorescens*, two strains of *E. coli*, *S. flexneri*, and *E. aerogenes* also were grown overnight in tryptic soy broth. One-tenth milliliter of each nonsalmonella culture (approximately 10^7 organisms) was added to a sterile test tube. An appropriate dilution of a *Salmonella* serotype was added to the mixed culture to give a concentration of approximately 10, 100, or 1000 cells in 0.02 ml of the mixture. The total volume was made up to 1 ml in each tube with PBS. The mixed culture (0.02 ml) containing known numbers of salmonellae was placed on the surface of SSM medium, HEM medium, or DBN medium contained in a Petri dish. An antiserum disc was placed on the surface of the medium in the Petri dish approximately 5 cm from the site of inoculation. The plates were carefully stacked in an upright position inside an air tight plastic container. A beaker containing tap water was placed inside the container to maintain high humidity during incubation. The plastic containers were placed in an incubator at 37°C and observed after 12, 16, and 24 hr.

MFDI procedure for foods

The following procedure was used for the detection of salmonellae in foods by the membrane filter-disc immunoimmobilization (MFDI) procedure. Twenty-five grams of the food samples were weighed aseptically into a sterile 474 ml canning jar and 225 ml of selenite cystine broth was added. In order to facilitate emulsification of the fat in the samples, 2.2 ml of Tergitol 7 (Aloe Scientific Co., St. Louis, MO) was added to the enrichment broth. The samples were blended at low speed for 2 min in an Osterizer blender (John Oster Mfg. Co., Milwaukee, WI) or were shaken thoroughly by hand. The enrichment broth cultures were incubated in a water bath at 37°C for 6 hr. At the end of the incubation period, 1 ml of the enrichment culture was transferred to 9 ml of pretempered selenite cystine broth and incubated for an additional 2-hr period. One milliliter of the post enrichment culture was filtered through a 0.45 μm, 25 mm diameter, membrane filter (Millipore Filter Corp., Bedford, MA) in a special filtration assembly capable of handling 30 filtrations at one time. The membrane containing the bacteria was placed in an inverted position on the surface of the semi-solid medium contained in a Petri dish. An antiserum disc was placed on the surface of the semi-solid medium approximately 2.5 cm from the nearest edge of the membrane (See Fig. 4). The plates were incubated as described earlier and observed after 16 and 24 hr.

RESULTS & DISCUSSION

THE SENSITIVITY of the disc immunoimmobilization technique for the detection of salmonellae in mixed culture containing large numbers (>10^6 cells/ml) of other bacteria was determined using SSM, HEM, and DBN media. Results shown in Table 1 indicate that HEM and DBN media were more sensitive than SSM medium in detecting low numbers of salmonellae. Four serotypes of *Salmonella* were detected using the DBN medium at a concentration of 8–12 cells in the inoculum while 3 were detected with the HEM medium at this level. All 6 serotypes gave positive results using HEM and DBN media at a concentration of approximately 80–122 cells in the inoculum. In contrast, 3 serotypes were detected using the SSM medium at a concentration of approximately 8–10 cells, 4 were detected at the 80–122 cell level, and all the 6 were detected at a level of approximately 1000 cells. Clear lines of immobilization were observed in the case of positive samples on SSM medium and DBN medium. The line of immobilization was not very clear on the HEM medium.

Table 1—Sensitivity of the disc immunoimmobilization technique for the detection of salmonellae in mixed cultures

Serotype of *Salmonella*	Minimum number of cells yielding a positive result in the immunoimmobilization test on different semi-solid media		
	SSM[a]	HEM[b]	DBN[c]
S. anatum	8	80	8
S. infantis	122	12	12
S. javiana	860	86	86
S. newport	10	104	10
S. senftenberg	940	94	94
S. typhimurium	8	8	8

[a] SSM — *Salmonella-Shigella* motility medium
[b] HEM — Hektoen enteric motility medium
[c] DBN — Dulcitol-brilliant green-novobiocin motility medium

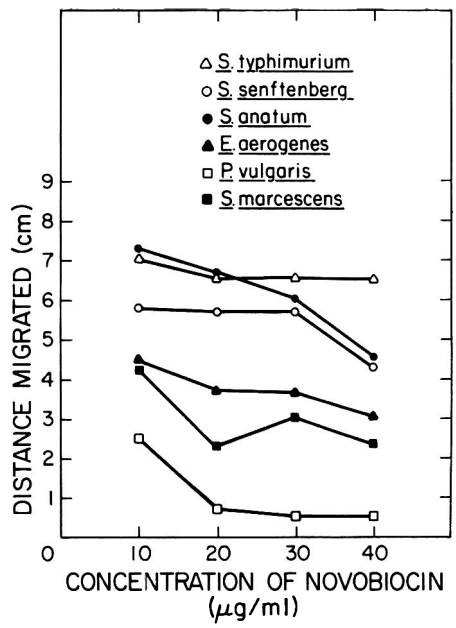

Fig. 1—Effect of novobiocin on the growth and motility of salmonellae and other microorganisms.

The effect of novobiocin, a component of DBN medium, on the growth and motility of 3 serotypes of *Salmonella* and 3 nonsalmonellae is shown in Figure 1. At 20 μg/ml, novobiocin did not cause any significant inhibition of salmonellae. However, at this concentration of novobiocin, there was a pronounced inhibitory effect on the spreading of *P. vulgaris*. The effect on *E. aerogenes* and *S. marcescens* was much less pronounced. Chau and Huang (1976) incorporated novobiocin into a motility medium that they had developed for the rapid detection of salmonellae in clinical specimens. Novobiocin, at a concentration of 20 μg/ml in the medium, was found to inhibit the migration of 83% of the strains of *C. freundii* tested but none of the *E. cloacae*, *Arizona* and *Salmonella* strains were inhibited. The results reported here are in agreement with the observations of Chau and Huang (1976). The concentration of 20 μg/ml novobiocin appears to be optimal for use in DBN medium.

The DBN medium contains dulcitol as the fermentable carbohydrate. Dulcitol is utilized fermentatively by most strains of salmonellae (86.7%) but is not fermented by most other Enterobacteriaceae except for some strains of *Escherichia* (49.3%), *Shigella* (5.3%), *Citrobacter* (57.3%), *Klebsiella* (33.0%), and a few strains of *Enterobacter cloacae* (Edwards and Ewing, 1972). Raj (1966) observed that the

incorporation of dulcitol in a medium containing selenite greatly enhanced the selectivity of the medium for salmonellae. Dulcitol selenite medium was reported to be highly sensitive for recovering as few as 2 to 7 cells of *Salmonella* even in the presence of large numbers of mixed flora common to sea foods. Abrahamsson et al. (1968) used the dulcitol selenite medium in a 'motility flask' to isolate salmonellae and reported that the new procedure was significantly more sensitive than the conventional cultural method for detection of salmonellae in 8 different types of foods. The DBN medium contains high concentrations of proteose peptone to offset the inhibitory effects of bile salts on salmonellae. Proteose peptone is a source of bound cystine and enhances the selectivity of a medium for salmonellae (Raj, 1966).

Table 2—Comparison of the efficiency of various semi-solid media in detecting salmonellae in chicken samples

	Semi-solid media		
	SSM[a]	HEM[b]	DBN[c]
Total samples	84	84	84
Salmonella positive	9	14	19
Detection efficiency (%)[d]	46	74	100
Line of immobilization	Clear when formed	Hazy; overgrowth by other bacteria	Black clear
Inhibition of non-salmonellae	Good	Poor	Good

[a] SSM – *Salmonella-Shigella* motility medium
[b] HEM – Hektoen enteric motility medium
[c] DBN – Dulcitol-brilliant green-novobiocin medium
[d] Detection efficiency = $\frac{\text{no. positive by the trial method}}{\text{no. positive by the cultural technique}} \times 100$

Table 3—Comparison of MFDI, FA and cultural techniques for the detection of salmonellae in chicken samples

	MFDI[a]	FA[b]	Cultural
Total samples	84	84	84
Salmonella positive	19	21	19
False positives[c]	0	2	–
False negatives[c]	0	0	–

[a] MFDI – Membrane filter-disc immunoimmobilization
[b] FA – Fluorescent antibody
[c] Using cultural method as the reference

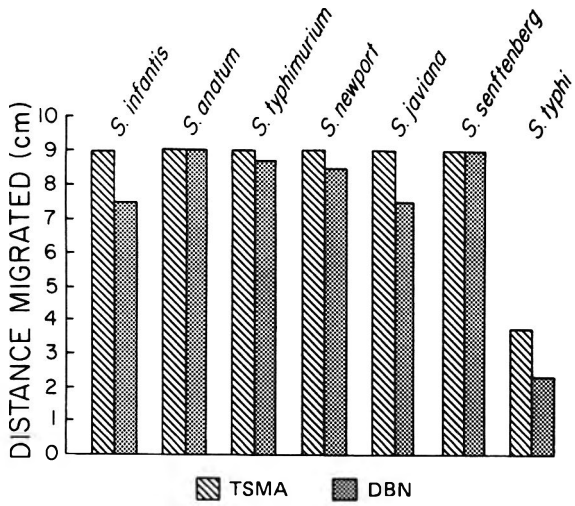

Fig. 2—Relative growth and motility of salmonellae on the DBN medium and TSMA medium.

The growth and motility of 7 serotypes of salmonellae on the DBN medium are compared to their growth and migration in a nutritionally complete nonselective medium (TSMA); see Figure 2. All the serotypes, except *S. senftenberg* and *S. anatum*, were inhibited slightly on the DBN medium as compared to the nonselective medium. The extent of inhibition ranged from 3% for *S. typhimurium* to 17% for *S. javiana* and *S. infantis*. The rate of migration of *S. typhi* on TSMA medium was much lower than that of other serotypes. It was retarded further (31%) on the DBN medium.

The relative rates of growth and migration of nonsalmonellae on DBN medium and TSMA medium are shown in Figure 3. All of the nonsalmonellae tested, except *C. freundii*, were significantly inhibited on the DBN medium. *P. fluorescens* and 2 strains of *E. coli* were completely inhibited on the DBN medium. *P. vulgaris* and *S. marcescens* were inhibited 75% and 79% respectively. Two strains of *C. freundii* grew well on the DBN medium but one H_2S strain was inhibited to the extent of 67%. Two strains of *A. hinshawii* were inhibited to the extent of 50% and 46% respectively on the DBN medium.

A comparison of the effectiveness of SSM, HEM, and DBN media in detecting salmonellae in chicken samples obtained from a poultry processing plant is given in Table 2. SSM medium proved to be unsatisfactory for the detection of salmonellae. Only 9 of 84 samples were positive on the SSM medium as against 19 of 84 by the cultural technique giving a detection efficiency of 46%. Hektoen enteric motility medium was also unsatisfactory with a detection efficiency of 74%. This medium was not sufficiently selective and other bacteria outgrew the salmonellae. After 24 hr of incubation, there was excessive growth on the medium and, with samples that were *Salmonella* positive, the line of immobilization was difficult to discern. The detection efficiency of DBN medium was 100%; in addition, the medium was significantly inhibitory to organisms other than salmonellae. Blackening caused by salmonellae that were H_2S positive rendered the line of immobilization clear and unambiguous (Fig. 4).

Nineteen of the 84 chicken samples tested by the MFDI technique using the DBN medium were positive for salmonellae (Table 3). The same number were positive by the cultural technique. Twenty-one samples were positive by the FA procedure. No false positives were obtained by the MFDI procedure. This is not surprising since H antigens are shared only among the *Salmonella-Arizona* group and thus tests involving H antigens and antibodies are extremely specific (Silliker et al., 1966).

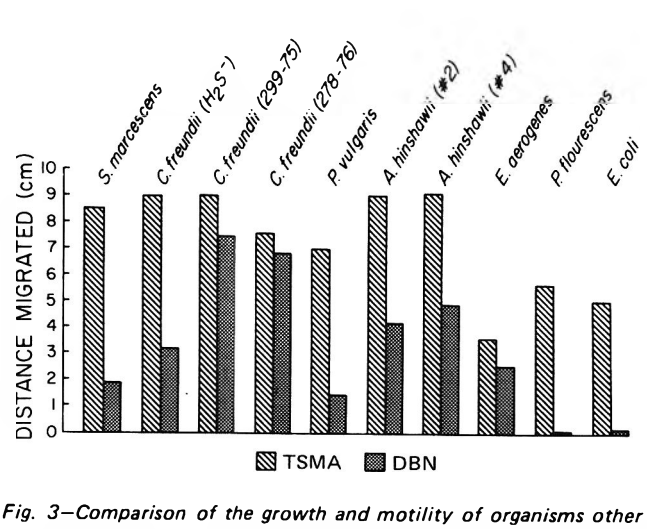

Fig. 3—Comparison of the growth and motility of organisms other than salmonellae on DBN medium with their growth and migration on TSMA medium.

The MFDI procedure also was evaluated against the cultural method in an examination of 142 samples of raw meats and poultry obtained from retail stores (Table 4). The agreement between the two methods was 91.6% for 36 beef samples, 92.3% for 65 pork samples and 97.5% for 41 poultry samples. The overall agreement for all 142 samples was 93.6%. Seven samples which were positive by MFDI were negative by the cultural procedure.

The successful application of the immunoimmobilization technique depends on the humidity at which the plates with semi-solid medium are stored and incubated. Any loss of moisture from the plates during storage or incubation tends to affect results adversely. Mohit et al. (1975) overcame the problem by sealing the lid on individual Petri dishes to the lower plates with stop-cock grease. This is a messy, cumbersome and time-consuming procedure and may lead to contamination of the medium. We solved the problem by storing and incubating plates in airtight plastic containers in which high humidity could be maintained at incubation temperatures of 37°C.

The membrane filtration step has been introduced to overcome the problem of detecting very low numbers of salmonellae in foods and feeds. In the original technique of Mohit et al. (1975), only 0.02 ml of the inoculum could be placed in the semi-solid medium. The bacteria in 1 ml of the broth could be used for MFDI by utilizing a membrane filter. The post-enrichment step is included primarily to facilitate the filtration of the sample through the membrane. Without post-enrichment, the membrane tends to get clogged, thus rendering filtration difficult.

Table 4—Comparison of the MFDI procedure with the cultural method in the detection of salmonellae in raw meat and poultry samples[a]

Sample category	No. analyzed	CUL + MFDI +	CUL − MFDI +	CUL + MFDI −	CUL − MFDI −	Percent agreement between CUL & MFDI
Beef	36	3	30	1	2	91.7
Poultry	41	3	37	0	1	97.6
Pork	65	13	47	1	4	92.3
Total	142	19	114	2	7	93.7

[a] CUL: cultural method; MFDI: membrane filter-disc immunoimmobilization procedure; CUL + positive by cultural method; CUL − negative by cultural method; MFDI + positive by MFDI technique; MFDI − negative by MFDI technique

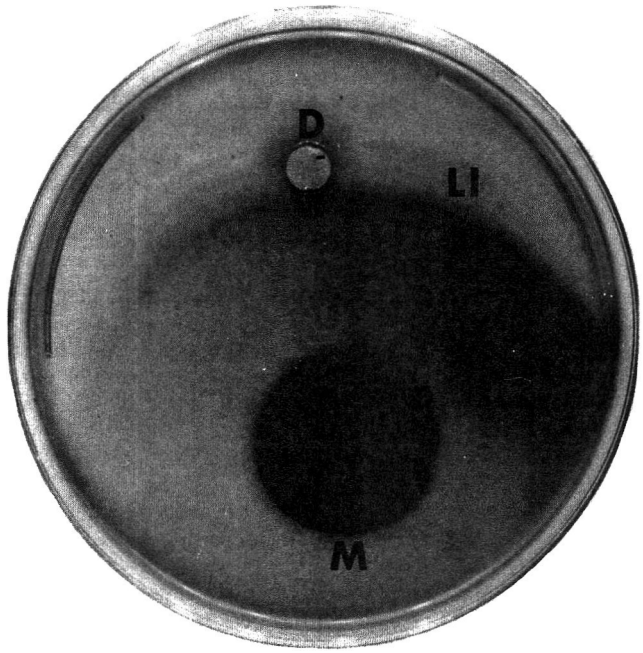

Fig. 4—Positive reaction for Salmonella on the DBN medium M = membrane filter; D = disc containing Salmonella polyvalent H antiserum; LI = line of immobilizaton.

CONCLUSIONS

A SEMI-SOLID MEDIUM (DBN) containing dulcitol, proteose peptone, bile salts, brilliant green, and novobiocin is more efficient than semi-solid modifications of *Salmonella-Shigella* agar and Hektoen enteric agar for the selective motility enrichment of salmonellae from mixed cultures. Use of the DBN medium in a membrane filter-disc immunoimmobilization procedure enables detection of salmonellae in foods within 24–30 hr after sampling. The membrane filter-disc immunoimmobilization (MFDI) procedure shows good correlation with conventional cultural methods for the detection of salmonellae in raw meats and poultry.

The MFDI procedure does not involve the use of any specially fabricated glassware. Plastic Petri dishes commonly used in microbiological laboratories are utilized. No expensive instrumentation is required. The results are obtained in 24–32 hr after sampling. The only drawback of the procedure is that it cannot detect nonmotile salmonellae. However, since the isolation of nonmotile salmonellae from human and non-human sources is rare (Anon, 1977), this should not cause undue concern. The MFDI procedure will be particularly useful in the routine screening of foods and feeds for the presence of salmonellae.

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Ms received 9/3/77; revised 3/30/78; accepted 4/5/78.

STABILITY OF CONCORD GRAPE (V. labrusca) ANTHOCYANINS IN MODEL SYSTEMS

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ABSTRACT

An anthocyanin extract was prepared by treating Concord grape filter trim with methanol acidified with 0.01% citric acid. The extract was concentrated to provide an aqueous extract for testing as a colorant for beverages. The concentrate was freeze-dried on a dextran carrier for use with a dry beverage mix. The stability of the anthocyanins in beverages with added glucose or sucrose was not pH dependent over the range from pH 2.8–3.6. Presence of ascorbic acid increased the pigment degradation rate. 5-hydroxymethyl-2-furfural (HMF) did not accumulate on storage in sufficient amounts to affect appreciably the degradation rate. D_{212} and z values were determined under aerobic conditions for several systems. The colorimetric "a/L" function correlated well with anthocyanin content at higher storage temperatures. The pigment was very stable in carbonated beverages, jelly and dry beverage base mix.

INTRODUCTION

CURRENTLY there is great interest in finding and evaluating natural sources of colorants as alternatives and replacements for coloring manufactured foods. Anthocyanins have been mentioned prominently in this regard. However, the idea of such commercial usage is not new. The suggestion that pigment remaining in grape skins after wine production be extracted for commercial utilization was first made by dal Piaz in 1885 (Singleton and Esau, 1969). Coste-Floret (1901) noted the commercial production of enocyanin in Italy by the turn of the century.

In the United States, Chiriboga and Francis first demonstrated a method for extracting anthocyanins from cranberry pomace in 1970. Since then other investigators have worked with anthocyanins from several sources and studied various extraction methods. The potential of roselle (*Hibiscus sabdariffa*) as an anthocyanin source was demonstrated by Esselen and Sammy in 1973. Its major advantage is ease of pigment extraction using an aqueous solvent system. Buckmire and Francis (1978) tested the anthocyanins from miracle fruit (*Synsepalum dulcificum, schum.*), and Du and Francis (1975) pointed out that berries of *Viburnum dentatum* contain 1% of their fresh weight as anthocyanin. Philip (1974) proposed a tartaric acid acidified methanolic extraction procedure for extraction of anthocyanins from grape wastes. Palamidis and Markakis (1975) tested anthocyanins that they extracted from grape wine pomace in a carbonated beverage model system. Shewfelt and Ahmed (1977) using red cabbage and blueberry sources demonstrated stability differences attributable to differences in extraction methods.

The anthocyanins of Concord grapes have been investigated by a number of workers (Anderson, 1923; Sastry and Tischer, 1952; Ingalsbe et al., 1963; Robinson et al., 1966; Shewfelt and Ahmed, 1966; Van Buren et al., 1970; Niketic-Aleksic and Hrazdina, 1972). In more recent work, Hrazdina (1975) isolated 20 anthocyanin pigments from Concord grapes.

The major anthocyanidins of Concord grapes were reported to be cyanidin and delphinidin by Ingalsbe et al. (1963); Robinson et al. (1966); Shewfelt and Ahmed (1966); and Hrazdina (1975) and malvidin by Sastry and Tischer (1952) and Van Buren et al. (1970). The pigments in grape filter trim would be expected to have the same qualitative but probably not the same quantitative composition. The pigments in filter trim would depend on their relative stability, solubility and absorption characteristics. Hrazdina (1975) indicated that increasing methylation increases stability, whereas increasing glycosylation decreases stability. Malvidin-3,5-diglucoside is likely to be a major pigment in grape products after long storage, hence the suggestion of Niketsic-Aleksic and Hrazdina (1972) that it be used as a standard for quantitative analysis of nonvinifera grapes is probably as appropriate as possible. The choice of any single pigment for quantitative analysis of a complex mixture will involve a compromise.

Two major goals of this endeavor were to study the potential of Concord grape pigments as natural colorants, and to develop a mechanism that would allow for easy comparison of different anthocyanin sources and preparation methods with respect to their relative thermal stability.

METHODS & MATERIALS

DURING the detartration step in the production of Concord grape juice a heavily pigmented precipitate is produced. Subsequent filtration with the use of diatomaceous earth as a filtering aid leaves what is referred to as grape filter trim. Grape filter trim, which incidently contains 10 times the pigment found in wine pomace, was the source of anthocyanin for this study. Homogenized grape filter trim was sealed in 404 × 700 fruit juice cans and frozen at Welch Foods, Inc., Westfield, NY 14787. It was transported to Amherst and stored in the −20°F room until needed.

Extraction was carried out using methanol acidified with 0.01% citric acid as the extracting solvent. Concentration and conversion to an aqueous solution was performed with a rotary vacuum flash evaporator at temperatures not exceeding 40°C. The aqueous concentrate was used for all testing with the exception of the dry beverage base mix for which a freeze-dried anthocyanin powder was prepared. Chiriboga (1972) freeze-dried cranberry anthocyanins with little difficulty. The grape system proved to be very hygroscopic, possibly due to the presence of sugars. It was necessary to incorporate the use of a low dextrose equivalent corn syrup solids support in order to produce a satisfactory product. The Hubinger Co. (Keokuk, IA 52632), provided 25 dextrose equivalent corn syrup solids for this purpose. The aqueous anthocyanin concentrate produced for use in this study contained 400–500 mg/liter of anthocyanin calculated as malvidin-3,5-diglucoside.

High temperature aerobic studies were performed using the flask method similar to that used by Tinsley and Bockian (1960) in their study of the strawberry anthocyanin system, and more specifically as described by Stumbo (1965). A 1000-ml three-neck ground glass round-bottom flask was immersed in an oil bath so that the level of the oil was always above the level of the reaction mixture. A plexiglass stirrer was installed through the center port; through one side port a mercury in glass thermometer was affixed with the aid of a rubber stopper that had a groove cut in it to serve as a vent; and the remaining port was closed with a ground glass stopper and used for sampling purposes. 500 ml of the medium being tested was added to the flask and heated to several degrees above the experimental temperature. A sample of the hot test medium was removed and approximately 50 ml of pigment concentrate was added to the reaction medium. Within

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0022-1147/78/0005-1448$02.25/0

seconds a homogenous appearance would develop and a "zero" time sample was taken. Sampling was then undertaken at 30- or 60-min intervals, and samples were stored in ice water until analyses could be performed.

Storage studies

A major user of colorants in the high acid food range is the fruit juice drink industry. The pigment's performance in fruit juice drink model systems composed the major emphasis of the storage studies. Storage studies were also conducted in carbonated beverage, jelly and dry beverage base model systems. Pasteurization of juice drink models was accomplished by pumping the juice drink model through a three foot ¼ in. o.d. coiled stainless steel tube that was immersed in the oil bath. Oil bath temperature and pumping speed were regulated to allow for the solution to attain a minimum temperature of 190°F. Although three different capacity (68, 84 and 96 ml) 1½ in o.d. cylindrical glass jars were used in preparing the storage test packs, only one size was used for any individual test pack. After the samples were filled, a nitrogen sweep of the headspace was performed before the screw caps were applied. The bottles were inverted for at least five minutes to allow for pasteurization of the caps before being immersed in ice water to facilitate cooling.

The composition of the carbonated beverage model was 0.1M citrate-phosphate buffer adjusted to pH 3.2, 15% sucrose, anthocyanin extract and 0.1% sodium benzoate. The batch was carbonated to 2.2 volumes of CO_2, and filled into 12-oz carbonated beverage bottles.

With slight modification, the standard formula for the production of a synthetic jelly described by Livingston et al. (1955) was used in the preparation of the jelly model. Briefly, the ingredients were mixed and dissolved as the temperature of the batch was raised to 180°F, and filled into 4½-oz baby food jars. Because of pilot plant limitations, it was necessary to produce two "identical" batches in order to provide enough samples for duplicate analyses at each storage time-temperature combination. Because of possible processing variations the two were coded separately and one sample from each batch was tested at each storage time-temperature combination.

A dry beverage base mix, lacking only the colorant was provided for testing purposes by a commercial company. For rehydration, it was decided that a 10% solution produced the most acceptable end product. Ten grams of the base and one gram of the powdered anthocyanin extract were added to the same type of jars used in the production of the fruit juice drink models.

The prepared test packs were stored in the dark at 34° ± 2°F ambient and 100° ± 4°F for up to 8 months.

Coding

Table 1 shows the coding system used for this study. All the liquid systems were composed of a base consisting of 0.1M citrate-phosphate buffer adjusted to the appropriate pH.

Analysis

Analyses were performed on the liquid systems without any prior preparation. The jelly required melting of the pectin gel and dilution with an equal volume of distilled water to prevent reformation of the gel upon cooling. The dry beverage base was rehydrated in a 100 ml volumetric flask and filtered to remove turbidity which would have interfered with the analyses.

Total anthocyanin analysis was performed by the pH differential spectrophotometric technique described by Fuleki and Franics (1968) and reported as malvidin-3,5-diglucoside.

Transmission colorimetric measurements were obtained in L, a, b color units using a Gardner XL-10 colorimeter (Gardner Laboratory, Inc., Bethesda, MD 20014) and a 1.0 cm light path.

Ascorbic acid determinations were made using a slight modification of the xylene extraction method devised by Pepkowitz (1943) as described by Starr and Francis (1968). This method utilized 2,6-dichlorophenol-indophenol as the indicator.

HMF measurement

Measurement of the accumulation of 5-hydroxymethyl-2-furfuraldehyde (HMF) is a routine procedure when conducting studies with sugar solutions. HMF is one of the stable primary degradation products of glucose, fructose, and sucrose, and its capability to increase the degradative rate of anthocyanins in solution has been demonstrated by Daravingas and Cain (1968), Tinsley and Bockian (1960), Markakis et al. (1957) and Meschter (1953).

In heterogeneous solutions, absorbance by other compounds will contribute to error in calculating HMF concentration from measurements at 285 nanometers (nm). Harborne (1967) lists individual anthocyanins that exhibit absorption maxima between 269 and 289 nm in methanolic solution. The aqueous anthocyanin mixture used in this study displayed a collective absorption maximum at 280 nm. The anthocyanin contribution to absorbance at 285 nm would be expected to change proportionately with its contribution at 520 nm. It is not possible to determine exactly the portion of 285 nm absorbance contributed by anthocyanin, but by assigning it the same initial proportion at 285 nm as is found at 520 nm with the pH differential spectrophotometric technique, a correction for anthocyanin contribution can be calculated. The four formulae that enable calculation of the correction term at each sampling interval are as follows:

Table 1—Coding system for anthocyanin storage studies

Code	System	Contents
CON	Control	0.1M Citrate-Phosphate buffer
GLU	15% Glucose	Buffer + 15% Glucose
SUC	15% Sucrose	Buffer + 15% Sucrose
FJD	10% Fruit juice drink	Buffer + 15% Sucrose + 10% White grape juice
CBS	Carbonated beverage	Buffer + CO_2 + 0.1% Sodium benzoate + 15% Sucrose
JEL	Standard jelly	Sugar-Acid-Pectin-Gel
DBM	Dry beverage base	Commercial formulation
+AA	—	Added ascorbic acid

Calculation of zero time anthocyanin peak height:

$$\text{ACY } A_{t_o}^{285} = \frac{A_{\text{pH 1.0}}^{520} - A_{\text{pH 4.5}}^{520}}{A_{\text{pH 1.0}}^{520}} \times A_{t_o}^{285} \quad (1)$$

Calculation of anthocyanin portion at times other than zero time:

$$\text{ACY } A_{t_n}^{285} = \text{ACY } A_{t_o}^{285} \times \frac{\text{ACY}_{t_n}}{\text{ACY}_{t_o}} \quad (2)$$

Corrected 285 nm peak height to be interpreted as being contributed by HMF:

$$\text{HMF } A_{t_n}^{285} = A_{t_n}^{285} - \text{ACY } A_{t_n}^{285} \quad (3)$$

Calculation of HMF concentration:

$$\text{HMF}_{t_n}\ (\text{mg/liter}) = \frac{\text{HMF } A_{t_n}(\text{cm}) \times \text{MW (g/m)} \times 1000\ (\text{mg/g})}{\epsilon\ (1/\text{m} - \text{cm})} \quad (4)$$

[The molar absorptivity (ϵ) using a molecular weight of 126 was 16,500 (Wolfrom et al., 1948).]

In summary, after adjusting the 285 nm absorbance for the anthocyanin contribution the remaining 285 nm absorbance value was interpreted as being contributed solely by HMF.

Statistical analyses were performed employing Student's t test for comparison of the differences between slopes (β's) as described in Steel and Torrie (1960). Regression analyses using the least-squares best fit criterion correlation coefficients were also calculated.

RESULTS & DISCUSSION

Effect of temperature

High temperature aerobic degradation data for the anthocyanin system were obtained at three different treatment temperatures in four different model systems at pH 3.2. Besides the citrate-phosphate buffered control (CON), the glucose (GLU) system, the sucrose (SUC) system and the 10% fruit juice drink (FJD) system were tested. Treatment temperatures were 185°, 194° and 203°F. Data obtained at each treatment temperature for each of the four systems were found to adhere well to first order kinetics. An example of the regression line

Table 2—Aerobic first order rate constants at pH 3.2

System	Temp °F	Rate k/hr	Correlation coefficient r
SUC	176	–0.120	–0.995
CON	185	–0.159	–0.996
GLU	185	–0.152	–0.992
SUC	185	–0.198	–0.989
FJD	185	–0.266	–0.995
CON	194	–0.216	–0.995
GLU	194	–0.222	–0.996
SUC	194	–0.287	–0.999
FJD	194	–0.396	–0.998
CON	203	–0.328	–0.979
GLU	203	–0.323	–0.995
SUC	203	–0.494	–0.997
FJD	203	–0.527	–0.997

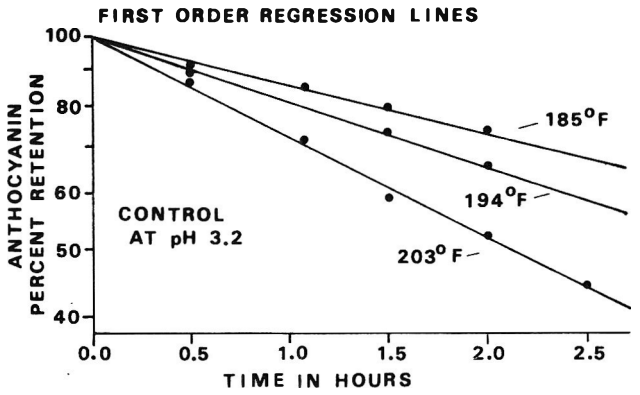

Fig. 1—Regression lines for degradation of anthocyanins at elevated temperatures.

Table 3—Aerobic degradation parameters for Concord grape anthocyanins in model systems at pH 3.2

System	Degradation parameters				
	k_{212} (k/hr)	D_{212} (hr)	z (°F)	β/°F	r
CON	–0.463	4.97	57	–0.0404	–0.993
GLU	–0.470	4.90	55	–0.0418	–0.993
SUC	–0.766	3.01	45	–0.0515	–0.997
FJD	–0.756	3.04	60	–0.0380	–0.993

Table 4—Effect of pH on aerobic first order degradation rate constants at 194°F

System	pH 2.8	pH 3.2	pH 3.6
		Rate k/hr	
CON	–0.264	–0.216	–0.232
GLU	–0.239	–0.222	–0.211
SUC	–0.280	–0.287	–0.308

obtained for each treatment temperature is shown in Figure 1 for the results from the control system. Table 2 summarizes the first order rate constants on a per hour basis, calculated from the data for each model at each treatment temperature. Also shown are the correlation coefficients that indicate the degree to which the raw data adhere to first order kinetics. Regression analysis of a plot of the log of these first order rate constants versus temperature for each system also produced regression lines with good linearity. From these regression lines it is possible to calculate the z value, D_{212} value and first order rate constant at 212°F for each system studied. These along with the regression coefficient and correlation coefficient for each system are shown in Table 3.

Statistically the results from the presence of 15% glucose in the glucose system did not produce a significant difference in the first order rate constants calculated for any of the treatment temperatures as compared to the corresponding rate constants calculated for the control system, nor was the rate of change with respect to temperature significantly different.

A significant difference at the 1% confidence level was observed both when the first order degradative rate constants for the sucrose system were compared with the corresponding rate constant for the control system, and when their rate of change with respect to temperature, or z values, were compared. As judged by the k value, the presence of sucrose served to increase the anthocyanin degradative rate at the temperatures studied, and to alter the z value from 57°F for the control system and 45°F for the sucrose system. The FJD system produced first order degradative rates greater than those produced by the control system at the temperatures studied that were significant at the 1% confidence level. The combined presence of fruit juice (10% white grape juice) and sucrose (FJD) in the system produced a z value of 60°F which was not significantly different from the z value of the control but the difference between this system and the sucrose system was significant at the 1% confidence level. Variation in z value emphasizes the importance of establishing controlled test criteria when comparing different anthocyanin sources or extraction methods.

Effect of pH

Hrazdina et al. (1970), Daravingas and Cain (1968), Tinsley and Bockian (1960), Markakis et al. (1957), Lukton et al. (1956) and Meschter (1953) all studied the effect of pH on the degradative kinetics of anthocyanin systems under either aerobic or oxygenated conditions, and observed that, as the pH was lowered to pH 2.0, the stability of the anthocyanin to thermal degradation was increased. Tinsley and Bockian studied the effect of change in pH on the degradative rate in a number of pelargonidin-3-glucoside model systems under aerobic conditions at 90°C (194°F) from pH 1.8 to pH 4.0. They reported an increase in degradative rate with increase in pH for all systems studied. The effect of pH on the first order kinetics was tested at 194°F on the three simple systems. The results (Table 4) from this grape anthocyanin system are not in accord with previously reported results obtained for either aerobic or oxygenated systems. The results from the glucose system show a slight increase in degradative rate with decreasing pH. Conversely, the results from the sucrose system indicate an increase in the degradative rate with increasing pH. The control system exhibited a pH optimum at pH 3.2 with change in either direction showing a decrease in stability. No explanation is offered to explain these observations, but it should be remembered that this system possesses a much more complex anthocyanin make up than other systems previously studied.

Effect of HMF

Data from the 285 nm absorbance measurements taken at pH 4.5 were adjusted to remove the anthocyanin influence as described earlier, and the adjusted readings were calculated as HMF concentration in milligrams per liter. Table 5 lists the accumulated HMF concentration for each system and temperature combination after 3 hr of heating. Also shown are the zero time concentrations, and the net production of HMF for the 3-hr period. Regression analysis of HMF concentration versus time using the least-squares fit criterion produced a line with good linearity in all cases. Figure 2 shows HMF accumulation for the four systems at pH 3.2 and 194°F. For the purpose of this calculation the zero time points were omitted because in most cases the rate for the first half-hour varied

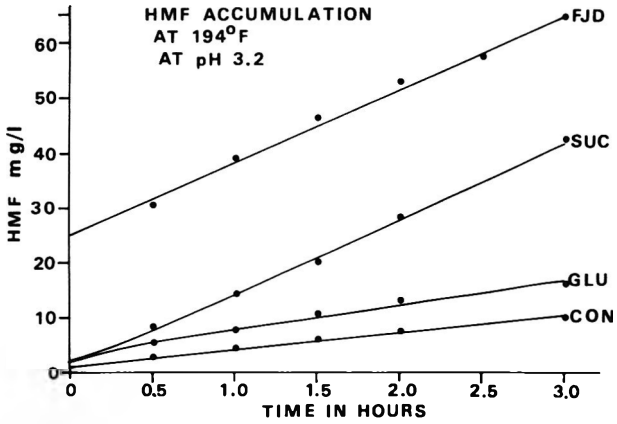

Fig. 2—Accumulation of 5-hydroxymethyl-2-furfuraldehyde (HMF) in anthocyanin systems containing different sugars.

considerably from the ensuing constant rate conditions. The net rate of accumulation as HMF is given in milligrams per hour. Under these conditions the net accumulation calculated as HMF followed pseudo zero order kinetics.

Since the anthocyanin concentrate contained less than 0.1% reducing sugars calculated as glucose, the increase in 285 nm absorbance is not necessarily due entirely to the presence of HMF. The increase is indicative of the formation of products containing carbonyl groups that absorb in this area. Singh et al. (1948) reported that glucose in solution exhibited a stability optimum to thermal degradation between pH 2.5 and pH 3.5. Is is not surprising that the 285 nm absorbance measurements indicate a low rate of HMF production for the glucose system. Probably a portion of the calculated HMF concentration for this system, as indicated by the increase in 285 nm absorbance for the control system, is in fact due to formation of other interfering compounds that absorb in this area.

In the sucrose system, the rate of HMF production was significantly greater than the rates observed for either the control or glucose systems. Presumably the increased rate is related to the presence of fructose which is one of the hydrolysis products of sucrose under acidic conditions. Haworth and Jones (1944) studied the rapid degradation of fructose that accounts for most of the HMF produced.

The rate of production of HMF in the 10% fruit juice system is essentially the same as that exhibited by the sucrose system for corresponding treatment conditions. It appears that sucrose accounts for the production of HMF, and that the presence of 10% white grape juice had no net effect on the accumulation of HMF (Fig. 2).

The above data indicate that relatively little HMF is accumulated. Studies that demonstrate the detrimental effect of HMF on anthocyanin degradative rate were conducted using from 0.01M–0.1M HMF added to the system which is equivalent to 1260 mg/liter to 12,600 mg/liter HMF. Tinsley and Bockian (1960) working with pelargonidin-3-glucoside in model systems at 90°C (190°F) added 0.05M HMF, which is in excess of 6000 mg/liter, when they demonstrated the effect of HMF on anthocyanin degradative rate. Under similar conditions in this study less than 100 mg/liter HMF was observed in almost all cases. This amount of HMF is probably too low to have much effect on the degradative rate of anthocyanins!

Storage studies

Conditions under which the fruit juice drink model storage studies were conducted were not identical to those of the high temperature studies with respect to available oxygen. While the high temperature studies were conducted in a system that was open to the atmosphere, the storage studies were conducted in sealed containers with a small amount of oxygen in each bottle. When Markakis et al. (1957) conducted thermal degradation studies of pelargonidin-3-glucoside in sealed tubes at high temperatures (176–212°F), they observed first order kinetics for those runs where meticulous care had been taken to remove all molecular oxygen. The runs conducted with aerobic headspace exhibited a pronounced tailing effect in logarithmic degradative rate which Tinsley and Bockian (1960) attributed to the rate limiting influence of the finite amount of oxygen present in the sealed tubes. Since the storage study models were prepared in a manner designed to simulate industrial processing conditions, no attempt was made to remove dissolved oxygen. Additionally, a small amount of oxygen may have remained in the headspace following the nitrogen sweep procedure.

Table 5—Production of HMF in Concord grape pigment model systems

		Temperature				
		185°F	194°F	194°F	194°F	203°F
System	Conc[a]	pH 3.2	pH 2.8	pH 3.2	pH 3.6	pH 3.2
CON	Initial	1.5	1.9	1.6	1.7	1.8
	3-hr	9.2	11.7	10.0	11.5	14.6
	ΔHMF	7.7	9.8	8.4	9.8	12.8
Rate (mg/hr)		2.5	2.9	2.7	3.0	3.9
GLU	Initial	1.7	1.8	1.9	1.9	1.8
	3-hr	10.9	16.0	16.1	15.0	22.5
	ΔHMF	9.2	14.2	14.2	13.1	20.7
Rate (mg/hr)		2.6	4.5	4.2	4.4	6.3
SUC	Initial	1.5	1.7	2.0	1.8	2.1
	3-hr	22.2	46.8	42.3	27.1	82.1
	ΔHMF	20.7	45.1	40.3	25.3	80.0
Rate (mg/hr)		6.7	15.9	13.6	8.7	28.8
FJD	Initial	21.6		25.1		21.8
	3-hr	45.2		64.3		103.5
	ΔHMF	23.6		39.2		81.7
Rate (mg/hr)		7.5		13.1		27.7

[a] Concentrations in milligrams per liter

Results from the storage study test packs at both 100°F and at ambient temperature (75°F) showed first order kinetics for the control, glucose and sucrose systems. The 10% fruit juice drink (FJD) system and the three systems containing concentrations after processing ranging from 86–95 mg per liter of added ascorbic acid underwent a change in their logarithmic degradative rate constants which occurred near the eighth week of storage. Where this happened it was necessary to calculate two rate constants. Regression lines possessing good linearity were obtained in all cases with first rate constant, k_1, representing the degradative rate for the first 8 wk, and the second rate constant, k_2, representing the degradative rate from the 8th to the 32nd week. Degradation at the 34°F storage temperature did not always adhere well to first order kinetics. This was not considered unreasonable, as this was a very complex system, and when one of the major degradative forces (heat) was minimized, then the multiple minor reactions that would be simultaneously occurring became magnified, and good adherence to logarithmic decline would not be expected to be observed. Table 6 lists the rate constants and corresponding correlation coefficients calculated for the pH 3.2 portion of the fruit juice drink model system storage studies; and Figure 3 depicts the change in anthocyanin retention versus time for the seven systems stored at 100°F.

While the more complex systems exhibited an obvious decrease in degradative rate, a plot of anthocyanin retention versus time for the three simple systems revealed a slight tailing off effect in their logarithmic degradative rates. At least two factors would be expected to affect the anthocyanin degradative rate in this manner. According to Ingalsbe et al. (1963), and Hrazdina (1975) the anthocyanin extract used in this

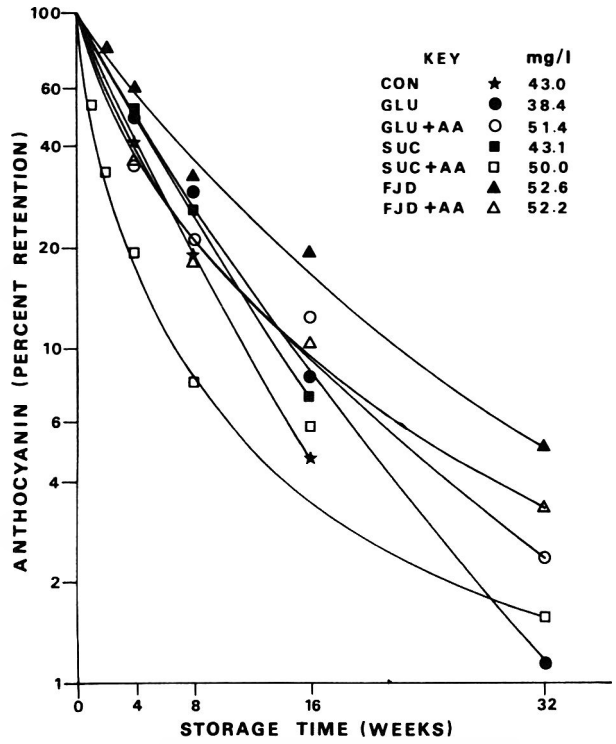

Fig. 3—Anthocyanin retention in model systems with various additives. The mg/liter refers to concentrations of anthocyanin.

Table 6—Logarithmic rate constants for degradation of Concord grape anthocyanins at pH 3.2

System	Temp (°F)	Rate[a] k_1/wk	Corr coef r_1	Rate k_2/wk	Corr coef r_2
CON	100	-1.92×10^{-1}	−0.998	–	–
	75	-5.76×10^{-2}	−0.990	–	–
	34	-5.17×10^{-3}	−0.925	–	–
GLU	100	-1.53×10^{-1}	−0.997	–	–
	75	-4.44×10^{-2}	−0.988	–	–
	34	-8.11×10^{-3}	−0.891	–	–
GLU+AA	100	-1.91×10^{-1}	−0.980	-9.59×10^{-2}	−0.982
	75	-1.13×10^{-1}	−0.962	-7.79×10^{-2}	−0.999
	34	-3.30×10^{-2}	−0.992	-5.98×10^{-3}	−0.719
SUC	100	-1.65×10^{-1}	−0.999	–	–
	75	-4.26×10^{-2}	−0.987	–	–
	34	-2.88×10^{-3}	−0.793	–	–
SUC+AA	100	-2.99×10^{-1}	−0.972	-6.93×10^{-2}	−0.982
	75	-1.42×10^{-1}	−0.963	-7.47×10^{-2}	−0.993
	34	-4.89×10^{-2}	−0.992	-1.12×10^{-2}	−0.929
FJD	100	-1.41×10^{-1}	−0.995	-7.82×10^{-2}	−0.998
	75	-5.94×10^{-2}	−0.992	–	–
	34	-4.51×10^{-3}	−0.825	–	–
FJD+AA	100	-2.13×10^{-1}	−0.992	-7.02×10^{-2}	−0.990
	75	-1.20×10^{-1}	−0.990	-6.74×10^{-2}	−0.996
	34	-1.28×10^{-2}	−0.970	–	–
CBS	100	-4.63×10^{-2}	−0.954	–	–
	75	-2.32×10^{-2}	−0.934	–	–
	34	-3.09×10^{-3}	−0.694	–	–
JEL[b]	100	-7.44×10^{-2}	−0.979	–	–
	75	-2.96×10^{-2}	−0.959	–	–
	34	-5.13×10^{-5}	−0.009	–	–

[a] When both k_1 and k_2 are given, k_1 represents rate constant calculated for the first 8 wk of the study and k_2 indicates the degradative rate for weeks 8 through 32. If only k_1 is given, it represents degradative rate for the entire study.

[b] JEL systems were at pH 2.6.

study would be expected to be composed of the mono and diglycosides of at least five anthocyanidins along with some of their acylated derivatives. Stability of the components of the anthocyanin mixture would be expected to vary, and as the more labile ones were depleted a slowing in the overall degradative rate would be anticipated. Second, while oxygen utilization would not be expected to be as rapid in these systems as in those containing ascorbic acid, a decrease in oxygen concentration should provide a rate limiting effect that should eventually be evident in terms of a slowing of the anthocyanin degradative rate.

Of those systems that exhibited a more pronounced logarithmic rate change, three of them contained added ascorbic acid, which produced an after processing concentration ranging from 86–95 mg of ascorbic acid per liter. The white grape juice used in formulating the FJD system contained some ascorbic acid which provided the system with a final concentration of 9 mg of ascorbic acid per liter. Sondheimer and Kertesz (1953), Markakis et al. (1957), and Starr and Francis (1968) studied the effect of oxygen and ascorbic acid on anthocyanin degradative rates. Markakis demonstrated that the combined presence of oxygen and ascorbic acid accelerated the rate of anthocyanin degradation in a synergistic manner. From Starr's work, oxygen concentration would be expected to approach zero after approximately 8 wk of storage.

Prediction of the magnitude of the effect of change in ascorbic acid concentration on the degradative rate of anthocyanin is difficult when coupled with a changing oxygen concentration. Reports by Shrikhande and Francis (1974), Starr and Francis (1968), Markakis et al. (1957) and Meschter (1953) indicated that the effect of ascorbic acid on the anthocyanin degradation rate was primarily due to an interaction of ascorbic acid oxidation products with anthocyanins and not merely ascorbic acid with anthocyanins. Hence, the decrease in oxygen concentration would be expected to play a major role in the slowing of the anthocyanin degradative rate, and decrease in ascorbic acid concentration would exert a lesser influence on the degradation rate of anthocyanin. Not only does ascorbic acid in the presence of oxygen serve to increase markedly the rate of anthocyanin degradation, but it also should serve to decrease the oxygen concentration more rapidly than would be expected without the presence of ascorbic acid.

At both 100°F and 75°F storage, the anthocyanin degradative rates exhibited by the glucose and sucrose systems were significantly different from the degradative rate calculated for the control system at the 1% confidence level. This concurs with the results Meschter (1953) reported for similar model systems of pelargonidin-3-glucoside. Tinsley and Bockian (1960) speculated that the protective effect observed by Meschter may have been due to the lower oxygen solubility in the sugar systems as compared to the sugarless control.

At 100°F comparison of the logarithmic rate constant, k_1, for the FJD system showed it to be significantly different at the 1% confidence level from the rate constants for both the control and sucrose systems. With respect to the protection afforded anthocyanins in this system compared to the sucrose system, it seems that the added protection was attributable to one or more components of the white grape juice which was present in the FJD system but not in the sucrose system. Presumably, this protection was in the form of competition for the finite amount of oxygen present in the system. The break in degradative rate near the 8th week of storage was attributed to depletion of oxygen, and the observed rate k_2 was probably what would be observed under anaerobic conditions. At 75°F, no break in rate was observed, nor was there a protective effect exhibited. The rate was significantly greater at the 1% confidence level from that exhibited for the sucrose system. Evidently the antioxidant effect imparted by the white grape juice was lessened as the storage temperature was lowered to a greater extent than the effect of those compo-

nents that act to increase the degradative rate of anthocyanin.

In all three of the systems containing added ascorbic acid, (GLU+AA, SUC+AA, and FJD+AA) the k_1 rates were significantly greater at the 1% confidence level than those exhibited by the corresponding systems without added ascorbic acid. The antioxidant effect exerted by white grape juice was again observed. The anthocyanin degradative rate k_1 for the FJD+AA system was significantly less at the 1% confidence level than that exhibited by the SUC+AA system at 100°F. However, while the k_1 rate of anthocyanin degradation at 75°F for the FJD+AA system was slower than that observed for the SUC+AA system, the difference was not significant at the 5% confidence level. Although these results are not exactly the same as were seen in the two corresponding systems without added ascorbic acid, the same trend with lowering of storage temperature is noted.

Small unavoidable variations in processing conditions from 190°F to 194°F would be expected to exhibit a greater influence on data variation at low temperature storage (34° ± 2°F) where the rate of anthocyanin degradation was much slower. When poor correlation coefficients were obtained, comparison of the calculated first order degradation rate constants was not considered to be appropriate. Clearly, degradation of anthocyanins in the systems not containing added ascorbic acid proceeded at a slower pace than those corresponding systems with added ascorbic acid. While anthocyanin loss for the systems without added ascorbic acid was not well explained in general by first order kinetics, the data showed that more than 90% of the anthocyanin was retained after 8 wk storage at 34°F. Addition of ascorbic acid exhibited a pronounced effect on anthocyanin retention in the two simpler systems. After 8 wk storage, nearly one-fourth of the anthocyanin present in the GLU+AA system and almost one third of the anthocyanin in the SUC+AA system was destroyed. In the FJD+AA system less than 15% of the anthocyanin was destroyed after eight weeks storage. Compared to the SUC+AA system this difference was significant at the 1% confidence level. Here again is evidence of the protective effect exerted by white grape juice towards anthocyanin degradation.

The literature is in general agreement that a decrease in pH leads to an increase in anthocyanin stability. Some exceptions do exist. Meschter (1953) reported that a strawberry syrup which was made up to 67% solids via addition of sucrose exhibited optimum anthocyanin stability at pH 1.8 when held under aerobic conditions at 38°C (100°F). Strawberry juice showed continued improvement in anthocyanin stability as pH was lowered from pH 5.0 to pH 1.0. Daravingas and Cain (1968) tested the stability of a black raspberry anthocyanin extract and of purified cyanidin-3-diglucoside in buffered solutions under oxygen atmosphere at 50°C from pH 4.15 to pH 0.95. When the pH was lowered from pH 2.15 to pH 0.95, both systems underwent an increase in their first order degradative rate constants.

Starr and Francis (1973) compared the stability of cranberry anthocyanins in cranberry juice cocktail, which is customarily manufactured at pH 2.7, to cocktail adjusted to pH 2.2. Batches were prepared both with and without added ascorbic acid. Because the same brix-acid ratio was desired, it was necessary to add extra sucrose to the pH 2.2 formulation. The methods that he employed for pasteurization, filling and ambient storage were almost identical to those used in this study. Examination of the first order degradative rate constants revealed that decrease in pH produced a slight increase in the degradative rate for the cocktail system without added ascorbic acid, and a slight decrease in the degradative rate for cocktail with added ascorbic acid. However, statistical analysis showed that the observed differences were not significant at the 5% level. It is not clear whether the extra sucrose present in the pH 2.2 formulations negated the anticipated improvement in anthocyanin stability. At 45°C, Lukton et al. (1956) demonstrated with both strawberry juice and buffered pelargonidin-3-glucoside systems that the decrease in anthocyanin stability observed under an oxygen atmosphere as pH was raised was not nearly as dramatic under a nitrogen atmosphere. Because only a finite amount of oxygen was present in Starr's samples, the potential for improved anthocyanin stability at lower pH may have been diminished. With the previously mentioned exceptions in mind, the amended generalization would be that as pH is lowered from pH 4.0 to pH 2.0 where pH is the only independent variable that anthocyanin stability is increased.

Table 7—Effect of pH on first order degradation rate constants at 100° F

System	pH 2.8	pH 3.2	pH 3.6
		Rate (k/wk)	
CON	−0.185	−0.192	−0.206
GLU	−0.156	−0.153	−0.156
SUC	−0.162	−0.165	−0.175

Table 7 shows the first order degradative rate constants on a per week basis for the three simple systems at the three pH's studied at 100°F. The data show that the effect of change in pH was minimal over the pH range tested. The control and the sucrose systems exhibited a slight trend towards decreased anthocyanin stability with increase in pH. The degradative rate of anthocyanin was unaffected by change in pH in the glucose system. While the difference in the rates between pH 2.8 and pH 3.6 for the control system was not significant, the difference in the anthocyanin degradative rate for the same pH range was significant at the 5% confidence level for the sucrose system. The significance in the sucrose system was due to smaller variations in the data. Only the sucrose system followed the same trend at 100°F, with a finite amount of oxygen, as it did at 194°F under aerobic conditions. The results obtained at 100°F in 0.1M citrate-phosphate buffer closely resemble the results Lukton et al. (1956) obtained for pelargonidin-3-glucoside in 0.1M citrate buffer under nitrogen atmosphere at 45°C with respect to the effect of change in pH on anthocyanin stability.

Table 8 shows the initial HMF concentration for the storage study test packs along with the concentration after 8, 16 and 32 wk of storage. Generally the rate of accumulation of HMF decreased with increase in storage time. With the exception of the synthetic jelly which was made up to 65% solids via the addition of sucrose, accumulation of HMF did not exceed a concentration of 100 mg per liter in any of the other test packs after 32 wk of storage under any of the storage conditions. Again the question is raised as to whether this is enough HMF to affect the degradative rate of anthocyanins.

It was desired to test the performance of the colorant in food systems other than fruit juice drink models, such as a jelly (JEL) system, a powdered dry mix (DBM) system and a chemically preserved liquid (CBS) system. Good adherence to first order degradation was observed in the carbonated beverage and jelly systems at 100°F and 75°F storage. First order degradative rate constants calculated for these two systems along with the respective correlation coefficients which indicate how well the data fit first order kinetics at each storage temperature are shown in Table 6.

Examination of results from the jelly system shows that the anthocyanin extract exhibited better stability in it than it did in any of the pasteurized liquid models. Also, the jelly samples retained under ambient conditions after testing was discontinued were deemed to possess "good color" two years after production. For comparison of its performance relative to other jelly systems, first order degradative rate constants were calculated from anthocyanin retention data reported by Decareau et al. (1956) for several batches of strawberry jelly stored

at 100°F. On a per week basis the strawberry jelly anthocyanin exhibited first order degradative rate constants ranging from −0.30 to −0.52.

The carbonated beverage system exhibited much better anthocyanin retention than did the other liquid systems. This was most probably due to the reduced oxygen concentration caused by the 2.2 volumes of carbon dioxide present in the system. Palamidas and Markakis (1975) produced carbonated beverage systems using a different anthocyanin source (wine pomace), different extraction methods and a different formulation. When their anthocyanin first order degradative rate constants were converted to a per week basis, the anthocyanin stability exhibited by their carbonated beverage system was similar to the stability exhibited by the anthocyanin in this carbonated beverage system. Their samples, stored at 38°C (100°F), produced anthocyanin first order degradative rate constants of −0.0608 and −0.0779, and samples stored at 20°C (68°F) produced rate constants of −0.0116 and −0.0188.

In the dry beverage base (DBM) system the Concord grape anthocyanin exhibited their best stability. Virtually no anthocyanin loss was detected at any of the storage temperatures. A random pattern of analyses was observed throughout the 32-wk study for each of the three storage temperatures. Results ranged from a low of 18.1 mg to a high of 22.4 mg per 100 ml after rehydration for individual samples. Duplicate analyses of zero time samples had indicated an initial anthocyanin concentration of 21.1 mg per sample as malvidin-3,5-diglucoside. The three storage temperatures (100°F, 75°F and 34°F) exhibited overall average anthocyanin concentrations for all samples tested over the 32-wk storage period of 20.6 mg, 20.5 mg and 20.2 mg of anthocyanin per 100 ml of liquid respectively. Differences in these averages were not statistically significant at the 5% confidence level. What small loss in anthocyanin there may have been in this dry system, was probably overshadowed by unavoidable small variations between samples attributable to the preparation method.

Table 8—Accumulation of HMF (mg/liter) in model systems with different temperatures and pH values

		Temperature (°F)				
		34	75		100	
	Storage			pH		
System	(wk)	3.2	3.2	2.8	3.2	3.6
CON	0	1.9	1.9	1.8	1.9	1.9
	4	1.8	4.6	12.5	13.1	14.0
	8	2.3	9.7	16.2	17.4	19.3
	16	4.3	13.5	18.8	19.5	19.6
	32	–	–	–	–	–
GLU	0	1.5	1.5	1.7	1.5	1.3
	4	1.7	6.0	12.3	11.0	11.9
	8	3.4	8.4	19.0	16.9	17.7
	16	4.2	11.3	21.7	20.5	20.6
	32	2.4	14.5	–	24.2	–
SUC	0	2.5	2.5	2.0	2.5	2.1
	4	2.4	4.2	10.6	13.8	13.0
	8	2.0	8.0	25.5	22.6	19.0
	16	4.3	9.6	46.5	39.1	32.9
	32	–	–	–	–	–
FJD	0	13.5	13.5	–	13.5	–
	4	13.9	16.0	–	24.4	–
	8	14.2	20.6	–	32.9	–
	16	13.6	28.5	–	39.0	–
	32	13.9	38.7	–	63.4	–
GLU+AA		0	2.0	2.0	–	2.0
	4	9.1	17.8	–	27.9	–
	8	6.3	21.7	–	27.9	–
	16	7.9	29.6	–	32.7	–
	32	10.4	33.5	–	36.7	–
SUC+AA	0	2.1	2.1	–	2.1	–
	4	10.0	21.2	–	35.0	–
	8	9.8	26.7	–	39.1	–
	16	10.5	33.2	–	42.6	–
	32	14.1	35.2	–	66.4	–
FJD+AA	0	14.7	14.7	–	14.7	–
	4	17.3	30.4	–	39.4	–
	8	18.6	37.1	–	46.5	–
	16	21.1	44.1	–	52.3	–
	32	24.6	48.0	–	77.4	–
CBS	0	2.6	2.6	–	2.6	–
	4	4.7	5.5	–	8.6	–
	8	6.3	8.3	–	17.6	–
	16	3.8	13.2	–	24.8	–
	32	1.4	14.6	–	43.4	–
JEL[a]	0	4.7	4.7	–	4.7	–
	4	10.1	13.8	–	54.3	–
	8	10.6	14.3	–	84.6	–
	16	11.4	29.7	–	121.0	–
	32	−2.2	62.4	–	263.2	–

[a] Jelly system was at pH 2.6

Colorimetric analysis

The transmission colorimetric measurements were examined for the purpose of finding a colorimetric function that could be used to follow anthocyanin degradation. Such a function could be useful as either a research or quality assurance tool. Regression analysis of semi-log plots of either the function "a" versus time or the function "a/L" versus time produced regression lines possessing excellent linearity ($r < -0.99$) for data from the high temperature studies. An example of the regression lines produced from both colorimetric and spectrophotometric data for the control system at pH 3.2 and subjected to 194°F is shown in Figure 4.

The function "a/L" was deemed preferable because its slopes were nearly identical to those for corresponding anthocyanin first order rate constants, and differences were not significant at the 1% confidence level in most cases. Table 9 lists the semi-log slopes obtained for each of the high temperature treatments conducted. In all cases the "a/L" slopes at pH 2.8 were significantly different at the 1% confidence level. This was attributed to the change in hue exhibited by the anthocyanin as pH was lowered which caused more of the color to be represented by Hunter "b" value which is not accounted for the "a/L" function. Zero time colorimetric values for each system and pH studied are given in Table 10 along with the corresponding initial anthocyanin concentrations.

When data from the juice drink model storage test packs were tested, the semi-log plot of the "a/L" function versus time produced regression lines with good linearity for both

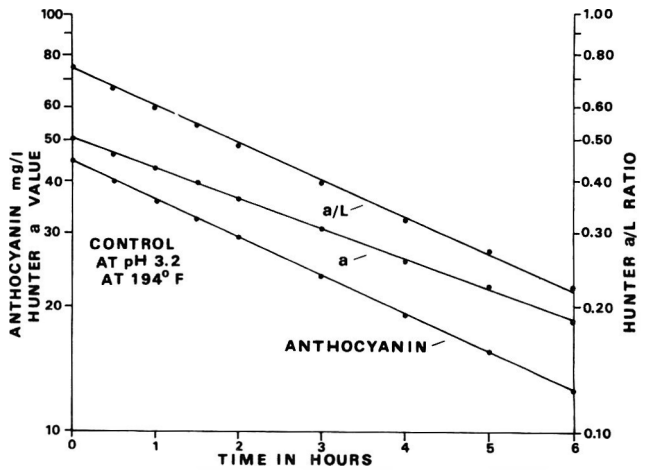

Fig. 4—Regresssion lines for anthocyanin content and color vs time.

Table 9—Color changes in Concord grape anthocyanin systems at higher temperatures

System	pH	a/L Semi-log rate constants Temp (°F)	a/L slope (β/hr)
CON	3.2	185	−0.149*
CON	2.8	194	−0.223**
CON	3.2	194	−0.202
CON	3.6	194	−0.223
CON	3.2	203	−0.301
GLU	3.2	185	−0.148
GLU	2.8	194	−0.195**
GLU	3.2	194	−0.209
GLU	3.6	194	−0.229
GLU	3.2	203	−0.300*
SUC	3.2	176	−0.129**
SUC	3.2	185	−0.192
SUC	2.8	194	−0.255**
SUC	3.2	194	−0.289
SUC	3.6	194	−0.296
SUC	3.2	203	−0.483
FJD	3.2	185	−0.256
FJD	3.2	194	−0.380
FJD	3.2	203	−0.539

*Indicates significant difference from anthocyanin degradative rate at the 5% level

** Indicates significant difference from anthocyanin degradative rate at the 1% level

Table 10—Initial color and anthocyanin content for model systems

System	pH	Color values L	a	b	Anthocyanin conc mg/liter
CON	2.8	59.8	62.7	13.1	44.4
CON	3.2	66.9	50.3	6.5	44.2
CON	3.6	72.5	38.4	2.8	43.6
GLU	2.8	58.6	63.0	10.5	42.3
GLU	3.2	64.4	52.9	5.1	41.4
GLU	3.6	70.7	39.2	0.9	42.3
SUC	2.8	58.6	65.0	10.6	41.2
SUC	3.2	64.4	54.0	4.5	41.4
SUC	3.6	69.4	42.0	0.9	42.0
FJD[b]	3.2	99.0	0.0	3.1	0.0
FJD	3.2	64.4	50.7	5.7	39.6
FJD	3.2	60.0	56.8	5.1	53.5
GLU+AA	3.2	55.7	63.3	8.7	55.0
SUC+AA	3.2	55.5	63.7	7.9	54.6
FJD+AA	3.2	58.8	58.5	5.7	52.6
CBS	3.2	55.5	60.6	4.0	60.3
JEL[a]	2.6	54.0	66.3	9.1	71.1
DBM	2.7	19.9	56.6	14.3	210

[a] Color values for JEL system were taken after 1:1 dilution with distilled water.

[b] FJD system before anthocyanin extract was added

Table 11—Color changes in Concord grape anthocyanin systems at lower temperatures

System	pH	Temp	a/L Semi-log rate constants a/L (β_1)[a]	r_1	a/L(β_2)	r_2
CON	2.8	100	−0.126	−0.991	–	–
	3.2	100	−0.148	−0.998	–	–
	3.6	100	−0.132	−0.949		
	3.2	75	−0.053	−0.994	–	–
GLU	2.8	100	−0.011	−0.992	–	–
	3.2	100	−0.099	−0.982	–	–
	3.6	100	−0.089	−0.950	–	–
	3.2	75	−0.033	−0.983	–	–
SUC	2.8	100	−0.115	−0.992	–	–
	3.2	100	−0.117	−0.989	–	–
	3.6	100	−0.102	−0.965	–	–
	3.2	75	−0.038	−0.986	–	–
FJD	3.2	100	−0.070	−0.909	−0.034	−0.984
	3.2	75	−0.034	−0.959	–	–
GLU+AA	3.2	100	−0.140	−0.972	−0.044	−0.999
	3.2	75	−0.086	−0.933	−0.049	−0.986
SUC+AA	3.2	100	−0.204	−0.916	−0.028	−0.959
	3.2	75	−0.101	−0.919	−0.056	−0.979
FJD+AA	3.2	100	−0.144	−0.975	−0.030	−0.966
	3.2	75	−0.103	−0.985	−0.036	−0.966
CBS	3.2	100	−0.032	−0.927	–	–
	3.2	75	−0.018	−0.908	–	–
JEL	2.6	100	−0.050	−0.995	–	–
	2.6	75	−0.021	−0.960	–	–

[a] When both β_1 and β_2 are listed, β_1 represents a/L slope for first 8 wk of storage, and β_2 represents a/L slope for weeks 8 through 32 of storage. When only β_1 is given it represents a/L slope for entire storage period.

100°F and ambient storage conditions. Data from the carbonated beverage and jelly systems also produced regression lines with good linearity. These "a/L" slopes and the correlation coefficients that indicate the degree of fit of the regression line to the raw data are given in Table 11. While the "a/L" function produced semi-log regression lines with good linearity, the "a/L" slopes (β's) in this temperature range proved to be significantly different from the corresponding logarithmic degradative rate constants (k's) at the 1% confidence level in all cases. Hence, in order to utilize the "a/L" function for prediction of change in anthocyanin concentration in this temperature range, it would be imperative to use a standard curve.

Change in the colorimetric function "a/L" should accurately describe change in anthocyanin concentration where such change is manifested primarily as a bleaching action, and pH remains constant. To the extent that "a" and "L" values are affected by change in concentration of other colored compounds, the function would become a less reliable indicator.

SUMMARY & CONCLUSIONS

THIS STUDY has investigated several areas pertaining to use of anthocyanin as a natural colorant. Specifically, the stability and potential of Concord grape anthocyanin has been demonstrated. The following is a summation of observations and conclusions.

1. The colorimetric function "a/L" could be used to follow changes in anthocyanin concentration, and it was most applicable where degradation of anthocyanin was manifested as a bleaching action.

2. The stability of the anthocyanin from this extract did not prove to be pH dependent in the range tested from pH 2.8 to pH 3.6. Hence, with this anthocyanin source, stability would not be a factor for consideration when choosing the pH for a product. This would allow greater emphasis to be placed on flavor, hue, tinctorial power and cost aspects of product design.

3. In liquid systems, complete elimination of oxygen is probably the most important single factor towards producing improved anthocyanin stability.

4. Consideration should also be given to lowering the concentration of added ascorbic acid in formulated foods that are designed to utilize anthocyanin as the natural colorant, with due regard for its nutritional implications.

5. Examination of HMF data from this study suggested that the HMF concentration may not have been high enough to produce a significant effect on the anthocyanin degradative rate.

6. Significant variations in D_{212} and z values for the different systems tested clearly demonstrated the need for carefully

controlling the test criteria when comparing the relative stability of different anthocyanin sources or extraction methods.

7. This source of anthocyanin from Concord grape filter trim performed well in carbonated beverage and jelly systems; but its best potential may be for use in dry beverage base drinks.

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Ms received 10/29/77; revised 3/16/78; accepted 3/20/78.

Contribution No. 2169 from the University of Massachusetts Agricultural Experiment Station. Appreciation is expressed to the Glass Packaging Institute, Washington, DC and General Foods, Inc., Tarrytown, NY for financial support.

RETENTION OF SYNTHETIC PHENOLIC ANTIOXIDANTS IN MODEL FREEZE-DRIED FOOD SYSTEMS

A. W. KIRLEIS and C. M. STINE

ABSTRACT

The retention of butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and mono-tertiary butylhydroquinone (TBHQ) was investigated in model freeze-dried systems designed to simulate a high lipid food. Model systems initially containing 400 μg antioxidant/g soybean oil were slowly frozen (−22°C) or rapidly frozen (−196°C) as slabs and freeze-dried for 12.5 or 26.5 hr., respectively. Under these conditions TBHQ was retained to the greatest extent and BHA to a greater degree than BHT. On the average, slowly frozen samples retained 32, 27 and 16% TBHQ, BHA and BHT, respectively, which was about 1.2 times higher than rapidly frozen samples. It was found that the retention of BHA and BHT was greatly influenced by the final moisture content of the freeze-dried system in the range between 1–5% moisture. TBHQ retention, however, was found to be less dependent on the final moisture content of the freeze-dried system over the same moisture range. The effect of extending the normal freeze-drying time (12.5 hr) of slowly frozen samples on antioxidant retention was determined. When the normal drying time was doubled BHA and BHT were completely removed and TBHQ content was reduced by an additional 19%. Antioxidant retention was interpreted on the basis of current mechanisms to explain the retention behavior of volatile organic substances during freeze drying.

INTRODUCTION

FREEZE DRYING is generally considered to be the dehydration process which will result in the highest quality dehydrated products (Calloway, 1962; King, 1970). Regardless of the success of the process itself one of the barriers to large scale application of freeze drying has been problems associated with the storage stability of foods that are susceptible to lipid oxidation. In order for such foods to have reasonable shelf life and acceptable flavor characteristics, protective additives which retard oxidation, are often added before dehydration. A fundamental property required of any antioxidant used in freeze-dried foods is that it must carry-through the process and not be lost due to volatilization.

It has been shown that monomeric antioxidants, particularly those with phenolic structures are quite volatile (Sherwin, 1972; Furia and Bellanea, 1977). In a study by Bishov et al. (1960) carried out to investigate the protective action of butylated hydroxytoluene (BHT) in freeze-dried model systems, it was found that BHT had only a slight effect on decreasing the rate of lipid oxidation. Karel et al. (1966) found that propyl gallate (PG) was effective in retarding oxidation in a freeze-dried model system. Labuza et al. (1969) confirmed the effectiveness of PG but found that BHT was not effective in controlling lipid oxidation in a freeze-dried system. In a series of recent studies, lipid oxidative stability of humidified freeze-dried model and food systems was investigated (Labuza et al., 1971, 1972; Chow and Labuza, 1974). In general, their results showed butylated hydroxyanisole (BHA) to be an effective antioxidant for delaying lipid oxidation under the experimental conditions used.

The variable effectiveness of the monomeric antioxidants studied in the freeze-dried systems presents a question as to the amount of antioxidant actually present in the dried system and available to combat oxidation. Since antioxidants are being continuously proposed for addition to freeze-dried foods there is a need to determine if the level of antioxidant remaining in these foods after processing is sufficient to reduce oxidative deterioration. The present study was, therefore, undertaken to determine and interpret the retention behavior of three commonly used synthetic phenolic monomeric antioxidants in a model freeze-dried system.

EXPERIMENTAL

Materials

The synthetic phenolic monomeric antioxidants studied included: butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and mono-tertiary butylhydroquinone (TBHQ) (Eastman Chemical Products, Kingsport, TN). Refined soybean oil (SBO), free of added stabilizers, was obtained from PVO International (Boonton, NJ).

The matrix used for the model system was wheat starch, obtained from Far-Mar-Co. (Hutchinson, KS). Before use the starch was defatted according to the method of Schock (1964), gelatinized and freeze-dried. Gelatinization was carried out by slurrying 70g of starch in 375 ml water and heating, with continuous agitation, in a 60°C water bath for 30 min. The dried gelatinized starch was then sized by grinding in a Wiley mill equipped with a stainless steel U.S. no. 60 sieve.

Preparation of model systems

The retention of phenolic antioxidants was studied in model freeze-dried systems designed to simulate a high lipid food. The system consisted of 15% pregelatinized wheat starch, 5% antioxidant spiked SPO and 80% water on a dry weight basis. The emulsion systems were prepared by incorporating the desired amount of starch into water and then adding the correct amount of antioxidant spiked SBO. Each component was mixed into the emulsion system by blending for 1 min at high speed in a Waring Blendor. Emulsions were then homogenized on a Logeman laboratory homogenizer model C-8 (Chase-Logeman Corp., Brooklyn, NY) at a pressure of 2500 psi.

After homogenization, 95-g portions of the emulsion were placed into two stainless steel trays (10 × 10 × 4 cm), frozen at −22°C or at liquid nitrogen temperature (−196°C) and freeze dried as slabs of about 10 mm in a Virtis RePP model 42 freezer dryer. The dehydration process took place with the heating platen set at 52°C, the condenser temperature set at −60°C and at a chamber pressure below 5×10^{-3} torr (McLeod guage) for most of the drying cycle. Sample drying time was 12.5 hr and 26.5 hr for slow (−22°C) and rapid (liquid nitrogen) freezing, respectively. After freeze drying the chamber vacuum was always released with nitrogen gas, in order to minimize lipid oxidation, leading also to possible antioxidant degradation in the dried system. In some experiments, the effect of extending the drying time of slow frozen samples was studied; in these cases the altered drying times are noted at appropriate sections under Results and Discussion.

Extraction of antioxidants

Immediately after drying the model system was macerated to a fine powder and placed in a sealed glass jar. BHA and BHT were extracted from the starch matrix with pentane. About 5g of the dry system was weighed (to the nearest mg) into a beaker, 60 ml of pentane were added and the beaker was covered with a watch glass. The mixture was stirred with a magnetic stirrer for 15 min and allowed to stand overnight in the dark at room temperature. The sample was brought back to its initial volume with pentane, mixed for 15 min and suction-filtered through a Buchner type funnel, with a fine fritted disc. The beaker was rinsed with 2–20 ml portions of pentane and each was added to the filter

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0022-1147/78/0005-1457$02.25/0

Table 1—Recoveries of antioxidants added[a] to freeze-dried model systems

Antioxidant	% Recovery		
	Trial 1	Trial 2	Average
BHA	97.2	98.0	97.6
BHT	95.9	96.9	96.4
TBHQ	100.0	99.6	99.8

[a] 100 μg antioxidant added to about 5g system

cake. An appropriate amount of 3,5-di-tert-butyl-4-hydroxyanisole (diBHA), which was used as an internal standard, was added to the filtrate and the pentane was removed under vacuum (water aspirator) on a rotary evaporator. During evaporation the flask was partially immersed in a water bath maintained at 24 ± 2°C. Evaporation was continued for about 10 min to an oily residue. The oil was filtered through Whatman No. 1 filter paper and anaylzed for BHA or BHT content by high performance liquid chromatography (HPLC) as described below. TBHQ was extracted from the freeze-dried systems by exactly the same procedure used for BHA or BHT except ethyl ether was used in place of pentane.

High performance liquid chromatographic method

Antioxidants (BHA, BHT or TBHQ) and internal standard (diBHA) were separated from oil extracts, without prior steps of sample preparation, by reversed phase liquid/solid high resolution liquid chromatography as outlined by Dark (1973). In this method, two columns (1/8 in. o.d. × 2 ft) packed with Bondapack™ C_{18}/Corasil (Waters Associates, Milford, MA), operated at ambient temperature, were the stationary phase. The mobile phase, consisting of distilled water: acetonitrile: n-butanol (5:3:2 v/v), had a flow rate of 0.6 ml/min. The appropriate size sample was injected in duplicate and the UV detector (280 nm) sensitivity was adjusted to keep all peaks on scale. After separation and detection of the antioxidant-internal standard about 20 ml of n-butanol was introduced into the system through a valve loop accessory, to wash the oil from the column. Subsequent injections were not made until the column had reestablished equilibrium with the mobile phase. Antioxidants were quantitatively analyzed by means of an internal standard technique described by McNair and Bonelli (1969).

The emerging peaks were identified by comparing retention times of those of known standard and by comparing the UV absorption spectrum of the column effluent, corresponding to a specific peak, to the spectrum of antioxidant standards.

Other analyses

The macerated freeze-dried systems were analyzed for moisture content by a vacuum oven procedure according to AOAC (1975) method 14.002–14.003.

Table 3—Retention of BHA by freeze-dried model systems placed at various platen locations[a]

Run no.	Sample position[b]	% Moisture	% BHA retention[c]
1	F	3.62	25.5
1	B	3.12	23.0
2	F	5.84	33.0
2	B	7.56	40.5
2	C	3.64	18.5
2	C	3.58	24.8
3	F	2.48	15.5
3	B	1.98	18.8
4	F	1.94	14.5
4	F	1.40	10.0
4	B	2.44	21.5
4	B	1.78	18.2
			Mean[d]: 21 ± 8

[a] Initial BHA content: 400 μg BHA/g SBO
[b] F = front; B = back; C = center.
[c] Average of duplicate analysis
[d] Variation indicated by standard deviation

Table 2—Peroxide value of SBO used to prepare model systems and of the same oil after extraction from freeze-dried model system

Freezing rate	Freeze-drying time (hr)	Peroxide value (meq/Kg)[a]	
		Parent oil	Oil extracted from F-D system
Slow	12.5	0.88	0.96
Rapid	26.5	0.95	1.02

[a] Average of duplicate analyses

Peroxide value determinations on SBO were done by AOCS (1974) method Cd8-53.

RESULTS

Antioxidant recovery

Before investigating the retention of antioxidants during freeze-drying, it was necessary to confirm the reliability of the extraction and chromatographic procedure used for the antioxidant determination. This was accomplished by adding 100 μg of a single antioxidant (BHA, BHT or TBHQ) to 5g of antioxidant-free freeze-dried system, extracting with pentane or ethyl ether, and measuring the antioxidant by HPLC as described in the Experimental section. Results of these antioxidant recovery experiments (Table 1) indicate average recoveries of duplicate determinations were greater than 95% for BHA, BHT or TBHQ. Consequently, the extraction and analytical procedure were considered satisfactory for their intended use.

Oxidative changes in oil during model system preparation and freeze drying

In order to minimize lipid oxidation, which would also lead to antioxidant degradation during model system preparation and freeze-drying, process conditions were carefully controlled. To determine the efficiency of these precautionary measures the peroxide value of SBO extracted from the freeze-dried model system immediately after drying was compared to the parent SBO used to prepare the system. The SBO after freeze-drying, as seen in Table 2, showed no significant change in peroxide value from its parent oil value. These results indicate that no significant oxidative losses of antioxidant occurred during model system processing.

Retention of antioxidants in freeze-dried model systems

In a series of freeze-drying experiments, BHA retention (initial concentration 400 μg BHA/g SBO) was studied. For the purpose of comparison, samples were placed at various positions on the heating platen. As shown by the data presented in Table 3, the retention of BHA was highly variable, both among samples at different platen locations from the same run and between samples at the same position of different runs. BHA retention appears to be greatest in samples containing the highest final moisture content.

Figure 1 presents a scattergram and correlation coefficient for the relationship between final moisture content and BHA retention by the freeze-dried starch emulsions. The plot shows that BHA retention exhibited a high degree of correlation with moisture content ($r = 0.94$) over the moisture range examined (1.40–7.56%). In fact, the linear trend shown accounts for 88.4% of the variation in BHA retention among the freeze-dried samples. This indicates that the sample position probably had only a minor influence on the retention behavior of BHA while the final moisture content of the freeze-dried system appears to be closely related to BHA retention. In order to eliminate the possiblity of any sample position effects, all subsequent freeze-drying experiments were conducted with two samples per run positioned at fixed heating platen location.

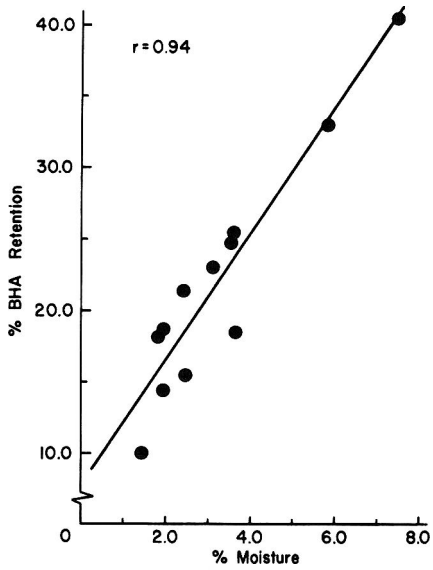

Fig. 1—Effect of final moisture content on BHA retention in freeze-dried model systems initially containing 400 μg/g SBO.

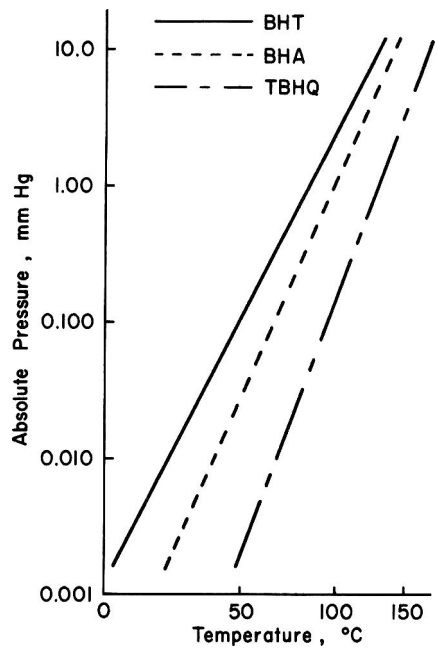

Fig. 2—Vapor pressure of BHA, BHT and TBHQ at various temperatures.

Results of experiments in which BHA, BHT and TBHQ retention was determined after freeze-drying of model starch emulsions initially containing 400 μg antioxidant per g SBO are shown in Table 4. On the average, when samples were given equal treatments the order of antioxidant retention, starting with the highest, was TBHQ, BHA and BHT. This order of retention correlates well with the volatility of the antioxidants (Fig. 2), e.g., the more volatile antioxidants were retained to a lesser extent. It is also apparent that the mean percentage retention for all treatments was more variable for BHA and BHT than for TBHQ. As was previously found for BHA, whenever high loss of BHA or BHT occurred during freeze-drying, it was accompanied by a low moisture content in the dried system. It should be noted that this trend was not demonstrated by the TBHQ samples. Correlation coefficients were not calculated for data shown in Table 4, to establish relationships between the final moisture content and antioxidant retention by the freeze-dried system, because, the moisture range was less than 2% and only four experimental values were available for each treatment.

Table 4—Retention of antioxidants by freeze-dried model systems, frozen at different rates[a]

Anti-oxidant	Sample position[b]	Slow freezing[c] Moisture %	Slow freezing[c] Retention %	Rapid freezing[c] Moisture %	Rapid freezing[c] Retention %
BHA	L	3.72	31.8	2.36	23.2
BHA	R	2.53	23.5	1.78	19.0
BHA	L	2.05	21.0	2.95	22.0
BHA	R	3.12	30.2	2.99	24.0
		Means[d]:	27 ± 5		22 ± 2
BHT	L	2.12	14.8	3.60	17.0
BHT	R	2.73	18.2	2.88	12.8
BHT	L	3.50	24.2	3.00	15.5
BHT	R	1.84	10.5	2.68	10.8
		Means[d]:	16 ± 6		14 ± 3
TBHQ	L	2.30	33.8	2.32	26.5
TBHQ	R	2.39	33.8	2.06	26.0
TBHQ	L	2.66	32.2	2.00	26.5
TBHQ	R	2.36	28.5	1.58	25.5
		Means[d]:	32 ± 2		26.1 ± 0.5

[a] Initial antioxidant content: 400 μg/g SBO
[b] L = left; R = right
[c] Average of duplicate analysis
[d] Variation indicated by standard deviation

Effects of freezing rate

In the present work using starch emulsions the influence of freezing rate an antioxidant retention was investigated. It was found that on the average slow freezing improved antioxidant retention over the rapid freezing by only about 1.2 times (Table 4). In fact, it is questionable if any real retention differences exist between the slowly and rapidly frozen samples containing BHA or BHT.

Effects of extending the freeze-drying time.

Results of experiments in which antioxidant retention was determined after extended freeze-drying of model systems initially containing 400 μg antioxidant/g SBO subjected to slow freezing are shown in Table 5. The greatest amount of antioxidant was lost in all cases, during the standard time of freeze drying (12.5 hr). When the drying period was doubled to 25 hr BHA and BHT were completely lost from the system and TBHQ content was lowered an additional 25%. During the extended drying process the final moisture content of the system was reduced to about 1%.

DISCUSSION

RECENTLY Massaldi and King (1974) proposed a mechanism to explain the retention behavior of volatile organic substances having limited solubility and forming droplets in aqueous solutions, i.e., an emulsion, as used here. Their model predicts three locations for the volatiles in the frozen slab: (1) droplets of volatiles adjacent to the ice-crystal interface; (2) droplets of volatiles in the solids region; and (3) homogenously dissolved volatiles in the solids region. Based on this model of volatile location the predicted retention characteristics of the volatiles by location are: (1) completely lost through vaporization after the sublimation of ice; (2) lost only through a relatively slow diffusion process; and (3) lost in the same way as substances below their solubility limit, i.e., by the selective diffusion (Menting and Hoogstad, 1967; Thijssen and Rulkens, 1968; King and Chandrasekaran, 1973) or microregion entrapment (Flink and Karel, 1970a, b) mechanism.

Thus, by extending this model to the starch emulsions used in the present work it can be predicted that antioxidants present in the oil droplets are lost from the oil phase at and above the sublimation front by a diffusion process. Accordingly

more antioxidant loss should occur from oil droplets located adjacent to the ice-crystal interface than from the oil droplets located in the solids region. When investigating the structural relationship of fat and carbohydrate in a freeze-dried matrix of oil-in-water model emulsions Gejl-Hansen and Flink (1977) showed that in emulsions containing a water insoluble carbohydrate solid support, all of the oil is present as a free-fat coating on the surface of the solid support crystals. Under these conditions, as in the present work, no oil droplets would be located in the solids region. Thus, antioxidant would be continuously and more rapidly lost from the oil droplets coating the starch surface than if the oil droplets were located in the solids region, after ice is removed from the system. Under these conditions the rate of water removal from the starch matrix, by evaporation after passage of the sublimation front, influences antioxidant loss from the oil phase. Thereby, higher antioxidant retention would be expected at higher system moisture content as a result of a less severe sample temperature history. The observed correlation between BHA retention and final freeze-dried system moisture content (Fig. 1) supports this interpretation. Lower system moisture contents in all cases lead to greater loss of BHA and vice versa.

Additional evidence indicating that antioxidant loss occurs by a continuous diffusion from the oil phase of the starch matrix was obtained through extended freeze-drying experiments (Table 5). Notice that BHA and BHT, present only in the oil phase of the system, were completely lost when the normal freeze-drying time was doubled. This indicates that indeed all the oil was present as a free-fat coating on the surface of the starch matrix which allowed antioxidant to continuously diffuse from the oil phase until completely removed, after passage of the sublimation front. The question, however, arises as to why under the same conditions of extended freeze-drying TBHQ was retained when BHA and BHT were not. The major difference that may account for the observed retention behavior of these antioxidants is that TBHQ has limited water solubility while BHA and BHT have not (Table 6). This allows TBHQ to be present not only in the oil phase but for a small fraction, extracted during system preparation to be dissolved in the aqueous phase of the starch emulsion. The fraction of TBHQ dissolved in the aqueous phase is most likely retained by a selective diffusion or microregion entrapment mechanism (Massaldi and King, 1974). Thus, the retention of this fraction of TBHQ would not be lost from the system even under the extended freeze-drying conditions (Flink and Karel, 1970a, b). Nevertheless the major fraction of TBHQ, present in the oil, is lost continuously the same as BHA or BHT. This implies that the percentage loss of TBHQ after 25 hr of freeze-drying represents the fraction of TBHQ in the oil phase and the percentage retention of TBHQ represents the fraction dissolved in the aqueous phase of the system (Table 5).

Table 5—Effect of extended freeze-drying on the retention of BHA, BHT and TBHQ by freeze-dried model systems[a]

Antioxidant	Sample position[b]	Drying time (hr)	% Moisture[c]	% Retention[c]
BHA	L	12.5	3.72	31.8
BHA	R	12.5	3.12	30.2
BHA	L	25.0	1.04	—0—
BHA	R	25.0	0.92	—0—
BHT	L	12.5	3.50	24.2
BHT	R	12.5	3.47	23.5
BHT	L	25.0	0.98	—0—
BHT	R	25.0	0.91	—0—
TBHQ	L	12.5	2.66	32.2
TBHQ	R	12.5	2.36	28.5
TBHQ	L	25.0	0.88	5.2
TBHQ	R	25.0	0.90	6.2

[a] Initial antioxidant content: 400 μg/g SBO
[b] L = left; R = right
[c] Average of duplicate analyses

Table 6—Solubility of BHA, BHT and TBHQ in water and soybean oil

	Solubility, % sol., (°C)		
Antioxidant	BHA	BHT	TBHQ
Water	insol, (0–50)[a]	insol, (0–60)[a]	1, (25)[b]
Soybean oil	50, (25)[a]	30, (25)[a]	10, (25)[b]

[a] Eastman, 1963
[b] Eastman, 1972

The present interpretation of antioxidant loss during freeze drying of starch emulsions is also an explanation for the differences observed between BHA, BHT and TBHQ retention behavior using normal freeze-drying times. Simply from a retention mechanism standpoint, volatiles homogeneously dissolved will be retained to a greater extent than those dissolved in the oil phase of a system. This would increase the retention of TBHQ when compared to BHA or BHT. Furthermore, due to vapor pressure differences between BHA, BHT and TBHQ the rate of antioxidant diffusion from the oil phase of the system should correlate with the volatility of these antioxidants, i.e. BHT > BHA > TBHQ (Fig. 2). This prediction was confirmed experimentally, as shown in Table 4, on the average TBHQ retention was highest and BHA and BHT retention correlated with their volatility.

The rate of freezing influences the structure of the freeze-dried material, and controls the size of the ice crystals and the degree of solute concentration achieved in the matrix phase (King, 1970). The rate of freezing is one of the most investigated process variables and it can be noted that in all cases reported, slow freezing results in improved retention of the volatile components. Antioxidant retention on the average was improved by only about 1.2 times in starch emulsions by the slow freezing treatment (Table 4). The explanation for this result probably lies in the location of the antioxidant in the system and the effect of freezing rate on volatile retention according to location. Fast freezing gives smaller ice crystals, thinner webs and lower gas permeability during drying thus, lower retention of volatiles located in the solids region (Flink and Karel, 1970a, b). Accordingly, freezing rate has the greatest effect on the retention of the TBHQ dissolved in the aqueous phase of the system. Since the major fraction of TBHQ probably is present in the oil phase, as is the case with BHA and BHT, only small retention differences were observed as a result of freezing rate (Table 4).

The overriding objective of this study was to determine and interpret the retention of some commonly used synthetic phenolic monomeric antioxidants in a model freeze-dried system. Unlike most current antioxidant research which deals with antioxidant effectiveness in freeze-dried food systems, these explorations were designed to determine the antioxidant level remaining in a freeze-dried model food system after processing. The true worth of these studies is reflected in an increased understanding of antioxidant retention functions which in several cases may now be used to interpret results obtained in antioxidant effectiveness studies.

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ANALYSIS OF INCOMPLETE BLOCK DESIGNS WITH REFERENCE SAMPLES IN EVERY BLOCK

M. C. GACULA, JR.

ABSTRACT

Sensory scores are relative and their magnitudes are highly dependent on the test samples being compared. In order to anchor the scores, the test samples are evaluated with reference to a control or standard. In this paper, balanced incomplete block and complete-incomplete block designs modified by the incorporation of a reference sample in every block are reviewed in view of their potential use in sensory evaluation. Examples of the design and the statistical analysis are provided.

INTRODUCTION

THE EXPERIMENTAL DATA derived from sensory evaluation techniques are correlated with quality, that is, the score a sample receives is highly dependent on the quality of the samples to which it is compared in a given set or block. In order to anchor the scores, a reference or standard sample is incorporated in each block. In incomplete block designs, not all samples are contained in each block, hence a reference standard is highly recommended.

When one of the objectives of a study is to compare the reference standard or control to every other treatment, then it is necessary that the reference sample appear in every incomplete block.

The purpose of this paper is to describe the balanced incomplete block (BIBD) and complete-incomplete block (CIBD) designs and their statistical analysis when a reference standard is contained in each block. From the author's knowledge, such designs are not available in textbooks today.

DISCUSSION

Balanced incomplete block design (BIBD)

Since the introduction of incomplete block designs by Yates (1936, 1939, 1940), numerous publications dealing with these designs have appeared and textbooks by Kempthorne (1952), Federer (1955) and Cochran and Cox (1957) have become the standard references in this area. The potential usefulness of BIBD in sensory evaluation has been described (Galinat and Everett, 1949; Hanson et al., 1951; Marquardt et al., 1963; Gacula and Kubala, 1972). In order to comprehend the sections to follow, we shall briefly review some properties of the BIBD.

The BIBD, as is known today, is defined by five integer values called design parameters:

t = Number of treatments.
k = Number of treatments appearing in a block, $k < t$.
b = Number of blocks or panelists.
r = Number of replications or judgments per treatment.
λ = Number of times a pair of treatments appear in the same block.

The value of these parameters should be known before the statistical analysis can proceed. When t and k are given, Yates (1936) showed that r, b and λ are obtained by taking all possible selections of k from t as follows:

$$b = \left\{ \begin{matrix} t \\ k \end{matrix} \right\} = \frac{t!}{k!\,(t-k)!} \quad (1)$$

$$r = \left\{ \begin{matrix} t-1 \\ k-1 \end{matrix} \right\} = \frac{(t-1)!}{(k-1)!\,(t-k)!} \quad (2)$$

$$\lambda = \left\{ \begin{matrix} t-2 \\ k-2 \end{matrix} \right\} = \frac{(t-2)!}{(k-2)!\,(t-k)!} = r(k-1)/(t-1) \quad (3)$$

Consider a BIBD with t = 4 and k = 2. Substituting the value of t and k into formulae (1) through (3), we obtain r = 3, b = 6 and λ = 1. The layout of the design with these parameters is given in the upper half of Table 1, where the letter X denotes the particular treatments that are compared within a block. This layout is often referred to as the *basic design*, (Federer, 1955; Li, 1964). If the basic design is repeated p times to achieve adequate replication, the design parameters b, r and λ are multiplied by p to become pb, pr and pλ. A catalogue of BIBD plans is found in Cochran and Cox (1957) and in Fisher and Yates (1963).

The calculation of the design parameters for BIBD and CIBD augmented with a reference standard is complex; therefore, it is appropriate to introduce a simpler general procedure applicable to both incomplete block designs with and without a reference standard (Pearce, 1960; Searle, 1971; Trail and Weeks, 1973). The general procedure starts by constructing the *incidence matrix* of the design layout. Using the BIBD layout in Table 1, the incidence matrix $\underset{\sim}{N}$ is

$$\underset{\sim}{N} = \begin{bmatrix} 1\,0\,1\,0\,1\,0 \\ 1\,0\,0\,1\,0\,1 \\ 0\,1\,1\,0\,0\,1 \\ 0\,1\,0\,1\,1\,0 \end{bmatrix} \quad (4)$$

where a 1 denotes the presence of the treatment and 0, its absence in the layout.

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Table 1—Construction of design with reference samples

Basic design for BIBD:

Treatments	Blocks (Panelists) 1	2	3	4	5	6
A	X		X		X	
B	X			X		X
C		X	X			X
D		X		X	X	

Design parameters: t = 4, k = 2, r = 3, b = 6, λ = 1, N = bk = 12

Basic design augmented with reference sample R:

Treatments	Blocks (Panelists) 1	2	3	4	5	6
R	X	X	X	X	X	X
A	X		X		X	
B	X			X		X
C		X	X			X
D		X		X	X	

Design parameters: t + 1 = 5, k + 1 = 3, r = 3, b = 6, λ = 1, N = b (k + 1) = 18

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0022-1147/78/0005-1461$02.25/0

The *transpose* of $\underset{\sim}{N}$, symbolized by $\underset{\sim}{N}'$, is obtained by interchanging the rows and columns (Searle, 1966) to read,

$$\underset{\sim}{N}' = \begin{bmatrix} 1 & 1 & 0 & 0 \\ 0 & 0 & 1 & 1 \\ 1 & 0 & 1 & 0 \\ 0 & 1 & 0 & 1 \\ 1 & 0 & 0 & 1 \\ 0 & 1 & 1 & 0 \end{bmatrix} \quad (5)$$

and the product between $\underset{\sim}{N}$ and $\underset{\sim}{N}'$, if all elements are either 0 or 1 (Pearce, 1960), yields a matrix with the main diagonal elements (underlined) equal to r and the off-diagonal elements equal to λ:

$$\underset{\sim}{N}\underset{\sim}{N}' = \begin{bmatrix} \underline{r} & \lambda & \lambda & \lambda \\ & \underline{r} & \lambda & \lambda \\ & & \underline{r} & \lambda \\ & & & \underline{r} \end{bmatrix} = \begin{bmatrix} \underline{3} & 1 & 1 & 1 \\ & \underline{3} & 1 & 1 \\ & & \underline{3} & 1 \\ & & & \underline{3} \end{bmatrix} \quad (6)$$

Only the upper half in (6) is shown because the matrix is *symmetrical*.

BIBD with reference sample

The idea of having a reference sample in every block of BIBD's traces back to the independent work of Pearce (1960) and Basson (1959). The construction of BIBD with a reference sample is achieved by simply adding the reference sample to all blocks of BIBD in a manner shown in the lower half of Table 1. The order of tasting of the three samples within each block is determined at random and not as shown in the layout. The addition of the reference sample modifies the parameters t and k to t + 1 and k + 1, respectively. The BIBD's are widely catalogued, therefore, the construction of the augmented design, that is, the BIBD with the added reference sample is greatly facilitated. Note that formulae (1) through (3) do not apply to the augmented design.

The incidence matrix of the augmented design (lower half, Table 1) is

$$\underset{\sim}{N} = \begin{bmatrix} 1 & 1 & 1 & 1 & 1 & 1 \\ 1 & 0 & 1 & 0 & 1 & 0 \\ 1 & 0 & 0 & 1 & 0 & 1 \\ 0 & 1 & 1 & 0 & 0 & 1 \\ 0 & 1 & 0 & 1 & 1 & 0 \end{bmatrix} \quad (7)$$

and its transpose,

$$\underset{\sim}{N}' = \begin{bmatrix} 1 & 1 & 1 & 0 & 0 \\ 1 & 0 & 0 & 1 & 1 \\ 1 & 1 & 0 & 1 & 0 \\ 1 & 0 & 1 & 0 & 1 \\ 1 & 1 & 0 & 0 & 1 \\ 1 & 0 & 1 & 1 & 0 \end{bmatrix} \quad (8)$$

Table 2—Intrablock analysis of variance for a BIBD with reference samples

Source of variance	DF	Sum of squares
Total	N − 1	$SS_T = \Sigma X_{ij\ell}^2 - CF$, $i = 1, 2, \ldots, t+1$; $j = 1, 2, \ldots, b$; $\ell = 1, 2, \ldots, p$
Repetitions	p − 1	$SS_p = \frac{\Sigma X^2_{ij\ell}}{b(k+1)/p} - CF$
Panelists within repetition	p(b − 1)	$SS_{b:p} = \frac{\Sigma B_j^2}{b(k+1)/p}$
Treatments (adj. for panelists)	t	$SS_{t\ adj.} = \Sigma \hat{t}_i Q_i$
Error	By difference	SS_e = By difference

and $\underset{\sim}{N}\underset{\sim}{N}'$ is shown by Pearce (1960) to be,

$$\underset{\sim}{N}\underset{\sim}{N}' = \begin{bmatrix} \underline{s_R} & \lambda_R & \lambda_R & \lambda_R & \lambda_R \\ & \underline{s} & \lambda & \lambda & \lambda \\ & & \underline{s} & \lambda & \lambda \\ & & & \underline{s} & \lambda \\ & & & & \underline{s} \end{bmatrix} = \begin{bmatrix} \underline{6} & 3 & 3 & 3 & 3 \\ & \underline{3} & 1 & 1 & 1 \\ & & \underline{3} & 1 & 1 \\ & & & \underline{3} & 1 \\ & & & & \underline{3} \end{bmatrix} \quad (9)$$

In (9), the value of the design parameters are $b = s_R = 6$, $r = s = 3$, $\lambda = 1$ and $\lambda_R = 3$. The parameter λ_R is used later in the analysis of CIBD with reference sample. The number of replicates for the reference standard is always equal to b.

Table 2 contains the intrablock analysis of variance for a BIBD with a reference sample. In this table, CF (correction factor), SS_T, SS_p, $SS_{b:p}$, $SS_{t\ adj.}$ and SS_e are computed by the standard procedure described by Yates (1936), Rao (1947) and Cochran and Cox (1957). Because of incomplete blocking, that is, not all treatments are contained in a given block ($k < t$), it is well known that the treatment totals T_i should be adjusted for block effects accomplished by calculating,

$$Q_i = T_i - \frac{B_{(i)}}{k+1}, \quad i = 1, 2, \ldots, t+1 \quad (10)$$

where $B_{(i)}$ refers to block totals in which treatment i occurs.

The calculations of effects due to treatments and reference sample, as well as their variances, are obtained by the formulae derived by Basson (1959) and are given as formulae (11) through (15) below. The estimate of effect due to the reference sample (t_R) is

$$\hat{t}_R = \left(\frac{k+1}{bk}\right) Q_R \quad (11)$$

with variance,

$$\left(\frac{k+1}{bk}\right) \sigma_e^2 \quad (12)$$

and that for the ith treatment,

$$\hat{t}_i = (Q_i + \frac{Q_R}{t}) / \left\{(rk + \lambda)/(k+1)\right\} \quad (13)$$

with variance,

$$\frac{(t-1)^2 (k+1)}{tr(kt-1)} \sigma_e^2. \quad (14)$$

If contrasts between the ith treatment and the reference sample are desired, the variance of the contrast is:

$$\frac{(k+1)(k+t-2)}{r(kt-1)} \sigma_e^2. \quad (15)$$

The standard error of an effect is the square root of its variance. The value of σ_e^2 in the above formulae is estimated by the error mean square in the analysis of variance. It should be recognized that when the basic design is repeated p times, the value of b, r and λ where these appear in formulae (11) through (15) should be multiplied by p.

The adjusted treatment mean is obtained by $\bar{X}_i = \hat{\mu} + \hat{t}_i$, $i = 1, 2, \ldots, t$, where $\hat{\mu}$ is the grand mean excluding the reference sample, and the reference sample by $\bar{X}_R = \hat{\mu} + \hat{t}_R$. Standard procedures, such as the Duncan's Multiple Range Test, the Least Significant Difference Test (LSD), are used to perform multiple comparisons of effects or the adjusted means.

Example of BIBD with reference sample

The data in Table 3, taken from the author's file pertaining to a nitrate/nitrite study on canned chopped ham, are a part of a larger body of sensory data. For each panelists, the order of

Table 3—Nitrate-nitrite study designed as a BIBD with reference samples. Design parameters: t + 1 = 5, k + 1 = 3, p = 2, pr = 6, pb = 12, pλ = 2

Repetition	Panelists	R	1	2	3	4	B_j	R_ℓ
I	1	4	5	4			13	
	2	5			3	6	14	
	3	5	7		6		18	
	4	4		4	4		12	
	5	5	6			5	16	
	6	4		5		3	12	85
II	7	5	6	3			14	
	8	3			4	4	11	
	9	5	6		7		18	
	10	4		6	5		15	
	11	4	7			5	16	
	12	6		5		2	13	87
T_i		$T_R = 54$	37	27	29	25	G = 172	
$B_{(i)}$		172	95	79	88	82		
$B_{(i)}/k + 1$		57.3333	31.6667	26.3333	29.3333	27.3333		
$Q_i = T_i - \frac{B_{(i)}}{k+1}$		−3.3333	5.3333	0.6667	−0.3333	−2.3333	$\sum_{t+1} Q_i = 0$	
$\hat{t}_i$		$t_R = -0.4167$	0.9643	−0.0357	−0.2500	−0.6786	$\sum_{t} \hat{t}_i = 0$	
$\bar{X}_i = \hat{\mu} + \hat{t}_i$		$\bar{X}_R = 4.50$	5.88	4.88	4.67	4.24		

$CF = (172)^2/36 = 821.7778$

$SS_T = (4^2 + 5^2 + \ldots + 2^2) - CF = 874.000 - 821.7778 = 52.2222$

$SS_p = \frac{(85^2 + 87^2)}{18} - CF = 821.8889 - 821.7778 = 0.1111$

$SS_{b:p} = \frac{13^2 + 14^2 + \ldots + 16^2 + 13^2}{3} - SS_p - CF$

$= 841.3333 - 0.1111 - 821.7778 = 19.4444$

$SS_{t\ adj.} = (-3.3333)(-0.4167) + (5.3333)(0.9643) + \ldots + (-2.3333)(-0.6786) = 8.1748$

$SS_e = 52.2222 - 0.1111 - 19.4444 - 8.1748 = 24.2919$

sample presentation was randomized.

The initial step in the analysis is to calculate the marginal totals and the quantities $B_{(i)}$ and Q_i, i = 1, 2, . . ., t + 1. For instance, for treatment 1:

$$B_{(1)} = 13 + 18 + 16 + 14 + 18 + 16 = 95$$
$$Q_1 = 37 - (95/3) = 5.3333$$

From formula (13),

$$\hat{t}_1 = \frac{5.333 - \frac{3.333}{4}}{4.667} = 0.9643$$

and from formula (11),

$$\hat{t}_R = \left\{3/(12)(2)\right\}(-3.333) = -0.4167.$$

The adjusted mean for treatment 1 is $\bar{X}_1 = 4.9167 + 0.9643 = 5.88$, and for the standard is $\bar{X}_R = 4.9167 - 0.4167 = 4.50$.

The analysis of variance for flavor in the chopped ham is displayed in Table 4. At 4 and 20 degrees of freedom, the 5% tabular F value is 2.87 which is not exceeded by 2.04/1.22 = 1.67. Thus, there is no evidence that the five means are statistically different.

Table 4—Analysis of variance of chopped ham flavor

Source of variance	DF	SS	MS	F-ratio
Total	35	52.2222		
Repetitions	1	0.1111	0.1111	
Panelists within repetition	10	19.444	1.944	
Treatments (adjusted)	4	8.1748	2.0437	1.67
Error	20	24.4919	1.2246	

Calculation of standard errors:

Standard error of treatment means (square root of formula 14):

$$SE_{\bar{x}} = \sqrt{\left[\frac{3^2\ (3)}{4(6)\ (8-1)}\right] 1.2246} = 0.4436$$

Standard error of treatment versus reference standard (square root of formula 15):

$$SE_{t-R} = \sqrt{\left[\frac{3(6-2}{6(8-1)}\right] 1.2246} = 0.5915$$

CIBD with reference sample

Cornell and associates (1972, 1975) developed the composite complete-incomplete block designs for use in sensory evaluation. These designs belong to a family of designs known in the statistical literature as the extended complete block designs first reported by John (1963). The CIBD also may be augmented with controls. However, attention should be given to the increase in the size of the block which may defeat the main purpose of incomplete blocking, i.e., to reduce block size. The block size increases from k + 1 to k + 1 + d for the augmented CIBD, where d is the number of duplicate observations within blocks.

The development of the CIBD is an attempt to segregate the nonadditive effects from the experimental error and thus obtain an estimate of pure error which is not possible in a randomized complete block design (RCBD) with one observa-

tion per cell. The result of this development is a more precise test of statistical significance of contrasts. A CIBD is formed by an appropriate combination of RCBD and incomplete block designs. We shall consider the CIBD generated from BIBD designs as reported by Trail and Weeks (1973) and then show the resulting design obtained when a reference standard is added in every block.

According to Trail and Weeks (1973), a CIBD has the following properties: (a) each treatment is applied either n_0 or n_1 times in a block; a value of 1 or 2 for n_0 and n_1 is practical from the standpoint of smaller block size; (b) replacement in the RCBD incidence matrix, of n_0 by zero and n_1 by 1 results in the incidence matrix of a BIBD. It follows from (a) and (b) that the incidence matrix for CIBD is

$$\underset{\sim}{N} = n_1 \underset{\sim}{N}^* + n_0 (\underset{\sim}{M} - \underset{\sim}{N}^*) \tag{16}$$

where $\underset{\sim}{N}^*$ = incidence matrix of the generating BIBD, and $\underset{\sim}{M}$ = incidence matrix of the generating RCBD with matrix elements all 1's.

Using matrix (4) as the generating BIBD, and substituting into formula (16), we have

$$\underset{\sim}{N} = n_1 \begin{bmatrix} 1\,0\,1\,0\,1\,0 \\ 1\,0\,0\,1\,0\,1 \\ 0\,1\,1\,0\,0\,1 \\ 0\,1\,0\,1\,1\,0 \end{bmatrix} + n_0 \left\{ \begin{bmatrix} 1\,1\,1\,1\,1\,1 \\ 1\,1\,1\,1\,1\,1 \\ 1\,1\,1\,1\,1\,1 \\ 1\,1\,1\,1\,1\,1 \end{bmatrix} - \begin{bmatrix} 1\,0\,1\,0\,1\,0 \\ 1\,0\,0\,1\,0\,1 \\ 0\,1\,1\,0\,0\,1 \\ 0\,1\,0\,1\,1\,0 \end{bmatrix} \right\} \tag{17}$$

Taking $n_1 = 1$ and $n_0 = 2$, the CIBD incidence matrix is

$$\underset{\sim}{N} = \begin{bmatrix} 1\,2\,1\,2\,1\,2 \\ 1\,2\,2\,1\,2\,1 \\ 2\,1\,1\,2\,2\,1 \\ 2\,1\,2\,1\,1\,2 \end{bmatrix}$$

and the layout of this matrix is given in Table 5. The design parameter λ is the off-diagonal elements of matrix $\underset{\sim}{N}\underset{\sim}{N}'$, which is equal to 13:

$$\underset{\sim}{N}\underset{\sim}{N}' = \begin{bmatrix} \underline{15} & 13 & 13 & 13 \\ & \underline{15} & 13 & 13 \\ & & \underline{15} & 13 \\ & & & \underline{15} \end{bmatrix}$$

The main diagonal, to be defined later, is a design parameter for CIBD with a reference standard. The parameter λ is also obtained by (Trail and Weeks, 1973)

$$\lambda = \lambda^* (n_1 - n_0)^2 + n_0(2r - bn_0) \tag{18}$$

where λ^* is the parameter of the generating BIBD (upper half, Table 1).

The analysis of variance for the CIBD layout in Table 5 is shown in Table 6 and follows closely the standard calculations in incomplete block designs. A numerical example of CIBD is found in Cornell and Schreckengost (1975).

When a CIBD is augmented with a reference sample, the result is a supplemented balance design (Type S) described by Pearce (1960) as shown in Table 7. The augmented CIBD has 12 samples less, per basic design, than the RCBD with two observations per cell. As in the BIBD's, the sample presentation within panelist is randomized. The incidence matrix of the augmented CIBD is

$$\underset{\sim}{N} = \begin{bmatrix} 2\,2\,2\,2\,2\,2 \\ 1\,2\,1\,2\,1\,2 \\ 1\,2\,2\,1\,2\,1 \\ 2\,1\,1\,2\,2\,1 \\ 2\,1\,2\,1\,1\,2 \end{bmatrix} \tag{19}$$

and,

$$\underset{\sim}{N}\underset{\sim}{N}' = \begin{bmatrix} \underline{24} & 18 & 18 & 18 & 18 \\ & \underline{15} & 13 & 13 & 13 \\ & & \underline{15} & 13 & 13 \\ & & & \underline{15} & 13 \\ & & & & \underline{15} \end{bmatrix} \tag{20}$$

Table 5—Design layout for a CIBD

Treatments	Panelists (Blocks) 1	2	3	4	5	6
A	X	X	X	X	X	X
		X		X		X
B	X	X	X	X	X	X
		X	X		X	
C	X	X	X	X	X	X
	X			X	X	
D	X	X	X	X	X	X
	X		X			X

Design parameters: $t = 4$, $k = 6$, $r = 9$, $b = 6$, $\lambda = 13$, $N = bk = 36$, $n_0 = 2$, $n_1 = 1$

Table 6—Analysis of variance of CIBD

Source of variance	DF	Sums of squares	
Total	$N - 1$	$SS_T = \Sigma X_{ij}^2 - CF$	$i = 1, 2, \ldots, t$; $j = 1, 2, \ldots, b$
Panelists (blocks)	$b - 1$	$SS_b = \Sigma B_j^2/k - CF$	
Treatments (adjusted)	$t - 1$	$SS_{t\ adj.} = (k/t\lambda)\Sigma Q_i^2$	
Interaction	$(b - 1)(t - 1)$	$SS_{bt} = \Sigma X_{ij}^2/n_{ij} - CF - SS_b - SS_{t\ adj.}$	
Pure error	$N - bt$	$SS_e = \Sigma d_{ij}^2/2$	

$CF = (\Sigma X_{ij})^2/N$, correction factor; N is the total number of observations.

$$Q_i = T_i - \frac{B_{(i)}}{k}$$

n_{ij} = Number of times the ith treatment occurs in the jth block and is equal to the elements of the incidence matrix $\underset{\sim}{N}$.

d_{ij} $X_{ij1} - X_{ij2}$, difference between duplicate samples where (i,j) cell is duplicated.

Table 7—Layout of CIBD with reference R

Treatments	Panelists (Blocks) 1	2	3	4	5	6
R	X	X	X	X	X	X
	X	X	X	X	X	X
A	X	X	X	X	X	X
		X		X		X
B	X	X	X	X	X	X
		X	X		X	
C	X	X	X	X	X	X
	X			X	X	
D	X	X	X	X	X	X
	X		X			X

Design parameters:
$t + 1 = 5$, $k = 8$, $b = 6$, $N = bk = 48$, $r = 9$, $r_R = 12$, $\lambda = 13$, $\lambda_R = 18$, $s = 15$, $s_R = 24$

Table 8—Analysis of variance of CIBD with reference samples

Source of variance	DF	Sums of squares
Total	$N - 1$	$SS_T = \Sigma X_{ij}^2 - CF$ $i = 1, 2, \ldots, t+1$ $j = 1, 2, \ldots, b$
Panelists (blocks)	$b - 1$	$SS_b = \Sigma B_j^2/k - CF$
Treatments (adjusted)	t	$SS_{t\ adj.} = \Sigma \hat{t}_i Q_i$
Interaction	$t(b-1)$	$SS_{bt} = \Sigma X_{ij}^2/n_{ij} - CF - SS_b - SS_{t\ adj.}$
Pure error	$N - b(t+1)$	$SS_e = \Sigma d_{ij}^2/2$

where each matrix element is given by (9) and equals the following design parameters (Pearce, 1960): $s_R = 24$, defined as the number of self-concurrences for the reference standard; $\lambda_R = 18$, the number of times that the reference sample and treatment appear together in a block; and $s = 15$, the number of self-concurrences for the treatments. When the design layout in Table 7 is repeated p times, b, r, r_R, λ, λ_R, s and s_R are each multiplied by p. Note that r_R is the number of replicates for the reference standard.

The analysis of variance of the layout in Table 7 is shown in Table 8. With repetitions of the basic design layout by different panelists, the source of variance will include repetitions (SS_p), and the panelists sum of squares (SS_b) become nested with repetitions ($SS_{b:p}$). Both SS_p and $SS_{b:p}$ are obtained by the formulae given in Table 2; however, a minor change in the denominator of these formulae should be made by using k instead of k + 1. The estimates of standard errors and effects due to treatments including the reference standard are obtained by the formulae given in Pearce (1960); these formulae are given by (21) through (24).

The estimate of effect due to the reference standard is determined by

$$\hat{t}_R = rQ_R/b\lambda_R \qquad (21)$$

and that due to the ith treatment by

$$\hat{t}_i = \frac{(bk\lambda_R)(kQ_i) - \left\{(r_R\lambda - r\lambda_R)(kQ_R)\right\}}{(t\lambda + \lambda_R)(bk\lambda_R)}, \quad i = 1, 2, \ldots, t \qquad (22)$$

The adjusted mean for the ith treatment or reference standard is calculated by $\hat{\mu} + \hat{t}_i$, i = 1, 2, . . ., t + 1, where $\hat{\mu}$ is the overall mean. The standard error of the difference between two adjusted treatment means is

$$SE_{\bar{x}_i - \bar{x}_{i'}} = \sqrt{2MS_e/\left(\frac{t\lambda + \lambda_R}{k}\right)}, \quad i \neq i' \qquad (23)$$

and that between treatment – reference contrast is

$$SE_{t-R} = \sqrt{\left(1 + \frac{\lambda}{\lambda_R}\right) MS_e/\left(\frac{t\lambda + \lambda_R}{k}\right)}. \qquad (24)$$

Example of CIBD with reference sample

Table 9 contains the data pertaining to visual color evaluation of pork chops seven days after storage in display cases. A five-point color scale is used where 1 = no discoloration and 5 = extremely gray or brown; score of 1 is most desirable.

The statistical calculations are given in the bottom half of Table 9. The analysis of variance shows that the differences between the five groups of pork chops are statistically significant at the 1% level. The reader may perform a multiple comparison test on the effects or adjusted means by using the standard errors given in Table 10.

Table 9—Data and statistical calculations. Design parameters: $t + 1 = 5$, $k = 8$, $b = 6$, $r = 9$, $r_R = 12$, $\lambda = 13$, $\lambda_R = 18$, $s = 15$, $s_R = 24$

	Treatments					
Panelists	R	A	B	C	D	B_j
1	2	3	4	2	1	16
	1			2	1	
2	3	3	2	3	2	19
	2	2	2			
3	2	2	2	4	2	19
	1		3		3	
4	1	3	3	3	2	18
	1	2		3		
5	2	1	1	3	2	14
	1		2	2		
6	1	2	1	2	1	13
	2	2			2	
T_i	19	20	20	24	16	G = 99
						$\hat{\mu} = 2.0625$
$n_{ij}B_j$	198	149	151	147	147	t+1
$kQ_i = kT_i - n_{ij}B_j$	−46	11	9	45	−19	$\Sigma kQ_i = 0$
						t+1
Q_i	−5.750	1.375	1.125	5.625	−2.375	$\Sigma Q_i = 0$
$\hat{t}_i$	−0.4792	0.1526	0.1240	0.6383	−0.2760	
$\bar{X}_i = \hat{\mu} + \hat{t}_i$	1.58	2.22	2.19	2.70	1.79	

$CF = 99^2/48 = 204.1875$

$SS_T = (2^2 + 1^2 \ldots + 1^2 + 2^2) - CF = 235 - CF$
$= 30.8125$

$$SS_b = \frac{16^2 + 19^2 + \ldots + 13^2}{8} - CF$$

$= 4.1875$

$bk\lambda_R = 6(8)18 = 864$ $r_R\lambda - r\lambda_R = 12(13) - 9(18) = -6$
$t\lambda + \lambda_R = 4\ (13) + 18 = 70$

$\hat{t}_R = 9(-5.75)/6(18) = -51.75/108 = -0.4792$
$\hat{t}_A = [864(11) + 6(-46)]/(70)\ (864) = 0.1526$
$\hat{t}_B = [864(9) + 6(-46)]/60{,}480 = 0.1240$
$\hat{t}_C = [864(45) + 6(-46)]/60{,}480 = 0.6383$
$\hat{t}_D = [864(-19) + 6(-46)]/60{,}480 = -0.2760$

$SS_{t\ adj.} = (-0.4792)\ (-5.750) + \ldots + (-0.2760)\ (-2.375)$
$= 7.3507$
$SS_{bt} = [(3^2/2) + (5^2/2) + \ldots + (2^2/1) + (3^2/2)] - CF - SS_b - SS_{t\ adj.}$
$= 13.2743$
$SS_e = [(2-1)^2 + (3-2)^2 + \ldots + (1-2)^2]/2$
$= 6.000$

The reference-treatment comparisons can be determined by using the least significant difference test (LSD). In this example, the critical value of the LSD test is

$$t_{.01,\,18}\,(SE_{t-R}) = 2.878\ (0.2561) = 0.7371$$

where t is the value of the Student's t distribution at the 1% significance level with 18 degrees of freedom for error. The

Table 10—Analysis of variance of pork chop experiment

Source of variance	DF	SS	MS	F-ratio
Total	47	30.8125		
Panelists	5	4.1875	0.8375	
Treatments (adjusted)	4	7.3507	1.8377	5.514, $P < 0.01$
Interaction	20	13.2743	0.6637	
Pure error	18	6.0000	0.3333	

Standard error of two adjusted treatment means:

$$SE_{\bar{x}_i - \bar{x}_{i'}} = \sqrt{2\,(0.3333)/(70/8)} = 0.2760,\ i \neq i'$$

Standard error of treatment versus reference standard:

$$SE_{t-R} = \sqrt{1.7222(0.3333)/(70/8)} = 0.2561$$

differences that exceed 0.7371 are declared significant. The comparisons of interest are:

$$R - A = 1.58 - 2.22 = -0.64$$
$$R - B = 1.58 - 2.19 = -0.61$$
$$R - C = 1.58 - 2.70 = -1.12^{**}$$
$$R - D = 1.58 - 1.79 = -0.21$$

Thus, the comparison between R and C is statistically significant. Based on the scale used, the negative difference indicates that treatment C is significantly inferior to the reference standard.

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Michigan Agricultural Experiment Station Journal Article No. 8397. Supported in part by Dairy Research Inc.

ETHANOL INHIBITION OF Staphylococcus aureus AT LIMITED WATER ACTIVITY

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ABSTRACT

The effectiveness of ethanol as an antimicrobial agent in model systems at limited water activity (a_w) was tested. The effectiveness of ethanol against *Staphylococcus aureus* was a function of a_w and test medium, but the antimicrobial effect was more than from just the a_w lowering ability of ethanol. The results indicate that in a_w lowered foods, low concentrations of ethanol (2–4%) have a specific antimicrobial effect, thus making it a useful agent to be used in conjunction with other humectants.

INTRODUCTION

THE PURPOSE of this study was to determine the effectiveness of ethanol as an antimicrobial agent in intermediate moisture food (IMF) systems. Many studies have evaluated the effect of water activity (a_w) on the growth and inhibition of microorganisms in IMF systems. Formulations designed to lower the a_w of a food system often include antimicrobial agents such as potassium sorbate, propylene glycol, and sodium benzoate. The present study was designed to evaluate the usefulness of ethanol in these food systems, based on the use of ethanol as an antiseptic agent.

A literature review indicates that past work has been divided into two areas: (1) evaluation of ethanol as a germicidal agent and (2) studies evaluating ethanol as an antimicrobial agent in pharmaceutical and food products. Krönig (1894) found that staphylococci, on dried threads did not die with an application of 95% ethanol, while a 60–70% concentration was effective. Epstein (1897), using various microorganisms applied to moist threads, found a 50% solution of ethanol most germicidal. Harrington (1903) also found greater susceptibility of microbes on moist than on dry threads. Harrington (1903), Beyer (1911), and Gregersen (1916) found a 70%/wt solution of ethanol most effective against *Staphylococcus aureus* on dried threads. Price (1938) concluded that a 60–70%/wt solution of ethanol was an effective germicide while a 95%/wt solution was much less effective. Morton (1951) demonstrated that 42%/wt and greater concentrations of ethanol killed *S. aureus* in ten seconds or less in liquid media. Morton (1951) concluded that a 95%/wt solution of ethanol was effective against *S. aureus*.

Few studies have evaluated ethanol as an antimicrobial agent or preservative in pharmaceuticals and food products. Bandelin (1951), when evaluating the use of ethanol in pharmaceuticals, concluded that in concentrated sugar solutions ($a_w \cong 0.90$), a 15–18% solution of ethanol was an effective preservative. Bramachari (1927) indicated that a 12.7% level of ethanol was necessary in beverages to assure sterility with 24 hours. Perry and Beal (1920), evaluating the levels of ethanol necessary to prevent the growth of yeasts and molds in dextrose broth, found that an 11% solution inhibited *Saccharomyces cereviseae* and that an 8% solution inhibited *Penicillium glaucum*. Recently, the Federal Register (1974) affirmed that ethanol was GRAS "under the FFD & C Act when used as an antimicrobial agent on pizza crusts prior to final baking at a maximum level of 2.0% by product weight." In addition, U.S. Patent No. 3,979,252 describes a process for retarding mold growth in partially-baked pizza crusts in which a 95% solution of ethanol is sprayed on pizza crusts immediately prior to packaging. The optimum usage levels of ethanol are stated as being from 0.002–0.004 ml per square cm of crust surface or <2%/product weight at a crust a_w of 0.96.

Table 1—Level of effectiveness of ethanol from literature

Study	Effective % ETOH level	[a]Alcohol (alcohol soln)	[a]Final system	Method	Effective exposure time
Krönig	60–70	0.521	0.421	dried thread	1 min
Harrington	60–70	0.521	0.421	dried thread	1 min
Beyer	60–70	0.521	0.421	dried thread	1 min
Gregersen	60–70	0.521	0.421	dried thread	1 min
Price	60–70	0.521	0.421	dried thread	1 min
Epstein	50	0.718	0.718	moist thread	1 min
Morton	>42	0.757	0.757	solution	10 sec
Bandelin	17-18	0.921	0.829	sugar soln	?
Bramachari	12.7	0.946	0.946	solution	24 hr
Perry	11 (yeasts)	0.936	0.936	broth soln	24 hr
	8 (molds)	0.940	0.940	broth soln	24 hr
Plenons	2 (molds)	0.992	0.952	surface pizza crust	?
Fed. Reg.	2	0.992	0.992	not specified	?

As may be noted from past work, the levels of ethanol which have been found to be effective against microorganisms depend upon: (1) the medium in which the microorganisms were tested, (2) the length of time the microorganisms were exposed to the ethanol, (3) the type of microorganisms tested and (4) the ethanol concentration.

Because ethanol is volatile and would interfere in any present method, it is impossible to measure the a_w of the media into which ethanol is added. It is possible, however, to theoretically calculate the a_w lowering effect of the ethanol from Raoult's Law as in Equation 1:

$$a_w = \frac{g\ H_2O/18}{(g\ H_2O/18) + (g\ \text{alcohol}/46)} \quad (1)$$

where: g H_2O is g H_2O in mixture and g alcohol is g alcohol added. If the a_w of the mixture is known before the addition of ethanol, then the final a_w can be calculated from the Ross equation (Ross, 1975). Table 1 shows the calculated a_w of the alcohol/water mixture at the effective ethanol concentration found in the different studies along with the predicted a_w of the final test system and effective exposure time.

As seen, the effective a_w for inhibition or death also varies with the test method and microbes used. The present study was designed to determine more specifically the interaction between a_w and ethanol in inhibiting *S. aureus*.

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0022-1147/78/0005-1467$02.25/0

MATERIALS & METHODS

STAPHYLOCOCCUS AUREUS F265 was used as the challenge microorganism because of its ability to grow down to and form toxin in the a_w 0.84–0.85 range (Troller, 1973; Tatini, 1973). Tryptic soy broth and Tryptic soy agar, both enriched (with yeast extract), were used as growth media to which glycerol was added to lower the a_w of the broth media. Glycerol has been shown to have no specific antimicrobial effect on the growth of *S. aureus* in lowered a_w systems (Hsieh, 1975). Ethanol (95% by wt-grain alcohol-undenatured) was added to the media and adjusted to the desired water activity, at levels from 1%/wt to 40%/wt.

Three methods were employed to evaluate ethanol as an antimicrobial agent. The first method involved preparing Tryptic soy agar plates with 15 ml of agar per plate. Then 1.0 ml aliquots of ethanol solutions, ranging in concentration from 2–40%/wt were aseptically spread on the plates and allowed to soak in. (This concentration range would be equivalent to a range of 0.0001–0.0023 ml ethanol/cm^2 plate surface area.) *S. aureus* F265 at an initial inoculum of 8.9×10^2 cfu was applied to the plates by spread plate method and the plates subsequently incubated at 37°C for 24 hr. The cfu were then evaluated on each plate.

The second method involved preparing double strength Tryptic soy agar, cooling this agar to 55°C after autoclaving, and diluting 150 ml portions to 300 ml with sterile diluent-ethanol solutions and/or glycerol, depending upon the final a_w desired. The ethanol solutions ranged in concentration from 1–20%/wt. The a_w levels before ethanol addition were 1.0 (0% glycerol), 0.95 (17.8% glycerol), 0.93 (22.2% glycerol), 0.90 (31.7% glycerol) and 0.88 (36.4% glycerol). After determining that plates made from agar mixtures prepared as above should be stored and incubated in sealed plastic bags to prevent loss of ethanol, 15 plates were made from each agar mixture. These plates were inoculated with approximately 10^3 cfu/plate of *S. aureus* F265, sealed in plastic bags and incubated at 37°C for approximately 100 hr.

The thread method involved the preparation of Tryptic soy broth at ethanol levels ranging from 1–10%/wt and at water activities, before ethanol addition, of 1.0, 0.95, 0.93, 0.90 and 0.88 (same % glycerol levels as for second method a_w levels). From each treatment, 100 ml aliquots were then placed in sterile flasks and sealed after being inoculated with approximately 1.6×10^7 cfu/ml broth *S. aureus* F265. The treatments were then incubated at 37°C with shaking at 125 rpm in a New Brunswick Incubator Shaker for 48 hr. Growth was measured both by plating out samples and counting cfu/plate and turbidimetrically at 450 nm on a Beckman Spectrophotometer 20. Growth curves were constructed, plotting either O.D. or cfu/ml versus time (θ).

Table 2–Effect of ethanol on growth of S. aureus F265 (Method I)

Ethanol %/wt Soln Applied to Agar	cfu/Plate X 10^{-1}	Theoretical 95% ethanol conc (ml/cm^2 X 10^4)
0 (control)	8.90	0
2	10.00	1
5	10.00	3
7	9.00	4
10	9.30	5
15	7.10	9
17	9.20	10
22	7.65	12
25	6.85	14
28	4.30	16
30	4.45	17
35	3.00	20
40	4.40	23

Table 3–Effectiveness of ethanol on S. aureus growth in solid media adjusted to various a_w's

Initial a_w[a]		% Ethanol/Agar	cfu/Plate X 10^{-1}
Initial a_w	1.0	0 (control)	10.00
Actual a_w	0.980	5	7.17
	0.967 to 0.910	8 to 20	NG[b]
Initial a_w	0.950	0 (control)	19.8
Actual a_w	0.946	1	16.7
	0.942	2	11.3
	0.939	3	9.9
	0.931	5	9.9
	0.927	6	2.5 (tiny colonies)
	0.922 to 0.888	7 to 15	NG
Initial a_w	0.930	0 (control)	15.7
Actual a_w	0.926	1	14.8
	0.923	2	9.85
	0.919	3	9.77
	0.911	5	10.8
	0.907	6	0.43
	0.903 to 0.870	7 to 15	NG
Initial a_w	0.900	0 (control)	7.87
Actual a_w	0.896	1	2.51
	0.893	2	1.68
	0.889 to 0.862	3 to 10	NG
Initial a_w	0.880	0	3.2
Actual a_w	0.877 to 0.843	1 to 10	NG

[a] Prior to addition of ethanol
[b] NG = No growth

RESULTS & DISCUSSION

METHOD I was designed to test the effectiveness of an ethanol spray on a ,surface. According to Patent #3,979,525, (Plenons et al., 1976) the optimum mold inhibiting ethanol levels for a pizza crust range from 0.002–0.004 ml/cm^2 of crust surface at a_w 0.96. The results for test Method I, in which dilute ethanol was applied to agar, are shown in Table 2. It appears that ethanol applied in this manner does not significantly inhibit *S. aureus* F265 up to 25%/wt. Above this concentration there appears to be partial inhibition. The table indicates that, based on the equivalent concentrations of 95% ethanol per square cm of agar, the level of ethanol necessary for inhibiting *S. aureus* at a_w 1.0 is higher than the required level for mold inhibition on a pizza at an a_w of 0.96.

The results of test Method II, which was designed to evaluate ethanol at various water activities in agar, are shown in Table 3. It can be seen that at a_w 1.0 inhibition occurred at the 8%/wt ethanol level, at a_w's 0.95 and 0.93 at the 7% level, at a_w 0.90 at the 3% level and at a_w 0.88 at the 1% level. The calculated water activity of the system at the ethanol effectiveness level is shown in Table 4. As seen, inhibition at high water activities is not related just to a_w lowering but also to the definite antimicrobial action of ethanol.

The growth curves from test Method III, constructed by plotting cfu/ml versus time as shown in Figures 1, 2, and 3. At an initial a_w of 1.0 as shown in Figure 1, inhibition occurs at the 9–10%/wt ethanol level with the 5–8% levels showing some inhibition. The results for a_w 0.95, shown in Figure 2, indicate that growth inhibition occurs at about the 7%/wt ethanol level and above, although there is a slight depression of growth rate at the 5%/wt level. The results for a_w 0.93 were similar to those for a_w 0.95. At a_w 0.90 growth inhibition occurs at a 4–5%/wt ethanol level. The results for a_w 0.88 are similar to a_w 0.90 for complete inhibition along with some inhibition at the 3%/wt level. The actual a_w at the effective

Table 4–A_w lowering effect of ethanol at effective antimicrobial levels in broth and agar systems

	Broth systems			Agar systems			
Broth a_i	ETOH Level	$a_{alcohol}$[a]	a_{final}[b]	a_i[c]	ETOH Level	$a_{alcohol}$	a_{final}
1.0	9%	0.963	0.963	1.0	8%	0.967	0.967
0.95	7%	0.971	0.922	0.95	7%	0.971	0.922
0.93	7%	0.971	0.903	0.93	7%	0.971	0.903
0.90	5%	0.980	0.882	0.90	3%	0.988	0.889
0.88	4%	0.984	0.866	0.88	1%	0.996	0.877

[a] a_w of alcohol/water
[b] Final a_w of system
[c] Initial a_w of system

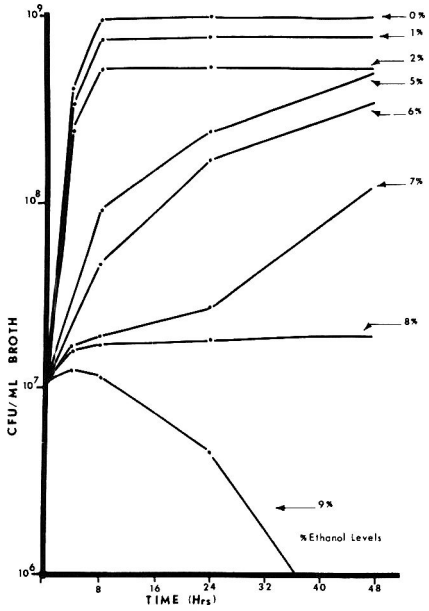

Fig. 1—Growth curves of S. aureus F265 at a_w 1.0 at various concentrations of ethanol.

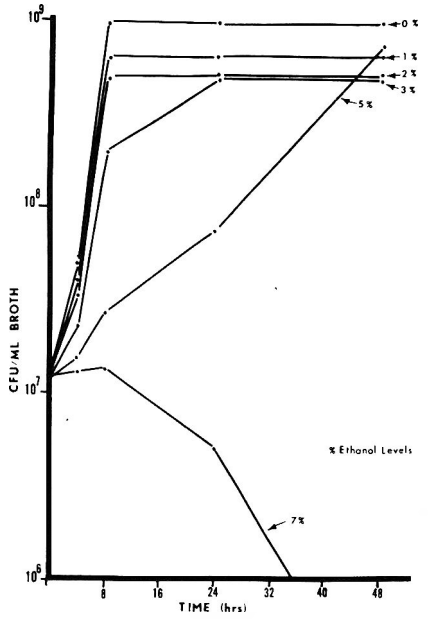

Fig. 2—Growth curves of S. aureus F265 at a_w 0.95 at various concentrations of ethanol.

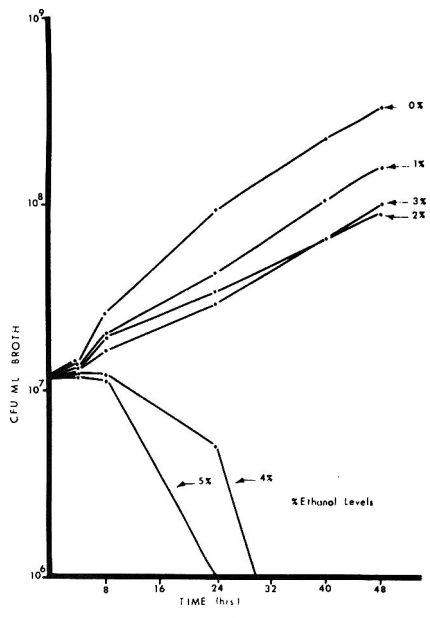

Fig. 3—Growth curves of S. aureus F265 at a_w 0.90 at various concentrations of ethanol.

ethanol levels are summarized in Table 4.

It appears that as the a_w of either the agar or broth systems is lowered, the amount of ethanol required to have a complete inhibitory effect on the growth of *S. aureus* F265 is decreased, showing synergism as has been found with glycols and potassium sorbate (Acott et al., 1976). At an initial a_w of 0.88, however, the ethanol may be acting only to lower the a_w to a level at which the *S. aureus* F265 is normally inhibited. In the agar system, the inhibition of *S. aureus* could be due, in part, to the different nutrient composition of the medium, the degree of oxygen availability (i.e. shaking in broth vs. surface growth on agar), as well as to the water activity lowering effect of ethanol. This would explain the difference in effective ethanol concentration levels between the broth and agar systems.

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Ms received 10/22/77; revised 4/15/78; accepted 4/20/78.

This is paper no. 10095 from the University of Minnesota Agric. Exp. Station. This study was supported in part by the University of Minnesota Agricultural Experiment Station Project 18-72 and Contract NAS 9-12560, Lyndon Johnson Space Center, Houston, TX.

LAXATION AND SERUM CHOLESTEROL IN RATS FED PLANT FIBERS

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ABSTRACT

Source and particle size of fiber were compared as laxative and hypocholesteremic agents in rats. Particle size of wheat bran and cellulose had no effect on fecal mass, moisture or dry matter. However, the cellulose with the larger particle size decreased intestinal transit time. Fibers which were extensively fermented by gut microflora increased fecal moisture and decreased intestinal transit time. No fiber decreased serum cholesterol compared to a fiber-free group. However, serum cholesterol was increased in rats fed 16 and 30 mesh wheat bran. This study indicates that each fiber source has a highly individualized effect on laxation but little effect on serum cholesterol in rats.

INTRODUCTION

DIETARY FIBER is generally defined as any plant material that is not degraded by mammalian gut enzymes. The physiological effects of materials which fit this description are difficult to predict because of the wide variation in chemical composition of dietary fiber. As the source of fiber is changed, the proportions of cellulose, hemicellulose, lignins, pectins and gums change. Moreover, in vitro chemical properties of fiber may not predict the properties of fiber in vivo, since physiological as well as chemical actions occur in the gastrointestinal tract. The fiber is exposed to the effect of other diet components, mastication, pH changes, gut enzymes and microbial fermentation. These factors modify the physiological action of a fiber to varying degrees depending on the original chemical composition of the fiber. For example, vegetable cell walls are generally more fermentable than cereal brans (VanSoest and Robertson, 1977). Thus, vegetable fibers would yield more volatile fatty acids (VFA) than cereal grains. Changes in VFA content of the colon could effect laxation parameters. The purpose of the present study was to evaluate the physiological effects of a wide range of fibers using the rat as the animal model. The specific objectives of this study were: (1) to compare the laxation effect of a variety of plant fibers, (2) to examine the effect of particle size on laxation and (3) to evaluate the effect of the plant fibers on serum cholesterol.

EXPERIMENTAL

Diets

The sources of fiber were: cellulose, wheat bran, wheat midlings, oat bran, oat flour, sugar beet pulp, soybean hulls and psyllium seeds. Fiber sources, except those used to evaluate particle size, were ground to pass through an 80 mesh screen. Particle sizes which were compared were: wheat bran (Official AACC White Wheat Bran, R07-3691) ground through 16, 30 or 80 mesh screens (WB16, WB30 and WB80, respectively) and an 80 mesh floc-type cellulose (Cel) versus an air classified cellulose (BW200, 75% passes through a 200 mesh screen). The compositions of the diets are shown in Table 1. All diets were formulated to have similar amounts of nitrogen, fat and carbohydrate per kcal. It was assumed no calories were derived from acid detergent fiber.

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0022-1147/78/0005-1470$02.25/0

Table 1—Composition of diet

Diet	Basal mix[a]	Casein	Fat[b]	White flour	Fiber source	ADF[c]	NDF[d]
	g/100g diet						
Control	6.5	11.5	22.0	60.0	0	0.1	0.1
WB 16	6.0	7.9	20.2	33.8	32.2	7.8	12.5
WB 30	6.0	7.9	20.2	33.8	32.2	7.8	12.5
WB 80	6.0	8.1	20.3	37.0	28.6	7.4	11.6
Wheat midlings	6.2	8.1	20.9	0	64.9	7.2	23.8
Oat flour	5.9	5.5	16.9	17.3	54.4	4.3	8.3
Oat bran	6.0	7.9	16.2	0	69.9	7.1	14.7
BW 200	6.0	10.7	20.5	55.8	7.0	7.0	7.0
Cel	6.0	10.7	20.5	55.8	7.0	7.0	7.0
Psyllium seed	6.0	11.0	21.0	57.0	5.0	ND[e]	ND[e]
Sugar beet pulp	6.0	8.2	20.3	48.7	16.8	7.3	11.9
Soyhull fiber	6.0	6.7	20.3	55.3	11.7	7.7	10.7

[a] 6.5g Basal mix contains: 1.0g cholesterol, 0.2g choline chloride, 0.3g methionine, 0.6 glucose, 0.4g vitamin mix (Yeh & Leveille, 1969) and 4g of mineral mix (Leveille and O'Hea, 1969).
[b] Mixture of corn oil and tallow with ratio of polyunsaturated fat of one.
[c] Acid-detergent fiber
[d] Neutral-detergent fiber
[e] Not determined

Animals and housing

Young adult (200–250g) male, Sprague-Dawley rats were housed individually in hanging wire cages. The animal room was temperature controlled (20 ± 1°C) and was on a 12 hr light-dark cycle. Water and food was provided ad libitum. Food consumption and body weights were recorded over the 28 day experimental period. Diets were fed to 10 rats per dietary treatment.

Laboratory analysis

Neutral detergent (NDF) and acid-detergent (ADF) fiber were determined as described by Georing and VanSoest (1970).

Three 24-hr quantitative fecal collections were pooled for individual rats to determine fecal VFA excretion. Feces were collected at 8 a.m. and frozen for subsequent analysis. Feces were air dried to a constant weight at 60°C for dry weight determination and VFA analysis. The pH of the feces were near neutrality. Thus, the VFA would be present primarily as salts of the acids and only negligible quantities of the VFA would be lost during drying. To extract the VFA, barrels from 5 ml plastic syringes were placed in centrifuge tubes. A piece of filter paper was placed in the bottom of the syringe barrel and 0.3g of dried, ground feces were transferred to the syringe barrel. One ml of 0.5N H_2SO_4 was pipetted into the syringe and the tube was gently vortexed. The moistened feces were held at 4°C for 12 hr and centrifuged at 12,000 × G for 15 min. The filtrate was used for VFA analyses. The VFA content of the filtrate was determined by a gas-liquid chromatograph (GLC) equipped with a flame ionization detector (FID). GLC conditions were: injector temperature, 135°; detector temperature, 150°; column temperature programmed from 100–125° at 4°/min; N_2 carrier gas flow 30 ml/min; H_2 flow, 15 ml/min; and air flow, 240 ml/min. VFA were separated on a 6 ft long, 1/8 in. diameter stainless steel column packed with 15% SP 1220/1% H_3PO_4 coated on 100/120 chromasorb W AW. The determined quantities of acetic, propionic, butyric, isobutyric, valaric and isovalaric acids were summed and the resultant values are expressed as μmoles of VFA excreted per g of feces. Standard solutions of the sodium salts of acetic, propionic and butyric acid containing 0.1 mg or 0.2 mg of the salts were pipetted onto 0.3g of

Table 2—Growth, laxation and serum cholesterol for rats fed plant fibers[a]

	Wt gain[b] (g)	Food Intake/g wt gain	Fecal wet mass (g/day)	Fecal dry mass (g/day)	Fecal water-holding capacity[c]	Volatile fatty acids (μmole/day)	Transit time (hr)[d]	Serum cholesterol (mg/100 ml)
Control	120 ± 10ab	4.7 ± 0.4ab	1.0 ± 0.1a	0.9 ± 0.1a	19 ± 1abc	135 ± 26a	43.1 ± 2.8ef	138 ± 10ab
WB 16	119 ± 10ab	5.3 ± 0.5ab	4.4 ± 0.4d	3.7 ± 0.3fg	32 ± 2cde	403 ± 59c	22.5 ± 2.0ab	162 ± 10b
WB 30	107 ± 4a	5.6 ± 0.1ab	4.3 ± 0.1d	3.2 ± 0.1fg	33 ± 2cde	394 ± 33c	25.8 ± 1.8abc	157 ± 6b
WB 80	105 ± 7a	5.8 ± 0.3b	3.8 ± 0.1cd	2.9 ± 0.1ef	32 ± 2cde	429 ± 22cd	26.1 ± 1.8abc	144 ± 3ab
Wheat Midlings	116 ± 7ab	5.3 ± 0.6ab	8.3 ± 0.3f	5.8 ± 0.2h	44 ± 2def	978 ± 49f	20.4 ± 2.2a	144 ± 12ab
Oat Flour	158 ± 10b	4.2 ± 0.2ab	1.9 ± 0.1ab	1.4 ± 0.1ab	29 ± 1bcd	189 ± 23ab	41.6 ± 2.3def	140 ± 7ab
Oat Bran	133 ± 14ab	4.4 ± 0.3ab	6.5 ± 0.5e	3.7 ± 0.1g	72 ± 1g	589 ± 39de	46.9 ± 3.1f	131 ± 6ab
BW 200	126 ± 14ab	4.4 ± 0.6ab	1.9 ± 0.1ab	1.7 ± 0.1bc	15 ± 2ab	120 ± 22a	45.3 ± 2.4f	120 ± 5a
Cellulose	149 ± 5ab	3.8 ± 0.1a	2.5 ± 0.1b	2.2 ± 0.1cd	12 ± 1a	144 ± 12a	33.4 ± 1.7bcde	122 ± 9a
Psyllium Seed	109 ± 8a	5.2 ± 0.3ab	2.5 ± 0.2b	1.6 ± 0.1b	55 ± 5f	456 ± 37cde	31.1 ± 2.1abcd	122 ± 5a
Sugar Beet Pulp	123 ± 8ab	5.2 ± 0.3ab	3.7 ± 0.3cd	2.5 ± 0.1de	45 ± 3ef	597 ± 46e	21.6 ± 2.4a	122 ± 9a
Soyhull Fiber	129 ± 7ab	4.9 ± 0.2ab	2.9 ± 0.1bc	2.3 ± 0.1cde	26 ± 1abc	332 ± 18bc	24.8 ± 2.1abc	133 ± 4ab

[a] Values followed by different letters are significantly different ($P < 0.05$)

[b] Mean ± s.e. for 10 rats per group

[c] Water-holding capacity = (g fecal H_2O/g fecal dry mass) X 100

[d] Hours required for 90% of recovered ^{14}C polyethylene glycol to be excreted in the feces

BW200 in syringe barrels and dried at 60°C. Extraction was carried out as above, recovery of the standards was 98 ± 3%.

Serum cholesterol was determined spectrophotometrically by the method of Searcy and Berquist (1960) with modification by Leveille et al. (1962).

Intestinal transit time was determined with ^{14}C-polyethylene glycol, a nondigestible, nonabsorbable water soluble marker. One ml of water containing 1 μCi of ^{14}C-polyethylene glycol/10 mg of polyethylene glycol (MW 4000) was intubated. Feces were collected at intervals after intubation and homogenized in water. Aliquots were added to scintillation cocktail and radioactivity was determined by liquid-scintillation counting. Scintillation cocktail consisted of 4g 2,5-diphenyloxazole and 200mg 1-4 bis(2-(4-methyl-5-phenyoxazolyl))-benzene in 667 ml toluene and 333 ml of triton-x. Intestinal transit time is defined as the hours required to excrete 90% of the recovered radioactivity.

Treatment means were compared by standard analysis of variance statistics. Where significant f values were detected results were further analyzed by Tukey's multiple range criterion.

RESULTS & DISCUSSION

General consideration

There were significant differences in both weight gain and food intake per g weight gain (Table 2). Rats fed the WB 30, WB 80 and psyllium seed diets had lower weight gains than rats fed the oat-flour diet. Rats fed the WB 80 diet required more food per unit weight gain than rats fed the Cel diet. It would be expected that a diet with added fiber would require more food intake per g of weight gain than the non-fiber containing control diet since energy from fiber is considered unavailable. This was not the case. The range in weight gain efficiencies can be most readily explained on the basis of variable digestion of the different fibers. Variable digestion would result in different quantities of available energy from the variety of fibers studied. Wheat bran is one of the least digestible fibers (VanSoest and Robertson, 1977) and in the present study the WB 80 diet had the lowest weight gain efficiency. Moreover, VanSoest and Robertson (1977) suggest that the fermentation of a fiber depends upon the chemical make-up of the fiber (i.e., the greater the lignification, the less fermentable) with the rate of fermentation of cellulose varying by as much as an order of magnitude. Variability of fermentation can explain the range of efficiencies in this study but doesn't answer the underlying question of the energy availability of a fiber. Various chemical determinations of fiber (neutral detergent fiber, acid detergent fiber, crude fiber, etc.) are estimates of unavailable carbohydrate and therefore, unavailable energy. However, the unavailability of energy as determined by chemical methods do not always agree with biological estimates. For instance, the NDF content of the wheat midlings diet was 24% but rats fed the wheat midling diet had similar weight gain as the control and with only a small decrease in weight gain efficiency.

Fecal weights

Many reviews have been written on the effects of fiber on increases in fecal weight (Cummings, 1973; Eastwood, 1974; Burkit et al., 1975). In general increases in fecal weight and volume are attributed to an increased amount of water in the feces with little importance given to increases in dry weights (Connell, 1976). In this study daily fecal wet weight ranged from 1.0g for the control diet to 8.3g for the wheat midling diet (Table 2). Only the oat flour and BW 200 diets had wet masses similar to the control diet. Fecal dry weights ranged from 0.9 g/day to 5.8 g/day, with only the oat-flour diet having a similar dry weight as the control diet. Therefore, when a variety of fibers are compared, a significant increase in both wet and dry fecal weights can occur.

The ability of fiber to increase fecal water is well documented (Williams and Olmsted, 1936; Eastwood et al., 1973). The major hypothesis put forth for this increased water retention by the feces is the physical adsorption of water molecules to the fibers, especially the hemicellulose component (Eastwood, 1974; Tasman-Jones, 1976; Leveille, 1976). The basis of this hypothesis is the in vitro adsorption of water by fiber and then extrapolation to in vivo situations. Williams and Olmsted (1936) suggested that microbial fermentation of fiber to VFA should be considered as an important parameter which influences the water content of the stool. The volatile fatty acid content of feces is shown in Table 2. Figure 1 illustrates the relationship between the volatile fatty acid content of the feces and the amount of fecal water excreted. While high correlation ($r = 0.95$) does not show cause and effect, we hypothesize that gut fermentation of the fibers, particularly hemicellulose, results in an osmotic gradient which retains and/or promotes water secretion into the colon. Volatile fatty acids are absorbed by the rat colon (VanSoest and Robertson, 1977), but sufficient quantities of volatile fatty acids appear to remain in the gut to act as an osmotic agent and to increase fecal water excretion. You cannot determine the extent of fiber fermentation from the present data since VFA absorption was not estimated. Acetic acid was the primary VFA found in the feces and there was little variation among treatments in the percentage of constituent acids that were excreted.

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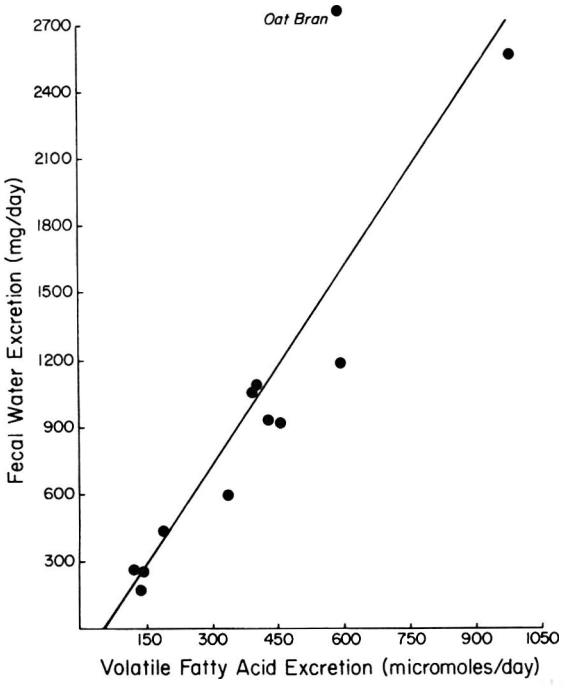

Fig. 1—Relationship between fecal volatile fatty acid excretion and fecal water excretion in rats fed plant fibers. Fiber sources other than oat bran and volatile fatty acid excretion data are listed in Table 2.

Laxation

The mechanism by which fiber induces laxation is not fully understood. There are five classes of laxatives: (1) irritant or stimulant, (2) wetting agent, (3) saline or electrolyte, (4) bulk and (5) lubricant (Binder and Donowitz, 1975). The ability of fiber to increase gastrointestinal motility is probably due to a combination of the above classes. The physical presence of the fiber provides bulk. Microbial fermentation of the fiber results in an increased electrolyte (solute) content of the water in the colon. Similarly, an increased excretion of bile salts which occurs with some types of fibers could also increase the solute concentration in colon water and the amount of fecal water excreted.

Many fecal parameters have been proposed as important in promoting laxation. Among these are the water holding capacity (WHC) of the feces (g of water/g of dry feces), the absolute amount of water associated with the feces, and the wet or dry mass of the feces. Values for these parameters are shown in Table 2. Correlations between these factors and intestinal transit time (considered a result of laxation) are similar ($r = -0.75$) with the exception of WHC which has a less significant correlation ($r = -0.55$). Consequently, the predictive value (based on transit time) of each of the factors is about equal. These correlations are calculated excluding the oat bran data. Rats fed the oat bran diet exhibited from slight to marked diarrhea. Upon autopsy the cecums of these animals were from 2 to 3 times larger than the cecums of other animals consuming similar quantities of fibers.

Particle size

The effect of particle size of a fiber has not been extensively investigated. In this study three particle sizes of wheat brans (16, 30 and 80 mesh) and two sizes of cellulose (ca 80 and ca 200 mesh) were compared. No differences were observed for any of the parameters shown in Table 2 for either the wheat bran or cellulose diets. Fecal wet and dry weight decreased as particle size was decreased but this was not significant. The only effect of particle size was on intestinal transit time (Table 2). The transit time for BW 200 was significantly greater than that of the cellulose diet. As the VFA, WHC and water content of the feces were similar for Cel and BW 200, a possible explanation for the difference in transit time could be the fecal mass was too small in the BW 200 diet to sufficiently stimulate a faster rate of passage. Kirwan et al. (1974) reported a decrease in transit times and increased intestinal motility when coarse wheat bran (approximately 16 mesh) was fed as opposed to a fine wheat bran (approximately 40–50 mesh). The authors attributed this to an increase in water-holding capacity, due to the ability of the coarse bran to hold more water. In this study there was no difference between water content or fecal WHC in either the wheat bran diets or cellulose diets.

Serum cholesterol

There were no differences found in serum cholesterol values when compared to control values (Table 2). However, animals fed two of the wheat bran diets (16 and 30 mesh) had significantly greater serum cholesterol values than animals fed the cellulose diets (Cel and BW 200), psyllium seed or sugar beet pulp diets. The ability of a fiber to affect serum cholesterol depends on the composition of the fiber, the level of fiber fed and other dietary components. DeGroot et al. (1963) reported rolled oats and wheat bran significantly decreased serum cholesterol levels when fed at 25% of the diet (1% cholesterol added). More recently Tsai et al. (1976) reported neither wheat bran nor cellulose lowered serum cholesterol when fed at fiber levels and under dietary conditions similar to our study. Supplementation of human diets with wheat bran have generally been ineffective in lowering serum cholesterol (Heaton and Pomare, 1974; Truswell and Kay, 1975; Connell et al., 1975; Jenkins et al., 1975). However, Sanstead (1977) reported the variety of wheat bran influences serum cholesterol. Hard red spring wheat bran lowers serum cholesterol, whereas the AACC soft wheat bran does not affect cholesterol values.

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SOME EFFECTS OF DIFFERENT PHOSPHATE COMPOUNDS ON IRON AND CALCIUM ABSORPTION

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ABSTRACT

Different phosphates commonly used in food processing have been evaluated for their effects on iron and calcium absorption. In growing rats, addition of phosphates to diets caused decreased iron absorption, decreased hemoglobin concentrations and depressed liver iron values in both the control and achlorhydric animals. Liver iron values were somewhat depressed in the mature rats which was slightly more severe in the achlorhydric animals. Sodium pyrophosphate and sodium tripolyphosphate impaired iron metabolism more severely than dibasic sodium phosphate and sodium metaphosphate in both age groups; this effect tended to be greater in the achlorhydric animals. In the growing rats, addition of phosphates to the diets decreased calcium absorption and femur calcium content, the severity of which was greater in the achlorhydric animals. Sodium tripolyphosphate caused consistent decreases in bone calcium content. The phosphate effects were definitely more severe in the achlorhydric growing rats and tended to be more severe in the achlorhydric mature rats. The data presented indicate that the various phosphates have different effects on calcium and iron metabolism and that these effects are magnified by achlorhydria.

INTRODUCTION

BECAUSE of the high incidence of osteoporosis in elderly people, several investigators have made cautionary remarks concerning phosphorus consumption (Lutwak, 1969, 1974; Albanese et al., 1975; Jowsey, 1977). This particular concern comes from the fact that the American diet may have a calcium:phosphorus ratio of about 1:4 (ARS, 1968). For elderly people in Utah, individual calcium:phosphorus ratios ranged from 1:1 to 1:4 (Fisher, Hendricks and Mahoney, unpublished data) which is in agreement with ratios calculated from the data of Jowsey et al. (1972). Dietary calcium:phosphorus imbalance is known to impair the utilization of calcium in animals. Jowsey et al. (1972) found that bone resorption was positively correlated ($P < 0.05$) with pretreatment dietary phosphorus in osteoporotic patients. The calcium:phosphorus ratio is particularly important when one of the elements is low in the diet (Wasserman, 1960) which is the case with the average American diet (Household Food Consumption Survey, 1965–66).

In addition to the phosphorus naturally present in food, the food manufacturing industry adds phosphorus in a variety of forms to an increasing number of foods (Deman and Melnychyn, 1971). Bell et al. (1977) presented evidence that substituting foods containing phosphate additives for similar foods that did not contain phosphate additives resulted in abdominal distress and mild diarrhea in some adult test subjects. The subjects exhibited increased urinary hydroxproline, cyclic AMP and phosphorus excretions and changes in serum calcium and phosphorus concentrations that were similar to the physiological changes of adult rats (Sie et al., 1974) and ponies (Argenzio et al., 1974) fed high phosphorus diets. Feeding high phosphorus diets to animals results in bone loss (Krook, 1968; Laflamme and Jowsey, 1972; Jowsey et al., 1974; and Sie et al., 1974) and hyperparathyroidism (Sie et al., 1974; Jowsey et al., 1974).

The condensed phosphates used in food processing have greater affinity for calcium at low pH's than the uncondensed phosphates such as dibasic sodium phosphate (Scharpf, 1971; Van Wazer, 1971). These phosphates would be expected to cause a more severe impairment in the utilization of food calcium than the uncondensed phosphates.

Although the condensed phosphates undergo hydrolysis to orthophosphate in aqueous conditions, the process is relatively slow (Van Wazer, 1971). From the chart presented by Van Wazer (1971), it is estimated that half-lives for hydrolysis of pyrophosphate and tripolyphosphate at pH 2 and 39°C (normal gastric conditions) are 400 and 150 hr, respectively. However, at pH 6 and 39°C (achlorhydric gastric conditions) these respective half-lives are approximately 5200 and 2100 hr (Van Wazer, 1971). Thus, significant amounts of salt would be expected to remain intact during the process of digestion and absorption in man and remain available to interact with divalent ions in the gastrointestinal tract.

Since various phosphate salts are incorporated into many commonly consumed foods, and because the salts differ in ion binding affinity according to the acidity of the medium, we evaluated the effects of feeding phosphate salts on mineral metabolism in normal and achlorhydric rats.

EXPERIMENTAL

IN BOTH EXPERIMENTS 1 and 2, a basal diet was prepared which contained (g/kg): casein 200, dextrose 510, corn oil 100, cellulose 20, vitamin mixture 20, and mineral mixture 150. The composition of the Vitamin Diet Fortification Mixture (United States Biochemicals) has been described by Hendricks et al. (1977). The mineral mixture contained (g/kg): $CaCO_3$ 104.1, $Na_2HPO_4 \cdot H_2O$ 206.7, KCl 22.9, $MgCO_3$ 9.27, $ZnSO_4 \cdot 7H_2O$ 0.587, $CoCl_2 \cdot 6H_2O$ 0.004, $Na_2MoO_4 \cdot 2H_2O$ 0.012, $CuSO_4 \cdot 5H_2O$ 0.1207, $MnSO_4 \cdot H_2O$ 0.9847, KI 0.0213 and dextrose 654.63. Iron was added directly to the diet during mixing as 100 mg of $FeSO_4 \cdot 7H_2O$ dissolved in 10 ml demineralized water. The test diets were prepared by adding to the basal diet one of the following salts at the expense of dextrose (g/kg): $Na_2HPO_4 \cdot 7H_2O$ 52.0, $NaPyroPO_4$ 43.7, $NaTriPolyPO_4$ 23.9 or $NaMetaPO_4$ 19.9. The calcium:phosphorus ratio of the basal and test diets were 1:1 and 1:2, respectively.

Experiment 1

Fifty, weaning, male, Sprague-Dawley rats (Simonsen Laboratories, Inc., Gilroy, CA) were surgically prepared as described by Mahoney and Hendricks (1974). Twenty-five of these rats were surgically prepared and X-irradiated to cause achlorhydria. The remaining twenty-five rats were sham-operated without X-irradiation to serve as controls. Three days postoperative, 5 achlorhydric and 5 control rats were randomly selected and assigned to each of the experimental diets. Each of the achlorhydric rats was paired with a control rat which received each day an amount of food equal to that consumed the previous day by his pairmate. The achlorhydric rats received food ad libitum. Fresh demineralized water was continuously available to all rats. All rats were housed in metabolism cages designed to quantitatively separate and collect urine and feces.

Food intakes, body weights and hemoglobin values were determined and feces were collected weekly for 4 consecutive weeks, after which the animals were killed. Femurs were obtained by removing the entire rear legs from the carcass; the legs were boiled in water for 20 min, and

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0022-1147/78/0005-1473$02.25/0

Table 1—Effect of different dietary phosphate salts and achlorhydria on apparent iron and dry matter adsorption (percent) and 4-wk weight gain (g) by growing male rats in experiment 1

	Iron[a]		Dry Matter[a]		Weight gain[a]	
No. of observations	Control 5	Achlorhydric 5	Control 5	Achlorhydric 5	Control 5	Achlorhydric 5
Basal	77.4c	70.9**,e	94.6c	94.6NS,ed	101c	93NS,ef
Na_2HPO_4	63.5(4)	46.2(4)**	95.0cd	94.1NS,e	86(4)cd	79(4)NS,fg
Na meta PO_4	73.8cd	71.2NS,e	94.8c	94.4NS,e	79d	72NS,g
Na pyro PO_4	53.9(4)	53.9(4)NS	95.0cd	95.2NS,d	93cd	87(4)NS,efg
Na tripoly PO_4	69.4d	65.5NS	95.5d	96.3NS	89cd	96(6)NS,e
$LSD_{.05/.01}$[b]	4.7/6.4		0.7/1.0		15/20	
Statistical summary						
Diet	F(4,36)=28.92(P < 0.01)		F(4,36)=9.60(P < 0.01)		F(4,38)=2.62(P < 0.05)	
Achlorhydria	F(1,36)=13.92(P < 0.01)		F(1,36)=0.03(NS)		F(1,38)=0.66(NS)	
Interaction	F(4,36)=3.25(P < 0.05)		F(4,36)=2.94(P < 0.05)		F(4,38)=0.37(NS)	

[a] Values in ( ) indicate the number of observations when different from 5.

[b] Differences must equal or exceed LSD values for means to be significantly different at the 5 or 1% levels of probability. Astericks and NS indicate achlorhydric effects (*means P < 0.05; **means P < 0.01; NS means not statistically significant). For phosphate effects, means having the same small letter are not significantly different (P < 0.05).

then scraped with the fingers to remove the soft tissue. The femurs were air-dried for at least 2 wk before analysis.

Experiment 2

Fifty 250g, male, Sprague-Dawley rats (Simonsen Laboratories) were surgically prepared, treated and fed the same diets as described in Experiment 1. They were killed after being fed the diets for 3 wk. All analyses were the same as in Experiment 1.

Analytical procedures

Gastric pH was measured by narrow range pH paper. The feces were dried at 105°C overnight before weighing for dry matter determination. The femurs, livers, diets, and feces were ashed (500–600°C for 24 hr), and the ash was solubilized in 6N HCl. The calcium and iron (liver only) contents of the ash solutions were quantitated by atomic absorption spectrophotometry (Nalder et al., 1972). The hemoglobin was determined spectrophotometrically as cyanomethemoglobin (Crosby et al., 1954). The data were processed by analysis of variance using the least significant difference (LSD) test to determine differences among group means. The LSD value was utilized to identify treatment differences only when the appropriate F value was significant at the 5% alpha level (Carmer and Swanson, 1973).

RESULTS & DISCUSSION

THE EFFECTS of incorporating different phosphate salts into diets fed to rats and of gastric achlorhydria on growth and iron metabolism are shown in Tables 1, 2 and 3. In almost every case, achlorhyria resulted in lower values. This is in agreement with previous findings (Murray and Stein, 1970; Mahoney and Hendricks, 1974; Mahoney et al., 1975).

Additional phosphate also depressed weight gain significantly (Table 1); however, it did not meaningfully depress dry matter absorption. Thus, in the growing rat, phosphate treatment nor achlorhydria altered digestion and absorption of the over-all diet enough to limit the digestible energy sufficiently to account for the impaired growth performance observed.

The addition of phosphate salts to the diets of growing rats resulted in decreased iron absorption (Table 1), decreased hemoglobin concentrations (Table 2), and slightly depressed liver iron values (Table 3). These decrements were most apparent when sodium pyrophosphate was incorporated into the diets. Sodium pyrophosphate resulted in a decreased hemoglobin concentration in the mature achlorhydric rat (Table 2). From the data presented from these 3- and 4-wk experiments, it appears that sodium pyrophosphate and sodium tripolyphosphate impair iron metabolism in control and achlorhydric rats and that dibasic sodium phosphate and sodium metaphosphate affect iron metabolism minimally. It is well known that phosphorus can interfere with the utilization of dietary iron (Hegsted et al., 1949; Sell, 1965). Also, phos-

Table 2—Effect of different dietary phosphate salts and achlorhydria on final hemoglobin concentration (g/dl) of growing and mature male rats

	Growing – Exp. 1		Mature – Exp. 2	
No. of observations	Control 5[a]	Achlorhydric 5[a]	Control 5[a]	Achlorhydric 5[a]
Basal	11.02	9.28**,e	14.20c	13.00NS,e
Na_2HPO_4	12.12(4)c	10.10(3)**,e	15.92d	13.66**,ed
Na meta PO_4	12.80c	9.80**,e	14.14c	15.26(6)*,d
Na pyro PO_4	9.62(4)d	9.43(4)NS,e	15.16cd	9.36(6)**
Na tripoly PO_4	9.48d	9.78NS,e	15.30cd	12.78**,e
$LSD_{.05/.01}$[b]	0.91/1.22		1.64/2.20	
Statistical summary				
Diet	F(4,35)=6.38(P < 0.01)		F(4,42)=4.27(P < 0.01)	
Achlorhydria	F(1,35)=20.91(P < 0.01)		F(1,42)=18.46(P < 0.01)	
Interaction	F(4,35)=4.01(P < 0.05)		F(4,42)=5.02(P < 0.01)	

[a] Values in ( ) indicate the number of observations when different from 5.

[b] Differences must equal or exceed the LSD value for means to be significantly different at the 5 or 1% levels of probability. Asterisks and NS indicate achlorhydria effects (* means P < 0.05; ** means P < 0.01; NS means not statistically different). For phosphate effects, means having the same small letter are not significantly different.

Table 3—Effect of different dietary phosphate salts and achlorhydria on total liver iron content (μg) of growing and mature rats

	Growing – Exp. 1		Mature – Exp. 2	
No. of observations	Control 5[a]	Achlorhydric 5[a]	Control 5[a]	Achlorhydric 5[a]
Basal	505c	415NS,d	1252c	801*,d
N_2HPO_4	478(4)c	328(4)**,d	1180c	924NS,d
Na meta PO_4	500c	329**,d	928(6)c	1080(6)NS,d
Na pyro PO_4	359(4)c	331(4)NS,d	1147c	769*,d
Na tripoly PO_4	412c	384NS,d	775c	737NS,d
$LSD_{.05/.01}$[b]	92/124		275/–	
Statistical summary				
Diet	F(4,45)=1.44(NS)		F(4,43)=1.54(NS)	
Achlorhydria	F(1,45)=9.71(P < 0.01)		F(1,43)=4.86(P <0.05)	
Interaction	F(4,45)=0.98(NS)		F(4,43)=1.83(NS)	

[a] Values in ( ) indicate number of observations when different from 5.

[b] Differences must equal or exceed the LSD value for means to be significantly different at the 5 or 1% levels of probability. For the achlorhydria effects, **means P < 0.01, * means P < 0.05, and NS means not statistically significant. For the phosphate effects, no significant differences exist among the means since the "F" values are too small. (Diet means having the same small letter are not significantly different.)

phorus salts of iron are poorly utilized relative to other forms of iron (Blumberg and Arnold, 1947; Amine et al., 1972; Cook et al., 1973; Waddell, 1974; Mahoney and Hendricks, 1976). Thus, it is not surprising that the phosphate salts impaired the utilization of dietary iron in these experiments. Of the phosphate salts tested, sodium pyrophosphate consistently caused the most adverse effects on iron metabolism.

The effects of the different phosphate salts on calcium metabolism are presented in Tables 4 and 5. In the growing rats, each of the salts caused significant ($P < 0.05$) decreases in calcium absorption. This decrease was more pronounced in the achlorhydric animals. No decrements in calcium absorption observed in the mature rats were attributable to phosphate or achlorhydria. That the calcium absorption of the sham-operated mature rats was less than that of the growing ones might account for the lack of phosphate and x-ray effects on the calcium absorption of the mature rats. No consistent differences in calcium absorption were observed among the phosphate salts; each salt impaired calcium absorption significantly ($P < 0.05$) in the growing rats when incorporated into the basal diet. Although the phosphate salts tended to impair calcium absorption in the mature rats, this impairment was not statistically significant. Shah and Meranger (1970) also observed impaired calcium absorption in young rats fed semi-purified diets in which the calcium to phosphorus ratio was changed from 2:1 to 1:1 by increasing the phosphorus content. Upon increasing the calcium to phosphorus ratio from 1:1 to 1:2 in medium calcium (7.8g/kg) or high calcium (16g/kg) diets, Whittemore et al. (1973) observed depressed calcium retentions in growing rats. Absorption of calcium from the intestine of the chick has been shown to be inversely related to the dietary phosphate concentration (Hurwitz and Bar, 1971; Tanaka et al., 1973). High phosphorus diets cause negative calcium balance in ponies (Argenzio et al., 1974).

The femur calcium contents of the growing and mature rats (Table 5) reflected, in general, their calcium absorption values (Table 4). All of the phosphate salts caused decreased bone calcium contents in the achlorhydric rats ($P < 0.05$). Only sodium tripolyphosphate caused consistent decreases in bone calcium content in the control rats ($P < 0.01$). This reflects the greater binding affinity for calcium of the more polymerized phosphate (Scharpf, 1971; Van Wazer, 1971).

In spite of the fact that these experiments were of short duration (4 and 3 wk, respectively) and the dietary phosphate treatment was mild (1:2 Ca:P ratio), phosphate effects were observed that agree with the results of experiments which were of longer duration and of greater severity (Shah et al., 1967; Draper et al., 1972; Krishnaro and Draper, 1972). One can only speculate that these effects would have become larger had the experiments been long-term. These observations become particularly important when one considers that it requires several years of continuous calcium loss from the skeleton of man before osteoporosis is observed (Lutwak, 1964; Nordin et al., 1966; Garn et al., 1967; Albanese et al., 1975).

The data presented here and elsewhere (Dymsza et al., 1959) indicate that different phosphate salts when consumed have differing effects on iron and calcium utilization and that these effects may be magnified in clinical conditions in which gastric acid secretion is prevented. Additional research needs to be done before definitive statements can be made about these differences. It is particularly important that this information be obtained. The physiological effects of phosphate additives in food products on human subjects may be deleterious (Bell et al., 1977) and the phosphorus content of a menu may readily be doubled by selecting those foods that include phosphate additives compared to similar foods that do not contain additives (Bell et al., 1977).

Table 4—Effect of different dietary phosphate salts and achlorhydria on percent calcium absorption of growing and mature male rats

	Growing – Exp. 1		Growing – Exp. 2	
No. of observations	Control 5[a]	Achlorhydric 5[a]	Control 5[a]	Achlorhydric 5[a]
Basal	83.6	72.7**	64.9	66.6
Na_2HPO_4	70.7(4)c	62.1(4)**,e	68.0	62.9
Na meta PO_4	75.6cd	63.7**,e	59.9	64.3(4)
Na pyro PO_4	77.6(4)d	64.4(4)**,d	60.1	58.0(4)
Na tripoly PO_4	72.6cd	65.8*,e	68.1(4)	58.2
$LSD_{.05/.01}$[b]	5.3/7.2		NS	
Statistical summary				
Diet	F(4,36)=5.21(P <0.01)		F(4,37)–0.58(NS)	
Achlorhydria	F(1,36)=34.51(P<0.01)		F(1,37)=0.38(NS)	
Interaction	F(4,36)=0.42(NS)		F(4,37)=0.61(NS)	

[a] Values in ( ) indicate the number of observations when different at the 5 or 1% levels of probability. For the achlorhydria effects, ** means $P < 0.01$ and * means $P < 0.05$. For the phosphate effects, means having the same small letter are not significantly different.

Table 5—Effect of different dietary phosphate salts and achlorhydria on fermur calcium (mg/g bone) content of growing and mature male rats.

	Growing – Exp. 1		Mature – Exp. 2	
No. of observations	Control 10[a]	Achlorhydric 10[a]	Control 10[a]	Achlorhydric 10[a]
Basal	159	140(9)**,f	165c	171NS
Na_2HPO_4	155(8)d	129(8)**,e	163c	152NS
Na meta PO_4	151cd	131(9)**,e	165c	160(12)NS,d
Na pyro PO_4	151cd	137(8)**,f	162c	159(11)NS,d
Na tripoly PO_4	144(9)c	124(11)**,e	156	158NS,d
$LSD_{.05/.01}$[b]	8/10		6/8	
Statistical summary				
Diet	F(4,82)=5.00(P < 0.01)		F(4,93)=4.58(P < 0.01)	
Achlorhydria	F(1,82)=64.50(P < 0.01)		F(1,93)=1.58(NS)	
Interaction	F(1,82)=0(NS)		F(4,93)=2.56(P < 0.05)	

[a] Values in ( ) indicate the number of femurs analyzed when different from 10.

[b] Differences must equal or exceed LSD values for means to be significantly different at the 5 or 1% levels of probability. Asterisks or NS indicate statistical significance ($P < 0.01$) or nonsignificance for the achlorhydria effects. For the phosphage effects, means having the same small letter are not significantly different ($P < 0.05$).

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Ms received 10/15/77; revised 3/6/78; accepted 3/13/78.

We thank Margaret Chambers for expert animal care and technical assistance. Utah Agriculture Experiment Station paper number 2239.

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Ms received 12/9/77; revised 2/21/78; accepted 2/23/78.

Michigan Agricultural Experiment Station Journal Article No. 8326. This research was funded in part by grants from USDA grant #516-15-102 and General Mills, Inc. WAF is a NIH trainee, grant # GM 01818. Fibers were generously provided by General Mills, Inc., Minneapolis, MN (wheat bran, oat flour, sugar beet pulp prepared for human consumption and psyllium seed), Quaker Oats Co., Barrington, IL (oat bran), Mennel Milling Col, Fostoria, OH (wheat midlings), FMC Corporation, Marcus Hook, PA (Cel #174-2) and Brown Company, Berlin, NH (BW 200). Reference to a company or product name does not imply approval or recommendation of the product by the U.S. Department of Agriculture to the exclusion of others that may be suitable. Standard Wheat Bran is available from the American Association of Cereal Chemists (AACC). St. Paul, MN.

COTTONSEED PROTEIN MODIFICATION IN AN ULTRAFILTRATION CELL

S. D. CUNNINGHAM, C. M. CATER and K. F. MATTIL

ABSTRACT

Cottonseed storage protein was modified by proteolytic enzymes contained within a semipermeable membrane reactor. An ultrafiltration cell was used as the enzyme reactor vessel. The protein was hydrolyzed by the enzyme and the resulting lower molecular weight peptides (modified protein or hydrolysate) were removed from the reactor through the membrane. Only about 2% of the original protein was removed through the membrane when no enzyme was present. When pepsin and molsin were added to the cell, protein hydrolysis occurred very rapidly. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to monitor hydrolysis. The hydrolysate contained many peptides below 4,000 daltons. Addition of pepsin was shown to precipitate about 4% of the SP and the precipitated protein rapidly clogged the membrane and reduced membrane flux rate. The precipitated protein was insoluble except in strongly alkaline solutions but its formation was shown to be influenced by pH. Use of hollow fibers as the enzyme reactor increased membrane surface area and improved hydrolysate removal from the reactor. Centrifugation of the precipitate was unnecessary with hollow fibers because of the high velocity of retentate through the fibers.

INTRODUCTION

THE CONTAINMENT of an enzyme within a semipermeable, controlled-pore membrane reactor is one form of immobilized enzyme technology. The membrane serves as a physical barrier through which the product of hydrolysis passes, but the unreacted (unhydrolyzed) substrate and the enzyme are retained inside the reactor. Several workers have reported using enzyme reactors to convert a cellulose substrate to glucose and the continuous removal of glucose through the ultrafiltration (UF) membrane (Ghose and Kostick, 1970; Butterworth et al. 1970). Little work has been published on the use of enzyme reactors to modify proteins. Blatt and coworkers (1968) used an ultrafiltration cell (UFC) to separate chymotrypsin hydrolyzed peptides from bovine milk whey protein. Removal of peptides from soybean protein after enzymatic hydrolysis in UFC membrane reactors has been reported (Roozen and Pilnik, 1973; Moretti et al., 1976).

Enzymatic modification of cottonseed proteins may offer a viable alternative to the use of functionally limited native proteins. Proteolytic enzymes are capable of reducing the size of protein molecules and increasing their solubility. This increase in solubility may provide new and interesting functional properties for the modified proteins. On the other hand, this protein solubility increase may introduce new difficulties to conventional oilseed protein processing. Thus, protein modification and protein product recovery must be considered as a single multifaceted problem.

In practice, proteins are enzymatically modified in batch systems and the resulting mixture is used as 'modified protein.' A wide range in polypeptide molecular weight is possible but very often excessive hydrolysis results in bitter, nonfuctional protein hydrolysates. If proteins can be successfully modified in a controlled-pore membrane reactor perhaps an hydrolysate having a specific molecular weight range would have improved sensory and functional properties. In addition, a membrane reactor may be useful to address both problems of protein modification and protein product recovery. UF might be used to separate a specific molecular weight range of modified proteins and reverse osmosis applied to concentrate the ultrafiltrate. This paper presents the results of experiments involving the application of ultrafiltration as a means to continuously modify and recover cottonseed protein.

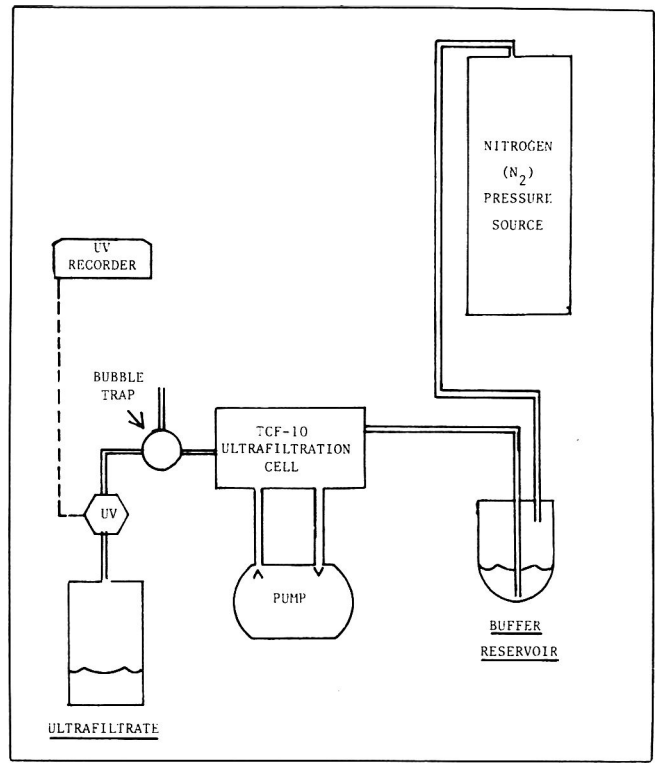

Fig. 1—Schematic diagram of ultrafiltration cell and ultrafiltration system (Amicon, Inc.)

MATERIALS & METHODS

Enzymes and substrate

Three enzyme preparations were used; Pepsin (Sigma, P-7000) a 'crude' pepsin powder, Pepsin (Sigma, P-7012) twice 'crystallized' pepsin and Molsin (Calbiochem, #475889), and acid protease from the mold *Aspergillus saitoi.* Cottonseed storage protein (SP) was prepared from glandless cottonseed flour according to Berardi et al. (1969).

Ultrafiltration

Cottonseed SP was modified (hydrolyzed) by enzymes contained within a semipermeable membrane reactor. A UFC was used as the enzyme reactor. Figure 1 contains a schematic diagram of the UF sys-

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0022-1147/78/0005-1477$02.25/0

tem. The protein was modified by the enzyme and the lower molecular weight peptides (modified protein or hydrolysate) which resulted were removed from the reactor chamber through the membrane. The Amicon TCF-10 Thin-Channel UFC and Amicon LP-1A reversible peristaltic pump were used in these experiments. PM-10 (10,000 nominal molecular weight cut-off) membranes were also supplied by Amicon, Inc. DP-02 membranes (Amicon, Inc.) having a pore size of 0.2 μm were used as prefilters. Experiments were performed at ambient temperature (22°C). The ultrafiltrate was passed through a bubble collector and a UV spectrophotometer recording absorbance at 280 nm (A^{280}) before collection. The ultrafiltrate leaving the cell through the membrane was continually and automatically replaced by buffer contained in a pressurized storage reservoir. This continuous buffer infusion system provided a constant operating pressure which was maintained at 40 psi.

Initial experiments to determine retention of native SP within the UF cell employed a 5% (w/v) SP slurry. A 1% (w/v) SP slurry was used when an enzyme preparation was to be incorporated into the UFC.

Enzymatic hydrolysis

SP was hydrolyzed with proteolytic enzymes in a batch system as well as within the UFC. Batch hydrolysis was performed in covered Erlenmeyer flasks placed on a magnetic stirrer inside an incubator at 37°C. Batch hydrolysis was accomplished by incorporating 1.0% (w/v) enzyme into a 1.0% (w/v) slurry of SP in 0.1N HCl-KCl at pH 2 (molsin at pH 2.8) after equilibration of the protein slurry for 1 hr.

Aliquots of hydrolysate were adjusted to pH 7 with 0.5M NaOH, then centrifuged at 6,000 × G for 15 min. The resulting supernatant was assayed for nitrogen (Kjeldahl) and nitrogen soluble in 10% trichloroacetic acid (TCA) (AOCS, 1975; Lowry et al., 1951).

Initial studies involved direct addition of the enzyme solution to the SP slurry within the reactor. This method of enzyme incorporation proved inadequate because a precipitate formed and clogged the reactor membrane just after enzyme addition. Precipitation problems were largely overcome by centrifugation of the slurry after enzyme addition and then using only the supernatant in the reactor.

Electrophoresis

Enzymatic hydrolysis was also monitored by electrophoresis. The sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) procedure of Weber et al. (1972) for molecular weight determination was used except that isobutyl alcohol was used to layer the gel surface rather than water. Gel tubes (0.5 cm × 10.0) were filled with 10% acrylamide. Samples were extracted for electrophoresis with 0.01M phosphate-SDS buffer (pH 7). Gels were run at 6 ma/tube for 5 hr. Molecular weights of peptides were calculated from a plot of log molecular weight vs relative mobility of BSA (68,000), Ovalbumin (45,000), Pepsin (35,000), Trypsin (23,300) and Cytochrome C (11,700).

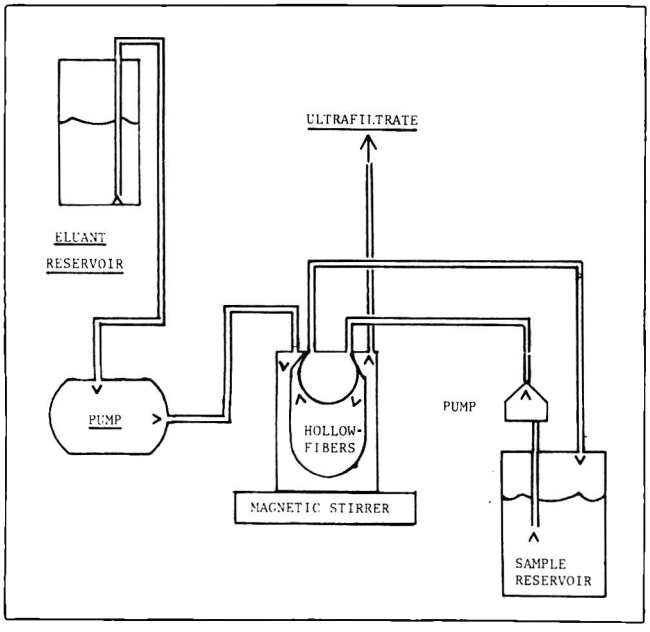

Fig. 2—Diagram of hollow-fiber system.

Hollow-fiber device

A Bio-Fiber 80 Beaker (Bio-Rad, Inc.) with a nominal molecular weight cut-off of 30,000 was used in these experiments. Fibers were operated in a continuous mode at 22°C using an Amicon LP-1A reversible peristaltic pump. Figure 2 contains a diagram of the hollow-fiber (HF) system. Sample was pumped from sample reservoir, through the lumen of the fibers and back to the reservoir. The exterior of the fiber was continually washed with 0.01N HCl or tap water.

RESULTS & DISCUSSION

THE UFC MEMBRANE REACTOR was not suitable for hydrolysis of insoluble materials such as oilseed flours. Although the UFC recirculated the substrate across the membrane surface, the membrane clogged quickly and membrane flux rate rapidly dropped to near zero. Since SP is soluble in dilute acid solutions, experimentation was continued using SP in acidic solutions. Two acid proteases, pepsin and molsin, both having low pH optima, were used as modifying enzymes.

Hydrolysis of SP by pepsin and molsin was monitored by determination of the amount of protein soluble at its least soluble pH (pH 7). Figure 3 shows the effect of pH on SP solubility. SP is 95% soluble at pH 3 but only 3% soluble at pH 7. Solubility increases at pH 7 were attributed to enzymatic hydrolysis of SP primary structure.

Effects of pepsin and molsin on storage protein

Experiments were performed to examine the effects of the selected proteolytic enzymes on SP solubility. Figure 4 contains solubility data of SP as a function of hydrolysis time at 37°C. The solubility of untreated SP at pH 7 was only 12%, but after enzyme was added, a fourfold increase in soluble protein was observed within 1 hr. The difference in SP solubility at pH 7 in Figure 3 as compared to Figure 4 was considered to be due to the higher ionic strength of the buffer used in Figure 4. All enzyme preparations were effective in increasing protein solubility. Very little difference in protein solubilizing capacity was demonstrated by the three enzyme preparations and, after 2 hr approximately 70% of the SP was soluble.

Figure 5 contains solubility data on the use of both pepsin and molsin together. When used together the enzymes had a greater solubilizing effect on SP than either independently (Fig. 4). With this information, experiments were designed to include these enzymes, both together and separately in an enzyme reactor to hydrolyse SP.

Initial experiments using the membrane reactor were designed to determine retention of unhydrolyzed SP within the reactor. After 5 hr, with only SP in the reactor (no enzyme), only 1.8% of the protein permeated the UM-10 membrane in 570 ml of ultrafiltrate. The remainder of the SP was retained (retentate) by the membrane. This preliminary step was used to 'wash' the SP of membrane permeable peptides.

If the enzyme (pepsin or molsin) preparation were added directly to the washed SP solution within the reactor, a precipitate would form clogging the membrane. For example, when pure pepsin was added directly to the washed SP, a total of only 645 ml of ultrafiltrate was collected in 6 hr but only 13% of the SP was passed through the membrane. Addition of more pepsin to the reactor after 6 hr had no detectable effect on A^{280} of the ultrafiltrate. Only 19% of the protein originally placed within the membrane reactor passed through the membrane in 9 hr. The reduction in membrane flux rate was due to membrane clogging caused by the white precipitate.

In order to determine the effect of the enzyme, SDS-PAGE was used to monitor the hydrolysis of SP. Figure 6 contains SDS-PAGE gels loaded with samples taken from the enzyme reactor at various times. Primary structure of SP is substantially modified during the first 10 min (6B) of enzyme hydrolysis and the majority of the hydrolysate applied to the gel appears to be very low molecular weight peptides. The hydrol-

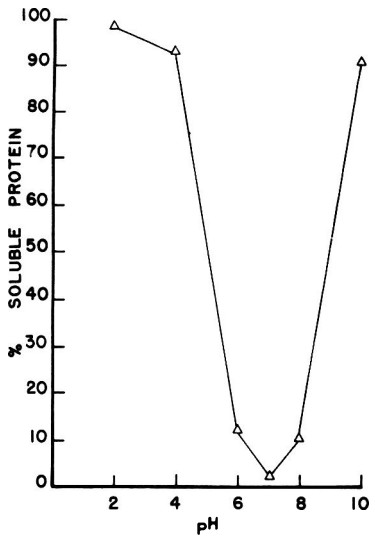

Fig. 3—Effects of pH on SP solubility.

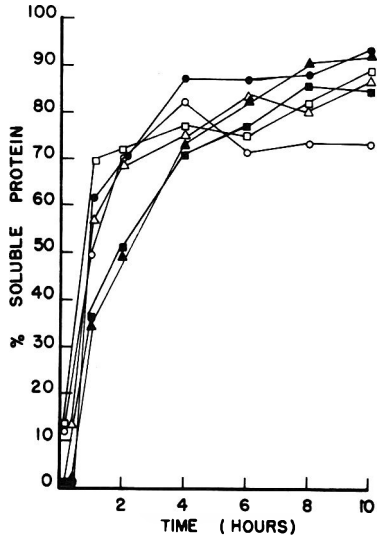

Fig. 4—The effect of crude pepsin (□), crystallized pepsin (○) and Molsin (△) on solubility of SP at pH 7 in 'batch' system. Open symbols are percent soluble protein and closed symbols are percent protein soluble in 10% TCA. Enzyme proportion was 1% of SP weight.

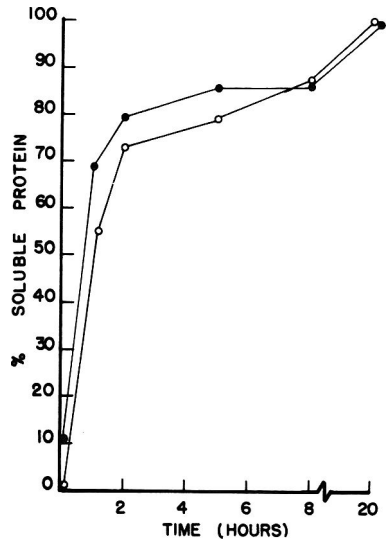

Fig. 5—The effect of both Crystallized Pepsin and Molsin on the solubility of SP at pH 7. Total enzyme concentration was 1% of SP weight and SP was dissolved (1g/100 ml) in 0.1M citrate-HCl buffer (pH 2.5). Open symbols are percent soluble protein and closed symbols are percent soluble in 10% TCA.

ysate in gel 6C (1 hr) shows even more extensive degradation of primary structure. Separation of the product of hydrolysis (peptides) from the enzyme and substrate appears to be limited by UF system design and by formation of the precipitate which clogs the membrane rather than incomplete hydrolysis of SP.

Precipitate formation

It was generally observed that a white precipitate occurs upon the addition of pepsin and molsin to SP. No differences were observed in method of enzyme addition that is, between dry and predissolved enzyme. This precipitate which amounts to about 4% of the SP by weight lowers removal of modified proteins by reducing membrane flux to nearly zero. Initially, it appeared as though the hydrolysate might be incompatible in the buffer used but precipitation occurred when enzymes and SP were prepared in all of the following solutions: (1) distilled water; (2) 0.1N HCl-KCl (pH 2.2); (3) 0.1M citrate-HCl (pH 2.7); (4) 0.1M citrate phosphate (pH 3); and (5) 0.1M glycine-HCl (pH 2.8). The 0.1M citrate-HCl (pH 2.7) was first to turn cloudy but all turned cloudy and produced a precipitate within 30 min.

Since previous work involved the use of both molsin and pepsin in equal amounts, an experiment in which each enzyme was added independently to the SP supernate was undertaken. Pepsin formed a precipitate immediately but the molsin-treated supernatant was only partially cloudy after 6 hr. Two additives were incorporated to see their effect on precipitate formation. Addition of 2-mercaptoethanol (2-ME) to a final concentration of 0.5M, prior to addition of pepsin, only slightly delayed precipitation. Pepsin contains 3 disulfide bonds (Fruton, 1970) but apparently was not sufficiently affected by 2-ME to substantially influence precipitation. When the solution was made 1M with NaCl, instantaneous precipitation occurred upon addition of pepsin. Neither NaCl nor 2-ME had any effect on the precipitate once formed. Dropwise addition of 1M NaOH caused the precipitate to redissolve and become clear but only when the pH had been adjusted to above pH 10. If the precipitate were removed by centrifugation, all nitrogen in the supernatant was soluble in 10% TCA. After centrifugation, addition of more pepsin to the supernatant did not result in additional precipitation.

In later experiments, the enzyme was not added directly to the reactor but to supernatant a as indicated in Figure 7. After the precipitate formed, it was centrifuged off and the resulting supernatant (supernatant b) was loaded into the reactor. It also was observed that formation of the precipitate could be partially controlled by pH adjustment. When pepsin was added to supernatant a at pH 2, very little precipitate formed and it was difficult to sediment by low speed centrifugation. At pH 2.5, the precipitate was easily sedimented by low speed centrifugation. Before the second centrifugation step (resulting in supernatant b), a pH adjustment to pH 3 resulted in a better yield in precipitate (solids b), and the supernatant b could be titrated to the optimum pH of the enzyme.

Figure 8 is a photograph after SDS-PAGE of several fractions of SP. SP is shown in gel 8A in its native form (before enzyme addition) and is similar to native SP in gel 6A. In 8B, the enzyme has degraded the sturcture of SP to the extent that the most of the protein (peptides) material has migrated farther than the tracking dye. Two very diffuse protein staining zones are apparent. The precipitate (supernatant b) loaded onto gel 8C also displays at least 2 peptide zones and the precipitate (8C) appears to contain a polypeptide band with a similar relative mobility as that of pepsin (8D).

Hollow fibers

The major difficulty with the UFC seemed to be the extremely low permeation rates for lower molecular weight peptides through the membrane. The membrane in the UFC had a 90 mm diameter but the effective membrane area was only 40 cm^2. The use of HF as an enzyme reactor has been reported (Lewis and Middleman, 1974). The primary advantage in the use of HF over flat membranes (UFC) is the significant increase in membrane surface area. The HF beaker had greater than 1000 cm^2 of membrane surface area and had a hold-up volume of only 10 ml.

In a preliminary experiment, 300 ml of SP supernatant a (containing 1% pepsin w/w) was loaded into the reservoir and pumped through the fibers. A total of 2L of tap water was

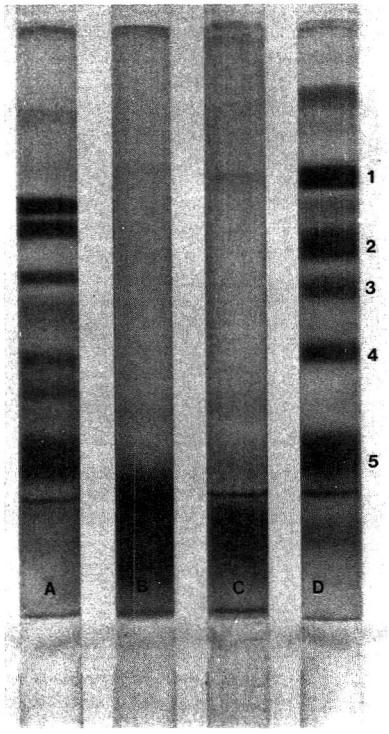

Fig. 6—SDS-PAGE gels (10% acrylamide) of (A) supernatant a prior to enzyme addition, (B) 10 min after addition of crystallized pepsin, (C) 1 hr after enzyme addition and (D) the standard marker proteins (1) BSA, (2) Ovalbumin, (3) Pepsin, (4) Trypsin and (5) Cytochrome C.

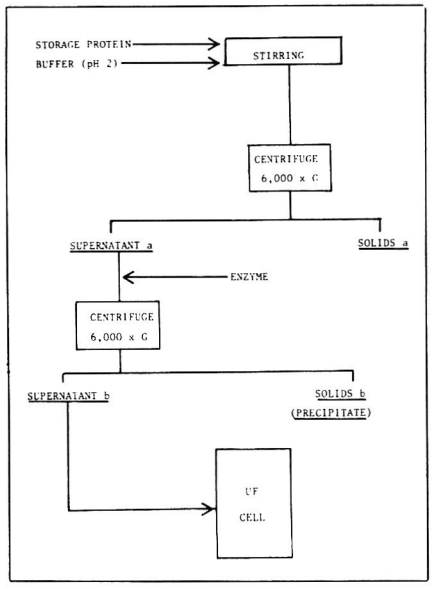

Fig. 7—Diagram of steps in preparation of protein solution to be loaded into Amicon ultrafiltration cell.

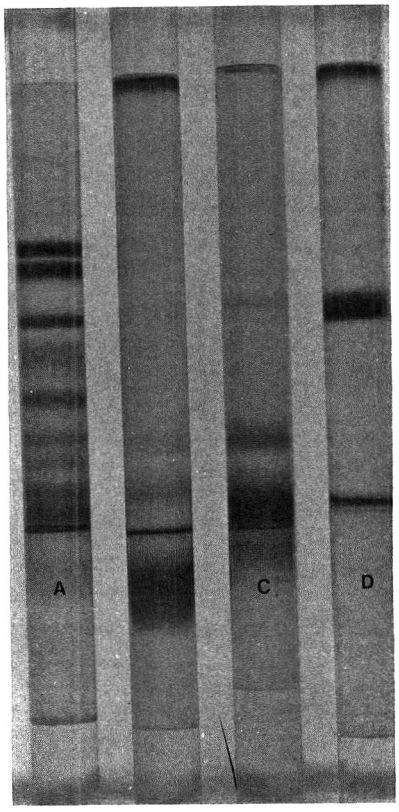

Fig. 8—SDS-PAGE gels (10% acrylamide) of (A) supernatant a, (B) retentate, (C) precipitate and (D) pepsin.

pumped against the membrane (refer to Fig. 2). In 2 hr, 42% of the hydrolyzed protein permeated the membrane. A disadvantage of the HF device is the large volume of wash solution required. As much as 65% of the protein can be removed through the membrane in 6 hr, but a total of 2.75L of eluant was collected which contained less than 0.1% (w/v) protein.

In additional experiments with HF, it was determined that centrifugation of supernatant a to remove the precipitate was unnecessary as compared to the ultrafiltration cell. When 1% (w/w) pepsin was added to a 5% SP solution and loaded into the hollow-fiber device, 74% of the protein passed through the membrane with 1,700 ml UF in 6 hr. The retentate was very cloudy and had a white precipitate but it did not seem to interfere with the membrane flux rate because of the high velocity of the retentate through the fibers.

CONCLUSIONS

TWO ENZYMES, pepsin and molsin, were shown to substantially increase protein solubility of SP at pH 7. When enzymes were incorporated into a UFC membrane reactor containing an SP slurry, a precipitate formed which rapidly clogged membrane pores and decreased membrane flux rate. When the precipitate is centrifuged off, as much as 80% SP is hydrolyzed and passes through the membrane. SDS-PAGE indicated that pepsin rapidly hydrolyzed SP but the nature of the UFC and formation of the precipitate may prevent continuous modification of cottonseed SP in UFC enzyme reactors. Cost of an industrial product might be reduced by eliminating the first clarifying centrifugation step and separating precipitate from the enzyme and substrate with a single centrifugation. Efficient UF will require large ultrafiltrate volumes, greater membrane flux and use of reverse osmosis to concentrate the ultrafiltrate. Use of HF as the enzyme reactor appeared more feasible because the greater membrane surface area increases membrane flux but use of HF also produced large volumes of dilute protein solution which may require concentration by reverse osmosis.

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Ms received 2/18/78; revised 5/1/78; accepted 5/10/78.

THERMALLY INDUCED GELATION OF THE 12S RAPESEED GLYCOPROTEIN

T. A. GILL and M. A. TUNG

ABSTRACT

Scanning electron microscopy (SEM) and rheological characterization were used to study gelation of the 12S rapeseed glycoprotein. Dispersions at pH $\geq$ 4 readily self-associated on heating with gelation at 4.5% protein concentration and measurable thickening at 1% protein. The SEM studies revealed a progression in three dimensional ordering and a decrease in pore size as pH was increased from 6 to 10. At pH 4, the gel was amorphous and readily reverted to a sol. Gel strength was affected by changes in pH and sodium chloride concentration, the strongest gels being formed at high pH and ionic strength. Aging the dispersion in the cold prior to thermally induced gelation increased apparent viscosity. Urea and dithiothreitol had little effect on gelation although 0.15M dithiothreitol gels were less elastic in nature, suggesting intermolecular disulfide bonding may have a role in matrix formation. Selective reduction alkylation prevented gelation but the content of ϵ-amino groups of lysine was unchanged indicating that modification of some other functional group essential for gelation had preferentially occurred. Gel formation in the rapeseed protein system is obviously a complex phenomenon which may involve covalent, ionic, disulfide, hydrophobic and hydrogen bonding. The presence of a high level of carbohydrate (12.9%) suggests the possibility of protein-carbohydrate interaction during gel formation.

INTRODUCTION

ALTHOUGH the entrapment of water in a three-dimensional protein matrix is an important functional property often utilized in fabricated foods, the mechanisms involved in the gelation of globular proteins are not well understood (Tombs, 1974). Soybean protein gel systems have been studied by Circle et al. (1964), Wolf (1970) and Catsimpoolas and Meyer (1970; 1971a, b) while recently Gill and Tung (1976) reported the thermally induced gelation of a major alkali soluble protein derived from rapeseed meal. Gelation of this glycoprotein was readily obtained at the 5.4% protein level, lower than that previously reported for soy protein by Circle et al. (1964).

Rapeseed meal, a major by-product of the vegetable oil industry in Canada, has great potential in human nutrition as protein levels of 40% (d.b., N X 6.25) are not uncommon. The chemistry and ultrastructure of the 12S rapeseed protein have been described by Stanley et al. (1976) and Gill and Tung (1976; 1977a, b). The present study was initiated to gain more information concerning rapeseed protein gelation. Rheological characterization and scanning electron microscopy (SEM) were used for this purpose.

EXPERIMENTAL

DISPERSIONS of the 12S rapeseed protein were derived by gel filtration of commercial rapeseed (*Brassica campestris* L. var. Span) meat extracts at pH 9.2. Details of this procedure were reported by Gill and Tung (1976). The 12S fraction was collected and dialyzed against running water at 4°C for 72 hr. Contents of the dialysis bags were concentrated to approximately 5% protein (w/v) by pervaporation and subsequently dialyzed against Britton and Robinson type universal buffers (Dawson et al., 1969) of pH 2, 4, 6, 8 and 10. Protein concentrations were determined by the rapid micro-Kjeldahl method of Concon and Soltess (1973), (% N × 6.25). The final concentration of each dispersion was adjusted to 4.5% with buffer. A sample of protein dispersed in 0.01M borate buffer pH 9.2 was also prepared and aliquots of this dispersion adjusted to 0, 0.5 and 1.0M sodium chloride, 1M urea and 0.15M dithiothreitol in order to observe the effects of varying ionic strength, the presence of dissociating agent, and reducing agent, respectively.

A formaldehyde and sodium borohydrate treatment described by Means and Feeney (1968, 1971) was used for the reductive alkylation of the amino groups of the protein. A protein suspension (10 mg/ml) was prepared in 0.2M borate buffer, pH 9.0, and 0.5 mg/ml $NaBH_4$ added at 0°C. Aqueous formaldehyde (37%) was added in five increments totalling 0.5 μl/ml suspension over a period of 30 min. The suspension was dialyzed against water to remove excess formaldehyde, pervaporated to approximately 5% protein (w/v), dialyzed against 0.01M borate pH 9.2 and the final concentration adjusted to 4.5% protein in preparation for rheological characterization. Samples of water-dialyzed, derivatized protein as well as underivatized 12S protein were lyophilized and the ϵ-amino groups determined by the trinitrobenzenesulfonic acid (TNBS) method of Eklund (1976).

All samples were gelled in stoppered 12 × 125 mm test tubes by heating for 5 min in boiling water and cooling in ice water for 2 min.

Scanning electron microscopy

Pieces of gel were immersed in liquid nitrogen and cryofractured. The frozen fragments were transferred immediately into a 2.5% glutaraldehyde solution buffered to pH 7.0 and fixed for 2 hr. The gels were dehydrated in ethanol solutions of increasing strength (30, 50, 70, 95 and 100%) and transferred into ascending concentrations of propylene oxide in ethanol (1:1, 2:1, 3:0). The specimens were then dried in a critical point dryer, glued to aluminum specimen stubs and coated with a gold-palladium alloy in a sputter coating device (Technics Inc). An ETEC Autoscan scanning electron microscope at 20kV was used to examine the gels.

Rheological characterization

Steady shear flow behavior of the various treated gels was evaluated over several decades of shear rate with a Model R.18 Weissenberg Rheogoniometer (Sangamo Controls Ltd., 1971). The gels were also evaluated for dynamic shear stress response to small amplitude oscillation in order to determine viscoelastic properties. Both 10 cm diameter 0.25° and 5 cm 2° cone/plate fixtures were employed. The shear strain input signals, the resultant shear stress output signals and the phase difference between the two sinusoidally varying functions were monitored with a Tronotec Model 703A digital analyzer (Tronotec Inc., Franklin, NJ) in the dynamic shear experiments. Because these tests required relatively large quantities of the purified protein, only one sample was tested for most treatments.

RESULTS & DISCUSSION

THE 12S RAPESEED glycoprotein readily self-associated upon heating dispersions at pH > 4. Thermally induced gelation was observed in dispersions at 4.5% protein concentration and measurable thickening occurred at the 1% protein level.

Gel microstructure

The effects of varying pH on the ultrastructure of the thermally induced gels is illustrated in Figures 1 and 2. At pH 4, the 12S protein was only partially soluble and a thermally induced gel was unstable, reverting to a sol in a few minutes.

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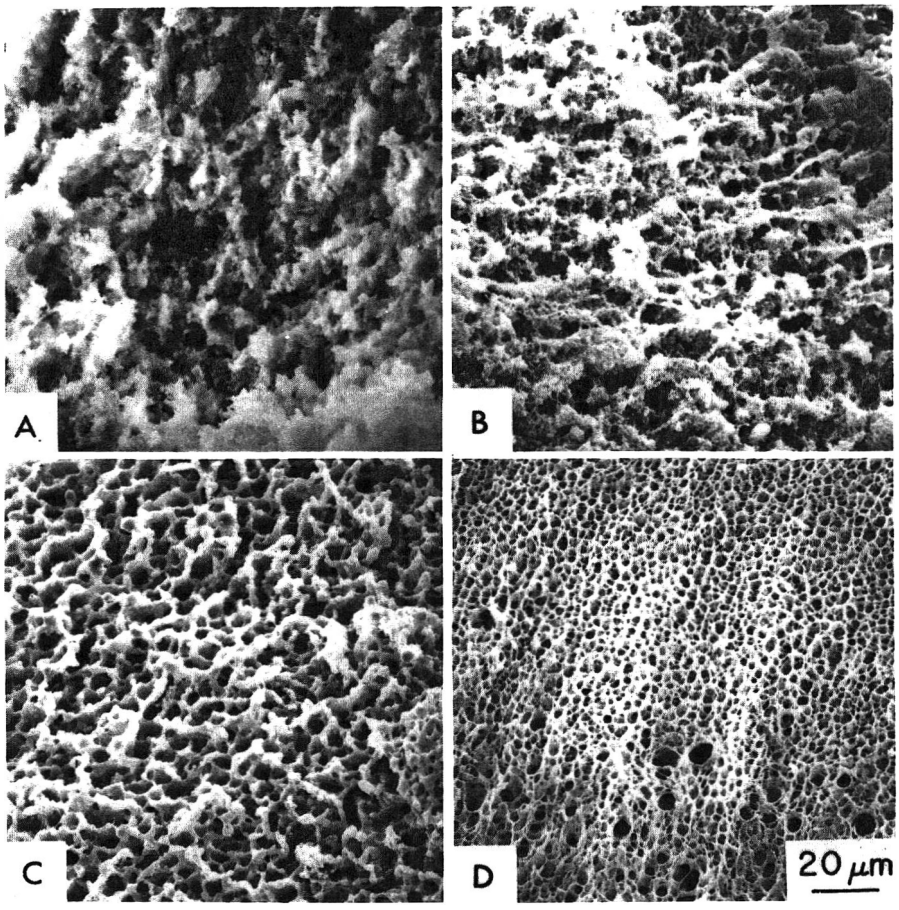

Fig. 1—Scanning electron micrographs of 4.5% rapeseed protein gels prepared at various pH levels (A, pH 4; B, pH 6; C, pH 8; D, pH 10).

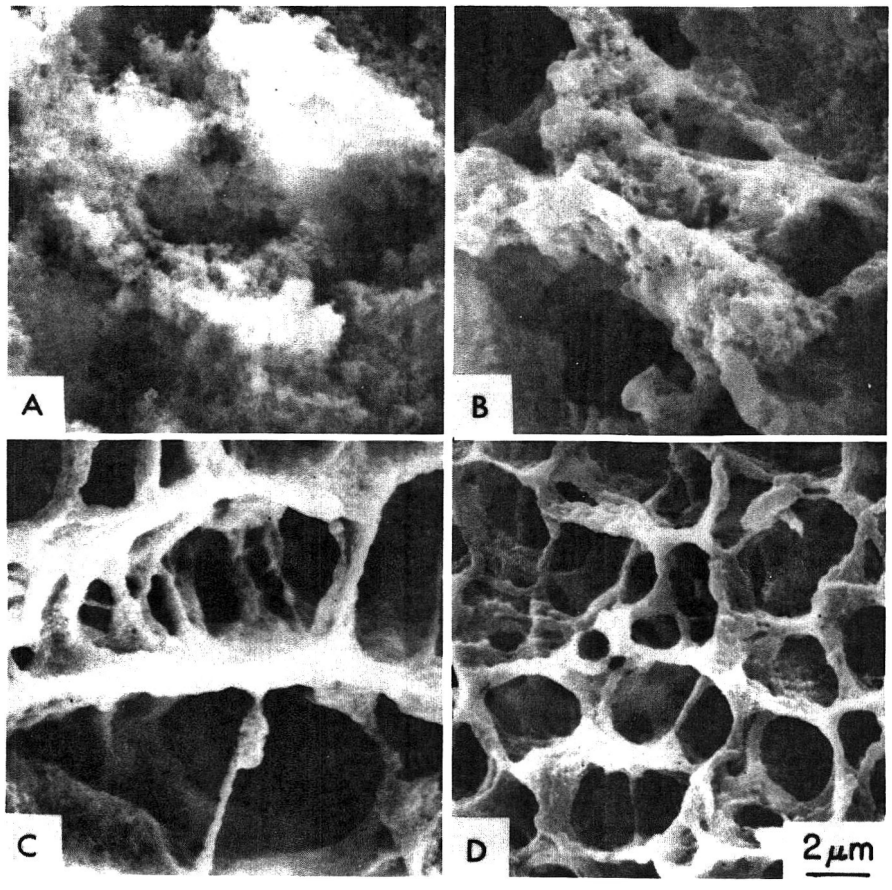

Fig. 2—Higher magnification scanning electron micrographs of 4.5% rapeseed protein gels prepared at various pH levels (A, pH 4; B, pH 6; C, pH 8; D, pH 10).

The electron micrographs reveal the amorphous nature of the pH 4 gel (Fig. 1A, 2A). The protein is highly aggregated at this pH (presumably near its isoelectric point), and little space is provided between protein particles for entrapment of water.

At pH 6, the beginning of a three-dimensional structure is evident (Fig. 1B, 2B). With an increase in pH to 8 and then 10 (Fig. 1C, 2C; Fig. 1D, 2D) there was a progression in three dimensional ordering and a decrease in pore size.

Rheological properties

Results of a preliminary study of the flow properties of rapeseed 12S protein dispersions and gels (Gill and Tung, 1976) indicated that both heated and unheated dispersions displayed shear-thinning flow behavior characteristic of pseudoplastic power law fluids. That is, apparent viscosity (η, poise) decreaed with increasing shear rate ($\dot{\gamma}$, sec^{-1}) according to the relation:

$$\eta = m \dot{\gamma}^{n-1}$$

where m is the consistency index (dyne sec^n cm^{-2}) and n is the flow behavior index.

In the present study, the effects of pH and various additives on the steady shear flow parameters were studied in order to gain insight into the nature of the bonding involved in gel formation. Figure 3 demonstrates the effects of various additives on the steady shear flow behavior of pH 9.2 gels. Perhaps the most notable feature of the rheogram is that samples of 4.5% protein gelled on different days yielded different flow properties indicating the complexity of the gelation phenomenon for this rapeseed protein. A gel which was formed from a dispersion that had been stored for 4 days at 4°C had a consistency index of 104 dyne sec^n cm^{-2} whereas the consistency index of a gel (no additives control) produced 4 days earlier from the same stock dispersion was only 16.3 dyne sec^n cm^{-2}. Moreover, the stored dispersion had a much lumpier texture. It is possible the protein self-associates in the cold to form higher molecular weight complexes which in turn produce firmer gels. The temperature dependent self association of the 12S protein of *B. juncea* has been reported (MacKenzie, 1975).

As the treated dispersions were also aged in the cold, the "aging" effect made the assessment of contributions of hydrogen and disulfide bonding in gelation difficult. However, neither urea nor dithiothreitol, at the levels used, prevented gelation. At the shear rates tested, gels containing urea or dithiothreitol exhibited apparent viscosities intermediate between the "unaged" and "aged" controls which contained no additives.

The relative ineffectiveness of the hydrogen bond disrupting agent in the rapeseed protein gels is in contrast to results with gelatin gels which are believed to be cross-linked primarily with hydrogen bonds (Bello and Vinograd, 1958). Although 1M urea is a relatively low concentration, gelatin gels (4.5% protein) containing 1M urea reverted to sol forms when warmed from refrigerator temperature to 22°C. The pH 9.2 rapeseed protein gels were observed to be thermally irreversible, at least up to 100°C, although measurements of gel strength were not taken at elevated temperatures. Moreover, attempts to dissolve the rapeseed protein gels in 8M urea were unsuccessful although slow disruption of the gel took place when the temperature of the dissociating agent approached 100°C. These observations suggest that although hydrogen bonding may occur, it is not a major factor in intermolecular cross-linking during gelation of rapeseed 12S protein.

The validity of concluding the absence of sulfhydryl-disulfide interchange, although tempting since 0.15M dithiothreitol did not prevent gelation, is questionable. Catsimpoolas and Meyer (1970) found that while low concentrations of mercaptoethanol (0.1%) inhibited the gelation of soy protein, high concentrations (10%) actually enhanced it. The same authors

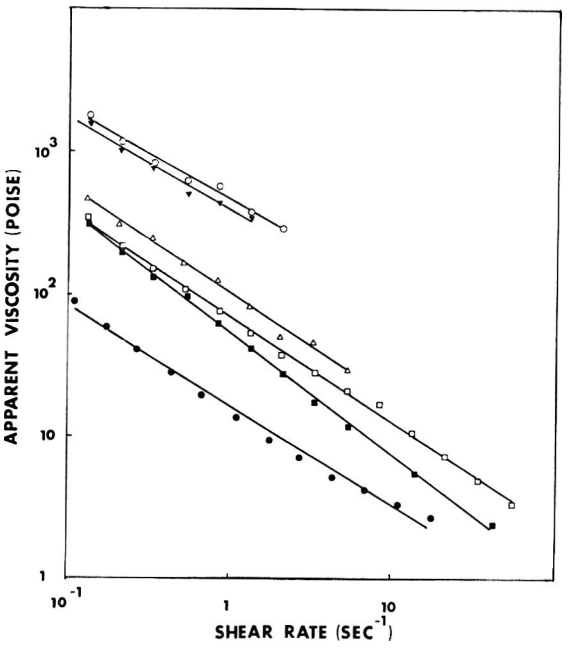

Fig. 3—Apparent viscosity as a function of shear rate for 4.5% rapeseed protein gels at pH 9.2 and 23° C (● no additives; ■ + 1M urea; □ + 0.15M dithiothreitol; ▲ + 0.5M NaCl; ○ + 0.1M NaCl; △ no additives but aged 4 days at 4° C).

reported that the addition of 0.1% N-ethylmaleimide (a sulfhydryl-blocking reagent) to soybean dispersions had no effect on gelation. In a previous report, Gill and Tung (1976) found that treatment of 12S rapeseed protein dispersions with p-mercuribenzoate did not affect apparent viscosity. Although the existence of intermolecular disulfide bonding has been demonstrated in the 12S glycoprotein complex (Gill and Tung, 1977a), it is difficult to imagine that the small amount of cystine reported in the amino acid profile could reflect such a highly cross-linked network within the gel. It is possible that disulfide interchange occurs at levels so low as to not significantly alter the rheological properties of the protein gel.

The effect of ionic strength on the apparent viscosity of the gels is shown in Figure 3, although it must be emphasized that this effect is confounded by the differences between the "aged" and "unaged" control gels. Gels adjusted to 0.5M and 1.0M NaCl demonstrated dramatically higher apparent viscosities. These results contrast with those for soybean protein dispersions (Catsimpoolas and Meyer, 1970; Hermansson, 1972) where increases in ionic strength resulted in lower gel viscosities at temperature above 70°C. If ionic bonds were of major importance in 12S rapeseed protein gels, ionic strength would be expected to result in reduced gel strength as a consequence of ion competition for the interacting functional groups of the protein. However, Gillberg and Törnell (1976) found that NaCl increased rapeseed protein solubility. It may be that greater solubility of the 12S aggregate at high ionic strengths permits more effective overlapping of functional groups.

Figure 4 demonstrates the effect of pH on apparent viscosity over a limited range of shear rates. The highest apparent viscosities were obtained from gels prepared at pH 10. No gel formation was observed at pH 2 although the 12S glycoprotein was much more soluble at pH 2 than at any of the other pH levels. A gel formed at pH 4 was unstable and quickly reverted to sol form; it is not shown in Figure 4. The gel strengths, as reflected by the steady shear flow properties, indicate little difference between pH 6, 8 and 9.2 gels although this result does not appear consistent with the ultrastructural evidence of

Figures 1 and 2. It is possible that the relatively high apparent viscosities observed for the pH 6 gel resulted from protein insolubility rather than an increase in structural integrity.

In order to determine the possible involvement of lysine in some form of cross-linking reaction, a sample of the 12S protein was reduced with low levels of sodium borohydrate and subsequently treated with formaldehyde to methylate free amino groups (Means and Feeney, 1971). The 12S protein modified by this reductive alkylation procedure did not gel to form a solid self-supporting matrix (Fig. 4). However, determination of the content of free ϵ-amino groups of lysine, as ϵ-N, N-dimethyl-lysine residues are the principal reaction products (Means and Feeney, 1971), revealed no detectable differences between modified and unmodified 12S protein, indicating that modification of some other functional group essential for gelation had taken place preferentially. Note that the concentration of the reductant ($NaBH_4$) is low so as not to cleave disulfide bonds (Means and Feeney, 1971). It may be that the cross-linking reactions involve carbohydrate as well as amino acid functional groups, however, more research would be required to establish such a relationship.

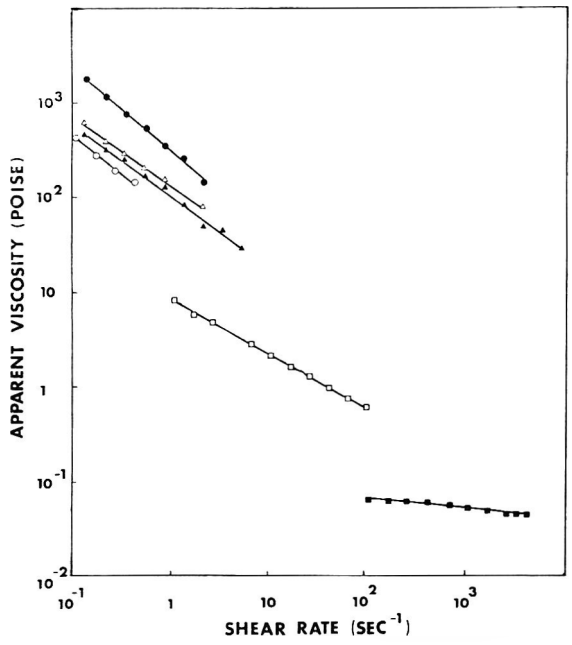

Fig. 4—Apparent viscosity as a function of shear rate for 4.5% rapeseed protein gels at 23°C (■ pH 2; Δ pH 6; ○ pH 8; ● pH 10; ▲ aged 4 days at 4°C, pH 9.2; ⊔ modified by reductive alkylation).

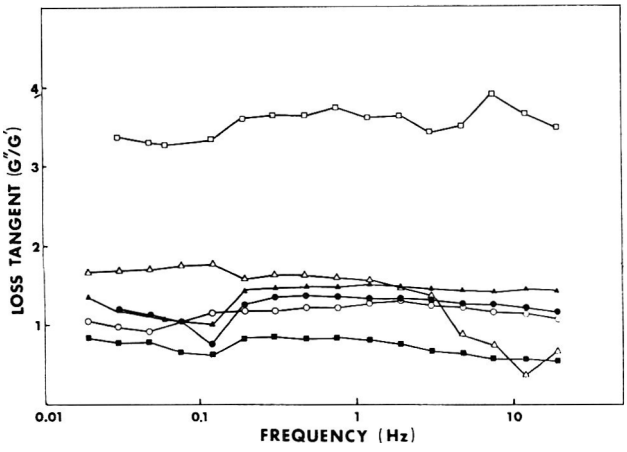

Fig. 5—Loss tangent (G''/G') as a function of oscillatory frequency for 4.5% rapeseed protein gels at pH 9.2 and 23°C (● no additives; ■ + 1M urea; □ + 0.15M dithiothreitol; ▲ 0.5M NaCl; ○ + 1M NaCl; Δ modified by reductive alkylation).

Steady shear rheological data are commonly used for characterization of gelatinous semi-solids. However, all gels exhibit viscoelastic behavior (Mitchell, 1976) and, since the response to external stress may be dominated by viscoelastic phenomena, an understanding of these properties is an aid in elucidating the mechanism of gelation as well as being of practical importance in clarifying the mechanical behavior of gels in processing and utilization. Viscoelastic response of the various rapeseed protein gels was determined by evaluation of dynamic shear stress response to small amplitude oscillation. The sinusoidally varying shear stress and strain signals were separated by a phase difference, Φ, the tangent of which, called the loss tangent, is a direct measurement of the ratio of G'', the loss modulus, a measure of energy lost as heat due to viscous flow, and G'', the storage modulus, a measure of the energy stored due to elastic deformation. Both moduli may be expected to depend on the oscillatory frequency of the dynamic tests.

The effects of frequency of oscillation on the loss tangents (G''/G') for the pH 9.2 gels containing various additives is illustrated in Figure 5. The small inflection points observed at low frequency may be the result of entanglement coupling (Ferry, 1970) in which extended linear fragments interact in a specific frequency range such that an increase in elastic behavior is observed. The gels containing no additives and NaCl exhibited no relative changes in dissipated/stored energy with increasing frequency of oscillation. Although the addition of NaCl increased the absolute strength of the pH 9.2 gel system, its effect on elasticity was minimal. The urea treated sample appeared to show a slight relative increase in elastic component with increasing frequency and exhibited the largest proportional elastic response overall. The sample modified by reductive alkylation, which did not form a solid self-supporting matrix, demonstrated a much greater dependency on frequency of oscillation. The most interesting result was that for the 0.15M dithiothreitol treatment where the gel was unable to store proportionally as much energy in oscillatory shear as the other gels; its loss tangent is thus considerably higher. The dithiothreitol gel then, while comparably viscous in relation to the control gel, is considerably less elastic. This suggests that disulfide bonding is involved in matrix formation but is not the only type of cross-linkage.

The effect of oscillatory frequency on the loss tangent of samples at different pH's is demonstrated in Figure 6. The loss tangent of the pH 2 sol is high with dissipated energy increasing with increased oscillation frequency. The pH 10 gel exhibited a tendency toward greater elasticity at higher oscillatory frequencies. The highly elastic recovery of the pH 6 gel is puzzling in view of the relatively amorphous structure indicated by the scanning electron micrographs. Moreover, values of the storage and loss moduli for the pH 6 gel were generally higher than those for the pH 10 gel, in contrast to the steady shear results presented in Figure 4 where the pH 6 gel exhibited lower apparent viscosities. It may be that a significant amount of structure was lost during steady shear which would account for the lower observed apparent viscosities of the pH 6 gels. It also may be that since the pH 6 gel had a much "lumpier" texture than the gels at higher pH, highly elastic localized aggregates could have been formed as a result of electrostatic forces in the pH 6 gel. Such aggregates could have been responsible for the highly elastic recoveries illustrated in oscillatory shear. Upon steady shearing, however, the aggregates may have been able to move with respect to one another since each aggregate would not necessarily be cross-linked with others. Thus, under conditions of high coulombic attraction

and minimum solubility, the moduli observed need not necessarily reflect the true gel strength. The scanning electron micrographs would tend to support this explanation and suggest that an increase in three dimensional gel structure occurs with increase in pH rather than a maximum observable structure at pH 6.

It should be noted that it was necessary to cut the gels several times and then force them into the narrow gap of the rheometer by compression so that some of the three dimensional structure was likely destroyed before rheological measurement could be made. Formation of the gel in the narrow gap between platens might eliminate this problem in future studies of gelation phenomena.

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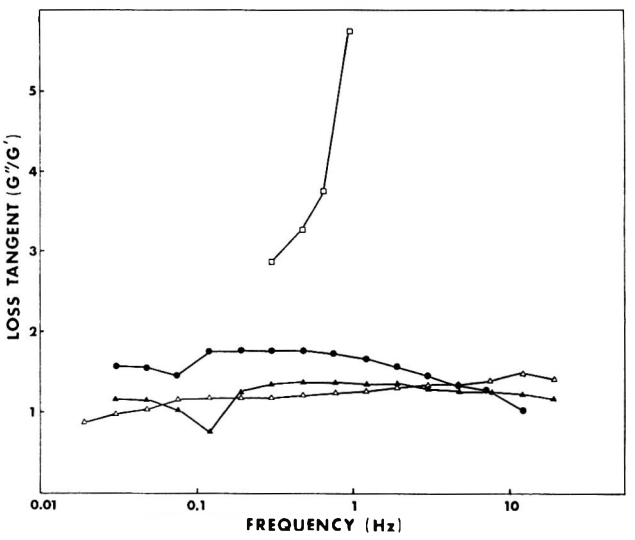

Fig. 6—Loss tangent (G"/G') as a function of oscillatory frequency for 4.5% rapeseed protein gels prepared at various pH levels and measured at 23°C (□ pH 2; Δ pH 6; ▲ pH 9.2; ● pH 10).

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Ms received 12/13/77; revised 4/6/78; accepted 4/10/78.

This research was supported in part by the National Research Council of Canada.

COMPARISONS BETWEEN MODEL PREDICTIONS AND MEASURED VALUES FOR AVAILABLE LYSINE LOSSES IN A MODEL FOOD SYSTEM

J. C. WOLF, D. R. THOMPSON and G. A. REINECCIUS

ABSTRACT

A mathematical model, developed to predict available-lysine losses in a model food system which had undergone an isothermal-nonstirred process, was tested under different process conditions. The model predictions were compared to measured available-lysine losses in both jacketed-mixer processing and extrusion processing. Statistical analysis using a paired t-test analysis indicated a significant correlation between predicted and actual results for the jacket-mixer process. In the extrusion experiment a statistically significant correlation (0.7) occurred when the predicted values were plotted against observed. However, variation due to product backmixing and inaccurate temperature measurement prevented a rigorous test of the model. The reaction order of available lysine loss in casein, single-cell protein and a soy protein isolate were determined at an elevated temperature. The casein and single-cell protein followed first-order reaction kinetics. The soy isolate initially follows first-order loss but after an approximate loss of 40–50% available lysine, the loss abruptly stops and a no-loss phase occurs.

INTRODUCTION

EXTRUSION or other thermal processes can cause large decreases in both protein nutritional quality (Bender, 1972) and available lysine (Lea and Hannan, 1949). These decreases can result from chemical reactions such as protein-protein interactions, protein-amino acid interactions and protein-sugar interactions. The protein-sugar interactions involving lysine and reducing sugars appear to be a significant contributor to the decreases which occur in available lysine. Jokinen et al. (1976), Thompson et al. (1976) and Wolf et al. (1977) studied the losses of available lysine which occurred during thermal processes and developed a mathematical model that predicts these losses. The model predicts activation energy and reference reaction rate in an Arrhenius equation and then the available-lysine loss is predicted from these values.

The model development, a multistep process, initially involved determining the reaction order for available lysine in the food system. Then available-lysine loss was measured as a function of pH, water activity and glucose at elevated temperatures (80–130°C). The activation energy (E) and reference reaction (k_r) rate were determined for each experimental composition. Then the equations for predicting activation energy and reference reaction rate as functions of pH, water activity and glucose were determined by regression analysis (Nie et al., 1975). The prediction equations, the reaction order and the Arrhenius equation form the mathematical model.

The present objective is to determine if model predictions are an accurate reflection of available lysine losses in a model food system under both mixing (extrusion-like) and extrusion-processing conditions. In addition, a preliminary study determined reaction order for available lysine losses at elevated temperatures for three types of protein.

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0022-1147/78/0005-1486$02.25/0

Table 1—Measured and predicted fraction available lysine remaining in the mixing experiment

	Composition			Process condition		Fraction Available lysine	
Case	Glucose %	pH	Water activity	Temp (°C)	Time (min)	Observed	Predicted
1	3.0	8.0	0.44	105	15	0.71 ± 0.10[a]	0.69 ± 0.06[a]
2	3.0	8.0	0.44	80	360	0.70 ± 0.12	0.76 ± 0.05
3	2.0	5.3	0.68	105	15	0.85 ± 0.09	0.76 ± 0.04
4	3.7	7.0	0.68	105	15	0.63 ± 0.14	0.73 ± 0.02
5	1.0	6.0	0.44	105	15	0.91 ± 0.14	0.73 ± 0.04
6[b]	1.0	8.0	0.44	105	15	1.01 ± 0.13	0.80 ± 0.04

[a] 95% Confidence interval
[b] Not used in t-test (See text)

MATERIALS & METHODS

Experimental design

All experiments were completely randomized designs. Treatment allocations were either fractional factorial (mixing and extrusion-processing experiments) or full factorial (various protein-sources experiment).

The mixing experiment, stirred nonisothermal-processing conditions, consisted of food system compositions chosen at random from the formulation utilized by Wolf et al. (1977). The experiment was designed to provide a quick assessment of the model's potential to accurately predict available-lysine losses during experimentally controlled conditions which approached extrusion processing (i.e. mixing, nonisothermal temperature). If the model failed to accurately predict available-lysine loss, it would not be necessary to expend considerable time and effort on the extrusion experiment. Therefore only six sample compositions were selected. Each treatment had three replications. Six controls, samples prepared and analyzed as treatments but not processed, were included to provide a check for block effects (Table 1).

The extrusion experiment used a 1/9 replication of a 3^4 factorial in water activity, glucose, pH and time at two temperatures. Each treatment was replicated three times for a total of 54 points with six control samples (Table 2).

The protein source experiment was a full factorial employing three proteins at two levels, 10% and 20%, and processed for four time intervals (1, 2, 4 and 8 min). Two replications per treatment were utilized and six controls were included for a check of the block effect. This design allows two different methods for determining reaction order, initial rate at different protein levels and determination of the best-fit line by linear least squares. This is important for reactions which have less than 50% loss (Thompson et al., 1976).

Sample preparation

A food system consisting of 15% soy protein isolate (Promine D, Central Soya), glucose and microcrystalline cellulose (Avicel PH 101, FMC Corporation) was utilized for experiments which measured model accuracy. The available lysine level was 11.2 mg/G sample. The dry ingredients were mixed and then slurried with 0.1M buffer (V/W 1.5:1). Phosphate buffer was used for the pH 6 and 7 samples and borate buffer was used for pH 8 samples. The samples were placed into shallow pans, frozen in a blast freezer (–30°C) and then freeze dried. After freeze drying, the samples for the mixing experiment were crushed with a mortar and pestle to a fine powder and then equilibrated to the desired water activity in partially-evacuated desiccators containing saturated salt solutions. The samples were allowed to equilibrate for 4

days. The water activity of the samples was checked with a Beckman SMT humidity measuring instrument to assure that the desired water activity was obtained. A 4-day equilibration period was sufficient to bring the samples to the desired level. In the extrusion experiment, the design moisture level was achieved by the direct addition of moisture to the samples and then mixing the samples with a twin shell mixer to achieve an approximate homogenous moisture distribution.

The model systems used in the various-type protein experiment were prepared as described for the mixing experiment. However, the protein type was varied. Casein (sodium caseinate), single-cell protein (Torula, Amoco) and Soy Protein Isolate (Supro 620, Ralston Purina) were substituted for Promine D. Protein levels (10 and 20% of the final composition) were used while glucose was held at 4%, pH at 7.0 and water activity at 0.68. Microcrystalline cellulose was used to bring all samples to 100%.

Sample processing

Mixing experiment. The samples were processed in a Brabender-jacketed mixer. All external surfaces of the mixer are double walled and were maintained at the process temperature by circulating a heated glycerol-solution between them. The liquid temperature was maintained by a circulating constant temperature bath. Immediately after processing, the samples were removed and divided into six subsamples. The subsamples and controls were randomized before analysis. A time-temperature profile was recorded for each sample. A typical profile is presented in Figure 1. Sample temperature was monitored by placing a thermocouple into the sample.

Extrusion processing. The samples for the extrusion experiment were processed in a Brabender 1.9 cm diameter screw extruder. Since microcrystalline cellulose is an excellent water-binding agent and readily plugged the extruder, two different procedures were developed for the high- and low-temperature extruder runs. For the high-processing temperature (130°C) the barrel was brought to 90°C before feeding the sample. The sample was then fed to the extruder and the barrel temperature was allowed to rise to 130°C. After the extruder barrel equilibrated at 130°C, samples were collected and stored in vacuum-sealed foil pouches at 4°C.

For the low-temperature runs, the same initial procedure was utilized. However, after reaching 130°C, the barrel temperature was brought down to 110°C for the section closest to the feed and to 105°C for the other barrel sections. After the sample had equilibrated at these lower temperatures, subsamples were collected and stored as described previously. All temperature measurements were from the thermocouples mounted in the extruder barrel and extending into the product chamber. The temperature profiles were estimated from the measurements.

The extrusion process time was determined with dyed samples. A small quantity of red dye was incorporated into an aliquot of sample, the sample dried and brought to the desired moisture level. To determine minimum and maximum residence time for each process time at the desired temperature, an aliquot of the dyed sample was introduced to the extruder barrel as a sample was being processed. The minimum residence time, which is the desired time, is measured from introduction of the dye sample to its exit from the extruder barrel. The maximum residence time is from dye sample introduction to when no further detectable dye is present in the extrudate. The minimum and maximum time values are the mean values of three replications of each process time and temperature.

Various protein-source experiment. The samples were sealed in retortable foil pouches (50 M-35 f-300079 R2 retort stock, Continental Can Company) and heat processed in a miniature retort at 125°C for up to eight minutes. Each pouch contained no more than 0.07g sample per sq cm of surface area. Immediately after processing the samples were cooled to room temperature. Heating and cooling lags which occurred at this high temperature were corrected using a method similar to that described by Gondo et al. (1972).

Chemical analysis

The samples were analyzed for available lysine and moisture. Available lysine was determined using the flourodinitrobenzene method (FDNB) of Carpenter (1960) with the modifications proposed by Booth (1971). The method involves reacting the sample with FDNB to form DNP-lysine, acid-hydrolyzing the sample and then measuring available lysine by a different technique.

Moisture content was determined using the gas chromatographic method of Hollis and Hayes (1966) as modified by Reineccius and Addis (1973), but with a 4-hr extraction time to insure complete moisture removal. The moistures were utilized to adjust all available-lysine levels to a dry-weight basis.

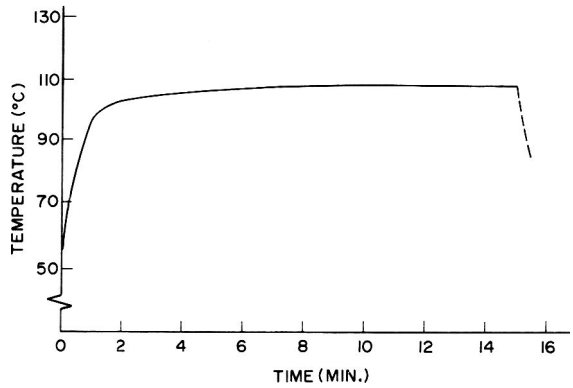

Fig. 1—Time-temperature profile for a sample during extrusion-like processing.

Model prediction

The mathematical model (Wolf et al., 1977) consists of the following equations:

$$E = 32.9 + 1.1*G^3 - 1.77a_w*G - 1.32*G^2 + 1.50*pH*G$$

$$k100 = 0.0122 + 0.00175*G^3 + 0.00104*G*a_w$$

$$k = k_{100}\exp\left[\frac{-E}{R}\left(\frac{1}{T_a} - \frac{1}{373.15}\right)\right]$$

$$\frac{dc}{dt} = -kc$$

where E = activation energy (Kcal/mol · K); R = gas constant (0.00198717 Kcal/mol K); G = coded level of glucose; a_w = coded level of water activity; pH = coded level of pH; k_{100} = reference reaction rate at 100°C (min^{-1}); k = specific reaction rate (min^{-1}); c = concentration of available lysine per gram of sample (mg/g); and t = time (min).

Coded values for glucose, water activity and pH are utilized because they facilitate both the detection and the evaluation of dominant effects.

Although E and k_r are predicted values, there is variability associated with their values. This variability is due to experimental variation encountered during the development of the prediction equations. It is necessary to calculate the variability in predicted available-lysine concentrations to compare these values with the new experimental values by a t-test. The varying temperature during this process prohibits the direct prediction of available lysine model variation from the known variation in E and k_r. Therefore, a Monte Carlo method (Snedecor and Cochran, 1967) of predicting variability was utilized as follows. The equations (Wolf et al., 1977) were solved 10 times for each set of processing conditions to simulate predicted loss variability. Thus the solutions were replicated 10 times for each set of processing conditions. Activation energy and reference reaction rate were adjusted each time the differential equation was solved by the addition of a random number. These numbers had a normal distribution with a mean 0 and a standard deviation equal to that reported by Wolf et al. (1977) for E and k_r. These random numbers were computer generated by the methods of Marsaglia and Gray (1964).

The differential equation was integrated using a Runge-Kutta-Gill procedure (Romanelli, 1960). Step size was held constant at twice the time interval between recorded sample temperatures. This allowed the program to use all the temperature data without requiring interpolation between data values. The mean and 95% confidence interval were calculated from the ten solutions.

Reaction order determination

Two methods were used to determine reaction order. Method one involved determining reaction rates assuming zero through fourth-order reaction kinetics at each time interval for both protein levels. The reaction rates for each order were paired and then pooled and a one-way analysis of variance compared reaction rate by level. Since the reaction rate at a particular time is independent of concentration, a nonsignifi-

cant difference between rates at the different protein levels indicates the correct reaction order has been assumed. Method two involved construction of regression lines (least square criteria) for zero through fourth-order reaction kinetics. The r value was then used as a measure of fit.

RESULTS & DISCUSSION

Mixing experiment

The mixing experiment was included to measure the efficacy of testing the model using extrusion processing. Since the model was developed under very controlled conditions where mixing did not occur and temperature was nearly isothermal, it was necessary to determine if the model functioned under conditions which approximated extrusion processing. The mixing experiment, with its mixing and non-isothermal conditions, approximated extrusion processsing but did not have potential problems of backmixing and pressure. Only six points were tested since the experiment was to demonstrate statistical relationships between predicted and observed values. The critical test, extrusion processing, would provide the in-depth test of the mathematical model (Wolf et al., 1977).

One drawback in a test of this size is that individual contrasts are precluded if no significant differences in the over all analysis are seen. This is because there is a 30% probability of finding a significant difference due to chance in one of the contrasts.

Based upon previous experimentation using soy protein isolates, coefficients of variation from 0.10–0.13 were expected. With this level of variation, two replications per treatment would be needed to demonstrate possible significant differences at a 95% level. In order to insure that potential differences would be noted, significant differences would reject the model, three replications per treatment were used. Therefore, ample replication was built into the experiment.

The 95% confidence intervals for predicted and measured available lysine overlap (Table 1). This result suggests that there is no significant difference between predicted and the measured value. The hypothesis that there is a significant difference between predicted and measured values was tested using a paired t-test ($p < 0.05$). The calculated t-value (0.54) is less than the t-value (2.15) needed to reject the null hypotheses. Therefore it appears that the model does predict available lysine loss. However, the 95% confidence intervals are wide and this increases the difficulty of rejecting the model. Also, the possibility of a type II error accepting the null (i.e. accepting the model) when it should be rejected, exists. To reduce the chance of a type II error, p was changed to 0.5 and the paired t-test value compared to the calculated. Even at this level, there are no significant differences between predicted and observed. Therefore the null hypothesis is not rejected and this supports the contention that the model predicts available lysine losses under processing conditions where mixing occurs and temperature is nonisothermal.

Sample 6, not used in analysis, demonstrated a problem which occasionally occurs when analyzing for DNP-lysine over long time periods. Although sugar analysis (30% loss) and brown pigment formation (visual observation) indicated that lysine loss occurred, the available lysine analysis does not demonstrate the loss.

Table 2—Predicted and measured fraction available lysine remaining in the extrusion precess experiment

Glucose %	pH	Temp (°C)	Process time[d] (minutes) Min	Process time[d] (minutes) Max	Fraction available lysine remaining: Actual	Predicted[c] Min	Predicted[c] Max
1.0	6.0	106	3.8	5.6	0.88	0.92	0.88
1.0	7.0	106	6.6	8.8	0.79	0.87	0.83
1.0	8.0	106	6.5	8.1	0.80	0.88	0.85
4.0	6.0	107	2.9	4.0	0.82	0.83	0.76
4.0	7.0	106	6.5	8.7	0.62	0.65	0.55
4.0	8.0	106	6.6	8.8	0.51	0.62	0.52
7.0	6.0	108	10.2	13.0	0.25	–[b]	–
7.0	7.0	106	3.2	4.3	0.51	–	–
7.0	8.0	106	7.8	10.3	0.34	–	–
1.0	6.0	130	2.1	2.7	0.94	0.60	0.50
1.0	7.0	130	0.9	1.9	0.81	0.82	0.64
1.0	8.0	130	0.9	1.8	0.79	0.85	0.69
4.0	6.0	130	1.8	3.1	0.43	0.56	0.33
4.0	7.0	130	0.9	1.7	0.56	0.68	0.43
4.0	8.0	130	0.9	2.0	0.34	–	–
7.0	6.0	130	0.9	1.8	0.39	–	–
7.0	7.0	130	2.7	4.5	0.29	–	–
7.0	8.0	130	0.9	2.0	0.35	–	–

[a] Moisture levels were from 28–36%

[b] Value not predicted, sugar level beyond range of model

[c] Standard deviation on predicted value is 0.10

[d] The minimum and maximum fractions are based on the minimum and maximum process times.

Extrusion processing

Measuring model accuracy using extrusion processing does not appear possible at this time because of inadequate control over system variables. When predicted values were plotted against observed (Fig. 2) and a best fit line constructed, the regression F-ratio was 24.9 which indicates a significant relationship. A fair correlation, r = 0.7, was determined and both the slope (0.88) and intercept (0.08) confidence intervals overlap the theoretical slope and intercept (1.0 and 0.0). However, in several cases there are clear deviations from predicted and measured. It appears that the inability to control product backmixing and inaccurate temperature measurement are the limiting factors which prevent testing the model. The extent of backmixing is indicated by the minimum and maximum process times (time needed for the sample to pass through barrel (Table 2). This product backmixing causes large variations in available lysine losses in the collected samples. Portions of the sample are processed for different lengths of time. Due to the speed of the chemical reactions at the temperatures used, even a 1/2 minute variation in the process time can cause large differences in the available lysine loss. When mean values were utilized, thus eliminating some variation due to unequal process time and analysis error, the experimental values in most cases fell within the predicted limits.

A second explanation why predicted and measured values differ is an inaccurate measured temperature profile. Thermocouples were inserted into the sample through the barrel. Therefore, barrel temperature influences the thermocouple measurement. Even a change of 3°C would cause the experimentally determined value to fall outside the range of the predicted available lysine levels. Because of backmixing and temperature sensing problems, it is not possible to determine model accuracy in predicting available lysine loss under extrusion processing conditions.

In an earlier publication, Thompson et al. (1976) noted that under nonstirred-isothermal processing conditions, available lysine loss changes from a first-order loss to a no-loss phase after a 35–55% loss. This observation was not always valid during extrusion processing. Samples with glucose levels initially at 7% dry sample weight did not exhibit the no-loss phase. These samples were not used in the regression analysis, since the model is valid only for less than 4% sugar in a food system, but the influence on lysine loss was observed. Interestingly, the failure to enter the no-loss phase occurred only in the extrusion process. Apparently in the presence of high sugar levels during extrusion processing, the no-loss phase can be circumvented. During isothermal processing high levels of sugar will not prevent the no-loss phase (Wolf et al.,

Table 3—Reaction orders and intital reaction rate (k_{120}) for the various protein sources

Protein type	Initial available-lysine level in sample (mg/g sample)		Order	F-ratio	Reaction Rate (k)		Units
	10% protein	20% protein			10% protein	20% protein	
Casein	16.3	33.3	0	10.20	2.12 ± 0.76[a]	4.76 ± 1.80[a]	$min^{-1}(mg/g)$
			1	0.02	0.185 ± 0.056	0.180 ± 0.056	min^{-1}
			2	12.07	0.017 ± 0.07	0.072 ± 0.005	$min^{-1}(mg/g)^{-1}$
			3	2.68	0.041 ± 0.035	0.082 ± 0.007	$min^{-1}(mg/g)^{-2}$
			4	2.96	0.054 ± 0.004	0.005 ± 0.000	$min^{-1}(mg/g)^{-3}$
Single Cell	16.1	31.4	0	6.96	2.11 ± 0.92	4.81 ± 2.33	$min^{-1}(mg/g)$
			1	0.37	0.161 ± 0.076	0.188 ± 0.081	min^{-1}
			2	2.81	0.013 ± 0.002	0.076 ± 0.003	$min^{-1}(mg/g)^{-1}$
			3	1.26	0.151 ± 0.017	0.15 ± 0.004	$min^{-1}(mg/g)^{-2}$
			4	1.68	0.002 ± 0.000	0.002 ± 0.000	$min^{-1}(mg/g)^{-3}$
Soy Isolate	11.6	23.0	0	0.81	2.87 ± 1.36	3.60 ± 1.61	min^{-1} (mg/g)
			1	3.77	0.185 ± 0.078	0.314 ± 0.152	min^{-1}
			2	11.36	0.009 ± 0.004	0.363 ± 0.020	$min^{-1}(mg/g)^{-1}$
			3	5.52	0.028 ± 0.003	0.028 ± 0.003	$min^{-1}(mg/g)^{-2}$
			4	5.21	0.000 ± 0.000	0.004 ± 0.003	$min^{-1}(mg/g)^{-3}$

[a] 95% confidence interval

1977). This indicates that under certain operating conditions the limits of available lysine loss set by the equations of Thompson et al. (1976) using Promine D can be exceeded.

Presently there is no data to explain why the no-loss phase did not occur during extrusion when sugar levels were initially at seven percent, but did occur when sugar levels were initially at four percent. Ample glucose was present for lysine loss to occur at the end of the process for both the four and seven percent glucose systems. Pressure and shear, which were not present during the isothermal, non-mixing experiment, where the no-loss phase is seen, may in combination with excessive heat alter protein configuration or conformation to expose additional lysine molecules to the sugar. However, when sugar is at four percent the no-loss phase did occur. This is interesting since Wolf et al. (1977) have shown that the induction of the no-loss phase occurs when sugar levels are great enough to cause available-lysine loss. Therefore, there may be additional factors responsible for preventing the no-loss phase. Wolf et al. (1978) have shown that once the no-loss phase is induced the application of pressure (>3450 kPa) or shear (>1000 sec^{-1}) in the absence of excessive heat will not reverse the no-loss phase. Thus it appears that possibly the extrusion process prevents the induction of the no-loss phase rather than reversing it. Another possibility is that the available lysine loss reaction may be an equilibrium reaction involving two mechanisms. The first is the standard available lysine loss due to nonenzymatic browning. The second, is a reaction where the polymers formed during the reaction are freed from the lysine and leave the lysine intact, thereby causing an equilibrium between loss and addition of lysine. This could result in an equilibrium, no-loss phase. However, during the extrusion process, the dual mechanism may be altered to prefer the first reaction and greater losses would occur. Such a change in mechanisms might be measured by a change in the reaction rate for polymer release during this time period but data to check this supposition is not available.

Reaction order for available lysine loss in three proteins

Available lysine losses in casein and the single-cell proteins followed first-order reaction kinetics at elevated temperatures. This agrees with casein data obtained at lower temperatures by Lea and Hannan (1949). The F-statistic (Table 3) clearly indicates only a nonsignificant difference between protein levels if first order is utilized. In addition, when best-fit specific reaction rates are calculated for both levels of protein using zero

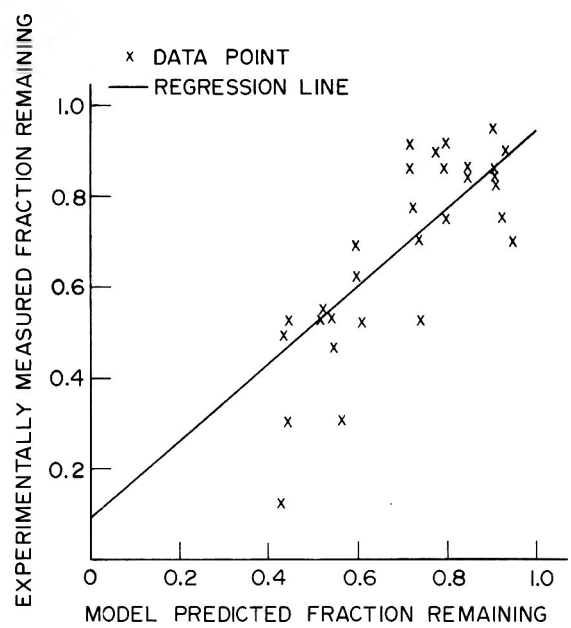

Fig. 2—Linear regression of mathematical prediction against measured fraction available lysine remaining in a model food system.

through fourth-order reaction kinetics, they are equal only if first-order kinetics are assumed. When first-order is plotted, a good fit occurs (Fig. 3).

It was not possible to conclusively determine the reaction order for the soy protein isolate (Table 3). A plot of the results (Fig. 3) demonstrates that available-lysine loss stops before 35% destruction occurs. Previous experimentation (Wolf et al., 1977) with Promine D, another soy isolate, would indicate that the order is first-order, but more data are needed. Like Promine D, the loss of available lysine fails to follow first-order kinetics after an initial loss period. Analysis of variance indicates that there are no significant differences between the k_{120}'s for casein, single-cell protein or for protein levels. This suggests that since two dissimilar protein types have no significant differences in k_{120}, the model might also be applied to them. However, the model predicts a k_{120} of 0.32

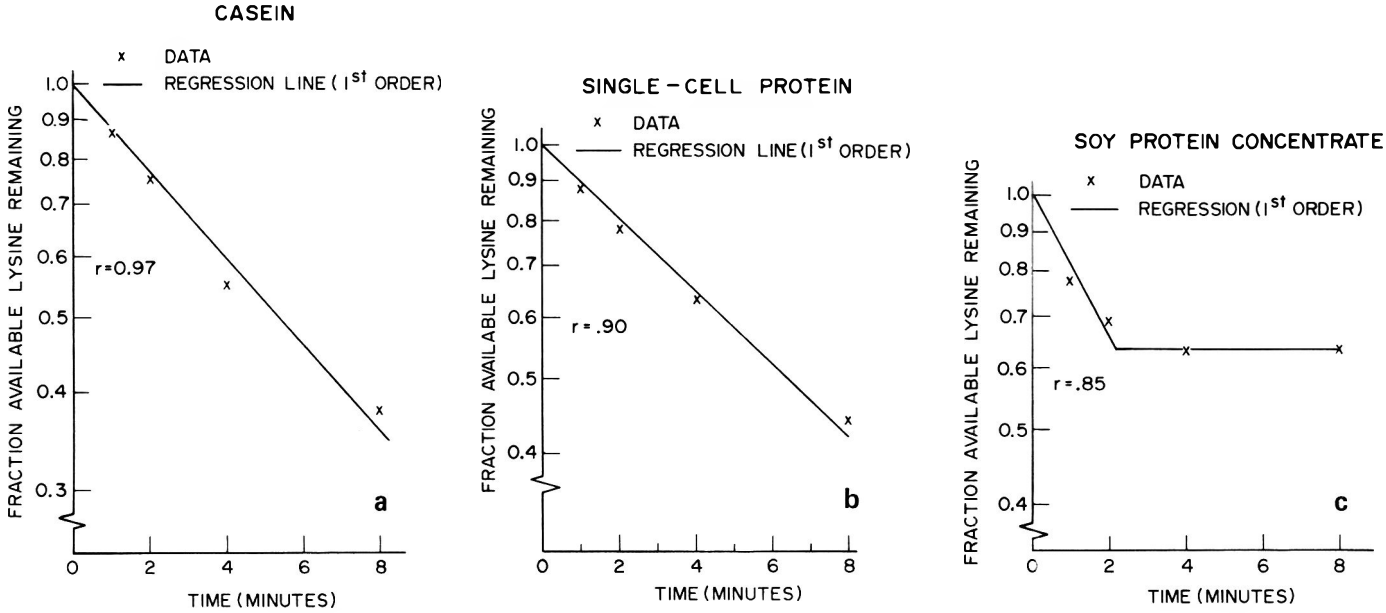

Fig. 3—First-order plots of available lysine loss at 120° C, pH = 7, a_w = 0.68, protein = 20%, glucose = 4% and cellulose (total 100%). Each point is mean of two replications; (a) Casein; (b) Single cell protein; and (c) Soy protein concentrate.

± 0.07 using Promine D which is different than the observed k_{120}'s for either the single-cell or casein-protein sources. At this time it is not known if the coefficient values in the model change with protein type or if the variables in the equation are altered. Experiments to determine if the model can be applied to various protein types are being performed.

CONCLUSIONS

1. There is no significant difference between the mathematical model predictions and experimental measurements of available lysine in a model food system subjected to a stirred-nonisothermal process.
2. Mathematical predictions of available-lysine losses during the rapid-loss phase are within the experimental error of values measured in extrusion-processed samples. However, the model should be tested in a system where conditions can be accurately monitored and uniform sample processing can be achieved.
3. The maximum loss level of available-lysine encountered under nonstirred-isothermal conditions can be exceeded using extrusion processing.
4. Casein and a single-cell protein (Torula) lose available lysine according to first-order reaction kinetics at elevated temperatures.

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Scientific Journal Series Paper No. 10118 of the Minnesota Agricultural Experiment Station, St. Paul, MN 55108.

The Authors express their appreciation to Dr. I. Pflug, University of Minnesota for use of the miniature retorts in his laboratory for processing the various proteins.

HYDROPHOBIC CHROMATOGRAPHY: DEBITTERING PROTEIN HYDROLYSATES

J. F. ROLAND, D. L. MATTIS, S. KIANG and W. L. ALM

ABSTRACT

A process for debittering casein and soy protein hydrolysates based on the application of hydrophobic chromatography to peptide solutions is described. During chromatography, binding forces occurring between the structurally similar phenolic resin and peptide amino acid residues containing aromatic/heterocyclic side chains delay the emergence of these bitter components and permits the selective preparation of a nonbitter peptide hydrolysate. Characterization of the nonbitter and bitter peptide fractions, based on amino acid content is reported. Applications involving the use of the nonbitter peptide fractions as a protein supplement to foods and beverages are evaluated.

INTRODUCTION

THERE IS A VARIETY of food and biomedical applications for proteins which have been solubilized by enzymatic hydrolysis. Their enhanced solubility, heat stability, and resistance to precipitation in acidic environs, where many proteins are insoluble, offer attractive features to biochemists and nutritionists involved in the research and development of high protein food formulations.

Applications of these valuable protein supplements may have merit in the diets of persons with digestive disorders, pre- and post-operative abdominal surgical patient, geriatric and convalescent feeding, and for others who for various reasons do not ingest a well-balanced diet (Russell, 1975). Unfortunately, the use of enzyme-treated hydrolysates in dietary food applications has, in many instances, been limited due to the presence of bitter flavor components. The unpalatability of these hydrolysates arises mainly from the formation of bitter peptide and amino acids liberated during the hydrolytic process (Eriksen and Fagerson, 1976). The bitterness appears to be closely related to the content and sequence of hydrophobic amino acids in the peptides (Sullivan and Jago, 1972).

Studies by Fujimaki et al. (1971) have shown that further hydrolysis of pepsin digested soy protein using a bacterial proteinase or an exopeptidase, reduced bitterness. Also, chymotryptic plastein reactions reduced bitterness in a number of protein hydrolysates. Similarly, Clegg and McMillan (1974) have reported that a combination enzyme treatment of casein using papain for 18 hr followed by the addition of a homogenate of swine kidney cortex, also produced a hydrolysate with reduced bitterness.

As another approach to resolving the bitter flavor problem, it seemed reasonable to attempt flavor improvement of protein hydrolysates by reducing the hydrophobic peptide and amino acid content of the digests. It was recognized many years earlier that activated carbon would absorb the aromatic amino acids tryptophan, tyrosine, and phenylalanine (Sayhun, 1947). At a later date, Murray and Baker (1952) utilized carbon to treat a commercial enzymic hydrolysate of casein and reported the taste was greatly improved. A bitter tasting polypeptide fraction was eluted from the carbon.

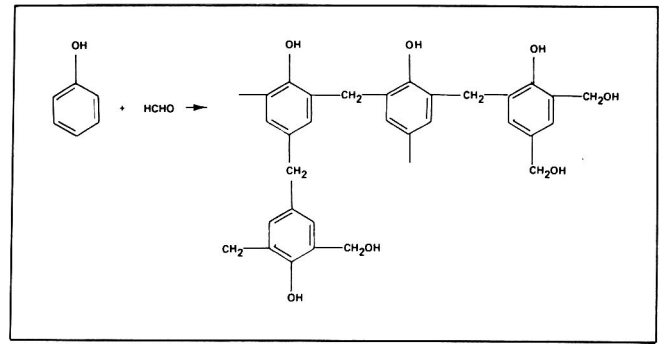

Fig. 1—Phenol-formaldehyde condensation reaction to form macroporous resin.

Various phenol-formaldehyde resins with structures similar to carbon (Garten and Weiss, 1957) are available commercially and are used in a wide variety of ion-exchange and absorbent applications. Therefore, studies to determine the ability of a phenol-formaldehyde resin polymer to interact preferentially with the nonpolar groups present in hydrophobic peptides were undertaken. From these findings, a hydrophobic chromatography process for debittering protein hydrolysates was developed and is reported here.

MATERIALS & METHODS

Protein hydrolysates

A series of commercially prepared casein and soy hydrolysates supplied by Humko-Sheffield Chemical, Norwich, NY, were used for these studies. Among the products examined were N-Z-Amine A (amino nitrogen/total nitrogen) (AN/TN 50.0), N-Z-Amine AS (AN/TN 49.5), N-Z-Amine HD (AN/TN 70.3), N-Z Soy (AN/TN 46.8) and also an acid hydrolyzed casein hydrolysate, Hy-Case SF-70 (AN/TN 68.0). Data derived from N-Z-Amine A, N-Z Soy and Hy-Case SF-70 are presented in this report.

Resinous absorbent

Duolite S-761, a granular resin polymer prepared by phenol-formaldehyde condensation, was obtained from Diamond Shamrock Ion Exchange Functional Polymers Division, Redwood City, CA. The resin is hydrophilic due to its high proportion of phenolic hydroxyl and methylol groups. The chemical structure of the resin polymer is presented in Figure 1 (Abrams and Benezra, 1967).

Analytical determinations

Protein (Kjeldahl N × 6.25) and α-amino nitrogen (U.S. Pharmacopeia XVIII) formol titrations were conducted on various hydrolysates and subfractions. Amino acid determinations were carried out on acid hydrolysed samples by ArRo Laboratories, Joliet, IL, and by Biopolymer Corp., Moreland Hills, OH. Spectrophotometric tryptophan determinations were performed using the method of Gaitonde and Dovey (1970). Conductivity, pH, and chloride measurements were made using a precalibrated Markam Electromark Analyzer MA4403. Duolite S-761 resin column fractions were monitored at 280 nm on a Gilford Model 250 spectrophotometer.

Experimental procedures

Prior to debittering fractionation, prehydrated Duolite S-761 resin (16–50 mesh) was filled into a 7.62 cm diameter glass column to a bed depth of 75 cm (0.12 ft^3). The resin was twice cycled with 2% NaOH

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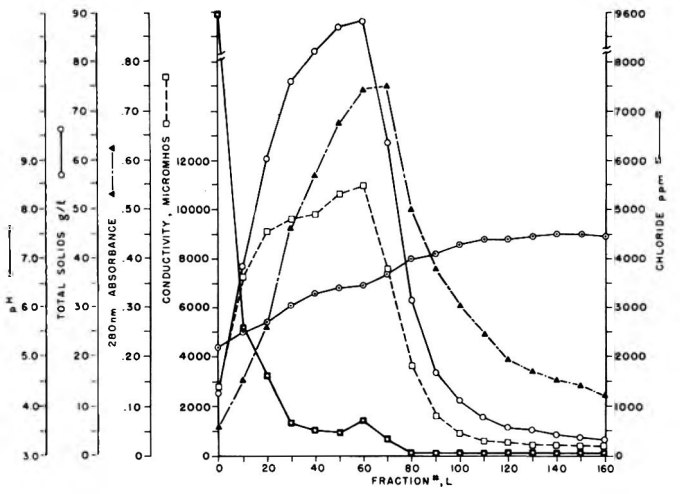

Fig. 2—Hydrophobic chromatography pattern of N-Z Soy enzymic digest of soy protein. See text for details.

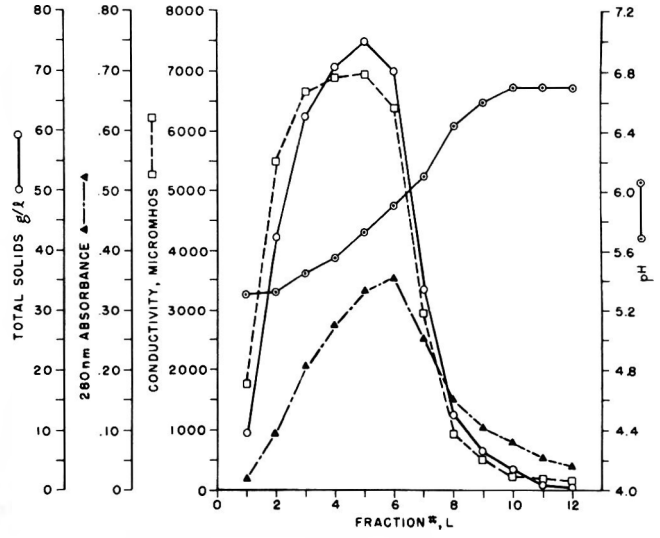

Fig. 3—Hydrophobic chromatography pattern of N-Z-Amine A casein hydrolysate on Duolite S-761 resin. See text for details.

upflow, followed by a downflow rinse with water, then followed by a downflow treatment with excess 1% HCl, and finally followed by a soft water rinse to an effluent flow at pH 5.2.

Five liters of N-Z-Amine A (19%TS) or other protein hydrolysates were applied to the top of the resin bed and allowed to flow through the column at 400 ml per min. One liter fractions of effluent were collected. When the 5.0L had almost completely entered the resin bed, water elution (400 ml/min) was initiated and continued until conductivity values of the effluent were below 50 μohms. Subsequently, the liquid fractions of the hydrolysate were evaluated for bitter flavor, freeze-dried, and weighed. The dried fractions were then analyzed individually or as pooled materials for molecular weight distributions, amino acid content, and peptide mapping. Only the amino acid data is presented in this text.

Biological evaluations

PER determinations were performed on the −NB hydrolysate using the standard method (AOAC, 1975). The −NB hydrolysate was supplemented with l-tyrosine (1.0%) and with dl-tryptophan (1.3%) prior to feeding trials. The assays were conducted by the Nutrition Research Laboratory, Research and Development, Kraft, Inc.

Pilot plant operations

The protein hydrolysate debittering operation was scaled up from experimental bench level trials (0.12 ft^3 resin bed) to resin bed columns containing 2.0 ft^3 and finally 10.0 ft^3 Duolite S-761 absorbent. In either case, only routine processing problems were encountered as long as plug-flow conditions were maintained in the resin column during service and regeneration cycles. A specially designed polypropylene radial arm distributor (Techni-Chem, Inc., Cherry Valley, IL) was installed in the 10.0 ft^3 resin column in order to minimize fluid channelization in the bed. Data from a pilot scale process run using N-Z Soy protein hydrolysate [service charge 75.0L (7491g)] in the 2.0 ft^3 plexiglass column (30.5 cm × 140.0 cm) is illustrated in Figure 2. Yield of −NB fraction was 4994g, −B fraction was 1816g.

RESULTS & DISCUSSION

Flavor evaluations

An experienced three-man panel was used to judge flavor and bitterness contributions of the eluted hydrolysate column fractions. Based on flavor trials of a series of six hydrolysate fractionations (0.12 ft^3 resin bed), it was the group consensus that bitter flavor consistently appeared first in the hydrolysate fraction containing the peak 280 nm absorbance (fraction 5, Fig. 3). Prior samples had a clean, meaty-brothy flavor. All subsequent fractions increased in their bitter flavor intensity. Flavor evaluations of pooled fractions 1–4 also gave a hydrolysate with a clean, completely nonbitter, meaty flavor (−NB fraction). Pooling fractions 5–12 gave a product with an intensely bitter flavor (−B fraction).

Food product applications

The flavor of the debittered hydrolysates has been found to be compatible in all foods or beverages where a beefy, brothy character is desirable. Additions of debittered hydrolysate to tomato or onion based soups, sauces, drinks, or extruded products were ranked most acceptable at the 1–3% w/v level. The debittered product has also been compounded into tablet or wafer form with various flavoring additives. Likewise, the addition of these debittered hydrolysates to bland soy based protein foods or to various cheese products has proven to be desirable.

Hydrophobic chromatography

The absorptive effect of the phenol-formaldehyde resins (Duolite S-761) on aromatic amino acids and peptides present in the protein hydrolysates is illustrated in Figure 2 and Figure 3. As can be observed (Fig. 3), the shape of the total solids and conductivity curves follow normal Gaussian-shaped distributions with peak concentrations occurring in fraction 5. However, the 280 nm absorption curve has its concentration peak appearing in fraction 6 and shows positive skewness. A similar retardation of the 280 nm absorption peak was consistently found in other hydrolysate fractionations and was also observed for the larger scale fractionation of N-Z Soy protein hydrolysate (Fig. 2). In this instance, the total solids/conductivity curves show peak values after 60L of hydrolysate have been eluted, followed by the 280 nm absorption values which peaks after 70L of processed material have been eluted.

In order to gain further insight into the hydrophobic binding interactions occurring between the phenolic structured resin and the mixed distribution of aromatic and heterocyclic peptides and amino acids in the hydrolysates, various amino acid analyses of individual and pooled fractions of nonbitter and bitter materials were undertaken.

Amino acid comparisons

The amino acid results are presented in Table 1 and 2. These data clearly indicate the reduction of aromatic amino acids occurring in the −NB fraction and the increases in the −B fraction when both fractions are compared with unprocessed NZ-Soy. Similar amino acid data were obtained for both N-Z-Amine HD, and NZ-Amine A hydrolysates.

In Table 2 are presented amino acid analyses of the

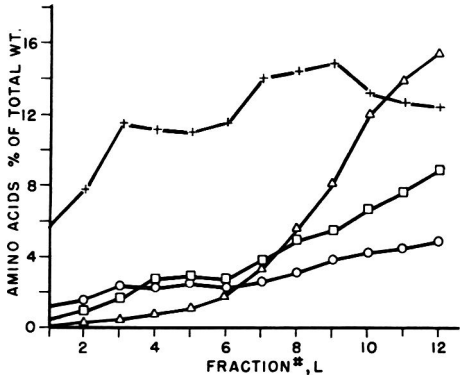

Fig. 4—Aromatic and heterocyclic amino acid content of fractions derived from Hy-Case SF-70 phenol-formaldehyde resin chromatography. X—proline, Δ—phenylalanine, □—tyrosine, ○—histidine.

NZ-Amine A, –NB, and –B fractions. Again, these data indicate the reduction which occurs in aromatic (phenylalanine and tyrosine), aromatic-heterocyclic (tryptophan) and also heterocyclic (proline and histidine) amino acid content in the –NB fraction.

Biological evaluations

Results of animal feeding trials to establish PER values for the supplemented hydrolysate are presented in Table 3. It can be seen from the amino acid data in Table 2 that tryptophan supplementation is required in order to provide nutritionally adequate amounts of the essential amino acid. However, additions of tyrosine did not improve rat growth performance beyond those provided by tryptophan supplementation. Further feeding trials to establish other potentially limiting amino acids are in progress.

In another experiment, HyCase SF-70 (acid hydrolyzed casein, 3 hr at 190°C, in 6N HCl) was fractionated on a Duolite S-761 column (0.12 ft^3) and individual 1-L fractions were collected and dried. Subsequently, they were assayed for their amino acid content after an 18-hr acid hydrolysis at 105°C. The resin adsorption pattern was similar to data (Fig. 2 and 3) previously presented for enzymically hydrolyzed casein hydrolysates. Subsequently, amino acid data of the (12) individual hydrolysate fractions were analyzed for concentration trends. Data on aromatic and heterocyclic amino acids which showed significant concentration changes were plotted and results of these changes are presented in Figure 4. The selectivity of the phenolic resin column for ring structured peptides and amino acids is most evident in the terminal fractions. As can be seen from the retention curves, hydrophobic binding occurring between the phenolic structure of the resin polymer and the nonpolar side chains of the aromatic and heterocyclic amino acids present in the peptides appear to provide a basis for the functional selectivity of the resin. Nonpolar aliphatic hydrocarbon side chains showed no significant binding affinity for the resin.

When a more fully hydrolyzed casein hydrolysate, e.g. NZ Amine-HD (AN/TN 70.3) was processed through Duolite S-761 resin, a nonbitter, low phenylalanine product with potential application in the dietary management of phenylketonuria was produced.

Table 1—Aromatic amino acid[a] analyses of soy protein hydrolysates

Sample	Phenylalanine	Tyrosine	Tryptophan
N-Z Soy	4.72	1.49	0.99
N-Z Soy–NB	2.15	1.14	0.36
N-Z Soy-B	8.81	1.83	1.36

[a] Grams of amino acid residue per 100g protein

Table 2—Amino acid[a] analyses of enzymic hydrolysates of casein

Amino acid	N-Z-Amine A	N-Z-Amine-NB[b]	N-Z-Amine-B[b]
ASP	5.32	5.61	4.92
GLU	15.68	17.32	14.83
PRO	7.82	5.26	8.48
PHE	2.45	0.96	4.11
TYR	2.04	1.10	1.95
TRP[c]	1.20	0.28	0.97
HIS	2.17	1.46	1.05
LYS	5.78	5.54	5.18
ARG	2.24	1.74	1.82
SER	3.94	4.36	3.46
MET	1.81	1.66	2.25
CYS[d]	ND	ND	ND
GLY	1.34	1.79	1.28
ALA	2.24	2.43	1.96
VAL	4.64	4.47	4.73
LEU	6.53	6.01	6.68
ISO	3.40	3.62	4.34

[a] Grams of amino acid residue per 100g protein

[b] –NB Pool of fraction 1–5 from Duolite S-761 processing; –B pool of fraction 6–12 from Duolite S-761 processing

[c] Calculated from ultraviclet absorption

[d] Not determined

Table 3—PER values of –NB[a] casein hydrolysate prepared by hydrophobic chromatography

Sample	Supplement	PER	
		As is	Corrected
–NB Casein hydrolysate	TRP (1.3%)	2.02	2.04
–NB Casein hydrolysate	TRP (1.3% + TYR (1.0%)	2.05	2.07
ANRC casein	–	2.47	2.5

[a] Pooled fractions 1–5 of N-Z-Amine A hydrolysate processed through Duolite S-761

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INFLUENCE OF BUTTERFAT CONTENT ON METHYL KETONE RETENTION DURING SPRAY DRYING AS DETERMINED BY HIGH PRESSURE LIQUID CHROMATOGRAPHY OF 2,4–DINITROPHENYLHYDRAZONES

G. A. REINECCIUS, H. C. ANDERSON and B. J. FELSKA

ABSTRACT

The retention of a homologous series of saturated n-methyl ketones during the spray drying of milk systems of different fat contents was examined. Retention of the ketones increased with molecular weight, particularly in the fat-free system. The addition of fat up to 21% (solids basis) improved retention. At higher fat contents, retention of the methyl ketones was diminished. Results suggest that the dispersed fat droplets serve as a reservoir for the ketones thereby lowering their vapor pressure and inhibiting their diffusion to the droplet surface. An increased loss of ketones at higher fat contents may be due to the fat forming a second continuous phase providing a diffusion pathway for the ketones to the drying surface.

INTRODUCTION

THE REDUCTION of volatile flavors during spray drying has received substantial attention since the early work of Sivetz and Foote (1963) on drying of coffee. Other research in this area has been conducted by Thijssen and co-workers (Bomben et al., 1973; Kerkhof and Thijssen, 1975, 1977; Rulkens and Thijssen, 1972; Thijssen and Rulkens, 1968, 1969; Menting et al., 1970a, 1970b), King and Massaldi (1974), and Reineccius and Coulter (1969).

The work cited above involved studies of the mechanisms governing flavor retention during spray drying of model systems and an evaluation of the influence of dryer operating parameters on flavor retention. Previous work has primarily focused upon the retention of water soluble (or partially so) components in relatively high concentrations (>0.1%) compared to what exists in nature. The model systems cited have most frequently been comprised of carbohydrates or other water soluble polymers. The only reference to flavor retention where the model system contained a lipid phase was a theoretical treatment presented by King and Massaldi (1974). They did not present any data to validate their theoretical predictions.

The objective of our study was to evaluate flavor retention during drying of model systems which contained a lipid phase. The loss during drying of a homologous series of compounds that represented a broad range of solubilities and vapor pressure at concentrations closer to what is found in food products was evaluated.

METHODS

THE INFLUENCE of fat on the retention of volatile flavors was evaluated by adding 4 ppm of a homologous series of saturated 2-methyl ketones to 4 lots of milk (0, 25, 50, and 75% fat dry basis; 30% total solids), drying the samples and analyzing the powders for residual amounts of added compounds.

Stock solutions of methyl ketones (C_4–C_6, C_8–C_{11}; Aldrich Chemical Company, Milwaukee, WI) were made in alcohol. A ten ml aliquot of stock solution was then blended into butter warmed to 57°C. Four lots of condensed bulk skim milk and sufficient amounts of water and butter were weighed and mixed at 57°C. Each sample was immediately homogenized in a two stage Manton-Gaulin homogenizer at 176 and 35 kg/cm² pressure. Portions of each lot were saved and analyzed to provide initial concentrations before drying. The samples were then spray dried (infeed temperature 40°C) using pilot plant Coulter spray dryer (inlet air 204°C, exit air 88°C; pressure nozzle orifice: 0.57 mm i.d., flow rate 38 l/hr; tower size: 143 cm i.d. × 712 cm long).

Sample analysis

Pentane and hexane were rendered carbonyl-free by refluxing 4 hr over H_2SO_4, distilling over KOH, and passing the distillate through a 2,4-dinitrophenylhydrazine (DNPH) column described by Schwartz and Parks (1961). Benzene was purified by passing it through the DNPH column and distilling over KOH. Celite 545 and Adsorptive Magnesia were dried for 48 hr at 150°C. All other materials were used as supplied.

Extraction of the methyl ketones from the dried samples was accomplished by adding 5g NaCl, 25 ml sample adjusted with water to 10% total solids, 5 ml internal standard (2 ppm 2-heptonone in absolute ethanol), 20 ml absolute ethanol, and 75 ml pentane to 250 ml Erlenmeyer flasks fitted with glass stoppers. The samples were extracted for 3 hr at 4°C on a rotatory shaker (200 rpm). The decanted pentane was dried over anhydrous $MgSO_4$ for 30 min and filtered (Whatman No. 1). Stable 2,4-dinitrophenylhydrazones were prepared with the DNPH column (Schwartz and Parks, 1961) followed by a hexane rinse until 100 ml of effluent was collected. The fat was removed by passing the hexane rinse through a Magnesia-Celite (5g:5g) column (Schwartz et al., 1963) and eluting the fat with 100–150 ml dichloromethane, and then the hydrazones with methanol. The hexane and nitromethane-chloroform elution sequence used by Schwartz et al. (1963) was changed as described since it did not adequately separate the hydrazones from the fat.

The hydrazone fraction was evaporated to dryness on a hot plate under N_2. The hydrazones were dissolved in CH_3CN/H_2O (30:10) and analyzed by high pressure liquid chromatography (HPLC). The HPLC system consisted of a Waters and Associates Model 440 Absorbance Detector with a 340 nm cell, 6000 A delivery Pump, U6K injector, a μ-Bondapak C_{18} column (0.64 × 30 cm) at room temperature and a Hewlett-Packard Model 3380 A integrator. A 10 μl sample was injected into the system with a flow rate of 1.0 ml CH_3CN/H_2O (30:10) per minute.

Methyl ketone concentrations were corrected for differences in extraction efficiencies, DNPH derivatization, HPLC injection volumes, etc. by adjusting the data using the internal standard curves prepared with DNPH derivatives.

RESULTS & DISCUSSION

MOST WORK PUBLISHED evaluating flavor retention during spray drying has used levels of volatile compounds in the drier feed at 0.1% or higher. This is certainly not typical or representative of concentrations of flavor compounds normally present in food product. The binding of organic molecules to major food constitutents is well documented in the literature (Solms et al., 1973; Maier, 1972, 1975). One would expect those volatile compounds which are bound to food constituents to be lost to a lesser extent during drying than those which are free of binding. Therefore, one might expect volatile concentration to have an effect on volatile retention during drying. Rulkens and Thijssen (1972) reported that flavor retention was independent of volatile concentration up to 1%. However, the range of concentrations evaluated went only

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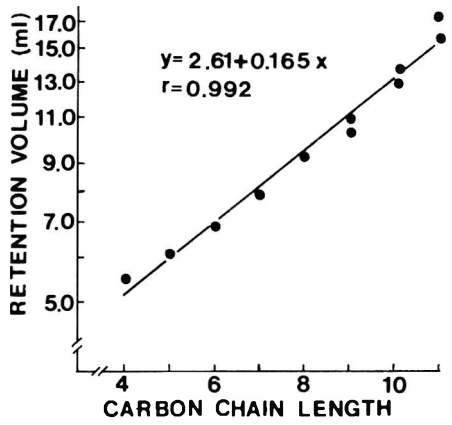

Fig. 1—Retention volumes of standard DNP-methyl ketones on a μ-Bondapak C_{18} column.

Table 1—Physical constants and recovery of n-methyl ketones added to 10% total solids skim milk

	MWT g/mole	bp 760	Solubility Water	Solubility Ether	Recovery (%)
2-Butanone	72.10	79.6	v[b]	00[a]	54.7 ± 1.80[g]
2-Pentanone	86.13	102	sl[d]	00	75.9 ± 0.05
2-Hexanone	100.16	126	sl	00	84.7 ± 4.40
2-Heptanone[h]	114.19	151	vsl[e]	00	100.0 ± zero
2-Octanone	128.22	173	vsl	00	100.9 ± 1.30
2-Nonanone	142.24	193[749]	i[f]	s[c]	103.8 ± 4.00
2-Decanone	156.27	210-1[767]	i	s	108.2 ± 1.30
2-Undecanone	170.29	228[748]	i	s	107.8 ± 2.45

[a] 00 = soluble in all proportions
[b] v = very soluble
[c] s = soluble
[d] sl = slightly so uble
[e] vsl = very slightly soluble
[f] i = insoluble
[g] mean ± SEM, n = 2
[h] internal standard

down to 0.1% which may still be above the amount needed for saturation of adsorption sites. There is another concern about using high levels of volatiles when studying flavor retention. If solubility of the volatile test compounds is exceeded, vapor pressure is independent of the concentration of this compound. One would not expect to find a relationship between volatility and retention during drying if the compound is present above its solubility level.

The two concerns discussed above motivated us to develop analytical methods suitable for quantitative analysis of test compounds at very low levels (>100 ppb). The resolving power of high pressure liquid chromatography (HPLC) and sensitivity of UV adsorption methods suggested HPLC of dinitrophenyl hydrazones as a method of choice.

Optimum HPLC conditions were first determined by using a C_5 and a C_{11} methyl ketone DNP and varying the HPLC solvent composition (CH_3CN/H_2O) until suitable retention volumes were attained. Figure 1 shows the relationship between the natural logarithm of solvent volume required to elute a particular DNP from the μ-Bondapak C_{18} column versus the DNP's carbon chain length. Two peaks appeared for each individual methyl ketone, nine carbons and greater. The double peaks were found to be isomers consistent with the work of Weihrauch and Schwartz (1972). Therefore, the sum of both peaks associated with a particular DNP was used in quantitative determinations. A least squares regression line was plotted using the retention volume of the corresponding peak areas. Some deviation from linearity is evident although slight (r = 0.992).

Extraction efficiencies were determined by adding 4.0 ppm n-methyl ketones to reconstituted nonfat dry milk and performing the analyses. The recoveries are presented in Table 1 and were means of triplicate analyses performed on two different days. The increased recovery with increased carbon chain length is most likely due to improved extraction efficiencies of the less water soluble methyl ketones. Table 1 also lists the solubility of each methyl ketone in water and ether (*Handbook of Chemistry and Physics*, CRC Press). Since the extracting solvent is pentane, one would expect higher extraction efficiencies of the longer chain methyl ketones. Recoveries greater than 100% are possible since 2-heptanone (internal standard) was given a value of 100 and recoveries of the other ketones are based on the 2-heptanone. While the recoveries of C_4-C_6 methyl ketones are low, they are reproduceable as is evidenced by the low standard errors.

The retention of methyl ketones during spray drying is presented in Table 2. Methyl ketone retention increased as carbon chain length increased. This is as one would expect, for retention is primarily determined by diffusivity of the volatile compounds through the drying matrix. The larger volatile

Table 2—Retention of methyl ketones added to milk systems of different fat contents following spray drying

Ketone	Fat content (% dry basis) of powder 0.67 ± 0.07	20.6 ± 1.09	43.0 ± 2.06	63.0 ± 5.00
	Percent Recovery			
2-butanone	23.7 ± 3.62	23.0 ± 7.08	17.0 ± 7.42	7.20 ± 7.20
2-pentanone	26.6 ± 2.34	28.9 ± 5.10	23.0 ± 5.26	7.45 ± 7.45
2-hexanone	37.1 ± 3.19	51.3 ± 7.58	46.3 ± 6.17	21.0 ±15.9
2-octanone	54.5 ± 4.53	93.9 ± 6.80	86.5 ± 9.06	46.4[b]
2-nonanone	67.2 ± 4.61	99.0 ± 8.29	84.6 ±10.1	63.0[b]
2-decanone	81.8 ± 5.65	90.4 ± 3.28	84.0 ± 9.26	64.1 ±10.5
2-undecanone	88.3 ± 7.09	92.7 ± 6.32	83.2 ±10.5	69.2 ± 7.60
number of trials	4	4	5	2

[a] x ± SEM
[b] One trial

compounds are "locked in" or unable to move through the matrix at a higher moisture content than the smaller volatiles. The smaller compounds can diffuse through the drying matrix for a longer period during drying and therefore, are lost to a greater degree. Although retention appears to parallel boiling point (Table 1), it has been shown that molecular size, not vapor pressure, determines volatile retention during drying of nonfat containing systems (Rulkens and Thijssen, 1972).

The dominance of molecular size in determining retention is particularly obvious if one considers the work of Buttery et al. (1973). They found vapor pressure of a homologus series of aldehydes to increase with increasing chain length in an aqueous system. Therefore, our results showing an increase in retention (fat-free system) with increasing carbon chain length is particularily significant.

The incorporation of fat into the drying matrix had a positive effect upon retention of most of the methyl ketones at the 25% fat level and a negative effect at 50 and 75% fat levels. The powder collected from the dryer was never as high in fat as expected. The 25, 50 and 75% fat infeed materials averaged 21, 45 and 63% fat in the powder following drying. The only obvious explanation for the difference in fat contents between the infeed material and dried powder would be that there was a dilution of each following run by the previous run. The drier was not washed up between samples and samples were dried in order of increasing fat content. This explanation is improbable, however, for the 25% fat powder would have to be diluted with nearly 20% of the previous nonfat powder run. Since the nonfat samples dried very well in nearly 100% recovery of solids from the drier, there would be no powder available for dilution of the 25% fat sample. We are unable to

find a reasonable explanation for the observed loss of fat from the samples.

The observed maximum in flavor retention which occurred at a fat content of 21% was expected. At low fat contents, the fat exists as discrete droplets dispersed throughout the atomized particle (Buma, 1971). The methyl ketones would be partitioned between the fat and aqueous phase with the majority of ketones in the fat phase (see Table 1). The fat would tend to hinder the diffusion of the ketones to the drying interface surface. As fat content is increased, a second liquid continuous phase consisting of fat would form. Buma (1971) reported that a continuous fat phase would theoretically occur at about 40% fat. At this point, there would be a direct diffusion pathway for ketones to reach the drying surface by transmission through the fat channels. Retention would be governed by diffusion in the fat and vapor pressure. The rate of migration to the surface and distillation from the fat:air drying interface would primarily determine retention. Since the smaller ketones would have the highest diffusion rates and highest volatility, (fat containing systems), they would again be retained in lesser amounts than the longer chain ketones.

Another effect of fat content on volatile retention would be through its influence on surface fat. Since all of the ketones were dissolved in, and probably remained in, the fat phase, the greater the amount of fat on the surface of the drying droplet, the lesser the retention of ketones. The ketones would be lost from surface fat via simple distillation. This mechanism would again favor retention of the less volatile ketones. Processing parameters that would increase free fat would logically reduce ketone retention during drying (Buma, 1971).

CONCLUSIONS

A METHOD for the quantification of methyl ketones in milk at ppb levels has been developed. This method has been applied to studying the retention of methyl ketones during the spray drying of fat containing milk systems. Ketone retention was shown to reach a maximum when 25% fat (solids basis) was included in the drying matrix. Ketone retention was less in the 75% fat drying matrix than in the nonfat system. It is hypothesized that the fat forms a second continuous phase at concentrations greater than 40% and the fat provides a direct pathway for diffusion of ketones from the interior of the drying particle to the drying surface. Since there is no diffusion limiting membrane of high solids solubles to hinder passage of ketones to the drying surface, a greater percentage of ketones is lost as compared to the nonfat system.

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Ms received 8/1/77; revised 2/6/78; accepted 2/15/78.

Scientific Journal Series Paper No. 9793, Minnesota Agricultural Experiment Station, St. Paul, MN 55108.

DETERMINING THE OPTIMAL RANGES OF FACTORS AFFECTING THE SENSORY ACCEPTABILITY OF A MINCED MULLET SAUSAGE

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ABSTRACT

Four factors (liquid smoke flavoring, textured soy flour, sodium tripolyphosphate, and water) were studied to establish approximate ranges for maximum acceptability of a minced mullet product. The optimum level of liquid smoke flavoring was found at 0.5% and the optimum level of sodium tripolyphosphate was 0.3%. The optimum range for soy was approximately 15% while the best water levels occurred between 10 and 15%. An acceptable product could be produced providing the extreme ranges of each factor were avoided.

INTRODUCTION

POPULATION GROWTH is expected to greatly increase the price of animal protein foods in the future. Inflation in the economy (John, 1974) and a rising demand for animal products from other increasingly prosperous countries such as Japan (Vickery, 1971) may also boost prices, providing incentive to develop new products from less expensive protein sources.

Underdeveloped fish species are a potential source of inexpensive protein. Fish are generally recognized as an excellent protein source (17–24% by weight). The fish proteins are high in the essential amino acids and have a good amino acid profile for human requirements (Finch, 1970).

Yearly harvests of the black mullet species (*Mugil cephalus*) are approximately 30 million pounds in the Gulf Coast area of the United States. However, mullet utilization has been limited for several reasons. Mullet has distinctive flavor characteristics and is subject to oxidative rancidity (Deng and Dennison, 1976) which limits long-term frozen storage and further processing. The mullet industry is hampered by an image problem and by the shift in consumer purchases toward processed convenience foods (Cato et al., 1976). Mullet also has a low meat yield (33% for boneless fillets or 60% headed and gutted) which increases labor processing costs. The recent development of fish deboning machines permits the recovery of flesh from filleting wastes to allow for economical processing of species with low flesh yield.

Minced fish therefore represents an inexpensive source of quality protein for human food providing it can be incorporated into acceptable products. Although the particular fish species used in this research was mullet, other underutilized species could be made into fish sausages using similar methods.

The purpose of this study was to tentatively identify the optimum levels of liquid smoke flavoring (Charsol), textured soy flour (soy), sodium tripolyphosphate (TPP), and water. These data were required for designing the subsequent statistical analysis using response surface methodology (a maximization procedure which is meaningful only if the ranges considered include the optimum levels). Also, the accuracy of the predictions resulting from a response surface analysis can be increased by setting the optimum ranges at the central points of the response surface design.

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0022-1147/78/0005-1497$02.25/0

Incremental levels of each of the four factors (with all other factors set at constant values specified in each section) were evaluated in various experiments. Liquid smoke flavoring (Charsol) was studied because it can increase sensory acceptability, retard rancidity development (Watts, 1954) and inhibit microbiol growth (Erdman et al., 1954). Textured soy flour was included since it can contribute nutritional, economic, and functional advantages (Seideman et al., 1977). Both water and sodium tripolyphosphate (TPP) levels were studied because of their effect on the textural properties of the product (Kramlich et al., 1973; Shults et al., 1972).

EXPERIMENTAL

Materials

Mullet were purchased fresh from Cedar Key, Florida and transported on ice to Gainesville where they were filleted, skinned, and washed. The cleaned fillets were stored in a 2°C room packed in ice for approximately 3 hr before use. Textured soy flour (Centex 400, caramel color) was purchased from the Central Soya Co. The Red Arrow Co. (Manitowoc, WI) supplied a liquid smoke flavoring (Charsol C-6). Food grade sodium tripolyphosphate (TPP) was obtained from the FMC Corp. Spices, sugar, and salt were purchased at local markets. Meat flavorings were supplied by Fries and Fries (Cincinnati, OH) and F.I.D. (White Plains, NY).

Methods

Sausage preparation. Sausages were always prepared on the day of fish purchase to assure uniform freshness. The chilled mullet fillets were diced, ground (to simulate deboned flesh), and mixed with constant amounts of sodium nitrite (100 ppm), salt (1.6%), sugar (0.9%), and 1.1% spices for all trend analyses. (All percentages were based on the total raw weight and were determined by preliminary studies.)

Sausages were formulated using varying percentage levels of Charsol, water (as ice), TPP, and rehydrated textured soy flour (1 part soy to 3 parts water) in addition to the constant factors. The TPP levels ranged from 0–1.2% while both the water and soy levels ranged from 0–30%. Charsol was initially added at levels up to 4%, but the resulting soupy batter made stuffing so difficult that the maximum level of Charsol was reduced to 2%.

All ingredients were stirred by hand (50 strokes) in a mixing bowl and ground through an electric food grinder (Hamilton Beach Co., Model 233) using a coarse plate with triangular holes. The ground batter was again stirred 50 times in a mixing bowl and passed through the same grinder fitted with a stuffing horn attachment, and stuffed into edible collagen casings (Teepak Coria®, No. 6730, 30 mm diameter). Sausages were baked at 185°C (375°F) for 48 min in a standard electric oven on a rack to allow for drainage of cooking juices. After cooling for 30 min, the sausages were weighed and stored at 2°C in a barrier bag (Cryovac®) to prevent moisture loss before sensory evaluation.

Sensory evaluation. The panels employed a seven-point hedonic scale including the categories of fishy flavor, smoke flavor, texture, and overall acceptability. The smoke flavor category ranged from imperceptible (1) to just right (4) to extremely in excess (7). Textures ranged from extremely soft (1) to just right (4) to extremely tough (7), while overall acceptability varied from undesirable (1) to medium (4) to excellent (7). Fishy flavor ranged from none (1) to extremely in excess (7). Eight to sixteen experienced panelists were used to evaluate three to six samples, but the same number of panelists were used in replicate experiments.

Sensory panels were always held the day following the sausage preparation. Before evaluation, the sausages were heated for 20 min at 205°C (400°F) in an electric oven. The sausage samples were sliced, allowed to equilibrate to room temperature, numerically coded (with three random digits), and served to judges in individual booths. Red lighting in the booths was used to mask color differences among samples. Experiments were repeated several times to minimize variations.

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Cooking loss. The percent cooking loss (as an indicator of product texture) was calculated as the difference between the raw and cooked weights of a sausage, divided by the raw weight and multiplied by 100. Measurements were done in duplicate or triplicate using a Toledo balance, Model 4020.

Statistical analysis. The data were analyzed by either one-way analysis of variance or linear regression statistical procedures. The one-way analysis of variance was used for determining differences in sensory scores of qualitatively different samples. Regression analysis was performed on samples with varying quantitative levels of a component by computer using the Statistical Analysis System (SAS) program package.

RESULTS & DISCUSSION

Preliminary sausage formulation experiments

The initial work involved the preparation of a good sausage recipe from fish using informal sensory evaluation procedures. The first sausages were very bland in flavor with no fishy flavor if smoked or made into patties and fried. Fishy flavors were apparent in sausages that were baked or boiled in water, but the addition of 0.5% liquid smoke flavoring (Charsol) almost completely masked the fishy flavor in baked sausages. Sausages prepared with 15% added pork fat were similar in flavor and texture to sausages without added fat. Pork fat was omitted from subsequent sausage formulations, since high fat foods are not suitable for the current American market and diet and the texture was satisfactory without the modifying effect of fat.

Since the sausages were so bland, imitation meat products were developed. Addition of a commercial spice mix (obtained from Copeland Sausage Co., Alachua, FL) at 0.85%, produced fish sausages very similar in flavor to typical pork sausages. An original spice combination was developed by trial-and-error to produce a unique sausage flavor for further experimentation. An experiment was performed incorporating meat flavors into the sausages, using a pork flavor (Fries and Fries), another pork flavor (F.I.D.), and a smoked meat flavor (F.I.D.) to enhance the similarity to meat sausages. The samples containing meat flavor were very similar to the control made with original spice combination. A similar experiment involving a comparison of sausages with Italian flavoring, a country sausage flavoring (both from Fries and Fries), and a control with only the original spice combination was performed. Although the products were distinctly different, there was no significant difference ($\alpha < 0.05$) in the overall acceptability. Many flavorings are commercially available and a very good meat sausage imitation could probably be produced, but no further research was done on this aspect because preference was given to developing a distinctive new product.

Identical samples using 1% Charsol, 20% textured soy flour, and 10% water in addition to the constant factors, were prepared using either mullet flesh or pork meat to evaluate the panel response for fishy flavor (the type of flesh was the only variable). A mullet sausage sample (baked) was evaluated with the pork sausage. The panel results showed no significant differences ($\alpha < 0.05$) in fishy flavor, texture, or overall acceptability. The average score for fishy flavor was 1.43 for pork, 2.07 for fish (baked), on a scale where 1 = no fishy flavor, 3 = slight fishy flavor, 5 = moderate fishy flavor, and 7 = extreme fishy flavor. The fishy flavor score for pork was probably the result of a tendency of panelists to avoid the extremes of the scale (Cloninger and Baldwin, 1976). The net difference was only 0.64 (a value one-fifth of slight fishy flavor) showing that fishy flavors were barely detectable. The average panel score for fishy flavor from all the preliminary experiments with sausages using 0.5% Charsol, 10% water, and 20% soy (a total of 122 observations from 22 panelists and 11 samples) was slightly lower (1.52). In this case the net difference between the average score and the pork score for fishy flavor was only 0.09, demonstrating that fishy flavor was not a problem.

The addition of sodium nitrite colored the dark flesh red but had less effect on the white flesh which lacks myoglobin. The color difference could be masked if desired (this was not necessarily detrimental to appearance) by adding food colorants.

Charsol effect

All sausages in the Charsol level experiments contained 10% water, 20% soy, and no TPP in addition to the constant factors. The cooking loss consistently increased as the Charsol level increased (Fig. 1). The general trend was an almost linear increase from 0–1% Charsol followed by a leveling off from 1–2%. However, statistical analysis showed that the linear trend for cooking loss was significant ($\alpha < 0.05$).

The average sensory panel scores (Table 1) showed increasing toughness (possibly from increased cooking loss) with increasing Charsol level with both highly significant linear and quadratic trends ($\alpha < 0.01$). The calculated correlation coefficient (r) for the cooking loss data and the texture panel response was 0.92. Smoke flavor scores also significantly increased ($\alpha < 0.01$) in a linear response while the overall acceptability scores showed no significant trends ($\alpha < 0.05$).

In Table 1, the optimum value for texture (4.0) was closest to the 0.5% Charsol level (4.16). In contrast, smoke flavor scores were highest at 2% Charsol. Because of the toughening effect and dramatically decreased yield using high Charsol levels, and since the values for overall acceptability were not significantly different, the Charsol level was fixed at the 0.5% level for the response surface analysis (Daley et al., 1978) as recommended by the manufacturer (Anonymous).

Soy effect

Three different types of soy (textured flour, textured concentrate, and concentrate) were included in sausage formulations at the 15% substitution level. No significant differences ($\alpha < 0.05$) for any of the soy blends or the all-fish control were found for the sensory panel evaluations of texture, flavor, or overall acceptability. Textured soy flour (soy) was chosen for all further experiments since it is least expensive.

Table 1—Effect of Charsol level (0–2%) on average sensory scores for mullet sausages from three experiments

	Smoke flavor[a]				Texture[b]				Overall acceptability[c]			
Experiment	0%	0.5%	1%	2%	0%	0.5%	1%	2%	0%	0.5%	1%	2%
A	2.70	3.40	3.50	4.10	3.10	4.10	4.30	4.10	4.20	5.00	4.90	4.00
B	3.07	3.00	3.36	3.64	4.07	4.50	4.50	4.64	5.07	4.57	4.86	4.86
C	3.08	3.85	3.62	4.23	3.23	3.85	4.54	4.54	4.77	5.15	5.08	4.85
Average	2.97	3.41	3.49	3.97	3.51	4.16	4.46	4.46	4.73	4.89	4.95	4.62

[a] Scoring system: 1 = imperceptible, 4 = just right, 7 = extremely in excess.
[b] Scoring system: 1 = extremely soft, 4 = just right, 7 = extremely tough.
[c] Scoring system: 1 = undesirable, 4 = medium, 7 = excellent.

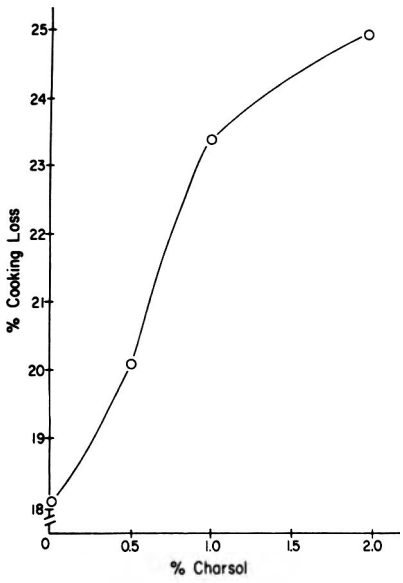

Fig. 1—Effect of Charsol level (0–2%) on cooking loss. Each point is an average value of six measurements from two experiments.

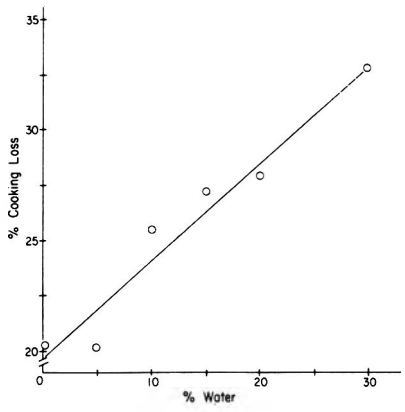

Fig. 2—Effect of added water level (0–30%) on cooking loss. Each point represents an average value for eight measurements.

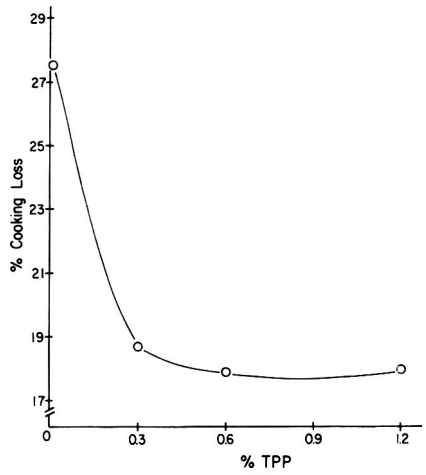

Fig. 3—Effect of TPP level (0–0.6%) on cooking loss. Each point represents an average of nine measurements

Textured soy flour was added to sausages at the 0, 10, 20, and 30% levels and evaluated by a sensory panel. (The sausages also contained 1% Charsol, 10% water, and no TPP.) Soy had a toughening effect from 0–10% which leveled off from 10–20% and a further toughening effect from 20–30% (Table 2). Statistical analysis showed a significant linear trend ($\alpha < 0.01$) for the texture score and for the fishy flavor score ($\alpha < 0.05$). No significant trends were observed for any of the samples for overall acceptability, but higher values were found for 0–10% soy. The optimum texture value (4.0) corresponded to the 10–20% range of soy substitution while the lowest values for fishy flavor were found in the 20–30% soy range. Since the average of these ranges predicted an optimum of approximately 15% soy, this value was used as the center of the response surface design (Daley et al., 1978) because the best predictions occur at the design center.

Table 2—Effect of soy levels (0–30%) on average sensory scores for mullet sausages

Panel category	Percent textured soy flour 0%	10%	20%	30%
Fishy flavor[a]	1.80	2.73	1.27	1.33
Texture[b]	3.40	4.27	4.07	4.47
Overall acceptability[c]	4.93	5.00	4.60	4.67

[a] Scoring system: 1 = none, 7 = extremely in excess.
[b] Scoring system: 1 = extremely soft, 4 = just right, 7 = extremely tough.
[c] Scoring system: 1 = undesirable, 4 = medium, 7 = excellent.

Water effect

In addition to the constant factors, all the sausages for the water level experiments contained 0.5% Charsol, 20% soy, and no TPP. The effect of percent water added to the batter on cooking loss are shown in Figure 2. Statistical analysis showed a highly significant ($\alpha < 0.01$) linear trend.

The correlation coefficient ($r = 0.89$) between the average cooking loss data and the mean texture panel scores (Table 3) was significant ($\alpha < 0.05$) (Steel and Torrie, 1960). Statistical analysis showed a highly significant ($\alpha < 0.01$) linear trend for the texture panel scores. The optimum texture response (4.0) was closest to 4.07 for 5% water and 4.22 for 10% water (Table 3). Overall acceptability scores showed a quadratic trend significant at 0.05 level. The optimum score for overall acceptability (7.0 on a seven-point hedonic scale) was closest to 5.08 for 15% water followed by 4.96 for 10% water and 4.73 for 5% water. Since the ideal water level for overall acceptability occurred at approximately 15%, this level was chosen for the center point of the response surface design for further product development studies (Daley et al., 1978).

—Text continued on page 1500

Table 3—Effect of water level (0–30%) on average sensory scores for mullet sausages from five experiments

Experiments	Texture[a] 0%	5%	10%	15%	20%	30%	Overall Acceptability[b] 0%	5%	10%	15%	20%	30%
A	5.17	4.25	4.08	3.67	3.58	3.92	4.58	4.83	4.83	4.83	4.50	4.08
B	5.18	3.28	4.18	–	3.45	2.36	–	–	–	–	–	–
C	4.60	3.40	4.00	–	2.60	2.00	–	–	–	–	–	–
C	–	4.67	4.58	3.67	2.92	2.83	–	–	–	–	–	–
D	–	4.67	4.58	3.67	2.92	2.83	–	–	–	–	–	–
E	–	–	–	–	–	–	–	4.64	5.07	5.29	4.71	–
Average	5.00	4.07	4.22	3.67	3.16	2.56	4.58	4.73	4.96	5.08	4.62	4.62

[a] Scoring system: 1 = extremely soft, 4 = just right, 7 = extremely tough.
[b] Scoring system: 1 = undesirable, 4 = medium, 7 = excellent.

Table 4—Effect of TPP level (0—1.2%) on average sensory scores for mullet sausages from four experiments

Experiments	Texture[a]			
	0%	0.3%	0.6%	1.2%
A	4.00	4.60	4.00	–
B	4.90	4.10	3.60	3.50
C	5.00	3.83	4.42	3.58
D	4.88	2.88	3.63	3.50
Average	4.70	3.90	3.95	3.53

[a] Scoring system: 1 = extremely soft, 4 = just right, 7 = extremely tough.

TPP effect

All sausages for TPP effect contained 0.5% Charsol, 20% soy, and 10% water in addition to the constant factors. The general trend in Figure 3 shows a drastic decrease in cooking loss from the addition of 0.3% TPP followed by a leveling off from 0.3–0.6%. A regression analysis showed highly significant ($\alpha < 0.01$) linear and quadratic trends confirming these observations. This agrees with the reports of Cohen et al. (1977), Pepper and Schmidt (1975), and Neer and Mandigo (1977) who found decreased cooking losses or increased yields with TPP additions. An interesting observation was a slight but consistent increase in water loss from 0.6–1.2%. Possible explanations for the decrease in cooking loss with TPP additions are that TPP can dissociate actomyosin and/or increase pH resulting in an increased electrostatic repulsion force which will increase available binding sites for water. Reasons for the increase in water loss with addition of TPP from 0.6–1.2% are not known.

A correlation coefficient of 0.93 was calculated for the average cooking loss data and the average texture panel scores (Table 4). Statistical analysis of the texture panel shows a significant linear trend ($\alpha < 0.05$). The value of 3.95 for 0.6% TPP and 3.90 for 0.3% TPP were very close to the optimum value for texture (4.00) (Table 4). The difference between these two scores was too small to be important in a practical application, and the cooking loss showed almost no change between 0.3 and 0.6% TPP. A value of 0.3% TPP was therefore considered the possible optimum level for TPP.

SUMMARY

THE OPTIMUM LEVELS of Charsol, soy, TPP, and water were determined using sensory evaluation and cooking loss data as criterion. Small amounts of either Charsol or TPP significantly improved the texture of the mullet sausages. Both water and soy significantly altered the texture of the product. Substantial levels of soy (in the 20% substitution range) could be incorporated into the sausages without decreasing sensory acceptability. Changes in texture could be correlated to cooking losses. The true optimum level of each factor was determined using these data as a basis for a response surface design in further research (Daley et al., 1978).

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Florida Agricultural Experiment Stations Journal Series No. 1118.

DEVELOPMENT OF A SAUSAGE–TYPE PRODUCT FROM MINCED MULLET USING RESPONSE SURFACE METHODOLOGY

L. H. DALEY, J. C. DENG and J. A. CORNELL

ABSTRACT

A sausage-type product was developed in an attempt to increase the acceptability and utilization of mullet. Seventeen formulations using varying proportions of fish, soy, H_2O, and sodium tripolyphosphate (TPP) were prepared and analyzed for cooking loss, shear force, expressible water and sensory acceptability. A response surface analysis was performed to determine the optimum combinations of ingredients to produce the most acceptable product. Results showed that all the responses were significantly influenced by the water level ($\alpha < 0.01$). Sensory ratings were affected by the soy level ($\alpha < 0.05$), and the objective results were strongly influenced by the TPP level ($\alpha < 0.01$). The analysis demonstrated that many combinations of ingredients could produce an acceptable product. The levels of each ingredient could be chosen according to economic or other considerations.

INTRODUCTION

ALTHOUGH LARGE NUMBERS of mullet are available in the Gulf Coast area (USDC, 1973), this fish has a low economic value because of flavor characteristics of the species and the rapid development of rancidity (Deng and Dennison, 1976). Recently, foreign countries have purchased large qualities of mullet roe (Cato et al., 1976), but the market demand for the leftover carcasses was small. It is therefore desirable to develop a mullet product with increased acceptability.

In this study, a sausage-type product was developed in an attempt to increase the acceptability and marketability of mullet. Mullet is not suitable for utilization as fish sticks or fish portions because the flesh contains both white and dark muscle (Camber, 1955; Deng et al., 1977). By grinding the flesh as in sausage manufacture, small pieces of dark flesh are distributed through the sausages. Since visibly nonhomogenous particles are typical to sausage products, the dark flesh is not detrimental to acceptability. A sausage-type product has the further advantage of a relatively high spice level which can mask the typical mullet flavor and the fishy flavor which may develop. Another advantage of a sausage product is consumer convenience, an important marketing consideration (Holahan, 1975). The sausage described in this paper requires no special equipment and could easily be prepared at home. This is particularly applicable to the Florida area where most of the individuals in the seafood industry are small businessmen who cannot afford large capital expenses. A final advantage of the sausage product is the possible incorporation of mechanically deboned fish should it become commercially available in the future.

Many studies concerning fish sausages have been done in Japan, but these products contain up to 10% starch (Tanikawa, 1963), imparting a rubbery texture unsuitable for the American market. In the United States, a fish-beef patty was developed by King and Flick (1973) and a fish frankfurter was developed by Carver and King (1971) which contained 10% fat and was an emulsion product. The sausage described in this paper differs from the previous studies as it contains neither added starch nor fat. Another difference from the previous work is the addition of textured soy flour as an extender and binder. Although much work has been done concerning the effects of soy on red meats, the interaction of fish and soy has not been adequately studied.

The purpose of this study was to determine the effect of three factors (soy, sodium tripolyphosphate, and water) on the organoleptic acceptability of the product. The effect of soy was studied because it is less expensive than fish, and the price of all muscle foods is expected to increase in the future. Sodium tripolyphosphate (TPP) was considered since it has been shown to increase cooking yields in red meats (Shults et al., 1972, 1976), poultry (Shults and Wierbiki, 1973), and fish (Lee and Toledo, 1976). The effect of water was also studied since water is a long-established factor in shrinkage and textural characteristics of the product (Kramlich et al., 1973). Liquid smoke flavoring (Charsol) was included in the sausage as a constant since it retards rancidity development, and improves sensory acceptability.

EXPERIMENTAL

Materials

Mullet (*Mugil cephalus*) were purchased fresh for each experiment from Cedar Key, FL during November and December when the fish are spawning (mature roe) and the largest landings occur. Fish were transported on ice to Gainesville where they were filleted, skinned, and washed on the same day. The cleaned fillets were packed in ice and stored in a 2°C refrigerated room until used. A liquid smoke flavoring (Charsol C-6) was supplied by the Red Arrow Company, Manitowoc, WI. Textured soy flour (Centex 400, caramel) was purchased from the Central Soya Company, Chicago, IL. Food grade sodium tripolyphosphate (TPP) was obtained from the FMC Corp., Philadelphia, PA. Spices, sugar, and salt were purchased from a local market. The spices used were mustard, ginger, paprika, mace, allspice, nutmeg, red pepper, cloves, sage, and monosodium glutamate.

Methods

Sausage manufacture. Sausages were always prepared on the day of fish purchase. Mullet fillets stored in ice for approximately 3 hr were cut into 1-inch squares and mixed with constant amounts of sodium nitrite, Charsol, salt, sugar, and spices. Sodium nitrite was added at a level of 100 ppm of the total raw weight. All other percentages were calculated on the same basis. A level of 0.5% Charsol C-6 was chosen based on a previous study (Daley and Deng, 1978) and the manufacturer's recommendations (Anonymous). Salt was added at a level of 1.6%, sugar at 0.9%, and spices at 1.1% as determined by preliminary sensory panels. The spice combination was also established by sensory panel experiments.

Varying amounts of rehydrated soy flour, water (as ice), and TPP were added to the constant factors according to a statistical response surface design. The soy and water levels both varied from 0–30% while TPP was added at levels ranging from 0–0.6%. The textured soy flour was rehydrated as 1 part soy to 3 parts water by weight. All the ingredients for a specific combination were stirred by hand in a mixing bowl (50 strokes) and ground through an electric food grinder (Hamilton Beach Co., Model No. 223) using a coarse plate. The ground batter was stirred 50 times more and again passed through the same grinder fitted with a stuffing horn attachment, and stuffed into edible collagen casing,

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0022-1147/78/0005-1501$02.25/0

Table 1—Effect of combinations of three independent variables on six dependent variables

Independent variables			Dependent variables					
% Soy	% TPP	% H_2O	Shear force (lb)	% Cooking loss	Exp. H_2O (Cm^2)	Flavor	Texture	O.A.[a]
6.08	0.122	6.08	18.90	19.07	11.26	4.89	5.00	4.89
23.92	0.122	6.08	19.15	21.99	10.26	4.44	4.56	4.44
6.08	0.478	6.08	18.42	16.84	12.48	4.56	4.56	4.67
23.92	0.478	6.08	18.08	18.78	11.70	4.33	4.78	4.67
6.08	0.122	23.92	14.53	22.69	11.55	4.67	4.67	5.00
23.92	0.122	23.92	17.53	22.45	11.06	4.11	3.11	3.67
6.08	0.478	23.92	13.38	20.32	10.91	4.44	3.67	4.00
23.92	0.478	23.92	12.41	20.33	13.40	4.00	3.33	3.67
0.00	0.300	15.00	17.60	18.74	11.77	4.44	5.00	4.67
30.00	0.300	15.00	19.22	19.94	10.63	4.22	4.78	4.67
15.00	0.000	15.00	19.93	25.83	9.75	3.44	3.67	3.67
15.00	0.600	15.00	16.15	21.59	11.32	4.78	4.33	4.67
15.00	0.300	0.00	19.23	18.01	11.14	4.44	4.67	4.78
15.00	0.300	30.00	13.30	24.16	10.64	4.22	3.56	3.44
15.00	0.300	15.00	17.36	19.83	10.22	4.44	4.00	4.00
15.00	0.300	15.00	17.00	19.29	10.21	4.11	5.00	4.44
15.00	0.300	15.00	17.28	19.68	10.53	4.56	4.56	4.67

[a] O.A. = Overall acceptability

(Teepak Coria®, No. 6730, 30 mm diameter). The sausages were allowed to equilibrate to 18°C at room temperature and were then baked at 185°C (375°F) for 50 min in a standard electric oven on a rack to allow for drainage of any drip. The sausages were cooled 30 min, weighed, and stored at 2°C in a barrier bag (Cryovac®, W.R. Grace & Co.) to prevent moisture loss.

Subjective evaluation. Sensory panels were always held the day following the purchase of the fish and preparation of the sausage to assure uniform freshness. The product was evaluated by the same 9 experienced judges. Before evaluation, the sausages were taken from the 2°C room and heated for 20 min at 205°C (400°F) in an electric oven. The sausage samples were sliced, allowed to equilibrate to room temperature, numerically coded, and served in random order to judges in individual booths. Three experimental combinations and a reference sample were included in each panel session. The reference was chosen from previously developed experimental products (Daley and Deng, 1978) and contained 20% soy, 10% water and no TPP. Red lighting in the panel booths was used to mask color differences among samples. Categories judged were flavor, texture, and overall acceptability. A seven-point hedonic scale with a reference was used, in which 4 = same as reference, 7 = extremely better than reference, and 1 = extremely poorer than reference.

Objective evaluation. Heat penetration was determined by placing copper-constantan thermocouples into the geometric centers of three sausages from both ends to give duplicate readings. The thermocouples were linked to an automatic temperature recorder (Westronics, Inc., No. M11B) to record the heat penetration. F_{180} values were calculated according to procedures in Ball and Olson (1957).

Percent cooking loss was calculated as the difference between the raw weight and cooked weight of a sausage, divided by the raw weight and multiplied by 100. Measurements were done in triplicate.

Shear force values were obtained with a Food Technology Corporation Texture Test System® Model TP1 using a 136 kg ring and maximum descent speed of 0.61 cm/sec. The cell used was a CS-1 standard blade shear-compression cell®. Core samples were 2 cm x 2 cm cylinders. Testing was always done the day following fish purchase and sausage preparation. Four to eight replicates were performed on each sample and data were reported as direct readings in pounds.

Expressible water was also evaluated the day following preparation, using a modification of the procedure reported by Hamm (1960). The major difference was a larger sample size to accommodate the non-homogeneity of the samples.

Salt-soluble protein was extracted at 4°C as follows: Twenty grams of minced mullet were blended for 30 sec with 200 ml of 0.6M KCl containing 0.01M $NaHCO_3$, and centrifuged at 10,000 rpm for 30 min; the supernatant was removed, and the precipitate was re-extracted with 160 ml of the same buffer and recentrifuged; the combined supernatants were analyzed for protein content by the biuret method (Gornall, et al., 1949).

Statistical–Theory. A response surface analysis (RSA) was performed on the data. This type of analysis permits the empirical prediction of the response for combinations of the factors not necessarily included in the actual experiment. In addition, the RSA provides a complete summary of the experimental results. If all the independent factors are quantitative variables, the response can be considered as a function of the levels of the variables. Thus

$$y = \phi (x_1, x_2 \ldots x_k) + e$$

where y is the response, ϕ is the response function or surface, k is the number of independent factors, and e is experimental error (Cochran and Cox, 1957).

In this experiment, three factors (soy, water and TPP) were chosen for their effects on the various responses and the function was expressed in terms of a quadratic polynomial equation (b represents a beta value), $\hat{y} = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{11}x_1^2 + b_{22}x_2^2 + b_{33}x_3^2 + b_{12}x_1x_2 + b_{13}x_1x_3 + B_{23}x_2x_3$, which measures the linear effect, quadratic effect, and interaction effect. Contours of a constant response were calculated by fixing one factor at a constant value and solving the equation for combinations of the other two factors. By changing the value of the fixed factor and solving the equation at the new level, the effect of the third factor was estimated. As with all statistical designs, responses cannot be extrapolated beyond the regions of experimentation.

Statistical–Design and analysis. Five levels of each of the three factors were chosen for study. Seventeen combinations (including three replicates of the center point) were chosen in random order according to a central composite rotatable design configuration for three factors (Cochran and Cox, 1957). The actual values of the three independent variables are shown in Table 1 with the average values of the raw data for the sensory and objective analyses.

The data were analyzed using the Statistical Analysis System (SAS) program package for the analysis of variance calculations. The surface contours were also calculated by computer. Computer coding assigned a value of −1.682 to the 0% level, −1 to 6.08%, 0 to 15%, +1 to 23.92%, and +1.682 to 30% for both soy and water. For tripolyphosphate, −1.682 corresponded to 0.000%, −1 to 0.122%, 0 to 0.300%, +1 to 0.478%, and +1.682 to 0.600%.

Soy was coded as X_1, TPP as X_2, and H_2O as X_3.

RESULTS & DISCUSSION

Heat penetration

Clostridium botulinum type E spores occur naturally in seafoods. U.S. Government regulations require an F_{180} = 30 min in smoke flavored fish (FDA, 1970) to control *C. botulinum* type E, which has a Z value of 16.5 (Schmidt, 1964). For this study, the sausages were baked at 185°C (375°F) for 50 min. Lethality calculations from the heat penetration data for this treatment yielded a slightly larger F_{180} value than the required internal heating time of 30 min at 82°C (180°F). Calculations were based on the slowest heating point of the sausages (geometrical center) in the

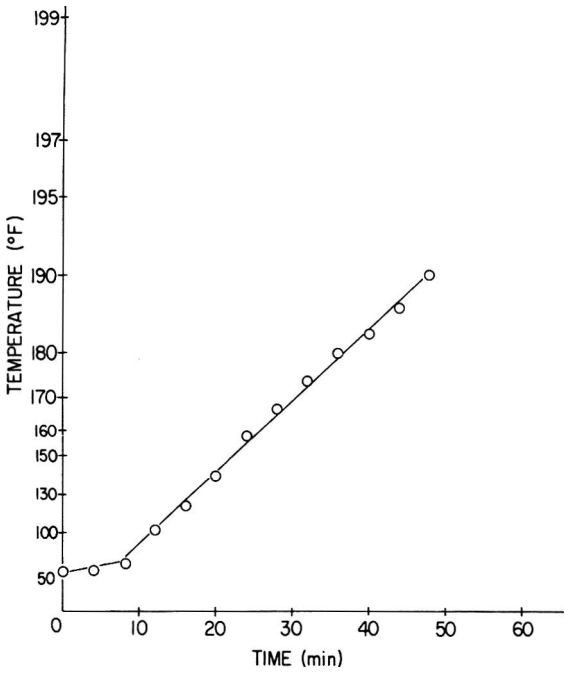

Fig. 1—Internal temperature of fish sausage baked 185°C (375°F) for 50 min.

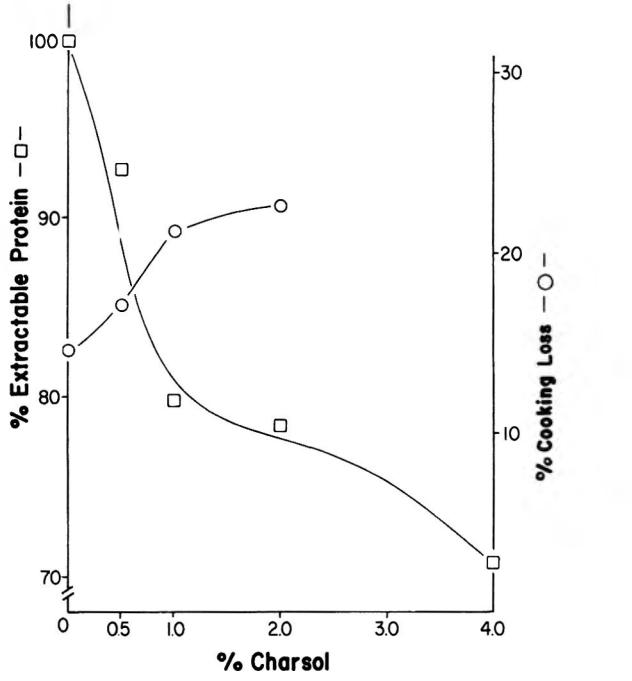

Fig. 2—Effect of Charsol level on cooking loss and extractability of salt-soluble protein in mullet sausages and mullet flesh.

coldest spot of the oven without including the effects of the cooling curve. This process should be sufficient, but microbial studies with inoculated samples should be done before marketing. Figure 1 shows the internal temperature of fish sausage when baked at 185°C (375°F); data are plotted on semi-log coordinates following the procedure of Ball and Olson (1957). A linear increase in temperature was observed after 9 min for the center part.

Charsol effect

Figure 2 shows the effect of various Charsol levels on the extractability of salt-soluble protein (from mullet flesh) and the cooking loss from sausages. Increasing the level of Charsol (0–2%) in the sausage batter causes an increase in the cooking loss. A possible explanation of this trend may be protein denaturation by the hydrophobic phenol compounds in the Charsol. This theory was tested by adding various amounts of Charsol to minced mullet flesh and allowing it to equilibrate for 30 min at 2°C before the protein extraction procedure. Analysis of duplicate samples showed that increasing Charsol levels resulted decreasing quantities of extractable salt-soluble protein, an indication of protein aggregation (denaturation) which reduces the number of water-binding sites. The correlation coefficient between extractable protein and the Charsol level was 0.989 which is highly significant ($\alpha < 0.01$).

Regression analysis

Summaries of the analysis of variance tables for each of the dependent variables are shown in Table 2 with their corresponding coefficients of determinations (R^2) (Mendenhall, 1975). The closer the value of R^2 is to unity, the better the empirical model fits the actual data. The smaller the value of R^2, the less influence the independent variables in the model have in explaining the behavior variation. In this study, only R^2 values greater than 0.900 were considered accurate enough for prediction purposes.

An R^2 value of 0.756 was calculated for expressible water. A lack-of-fit test produced an F value of 38.45 which is highly significant ($\alpha < 0.01$). This indicates that a higher degree model is probably required to explain water expressibility. Another possible explanation for this low R^2 value may be the method alteration. The original method used a sample weight

Table 2—Analysis of variance summaries for six dependent variables

Dependent variables	Code	R^2	Mean square regression	Mean square error	F	Probability
Shear force	Y_1	0.935	8.871	0.797	11.13	0.002*
Cooking loss	Y_2	0.923	8.659	0.924	9.37	0.004*
Expressible water	Y_3	0.756	1.106	0.459	2.41	0.130^{ns}
Flavor (panel)	Y_4	0.367	0.074	0.163	0.45	0.867^{ns}
Texture (panel)	Y_5	0.900	0.613	0.087	7.04	0.008*
Overall acceptability (panel)	Y_6	0.790	0.351	0.119	2.95	0.084^{ns}

* Significant at 0.01 level

of 300 mg which was not suitable for this nonhomogeneous product. The average sample weight used here was 1.275 grams, over four times as large. The data from this method showed differences between replicates that often exceeded differences between samples. The reproducibility was not improved when the experiment was repeated, and this response was omitted from further experiments since it was not suitable for this product.

An extremely low R^2 (0.367) was observed for the flavor panel evaluation, indicating that flavor panel results are not adequately described by the three factors in the form of the polynomial model used. Since the lack-of-fit test did not produce a significant F value, it is likely other independent variables are necessary if flavor is to be described in model form. This result was expected, since neither water nor TPP at the levels used should affect the flavor. This category was included in the analysis only to confirm the prediction that even high levels of soy cause only subtle flavor changes. No further statistical analyses were performed on the flavor panel responses because of the poor model fit.

A value of 0.790 was observed for the overall acceptability (panel) and the F value was significant only at the 0.084 level. The model was considered approximate and used for trend analysis, but not for prediction of optimum values.

The R^2 values for the shear force, texture panel, and

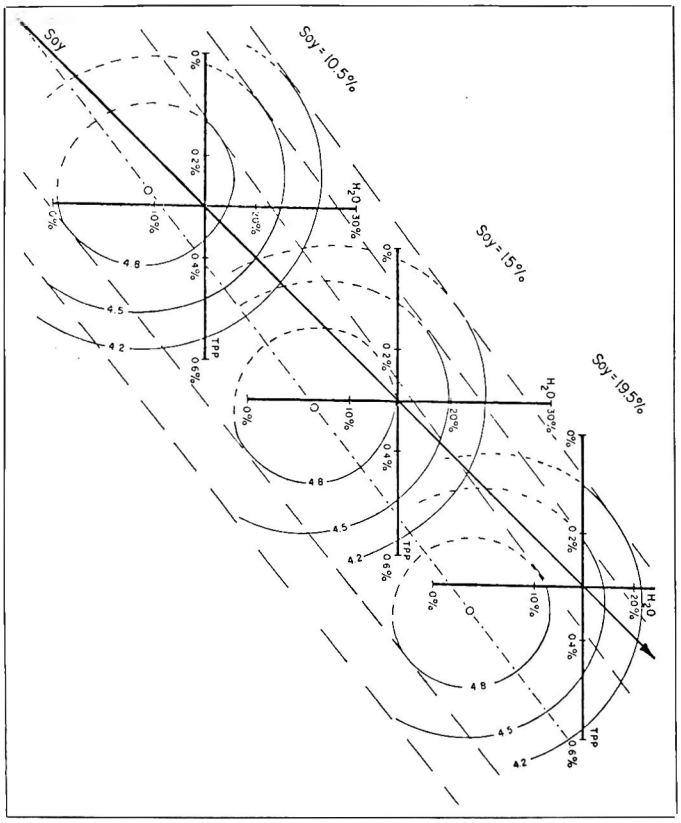

Fig. 3—Response surface contours for TPP and H_2O effect on the texture panel response at various soy levels (three dimensions).

Table 3—Regression coefficients for four dependent variables

Coefficient[a]	Shear force	Cooking loss	Texture panel	Overall acceptability
b_0 (intercept)	17.28**	19.67**	4.78**	4.58**
b_1	0.34	0.49	−0.26*	−0.24*
b_2	−1.04**	−1.25**	−0.01	0.05
b_3	−1.95**	1.42**	0.42**	−0.34**
b_{11}	0.19	−0.35	0.10	−0.05
b_{22}	0.06	1.19**	0.27*	−0.11
b_{33}	−0.57	0.27	0.23*	−0.13
b_{12}	−0.57	−0.09	0.26*	0.18
b_{13}	0.26	−0.64	−0.24	−0.15
b_{23}	−0.59	0.12	−0.04	−0.13

[a] 1 = soy level, 2 = TPP level, 3 = water level
* Significant at 0.05 level.
** Significant at 0.01 level.

Table 4—Predicted and observed values for reference sample

Dependent variable	Observed value	Predicted value	Difference
Shear force (lb)	19.02	20.27	1.25
Cooking loss (%)	25.35	24.98	0.37
Texture (panel)	4.00	3.88	0.12

Table 5—Some combinations of soy, TPP and water resulting in a predicted texture value of 5.01 as observed in Fig. 3

% Soy	% TPP	$\%H_2O$
10.5	0.27	9.65
15.0	0.31	7.06
19.5	0.35	4.30

cooking loss were all high (greater than 0.90) and predictions concerning these responses were made with confidence ($\alpha < 0.01$).

Coefficient estimates for four regression models and the results of significance tests on the coefficients are shown in Table 3. The estimates of the model coefficients (beta values) were calculated by the method of least squares and are in terms of the computer coding. According to the significance tests on the estimates, the H_2O level is probably the primary factor influencing texture since b_3 is highly significant for all four responses. TPP (b_2) has a significant influence on both shear force and cooking loss ($\alpha < 0.01$), but not on the panel responses for texture and overall acceptability. The two panel responses were influenced by the soy level ($\alpha < 0.05$) although the objective responses were not. This interesting contrast between the subjective and objective data has also been observed by other researchers. Cohen et al. (1977) reported reduced cooking loss in ground beef patties with no significant differences in the sensory scores.

To use the beta values to predict a response, values must first be converted into the computer code. The predicted responses for the reference sample were calculated and compared to the observed responses in Table 4. The estimates are close to the actual values, particularly the cooking loss and texture panel scores, demonstrating the usefulness of the response surface analysis in a practical application.

Contours of constant response values were computed for the three significant responses (texture panel, shear force, and cooking loss) and for overall acceptability for further analysis. Some representative contours for the texture panel response are shown in Figure 3 for three fixed levels of soy to demonstrate the three-dimensionality of the surface. The geometrical surface for texture panel is depicted as a cylinder with a slight tapering in the diameter of the cylinder as the soy level increases. The TPP and H_2O axes are in the plane of the paper; the soy axis passes through the plane of the paper. All three axes are drawn in heavy ink. Long dashed lines represent the longitudinal cylindrical surfaces. The short dashed lines curve around the cylinder circumference. A long and short dashed line connects the center points of the contours which represent the maximum responses (approximately 5.0) for given levels of soy. The contour lines around the center points represent cylinders within a cylinder in three dimensions. In two dimensions, the contour lines represent estimated taste panel responses. For example, any point on a 4.8 contour line should represent a combination of TPP and H_2O which will give a texture panel score of 4.8 at a fixed level of soy. It is also evident that different combinations of H_2O and TPP can yield the identical texture panel response of 4.8.

A general trend observed with increasing soy level was the shift of the contours downward and toward the right of the central axis. As the soy level increased, an optimum product could be maintained only by increasing the percent TPP while decreasing the percent H_2O (actual values shown in Table 5). As increasing soy levels caused more and more of the contours to shift out of the region considered in the statistical design, fewer combinations were possible for a response of 4.8 or better. A maximum response for panel texture could not be attained at 30% soy, confirming the significant beta value found for soy by the panelists.

General trends for TPP and H_2O can also be seen in Figure 3. For both H_2O and TPP, as the level added increased, the texture panel response increased to a maximum and then decreased. This trend was expected since water has a tenderizing effect on sausage products (Kramlich, 1971) and TPP decreases cooling losses from most products (Shults et al., 1972; Cohen et al., 1977). So at low levels of H_2O (or TPP), the product was too tough; when the H_2O (or TPP) level was high, the sausage was too soft. In either case, a lower score resulted

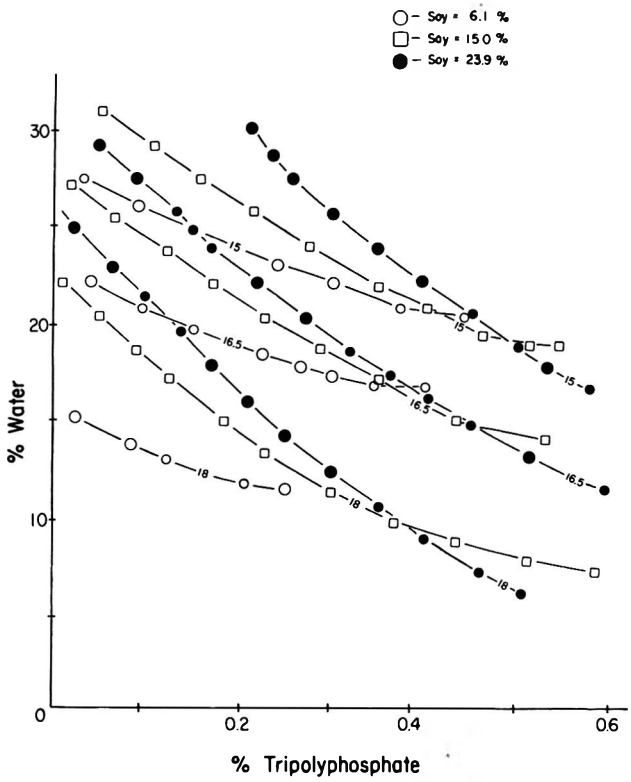

Fig. 4—Response surface contours for TPP and H_2O effect on shear force values at various soy levels.

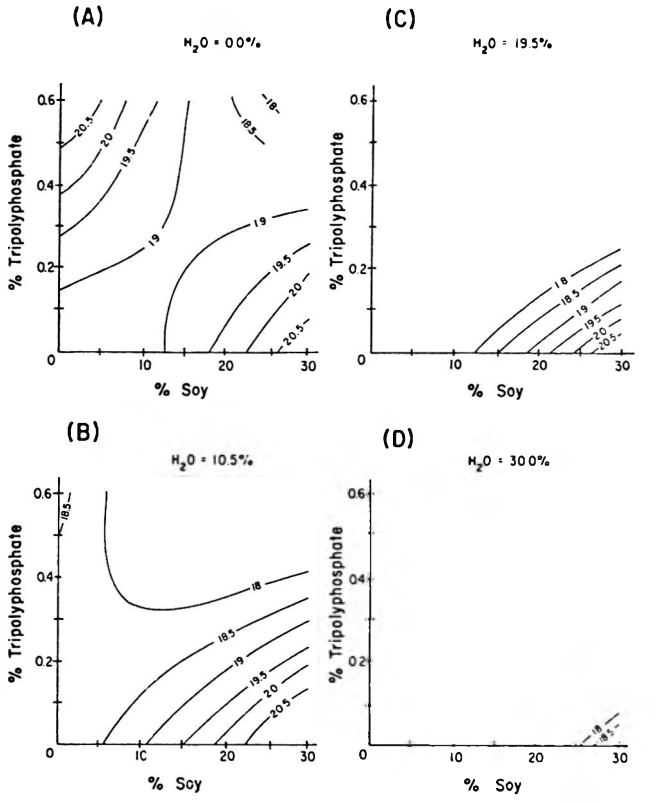

Fig. 5—Response surface contours for TPP and soy effect on shear force values at various H_2O levels.

as defined in the panel scale which assigned lower scores to samples less desirable than the reference regardless of the cause. The tenderizing effect of both H_2O and TPP confirmed the results of a previous study (Daley and Deng, 1978) which showed a general increase in tenderness with increasing levels of either factor.

Figure 4 shows some contours for the shear force responses. Shear force is a measure of the force required to shear a sample of standard size; the shear force values are therefore expected to increase as the product becomes tougher. The general trend observed was a decreased shear force value (implying increased tenderness) with increasing levels of both H_2O and TPP, in agreement with the texture panel results. To compare the shear force values with the previous texture panel scores, a correlation must be made, keeping in mind that although shear force values increase as the product becomes tougher (a linear response), the texture panel score increases to a maximum and then decreases (a quadratic response).

By substituting the optimum values for texture panel in Table 5 into the graphs for shear force (Fig. 5), predictions of the optimum value for shear force were made. The graph for 10.5% soy predicts an optimum of 18.0 lb; 15% predicts 18.7 lb, and 19.5% soy predicts 18.5 lb. This agrees with an experimentally derived estimate of 18–20 lbs for optimum texture (Daley, 1977).

Since the desired response of approximately 19 lb could not be best represented in Figure 4, the H_2O level was fixed and new surface response contours were generated for shear force values. The results of several representative levels are shown in Figure 5. The graphs of Figure 5A and 5B are typical minimax responses; the center is a minimum for some of the variables and a maximum for others. This is a complicated figure which cannot be easily represented geometrically. A general trend from 0–30% water was increased tenderness as observed before in Figure 4.

At H_2O levels above 10%, optimum texture resulted only from high soy and low TPP combination. Soy had only a toughening effect, while TPP had only a tenderizing effect. A decrease in shear force values with increasing TPP levels was also observed in pork by Shults et al. (1976).

At H_2O levels below 10% (Fig. 5A), the responses were more complicated and variations from the general trends were observed for specific factor combinations. Moderate levels of TPP with either high or low soy levels could produce an optimum response of approximately 19 lbs. At 0% H_2O, the general trend from 0–10% soy showed a tenderizing effect, while soy values of 10–30% had a toughening effect. Yet, at TPP levels above 0.47%, soy had only a tenderizing effect. TPP had the usual tenderizing effect at soy levels above 10% but actually toughened the product at soy levels below 10%. This interaction between soy and TPP was not expected.

Figure 6 shows the response surface contours for cooking loss at the 15% soy level. For all levels of TPP, as the level of added H_2O was increased, the cooking loss was also increased. The effect of water was still tenderizing, however, since the net retention of water was greater with higher water levels. For a fixed level of added H_2O, increasing TPP resulted in a decrease and then a slight increase in cooking loss. Interestingly, the transition point was almost exactly the same for all five levels of soy ranging from 0–30%; the transition level of TPP varied from only 0.37–0.41% for all observed levels of soy. This means that TPP levels above approximately 0.5% actually increase the cooking loss. The results of a previous study (Daley and Deng, 1978) also showed increased cooking losses between 0.5 and 1.2% TPP. Decreased yields at specific TPP levels were also observed in pork by Neer and Mandigo (1977) and by Schwartz and Mandigo (1976), but the decrease in yield occurred at 0.25% TPP and 0.375% TPP, respectively.

Figure 7 shows contours for overall acceptability (panel) with the soy level fixed at 15%. Since the R^2 was not quite large enough for accurate predictions, only general trends need be considered. Increasing TPP levels generally increased acceptability, although a slight decrease in acceptability was

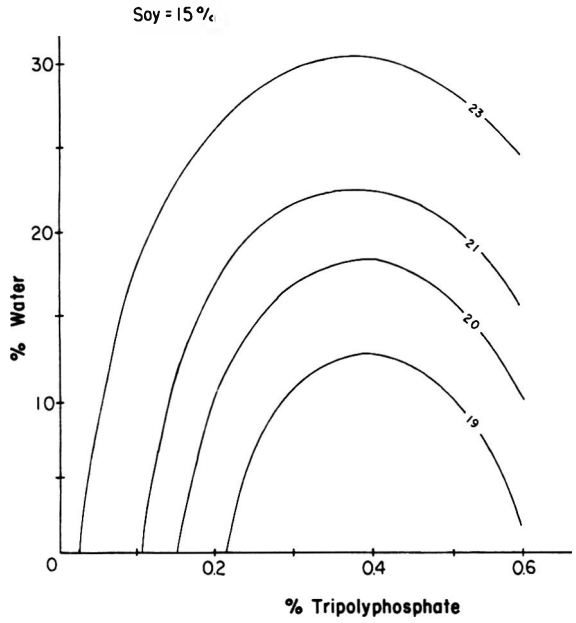

Fig. 6—Response surface contours for TPP and H_2O effect on cooking loss at a soy level of 15%.

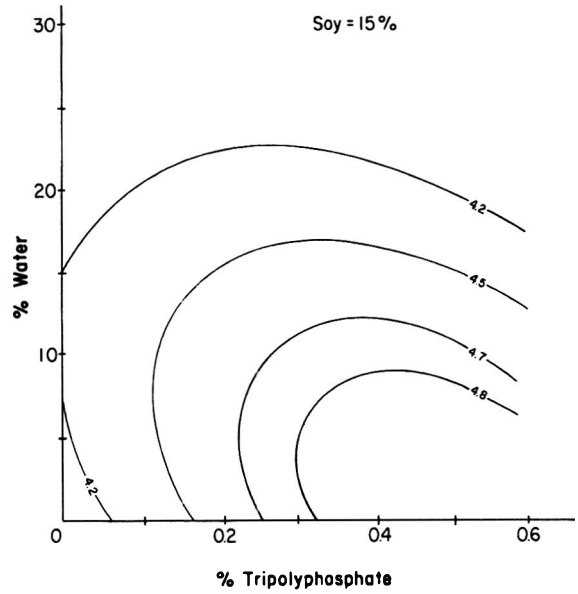

Fig. 7—Response surface contours for TPP and H_2O effect on panel overall acceptability scores at a soy level of 15%.

observed at TPP levels greater than 0.5%. This confirmed the transition point for the cooking loss data in Figure 6. Increasing H_2O levels generally decreased the overall acceptability score, but an increasing then decreasing trend was also observed for certain H_2O levels. Also at 30% soy (not shown), an optimum combination could not be obtained, agreeing with the texture panel results and the shear force values (Fig. 5D). At the high soy levels (not shown), the best product could be produced only with low H_2O and moderately high levels of TPP, the same result as for texture panel scores (Fig. 3).

CONCLUSIONS

RESPONSE SURFACE ANALYSIS could successfully be applied to subjective as well as objective data, although sensory panel results are often difficult to reproduce. Accurate predictions could be made concerning texture panel scores, shear force values, and percent cooking loss. The predictions from these three responses were in good agreement.

Many optimum combinations were possible, and an acceptable mullet sausage could be produced using a range of proportions of fish:soy:TPP:H_2O. A particular combination could be chosen on a basis of acceptability, economics, availability of raw materials, or other considerations.

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Ms received 6/8/77; revised 4/6/78; accepted 4/10/78.

Presented at the 37th Annual Meeting of the Institute of Food Technologists, Philadelphia, PA. June 5-8, 1977.

Florida Agricultural Experiment Stations Journal Series No. 599.

The authors thank Dr. D.M. Janky and the Poultry Science Dept. of the University of Florida for use of the Texture Test System®.

EFFECT OF DESINEWING VERSUS GRINDING ON TEXTURAL PROPERTIES OF BEEF

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ABSTRACT

The objective of this study was to evaluate the effect of mechanical desinewing (a new process whereby the major portion of the sinew or connective tissue and foreign particles is removed) vs grinding on textural properties of comminuted beef. Desinewing generally improved textural properties of beef patties with greatest effects on meat from mature animals. The 0.19 cm desinewing head was superior to 0.25 or 0.32, but further research is needed to refine head selection.

INTRODUCTION

IN THE UNITED STATES the consumption of ground beef has increased steadily over the past decade. Cross et al. (1976a, 1977) reported that connective tissue was and is a major problem associated with the acceptance of ground beef. They found that meat from U.S. Utility or lower quality or from minor cuts of any grade, produced a ground product that was unacceptably high in connective tissue. Cross et al. (1976b) reported that method of grinding affected textural properties of ground beef, especially the amount of connective tissue remaining in the cooked product. These results indicated that the size of the plates in the initial grind were directly related to the amount of connective tissue in the cooked product. The major sources of lean for ground beef are minor cut trimmings from young cattle and major cuts from older, lower quality cattle. Since it is not economically feasible for the industry to alter its source of lean, we are investigating methods of comminution that would remove a portion of the objectionable connective tissue.

A new process has been developed whereby the major portion of the sinew or connective tissue and foreign particles can be removed. The process is called "desinewing" and the device is an adaptation of the Beehive mechanical deboning machine. Gillett and Tanatikarnjathep (1976) reported that mechanical desinewing removed about half of the connective tissue from beef shanks and plates. The objective of this study was to evaluate the effect of desinewing versus grinding on textural properties of comminuted beef.

EXPERIMENTAL

EIGHTEEN FORMULATIONS of comminuted beef were prepared as outlined in Table 1. All raw materials were selected from the same day's slaughter. The various cuts were removed from each carcass, boned and shipped about 10 miles in a refrigerated truck to the commercial research laboratory. The plates and flanks from the U.S. Choice minor cuts were partially trimmed of fat prior to communition. About 40% of the Choice minor cuts consisted of undefatted foreshank lean and fat. Fat from U.S. Choice plates was added to the bull triangle and U.S. Utility triangle meat to increase the fat content.

For each treatment, a minimum batch of 50 kg was prepared. All raw material was passed through a grinder plate having 5.08 cm diameter holes. Grab samples were collected for fat analysis, and were further comminuted by passing them through a colloid mill. Fat content was determined by the Modified Babcock procedure. Fat content was standardized at 24 ± 2% by the addition of appropriate amounts of fat or lean. Based on previous determinations, the desinewing process was expected to remove 2–3% of the fat; therefore, the fat content for the raw materials to be desinewed was adjusted to about 26%.

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Table 1—Treatment formulations and listing of tables where comparisons are made

Raw beef material[a]	Methods of comminution	Table 2	3	4	5
Choice Minor Cuts (CM)	Grind (G)		X	X	
Choice Minor Cuts	Desinew 1 (D1)	X	X	X	
Choice Minor Cuts	Desinew 2 (D2)	X			
Choice Minor Cuts	Desinew 3 (D3)	X			
Utility Triangles (UT)	Grind		X	X	
Utility Triangles	Desinew 1	X	X	X	
Utility Triangles	Desinew 2	X			
Utility Triangles	Desinew 3	X			
Choice Flanks	Grind		X		
Choice Flanks	Desinew 1		X		
Bull Triangles (BT)	Grind		X		
Bull Triangles	Desinew 1		X		
CM/CM 50/50	G/D			X	
UT/UT 50/50	G/D			X	
CM/UT 50/50	G/G				X
CM/UT 50/50	G/D				X
CM/UT 50/50	D/G				X
CM/UT 50/50	D/G				X

[a] CM = flanks, plates and foreshanks; UT and BT = plates, foreshanks and chucks; G = Three stage grind, 5.08, 1.90 and 0.32 cm plates; D1 = 0.19 cm head; D2 = 0.25 cm head; and D3 = 0.32 cm head

Grinding

Following the initial grind and fat adjustment the meat was mixed for 2 min in a mechanical mixer. Temperature was monitored during the mixing process and if it exceeded 2–3°C, sufficient CO_2 snow was added to bring the internal temperature of the ground product below 3°C. After mixing, the product was ground through a 1.90 cm plate and finally through a 0.32 cm plate.

Desinewing

Meat was passed through a Beehive Desinewing Machine (Model AU 6173). Heads of three different sizes were used and had holes with diameters of 0.19, 0.25 and 0.32 cm. Prior to the desinewing, fat content was adjusted by the addition of fat that had been passed through the 0.19 cm desinewing head. The product was mixed for 2 min and CO_2 snow was added if necessary.

Combinations

Various combinations of raw meat materials (U.S. Choice minor cuts and U.S. Utility triangles) and methods of communition (grind and desinew) were prepared. Desinewed meat (0.19 cm head) was added in equal amounts to ground meat after it had been passed through the 1.90 cm plate. Following a 2-min mix, the product was ground through the 0.32 cm grinder plate. Desinewed Utility triangles and Choice minor cuts were combined in equal amounts and mixed for 2 min but were not ground.

Patties

Communited meat from each treatment was formed into 142g patties with a Hollymatic Patty Machine. Patties were placed in plastic bags, covered with CO_2 snow and placed in a −20°C freezer. After 72 hr, patties were packed in dry ice and shipped via air freight to Beltsville, MD.

Cooking

Patties were broiled from the frozen state on electric Farberware grills (model 450) for 6 min per side. Cooked patties were quartered and served immediately to panelists for sensory evaluation. Degree of

Table 2—Effect of desinewing method on the textural and chemical properties of beef[a]

Desinewing head size[b] (cm)	Tenderness		Panel connective tissue amount		Instron shear force (kg)		Total collagen (mg/g, wet basis)	
	CM	UT	CM	UT	CM	UT	CM	UT
0.19	6.31b	6.61b	6.01b	6.36b	6.64b	6.26b	14.39c	16.61c
0.25	5.70b	5.50b	5.26b	4.40c	6.62b	10.07c	16.00c	27.20b
0.32	5.77b	4.78c	5.51b	3.92c	8.05b	11.18c	25.47b	22.31b

[a] Means in the same column followed by the same letter are not significantly different ($P < 0.10$).
[b] Desinewing methods differed according to diameter of holes in the heads of the machine.

doneness was evaluated, by comparison with color photographs, on all cooked patties 5 min after cooking.

Panel selection and training

A 10-member panel was selected and trained in descriptive attributes by the procedures of Cross et al. (1978). The panel rated the following attributes on an 8-point structured scale: (a) tenderness and juiciness, with 8 = extremely tender and juicy; and 1 = extremely tough and dry; and (b) connective tissue amount with 8 = none and 1 = abundant. Panelists rated five samples at each of 22 sessions. Each treatment was replicated six times and samples to be evaluated in each session were selected via a table of random numbers.

Instron shear

Ten patties from each treatment were broiled for determination of shear values. The cooked patties were cooled for at least 2 hr, and shear force was measured on each quarter of the patty with the single blade shearing device attached to the Instron as described by Cross et al. (1976b). Data were recorded for the maximum force required to shear through the meat patty.

Hydroxyproline

Duplicate samples (4g) of frozen powdered meat from each treatment were heated for 70 min at 77°C in 0.25% strength Ringer's solution and separated into supernatant and residue fractions according to the procedures of Hill (1966). Each fraction was individually hydrolyzed in 6N HCl at 102°C for 16 hr at 1 atm pressure.

The hydroxyproline content was determined as outlined by Bergman and Loxley (1961). Total collagen content (mg/g) was calculated by multiplying the hydroxyproline content of the residue by 7.25 and that of the supernatant by 7.52.

Statistical analysis

The experimental design was structured so that each treatment occurred once in each replication and once in each order (1 to 5). The design was a partially balanced incomplete block design according to Clatworthy (1973). Each observation used in the analysis was the average of the scores of all panelists. Data were subjected to statistical analysis of variance according to Snedecor (1956) and to mean separation by Scheffe's method (1959). Means for the taste panel traits were adjusted for session/replication effects. Means for degree of doneness were evaluated by nonparametric analysis of variance (Kraskal-Wallis procedure) discussed in Hollander and Wolfe (1973).

RESULTS & DISCUSSION

DATA IN TABLE 2 describe the effect of desinewing method on textural and chemical properties of beef. Since interactions between type or grade of meat and method of communition were significant, the data are presented separately for each type and grade of meat. Three desinewing methods (0.19, 0.25 and 0.32 cm hole sizes) were applied to Choice minor cuts (CM) and Utility triangles (UT). No significant differences among methods were evident for CM cuts in either panel or Instron ratings (Table 2). For UT, the methods with the 0.19 and 0.25 cm head sizes were superior in terms of panel ratings of tenderness, and the 0.19 cm head was best for connective tissue amount and Instron shear force.

Degree of doneness, cooking losses, and juiciness were not significantly affected ,by method of desinewing (data not shown). As the hole size increased from 0.19 to 0.25 and 0.32 cm, the amount of total collagen in the final product increased significantly. With CM or UT, the amount of collagen in the product was significantly less from the 0.19 than the 0.32 cm head. These differences in collagen were also reflected by the differences in the panelists' ratings for tenderness and connective tissue.

Based on the results in Table 2, the desinewing method with the 0.19 cm head was used to prepare meat for comparison with ground meat. Data in Table 3 describe the effect of grinding or desinewing on the textural and chemical properties of beef. The two methods of communition (grinding and desinewing—0.19 cm head) were applied to Choice minor cuts (CM), Utility triangles (UT), Choice flanks (CF) and bull triangles (BT). Bull triangles were from 'C' maturity carcasses and UT from D/E maturity carcasses. Although not all differences were significant, desinewed patties from CM cuts consistently tended to be rated more tender (panel and Instron) and lower in detectable connective tissue than ground patties (Table 4). Patties from desinewed UT and BT cuts were rated significantly more tender (panel) and lower in connective tissue than patties from ground UT and BT. Patties from desinewed BT were also more tender, as indicated by lower Instron values, than patties from ground BT. Desinewed UT patties also had significantly higher juiciness ratings than ground patties (not shown in table).

Apparently, the effect of desinewing was greatest on cuts from older (C-E versus A maturity) carcasses, which usually

Table 3—Effect of grinding or desinewing method[a] *on the textural and chemical properties of beef*[b]

Type of cut[c]	Tenderness		Panel connective tissue amount		Instron shear force (kg)		Total collagen (mg/g, wet basis)	
	Grind	Desinew	Grind	Desinew	Grind	Desinew	Grind	Desinew
CM	5.61c	6.31c	5.56c	6.01c	7.35c	6.64c	33.52c	14.39d
UT	5.36d	6.61c	4.95d	6.36c	7.49c	6.26c	29.86c	16.61d
CF	5.18c	5.49c	5.90c	5.66c	6.09c	7.72c	10.86c	12.47d
BT	5.08d	6.83c	4.63d	6.97c	8.72c	5.55d	13.80c	7.67d

[a] Grinding = 5.08; 1.90 X 0.32 cm plates and desinewing = 0.19 cm diameter holes.
[b] Paired means in the same row followed by the same letter are not significantly different ($P < 0.10$).
[c] CM = Choice minor cuts; UT = Utility triangles; CF = Choice flanks; BT = bull triangles.

Table 4—Textural and chemical properties of various mixtures of ground and desinewed beef[a]

Percent desinewed beef[b]	Panel tenderness		Instron shear force, kg		Panel connective tissue amount		Total collagen (mg/g, wet basis)	
	CM	UT	CM	UT	CM	UT	CM	UT
0	5.61b	5.36bc	7.35b	7.49bc	5.56b	4.95c	33.52b	29.86b
50	6.08b	4.84c	6.94b	9.94b	6.36b	4.76c	25.96c	29.30b
100	6.31b	6.61b	6.64b	6.26c	6.01b	6.36b	14.39d	16.61c

[a] Means in the same column followed by the same letter are not significantly different ($P < 0.10$).
[b] Desinewed beef was passed through the 0.19 cm head.

have large amounts of tough connective tissue. This is important because mature animals are the major source of beef for hamburger. Cross et al. (1976a, 1977) indicated that ground beef from old animals is unaceptably high in connective tissue. The panel rated patties made from ground BT lowest in quality in terms of tenderness and connective tissue, but rated those from desinewed BT highest for these same traits (Table 3).

The effects of desinewing versus grinding on total cooking loss and degree of doneness were not significant (data not shown). From all cuts except CF, desinewed beef contained significantly less collagen than ground beef. These differences were reflected in the ratings for tenderness and connective tissue (Table 3).

Three mixtures of desinewed and ground beef from two classes of cuts (CM and UT) were prepared as outlined in Table 4. Generally, as the percent desinewed meat of the CM formulation increased, ratings for tenderness increased and Instron shear force decreased. Although these differences were not significant, the trend is similar to that in Table 2. For UT, 100% desinewed beef was the best in terms of tenderness, connective tissue and Instron shear force. It is impossible to explain why UT containing 50% ground/desinewed beef were rated tougher than either of the other formulations. This segment of the experiment will be repeated to determine whether that effect was real. Juiciness, cooking losses, and degree of doneness ratings were not significantly affected by the amount of desinewed beef in the formulation (data not shown). As expected, total collagen decreased as the percentage of desinewed beef increased. The change was greatest between 100% and 50% desinewed.

Various combinations of desinewed and ground cuts were prepared and the results are presented in Table 5. From CM and UT cuts, four 50/50 mixtures in all combinations, were prepared of ground and desinewed meat. The CM ground/UT desinewed and CM desinewed/UT ground treatments were more tender, according to the Instron, than the CM ground/UT ground mixture. Juiciness, total cooking loss, and degree of doneness did not differ significantly among the four mixtures (data not shown). Mixtures containing UT desinewed beef contained significantly less total collagen than other mixtures. In mixtures with ground UT beef, desinewing of CM did not significantly reduce total collagen. Generally, there appears to be few differences in tenderness between mixed ground beef regardless of the desinewing treatment.

CONCLUSIONS

DESINEWING generally improved the textural properties of beef patties. The effects of desinewing were greatest on meat from mature animals that were high in connective tissue. That finding is economically important because most ground beef is now prepared with meat from mature animals or with minor cuts from younger animals. Meat desinewed through the 0.19 cm head was superior to that desinewed through the 0.25 and 0.32 cm heads. Choice of the method of communition probably should be based on the nature of the raw material, but further research is needed to refine the selection of heads.

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Table 5—Effect of combinations of desinewed and ground cuts on textural and chemical properties of beef[a]

Formulation[b]	Panel tenderness	Instron shear force, kg	Panel connective tissue amount	Total collagen (mg/g, wet basis)
CM ground/UT ground	5.76c	7.95c	5.41c	18.53c
CM ground/UT desinewed	5.32c	5.97d	5.47c	14.08d
CM desinewed/UT ground	5.75c	5.22d	5.76c	18.00c
CM desinewed/UT desinewed	6.07c	6.85cd	5.82c	13.56d

[a] Means in the same column followed by the same letter are not significantly different ($P < 0.10$).
[b] Desinewed beef was passed through the 0.19 cm head. Beef from Choice minor cuts and Utility triangles were combined in equal amounts.

OBJECTIVE MEASUREMENTS OF TEXTURE IN GROUND BEEF PATTIES

H. R. CROSS, MARILYN S. STANFIELD and W. J. FRANKS JR.

ABSTRACT

Beef patties were prepared from carcasses ranging in quality grade from U.S. Prime to Cutter and formulated into 16 grade/cut combinations. Texture was evaluated for each sample with three devices (two shear and one compression) adapted to the Instron Universal Testing Machine. The single blade shear (SBS) and circle blade shear (CBS) were used to determine the maximum force required to shear the sample and to measure the area under the curve, i.e., work. The maximum force required to compress a core to one-half its original thickness also was determined. In simple correlations, machine readings for single blade area under the curve and for maximum single blade shear force were the most highly correlated with the subjective panel evaluation of connective tissue amount (−0.93 and −0.92) and tenderness (−0.89 and −0.88). Correlations for CBS were highly significant but had lower coefficients (−0.86, −0.78; −0.82, −0.76; respectively) than the SBS. Regression equations containing single blade area, circle blade area and compression force accounted for 95.7% of the variability in subjective panel evaluations for amount of connective tissue and when circle blade shear replaced circle blade area, accounted for 92.6% of the variability in panel ratings for tenderness.

INTRODUCTION

RESEARCH on objective methods of measuring texture is not a new development. Lehman (1907) described two instruments for testing the tenderness of meat. Schultz (1957) summarized the advantages and disadvantages of various mechanical methods that had been used to measure meat tenderness. Voisey (1976) provides an excellent review of instruments used for meat tenderness evaluation. Interest continues in the development of new devices and the modification of existing devices, for the measurement of texture in meat (Szczesniak and Torgeson, 1965; Burrill et al., 1962; Sperring et al., 1959; Kelley et al., 1960; Spencer et al., 1962; Smith and Carpenter, 1973; Segars et al., 1975).

Tenderness is the principal attribute associated with texture in meats as evidenced by consumer preferences for tender meats. Of the many mechanical devices used to simulate or measure tenderness or texture as perceived by the sensory panel, the Warner-Bratzler instrument is probably the most popular (Szczesniak and Torgeson, 1965). That device is simple and inexpensive but generates data that may not always correlate highly with sensory tenderness. Szczesniak and Torgeson (1965) stated "that 41 of 51 research studies established good to highly significant relationships between Warner-Bratzler shear and sensory tenderness with the remaining studies showing poor to no significant correlations with sensory tenderness."

Many researchers have adapted the meat shear principle of the Warner-Bratzler apparatus to use with the Instron Universal Testing Machine. Because of its basic design and its capability to record force-distance relationships accurately, the Instron may be used to quantify other parameters of texture other than maximum shear force.

Few researchers have used instruments to evaluate texture in ground beef. Cross et al. (1976) demonstrated sensory panel differences in texture (tenderness and amount of connective tissue) in ground beef formulated from different U.S. quality grades. We now report a secondary phase of that study. We evaluated three devices that were designed for use on the Instron to objectively measure texture in ground beef patties. The different U.S. quality grades and cut combinations provided ranges of tenderness, juiciness and connective tissue so that we could evaluate any possible relationships between mechanical measurements of texture and the sensory responses of a trained panel. We wanted to determine whether the mechanical devices could accurately evaluate, with respect to human evaluations, the textural characteristics of cooked ground beef patties.

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Table 1—Sample combination designations

Combination no.	Grade/Cut
1	Prime/chuck
2	Choice/chuck
3	Good/chuck
4	Utility/chuck
5	Cutter/chuck
6	Prime/plate
7	Choice/plate
8	Good/plate
9	Utility/plate
10	Cutter/plate
11	Prime/combination[a]
12	Choice/combination
13	Good/combination
14	Utility/combination
15	Cutter/combination
16	Choice and Cutter/combination

[a] Combination consisted of chuck and plate in equal proportions

EXPERIMENTAL

GROUND BEEF SAMPLES were prepared from 16 different grade/cut combinations (Table 1). Carcasses which were selected and fabricated as described by Cross et al. (1976), ranged in quality grade from U.S. Prime through U.S. Cutter to provide variation in texture. Fat content was standardized at 24 ± 2% by the addition of appropriate amounts of fat or lean and was determined with the Modified Babcock procedure. The ground meat was formed into 75-g patties which were 10 cm in diameter and 0.95 cm thick with a Hollymatic Patty Machine. All patties were placed in boxes (4.5 kg to a box), frozen in a blast freezer (−30°C), and shipped from Oklahoma City, OK, to Beltsville, MD, via air freight.

Cooking

Patties were roasted from the frozen state in a 200°C oven for 9 min, quartered, and served as hot as possible to the panelists. Patties were allowed to cool for 60 min after cooking before sampling for shear and compression.

Panel selection and training

Male and female panelists were selected from the scientific and ancillary staff of the Agricultural Research Center, Beltsville, MD. Panelists were selected and trained according to the procedure outlined by Cross et al. (1978). Panelists individually evaluated the samples under red lights in booths.

The trained panel rated each patty for tenderness and juiciness on a

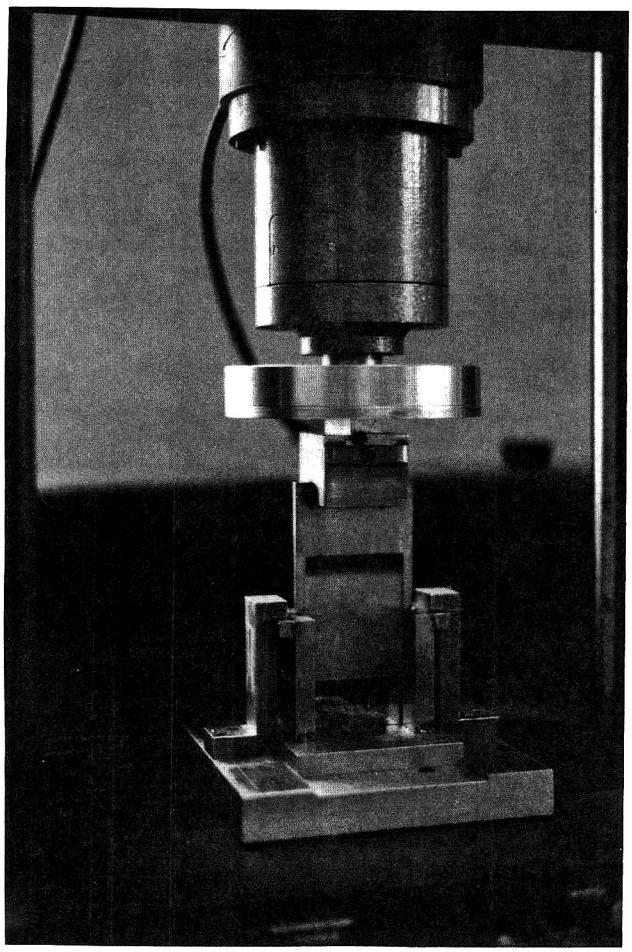

Fig. 1—Single blade shear.

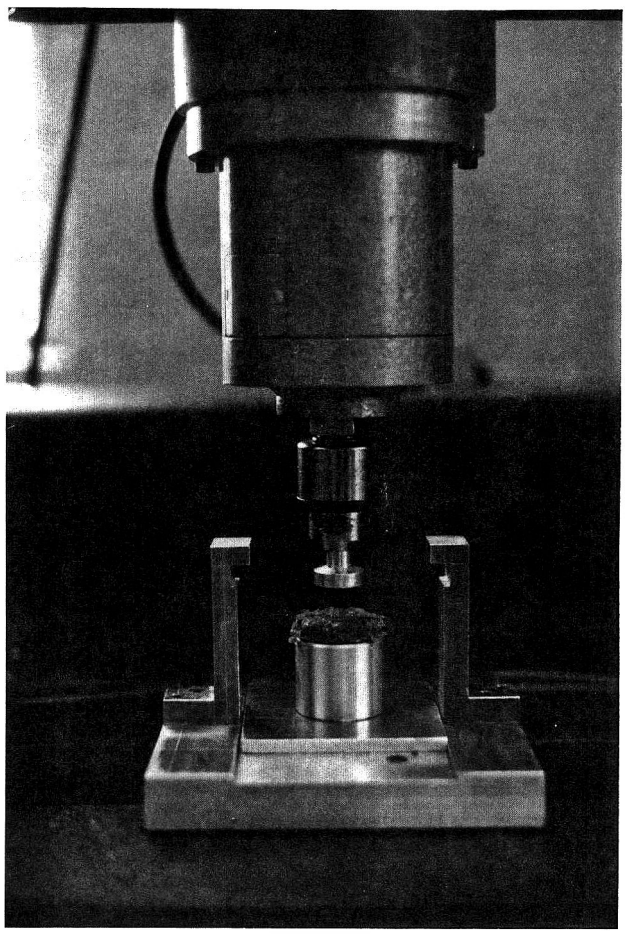

Fig. 2—Circle blade shear.

9-point scale (9 = extremely tender or juicy and 1 = extremely tough or dry). Tenderness and juiciness were evaluated during the first 5–10 chews. The amount of connective tissue residue remaining at the end of mastication was rated on a 9-point scale (9 = none and 1 = very abundant amount). The taste panel evaluations were conducted over eight 3-day periods. Panelists evaluated six ground beef samples each day so that during each 3-day period all 16 grade/cut combinations were tested. In addition, two combinations were repeated once during each 3-day period. If each panelist had been present for every session, there would have been an equal number (9) of evaluations by each panelist on every ground beef combination. In the "ideal" panel situation each combination would have received 144 evaluations, but panelists' attendance was not perfect. Three panelists had fewer than 50 evaluations and averaged 120 evaluations out of a possible 144. To avoid problems in statistical analysis, created by a side disparity in the number of evaluations, we eliminated the data from these three members.

Mechanical measurements

Shear and compressive force tests were carried out with the Instron Universal Testing Machine (type TM). Two shear and one compression devices were adapted to the Instron machine. The rate of crosshead descent for all three tests was 25.54 cm per minute. All test cells were manufactured in-house. The single blade shear (SBS) device used in this study (Fig. 1) consisted of a rectangular dull-edged blade 6.00 cm wide and 0.11 cm thick. There was a 0.04 cm clearance between the shearing edge of the blade and the corresponding edge of the hole in the base plate where the shearing action occurred. Two cooked patties from each grade/cut combination were tested with the SBS. Each patty was cut into four equal sections (3.15 cm square × 0.95 cm thick) to give eight samples per combination (Table 1).

The circle blade shear (CBS) device (Fig. 2) was similar to the slice tenderness evaluator (STE) described by Kulwich et al. (1963) and Segars et al. (1975), except that the CBS device did not have a mechanism to puncture the sample prior to shear. The CBS device consisted of a flat plunger or coin shaped device, 2.52 cm in diameter and 0.72 cm thick, that sheared a sample placed over a cylindrical base. There was 0.01 cm clearance between the shearing edge of the device and the corresponding edge of the hole in the cylinder. Two cooked patties from each grade/cut combination were tested with the CBS. Each patty was cut into three cores (3.81 cm in diameter × 0.95 cm thick) to give six samples per combination (Table 1). Data were recorded for maximum shear force (SBSHEAR and CBSHEAR) and for work or area under the peak curve (SBSAREA and CBSAREA).

The compression device consisted of a flat plate (9.68 cm × 10.16 cm) and a pedestal-like device 7.62 cm in diameter and 0.65 cm thick (Fig. 3). A core (3.81 cm in diameter × 0.95 cm thick) was placed on the plate and the force required to compress the cooked ground beef core to one-half its original thickness was measured. Eight cores were from each of two patties. Ten consecutive "strokes" or "chews," were made on each of the eight cores to give a total of 80 readings for each grade/cut combination (Table 1). Plots (not shown) of compression force (COMFORCE) versus stroke number (1–10) for each core within a combination were obtained. COMFORCE tended to steadily decrease from the first stroke through the tenth, with the rate of decrease becoming less with increasing stroke number. For any given combination, a relatively consistent difference existed between the COMFORCE plots of the eight cores. Examination of these plots indicated that for each stroke the average value of COMFORCE of the eight cores was meaningful in describing the relationships between COMFORCE and stroke number. For purposes of statistical analysis, we chose to average COMFORCE over all cores and strokes for each combination.

The overall average for sensory panel evaluations were used for each grade/cut combination, so there is one observation for each. The average for each combination was the best estimate of the "true" panel response. In this study, our primary interest was that "true," yet unknown, response. The rationale was similar for the use of the average machine reading to represent each combination.

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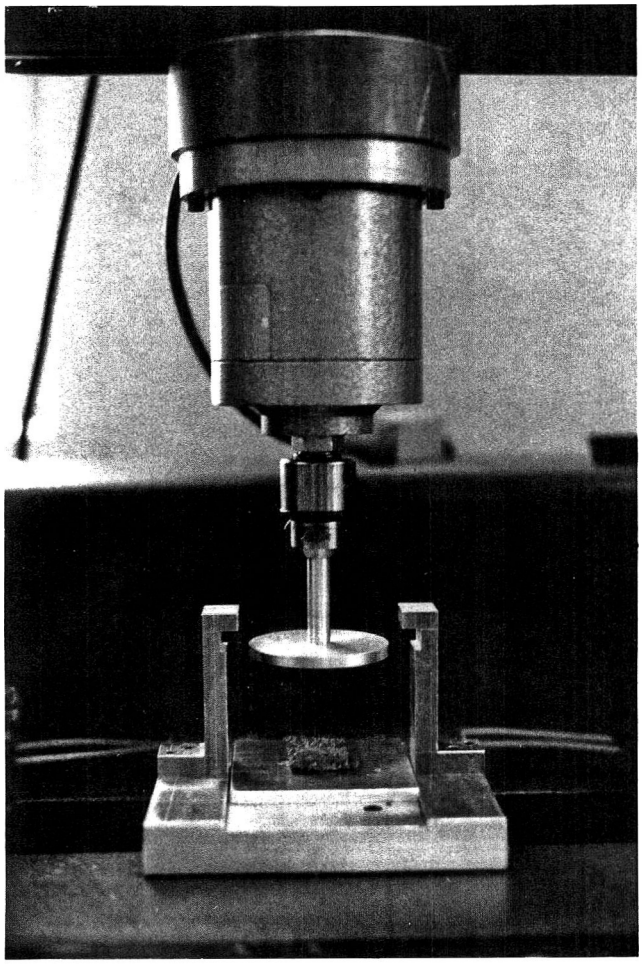

Fig. 3—Compression device.

Table 2—Simple correlation coefficients of the Instron readings and palatability characteristics

Instron	Palatability characteristics		
	Tenderness	Juiciness	Connective tissue
SBSHEAR[a]	−0.88**	−0.58*	−0.92**
SBSAREA[a]	−0.89**	−0.52*	−0.93**
CBSHEAR[a]	−0.76**	−0.56*	−0.78**
CBSAREA[a]	−0.82**	−0.47	−0.86**
COMFORCE[a]	−0.19	−0.14	−0 08

[a] SBSHEAR = single blade shear; SBSAREA = single blade area; CBSHEAR = circle blade shear; CBSAREA = circle blade area; and COMFORCE = compression force.
* Significant at the $P < 0.05$ level.
** Significant at the $P < 0.01$ level.

Table 3—Overview of regression analyses with all five Instron readings as potential independent variables

Independent Instron	Dependent variables[a,b]		
	Tenderness	Juiciness	Connective tissue
SHSHEAR		X	
SBSAREA	X		X
CBSHEAR	X		
CBSAREA			X
COMFORCE	X		X
R^2 (%)	92.6	33.2	95.7
S.E.E.	0.312	0.235	C.329

[a] An "X" indicates the independent variables that were ultimately selected by the stepwise procedure when the appropriate levels of significance were set at 5%.
[b] The overall sensory panel average evaluation and average Instron reading were used for each grade/cut combination. Consequently, there is one observation for each combination.

Statistical analysis

Results of this study apply only to the average panel responses and to the average readings obtained from the Instron machine and do not necessarily represent evaluations by a single panelist or a single machine measurement. We used the stepwise regression procedure, described by Draper and Smith (1966), to select the best regression equations. We also investigated results from the forward selection and backward elimination regression procedures of Draper and Smith (1966). Those two procedures usually produced the same regression equations as the stepwise procedure. Instron readings were used as independent variables and sensory panel traits as dependent variables. The appropriate levels of significance were set at 5%. Regression analysis was used solely to indicate which combinations of Instron readings were meaningful in predicting sensory panel traits. We place no particular importance on the specific values of the partial regression coefficients and have not included them in our results and discussion.

RESULTS & DISCUSSION

CROSS ET AL. (1976) reported significant differences at the 5% level of significance between the 16 grade/cut combinations for panelist evaluations of tenderness, juiciness and connective tissue (Table 1). Results of analysis of variance (not shown) indicated that the means of the 16 combinations differed at the 5% level of significance, for each type of Instron measurement. Evaluation of the relationship between palatability characteristics and Instron readings begins with the simple correlation coefficients in Table 2. SBSAREA and SBSHEAR were most highly correlated with sensory panel scores for tenderness and connective tissue amount. The relationship between compression force and sensory panel traits was low and nonsignificant.

In the first set of regression analyses, all five Instron readings were considered as potential independent variables (Table 3). The Instron readings explained 92.6% and 95.7%, respectively, of the variability of tenderness and connective tissue amount. The low R^2 for juiciness apparently reflects the inability of the Instron shear and compressive force measurements to meaningfully correlate with sensory juiciness.

In stepwise regression analysis, the resulting equation is highly dependent upon the specific potential independent variables that are submitted for analysis. Any changes in the number and type of potential independent variables can result in different equations. Because of this situation, different sets of independent variables were used. Overviews of the results of these analyses for tenderness and connective tissue amount are provided in Tables 4 and 5, respectively.

In Tables 4 and 5 use of area readings in combination (SBSAREA and CBSAREA) usually gave slightly higher values of R^2 than use of shear readings (SBSHEAR and CBSHEAR)—equations 2 and 6 versus equations 1 and 5. The same situation existed when a third Instron variable (COMFORCE) was added—equation 4 versus equation 3 in Table 4—and the values of the R^2's were not markedly increased.

For readings from either the single blade or the circle blade, either the area or shear values are meaningful alone, but not together. That finding is a direct result of the high correlation (not shown) between shear and area readings from the same device.

Some plots (not shown) of Instron readings versus palatability characteristics indicated that the relationship was slightly curvilinear and, therefore, suggested that the squares of those readings might improve the regression equations (data

Table 4—Overview of regression analyses for sensory panel tenderness

	Independent variable[a,b]						
Eq	SBSHEAR	SBSAREA	CBSHEAR	CBSAREA	COMFORCE	R^2 (%)	S.E.E.
1	X		X			84.0	0.440
2		X		X		84.3	0.436
3	X		X		X	90.4	0.356
4		X		X	X	91.6	0.333

[a] An "X" indicates the independent variables that were ultimately selected by the stepwise procedure when the levels of significance were set at 5%.

[b] The overall sensory panel average evaluation and average Instron reading were used for each grade/cut combination. Consequently, there is one observation for each combination.

Table 5—Overview of regression analyses for sensory panel connective tissue

	Independent variable[a,b]						
Eq	SBSHEAR	SBSAREA	CBSHEAR	CBSAREA	COMFORCE	R^2 (%)	S.E.E.
5	X		X			91.2	0.451
6		X		X		93.0	0.403
7		X		X	X	95.7	0.329

[a] An "X" indicates the independent variables that were ultimately selected by the stepwise procedure when the levels of significance were set at 5%.

[b] The overall sensory panel average evaluation and average Instron reading were used for each grade/cut combination. Consequently, there is one observation for each combination.

not presented). Squared terms were tested for the different regression procedures. Results of the regression analysis, however, indicated that the addition of squared machine readings as independent variables increased R^2 only slightly. We can, therefore, accept the basic machine readings as adequate for the regressions.

The shear force devices that we used for characterizing the texture of ground beef patties were acceptable and gave readings that were highly correlated with the sensory evaluation of patties. They were easy to use, allowed rapid testing of many samples and required relatively small samples. SBSAREA accounted for 79.2% and 86.5% of the variability in sensory panel tenderness and connective tissue amount, respectively (r^2, Table 2). Regression equations for data from three Instron measurements from three different devices, accounted for 92.6% and 95.7% of the variability in tenderness and connective tissue amount, respectively (Table 3). The best two-variable, two-device equations accounted for 84.3% (#2—Table 4) and 93.0% (#6—Table 5) of the variability in tenderness and connective tissue amount, respectively.

The use of more than one device gave meaningful increases in R^2 with associated decreases in the standard error. Measurements of the area under the peak curves for shear forces, as measured by the circle and single blades, seemed to offer the best compromise between the use of only one device or the use of three different devices.

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Ms received 12/15/77; revised 2/23/78; accepted 2/28/78.

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EFFECT OF TUMBLING METHOD, PHOSPHATE LEVEL AND FINAL COOK TEMPERATURE ON HISTOLOGICAL CHARACTERISTICS OF TUMBLED PORCINE MUSCLE TISSUE

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ABSTRACT

Six groups of hams were cured, tumbled, canned and cooked. Duplicate histological samples were removed from both the surface and deep muscles of fresh, cured and cooked hams, prepared and stained both with haematoxylin and trichrome staining solutions. Results indicated that tumbling significantly ($P < 0.01$) increased cell membrane disruption, and phosphate level had a significant ($P < 0.01$) quadratic effect on decreasing clarity of striation patterns. On a tumbling time constant basis, intermittent tumbling resulted in more alterations in cell structure than did continuous tumbling. Tumbling also had a significant ($P < 0.01$) effect on disorganizing nuclei as well as a significant ($P < 0.05$) effect on decreasing clarity of striation patterns of deep muscle samples.

INTRODUCTION

THE RESULTS of tumbling meat have been studied by several investigators. According to Viskase Limited (1971), tumbling is defined as the massaging of meat surfaces; however, many meat processors now make a distinction between "tumbling" and "massaging." Tumbling involves the physical process of meat rotating in a drum, falling and making contact with metal walls and paddles. This process involves a transfer of kinetic energy and consequently causes alterations in muscle tissue.

In contrast, the process of "massaging" is considerably less rigorous. It usually involves a stationary drum with paddles rotating around a vertical axle. This process does not involve free falling of meat contents. Consequently, the process mainly involves muscle tissue rubbing other muscle tissue and the smooth surface of the drum. Theoretically, this results in less transfer of kinetic energy and therefore less heat rise in the product.

Tumbling has many beneficial effects, some of which are due to the formation of a protein exudate. According to Rust and Olson (1973), this protein exudate acts as a sealer when the protein is denatured during thermal processing. Vartorella (1975) and Krause (1976) add that this sealer helps hold in juices during smoking and cooking, and results in increased yields, increased juiciness, and improved slicing characteristics of the finished product. Other benefits of tumbling include improved tenderness and more uniform cured meat color (Krause, 1976).

Theno et al. (1977, 1978a, b, c) report that massaging shredded the muscle fiber, ruptured the sarcolemma and dislocated the nuclei. Siegel et al. (1976) claim that as massaging time increases, the amount of protein and fat in the exudate also increases. They note that this effect is more pronounced in the presence of salt and phosphates (Siegel et al., 1978a, b).

With all of the aforementioned physical changes caused by tumbling, or massaging, it seems logical that there must be several significant changes occurring at the cellular level. This study was conducted to determine the effects of tumbling method, phosphate content, and internal cooked temperature on selected histological characteristics of porcine muscle tissue as viewed under the light microscope.

MATERIALS & METHODS

FOURTEEN PAIRED HAMS were boned, external skin removed and most of the intermuscular and external fat removed in a commercial fashion to produce canned hams. The total mass of the boneless, skinned, and trimmed hams from pigs of approximately the same weight and quality were randomly assigned to seven treatments to study the effect of: tumbling method; phosphate level and internal cooked temperature on the histological quality of tumbled muscle tissue (see Fig. 1 and Table 1).

Tumbling methods include: 3-hr continuous cycle; 18-hr intermittent cycle (10 min per hour); and nontumbled. Brine phosphate levels of 0%, 2% and 4% were studied, and internal cooked temperature treatments included 66°C, 69°C and 72°C.

In experiment I (tumbling method), duplicate samples for histological evaluation (2 cm × 2 cm × 0.5 cm) were removed from both the surface and deep (2 cm below surface) region of the semimembranosus muscle in the fresh boneless hams to establish a standard for histological comparison and to serve as a reference for this muscle location after processing. Subsequent histological samples were taken from the same muscle in the same general area. Hams were then subjected to a multiple injection stitch cure process with a 15% pump. The curing brine was composed of 75.5% water, 16.5% salt, 4% glucose, 4% sodium phosphate ("Briphos" produced by Kalle and observed spots from thin-layer chromatography would suggest a phosphate mixture of orthophosphate and sodium diphosphate as the major compounds followed by sodium triphosphate and sodium tetraphosphate), and 0.147% sodium nitrite. After pumping the hams were held for 18 hr at 4° ± 2°C.

Boneless hams were then tumbled with a Karn Tumbler at a speed of 12 revolutions per minute either continuous for 3 hr or intermittent (10 min/hr) for 18 hr. Nontumbled, cured hams were held in a cover pickle (same solution used in pumping) in the cooler for the entire 18 hr tumbling duration. The hams tumbled for 3 hr (continuous) were held in the cooler for 15 hr following tumbling. Following curing and tumbling, duplicate samples for histological evaluation were removed from both the surface and deep regions of the semimembranosus muscle.

Hams were then placed in a polyethylene lined number 145 pear-shaped can. The polyethylene liner was pulled around the tissue to exclude the major portion of the air around the meat. Lids were sealed, and cans pasteurized in a 75°C water bath until an internal temperature of 69°C was obtained using thermocouples. Cans were immediately cooled in a 7° ± 3°C water bath for 90 min, then tranferred into a 4° ± 2°C cooler for 18 hr. Cans were opened, and duplicate surface and deep samples for histological evaluation were removed from the semimembranosus muscle of the cooked hams.

In experiment II, comparisons were made to determine the effect of phosphate level. The salt, glucose, and nitrite content in the brine was the same as in experiment I. The sodium phosphate level in the brine was 0%, 2% and 4%, and the water content was adjusted to keep other ingredients in the brine constant.

Hams for all three phosphate treatments were tumbled continuously for 3 hr at a speed of 12 revolutions per minute. They were then canned and pasteurized to 69°C the same as in experiment I.

Work reported in this paper was done by the Dept. of Animal Science, Ohio State University, Columbus, OH 43210 and the Ohio Agricultural Research & Development Center, Wooster, OH 44691 in cooperation with the Central Institute for Nutrition & Food Research TNO, Zeist, The Netherlands, and the Dept. of Science of Food of Animal Origin, Faculty Veterinary Medicine, State University of Utrecht, Utrecht, The Netherlands. Authors Cassidy, Ockerman, Plimpton and Cahill are with Ohio State Univ.; Author Krol is with the Central Institute for Nutrition & Food Research; and Author van Roon is with the Univ. of Utrecht.

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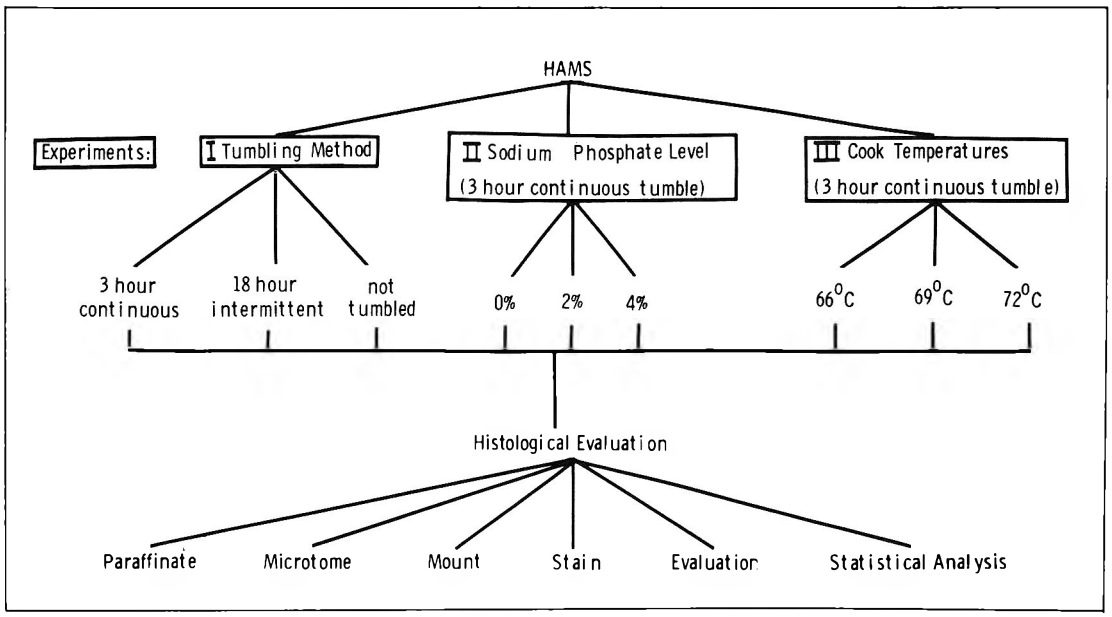

Fig. 1—Experimental design.

Duplicate surface and deep samples for histological evaluation were removed from fresh, cured (tumbled), and cooked hams to observe the effects of all phosphate treatments.

In experiment III, tumbled hams were cooked to three different internal temperatures (66°C, 69°C and 72°C).

All of the hams in this experiment were pumped 15% with the same brine formulation used in experiment I. After pumping, hams were tumbled continuously for 3 hr at a speed of 12 revolutions per minute. They were then canned and pasteurized to three different internal temperatures using thermocouples. Duplicate surface and deep samples for histological evaluation were removed from fresh, cured (tumbled), and cooked hams to observe the effect of internal cooked temperature.

A summary of the treatments can be found on Table 1. It is important to note that there are only seven treatments listed, even though it appears that each of the three experiments has three treatments. This is because treatment 1 is common to all three experiments (i.e. 3 hr continuous tumbling, 4% phosphate, 69°C cooked temperature).

All histological samples for each of the three experiments were parafinated, microtomed, and mounted on coverslips as outlined by Cassidy (1977). Half of the slides were stained using the haematoxylin-eosin method and the other half using the trichrome method (Humason, 1967). The haematoxylin-eosin method was used to accentuate the nuclei, and the trichrome method was used for viewing the striation patterns and cell membranes.

Five fields of each slide were randomly selected and evaluated for the following cellular characteristics:

1. clarity of striations
2. cell membrane disruptions
3. clarity of nuclei
4. disorganization of nuclei

Each field was scored on a scale of 1.0–3.0 for each cellular characteristic. A score of 1.0 indicates the field was typical for a normal fresh muscle cell. A score of 3.0 suggests the field was characterized by either complete loss of striation, total disruption of cell membranes, nuclei very faint or indistinguishable, or clustered or randomly disorganized nuclei.

There were 84 [7 treatments × 2 locations (surface and deep) × 2 hams × 3 processing stages (fresh, cured or cured and tumbled, and cooked)] combinations of treatments, locations and processing stages which resulted in a total of 1,680 (84 histological observation points × 5 fields per point × 4 characteristics scored per field) histological scores. This information was analyzed using Harvey's (1975) analysis of data with unequal subclass numbers.

RESULTS & DISCUSSION

Experiment I—Effect of tumbling

The effect of tumbling on all cell characteristics can be found in Tables 2 and 3. Tumbling caused a significant increase in cell membrane disruption in both surface and deep muscle regions for both cured, uncooked, and cured cooked samples. This finding helps to explain why tumbling improves tenderness (Rahelic et al., 1974). Once the cell membranes have been broken, the myofibrillar protein can migrate to the surface since salt and phosphate aid in solubilization (Yasui et al., 1964a, b). This protein exudate promotes cohesion in cooked product (Vartorella, 1975).

Tumbling also results in a highly significant decrease in clarity of striation patterns in deep, cured muscle samples. This effect was also observable in cured, cooked surface

Table 1—Summary of treatments, two hams per treatment cell

Treatment #	Description	% PO_4 in brine	Cooked temp	Tumbling method	Fig. 1 Description	
					Experiment	Conditions
1	Standard (tumbled 3 hr continuously)	4%	69°C	3 hr continuous	I	Continuous
					II	4%
					III	69°C
2	Control	4%	69°C	Nontumbled	I	Nontumbled
3	Intermittent tumbling	4%	69°C	18 hr intermittent	I	18 hr intermittent
4	No phosphate	0%	69°C	3 hr continuous	II	0% PO_4
5	Low phosphate	2%	69°C	3 hr continuous	II	2% PO_4
6	Low cook temperature	4%	66°C	3 hr continuous	III	66°C
7	High cook temperature	4%	72°C	3 hr continuous	III	72°C

Table 2—Least square means and standard errors for comparison of the effects of tumbling on cell characteristics of cured porcine muscle tissue

Characteristics[b]	Surface cells			Deep cells[a]		
	Non-tumbled	Tumbled	S.E.	Non-tumbled	Tumbled	S.E.
Clarity of striation	2.80	3.00	0.24	2.00	2.30*	0.23
Cell membrane disruption	2.10	2.90*	0.46	2.00	2.60**	0.26
Clarity of nuclei	1.00	1.45	0.40	1.00	1.25	0.24
Disorganization of nuclei	1.20	1.80	0.52	1.10	1.50**	0.16

[a] 2 cm below the surface
[b] Scoring system: 1.0 = normal, 3.0 = either complete loss of striation, total disruption of cell membranes, nuclei very faint or indistinguishable or clustered or randomly disorganized nuclei.
* Significant ($P < 0.05$)
** Highly significant ($P < 0.01$)

Table 3—Least square means and standard errors for comparison of the effects of tumbling on cell characteristics of cured and cooked porcine muscle tissue

Characteristics[b]	Surface cells			Deep cells[a]		
	Non-tumbled	Tumbled	S.E.	Non-tumbled	Tumbled	S.E.
Clarity of striation	2.10	2.60*	0.31	2.00	2.20	0.19
Cell membrane disruption	1.70	2.75**	0.45	1.60	2.45**	0.40
Clarity of nuclei	2.70	2.95	0.23	2.30	3.00**	0.23

[a] 2 cm below the surface
[b] Scoring system: 1.0 = normal, 3.0 = either complete loss of striation, total disruption of cell membranes, nuclei very faint or indistinguishable or clustered or randomly disorganized nuclei.
* Significant ($P < 0.05$)
** Highly significant ($P < 0.01$)

muscle samples and approached significance in the cured, cooked deep samples.

The nuclei of the tumbled, deep samples appeared significantly ($P < 0.01$) more disorganized in both the cooked and uncooked tissue than the nuclei in the nontumbled deep samples.

In Tables 4 and 5, comparisons were made between the 3-hr continuous tumbling schedule and the 18-hr intermittent tumbling schedule (10 min/hr). Although both schedules had 180 min of actual tumbling time, the intermittent schedule caused more changes especially in the deep tissue. The nuclei of intermittent tumbled hams were significantly more disorganized than the nuclei of the continuous tumbled hams in both surface and deep cured samples.

The striation patterns of intermittent tumbled hams were significantly ($P < 0.01$) less clear than the striation patterns of continuous tumbled hams in the deep cured and the deep cured and cooked samples.

These findings indicate that the rest period in the intermittent schedule does play a role. Perhaps the role involved greater diffusion of curing agents, as well as providing for a more favorable time and temperature combination for the phosphate and salt to exhibit maximum effect. Yasui et al., (1964a, b) indicated that tripolyphosphate could be of use when a long curing period is used since it exhibits its influence for a long time due to the hydrolysis by the tripolyphosphatase activity.

Figures 2–5 follow photomicrographs that illustrate differences between fresh hams, cured hams (not tumbled), hams tumbled for 3 hr continuously, and hams tumbled for 18 hr intermittently.

Experiment II—Influence of sodium phosphate level in tumbled hams

In this phase of the research, the effect of the level of sodium phosphate in the brine (0%, 2% and 4%) on the cell structure of tumbled hams was studied (see Tables 6 and 7). All three phosphate treatments involve hams tumbled continuously for 3 hr but were statistically analyzed with tumbling effects absorbed.

Sodium phosphate had a highly significant ($P < 0.01$) quadratic effect of decreasing the clarity of striation patterns in both surface and deep, cured, tumbled muscle samples. For cured, tumbled and cooked samples, this effect was significantly ($P < 0.01$) linear. Yasui et al. (1964a, b) reported that tripolyphosphate effectively results in the disassociation of actomyosin to actin and myosin, but that this occurs only after it (tripolyphosphate) is first hydrolyzed by tripolyphosphatase in the presence of divalent cations at a high ionic strength.

There was a significant effect of phosphate on cell mem-

Table 4—Least square means and standard errors for the effects of tumbling method on cell characteristics of cured porcine muscle tissue

Characteristic[b]	Surface cells			Deep cells[a]		
	3-hr Cont.	18-hr Inter.	S.E.	3-hr Cont.	18-hr Inter.	S.E.
Clarity of striation	3.00	3.00	0.14	2.10	2.50*	0.13
Cell membrane disruption	2.80	3.00	0.27	2.50	2.70	0.15
Clarity of nuclei	1.20	1.70	0.23	1.20	1.30	0.14
Disorganization of nuclei	1.30	2.30*	0.30	1.20	1.80*	0.09

[a] 2 cm below the surface
[b] Scoring system: 1.0 = normal, 3.0 = either complete loss of striation, total disruption of cell membranes, nuclei very faint or indistinguishable or clustered or randomly disorganized nuclei.
* Significant ($P < 0.05$)

Table 5—Least square means and standard errors for the effects of tumbling method on cell characteristics of cured and cooked porcine muscle tissue

Characteristic[b]	Surface cells			Deep cells[a]		
	3-hr Cont.	18-hr Inter.	S.E.	3-hr Cont.	18-hr Inter.	S.E.
Clarity of striation	2.50	2.70	0.18	1.90	2.50*	0.11
Cell membrane disruption	2.60	2.90	0.26	2.20	2.70	0.23
Clarity of nuclei	2.90	3.00	0.13	3.00	3.00	0.13

[a] 2 cm below the surface
[b] Scoring system: 1.0 = normal, 3.0 = either complete loss of striation, total disruption of cell membranes, nuclei very faint or indistinguishable or clustered or randomly disorganized nuclei.
* Highly significant ($P < 0.01$)

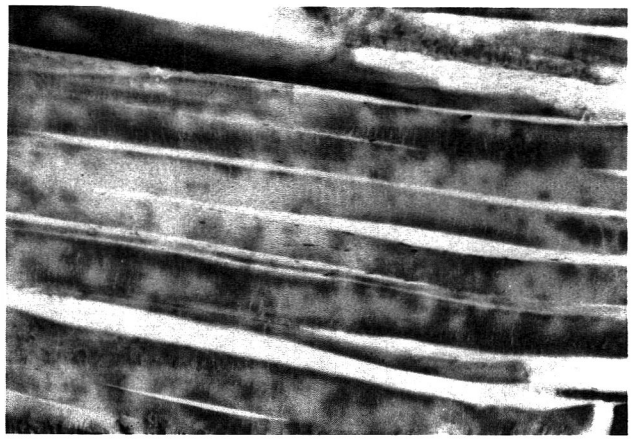

Fig. 2—Photomicrograph of fresh ham muscle cells (320X).

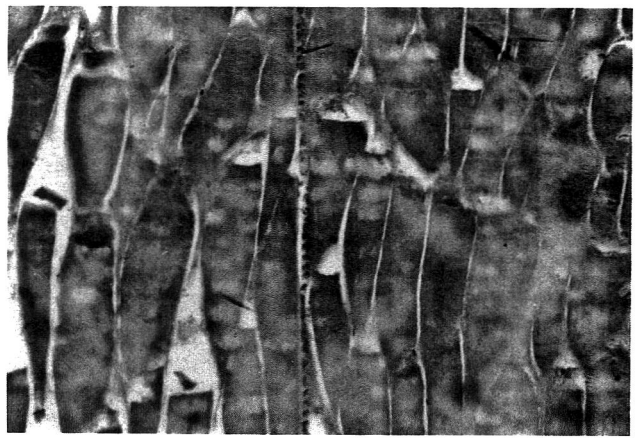

Fig. 3—Photomicrograph of cured—not tumbled ham muscle cells (320X).

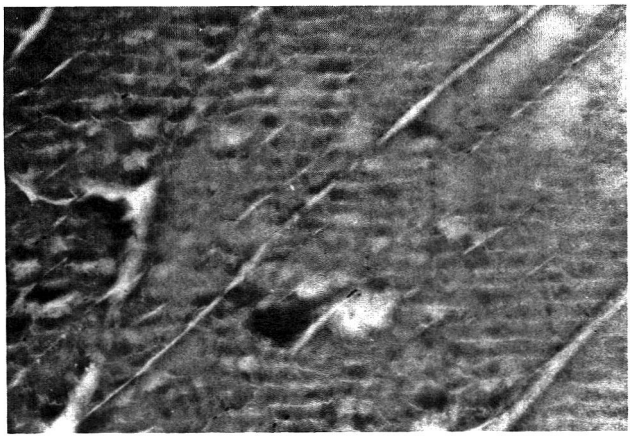

Fig. 4—Photomicrograph of 3-hr continuously tumbled ham muscle tissue (320X).

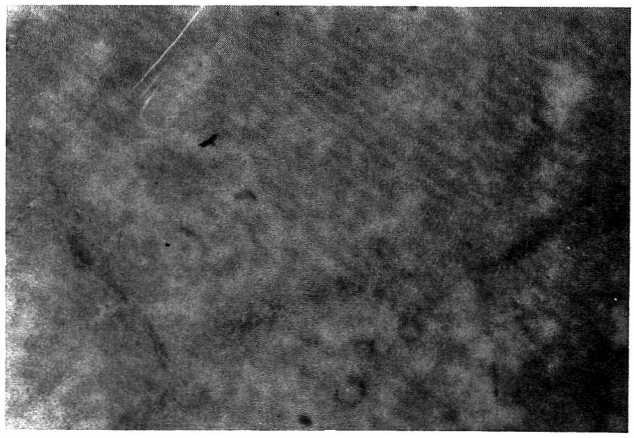

Fig. 5—Photomicrograph of 18-hr intermittently (10 min/hr) tumbled ham muscle tissue (320X).

brane disruptions in deep, cured, tumbled tissue. Mahon (1961) and Weiss (1973) both discussed a synergistic effect of salt and phosphate on the extraction of actin and myosin type proteins. The present experiment was not designed to measure a synergistic relationship.

The level of phosphate in the brine did not appear to have any significant effect on the clarity of nuclei in tumbled or cooked muscle tissue. There was, however, a highly significant ($P < 0.01$) quadratic effect on the disorganization of nuclei in the tumbled deep uncooked samples.

Experiment III—Effect of cooking temperature

The process of cooking brings on several dramatic changes in the appearance of muscle tissue. Heat denatures protein and results in changes of color and texture of sarcoplasm. Nuclei become so faint in cooked tissue that it is difficult to distinguish them from the rest of the muscle cell. For this reason it was impossible to evaluate the degree of disorganization of nuclei in cooked samples.

The aforementioned changes occurred in samples from all three internal cooked temperatures (66°C, 69°C and 72°C). Consequently, the statistical analysis showed no significant difference in clarity of nuclei over the temperatures of this research.

In this research the range of internal cooked temperatures was only 6°C (66–72°C) because it was felt that this was a practical range for pasteurization of hams, and temperatures above or below this range would not be used by many pro-

Table 6—Least square means and standard errors for the effect of phosphate level on cell characteristics of cured and tumbled porcine muscle tissue

	Surface cells				Deep cells[a]			
Characteristic[b]	0% PO_4	2% PO_4	4% PO_4	S.E.	0% PO_4	2% PO_4	4% PO_4	S.E.
Clarity of striation	2.30	2.50	3.00[c]	0.24	1.50	1.90	2.10[c]	0.23
Cell membrane disruptions	2.70	2.70	2.80	0.25	1.30	1.70	2.50[c]	0.26
Clarity of nuclei	1.50	1.30	1.20	0.23	1.30	1.10	1.20	0.14
Disorganization of nuclei	1.50	1.70	1.30	0.30	1.30	1.50	1.20[c]	0.16

[a] 2 cm below the surface

[b] Scoring system: 1.0 = normal, 3.0 = either complete loss of striation, total disruption of cell membranes, nuclei very faint or indistinguished or clustered or randomly disorganized nuclei.

[c] Highly significant quadratic ($P < 0.01$)

Table 7—Least square means and standard errors for the effect of phosphate level on cell characteristics of cured, tumbled and cooked porcine muscle tissue

Characteristic[b]	Surface cells				Deep cells[a]			
	0% PO_4	2% PO_4	4% PO_4	S.E.	0% PO_4	2% PO_4	4% PO_4	S.E.
Clarity of striation	2.00	2.00	2.50[d]	0.18	2.30	1.90	1.90[d]	0.11
Cell membrane disruptions	1.80	1.90	2.60[c]	0.45	1.70	1.40	2.20[d]	0.23
Clarity of nuclei	2.70	2.90	2.90	0.13	2.90	2.80	3.00	0.13

[a] 2 cm below the surface

[b] Scoring system: 1.0 = normal, 3.0 = either complete loss of striation, total disruption of cell membranes, nuclei very faint or indistinguished or clustered or randomly disorganized nuclei.

[c] Significant quadratic ($P < 0.05$)

[d] Highly significant linear ($P < 0.01$)

Table 8—Least square means and standard errors for the effect of cooking temperature on cell characteristics of tumbled porcine muscle tissue

Characteristic[b]	Surface cells				Deep cells[a]			
	66°C	69°C	72°C	S.E.	66°C	69°C	72°C	S.E.
Clarity of striation	2.00	2.50	3.00[d]	0.18	2.00	1.90	3.00[e]	0.19
Cell membrane disruptions	2.20	2.60	2.90[c]	0.26	2.10	2.20	2.40	0.23
Clarity of nuclei	2.90	2.90	2.90	0.13	3.00	3.00	3.00	0.13

[a] 2 cm below the surface

[b] Scoring system; 1.0 = normal, 3.0 = either complete loss of striation, total disruption of cell membranes, nuclei very faint or indistinguished or clustered or randomly disorganized nuclei.

[c] Significant linear ($P < 0.05$)

[d] Highly significant linear ($P < 0.01$)

[e] Highly significant quadratic ($P < 0.01$)

cessors. Perhaps if a wider range of temperatures was examined, there might have been significant changes in the nuclei. However, over the 6°C temperature range, clarity of striation patterns did show a significant ($P < 0.01$) change. As internal cook temperature increased, the clarity of striations decreased in both surface and deep cooked samples.

There was also a significant relationship ($P < 0.05$) between the degree of cell membrane disruptions and internal cooked temperature. The higher the internal cooked temperature, the more disruption was observed.

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Ms received 8/21/77; revised 2/16/78; accepted 2/25/78.

Journal Article 95-77 of the Ohio Agricultural Research & Development Center, Wooster, OH 44691.

Acknowledgment is made to Dr. J.A.A. Snyders and Mr. G.C. Ammerlaan from the Dept. of Science of Food of Animal Origin at Utrecht for technical assistance in histological slide preparation and interpretation.

INCREASING SHELF LIFE BY CARBON DIOXIDE TREATMENT AND LOW TEMPERATURE STORAGE OF BULK PACK FRESH CHICKENS PACKAGED IN NYLON/SURLYN FILM

EUGENE H. SANDER and HONG-MING SOO

ABSTRACT

Simulated commercial bulk pack, whole chickens were subjected to conventional ice pack and three modified atmospheric, vacuum and two levels of CO_2 within a Nylon/Surlyn film, storage environments. Pathogenic and nonpathogenic microbial growth, void space gas composition (except ice pack) and off-odor development were monitored on samples stored at 1.1°C. Polynomial regression analysis of aerobic growth curves revealed that the lag phase was extended to 8–10 days by using a CO_2 addition rate of 3.61×10^{-4} and 7.22×10^{-4} m^3/kg, respectively. The transition from lag to log phase appeared when the residual level of CO_2 in the void space reached 15–55%. Following initial replacement of the air in the pouch with 95% CO_2, the growth of anaerobic microorganisms was limited by the presence of CO_2. The growth of potential pathogens, salmonella, coliforms, *Staphylococcus aureus* and *Clostridium perfringens* in the presence or absence of CO_2 was negligible at 1.1°C. Off-odor development can be detected when the colony forming units (CFU) numbers reach 10^6/g of body weight. Carbon dioxide restricts the growth of most objectionable putrefactive off-odor bacteria; lactic acid organisms are encouraged by its presence on the surface of the sample and in the pouch void space. A recommended usage rate of 7.22×10^{-4} m^3 CO_2/kg of body weight extends shelf life quality to 27 days at 1.1°C.

INTRODUCTION

CONCENTRATION of consumers in urbanized areas, away from major production and food processing regions, characterizes the United States today. As a result, opportunities exist for improvement of the shelf life of perishable fresh products during transport, distribution and merchandising.

Sixty-one percent of total chicken production in the United States is concentrated in the southern states. Bulk packaging of fresh chicken products (whole or parts) in ice permits distribution from this area to major markets on the east and west coasts. However, ice pack provides limited shelf life. Finding an alternative to ice, that also extends shelf life would: (1) adapt handling of fresh chickens to a complex retail distribution system; (2) reduce unit freight costs by replacing ice with salable product; (3) improve the physical handling characteristics of bulk, fresh chickens by removing melting ice; and (4) provide a packaging environment which enables fresh chickens to withstand abuse in the travel from processor to the consumer's table.

As early as 1899, Hines determined that concentration of carbon dioxide (CO_2) in the environment selectively inhibited growth of bacteria. More recently, investigators have demonstrated that a combination of low temperature, 0–10°C, and the presence of CO_2 in a controlled environment, effectively extends the shelf life of red meat, poultry, and fish (Killeffer, 1930; Ayres et al., 1950; Ogilvy and Ayres, 1951a, b; Kraft and Ayres, 1952; Wabeck et al., 1968; Clark and Lentz, 1969; 1972; Baran et al., 1970; Adams and Huffman, 1972). With the exception of application of primal cuts of red meats, this knowledge has not been utilized commercially. Therefore, the objectives of this investigation were:

1. To evaluate a practical package and commercial process for extending the shelf life of fresh chickens to replace conventional ice pack.
2. To evaluate the performance of co-extruded Nylon/Surlyn film as a barrier to CO_2 loss and as a suitable material for packing fresh chickens.
3. To monitor the effect of CO_2 level on the growth patterns of pathogenic and nonpathogenic bacteria during storage at 1.1°C.

EXPERIMENTAL

Sample preparation and storage

A commercial processor, bulk packaging fresh chickens in ice, was selected for in-plant sampling and packaging of experimental samples.

Whole chickens containing 11% added moisture were randomly selected as they left the continuous spin chill tank. Birds were held under ice until packed and packaged birds were held under ice until they reached the controlled temperature room for sustained storage. A commercial flexible packaging machine (CVP Systems, Chicago, IL) capable of handling a 29.5 kg bulk pack box with film liner, was used. Simulated bulk packs were prepared by placing three chickens in a 3 mm Nylon/Surlyn, 20 GA Nylon and 60 GA Surlyn respectively, preformed pouch; samples ranged in weight from 4199–4962 g/pouch.

Three treatments were used: vacuum pack-no CO_2 flush and vacuum pack followed by a flush with two addition rates of CO_2, 3.61×10^{-4} m^3/kg body weight and 7.22×10^{-4} m^3/kg. Whole birds, packed 25 to a box, were covered with ice, and served as a fourth treatment. A controlled temperature room was used to hold all four treatments at 1.1°C. Carbon dioxide-treated birds were randomly selected and frozen at −28.9°C to serve as controls.

In this commercial operation, giblets and necks were wrapped in parchment paper and added (by count) to the bulk pack box before the contents were iced. Packaged giblets were randomly selected, placed in Nylon/Surlyn pouches, flushed with 7.22×10^{-4} m^3 CO_2/kg and carried through the storage period to determine their potential contribution to spoilage.

Microbiological analysis

Every 2 days, duplicate samples from each treatment were evaluated for numbers of aerobic and anaerobic Colony Forming Units (CFU). *Clostridium perfringens*, salmonella, *Staphylococcus aureus* and presumptive coliform counts were determined at 4-day intervals using standard microbiological testing methods (Speck, 1976); giblets were evaluated every 4–5 days for numbers of aerobic and anaerobic CFU only. Each simulated bulk pack sample was weighed before opening. Sterile, 0.1% peptone solution was poured into opened pouches in controlled and sufficient quantity to thoroughly rinse the surface and body cavities of the contained birds. Aliquots were pipetted directly from the peptone rinse for subsequent dilutions. All results are reported as CFU/gram of body or giblet weight.

Numbers of aerobic and anaerobic CFU were determined by direct plating on Trypticase Soy Agar (TSA). For total numbers, aerobic CFU plates were incubated at 20°C for 72 hr; however, for total numbers, anaerobic CFU plates were incubated in a Gas Pak (BIO Quest, Cockesville, MD) anaerobic system for 48 hr at 20°C (Gardner and Martin, 1971). The *C. perfringens* population was determined by plating on sulfite-polymyxin-sulfadiazine (SPS) agar and incubating anaerobically at 37°C for 24 hr (Angelotti et al., 1962). The presumptive tests for *C. perfringens* were conducted by inoculating black colonies from incubated SPS plates into iron milk, indole nitrate medium and incubating at 37°C for 24 hr. Salmonellae analysis was carried out as outlined by

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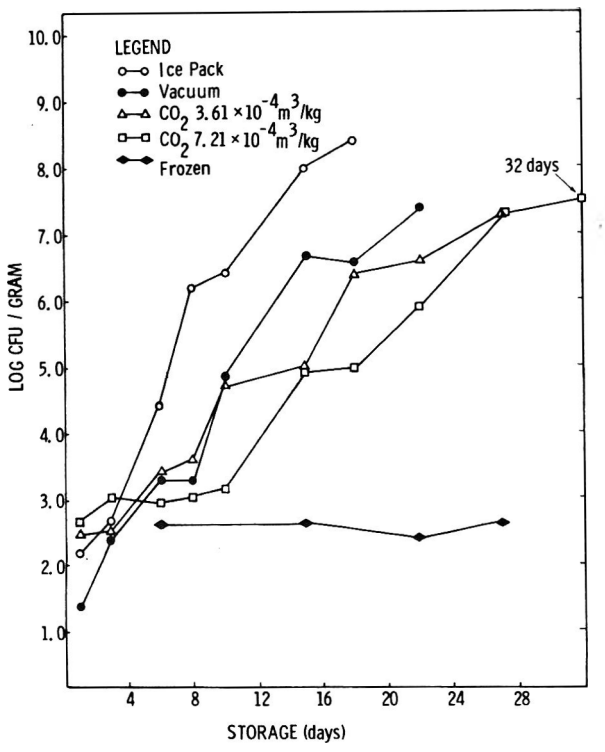

Fig. 1—Aerobic CFU growth on whole chickens stored at 1.1° C.

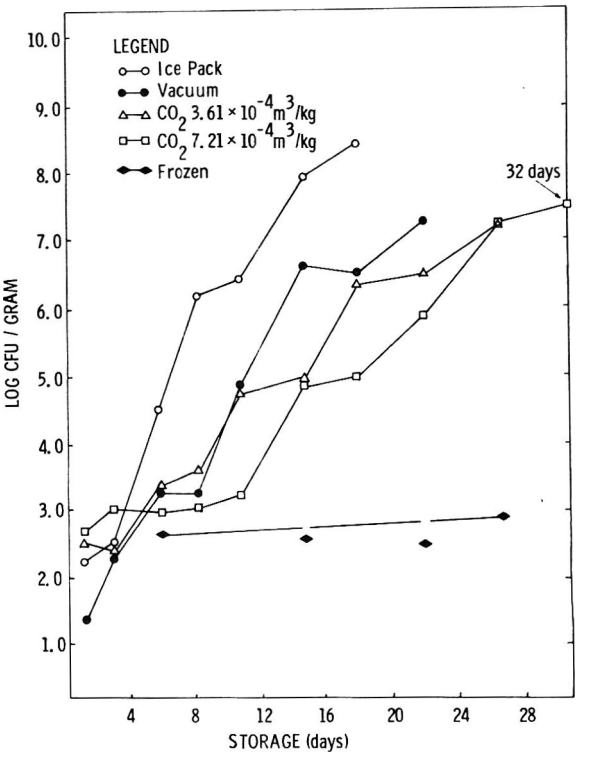

Fig. 2—Anaerobic CFU growth on whole chickens stored at 1.1° C.

Galton et al. (1968). A 10-ml aliquot of 0.1% peptone rinse was enriched in 100 ml of tetrathionate broth and streaked on brilliant green sulfadiazine agar (BGS). Suspect colonies were tested biochemically in triple sugar iron agar, lysine iron agar, dulcitol broth and urea broth. Presumptive salmonellae were confirmed serologically with O-antigen and Spicer-Edwards H-antigen antisera schemes. *S. aureus* was determined by plating aliquots on Baird-Parker medium and incubating at 37°C for 48 hr. The coagulase positive *S. aureus* was confirmed by using the coagulase plasma and heat-stable nuclease (DNase) test as outlined by Tatini et al. (1976). Total number of coliform organisms was determined by direct plating on Violet Red Bile agar (VRB) as suggested in Sharf (1966).

Head space analysis for residual carbon dioxide and air

Approximately every 4 days two randomly selected samples from each treatment were analyzed for void or head space gas composition. A self sealing septum consisting of a drop of silicone cement dried onto two pieces of electricians tape was placed on the pouch. The film was punctured through the septum using a 2.5 ml gas tight syringe with a push button valve. A Hewlett-Packard Model 7620 gas chromatograph equipped with 12-inch Porpak Q columns and an automatic integrator was used to quantify the air and CO_2 levels.

Sensory evaluation

Following head space compositional analysis, the pouches were opened. Single drum sticks were removed from the birds and retained along with a small vial of the accumulated drip in the pouch for subsequent sensory evaluation. Triangle testing together with a 10-member taste panel was used to determine off-odor differences among drip and drum sticks from various treatments. The informal off-odor test (by smelling) as suggested by Clark and Lentz (1972) and Cox et al. (1975), was also conducted. Immediately after opening the pouches and prior to the peptone solution rinse, the samples were evaluated for off-odor development by a 3-member panel.

RESULTS & DISCUSSION

THE GROWTH of aerobic and anaerobic CFU during storage of simulated bulk pack chickens is presented in Figures 1 and 2, respectively. Aerobic CFU growth (Fig. 1) was indeed limited by the absence of air (vacuum treatment) and increasing levels of CO_2 in the Nylon/Surlyn pouch. Enrichment (95% replacement of air) of the package environment with CO_2 delayed the onset of the log growth phase, up to 10 days, when a rate of 7.22 × 10^{-4} m^3/kg was used. The ability of aerobic CFU to grow rapidly in the ice pack treatment is indicated by the absence of an initial lag phase and linear growth from the first day in storage. Entry into the stationary phase of growth was signaled by aerobic CFU reaching 10 million/g in vacuum and CO_2 treatments, and 100–150 million in ice pack treatment within comparable storage periods at 1.1°C.

Anaerobic CFU (Fig. 2) among the treatments used, show growth patterns similar to those of aerobic CFU; addition rates of 3.61 × 10^{-4} and 7.22 × 10^{-4} m^3 CO_2/kg appears to extend the shelf life to 8–10 days, respectively; vacuum treatment produced an oxygen depleted environment favorable to aerobic CFU growth. Anaerobic CFU grew to a rate higher on chickens (vacuum treatment) than on chickens exposed to high concentrations of CO_2. Inhibition of anaerobic CFU growth by the presence of CO_2 is evident from these results. Investigators have postulated that the mechanism whereby CO_2 limits growth is metabolic and not the lowering of surface pH resulting from dissolved CO_2 (Coyne, 1932; Haines, 1933).

High concentrations of CO_2 increase both the generation time and lag phase in the growth cycle of microorganisms, (Tomkins, 1932). In order to establish the lag phase in the growth cycle, polynomial regression and analysis of variance were used to determine the statistical significance of lag growth phases for aerobic CFU among the various treatments (Snedecor and Cochran, 1976); "F" values for coefficient of regression equation, degree 1 and 2 analysis, are presented in Table 1. "F" values for degree 1 are all significant at the 1% level. Only one "F" value for degree 2 analysis is significant for the CO_2 treatment using an addition rate of 7.22 × 10^{-4} m^3/kg. Clearly a lag phase is statistically defined for this treatment (7.22 × 10^{-4} m^3/kg) and is absent in the other treatments. Evaluation of numbers of anaerobic CFU (data not shown) found that "F" values for degrees 2 analysis were not significant for any of the treatments, despite graphical evidence of a CO_2-induced lag phase among anaerobic CFU (Fig.

Table 1—Polynomial regression and analysis of variance (ANOVA): Aerobic CFU growth on bulk pack whole chickens treated with and without CO_2 and stored at 1.1°C

	D.D.[a]				"F" value			
Source	Ice pack	Vac.	CO_2 (3.61 X 10^{-4} m^3/kg)	CO_2 (7.22 X 10^{-4} m^3/kg)	Ice pack	Vac.	CO_2 (3.61 X 10^{-4} m^3/kg)	CO_2 (7.22 X 10^{-4} m^3/kg)
Degree 1	1	1	1	1	184.65**	142.1**	194.0**	226.1**
Degree 2	1	1	1	1	6.66	2.3	1.15	10.6*
Error	4	5	6	6				

[a] Degree of freedom
** Significant at 1% level ($P \leq 0.01$)
* Significant at 5% level ($P \leq 0.05$)

2). Polynomial regression and ANOVA interpretation show that anaerobic CFU growth is linear from initiation of storage.

Generation times for aerobic and anaerobic CFU growth on bulk pack chickens for various treatments and storage at 1.1°C, were calculated using numbers of CFU obtained during the log growth phase; results are presented in Table 2. Vacuum and CO_2 treatments produced significant increases in generation time for aerobic CFU compared to values for ice pack treatment. Aerobic CFU generation time was increased 18% when CO_2 was used at a rate of 7.22 X 10^{-4} m^3/kg in place of vacuum treatment. A comparison of anaerobic CFU generation times shows that the vacuum treatment favors anaerobic growth to a greater degree than does ice pack. It appears that rapid aerobic CFU growth during ice pack treatment creates an intolerant environment for anaerobes, whereas vacuum treatment reversed the situation. CO_2, added at rates of 3.61 X 10^{-4} and 7.22 X 10^{-4} m^3/kg increased generation times over vacuum treatment by 68 and 81%, respectively. This effect of CO_2 suggests a treatment is possible whereby the growth of anaerobic pathogens can be retarded.

Giblets and necks represent a variable source of contamination depending on in-plant handling practices and sanitation. The practice followed in the commercial processing plant is to pack giblets and necks separately in a parchment type pouch. The pouches are then counted into the bulk pack box directly on top of the chicken carcasses before icing.

Results of aerobic and anaerobic CFU evaluations of packaged giblets are presented in Table 3. The results of this pilot study on giblets indicate that aerobic and anaerobic CFU growth parallels lag/log growth rates of the whole birds and does not represent a source of unexpected microbial contamination to the total bulk pack. Informal odor testing indicated no unusual development up to 24 days; after 24 days atypical odors developed which were not rancid/proteolytic types of decomposition odors as detected on the whole birds.

Changes in numbers of coliform organisms on bulk pack whole chickens, subjected to various treatments, are presented in Figure 3. The results show that coliforms will grow rapidly on ice pack chickens; the presence of CO_2 reduced the rate of growth significantly as shown by the two log number difference between CO_2 treatments and vacuum treatment after 22 days of storage. A CO_2 level of 7.22 X 10^{-4} m^3/kg restricts the growth of coliforms up to 22 days of storage.

The results from the monitoring of bulk pack chickens for the presence and growth of potentially pathogenic microorganisms is presented in Table 4. Growth of *C. perfringens* was monitored to assess the potential for growth of *C. botulinum* in anaerobic environments created by vacuum or CO_2 treatments. *C. perfringens* was detected in two ice pack samples on days 10 and 14. Surprisingly, no salmonella were found among any samples. Coagulase positive *S. aureus* were detected among samples from all treatments; no significant increases in numbers were detected as storage progressed; numbers were not high enough to generate significant levels of harmful toxin (Tatini et al., 1976).

Table 2—Generation times for aerobic and anaerobic CFU on bulk pack whole chickens stored at 1.1°C

Treatment	Generation time (hr)	
	Aerobic	Anaerobic
Ice pack	18.4	21.6
Vacuum	27.4	19.3
CO_2 (3.61 X 10^{-4} m^3/kg)	30.6	32.5
CO_2 (7.22 X 10^{-4} m^3/kg)	32.3	34.9

Table 3—Aerobic and anaerobic CFU growth on parchment wrapped giblets packed in Nylon/Surlyn pouches, flushed with 3.61 X 10^{-4} m^3/kg of CO_2 and stored at 1.1°C

Day	CFU/g of giblets	
	Aerobic	Anaerobic
6	6.0 X 10^3	1.3 X 10^3
15	1.9 X 10^5	7.8 X 10^4
20	1.4 X 10^6	1.4 X 10^6
26	7.4 X 10^6	3.7 X 10^6
32	6.9 X 10^7	6.4 X 10^7

Table 4—Detection of potentially pathogenic bacteria on bulk pack whole chickens during controlled atmospheric storage at 1.1°C

Organisms	Storage (Day)	CFU/g	Treatment
C. perfringens	10	1	Ice pack
	14	29	Ice pack
Salmonella	None found		
S. aureus	3	43	Ice pack
	8	5	Vacuum
	1	1	CO_2 (3.61 X 10^{-4} m^3/kg)
	3	3	CO_2 (3.61 X 10^{-4} m^3/kg)
	8	8	CO_2 (3.61 X 10^{-4} m^3/kg)
	3	18	CO_2 (7.22 X 10^{-4} m^3/kg)

The failure to detect salmonella among bulk pack chickens early in the storage was surprising. Cox and Blankenship (1975) reported on the variability of salmonella occurrence among broiler carcasses taken from several Georgia processing plants. These investigators established that incubation of the carcass in 500 ml of lactose broth for 24 hr at 37°C (Method 1) compared with removal of a 10 ml aliquot from the sterile lactose broth rinse (Method 2), followed by incubation under similar conditions, showed that 46% compared with 3.8%, respectively, of the samples carcasses were positive. Sensitivity of both methods was verified by selective inoculation of carcasses with salmonella. As few as 8 cells could be detected in

Table 5—Relationship between off-odor development and total numbers of aerobic/anaerobic CFU

Treatment	Day	Aerobic/Anaerobic CFU/g	Descriptive off-odor
Ice pack	10	$2.3 \times 10^6/5.9 \times 10^4$	Proteolysis/slimy surface
Vacuum	15	$4.3 \times 10^6/2.2 \times 10^6$	Rancid/proteolysis
CO_2 (3.61×10^{-4} m^3/kg)	22	$4.0 \times 10^6/1.4 \times 10^6$	Rancid/lactic acid
CO_2 (7.22×10^{-4} m^3/kg)	27	$3.0 \times 10^7/1.6 \times 10^7$	Rancid

10 out of 10 samples using Methods 1 whereas the cells present on the carcass had to number 52 before 100% detection was achieved using Method 2.

Success of the proposed controlled atmospheric packaging concept depends not only on microbiological changes but also on organoleptic changes during storage at 1.1°C. As indicated earlier, a 10-member panel was asked to evaluate off-odors occurring on the chicken drum sticks and drip collected from the opened pouch. Significant differences among treatments and within storage days could not be detected by the 10-member taste panel. The informal 3-member panel which smelled the packaged chickens immediately after opening, were able to distinguish between acceptable and unacceptable odors. This sensory method was subsequently used to help establish shelf life limits among the various treatments since it represented the realism with which a customer would immediately accept or reject the product. The relationship between aerobic and anaerobic CFU growth and off-odor development is presented in Table 5. Proteolysis and slime development characterized ice pack chickens after 10 days of storage. Proteolytic off-odors were detected in vacuum pack chickens after 15 days; proteolysis was definitely absent in chicken treated with CO_2; a lactic acid "sour" odor dominated the chickens treated with 3.61×10^{-4} m^3/kg after 22 days of storage. Objectionable lactic acid odor was not detected in packaged chickens treated with 7.22×10^{-4} m^3/kg until the 32nd day of storage. Aerobic and anaerobic CFU numbers reached 10 million before lactic acid off-odors were detected compared to 1 million in other treatments. The differences in off-odors suggest that selective inhibition of certain organisms by CO_2 leads to domination by lactic acid production types. This observation is supported by the work of other investigators (Ogilvy and Ayres, 1951a; Kraft and Ayres, 1952; Clark and Lentz, 1969; Baron et al., 1970) who have demonstrated that CO_2 encourages the growth of lactic acid types. Detected rancid off-odors were attributed to oxidative rancidity, typical in stored poultry.

Properties of the Nylon/Surlyn film used as the packaging material were evaluated. Two important properties of the film are: the absence of flex cracking at low temperatures and the low transmission rates of CO_2 (5–5.9 cc CO_2/m^2/mm/24 hr/20°C/65% RH). Based on this CO_2 transmission rate, the time required to lose added CO_2 through the film (3.2 mm thickness) was calculated. Using a CO_2 addition rate of 28.88 m^3/8 kg (80 in^3/8 lb) to chickens in the pouch which measured 0.3189 m^2 of total surface area, 754 days would be required to lose all of the added CO_2.

Within 24 hr following addition of CO_2 to the pouch, at least 50% or more is adsorbed by the moisture on the surface of the chickens. A skin-tight pouch forms as the CO_2 is adsorbed causing the film to collapse onto the surface of the chickens. Changes in void or headspace gas composition which occurred during storage at 1.1°C are presented in Figures 4 and 5. The CO_2 level is plotted on the right hand ordinate. Immediately following the air evacuation, gas flush, and seal-

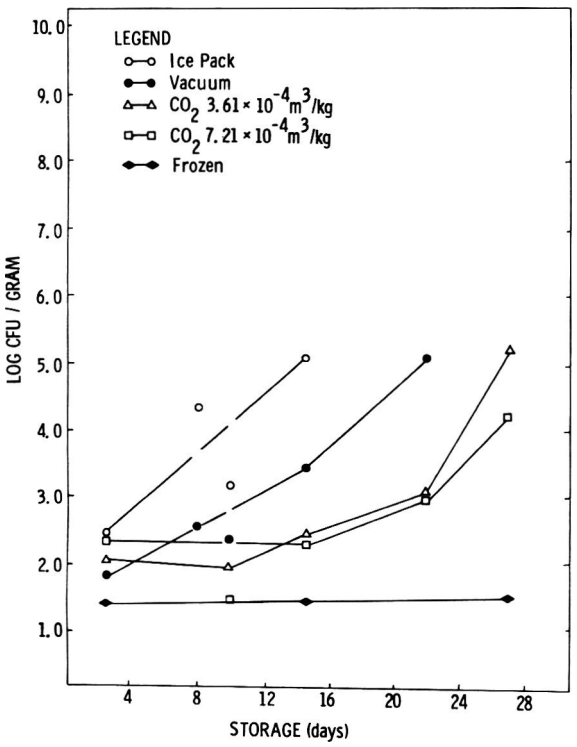

Fig. 3—Changes in coliform numbers on VRB agar in bulk pack whole chickens with various treatments and stored at 1.1°C.

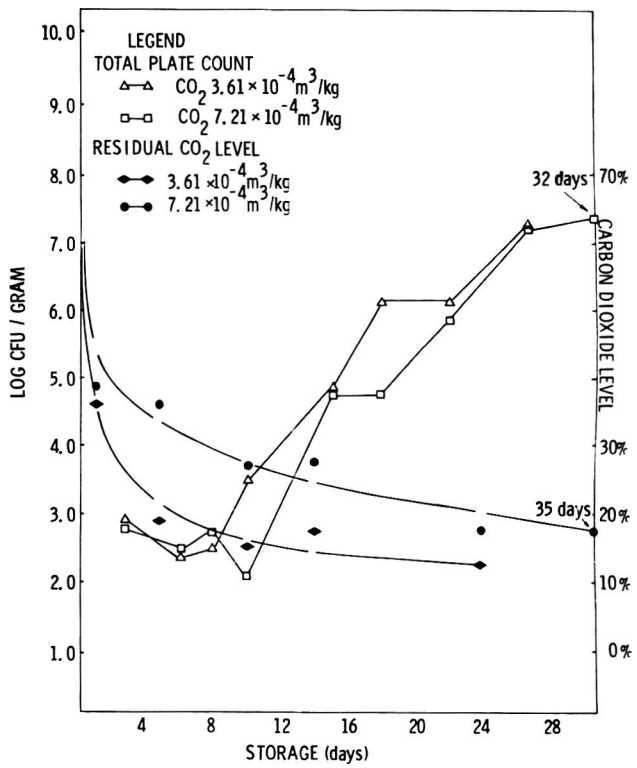

Fig. 4—Comparison of the effect of residual CO_2 in the pouch on limiting aerobic CFU growth.

ing sequence, the CO_2 content in the pouch was 96–98%, regardless of the addition rate used, 3.61×10^{-4} or 7.22×10^{-4} m^3/kg. Within 24 hr the CO_2 content in void space fell to 37–39% indicating that 61% (by difference) was adsorbed. A check of the skin surface pH showed a value of 6.9–7.1 on fresh, untreated chickens compared to 5.8–5.9 for the CO_2-treated birds. The CO_2 level in the void space continued to decrease at a slower rate through the remainder of the storage period. In Figure 4, the total number of aerobic CFU have been superimposed on the CO_2 composition of the void space. It appears that when the level of CO_2 reached 25% in the pouch void space, regardless of the addition rate of CO_2 used (3.61×10^{-4} or 7.22×10^{-4} m^3/kg), aerobic CFU enter the log growth phase. Figure 5 shows a similar presentation for CO_2 changes in the void space as related to changes in anaerobic CFU growth. Using CO_2 addition rates of 3.61×10^{-4} and 7.22×10^{-4} m^3/kg, the levels of CO^2 reached 18 and 26% on days 8 and 11, respectively, before anaerobic CFU appeared to enter the log growth phase. This observation, supported by higher generation times, suggests that anaerobic CFU are more sensitive than are aerobic CFU to CO_2 content in the pouch void space.

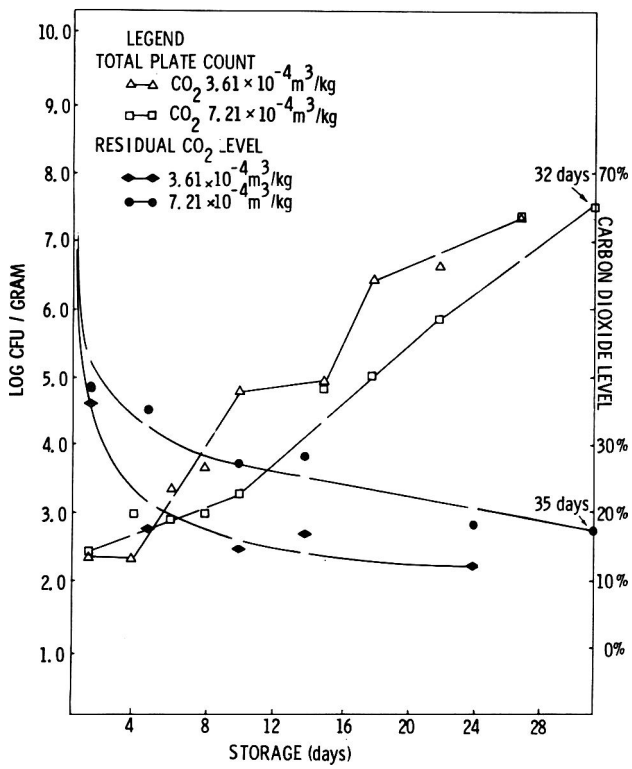

Fig. 5—Comparison of the effect of residual CO_2 in the pouch on limiting anaerobic CFU growth.

Table 6—Cost comparisons of three methods used for bulk packaging for shipment of fresh whole chickens or parts

Item[a]	Ice pack	CO_2 snow	Modified atm
Corrugated box	$712	$712	$599
Ice	50		
CO_2 (snow)		200	
CO_2 (gas)			10
Closure[b]			20
Nylon/Surlyn liner	___	___	320
Total	762	912	949
Cost/kg[c]	$0.0258	$0.0309	$0.0321

[a] Basis: 1000 units
[b] Mechanical evacuation of air, CO_2 flushing and heat sealing liner
[c] Based on a net weight of 29.5 kg/box

In view of the above discussion, a shelf life to 30 days is a reality for fresh, whole chickens treated with CO_2 with a Nylon/Surlyn film and held at 1.1°C. Cost/value relationships for controlled atmospheric storage, known as "modified atmosphere," were compared with those for ice pack and CO_2 snow (placed on top of the contents before closing) methods for packaging and preservation and are presented in Table 6.

Modified atmosphere (commercial trade name, International Paper Co.) packaging of bulk pack, whole chickens or parts offers the processor some distinct marketing opportunities. Initially, modified atmosphere packaging requires capital for equipment and higher costs for packaging materials. Although the modified atmosphere technique requires a cheaper corrugated shipper (water protection unnecessary) the Nylon/Surlyn film (liner) adds $320/1000 boxes to the materials costs. Mechanical air evacuation, gas flushing and heat sealing (closure) of the liner adds $20/1000 boxes. The final unit cost, $0.0322/kg, is higher than the two conventional methods, $0.0309/kg for CO_2 snow and $0.0258/kg for ice. However, the processor can look forward to significant freight savings (up to 30%) resulting from replacement of ice with salable product in transport. In one case, a commercial processor is able to charge a premium of $0.022/kg for modified atmosphere packaged chickens. Because of the handling conveniences provided to the customer, i.e. supermarket chain, the premium reduces the packaging cost below the cost of ice pack. In addition, the capital required to purchase ($36,000) or lease ($1000/month for 36 months) a machine for closure ($0.0011/kg) is also covered by the premium (ex operator labor). This cost, $0.0011/kg, is based on a throughput rate of 3 boxes/min or 88.6 kg/min.

CONCLUSION

TREATMENT of simulated bulk pack chickens in Nylon/Surlyn film with CO_2 addition rates of 3.61×10^{-4} and 7.22×10^{-4} m^3/kg of chickens extends shelf life to 22 and 27 days, respectively at 1.1°C. Ice pack and vacuum packaging (in Nylon/Surlyn film) provide limited shelf life—10 and 15 days, respectively.

The presence of CO_2, dissolved on the surface of the bird and present in the pouch void space, limits microbial growth. It extends the lag phase of aerobic CFU to 8–10 days or until the residual CO_2 in void space reaches 15–25% of the total gas composition. Carbon dioxide also limits anaerobic growth; generation times are higher than for aerobic types at both levels of CO_2 addition. Generation times for anaerobic CFU are considerably higher when CO_2 is present (32.5 and 34.9 hr, at usage levels of 3.61×10^{-4} and 7.22×10^{-4} m^3/kg, respectively) than in the presence of ice, (21.6 hr) and vacuum (19.3 hr). The presence and growth of critical pathogenic organisms are negligible regardless of the storage environment used, provided that the storage temperature is maintained at 1.1°C.

Numbers of aerobic and anaerobic CFU reach 1,000,000 CFU/g in ice pack and vacuum treatments and 10,000,000 CFU/g in CO_2 treatments before off-odors are detected. The presence of CO_2 selectively limits more objectionable proteolysis or putrefactive off-odor causing organisms while encouraging lactic acid-producing types.

A usage level of 7.22×10^{-4} m^3 CO_2/kg of whole chickens in a Nylon/Surlyn pouch with a minimum replacement of 95% of the air creates the optimum modified atmosphere required

—Continued on page 1527

EFFECT OF LEVEL OF STRUCTURED PROTEIN FIBER ON QUALITY OF MECHANICALLY DEBONED CHICKEN MEAT PATTIES

C. E. LYON, B. G. LYON, W. E. TOWNSEND and R. L. WILSON

ABSTRACT

Mechanically deboned chicken meat (without skin) and chicken (with skin) were combined with 15 or 25% structured protein fiber (SPF) to subjectively and objectively evaluate the quality of patties. Proximate composition, water-holding capacity, color, texture, and cooking loss were objectively determined. Products were characterized by the Quantitative Descriptive Analysis sensory technique. Raw patties containing 15% SPF had a higher water-holding capacity than patties with 25% SPF. Cooked patties with 25% SPF required significantly more force to shear, exhibited higher cooking losses, and were characterized as more chewy and elastic, less moist, and more desirable than 15% SPF patties. Textural properties of patties containing mechanically deboned poultry meat were improved by adding 25% SPF; however, cook yield was greater for patties with 15% SPF.

INTRODUCTION

MECHANICALLY DEBONED poultry meat has few, if any, intact muscle fibers (Vadehra and Baker, 1970) and resembles a meat paste. Mechanical deboning may also cause considerable cellular disruption, possible protein denaturation, and increased lipid and heme oxidation (Froning, 1976). This lack of texture and instability of flavor and color during storage have hampered optimum utilization of this economical and nutritious meat source.

The paste-like consistency of mechanically deboned poultry meat should make it adaptable to emulsified products; however, Pauly (1967) indicated that mechanically deboned poultry meat is most suitable in combination with other meat sources. Froning (1970) tested the emulsifying properties of various meat sources and reported that combining hand deboned chicken and turkey meat with mechanically deboned meat should enhance the stability of the final emulsion. Froning (1966) also listed the beneficial characteristics of a binder for poultry meat products as the ability to absorb moisture and impart a desirable color and the absence of undesirable flavors. A multi-tubular buff colored, flesh-like fiber structured from a soy isolate (structured protein fiber, SPF, supplied by Ralcon Foods, Chicago, IL 60606) might meet those requirements as a binder and also improve the texture of a mechanically deboned poultry meat product.

The purpose of this study was to evaluate the qualtiy characteristics of patties made from two formulas containing mechanically deboned chicken (MDC), mechanically deboned chicken meat (MDCM), and two levels of SPF.

EXPERIMENTAL

Preparation of product

Mechanically deboned chicken (product with skin – MDC) and mechanically deboned chicken meat (skinless – MDCM) were purchased from a processor in the north Georgia area. The meat was mechanically deboned and held at −23°C for 2 days, then transported to the laboratory, and stored at −40°C until product preparation approximately a week later. For patty preparation the MDC, MDCM, and SPF were tempered to 2°C. The MDC and MDCM were mixed in a Keebler Mixer with Morton's Meat, Poultry and Sausage Seasoning and a phosphate salt (Kena FP-37) for 5 min. SPF was then added and the ingredients mixed for two additional min. Products were stuffed into 101 mm diam fibrous casings, tempered overnight at −3°C, sliced 1.27 cm thick, packaged in plastic bags, and stored at −40°C until evaluated within one month's time. A total of 13.6 kg of each formula was made. Ingredients of the formulas are shown in Table 1.

Table 1—Percent ingredients of two poultry patty formulas

Ingredients	Formulas 1 %	Formulas 2 %
Mechanically deboned chicken (with skin)	42	37
Mechanically deboned chicken meat (skinless)	42	37
Structured protein fiber	15	25
Salt, seasoning and phosphates[a]	1	1

[a] Salt seasoning mix: Morton's Meat, Poultry and Sausage Seasoning. Phosphate: Kena FP-37.

Proximate composition

Moisture, fat and protein contents of the MDC, MDCM and patty formulas were determined in triplicate by standard AOAC (1965) procedures.

Calcium and bone analysis

Calcium content of raw meat and formulas was determined in duplicate by the Wilson and Co. method (1964) as outlined in the Beehive Model BX-66 Instruction Manual. Calcium content was converted to bone content as described by Satterlee et al. (1971).

Water-holding capacity (WHC)

The WHC of meat sources and formulas was determined by a modified procedure of Mast and MacNeil (1976). Samples (20g) were weighed into 250 ml polypropylene centrifuge tubes. Mast and MacNeil (1976) used 12,000 × G. We found in preliminary work, however, that 12,000 rpm (23,300 × g) was required to separate the liquid from the fat and meat. Samples were spun 10 min at 0°C in a Sorvall Superspeed RC2-B refrigerated centrifuge. Radius of the rotar was 14.6 cm. Any free liquid was decanted and the remaining meat reweighed. MDC, MDCM, Formula 1 (15% SPF), and Formula 2 (25% SPF) contained 57.97, 71.42, 64.83, and 63.85% moisture, respectively; therefore, 20-g samples contained 11.59, 14.28, 12.96 and 12.77g of moisture, respectively. Liquid lost during centrifugation was subtracted from the g of moisture present and WHC expressed as g of total moisture retained in a 20-g sample after centrifugation.

Color

Objective color differences were measured with the Hunter Color and Color Difference Meter (Model D-25-D), which was standardized with the white plate (W783). L, a_L and b_L color values were determined on 15 patties from each formula, frozen and heated.

Cooking loss

Frozen patties were placed on racks in aluminum pans and cooked in a rotary reel oven set at 177°C to an end-point temperature of 77°C, which was monitored with a digital thermometer. Patties were turned after 30 min and cooking time ranged from 55–65 min. Aluminum pans were weighed without and with four frozen patties, then reweighed after heating with and without the heated patties. Evaporative and drip losses were determined from these weights. The cooking procedure was replicated 10 times.

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Table 2—Moisture, fat, protein, calcium, bone equivalent and water-holding capacity of two meat sources and patties containing two levels of structured protein fiber

Meats and formulas	Analyses					
	Moisture[a] %	Fat[a] %	Protein[a] %	Calcium[b] %	Bone[b] equivalent %	Water-holding[a,c] capacity
Mechanically deboned chicken — with skin	57.97	30.53	11.18	0.14	0.78	5.37
Mechanically deboned chicken meat — skinless	71.42	14.04	14.21	0.17	0.96	7.30
Formula 1 (15% structured protein fiber)	64.83	18.14	15.78	0.12	0.65	8.63
Formula 2 (25% structured protein fiber)	63.85	16.96	17.67	0.12	0.65	7.65

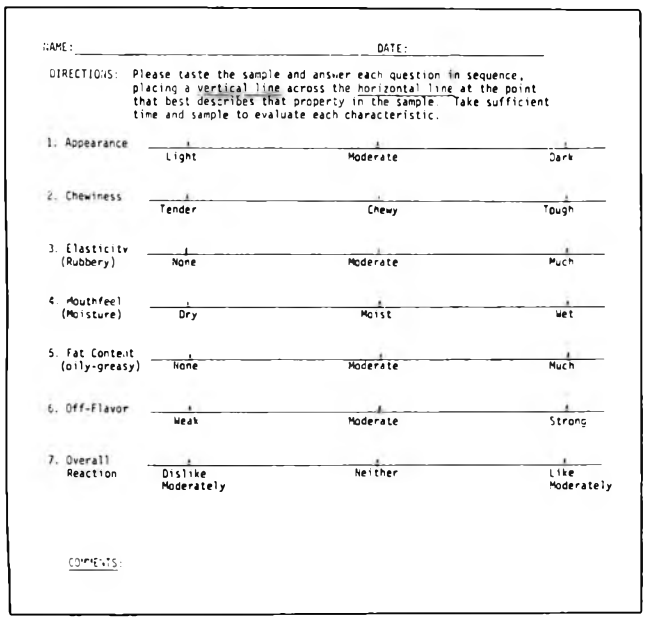
[a] Each number is the mean of three observations.
[b] Each number is the mean of two observations.
[c] Water-holding capacity is expressed as g of total moisture retained in a 20-g sample after centrifugation.

Texture evaluation—Instron

An Instron equipped with a single blade attachment was used to measure the force in kg required to shear a 2.5 cm wide slice of the heated patty. Each slice was sheared twice. Shear force was determined on five slices from each formula and replicated three times for a total of 30 observations per formula. Cross-head speed up and down and chart speed were 50 cm per min.

Sensory evaluation

Patties were evaluated by triangle tests and Quantitative Descriptive Analysis (QDA). A triangle test was used initially to determine if panel members with previous training and known discriminative ability could detect differences between patties from the two formulas. Nine panelists were served quarter pieces of cooked patties on coded white plates under white light in individual booths. Panelists were asked to report their bases for selection of the odd sample. The triangle test was replicated twice and significant product differences were found. In the second phase of the sensory analysis, sensory characteristics of the patty products were profiled using the QDA technique (Stone et al., 1974). In the QDA approach, preceptible factors, intensities, and order in which factors are perceived are determined during group training by the panel. Metric scales are used to record panel responses of intensity of a specific factor. Five panelists who had been trained in the QDA technique with chicken patties participated in this phase. Panelists evaluated six sensory attributes that had been previously determined during training to characterize the products. In addition, a scale to reflect overall impression of the products was included (Fig. 1).


NAME: _____ DATE: _____

DIRECTIONS: Please taste the sample and answer each question in sequence, placing a vertical line across the horizontal line at the point that best describes that property in the sample. Take sufficient time and sample to evaluate each characteristic.

1. Appearance — Light — Moderate — Dark
2. Chewiness — Tender — Chewy — Tough
3. Elasticity (Rubbery) — None — Moderate — Much
4. Mouthfeel (Moisture) — Dry — Moist — Wet
5. Fat Content (oily-greasy) — None — Moderate — Much
6. Off-Flavor — Weak — Moderate — Strong
7. Overall Reaction — Dislike Moderately — Neither — Like Moderately

COMMENTS:

Fig. 1—Scoresheet for sensory evaluation of poultry patties made from mechanically deboned broiler meat and two levels of Structured Protein Fiber.

Quartered slices of the products were presented to the panelists in individual booths under white light. Duplicate samples from each formula were evaluated at each session for a total of four coded samples per session. Order of presentation of the four samples was randomized at each session for each panelists. In all, six sessions were held. Panelists marked their score sheets with a vertical line across the 6-in. horizontal continuum. A transparent overlay of a 6-in. line divided into 60 units was super-imposed on the score sheet and a numerical score between 0–60 was assigned to the panelists' marks.

Statistical analyses

Objective data were treated by analysis of variance, and sensory data were analyzed by one- and two-way analysis of variance for individual and panel performance. Where significant differences were found, means were separated by Duncan's multiple range test (Steel and Torrie, 1960).

RESULTS & DISCUSSION

PROXIMATE COMPOSITION, % calcium and bone, and WHC of the MDC, MDCM, and resulting formulas are shown in Table 2. MDC exhibited lower moisture and protein and higher fat than MDCM. Presence of skin in MDC would account for these differences. Percent protein was higher in Formula 2 (17.67%) than in Formula 1 (15.78%). The difference in SPF levels would account for this difference. Bone content was higher in MDCM (skinless) than in MDC (with skin); values were 0.96 and 0.78%, respectively. Satterlee et al., (1971) observed that bone content of deboned poultry meat decreased as percent skin increased, and this trend is evident in our data. The WHC was lower for MDC (5.37) than for MDCM (7.30). The WHC was related to percents of fat and protein in the meat sources, and was higher for Formula 1 (8.63) than for Formula 2 (7.65). The lower WHC of Formula 2 might be explained by the acid pH of SPF (5.2). Perhaps pH was lower in Formula 2 than in Formula 1. Small changes in meat pH may cause relatively great changes in WHC (Hamm, 1960), and the additional 10% SPF in Formula 2 could account for this difference in WHC. Possibly SPF should be buffered with food grade sodium carbonate to increase the p*H* of the product (Moller, 1977).

Hunter color values of frozen and cooked patties are shown in Table 3. Frozen patties containing 15% SPF (Formula 1) were significantly redder and yellower (higher a_L and b_L values) than patties containing 25% SPF. The larger amounts of MDC and MDCM in Formula 1 would account for these color differences. We attribute the difference in redness to extra heme pigments, and the difference in yellowness to extra fat.

—Text continued on page 1526

Table 3—Hunter color values and standard errors to two poultry patty formulas containing two levels of structured protein fiber[a,b]

Formulas and percent structured protein fiber	Frozen			Cooked		
	L	a_L	b_L	L	a_L	b_L
1. 15% structured protein fiber	46.66 ± 0.41a	17.51 ± 0.45a	13.44 ± 0.14a	40.35 ± 0.29a	6.22 ± 0.09a	13.00 ± 0.09a
2. 25% structured protein fiber	49.18 ± 0.39a	12.46 ± 0.24b	11.11 ± 0.23b	43.21 ± 0.55b	5.41 ± 0.12a	12.52 ± 0.08a

[a] Each number in the table is the mean value of 15 observations.
[b] Means within columns with different letters differ significantly ($P < 0.05$).

Table 4—Cooking losses and objective texture measurements plus standard errors of two poultry patty formulas containing two levels of structured protein fiber[a]

Formulas and percent structured protein fiber	Shear force[b]	% Cooking loss[c]		
		Evaporative	Drip	Total
1. 15% structured protein fiber	2.00 ± 0.05a	17.91 ± 0.63a	1.23 ± 0.09a	19.14 ± 0.63a
2. 25% structured protein fiber	3.05 ± 0.09b	19.65 ± 0.69a	5.24 ± 0.33b	24.89 ± 0.59b

[a] Means within columns with different letters differ significantly ($P < 0.05$).
[b] Kilograms of force required to shear a 2.5 cm wide slice of a cooked patty. Each number is the mean of 30 observations.
[c] Each number is the mean of 10 observations.

Cooked patties containing 15% SPF were significantly darker (lower L values) than patties containing 25% SPF. Proteins were denatured by cooking and redness did not significantly differ between the formulas. In Formula 1 the extra amounts of MDC and MDCM and of heme pigments from the muscle tissue would probably account for the dark appearance of the cooked patties.

Percent cooking losses and objective texture measurements of the formulas are shown in Table 4. Shear force was significantly greater for Formula 2 than for Formula 1 (3.05 and 2.00 kg, respectively). The 25% SPF in Formula 2 did not bind the fat and moisture as efficiently as did the 15% SPF in Formula 1. Formula 2 had significantly higher drip and total cooking losses than Formula 1. The addition of a buffer, as mentioned earlier, might lower cooking loss by increasing pH and subsequent product hydration.

All nine panel members correctly identified the odd sample in the triangle tests for both replications. These results indicate a definite difference between the formulas. Panelists' comments for selection of the odd sample centered on textural differences. Formula 1 was characterized as "spongy" while Formula 2 was characterized as "firm, coarse, and chewy." Results of the QDA portion of the sensory data are presented in Table 5. Panelists characterized patties with 25% SPF as significantly lighter, more chewy, elastic or springy, and off-flavored and significantly less moist than patties with 15% SPF. The subjective textural categories of chewiness and elasticity seem to agree with the objective texture measurement (Instron) in that patties with 25% SPF required significantly more force to shear and were characterized by the panel as more chewy and elastic than patties with 15% SPF. The significant difference in mouthfeel (moisture) between the products may reflect the lower WHC of Formula 2 (25% SPF) as compared to Formula 1 (15% SPF) in Table 2 and the increased cooking loss of Formula 2 as shown in Table 4.

The off-flavor scores were in the weak-to-moderate intensity range. Panelists characterized patties with 25% SPF as more off-flavored and ranked them higher on the scale for overall impression than patties with 15% SPF. Lyon et al. (1978) noted that off-flavor score was significantly higher for poultry patties (again in the weak-to-moderate intensity range)

Table 5—Panel responses to poultry patties containing two levels of structured protein fiber[a,b]

Panel category[c]	Formulas 1 15% structured protein fiber	Formulas 2 25% structured protein fiber	Standard error
Appearance	34.54a	30.86b	0.70
Chewiness	10.66a	26.32b	0.92
Elasticity (rubbery)	14.11a	23.84b	1.16
Mouthfeel (moisture)	31.83a	22.24b	0.79
Fat content (oily-greasy)	16.38a	16.36a	0.85
Off-flavor	8.53a	13.13b	0.93
Overall impression	25.12a	32.48b	1.12

[a] Each number is the mean of 60 observations.
[b] Means within rows with different letters differ significantly ($P < 0.05$).
[c] Numerical range for each scale is 0–60. For interpretive purposes, appearance scale is light to dark; chewiness is tender to tough; elasticity is none to much; mouthfeel is dry to wet; fat content is none to much; off-flavor is weak to strong; and overall impression is dislike moderately to like moderately.

containing 16% SPF than for patties containing either 8% or no SPF. They theorized that high levels of SPF in the patties imparted a flavor that differed from a typical all meat flavor. A slight off-flavor score apparently is not objectionable. However, Moller (1977) stated that this off-flavor might be eliminated by the buffering effect of food grade sodium carbonate added to the SPF prior to incorporation in the meat system.

The products were profiled for visual comparisons of sensory characteristics by constructing axes around a central point to represent the sensory characteristics and plotting mean scores at a central point to represent the sensory characteristics and plotting mean scores at a distance from the center to represent the magnitudes of intensity (Fig. 2). The most important differences between the formulas were in the textural categories of chewiness and elasticity (springiness). Patties were significantly more chewy and elastic, less moist

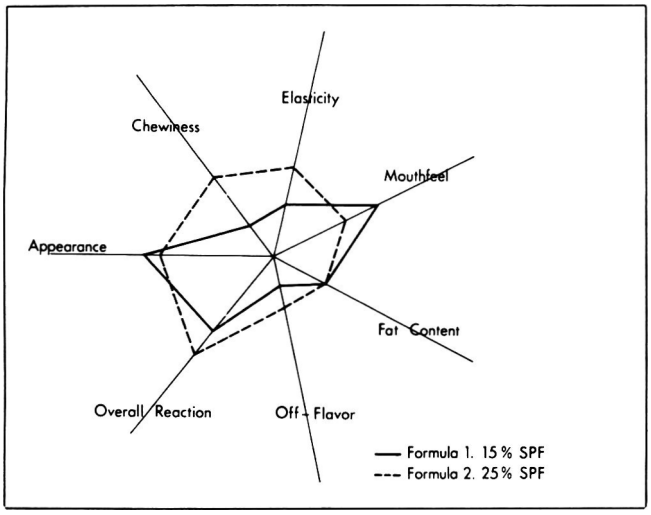


Fig. 2—Profiles of the panel responses for Formulas 1 and 2.

(mouthfeel) and more off-flavored with 25% than with 15% SPF, but ranked higher on the QDA scale for overall impression. The product profiles in Figure 2 suggest that texture of the product is more important to overall reaction than moisture or off-flavor if the off-flavor scores are in the weak-to-moderate range and do not reflect product deterioration.

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Presented at the 38th Annual Meeting of the Institute of Food Technologists, Dallas, TX, June 4-7, 1978.

Reference to a company or product name does not imply approval or recommendation by the U.S. Department of Agriculture to the exclusion of others that may be suitable.

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to limit microbial growth and insure organoleptic quality to 27 days of storage at 1.1°C.

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Ms received 10/22/77; revised 5/5/78; accepted 5/10/78.

Scientific Journal Series Paper No. 10,088, Minnesota Agricultural Experiment Station, St. Paul, MN 55108.

This research investigation was undertaken at the request of and with the financial support of International Paper Company. We are grateful for the opportunity and experience gained. Mention of commercial names of equipment, films, ingredients, and chemicals in this paper does not constitute an endorsement by the investigators, the Department of Food Science and Nutrition, or the University of Minnesota.

We are grateful for the technical help and consultation provided by Dr. S.R. Tatini of this department.

IMPROVING QUALITY OF BIRD CHILLER WATER FOR RECYCLING BY DIATOMACEOUS EARTH FILTRATION AND CHLORINATION

H. S. LILLARD

ABSTRACT

Bird chiller water was filtered in a vertical tank pressure leaf filter by use of two grades of Diatomaceous Earth (DE). With both grades of DE, significant reductions were obtained in suspended solids, dissolved matter, grease, COD, BOD and total Kjeldahl nitrogen so that effective bactericidal treatment was accomplished with 26–28 ppm chlorine gas. Total aerobic counts, fecal coliforms and salmonellae were essentially eliminated from filtered, chlorinated water. The pH of filtered chiller water was similar to the pH of the unfiltered water. Clarity and light transmission compared favorably with potable water. Filtered, chlorinated chiller water was neither esthetically nor microbiologically objectionable. It was concluded that filtered chiller water could safely be reused to flume broiler giblets.

INTRODUCTION

THE AVERAGE BROILER processing plant in the United States uses about 1 million gallons of potable water per day. In addition to water shortages experienced in certain areas of the United States, the cost of potable water and effluent discharge is expected to continue to rise. One of the largest processors in Georgia reports using 30 million gallons of potable water per month at a cost of about $27,000 including cost of effluent discharge. The high cost of potable water and effluent discharge in certain areas is either passed on to the consumer or is absorbed by the processor who must compete with processors in areas where cost of water and effluent discharge is lower.

Regulatory agencies require the use of potable water in all phases of processing except, by petition, in scalding. The demand for potable water could be reduced by reusing water in certain phases of processing. Lillard (1978a) showed that necks flumed with untreated water from the bird chiller were microbiologically comparable to necks flumed with potable water: there was no difference in shelf-life, incidence and level of fecal coliforms or salmonellae, but total aerobic counts were slightly higher for necks flumed with untreated bird chiller water. Bactericidal treatment of bird chiller water could eliminate microbiological differences between products flumed in bird chiller or potable water. However, the high level of organic matter in bird chiller water could interfere with effective bactericidal treatment. Even if effective bactericidal treatment of chiller water were possible, regulatory approval for reuse of chiller water without further improvement probably would be denied on the basis of esthetic considerations.

Rogers (1975) screened various methods for removal of organic matter from bird chiller water. His unpublished report indicated that filtration through diatomaceous earth (DE) in a pressure leaf filter was the most promising of the methods he tested for organic matter removal from chiller water. Although the most common applications for DE filters are in the chemical industry, brewers and vintners use DE filters as primary and final polishing filters. Major manufacturers of oils from corn, peanuts and vegetables use DE filters to remove husk as well as for final polishing, and sugar and bottling industries use them for sugar filtration. Some manufacturers of frozen cooked chicken use DE filters to recycle grease.

This study was undertaken to evaluate the microbiological, chemical and physical properties of DE-filtered bird chiller water in order to determine whether the esthetic and microbiological qualities could be improved for recycling.

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MATERIALS & METHODS

Diatomaceous earth filtration

Water from the bird chiller overflow was collected and pumped away from the main broiler processing area of a commercial plant to a pilot size vertical tank pressure leaf filter (Durco, The Duriron Co., Inc.) containing 6 leaves with a total filter area of 30 sq ft (Fig. 1). Two grades of DE filter aid were tested: Dicalite 4200 and Speedflow. Preliminary results showed that some filter aid was recovered in the filtered water when Speedflow was used. Therefore, leaves were coated either with Dicalite 4200 or with a 1:1 w/w mixture of Dicalite 4200 and Speedflow. When the two grades were mixed, Dicalite, which is coarser, coated the leaves first and prevented Speedflow from contaminating the filtered water.

The leaves were precoated with 0.13 lb DE per sq ft filter area when Dicalite 4200 was used (resulting in a flow rate of 16.9 gal/min) and with 0.26 lb DE per sq ft filter area when the mixture of two grades was used (resulting in a flow rate of 14.7 gal/min). Operating time of the filter was extended without recoating the leaves, by addition of a DE slurry to the chiller water. A body feed rate consisting of 0.03% DE (w/v) was used with Dicalite 4200 but was increased to 0.05% Speedflow when the two-grade mixture was used. The precoat tank, filter, leaves, all pipes and connections were sanitized with 2% Lysol for 15–20 min then rinsed with potable water for 15–20 min prior to each use.

Chlorination

An Advance cylinder-mounted gas chlorinator (Capital Controls Co., Inc.) was used. The chlorinator ejector was installed in the line carrying the filtered water (Fig. 1). Measured amounts of chlorine were added until the free residual was 0.4–0.6 ppm. Total and free residual chlorine were measured with a Fisher and Porter Model 17T1010 amperometric titrator.

Sampling

Two water samples (500 ml) were collected from each of two sampling points on each sampling day: (1) The commercial bird chiller overflow; (2) the DE filter outlet for filtered chiller water or a collection tank for filtered, chlorinated chiller water. An appropriate amount of sodium thiosulfate (105 mg) was added to each container in which chlorinated water was collected.

All samples were kept on ice and transported immediately to the laboratory where microbiological tests were started within 1½ hrs of sampling. Both water sources were sampled on the same day for each treatment and all tests were performed on the same sample. The number of sampling days varied (5–17 days) among treatments as shown in Tables 1 and 2.

Microbiological analyses

For total aerobic plate counts samples were decimally diluted in 1% peptone, pour-plated in duplicate in Difco Plate Count Agar and incubated at 20°C for 72 hr.

For enhanced recovery of damaged cells, fecal coliform densities were determined by the five-tube MPN procedure described in Standard Methods for the Examination of Water and Wastewater (APHA, 1971). Fermentation tubes with inoculated Difco EC Medium were incubated in a Precision Scientific Coliform Incubator Bath at 44.5°C for 24 hr.

For salmonellae, incidence was estimated by enrichment methods, and levels by a five-tube MPN procedure (Lillard, 1977).

Chemical and physical analyses

Suspended solids (nonfilterable residues), dissolved matter (filterable residues), total grease, total Kjeldahl nitrogen, Biochemical Oxygen Demand (BOD) and Chemical Oxygen Demand (COD) were determined by Standard Methods (APHA, 1971). Turbidity was measured in a Coleman nephelometer Model 9. Turbid samples were diluted with distilled water before they were measured and turbidity was reported as recommended by APHA (1971). Percent light transmission was compared to transmission of potable water and was measured at two wavelengths: 400 nm, the Soret band of maximum absorption (or minimum transmission) of all heme proteins, and 540 nm, the wavelength of maximum absorption of hemoglobin (White et al., 1964).

Statistical analyses

All microbiological data including shelf-life, and data for suspended solids, dissolved matter, grease, COD, BOD and total Kjeldahl nitrogen were treated by Standard Analysis of Variance. Significance is reported at the 5% level; the term highly significant is used to denote differences at the 1% level.

RESULTS & DISCUSSION

LEVELS of organic matter in untreated bird chiller water differed for the two test periods in which the different grades of DE were used (Table 1). However, statistical analyses of the data showed that for both test periods DE filtration of chiller water with either grade filter aid resulted in highly significant reductions of suspended solids, grease, BOD and total Kjeldahl nitrogen, and statistically significant reductions in COD and dissolved matter. Filtration with the combination of Speedflow plus 4200 removed a higher percentage of these organic contaminants from chiller water and produced water of greater clarity, less color and turbidity, at times transmitting 100% of light at 540 nm, than water filtered with Dicalite 4200 alone.

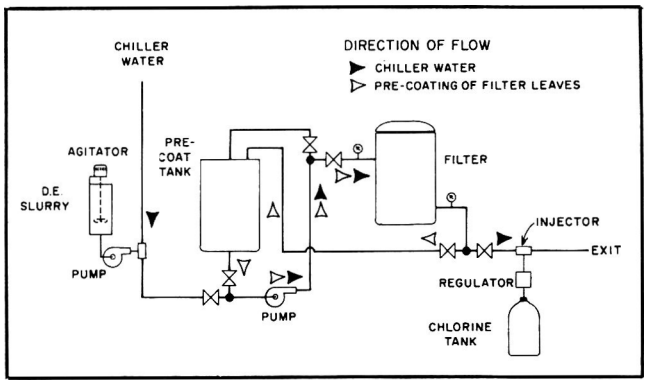

Fig. 1—Schematic diagram of pre-coat tank, pressure leaf filter, and chlorinator. Arrows show the line of flow.

Differences in organic matter removal from chiller water filtered with either grade DE were not statistically significant except for COD. However, fewer data were available for tests with Speedflow plus 4200 than for tests with 4200 alone. In general pH did not differ between filtered and unfiltered chiller water (Table 1).

Aerobic counts and fecal coliform levels were lowered significantly when chiller water was filtered with Dicalite 4200 (Table 2). When Speedflow was combined with 4200 reduction in counts for filtered chiller water were highly significant but still not equivalent to standards set for fecal coliforms in potable water. Salmonellae were not recovered frequently during this study. Therefore, a good evaluation of the effect of

Table 1—Chemical, biochemical and physical properties of untreated and DE filtered bird chiller water

Sample Source		mg/L[a] Suspended solids	Dissolved matter	Total grease	COD	BOD	Total Kjeldahl/N_2	pH	% Light Transmission 400 nm	540 nm	Turbidity
Bird chiller water[b]	Mean	114	471	172	582	359	28.55	NA[f]	36	51	287
	High	273	746	319	1095	606	32.70	7.56	50	68	654
	Median	112	427	155	555	373	29.30	7.23	35	51	210
	Low	29	353	83	290	161	23.60	6.51	22	36	146
Filtered bird chiller water (Dicalite 4200)[c]	Mean	15	407	32	343	208	24.65	NA[f]	69	86	62
	High	50	521	115	698	422	28.50	7.51	81	91	107
	Median	12	395	15	340	198	23.60	7.34	68	87	53
	Low	3	324	6	185	110	22.30	7.22	60	81	35
Percent reduction due to filtration (based on means)		87	14	81	41	42	14	NA[f]	NA[f]	NA[f]	NA[f]
Bird chiller waters[d]	Mean	160	414	137	623	407	31.49	NA[f]	37	56	445
	High	189	526	243	752	770	35.70	7.70	49	66	460
	Median	161	432	138	616	369	31.69	7.54	37	56	450
	Low	125	198	73	544	226	26.18	6.88	26	44	420
Filtered bird chiller water (1:1 w/w Speedflow + Dicalite 4200)[e]	Mean	9	324	16	259	207	20.86	NA[f]	81	96	30
	High	15	413	43	451	328	31.99	7.64	89	100	39
	Median	9	338	14	230	179	20.47	7.54	82	95	29
	Low	2	102	2	79	124	15.71	7.33	70	91	23
Percent reduction due to filtration (based on means)		94	22	88	58	49	34	NA[f]	NA[f]	NA[f]	NA[f]

[a] By all parameters filtered chiller water had values lower than chiller water; all these differences were either statistically significant or highly significant. However, there were no significant differences between samples of chiller water filtered by either grade DE.

[b] Means based on the following number of samples: Suspended solids 32, Dissolved matter 28, Grease 27, COD 31, BOD 12, Total Kjeldahl nitrogen 10, pH 11, Light transmission 16, Turbidity 20.

[c] Means based on the following number of samples: Suspended solids 32, Dissolved matter 27, Grease 27, COD 32, BOD 12, Total Kjeldahl nitrogen 10, pH 9, Light transmission 12, Turbidity 20.

[d] Means based on the following number of samples: Suspended solids 18, Dissolved matter 18, Grease 17, COD 18, BOD 12, Total Kjeldahl nitrogen 18, pH 22, Light transmission 18, Turbidity 4.

[e] Means based on the following number of samples: Suspended solids 18, Dissolved matter 18, Grease 18, COD 18, BOD 12, Total Kjeldahl nitrogen 18, pH 22, Light transmission 18, Turbidity 4.

[f] NA = not applicable.

Table 2—Microbiological characterization of untreated, filtered, filtered and chlorinated bird chiller water

Sample source	Mean $\log_{10}$/ml		Salmonellae	
	Total aerobic count	Fecal coliforms	Incidence +/total	Level & Range # salmonellae/ml
Bird chiller water	4.16	2.84	3/34	0–<0.4
Filtered bird chiller water[a] (Dicalite 4200)	3.96	2.59	2/34	0–<0.4
Bird chiller water	5.12	3.13	0/18	NA[c]
Filtered bird chiller water[b, d] (Speedflow + Dicalite 4200)	3.72	1.33	0/18	NA[c]
Bird chiller water	4.71	2.74	2/12	0–<0.4
Filtered, chlorinated bird chiller[b, e] water (Dicalite 4200)	0.16	<0	0/12	NA[c]
Bird chiller water	5.12	3.13	0/10	NA[c]
Filtered, chlorinated bird chiller[b, f] water (Speedflow + Dicalite 4200)	1.45	<0	0/10	NA[c]

[a] The reduction in levels of aerobic organisms and fecal coliforms in filtered chiller water is significant at the 5% level. Means in this group based on 34 samples taken on 17 sampling days.
[b] The reduction in levels of aerobic organisms and fecal coliforms in filtered, chlorinated water is highly significant (1% level).
[c] NA = Not applicable.
[d] Means in this group based on 18 samples taken on 9 sampling days
[e] Means in this group based on 12 samples taken on 6 sampling days
[f] Means in this group based on 10 samples taken on 5 sampling days

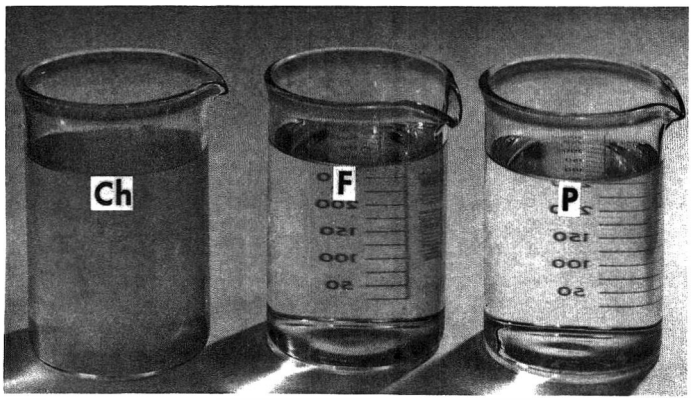

Fig. 2—Water samples (left to right) from the bird chiller, filtered chiller water and potable water.

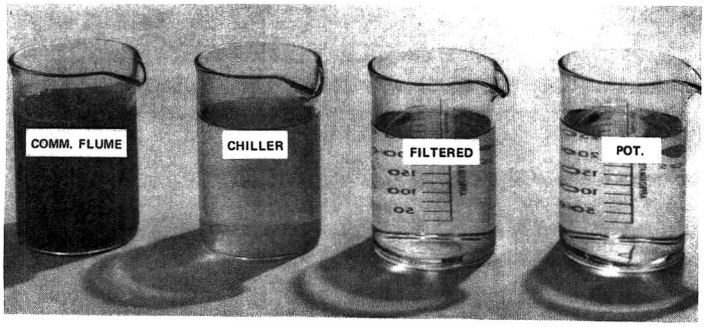

Fig. 3—Water samples (left to right) from the commercial neck flume, bird chiller, filtered chiller water and potable water.

filtration with Speedflow plus 4200 was not possible since all chiller and filtered water samples were negative during that period. However, Table 2 shows that salmonellae were recovered from 2/34 water samples filtered with Dicalite 4200; bird chiller water was positive on the same sampling dates. Salmonellae levels in all positive samples were shown to be very low by the MPN procedure (Table 2).

In water purification, a free residual of 0.5–1 ppm chlorine is an effective bactericide for *E. coli* and the various species of Salmonella and Shigella (Smith and Martin, 1948). Chlorination of untreated bird chiller water to a free residual of 0.4–0.6 ppm required about 48–50 ppm chlorine. Removal of organic matter from chiller water by filtration with either grade DE made it possible to chlorinate to a free residual of 0.4–0.6 ppm with 26–28 ppm chlorine gas. Chlorination of the filtered water at these levels reduced bacterial levels drastically so that the results were not included in the statistical analyses. With Dicalite 4200, aerobic counts were reduced from an arithmetic average of 55,000/ml in chiller water to 1.5/ml in filtered, chlorinated chiller water; fecal coliforms were reduced from an arithmetic average of 613/ml to 0/100 ml. When chiller water was filtered with Speedflow plus 4200, then chlorinated to a free residual of 0.5–0.6 ppm, total aerobic counts were reduced from an arithmetic average of 670,000/ml in chiller water to 28/ml in filtered, chlorinated chiller water, and fecal coliforms were reduced from an average of 3000/ml to an average of 1.5/ml. Table 2 shows these results expressed as means of the logarithmic values. Salmonellae were not isolated, even by enrichment methods, from chiller water filtered with either grade DE and chlorinated, even on dates when unfiltered and filtered chiller water samples were salmonellae positive.

These results show that DE filtration of chiller water without chlorination effectively reduces organic matter and bacterial levels in bird chiller water. DE filtration plus chlorination results in highly significant reductions in bacterial populations and produces water which visually resembles potable water and is neither esthetically nor microbiologically objectionable.

Figures 2 and 3 show the extremes in color and clarity of filtered chiller water compared to potable water. Figure 2 shows the maximum color obtained, Figure 3 shows the

clearest water obtained with or without chlorination (see 100% light transmission, Table 1). On days when filtered water had some color compared to potable water, chlorination bleached the color. The color of filtered, unchlorinated water was generally not discernable unless placed next to potable water.

Clarity of the filtered chiller water, unfiltered chiller water and commercial flume water are best compared to potable water by the ease with which markings on the beakers can be seen through the water (Fig. 3). When necks are flumed with potable water the appearance of the water changes within seconds of the necks entering the flume. Figure 3 shows the dark (bloody) characteristic of commercial flume water and the grease floating at the surface. By contrast, filtered chiller water is relatively clear, colorless and free of grease. Considering the chemical, physical and microbiological properties of the filtered chiller water discussed above and the rapid deterioration of potable water in the neck flume (Lillard, 1978b) there seems to be no logical basis for the requirement for use of potable water in neck flumes. In a companion paper, necks flumed commercially will be compared with necks flumed in filtered and filtered, chlorinated chiller water.

Finally, in order for a process to be commercially practical it is important that it be effective and financially feasible. Variations in grease and solids content of chiller water in processing plants can be effectively controlled by varying the size and filtering capacity of a pressure leaf filter. Cost to the processor, of course, will vary in different areas. However, based on surcharges, cost of potable water and discharge in the Gainesville, Georgia area, a plant the size of the one cooperating in this study (200,000 birds/day), and the means for organic matter shown in this study for chiller water and for chiller water filtered with Dicalite 4200, the following annual savings were estimated: $3200 on surcharges for BOD, solids and grease (surcharge 4¢/lb > 250 BOD, 2¢/lb > 250 solids, 4¢/lb > 100 grease); $23,000 on cost of water and sewage discharge saved by reusing 26 million gallons of chiller water per year (66¢/100 cu ft or 88¢/1000 gallons); $550–$1,100 from selling to rendering plants the grease and solids removed by the filter (at 1–2¢/lb) for an annual total of about $27,000. This does not include energy savings derived from cooling the giblets with chiller water in the flume with a concomitant reduction in energy requirements in the giblet chiller.

For reuse of water from 2 chillers for 2 shifts without recoating the leaves, a filter with about 150 sq ft filter area would be required at a cost of about $45,000. In general 1 lb of DE removes 1 lb of organic matter (Colwell, 1977). Therefore, annual cost for DE would be about $4,000 (based on removing about 55,000 lb of organic matter at $140/ton of DE). Energy requirements for operating the filter were not included in the calculations. The energy requirement would be very low because no parts to the filter move during operation and the supply pump to the filter is the only energy source required.

Cost of manpower required for operating the filter was not included because it would vary with the degree of automation obtained in a pressure leaf filter. Tax advantages for equipment depreciation were not included in savings derived. However, in general, these estimates indicate that a plant could recover the cost of such a filter in about 2 years by reusing water from the bird chiller to flume giblets.

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Ms received 1/30/78; revised 3/20/78; accepted 4/5/78.

The author acknowledges R.L. Wilson for the statistical analyses, Jack L. Witherow, Project Officer, for making the unpublished EPA report available, Larry Miller and Country Pride Foods for their unfailing cooperation and interest in this project, Kelly Colwell and the Duriron Company for making the pressure leaf filter available.

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EVALUATION OF BROILER NECKS FLUMED WITH DIATOMACEOUS EARTH FILTERED CHILLER WATER

H. S. LILLARD

ABSTRACT

In order to conserve water and energy the feasibility of fluming necks with treated bird chiller water instead of potable water was examined. No significant differences were found in shelf-life, total aerobic counts, levels of fecal coliforms, or salmonellae incidence between necks flumed with potable water and necks flumed with diatomaceous earth-filtered chiller water or filtered, chlorinated chiller water. It was concluded that the use of potable water is not essential in all phases of broiler processing and that necks could be flumed safely with either filtered chiller water or filtered, chlorinated chiller water.

INTRODUCTION

REGULATORY AGENCIES require the use of potable water in all phases of broiler processing except, by petition, in scalding. Broiler processing plants in the United States are required to use an overflow of 1/2 gallon/bird. An average plant in the United States processes 70,000 birds per day. Some of the largest plants process 200,000 broilers per day. By reuse of water from the bird chiller in other phases of processing, a plant could save 35,000–100,000 gallons of potable water per day or 9–26 million gallons per year. There are over 400 federally inspected broiler processing plants in the United States. The reuse of chiller water could result in an average annual saving to the whole industry of about 3.6 billion gallons of potable water and discharge. Furthermore, water from the bird chiller, if used to flume giblets, would also cool the giblets in the flumes and reduce the energy requirement for cooling in the chillers.

Lillard (1978a) showed that necks flumed with untreated bird chiller water were 1/4 log higher in total aerobic counts than necks flumed with potable water but were microbiologically equal in other respects (fecal coliform levels, salmonellae incidence and levels, shelf-life). In order to eliminate the difference in total aerobic counts, Lillard (1978b) filtered bird chiller water through diatomaceous earth in a pressure leaf filter to remove organic matter, so that the water could be effectively chlorinated. The filtered chlorinated chiller water was very clear, visually very similar to potable water and was neither esthetically nor microbiologically objectionable.

This study was undertaken to determine the feasibility of fluming giblets with treated bird chiller water instead of potable water. Under present regulations, products flumed with other than potable water may not be sold. Therefore, necks were used for this study because they would cause the least financial loss to the cooperating processor. The microbiological quality of necks flumed commercially with potable water was compared to quality of necks flumed in a simulated giblet flume with potable water, filtered bird chiller water and filtered, chlorinated chiller water.

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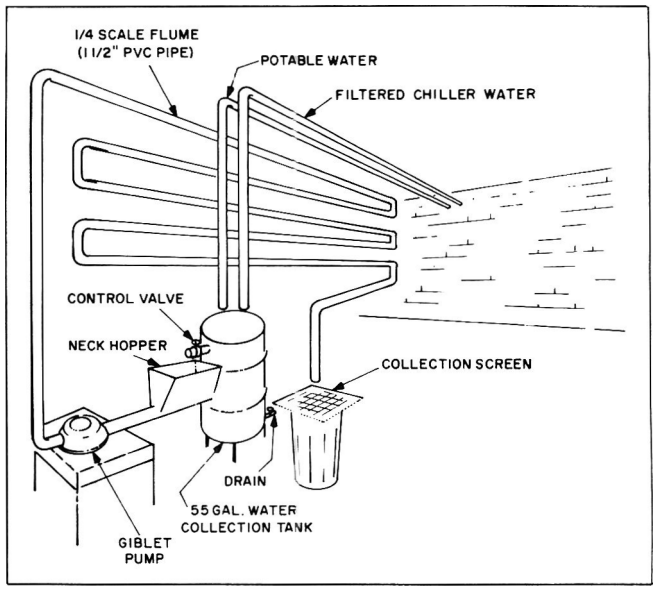

Fig. 1—Simulated giblet flume (1/4 scale).

MATERIALS & METHODS

Fluming giblets

The commercial broiler processor who cooperated in this study flumes gizzards and necks separately but flumes livers and hearts together. Because current regulations prohibit the use of bird chiller water in giblet flumes, cooperation of the commercial processor was obtained and a 1/4 scale simulated giblet flume was constructed in an area removed from the main processing area of the plant (Fig. 1). The simulated flume was constructed so that giblets could be flumed with water from a potable source or with chiller water filtered with the diatomaceous earth (DE) filter described by Lillard (1978b). Holding time of product in the flume (about 80 sec) and proportion of product to water (about 100 necks:10 gal water) were the same as in the commercial neck flume of the plant. The water collection tank, giblet pump and simulated flume were sanitized with 2% lysol for 15 min then rinsed for 15–20 min with potable water prior to each use.

Water from the bird chiller overflow was pumped to the DE filter. Chiller water was filtered with 2 grades of DE: Dicalite 4200 and 1:1 (w/w) Speedflow plus 4200 (Lillard, 1978b). Filtered water with and without chlorination was collected in a tank as shown in Figure 1 for the simulated line.

A large-mesh screen covered with sterile cheesecloth was placed over a barrel for aseptic collection of giblets from the simulated flume (Fig. 1).

Sampling for microbiological analyses

Two samples (each consisting of 3 necks) were collected aseptically from each of 3 sampling points on each sampling day: (1) The commercial flume with potable water; (2) the simulated flume with potable water, (3) the simulated flume with filtered or filtered, chlorinated water. About 90g (skin and flesh) were diluted 1:3 (w/w) with sterile 1% peptone and the mixture was blended for 1 min at medium speed. All tests were performed on all samples from each of the 3 sampling points on each sampling date. Sampling dates were divided into 4 periods depending on the type of filtered chiller water used in fluming: (1) 22 samples from 11 sampling days with Dicalite 4200, (2) 12

Table 1—Microbiological characterization of necks flumed commercially, and necks flumed in a 1/4 scale simulated giblet flume with potable water, DE filtered and DE filtered, chlorinated bird chiller water

Fluming method and water source	Mean $\log_{10}$/ml[a]		Salmonellae	Shelf-life[a]	
	No. of samples	Total aerobic count	Fecal coliforms	Incidence	Mean no. of days	No. of samples
Commercial flume:						
Potable water	22	4.90b	3.77f	0/22	17.3k	40
Simulated flume:						
Potable water	22	4.78b	3.56f	0/22	20.3k	40
Filtered water (4200)	22	4.81b	3.63f	0/22	16.2k,p	40
Commercial flume:						
Potable water	12	5.40c	3.84g	0/12	16.4	30
Simulated flume:						
Potable water	12	5.48c	3.75g	0/12	14.2l	30
Filtered water (Speedflow + 4200)	12	5.45c	3.47g	0/12	14.2l,p	30
Commercial flume:						
Potable water	12	5.29d	3.89h	0/18	18.8	25
Simulated flume:						
Potable water	12	5.29d	3.93h	0/18	14.3	25
Filtered, chlorinated water (4200)	12	5.15d	3.78h	0/18	16.1p	25
Commercial flume:						
Potable water	10	5.51e	3.84j	0/10	17.4	25
Simulated flume:						
Potable water	10	5.17e	3.50j	0/10	11.7m	25
Filtered chlorinated water (Speedflow + 4200)	10	5.27e	3.53j	0/10	13.0m,p	25
Commercial flume: potable water (based on total observations from 4 experiments)					17.4n	120
Simulated flume: potable water (based on total observations from 4 experiments)					15.7n	120

[a] The same lower case letter follows values which are not significantly different at the 5% level. The letter p indicates no differences in shelf-life for the 4 treatments (10% level).

samples from 6 sampling days with Dicalite 4200 chlorinated to a free residual of 0.4–0.6 ppm, except 18 samples were analyzed for salmonellae, (3) 12 samples from 6 sampling days with 1:1 (w/w) Speedflow plus 4200, (4) 10 samples from 5 sampling days with 1:1 (w/w) Speedflow plus 4200 chlorinated to a free residual of 0.5–0.6 ppm (Lillard, 1978b).

Microbiological analyses

For total aerobic plate counts, blended neck samples were decimally diluted in 1% peptone, pour-plated in duplicate in Difco Plate Count Agar and incubated at 20°C for 72 hr.

For enhanced recovery of damaged cells, fecal coliform densities were determined by the five-tube Most Probable Number (MPN) procedure described in Standard Methods for the Examination of Water and Wastewater (APHA, 1971). Fermentation tubes with inoculated Difco EC Medium were incubated in a Precision Scientific Coliform Incubator Bath at 44.5°C for 24 hr.

For salmonellae, incidence was estimated by enrichment methods, and levels by a five-tube MPN procedure (Lillard, 1977).

Shelf-life

For shelf-life determinations 5 necks (skin on) from each of the 3 sampling points on each sampling day were placed in Whirl–pak bags, loosely sealed and stored at 2°C. The number of sampling days were: 8 when Dicalite 4200 was used, 6 for Speedflow + 4200, 5 for 4200 plus chlorine and 5 for Speedflow + 4200 with chlorine. Odor of samples was evaluated daily until an off-odor was detected; that marked the end of shelf-life of a sample. Shelf-life was determined for all samples at 2°C rather than at commercial refrigeration temperatures so that small differences between treatments could be detected more easily (Thomson et al., 1975).

Statistical analyses

Data for total aerobes, fecal coliforms and shelf-life were treated by the Standard Analysis of Variance. Significance is reported at the 5% level unless stated otherwise.

RESULTS & DISCUSSION

TABLE 1 shows that, within each of the 4 experimental time periods, there were no significant differences in incidence of salmonellae, levels of total aerobic organisms or fecal coliforms between necks flumed commercially and necks flumed in the simulated line with potable, filtered or filtered, chlorinated chiller water. These data show that the simulated flume was comparable to the commercial flume. Lillard (1978a) showed that the similarity in microbiological quality of necks flumed with untreated chiller water and necks flumed with potable water was not due to a temperature difference of the waters. During experiments performed in the winter, the temperatures were about the same for potable water and chiller water. Yet, despite microbiological difference in water quality necks flumed with either water were comparable in quality.

Table 1 also shows that the microbiological quality of necks was not improved by chlorination of the filtered chiller water even though Lillard (1978b) showed that chlorination improved the microbiological quality of the water itself. These results confirm previous findings (Lillard, 1977, 1978a) that showed no direct relationship between the quality of broiler processing water at certain sampling points and product exposed to it.

Salmonellae were not isolated frequently from the filtered chiller water (Lillard, 1978b). However, all neck samples were salmonellae negative (Table 1) even on days when the filtered water was salmonellae positive. Again, this confirms previous results that showed no direct relationship between quality of broiler processing water at certain sampling points and product at these points (Lillard, 1977, 1978a).

When data from all four experiments were pooled, mean days of shelf-life did not differ significantly between necks flumed commercially and necks flumed with potable water in the simulated line (Table 1). Absence of difference was attributed to the high mean shown in the first experiment for necks flumed with potable water in the simulated line. In the other 3 experiments, however, mean days of shelf-life did differ between necks flumed commercially and necks flumed with potable water in the simulated line. Prior to fluming in the simulated line, unlike the commercial flume, necks had to

be collected, handled and taken to the simulated flume. Therefore, the best comparison, especially if based on a small number of observations, is between necks handled in the same manner, i.e. necks flumed in the simulated line with potable water and necks flumed in the simulated line with chiller water filtered with Dicalite 4200 or Speedflow with or without chlorination. Table 1 shows that mean days of shelf-life did not differ significantly between necks flumed in the simulated line with potable water and necks flumed with bird chiller water treated by the following 3 methods: filtered with Dicalite 4200, filtered with Speedflow plus 4200, filtered with Speedflow plus 4200 and chlorinated. However, the mean days of shelf-life was significantly longer for necks flumed with chiller water filtered through 4200 and chlorinated than for necks flumed with potable water. No chlorine odor was detected in any of the samples flumed with filtered, chlorinated chiller water.

Lillard (1978a) showed that necks flumed with untreated chiller water were of comparable quality to necks flumed with potable water when fecal coliforms, salmonellae (incidence and levels) and shelf-life were considered but had slightly higher (about 1/4 log) total aerobic counts. The data in Table 1 show that this difference in level of aerobic organisms was eliminated by fluming necks with DE filtered bird chiller water. Necks flumed with chiller water filtered with either grade DE were microbiologically comparable in all respects to necks flumed with potable water. Chlorination of the filtered chiller water improved the microbiological quality of the water itself (Lillard, 1978b) but had no apparent effect on the microbiological quality of the product.

Processing water exerts a washing effect on poultry products (Salzer et al., 1965; Gardner and Golan, 1976). Attached bacteria are best recovered by maceration (Notermans et al., 1975). In this study necks were blended prior to testing which should give results comparable to maceration. Data in this study indicate that loosely attached microorganisms were probably washed off when processing water was improved even though not to potable standards. However, microorganisms that were attached to the product probably remained attached regardless of processing water source. Notermans and Kampelmacher (1974) found that nonflagellated bacteria rarely attach during processing of broilers, but flagellated bacteria attach and the rate of attachment is optimum at about 21°C. They found that attachment occurs at temperatures above and below 21°C but to a lesser extent. Whether the rate of attachment decreases or increases at temperatures higher or lower than 21°C seems to depend on the genus and species of flagellated microorganisms. However, the above authors did show that at a given temperature the rate of attachment is pH dependent, and is optimum at pH 7.1–8.4. Table 2 shows that temperatures of water in the commercial flume and of potable water in the simulated line were above the optimum for attachment whereas temperatures of the filtered chiller water were below optimum for attachment; however, pH of all three water sources was optimum for attachment of flagellated bacteria such as fecal coliforms and salmonellae.

Table 2 also shows that the temperature of necks was about 12°C lower for those flumed with filtered chiller water than for those flumed with potable water. Regulatory agencies require the cooling of giblets to 40°F within 2 hr (USDA, 1973). In order to meet this requirement, many processors have to precool giblets before they reach the chiller by cooling the flume water or else place a heavy energy load on the giblet chillers. The use of chiller water in fluming could reduce this additional demand for energy.

Table 2—Temperature and pH of water from the commercial flume, potable water, chiller water filtered with Dicalite 4200, and temperature of necks after fluming in these waters

Source	Water temp °C Mean[a]	Water temp °C Range[a]	pH of water Range[b]	Temp of flumed necks °C Mean[c]	Temp of flumed necks °C Range[c]
Commercial flume:					
Potable water	25.7	23–27	7.11–7.51	26.7	24–29
Simulated flume:					
Potable water	25.9	23–27	7.30–7.71	27.1	24–29
Filtered chiller water[d]	11.5	10–17	7.22–7.51	14.7	12–18

[a] Based on 12 water samples taken on 12 sampling dates
[b] Based on 10 water samples taken on 5 sampling dates
[c] Based on 36 necks taken on 12 sampling dates
[d] Mean temperature of the filtered chiller water at the filter exit was 1.3°C lower than the temperature indicated in the table (mean 10.2°, range 9–13°). Temperatures listed in the table are for the filtered chiller water in the collection tank as used for fluming necks in this experiment.

Fluming with potable water represents the best currently available technology. Therefore, necks from the commercial flume represent the best currently attainable standard of quality and safety. This study has shown that this standard can be met with water of somewhat less than potable quality, and that necks flumed with DE-filtered chiller water are microbiologically equal in all respects to necks flumed with potable water. Fluming giblets with filtered chiller water would reduce the demand by poultry processors for potable water and energy, and also would reduce the amount of water and level of organic matter discharged into municipal waste treatment systems.

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The author acknowledges R.L. Wilson for the statistical analyses.

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PROPERTIES OF RESTRUCTURED PORK PRODUCT AS INFLUENCED BY MEAT PARTICLE SIZE, TEMPERATURE AND COMMINUTION METHOD

M. S. CHESNEY, R. W. MANDIGO and J. F. CAMPBELL

ABSTRACT

The process of flaked, formed and sectioned meat products could change the identity of lesser valued meat cuts via comminution, shaping and slicing. This study of flaking versus grinding included comparisons of three different meat temperatures prior to processing and three different comminution particle sizes on the chemical, physical and organoleptic analysis of the fabricated product. Flaked product did not differ from ground product regarding proximate composition. Temperature prior to comminution did not affect percentages of ash, moisture or crude fat in the fabricated product. The protein percent was lower for the −5.6°C product ($P < 0.05$) than the 32.2 or 2.2°C product, and fabricated product from −5.6°C meat had a significantly lower ($P < 0.01$) water-holding capacity than the two higher temperatures. Cooking loss increased significantly ($P < 0.01$) as meat processing temperature decreased. Comminution particle size had no influence on chemical properties of the fabricated product; however, percent cook-out significantly decreased ($P < 0.05$) as particle size became smaller. Taste panel results showed that the flaked product was more cohesive ($P < 0.01$) and more acceptable ($P < 0.01$) than the ground product.

INTRODUCTION

INTEREST has been generated by increased consumer demands for less expensive and more convenient meat items in various comminuted and fully processed meat products. Transforming meat cuts of lower economic value into uniform, consumer-ready products can provide greater variety, increased convenience and maintain food budget economy. Flaking, forming and sectioning can change the identity of the lower value meat cuts via comminution, shaping and slicing.

Flaking is a development in meat comminution whereby tissue is cut in a shaving-like manner, into flakes of varying particle sizes and texture (Fenters and Ziemba, 1971; Mandigo, 1972; Pietraszek, 1972). Tillman and Ziemba (1972) described a process in which flaked poultry meat was shaped into tempered loaves. Hansen and Mandigo (1972) compared flaked pre-rigor pork to conventionally chilled flaked pork and found that as the quantity of warm pork increased, when blending with chilled meat, a decrease in percent fat occurred in the uncooked samples.

Acton (1972) found that increased cell disruption and breakage of muscle fibrils resulted in the release of intracellular contents. Percent extractable protein significantly increased with each step of muscle destruction during grinding and closer contact between meat surfaces aided in reducing cooking loss and increasing binding strength. An important factor in the preparation of restructured or fabricated meat items is the process of comminution. Grinding has been the most accepted form of comminuting meat and flaking has recently been involved in manufactured meat products.

This study was conducted to examine the manufacturing properties of pork muscle and to determine sensory panel response to fabricated pork products as influenced by: (1) method of comminution: flaking versus grinding; (2) temperature of the meat prior to processing; and (3) comminution particle size during processing.

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0022-1147/78/0005-1535$02.25/0

EXPERIMENTAL

PORK UTILIZED in this study was derived from sows slaughtered at the University of Nebraska Meat Laboratory. Left sides were boned immediately after slaughter and comprised the pre-rigor (32.2°C) pork trim which consisted of the ham, loin, shoulder and belly. The pork trim was thoroughly mixed by hand and 68 kg of the pre-rigor meat was used at the 32.2°C. Less than 3 hr lapsed from time of slaughter to completion of processing.

The right sides were chilled conventionally in a 2.2°C cooler for 24 hr and the ham, loin, shoulder and belly boned into pork trim, excess fat and trimmings. Two 68 kg portions were selected for study. One batch, representing the 2.2°C temperature, was processed immediately following the boning operation; the remaining batch (68 kg) was placed in plastic bags and crust frozen (−17.8°C) overnight and then freeze-tempered for 8 hr until the internal temperature reached −5.6°C. Meat from each of the three temperature groups (32.2, 2.2 and −5.6°C) was comminuted by flaking or by grinding. Flaking heads of 12.7, 6.9 and 3.0 mm and grinder plates of 9.5, 6.4 and 3.2 mm were used to prepare particles of differing size. Correspondingly, 18 treatments (3 × 2 × 3; temperature × comminution method × particle size) of meat were studied. Each individual sample was mechanically blended for 10 minutes following comminution.

The comminuted meat was freeze-tempered until an internal temperature of −5.6°C was reached. Meat was then pressed and formed into a cylindrical "log" 9.9 cm in diameter. Approximately 28.0 kg/cm² (400 psi) pressure was exerted to compress the comminuted meats. All treatments were formed in the same manner. The processed meat "logs" were cleaved with a power cleaver into uniform slices (2.5 cm thick × 9.9 cm diameter). Random samples were removed for proximate analysis (AOAC, 1965), cooking loss, Warner-Bratzler shear and taste panel determinations. Water holding capacity was determined as percent liquid loss according to Wierbicki et al. (1957).

Fabricated chops were stored 4 wk, then removed from the freezer 24 hr before cooking and were allowed to thaw at 1.7°C. Samples were cooked at 177°C in an electric rotary hearth oven until an internal temperature of 70°C was reached. Samples were blotted dry and re-weighed to determine cooking loss. Shear values were taken on cores removed from the cooked samples. Six cores, 2.54 cm in diameter were sheared and shear values were interpreted as a measure of binding ability rather than as a measure of tenderness.

An untrained panel of ten people evaluated the raw product for visual appearance using a scale ranging from 1 to 5, with 1 being least desirable, 5 being most desirable. The panel evaluated the cooked product for cohesion, juiciness, tenderness and overall acceptability. Samples were served at 50°C to the panelists. Cohesion and overall acceptability were rated on a 5 point scale while juiciness and tenderness were based on a 9 point scale (see Table 5 footnotes for scales). Two preliminary practice sessions were conducted to acquaint the panelists with the characteristics being rated and with procedures of evaluation. Three random samples were evaluated during each testing period.

This experiment was a cross classified design with a factorial arrangement of treatments (3 × 2 × 3). Analysis of variance was conducted according to appropriate procedures for the data (Snedecor and Cochran, 1967).

RESULTS & DISCUSSION

Comminution method

Proximate analysis for percent moisture, ash, ether extract and protein of the formed and sectioned product was not

affected by flaking or grinding and no significant differences were determined between the two methods (Table 1). Water-holding capacity, cooking loss and shear values were not different between the flaked and ground methods of comminution.

Temperature

There were no significant differences (Table 2) between processing temperatures for percent ash, moisture, and crude fat for the fabricated product. Percent protein was found to be significantly ($P < 0.05$) lower in the tempered (−5.6°C) patties which may reflect less bind in the samples. As shown in Table 2 no significant differences were found between the pre-rigor (32°C) and the chilled (2.2°C) product with regard to protein percent. There was a significant decrease ($P < 0.10$) in water-holding capacity for the frozen meat, while no difference existed for the pre-rigor and chilled treatments. Miller et al. (1968) found a decrease in water-holding capacity as fat level increased due to the increase in the moisture:protein ratio. Cooking loss increased ($P < 0.01$) as the processing temperature of the fabricated pork product decreased which is in agreement with the findings of Miller et al. (1968) and Popenhagen et al. (1973). As the moisture:protein ratio increased, water-holding capacity increased and percent cooking loss decreased. Hansen and Mandigo (1972) reported less cooking loss for blends containing greater quantities of pre-rigor pork.

Warner-Bratzler shear values (Table 2) were highest ($P < 0.01$) in the pre-rigor product (32.2°C) and decreased with decreased processing temperature. These values are interpreted as a function of binding ability rather than as an objective measure of tenderness. In this instance the higher values indicate a superior bind while the lower values represent a crumbly product.

Particle size

Particle size had no significant influence on the chemical properties, water-holding capacity or shear force values measured in the fabricated pork products (Table 3). There was a decrease ($P < 0.05$) in percent cooking loss as the particle size decreased. Acton (1972) also reported that cooking loss significantly decreased when the meat surface area was increased by reducing the particle size during grinding. Finely grinding the meat five times resulted in the least cooking loss with the greatest binding strength, however, emulsification may have been the reason for the improved values (Acton, 1972).

Taste panel

The flaked product was more cohesive ($P < 0.01$) than the ground product as indicated by the panel evaluations (Table 4). Cohesion refers to the ability of the product to hold together while cutting or chewing. Overall acceptability values (Table 4) were higher ($P < 0.01$) for the flaked product. The mean values for acceptability, however, are not high and approach the neutral category on the five point scale. This can be accounted for by the bland taste of the fabricated product due to the lack of salt or seasoning. Mandigo et al. (1972) determined that taste panelists preferred restructured pork products with salt added as opposed to those products without salt.

Panelists found the chilled (2.2°C) product to be more cohesive than either the pre-rigor (32.2°C) or tempered (−5.6°C) pork products. Pre-rigor products possessed a higher ($P < 0.01$) mean cohesion value than did products processed at the lowest temperature (2.2°C). Juiciness scores were greater ($P < 0.01$) for the two higher temperatures but were not significantly different from one another; the product processed at the lower temperature had the lowest mean juiciness score. These results correspond to the water-holding capacity values presented in Table 2 and the panel rated juiciness similarly

Table 1—Influence of comminution method on the chemical and physical properties of formed and sectioned pork products

Trait		Comminution method		Std error
		Flaked	Ground	
Moisture	%	61.99	62.59	0.38
Ash	%	0.85	0.79	0.24
Ether extract	%	19.09	19.01	0.57
Protein	%	17.18	17.38	0.13
Water-holding capacity[a]	%	9.53	9.59	0.20
Cooking loss	%	34.70	34.40	0.97
Warner-Bratzler shear	(kg/2.54 cm core)	2.56	2.31	0.21

[a] Percent liquid loss

Table 2—Influence of temperature on the chemical and physical properties of formed and sectioned pork products

Trait		Temperature (Mean Values)			Std. error
		32.2°C	2.2°C	−5.6°C	
Moisture	%	62.31	62.36	62.20	0.47
Ash	%	0.84	0.81	0.81	0.03
Ether extract	%	18.66	18.84	19.67	0.70
Protein	%	17.58[a]	17.38[a]	16.87[b]	0.16
Water-holding capacity[h]	%	9.26[c]	9.36[c]	10.07[d]	0.24
Cooking loss	%	30.46[e]	33.56[f]	39.62[g]	1.19
Warner-Bratzler shear	(kg/2.54 cm core)	3.53[e]	2.72[f]	1.06[g]	0.26

[a,b] Means with different superscripts on the same line are significantly different ($P < 0.05$)

[c,d] Means with different superscripts on the same line are significantly different ($P < 0.01$)

[e,f,g] Means with different superscripts on the same line are significantly different ($P < 0.01$)

[h] Percent liquid loss

Table 3—Influence of comminution size on the chemical and physical properties of formed and sectioned pork products

Trait		Comminution size[a] (Mean Values)			Std error
		Large	Medium	Small	
Moisture	%	62.59	62.43	61.84	0.47
Ash	%	0.82	0.81	0.83	0.03
Ether extract	%	18.65	18.92	10.59	0.70
Protein	%	17.37	17.37	17.09	0.16
Water-holding capacity[b]	%	9.56	9.56	9.56	0.24
Cooking loss	%	34.26[c]	34.26[c]	32.34[d]	1.19
Warner-Bratzler shear	(kg/2.54 cm core)	2.66	2.60	2.05	0.26

[a] Flake particle size: Large = 12.7 mm, Medium = 6.9 mm, Small = 3.0 mm.

[b] Percent liquid loss

[c,d] Means with different superscripts on the same line are significantly different ($P < 0.05$).

with the amount of moisture retained in the fabricated product (Table 4). The panel preferred the chilled product (2.2°C) over the tempered product (−5.6°C) for overall acceptability. This is in agreement with the results of Popenhagen et al. (1973) in which juiciness, cohesiveness, and overall acceptability were superior for 2.2°C flaked meat. Raw product acceptability for pre-rigor products was higher ($P < 0.05$) than for the two lower processing temperatures. This was due to the bright red and more acceptable color exhibited by the pre-rigor product when appraised for visual appearance.

Taste panel means as influenced by particle size were significantly ($P < 0.01$) different for cohesion, juiciness, tenderness

Table 4—Mean taste panel scores for method of comminution, comminution size and processing temperature

Treatment	Taste panel traits				
	Cohesion[a]	Juiciness[b]	Tenderness[b]	Overall acceptability[a]	Raw product acceptability
Comminution Method					
Flaked	3.08[f] ± 0.05	4.80 ± 0.11	4.96 ± 0.11	2.91[f] ± 0.07	2.49 ± 0.11
Ground	2.34[g]	4.97	4.70	2.54[g]	2.62
Temperature					
32.2° C	2.87[f] ± 0.07	5.25[f] ± 0.14	4.70 ± 0.14	2.90[f] ± 0.08	2.88[d] ± 0.14
2.2° C	3.38[g]	5.23[f]	4.95	3.08[f]	2.45[e]
−5.6° C	1.88[h]	4.14[g]	4.83	2.20[g]	2.33[e]
Comminution Size[c]					
Large	2.45[f] ± 0.07	4.52[f] ± 0.14	4.00 ± 0.14	2.35[f] ± 0.08	2.58 ± 0.14
Medium	2.87[g]	4.82[f]	4.88[g]	2.90[g]	2.53
Small	2.82[g]	5.32[g]	5.60[h]	2.93[g]	2.55

[a] Cohesion, overall acceptability and raw product acceptability based on five point hedonic scale (1 = least desirable and 5 + most desirable).

[b] Juiciness and tenderness based on nine point hedonic scale. (1 = least desirable and 9 = most desirable)

[c] Flake Particle Size: Large = 12.7 mm, Medium = 6.9 mm, Small = 3.0 mm. Grind Particle Size: Large = 9.5 mm, Medium = 6.4 mm, Small = 3.2 mm.

[d,e] Means bearing different superscripts in the same column are significantly different ($P < 0.05$).

[f,g,h] Means bearing different superscripts in the same column are significantly different ($P < 0.01$).

and overall acceptability. Products prepared from larger size particles were less cohesive than the medium or small size with no difference between the latter. This is in agreement with work conducted by Acton (1972) and Popenhagen et al. (1973). Juiciness scores were higher for products manufactured from the smaller particles and decreased as particle size increased. Tenderness was rated higher for small comminution sizes and decreased as particle size increased. No differences were found for the comminution sizes when raw product acceptability was appraised.

From a processing viewpoint, flaking has little advantage over grinding in terms of chemical and physical properties. However, taste panel results show flaked meat to be more cohesive and more acceptable than the ground product. The practical significance for flaked meat may rest solely with texture of the finished consumer product. Meat processed pre-rigor (32°C) had a greater percentage of protein with less cookout and was more cohesive according to shear measurements, however, panelists found the chilled (2.2°C) meat to be more cohesive. Percent cooking loss decreased as comminution size decreased and taste panelists favored the smaller particle sizes.

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Ms received 10/27/76; revised 4/21/78; accepted 4/25/78.

Paper Number 3660, Journal Series, Nebraska Agricultural Experiment Station, Project 13-23.

Supported in part by a cooperative agreement with the National Pork Producers Council; Bettcher Industries Inc., Vermilion, OH and Urschel Laboratories, Valparaiso, IN.

IMPLICATIONS OF THE RICE KERNEL STRUCTURE IN STORAGE, MARKETING, AND PROCESSING: A REVIEW

D. B. BECHTEL and Y. POMERANZ

ABSTRACT

In rice, resistance to insect infestation is related to the silica-rich husk tissues (palea and lemma) that are held together by two hook-like structures. The structure and composition of the pericarp, seed coat, nucellus, and the seed coat and nucellar cuticles govern, in part, entry of water into the kernel, resistance to mold damage, and resilience during grain handling and transportation. Ease of disruption of aleurone cells, and accompanying fusion of lipid bodies, may be responsible for rancidity in undermilled rice. Uneven distribution of protein in the subaleurone and central starchy endosperm governs the nutritive value of milled rice and makes possible preparation of protein-rich flours. Separation of the germ during rice milling is related to the structure of a fibrous zone separating the scutellar epithelium from the starchy endosperm. Modifications of the scutellum and aleurone layers during parboiling affect milling of rice.

THE STRUCTURE of rice has been well established, but little is known about the implications of kernel structure on grain utilization (Bechtel and Pomeranz, 1977, 1978a, b). Rice is harvested as a covered caryopsis (grain, kernel) in which the threshed grain (rough rice) is enclosed in a tough siliceous husk. A caryopsis is a single seeded fruit in which the pericarp is fused to the seed. When the husk is removed during milling, the kernel, comprised of the pericarp (outer bran), seed coat, nucellus and aleurone (inner bran), endosperm, and germ, is known as "brown" rice or sometimes as "unpolished" rice. Brown rice is in little demand as a food, but when the bran (pericarp, seed coat, nucellus and aleurone) and germ are removed this milled rice is marketed as white or polished rice.

Some twenty species of the genus *Oryza* are botanically recognized but practically all cultivated rice today is *Oryza sativa* L. The important subspecies are: *indica* with slender, somewhat flat grains, *japonica* with short, roundish grains, and *javanica* with broad thick grains (Houston and Kohler, 1970). In the United States rice is marketed under three grain types: long (over 6 mm long), medium (5–6 mm), and short (less than 5 mm). Each type has specific cooking and processing attributes (Webb, 1975). Parboiled rice is rice that has been soaked, steamed, cooked, dried while in the husk, and then milled to produce white rice.

We now review some of the structural implications of the rice kernel as they relate to grain handling, pest resistance, storage, and processing. The figures (some not previously published) are from our studies.

Husk

Figure 1 is a diagram of the mature rice caryopsis for use in structure identification. The harvested rice caryopsis is surrounded by a husk, composed of two covering structures, the palea and the larger lemma. The husk accounts for 16–26% of the weight of the rough rice and usually weighs more in the slender, long- than in the round, short-grain varieties (Gariboldi, 1974a). The palea and lemma are held together by two hook-like structures (Fig. 2). The ability of these structures to hold the palea and lemma together without gaps is probably variety dependent. The husk provides primary resistance to insect infestation. Insect infestations were more common in varieties of rice that had many gaps and separations than in varieties with tight husks (Breese, 1960; Russell, 1968; Cohen and Russell, 1970; McGaughey, 1970). The high silica content of the husk may enhance protection against insects but at the same time may cause excessive wear on milling equipment (Gariboldi, 1974b). The silica is located in and on the epidermal cells. The husks also may protect rice indirectly from fungal damage. Christensen and Lopez (1965) reported that in rice stored at 80% relative humidity and 22–25°C, the equilibrium moisture content was about 13.9% for rough rice and 14.9% for brown and polished rice. The lower equilibrium moisture of the husk may help in protecting the kernel by acting as a barrier to the penetration of fungi. The husk, apparently, also prevents the grain from becoming rancid by protecting the bran layers from mechanical damage during harvesting and subsequent handling (see section on the bran).

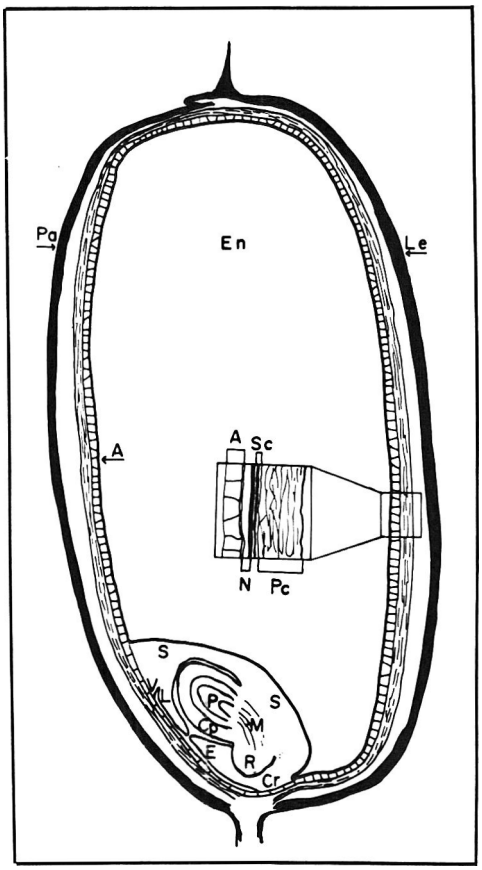

Fig. 1—Diagram of mid longitudinal section through the rice caryopsis: Endosperm (En), palea (Pa), lemma (Le), pericarp (Pc), seed coat (Sc), nucellus (N), and aleurone (A); germ = scutellum (S), ventral scale (V), lateral scale (L), coleoptile (Cp), plumule (P), mesocotyl (M), radical (R), epiblast (E), and colerohiza (Cr).

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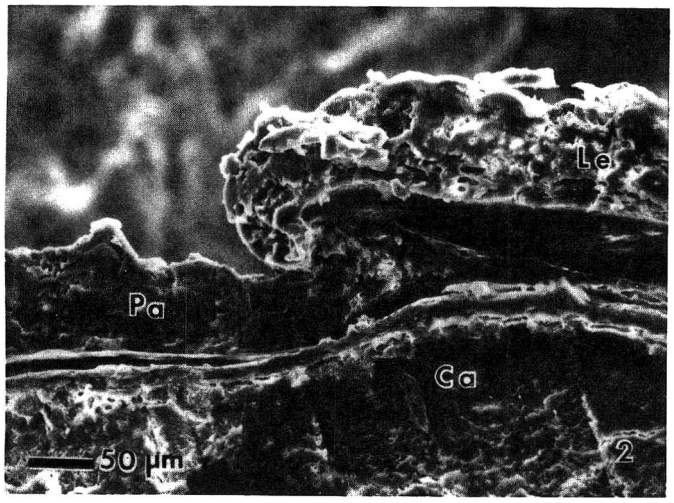

Fig. 2–SEM of palea (Pa) and lemma (Le) surrounding rice caryopsis (Ca). Note junction between palea and lemma. X 152.

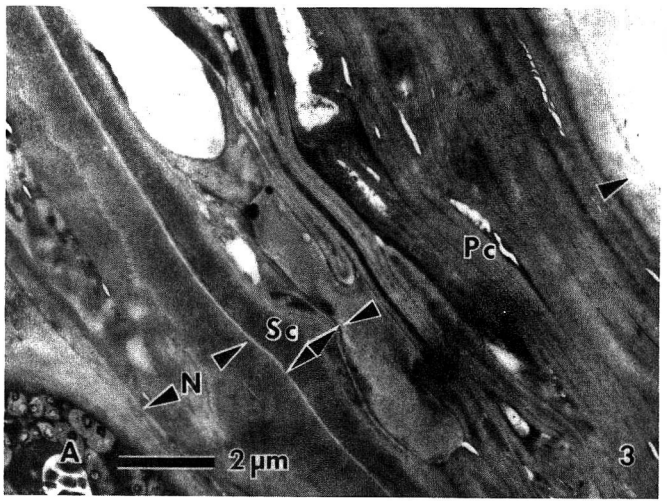

Fig. 3–TEM showing pericarp (Pc), seed coat (Sc), nucellus (N), cuticles (*), and aleurone cell (A). (X 5915)

Large amounts of rice husks are produced during milling. It is estimated that about 50 million tons of rice husks are produced in the milling of paddy rice (Hawkey, 1977). The abrasive husks contain about 15% silica, have a poor nutritional value, are difficult to degrade, and have a low bulk density (Mehta and Pitt, 1977). At the current rate of world production, the husks–if undisposed–would need about 500 million cubic meters of storage space every year. Based on the structure and composition of the husk, the following uses have been recommended: as a fuel, after incineration (as fertilizer, cement and brick admix, mulch, food packaging, insulator), after granulation (as polishing and blasting powder, brick admix), after silica extraction (in glass and board making, coatings, fibers), and in production of furfural (Hawkey, 1977). According to Bartha and Huppertz (1977), the high silica content in rice husks imparts excellent refractory properties to the husk ash; the ash has a low bulk density and a high porosity. While the silica in the husks has a cristobalite structure, it is possible to obtain ashes of different silica structure by controlled industrial processing. The resultant structure is important in manufacture of refractory products in which thermal expansion governs utilization.

Bran (pericarp, seed coat, nucellus and aleurone)

Rice bran is about 5–7% by weight of the brown rice. The term bran is used in trade to describe a mixture of several botanical tissues such as pericarp, seed coat (only one seed coat is present at maturity; Bechtel and Pomeranz, 1977), nucellus, and the outermost portion of the endosperm, the aleurone layer. Apparently the rice bran layers afford little protection against insects, because higher numbers of insects consistently develop in brown rice than in either rough rice or milled rice (McGaughey, 1973). Furthermore, infestation in brown rice was unaffected by variety. Lack of resistance to infestation is probably due to the thinness of the bran, which allows easy insect penetration into the starchy endosperm and/or to the large quantities of lipid and protein that are present in the aleurone and provide nourishment to the insects.

The structure of the pericarp, seed coat, and nucellus also explain how the kernel reacts to water. The pericarp is composed of crushed parenchyma cells that probably act as a sponge and readily absorb water. Below the pericarp are the seed coat and nucellus; each layer has a cuticle. Morrison (1975) found that the wheat seed coat cuticle was thicker than the nucellar cuticle. In rice, however, nucellar cuticle is thicker than seed coat cuticle (Fig. 3) and the arrangement of the cuticles differ from that in wheat. Possibly these cuticles prevent water imbibition into the caryopsis through the caryopsis coat. Jones (1949) reported that in wheat the rate of water adsorption (by pericarp ?) initially was rapid and then decreased. Hinton (1955) found that in wheat the seed coat offered more water resistance than the nucellus, possibly because the seed coat cuticle is thicker. Since the cuticles are deficient in the attachment region of some cereal grains, water can enter the kernel readily only via the germ (Krauss, 1933).

Rice is usually stored as the relatively protected rough rice (Schroeder and Calderwood, 1972). The best safeguards against attack by molds and production of aflatoxins during storage are low moisture content, low temperature, and adequate aeration. Under optimum conditions for fungal growth aflatoxins can develop to significant levels within a few days. Schroeder et al. (1968) showed in vitro that in rough rice inoculated with *Aspergillus parasiticus* 60–80% of the aflatoxins was in the bran and polish fractions. Consequently, the milled rice contained much less aflatoxin than the rough rice.

According to Houston and Kohler (1970), nutritional losses during storage may result from insect or microorganisms attack, oil rancidity, protein modification, or vitamin losses. Once rough rice is dehulled the brown rice may become rancid, primarily due to free fatty acid production by the action of lipase. The fatty acids, in turn, become oxidized and cause rancidity (Houston, 1972). Oxidative rancidity, however, does not apparently take place in viable grain (Mitchell and Henick, 1964). Hydrolytic rancidity can become a consequence in whole grain stored at high moisture or grain damaged by crushing or milling. According to Desikachar (1977) the difference in storability can be explained by compartmentalization of enzyme and substrate; the oil is localized in the aleurone and germ fractions and the lipase in the seed coat-cross layers of rice (Shastri, 1973; Viraktamath et al., 1973). As long as the bran is intact and protected by the husk, there is little lipase activity. We (Bechtel and Pomeranz, 1977) have shown that rice aleurone cells are susceptible to disruption during dissection and that the disruption resulted in lipid body fusion. Possibly, the dehulling process and subsequent milling and handling of the rice disrupts the aleurone cells which leads to rice rancidity. For example, Houston et al. (1952) found that brown rice from rubber-roller shellers stored better than rice dehusked by an emery coated disc sheller.

It is known that well milled rice keeps better than undermilled rice. Deteriorative changes (discolorations, rancidity, odors, etc.) which occur in undermilled rice reside primarily in the bran layers left on the rice (Barber, 1972). Barber (1972)

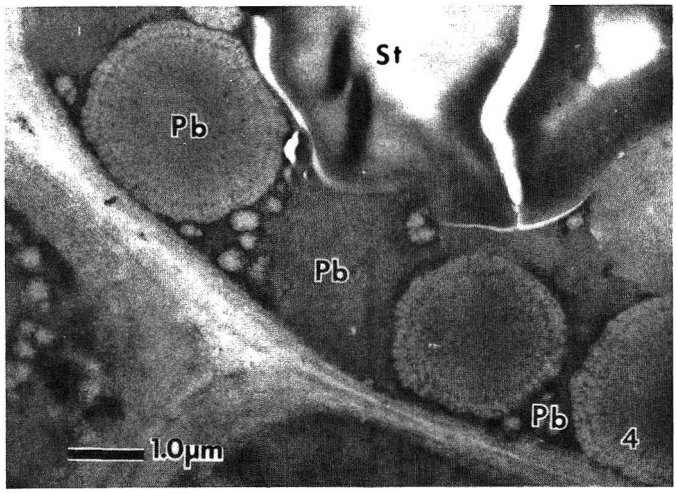

Fig. 4—TEM of subaleurone region of endosperm. Note starch grains (St) and various protein bodies (Pb). (X 13,832)

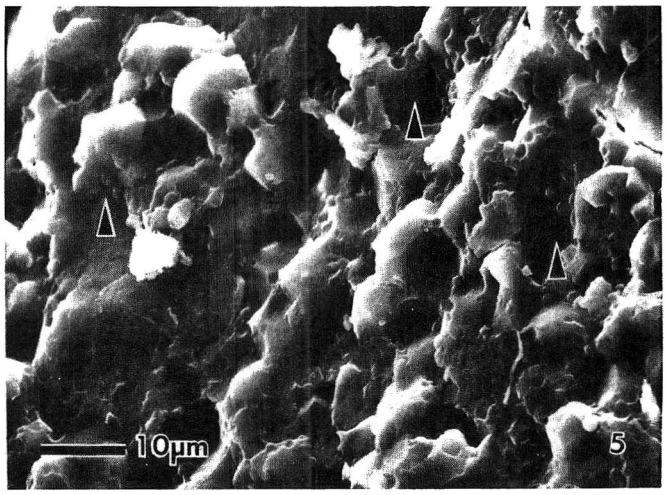

Fig. 5—SEM of subaleurone endosperm with protein bodies (arrows) located between starch grains. (X 1037)

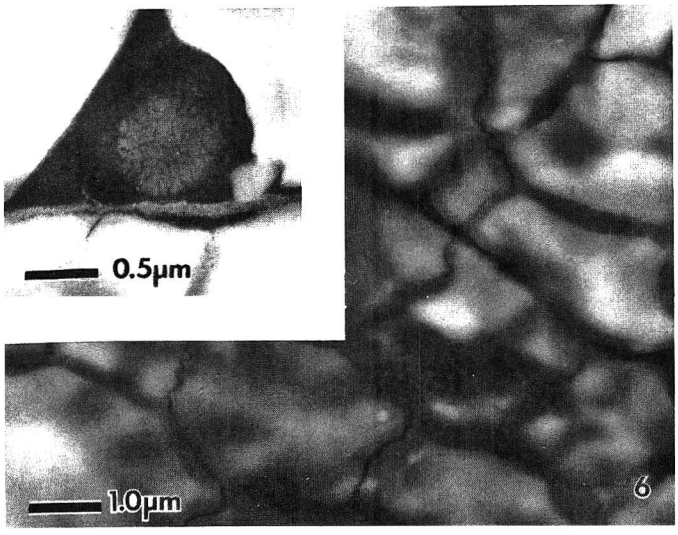

Fig. 6—TEM of central endosperm with no protein bodies apparent in field of view. X 10,000; insert shows protein body (X 16,653).

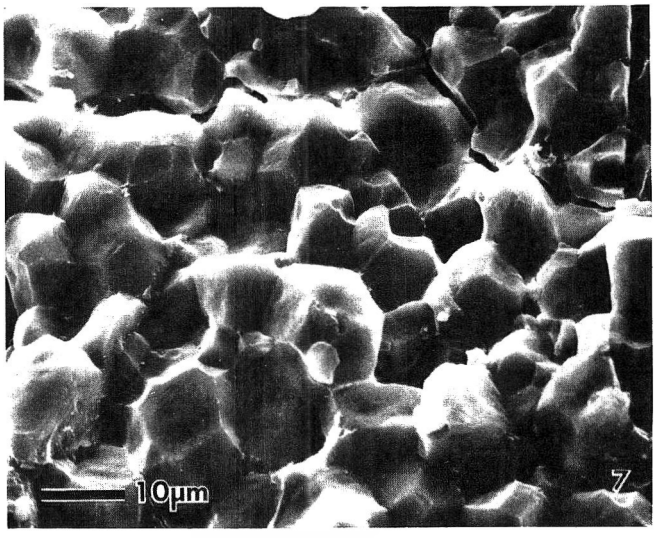

Fig. 7—SEM of central endosperm. Protein bodies not evident, indicating few protein bodies in the central endosperm. (X 1037)

stated "relatively small differences in milling degree might result in noticeable differences in lipid content and hydrolytic, oxidative, and enzymatic activities of the surface layer and consequently in different stabilities." Watson et al. (1975) found that small differences in degrees of milling resulted in substantial differences in surface lipid content (0.99% for undermilled vs 0.63% for lightly milled).

The bran layers may also have an effect on rice breakage. Louvier and Calderwood (1972) showed that medium-grain rice is more resistant to breaking during handling and transportation than long-grain rice, indicating a kernel size and shape effect. For the long-grain type, brown rice was more resistant to breakage than milled rice and parboiled rice was the most resistant. The high resistance of parboiled rice to breakage is due to the toughening effect of heat treatment; the difference between the brown and milled rice may be due to the combined effects of decrease (during milling) of the diameter/length ratio and to the contribution of the bran to grain resiliency.

Rice bran contains 15–20% oil. If the lipase in the bran is not heat inactivated promptly after milling free fatty acids may increase about 5% per day and reach 70% (of the oil) in a month (Desikachar, 1977). High fat acidity affects adversely organoleptic acceptability, recovery of edible grade oil, and suitability of the bran as a food or feed. In addition to lipase, rice bran and germ also contain lipoxygenase. The lipoxygenase is more heat resistant than the lipase. Inactivation of the lipoxygenase is wrought with difficulties because the treatment can destroy tocopherols and other antioxidants. This may be of particular significance in parboiled rice bran or bran from steamed paddy (Desikachar, 1977). Parboiling is described briefly later in this review.

Parboiled rice bran contains 20–28% oil as compared with 16–18% in ordinary bran (Pe, 1977). Pe (1977) and Sarda (1977) described difficulties encountered in extracting oil from parboiled rice bran and methods of overcoming some of the problems.

There is a yearly potential of 30 million tons of rice bran (Barber and de Barber, 1977). The physical and chemical characteristics of rice bran depend on several factors which include the rice, the milling process, and the treatment of the rice and/or the bran. The variations in the rice have two origins: Among the chemical effects are composition and distribution of components in the kernel. Among the second

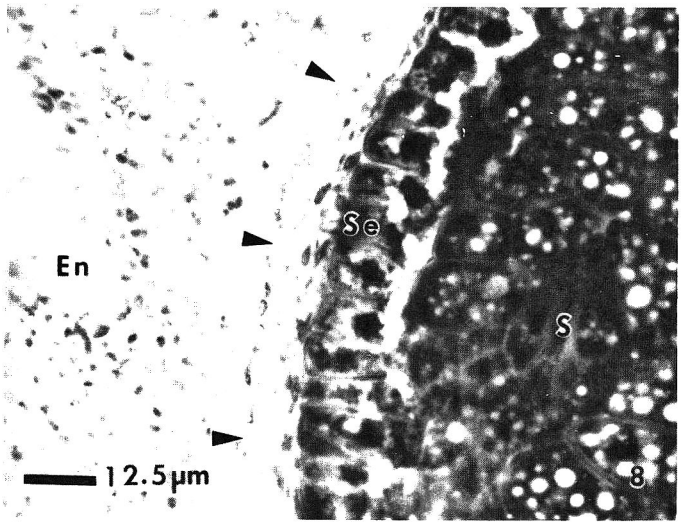

Fig. 8–LM of germ and endosperm. Scutellar epithellium (Se) and fibrous zone (arrows) separate the scutellum (S) from endosperm (En). (X 728)

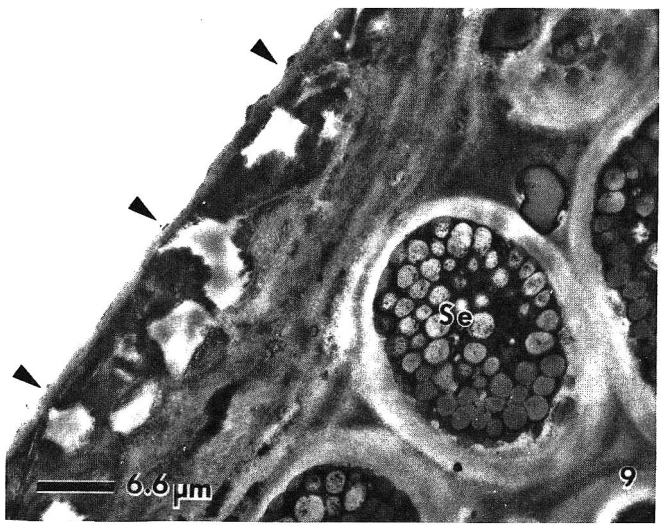

Fig. 9–TEM of germ that was dissected from whole caryopsis. Note that germ fractured between endosperm and fibrous zone (arrows). Scutellar epithellium (Se). (X 6006)

effects are included: (a) size and shape of the kernel, (b) size of the husk, (c) thickness of anatomical layers in the caryopsis, (d) physical properties–including resilience, breakage, resistance to abrasion during milling, and (e) effects of treatment prior to, and during, milling.

Starchy endosperm

The starchy endosperm of rice is divided into two regions, the subaleurone, located just beneath the aleurone layer and the central region consisting of the rest of the starchy endosperm. Comparison of these two regions was studied using light and transmission electron microscopy coupled with proteolytic enzyme digestions (pepsin and pronase; Bechtel and Pomeranz, 1978b). Three types of membrane bounded protein bodies were found in the subaleurone region whereas only one was observed in the central region (Fig. 4–7). Large spherical protein bodies were common to both regions, measured 1–2 μm in diameter, displayed concentric rings and/or radial rays, had dense centers, were susceptible to pepsin hydrolysis, and were digested only partially by pronase. The small spherical protein bodies of the subaleurone region measured 0.5–0.75 μm in diameter, had concentric rings and/or radial rays, and were completely digested by pepsin and pronase. The third type of protein body of the subaleurone region was crystalline. The crystalline protein bodies displayed crystal lattice fringes, had a rounded shape, were composed of small angular components, measured 2–3.5 μm in diameter, were removed completely by pepsin, and were resistant to pronase degradation under conditions which were optimum with regard to pH for the two enzymes.

In 1967, Mitsuda et al. described protein bodies isolated from rice polish. Subsequently, they described protein bodies isolated from the endosperm (Mitsuda et al., 1969) that had concentric rings and were similar to those shown in Fig. 4.

The proportions of albumin and globulin in the protein are highest in the outer layers of the milled rice kernel and decrease toward the center; proportions of glutelin have an inverse distribution (Houston and Kohler, 1970). In rice, as in other cereal grains, the proteins differ considerably in their amino acid composition and biological value. The most notable differences are in the high concentration of lysine in albumins and of cystine in globulins, and in the very low lysine and cystine concentrations in the prolamines. Rice protein is not ideally balanced; it is appreciably low in lysine concentration in comparison with the FAO Reference Pattern. Supplementation with lysine and threonine significantly increases the biological value of rice protein (Houston and Kohler, 1970). It is not known whether the three types of protein bodies in rice contain different types of protein or whether the bodies differ in amino acid composition and biological value.

The subaleurone region is only several layers thick and lies directly below the aleurone and is subject to relatively easy removal during milling. It is, therefore, desirable either to mill rice as lightly as possible and thus retain some of the protein in the subaleurone, or to breed cultivars that have an increased number of subaleurone layers or cultivars that have the protein more evenly distributed throughout the endosperm.

Alternatively, the nutritious subaleurone region in milled rice kernel can be collected by further milling white or parboiled-milled rice or used to prepare protein rich flour. That flour contains about 20% protein (Houston and Kohler, 1970). The main problem is, however, that removing the protein rich subaleurone reduces further the nutritive value of the milled rice.

Germ

The ease with which the rice germ is removed from the caryopsis during milling, probably depends upon two structural factors. The first is the fibrous zone separating the scutellar epithelium from the starchy endosperm (Fig. 8 and 9). This zone provides a line of easy fracture so that the germ can be removed intact with minimum energy expenditure (Fig. 9). The second factor is that the starchy endosperm in this region does not have well defined cell walls (the endosperm appears coenocytic–without cell walls) and, therefore, provides a structural defect that allows the germ to be removed easily (Fig. 8).

Parboiling

The purpose of parboiling is to produce modifications in the grain that improve its nutritional, cooking, and storage attributes. The main modifications are the transfer of some vitamins and minerals from the aleurone and germ into the starchy endosperm, dispersion of lipids from the aleurone and germ layers, inactivation of enzymes, and destruction of molds and insects (Gariboldi, 1974b). Those changes are accompanied by reduction of chalkiness and increase in vitreousness and translucency of the milled rice, and improved digestibility and cooking properties. Parboiling strengthens the attachment of the germ to the starchy endosperm and prevents separation

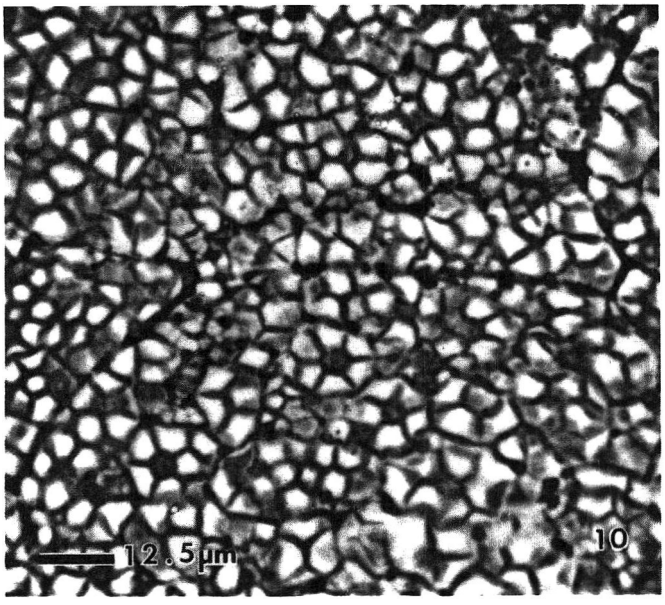

Fig. 10—LM of untreated endosperm from region beneath subaleurone. (X 728)

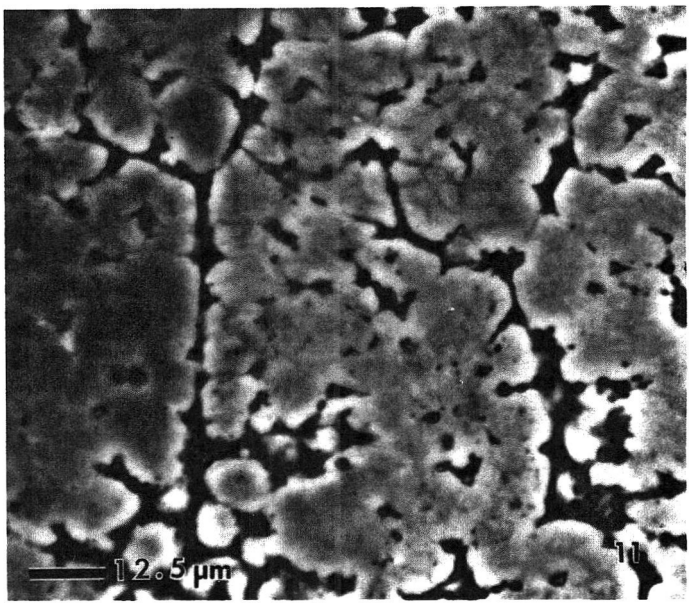

Fig. 11—LM of parboiled endosperm from region beneath subaleurone. (X 728).

of the germ during husking. At the same time, milling of the husked grain from parboiled rice is made more difficult by the hardening of the endosperm and cementing of the aleurone and germ to the starchy endosperm.

Parboiling in commercial rice modified the starch (Fig. 10 and 11). We found that modification is extensive in about one-third to one-half the distance from the aleurone toward the center of the endosperm in commercially parboiled rice. Watson and Dikeman (1977), however, found using SEM that all portions of the grain were altered. Starch in the central endosperm may be modified little if at all. The protein bodies in the endosperm, apparently, were not affected by parboiling; the structure and the crystal lattice fringes and rings were unchanged according to examination by TEM (unpublished data). Feeding and in vitro enzymatic studies showed that protein bodies are less digestible in parboiled milled than in raw milled rice, however, the decrease in digestibility was compensated by an increase in biological value (B.O. Juliano, private communication).

In conclusion, the rice caryopsis (in common with other cereal grains) has distinct structural features that seem to influence its harvesting, handling, and milling characteristics. Some of the structural features are beneficial to the consumer while others may hinder processing. Understanding of the desirable features should be helpful in designing plant breeding programs to develop new cultivars with improved properties for rice handling, storage, processing, and nutrition.

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EFFECTS OF HEATING METHODS ON PROTEIN NUTRITIONAL VALUE OF FIVE FRESH OR FROZEN PREPARED FOOD PRODUCTS

C. E. BODWELL and M. WOMACK

ABSTRACT

Lyophilized samples of instant mashed potatoes, peas with onions, beans with frankfurters, beef pot roast with gravy, and breaded fish portions were evaluated for effects on protein nutritional value of treatments simulating conventional institutional procedures (fresh preparation followed by holding at 82°C for 0, ½, 1½, and 3 hr) and convenience food system handling procedures (preparation, freezing, reheating to 82°C using microwave, high pressure steam, infrared or hot air convection oven heating and holding at 82°C for ½ hr). For the five products studied, the treatment means for Shortened Pepsin Digest Index values ranged from 66–69, for percent available lysine (excluding values for the fish product) from 82–86% (FDNB extractive method) or 59–65% (TNBS extractive method) and for Modified Protein Efficiency Ratio from 2.99–3.07. Lysine availability, when estimated by the "difference" method, for the fish product was over 95% for all treatments. Amino acid compositions of selected samples did not vary markedly with treatment. With rat bioassays, designed to detect decreases in selected amino acids, several significant differences were observed but they were not consistently related to treatment. Neither conventional nor convenience food system handling procedures seriously decreased estimated protein nutritive values of the products studied.

INTRODUCTION

FEW DATA are available on the comparative effects of various reconstitution methods on protein nutritive value. In foods prepared in large quantity and reheated in convection ovens in a "cook/freeze" system, chemical estimates of available lysine were slightly higher than in foods conventionally prepared (Catering Research Unit, 1970). Harwood et al. (1971) studied the effects of method of reconstitution on relative nutritive value (RNV) of beef stew, chicken and noodle casserole, and creamed spinach. RNV values were based on 96 hr *Tetrahymena pyriformis W.* counts expressed as a percent of the counts obtained with casein. The products were reheated by conventional range-oven, compartment steamer, microwave oven, forced air convection oven and "a special reconstituting oven." For creamed spinach, RNV values did not differ significantly among reheating methods. For the chicken casserole, the RNV value (81) for the product reheated in the microwave oven was significantly lower than the RNV values (86–88) for the products reheated by the other methods. RNV values for beef stew ranged from 71 for the product reheated in the conventional oven to 74 for the product reheated in the microwave oven.

The objective of the present study was to evaluate the effects of various reconstitution methods on the protein quality of five bulk-packed frozen prepared food products. The study was conducted in collaboration with the Institute of Human Nutrition, Columbia University, New York, NY. Preparation and reconstitution of the foods were carried out and reported by the Columbia University collaborators (Ang et al., 1975). The products studied were instant mashed potatoes, peas with onions, beans and frankfurters, beef pot roast with gravy and frozen fried fish portions. A preliminary report has been presented (Bodwell and Womack, 1974).

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EXPERIMENTAL

Materials

The materials and procedures used in preparing, assembling, and handling products were described by Ang et al. (1975). After preparation, freezing and/or reconstitution, sufficient quantities of each product for protein nutritional evaluation were frozen and lyophilized. Batches of each lyophilized product were ground in a Wiley mill, thoroughly mixed, and then stored at −20°C.

Amino acid analyses

Approximately 200 mg samples of the peas with onions, instant mashed potatoes, beef pot roast with gravy, beans with frankfurters, raw unbreaded fish portions, and raw breaded fish portions were acid hydrolyzed for 22 hr at 110°C in 40 ml of 6N HCl and analyzed by ion-exchange chromatography as described (Bodwell et al., 1971). Tryptophan in the beef pot roast with gravy and beans with frankfurters products was determined on alkaline hydrolysates by the spectrophotometric method of Spies and Chambers (1948) as modified by Miller (1967). For ion-exchange chromatography analyses of tryptophan in the other products, 16-hr hydrolyses at 110°C were done by using approximately 3.1M $Ba(OH)_2$ (Knox et al., 1970).

The freshly prepared frozen and thawed breaded fish portions were analyzed by a commercial laboratory (WARF, Inc., Madison, WI) by ion-exchange chromatography. The same laboratory also determined cystine in all four fish products and methionine in two of the fish products by microbiological assay of alkaline hydrolyzates according to the method of Henderson and Snell (1948).

Available lysine

Chemical estimates of available lysine were made on four of the products by the 1-flouro-2,4-dinitrobenzene (FDNB) extractive method of Carpenter (Carpenter and Booth, 1973) and the 2,4,6-trinitrobenzene sulfonic acid (TNBS) method of Kakade and Liener (1969). For the FDNB method, FDNB reacted samples were heated for 16 hr at 110°C in sealed tubes instead of being acid-refluxed for 16 hr (Carpenter procedure). The 1-hr autoclaving step of Kakade and Liener (1969) for partial hydrolyses of TNBS reacted samples was replaced by heating the reacted samples at 110°C for 4 hr in sealed tubes. These substitutions give equivalent results to those obtained by use of the original procedures (S.N. Hagan and C.E. Bodwell, unpublished data). For the fish portions, available lysine was estimated by the "difference" method (total lysine minus non-FDNB reactive lysine; Williams, 1967; Roach et al., 1967). The procedural modifications of Ostrowski et al. (1970) were used (including a modified citrate buffer) except that Technicon Chromobeads Type A resin was used, the resin bed was increased to 20 cm in height, and buffer pumps were used instead of gravity flow for elution at flow rates of 29–30 ml/hr.

Shortened pepsin digest residue index assays

The procedures of Sheffner (1967) were used for digestion and preparation of samples for amino acid analyses. For measurement of total lysine, methionine and tryptophan, duplicate hydrolyzates were analyzed microbiologically by the method of Henderson and Snell (1948) by WARF, Inc. (Madison, WI). Indexes were calculated as indicated by Sheffner (1967).

Rat assays

Possible changes in the availability of selected essential amino acids were studied by use of a modified Protein Efficiency Ratio (M-PER) method (Womack et al., 1974). In this approach, the diets supply selected amino acids (preferably entirely provided by the protein source) at "critical" levels. The critical level is defined as that level of a specific amino acid which maintains a moderately rapid rate of growth

but which, when reduced by a known amount (i.e., 20%), results in either significantly lower weight gains, food efficiencies, or M-PER. Each amino acid studied, the dietary levels, critical levels and the percent of the critical level provided by the protein sources are listed in Table 1.

All diets were analyzed for nitrogen by the macro-Kjeldahl method and actual nitrogen content was used for calculating M-PER values.

Table 1—Amino acids studied in five food products by a Modified Protein Efficiency Ratio Assay[a]

Product	Amino acids studied	Level in diet (%)	Critical level[a] (%)	% of critical level provided by test food (%)
Peas with onions	Lysine	0.60	0.60	98
	Tryptophan	0.07[b]	0.09	78
Instant mashed	Lysine	0.60	0.60	100
potatoes	Methionine	0.16	0.16	100
Beef pot roast	Lysine	0.60	0.60	97
with gravy	Methionine	0.16	0.16	100
Beans with	Isoleucine	0.33[b]	0.32	104
Frankfurters	Lysine	0.60	0.60	93
Experiment 1	Threonine	0.35	0.35	100
	Valine	0.40	0.40	94
Experiment 2	Methionine	0.16	0.16	100
Fried breaded	Isoleucine	0.32	0.32	97
fish portions	Lysine	0.60	0.60	100
Experiment 1	Phenylalanine	0.30	0.30	95
	Valine	0.40	0.40	92
Experiment 2	Methionine	0.16	0.16	100

[a] See text for definition of M-PER
[b] All from test food

Table 2—Amino acid composition of peas with onions and instant mashed potatoes

Amino acid	Peas with onions		Instant mashed potatoes
	Freshly prepared, frozen and thawed (g/g N)	Freshly prepared, held 3 hr @ 82°C (g/g N)	Freshly prepared (g/g N)
Alanine	0.37 ± 0.01[a,b]	0.37 ± 0.01[a,b]	0.23 ±0.01[a,c]
Arginine	0.75 ± 0.01a	0.76 ± 0.01a	0.21 ±0.01
Aspartic acid	0.57 ± 0.01a	0.59 ± 0.02a	0.84 ±0.07
Cystine	0.05 ± 0.01a	0.05 ± 0.01a	0.04 ±0.01
Glutamic acid	0.97 ± 0.08a	1.01 ± 0.11a	1.22 ±0.01
Glycine	0.21 ± 0.01a	0.22 ± 0.01a	0.18 ±0.01
Histidine	0.11 ± 0.01a	0.11 ± 0.01a	0.13 ±0.01
Isoleucine	0.23 ± 0.01a	0.24 ± 0.01a	0.28 ±0.01
Leucine	0.43 ± 0.03a	0.42 ± 0.01a	0.53 ±0.02
Lysine	0.39 ± 0.01a	0.40 ± 0.01a	0.46 ±0.01
Methionine	0.07 ± 0.01a	0.06 ± 0.01a	0.13 ±0.01
Phenylalanine	0.23 ± 0.01a	0.25 ± 0.01a	0.29 ±0.01
Proline	0.18 ± 0.01a	0.19 ± 0.03a	0.38 ±0.05
Serine	0.25 ± 0.01a	0.26 ± 0.01a	0.32 ±0.01
Threonine	0.28 ± 0.01a	0.28 ± 0.01a	0.28 ±0.01
Tryptophan	0.05 ± 0.01a[d]	0.05 ± 0.01a[d]	0.14 ±0.02[d]
Tyrosine	0.18 ± 0.01a	0.18 ± 0.01a	0.31 ±0.01
Valine	0.28 ± 0.01a	0.29 ± 0.01a	0.37 ±0.01

[a] Means ± standard deviation; standard deviations of less than 0.01 reported as 0.01.
[b] For the peas with onions products, values followed by the same letter within a row, are not significantly different at the 5% level; values (except for tryptophan) based on two analyses for the freshly prepared, frozen and thawed product and on three analyses for the freshly prepared, held 3 hr product.
[c] Values based on three analyses
[d] Determined on duplicate alkaline hydrolysates

Glutamine was added as needed to maintain the nitrogen content at a level equivalent to approximately 10% protein (6.25 × N). For the various diets, glutamine varied from none to 2.9% and the added amino acid mixture, from 0.10–1.66%. Dietary levels of protein provided by each product were 6.88% (instant mashed potatoes), 9.19% (peas with onions), 7.02% and 9.43% (beans and frankfurters, Experiments 1 and 2, respectively), 7.61 and 5.35% (fried breaded fish portions, Experiments 1 and 2, respectively) and 7.50% (beef pot roast with gravy). Other non-protein dietary ingredients were as described (Womack et al., 1974) except that in diets containing greater than 10% fat, the level of cornstarch was proportionally reduced. Since dietary fat level may alter rat growth rates, within each product studied, fat levels were held constant. This was done by adding corn oil to the different treatments of a specific product to equalize each treatment diet at the same fat level for each product. Actual fat contents of diets so adjusted were 10% (peas with onions and fried breaded fish diets), 12% (beef pot roast with gravy and beans with frankfurters diets) and 24% (instant mashed potatoes diet).

Statistical analysis

Duncan's new multiple-range test (Steel and Torrie, 1960) or Students' t-test (Snedecor, 1959) was used for testing for possible significance of differences between treatment means.

RESULTS & DISCUSSION

Amino acid composition

Amino acid composition did not differ for the freshly prepared-frozen-thawed peas with onion product and the freshly prepared-held 3 hr at 82°C products (Table 2). For the beef pot roast with gravy products (Table 3), holding for 3 hr at 82°C significantly decreased several amino acids including the essential amino acids isoleucine, leucine, lysine, methionine and threonine. These differences, however, may not be of practical importance since for each of these amino acids the values for the product held for 3 hr are not significantly different from the values for the cooked beef pot roast. For beans with frankfurters, the few significant differences in values for any amino acid, between the freshly prepared, freshly prepared-frozen-thawed, and the freshly prepared-held 3 hr at 82°C are likewise not of practical significance.

Values for the raw fish portions and the raw, the freshly prepared-frozen-thawed, and the freshly prepared-held 3 hr at 82°C breaded fish portions are listed in Table 4. As expected, the effects of "breading" were minimal; significant increases were observed for glutamic acid, phenylalanine, serine and threonine and decreases were observed for cysteine and tryptophan. The preparation procedures used resulted in apparent increases in the values for most of the amino acids. Holding for 3 hr at 82°C, however, apparently caused significant decreases in values for several of the amino acids including those for the essential amino acids isoleucine, leucine, lysine, methionine, threonine, and valine.

Available lysine

Chemical estimates of nutritionally available lysine for the peas with onions and the instant mashed potato products are given in Table 5. All estimates were much higher by the FDNB method than by the TNBS method. For the estimates from either method, significant differences between treatments were observed, but were not always consistent between the two methods. Estimates by both methods suggest that available lysine was moderately decreased during holding for up to 3 hr at 82°C. For the convenience food system treatments, the FDNB estimates suggest that the highest level of available lysine was retained by the product reheated in the convection oven. However, for the TNBS method estimates, the differences in available lysine were small with the values observed representing from 63–72% of the total lysine. For the instant mashed potato product, by the FDNB method, the value was lowest for the freshly prepared product and highest for the freshly prepared-held 3 hr product. The range in percent available lysine was 70–81%. For estimates by either method,

Table 3—Amino acid composition of cooked beef pot roast, beef pot roast with gravy, and beans with frankfurters

Amino acid	Cooked pot roast (g/g N)	Beef pot roast with gravy		Beans with frankfurters		
		Freshly prepared (g/g N)	Freshly prepared, held 3 hr @ 82°C (g/g N)	Freshly prepared (g/g N)	Frozen and thawed (g/g N)	Freshly prepared, held 3 hr @ 82°C (g/g N)
Alanine	0.29 ± 0.01a[a,b]	0.34 ± 0.01a[a,b]	0.31 ± 0.01a[a,b]	0.33 ± 0.01a[a,c]	0.32 ± 0.03ab[a,c]	0.37 ± 0.01b[a,c]
Arginine	0.33 ± 0.01	0.36 ± 0.01	0.34 ± 0.01	0.34 ± 0.01a	0.39 ± 0.02a	0.44 ± 0.02a
Aspartic acid	0.51 ± 0.04ab	0.50 ± 0.01a	0.46 ± 0.01b	0.79 ± 0.19a	0.98 ± 0.12a	0.84 ± 0.01a
Cystine	0.08 ± 0.01ab	0.10 ± 0.01a	0.08 ± 0.01b	0.03 ± 0.01a	0.03 ± 0.01a	0.04 ± 0.01a
Glutamic acid	0.72 ± 0.02a	0.69 ± 0.01a	0.69 ± 0.01a	1.00 ± 0.06a	0.94 ± 0.08a	0.99 ± 0.14a
Glycine	0.25 ± 0.01	0.31 ± 0.01	0.28 ± 0.01	0.35 ± 0.02a	0.33 ± 0.03a	0.43 ± 0.02
Histidine	0.18 ± 0.01	0.22 ± 0.01	0.20 ± 0.01	0.19 ± 0.01a	0.20 ± 0.01a	0.21 ± 0.03a
Isoleucine	0.24 ± 0.01a	0.28 ± 0.01	0.23 ± 0.01a	0.29 ± 0.04a	0.30 ± 0.05a	0.30 ± 0.03a
Leucine	0.43 ± 0.03a	0.48 ± 0.01	0.45 ± 0.01a	0.56 ± 0.01a	0.54 ± 0.02a	0.56 ± 0.07a
Lysine	0.46 ± 0.02a	0.51 ± 0.02	0.48 ± 0.01a	0.50 ± 0.03a	0.48 ± 0.03a	0.51 ± 0.04a
Methionine	0.12 ± 0.01a	0.16 ± 0.02	0.13 ± 0.01a	0.10 ± 0.01a	0.11 ± 0.02a	0.11 ± 0.01a
Phenylalanine	0.21 ± 0.01a	0.23 ± 0.01a	0.22 ± 0.02a	0.34 ± 0.01a	0.31 ± 0.04a	0.35 ± 0.07a
Proline	0.19 ± 0.01	0.23 ± 0.01a	0.23 ± 0.01a	0.23 ± 0.04a	0.22 ± 0.04a	0.23 ± 0.02a
Serine	0.19 ± 0.01a	0.21 ± 0.01b	0.20 ± 0.02ab	0.36 ± 0.02a	0.35 ± 0.05a	0.38 ± 0.04a
Threonine	0.22 ± 0.01	0.26 ± 0.01	0.23 ± 0.01	0.31 ± 0.01a	0.29 ± 0.03a	0.33 ± 0.05a
Tryptophan	0.09 ± 0.01a[d]	0.08 ± 0.01a[d]	0.08 ± 0.01a[d]	–	0.07 ± 0.01a[d]	0.07 ± 0.01a[d]
Tyrosine	0.17 ± 0.01a	0.18 ± 0.01a	0.18 ± 0.01a	0.25 ± 0.01a	0.23 ± 0.02a	0.23 ± 0.04a
Valine	0.25 ± 0.01a	0.27 ± 0.02a	0.25 ± 0.01a	0.33 ± 0.03a	0.32 ± 0.04a	0.36 ± 0.02a

[a] Means ± standard deviation. standard deviations of less than 0.01 reported as 0.01; for each product, values followed by the same letter, within, a row, are not significant y different at the 5% level.
[b] Values based on three analyses of each product.
[c] Values based on two or, in most cases, three analyses.
[d] Tryptophan values based on spectrophotometric analyses of duplicate alkaline hydrolysates.

the significant differences observed probably have little practical importance.

For the beef pot roast with gravy products (Table 6), except for the freshly prepared-held ½ hr at 82°C product, values from the FDNB method suggested that the level of available lysine was high (96–102% of total lysine). Values from the TNBS method were much lower and suggested decreased available lysine in all of the treated products rela-

Table 4—Amino acid compositions of raw fish portions, breaded raw fish portions, and fried breaded fish portions subjected to two different treatments

Amino acid	Raw fish portions (g/g N)	Breaded fish portions		
		Raw (g/g N)	Freshly prepared, held 3 hr @ 82°C (g/gN)	Freshly prepared, frozen and thawed (g/g N)
Alanine	0.34 ± 0.03a[a,b]	0.35 ±0.01a[a,b]	0.34 ±0.01a[a,c]	0.39 ±0.01a[a,c]
Arginine	0.32 ± 0.02a	0.33 ±0.02a	0.32 ±0.03a	0.38 ±0.01
Aspartic acid	0.51 ± 0.02a	0.55 ±0.03a	0.52 ±0.01ab	0.65 ±0.01b
Cysteine	0.05 ± 0.01a[d]	0.04 ±0.01[d]	0.06 ±0.01[d]	0.05 ±0.01a[d]
Glutamic acid	0.83 ± 0.03	1.02 ±0.04ab	0.96 ±0.01a	1.06 ±0.01b
Glycine	0.27 ± 0.01a	0.27 ±0.01a	0.27 ±0.01a	0.32 ±0.01
Histidine	0.09 ± 0.04a	0.12 ±0.01a	0.11 ±0.01a	0.12 ±0.01a
Isoleucine	0.25 ± 0.01ab	0.27 ±0.01a	0.25 ±0.01b	0.29 ±0.01
Leucine	0.45 ± 0.05abc	0.49 ±0.01a	0.45 ±0.01b	0.51 ±0.01c
Lysine	0.49 ± 0.03ab	0.52 ±0.01a	0.49 ±0.01b	0.53 ±0.01a
Methionine	0.17 ± 0.01a	0.17 ±0.01a	0.18 ±0.01a[e]	0.21 ±0.01[f]
Phenylalanine	0.21 ± 0.01	0.25 ±0.01a	0.23 ±0.01	0.26 ±0.01a
Proline	0.18 ± 0.01a	0.23 ±0.04ab	0.22 ±0.01b	0.34 ±0.01
Serine	0.24 ± 0.01	0.27 ±0.01a	0.26 ±0.01a	0.28 ±0.01
Threonine	0.23 ± 0.01	0.26 ±0.01a	0.25 ±0.01a	0.28 ±0.01
Tryptophan	0.07 ± 0.01[g]	0.06 ±0.01a[g]	0.06 ±0.01a[g]	0.06 ±0.01a[g]
Tyrosine	0.17 ± 0.02ab	0.14 ±0.01a	0.21 ±0.01b	0.22 ±0.01
Valine	0.27 ± 0.01a	0.33 ±0.03abc	0.30 ±0.01b	0.32 ±0.01c

[a] Means ± standard deviation; standard deviation of less than 0.01 reported as 0.01; values followed by the same letter within a row are not significantly different at the 5% level.
[b] Values from three analyses
[c] Values from two analyses; hydrolyses and ion exchange analyses done by commercial laboratory
[d] All cysteine values from base hydrolyzate; determined by duplicate microbiological assays (commercial laboratory)
[e] Value of 0.187 ± 0.001 obtained from duplicate microbiological analyses of base hydrolysate (commercial laboratory)
[f] Value of 0.188 ± 0.001 obtained from duplicate microbiological analyses of base hydrolysate (commercial laboratory)
[g] Determined by ion exchange analyses of alkaline hydrolysates

Table 5—Chemical estimates of nutritionally available lysine in peas with onions and instant mashed potatoes

No.	Treatment	Peas with onions				Instant mashed potatoes			
		Available lysine content				Available lysine content			
		FDNB Method		TNBS Method		FDNB Method		TNBS Method	
		mg/g N	% of total lysine	mg/g N	% of total lysine	mg/g N	% of total lysine	mg/g N	% of total lysine
Conventional institutional handling									
3	Freshly prepared, no holding	374 ± 2a[a,b]	93	283 ± 3ab[a,b]	71	320 ± 13a[a,b]	70	250 ± 14abcd[a,b]	55
4	Freshly prepared, held ½ hr	371 ± 3	92	285 ± 2ab	71	336 ± 8abc	74	232 ± 1ae	51
5	Freshly prepared held 1½ hr	326 ± 46b	81	259 ± 3	65	343 ± 13bcde	75	224 ± 8ef	49
6	Freshly prepared, held 3 hr	332 ± 1bc	83	251 ± 1	63	369 ± 23e	81	264 ± 2b	58
Convenience food system handling									
7	Frozen and thawed	349 ± 6	87	283 ± 3ab	71	331 ± 5bd	73	195 ± 11	43
8	Frozen, reheated in convection oven, held for ½ hr	376 ± 1a	94	285 ± 5a	71	335 ± 17acde	73	223 ± 3cf	49
9	Frozen, reheated in infrared oven, held ½ hr	334 ± 3c	83	283 ± 2b	70	341 ± 16acde	75	233 ± 1ae	51
10	Frozen, reheated in steamer, held ½ hr	322 ± 3b	80	287 ± 2ab	72	340 ± 6bcd	75	232 ± 26abcde	51
11	Frozen, reheated in microwave oven, held ½ hr	312 ± 2	78	276 ± 1	69	341 ± 2c	75	250 ± 10d	55

[a] Means ± standard deviation; each mean is based on at least four values obtained on two or more samples.
[b] Values followed by the same letter, within the same column, are not significantly different at the 5% level.

tive to the freshly prepared product. It is unlikely, however, that merely freezing and thawing would cause a decrease in available lysine and the practical importance of the differences is questionable. For the beans with frankfurters product, only one treatment (holding for 3 hr at 82°C), apparently caused a decrease in available lysine as estimated by both methods.

For the conventional institutional handling treatments, mean values of available lysine, for the products listed in

Table 6—Chemical estimates of nutritionally available lysine in beef pot roast with gravy and in beans with frankfurters

No.	Treatment	Beef pot roast with gravy				Beans with frankfurters			
		Availably lysine content				Available lysine content			
		FDNB Method		TNBS Method		FDNB Method		TNBS Method	
		mg/g N	% of total lysine	mg/gN	% of total lysine	mg/g N	% of total lysine	mg/g N	% of total lysine
Conventional institutional handling									
3	Freshly prepared, no holding	464 ± 22a[a,b]	96	406 ± 13a[a,b]	84	388ab[a,b]	78	241 ± 6a[a,b]	48
4	Freshly prepared, held ½ hr	434 ± 23	90	355 ± 12a	73	374 ± 9c	75	242 ± 3ab	49
5	Freshly prepared, held 1½ hr	465 ± 16a	96	354 ± 8ab	73	382 ± 6acd	77	233 ± 15a	47
6	Freshly prepared, held 3 hr	493 ± 20a	102	365 ± 9cd	76	361 ± 8	72	203 ± 8	41
Convenience food system handling									
7	Frozen and thawed	490 ± 35a	101	361 ± 8cef	75	389 ± 6ad	78	236 ± 13a	47
8	Frozen, reheated in convection oven, held for ½ hr	474 ± 11a	98	360 ± 3e	74	391 ± 4ad	78	236 ± 9a	47
9	Frozen, reheated in infrared oven, held ½ hr	492 ± 21a	102	365 ± 5cg	75	381 ± 8ac	76	234 ± 7a	47
10	Frozen, reheated in steamer, held ½ hr	470 ± 10a	97	351 ± 36bf	72	387 ± 6bd	78	252 ± 4bc	51
11	Frozen, reheated in microwave oven, held ½ hr	478 ± 21a	99	366 ± 3dg	76	385 ± 8acd	77	253 ± 7c	51

[a] Means ± standard deviation; each mean is based on at least four values obtained on two or more samples.
[b] Values followed by the same letter within a column are not significantly different at the 5% level.

Table 7—Shortened pepsin digest residue indexes for five food products

No.	Treatment	Beef pot roast with gravy	Beans with frankfurters	Peas with onions	Instant mashed potatoes	Fried breaded fish portions	
Raw materials							
1	Raw beef, raw fish	64	–	–	–	81	
2	Cooked beef, raw breaded fish portions	62	–	–	–	82	
Conventional institutional handling							
3	Freshly prepared, no holding	62	61	59	75	79	(Frozen and reheated in convection oven, no holding)
4	Freshly prepared, held ½ hr	61	62	57	74	78	(Frozen and reheated in convection oven, held ½ hr)
5	Freshly prepared, held 1½ hr	59	61	58	76	79	(Frozen and reheated in convection oven, held 1½ hr)
6	Freshly prepared, held 3 hr	60	60	57	78	79	(Frozen and reheated in convection oven, held 3 hr)
Convenience food system handling							
7	Frozen and thawed	62	64	62	76	81	
8	Frozen, reheated in convection oven, held for ½ hr	60	63	57	75	–	(Same as #4)
9	Frozen, reheated in infrared oven, held ½ hr	60	64	63	76	77	
10	Frozen, reheated in steamer, held ½ hr	60	67	62	76	79	
11	Frozen, reheated in microwave oven, held ½ hr	60	63	60	76	77	

Tables 5 and 6, were 84 and 61% by the FDNB and TNBS methods, respectively. For the same products, treatment means were identical for the convenience food handling system treatments. For the breaded fish portions, over 95% of the total lysine was available, as estimated by the "difference" method, in the products from all of the treatments. These results differ from those of Tooley (1972) who observed 17–25% losses in available lysine following deep fat frying.

—Text continued on page 1548

Table 8—Food efficiencies, and modified protein efficiency ratios (M-PER) of rats fed diets used to study effects of various treatments on lysine and tryptophan availability in peas with onions and of lysine and methionine availability in instant mashed potatoes

		Peas with onions		Instant mashed potatoes	
No.	Treatment	Food efficiencies (g gain/g food)	M-PER[a]	Food efficiencies (g gain/g food)	M-PER[b]
Conventional institutional handling					
3	Freshly prepared, no holding	0.35 ± 0.01a[c,d]	3.47 ± 0.11a[c,d]	0.24 ± 0.01[c,d]	2.39 ± 0.10a[c,d]
4	Freshly prepared, held ½ hr	0.34 ± 0.01ab	3.35 ± 0.09ab	0.29 ± 0.01a	2.85 ± 0.14b
5	Freshly prepared held 1-½ hr	0.32 ± 0.01bc	3.27 ± 0.08abc	0.28 ± 0.01a	2.88 ± 0.09b
6	Freshly prepared, held 3 hr	0.30 ± 0.02c	3.01 ± 0.16bc	0.29 ± 0.01a	2.98 ± 0.17b
Convenience food system handling					
7	Frozen and thawed	0.32 ± 0.02bc	3.18 ± 0.23abc	0.27 ± 0.01a	2.77 ± 0.09b
8	Frozen, reheated in convection oven, held for ½ hr	0.30 ± 0.01c	3.03 ± 0.10c	0.28 ± 0.01a	2.89 ± 0.09b
9	Frozen, reheated in infrared oven, held ½ hr	0.32 ± 0.02bc	3.30 ± 0.18abc	0.28 ± 0.01a	2.77 ± 0.13b
10	Frozen, reheated in steamer, held ½ hr	0.31 ± 0.01cd	3.17 ± 0.14abc	0.27 ± 0.01a	2.70 ± 0.11ab
11	Frozen, reheated in microwave oven, held ½ hr	0.33 ± 0.01abd	3.30 ± 0.03ab	0.27 ± 0.01a	2.69 ± 0.12ab

[a] In 14 days; average initial weights: 57–59g
[b] In 14 days; average initial weights: 63–64g
[c] Means ± standard errors; standard errors less than 0.01 reported as 0.01.
[d] Values within a column followed by the same letter are not significantly different at the 5% level.

However, as noted by Tooley (1972), 10-g samples were used for frying and neither crumb nor batter coatings were used.

Shortened pepsin digest residue indexes

Indexes are listed for the five food products, by treatments, in Table 7. Within products, very little variation was observed between treatments. Mean treatment values for all five products were 67 for the conventional institutional handling treatments and 68 for the convenience food system handling treatments.

Rat bioassay

Food efficiencies and M-PER values of rats fed diets used to study the effects of the various treatments on the availability of selected essential amino acids in each product are listed in Tables 8–11. Eight to 12 rats per treatment group were used. For some products significant differences were observed between treatments. For example, for the peas with onions product (Table 8), the M-PER value was significantly lower for the freshly prepared-held 3 hr at 82°C product than for the freshly prepared product. Among the convenience food system handling procedures the product reheated in the convection oven had a significantly lower M-PER than the freshly prepared product. These results suggest that holding the product at 82°C or re-constituting by use of convection oven reheating caused a decrease in available lysine or tryptophan. Likewise, for the beans with frankfurters product (Table 10), the significantly lower M-PER value for the freshly prepared-held 3 hr at 82°C product (when compared to the M-PER value for the freshly prepared product) suggests that this treatment caused a significant decrease in available methionine. Other significant differences were observed (Tables 10, 11).

In general, however, if the overall values for the five products and the severity of the various treatments are considered, we suggest that the differences among treatments are probably of little practical importance. For the five products, the mean M-PER was 3.04 for the conventional institutional handling and 3.02 for the convenience food system handling treatments. Thus if availability changed for the amino acids studied, the changes were not consistently detected by the methods used.

Table 9—Food efficiencies and modified protein efficiency ratios (M-PER) of rats fed diets used to study effects of various treatments on the availability of methionine and lysine in beef pot roast with gravy

		Food efficiencies (g gain/g food)	M-PER[a]
Conventional institutional handling			
3	Freshly prepared, no holding	0.40 ± 0.01a[b,c]	4.14 ± 0.06a[b,c]
4	Freshly prepared, held ½ hr	0.39 ± 0.01a	3.98 ± 0.10a
5	Freshly prepared, held 1½ hr	0.38 ± 0.01a	3.82 ± 0.06a
6	Freshly prepared, held 3 hr	0.38 ± 0.01a	3.88 ± 0.06a
Convenience food system handling			
7	Frozen and thawed	0.40 ± 0.01a	3.95 ± 0.08a
8	Frozen, reheated in convection oven, held for ½ hr	0.38 ± 0.01a	3.90 ± 0.08a
9	Frozen, reheated in infrared oven, held ½ hr	0.38 ± 0.01a	3.90 ± 0.07a
10	Frozen, reheated in steamer, held ½ hr	0.39 ± 0.01a	3.91 ± 0.05a
11	Frozen, reheated in microwave oven, held ½ hr	0.37 ± 0.01a	4.13 ± 0.11a

[a] In 21 days; average initial weights: 60g
[b] Means ± standard errors; standard errors less than 0.01 reported as 0.01.
[c] Values within a column followed by the same letters are not significantly different at the 5% level.

CONCLUSIONS

FOR THE FIVE PRODUCTS studied, the treatment means for the Shortened Pepsin Digest Residue Indexes varied from 66–69, for percent available lysine (excluding values for the fish product) from 82–86% (FDNB method) or 59–65% (TNBS method), and for M-PER from 2.99–3.07. By use of the "difference" method, lysine was estimated to be over 95% available in all of the fish products. Amino acid compositions

Table 10—Food efficiencies, and modified protein efficiency ratios (M-PER) of rats fed diets used to study effects of various treatments on the availability of isoleucine, lysine, threonine and valine (Experiment 1) and of methionine (Experiment 2) in beans with frankfurters

		Experiment 1		Experiment 2	
No.	Treatment	Food efficiencies (g gain/g food)	M-PER[a]	Food efficiencies (g gain/g food)	M-PER[a]
Conventional institutional handling					
3	Freshly prepared, no holding	0.26 ± 0.01ab[b,c]	2.62 ± 0.08ab[b,c]	0.31 ± 0.01a[b,c]	2.95 ± 0.07ab[b,c]
4	Freshly prepared, held ½ hr	0.26 ± 0.01ab	2.52 ± 0.05ac	0.28 ± 0.01bc	2.69 ± 0.10ac
5	Freshly prepared, held 1½ hr	0.26 ± 0.01ab	2.52 ± 0.05ac	0.27 ± 0.01b	2.61 ± 0.09cd
6	Freshly prepared, held 3 hr	0.24 ± 0.01a	2.42 ± 0.01a	0.27 ± 0.01b	2.58 ± 0.08c
Convenience food system handling					
7	Frozen and thawed	0.28 ± 0.01b	2.81 ± 0.11bd	0.27 ± 0.01b	2.63 ± 0.06cd
8	Frozen, reheated in convection oven, held for ½ hr	0.25 ± 0.01ac	2.41 ± 0.11a	0.29 ± 0.01ab	2.77 ± 0.07ace
9	Frozen, reheated in infrared oven, held ½ hr	0.27 ± 0.01bc	2.63 ± 0.08ad	0.30 ± 0.01ac	3.00 ± 0.07be
10	Frozen, reheated in steamer, held ½ hr	0.27 ± 0.01abc	2.61 ± 0.10ad	0.28 ± 0.01bc	2.68 ± 0.12ac
11	Frozen, reheated in microwave oven, held ½ hr	0.28 ± 0.01b	2.72 ± 0.07bcd	0.30 ± 0.01ac	2.84 ± 0.10ade

[a] in 14 days; average initial weights: 60–62g
[b] Means ± standard errors; standard errors less than 0.01 reported as 0.01.
[c] Values within a column followed by the same letter are not significantly different at the 5% level.

Table 11—Food efficiencies, and modified protein efficiency ratios (M-PER) of rats fed diets used to study effects of various treatments on the availability of isoleucine, lysine, phenylalanine and valine (Experiment 1) and of methionine (Experiment 2) in fried breaded fish portions

No.	Treatment	Experiment 1		Experiment 2	
		Food efficiencies (g gain/g food)	M-PER[a]	Food efficiencies (g gain/g food)	M-PER[a]
Conventional institutional handling					
3	Frozen, reheated in convection oven, no holding	0.37 ± 0.01abc[b,c]	3.72 ± 0.09ab[b,c]	0.35 ± 0.01a[b,c]	3.45 ± 0.11ab[b,c]
4	Frozen, reheated in convection oven, held ½ hr	0.36 ± 0.01ab	3.68 ± 0.10ab	0.36 ± 0.01a	3.50 ± 0.13ab
5	Frozen, reheated in convection oven, held 1½ hr	0.38 ± 0.01acd	3.73 ± 0.09ab	0.37 ± 0.01a	3.60 ± 0.09a
6	Frozen, reheated in convection oven, held 3 hr	0.38 ± 0.01ac	3.71 ± 0.05ab	0.35 ± 0.01a	3.31 ± 0.13ab
Convenience food system handling					
7	Frozen and thawed	0.35 ± 0.01b	3.55 ± 0.07ab	0.36 ± 0.01a	3.42 ± 0.07ab
8	(Same as 4)	–	–	–	–
9	Frozen, reheated in infrared oven, held ½ hr	0.35 ± 0.01bd	3.44 ± 0.12a	0.33 ± 0.01a	3.19 ± 0.13b
10	Frozen, reheated in steamer held ½ hr	0.37 ± 0.01abe	3.57 ± 0.09ab	0.34 ± 0.01a	3.38 ± 0.12ab
11	Frozen, reheated in microwave oven, held ½ hr	0.39 ± 0.01ce	3.76 ± 0.09b	0.33 ± 0.02a	3.13 ± 0.11b

[a] In 14 days; average initial weights: 62g
[b] Means ± standard errors; standard errors less than 0.01 are reported as 0.01.
[c] Values within a column followed by the same letter are not significantly different at the 5% level.

of selected samples were not consistently altered by the various treatments. These results suggest that none of the conventional or convenience food handling procedures studied caused substantial changes in protein nutritive value as estimated by the methods described. The methods used might lack precision and sensitivity, but it would seem more likely that protein nutritional value was not substantially decreased by the various treatments. This is in contrast to the significant effects observed on the retention of vitamins (Ang et al., 1975).

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Appreciation is expressed to Mrs. S.N. Nelson, Mrs. Bernice Brooks and Mrs. Ethel Hall for technical assistance and to Dr. S.H. Lipton, Dr. P.E. McClain and Mr. Ernest Schuster for amino acid analyses.

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DEVELOPMENT OF INDICES FOR SORTING IRANIAN PISTACHIO NUTS ACCORDING TO FLUORESCENCE

ALI FARSAIE, W. F. McCLURE and R. J. MONROE

ABSTRACT

Study of the excitation and emission spectra of Iranian pistachio nuts was made in order to develop an index for sorting aflatoxin contaminated nuts. The method is based on previous work which discovered that nuts emitting bright-greenish-yellow (BGY) fluorescence under LW-UV light had a high probability of aflatoxin contamination. Six different indices were tested; I_{490}, I_{490}/I_{470}, I_{490}/I_{420} with excitation at 360 nm; and I_{490}, I_{490}/I_{450}, I_{490}/I_{550} with excitation at 420 nm (I = fluorescence intensity at indicated wavelengths). Tests clearly indicated that BGY fluorescent nuts can be separated from other nuts by electronic methods.

INTRODUCTION

DETECTION of bright-greenish-yellow fluorescence (BGY) on agricultural commodities under long-wavelength ultraviolet excitation (LW-UV) has long been considered a presumptive indicator of the presence of aflatoxin (Ashworth and McMeans, 1966; Dickens and Welty, 1975; Fennell et al., 1973). Dickens and Welty (1975) observed samples from 46 lots of Iranian pistachio nuts and found that 7% of the nuts exhibited BGY and that kernels from the fluorescent nuts contained 50% of the aflatoxin in the samples. Although this work does not conclude that aflatoxin is always present where BGY exists, the results do show that the removal of BGY nuts from a lot would significantly reduce the total concentration of aflatoxin in that lot.

Preliminary results of efforts to automatically separate BGY nuts using the ICORE Model 5140 UV (ICORE, Inc., 485 Clyde Avenue, Mountain View, CA 94043) sorter with long-wave ultraviolet (LW-UV) excitation were unsatisfactory. These results implied that improved separation would depend on a knowledge of the fluorescence spectra of pistachio nuts. The objective of this paper is to report the characteristic fluorescence spectra of Iranian pistachio nuts and to discuss the results in terms of developing an index for automatically separating the nuts according to their fluorescence characteristics.

EXPERIMENTAL

SAMPLES were taken from six lots of Iranian pistachio nuts which, under tests by Agricultural Marketing Service, USDA, had been rejected for aflatoxin contamination. The samples weighed approximately 50 kg each.

During examination of individual nuts under LW-UV light (UVL-21 lamps, Ultra-Violet Products, Inc., San Gabriel, CA), it was discovered that the nuts could be classed, according to fluorescence, into four basic categories. Some nuts had no fluorescence and were called normal (N). Some of the darker nuts gave a purple (P) emission; still others fluoresced bright-yellow (BY) over the whole surface of the nut, unlike the bright-greenish-yellow (BGY) which almost never covered the total surface of the nuts.

Subsamples of approximately 1000 nuts were visually selected for each of the four categories of fluorescence. Spectral curves were obtained on individual nuts from each of the subsamples.

Excitation and emission spectra were taken with an Aminco-Bowman Spectrophotofluorometer (SPF, American Instrument Company, 8030 Georgia Avenue, Silver Spring, MD 20910) with 1.0 mm slits, giving a band pass of approximately 8 nm. The solid-sample accessory was a convenient means of positioning the nuts in the SPF. BGY nuts were positioned so that the BGY fluorescence was viewed by the instrument. Fluorescence of the other categories was generally over the entire surface of the nuts so that little care was needed in positioning the nuts in the sample holder.

The average spectrum for each of the categories was obtained by plotting the average fluorescence intensity of the individual spectra at every 10 nm interval and connecting the points with a smooth curve.

Selection of sorting criteria for excitation at 360 nm and 420 nm was based on detailed studies of both the individual and average spectra. The validity of a particular selection was based on the F-ratio test of the difference between the means of the four categories, and the performance was based on the LSD test of the means of the fluorescence of each category. Two sorting parameters were tested: (1) I_i and (2) I_{490}/I_i, where i = wavelength of the fluorescence intensity I.

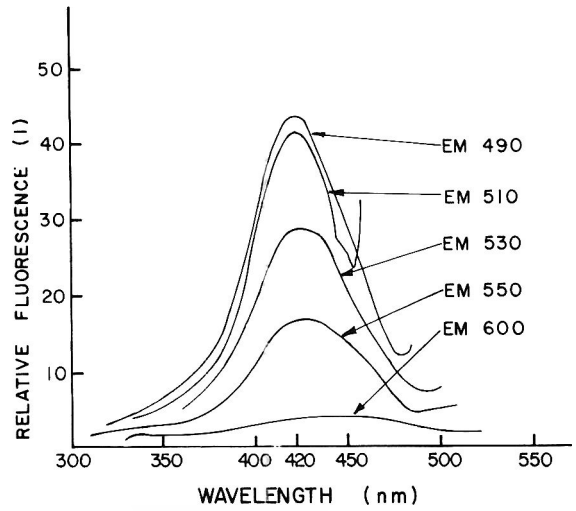

Fig. 1—Typical excitation spectra of BGY nuts for various emission wavelengths.

RESULTS

Excitation and emission spectra of BGY nut

It was determined that the maximum fluorescence of BGY occurs at an excitation wavelength of 420 nm (Fig. 1). Since this maximum occurs for all emission wavelengths measured between 490 and 600 nm, we believe that BGY fluorescence is caused by a rather pure substance.

The emission spectra of BGY nut peaks at approximately 490 nm as shown in Figure 2. The excitation wavelength influences this maximum very little until the excitation nears the vicinity of the emission maximum. The changes which occur for excitation at 460 and 480 are probably due to excitation and emission overlap. The emission spectra in Figure 2 further indicates that maximum quantum efficiency occurs for the

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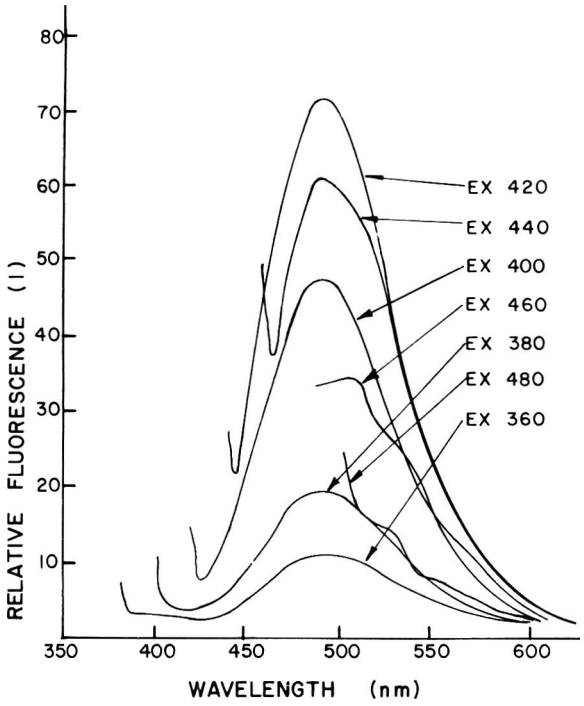

Fig. 2—Typical emission spectra of BGY nuts for various excitation wavelengths.

420 nm excitation-490 nm emission combination for BGY fluorescence.

Characteristic fluorescence spectra

Development of an electronic sorting criteria for automatically removing BGY nuts requires selection of the optimum wavelengths for excitation and for emission measurements. As discussed above, 420 nm is the best wavelength for excitation, but it is rather difficult to economically achieve narrowband excitation at 420 nm. The use of monochromators to isolate the 420 nm band would definitely be too expensive and cumbersome. Narrow-band interference filters are relatively inexpensive but do require parallel light for achieving narrow pass bands and the background transmission of these filters quite often exceeds 0.01%. LW-UV with a nominal peak emission of 360 nm is readily available and inexpensive. Therefore, it was decided to investigate both excitation sources even though 360 nm excitation is less efficient than 420 nm.

Figure 3 shows the average emission spectra (Ex - 360 nm) of the four fluorescence categories of pistachio nuts. Each spectrum in Figure 3 is the average of 10 spectra from individual nuts within a category. The shape of the spectra verifies our visual observation of at least four classes of fluorescence in Iranian pistachio nuts when observed under LW-UV. The purple nuts have a peak at 400 nm with a lower peak at 450 nm which accounts for the purple appearance of these nuts. The normal nuts fluoresce considerably less in the blue region and the peak, which occurred at approximately 450 nm for the purple, has shifted to approximately 460 nm. The bright-yellow nuts have the highest peak at 460 nm with a slightly lower peak at 420 nm. There is less blue emission for the BGY nut since the peak emission occurs at 490 nm.

Several samples were sent to a commercial laboratory to obtain corrected emission spectra. The results were that the corrected spectra of BGY would shift toward the long wavelength side by approximately 10 nm. A 10 nm shift of the BGY peak would bring it to 500 nm, still short of the greenish-yellow region (555 nm). At 490 nm the human eye is only 30% of its peak sensitivity at 550 nm. This insensitivity of the

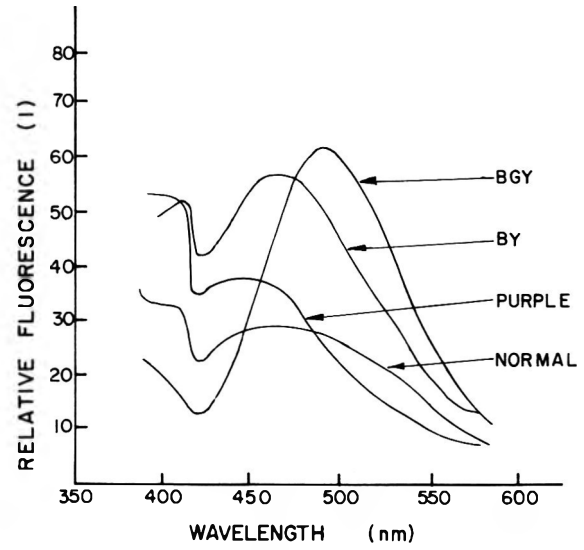

Fig. 3—Average emission spectra for excitation at 360 nm. Each spectrum is the average of 10 spectra.

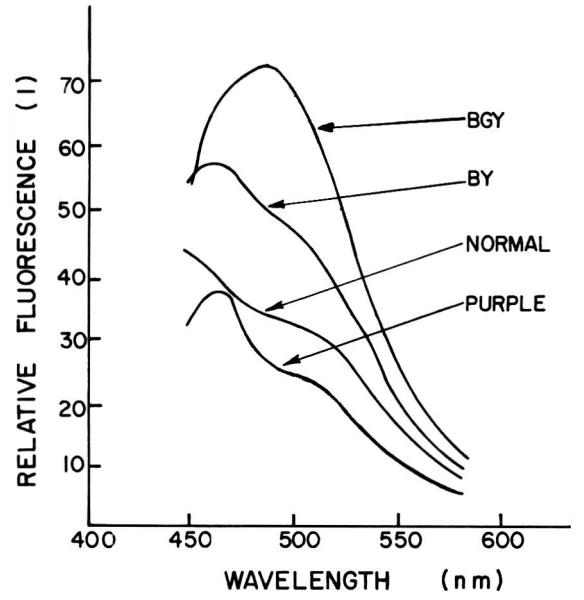

Fig. 4—Average emission spectra for excitation at 420 nm. East spectrum is the average of 20 spectra.

human eye to blue causes BGY to appear bright-greenish-yellow when in fact the peak emission occurs at the boundary between the blue and green regions.

Figure 4 shows the average emission spectra of the four fluorescence categories excited at 420 nm. BGY nuts exhibit a single peak at 490 nm while BY and N nuts have a peak at 460 nm. The spectra of BY, N, and P show a shoulder occurring at 510 nm.

Sorting indices

Performance data of the two sorting parameters are given in Table 1. The I_{490} parameter was chosen because it occurred at the peak of the BGY spectra. The means of this parameter for the four categories of nuts were significantly different from excitation at 420 nm. However, for excitation at 360 nm the only adjacent categories that had a significant difference in I_{490} were BY and N.

The index I_{490} with excitation at 360 nm would not separate BGY nuts from other nuts. Experience with optical

Table 1—A comparison of sorting indices for four categories of nuts[a]

Category	Excitation = 360 nm			Excitation = 420 nm		
	I_{490}	I_{490}/I_{470}	I_{490}/I_{420}	I_{490}	I_{490}/I_{450}	I_{490}/I_{550}
BGY	59.000	1.156	6.344	69.425	2.316	3.113
BY	47.950	0.872	1.173	45.900	1.980	2.580
N	25.350	0.906	1.243	30.500	1.797	2.317
P	24.600	0.788	0.740	23.325	2.152	2.768
$LSD_{.01}$	17.154	0.056	1.186	7.999	0.128	0.205
$LSD_{.05}$	12.793	0.042	0.884	6.030	0.097	0.155

[a] Values shown are average measurements on 40 nuts for excitation at 360 nm and 80 nuts for excitation at 420 nm.

measurements on other agricultural products indicated a ratio of the fluorescence intensities at two wavelengths would improve the selectivity over the single wavelength measurement. The F-test chose I_{470} and I_{450} for the second wavelength in the ratio I_{490}/I_i for excitation at 360 nm and 420 nm respectively. The ratio I_{490}/I_{550} was chosen subjectively to determine if a reference wavelength on the right side of the peak would improve dual wavelength sorting. Observation of the average spectra for excitation at 360 nm suggested the ratio I_{490}/I_{420}.

The performance of the dual wavelength measurements is given in Table 1. I_{490}/I_{420} and I_{490}/I_{470} for excitation at 360 nm did distinguish between BGY and all other categories. However, the difference between BY and N and N and P were not significant at the 0.05 level.

The dual wavelength measurements I_{490}/I_{450} and I_{490}/I_{550} for excitation at 420 nm did not improve the performance over the single measurement of fluorescence at 490 nm (I_{490}). I_{490}/I_{450} did distinguish between BGY and all other categories but the other adjacent categories were not significantly different from each other.

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Paper No. 5448 of the Journal Series of the North Carolina Agricultural Experiment Station, Raleigh, NC. The use of trade names in this publication does not imply endorsement by the North Carolina Agricultural Experiment Station of the products named, nor criticism of similar ones not mentioned.

The authors acknowledge the contributions of Dr. D.E. Moreland and Prof. J.W. Dickens, USDA-SEA in conducting the research and preparing the manuscript. The research was supported in part by Rafsanjan Pistachio Producers Cooperative, Teheran, Iran.

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FUNCTIONAL CHARACTERIZATION OF PROTEIN STABILIZED EMULSIONS: STANDARDIZED EMULSIFYING PROCEDURE

E. TORNBERG and G. LUNDH

ABSTRACT

In order to evaluate the emulsifying properties of proteins it is important to quantify and to more exactly describe the emulsification process. This has been done by utilizing a recirculating emulsification system, where the flow velocity is controlled. The power and energy input during the emulsification process has been measured, thereby providing a tool for the comparison of the emulsifying efficiency of various kinds of emulsifying equipment. A description of such an emulsification system on a laboratory scale is given for emulsifying with a turbo-mixer, a valve homogenizer and an ultrasonic device.

INTRODUCTION

THE IMPORTANCE of the emulsifying procedure in the evaluation of a protein as an emulsifier has previously been shown (Tornberg and Hermansson, 1977). Consequently, the need for better understanding and control of the emulsification process is of vital importance. Firstly, the emulsifying equipment should be standardized so that the flow conditions are comparable. Secondly, it would be desirable to measure power and energy input during the emulsification process, thereby being able to compare the emulsifying efficiency of various kinds of emulsifying equipment.

The purpose of this paper is to describe standardized laboratory apparatus for preparing emulsions, where the flow conditions, power and energy input are controlled and measured.

Mixers of different types, colloid mills, pressure homogenizers and ultrasonic devices are the main tools for emulsification in the food industry. Various mixers find wide application in emulsion technology, and the high speed turbo-mixer is among the most efficient (Brennan, 1970). To produce highly dispersed emulsions both the valve homogenizer and the ultrasonic device can be useful. We have chosen to work with three different types of emulsifying equipment, namely a valve homogenizer, an ultrasonic device and a turbo-mixer.

THE CONTINUOUS FLOW SYSTEM

INSUFFICIENT MIXING throughout the emulsifying chamber during emulsification can easily cause reproducibility problems. These problems can be reduced in a continuous recirculating system, where the flow velocity is controlled. Continuous emulsification also gives a way to control and automate the emulsifying procedure; thus, data obtained from this small scale equipment can more easily be scaled up for production requirements. For example, temperature control can be much improved compared to batchwise emulsification. An isothermal emulsification process is obtained by incorporating a heat exchanger unit into the continuous flow system.

Considering these facts, each emulsifying apparatus used is part of an isothermal continuous-flow system with adjustable flow rate and temperature. This system is schematically represented in Figure 1, and consists of a gear pump (1) connected to a reversible motor (2) and a revolution counter (3), an emulsifying chamber with inlet and outlet (4), a heat exchanger (5) with a thermometer (6) and plastic tubings (7), which complete the recirculating system. The speed of revolution can be varied up to 2,800 rpm with the Multiflex motor (80W) registered on the Jaquet speed indicator, giving rise to a flow rate of the emulsion up to 1,050 ml min^{-1}. The flow rate of the emulsion through the recirculating system was found to be proportional to the revolution rate of the gear pump.

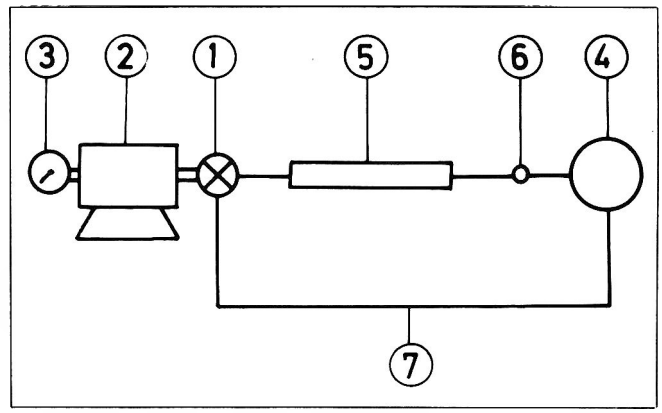

Fig. 1—A schematic representation of the isothermal recirculating, emulsification system: (1) gear pump; (2) reversible motor; (3) revolution counter; (4) emulsifying chamber; (5) heat exchanger; (6) thermometer; and (7) plastic tubings.

The heat exchanger is designed as a jacketed cylinder with an inner diameter of the same dimensions as the plastic tubing. The cooling liquid circulates through the jacket and is thermostated by a cryostat (Heto, Denmark). The temperature range employed so far is between 20 and 30°C, and the accuracy of the temperature control is ± 2°C.

The emulsifying part (4) can be varied from emulsification with a turbo-mixer, to sonication and to valve homogenization. The total volume of the circulating system is 52 ml, whereas the volume in the emulsifying chamber can vary from 4.6 ml in the valve homogenizer to 25.2 ml in the turbo-mixer and to 17.5 ml in the ultrasonic device.

The net energy consumption, E, during emulsification in the recirculating system is given by:

$$E = P \cdot N_p \cdot t_p \qquad (1)$$

where P = net power input (W); N_p = the average number of passes of an emulsion droplet through the recirculating system; and t_p = the average residence time of an emulsion droplet during one passage(s).

The determination of net power consumption varies with the emulsifying apparatus used, and the description of the different procedures is given below; t_p is calculated from the flow rate and the total volume of the recirculating system.

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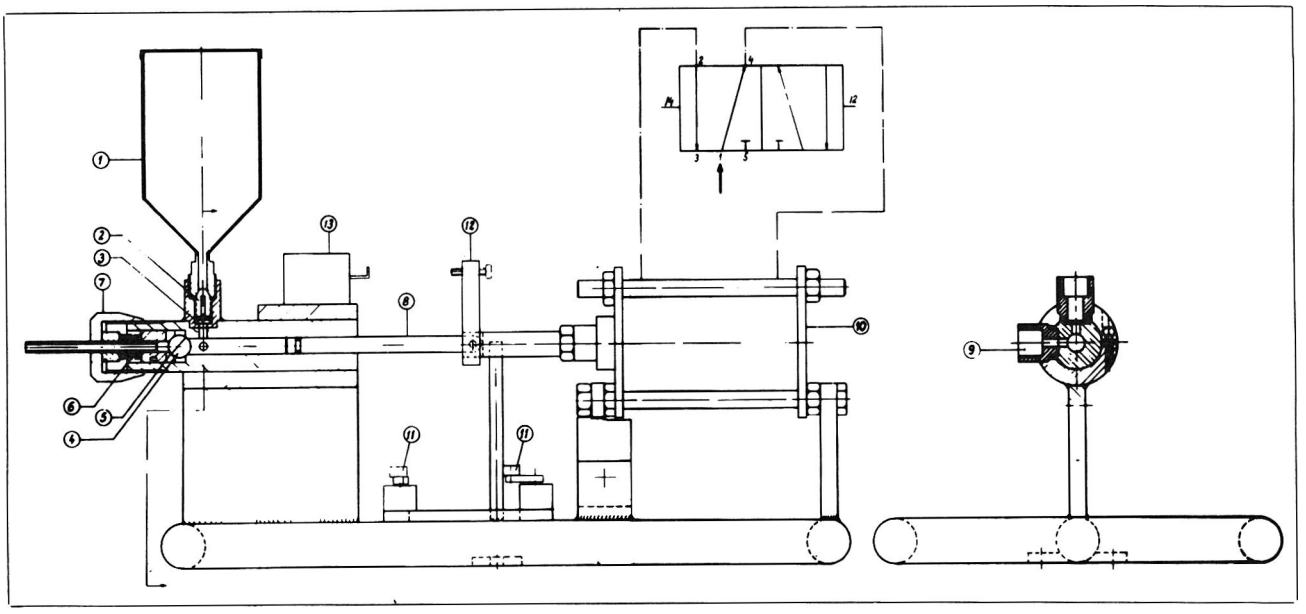

Fig. 2—A Construction drawing of the valve homogenizer.

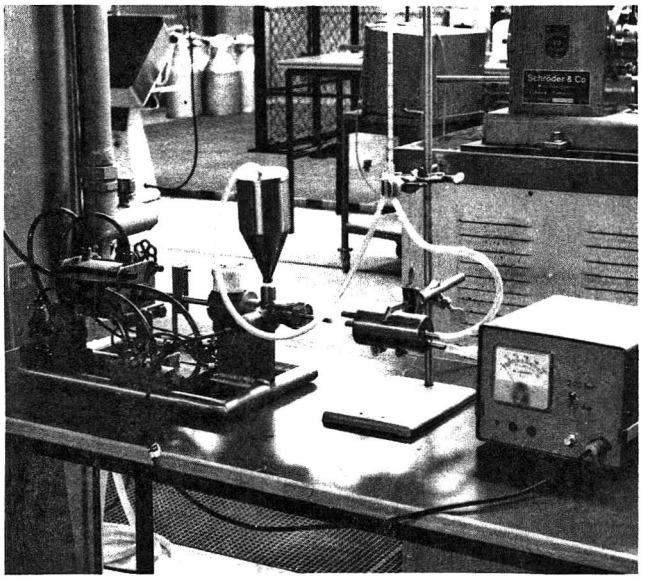

Fig. 3—The apparatus set-up of the recirculating emulsification system using the valve homogenizer.

THE EMULSIFYING CHAMBER

Valve homogenizer

Description of the apparatus. After preliminary studies with a valve homogenizer consisting of a spring-loaded conical valve and a hand pump, an improved apparatus was built (Fig. 2). A spring-loaded (3) entrance valve (2) is situated just below the feeder funnel (1), to keep the stroke volume, V_s, constant within 4.6 ± 0.2 ml. The entrance valve is a conical, guided valve, to prevent vibration at high pressures (Walstra, 1974a) and thereby leaking of emulsion.

The crucial part of the homogenizer is the valve (4) and the valve seat (5). An earlier conical valve was changed to a ball valve of stainless steel, and this greatly improved the pressure maintenance during a stroke. The size of the gap between the valve faces depends upon the flow rate of the emulsion and the loading of the valve, which can be adjusted by compressing a package of disk springs (6) with a screw (7). By replacing the former coil spring with disk springs a better defined spring-load could be obtained.

The pressure applied is registered with an accuracy of ± 0.1 MPa by a strain gauge pressure transducer (Bell & Howell) inserted in the valve seat (9). The pressure transducer is connected to a voltmeter, working up to the pressure limit of the apparatus of 20 MPa. The pressure drop can easily be read off on a panel (see Fig. 3). When pressure changes often and abruptly, as in the case of valve homogenization, it is preferable to use this type of pressure gauge. Cleaning problems are also facilitated compared to other types of gauges.

The plunger (8) is sealed with an O-ring to prevent any leakage of the emulsion. The motion of the plunger is accomplished by a pneumatic system (10), which considerably decreases the size of the entire apparatus compared to a mechanically driven plunger with all its gear equipment. The pressure maintenance during one stroke is also improved by this type of power transmission. All the apparatus can be placed on a table, which makes it easy to operate and to clean. The back and forth motion of the plunger is controlled by two microswitches (12), which allow the pneumatic system to work continuously.

The continuously working homogenizer is pictured in Figure 3. The gear pump is not required in this case, as the homogenizer itself consists of a displacement pump. Provided the stroke volume is constant, the flow rate of the emulsion can be recorded as the number of strokes per unit time; this is achieved by a rod (12) fixed to the plunger, which hits a mechanical counter (13) at every stroke. The stroke rate can be modified by adjusting the air pressure feeding the pneumatic system.

In order to record the pressure profile generated per stroke as a function of the pressure drop and the stroke rate, the pressure transducer was coupled to a storage oscilloscope. Examples of pressure profiles obtained are given in Figure 4 at three different pressure drops at a stroke rate of 0.8 stroke/s. Due to the inertia of the system both the form of the pressure profile and the time during which the valve is lifted is dependent on the stroke rate and the pressure drop, as can be judged from Figure 4. In Figure 5 the time of valve lift during one stroke, t_v, is plotted as a function of the maximum

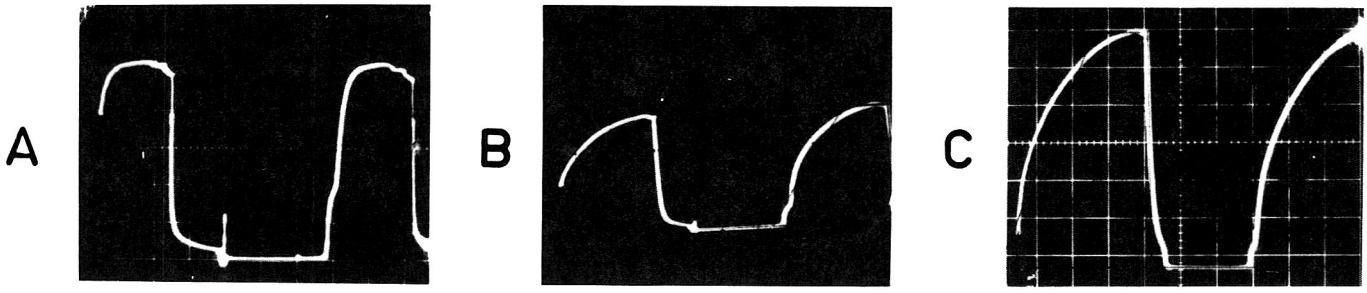

Fig. 4–Examples of pressure profiles obtained per stroke with the valve homogenizer at a stroke rate of 0.8 stroke/s. (A) Pressure drop = 2.5 MPa; Vertical 1 mV/division; Horizontal = 0.2 s/division. (B) Pressure drop = 7.5 MPa; Vertical = 5 mV/division; Horizontal = 0.2 s/division. (C) Pressure drop = 15 MPa; Vertical = 5 mV/division; Horizontal = 0.2 s/division.

homogenizing pressure, Δp_{max}. The figure shows that the longest times are obtained at the highest pressures and lowest stroke rates.

Measurement of the power input. The net power consumption, P, of the valve homogenizer is equal to the pressure drop, Δp, times the flow rate, Q, of the emulsion, which gives

$$P = \Delta p \cdot Q \quad (J/s, W) \tag{2}$$

where Δp is the pressure drop in N/m^2 and Q is the flow rate in m^3/s. Q can also be written as

$$Q = V_s \cdot \frac{1}{t_v} \quad (m^3/s) \tag{3}$$

where $V_s = (4.6 \pm 0.2) \cdot 10^{-6}$ m^3 and t_v = time when valve is open during one stroke. Insertion of (3) in (2) gives:

$$P = \Delta p \cdot V_s \cdot \frac{1}{t_v} \quad (W) \tag{4}$$

As shown by the pressure profiles in Figure 4, the pressure drop varies with time during one stroke, which can also be expected for the volume displacement. It is reasonable to assume that the volume displacement, v, is at any moment proportional to Δp, i.e.

$$v = k \cdot \Delta p \tag{5}$$

Integration over the time gives:

$$P = \frac{k}{t_v} \int_0^{t_v} \Delta p(t)^2 \, dt \tag{6}$$

$$V_s = k \int_0^{t_v} \Delta p(t) \, dt \tag{7}$$

If a pressure profile is approximated by a parallel trapezium with height Δp_{max}, and the shortest parallel side is denoted t_2 and the longest $t_v = t_1 + t_2$, Eq (6) and (7) can be written as follows:

$$P = \frac{k}{t_v}\left[\int_0^{t_1} \left(\frac{\Delta p_{max}}{t_1}\right)^2 \cdot t^2 \, dt + \int_0^{t_2} \Delta p_{max}^2 \, dt\right] \tag{8}$$

$$V_s = k\left(\int_0^{t_1} \frac{\Delta p_{max}}{t_1} \cdot t \, dt + \Delta p_{max} \cdot t_2\right) \tag{9}$$

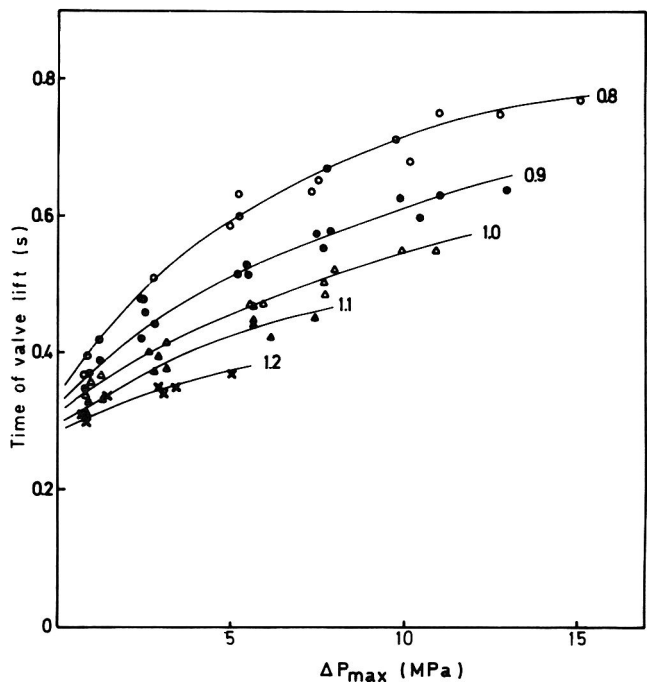

Fig. 5–The time of valve lift, t_v, in the valve homogenizer as a function of the maximum homogenizing pressure, Δp_{max}, and stroke rate in stroke/s as a parameter.

After integration on the following relationships are obtained:

$$P = \frac{k}{t_v}\left(\frac{\Delta p_{max}^2 \cdot t_1}{3} + \Delta p_{max}^2 \cdot t_2\right) \tag{10}$$

$$V_s = k\left(\frac{\Delta p_{max} \cdot t_1}{2} + \Delta p_{max} \cdot t_2\right) \tag{11}$$

The constant k is obtained from Eq (11) and put into Eq (10), which gives power, P, equal to:

$$P = \frac{V_s \cdot \Delta p_{max}}{t_v} \cdot \frac{\left(\frac{t_1}{3} + t_2\right)}{\left(\frac{t_1}{2} + t_2\right)} \tag{12}$$

Eq (12) differs from Eq (4) only with respect to the last factor $[(t_1/3)+t_2]/[(t_1/2)+t_2] = f$ in Eq (12). This factor was calculated at different maximum pressures and strokes rates, and a

linear relationship is obtained as a function of Δp_{max} with a correlation coefficient of 0.925:

$$f = -0.00404 \cdot \Delta p_{max} + 0.996 \quad (13)$$

where Δp_{max} is expressed in MPa.

The discrepancies in the factor f due to different stroke rates were within the limits of error.

Being a one-piston homogenizer, the transmission of power to the emulsion is made intermittently, hence during liquid flow the net energy input, E, is given by:

$$E = P \cdot N_s \cdot t_v \quad (W) \quad (14)$$

where N_s is the number of strokes and t_v is the time of valve lift and can be read off from Figure 5.

Estimation of the energy density. Though there are many theories of the mechanism of globule break-up in a valve homogenizer, probably the most important variable determining homogenization efficiency would be the energy density, ϵ_0, i.e. the power generated per unit volume, depending on pressure and time scale of the process.

Mulder and Walstra (1974) claim that at least in larger machines globules are mainly broken up by small turbulent eddies that cause locally high pressure gradients, which are effective in disrupting emulsion globules of the same order of size. According to Kiefer (1976) and Treiber (1976) the homogenizing efficiency of a valve homogenizer is mainly attributed to the amount of cavitation occurring. Cavitation is the formation and collapse of small cavities in a liquid, caused locally by pressure drops below the vapor pressure. Collapse of these cavities gives rise to high pressure gradients, and thereby disruption of emulsion globules. Phipps (1974) proposed that it is a viscous shear type of break-up in the elongational flow at the entrance of the valve that causes disintegration into smaller droplets.

According to Walstra (1969), the globule break-up at a certain pressure will primarily depend on the dissipation of the energy in the shortest possible time, which is mainly governed by the valve design. Most of the valve homogenizers commercially available have flat valves, differing in inner and outer radius, giving rise to passage times through the valve seat of about 50 μs (Walstra, 1974b). Most of the energy is probably dissipated within 10 μs (Walstra, 1974b), whereas during the rest of the time in the valve seat coalescence of freshly formed globules occurs (Phipps, 1974). The construction of the homogenizing valve presented here would be likely to have a very short time of the droplets within the valve, as the valve only consists of the circumference of the ball (4) in contact with the valve seat (5). Therefore some rough calculations of the valve lift (1), the time in the valve (τ) and the energy density (ϵ_0) have been performed. The formulas derived for the calculations of 1, τ and ϵ_0 are given in the appendix. The results are presented in Table 1 for some different pressure drops at a stroke rate of 0.9 stroke/s.

Table 1—Calculated values of the valve lift, time in the valve seat and energy density at different maximum pressure drops for a stroke rate of 0.9 stroke/s

Max pressure drop Δp_{max} (MPa)	Valve lift l (μm)	Time in the valve τ (μs)	Energy density ϵ_0 (kW/cm^3)
1.0	7.1	9.0	110
2.0	4.5	6.3	315
3.0	3.4	5.2	580
5.0	2.3	4.0	1250
7.5	1.7	3.3	2300
10.1	1.4	2.8	3570

From Table 1 it can be concluded that the valve clearance, l, diminishes with increasing homogenizing pressure, which is also the experience of Phipps (1974) and Mulder and Walstra (1974). It is also obvious from the table that the time passages through the valve are comparatively short, all below 10 μs, which give rise to high energy densities even at relatively low pressures. Walstra (1974b) has given an approximate value of ϵ_0 of 500 kW/cm^3 for a high-pressure homogenizer, which is surpassed already at a pressure drop of 3.0 MPa, thus, the homogenizing efficiency can be expected to be very good. As the valve clearances of the apparatus shown here are very small, the mechanism of globule disruption proposed by Phipps can be assumed to occur, which will probably differ from the mechanism taking place in a large-scale homogenizer, where turbulent eddies are supposed to be the main cause of the disruption of globules.

Turbo-mixer

Description of the apparatus. The active part of a turbo-mixer consists of a stationary (stator) and a rotating impeller (rotor), which are both toothed and separated by a small clearance. The liquid circulates through the impellers due to the pumping action exerted by the rotor; it is sheared between both impellers and it impinges upon the slots in them.

A drawing of the apparatus with a thermostating jacket (1) surrounding the emulsifying chamber (2) can be seen in Figure 6. In the case of emulsification with the turbo-mixer, the heat exchanger previously described is omitted and the thermostating box (1) is used instead. The filled arrows show the direction of flow of the cooling liquid, whereas the unfilled arrows indicate the flow of the emulsion. The slots of the stator (4) are somewhat tilted, whereas the slots of the rotor (3) are cut vertically. The shaft (5) to which the rotor is attached runs in bearings, a prelubricated slide bearing (6) and ball bearings (9). To avoid any emulsion squeezed up in the ball bearings, an outlet (7) is placed just below the bearings. The small clearance between the stator and the rotor is adjustable between 0.1 and 1.0 mm, giving rise to differing emulsification processes: both the stator and the rotor are conical, and by moving the shaft (5) upwards, the distance between the rotor and the stator is increased. The shaft is fixed in a position by the guidance screws (10) and the clamping rings (8), and the clearance obtained is read on a scale in vernier graduation (11).

Energy for emulsification comes from the rotor drive motor, a Janke-Kunkel TP 18-10, 170W, giving a revolution rate up to 20,000 rpm. The rotor speed is monitored by means of a stroboscope, and maintained at the same rate by adjusting a variable transformer.

Measurement of the power input. The measurement of the power generated by the turbo-mixer is not as straightforward as for the valve homogenizer. Usually the power from a mixer is derived by measuring the torque on the shaft and multiply it by the revolution rate. This is not easily done in our case because of the high rotation speed of the shaft. Another way to approach this problem is to measure the heat dissipated during emulsification. This has been done by incorporating a heater into the continuous recirculating system. The heater consists of a glass tube, on which a resistance wire is wound. Another glass tube with inlets and outlets is placed exterior to the first one, thereby forming a small gap between the glass tubes, wherein the rapidly circulating emulsion can be readily warmed up by the resistance wire. The product of the voltage (U) and the amperage (I) through the wire is assumed to determine the amount of power liberated in the heater. A schematic representation of the electronic regulation set up is shown in Figure 7. Directly after the heater a thermistor senses the temperature of the emulsion. The cooler is adjusted so that the

temperature of the emulsion is kept constant at 25°C. When emulsification takes place, the voltage and the amperage through the heater will diminish in accordance with the amount of heat dissipated in the emulsifying chamber, i.e. the power generated by the turbo-mixer.

Complications that may arise with this sort of apparatus are mainly dependent on the time constant of the system. The time constant can be kept low by a high circulation speed and with a high accuracy of the temperature measurement. Besides, fluctuations in the system, caused for example by entrapped air or by an inhomogeneous emulsion, should be minimized, otherwise the system starts to oscillate. Therefore no measurements could be performed on highly viscous emulsions.

Because of air inclusion and foaming, which sometimes could not be avoided, the accuracy of the measurements was rather low, and the power was determined with an accuracy of ± 1W. It was found that between 15,000 and 20,000 rpm the power transmitted to the emulsion by the turbo-mixer varied from 7–12W.

Estimation of the energy density. Mulder and Walstra (1974) claim that both turbulence and cavitation are produced, and the effective energy density is estimated to be around 200 W/cm^3 (Walstra, 1975).

Ultrasonic device

Description of the apparatus. A Branson Sonifier Cell Disruptor model B-12 (Branson Sonic Power Co.) was used. To fit it into the recirculating system a continuous-flow attachment of stainless steel from the same firm was used. The attachment is screwed onto the horn, and the distance from the tip of the horn to the outlet can be varied by unscrewing, with each complete revolution corresponding to a distance of 0.8 mm. By this procedure the size of the space where most of the emulsification takes place can be changed, and thereby the emulsification performance modified.

Measurement of the power input. The two most commonly used methods of measuring ultrasonic power are calorimetry and radiation force method (Zieniuk and Chivers, 1976). Although the calorimetric method has a better theoretical basis, the radiation force method is more rapid and convenient to use. For this reason we decided to mainly use the radiation force method, although some measurements of power were performed with the previously described calorimeter.

In the radiation force method one measures the radiation pressure force, F, exerted on a wall placed at a right angle in the path of an ultrasonic beam with a power, P. Eq (15) is obtained,

$$F = \frac{P}{c} \quad (N) \qquad (15)$$

where c is the velocity of sound in the medium in which the beam travels. Wemlén (1968) has calculated that if oil is used as the propagating medium with c equal to 7377 m/s the radiation force expressed in weight is 74 μg for an ultrasonic power of 1 mW. As the velocity of sound in water is 1,500 m/s, a radiation force of approximately 72 μg corresponding to a power of 1 mW, is obtained for an o/w-emulsion of 40% oil by weight.

Zieniuk and Chivers (1976) have discussed probable errors that can be introduced in the radiation force method, and they came to the conclusion that at present it would appear to be both convenient and correct for practical purposes to assume that Eq (15) is valid.

The experimental set up consists of an open vessel containing emulsion, and electronic balance (Sartorius 3700) with an analog output, a recorder, and the ultrasonic device. The plastic vessel has a bottom diameter of 65 mm and the walls are covered with a 15 mm thick plastic foam. Assuming that the bottom of the vessel behaves like a perfect reflector of the ultrasound beam, the plastic foam on the walls of the vessel

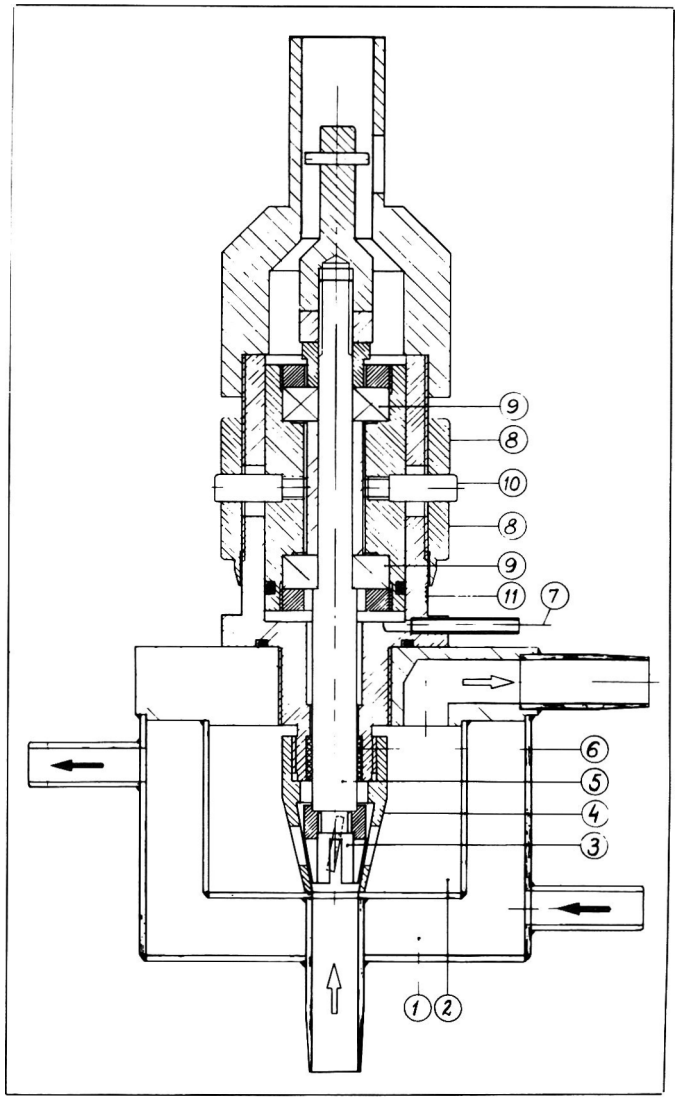

Fig. 6–Construction drawing of the turbo-mixer.

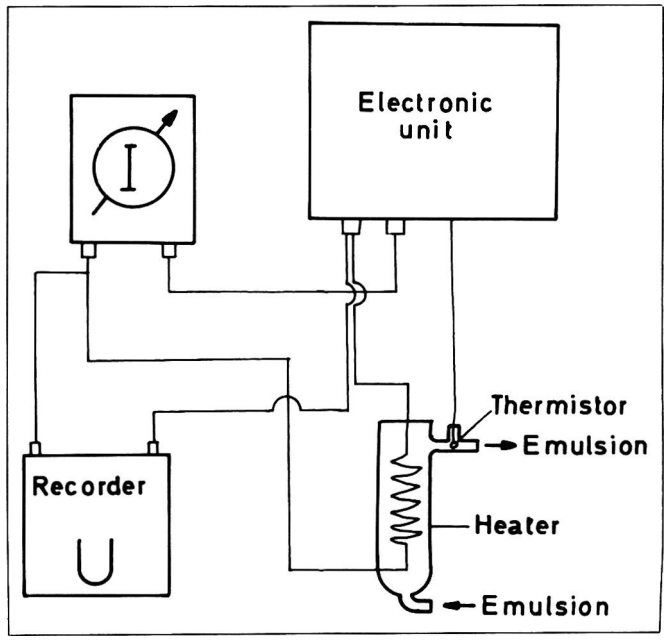

Fig. 7–Schematic representation of the electronic unit within the set-up for calorimetry.

should behave like a perfect absorbant. That this could be justified within the limits of error was found by preliminary experiments, in which the model of the vessel given above was independent of the thickness of the plastic foam and of the distance from the bottom to the ultrasonic horn dipped into the emulsion.

The analog output of the balance was coupled to the recorder, and the power generated by the ultrasonic horn could be detected with an accuracy of ± 1.5W. During the experiments the emulsion was cooled now and then in order to minimize the influence of temperature on the measurements.

In Figure 8 the power measured is plotted as a function of the apparatus parameter, control setting. Power has been measured for emulsions of soybean oil (40 wt %) in water, where soy protein isolate, whey protein concentrate (WPC) and sodium caseinate have been used as stabilizers. The number in brackets (0–7) and (0.2–7) denote when the protein is dispersed in water or in 0.2M sodium chloride solution at pH 7. Minor variation due to the protein used as stabilizer can be viewed from Figure 8, but if this is taken into account the net power consumption can be determined with an accuracy of ± 3W. For comparison, measurements made with the calorimeter are also given in Figure 8 for emulsions stabilized by caseinate (0–7) and caseinate (0.2–7). As can be seen from the figure, the values obtained from the calorimeter are higher at low control settings, whereas they more or less coincide for increasing values of the control setting. The discrepancies between the values obtained by the two different methods are most probably due to insufficient accuracy of the thermistor in the calorimeter.

Estimation of the energy density. An ultrasonic horn generates ultrasonic waves, which produce cavitation, i.e. the formation and collapse of small cavities in the liquids. When these cavities collapse high pressure gradients arise, which cause globule disruption. The energy density produced can be as much as approximately 1 MW/cm^3 (Walstra, 1974).

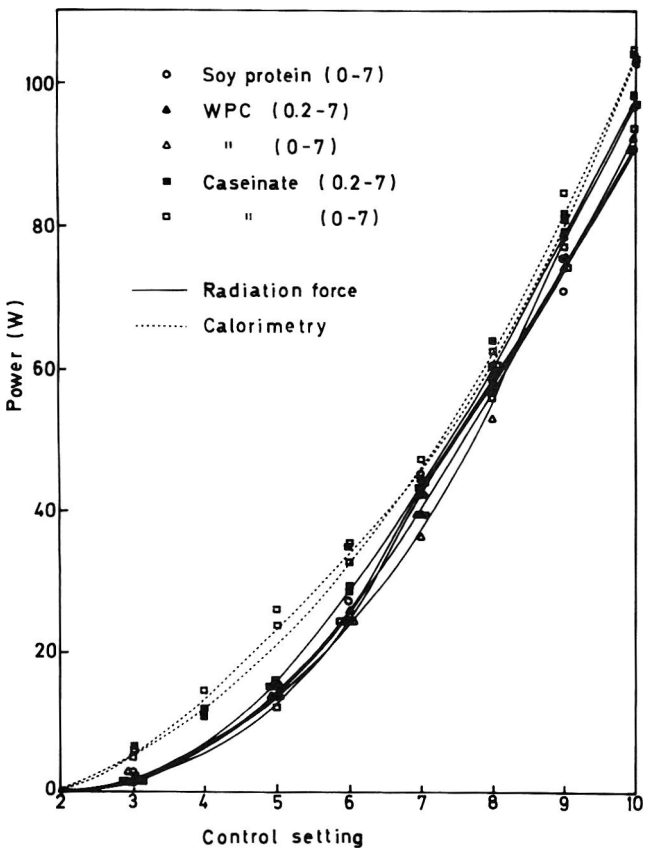

Fig. 8—Ultrasonic power as a function on control setting for different protein stabilized emulsions measured with both the calorimetry and the radiation force method.

APPENDIX

Calculation of l, τ and ϵ_o in the valve homogenizer

By assuming that pressure tends towards zero somewhere inside the valve, the average maximum velocity, v_m, can roughly be estimated from Bernoulli's law:

$$v_m \simeq \left(\frac{2\,\Delta p_{max}}{\rho}\right)^{1/2} \tag{16}$$

The average maximum velocity, v_m, can also be calculated from the flow rate, $Q = V_s/t_v$, divided by A_t, the minimum area the emulsion must pass when traversing the valve.

$$A_t \approx 2\,\Pi R l \tag{17}$$

In this equation, R is the radius of the circumference of the ball valve which is in contact with the valve seat and equals 0.5 cm. l can now be extracted from the following relationship:

$$v_m = \frac{V_s}{t_v \cdot 2\,\Pi R l} = \left(\frac{2\,\Delta p_{max}}{\rho}\right)^{1/2} \tag{18}$$

The volume inside the valve, V_i, is given by:

$$V_i = 2\,\Pi \cdot R \cdot a \cdot l \tag{19}$$

where R = 0.5 cm and a is the length of the valve seat and can be considered not to exceed 0.05 cm. The time in the valve seat, τ, can then be calculated according to:

$$\tau = \frac{V_i}{Q} = \frac{2\,\Pi R \cdot a \cdot l \cdot t_v}{V_s} \tag{20}$$

The energy density, ϵ_0, can be estimated from the following equation:

$$\epsilon_0 = \frac{\Delta p_{max}}{\tau} \tag{21}$$

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Ms received 6/18/77; revised 12/3/77; accepted 12/8/77.

Dr. Anne-Marie Hermansson is heartily thanked for her kind interest in this work, and Dr. P. Walstra is thanked for his valuable comments. The authors are also much indebted to Ing. Ernst Braun for valuable advice during the construction of the emulsifying apparatus and for making the construction drawings. The practical construction work of the valve homogenizer and the ultra-turrax by Jan Walter and Sune Jönsson, respectively, is gratefully acknowledged. The authors also thank Mr. Dennis Jansson for the construction of the electronic unit of the calorimeter. The investigations were made possible by grants from the Swedish Board for Technical Development, and this financial support is gratefully acknowledged.

FUNCTIONAL CHARACTERIZATION OF PROTEIN STABILIZED EMULSIONS: CREAMING STABILITY

E. TORNBERG

ABSTRACT

Protein stabilized emulsions have been prepared in a recirculating emulsification system, where flow velocity, power and energy input have been controlled and measured. Three different types of emulsifying equipment have been used, namely a turbo-mixer, an ultrasonic device and a valve homogenizer. The protein systems studied were a soybean protein isolate, a whey protein concentrate (WPC) and a sodium caseinate, and the emulsions obtained were characterized in terms of creaming stability. It was found that although the power and energy consumption during emulsification were the same, the creaming stabilities differed as a function of the emulsifying apparatus used. Increased power and energy input contributed in general to an improved creaming stability up to a certain limit, whereupon it leveled off. The emulsifying efficiency of the turbo-mixer is poorest in terms of creaming stability of the emulsions formed, whereas the ultrasonic device most generally is the best choice of equipment at lower power input. At an increase of power consumption the valve homogenizer is an equally good alternative, or even better.

INTRODUCTION

THE EMULSIFYING PROPERTIES of proteins have been subject to considerable study, but no clear picture has so far emerged from the findings. The fact that the mode of formation of the emulsions greatly influences their properties (Tornberg and Hermansson, 1977) may be one reason that so many contradictory results are to be found in the literature, since very little attention has been paid to this variable. To be able to deal with the parameters of emulsification, the process must be standardized and quantified. This has been done by using a recirculating system, where the flow velocity, power and energy input are controlled and measured. This emulsification system is described elsewhere (Tornberg and Lundh, 1978).

In this paper, the creaming stability of protein-stabilized emulsions has been investigated as a function of the energy input, the power input and the number of passes through a recirculating system during emulsification. Three different pieces of emulsifying equipment, a turbo-mixer (T-M), a valve homogenizer (V-H) and an ultrasonic device (U-S), have been used. The emulsions have been stabilized by three protein products, namely a soy protein isolate, a sodium caseinate and a whey protein concentrate (WPC).

MATERIAL

Soy protein isolate

A soy protein isolate produced under mild conditions was kindly provided by Central Soya. It was prepared from defatted soy bean flakes by extraction with deionized water, precipitation at pH 4.6, washing the curd with deionized water and neutralizing to pH 7.0.

Analysis: protein (N × 6.25) 96.0% (dry weight); solubility determined according to Hermansson (1973) in distilled water and in 0.2M sodium chloride solution at pH 7, denoted as (0–7) and (0.2–7), is 91.7% and 76.1%, respectively.

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0022-1147/78/0005-1559$02.25/0

Caseinate

Spray blend caseinate (DMV, Holland), a commercially available sodium caseinate, was used. Analysis: protein (N × 6.43) 92.6% (dry weight); solubility determined as above in distilled water and in 0.2M sodium chloride solution at pH 7, denoted as (0–7) and (0.2–7), is 97.4% and 95.4%, respectively.

When protein concentrate (WPC)

The whey protein concentrate was obtained by ultrafiltration and spray drying of cheese whey. UF was carried out at 5°C in a pilot plant unit from De Danske Sukkerfabriker (DDS) with a membrane area of 2.2 m^2 and with a membrane type designated as 600 DDS. Analysis: protein (N × 6.15) 68.4% (dry weight); solubility determined as above in distilled water and in 0.2M sodium chloride at pH 7, denoted as (0–7) and (0.2–7), is 100% and 98.2%, respectively.

Soybean oil

A commercially available soybean oil (AB Karlshamns Oljefabriker, Karlshamn, Sweden) was used. Analysis: fatty acid composition 18:2 53.3%, 18:1 23.0%, 16:0 10.8%.

METHODS

Preparation of samples

Protein dispersions (2.5% (w/w), based on the protein content) in distilled water or sodium chloride solution were made with the Sorvall omni-mixer. The pH was adjusted with 0.2M NaOH or 0.2M HCl. Soybean oil was added directly to the protein dispersion to attain 40% oil by weight.

Emulsion formation

A quantity of 50g of emulsion was emulsified in a recirculating system as previously described (Tornberg and Lundh, 1978). The emulsifying part was varied from a turbo-mixer, to a valve homogenizer and to an ultrasonic device. A thorough description of each the emulsifying units and the measurement of the power and energy input has been given by Tornberg and Lundh (1978). The flow rate of the emulsion through the recirculating system was held constant at 250 ± 25 ml min^{-1}, giving an average residence time of an emulsion droplet during one passage, t_p, of 12.5 ± 0.4s, and an average stroke rate of 0.9 stroke/s in the valve homogenizer. The distance from the tip of the ultrasonic horn to the outlet of the continuous flow attachment was set to 0.8 mm.

Cooling was performed during all the emulsification procedures to keep the temperature of the emulsion near 25°C during processing.

Emulsion characterization

The extent of creaming is called the stability rating (SR). It was determined on the basis of the percentage change of fat in the aqueous lower phase after creaming for 24 hr of the emulsions formed. The description of the method and the calculations are given elsewhere (Tornberg and Hermansson, 1977). The accuracy of the method is ± 1.5% SR.

RESULTS

Effect of energy input

In Figure 1 the creaming stabilities of differently emulsified WPC-(0.2–7)-stabilized emulsions are plotted as a function of the net energy input. The power consumption has been held constant at levels of 10 and 40W, and the number of passes has been varied during emulsification. Firstly, we can establish that although the same amount of energy has been transferred to the emulsion during emulsification, the creaming properties differ considerably as a function of the emulsifying equipment and the power generated. Secondly, an increase in energy input gives rise to better emulsions. At a

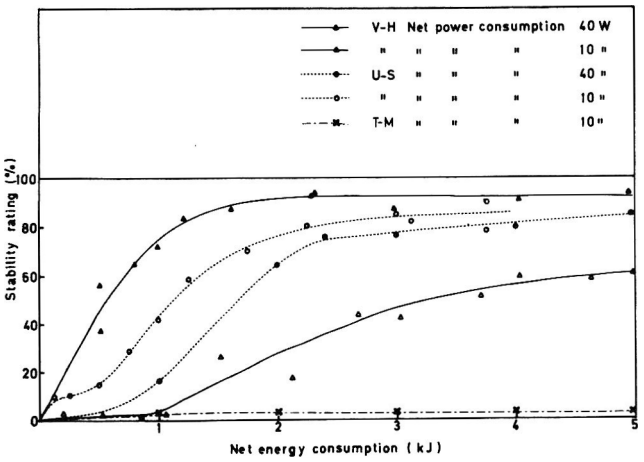

Fig. 1—Stability rating of WPC-(0.2—7)-stabilized emulsions as a function of net energy input, on emulsification with a turbo-mixer (T-M), a valve homogenizer (V-H) and an ultrasonic device (U-S) at a power input of 10 and 40W.

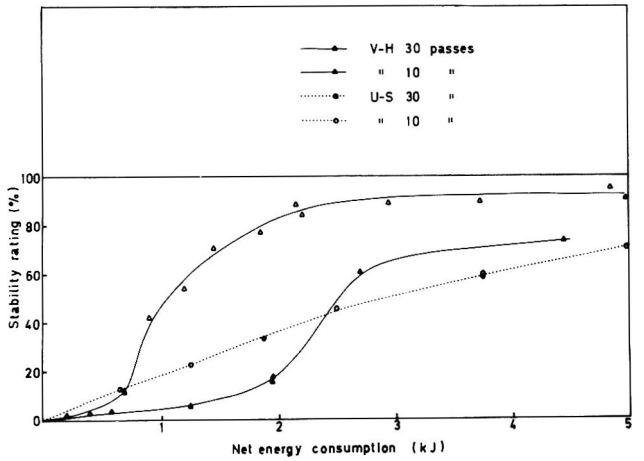

Fig. 2—Stability rating of caseinate-(0.2—7)-stabilized emulsions as a function of net energy input, on emulsification with a valve homogenizer (V-H) and an ultrasonic device (U-S) at 10 and 30 passages through the recirculating system.

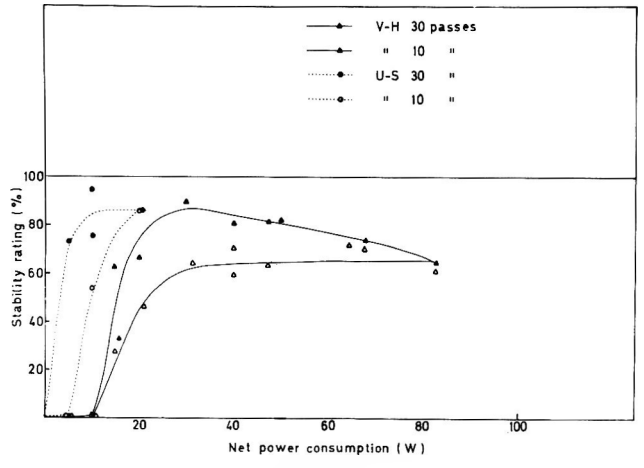

Fig. 3—Stability rating of soy protein-(0.2—7)-stabilized emulsions as a function of power consumption, on sonication (U-S) and valve homogenization (V-H) at 10 and 30 passages through the recirculating emulsification system.

power transmission of about 10W the sonicated emulsions are superior in creaming stability to the valve homogenized ones at all energy levels, and these are in turn better than the emulsions made in the turbo-mixer. Interesting to note is the difference in creaming behavior of the emulsions on sonication or valve homogenization as the power consumption is increased to 40W. Now the valve homogenizer is a better alternative, and the sonicated emulsions at this level of power consumption are even worse in creaming stability than the emulsion made at 10W for the same amount of energy input. Similar creaming behavior as a function of net energy consumption, at a constant power supply, has been observed for WPC-(0—7)-, caseinate-(0.2—7)- and soy protein-(0.2—7)-stabilized emulsions.

In Figure 2 the creaming stabilities of caseinate-(0.2—7)-stabilized emulsions are plotted as a function of net energy consumption. In this case, the number of passes has been held constant and the power consumption has been varied. Here again the ultrasonic equipment and the valve homogenizer behave very differently, when the number of passes is increased from 10 to 30. For the valve homogenizer it is more energetically favorable to use a lower number of passes, whereas there is nothing to gain in energy consumption by lowering the number of passes if the emulsions are sonicated. Another interesting feature visible in Figure 2 is the difference in form of the curves obtained by sonicating and valve homogenizing the emulsions; the former increases only slowly with higher energy input, whereas the latter changes more abruptly in a certain range of energy input. Similar curves to those in Figure 2, when using the same variables, have also been obtained for WPC-(0.2—7)- and soy protein-(0—7)-stabilized emulsions.

Effect of power input

In Figure 3 the stability ratings of soy protein-(0.2—7)-stabilized emulsions are plotted as a function of the net power consumption for sonicated and valve homogenized emulsions at 10 and 30 passes, respectively. The wide scatter of results derived is probably due to differences in viscosity, as the emulsions got very viscous when formed, as visually judged. In the ultrasonic device, the viscosity increase is so high that the emulsions could not circulate at a power input beyond 20W. It can also be deduced from Figure 3 that the valve-homogenized products at 30 passages tend to be overprocessed in terms of creaming stability.

As expected for the two types of emulsifying equipment, an increase in power input improved the creaming stability of the emulsions up to a certain level, whereupon it levels off. But the steep rise in stability rating occurs at a lower power input for the sonicated emulsions than for the valve-homogenized ones. In Figure 4 the emulsification efficiency of the valve homogenizer and the ultrasonic device at 30 passages have been compared for soy protein-(0—7)-, WPC-(0—7)- and caseinate-(0.2—7)-stabilized emulsions. It is obvious that the ultrasonic device is superior to the valve homogenizer in the low power region below 20W. Although all emulsions shown in Figure 4 reach the plateau value of $\approx$ 90% SR, the rate of increase of the creaming stability as a function of power input differs between emulsions stabilized with different proteins. As judged from Figure 4, WPC-(0—7)-stabilized emulsions reach the plateau value at the lowest power input, closely followed by soy protein-(0—7)-, whereas caseinate-(0.2—7)-stabilized emulsions have the lowest creaming stabilities up to 40W.

One protein that stabilized emulsions with completely different creaming behavior than the other proteins used, is caseinate-(0—7). This becomes evident on comparing Figures 4 and 5, where in the latter figure the stability rating of caseinate-(0—7)-stabilized emulsions is given as a function of power consumption at a constant number of passages of 10 and 30. It is clear from Figure 5 that the improvement in

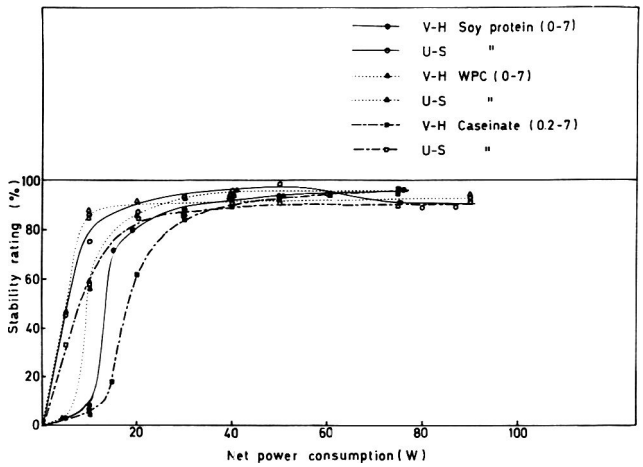

Fig. 4–Creaming stability (SR) of soy protein-(0–7)-, WPC-(0–7)- and caseinate-(0.2–7)-stabilized emulsions as a function of power input, on sonication (U-S) and valve homogenization (V-H) at 30 passes through the recirculating system.

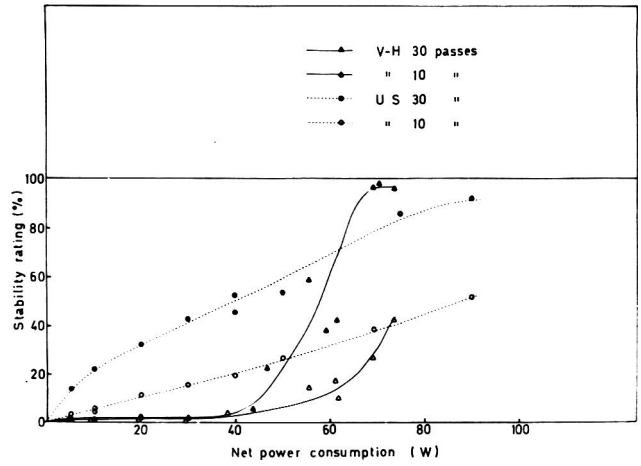

Fig. 5–Stability rating of caseinate-(0–7)-stabilized emulsions as a function of power consumption, on sonication (U-S) and valve homogenization (V-H) at 10 and 30 passages, respectively.

creaming stability on increasing the power input is comparatively slow for caseinate-(0–7). A value as high as about 70W is needed to reach the plateau value for 30 passages, whereas the other proteins only need 10–40W to attain the same level of creaming stability. The difference in emulsifying behavior of the ultrasonic device and the valve homogenizer is also very clear from Figure 5. Sonication gives emulsions with a creaming stability that almost linearly increases with power consumption, whereas valve homogenization gives rise to emulsions in which the stability rating increases more abruptly with power input.

All the protein stabilized emulsions investigated are compared for valve homogenization at 10 passages in Figure 6, where stability rating is given as a function of power consumption. It can be concluded from the figure that the addition of salt most favorably increases the creaming stability of caseinate-stabilized emulsions, whereas the opposite is found for the two other proteins. The same ranking order in creaming stability as found in Figure 4 can also roughly be estimated from Figure 6, i.e. WPC gives emulsions of highest SR followed by soy protein isolate and thereafter sodium caseinate.

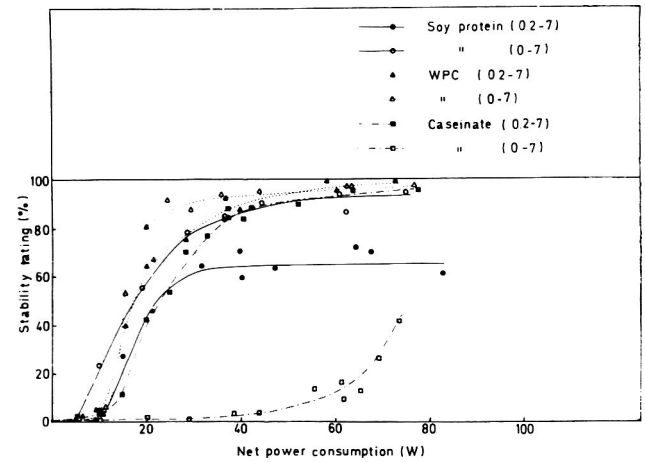

Fig. 6–Creaming stability (SR) of soy protein-(0.2–7)-, soy protein-(0–7)-, WPC-(0.2–7)-, WPC-(0–7)-, caseinate-(0.2–7)- and caseinate-(0–7)-stabilized emulsions as a function of power input, on valve homogenization at 10 passages through the recirculating system.

Effect of number of passes

In Figure 7 the stability ratings of WPC-(0–7)-stabilized emulsions are plotted as a function of number of passes at constant levels of about 10 and 40W power input. Some features, typical for all the protein stabilized emulsions, can be seen in this figure: At the lower power input the ultrasonic equipment gives emulsions in which the rate of growth of creaming stability is very high compared to the valve-homogenized emulsions; the emulsions obtained at 40W are always superior in creaming stability to those obtained at 10W for the same number of passes. Also, increasing the number of passes improves the creaming stability of the emulsions formed. A property of the valve-homogenized WPC-(0–7)-stabilized emulsions at 40W not observed to the same extent for the other protein stabilized emulsions is the extreme sensitivity of the creaming properties to an increase in the number of passes in the range 1–3.

A comparison of the emulsifying efficiency of both the emulsifying machines can be made at a power input of 40W in Figure 8, where stability ratings obtained for soy protein-(0–7)-, WPC-(0.2–7)- and caseinate-(0–7)-stabilized emulsions are plotted as a function of number of passes. For both the caseinate-(0–7) and the soy protein-(0–7), the ultrasonic device is more efficient, whereas the reverse is found for

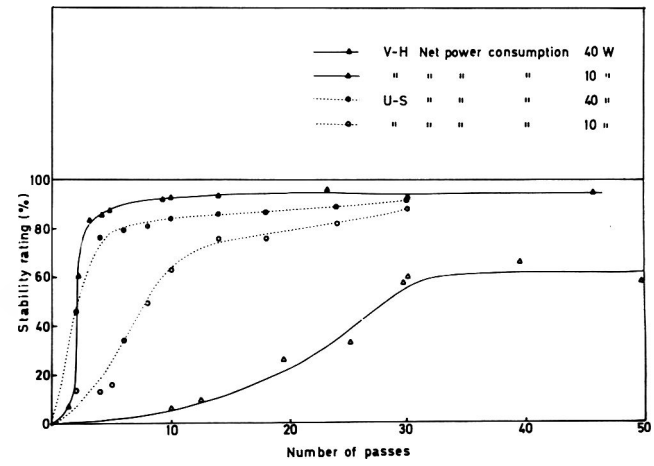

Fig. 7–Stability rating of WPC-(0–7)-stabilized emulsions as a function of number of passes, on emulsification with a valve homogenizer (V-H) and an ultrasonic device (U-S) at a power input of 10 and 40 W.

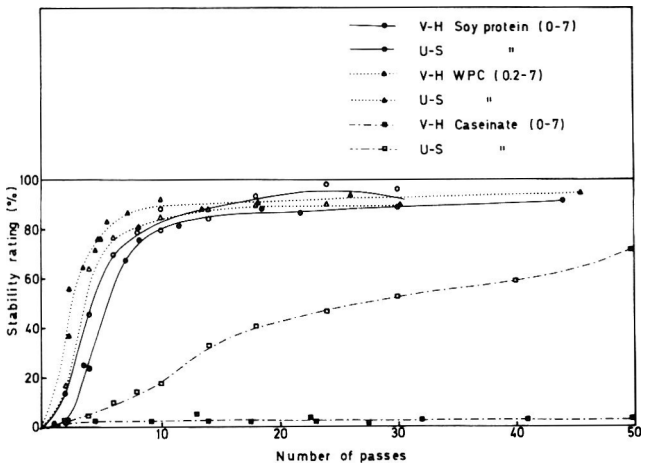

Fig. 8—Creaming stability of soy protein-(0—7)-, WPC-(0.2—7)- and caseinate-(0—7)-stabilized emulsions as a function of number of passes, on sonication (U-S) and valve homogenization (V-H) at a power input of 40W.

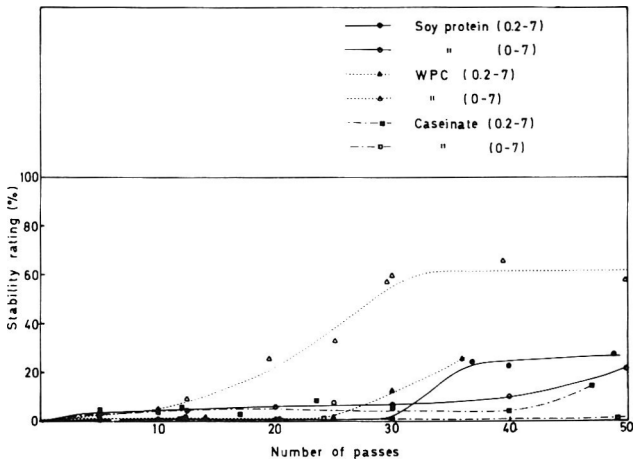

Fig. 9—Stability rating of soy protein-(0.2—7)-, soy protein-(0—7)-, WPC-(0—7)-, WPC-(0.2—7)-, caseinate-(0.2—7)- and caseinate-(0—7)-stabilized emulsions as a function of number of passes, on valve homogenization (V-H) at a power input of 10W.

WPC-(0.2—7). Apparently, the valve homogenizer is a good choice of emulsifying equipment for the preparation of creaming stable WPC-stabilized emulsions. This is also verified by Figure 9, where all the proteins can be compared as emulsifiers on valve homogenization at 10W. The axes are the same as in Figures 7 and 8. It is obvious from the figure that especially the WPC-(0—7)-stabilized emulsions more readily get a higher creaming stability as the number of passes is increased. The same roughly estimated ranking order as found in Figures 4 and 6 also becomes apparent in Figures 8 and 9.

DISCUSSION

IN THIS INVESTIGATION the creaming behavior of protein-stabilized emulsions formed in different ways under controlled conditions has been studied. An evaluation of the factors affecting the creaming behavior of the emulsions can be made by comparison of studies made on milk systems. Walstra and Oortwijn (1975) have found that viscosity and globule size are the most important variables in determining the creaming behavior of unclustered milk, and that increased polydispersity generally decreases the creaming rate.

To be certain that the protein itself was not severely affected by the high energy densities introduced during the emulsification process, some preliminary experiments were performed. The protein dispersions used in this investigation were subjected to the same emulsification procedures as the emulsions formed, and the turbidity and the solubility of the dispersions were measured before and after treatment. No profound irreversible changes in the properties of the protein dispersions were observed. Mulder and Walstra (1974) report that at high pressures and with large micelles disruption of casein micelles can take place in the homogenizer. But the preparation of sodium caseinate destroys the original properties of the casein micelle and possibly also the structure of the submicelles (Schmidt and Buchheim, 1975). Consequently, disruption of the sodium caseinate in the dispersion during emulsification can be considered negligible. It is assumed that the properties of the emulsion formed is mainly determined by the dynamic flow conditions during emulsification and by the way in which the proteins act at the interface under these circumstances.

The type of emulsifying equipment and power input determine the energy density, ϵ_0, generated i.e power input per unit volume, and these values have been estimated in a preceding paper (Tornberg and Lundh, 1978) for the three emulsifying machines used.

The emulsifying efficiency of the turbo-mixer is low compared to that of the other two pieces of emulsifying equipment (see Fig. 1). This could be expected as both the estimated energy density is low ($\approx$ 200 W/cm^3) and a very wide spread in globule size is often obtained (Mulder and Walstra, 1974). The differences in creaming behavior of emulsions produced by the valve homogenizer and the ultrasonic device is mainly due to the latter giving emulsions with very small particles but a wide spread in globule size, compared to the former (Mulder and Walstra, 1974). This can probably be one of the reasons that the sonicated emulsions have higher creaming stabilities in the lower power range around 10W, which is apparent in Figures 1, 3, 4, 5 and 7. Another feature observed, which probably also can be related to this difference in the particle size distribution between the ultrasonic device and the valve homogenizer, is the smoother curves obtained for the sonicated emulsions compared to the more abruptly changing curves obtained for the valve-homogenized products (see Fig. 2, 5 and 7).

The increase in creaming stability at higher levels of power input, seen in Figures 3 to 6, has also been observed by Walstra (1975) for pasteurized whole milk, when valve homogenized. It is interesting to note that the improvement in creaming stability for the valve homogenized emulsions does not occur until a power level of 10W has been passed. This is clearly visible in Figures 3 to 6.

The time of emulsification, as reflected in an increased number of passes, causes in general an enhanced creaming stability of the emulsions, as can be seen in Figures 7 to 9. This is in accordance with Walstra (1975), who demonstrated that on repeated homogenization, the size distribution of milk becomes narrower and globule size smaller. For some of the emulsions viewed in Figures 7 to 9, the increase in creaming stability levels off. Gopal (1968) also states that prolonging the emulsification beyond an optimum time interval does little to improve the quality of the emulsions.

If a comparison is performed between the proteins as emulsifiers, the WPC seems to give the best emulsions in terms of creaming stability, which can be judged from Figures 4, 6, 8 and 9. This is especially so for WPC dispersed in distilled water, whereas the addition of NaCl to 0.2M reduces the creaming stability of the WPC-stabilized emulsions (Fig. 6 to 9). Another interesting feature of the WPC as an emulsifier is its sensitivity to valve homogenization, which is evident from Figures 7 to 9.

—Continued on page 1565

PROTEIN QUALITY OF A WHOLE CORN/WHOLE SOYBEAN MIXTURE PROCESSED BY A SIMPLE EXTRUSION COOKER

R. BRESSANI, J. E. BRAHAM, L. G. ELÍAS, R. CUEVAS and M. R. MOLINA

ABSTRACT

The present study was undertaken to determine the effects of extrusion with a Brady Extruder, on a 70:30 corn:soybean blend prepared with whole corn grain ground to a fine, intermediate and coarse particle size, and pre-treated with water and heat prior to extrusion. The results indicated that it is preferable to use the flour with intermediate or coarse particle size, and that the addition of water up to 17% and the application of heat prior to extrusion yields a product of greater specific volume, high-water retention and lower trypsin inhibitor activity. Some improvement was also observed on the protein quality of the product. The simple extruder may be useful equipment for community or small cooperative agro-industries.

INTRODUCTION

THE INTEREST to produce foods with a high good-quality protein content is steadily increasing in various Latin American countries, as this measure will help meet the population protein needs, especially of the vulnerable groups. Although much work has been done towards the development of such supplementary foods, only a few of them are commercially available. This is due to several factors, but a very important one, particularly for small countries, is the lack of capital investment needed to build up the industry and to market the product for a relatively small potentially-buying sector of low economic resources. Therefore, if these food industries are to be successful, it will be necessary for them to blend and package their products as processed foods or to use simple and inexpensive technologies to process the various components of the mixtures.

Previous studies (Bressani et al., 1974; Tejerina et al., 1977) have shown that whole soybean and whole corn proteins mixed in a weight ratio of approximately 3:7 gave a higher protein-quality food than each individual component. This mixture contains around 18% protein and 10% fat; hence, it has a relatively high content of the two nutrients and a PER of 2.54. It was processed by an alkaline wet-cooking method applied to the mixture of the two ingredients, the end results being a nutritionally adequate and acceptable product. The process, however, although feasible, is too expensive.

Since the ground mixture referred to is not stable when blended raw, simple processing techniques were sought. Simple extrusion cooking, of which the Brady Extruder is an example, offered a promising opportunity. This extruder has been used with success for processing whole soybeans alone and in mixtures for animal feeding (Faber and Zimmerman, 1973; Noland et al., 1976). Its applicability to processed foods for human consumption is being tested at Colorado State University (Stone et al., 1976) and in other laboratories.

Although the extruder is capable of handling a variety of ingredients, the processing conditions need to be standardized as much as possible with respect to both equipment and ingredients. The purpose of our study was to determine the best physical form of the ingredients, and how to condition them so as to produce a food of the quality needed for human consumption.

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0022-1147/78/0005-1563$02.25/0

MATERIALS & METHODS

THE SOYBEANS and corn used in these studies were grown either at INCAP's experimental farm (1,480m above sea level) or in the lowlands of Guatemala.

Batches of 400 kg of whole corn were ground in a hammer mill to three particle sizes (3, 7 and 20 mesh) and whole soybeans were ground to a standard particle size (10 mesh). The distribution of particles in each batch, either of corn or soybeans, was determined by weighing five 100-g samples and screening them through 12, 20, 45, 60 and greater than 60 mesh screens. The amount passing each screen was recorded and the distribution calculated.

With each particle size of corn, 200 kg batches of the corn:soybean blend in a 70:30 weight ratio were mixed, and a sample was taken from each for chemical analysis. Water was added to the raw mixtures in a horizontal blender when the run with the extrusion cooker called for such treatment. The amounts used increased it from about 14 to 18%.

The mixture was then processed with the extruder and after 20 min of operation the extrusion conditions were recorded and a 50-kg sample was taken. When the sample cooled to room temperature its density was measured by weighing the amount placed in a one liter container. This was done five times per sample.

Each sample was analyzed for protein and fat content by the AOAC methods (AOAC, 1970), and trypsin inhibitor activity was determined by the method of Kakade et al. (1969); samples were also analyzed for water absorption (Anderson et al., 1969; Leach et al., 1959), and water retention was measured by mixing a 5-g sample with 100 cc of water (30°C) and shaken for 30 min. The suspension was then filtered through No. 4 Whatman filter paper into a 100 cc cylinder. After two hours the volume of the filtrate was measured. Water retention capacity was calculated by subtracting the volume of filtrate from 100 and dividing by the weight of the sample. PER was determined in rats according to the AOAC procedure (1970). Flavor and odor of the extruded flakes were rated as acceptable or unacceptable by a ten-member taste panel.

Table 1—Particle size distribution of corn and soybeans (g/100g)

Particle size mesh	Corn particle size			Soybeans
	Fine	Medium	Coarse	
12	1.36	27.28	84.82	25.17
20	35.81	33.80	9.56	44.70
45	31.88	20.75	3.64	17.22
60	10.72	5.30	0.70	6.26
<60	13.39	10.20	0.94	4.91
Recovery	93.16	97.33	99.66	98.26

RESULTS & DISCUSSION

TABLE 1 summarizes the particle size distribution of the ground soybeans and of the ground whole corn utilized. Each of the three particle size ground corn samples was blended in a 7:3 ratio by weight for subsequent studies.

The results obtained in the first series of experimental runs

Table 2—Physical and nutritional characteristics of mixtures of corn and soybeans of different particle size processed by the Brady Extruder

Description of sample	TUI[a]/g	Water retention (%)		Water absorption g gel/g sample		Specific wt (g/L)	Protein efficiency ratio[b]
		Raw	Extruded	Raw	Extruded		
Raw	14.1	–	–	2.71	–	638.6	0.81
Small particle size	11.1	3.00	510	2.55	4.64	183.9	2.07
Intermediate size	8.5	4.50	827	2.77	4.57	191.2	1.93
Coarse size	7.5	7.20	1013	2.82	5.12	206.5	1.96
Casein	–	–	–	–	–	–	2.50

[a] TUI = Trypsin Units Inhibited.
[b] PER for casein was 3.09; all values in the Table were adjusted to 2.50.

with the Brady Extruder, using the blends without any previous treatment before extrusion, are summarized in Table 2. Extrusion decreased trypsin inhibitor activity, and it appears that more was destroyed when the mixture extruded contained corn of a coarse particle size.

Table 3—Effect of water addition and heating of a soybean:corn mixture before extrusion, on density of extruded product

Corn particle size	Treatment before extrusion			Wt of extruded product (g/L)
	Water addition	Heat (°C)	Time (min)	
Fine	None[a]	no	–	184
	None[a]	60–65	8–10	149
	4%[b]	60–65	8–10	57
Intermediate	None[a]	no	–	191
Coarse	None[a]	no	–	207

[a] Moisture content, 14%
[b] Moisture content, 18%

Table 4—Conditions of extrusion[a] and some characteristics of extruded product

Particle size	H_2O (%)	Conditions of extrusion		Characteristics of extruded product	
		Cone aperture (cm)	Output rate (kg/hr)	Specific volume (cm/g)	Water absorbed (%)
Fine grind[b]	13.6	0.01	717	1.52 ± 0.05	3.97
Fine grind	17.4	0.02	680	2.69 ± 0.10	5.34
Fine grind	21.4	0.02	652	3.27 ± 0.21	4.83
Fine grind[c]	17.8	0.02	454	2.09 ± 0.07	5.03
Coarse	23.4	0.02	573	3.06 ± 0.25	4.78

[a] Input feeding rate and temperature of extrusion: 32 rpm and 143°C for all samples
[b] Refers to particle size of ground whole corn (3 and 20 mesh for coarse and fine, respectively)
[c] With 0.25% $Ca(OH)_2$ added

Table 5—Nutritional characteristics of extruded products

Particle size	H_2O (%)	TUI[a]/ml	Avg wt gain (g)	PER[b]
Raw product	–	7.2	18	0.77
Fine grind	13.6	6.1	69	2.18
Fine grind	17.4	3.4	77	2.16
Fine grind	21.4	1.4	84	2.22
Fine grind[c]	17.8	2.9	96	
Coarse	23.8	–	92	2.30
Casein	–	–	112	2.50

[a] TUI = Trypsin Units Inhibited.
[b] PER for casein was 2.88; all values in the Table were adjusted to 2.50.
[c] Refers to particle size of corn

Water retention increased in the extruded product for each particle size, and it was higher with the sample prepared with the coarse particle size corn. Water absorption increased by extrusion to about the same extent for the three particle sizes, while the specific weight decreased when the raw and extruded samples were compared. These results show an inverse relationship between particle size of corn and weight of extruded product per unit volume, indicating that a significant expansion took place upon extrusion. Finally, protein quality, as measured by a protein efficiency ratio (PER) assay, was increased by extrusion cooking but there was no apparent relationship to corn particle size.

Table 3 shows the effect of the addition of water and heat before extrusion on the specific weight of the extruded product per unit volume. A thermic treatment for 8–10 min decreased weight per liter from 184 to 149, while the application of both heat and moisture decreased it even further, inducing a relatively high expansion of the product. Such studies were not carried out with the other two corn particle sizes, but it is probable that the same results would have been obtained.

In a second series of experimental runs, fine and coar particle size corn were used. Experimental conditions are shown in Table 4. The fine particle size corn was adjusted in the mixture with soybeans to three moisture levels: 13.6, 17.4 and 21.4%, respectively; in an additional run, 0.25% lime was added and the moisture adjusted to 17.8%. The coarse grou mixture was adjusted to 23.4% moisture. The addition of water prior to extrusion decreased the output rate or increased retention time which apparently was also affected by particle size.

The specific volume of the extruded product increased with respect to water addition before extrusion in the fine grind corn samples. The addition of lime or the use of a coarse particle size corn tended to decrease specific volume of the extruded product, which was higher for samples of the mixture with more than 17% moisture than in those with 13.6% moisture.

Table 5 summarizes the level of trypsin inhibitor activity and the quality of the protein of the extruded product. Moisture level in the blend before extrusion helped decrease trypsin inhibitor activity, which dropped from a value of 7.2 in the raw product to 1.4 when the moisture level was 21.4%. Average weight gain showed a direct relationship to moisture level in the blend before extrusion, and an increased PER from the raw to the extruded product irrespective of moisture content. Since no change in PER in the extruded products was observed even though the rats gained more weight, extrusion probably increased the palatability of the product. Finally, some observations made on the physical quality of the flakes are shown in Table 6. The best product was sample No. 3 which contained 21.4% moisture before extrusion.

Even though some changes in the physical quality characteristics of the extruded product were observed with respect to particle size of the raw material, the results show no definite trend. However, they do suggest that the intermediate or coarse grind is better to use because less problems were en-

countered in running the extruder without getting it jammed.

On the other hand, the addition of water to the raw blend before extrusion induced some beneficial effects, such as increasing specific volume, water absorption and weight gain. Protein efficiency, however, did not change. This is really not a new finding, since it is a well-known fact that to destroy or inactivate trypsin inhibitors in soybeans, moisture is an essential factor (Rackis, 1974).

The results show that the Brady Extruder can play a useful role towards the solution of the nutrition problems faced by underdeveloped societies, since it is a simple inexpensive machine with a relatively good output per unit time. Because of these characteristics many units could be installed in a country, thus providing a better and cheaper distribution of the product. Furthermore, the equipment may be very useful for common or small cooperative agro-industries. Nevertheless, it is necessary to carry out additional studies in order to standardize processing conditions and ensure that these will result in high-quality products for human feeding.

Table 6—Physical and organoleptic characteristics of the flakes obtained

Particle size	Flakes	Panel testing	
		Odor	Flavor
Fine grind[a]	Hard, small nonporous	Acceptable	Not acceptable
Fine grind	Expanded, large porous, flexible	Acceptable	Acceptable
Fine grind	Expanded, large porous, brittle	Acceptable	Highly acceptable
Fine grind[b]	Expanded, large porous, flexible	Not acceptable	Not acceptable
Coarse	Expanded, large porous, brittle	Acceptable	Acceptable

[a] Refers to particle size of corn
[b] With 0.25% $Ca(OH)_2$ added

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Ms received 10/15/77; revised 2/17/78; accepted 2/25/78.

This research was carried out with funds from the Research Corporation, New York, NY. (Grant-in-aid INCAP PN-740/FDTEC) and from the Agency for International Development (AID) of the United States (Grant No. PN-171).

PROTEIN STABILIZED EMULSIONS – CREAMING STABILITY . . . From page 1562

The soy protein gives emulsions of relatively high creaming stability mostly in between those stabilized with WPC and sodium caseinate.

The sodium caseinate-stabilized emulsions have the lowest creaming stabilities, especially when the caseinate is dispersed in distilled water. It is interesting to note that the addition of salt up to 0.2M NaCl in the sodium caseinate stabilized emulsions improves the creaming stability, whereas the reverse is found for the two other proteins studied.

The approach of standardizing the emulsifying procedure and studying its influence seems to be promising as a method for evaluating the emulsifying characteristics of a protein product on a more general basis. But before a more thorough interpretation of the action of proteins as emulsifiers can be given, additional methods of characterizing the emulsions are needed. This is at present under investigation in our laboratories.

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Ms received 6/18/77; revised 12/3/77; accepted 12/8/77.

Dr. Anne-Marie Hermansson and Dr. P. Walstra are heartily thanked for valuable comments. The technical assistance of Mrs. Gunnel Lundh and Mr. Staffan Knutsson is gratefully acknowledged. The investigations were made possible by grants from the Swedish Board for Technical Development, and this financial support is gratefully acknowledged.

VITAMIN COMPOSITION OF FRESHLY HARVESTED AND STORED POTATOES

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ABSTRACT

The major US potato varieties grown in various locations were analyzed for their contents in water-soluble vitamins both at harvest time and during subsequent storage. Compositional ranges of each vitamin were quite large. Only a few varietal and location effects were detected. In the case of ascorbic acid, what appeared to be a location effect could likely have been the result of differences in harvest time of the samples. Prolonged storage had little overall effect on thiamine and riboflavin. It resulted in a sharp initial decrease in ascorbic acid, significant decreases in niacin and folic acid, and a significant and large increase in Vitamin B_6. Storage temperature in the range of 38°–45°F did not affect the vitamin composition, nor did reconditioning of the tubers at room temperature following cold storage.

INTRODUCTION

WITH A PER CAPITA consumption fluctuating between 110 and 120 lbs since 1968, potatoes rank number one of all vegetable crops consumed in the United States (ERS, USDA, 1977). This signifies that the potato plays an important role in the daily American diet.

Nutritional labeling and the increased public interest in the nutritional value of foods induced the Potato Board to assess the nutritional value of the potato in order to see just how much potatoes contribute in meeting the daily nutritional needs of man.

A subsequent search of the literature revealed some information to be available on the nutrient composition of potatoes (Adams, 1975; Augustin, 1975; Augustin et al., 1975; Pederson, 1972; Page and Hanning, 1963; Yamaguchi et al., 1960; Leichsenring et al., 1951). However, these studies were found to be limited to a few and often unimportant varieties, they covered only a few nutrients, and/or they did not include the effects of long term storage.

The literature also revealed considerable variations to exist in the composition of the raw material, both at harvest time and during subsequent storage.

In view of a lack of adequate background information, the Potato Board deemed it necessary to initiate a nationwide survey-type investigation of the nutrient composition of white potatoes encompassing the major U.S. varieties. This paper covers the water-soluble vitamins of this study. Data on proximate and mineral composition will be covered in subsequent papers.

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0022-1147/78/0005-1566$02.25/0

MATERIALS & METHODS

Varieties and locations

Table 1 summarizes the varieties chosen for this study as well as the locations in which they were grown. The cultural practices used in raising these potatoes were what is considered "normal" for each particular variety and location.

Sampling and post harvest treatments

At harvest time three replicate sample lots consisting of healthy, undamaged tubers were selected from each variety and from each location. Immediately following harvest, the samples were transported to one of the following locations for storage and sample preparation: samples grown in California, Idaho and Washington to the Food Research Center of the University of Idaho in Moscow, ID; samples grown in North Dakota to the ARS-USDA Red River Valley Potato Research Center in East Grand Forks, MN, and those grown in Maine to the Department of Food Science at the University of Maine in Orono, ME. All samples were placed into storage at 45°F at 95% relative humidity. After 30 days subsamples from each sample lot were removed for the initial analysis. All varieties grown in California, as well as Norgold from this point on were not subjected to any further storage treatment. The remainder of the samples were stored as outlined in Table 1.

Sample selection and preparation

At each laboratory for each sampling, 16 tubers were selected from each lot. The tubers were thoroughly cleaned in distilled water and allowed to dry in ambient air until the skin appeared dry. Each tuber was cut longitudinally into four wedges. One wedge from each tuber was selected for each of the following analyses, and treated as outlined below:

Dry matter. One sample wedge of each of 16 tubers was cut randomly into small sections. The combined cuttings from these wedges were macerated for 1 min in a Waring Blendor. A 25-g sample of the

Ascorbic acid. A thin wedge of approximately 2 mm maximum thickness was cut from one wedge of each of 16 tubers. These wedges were then pooled and cut in smaller size pieces. A 100-g subsample was then ground in 200 ml of 1:1 mixture of 5% metaphosphoric acid and acetic acid using a Waring Blendor, and analyzed.

Other vitamins. One wedge from each of the 16 tubers was cut crosswise into 1–2 mm thick slices. The slices were combined and frozen by immersion in liquid nitrogen, freeze dried, ground in a Wiley Mill using a 40 mesh screen, and shipped in sealed containers to the Moscow laboratory where they were stored at 0°F until analyzed on location.

Analytical

Analysis of the vitamins was done by the following methods: total ascorbic acid, thiamine and riboflavin by fluorometry, reduced ascorbic acid by indophenol titration, and niacin by colorimetry; vitamin B_6 and total folic acid 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 kidneys 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 37°C.

Total vitamin B_6 rather than the individual three forms were determined with pyridoxine as the standard.

Table 1—Varieties, locations of growth and storage conditions

Variety	Locations	Storage		
		Time (months)	Temp (°F)	Reconditioning
Centennial Russet	California	none		
Katahdin	Maine	4 & 8	38 & 45	W & W/O
Kennebec	California	none	–	–
	Maine	4 & 8	45	W
	North Dakota	4 & 8	45	W
	Washington	4 & 8	45	W
Norchip	Maine	4 & 8	45	W
	North Dakota	4 & 8	45	W
Norgold	Washington	none	–	–
Pontiac	North Dakota	4 & 8	45	W
Russet Burbank	Idaho	4 & 8	38 & 45	W & W/O
	Maine	4 & 8	38 & 45	W & W/O
	Washington	4 & 8	38 & 45	W & W/O
White Rose	California	none	–	–

RESULTS & DISCUSSION

TABLE 2 summarizes the ranges of the vitamin concentrations in potatoes established in this investigation as well as the corresponding U.S. Recommended Daily Allowance (USRDA) values. The data were based on a 150-g serving size, a value which was based on the U.S. per capita consumption of 120 lb (ERS-USDA, 1977). The results show potatoes to contain significant amounts of several of the vitamins listed. They also provide evidence of considerable variations to exist in the concentrations of these vitamins, the extent of which varied with the particular vitamin involved.

The compositional ranges shown in Table 2 cover the extreme values which were encountered during this investigation. Such values were found to be rather exceptional. Therefore, in order to more equitably describe compositional variations in potatoes, the data were computed on an arithmetic mean basis and listed together with the corresponding coefficients of variations in Table 3. These results covering freshly harvested tubers only clearly indicated that large compositional variations existed at the time of harvest. In comparing the coefficients of variations between the freshly harvested and freshly harvested and stored samples, it becomes obvious that at least in the case of ascorbic acid, niacin, folic acid and vitamin B_6, changes occurred in the concentration of these vitamins during storage which significantly affected their variability.

Since the data so far were computed on a wet basis, one has to consider the variability of the moisture or dry matter content as well, which as shown in Table 3 can be considerable. In order to eliminate this source of variability all data listed in the following tables are based on a dry weight basis (DWB).

Two other indirect sources of variabilities which have to be considered besides dry matter, are those associated with the analytical procedures and those with sample replication. Data concerning these are summarized in Table 4. While both the analytical and mean replicate coefficients of variation were small in combination, they can be significant, but not of the magnitude shown in Table 3. Computation of the coefficients of variation on a dry weight basis which was listed in parentheses show that the elimination of the moisture variability resulted in little change in comparison to the coefficients of variation based on a wet-basis. Therefore, as indicated in Table 3, the bulk of the variability is real and either associated with preharvest or postharvest factors, or both.

The vitamin contents of freshly harvested potato samples are summarized in Table 5. The results clearly show that the experimental design was such as not to allow in many instances to make valid comparisons in the concentration of a

Table 2—Ranges in vitamin composition of potatoes

	mg/150g (wet basis)	U.S. RDA (%)
Ascorbic acid	11.8–54.1	19.7–90.3
Thiamine	0.09–0.18	6.0–11.9
Riboflavin	0.03–0.14	1.9– 8.0
Niacin	1.0–3.9	4.8–19.7
Folic acid	0.01–0.03	3.2– 8.1
Vitamin B_6	0.19–0.60	9.5–29.9

Table 3—Mean values and coefficients of variation of freshly harvested and stored potatoes

	Freshly harvested and stored potatoes		Freshly harvested potatoes	
	Mean	Coeff.	Mean	Coeff.
	mg/150g (WB)	Var. (%)	mg/150g (WB)	Var. (%)
Dry matter	21.37*	10 (–)	20.96*	10 (–)
Ascorbic acid	21.15	47 (52)**	36.05	24 (27)
Thiamine	0.135	16 (14)	0.122	14 (11)
Riboflavin	0.057	36 (34)	0.066	30 (28)
Niacin	2.24	27 (28)	2.46	19 (21)
Folic acid	0.0191	21 (23)	0.0240	14 (15)
Vitamin B_6	0.386	29 (30)	0.258	19 (19)

* = %

** = CV based on data computed on a dry weight basis

Table 4—Accuracy of methods and variability of replications

	Method CV*	Replication	
		Mean CV*	Maximum CV*
Ascorbic acid	1	4	14
Thiamin	5	3	9
Riboflavin	2	7	29
Niacin	1	6	21
Folic acid	8	6	16
Vitamin B_6	9	6	21

* CV – Coefficient of variation

Table 5—Vitamin composition of freshly harvested potatoes (mg/100g DWB)

	Location	Ascorbic acid	B_1	B_2	Niacin	Folic acid*	B_6
Russet Burbank	ID	117.2	0.39	0.14	9.4	97.4	1.01
	ME	82.6	0.42	0.24	6.7	61.7	0.95
	WA	92.0	0.36	0.17	5.1	54.2	0.98
Katahdin	ME	108.2	0.45	0.33	8.4	84.8	0.92
Kennebec	CA	150.8	0.45	0.14	10.5	91.1	0.92
	ME	76.8	0.37	0.17	6.3	81.5	0.90
	ND	–	0.38	0.17	6.9	81.5	0.60
	WA	109.2	0.42	0.20	10.3	81.4	0.71
Norchip	ME	86.3	0.38	0.34	5.5	73.6	0.69
	ND	–	0.38	0.22	6.4	59.3	0.49
Superior	ME	129.8	0.31	0.24	7.1	74.9	0.71
	ND	–	0.35	0.18	9.6	77.7	0.90
Pontiac	ND	–	0.44	0.19	8.9	87.9	0.69
White Rose	CA	179.4	0.38	0.22	8.0	68.4	0.95
Centennial Russet	CA	148.8	0.33	0.18	8.3	80.6	0.99
Norgold	WA	142.9	0.36	0.19	8.8	75.5	0.80

* μg/100g DWB

particular vitamin between different varieties. This becomes very evident when one discovers the often large differences in concentration just in one variety which was grown in different locations. It is obvious that it is impossible to compare the ascorbic acid values of Kennebec potatoes grown in three location with that of White Rose grown only on one location, and reach the conclusion that the ascorbic acid content in White Rose tubers is higher compared to those of Kennebec potatoes. On the other hand it was not the purpose of this study to comparatively evaluate the vitamin content of different potato varieties. Rather, and as indicated earlier, the purpose of this investigation was to assess the vitamin content in potatoes in general. This meant selecting the major U.S. potato varieties from their predominant areas of growth. Nonetheless the data in Tables 3 and 5 clearly demonstrate some large differences to exist. The remainder of this paper is devoted to pinpoint, where possible, the nature of such differences.

A comparative analysis of the data in Table 3 revealed that storage is a major factor contributing toward the variability of the concentration of vitamins in potatoes. It plays a major role with regard to ascorbic acid. folic acid and vitamin B_6. Storage temperatures, within the ranges used in this study, and reconditioning appear to have had little effect on vitamin composition. If there were any, they were erratic. It was the storage time which exerted the major effect on the concentration of these vitamins.

Table 6—Mean ascorbic acid contents of freshly harvested and stored potatoes (mg/100g DWB)

Storage Time (Months)	Temp (°F)		RB 3**	KA 1	NC 1	KE 2	SU 1
1	45		82.6–117.2	108.2	86.3	76.8–109.2	129.8
4	38		44.2–50.3	66.1	–	–	–
	45		50.3–57.5	64.6	–	–	–
	38	R*	45.6–48.8	50.7			
	45	R*	48.9–57.8	52.0	44.8	49.31–51.81	52.8
8	38		39.7–51.2	38.2	–	–	–
	45		34.7–53.7	40.2	–	–	–
	38	R*	44.5–50.9	54.7	–	–	–
	45	R*	42.0–53.4	54.2	43.4	46.48–50.41	48.1

* R = Reconditioned
** = Number of locations sampled

Ascorbic acid (Tables 3, 5 and 6)

No statistically significant differences were found between varieties which are grown in more than one location: e.g. between Russet Burbank and Kennebec. Varieties grown in California, e.g. Kern County, showed highly significantly higher ascorbic acid values than those of the other growing areas. One of the reasons these samples were high in this vitamin was due to the fact that they were harvested early. It is for this same reason that the Russet Burbank samples grown in Idaho exhibited a higher ascorbic acid content than those grown in Washington. Developing potatoes reach a peak in ascorbic acid which seems to occur both in the White Rose and in the Russet Burbank varieties, and likely in all varieties, at or around 110 days after planting, followed by a decline which continues right into several months of the storage period until it finally eases somewhat (Augustin et al., 1975; Yamaguchi et al., 1960). This decrease of ascorbic acid occurs even if harvest is delayed. And this is what happened in the cases of the above

Table 7—Mean thiamine contents of freshly harvested and stored potatoes (mg/100g DWB)

Storage Time (Months)	Temp (°F)		RB 3**	KA 1	NC 2	KE 3 (4)	SU 2	PO 1
1	45		0.36–0.42	0.45	0.36–0.37	0.37–0.45	0.31–0.35	0.44
4	38		0.30–0.45	0.42	–	–	–	
	45		0.31–0.46	0.46	–	–	–	–
	38	R*	0.30–0.49	0.41	–	–	–	–
	45	R*	0.30–0.48	0.45	0.30–0.47	0.34–0.48	0.44–0.46	0.57
8	38		0.40–0.48	0.44	–	–	–	–
	45		0.42–0.47	0.50	–	–	–	–
	38	R*	0.43–0.46	0.50	–	–	–	–
	45	R*	0.43–0.45	0.54	0.39–0.40	0.41–0.48	0.37–0.39	0.50

* R = Reconditioned
** = Number of locations sampled

Table 8—Mean riboflavin contents of freshly harvested and stored potatoes (mg/100g DWB)

Storage Time (Months)	Temp (°F)		RB 3**	KA 1	NC 2	KE 3	SU 2	PO 1
1	45		0.14–0.24	0.33	0.22–0.34	0.17–0.20	0.18–0.24	0.19
4	38		0.11–0.20	0.17	–	–	–	–
	45		0.11–0.13	0.16	–	–	–	–
	38	R*	0.12–0.16	0.12	–	–	–	–
	45	R*	0.10–0.15	0.12	0.22–0.37	0.11–0.13	0.14–0.20	0.23
8	38		0.15–0.21	0.16	–	–	–	–
	45		0.14–0.24	0.19	–	–	–	–
	38	R*	0.12–0.18	0.20	–	–	–	–
	45	R*	0.11–0.14	0.18	0.27–0.37	0.14–0.21	0.15–0.17	0.17

* R = Reconditioned
** = Number of locations sampled

Table 9—Mean niacin content of freshly harvested and stored potatoes (mg/100g DWB)

Storage Time (Months)	Storage Temp (°F)		RB 3**	KA 1	NC 2	KE 3	SU 2	PO 1
1	45		5.1–9.4	8.4	5.5–6.4	6.3–10.3	7.1–9.6	8.9
4	38		5.3–8.2	7.4	–	–	–	–
	45		5.9–8.4	7.1	–	–	–	–
	38	R*	5.5–7.0	7.4	–	–	–	–
	45	R*	5.5–7.4	7.4	5.5–9.1	7.9–9.1	4.1–13.5	8.9
8	38		5.1–9.0	5.7	–	–	–	–
	45		4.3–7.3	6.0	–	–	–	–
	38	R*	4.2–6.9	6.7	–	–	–	–
	45	R*	4.7–6.3	5.5	4.5–6.3	3.5–7.6	3.2–11.9	7.2

* R = Reconditioned
** = Numbers of locations sampled

samples. The California samples were harvested relative early in the season. The Russet Burbank samples grown in south central Idaho too were harvested early, i.e. early September, whereas those grown in Washington were harvested late, i.e. in mid-October, and thus the relatively high values for the Idaho Russets and the low values for the Washington Russets.

While location, variety etc. could be factors affecting the ascorbic acid content at harvest time, and while data of harvest or physiological tuber maturity definitively is a factor influencing the content of this vitamin in potatoes at harvest time, there may be others. In terms of potaotes in general, i.e. both freshly harvested and stored undoubtedly the major factor contributing to the variability of ascorbic acid is storage time (Tables 3 and 6). There was a rather sharp decrease in ascorbic acid during the first 4 months of storage either followed by a complete leveling out or a less pronounced decrease. It appears to be immaterial, whether high or low at harvest time; after 8 months storage ascorbic acid values varied comparatively little regardless of variety or location of growth. Even when computing the data reported by Yamaguchi et al. (1960) on White Rose on a dry weight basis, its ascorbic acid content amounted to 52.6 mg/100g, a value remarkably close to the mean values reported for all tuber samples after 8 months' storage in Table 6.

Thiamine (Tables 3, 5 and 7)

This vitamin varied comparatively little in potatoes. No statistically significant differences were found in the content of this vitamin between varieties and locations. Prolonged storage of potatoes resulted in a slight but significant increase over an 8-month period. Only the Kennebec variety grown in Washington and the Norchip variety appeared to change little. The patterns of changes throughout the storage period were somewhat erratic, a fact that is in agreement with the findings of Augustin (1975), Yamaguchi et al. (1960) and Leichsenring et al. (1951).

Riboflavin (Tables 3, 5 and 8)

This vitamin varied considerably both in freshly harvested as well as in stored potatoes. However, variations were erratic and could not be attributed to any one factor, e.g. variety, location, and/or storage conditions. Changes during storage were erratic. The values obtained in general are higher than those reported earlier by Augustin (1975), Yamaguchi et al. (1960) and Leichsenring et al. (1951). The reason for this is the fact that in these earlier studies potatoes were peeled before analysis whereas in this case they were analyzed unpeeled. Augustin et al. (1977) reported the peel areas to contain significantly higher amounts of riboflavin than potato flesh. If computed on a dry weight basis, the amount of riboflavin in hand peelings exceeded that of the corresponding flesh by a factor of more than two. Pederson (1972) who analyzed riboflavin in peeled and unpeeled potatoes reported much higher values in the unpeeled tuber material, although his values were somewhat lower than those reported in this study.

Niacin (Tables 3, 5 and 9)

The results showed no differences to exist in the niacin concentration between varieties and location of growth. Storage in general resulted in a decrease in niacin values. The values in general are of the same magnitude as those reported by Pederson (1972). The results of the storage study do not agree with those reported earlier by Augustin (1975), probably because of the much smaller scope of the earlier study. However, they are in reasonably good agreement with those of Page and Hanning (1963).

Folic acid (Tables 3, 5 and 10)

No differences were found in the contents of this vitamin

Table 10—Mean folic acid content of freshly harvested and stored potatoes (μg/100g DWB)

Storage Time (Months)	Storage Temp (°F)		RB 3**	KA 1	NC 2	KE 3	SU 2	PO 1
1	45		54.2–97.4	84.2	59.3–73.6	81.4–81.5	74.9–77.7	87.9
4	38		50.5–64.2	79.1	–	–	–	–
	45		44.7–55.1	56.1	–	–	–	–
	38	R*	44.7–64.4	50.6	–	–	–	–
	45	R*	41.1–49.6	48.9	53.4–55.0	45.5–58.0	48.0–57.7	65.0
8	38		50.5–70.2	66.3	–	–	–	–
	45		44.7–52.8	60.8	–	–	–	–
	38	R*	45.2–54.3	49.6	–	–	–	–
	45	R*	44.3–64.1	50.7	51.3–52.5	44.0–71.4	46.2–62.5	72.8

* R = Reconditioned
** = Number of locations sampled

Table 11—Mean vitamin B_6 content of freshly harvested and stored potatoes (mg/100g DWB)

Storage Time (Months)	Storage Temp (°F)		RB 3**	KA 1	NC 2	KE 3	SU 2	PO 1
1	45		0.95–1.01	0.92	0.49–0.69	0.60–0.90	0.71–0.89	0.69
4	38		1.06–1.27	1.26	–	–	–	–
	45		1.07–1.17	1.24	–	–	–	–
	38	R*	1.00–1.35	1.33	–	–	–	–
	45	R*	1.08–1.35	1.51	0.62–0.93	0.66–0.99	1.05–1.12	0.76
8	38		1.56–1.69	1.43	–	–	–	–
	45		1.46–1.62	1.46	–	–	–	–
	38	R*	1.57–1.62	2.11	–	–	–	–
	45	R*	1.41–1.72	2.08	1.16–1.35	1.15–1.57	1.50–1.85	1.62

* R = Reconditioned
** = Number of locations sampled

between varieties and locations. However, losses during an 8-month storage period were substantial and ranged from 17–40% on a dry weight basis. In general the data are higher than those reported by Pederson (1972). This is because Pederson determined free folic acid only, whereas this study covered total folic acid. They fell somewhat short of the value reported by Perloff and Burton (1977) which possibly may have been derived from a single measurement only and thus can hardly be considered to be representative.

Vitamin B_6 (Tables 3, 5 and 11)

The variability of this vitamin in freshly harvested tubers was large, but generally not attributable to any one particular factor. Only freshly harvested Norchip potatoes contained significantly less at the 5% level vitamin B_6 than Russet Burbank, but they did not differ from any of the other varieties.

The vitamin B_6 content increased significantly during storage. This increase was consistent regardless of variety and storage conditions. Pederson (1972) showed this vitamin to be higher in 2-month old potatoes than in freshly harvested tubers. Whether the increase in vitamin B_6 was the result of a de novo synthesis during storage has not been established. It is possible that this vitamin exists in some bound form in the early stages of storage which may not be captured using current methods of extraction and/or analysis. Be this as it may, Page and Hanning (1963) reported similar increases in vitamin B_6 to occur during storage of Triumph and Bliss varieties.

NUTRITIONAL LABELING

ONE OF THE OBJECTIVES of this investigation was the establishment of a nutritional labeling system for raw potatoes.

Current federal regulations do not allow for large compositional variations which occur in fresh, i.e. nonprocessed fruits and vegetables such as potatoes. They require that the actual U.S. RDA value for any nutrient declared on the label be no more than 20% below the value listed on the label. Therefore, under existing federal regulations one is left but with one choice, i.e. to declare values at the 20% minimum levels. U.S. RDA values for potatoes based on this minimum concept are tabulated in Table 12 under the heading "minimum values." In order to contrast these, "maximum values" are also listed. It is obvious that neither one of these values adequately describes the vitamin value of potatoes. Neither would be the use of values based on arithmetic means, because such values could be unfairly weighted depending upon the number of measurements and the time of year the measurements would be taken. What all three methods of computation fail to take into account are consumption patterns of potatoes through the year. These patterns have been outlined in Table 13, and U.S. RDA values have been computed and properly weighted on the basis of these figures and the vitamin composition of potatoes after 1-month, 4-months and 8-months storage. The results of these computations are shown in Table 12 under the heading "corrected values." For comparative purposes U.S. RDA values have also been calculated on the basis of nutrient values listed in the USDA Handbook 8 (Watt and Merrill, 1963); these values as well as others are shown in Table 12.

It is quite obvious from the data in Table 12, that the corrected U.S. RDA values are in total agreement with those computed on the basis of the Handbook No. 8 data. As far as folic acid and vitamin B_6 are concerned, no data are available from Handbook 8. Comparative data on folic acid are scarce in the literature. Those obtained by Pederson (1972) cover free folic acid only, and are therefore considerably lower than the values obtained in this investigation. The value cited by Perloff and Butrum (1977) while representing total folic acid, appears to be the result of a single measurement only, and therefore can hardly be considered to be representative. As far as vita-

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Table 12—U.S. RDA values in potatoes (150g serving)

	Min	Max	Corrected	Handbook 8	Other
Ascorbic acid	20	100	60	60	–
Thiamine	6	10	10	10	10[a]
Riboflavin	2	10	4	4	2[a]
Niacin	8	20	10	10	10[a] 15[b]
Folic acid	2	10	6	–	2[a] 8[c]
Vitamin B_6	10	35	20	–	15[a] 20[b]

[a] Pederson (1972)
[b] Page and Hanning (1963)
[c] Perloff and Butrum (1977)

Table 13—Statistical data for computing vitamin values

	Fresh[a]	Percent consumed 0–2 months	2–6 months	>6 months
Russet Burbank	35.8	50	35	15
Kennebec	4.2	100	–	–
Katahdin	30.3	50	45	5
Superior	5.8	100	–	–
Norgold	12.8	100	–	–
Pontiac	5.6	50	50	–
White Rose	5.4	100	–	–

[a] Source, The Potato Board

PROPERTIES OF OGI POWDERS MADE FROM NORMAL, FORTIFIED AND OPAQUE–2 CORN

AYODEJI O. ADENIJI and NORMAN N. POTTER

ABSTRACT

Ogi powders were made from normal corn, corn plus lysine and tryptophan, and opaque-2 corn by grinding, steeping, fermenting, supplementing and drum drying. Compositional, nutritional, organoleptic, and storage properties of the powders were determined. Processing did not decrease protein content of the corn but total and available lysine were significantly reduced. Tryptophan levels were more stable to processing and in two of the powders increased, probably due to fermentation. All powders were acceptable to a Nigerian taste panel when made into ogi porridge, and storage of powders at 30°C for 18 wk caused only small changes in acceptability. The nutritional quality of the powders as judged by PER values, however, was substantially decreased by processing. The data suggest that supplementation with lysine and other amino acids following rather than preceding drying may be beneficial.

INTRODUCTION

CORN (*Zea mays*) is a principal source of calories and protein for many people of Latin America and some African countries. It is the predominant cereal crop of southern Nigeria, eaten as grains in combination with legumes, or wet milled and fermented to "ogi." Ogi is an important weaning food for children, and a breakfast cereal for adults. Corn is deficient in lysine and tryptophan (Mitchell and Smuts, 1932). Simultaneous supplementation with lysine and tryptophan improves nutritional quality (Scrimshaw et al., 1958; Truswell and Brock, 1961; Bressani et al., 1968). In predominantly corn-eating populations of East Africa, tryptophan deficiency and the highly bound nicotinic acid of corn have been implicated in the occurrence of pellagra (Latham, 1965). Feeding tests with rats have shown that no single amino acid is limiting for growth in opaque-2 corn protein (Mertz et al., 1965).

Ogi is produced from corn that is steeped in cold water, wet milled, sieved to remove hulls and fermented naturally for about 24 hr. Lactic and acetic acids are responsible for its sour taste (Banigo and Muller, 1972). Production is generally on a small scale. The wet paste is then cooked in water to a smooth textured porridge. When fed as a weaning food it is usually supplemented with milk for those who can afford the cost (Akinrele, 1967). During the traditional processing of corn to ogi, protein losses can be as high as 50% as overtails and in wash water (Banigo and Muller, 1972). An improved processing method capable of reducing nutrient losses and yielding organoleptically acceptable product has been described by Banigo et al. (1974).

The present study was undertaken to produce dehydrated convenience ogi powders from normal corn, lysine and tryptophan supplemented corn and opaque-2 corn and to determine compositional, nutritional, organoleptic and storage properties of the ogi powders.

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0022-1147/78/0005-1571$02.25/0

EXPERIMENTAL

Sample preparation

Normal endosperm dent corn (Lot 590) and opaque-2 corn (Lot HL3009) were supplied by Crow's Hybrid Corn Company, Milford, IL. The corn grains were cleaned by agitating in large quantities of water and dried at 57°C in a cabinet drier. The dried grains were milled twice in a laboratory Model No. 2 Wiley mill fitted with a 1 mm mesh screen to a final flour recovery of 73% in normal dent corn, and 81% in opaque-2 corn.

Two separate slurries of normal dent corn and one of opaque-2 corn were made from the flours with clean tap water to 45% solids in aluminum pots. The slurries were covered and allowed to undergo natural fermentation for a period of 24 hr at room temperature (28°C). L-Lysine-HCl and DL-tryptophan (ICN Pharmaceuticals, Inc.) dissolved in steep liquor were added at the end of fermentation to one of the fermented normal corn slurries to give 0.3% lysine and 0.1% tryptophan on a solids basis. Each fermented slurry was then thoroughly mixed in a Hobart mixer and aliquots taken for freeze drying. The remaining materials were drum dried with a laboratory atmospheric drum drier operated at a steam pressure of 50 psi, drum speed of 1.5 rpm, and drum spacing of 0.005 in. The dried flakes were comminuted into powder through a No. 18 standard sieve (U.S.A. Standard Sieve-Tyler equivalent 16 mesh), packed in approximately 0.5 kg quantities in medium density polyethylene bags and stored at 20°C and 30°C for 18 wk.

Chemical and organoleptic properties

Moisture, protein (N × 6.25) and total ash were determined on fresh and stored samples by AOAC methods (1975). Neutral detergent fiber and lignin were determined by the method of Goering and Van Soest (1970). Total amino acid profiles were obtained by acid hydrolysis of samples containing 18 mg protein with 6N HCl under vacuum at 110°C for 22 hr. Hydrolysates were filtered, evaporated to dryness and made to volume with pH 2.2 citrate buffer. Amino acids were determined by ion-exchange column chromatography with a Beckmann Model 120C amino acid analyzer. Tryptophan content was determined by the method of Horacio Hernandez and Bates (1969). Available lysine was determined by the method of Finley and Friedman (1973).

Organoleptic evaluation was based on color, texture, flavor and overall acceptability. The fresh and stored ogi powders were assessed by a taste panel consisting of 10 Nigerian male students at Cornell University. The ogi powders were reconstituted by mixing into water in a 1:7 powder to water ratio, warmed, served as coded randomized samples and scored on a 9-point hedonic scale. Tasters' preference scores were statistically analyzed by a split-plot design and means of significant characteristics were further analyzed by a protected Lsd test ($P < 0.05$).

Growth response

A measure of nutritional value was obtained by growth experiments using 21-day old male Sprague-Dawley rats weighing 50–60g. They were randomly assigned to experimental diets after feeding for 3 days on a commercial stock diet. The fresh and stored ogi powders were the sole protein sources contributing approximately 8% protein in the diets and were compared to an 8% casein diet. The diets further contained 4.0% mineral mix (Jones-Foster salt mixture, Nutritional Biochemicals Corp.), 2.2% vitamin fortification mix (ICN Pharmaceuticals, Inc.), corn oil as needed to provide 5.0% (Mazola, Best Foods), and 6.0% crude fiber (Alpha cel, ICN Pharmaceuticals, Inc.) plus corn starch (CPC International) to complete the casein diet. The rats were kept in individual wire-bottomed cages and fed the experimental diets from porcelain food cups covered with wire screens to prevent excessive spillage. Food and water were provided ad libitum. Individual feed consumption and body weights were recorded at 2- and 5-day intervals, respectively. Experimental feeding lasted 21 days after which PER values were calculated.

—Text continued on page 1572

Table 1—Compositions of raw corn and drum dried and freeze dried ogi powders

	% of Component[a]					
	Moisture	Dry matter	Protein (N X 6.25)	Neutral detergent fiber	Lignin	Total ash
Raw normal endosperm corn	9.33	90.67	9.71	11.55	1.48	1.26
Raw opaque-2 corn	9.51	90.49	9.83	13.98	1.82	1.46
Normal corn ogi – freeze dried	2.54	97.46	9.00	13.16	1.38	1.44
Supplemented normal corn ogi – freeze dried	2.93	97.07	8.33	13.46	1.32	1.70
Opaque-2 corn ogi – freeze dried	2.71	97.29	8.27	12.45	1.12	1.40
Normal corn ogi – drum dried	3.08	96.92	9.56	14.16	1.40	1.60
Supplemented normal corn ogi – drum dried	3.02	96.98	9.53	12.40	1.34	1.56
Opaque-2 ogi – drum dried	3.04	96.96	8.37	12.66	1.31	1.35

[a] Average of duplicate determinations. Data in last four columns are on dry basis.

Table 2—Amino acid compositions of corn kernels

Amino acid (g/16g N)	Normal corn	Opaque-2 corn
Lysine	2.29	3.88
Histidine	2.87	3.36
Ammonia	1.41	1.43
Arginine	4.29	6.79
Aspartic Acid	6.65	8.81
Threonine	3.50	3.75
Serine	4.61	4.84
Glutamic Acid	19.64	17.91
Proline	8.99	8.15
Glycine	3.49	4.91
Alanine	8.42	7.35
Half cystine	3.46	5.54
Valine	4.81	5.32
Methionine	1.80	1.94
Isoleucine	3.80	3.63
Leucine	15.11	11.16
Tyrosine	3.74	3.21
Phenylalanine	4.94	4.42
Tryptophan	0.40	0.80

Table 3—Total and available lysine contents of raw corn and drum-dried and freeze-dried ogi powders

Lysine (g/16g N)	Samples[a]							
	Freeze dried					Drum dried		
	I	II	III	IV	V	III	IV	V
Total	3.02	3.63	2.51	3.46	2.63	2.15	3.25	2.83
Available	2.49	3.32	2.36	3.16	2.41	1.46	2.36	1.97
% Available Lysine	82	91	94	91	92	68	73	70

[a] I – Raw normal endosperm corn; II – Raw opaque-2 corn; III – Normal corn ogi; IV – Supplemented normal corn ogi; and V – Opaque-2 corn ogi.

RESULTS & DISCUSSION

AT THE END of the 24-hr fermentation period the slurries dropped from an initial pH of 6.2 to a pH of 4.4 in normal endosperm corn and to 4.5 in opaque-2 corn. Acidity values were comparable to those obtained in a cultured ogi fermentation by Banigo et al. (1974).

Fermentation and drum drying did not result in appreciable changes in protein, fiber and total ash contents as seen in Table 1. Amino acid compositions of normal endosperm corn and opaque-2 corn (Table 2) were comparable to those found in the literature. Lysine and tryptophan contents of opaque-2 corn were about twice those of normal endosperm corn.

Total and available lysine contents of the raw corn, freeze-dried ogi and drum-dried ogi are presented in Table 3. Losses in total lysine in samples due to fermentation and drying ranged up to 29% and available lysine losses ranged up to 41%. The sugar-amine condensation reaction (Maillard reaction) is generously favored by the processing conditions, hence should be a major cause of lysine losses in the dried ogis because of the sensitivity of its epsilon amino group. Available lysine values were higher in the freeze dried ogi samples than in the drum dried ogi samples, reflecting the greater severity of drum drying. Tryptophan values shown in Table 4 increased slightly with fermentation and drying processes except in the supplemented normal corn ogis. The increases might have resulted from the fermenting organisms. The possible decrease in the supplemented normal corn drum dried ogi could reflect a loss of added tryptophan rather than of endogenous tryptophan.

Total and available lysine contents of ogi powders stored at 20°C and 30°C up to 18 wk are given in Table 5. Storage at both temperatures for 18 wk generally caused slight losses in total and available lysine values although the data were not consistent for the 30°C opaque-2 sample. Tryptophan contents of ogi powders stored at these temperatures are given in Table 6. There were no appreciable changes as a result of temperature or time.

The results of organoleptic evaluations indicate a general acceptance of all fresh and stored ogi powders. Tasters' preference scores on all samples were analyzed by a split-plot design as shown in Table 7. Preference differences based on storage time for color were significant at $P < 0.05$. Texture and overall acceptance perference differences were significant at $P < 0.01$. There were no significant differences with regards to flavor. Storage temperature X time interactions did not have any influence on preference. Tasters' preference for the different ogi types (Treatments) was highly significant in all the characteristics tested. Protected Lsd tests ($P < 0.05$) of the mean scores with respect to storage time (Table 8) indicate that there was no significant difference in preference for color and texture of ogi powders stored up to 12 wk. But preference declined significantly at 18 wk. The overall acceptance was significantly higher at 6 and 12 wk. Protected Lsd tests ($P < 0.05$) with respect to ogi types (Treatments) are given in Table 9. Tasters' preference was significantly higher for opaque-2 ogi in all the characteristics tested. Supplemented ogi scored

Table 4—Tryptophan contents[a] of raw corn and freeze-dried and drum-dried ogi powders

	Raw corn	Freeze-dried ogi	Drum-dried ogi
Normal corn	0.40	0.71	0.74
Supp. normal corn	1.50	1.57	1.40
Opaque-2 corn	0.80	0.87	1.10

[a] g/16g N

Table 5—Total and available lysine contents of stored ogi powders

	Sample I[a]			Sample II[a]			Sample III[a]		
	0	12	18	0	12	18	0	12	18
	Weeks								
	Stored at 20°C								
Total lysine (g/16g N)	2.15	1.80	1.73	3.25	2.49	2.56	2.83	2.67	2.11
Available lysine	1.46	1.45	1.19	2.36	2.01	1.21	1.97	2.15	1.39
% Available lysine	68	81	69	73	81	47	70	81	66
	Stored at 30°C								
Total lysine (g/16g N)	2.15	1.50	1.73	3.25	1.84	2.55	2.83	2.61	3.45
Available lysine	1.46	1.07	0.98	2.36	1.38	1.81	1.97	2.16	2.57
% Available lysine	68	71	56	73	75	71	70	83	74

[a] I – Normal corn ogi; II – Supplemented normal corn ogi; and III – Opaque-2 corn ogi.

significantly higher except for texture when compared to the unsupplemented normal corn ogi. The higher acceptance of opaque-2 corn ogi can be especially related to its softer texture and lighter color.

Data on average weight gains, food and protein consumed and protein efficiency ratios (PER) are presented in Table 10. The food consumption and PER values of all drum-dried ogi powders showed substantial reductions compared to these values for the corresponding milled raw corn diets. The stored ogi powders had PER values comparable to the freshly drum-dried powders. Losses of lysine, reduction in available lysine and other changes in ogi powders as a consequence of drum drying rather than storage appear to be the major factors responsible for reductions in consumption and nutritional quality.

From the above study it is concluded that ogi powders that are convenient to use and are organoleptically acceptable to

Table 6—Tryptophan contents[a] of stored ogi powders

	Weeks at 20°C			30°C	
	0	12	18	12	18
Normal corn ogi	0.74	0.66	0.85	0.65	0.97
Supp. normal corn ogi	1.40	1.25	1.52	1.37	1.65
Opaque-2 corn ogi	1.10	0.86	0.93	0.80	0.99

[a] g/16g N

Table 7—Analysis of variance on organoleptic data

Source	df	MS_{color}	$MS_{texture}$	MS_{flavor}	$MS_{overall\ acceptance}$
Whole plot					
Time	3	4.0579*	5.1804**	3.7762	6.3798**
Temp	1	0.6722	0.5014	0.2000	0.0125
Time X temp	2	0.5056	0.6681	0.3500	0.8042
Error	54	1.3907	0.8647	2.1774	1.3719
Split plot					
Treatments	2	62.0333**	13.4798**	8.5333**	29.7130**
Time X treatments	6	0.8222	0.5554	1.0500	0.8311
Temp X treatments	2	0.5056	0.1847	0.3167	0.3285
Time X temp X treatments	4	0.4639	0.1889	1.9167	0.2052
Error	126	1.1545	1.1753	1.5159	1.0609

* Significant at $P < 0.05$
** Significant at $P < 0.01$

Table 8—Storage time preference score means

Storage time (wk)	No. of observations	Means[a] Color	Texture	Overall acceptance
0	30	6.2000AB	6.6000C	6.0667E
6	60	6.4667A	6.7333C	6.6533F
12	60	6.5667A	6.7750C	6.6433F
18	60	5.9833B	6.1333D	6.0250E

[a] Means followed by the same letter are not significantly different by protected Lsd test $P < 0.05$.

Table 9—Treatment preference score means

Treatment	No. of observations	Means[a] Color	Texture	Flavor	Overall acceptance
Normal corn ogi	70	5.4429A	6.2143D	6.0143F	5.8086I
Supp. normal corn ogi	70	6.2000B	6.4000D	6.4714G	6.2600J
Opaque-2 corn ogi	70	7.3143C	7.0500E	6.7000H	7.0929K

[a] Means followed by the same letter are not significantly different by protected Lsd test $P < 0.05$.

Table 10—Growth response of rats fed corn and ogi diets[a]

	Experimental diet	Weight gain (g)	Food consumed (g)	Protein consumed (g)	Adjusted PER
I	Casein	92.07	338.87	27.11	2.50
II	Raw normal corn	47.54	294.49	25.17	1.39
III	Raw opaque-2 corn	64.73	321.31	25.61	1.86
IV	Freshly drum-dried normal corn ogi	−1.25	156.06	13.14	−0.07
V	Freshly drum-dried normal corn ogi + 0.3% lysine + 0.1% tryptophan	13.81	201.81	16.66	0.61
VI	Freshly drum-dried opaque-2 corn ogi	16.06	209.81	15.97	0.74
VII	Normal corn ogi stored 30°C, 18 wk	15.70	182.74	16.04	0.72
VIII	Normal corn ogi + 0.3% lysine + 0.1% tryptophan stored 30°C, 18 wk	6.78	160.80	13.86	0.36
IX	Opaque-2 corn ogi stored 30°C, 18 wk	17.90	194.07	15.68	0.84

[a] Eight rats per dietary treatment; duration of experiment 21 days.

Nigerian tasters can be produced from dry milled corn that is then fermented and drum dried. Such products can be organoleptically stable for at least 12 wk at 30°C, which is close to room temperature in the tropics. However, there was an appreciable decline in nutritional quality, as evidenced by decreased PER values, as a result of processing. Such products might be improved by fortifying ogi powders with lysine and other amino acids after drum drying. Though products were of low protein quality they would provide a good calorie source for consumption with other protein-containing foods.

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Ms received 10/15/77; revised 3/2/78; accepted 3/8/78.

The authors express their thanks to Dr. L.W. Schaible of Crow's Hybrid Corn Company for supplying the corn and to Mr. George E. Houghton, Dept. of Food Science, for assistance with statistical analysis of the data.

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min B_6 is concerned the U.S. RDA values derived from Pederson's data are low, but this is because his work only covered 2-months storage. Since this vitamin has been reported to increase in concentration in potatoes during storage, this value cannot be considered to be representative. The U.S. RDA value derived from the mean vitamin B_6 value reported by Page and Hanning (1963) is 25. However, this mean value appears to be the arithmetic mean of all measurements and did not take into account consumption patterns. If this were done, then the U.S. RDA values becomes 20.

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Ms received 11/12/77; revised 2/18/78; accepted 2/25/78.

The authors express their appreciation to the Potato Board for their financial support which made this work possible; to Ms. Bee Marks of Botsford and Ketchum and to Dr. Bernice K. Watts for their helpful suggestions in the design of this investigation.

Published as Research Paper No. 77512 of the Idaho Agricultural Experiment Station.

FUNCTIONAL PROPERTIES OF HYDROLYZED LACTOSE: RELATIVE SWEETNESS

N. O. SHAH and T. A. NICKERSON

ABSTRACT

A sensory panel of 11 judges determined the relative sweetness of lactose/glucose/galactose mixtures simulating hydrolyzed lactose syrups in a semi-model system similar to unflavored ice cream mix. The hydrolyzed syrups were evaluated at two levels of substitutions (25% and 50%) of the sucrose in the control mix, which contained 15% sucrose. A paired-comparison method was used, and the samples were served at 4.5 ± 0.7°C. The equisweetness point was determined graphically. The data showed a synergistic sweetness effect. A small amount of lactose enhanced sweetness, e.g., less sucrose was needed for equisweetness when 25% of the sucrose was replaced with 70%-hydrolyzed lactose syrup than with 100%-hydrolyzed syrup.

INTRODUCTION

THERE IS considerable interest in hydrolysis of lactose into glucose and galactose, both enzymatically (Mahoney et al., 1975) and chemically (Coughlin and Nickerson, 1975). The resulting sugars, glucose and galactose, are known to be sweeter than lactose itself (Amerine et al., 1965), but little is known about the sweetness of mixtures of the three sugars. The sweetness of a sugar is normally expressed in terms of units relative to sucrose, since sucrose is the criterion the public uses, consciously or unconsciously. Amerine et al. (1965) described many studies in which various sugars were compared with sucrose as to sweetness.

The relative sweetness of sugars is not a constant relationship, but depends on many factors: pH, temperature, and other constituents. Mixtures of sugars can give a different sweetness impression than individual sugars alone; synergistic sweetness results from a combination of sugars. This suggests that a sweetness advantage may result from a partial hydrolysis that yields a mixture of the three sugars: lactose, glucose and galactose.

This study evaluated the relative sweetness of simple syrups representing hydrolyzed lactose (lactose/glucose/galactose mixtures) in a semi-model system simulating an unflavored ice cream mix. The syrups were evaluated at two levels of substitution (25% and 50%) to a control mix of 15% sucrose. This is part of a study of the functional properties of hydrolyzed lactose (Shah and Nickerson, 1978a, b).

MATERIALS & METHODS

ALPHA-LACTOSE HYDRATE powder (USP grade) was obtained from Foremost Food Company, San Francisco; D-glucose anhydrous (analytical reagent) from Mallinckrodt; D-galactose anhydrous from Nutrition Biochemicals Corporation; and commercial sucrose.

Syrups were prepared from pure sugars (lactose, glucose, galactose) equivalent to lactose hydrolyzed 70, 90 and 100%. The two levels of hydrolyzed lactose used in the test mixes were 3.75% and 7.5% [this was a 25% and a 50% substitution of the sucrose (15%) in the control mix]. The test mixes also contained various levels of sucrose, in the range shown in Table 1, and were compared against the control mix.

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0022-1147/78/0005-1575$02.25/0

Table 1—Sugar constituents in the test mixes

Equivalent % hydrolysis	Level of substitution by hydrolyzed mixture for sucrose in control	Range of sucrose in test mixes (in 0.5% increments)
100% hydrolysis	3.75%[a]	11.5–13.5%
90% hydrolysis	3.75%	10.5–12.5%
70% hydrolysis	3.75%	11.5–13.5%
100% hydrolysis	7.50%[b]	7.5–9.5%
90% hydrolysis	7.50%	8.0–10.0%
70% hydrolysis	7.50%	8.5–10.5%

[a] Equal to 25% of the sucrose in the control mix (15%)
[b] Equal to 50% of the sucrose in the control mix (15%)

Relative sweetness was evaluated by sensory techniques. A paired-comparison method was used, with the judges presented simultaneously with two samples to judge which seemed sweeter. Samples were served in opaque blue 50-ml beakers with two- and three-digit coded aluminum lids. A 30-ml sample was poured into each beaker. Samples, served at 4.5 ± 0.7°C, were placed in ice and left in the refrigerator until the judges arrived. Each serving tray held five paired tests (10 beakers), and the judges usually completed five paired tests per day. Tasting took place 4 days of the week between 10 a.m. and 11 a.m., the first day of the week being the familiarization day; thus three replications were obtained for each sample. Samples were evaluated in individual booths, under red illumination to mask color differences. Distilled water at 23°C was provided for oral rinsing between pairs but not within pairs, which would destroy the sensitivity of the paired-comparison test. Judges were instructed not to swallow the samples, but to use the cuspidors supplied. The samples were randomized within each pair and between pairs in order to minimize the effect of one treatment upon another. Make-up tests were given to absentees by making a fresh batch of the solution the day before. To prevent sweetness fatigue, this test was delayed for an hour after the day's scheduled test. Initially the control mix was compared with the test mix, which had 100%-hydrolyzed mixture at 25% substitution, and then with the test mix, which had 100%-hydrolyzed mixture at 50% substitution. This procedure was then repeated with mixtures that were hydrolyzed 90% and 70%.

Screening trials

There were five screening-trial judges, including the experimenters. Each judge tasted each mix five times (n = 25). Screening trials were carried out a week before the judges were selected and before each main experiment. Screening trials were used to determine: (1) a concentration of sucrose just noticeably different from 15% sucrose in water, for use in selecting panel judges; and (2) the concentration range of sucrose in the test mixes, so that the range of sucrose concentration would be sure to bracket the equisweetness level (Table 1).

Judge selection

Judges were screened and selected on the basis of their ability to distinguish sweetness in a model system of 15% sucrose vs 14.25% sucrose in distilled water at refrigerator temperature (5°C). Selected from 22 prospects were 11 judges, five males and six females, aged 20–50 yr. These judges were able to differentiate the concentration of the stimulus at the 95% probability level.

Ice cream mix preparation

The ice cream mix (semi-model system) differed from commercial ice cream in that it had no added salt, color, stabilizer, or flavor. The ice cream mix was made from a commercial brand of "half and half" and concentrated milk, which were free of a noticeable cooked flavor. The desired composition was calculated by the "Pearson Square" method; the fat was 12%, and solids-not-fat was 11%.

The ice cream mix without the sweetener was prepared on the Friday before each week's experiment and stored at 0°C. Mojonnier and Babcock tests were carried out to determine % fat, % total solids (TS),

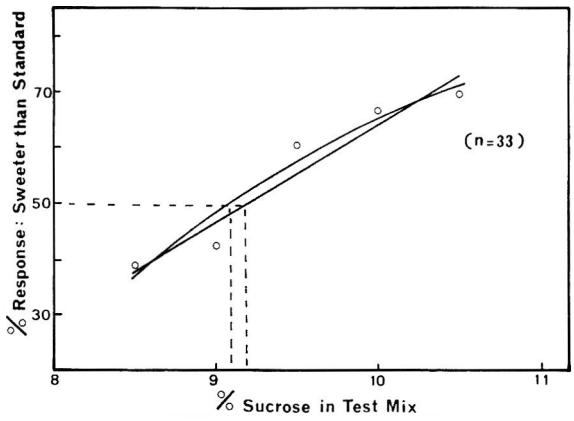

Fig. 1—Representative graph; sweetness of test mixes containing 7.50% hydrolyzed mixture (70% hydrolysis) relative to 15% sucrose in control mix. Serving temperature 4.5 ± 0.73° C.

Table 2—Sucrose level needed to produce equisweet test and control mixes

Mixes	Syrup	Sucrose	Total sugar solids in mix	Replacement ratio[b]
Control	—	15%	15%	—
25% Substitution level				
100% hydrolyzed mixture	3.75%	12.83%	16.58%	1.73
90% hydrolyzed mixture	3.75%	11.72%	15.47%	1.14
70% hydrolyzed mixture	3.75%	12.20%	15.95%	1.34
50% Substitution level				
100% hydrolyzed mixture	7.5%	8.55%	16.05%	1.16
90% hydrolyzed mixture	7.5%	8.56%	16.06%	1.16
70% hydrolyzed mixture	7.5%	9.10%	16.60%	1.27

[a] Composition: % SNF 10.91 ± 0.12; % Fat 11.91 ± 0.49; % TS 22.83 ± 0.46

[b] % hydrolyzed lactose to replace 1% sucrose at the level of substitution indicated.

and % solids-not-fat. At noon the day before tasting, the required amount of mix was brought rapidly to room temperature by placing it in a water-bath at 40°C and stirring continuously. The test mixes were prepared with percent sugars as required and as determined by the screening-trial judges. Prepared solutions were left standing in a dark room for 6 hr and then refrigerated overnight to slowly equilibrate to the serving temperature. This procedure also assures mutarotation equilibrium. After the solutions were measured into the beakers, the serving trays were refrigerated for at least 1 hr before serving. The temperature of the served samples was established by recording the temperature of the middle sample of the first tray served and the final tray served that day. From the responses of the first day (familiarization day), it was possible, if necessary, to adjust the percent sucrose in the test mixes so that they fell on either side of the 50% response.

Analyses

Judges' evaluations were decoded daily and recorded. The response was the number of times the judge found the test mix sweeter than the control mix. Equisweetness, i.e., the point of subjective equality, was the concentration where 50% of the judges perceived the test mix to be sweeter than the control mix. Equivalent sweetness levels were determined for each of the test mixes from the panel data. The mean temperature ± standard deviation of the serving samples was recorded. The data determined by the Mojonnier and the Babcock tests were also recorded, and standard deviations were calculated.

RESULTS & DISCUSSION

LACTOSE, glucose and galactose have α- and β-forms and therefore mutarotate. An equilibrium is essential between the two forms ($\alpha \leftrightharpoons \beta$) since Pangborn and Gee (1961) have shown that the sweetness of the mutarotating sugars depends on the sugar's form. It has also been shown that 90% of the equilibrium is reached within 1½ hr at room temperature, whereas the remaining 10% takes 10½ hr. At a higher temperature, 70°C, however, equilibrium is reached within 10 min (Hudson, 1908).

The method adopted consisted of leaving the prepared test mixes (including the control mix, which had the nonmutarotating sugar, sucrose, as the sweetener) at room temperature in a dark room for 6 hr and then refrigerating overnight, allowing the temperature to slowly reach 5°C. The composition of the ice cream mix was 11.91 ± 0.49% fat, 10.91 ± 0.12 solids-not-fat and 22.83 ± 0.46% TS.

Judge responses as to which was sweeter, the test mix or the control, were plotted as shown in Figure 1 to determine equisweetness. Linear and parabolic regression lines were drawn for all the graphs. In almost all cases, parabolic regression lines fit the data slightly better, although in most cases there was little difference between equisweetness points calculated from the two regression lines.

The sucrose level needed for equisweetness in each of the test mixes is shown in Table 2; for example, when 3.75% of the 100% hydrolyzed lactose syrup was used, it required 12.83% sucrose to give the same sweetness as in the 15% sucrose control mix. The relative sweetness of the hydrolyzed lactose was greater when it was used at the higher 50% substitution level, as shown by the lower replacement ratios (% syrup to replace 1% sucrose) in Table 2.

In each of the test mixes, the amount of sucrose needed for equisweetness was less than would be predicted from the relative sweetness of the individual sugars. At the concentration used in the mixes glucose and galactose have nearly the same sweetness and slightly less than 60% as sweet as sucrose. Lactose is even less sweet (about 40%) (Amerine et al., 1965). The 100% hydrolyzed mixture used at the 25% substitution level had a relative sweetness of 57% (1.73 replacement ratio), which is the expected value. However, all other mixtures show much higher relative sweetness levels (low replacement ratios, Table 2). The data thus indicate a synergistic enhancing of sweetness when these three sugars are mixed.

Overall, it can be concluded that sugar mixtures equivalent to hydrolyzed lactose syrups have good sweetening properties and appear acceptable for use in foods. Complete hydrolysis appears unnecessary, for results were good at both 70% and 90% hydrolysis levels.

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Ms received 12/16/77; revised 3/16/78; accepted 3/18/78.

Presented at the 36th Annual Meeting of the Institute of Food Technologists, Anaheim, CA June 6-9, 1976.

This work was supported in part by a grant from Whey Products Institute, Chicago, IL.

The authors thank Mr. E.E. Moore for excellent technical assistance.

SWEETENING OF COFFEE AND TEA WITH FRUCTOSE–SACCHARIN MIXTURES

LEA HYVÖNEN, RAKEL KURKELA, PEKKA KOIVISTOINEN and ANNI RATILAINEN

ABSTRACT

For reducing the calories from coffee and tea without reducing the conventional sweetness, drinks sweetened with mixtures of fructose and saccharin were prepared. Mixtures of fructose and saccharin, the sweetness of which in coffee, lemon tea and iced lemon tea corresponded to that of 5% sucrose in the drinks, were prepared. Magnitude estimation and forced-choice paired comparison tests were used as methods. Using a triangle test with a trained panel it was checked whether the mixture-sweetened drinks were distinguishable from those sweetened with sucrose. In consumer tests a single sample test was used. Energy saving of 50–70% compared to equally sweet sucrose-sweetened coffee and tea could be achieved without deterioration of other taste qualities.

INTRODUCTION

DIABETES, interest in low-calorie foods and fluctuation of the price of sucrose have awakened the need for sugar substitutes, especially for low-calorie sweeteners. Problems with aftertastes of the noncaloric sweeteners have focused the interest of investigators to combinations of noncaloric sweeteners, and to mixtures of noncaloric and new carbohydrate sweeteners which could provide lower cost, better taste quality and the necessary functional properties for low-calorie foods (Paul, 1921; Sale and Skinner, 1922; Cameron, 1947; Kamen, 1958; Stone and Oliver, 1969; Weickmann et al., 1969; Yamaguchi et al., 1970; Moskowitz and Klarman, 1975).

In our earlier studies (Hyvönen et al., 1978) the sweetness of fructose-saccharin and xylitol-saccharin mixtures was found to be enhanced in coffee compared to their sweetness in water solutions. In most instances mixtures of sugars exhibit some degree of synergism (Moskowitz and Toscano, 1975). In addition there may be optimal combinations of sweeteners as to the extent of synergism (Weickmann et al., 1969; Hyvönen et al., 1978). The enhanced sweetness can be used for reducing calorie intake.

The purpose of the present study was to search for a mixture of fructose and saccharin, which would sweeten coffee and tea to the sweetness level commonly used (about 5% sucrose). The energy content of the mixture would be as low as possible without noticeable aftertaste of saccharin.

EXPERIMENTAL

Materials

Sucrose (purity 99.9%) and fructose (purity 99.5%) (Finnish Sugar Co. Ltd. Finland) and Na-saccharin (purity >97%) (Apodan, Denmark) were used. Coffee was Finnish Kultamokka filter coffee and tea was Twining Ceylon Breakfast Tea.

Panels

The laboratory panel of 10 members, five women and five men, aged 20–35 years, who had 2 years' experience in the judging of sugars made their judgements daily between 10:30 and 11:00 a.m. in separate partitioned booths.

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0022-1147/78/0005-1577$02.25/0

Students of the Faculty of Forestry and Agriculture and the personnel of the Department of Food Chemistry and Technology (45 persons) participated in consumer tests as well as the consumer panel of the Finnish Co-operative Wholesale Society (SOK) consisting of 24 consumer members.

Tests

In the sensory evaluations by the trained judges magnitude estimation, paired comparison and triangle tests were used. In consumer tests a single sample test was applied.

Magnitude estimation was repeated three times and paired comparison and traingle tests twice by the panel.

Sample preparation

Coffee was prepared by filtering using 40g of coffee in 1 liter of boiling water. 20g of tea were steeped in 1 liter of boiling water. This was diluted 2:10 for tasting. 10 ml pressed lemon juice was added to 1 liter of tea for lemon tea.

Sample presentation

In laboratory tests, coffee and tea were served in three-digit coded 50 ml glasses at 55°C and tea was also served at 7°C. In consumer tests beverages were served in cups and the situation also resembled an otherwise normal occasion of drinking coffee.

In magnitude estimation tests eight coffee or tea samples were given to each judge per session. The drinks were sweetened with four different concentrations of fructose (2, 3, 4 and 5%) and sucrose (2, 3, 4 and 5%) or saccharin (0.007, 0.009, 0.012, 0.016%) and sucrose (2, 3, 4 and 5%), respectively. The presentation order of the samples was randomized.

The judges were asked to give relative values to the sweetnesses of coffee (or tea). The judges could select any of the coffee (or tea) samples as the standard and compare the sweetness of others to it using a ratio scale. The assigned values were afterwards normalized to the standard value 10, which represented the sweetness of the drink sweetened with 5% sucrose present in each test.

In forced-choice paired comparison tests four pairs, whose presentation order and order within a pair were randomized, were presented to each judge per session. The judges were asked to indicate the sweeter member of each pair. Coffee (tea) sweetened with fructose and saccharin was one member of the pair and the other was a sucrose-sweetened one (from 5–9% of sucrose). In the graphic evaluation the percentage of judges, who had regarded the sucrose-sweetened drink of the pairs sweeter, was plotted against sucrose concentrations in the drink. At the point where 50% of the judges regarded the sucrose-sweetened drink as sweeter, the drinks were considered isosweet (Fig. 1).

In magnitude estimation, paired comparison and triangle tests the judges were asked not to swallow the samples and to rinse their mouths with distilled water of room temperature between samples. In consumer tests the drinks were swallowed normally. A cup of coffee or tea without cream was served to the panelists. Beverages sweetened with a fructose-saccharin mixture and with sucrose were served in fully randomized order; they were equal in numbers in a test. The judges were asked whether the beverage was sweetened with sucrose or "saccharin" and the time it took for them to recognize the sweetener was observed.

Procedure for the determination of the equality of the sweetness of mixtures and sucrose in drinks

From the averages of the normalized responses in magnitude estimation tests graphs were drawn in which the abscissa represented fructose or saccharin concentrations and the ordinate the average sweetness values. Using these and extrapolated graphs for the concentrations below 2%, drinks, the sweetness of which was expected to correspond closely to the sweetness of commonly used coffee and tea, were prepared.

The proportions of fructose and saccharin in the mixture were varied and the isosweet sucrose concentration was determined by a forced-choice paired comparison test.

If the sweetness of the mixture in the drink was greater than that of 5% sucrose, the mixture was reconstituted taking into account the

Table 1—Composition of mixture-sweetened coffee, tea and iced tea, the sweetness of the mixture components from the relative sweetness graphs, the sum of the sweetness predicted, and the empirical sweetness of the drinks as isosweet sucrose concentration in a manner similar to Fig. 1. Sweetness value 10 refers to the sweetness of 5% sucrose in the drinks

Beverage (serving temp)	Mixture composition w/w	Empirical Component sweetness		Sum predicted	Empirical isosweet sucrose %
		Fructose	Saccharin		
	1% Fructose + 0.014% Saccharin	0.2	7.6	7.8	5.5
Coffee (55° C)	1.5% Fructose + 0.015% Saccharin	1.4	7.9	9.3	6.4
	2% Fructose + 0.0145% Saccharin	2.3	7.7	10.0	6.8
	2% Fructose + 0.014% Saccharin	2.8	7.2	10.0	6.6
Tea (55° C)	2.5% Fructose + 0.0126% Saccharin	3.6	6.4	10.0	6.8
	1% Fructose + 0.009% Saccharin	1.5	7.5	9.0	6.0
Iced Tea (7° C)	1% Fructose + 0.0102% Saccharin	1.5	8.5	10.0	6.3
	1.5% Fructose + 0.011% Saccharin	3.0	9.2	12.0	8.3
	2.0% Fructose + 0.0066% Saccharin	4.4	5.6	10.0	7.1

amount of extra sweetness and the ratio of the sweeteners in the mixture.

The reconstituted mixture-sweetened coffees and the sucrose-sweetened coffee (5%) were tested in a triangle test to find out if, for any reason they were distinguishable from each other.

Data analysis

The table by Roessler et al. (1948) and that by Bengtsson (1953) were used for estimation of the significance of the differences between drinks in triangle tests.

RESULTS

Coffee

The relative sweetness of fructose and saccharin by magnitude estimation at 55°C in coffee as a function of concentration is illustrated in Figure 2. The sweetness values were normalized to the standard value 10, representing the sweetness of the 5% sucrose in coffee which was included in every test.

Usually the sweetness of the mixture-sweetened coffee was greater than predicted (Table 1). The mixtures in coffees were reconstituted taking into account the extent of the synergism and the ratio of the sweeteners in the mixture (Table 2).

The reconstituted mixture-sweetened coffees and the coffee sweetened with 5% sucrose were tested in a triangle test. The experienced test panel could not distinguish coffee sweetened with 5% sucrose from the coffee sweetened with a fructose-saccharin mixture, when the proportion of saccharin in the mixture was 0.28% (w/w). With this mixture the resulting concentrations of fructose and saccharin in the coffee were 2.3 and 0.0065%, respectively. Similarly in the consumer tests, coffee sweetened with a fructose-saccharin mixture containing 0.3% saccharin was not distinguishable from the coffee sweetened with 5% sucrose.

Table 2—Composition of the mixture-sweetened test coffees compared to the reference coffee sweetened with 5% sucrose at 55° C and the results in triangle tests

	Conc of fructose and saccharin in test coffees	Wt-% saccharin in mixture	Proportion of correct responses
Drink 1	1% Fructose 0.016% Saccharin	1.6	24/40***
Drink 2	0.9% Fructose 0.013% Saccharin	1.4	19/40*
Drink 3	1.2% Fructose 0.012% Saccharin	1.0	23/40**
Drink 4	1.5% Fructose 0.0107% Saccharin	0.73	21/40*
Drink 5	1.7% Fructose 0.007% Saccharin	0.40	21/40*
Drink 6	2.3% Fructose 0.0068% Saccharin	0.30	11/20*
Drink 7	2.3% Fructose 0.0065% Saccharin	0.28	9/20
Drink 8	2.7% Fructose 0.0055% Saccharin	0.20	8/20

* Difference is significant at 5% level of probability
** Difference is significant at 1% level of probability
*** Difference is significant at 0.1% level of probability

Lemon tea, 55°C

The relative sweetness of fructose and saccharin in tea at 55°C as a function of concentration is illustrated in Figure 3. The sweetness values were normalized to the standard value 10, representing the sweetness of 5% sucrose in tea, which was included in every test.

Using these graphs lemon tea drinks, whose sweetness would theoretically correspond to the sweetness of lemon tea sweetened with 5% sucrose, were prepared (Table 1).

Sucrose-sweetened teas isosweet with mixture-sweetened lemon teas were determined by a paired comparison. Mixtures, where synergism existed were reconstituted and then tested in a triangle test with sucrose-sweetened lemon tea (Table 3).

Trained judges distinguished the mixture-sweetened lemon teas from that sweetened with sucrose (Table 3). Two of the

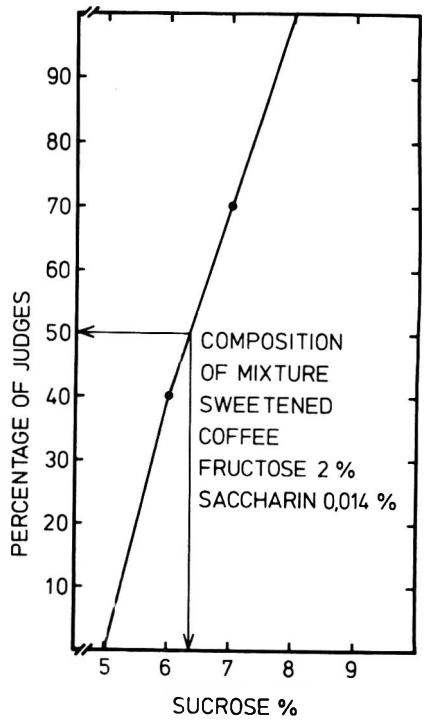

Fig. 1—Determination of the sucrose concentration of coffee which is isosweet to coffee sweetened with a fructose-saccharin mixture.

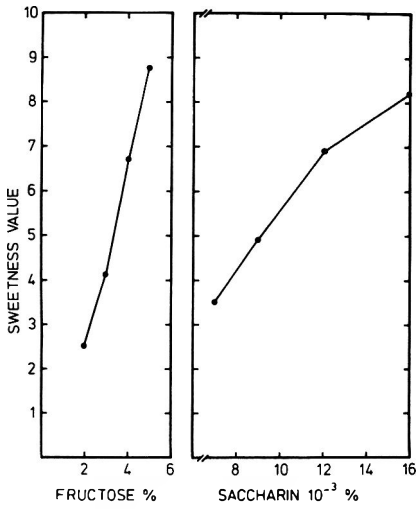

Fig. 2—Relative sweetness of fructose and saccharin in coffee at 55°C. The sweetness value 10 represents the sweetness of 5% sucrose.

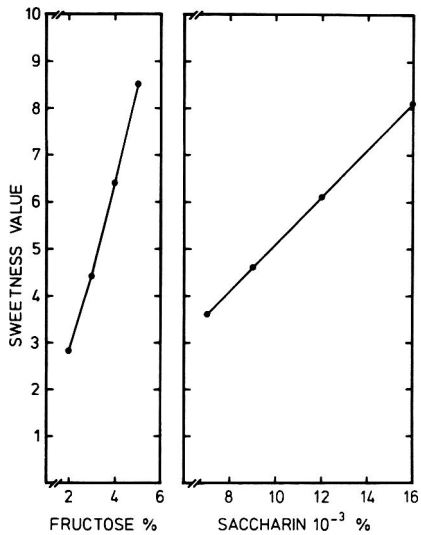

Fig. 3—Relative sweetness of fructose and saccharin in lemon tea at 55°C. The sweetness value 10 represents the sweetness of 5% sucrose.

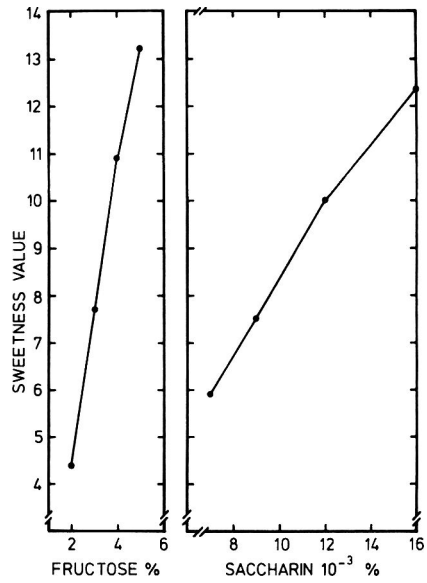

Fig. 4—Relative sweetness of fructose and saccharin in lemon tea at 7°C. The sweetness value 10 represents the sweetness of the 5% sucrose.

judges recognized the mixture-sweetened lemon tea on the basis of the bitter aftertaste almost without exception.

However, since most of the judges regarded the mixture-sweetened tea as pleasant tasting without any extra bitterness, lemon tea sweetened with fructose-saccharin mixture containing 0.73% saccharin was tested in a consumer test. Mixture-sweetened lemon tea and 5% sucrose-sweetened lemon tea were given to judges alternately in randomized order. The judges, who included both students and personnel of the University laboratories, recognized the sweetener used in 13 out of 33 cases. The sweeteners were not distinguishable in a normal situation of having a cup of tea.

The consumer panel of the Finnish Co-operative Wholesale Society (SOK), which consisted of 24 members, judged the lemon tea sweetened with fructose-saccharin mixture, where the proportion of saccharin was 0.3%, i.e. the same proportion as was indistinguishable from sucrose in coffee. Most of the judges, who could not positively identify, but rather guessed regarding the sweetener had considered the lemon flavor to be saccharin. Their proportion of correct answers was 17/24. The difference is significant at the 5% level of probability.

The panel that consisted of the personnel of the University laboratories and of students was unable to distinguish tea without lemon sweetened with fructose-saccharin mixture (0.3% saccharin) from tea sweetened with 5% sucrose at 55°C.

Table 3—Composition of the mixture-sweetened test lemon tea compared to the reference lemon tea sweetened with 5% sucrose at 55°C and the results in triangle tests

	Conc of fructose and saccharin in test teas	Wt-% saccharin in mixture	Proportion of correct responses
Drink 1	0.5% Fructose 0.016% Saccharin	1.6	30/40***
Drink 2	1.5% Fructose 0.011% Saccharin	0.73	26/40***
Drink 3	1.8% Fructose 0.0092% Saccharin	0.51	32/60**

** Difference is significant at 1% level of probability
*** Difference is significant at 0.1% level of probability

Iced tea

The relative sweetness of fructose and saccharin in lemon tea at 7°C as a function of concentration is illustrated in Figure 4. The sweetness values were normalized to the standard value 10, representing the sweetness of the 5% sucrose in iced lemon tea, which was present in every test. Using these graphs cold tea drinks, the sweetness of which corresponded closely to the sweetness of iced tea sweetened with 5% sucrose, were prepared (Table 1).

The sweetness of mixture-sweetened iced teas was determined by paired comparisons. If synergism was apparent the mixture was reconstituted. The cold teas sweetened with revised fructose-saccharin mixtures and with 5% sucrose were tested by a triangle test (Table 4). Iced tea sweetened with a fructose-saccharin mixture containing 0.73% saccharin was not distinguishable from the iced tea sweetened with 5% sucrose in a triangle test.

In a consumer test it was checked whether the proportion of saccharin in the mixture could be increased from 0.73%, without the trained panel noticing any aftertaste. Iced tea sweetened with a fructose-saccharin mixture containing 0.9% saccharin and iced tea sweetened with 5% sucrose were served randomly to the judges. Twelve out of 32 persons recognized

—Continued on page 1584

Table 4—Composition of the mixture-sweetened test lemon teas compared to the reference lemon tea sweetened with 5% sucrose at 7°C and the results in triangle tests

	Conc of fructose and saccharin in test teas	Wt-% saccharin in mixture	Proportion of correct responses
Drink 1	0.8% Fructose 0.0081% Saccharin	1.0	38/60***
Drink 2	0.8% Fructose 0.007% Saccharin	0.9	30/40***
Drink 3	0.9% Fructose 0.0066% Saccharin	0.73	17/40
Drink 4	1.4% Fructose 0.0046% Saccharin	0.33	20/40*

* Difference is significant at 5% level of probability
*** Difference is significant at 0.1% level of probability

SWEETENING OF SOFT DRINKS WITH MIXTURES OF SUGARS AND SACCHARIN

LEA HYVÖNEN, PEKKA KOIVISTOINEN and ANNI RATILAINEN

ABSTRACT

Low-calorie citrus base and cola-type soft drinks were prepared using 10% sucrose isosweet sugar-saccharin mixtures as sweeteners. The proportion of saccharin in the mixture, in which the aftertaste of saccharin was not noticed, was determined by an experienced taste panel. The equality of the sweetness of mixtures and 10% sucrose in the drinks was determined by the forced-choice paired comparison method. The noticeable differences between mixture-sweetened and sucrose-sweetened drinks were checked in the triangle test. The preferences of the drinks were tested by rank order and hedonic scaling tests. The test drinks were also compared with corresponding commercial beverages as well. In consumer tests it was checked, whether the mixture-sweetened drink was distinguishable from the sucrose-sweetened one in a normal condition of drinking, using single sample test. In consumer tests preferences were also tested by ranking. The following conclusions were drawn. To avoid aftertaste the proportion of saccharin in the sugar-saccharin mixture cannot be higher than 0.3% for sweetening cola-type and 0.4% for citrus-type soft drinks at the sweetness level of 10% sucrose. The test soft drinks sweetened with mixtures of sugar and saccharin were judged as good as or better than the corresponding conventional drinks on the market. Depending on the sugar component of the mixture the energy content of the drinks was 50–70% lower than that of the isosweet sucrose-sweetened one.

INTRODUCTION

CYCLAMATE, saccharin and their blends have been used to sweeten low-calorie beverages. Difficulties with aftertastes have been significant (Helgren et al., 1955; Bottle, 1964; Ziemba, 1969). In order to mask aftertaste and produce bulk and mouthfeel to the product many kinds of mixtures of sugars and synthetic sweeteners have been developed and patented (Weickmann et al., 1969). Among others starch and protein hydrolysates have been used as the bulking agent in low-calorie sweeteners to yield texture and flavor resembling sucrose.

Media have been found to have a profound effect on the sweetness of sweeteners and in addition synergistic effects have been noticed in proper combinations of sweeteners (Wicker, 1966; Kunst, 1971; Fricker et al., 1973; Hyvönen et al., 1978). The enhancement of sweetness can be utilized in developing low-calorie products. In our studies we have been able to sweeten coffee, tea and juice to conventional sweetness levels and achieving good sweetness quality with fructose-saccharin and xylitol-saccharin mixtures (Hynönen et al., 1978a, b).

The purpose of this study was to search for a mixture of fructose and saccharin, the sweetness of which in cola-type and citrus base soft drinks would correspond to the sweetness of commonly used 10% sucrose in the drinks. The mixture should have a low energy content, but the aftertaste of saccharin should not be detected in the saccharin concentration used. Later on it was tested, whether glucose, sucrose or invert sugar could be used in the corresponding mixtures.

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0022-1147/78/0005-1580$02.25/0

Table 1—Concentrations of fructose and saccharin in citrus base and cola-type soft drinks compared in pairs with sucrose-sweetened drinks of various concentrations, and concentrations of the respective isosweet sucrose-sweetened beverages

Mixture compositions	Concentrations of sucrose (%)	Isosweet sucrose conc (%)
Citrus base soft drink		
4.0% Fructose + 0.012% Saccharin	8, 10, 12, 14	12.0
5.0% Fructose + 0.02% Saccharin	12, 14, 16, 18	14.4
4.0% Fructose + 0.02% Saccharin	10, 12, 14, 16	12.5
3.2% Fructose + 0.0192% Saccharin	8, 10, 12, 14	11.3
Cola-type soft drink		
3.3% Fructose + 0.01% Saccharin	8, 10, 12, 14	10.2
3.5% Fructose + 0.0136% Saccharin	8, 10, 12, 14	11.7
3.2% Fructose + 0.016% Saccharin	8, 10, 12, 14 or 10, 12, 14, 16	12.0
2.7% Fructose + 0.0162% Saccharin	8, 10, 12, 14	10.8

EXPERIMENTAL

Materials

The sucrose, glucose and fructose used in the study were products of the Finnish Sugar Company Ltd. (Finland), and Na-saccharin was the product of Apodan (Denmark). The purity of sucrose was greater than 99.9% and that of glucose and fructose 99.5%. Invert sugar was made of glucose and fructose (50:50). Na-saccharin fulfilled the requirements of Farmakopea Nordica (1964). Citric acid (Merck, Darmstadt) of pro analyse purity and sodium benzoate (Riedel-de Haën A.G. Seelze b. Hannover) were used in extracts. For preparation of the citrus base soft drink, "Apelsin extract" (AB Fructus Fabriker, Sweden) was used. The cola-type soft drink was made using two different essences (Coca-Cola Export Corporation).

Commercial soft drinks used in rank order and hedonic scaling tests were as follows:

orange drink 1 sweetened with 0.025% saccharin
orange drink 2 sweetened with 0.016% saccharin
orange drink 3 sweetened with 2.0% sorbitol and 0.025% saccharin
orange drink 4 sweetened with 0.0126% saccharin
Jaffa 1 and Jaffa 2, two sucrose-sweetened orange drinks of two producers
Cola 1, Cola 2 and Cola 3, three sucrose-sweetened cola-type soft drinks of three producers

Methods

In the sensory evaluations paired comparison, triangle, rank order and hedonic scaling tests were used.

In paired comparison tests four pairs were given to each judge per session. The presentation order of the pairs and the order within pairs were randomized. The judges were asked to indicate the sweeter member of each pair. The mixtures were planned so that about one-half of the sweetness was derived from fructose and the other half from saccharin. The mixture-sweetened sample was one member of each pair and the other was a sucrose-sweetened sample whose sucrose concentration varied as shown in Table 1.

The percentage of judges who had regarded the sucrose-sweetened member of the pairs as sweeter was plotted against sucrose concentration in the test drink. The sucrose concentration, which was regarded by 50% of the judges as sweeter was determined from the curve, i.e. the mixture-sweetened and sucrose-sweetened drinks were found equally sweet at that point. The isosweet points were based on 20 judgements in two paired comparison tests.

In triangle tests the judges were asked to designate the odd sample within each triangle set.

In the rank order test the judges were asked to rank the beverages in order according to their preference. The preferred beverage hence had the lowest rank sum.

In the hedonic scaling test the judges estimated numerically the degree of beverage pleasantness: 1 = extremely unpleasant, 2 = very unpleasant, 3 = unpleasant, 4 = slightly unpleasant, 5 = slightly pleasant, 6 = pleasant, 7 = very pleasant and 8 = extremely pleasant.

In consumer tests the rank order method was used to determine preferences. A single sample test was used to identify the noticeable aftertaste of saccharin.

Panels

The laboratory panel consisted of 10 members, who had earlier experience in tasting corresponding sweetener solutions. They were people from the university laboratories, 20–35 years old. The judgements were made daily between 10:30–11:00 in the sensory evaluation laboratory of the Department of Food Chemistry and Technology.

There were two consumer panels. One was that of the Finnish Co-operative Wholesale Society (SOK). It consisted of 24 consumer members, who regularly taste test the products of SOK. The other panel consisted of the personnel and students of the Department.

Sample preparation

About 50 ml of the carbonated water from a prefilled bottle was decanted. Extract and sweetener were added into the bottle and sufficient amount of the carbonated water was poured back into the bottle to fill the bottle again. The bottle was closed immediately with a crown cork and after that shaken. The soft drinks were prepared and preserved in cold stores (4°C) in order to ensure that as little as possible CO_2 would be lost during the preparation. In general the drinks were judged the following day.

Citrus base soft drinks were prepared by adding 3 ml of "Apelsin extract," 0.55g of citric acid and the respective sweetener into the carbonated water (2.5–3.0 volumes CO_2) of 1/3 liter.

The extract for cola-type soft drink consisted of 60 ml of 25% sodium benzoate solution, 120 ml essence I, 480 ml essence II and 340 ml water. 2.5 ml of the extract were used in a 1/3-liter bottle of carbonated water (3.5–4.0 volumes CO_2).

Sample presentation

The soft drinks were served in three-digit coded 50 ml glasses at 7 ± 2°C. Samples were not swallowed and between samples the panelists rinsed their mouths with distilled water of room temperature. In single-sample consumer tests the drinks were served in normal glasses and samples were swallowed.

Data analysis

Analysis of variance and multiple range tests were used in estimating the significance of the differences between drinks in hedonic scaling tests. Expanded tables for determining significance of differences for ranked data (Kahan et al., 1973), the table by Roessler et al. (1948) and by Bengtsson (1953) for significance in triangle tests, and the table by Roessler et al. (1956) for paired tests were used in rank order, triangle and single sample tests, respectively.

Procedure of the tests

The sweetness of the soft drinks sweetened with mixtures of fructose and saccharin was determined by a forced-choice paired comparison method and expressed as isosweet sucrose concentration. The proportions of saccharin in the mixtures were respectively 0.3, 0.4, 0.5 and 0.6%. In order to obtain the equality of the sweetness of the mixtures to 10% sucrose the compositions of the mixtures were reconstituted by maintaining an unchanged ratio of the sweeteners in the mixture and assuming that the mixture concentration vs sweetness relation is linear within the range of small concentration differences. The sweetness of the soft drink thus obtained was compared to that of the beverage sweetened with 10% sucrose in a triangle test in order to discover noticeable differences. Reconstituted mixture-sweetened soft drinks were further tested in rank order and hedonic scaling tests.

The suitability of glucose-saccharin, sucrose-saccharin and invert sugar-saccharin mixtures for sweeteners in soft drinks was tested replacing the fructose component of the earlier mixtures by the same amount of glucose, sucrose or invert sugar. The isosweet sucrose concentration was determined by paired comparison. The reconstitution was carried out as before and the drinks were submitted to triangle tests to determine possible noticeable differences. The preferences were determined by rank order tests.

Table 2—Comparison of citrus base soft drinks sweetened with mixtures of fructose and saccharin with the isosweet drink sweetened with 10% sucrose in a triangle test

Fructose and saccharin conc in prepared beverages		Saccharin % (w/w) in sweetening mixture	Correct responses
Drink 1	3.3% Fructose 0.01% Saccharin	0.3	36/60***
Drink 2	3.5% Fructose 0.0136% Saccharin	0.4	15/40
Drink 3	3.2% Fructose 0.016% Saccharin	0.5	19/40*
Drink 4	2.8% Fructose 0.017% Saccharin	0.6	28/60*

*** Significant differentation P = 0.001
* Significant differentation P = 0.05

RESULTS

Fructose-saccharin mixtures in citrus base soft drinks

The concentrations of fructose and saccharin in the citrus base and cola-type soft drinks, which were compared in pairs with sucrose-sweetened drinks of various concentrations, and the resulting determinations of isosweet sucrose concentrations are given in Table 1.

Table 2 presents mixture-sweetened citrus base soft drinks reconstituted to isosweetness with 10% sucrose-sweetened drinks when the proportion of saccharin by weight in the sweetening mixture was respectively 0.3, 0.4, 0.5 and 0.6%. The responses to triangle tests of distinguishability between sucrose- and mixture-sweetened beverages are also given in Table 2.

On the basis of the triangle tests drink 2, which contained 3.5% fructose and 0.0136% saccharin was indistinguishable from the citrus base soft drink sweetened with 10% sucrose. Other mixture-sweetened citrus base soft drinks in this study were distinguished from the sucrose-sweetened soft drink on the basis of the quality of their sweetness.

In a consumer test, which was a single sample test, the tasters were asked, whether the soft drink was sweetened with either sucrose or "saccharin." Drink 2 (Table 2), which was indistinguishable from the sucrose-sweetened soft drink in a triangle test was tested in the consumer test. The panelists were personnel and students of the department. The sweetener was recognized correctly in 14 out of 21 cases. In other words the mixture-sweetened soft drink 2 was indistinguishable from the conventional sucrose-sweetened one in a normal situation of drinking soft drink.

The data of the rank order tests are given in Table 3. On the basis of the rank order tests the fructose-saccharin-sweetened citrus base soft drinks were regarded as better than the commercial soft drinks which were sweetened with either saccharin or a mixture of sorbitol and saccharin. The test soft drinks sweetened with the mixtures of fructose and saccharin were ranked as equally good or even better than the corresponding sucrose-sweetened commercial products. No significant difference between the citrus base soft drinks sweetened with different mixtures of fructose and saccharin were noticed, when only they were ranked, although two of them were found distinguishable from the sucrose-sweetened drink in the triangle test.

The average scores obtained in the hedonic scaling tests are given in Table 4. There was no significant difference between the commercial sucrose-sweetened Jaffa 1, Jaffa 2 and test drinks 1 and 2, which were sweetened with 3.3% fructose and 0.01% saccharin and with 3.5% fructose and 0.0136% saccharin, respectively. Test drink 3, which contained 3.2% fructose and 0.016% saccharin didn't differ significantly from industrial

Table 3—Data from the rank order tests of the citrus base soft drinks. Vertical comparisons, statistical significance according to superscript footnotes

Drink	Rank sum						
		Trained panel[a]					
	Consumer panel	Test 1	Test 2	Test 3	Test 4	Test 5	Test 6
Drink 1 3.3% Fructose 0.01% Saccharin	68	78	24			25	
Drink 2 3.5% Fructose 0.0136% Saccharin	61	73*	17*	32, 29[b]		29	
Drink 3 3.2% Fructose 0.016% Saccharin			32		25	20	
Drink 4 2.8% Fructose 0.017% Saccharin						26	18
Drink 5 10% Sucrose		73*					
"Jaffa" 1			36	22	14*		21
"Jaffa" 2	50*	112		30	21		22
Orange drink 1 (0.025% Saccharin)	79	138**					
Orange drink 2 (0.016% Saccharin)	100**	154**	46**				
Orange drink 3 (2.0% Sorbitol 0.025% Saccharin)				37			
Orange 4 (0.0126% Saccharin)					40**		39**

[a] Consumer panel = 24 judges; Test 1: 3 X 10 judgements; Test 2–6: 10 judgements
[b] 2 months in stock
* Significantly better than others at 1% level ($P < 0.01$)
** Significantly inferior to others at 1% level ($P < 0.01$)

Table 5—Comparison of the cola-type soft drinks sweetened with mixtures of fructose and saccharin with the isosweet drink sweetened with 10% sucrose in a triangle test

Fructose and saccharin contents in prepared beverages		Saccharin % (w/w) in sweetening mixture	Correct responses
Drink 1	3.2% Fructose 0.0097% Saccharin	0.3	16/40
Drink 2	3.0% Fructose 0.012% Saccharin	0.4	19/40*
Drink 3	2.7% Fructose 0.013% Saccharin	0.5	20/40*
Drink 4	2.5% Fructose 0.015% Saccharin	0.6	23/40**

* Significant differentation P = 0.05
** Significant differentation P = 0.01

Jaffa drinks or from the industrial orange drink 1 sweetened with 0.025% saccharin. On the other hand the test drink 2 and Jaffa 1 were significantly better than the commercial citrus base soft drink sweetened with 0.025% saccharin, but they did not differ significantly from test drink 1. Test drink 4, which was sweetened with 2.8% fructose and 0.017% saccharin was scored significantly better than the industrial orange drinks 2 and 4, which were sweetened with 0.016% saccharin and 0.0126% saccharin, when they and orange drink 1 were judged in the same session. Other drinks did not differ from each other significantly.

Fructose-saccharin mixtures in cola-type soft drinks

On the basis of triangle tests the cola-type drink 1, which

Table 4—Data from the hedonic scaling tests of the citrus base soft drinks[a]

Drink	Average panel scores			
	Test 1	Test 2	Test 3	Test 4
Drink 1 3.3% Fructose 0.01% Saccharin	5.6	5.4		
Drink 2 3.5% Fructose 0.0136% Saccharin	5.5	6.5*		
Drink 3 3.2% Fructose 0.016% Saccharin			5.6	
Drink 4 2.8% Fructose 0.017% Saccharin				5.8*
Jaffa 1	5.8	6.0*	4.3	
Jaffa 2	5.2		5.3	
Orange drink 1 (0.025% Saccharin)		4.3**	5.1	4.6
Orange drink 2 (0.016% Saccharin)				3.8**
Orange drink 4 (0.0126% Saccharin)				3.6**

[a] N = 10 judgements for each test
* Significantly better than **
** Significantly inferior to *

Table 6—Data from rank order tests of the cola-type soft drinks by a trained panel[a]

Drink	Rank sum		
	Test 1	Test 2	Test 3
Drink 1 3.2% Fructose 0.009% Saccharin	19	25	28
Drink 2 3.0% Fructose 0.012% Saccharin	26	24	
Drink 3 2.7% Fructose 0.013% Saccharin	23		
Drink 4 2.5% Fructose 0.015% Saccharin	32		36
Drink 5 10% Sucrose		27	
Cola 1		24	25
Cola 2		29	22
Cola 3			39

[a] N = 10 judgements for each test

contained 3.2% fructose and 0.0097% saccharin, was indistinguishable from the similar drink sweetened with sucrose. Other mixture-sweetened cola-type soft drinks in this study differed significantly from the sucrose-sweetened one. As the saccharin proportion in the mixture was increased the difference became more and more pronounced (Table 5).

In rank order tests the different mixture-sweetened cola-type soft drinks did not significantly differ from each other. The difference between mixture-sweetened test drinks and commercial sucrose-sweetened cola-type soft drinks was also insignificant (Table 6).

The same conclusion could be drawn on the basis of the hedonic scaling test, e.g. the mixture-sweetened cola-type soft drinks neither differed from each other nor from the commercial products included in the test. Only once (in the test 2) was a commercial Cola 3 judged significantly less pleasant than Cola 1, Cola 2 and the test drink, which was sweetened with 2.7% fructose and 0.0134% saccharin (Table 7).

Comparison of isosweet glucose-saccharin, sucrose-saccharin, invert sugar-saccharin and fructose-saccharin mixtures in soft drinks

The results from the comparison of the citrus base soft drinks sweetened with various sugar-saccharin mixtures with

Table 7—Data from the hedonic scaling tests of the cola-type soft drinks[a]

Drink		Average panel scores Test 1	Test 2	Test 3
Drink 1	3.2% Fructose 0.0097% Saccharin	4.9		5.4
Drink 2	3.0% Fructose 0.012% Saccharin	4.9		
Drink 3	2.7% Fructose 0.0134% Saccharin		5.2	
Drink 4	2.5% Fructose 0.015% Saccharin			5.7
Cola 1		5.0	5.1	5.0
Cola 2		5.3	5.4	
Cola 3			4.1**	5.7

[a] N = 10 judgements for each test
** Significantly inferior to others

Table 8—Comparison of citrus base soft drinks sweetened with mixtures of various sugars and saccharin with the isosweet drink sweetened with 10% sucrose in a triangle test

Conc of sweeteners in the drink	Correct responses
0.4% saccharin (w/w) in the mixture	
3.5% Fructose, 0.0136% Saccharin	15/40
4.9% Glucose, 0.0195% Saccharin	18/40
4.2% Sucrose, 0.0167% Saccharin	19/40*
3.3% Invert sugar, 0.0133% Saccharin	20/40*
0.6% saccharin (w/w) in the mixture	
2.8% Fructose, 0.017% Saccharin	28/60*
3.5% Glucose, 0.021% Saccharin	22/40**
3.3% Sucrose, 0.020% Saccharin	19/40*
2.8% Invert sugar, 0.017% Saccharin	20/40*

* Significant differentation P = 0.05
** Significant differentation P = 0.01

the isosweet drink sweetened with 10% sucrose in a triangle test are given in Table 8. When the proportion of saccharin in the mixture was 0.4% the experienced panel could not distinguish the soft drinks sweetened with glucose-saccharin or fructose-saccharin mixtures from the sucrose-sweetened one. The panel distinguished the citrus base soft drinks sweetened with sucrose-saccharin and invert sugar-saccharin mixtures from the sucrose-sweetened one. It was not the aftertaste of saccharin that was regarded as the reason for differentation in the triangle test, but rather the typical flavor of the different sugars. When the saccharin proportion of the mixture was 0.6%, all the sugar-saccharin mixtures in this study were distinguishable from sucrose in the citrus base soft drink.

Data from the preference tests of the citrus base soft drinks are given in Table 9. The drink sweetened with the mixture of fructose and saccharin was ranked as superior to, and the drink sweetened with the mixture of invert sugar and saccharin as inferior to the other ones. The proportion of saccharin in these mixtures was 0.4%. When the proportion of saccharin in the sugar-saccharin mixtures was 0.6%, the drinks did not differ significantly from each other.

In the cola-type soft drink the fructose-saccharin and sucrose-saccharin mixtures (0.3% saccharin) were not distinguished from sucrose by the trained panel. Corresponding glucose-saccharin and invert sugar-saccharin mixtures differed significantly from sucrose. The trained panel also was able to distinguish all the sugar-saccharin mixtures containing 0.6% saccharin from sucrose in the cola-type soft drink (Table 10).

Table 9—Data from the rank order tests of the citrus base soft drinks sweetened with isosweet various sugar-saccharin mixtures and corresponding commercial sucrose-sweetened drinks

Conc of sweeteners in the drink	Rank sum
0.4% saccharin in the mixture	
3.5% Fructose, 0.0136% Saccharin	22*
4.9% Glucose, 0.0195% Saccharin	35
4.2% Sucrose, 0.0167% Saccharin	27
3.3% Invert sugar, 0.0133% Saccharin	39**
Jaffa 1	28
0.6% saccharin in the mixture	
2.8% Fructose, 0.017% Saccharin	23
3.5% Glucose, 0.021% Saccharin	27
3.3% Sucrose, 0.020% Saccharin	25
2.8% Invert sugar, 0.017% Saccharin	25

[a] N = 10 judgements for each test
* Significantly superior to the other drinks at the 5% level P < 0.05
** Significantly inferior to the other drinks at the 5% level P < 0.05

No significant difference in preference was noticed between the cola drinks sweetened with different sugar-saccharin mixtures (Table 11). The commercial Cola drink 1 was ranked significantly inferior to the test drinks. The reason was probably the higher amount of CO_2, which was found less pleasant.

—Text continued on page 1584

Table 10—Comparison of cola-type soft drinks sweetened with mixtures of various sugars and saccharin with an isosweet drink sweetened with 10% sucrose in a triangle test

Conc of sweeteners in the drink	Correct responses
0.3% saccharin in the mixture	
3.2% Fructose, 0.0097% Saccharin	16/40
4.5% Glucose, 0.0135% Saccharin	23/40*
4.3% Sucrose, 0.013% Saccharin	17/40
3.7% Invert sugar, 0.0112% Saccharin	23/40*
0.6% saccharin in the mixture	
2.5% Fructose, 0.015% Saccharin	23/40*
3.4% Glucose, 0.0204% Saccharin	31/40**
3.2% Sucrose, 0.0193% Saccharin	27/40**
2.8% Invert sugar, 0.0168% Saccharin	27/40**

* Significant differentation P = 0.01
** Significant differentation P < 0.001

Table 11—Data from the rank order tests of the cola-type soft drinks sweetened with isosweet sugar-saccharin mixtures and a commercial Cola 1[a]

Conc of sweeteners in the drink	Rank sum
0.3% saccharin in the mixture	
3.2% Fructose, 0.0097% Saccharin	26
4.5% Glucose, 0.0135% Saccharin	33
4.3% Sucrose, 0.013% Saccharin	25
3.7% Invert sugar, 0.0112% Saccharin	28
Cola 1	38*
0.6% saccharin in the mixture	
2.5% Fructose, 0.015% Saccharin	31
3.4% Glucose, 0.0204% Saccharin	33
3.2% Sucrose, 0.0193% Saccharin	30
2.8% Invert sugar, 0.0168% Saccharin	31
Cola 1	34

[a] N = 10 judgements for each test
* Significantly inferior to the other drinks at the 5% level (P < 0.05)

CONCLUSIONS

A CITRUS BASE soft drink can be sweetened with a mixture of fructose and saccharin to a level which is isosweet with a 10% sucrose-sweetened beverage with neither bitterness nor aftertaste due to saccharin being noticed when the proportion of saccharin in the mixture is 0.4%. If the proportion of saccharin in the mixture is greater than 0.4%, the aftertaste begins to appear while drinking.

A cola-type soft drink needs a smaller amount of a fructose-saccharin mixture than a citrus base soft drink for producing sweetness equal to that of a 10% sucrose-sweetened beverage. To avoid the aftertaste of saccharin in cola-type beverages its proportion in the mixture cannot be higher than 0.3% at the sweetness level of 10% sucrose.

The mixtures of glucose and saccharin, sucrose and saccharin, and invert sugar and saccharin are useful as sweeteners in soft drinks. However, the citrus base soft drink sweetened with the mixture of fructose and saccharin (0.4% saccharin) was regarded as very good, even better than the sucrose-sweetened one. If the soft drinks are sweetened with the mixtures of sugar and saccharin, the energy content can be reduced to levels which are 50–70% lower than that of conventional sucrose-sweetened drinks while still maintaining good sweetness quality. Earlier Neumann (1924) drew similar conclusions from his studies in which he replaced different proportions of sucrose in foods by saccharin. A somewhat bitter taste was evident when more than 60–80% of the sugar was substituted.

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Ms received 12/23/77; revised 3/17/78; accepted 3/20/78.

We thank Oy Mallasjuoma (Finland) for carbonated water, and AB Fructus Fabriker (Sweden) and the Coca-Cola Export Corporation for beverage bases received as gifts for this study. The study was supported by the National Research Council for Agriculture and Forestry of the Academy of Finland and the Finnish Sugar Co. Ltd.

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the sweetener used. In other words the mixture-sweetened iced tea and iced tea sweetened with 5% sucrose were not distinguishable, when consumed in a "normal" situation.

The speed of sweetener identification (correct or incorrect) depended more on the mentality of the individual than on some other logical reason.

CONCLUSIONS

A FRUCTOSE-SACCHARIN MIXTURE, which contains 0.73% saccharin, can be used to sweeten lemon tea. Only a few people tasted any extra or unpleasant bitterness due to saccharin.

When coffee is sweetened with a fructose-saccharin mixture containing 0.73% saccharin, a number of people taste the sweetness of saccharin either as metalic or bitter. The mixture-sweetened coffee is not distinguishable from the coffee sweetened with sucrose until the proportion of saccharin in the fructose-saccharin mixture is 0.3%.

The mixture of fructose and saccharin, whose sweetness is equal to that of 5% sucrose-sweetened lemon tea containing 7g of sucrose, consists of 2.2g of fructose and 0.16g of saccharin. In this case the energy contents of the isosweet amounts of the sweetening mixture and of sucrose were respectively 36 and 118 kJ. By using the fructose-saccharin mixture (0.73% saccharin) instead of sucrose the energy content of a sweetened beverage can be lowered about 70%.

In general the amount of fructose-saccharin mixture that can be used instead of sucrose to sweeten coffee, tea or lemon tea to the conventional sweetness level without distinguishable difference in sweetness quality and acceptability consists of 2.4g of fructose and 0.010g of saccharin. The energy content of this amount of the fructose-saccharin mixture is 56 kJ, or about half of that of the isosweet amount of sucrose.

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Ms received 12/23/77; revised 3/17/78; accepted 3/20/78.

The study has been supported by the National Research Council for Agriculture and Forestry of the Academy of Finland and the Finnish Sugar Co. Ltd.

ASSESSMENT OF ROASTING EFFECTS ON VITAMIN B_6 STABILITY AND BIOAVAILABILITY IN DEHYDRATED FOOD SYSTEMS

J. F. GREGORY and J. R. KIRK

ABSTRACT

Dehydrated food systems were employed to study the stability and bioavailability of vitamin B_6 as affected by roasting at 180°C for 25 min. The roasting conditions were selected to permit estimation of the maximum effects of the roasting process on the B_6 vitamers and are above those which would be normally used for commercial processing. The relative degradation of pyridoxine, pyridoxamine, and pyridoxal phosphate was found to be 50–70% by both microbiological and semiautomated fluorometric assay methods. Estimates of biologically available vitamin B_6 in the roasted model systems, as determined by rat bioassay correlated closely with microbiological results. Thus, vitamin B_6 remaining after roasting at 180°C for 25 min was biologically available and active. Semiautomated fluorometric values for total vitamin B_6 in untreated and roasted systems were 1.65–3.63 times higher than corresponding microbiological assay data. Fluorescence spectra studies of samples prepared for the semiautomated assay revealed no detectable interfering compounds. Microbiological assay provided an accurate evaluation of available vitamin B_6 in the roasted model food system as shown by the rat bioassay. The consistently high results from the semiautomated fluorometric method indicate that further work is required before this method can be accurately used to quantitatively determine the level of vitamin B_6 in foods.

INTRODUCTION

THE STABILITY of vitamin B_6 during the thermal processing of foods has been examined in a variety of products. Relatively few studies have been carried out on the effects of thermal processing on the bioavailability of the B_6 vitamers. Furthermore, systematic investigations of vitamin B_6 stability and bioavailability as affected by food composition and processing conditions have never been reported.

Most studies concerning the stability of vitamin B_6 during food processing have dealt with retort processing of canned foods. Processing of beans, meats, and tomato juice concentrate at 120°C for 30–45 min has been reported to result in a maximum destruction of 20% of the total vitamin B_6 (Everson et al., 1964; Raab et al., 1973; Miller et al., 1973; Daoud et al., 1977). The retorting of evaporated milk and nonfortified liquid infant formula was found to induce varying apparent losses of the naturally occurring B_6 vitamers, depending on the specificity of the microbiological assay organism employed. Losses of 40–60% were observed using *Saccharomyces uvarum*, while assays with *Neurospora sitophilia* revealed little degradation (Hassinen et al., 1954; Gregory, 1959). Hassinen et al. (1954), demonstrated that milk fortified with pyridoxine (PN) exhibited little loss of the added vitamin during sterilization and storage. Milk samples experimentally fortified with pyridoxal (PL) or pyridoxamine (PM) were shown to exhibit the same rate of vitamin B_6 degradation as determined for the vitamin B_6 occurring naturally in milk.

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0022-1147/78/0005-1585$02.25/0

Table 1—Composition of dehydrated model system for determination of roasting effects

Component	Percent by weight
Casein, vitamin free test[a]	5
Corn syrup solids[b]	37
Starch[c]	50
Cellulose (Alphacel)[d]	7
Sodium chloride	1

[a] General Biochemicals, Inc.
[b] Malto-Dextrin (D.E. = 15). Provided approximately 5% reducing sugar (American Maize)
[c] Food grade powdered starch (A.E. Staley Mfg. Co.)
[d] ICN Pharmaceuticals, Inc.

The effects of thermal processing on the biological availability of vitamin B_6 in foods have been most thoroughly studied in dairy products. Tomarelli et al. (1955), observed excellent agreement between rat bioassay and the *S. uvarum* microbiological assay for sterilized infant formula which had been fortified with PN. In nonfortified sterilized infant formula and sterilized milk, rat bioassay data indicated that only 50% of the total vitamin B_6 as measured microbiologically was available to the rats (Tomarelli et al., 1955). In contrast, Davies et al. (1959), reported rat and chick bioassay data which were significantly greater than *S. uvarum* results for vitamin B_6 in retorted evaporated milk. These conflicting data indicate that the effect of retorting on the bioavailability of vitamin B_6 is unclear.

Recently, Yen et al. (1976) reported that the roasting of shelled corn in the production of animal feed induced large losses of available vitamin B_6. Whether these losses represented thermal destruction of the vitamin or decreased bioavailability could not be determined from their data.

In this study, a dehydrated model food system was fortified with various forms of vitamin B_6 and subjected to a roasting process to determine the effect of roasting on the B_6 vitamers. The stability of the added vitamers was determined by the microbiological method of Haskell and Snell (1970) and the semiautomated fluorometric procedure of Gregory and Kirk (1977a) to provide a direct comparison of the two analytical techniques. In addition the roasted model systems were analyzed by rat bioassay to determine the content of biologically available vitamin B_6. This provided an assessment of the effect of roasting on the bioavailability of vitamin B_6.

EXPERIMENTAL

Model system

The dehydrated food system simulating breakfast cereal composition (Table 1) was a modification of that designed by Kirk et al. (1977). Four model systems were prepared, differing only in the chemical form of vitamin B_6 used for fortification. Model systems were fortified with pyridoxal phosphate monohydrate and pyridoxamine dihydrochloride obtained from ICN Pharmaceuticals, Inc., and pyridoxine hydrochloride from Sigma Chemical Co. A nonfortified blank was also prepared. The ingredients of each model system were homogenized as a slurry of 40% solids by mixing all ingredients in distilled water in a Waring Blendor. Vitamin B_6 fortification to a level of 25% NAS/NRC RDA (0.5 mg) per ounce of dry model system was performed by

thoroughly mixing aqueous solutions of the respective vitamers with the slurry.

The slurries were transferred to shallow trays, cooled to −40°C, and dried in a Virtis Model FFD 42 WS Freeze-Dryer at a platen temperature of 43°C to a constant absolute pressure of 5 μm Hg.

After drying, the model systems were finely powdered in a Waring Blendor and layered on stainless steel trays at a thickness of approximately 3 mm. The trays were placed in a 180°C oven and held for 25 min, after which the model systems had a golden brown appearance. The roasted model systems and aliquots of each taken prior to roasting were stored in stoppered glass containers at 2°C until assayed for their vitamin B_6 contents.

Determination of Vitamin B_6

Total vitamin B_6 was determined microbiologically by the *Saccharomyces uvarum* turbidimetric method of Haskell and Snell (1970). The individual B_6 vitamers were quantitatively analyzed by the semiautomated fluorometric method of Gregory and Kirk (1977a), in which each of the B_6 vitamers is determined after its conversion to 4-pyridoxic acid lactone. Each vitamer was determined as its nonphosphorylated free base.

Rat bioassay methods

The concentration of biologically available vitamin B_6 in the roasted model systems was determined by a modification of the methods of Sarma et al. (1946), and Linkswiler et al. (1951).

All diets were of the composition shown in Table 2 and were prepared by thoroughly mixing the diet ingredients in a Hobart mixer. The composition of the standard diets was identical to that of the basal diet with the exception of the addition of a dry pyridoxine hydrochloride premix (prepared in casein) to a level of 0.25, 0.50, 0.75, and 1.00 μg PN per gram of diet. The test diets were prepared by blending the finely powdered roasted model systems into the basal diet at a level of 5% by weight. Replacement of basal diet ingredients was based on the model system proximate composition to maintain uniform dietary composition and caloric density with the addition of the roasted model systems.

Male weanling Sprague-Dawley rats (Spartan Research Animals, Inc., Haslett, MI) were randomly assigned into 9 groups of 8 rats each. The rats were individually housed in metal cages with raised wire mesh floors. Water was supplied ad libitum. The basal diet was fed to all rats for 14 days to induce a mild state of vitamin B_6 deficiency. The rats were weighed at the start of the assay period and weekly thereafter. Standard (0.00, 0.25, 0.50, 0.75, and 1.00 μg PN/g) and test (containing 5% roasted model systems) diets were fed to the respective groups ad libitum for 21 days. Feed consumption was carefully measured. Spillage was found to be low and, therefore, it was neglected.

Blood was prepared for assay of erythrocyte aspartate aminotransferase (AspAT) by the following procedure. After the 21 days assay period, the rats were killed by decapitation. Blood was collected into 16 × 100 mm screw cap test tubes containing several drops of a 1000 U/ml aqueous solution of heparin (Grade 1, sodium salt; Sigma Chemical Co.). Immediately after collection, the samples were gently mixed with the heparin solution and centrifuged at approximately 3000 rpm in a clinical centrifuge. The plasma of each sample was removed by aspiration, and the erythrocytes washed by suspension in cold 0.9% (w/v) saline and recentrifuged. After removal of the saline wash solution, the packed erythrocytes were stored at −25°C until assayed. On the day of the AspAT assays, the test tubes containing erythrocytes were warmed to ambient temperature and 10 ml of distilled water added to each. The contents were then thoroughly mixed on a Vortex mixer and cell debris sedimented by centrifugation in a clinical centrifuge at 3000 rpm. Aliquots of the supernatant hemolysates were taken for assay of AspAT activity and hemoglobin content without further dilution.

Table 2—Composition of diets for rat bioassay

Ingredient	Basal and standard diets	Test diets
Casein (vitamin free, test)[a]	19.80	18.85
DL-Methionine, N.R.C.[b]	0.20	0.20
Sucrose	60.50	56.45
Cellulose (Alphacel)[a]	9.40	9.40
Salt mix[c]	4.00	4.00
Vitamin mix[d]	1.10	1.10
Corn oil	5.00	5.00
Model system	—	5.00

[a] ICN Pharmaceuticals, Inc.

[b] Grand Island Biological Co.

[c] Teklad Test Diets; Wesson modified Osborne-Mendel; $ZnCO_3$ added to provide 15 ppm Zn in all diets.

[d] Vitamin mix (ICN Pharmaceuticals, Inc.) provided (per kg diet): vitamin A, 9900 units; vitamin D, 1100 units; α-tocopherol, 55 mg; choline chloride, 825 mg; menadione, 25 mg; niacin, 50 mg; riboflavin, 11 mg; calcium pantothenate, 33 mg; thiamine, 0.22 mg; folic acid, 0.99 mg; vitamin B_{12}, 0.015 mg.

The activity of erythrocyte AspAT was assayed, in the presence and absence of pyridoxal phosphate added in vitro, by a slight modification of the automated serum glutamic-oxaloacetic transaminase method of Levine and Hill (1966). This procedure is based on the coupling of malate dehydrogenase to the AspAT reaction, with fluorometric quantitation of the oxidation of reduced nicotinamide adenine dinucleotide. A standard curve for enzyme activity was determined by assaying serial dilutions of a hemolysate of high activity using a Technicon AutoAnalyzer. The AspAT activity of the standard hemolysate was independently determined spectrophotometrically by the Calbiochem (1975) procedure. After calibration of the AutoAnalyzer, all other hemolysates were assayed by the modified automated procedure. The stimulation of AspAT activity by the in vitro addition of PLP was performed by adjusting 0.9 ml of each hemolysate to 0.820 mM with respect to added PLP by the addition of 0.1 ml of an 8.20 mM aqueous solution of PLP (Bayoumi and Rosalki, 1976). Following incubation for 30 min at ambient temperature, the hemolysates were reassayed. The percent stimulation of AspAT activity induced by the in vitro addition of PLP was calculated from the observed enzyme activity in the presence and absence of PLP.

Determination of hemoglobin (Hb) in the hemolysates was performed according to the automated colorimetric method described by Technicon (1969). Working standards were prepared by dissolving bovine hemoglobin (2 × crystallized, type I; Sigma Chemical Co.) in distilled water and diluting over a range of 4–20 mg/ml.

The bioassay results were evaluated by comparison of the response of the animals on the test diets to the dose-response results of animals fed the standard diets. Dose-response curves were determined by linear regression techniques (Neter and Wasserman, 1974). The logarithm of the dietary PN concentration (μg/g) was plotted against rat growth (g) and rat growth per gram feed consumed (g/g). The dietary PN concentration data were coded to permit taking the log of 0.00 μg PN/g by the linear transformation of adding 1.00 to each standard concentration, as described by Bliss and White (1967). AspAT activity (mU/mg Hb) and PLP stimulation (%) dose-response curves were determined by plotting these enzyme data against linear dietary PN concentration, which provided a better fit than the log dose-response functions.

Statistical methods

The presence of significant differences among the rat bioassay estimates of available vitamin B_6 using the various response criteria of B_6 status (growth, growth per gram feed, AspAT activity, and PLP stimulation) was tested for each model system by analysis of variance using a randomized complete block design.

Completely randomized distribution analysis of variance (ANOVA) was used to compare estimates by any one of the bioassay criteria with the microbiological and fluorometric assay data. An ANOVA was run repeatedly for each model system for which the microbiological and fluorometric assay data were tested against the bioassay data based on each of the response parameters. This repeated analysis method was necessitated because of the lack of independence among the bioassay estimates; that is, for each model system, each of the four bioassay estimates was derived from the response of the same group of rats. To achieve a family error rate of $\alpha = 0.05$, the critical probability employed in each of the four ANOVA's for each model system was $\alpha/4 = 0.0125$. All statistical procedures were described by Neter and Wasserman (1974).

RESULTS & DISCUSSION

PYRIDOXINE, pyridoxamine, and pyridoxal phosphate were employed in the fortification of the dehydrated model food systems to determine the relative stability of these vitamers during roasting. Pyridoxine hydrochloride is routinely employed in food fortification because of its stability observed in retort processing (Hassinen et al., 1954) and storage (Bunting,

1965; Cort et al., 1976; Anderson et al., 1976). PM and PLP are the predominant naturally occurring forms of vitamin B_6. PLP has been observed to bind to protein during the thermal processing of liquid model food systems in a manner which may affect its bioavailability (Gregory and Kirk, 1977b). Through the interaction of the amino group of PM with carbonyl groups of reducing sugars in liquid systems, the bioavailability of PM may be reduced by a similar process (Gregory, 1977).

The stability and bioavailability of the B_6 vitamers as affected by roasting has not been previously determined. The severe process conditions of 180°C for 25 min were selected to permit estimation of the maximum losses occurring in foods during roasting and do not represent normal processing conditions.

The results of the semiautomated fluorometric determination of vitamin B_6 in control and roasted model systems are presented in Table 3. Thermally induced losses of total vitamin B_6 were observed in each model system. The decrease in PM concentration and corresponding increase in PL concentration in the PM-fortified system after roasting at 180°C for 25 min suggested the occurrence of transamination reactions in the model systems. No evidence of a conversion of PLP to PM or its phosphate (PMP) was observed. Previously reported transamination reactions in food processing have involved the formation of PM and PMP from PL and PLP (Hodson, 1956; Gregory, 1959; Gregory and Mabbitt, 1961; Polansky and Toepfer, 1969).

Comparison of the results of model system vitamin B_6 assays by semiautomated fluorometric and microbiological methods (Tables 3 and 4) indicated large differences in the apparent vitamin B_6 contents. Fluorometric values for total B_6 were greater in all cases. Thermally induced losses of total vitamin B_6 were comparable as assessed by either method, ranging from 53–73%, except in the case of the microbiological analysis of the PM-fortified system. Microbiological determination of thermal losses of vitamin B_6 in the PM-fortified model system indicated significantly lower losses than observed in all other systems analyzed. Previous researchers have reported that the assay organism, *S. uvarum*, exhibits a reduced growth response for PM, with the apparent B_6 potency of PM being only 50–60% of that of PN (Rabinowitz and Snell, 1948; Parrish et al., 1955; Gregory, 1959; Woodring and Storvick, 1960; Chin, 1975). Therefore, the microbiological assay of the PM-fortified system would yield erroneously low results. The reductions in PM concentration in the PM-fortified system by thermal destruction and partial transamination to PL (Table 3) would lessen the bias in the microbiological assay after roasting because of the lower PM content relative to the other vitamers. These factors would account for the apparent greater stability of PM as measured microbiologically. In total, these data suggest that the thermal stability of PN, PM, and PLP was comparable in the dehydrated model food systems during roasting at 180°C for 25 min.

—*Text continued on page 1588*

Table 3—Vitamin B_6 in control and roasted model systems, as determined by the semiautomated fluorometric assay procedure[a,b]

Fortification	PN[c]	PM[d]	PL[f]	Total	% Loss
Control					
Nonfortified	1.4 ± 1.6	15.1 ± 3.0	2.5 ± 0.7	19.0 ± 3.5	–
PN[c]	18.6 ± 1.1	13.9 ± 4.5	2.0 ± 1.3	34.5 ± 4.8	–
PM[d]	9.7 ± 1.8	24.5 ± 4.2	1.6 ± 0.9	35.2 ± 4.7	–
PLP[e]	2.1 ± 0.6	8.1 ± 2.6	15.1 ± 0.3	25.3 ± 2.7	–
Roasted					
Nonfortified	1.2 ± 1.1	2.8 ± 1.4	1.2 ± 0.0	5.2 ± 1.8	73
PN[c]	4.4 ± 0.4	2.1 ± 0.5	7.9 ± 1.2	14.4 ± 1.4	58
PM[d]	2.8 ± 1.2	2.1 ± 0.3	5.6 ± 1.2	10.5 ± 1.7	70
PLP[e]	3.7 ± 0.4	1.5 ± 1.1	5.8 ± 0.4	11.0 ± 1.2	57

[a] μg/g model system.
[b] Mean and standard deviation, triplicate determinations.
[c] Pyridoxine
[d] Pyridoxamine
[e] Pyridoxal-5-phosphate
[f] Pyridoxal

Table 4—Microbiological determination of total vitamin B_6 in control and model systems[a,b]

Model system fortification	Control	Roasted	% Loss
Nonfortified	1.1 ± 0.3	1.0 ± 0.4	–
PN[c]	19.6 ± 3.6	9.3 ± 0.8	53
PM[d]	9.7 ± 2.4	6.2 ± 1.6	36
PLP[e]	13.6 ± 0.8	5.1 ± 0.5	63

[a] μg total vitamin B_6 /g model system
[b] Mean and standard deviation; Duplicate determinations
[c] Pyridoxine
[d] Pyridoxamine
[e] Pyridoxal-5-phosphate
[f] Pyridoxal

Table 5—Rat bioassay of roasted model systems[a,b]

Diet	Rat growth	Feed consumed (g)	Growth (g) per g feed consumed (g/g)	Erythrocyte AspAT Activity (mU/mg Hb)	Erythrocyte AspAT PLP Stimulation (%)
Standard					
0.00 μg PN[c]/g	44 ± 3	179 ± 5	0.243 ± 0.009	7.00 ± 0.68	61.0 ± 5.7
0.25 PN[c]/g	78 ± 4	227 ± 4	0.346 ± 0.018	7.18 ± 0.72	59.4 ± 8.9
0.50 PN[c]/g	102 ± 6	261 ±10	0.387 ± 0.012	9.52 ± 1.52	53.0 ± 3.8
0.75 PN[c]/g	134 ± 5	333 ± 9	0.401 ± 0.008	9.27 ± 0.95	43.5 ± 2.5
1.00 PN[c]/g	140 ± 5	347 ±14	0.406 ± 0.010	14.17 ± 1.60	23.8 ± 3.3
5% Roasted model systems					
Nonfortified	51 ± 6	211 ± 14	0.236 ± 0.017	6.45 ± 0.51	65.4 ± 5.8
PN[c]	84 ± 4	248 ± 9	0.338 ± 0.005	9.20 ± 1.07	56.0 ± 4.4
PM[d]	77 ± 7	241 ± 5	0.305 ± 0.029	7.48 ± 1.31	57.9 ± 5.4
PLP[e]	90 ± 5	258 ± 9	0.351 ± 0.014	7.91 ± 0.62	54.1 ± 4.2

[a] Mean and standard error, 8 rats per dietary group
[b] Growth, feed consumption, and growth/g feed consumed were for the 21 day test period following a 2-wk depletion.
[c] Pyridoxine
[d] Pyridoxamine
[e] Pyridoxal-5-phosphate

Table 6—Linear regression parameters of the rat bioassay dose-response curves

X[a]	Y	Y-intercept	Slope	Correlation coefficient
log (PN[b] + 1)	Growth (g)	44.9 ± 3.7	334 ± 19	+0.9425
log (PN[b] + 1)	Growth (g)/g feed	0.270 ± 0.012	0.530 ± 0.059	+0.8235
(PN)	AspAT activity	6.15 ± 0.92	6.57 ± 1.50	+0.5778
(PN)	AspAT PLP stimulation	66.3 ± 3.8	−36.7 ± 5.6	−0.7660

[a] All X data represents the dietary concentration of added pyridoxine. When the log was taken, the concentrations were coded by the addition of 1 to each value.
[b] Pyridoxine

The results of the rat bioassay for available vitamin B_6 in the roasted model systems are presented in Table 5. The dose-response curves for the various response criteria were calculated from the responses of rats fed the standard (0–1.0 μg PN/g) diets and their regression parameters shown in Table 6. Inspection of the correlation coefficients and relative standard deviations indicated that the rat growth data provided the greatest precision of the dose-response curves and, thus, assay sensitivity. The dose-response curves for growth, growth per gram feed, and AspAT activity were similar to those previously reported (Sarma et al., 1946; Linkswiler et al., 1951; Lushbough et al., 1959; Beaton and Cheney, 1965; Brin and Thiele, 1967). The inverse relationship between PLP stimulation of AspAT activity and dietary vitamin B_6 concentration is in agreement with that first observed by Raica and Sauberlich (1964).

Rat bioassay estimates of biologically available vitamin B_6 in each roasted model system based on the various response criteria (Table 7) were not significantly different ($P < 0.05$). As suggested by the precision of the dose-response curves, the growth-based estimates of available vitamin B_6 provided the smallest relative errors. The excellent correlation between estimates of available B_6 based on growth and growth per gram feed indicates that potential biases in the bioassay results due to avoidance of, or preference for, certain test diets were not significant in this study. In addition, the low responses to the diet containing the nonfortified model system indicate that the incorporation of the model system at the 5% level did not interfere with the bioassay by altering the intestinal synthesis of vitamin B_6. Sensitivity to diet composition, particularly with regard to the source of carbohydrate, is a potential source of interference in animal vitamin B_6 bioassays (Sarma et al., 1946; Waibel et al., 1952).

The comparison of the vitamin B_6 contents of the roasted model systems, as assessed by microbiological and semiautomated fluorometric methods and rat bioassay, are presented in Table 8. No significant differences were observed between rat bioassay estimates of biologically available vitamin B_6 and microbiologically determined total B_6 values for each model system ($P < 0.05$). These results indicate that the vitamin B_6 remaining after the roasting process retained full biological availability and activity. By contrast, the results of the semiautomated fluorometric assay were significantly greater than microbiological and rat bioassay values for each model system ($P < 0.05$).

The results of these studies indicate that, although the semiautomated fluorometric procedure correlated reasonably well with the microbiological assay in determining the relative loss of the B_6 vitamers during roasting, it was not a satisfactory indicator of available vitamin B_6 in these dehydrated model systems. In thin-layer chromatographic studies, Gregory and Kirk (1977a) were unable to detect the presence of interfering

Table 7—Biologically available vitamin B_6 in roasted model systems.[a] Data represent μg available B_6/g model system

Model system fortification	Rat bioassay response criterion[b,c]			
			Erythrocyte AspAT	
	Growth	Growth (g) per g feed consumed	Activity	PLP stimulation
Nonfortified	1.4 ± 0.8	−2.4 ± 1.2	0.9 ± 1.6	0.5 ± 3.2
PN[d]	6.3 ± 0.7	7.0 ± 0.6	2.8 ± 2.5	5.6 ± 2.4
PM[e]	5.2 ± 1.2	4.7 ± 3.6	1.5 ± 2.7	5.1 ± 2.9
PLP[f]	7.5 ± 0.8	8.8 ± 1.8	4.5 ± 1.9	4.6 ± 2.0

[a] Mean and standard error, 8 rats per assay group
[b] Response criterion refers to the index of B_6 nutritional status used in calculation of the available vitamin B_6 in the model system.
[c] Estimates of available vitamin B_6 by each of the response criteria were not significantly different for each of the model systems ($P < 0.05$).
[d] Pyridoxine
[e] Pyridoxamine
[f] Pyridoxal-5-phosphate

Table 8—Comparison of rat bioassay estimates of biologically available vitamin B_6 in roasted model systems with microbiological and semi-automated fluorometric assay results for total vitamin B_6[a,b]

Model system fortification	Rat bioassay response criterion					
			AspAT[f]			
	Growth	Growth (g) per g feed consumed	Activity	PLP stimulation	Microbiological	Fluorometric
Nonfortified	1.4	−2.4	0.9	0.5	1.0	5.2
PN[c]	6.3	7.0	2.8	5.6	9.3	14.4
PM[d]	5.2	4.7	1.5	5.1	6.2	10.5
PLP[e]	7.5	8.8	4.5	4.6	5.1	11.0

[a] μg total B_6/g model system
[b] No significant difference between microbiological and rat bioassay estimates; fluorometric data were significantly greater than microbiological and rat bioassay ($P < 0.05$).
[c] Pyridoxine
[d] Pyridoxamine
[e] Pyridoxal-5-phosphate
[f] Erythrocyte aspartate aminotransferase

compounds in sample extracts after the preparative ion exchange chromatography of the semiautomated assay. However, the observed poor correlations of the fluorometric assay values with the microbiological and rat bioassay results suggest the presence of a compound(s) which interfere with the accurate measurement of the B_6 vitamers as 4-pyridoxic acid lactone.

In an attempt to identify the source of the interference, the fluorescence emission spectra of model system extracts treated for fluorometric analysis, and their assay blanks, were examined. As performed in the semiautomated assay, all samples were adjusted to pH 10.2 with 0.4M sodium carbonate, 0.2% Brij 35, prior to spectral analysis. The emission spectrum of the fluorphore, 4-pyridoxic acid lactone, was manually calculated by subtraction of the emission spectra of the assay blanks from those of chemically treated samples. All samples and blanks were prepared in a manner identical to that followed in the fluorometric assay. At the excitation wavelength of 355 nm, the fluorophore emission spectra for all model system extracts were found to be highly similar to that of 4-pyridoxic acid lactone formed from standards. No significant differences were observed in the emission maximum or peak width (full width at half maximum).

The fact that the emission spectra were highly similar suggests that the interfering compound is converted to 4-pyridoxic acid lactone or a very similar product during the formation of the fluorophore in the semiautomated assay. The results of Table 3 suggest that the interfering compounds possesses thermal sensitivity similar to that of the B_6 vitamers. The heat sensitivity and fluorescence spectra indicate that the interfering compound may be a vitamin B_6 derivative which possesses little or no biological activity. Further research is required to identify this compound and alleviate the problem associated with the semiautomated fluorometric assay.

In summary, the roasting of dehydrated model food systems at 180°C for 25 min was found to induce the loss of 50–70% of PN, PM, and PLP added in experimental fortification. These results, which were determined in dehydrated food systems, were in contrast to previous studies which reported greater thermal stability of PN than the other vitamers in retorting fluid milk (Hassinen et al., 1954). Because the thermodynamic parameters for the thermal degradation of B_6 vitamers in liquid and dehydrated food systems have not been determined, this difference cannot be explained and is under investigation. For the roasted dehydrated food systems, microbiological assays were found to provide accurate estimation of biologically available vitamin B_6 as assessed by rat bioassay.

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Ms received 2/11/78; revised 4/11/78; accepted 4/20/78.

Partially supported by NIH grant GMO 1818. Michigan Agricultural Experiment Station Journal Article No. 8430.

DIETARY FIBER SOURCES FOR BAKED PRODUCTS: BRAN IN SUGAR–SNAP COOKIES

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ABSTRACT

To study the feasibility of producing high fiber cookies, sugar-snap cookies were prepared following the Micro III Method in which 10, 20 and 30% red and white wheat bran was substituted for flour and compared to a control cookie (100% flour). Substitution of 10% bran contributed 0.7g of dietary fiber, 1.2g with 20% substitution and 1.7g with 30% substitution. Incorporation of bran did not significantly affect top grain but did reduce spread, darkened the color and produced a more tender, less crisp cookie. Sensory data revealed that at levels of 10 and 20% substitution, bran affected only surface and interior color. Flavor was adversely affected at the 30% level.

INTRODUCTION

ALTHOUGH the inclusion of a certain amount of fiber in the diet has been recognized for sometime as desirable in maintaining normal laxation, fiber has been largely neglected by human nutritionists and considered an insignificant part of the diet. Recent interest in the comparison of the incidence of disease in different countries and in the function of environmental factors in disease processes has focused attention on the role of fiber in the gastrointestinal system related to the maintenance of man's health. A hypothesis based largely on epidemiological evidence states that a lack of fiber in the diet may be a contributing factor in a number of noninfectious "diseases of civilization" including diverticular disease of the colon (Painter and Burkitt, 1971), colonic cancer (Burkitt, 1975), appendicitis (Walker et al., 1973), hiatus hernia (Burkitt, 1975), and coronary heart disease (Trowell, 1972).

According to 1960 figures, approximately 80% of flours and cereal products consumed in the United States comes from wheat products and these products contribute about 37% of the total carbohydrates in the diet (USDA, 1964). Thus, in an effort to increase dietary fiber consumption, wheat products may provide feasible fiber carriers. Rajchel et al. (1975) and Brockmole and Zabik (1976) demonstrated that bran and middlings may be successfully substituted for flour in white and flavored layer cakes. Pomeranz et al. (1977) reported that acceptable bread was produced from wheat flours in which about 7% of the flour was replaced by microcrystalline cellulose or wheat bran. Cookies, while not considered a staple food as is bread, may also be feasible fiber carriers because of their long shelf-life which makes large scale production and widespread distribution possible, and to their eating quality which makes cookies a generally well-liked bakery product particularly among children.

This paper presents information as to the effect on sugar-snap cookies of substituting 0–30% wheat bran from a soft red (Arthur 71) and soft white (Yorkstar) variety for flour.

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0022-1147/78/0005-1590$02.25/0

Table 1—Average particle size[a] of flour and cyclone milled bran[b]

U.S. Std. sieve size	Particle size (μ)	Flour (%)	Red bran (%)	White bran (%)
30	589	0	4	3
40	419	0	47	13
50	297	0	36	28
100	150	96	11	50
200	74	3	2	6
325	43	1	0	0

[a] Average of 3 replications
[b] Soft red wheat bran was from Arthur 71 while soft white wheat bran was from Yorkstar.

MATERIALS & METHODS

Cookie preparation

Sugar-snap cookies were prepared following a modification of the Micro III Method procedure described by Finney et al. (1950). The method was modified to include 2% soy lecithin based on the weight of flour or flour and bran as recommended by Kissell and Yamazaki (1975) and the formulation was increased to prepare six cookies per bake. The control formula contained 6 ml of deionized water; however, preliminary testing indicated water levels had to be increased to 8, 12, and 16 ml for bran levels of 10, 20, and 30%, respectively, for optimal dough handling.

Unbleached cookie flour milled from a soft red winter wheat and representing a 90% patent flour was donated by Mennel Mills of Fostoria, Ohio. The flour showed the following analysis according to Mennel Mills: 8.9% protein, 0.40% ash and 13.5% moisture. Soy lecithin representing 60% phosphatides was purchased through Central Soya in Chicago, Illinois. Common lots of all other ingredients were also procured. Five replications were made of the control cookie and of the variables with 10, 20 and 30% of the flour substituted with wheat bran from a soft red variety (Arthur 71) and a soft white variety (Yorkstar).

To eliminate mouthfeel differences, the bran was ground to a particle size similar to that of the control flour in a Udy Cyclone Sample Mill, model MS. Forsythe et al. (1978). reported grinding wheat bran had no effect on either laxation including the parameters of fecal wet mass, fecal dry mass, fecal water holding capacity and transit time or serum cholesterol levels in vivo studies with rats. Particle sizing of the control flour as well as the ground bran was done in a Roto-Tap Testing Sieve Shaker, model 4589 (Donelson and Yamazaki, 1972) (Table 1). Prior to preparation of the cookies, bran and flour for both types of bran at each of the three levels of substitution were mixed for 15 min in a Liquid-Solids blender, model S44EXAK-989, to insure an even distribution of the two components. Enough flour and bran mixture per level of substitution was prepared at the same time so that the ingredients for five replications could be portioned from one mixture. These portions were weighed to the nearest 0.01g, sealed in clear polyethylene bags, randomly numbered and held at −23.9°C until used. Five portions of flour representing the control replications were also weighed to the nearest 0.01g, sealed in polyethylene bags, coded and stored at −23.9°C until used.

Baking order of the five replications of the control and six variables was completely randomized and re-randomization was done to determine the order in which the cookies were to be evaluated. Humidities on each day of baking were recorded by a Weathermeasure Meteorograph, model M701-E.

The creamed mass consisting of shortening, sugar, sodium bicarbonate, and nonfat dry milk solids was prepared in a Kitchen Aid mixer, model K5-A. A portion of the creamed mass representing the quantity for preparation of six cookies was transferred to the bowl of a National nonrecording micromixer, model 100-200A with a head speed of 86

Table 2—Objective measurements[a] of sugar-snap cookies prepared with 10, 20 and 30% of the flour substituted with red and white bran[b]

Physical characteristic	Control	Red bran (%)			White bran (%)		
		10	20	30	10	20	30
W/T spread factor	14.12 ± 0.38	12.34 ± 0.12*	10.81 ± 0.53*	9.60 ± 0.22*	12.44 ± 0.13*	11.07 ± 0.15*	9.76 ± 0.13*
Top grain[c]	9.0 ± 0.0	9.0 ± 0.0	8.4 ± 0.6	7.4 ± 0.9*	9.0 ± 0.0	8.6 ± 0.9	7.6 ± 0.9*
Color values[d]:							
L	66.7 ± 1.9	57.8 ± 0.7*	54.1 ± 0.6*	51.8 ± 0.5*	63.6 ± 0.6*	60.6 ± 1.2*	59.0 ± 0.7*
a_L	5.1 ± 1.0	6.3 ± 0.1*	6.3 ± 0.3*	6.6 ± 0.3*	6.1 ± 0.4*	6.6 ± 0.4*	6.7 ± 0.6*
b_L	23.8 ± 0.3	19.9 ± 0.1*	18.2 ± 0.2*	17.4 ± 0.1*	22.4 ± 0.2*	21.7 ± 0.3*	21.5 ± 0.1*
Breaking strength (lb/cm²)	5.30 ± 0.42	4.54 ± 0.27*	3.66 ± 0.20*	3.10 ± 0.07*	4.46 ± 0.31*	3.52 ± 0.09*	3.40 ± 0.21*
Shear compression (lb/g)	24.02 ± 3.59	21.58 ± 1.42	18.74 ± 2.91*	16.82 ± 1.83*	21.71 ± 2.83	17.84 ± 1.92*	15.41 ± 0.90*
Moisture (%):							
Raw	14.70 ± 0.11	15.13 ± 0.18*	16.34 ± 0.23*	17.60 ± 0.42*	15.34 ± 0.34*	16.23 ± 0.26*	17.57 ± 0.17*
Baked	2.65 ± 0.24	2.73 ± 0.25	3.16 ± 0.45*	3.41 ± 0.25*	3.11 ± 0.45	3.15 ± 0.09*	3.24 ± 0.09*

[a] Mean and standard error of the mean based on five replications
[b] Soft white wheat bran was from Yorkstar while soft red wheat bran was from Arthur 71.
[c] Total possible score of 9, with 9 signifying the most desirable
[d] Color values: L = lightness, a_L = redness, b_L = yellowness.
* Significant difference from control at the 5% level of probability (Dunnett's, 1955)

rpm. Remaining steps in preparation of the cookies were carried out in the micromixer.

Baking was done immediately following rolling and cutting of the dough in a 12 1-lb loaf National Reel Type Test Baking Oven for 10 min. An oven temperature of 400°F (204°C) was maintained by a Partlow Indication Controller, model MF-2-665K1P220. After removal from the oven, the cookies were allowed to cool for 5 min on the cookie sheet and were then transferred from the sheet to wire racks and cooled an additional 30 min before being bagged in polyethylene bags and held at −23.9°C for later evaluation.

Objective measurements

After width and height determinations were made on the six cookies of each bake, the samples were cut for evaluation according to a preset pattern so that each taste panelist received a cookie half from the same location on the baking tray. All samples were wrapped in plastic film to prevent dehydration.

W/T spread factor. The width and height of the six cookies of each bake were taken to the nearest mm. Width divided by height or thickness (W/T) called the spread factor was calculated according to the AACC Method 10-50 (AACC, 1962).

Top grain. Using a series of cookies from the Soft Wheat Quality Laboratory in Wooster, Ohio as a reference standard, the top grain of a selected cookie representing each bake was scored from 0 for a compact cookie with no surface breaks to 9 which represented a well-broken top containing numerous small "islands" characteristic of an optimal sugar-snap cookie (Finney et al., 1950).

Color. The Hunter Color Difference Meter, model D25, was used to determine the color of the cookie's surface. The instrument was standardized before use against a yellow tile (L− 83.0, $a_L = -3.5$, $b_L = 26.5$). Three readings were taken on each cookie with 60° rotations between readings.

Tenderness. Values for tenderness were determined using the standard shear compression cell of the Allo-Kramer Shear Press, model SP12, equipped with an electronic recorder, model E2EZ using the method of Funk et al. (1965). The 3,000 lb proving ring with a range of 20 was used for each measurement.

Breaking strength. Values for breaking strength were determined using the single blade cell of the Allo-Kramer Shear Press, model SP12, equipped with an electronic recorder, model E2EZ. The 100 lb proving ring with a range of 20 was used for each measurement. The height and width of the two cookie halves designated for this measurement were determined at the midpoint of the cookie to the nearest 0.01 cm using a vernier caliper. An average taken from the two cookie halves designated for breaking strength measurement was recorded as breaking strength in pounds of force per square centimeter broken.

Moisture. Moisture determinations on the raw cookie dough and baked cookies were done using the AACC procedure 44-40 (AACC, 1962).

Neutral detergent fiber. Enzyme and neutral detergent fiber analyses of the baked cookies were determined as described by Goering and Van Soest (1970). Bacterial α-amylase was used to digest starch at room temperature for 30 min.

Sensory evaluation

Training sessions held prior to taste panel evaluation acquainted the six panel members with the sugar-snap cookie score card which was used to evaluate surface appearance, interior appearance and eating characteristics (Vratanina, 1978). Each characteristic was evaluated on a 7-point scale, with 7 being optimum.

Analyses of data

The data were analyzed for variance and the following orthogonal comparisons calculated using the procedures described by Snedecor and Cochran (1967): control vs test, red vs white, level effect – linear, level effect – quadratic, linear × red vs white, quadratic × red vs white. Dunnett's procedure (1955) was also applied to compare the control against each of the treatment means.

RESULTS & DISCUSSION

MEANS and standard deviations of the objective and sensory evaluations of the cookies prepared with varying levels of bran are presented in Tables 2 and 3, respectively. Statistical analyses of these data are presented in Tables 4 and 5, respectively.

Spread factor and top grain

Increasing fiber substitution levels lowered the spread factors in the cookies. The spread factors at each level of substitution were significantly different from the control by Dunnett's test (1955) at the 5% level of probability (Table 2). Increasing fiber substitution levels also resulted in lower top grain scores, however, a comparison of the control to the treatment means using Dunnett's test revealed a significant difference ($p < 0.05$) in top grain only at the 30% level for both red and white bran. No significant differences were noted for the top grain scores or spread factors of cookies prepared with red or white bran. Kissell and Yamazaki (1975) reported that addition of ingredients to a cookie system with increased water retention properties results in an increased competition for the limited amount of free water present in the cookie dough. The rapid partitioning of water to added sites of hydrophilicity within the system results in a decreased solution of sugar, an increased concentration of the solution and a greater internal dough viscosity. Yamazaki (1955) has shown that such conditions reduce cookie spread and limit top grain formation. Room relative humidities varied from 42 to 68%. Although relative humidities over 60% have been considered to have an adverse effect on spread and top grain (Yamazaki, 1977) no relationship between these humidities and recorded top grains or spread factors (Table 2) was noted.

Color

Dunnett's test revealed that the treatment means for both red and white bran at all three levels of substitution were

Table 3—Sensory evaluations[a] of sugar-snap cookies prepared with 10, 20 and 30% of the flour substituted with red and white bran[b]

Sensory characteristic[b]	Control	Red bran (%)			White bran (%)		
		10	20	30	10	20	30
Surface appearance:							
Shape (7)	6.08 ± 0.41	6.68 ± 0.18*	6.34 ± 0.30	5.98 ± 0.25	6.70 ± 0.07*	6.40 ± 0.25	6.22 ± 0.16
Color (7)	6.02 ± 0.16	5.83 ± 0.27	5.40 ± 0.23*	4.36 ± 0.34*	6.68 ± 0.11*	6.28 ± 0.30	6.04 ± 0.22
Characteristics (7)	5.80 ± 0.30	6.38 ± 0.31	5.84 ± 0.05	5.16 ± 0.80	6.52 ± 0.27*	6.34 ± 0.22	5.72 ± 0.26
Interior appearance:							
Distribution of cells (7)	5.66 ± 0.09	5.86 ± 0.22	5.20 ± 0.17	4.88 ± 0.37*	6.06 ± 0.27	5.62 ± 0.44	5.16 ± 0.30
Shape & Size of cells (7)	5.38 ± 0.38	5.76 ± 0.36	5.34 ± 0.21	4.98 ± 0.44	5.86 ± 0.29	5.56 ± 0.58	5.10 ± 0.19
Color (7)	5.66 ± 0.11	5.74 ± 0.29	4.94 ± 0.38*	4.00 ± 0.38*	6.32 ± 0.16*	6.12 ± 0.31	5.52 ± 0.37
Eating characteristics:							
Texture (7)	6.14 ± 0.55	6.18 ± 0.88	5.94 ± 0.65	6.04 ± 0.42	6.22 ± 0.58	6.72 ± 0.04	5.46 ± 1.02
Mouthfeel (7)	6.26 ± 0.50	6.08 ± 0.97	5.98 ± 0.63	5.56 ± 0.48	6.30 ± 0.47	6.66 ± 0.21	5.52 ± 0.61
Flavor (7)	5.80 ± 0.39	5.16 ± 1.13	5.34 ± 0.17	4.74 ± 0.50*	5.92 ± 0.27	5.76 ± 0.27	4.90 ± 0.47

[a] Mean and standard error of the mean based on five replications
[b] Soft white wheat bran was from Yorkstar while soft red wheat bran was from Arthur 71.
[c] Total possible points listed in parenthesis
* Significant difference from control at the 5% level of probability (Dunnett's, 1955)

significantly different from the control for L (lightness), a_L (redness), and b_L (yellowness) values (Table 2). As the percentage of fiber substituted increased, the lightness and yellowness values decreased. Although there was a significant difference ($p < 0.001$) (Table 4) between the control and the fiber substituted cookies for the a_L or redness value, no significant differences for that parameter were revealed between levels of substitution of bran or between types of bran. The red bran had a greater effect in decreasing the yellowness of the cookies than did the white bran. An examination of the treatment means (Table 2) revealed that above the 20% level of substitution for the white bran, additional bran had only a slight effect in decreasing yellowness.

Breaking strength and shear compression

Breaking strength was used as a measure of the crispness of the cookie and shear compression as indicative of cookie tenderness. Breaking strength values at all three levels of substitution and in both the cookies substituted with red and white bran were significantly different from the control at the 5% level of probability by Dunnett's test (Table 2). Shear compression values were significantly different from the control only at the 20 and 30% levels of substitution in both red and white bran substituted cookies (Table 2). Breaking strength and shear compression values analyzed for variance revealed significant differences ($p < 0.001$) between levels of bran substituted while no significant differences were revealed between types of bran (Table 4). The force required to break and shear the cookies decreased with increasing amounts of bran indicating a less crisp, more tender cookie as the level of fiber substituted increased. There was a greater decrease in crispness in the white bran substituted cookies betwen the 10 and 20% levels than between the 20 and 30% levels.

Moisture

Gilles (1960) reported on the increased water absorption capacity of the fiber components of wheat and Eastwood (1975) stated that 1g of raw cereal bran has the capacity to hold three to four times its weight of water. Therefore, as the levels of bran substituted in the cookie formula were increased, an increase in the amount of water required to produce a dough was necessitated with each level. The percentage moisture values in the raw dough and baked cookies revealed significant differences between levels of substitution of bran (Tables 2 and 4).

Sensory evaluation

The cookies substituted with red and white bran both received increasingly lower scores for shape with each increase in level of bran substituted (Table 3). Both types of bran, however, were scored higher than the control at the 10 and 20% levels and the only cookie which scored lower than the control

Table 4—Analyses of variance and orthogonal comparisons for objective data of sugar-snap cookies prepared with 10, 20 and 30% of the flour substituted with red and white bran

Source	df	Mean squares								
		W/T spread Factor	Top Grain	Hunter color values			Breaking strength	Shear compression	Moisture	
				L	a_L	b_L			Raw	Baked
Total	34									
Treatment	6	13.11***	2.30***	133.45***	1.41**	26.77***	3.09***	47.35***	6.63***	0.38***
Control vs rest	1	41.74***	1.90*	334.91***	7.17***	56.47***	9.88***	122.04***	11.98***	0.99**
Red vs white	1	0.24	0.13	318.83***	0.01	85.68***	0.00	3.94	0.01	0.03
Level effect (linear)	1	36.56***	11.25***	142.04***	0.99	14.45***	7.85***	152.74***	27.59***	0.83**
Level effect (quadratic)	1	0.06	0.42	2.77	0.01	0.86***	0.53**	2.35	0.10	0.01
Linear X red vs white	1	0.00	0.05	2.11	0.16	3.04***	0.18	2.94	0.07	0.39*
Quadratic X red vs white	1	0.03	0.02	0.00	0.15	0.10	0.11	0.12	0.06	0.02
Error	28	0.08	0.39	0.99	0.27	0.04	0.06	5.60	0.07	0.09

[a] Soft red wheat bran was from Arthur 71 while soft white wheat bran was from Yorkstar.
* Significant at the 5% level of probability
** Significant at the 1% level of probability
*** Significant at the 0.1% level of probability

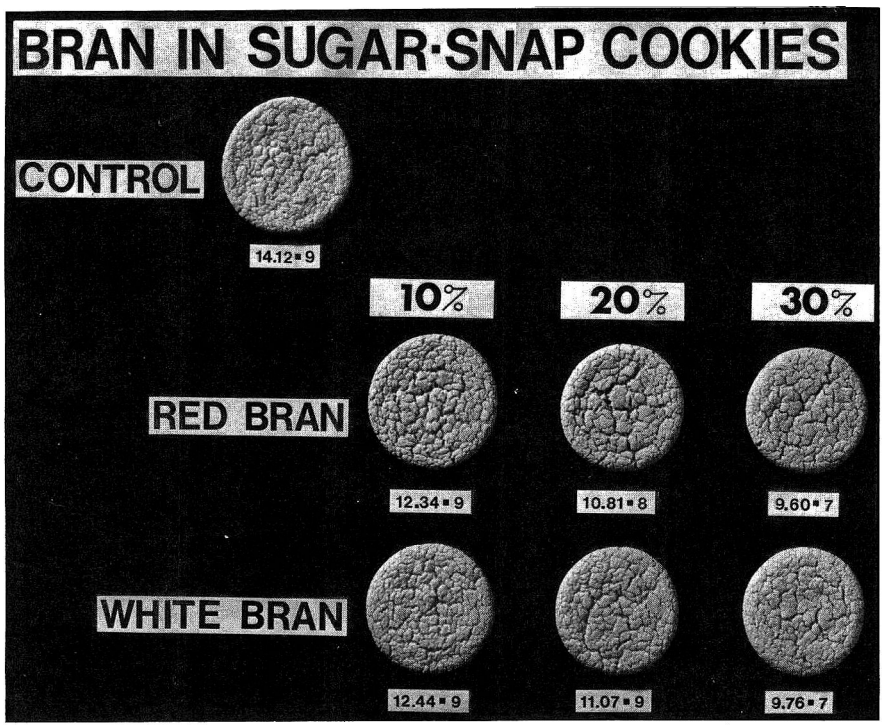

Fig. 1—Appearance of sugar-snap cookies prepared with increasing levels of red and white wheat bran substituted for flour.

was the red bran substituted at the 30% level. Significant differences were noted between the red and white bran substituted cookies for surface color ($p < 0.001$) and surface characteristics ($p < 0.01$) (Table 5). As the level of bran substituted increased, scores for cell distribution decreased with panelists noting cells to be more compact at higher levels of substitutions. Increasing levels of bran lowered acceptability of the shape and size of the cells, though red and white brans were not significantly different in this effect and none of the treatment means were found significantly different from the control by Dunnett's test (Table 3). The interior color of the cookies was observed to be affected by both the level of substitution and type of bran used (Table 5). Increasing levels of bran substituted decreased the acceptability of the cookies' interior color in both red and white bran substituted cookies. However, the interior color of both red and white bran substituted cookies at the 10% level and the white bran cookies at the 20% level was scored as more acceptable than the interior color of the control cookie (Table 3). No significant differences were revealed in any of the cookies for texture (Table 5). Mouthfeel was scored as most acceptable in the cookies substituted with white bran at the 10 and 20% level. The general descriptive term selected by the panelists for mouthfeel was crunchy; however, at higher levels of bran substitution, a slightly harsh or powdery mouthfeel was described. As the level of bran increased, the acceptability for flavor decreased. At the 30% level of substitution, the panelists described the flavor as distinctive of bran and/or grainy.

Based on the analyses of the objective and subjective data, a recommendation of bran substitution in sugar-snap cookies at the 10 and 20% levels using either red or white wheat bran appears to be feasible.

The visual characteristics of the sugar-snap cookies prepared can be seen in Figure 1.

Nutritional relationships

Substituting 10% of the cookie flour with red or white wheat bran increased the amount of dietary fiber in a 24g cookie to approximately 0.7g (Table 6). Twenty percent substitution of white wheat bran contributed 1.2g of dietary fiber and 30% substitution 1.7g of dietary fiber. Twenty percent substitution of red wheat bran contributed 1.3g of dietary

Table 5—Analyses of variance and orthogonal comparisons for sensory evaluations

		Surface appearance			Interior appearance			Eating characteristics		
Source	df	Shape	Surface color	Surface characteristics	Distribution of cells	Shape and size of cells	Interior color	Texture	Mouthfeel	Flavor
Total	34									
Treatment	6	0.39***	2.79***	1.14***	0.90***	0.53**	3.08***	0.71	0.84	1.09**
Control vs rest	1	0.40*	0.28*	0.16	0.17	0.01	0.21	0.01	0.25	1.06
Red vs white	1	0.09	9.75***	1.20**	0.68**	0.16	8.97***	0.05	0.62	1.50*
Level effect (linear)	1	1.74***	5.51***	5.10***	4.42***	2.96***	8.06***	1.01	2.11*	2.59**
Level effect (quadratic)	1	0.00	0.09	0.14	0.04	0.00	0.12	0.84	1.38	0.91
Linear X red vs white	1	0.06	0.84***	0.22	0.01	0.00	1.10**	0.48	0.08	0.45
Quadratic X red vs white	1	0.01	0.25	0.04	0.05	0.02	0.03	1.84	0.58	0.00
Error	28	0.06	0.06	0.15	0.08	0.14	0.09	0.44	0.35	0.30

* Significant at the 5% level of probability
** Significant at the 1% level of probability
*** Significant at the 0.1% level of probability

Table 6—Enzyme and neutral detergent fiber analyses (%) of cookies prepared with 0, 10, 20 or 30% red or white wheat bran

Percentage of flour substituted with bran	Bran type	
	White	Red
0	1.01 ± 0.04[a]	1.01 ± 0.04
10	3.05 ± 0.13	3.12 ± 0.10
20	4.94 ± 0.03	5.30 ± 0.14
30	6.96 ± 0.04	7.73 ± 0.37

[a] Based on triplicate determinations

fiber while 30% substitution contributed 1.9g. The ground red wheat bran (Arthur 71) has been reported to contain 40.45% dietary fiber while the ground white wheat bran (Yorkstar) contained 37.41% dietary fiber (Shafer and Zabik, 1978). The control cookie has 0.2g dietary fiber. Studies on patients given bran to cure constipation or symptoms of diverticular disease (Painter et al., 1972) reported that the amount of bran needed to prevent straining at stool in individuals varied from 3 to 45g. An average of approximately 15g, however, rendered the stools soft and easy to pass. Fifteen grams of unprocessed bran represents approximately 6.0g of dietary fiber. The addition of bran to these patients' diets reduced the transit time in most to just over 40 hr and yielded stool weights of about 180g per day.

Although the assignment of a nutritional requirement for fiber to protect against various diseases is not feasible at this time and will probably remain unlikely for some time to come, the recommendation has been made (Trowell and Burkitt, 1975) that cereal fiber be added to food in sufficient quantities to ensure the passage of at least one soft stool daily. Addition of fiber to multiple foods would allow division of the intake of fiber throughout the day. Cookies along with other baked products may provide feasible fiber carriers for increasing the consumption of dietary fiber in a manner acceptable to consumers.

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Ms received 2/4/78; revised 3/31/78; accepted 4/5/78.

Michigan Agricultural Experiment Station Journal Article No. 8425.

The authors thank Mennel Mills of Fostoria, Ohio for supplying the cookie flour and the Soft Wheat Quality Laboratory of the Ohio Agricultural Research Development Center in Wooster, Ohio for supplying the bran.

Partial support for the research was supplied by USDA Grant No. 516-15-102.

ANALYSIS OF TRYPTOPHAN UTILIZING ITS REACTION WITH ALPHA–KETOGLUTARIC ACID

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ABSTRACT

The interaction between tryptophan and α-ketoglutaric acid (α-KGA) was utilized for the quantitative estimation of tryptophan. Previous work enabled two sets of reaction conditions to be chosen which produced stable quantities of chromophore with wavelength of maximum absorbance (λmax) at 358 nm. Both conditions produced Beer's Law plots over the concentration range 0–1.8 mM tryptophan. This interaction was found to produce a specific analytical technique for tryptophan. Analysis of reaction mixtures revealed that a yellow chromophore was responsible for the production of absorbance with λmax at 358 nm. Measurement of N-terminal tryptophan dipeptides was successful whereas attempts to measure the tryptophan content of lysozyme and C-terminal tryptophan dipeptides were unsuccessful utilizing this reaction.

INTRODUCTION

DUE TO THE BIOLOGICAL importance of tryptophan, many analytical procedures and methods have been developed to assay for its presence in both its free and peptide-bound forms. However, most procedures have been time consuming and/or unreliable. Friedman and Finley (1971) have presented an excellent review on this subject.

The purpose of this investigation was to explore the interaction between tryptophan and α-ketoglutaric acid (α-KGA), first reported by Chu and Clydesdale (1975, 1976) as a possible technique for tryptophan analysis. Previous work by Anderson and Clydesdale (1977) involved the manipulation of four parameters, concentration of HCl, concentration of α-KGA, concentration of $NaNO_2$, and temperature, to produce stable amounts of a chromophore with λmax at 358 nm. Two sets of reaction conditions, RC-1 and RC-2, were chosen for the quantitative and qualitative work conducted in this study.

MATERIALS & METHODS

Preparation of standard tryptophan solutions

A 4.0 mM solution of l-tryptophan (Aldrich Chemical Co.) was prepared with distilled water in a one liter volumetric flask which was wrapped in aluminum foil and stored in the refrigerator to minimize destruction.

Preparation of α-KGA reagents

A 0.5M and a 0.05M solution of α-KGA (Eastman Kodak Company) were prepared with 6N HCl to formulate Reagent 1 (RC-1) and Reagent 2 (RC-2), respectively.

Reaction samples were prepared by mixing equal amounts of diluted standard tryptophan solution with RC-1 or RC-2. Blanks were made by replacing the tryptophan solution with distilled water.

Measurement of color formation

Measurement of chromophore production was made by measuring absorbance at 358 nm on a Hitachi-Perkin Elmer Model 139 Spectrophotometer (HPE-139) (Anderson and Clydesdale, 1977).

Preparation of standard curves

Test samples containing various dilutions of the standard tryptophan solution and either RC-1 or RC-2 were prepared. Samples containing RC-1 were incubated at 40°C whereas samples containing RC-2 were incubated at 80°C. Aliquots were taken at various times and analyzed for chromophore production.

Specificity of the reagents

2.0 mM solutions of selected amino acids, depeptides, and compounds chemically similar to tryptophan were prepared. These were reacted with RC-1 and RC-2 and analyzed for the development of chromophore. Tyrosine, phenylalanine, proline, tryptophan-HCl, tryptophol, indole-3-acetic acid, l-leucyl-l-tryptophan, l-phenylalanyl-l-tryptophan, l-trytophyl-l-leucine, and l-tryptophyl-l-phenylalanine were obtained from the Sigma Chemical Company and α-Methyl tryptamine and N_w-methyl tryptamine from the Aldrich Chemical Company.

Analysis of reaction mixtures by paper chromatography

Aliquots of the reaction mixtures were taken at various time intervals and spotted on Whatman 3 MM paper. They were developed with 5% NH_4OH (Chu and Clydesdale, 1975, 1976). Reaction products could be seen under a UV lamp and marked. α-KGA was analyzed by spraying the dried, previously marked chromatogram with a solution containing 15 ml Universal Indicator solution (Fisher Scientific Co.) plus 3 ml 0.1N NaOH.

Several chromatographic papers were streaked with the reaction mixture obtained from the RC-1-tryptophan interaction. After development the resulting yellow and blue bands (when observed under a UV lamp) were cut out and the chromophores eluted with water. They were then analyzed for absorption over the range 340–370 nm using the HPE-139.

Estimation of the tryptophan content of lysozyme

Egg white lysozyme (dessicated) (Sigma Chemical Co.) and thioglycolic acid (Sigma Chemical Co.) were stored in a freezer.

Samples of lysozyme and tryptophan were weighed out and placed into separate thermal death time (TDT) tubes. Nine tubes of each compound were prepared. To each tube was added three ml of 6N HCl which contained 4% thioglycolic acid (Matsubara and Sasaki, 1969). The samples were frozen using liquid nitrogen and then evacuated on a cold finger distillation apparatus to below 1 ml pressure.

The samples were hydrolyzed at 110°C (±2°C) for three different lengths of time. Three samples of each substance were analyzed after 22, 48 and 68-hr of hydrolysis. After incubation each sample was cooled, mixed, and opened. To each tube was added three ml distilled water and a few glass beads. Again the tubes were thoroughly mixed. Two 2.0 ml aliquots were taken from each sample and placed in separate flasks. Three ml of distilled water and five ml RC-1 or RC-2 were then added to each flask.

In this case RC-1 and RC-2 were made up with 3N HCl instead of 6N HCl. This was due to the fact that in the previous studies the reagents were mixed with equal amounts of tryptophan solutions which were made with distilled water. Therefore, the normality of the reaction mixture was one-half that of the reagents (3N). In this study the samples containing tryptophan were hydrolyzed with 6N HCl and then diluted with an equivalent amount of distilled water, such that, the normality of the reaction mixture would be three normal.

After the addition of the reagents, the samples were incubated and then analyzed for the development of chromophore as previously outlined. The standard curves prepared as previously outlined were used to determine the tryptophan content of the samples. The actual number of micromoles of tryptophan in the lysozyme samples was calculated using its molecular weight, the weight of the sample, the percent pro-

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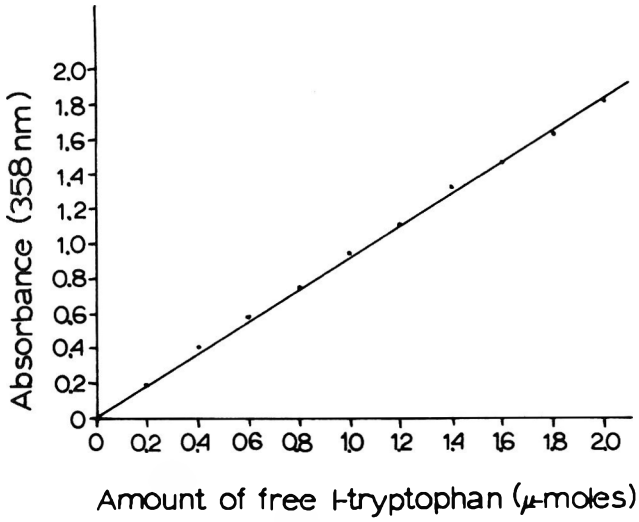

Fig. 1—Standard curve obtained from treatment of known amounts of 1-tryptophan using RC-1.

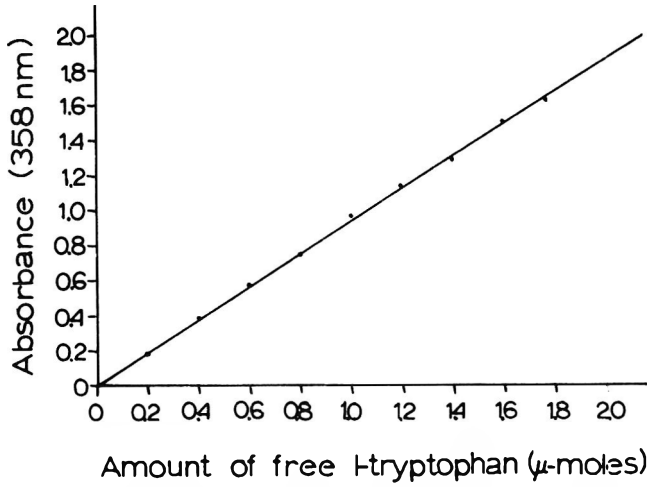

Fig. 2—Standard curve obtained from treatment of known amounts of 1-tryptophan using RC-2.

tein in the sample, and the theoretical value for the number of tryptophan residues per molecule of lysozyme. There were believed to be six tryptophan residues per molecule of lysozyme (Canfield and Liu, 1965).

Effect of thioglycolic acid on chromophore production. Due to the consistently low recoveries of tryptophan from the samples containing free tryptophan and thioglycolic acid, it became apparent that either the thioglycolic acid was not fully protecting the tryptophan or it was interfering with the production of chromophore. In order to test this latter hypothesis thioglycolic acid was added to several samples of free tryptophan. These were then reacted with RC-2 and analyzed for the production of chromophore without any heat treatment.

Ten samples containing approximately one micromole of tryptophan were weighed out. Four samples were analyzed for tryptophan content using RC-2 without any hydrolysis treatment. To two of these samples was added three ml 6N HCl and to the remaining two was added three ml 6N HCl which contained 4% thioglycolic acid. Three milliliters of distilled water were added to these samples and they were analyzed for tryptophan.

The remaining six samples were evacuated, sealed, and hydrolyzed for 22 hr as previously described. Two of these samples contained 3.0 ml 6N HCl and the remaining four samples contained 3.0 ml 6N HCl with 4% thioglycolic acid. All six were analyzed for tryptophan content using RC-2.

Attempts to remove thioglycolic acid. Since the thioglycolic acid interfered with the production of chromophore even without any heat treatment, attempts were made to remove it after hydrolysis and prior to reaction with RC-1 or RC-2.

Eight samples, containing approximately one micromole of tryptophan and 3.0 ml 6N HCl, were prepared in TDT tubes. Four of these samples also contained 4% thioglycolic acid. All eight samples were then evaporated on a rotary evaporator using a water bath temperature of 60°C as described by Matsubara and Sasaki, (1969).

Eight similar samples were prepared in the same manner. These were frozen and then lyophylized on a Virtis Freeze Drier.

RESULTS & DISCUSSION

Preparation of standard curves

RC-1. Figure 1 shows that RC-1 produces a Beer's Law plot over the concentration range 0–2.0 mM for free l-tryptophan.

Previous work by Anderson and Clydesdale (1977) indicated that RC-1 reached maximum chromophore production after 48 hr of incubation at 40°C and remained stable for approximately 60 hr thereafter. When the various concentrations of free tryptophan were analyzed for the development of chromophore after maximum absorbance had been obtained, each concentration of tryptophan yielded approximately the same absorbance at every time period at which they were tested (Table 1). This implies that readings can be made during the interval 48–108 hr after the beginning of incubation.

RC-2. As can be seen in Figure 2, RC-2 produces a Beer's Law plot over the concentration range 0–1.8 mM free l-tryptophan.

Previous work (Anderson and Clydesdale, 1977) indicated that RC-2 reaches maximum chromophore production after 24 hr of incubation at 80°C and remains stable for approximately

Table 1—Determination of time during which chromophore production remains constant using RC-1

Tryptophan (μmoles X10)	Absorbance at 358 nm (X1000)					
	Time (hours)					
	48	60	72	84	96	108
2	188	187	186	188	188	188
4	388	390	386	388	380	381
6	574	574	573	565	563	561
8	749	748	749	748	743	735
10	964	946	953	944	963	939
12	1140	1139	1139	1126	1125	1120
14	1284	1279	1285	1278	1269	1259
16	1504	1494	1507	1493	1480	1463
18	1671	1653	1662	1648	1624	1627
20	1746	1745	1783	1761	1733	1745

Table 2—Determination of time during which chromophore production remains constant using RC-2

Tryptophan (μmoles X10)	Absorbance at 358 nm (X1000)			
	Time (hours)			
	24	27	30	33
2	198	198	197	199
4	412	415	409	407
6	581	584	585	586
8	756	761	759	759
10	950	952	957	951
12	1118	1117	1118	1113
14	1340	1354	1346	1336
16	1473	1462	1479	1467
18	1634	1634	1645	1611
20	1816	1835	1835	1803

Table 3—Production of chromophore from the reaction of RC-1 and selected amino acids, similar compounds and dipeptides

Test compound	Tryptophan theorized (μmoles)	Tryptophan found at selected incubation times (μmoles)	
		48 hr	72 hr
Tyrosine	0	0	0
Phenylalanine	0	0	0
Proline	0	0	0
Tryptamine-HCl	0	0.15	0.24
Tryptophol	0	0.10	0.17
Indole-3-acetic acid	0	0.05	0.07
α-Methyltryptamine	0	0.08	0.16
N_w-Methyltryptamine	0	0.06	0.09
Leucyl-tryptophan	1.00	0.08	0.10
Phenylalanyl-tryptophan	1.00	0.07	0.12
Tryptophyl-alanine	1.00	1.02	0.97
Tryptophyl-leucine	1.00	1.04	0.92
Tryptophyl-phenylalanine	1.00	1.04	0.90

Table 4—Production of chromophore from the reaction of RC-2 and selected amino acids, similar compounds and dipeptides

Test compound	Tryptophan theorized (μmoles)	Tryptophan found at selected incubation times (μmoles)		
		24 hr	28 hr	32 hr
Tyrosine	0	0	0	0
Phenylalanine	0	0	0	0
Proline	0	0	0	0
Tryptamine-HCl	0	0.36	0.39	0.40
Tryptophol	0	0.10	0.10	0.10
Indole-3-acetic acid	0	0.18	0.19	0.19
α-Methyltryptamine	0	0.31	0.34	0.37
N_w-Methyltryptamine	0	0.37	0.37	0.43
Leucyl-tryptophan	1.00	0.38	0.40	0.46
Phenylalanyl-tryptophan	1.00	0.40	0.44	0.54
Tryptophyl-alanine	1.00	0.89	0.99	0.84
Tryptophyl-leucine	1.00	0.96	1.02	1.03
Tryptophyl-phenylalanine	1.00	1.03	1.06	1.07
Tryptophan	1.00	1.06	1.00	1.05

9 hr thereafter. When the various concentrations of free tryptophan were analyzed for chromophore production during these 9 hr, each concentration was found to yield approximately the same absorbance at every time interval tested (Table 2). Therefore readings can be made during the interval 24–33 hr after the beginning of incubation.

Specificity of RC-1 and RC-2

Reaction with selected amino acids. Tables 3 and 4 show that there was no production of chromophore when either RC-1 or RC-2 was reacted with solutions of tyrosine, phenylalanine, and proline. These amino acids were selected because they were chemically most similar to tryptophan. Since Chu and Clydesdale (1975, 1976) found no interaction between α-KGA and glycine, glutamine, phenylalanine, threonine, tyrosine, asparagine, arginine and histidine, it was believed that this reaction was very specific for the amino acid tryptophan.

Reaction with similar compounds. Tables 3 and 4 show that there was much less production of chromophore when either RC-1 or RC-2 was reacted with compounds which are chemically similar to tryptophan. α-Methyltryptamine differs from tryptophan in that a methyl group replaces the carboxylic acid moiety of the tryptophan molecule. Since this compound reacted only to a small extent, it implies that the free carboxylic acid group or a free carbonyl group may be essential for the production of chromophore. It was hypothesized that tryptamine · HCl failed to react for the same reason.

Indole-3-acetic acid lacks the α-amine group and one methyl group of the hydrocarbon side-chain of the indole group. Thus, it was theorized that the free α-amine group was also necessary for chromophore production.

N_w-methyltryptamine and tryptophol differ from tryptophan because both lack the carboxylic acid group, tryptophol lacks the α-amine group, and N_w-methyltryptamine has a methyl-substituted amine group. Since these substrates did not react very well with the reagents, it supported the hypothesis that both the free α-amine and carboxylic acid (or free carbonyl) groups are necessary for optimal chromophore production. Since neither tyrosine nor phenylalanine reacted with the reagents, it may be concluded that the indole portion of the tryptophan molecule is also essential.

Temperature did seem to affect these interactions. RC-1 (40°C incubation) reacted very slightly with these compounds whereas RC-2 (80°C incubation) reacted up to 50% in some cases. Thus RC-1 would be a more specific reagent for free tryptophan.

Reaction with selected dipeptides. Tables 3 and 4 show that both RC-1 and RC-2 react fully with N-terminal tryptophan dipeptides but react much less with C-terminal tryptophan dipeptides. The main difference between C- and N-terminal tryptophan residues is the lack of a free α-amine group in the C-terminal residue. Since the C-terminal tryptophan dipeptides failed to yield much chromophore, more evidence that the free α-amine group is necessary for chromophore production is obtained. Also it may be theorized that the reagents were unable to hydrolyze the peptide bonds in these dipeptides.

Thus, the presence of an indole ring, a free α-amine group, and a free carboxylic acid (or free carbonyl) group seem to be necessary for optimal chromophore production. These requirements would make these reagents fairly specific for free or N-terminal tryptophan.

Analysis of interaction products by paper chromatography

Early in the study it became evident that visually different end products could be obtained depending upon the conditions employed during the reaction. Under some conditions dark green precipitates resulted, in others the solution turned brown, and in still others the solution showed the expected yellow color. It became obvious that this was due to the production of more than one reaction product. Therefore, paper chromatography was used to separate these products and also to determine which ones were produced when RC-1 or RC-2 were used. More importantly, the chromophore which was being measured at 358 nm would hopefully be isolated.

Chu and Clydesdale (1975, 1976) used 5% NH_4OH to separate the reaction products. They obtained five different bands: three blue bands, a green, and a yellow band with R_f values of 0.01, 0.05, 0.15, 0.55 and 0.77, respectively. In this study it was found that RC-1 produced a blue band and a yellow band. This differed from RC-2 which produced three blue bands and a yellow band. Table 5 shows that the R_f values differed from those obtained by Chu and Clydesdale (1976, 1976). Only the yellow compound had approximately the same R_f value.

Reasons for the discrepancies of R_f values were not known. However, the conditions of the reaction (temperature, pH and concentration of α-KGA) varied between the two sets of work. Temperature had an obvious effect on chromophore production. RC-1 (40°C) produced only two compounds whereas RC-2 (80°C) produced four compounds.

Since the yellow band was unquestionably the brightest

Table 5—Production of interaction products between tryptophan and RC-1 and RC-2 with time using paper chromatography and 5% NH_4OH for development

	Time (hours)	R_f values (X100) Isolated compounds[a] Blue 1	Blue 2	Blue 3	Yellow 1
RC-1					
	48	–	–	88	82
	60	–	–	89	83
	72	–	–	87	84
	96	–	–	89	82
RC-2					
	12	–	–	86	74
	24	24	46	86	77
	28	25	48	87	76
	32	35	59	80	75

[a] Observed under UV light

Table 6—Recovery of free tryptophan (T) and peptide-bound tryptophan from Lysozyme (L) after hydrolysis with 6N HCl containing 4% thioglycolic acid in evacuated tubes using RC-1

Length of hydrolysis (hr)	Sample no.	Yield of Tryptophan (μmoles) Found	Theorized	% Recovery
22	T1	0.46	1.00	46
	T2	0.51	0.97	51
	T3	0.56	0.97	56
48	T4	0.54	1.05	52
	T5	0.56	0.98	57
	T6	0.66	1.05	62
68	T7	0.71	1.02	70
	T8	0.73	1.03	71
	T9	0.70	1.00	70
22	L1	0.63	0.88	72
	L2	0.72	0.88	82
	L3	0.72	0.88	82
48	L4	0.60	0.87	69
	L5	0.66	0.88	75
	L6	0.66	0.88	75
68	L7	0.73	0.88	82
	L8	0.68	0.88	77
	L9	0.73	0.87	84

Table 7—Recovery of free-tryptophan (T) and peptide-bound tryptophan from Lysozyme (L) after hydrolysis with 6N HCl containing 4% thioglycolic acid in evacuated tubes using RC-2

Length of hydrolysis (hr)	Sample no.	Yield of Tryptophan (μmoles) Found	Theorized	% Recovery
22	T5	1.10	1.16	95
	T6	1.10	1.18	94
48	T7	0.76	1.00	75
	T8	0.76	1.00	76
	T9	0.83	1.06	79
68	T3	0.40	0.98	41
	T4	1.08	1.23	88
	T10	0.93	1.00	93
22	L5	0.47	0.60	78
	L6	0.50	0.60	76
48	L7	0.39	0.60	64
	L8	0.40	0.60	68
	L9	0.43	0.60	72
68	L3	0.38	0.60	64
	L4	0.38	0.60	64
	L10	0.46	0.60	76

band when observed under a UV lamp, it was believed that this was the chromophore being measured at 358 nm. Figure 3 shows that upon elution with distilled water this chromophore had peak absorbance at 352 nm. Upon acidification, the peak absorbance shifted to 358 nm.

None of the blue compounds appeared to be present in sufficient quantities to be the chromophore of interest. The two blue bands which appeared closest to the origin on the chromatography paper when RC-2 was used definitely could not be the main chromophore since they were not produced when RC-1 was used. Upon elution and acidification, the third blue compound, which was produced by both reagents, showed very little absorbance near 358 nm. Therefore, it was concluded that it was this yellow compound which was being produced in proportion to the amount of tryptophan present. No work was done to identify this compound.

Analysis of tryptophan found in lysozyme

Since the α-KGA reagents did not produce the predicted amount of absorbance when reacted with C-terminal tryptophan dipeptides, it was believed that the reagents did not cleave the peptide bonds. Therefore, to analyze for the tryptophan residues in lysozyme, the protein samples were hydrolyzed prior to analysis. The lysozyme was hydrolyzed in evacuated tubes with 6N HCl containing 4% thioglycolic acid (Matsubara and Sasaki, 1969) in order to protect the tryptophan residues from destruction.

RC-1. Table 6 shows that the recovery of free tryptophan after 22, 48 and 68 hr of hydrolysis ranged from 46–71%. The recovery from lysozyme was higher, ranging from 63–84%. In all cases the recovery of tryptophan was lower than expected.

RC-2. Table 7 shows that the recovery of free tryptophan ranged from 41–95%. The recovery of tryptophan from

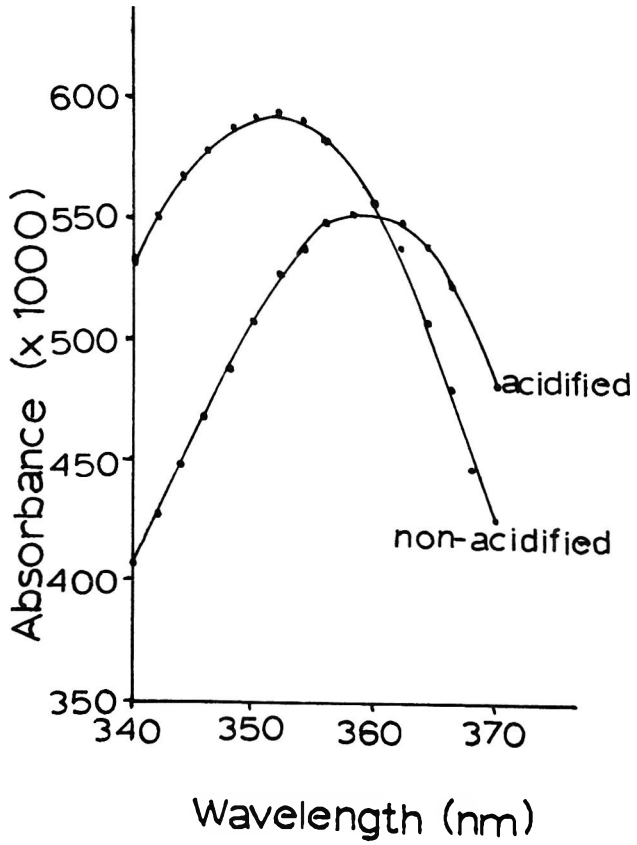

Fig. 3—Absorption spectra of yellow band after elution with distilled water before and after acidification with concentrated HCl.

lysozyme ranged from 64–78%. Again these values were lower than expected.

Matsubara and Sasaki (1969) reported recoveries of tryptophan in excess of 90% when samples of lysozyme were hydrolyzed for 22–64 hr at 110°C with 6N HCl containing 4% thioglycolic acid followed by analysis on an amino acid analyzer. Since the results obtained from this study were consistently low, it was theorized that the thioglycolic acid was interfering with the production of chromophore.

Effect of thioglycolic acid on chromophore production. Table 8 reveals that when samples of free tryptophan were analyzed for chromophore production using RC-2 plus 4% thioglycolic acid, only 72–75% of the expected amount of chromophore was produced. The two samples containing no thioglycolic acid developed 100 and 104% of the expected absorbance.

Table 8 also shows that when samples of free tryptophan were subjected to acid hydrolysis conditions for 22 hr using 6N HCl with 4% thioglycolic acid, only 49–74% of the expected increase in absorbance was obtained. Interestingly, the two samples that were subjected to hydrolysis conditions, without the aid of thioglycolic acid, produced 68 and 96% of the expected increase in absorbance. Therefore, it is suspected that the thioglycolic acid interferes with the production of chromophore.

Attempts to remove thioglycolic acid after hydrolysis. Both the rotary evaporator and Virtis freeze drier were unable to remove the thioglycolic acid from the samples containing 4% thioglycolic acid. The Handbook of Physics and Chemistry (1972–73 edition) lists the boiling point of mercapto-acetic acid (thioglycolic acid) as 120°C when a pressure of 20 mm Hg is used. Thus, thioglycolic acid is nonvolatile which explains why it was not removed from the hydrolysates.

In conclusion, two α-KGA reagents and reaction conditions were developed which can be used to estimate free l-tryptophan content in aqueous solutions. RC-1 was found to be very specific for free tryptophan and N-terminal tryptophan residues. Only two end products were produced by the interaction of tryptophan and RC-1. Attempts to measure the tryptophan content of lysozyme were unsuccessful.

Table 8—Effects of thioglycolic (Thio) content on production of chromophore with and without 22 hr of hydrolysis using RC-2

Hydrolysis treatment	Sample no.	Yield of tryptophan: Tryptophan found (μmoles)	Tryptophan theorized (μmoles)	% Recovery
None				
(+Thio)	T1	0.800	1.066	75
(+Thio)	T2	0.730	1.016	72
(NO Thio)	T3	1.040	1.000	104
(NO Thio)	T4	1.048	1.049	100
22 Hours				
(+Thio)	T5	0.490	0.967	51
(+Thio)	T6	0.485	0.984	49
(+Thio)	T7	0.760	1.033	74
(+Thio)	T8	0.500	0.984	51
(NO Thio)	T9	0.740	1.082	68
(NO Thio)	T10	0.960	1.000	96

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Ms received 11/17/77; revised 3/4/78; accepted 3/8/78.

Paper No. 2221, Massachusetts Agricultural Experiment Station, University of Massachusetts at Amherst. This research supported in part from Experiment Station Project No. 430 and the Glass Packaging Institute, New York, NY.

EVALUATION OF CHEESE TEXTURE

CHERL-HO LEE, ELAINE M. IMOTO and CHOKYUN RHA

ABSTRACT

The textural characteristics of cheese were surveyed for consumer preference and sensory perception. The important textural characteristics of cheese–hardness, chewiness, springiness and adhesiveness–were evaluated by a panel. The mechanical properties of cheese were measured with an Instron Universal Testing Machine. In making the Instron measurements, the effects of the conditions (i.e., rate of compression, rate of loading and cheese temperature) on the mechanical properties of cheese were determined. The melting property of cheese was determined by viscosity changes upon heating using a Brookfield Viscometer, Model RVT with T-F bar. The correlations between sensory evaluation and the mechanical properties of cheese were resolved. The panel concluded that the important textural characteristics of cheese were hardness, springiness and adhesiveness. The hardness evaluated by the panel was related to the Instron determinations of the compression force, work ratio, adhesive force, force at the inflection point and viscometer determination of the melting property. The sensory assessment of springiness correlated with elastic recovery and force at the inflection point. The sensory adhesiveness rating was related to the adhesive force but inversely related to force at the inflection point.

INTRODUCTION

THE OBJECTIVES of this study were to determine the important textural characteristics of cheese and to relate sensory parameters to objective measurements so that instruments may be used to evaluate texture in fabricated cheese.

The textural characterization of milk curd and cheese was studied extensively in Switzerland and England during the 1940s. The important textural characteristics considered were firmness, springiness and smoothness. These characteristics were measured objectively by a Ball Compressor, Meyer Hardness Tester, Borer and Needle Penetrometer. Studies in this period were comprehensive, as summarized by Baron and Scott Blair (1953). Since then, automated testing systems, texture profile analysis, and a variety of instruments and methods for determining textural parameters have been developed (Szczesniak, 1966; Bourne, 1968; Finney, 1969; Breene, 1975), which further refine the quantitative analysis of cheese texture.

In the present study, descriptive terms used to represent the textural characteristics of cheese were collected, frequently from survey questionnaires and organoleptic evaluations. The physical and mechanical properties of cheeses determined by the Instron Universal Testing Machine and by the Brookfield Viscometer were compared with sensory evaluations.

MATERIALS & METHODS

Cheese samples

Commercial cheeses representing a wide variety of textures were selected for this study. They were:

Cream cheese (S)	American cheese (K)
Cream cheese (P)	American cheese (P)
Camembert	Mild cheddar
Mozzarella	Sharp cheddar
Muenster	Processed cheddar (U)
	Swiss cheese

Letters in parentheses denote different brands of the same type of cheese.

General survey of cheese texture

The following questionnaire was submitted to approximately 50 students and staff members at the Massachusetts Institute of Technology who eat cheese regularly.

1. What kind(s) of cheese and cheese product(s) do you like most and why?
2. Are there any specific textural characteristics you like in cheese products?
3. What kind(s) of cheese and cheese product(s) do you dislike most and why?
4. Are there any specific textural characteristics you dislike in cheese products?

Sensory evaluation

A "taste panel" of 20–21 students of the Department of Nutrition and Food Science at MIT was selected on the basis of frequent consumption of cheese and cheese products. In a preliminary test, the ability of the panel to distinguish differences in the textural parameters of various types of cheeses was tested by a randomized complete block design. Six different types of cheese–Camembert, cream cheese, Mozzarella, American, cheddar and Swiss cheese–were tested by the panel. Each of the parameters, as defined by Szczesniak (1963) and Civille and Szczesniak (1973), was described to the panelists at each test as follows:

Hardness–	Force required to penetrate the sample with the molar teeth
Brittleness–	Breakability of the sample at the first bite,
Chewiness–	Number of chews required to swallow a certain amount of sample
Springiness–	Bouncing property of sample through several consecutive bites
Adhesiveness–	Stickiness of sample in the mouth throughout mastication
Lumpiness–	Heterogeneous mouth-feeling of sample throughout mastication

Each sample was cut in a cube weighing approximately 10g, wrapped in plastic film and kept in a refrigerator. Samples were exposed to room temperature for 30–60 min before being coded and served together in a dish.

The panel was asked to rank six different cheese samples in order of increasing hardness (from soft to firm), chewiness (from tender to tough), adhesiveness (from slippery to sticky), brittleness (from mushy to fragile), springiness (from plastic to elastic) and lumpiness (from smooth to grainy). Three parameters were measured in each test. In the first test, hardness, chewiness and adhesiveness were examined. Two to three days after the first test, the second test was carried out on brittleness, springiness and lumpiness. The results were analyzed by histogram and Ranking Difference Analysis (Larmond, 1970).

A sensory evaluation system, called the Milestone method in our laboratory, was devised as follows using the results of the preliminary sensory evaluation and a modification of the standard rating scale (Szczesniak et al., 1963; Boyd and Sherman, 1975). The standard rating scale for hardness rates samples from cream cheese to rock candy on a 1–9 scale. It is suitable for distinguishing among different types of

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0022-1147/78/0005-1600$02.25/0

foods, but it is not sensitive enough to distinguish among the various cheese products used in this study. Therefore, the hardness scale was adjusted so that cream cheese would have a standard rating of 2 and Swiss cheese a standard rating of 6 on a total scale of from 1–7.

Using these Milestones, each panelist was asked to set up his or her own reference scale to evaluate what a rating of 3 or 4, etc., means in terms of hardness. The test samples were provided immediately after the standards were tasted, and the panelists were asked to give the hardness number for each sample according to his or her reference scale. In this way, the sensory stimulation was quantified in relation to the given Milestones.

The chewiness scale was determined by counting the number of chews required to swallow a given amount of sample. Preliminary tests indicated that the number of chews required for most of the cheese samples ranged between 3 and 18. Therefore, a hedonic scale was made in which each level represented three chews, with the first level being less than three and the final being more than 18. Figure 1 shows the master chart used for the Milestone method of sensory rating.

Objective measurements

The mechanical properties of cheese samples were determined by a compression test using an Instron Universal Testing Machine Model 1122. Cylindrical samples (1 ± 0.1 cm tall and 1.5 cm in diameter) were bored from a 1-cm-thick slab of cheese. Once samples were cut, they were placed in air-tight containers and kept under refrigeration until the test.

In order to select a test condition applicable to all cheese samples, the effects of various crosshead speeds, compression ratios and cheese temperatures were determined with cheddar, American and cream cheeses. From the results of the preliminary study, a standard procedure was set up. The standard condition was a crosshead speed of 20 mm/min, chart speed of 50 mm/min and compression ratio of 0.8 (80% deformation). The cheese samples were taken from the refrigerator 1 hr before the measurements were made. The compression was carried out in two consecutive cycles.

The following mechanical properties were measured by the Instron Universal Testing Machine: the compression force required for the given deformation, the force at the inflection point on the force/compression curve (often called breaking force), the work ratio of the first two consecutive compressions (corresponding to cohesiveness [Bourne, 1968]), the adhesive force exerted on the ascending motion of the compressing plate after the first bite, and the elastic recovery (or the recovered height in mm) after the first bite (corresponding to springiness). All experiments were replicated more than three times, and the average value was calculated. The data were tested for correlation with sensory evaluation by using standard regression analysis and nonparametric rank correlation analysis (Snedecor and Cochran, 1967).

Melting property

Since cheese does not have a sharp melting point, the changes in the viscosity of the samples with respect to rising temperature were recorded using a Brookfield Sincro-Lectric Viscometer Model RVT with T-F bar and a helipath stand.

Cheese samples were first shredded, then tamped down into a test tube 16 mm in diameter and 100 mm in depth. Duplicate samples were made in two test tubes and immersed in a temperature-controlled water bath. A sealed Pasteur pipette, containing glycerine and a copper thermocouple wire, was inserted into the middle of one of the test tubes to monitor the temperature of the sample. The T-spindle of the viscometer was placed in the other test tube. The water bath was gradually heated, and the internal temperature of the cheese sample was checked by the thermocouple readout. The temperature was determined at which the viscometer reading at 10 rpm began to decrease from the full-scale reading to lower values, and this temperature was designated as the initial melting temperature. After the initial melting temperature was reached, the sample was equilibrated at a 2–4°C increment, and the viscometer reading was taken. All tests were carried out in triplicate, and the average values were calculated.

RESULTS

Important textural characteristics of cheese

The results of the survey showed that people do prefer certain typical textural characteristics of cheese. The two most frequently cited qualities were firmness and softness (Table 1). One group of answerers liked firmness in cheese, another group liked softness, and some liked both firmness and softness. Chewiness, smoothness, elasticity and good melting properties were generally favored, while crumbly, grainy, creamy, mushy, gooey, runny and waxy textures were disliked. Eight of the 50 respondents did not have any preference as to cheese texture.

Master Chart
(Use as answer sheet)

Panel rating	Hardness (Soft-Firm)	Chewiness (Tender-Tough)
1		less than 3 chew
2	Sample A	6
3		9
4		12
5		15
6	Sample B	18
7		more than 18 chew

Fig. 1—An example of the master chart used for the Milestone method of sensory evaluation.

Table 1—Descriptive terms of the textural characteristics of cheese (in order of higher frequency of appearance)

Like	Dislike
Firm	Crumbly
Soft	Grainy
Creamy	Firm
Chewy	Soft
Smooth	Creamy
Mixing property	Plastic
Elastic	Mushy
Melting	Gooey
	Runny
	Waxy

Critical textural parameters of cheese determined by sensory evaluation

From the results of the questionnaire, six textural parameters were selected to test the panelists' ability to identify them in six different kinds of cheese. A rank difference analysis of the data indicated that differences in hardness, brittleness, chewiness, lumpiness and springiness among the cheese samples were significant ($P < 0.01$), while the differences in adhesiveness were not significant. There was very little difference in the panel's evaluations of brittleness and hardness, and the ranking of lumpiness was related to that of chewiness and hardness.

Sensory evaluation

According to the above results, hardness and chewiness were selected to be the primary parameters to be evaluated by the Milestone method. Adhesiveness and springiness were evaluated again by rank method, since these parameters might contribute significantly to the overall textural quality and ac-

ceptability of the cheese, even though they are difficult to distinguish separately as primary stimuli in the sensory test.

Table 2 shows the sensory ratings of 11 cheese samples. It shows generally that the standard deviations of hardness and chewiness are smaller than those of adhesiveness and elasticity.

Critical test conditions for measuring mechanical properties of cheese using the Instron Universal Testing Machine

Figure 2 shows the effect of compression ratio on the mechanical properties of the cheese samples. When it increased, the compression force of the cheese samples increased steadily, rising most rapidly between 60–80% deformation (Fig. 2A). The work ratio of the cheese samples decreased from 70% to less than 20% when the degree of deformation increased from 10–70%, whereas the adhesive force showed the opposite tendency, increasing steadily with increasing degree of deformation (Fig. 2B and C). The elastic recovery of the cheese samples increased with an increasing compression ratio to a peak between 50 and 60% deformation for American cheese and between 30 and 50% for cheddar cheese (Fig. 2D), after which the elastic recovery decreased with increasing compression ratio. At low compression ratios (<50% deformation), the mechanical response of cream cheese was too low to be measured. The inflection point was observed at 40% deformation for cheddar cheese, 70% for American cheese and 60% for cream cheese. The force at the inflection point did not change with changes in compression ratio (Fig. 2E).

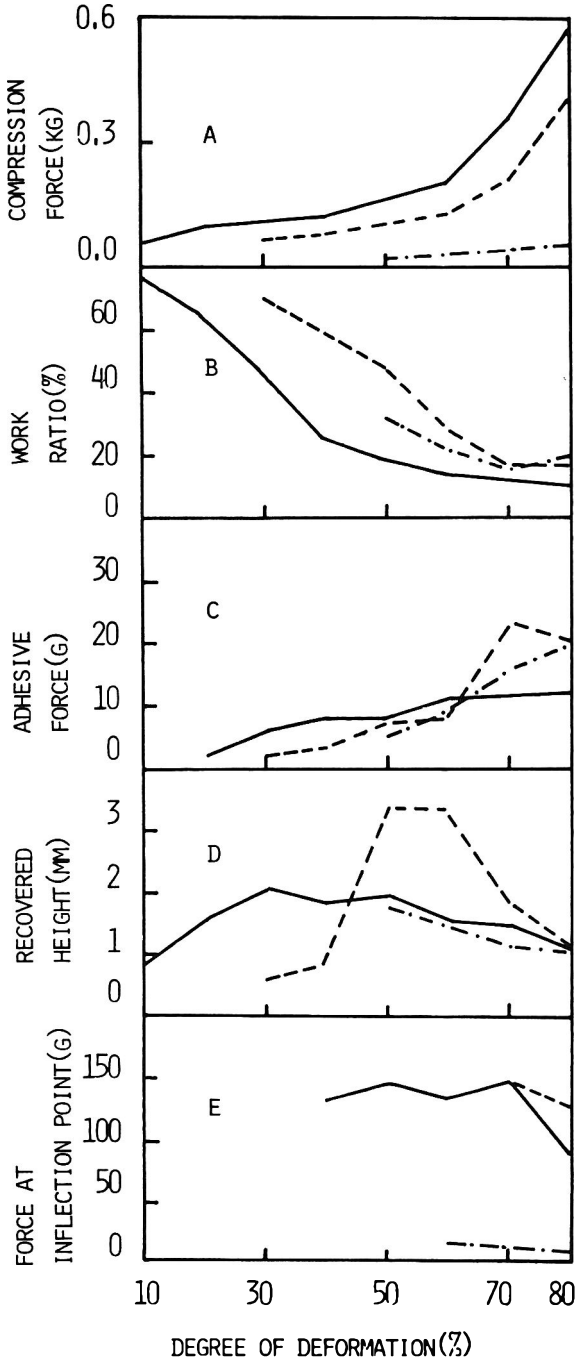

Fig. 2—Effect of compression ratio on the mechanical properties of cheese measured by Instron Universal Testing Machine (——Cheddar, --- American, — · — · — Cream cheese).

Figure 3 shows the effect of the rate of loading on the mechanical properties of three cheese samples. Changes in the crosshead speed of 1–100 mm/min increased the compression force slightly, but did not appreciably affect the elastic recovery or work ratio (Fig. 3A, B, D). Adhesive force did not appear at high crosshead speeds. The force at the inflection point increased rapidly for American and cheddar cheeses as the compression speed increased (Fig. 3E).

The cheese reached room temperature 1 hr after being taken from the refrigerator. The compression force, adhesive force and the force at the inflection point decreased with increasing temperature for the 60 min of equilibration time and then remained unchanged, as shown in Figure 4.

It was concluded that measurements of the mechanical properties of cheese required a degree of deformation greater than 70%, except for the measurement of the work ratio, which required a degree of deformation of less than 50%. The crosshead speed appeared to be critical only for measurements of adhesive force.

Mechanical properties determined by the Instron Universal Testing Machine

After the preliminary experiments, 11 cheese samples were tested for their mechanical properties by the compression test at 80% deformation and a crosshead speed of 20 mm/min. The refrigerated samples, stored in air-tight containers, were exposed to room temperature for 60 min before measurements were made. Table 3 shows the five mechanical properties of the 11 cheese samples and their standard deviations.

Melting property of cheese

Figure 5 shows the melting properties of the cheese samples as determined by the Brookfield Viscometer Model RVT with T-F spindle at 10 rpm. The changes in the viscometer readings under these conditions were plotted against the increasing temperature of the samples. Three temperatures indicating the melting properties of the cheeses were obtained: the initial melting temperature; the half melting temperature, where the viscometer reading reached 50; and the complete melting temperature, where the curve reached asymptote.

Table 2—Rating of cheese textures with sensory evaluation

Name of cheese	Hardness	Chewiness	Springiness	Adhesiveness
Cream cheese (S)	1.3 ± 0.5	1.3 ± 0.5	4.0 ± 4.1	4.2 ± 3.6
Cream cheese (P)	2.0 ± 0	1.4 ± 0.7	4.3 ± 3.8	5.2 ± 3.9
Mozzarella (C)	3.0 ± 0	2.8 ± 1.3	7.9 ± 3.6	4.1 ± 3.3
Muenster	3.7 ± 0.9	2.9 ± 1.4	7.5 ± 2.0	5.3 ± 2.1
Mozzarella (D)	3.8 ± 0.4	3.4 ± 1.3	6.0 ± 2.2	6.0 ± 2.4
American cheese (K)	4.3 ± 0.6	3.8 ± 1.1	5.9 ± 2.3	6.2 ± 3.6
Processed cheddar cheese (U)	4.9 ± 0.6	4.3 ± 1.4	6.5 ± 2.9	7.4 ± 2.3
Mild cheddar (L)	4.9 ± 0.4	3.8 ± 1.1	5.8 ± 2.1	6.0 ± 3.0
Sharp cheddar (S)	6.0 ± 0	4.0 ± 1.3	5.1 ± 2.6	7.3 ± 2.7
American cheese (P)	6.5 ± 0.8	3.4 ± 1.2	5.2 ± 2.9	6.9 ± 2.6
Swiss (S)	6.8 ± 0.4	5.4 ± 1.3	8.1 ± 3.7	4.9 ± 3.2

Correlation between sensory evaluation and the mechanical properties measured by the Instron Universal Testing Machine

The correlation between the hardness and chewiness ratings determined by the sensory Milestone method, and the mechanical properties measured by the Instron Universal Testing Machine were computed by standard regression analysis. Since the values obtained by the ranking method were neither random nor independent, the nonparametric rank correlation analysis was used to plot sensory springiness and adhesiveness ratings against the mechanical properties. Table 4 shows the coefficients of the parameters in the correlation between sensory evaluation and Instron measurements. Both compression force and work ratio, as assessed by the Instron, correlated

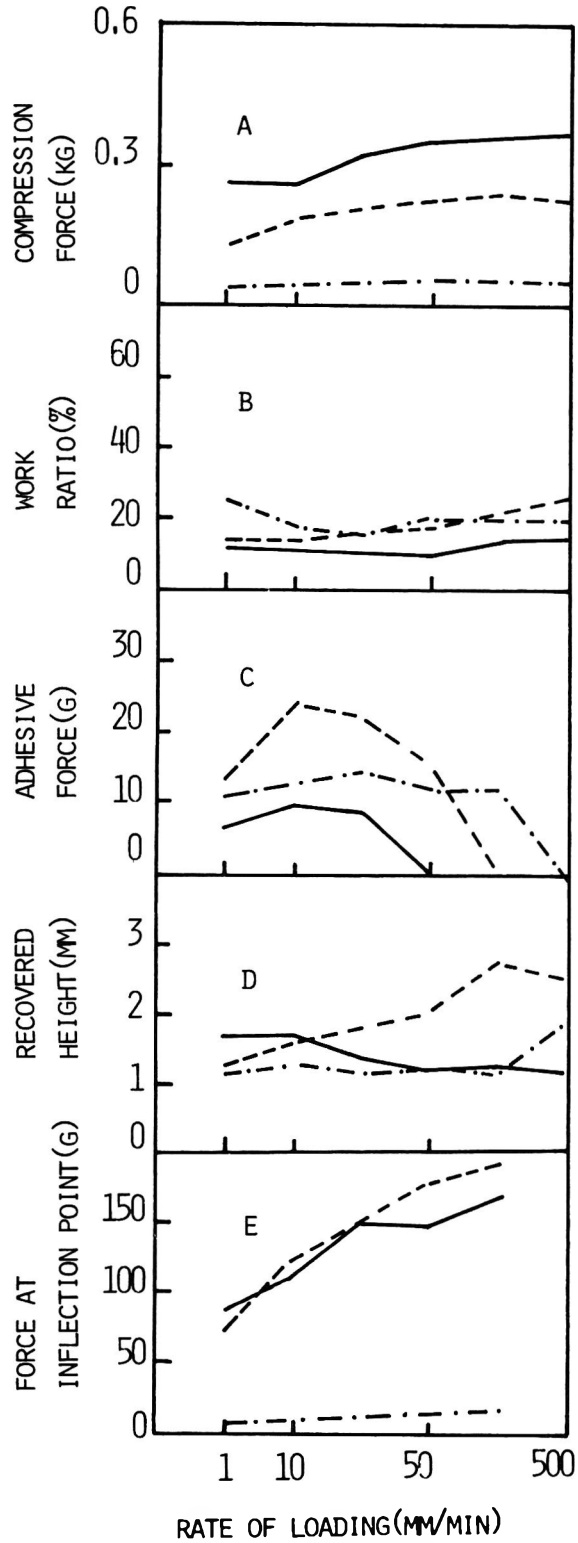

Fig. 3—Effect of rate of loading on the mechanical properties of cheese measured by Instron Universal Testing Machine (——Cheddar, --- American, — · — · — Cream cheese).

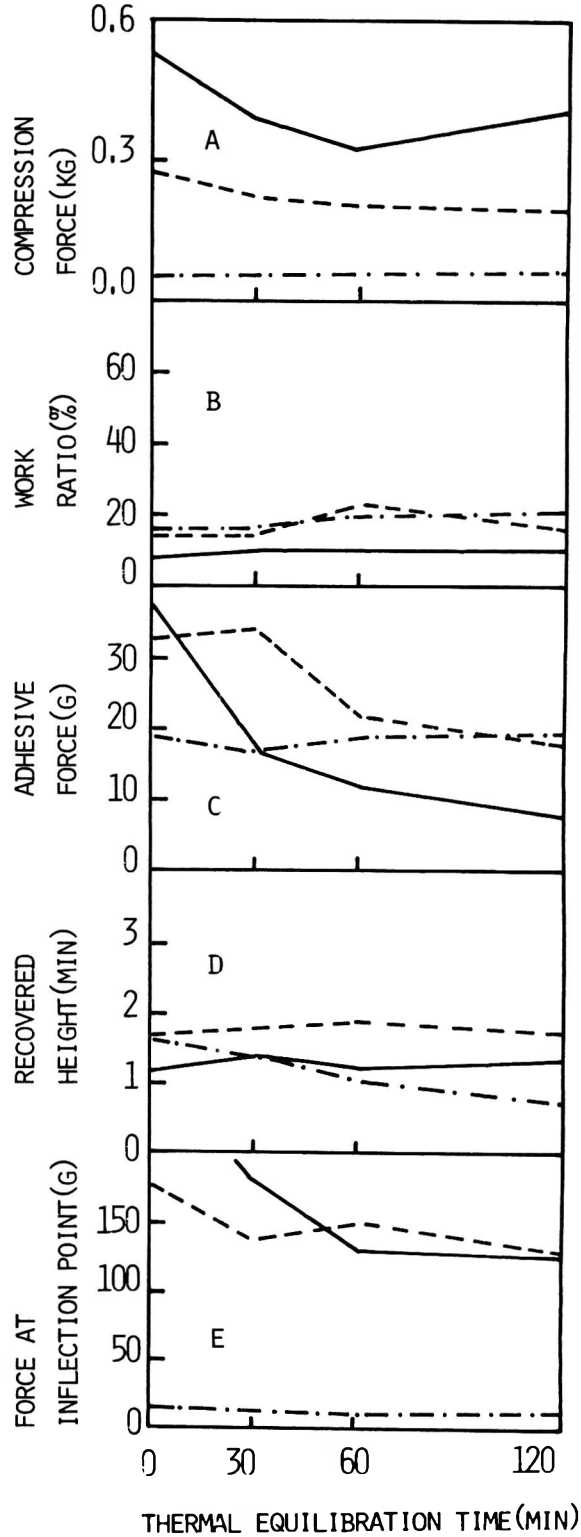

Fig. 4—Effect of cheese temperature on the mechanical properties of cheese measured by Instron Universal Testing Machine (—— Cheddar, --- American, — · — · — Cream cheese).

significantly with the sensory evaluation of hardness and chewiness.

The elastic recovery of the cheese samples and the springiness rating of the sensory evaluation panel correlated significantly. The adhesive force measured by the Instron also correlated significantly with the degrees of adhesiveness, hardness, and chewiness assessed by the panel. The force at the inflection point correlated significantly with the sensory evaluation of hardness and springiness but correlated inversely with adhesiveness.

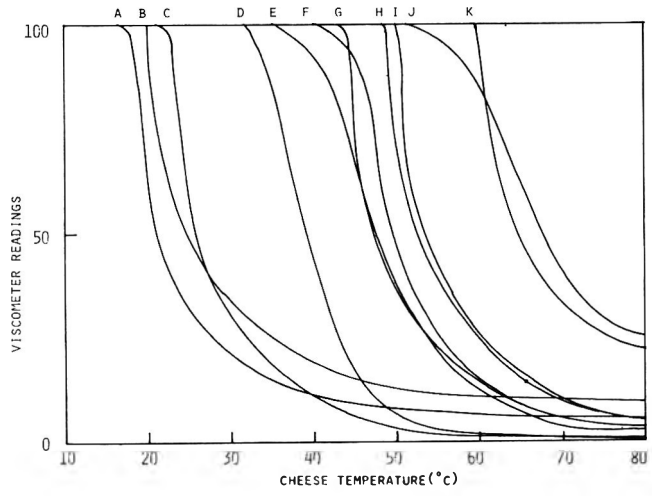

Fig. 5—Melting property of cheese as shown by viscosity change with rise in temperature [A—Cream cheese(S), B—Cream cheese(P), C—Mozzarella(C), D—Mozzarella(D), E—American(K), F—Cheddar(S), G—Muenster, H—Cheddar(L), I—Processed cheddar(U), J—American(P), K—Swiss cheese].

Table 3—Textural parameters of 11 cheese samples determined by the Instron Universal Testing Machine

Name of cheese	Compression force (g force)	Work ratio (%)	Recovered height (mm)	Adhesive force (g force)	Force at inflection point (g force)
Cream cheese (S)	61 ± 7	35.1	0.12 ± 0.04	25 ± 3	–
Cream cheese (P)	92 ± 10	27.2	0.18 ± 0.01	36 ± 7	–
Mozzarella (C)	212 ± 43	18.1	1.84 ± 0.21	58 ± 20	–
Muenster	242 ± 41	25.0	1.17 ± 0.32	29 ± 1	–
Mozzarella (D)	267 ± 24	19.1	1.02 ± 0.05	55 ± 4	–
American (K)	480 ± 30	15.7	0.75 ± 0.06	124 ± 21	133 ± 9
Sharp cheddar (S)	594 ± 41	12.2	0.39 ± 0.07	160 ± 39	–
Processed cheddar (U)	626 ± 61	21.4	0.82 ± 0.09	110 ± 25	–
Mild cheddar (C)	720 ± 43	16.7	0.78 ± 0.14	98 ± 36	110 ± 4
Swiss (S)	976 ± 55	14.4	1.10 ± 0.05	–	336 ± 15
American (P)	990 ± 60	10.7	0.55 ± 0.14	98 ± 48	240 ± 11

Table 4—Correlation coefficient between textural parameters evaluated by sensory evaluation and Instron measurement

Sensory	Hardness	Chewiness	Springiness	Adhesiveness
Instrumental compression force	0.9461*	0.8643*	0.2727	0.5909
Work ratio	0.8703*	0.8000*	0.2318	0.4409
Recovered height	0.1705	0.3606	0.9682*	−0.2273
Adhesive force	0.8020*	0.8037*	0.0788	0.8061*
Force at inflection point	0.7006*	0.8248*	0.8000*	−0.8000*

* $P < 0.01$

Correlation between sensory evaluation of cheese texture and melting property

The coefficients of the correlation between sensory texture evaluation and the melting temperatures of the cheese samples were computed by the same method used to compute the coefficients of the correlation between sensory evaluation and Instron measurements. Table 5 shows that the melting property of the cheese correlated significantly ($P < 0.01$) with the hardness and chewiness ratings of the sensory evaluation, but that no significant correlation was observed between the melting property and the sensory springiness or adhesiveness ratings.

DISCUSSION

IN EARLIER STUDIES, the important textural characteristics considered were firmness, springiness and smoothness (Baron and Scott Blair, 1953). The present study affirms that firmness (or hardness) is the most important characteristic of cheese with respect to consumer preference and sensory perception. According to the sensory evaluations, hardness can practically represent brittleness and is closely related to lumpiness and chewiness. Springiness (elastic or plastic behavior) appears to be important in the sensory perception of cheese texture, but it is more difficult to assess than hardness. Similarly, the adhesiveness of cheese samples cannot be easily detected as a separate sensory stimulus, although it is considered an important mechanical property during the handling and packaging of cheeses.

The Milestone method developed in this study appears to provide an adequate quantitative estimation of the hardness and chewiness of cheeses. It may reduce the variations among individuals in sensory evaluations by giving a certain reference scale. The mechanical properties assessed by instrument measurements can also be used as references for the rating scale, but rational division of the measurement scale and its correlation to the sensory rating require further study.

The present study indicated that the sensory evaluation of hardness by the panel correlated with the Instron measurements of the compression force, work ratio, adhesive force and force at the inflection point as well as with viscometer measurements of the melting properties. Similarly, the sensory assessment of springiness was related to elastic recovery and force at the inflection point, while adhesiveness was related to adhesive force but inversely related to force at the inflection point.

The sensory evaluation of hardness and chewiness corresponded so closely that one could represent the other. The fact that chewiness was unrelated to elastic recovery indicated that, as suggested by Szczesniak (1966), gumminess is a better term than chewiness for semi-solids like cheese.

The increase in compression force with increasing compres-

Table 5—Correlation coefficient between the sensory textural parameters and the melting property of cheese

Melting temp	Sensory: Hardness	Chewiness	Springiness	Adhesiveness
Initial	0.912*	0.894*	0.464	0.463
Half	0.839*	0.869*	0.291	0.536
Complete	0.921*	0.875*	0.391	0.482

* $P < 0.01$

sion ratio and crosshead speed, and the decrease at elevated temperatures observed in this study agree with results reported by Shama and Sherman (1973), Boyd and Sherman (1975) and Culioli and Sherman (1976). In addition, the work ratio, adhesive force and elastic recovery of cheese were influenced strongly by the compression ratio. Compression ratio, therefore, was the most important factor in the mechanical response of cheese. The rate of loading affected the adhesive force and force at the inflection point significantly. Raising the temperature generally decreased compression force, force at the inflection point and adhesive force. There was great variation in the responses of different types of cheese to the experimental conditions. Cream cheese was in most cases less affected by the temperature, rate of loading and compression ratio than were American and cheddar cheeses.

For the purpose of the present study, a combination of experimental conditions was selected that produced the largest differences among samples and fit most of the cheeses and most of the textural parameters i.e., a compression ratio of about 80% deformation, rate of loading of 20 mm/min and thermal equilibration for 60 min at room temperature. It would, however, be more desirable to use the optimum experimental conditions for measuring each parameter. These must be established by referring to the sensory evaluation data.

The melting property of cheese is important not only for the texture but also for the mixing property of cheese in food preparation. There are no established methods for determining the melting property of cheese. Chang (1976) used the percent increase in diameter of the cheese plug after it was heated at 450°F for 3 min as an index of the melting property. The method uses relatively high heating temperatures, and the actual temperature of the cheese is not known. The viscosity method used in the present study indicates the softening of the cheese as the temperature increases. Since this method uses only one shearing condition of the viscometer, it measures the temperature where the melt softening reaches a critical point and the melting property at a temperature higher than the critical point. At temperatures higher than 95–100°C, the cheese starts to liquidate (bubble), and the method is no longer valid.

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Ms received 12/4/77; revised 3/2/78; accepted 3/8/78.

The Instron Universal Testing Machine used in this study was obtained with a Biomedical Research Support Grant from the Office of the Provost, M.I.T.
This paper is Contribution No. 3388 from the Department of Nutrition and Food Science, M.I.T.

A Research Note

STRUCTURAL CHANGES IN ELECTRICALLY STIMULATED BEEF MUSCLE

J. W. SAVELL, T. R. DUTSON, G. C. SMITH and Z. L. CARPENTER

ABSTRACT

The right sides of five steers were electrically stimulated with 50 impulses of 0.5–1.0 sec duration, 100 volts (machine setting), 5 amps, 50–60 cycles per sec, producing an approximate 440 volt potential difference between electrodes; the left sides were used as untreated controls. Longissimus muscle samples were removed 20–24 hr postmortem from both sides of each carcass for palatability, cookery and sarcomere length determination as well as for light and electron microscopy. Steaks from electrically stimulated sides were less juicy, more tender and more flavorful, had less organoleptically detectable connective tissue and lower shear force values, and sustained greater cooking losses than did samples from control sides. Mean sarcomere length did not differ significantly between control and electrically stimulated samples. Light micrographs of electrically stimulated samples revealed contracture bands throughout the myofibers along with stretched areas on either side of the contracture bands. Electron micrographs also showed contracture bands along with physical disruption of the myofibrils on either side of the bands. These data suggest that electrical stimulation (performed in the manner described here) may improve tenderness by physical disruption and the formation of contracture bands and not by prevention of cold shortening.

INTRODUCTION

ELECTRICAL STIMULATION has been reported to be an effective method for improving meat tenderness (Chrystall and Hagyard, 1976; Davey et al., 1976; Grusby et al., 1976; Savell et al., 1977, 1978). Although the mechanism by which electrical stimulation improves tenderness has not been elucidated, postulations include reductions in cold shortening (Davey et al., 1976; Chrystall and Hagyard, 1976) and increased activity of acid proteases (Savell et al., 1977). The method of electrical stimulation used in studies reported by Savell et al. (1977, 1978) and Smith et al. (1977) enhances tenderness by mechanisms other than or in addition to minimization of cold shortening since sarcomere length does not normally differ between stimulated and nonstimulated sides.

A possible mechanism for the tenderness improvement associated with use of the electrical stimulation procedure used in our laboratory is physical disruption of muscle fibers resulting from the massive contractions during stimulation. The present study determined the effect of electrical stimulation on changes in the structure of beef muscle.

EXPERIMENTAL

FIVE STEERS were conventionally slaughtered, split longitudinally and the right side of each carcass was electrically stimulated within 1-hr postmortem and before chilling. Steers ranged in USDA quality grade from Average Good to Low Choice, yield grade from 2.4–4.3 and carcass weight from 565–881. An "Electro-Sting," model 160-ESS (50–60 cycles, 5 amps, AC) electrical stunner with a setting of 100 volts was used as the source of electrical stimulation. A machine setting of 100 volts produces a 440 volt potential difference between electrodes. The stunner was modified for use in electrical stimulation by attaching two cables to the lamb stunning unit in order to facilitate extension of the stunning probes. At the end of each cable a metal pin (approximately 0.6 × 20 cm) was attached; 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 right side of each carcass was stimulated 50 times (0.5–1 sec duration) while the left side served as an untreated control.

Microscopy

Samples were removed from both sides of each carcass 20–24 hr postmortem and were embedded for transmission electron microscopy according to procedures described by Dutson (1974). The embedded samples were sectioned at 1 μm for light microscopy and at 50 nm for electron microscopy using an LKB ultramicrotome. Thick sections were placed on glass slides, stained with toluidine blue (2 parts 0.5% toluidine blue in 2.5% $NaCO_3$ and 1 part Paragon epoxy tissue stain filtered through Whatman No. 3 paper) and covered with a coverslip using Permount (Fisher Chemical Co.). Thin sections were placed on copper grids and stained with uranyl acetate and bismuth subnitrate (Riva, 1974). Electron micrographs were taken using a Jeol 100-S electron microscope; light micrographs were taken using a Zeiss-WL light microscope.

Palatability tests

One steak (2.5 cm in thickness) was removed from the 11–12th rib section of the longissimus muscle of each side, 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 organoleptically detectable connective tissue and flavor. 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 20–24 hr postmortem from each side, 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

MEAN VALUES for certain cooking characteristics and palatability attributes of control and electrically stimulated beef are presented in Table 1. The small number of animals used in the present study reduced the likelihood that differences would be statistically significant at low alpha levels, but the differences between treatment means are of essentially the same magnitude as those reported by Savell et al. (1977). Steaks from electrically stimulated sides were less juicy, more tender and more flavorful, had less organoleptically detectable connective tissue and lower shear force values, and sustained greater cooking losses than did samples from control sides. Mean sarcomere length did not differ significantly between control and electrically stimulated samples. The latter finding agrees

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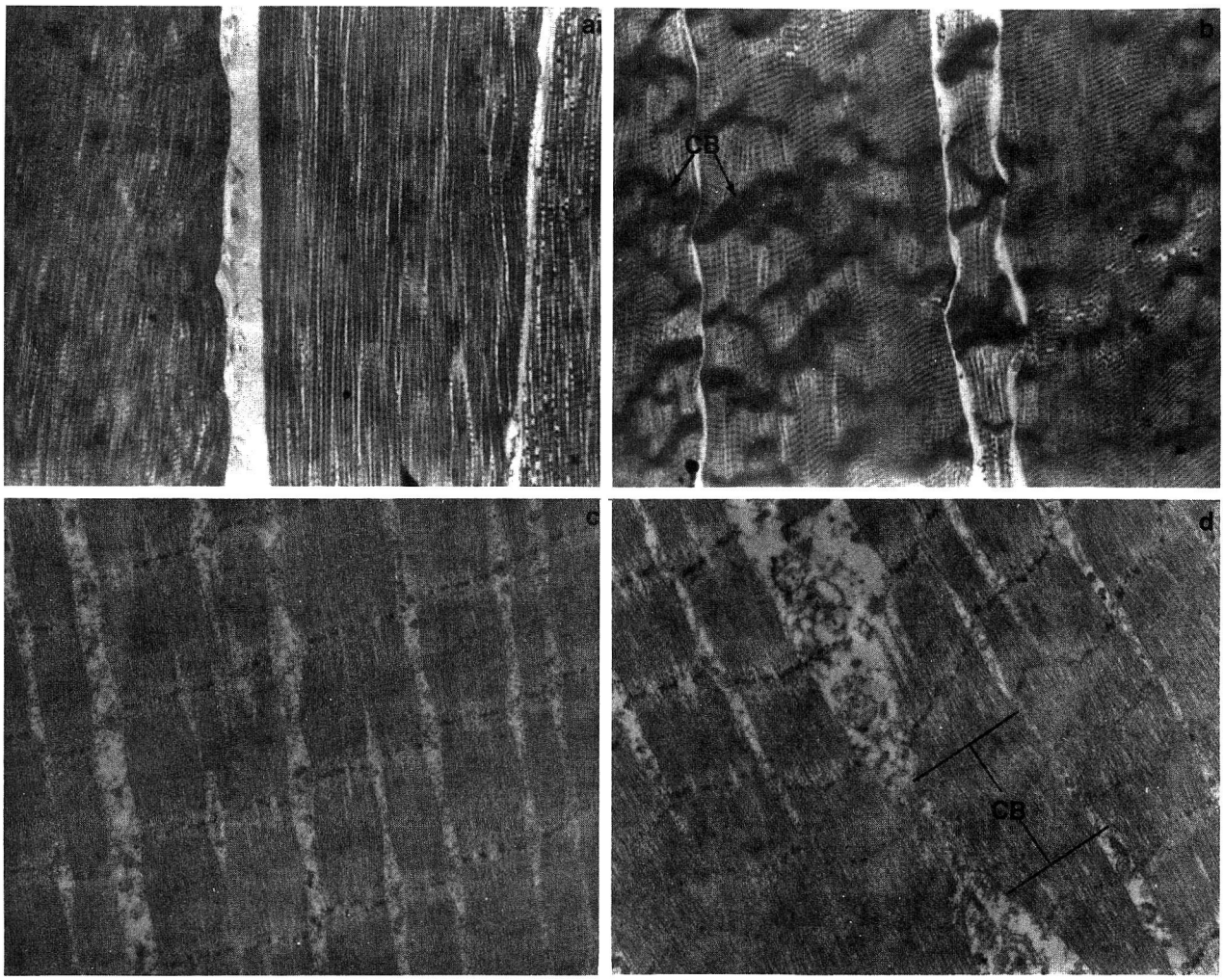

Fig. 1—(a) Light micrograph of muscle fibers from a control side (X ≈ 750). (b) Light micrograph of muscle fibers from an electrically stimulated side. CB = contractraction band (X ≈ 750). (c) Electron micrograph of a muscle fiber from a control side (X ≈ 9600). (d) Electron micrograph of a muscle fiber from an electrically stimulated side. CB = contraction band (X ≈ 9600).

with Savell et al. (1977) who reported that electrical stimulation of beef increased tenderness without an accompanying increase in sarcomere length. These results suggest that the tenderness improvement associated with use of electrical stimulation can be achieved by means other than prevention of cold shortening.

Table 1—Mean values for certain cooking characteristics and palatability attributes

Trait	Treatment: Control (untreated) Mean	Control (untreated) S.D.	Electrically stimulated Mean	Electrically stimulated S.D.	Level of probability[a]
Juiciness[b]	5.8	0.24	5.2	0.85	P<0.20
Tenderness[c]	4.8	1.38	6.1	0.43	P<0.20
Connective tissue amount[d]	6.2	0.82	7.2	0.13	P<0.05
Flavor	5.2	0.64	5.8	0.41	P<0.10
Warner-Bratzler shear, (N)	71.12	24.46	54.18	6.41	P<0.15
Cooking loss (%)	25.7	2.15	32.0	1.31	P<0.05
Sarcomere length (μm)	1.75	0.14	1.73	0.93	N.S.

[a] Probability that the difference between treatments is statistically significant based on paired-t analysis (Steel and Torrie, 1960).
[b] 8 = extremely juicy, 1 = extremely dry
[c] 8 = extremely tender, 1 = extremely tough
[d] 8 = none, 1 = abundant; evaluated organoleptically

Micrographs of muscle tissue from electrically stimulated and control sides are presented in Figure 1. The structural appearance of control and electrically stimulated samples differs greatly in the light micrographs; electrically stimulated samples (Fig. 1-b) displayed definite contracture bands in certain areas and slightly stretched sarcomeres in other areas. Cassens et al. (1963) described contracture bands which were formed by extremely rapid postmortem glycolysis and by physical disturbance such as pricking or cutting muscle fibers at the time of death. Both of these conditions (rapid postmortem glycolysis and physical disturbance) occur during or in association with electrical stimulation and may account for the contracture bands observed in the present study. In the electron micrographs of electrically stimulated samples (Fig. 1-d), definite structural differences are apparent when compared to those from the control sides (Fig. 1-c); electrically stimulated samples had less well defined I-bands and Z-lines through the contracture bands while sarcomeres on either side of the contracture bands seem to be stretched or even broken.

The similarity in sarcomere lengths of control and electrically stimulated samples despite significant tenderness improvements in this and other studies may result from the lesser amount of overlap in the stretched areas and physical damage in the contracture band region leading to less structural integrity and greater tenderness. The significantly higher

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A Research Note

PROTEIN EFFICIENCY RATIO (PER) OF COMBINATION HAM CONTAINING ISOLATED SOY PROTEIN

P. T. TYBOR, J. G. ENDRES and L. D. WILLIAMS

ABSTRACT

Regular hams, water-added hams and three different combination ham products were prepared by stitch injection, massaging, canning and water cooking. Pumping pickles for the combination ham products contained 10% or more of isolated soy protein. The combination hams were pumped with differing amount of soy protein. These hams differed as to actual ham content as follows; 76%, 71% and 66% ham. The protein efficiency ratios (PER) of the combination hams were statistically equivalent to both the regular and water-added hams. Thus, the extension of ham with isolated soy protein can result in a product having a protein nutritional value equal to traditional ham products as determined by the PER assay in rats.

INTRODUCTION

IN MAY, 1976, the United States Department of Agriculture published an interim regulation which defined the use of nonmeat proteins, such as isolated soy proteins, in cured meat products (USDA, 1976). The term "combination" was used to define the new meat product concept, and to differentiate this product from conventional and water-added cured meats.

A "combination ham" is defined as cured ham that has been processed such that the finished product contains nonmeat proteins which extend or replace meat proteins. The product contains a minimum of 17% total protein, and has a moisture-to-protein ratio less than or equal to 4:1. The nonmeat protein which is used to augment meat proteins must have a protein efficiency ratio (PER) of not less than 2.0, or an essential amino acid content (excluding tryptophan) of not less than 28% of the total protein. Furthermore, the nonmeat protein must be fortified with a specified quantity of vitamins and minerals. The vitamins and minerals required for fortification are: vitamin A, thiamine, riboflavin, niacin, pantothenic acid, vitamin B_6, vitamin B_{12}, iron, magnesium, zinc, copper and potassium. These parameters were prescribed by the United States Department of Agriculture so that the nutritive value of combination hams would be equivalent to traditional products.

Preliminary research on the utilization of isolated soy protein for the extension of cured meat pieces began in 1962. Moerman (1966) reported on the feasibility of augmenting the meat proteins of hams with isolated soy proteins. It was demonstrated that an increase in protein content was attainable with artery pumping or stitch injecting to 121% over raw weight. However, higher levels of injection resulted in an unacceptable product due to the limited functionality of the isolated soy protein. Since then, isolated soy proteins with improved protein functionality have been developed specifically for use in cured meats, including ham.

The composition and certain nutritional constituents of combination hams were described by Tybor et al. (1977). Combination hams were shown to be equivalent in essential amino acid, vitamin and essential mineral contents to both regular and water-added hams. Furthermore, it was concluded that the extension of boned and trimmed hams to at least 50% over raw weight (combination ham containing 66% ham) with isolated soy protein is possible without adversely affecting the nutrient composition of the finished product.

The work described in this report was initiated to verify, by means of rat feeding studies, that the protein quality of combination ham is equivalent to that of traditional ham products.

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0022-1147/78/0005-1608$02.25/0

Table 1—Pumping pickle formulations for the combination hams

Ingredient	130% Pump (76% Ham) percent	140% Pump (71% Ham) percent	150% Pump (66% Ham) percent
Water	76.051	76.391	76.590
Isolated soy protein	10.180	12.495	13.890
Sodium chloride	10.486	8.470	7.260
Sucrose	1.777	1.435	1.230
Sodium tripolyphosphate	1.387	1.120	0.960
Sodium nitrite	0.052	0.039	0.030
Sodium erythorbate	0.067	0.050	0.040

EXPERIMENTAL

WHOLE HAMS were skinned, trimmed to remove fat and boned. The muscles were sectioned and further trimmed in order to obtain lean meat pieces with a protein content of approximately 20%. Using the trimmed and sectioned meat, five ham variables (regular, water-added and three different combination products) were produced by the stitch injection and massaging process. Combination hams differed as to ham content in the finished product. For this investigation, the ham sections for the combination products were pumped with curing pickle to 30%, 40% and 50% over raw weight, which is equivalent to 76%, 71% and 66% ham content, respectively.

Pumping pickle formulations for the combination hams are presented in Table 1. The procedure for the preparation of the pumping pickles was as follows; (a) soy protein isolate was dispersed in 10°C water using a propeller mixer and allowed to mix for 30 min; (b) sodium tripolyphosphate was dissolved in hot tap water (9 parts water: 1 part phosphate) and then added to the soy isolate dispersion; and (c) salt, sugar, sodium nitrite and sodium erythorbate were added. The final pickle dispersion was allowed to mix for 30 min.

Approximately 100 lb of trimmed boneless ham sections were stitch injected using a multi-needle injector at 60 psig to slightly less than the desired level of pump. The ham sections were massaged in a vertical rotating arm massaging unit for 20 hr using additional pumping pickle to bring the meat to the necessary weight. Massaging was carried out at 3 rpm of the rotating arm with a 20-min clockwise, 20-min rest and 20-min counterclockwise cycle. The hams were then stuffed into 3-lb rectangular cans and vacuum sealed. The hams were cooked in water at 77°C to an internal temperature of 68°C. Finally, the canned hams were cooled and held under refrigeration.

The protein efficiency ratio (PER) of the regular, water-added and combination hams was determined according to the standard AOAC method (AOAC, 1975) in which weanling rats (10/variable) are fed a standard diet containing 10% protein for 28 days. Other nutritive elements of the diets were also adjusted and balanced as specified.

RESULTS & DISCUSSION

THE PER VALUES for the ham products are presented in Table 2. All ham products were shown to have corrected PER values greater than 2.5, and therefore can be considered to be

Table 2—Protein efficiency ratios of regular, water-added and combination canned hams

Products	PER[a]	Corrected PER[b]
Regular ham		
Lot 1	3.41 ± 0.22	3.03
Lot 2	3.48 ± 0.26	3.09
Water-added ham		
Lot 1	3.21 ± 0.18	2.85
Lot 2	3.29 ± 0.31	2.92
Combination hams		
76% ham		
Lot 1	3.28 ± 0.27	2.91
Lot 2	3.30 ± 0.18	2.93
71% Ham		
Lot 1	3.14 ± 0.30	2.79
Lot 2	3.20 ± 0.24	2.84
66% Ham		
Lot 1	3.45 ± 0.26	3.06
Lot 2	3.27 ± 0.22	2.90

[a] Protein efficiency ratio with standard deviation

[b] Corrected to a casein value of 2.50. The actual value for casein was 2.82 ± 0.25.

of good nutritive value. Augmenting the meat proteins with isolated soy protein did not adversely affect the PER of the hams. The combination hams containing isolated soy protein exhibited PER's statistically equivalent to both the regular and water-added products.

Tybor et al., (1977) described the similarities between combination and traditional hams as they relate to proximate composition and amino acid, vitamin and mineral contents. The feeding studies reported here substantiate the conclusion that the protein quality of combination hams is equivalent to both regular and water-added products when isolated soy protein is used to augment the meat proteins to the levels described.

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Ms received 3/2/78; revised 5/18/78; accepted 5/28/78.

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cooking loss sustained by electrically stimulated steaks may be due to the pH-temperature relationship causing reduced water-holding capacity as well as to physical disruption of fibers leading to greater moisture loss. Although this study definity shows that structural alterations of muscle fibers occur as a result of electrical stimulation of beef carcasses, other mechanisms, including increased autolytic proteolysis, could also be operative in increasing the tenderness of electrically stimulated beef.

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Ms received 11/28/77; revised 3/29/78; accepted 4/5/78.

Technical Article 13820 of the Texas Agricultural Experiment Station.

This study was partially supported by King Ranch, Inc., Kingsville, TX and Texas Cattle Feeders Assn., Amarillo, TX. X.

A Research Note

MAGNESIUM CONTENT OF SELECTED FOODS

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ABSTRACT

The magnesium content of about 150 foods was determined by atomic absorption spectrophotometry. Generally bread and cereal products and chocolate and licorice candies had two to three times the magnesium per wet weight as milk, meat or vegetable products. The ratios of magnesium to caloric content of these foods were also calculated using published values for caloric content of the foods. Generally the ratios were higher in vegetables than in meat, milk and cereal products.

INTRODUCTION

MAGNESIUM is a prosthetic ion in enzymes that hydrolyze and transfer phosphate groups. Hence it is essential for energy requiring biological functions such as membrane transport, generation and transmission of nerve impulses, contraction of muscles and oxidative phosphorylation. It is also essential for the maintenance of ribosomal structure and thus protein synthesis (Aikawa, 1971). Magnesium deficiency in humans has not been documented to occur except in cases of predisposing and complicating disease states (Shils, 1976). However, Seelig and Heggtveit (1974) have hypothesized that long term intakes of marginal dietary level of magnesium may be related to the incidence of ischemic heart disease among Western populations.

An allowance for magnesium has been included in the Recommended Dietary Allowance since 1968 (Foods and Nutrition Board, 1968). While there are several compilations of the magnesium content of foodstuffs (Watt and Merrill, 1963; Seelig, 1964; Schlettwein-Gsell and Mommsen-Straub, 1973), we found that we could not calculate with the published tables the magnesium content of diets consumed by adolescents who were eating a variety of processed and convenience foods.

Recently, Johnson et al. (1977) and Allen et al. (1977) have reported the metalocalorie ratios for the copper, iron, and zinc content of foods. Such information on nutrient density would be valuable for individuals calculating calorie restricted diets. However, no similar information has been published on magnesium to calorie ratios.

In this paper we report the magnesium content of about 150 commonly consumed foods. The ratios of magnesium to the calorie content of the foods were also calculated.

METHODS

FOODS were purchased at a local supermarket. The form of the foods analyzed is indicated in Table 1. Food items that were not homogeneous in nature were homogenized in deionized water. Food samples were dried at 90–95°C, dry ashed in a muffle oven at 450°C overnight, dissolved in 3N HCl, diluted with a strontium chloride solution so that the final concentration of strontium (w/v) was 0.1% and analyzed by atomic absorption spectrophotometry as described by Osis et al. (1972). Liquids were diluted with a 0.1% strontium chloride solution and analyzed by atomic absorption spectrophotometry directly. Triplicate analyses were performed on all foods. Three additional samples were analyzed if differences between the original three values were in excess of 5%. Replicates of commercially prepared products were selected from different packaging units. When known amounts of magnesium were added to food samples processed in this manner, the average recovery was 95%.

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0022-1147/78/0005-1610$02.25/0

Table 1—Magnesium content of foodstuffs

Food	mg Mg per 100g
Apricot nectar juice, canned	6.2
Barbecue sauce, commercially prepared	5.5
Beef, chipped, dry	37.6
Beef stew, homemade with vegetables	17.2
Beverages, carbonated:	
Club soda	0.6
Cola:	
(Coca Cola)	1.7
(Pepsi)	0.5
Grape	0.7
Lemon:	
(Seven Up)	0.7
(Sprite)	1.3
Orange	1.7
Root beer	1.5
Bouillon cubes:	
Beef	31.0
Chicken	8.9
Bun:	
Hamburger, enriched	20.5
Hot dog, enriched	19.8
Butterscotch sauce, commercially prepared	6.7
Cabbage, Chinese, raw	14.0
Cabbage, red, raw	13.8
Cabbage, white:	
Raw	13.5
Cooked, drained	6.3
Cake, commercially-prepared:	
Sponge with cream filling	7.5
Chocolate with fudge icing and cream filling	42.2
Roll of chocolate and cream filling coated with fudge icing	39.5
Candy:	
Nougat, caramel, and peanut bar coated with chocolate (Snickers)	52.8
Chocolate bar with rice puffs	51.9
Chocolate caramel roll (Tootsie Roll)	31.8
Licorice-flavored sticks:	
Red	21.8
Black	78.0
Peanut & nougat bar coated with chocolate (Baby Ruth)	89.9
Malted milk balls	55.3
Peanut butter & chocolate cup	84.6
Nougat & caramel bar coated with chocolate:	
(Milky Way)	31.9
(Milk Shake)	31.2
Carrots, cooked, drained	12.5
Cereal, ready-to-eat breakfast:	
Presweetened:	
Corn, fruit-flavored, nutrients added (Trix)	24.9
Corn, nutrients added (Quisp)	40.8
Corn & graham flour, nutrients added (Golden Grahams)	40.8

Table 1—Continued

Food	mg Mg per 100g
Corn & oat:	
Nutrients added:	
(Captain Crunch)	37.2
(Honey Comb)	33.7
Vitamin and mineral supplement (King Vitamin)	34.3
Corn, wheat, & oat, nutrients added:	
Apple flavored (Apple Jacks)	23.1
Fruit flavored (Fruit Loops)	26.8
Oat & corn, nutrients added (Alphabits)	57.7
Oat & corn, blueberry flavored, nutrients added (Boo Berry)	56.5
Oat, soy protein concentrate, sodium caseinate, nutrients added (Life)	32.4
Rice puffs, cocoa-flavored, nutrients added (Cocoa Pebbles)	31.5
Unsweetened:	
Corn, nutrients added (Corn Chex)	10.6
Corn, oat, wheat, & rice flakes, vitamin and iron supplement (Product 19)	31.4
Rice, nutrients added (Rice Chex)	24.6
Wheat flakes, nutrients added (Wheaties)	102.2
Wheat & malted barley granules, nutrients added (Grape Nuts)	67.0
Cheese:	
Cream	6.8
Mozzarella	21.9
Spread, pasteurized, canned	25.2
Chicken, canned, deboned	12.3
Chicken pot pie, commercially-prepared, frozen	11.1
Chocolate chips, semisweet	119.2
Cocoa beverage powder mix, without milk powder	70.1
Cone, ice cream, regular	26.0
Cookies:	
Brownies, commercially prepared	39.3
Caramel-peanut log coated with chocolate, commercially prepared	63.6
Chocolate covered graham crackers	41.2
Chocolate sandwich with vanilla filling, commercially prepared	50.8
Ginger, commercially prepared	53.2
Oatmeal, commercially prepared	50.8
Peanut butter, homemade	38.5
Sugar, soft, commercially prepared	13.1
Sugar wafer	8.9
Corn bread, homemade	32.8
Corn chips	77.4
Corn & cheese snack curls	32.0
Crackers:	
Cheese	26.1
Melba toast	62.7
Wheat, rye, malted barley (Ritz)	22.1
Cream substitute, dried	4.1
Croutons, commercially prepared	33.2
Doughnut, cake-type	22.5
Egg:	
Hard cooked	12.2
Scrambled	10.9
Fish sticks, frozen, raw	24.0
Frozen desserts:	
Vanilla ice cream bar with chocolate coating	18.4
Fudge bar	24.7
Fruit punch:	
Orange, canned, vitamin C fortified	3.3
Tropical mixture, canned, vitamin C fortified	4.1
Tropical mixture, powder	1.7
Gelatin dessert with peaches	2.7
Gravy mix:	
Beef, dried	56.2
Chicken, dried	37.8
Gum, peppermint	17.9
Haddock, fillet, frozen, raw	24.0
Herring, lake fillet	17.0

Table 1—Continued

Food	mg Mg per 100g
Ice cream, chocolate	22.8
Jelly, apple	3.9
Macaroni & cheese, homemade	20.2
Macaroni salad, homemade	22.4
Macaroni shells, dried, enriched	40.8
Marshmallows	3.8
Milk:	
2% Fat, dry milk solids added	11.6
Nonfat dry milk solids & vegetable oil, canned (Milnot)	25.7
Muffin, English	18.0
Noodles, enriched, dry	126.0
Onion, green, raw	20.6
Orange juice, dehydrated crystals	2.2
Pastry, commercially prepared:	
Apple, thawed and baked	4.6
Blueberry jam, toaster-sized	18.2
Cherry, individual	9.3
Chocolate, toaster-sized	41.3
Peas, green, cooked, drained	17.6
Popcorn:	
Whole kernel	156.2
Caramel covered, commercially prepared	83.8
Potato:	
Baked	18.2
Chips made from minced potatoes	54.6
Mashed, homemade	14.1
Scalloped, homemade	6.8
Pretzels	24.3
Pudding, commercially prepared, canned:	
Butterscotch	9.5
Chocolate	18.6
Vanilla	8.0
Rennet tablets	10.4
Roll, cinnamon Danish	19.8
Salad dressing, thousand island	5.6
Sausage:	
Beef, hard	14.9
Bologna, all meat	13.6
Frankfurter, raw	13.2
"Old-fashioned" loaf, with olives and pimentos	21.3
Polish	16.6
Pork, chopped, spiced, canned	15.2
Pork, raw, patties	9.8
Salami	10.9
Seaweed, canned with sugar & soy sauce	116.0
Sherbert, orange	4.7
Soup:	
Chicken noodle, canned, condensed	10.4
Cream of celery, canned, condensed	6.2
Cream of mushroom, canned, condensed	4.6
Vegetable, homemade	10.8
Sirup:	
Maple	23.5
Table, cane & maple	3.4
Taco shell	103.9
Tapioca, dry	3.8
Tartar sauce, commercially prepared	3.2
Topping mixture, whipped cream substitute, dried	14.2
Turnips, cooked, drained	6.7
Wheat starch	1.5
Worcestershire sauce	14.7

The caloric content of the foods as indicated in USDA Handbook 8 (Watt and Merrill, 1963) and on nutritional labels and the analyzed magnesium content of the foods were used to calculate magnesium to calorie ratios shown in Table 2. Standard errors of the means were calculated (Steel and Torrie, 1960).

RESULTS & DISCUSSION

TABLE 1 is an alphabetic listing of the foods analyzed in our laboratory for magnesium content. These foods were analyzed

Table 2—Distribution of magnesium in foods classified into food groups

Food group	No. of Samples	Magnesium concentration mg/100g wet wt Mean ± S.E.M.	mg/100g wet wt Range	µg/kilocalorie Mean ± S.E.M.	µg/kilocalorie Range
Milk products (cheeses, ice cream, milks, puddings)	9	16.7 ± 2.6	6.8–25.7	108 ± 21	18–198
Meat & meat alternates (chicken, dried beef, eggs, fish, sausages)	15	16.9 ± 1.9	9.8–37.6	93 ± 24	20–353
Vegetables (cabbages, carrot, onion, potato, turnip)	7	14.2 ± 2.2	6.7–20.6	495 ± 107	196–1000
Breads and Cereals (buns, cereals, cornbread, crackers, croutons, English muffin, pasta, taco shells)	28	42.2 ± 5.4	10.6–126.0	112 ± 14	27–325
Baked desserts (cakes, cookies, doughnuts, pastries, sweet rolls)	16	28.9 ± 4.6	4.6–53.2	86 ± 18	18–307
Candies (chocolates, licorice)	11	52.9 ± 7.7	21.8–89.9	117 ± 16	63–225

because they were the ones consumed by adolescents during nutrition surveys (Greger, 1976). In Table 2, these foods are grouped into six classifications: milk products, meat or meat alternates, vegetables, bread and cereals, baked desserts, and candies. Magnesium concentrations are expressed as mg magnesium per wet weight and as µg magnesium per kilocalorie in Table 2. Foods in Table 1 which did not logically fit into these food groups were not used for the calculations in Table 2.

The distribution of magnesium in foodstuffs is very different than the distribution of zinc in foodstuffs (Allen et al., 1977; Haeflien and Rasmussen, 1977; Johnson et al., 1977; Osis et al., 1972). Generally breads and cereals, baked desserts, and candies contained more magnesium per weight than milk products, meats and meat alternates, or vegetables. However, when magnesium concentrations were expressed as µg magnesium per kilocalorie, vegetables had four times as much magnesium as the foods in the other groups in Table 2.

Within each of the six groups, there were great variations in the magnesium content of the foods considered. However, many of the snack foods, i.e. candies and baked desserts, especially chocolate ones, contained high concentrations of magnesium. As these foods are extremely popular among adolescents (Greger, 1976), they may be a major source of magnesium in the diets of this age group.

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Ms received 12/20/77; revised 3/29/78; accepted 4/5/78.

Supported in part by Cooperative State Research Service, USDA Grant #516-15-101. Purdue University Experiment Station journal paper 7019.

A Research Note

EFFECT OF TEMPERATURE ON SHORTENING IN CHICKEN MUSCLE

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ABSTRACT

Chicken breast and thigh muscles were subjected to different environmental temperatures to determine if the phenomenon of cold shortening exists in chicken muscle. For both breast and thigh muscles, minimum shortening was observed in the 4–10°C range. Muscles held at 0°C showed a slightly higher extent of shortening than at 4°C; whereas muscles held at above 20°C showed a severe shortening effect. It was concluded that no apparent cold shortening was detected in chicken muscle except at 0°C and even at 0°C the extent of shortening was of a small magnitude compared to bovine muscles. Since high temperature induces a much greater shortening, muscle temperature must be lowered to below 20°C as early as possible to prevent excessive muscle shortening.

INTRODUCTION

IT IS NOW well recognized that the cold shortening phenomenon described by Locker and Hagyard (1963) in bovine muscle excised pre-rigor has a toughening effect in beef and lamb (Marsh and Leet, 1966). It is also generalized that mammalian white muscles, rabbit psoas for instance, display no cold shortening; whereas, red muscles–beef, lamb, rabbit semitendinosus, and rat soleus–exhibit severe cold shortening by as much as 50% or more (Marsh, 1977). Some cold shortening has been observed in porcine muscle, although its extent was much less than that observed in beef or lamb (Marsh et al., 1972).

Limited studies have been reported on the cold shortening effect in avian muscles and the results are controversial. Smith et al. (1969) reported that shortening in excised pectoralis major muscles of chicken and turkeys held at 0°C was significantly greater than those held in the 12–18°C temperature range. In contrast, Jungk and Marion (1970) detected no cold shortening in turkey breast muscle and the extent of postmortem shortening was linearly related to temperature. Yet another study (Welbourn et al., 1968) showed that the 0°C treatment for 3 hr resulted in a significant toughening for thigh muscle but only a slight toughening for breast muscle.

In poultry processing, the birds are rapidly chilled to 4°C after evisceration, and thus, subject to possible cold shortening. The objective of the present study was to determine whether the phenomenon of cold shortening truly exists in chicken muscle.

EXPERIMENTAL

FIVE ROASTER CHICKENS, weighing 1.8–2.2 kg, were obtained from a local processor. The birds were inverted in a metal cone to restrict struggle and killed by carotid severance. Immediately after exsanguination, the skin was incised and pectoralis major and thigh muscles were rapidly removed. Thin strips of muscle, 0.5 × 0.5 × 4–6 cm, were dissected parallel to the direction of the muscle fibers. Muscle strips were laid on filter paper in a petri dish and the initial muscle strip length was marked on the filter paper which was then moistened with saline solution. Petri dishes containing muscle strips were covered and placed at different storage temperatures of 0, 4, 10, 16, 22, 25, 35 and 43°C. In the case of 0°C samples, the muscle strips were placed in plastic bags and immediately immersed in crushed ice. Ten muscle strips of P. major and ten strips of thigh muscle were subjected to each temperature treatment. Final shortening measurements were taken at 5 hr and the percent shortening was calculated from the initial length. Final muscle pH was also measured after homogenization in 0.005M iodoacetate solution adjusted to pH 7.0.

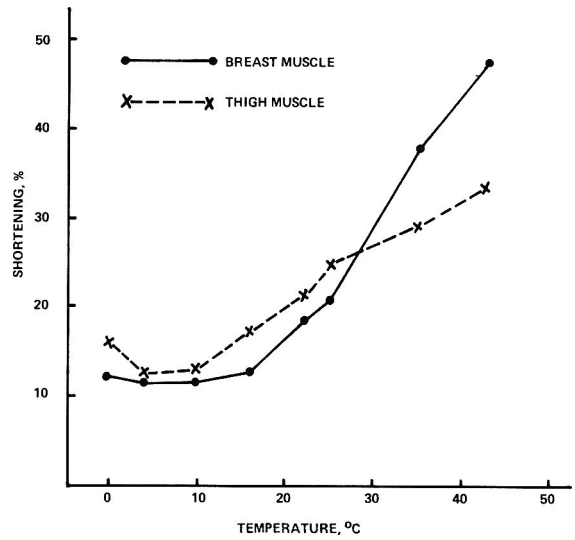

Fig. 1–Effect of temperature on the extent of shortening in chicken muscles excised pre-rigor.

RESULTS & DISCUSSION

THE EXTENT of shortening at different temperatures is illustrated in Figure 1. For both thigh (dark muscle) and breast muscle (white muscle), minimum shortening was observed in the 4–10°C temperature range. Thigh muscles held at 0°C showed an appreciably higher degree of shortening than those held at 4°C; whereas breast muscle showed little difference between the two temperatures. The percentage shortening sharply increased linearly with temperature over the temperature range of 10–43°C, and this shortening effect was much greater in breast muscle.

The data clearly indicated that chicken muscle shows some cold-shortening effect at 0°C, but not as extensive cold shortening effect as observed in bovine and ovine muscles. This observation generally agrees with the results of Jungk and Marion (1970) who reported no cold shortening, but rather a significant linear relation between the extent of shortening and temperature. However, these authors did not make measurements at 0°C, and thus, could not demonstrate a potential cold shortening effect at 0°C.

The shortening curve in Figure 1 is in good agreement with the relative toughness curve reported by de Fremery (1963) who showed minimum toughness at 10°C, some increased toughness at 0°C, but a dramatic increase in toughness above 20°C. These results strongly indicate that high postmortem muscle temperature (>20°C) induces more severe shortening and greater toughness than low temperature.

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0022-1147/78/0005-1613$02.25/0

A Research Note

MINOR FATTY ACIDS FROM PECAN KERNEL LIPIDS

S. D. SENTER and R. J. HORVAT

ABSTRACT

Twenty-three fatty acids have been identified in the oils from pecan (*Carya illinoensis* Wang) kernels. In addition to confirming the 10 previously reported, 13 newly identified acids were found in 2 years' sampling of four varieties of pecans. Newly identified acids are: decanoic, dodecanoic, dodecenoic, tetradecanoic, tetradecenoic, tetradecadienoic, pentadecanoic, pentadecenoic, pentadecadienoic, hexadecadienoic, heptadecadienoic, eicosenoic, and heneicosanoic acids. All but one were present in all oil samples in quantities of less than 0.1%; tetradecanoic acid averaged ca 1.2%.

INTRODUCTION

PREVIOUSLY, the oils from six varieties of pecan kernels were studied in relation to lipid classes and fatty acid composition (Senter and Horvat, 1976). Seven classes of lipids were found in all varieties and identified as complex lipids, monoglycerides, α- and β-diglycerides, sterols, free fatty acids and triglycerides. The oils were predominantly triglycerides that were mainly composed of the C_{18} unsaturated fatty acids. Ten fatty acids were identified, including four that had not been recognized previously (Woodroof and Heaton, 1961; French, 1961; Bailey et al., 1967; and Pyriadi and Mason, 1968).

We now report the presence of 23 fatty acids in the oils from four varieties of pecan kernels. Those reported previously were reconfirmed; additionally, 13 new acids were identified. This report supplements previous information on the nutritional composition of pecans in the compilation of data by the Nutrient Data Bank.

EXPERIMENTAL

Samples

Kernels from four of the varieties (Stuart, Mahan, Cheyenne and Shoshoni) previously analyzed (Senter and Horvat, 1976) were used in this study. Samples were from the 1975 and 1976 harvests in Brownwood, TX and were representative of standard and improved varieties. Samples were obtained in 45.4-kg lots from trees that were grown on similar soil types and had received similar levels of fertilization and sprays for insect and disease control. The nuts were stored in-shell at -35°C until analyzed.

Approximately 1 kg of nuts was selected from the above samples on the basis of full development, freedom from disease, and typical phenotypic characteristics. Selections were warmed to ambient temperatures and manually shelled. About 100g of kernels were placed in a perforated aluminum block, and the kernels were extruded with a plunger and Carver laboratory press. About 70 kg/cm^2 pressure was required to force the kernels through the holes (0.08 cm diam.) in the block. The macerate was collected in beakers, and the oils were separated by centrifugation at 10,000 × G for 10 min. The oils were decanted into glass vials, flushed with N_2 and stored at 4°C until analyzed.

GLC-MS analyses

About 150 mg of oil was taken from each stored sample and saponified with 4 ml of 0.5N methanolic-NaOH for about 5 min. Methyl ester derivatives of the free fatty acids were formed with 14 percent methanolicboron trifluoride (Regis Chemical Co.) according to established procedures (AOCS, 1974). The esters were dried over anhydrous sodium sulfate and the solvent was removed with a stream of N_2.

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Qualitative analyses were performed with a Perkin-Elmer Model 900 gas-liquid chromatograph (GLC) on samples ranging from 0.2–1.0 μl in size. The GLC was connected by means of an effluent splitter to a DuPont 21-490 B Mass spectrometer (MS) equipped with differential pumping on the analyzer section. Separations were made on a 91.44-m × 0.0762-cm stainless steel, open-tubular column, coated by the procedures of Mon (1971), with Carbowax 20M (Applied Science Co.). GLC conditions were: He inlet pressure, 1 kg/cm^2; injector, manifold and flame ionization detector, 250°C; and column, held at 210°C for 24 min then programmed to 245°C at 2°C/min. Effluent splitting and MS conditions were as previously reported (Senter and Horvat, 1976). The esters were identified by comparison of their mass spectra with those of known standards (Supelco, Inc.) and on the basis of parent ion and GLC retention time.

Peak areas were quantitated by means of an Autolab Computing Integrator.

RESULTS & DISCUSSION

BECAUSE of the diversity in quantities of component fatty acids in pecan oils, multiple runs were required for positive identification of the prepared esters. We used sample sizes of about 0.2 μl to resolve the major peaks and to prevent excessive carry over into succeeding peaks. However, we used as much as 1 μl of sample to identify the minor components. This resulted in column overload, but was permissible because of the more rapid clution of the minor peaks (up to C_{18}) and the excessive retention time of esterified acids greater than $C_{18:3}$.

These analyses have resulted in the positive identification of 23 fatty acids in pecan oils, including 13 that have not been previously reported. These acids, in order of elution as presented in Figure 1, are as follows: decanoic (10:0), dodecanoic (12:0), dodecenoic (12:1), tetracecanoic (14:0), tetradecenoic (14:1), tetradecadienoic (14:2), pentadecanoic (15:0), pentadecenoic (15:1), pentadecadienoic (15:2), hexadecanoic (16:0), hexadecenoic (16:1), hexadecadienoic (16:2), heptadecanoic (17:0), heptadecenoic (17:1), heptadecadienoic (17:2), octadecanoic (18:0), octadecenoic (18:1), octadecadienoic (18:2), octadecatrienoic (18:3), eicosanoic (20:0), eicosenoic (20:1), eicosodienoic (20:2) and heneicosanoic (21:0) acids.

The newly identified fatty acids (10:0, 12:0, 12:1, 14:0, 14:1, 14:2, 15:0, 15:2, 16:2, 17:2, 20:2, and 21:0) comprised a very small percentage of the fatty acid composition in the pecan oils. The integrated areas for each of these compounds except one were less than 0.2% in all varieties. Tetradecanoic acid (14.0) comprised an average of ca 1.2% in each variety. Average areas for the major components corresponded well with the amounts reported by Senter and Horvat (1976). No variation was found between the 1975 and 1976 samples in fatty acid composition.

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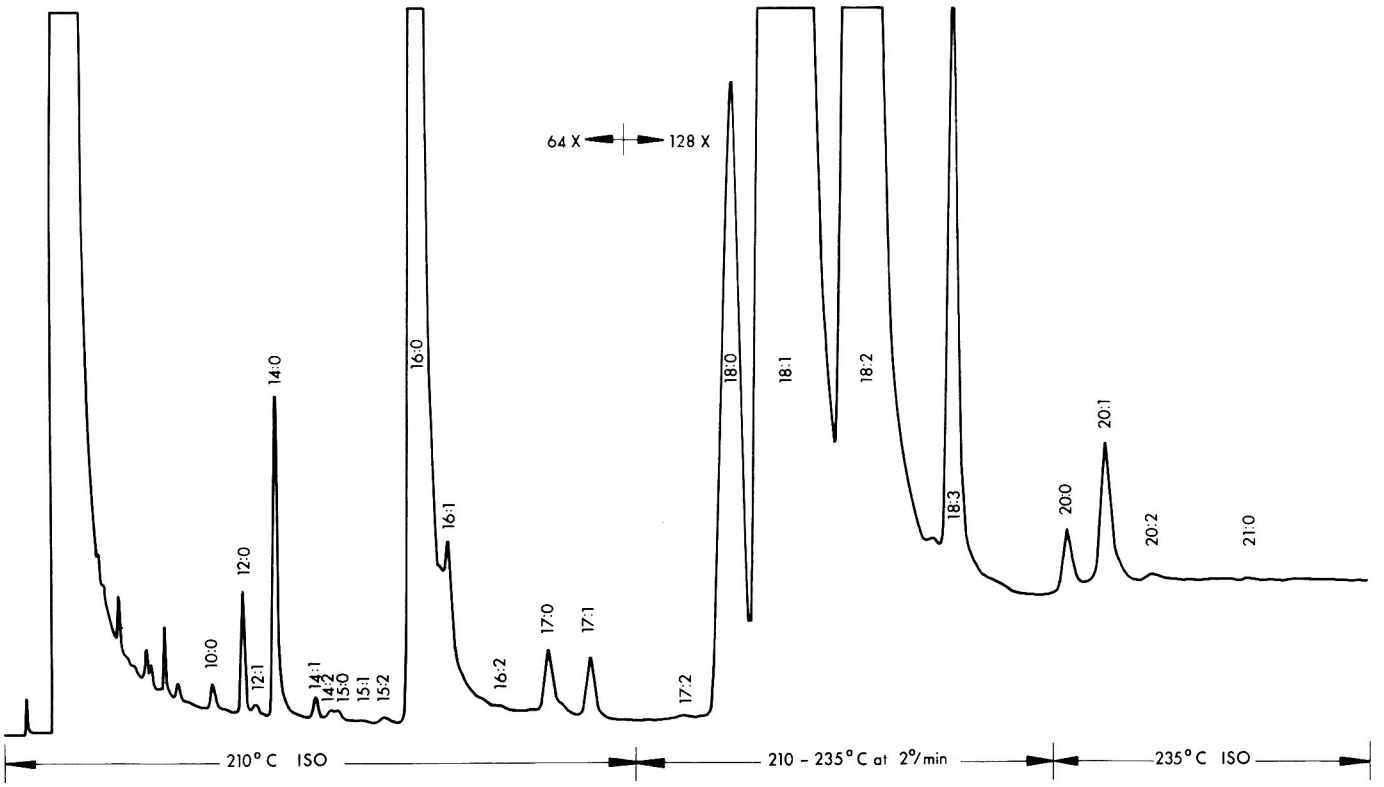

Fig. 1—Gas-liquid chromatogram of methyl esters of pecan kernel fatty acids on a 91.4-m X 0.0762-cm, open-tubular, Carbowax 20M column. Helium carrier gas at 1 kg/cm²; injector and detector temperature, 250° C; flame ionization detector; column temperatures and attenuations as marked.

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CHICKEN MUSCLE SHORTENING . . . From page 1613

There was no difference in final muscle pH among treatments, indicating that glycolysis was completed and muscles were in full rigor at the time of final shortening measurement (5 hr postmortem).

It can be concluded from the foregoing data that no appreciable cold shortening occurs for both breast and thigh chicken muscles except at 0°C. Even at 0°C, the extent of shortening was of a much smaller magnitude than that observed for bovine and ovine muscles. Yet, some toughening may be expected in thigh meat when the birds are directly immersed in crushed ice immediately after evisceration. However, no shortening and/or toughening would be expected when the birds are eviscerated, gradually chilled to 4°C in a mechanical chiller, and then aged in a 4°C cooler.

Since high temperature induces a greater shortening in chicken (heat shortening), it should be emphasized that muscle temperature must be lowered to below 20°C as early as possible in the processing line to prevent excessive muscle toughening.

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A Research Note

IMMOBILIZATION OF PEPSIN IN ACTIVE FORM IN PARAFFIN WAX

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ABSTRACT

This communication describes a new and simple method devised for immobilization of pepsin. Paraffin wax was used for immobilization. Pepsin was mixed with molten paraffin wax and the resultant mixture was solidified. The molten paraffin wax could be applied as a film on solid supports before solidifying. The excess pepsin on the surface, whenever present, was washed with an aqueous medium at or below pH 5.4 to leave, on the surface, immobilized pepsin in active and repeatedly usable form. The immobilized pepsin was used several times for clotting milk and for the hydrolysis of leaf protein concentrate.

INTRODUCTION

IMMOBILIZATION of enzymes has established itself as a distinct field of research activity in enzyme and food technology. The work in this field has been reviewed recently by Zaborskey (1973), Richardson (1974) and Weetall (1975). Although a considerable amount of work has been done on immobilization of enzymes, little work has been reported on proteolytic enzymes in general and pepsin (Rao et al., 1970; Line et al., 1971; Goldstein, 1973) in particular. The costs of various methods used for the immobilization of pepsin are very high when one takes into consideration the raw materials required, the amount of activity retained by immobilized pepsin and its subsequent life and mechanical properties. The present investigation was undertaken to find out whether a cheap and simple method could be devised using paraffin wax for the immobilization of pepsin.

MATERIALS & METHODS

ALL REAGENTS and glassware were maintained at 37°C throughout.

Preparation of pepsin-paraffin mixture and its deposition on a glass slide

Pepsin (Difco; 1:10,000) crystals were powdered in a mortar and equilibrated at 37°C for 30 min in an incubator. The powder was added to 4 times its weight of paraffin wax at 60°C and thoroughly mixed. This mixture is easily amenable for spreading in the form of a film on solid supports. A film (2 × 2 cm) was obtained on glass slide and allowed to solidify at room temperature.

Assay of soluble pepsin

To 2 ml of skim-milk powder (12.5% w/v in 0.01M $CaCl_2$) taken in test tube was added 0.5 ml of pepsin solution prepared in sodium acetate buffer (pH 5.4, 0.1M). The test tube was shaken continuously in an inclined position. Time required (in seconds) for the appearance of granules in the film of milk on glass surface of the test tube was noted and taken as the measure of pepsin activity.

Assay of immobilized pepsin

Slide coated with immobilized pepsin was taken in a container and 0.5 ml of sodium acetate buffer followed by 2 ml of skim-milk solution mentioned above were added. The dimensions of the container were such that the entire film came in contact with the solution freely. The time required (in seconds) for the appearance of milk granules was noted.

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0022-1147/78/0005-1616$02.25/0

Preparation of leaf protein concentrate of lucerne

The method described by Pirie (1969) was used for the preparation of leaf protein concentrate (LPC) from lucerne.

RESULTS & DISCUSSION

Evidence of immobilization of pepsin in wax in active form

The slide coated with wax-pepsin mixture was likely to have, on its surface, loosely as well as intimately bound pepsin. In order to get rid of all loosely bound pepsin, the slide was dipped into 50 ml of sodium acetate buffer (pH 5.4, 0.1M) at 37°C and shaken by hand. Aliquots (0.5 ml) were drawn from the buffer from time to time and subjected to milk clotting test, to determine the pepsin activity. Shaking was continued until there was no further rise of clotting activity in the buffer. This indicated that there was no loosely bound pepsin on the surface of the film. The slide was later washed with several aliquots of buffer and the slide itself was subjected to the milk clotting test. Milk clotted in 42.4 sec. This indicated the presence of active pepsin in the water insoluble state at the surface of the film.

Reusability of wax immobilized pepsin

The slide, after subjecting to the milk clotting test, described above, was washed with a jet of water, gently wiped with a moist pad of absorbant cotton and resubjected to milk clotting test in order to determine whether it could be used over and over again. It was noticed that with washings the slide could be used at least 14 times for clotting the milk. The film was still active when the experiment was stopped. The clotting time was, however, highly variable within the range 36.4–63.4 sec. The activity decreased gradually and steadily and deposits of milk clots appeared on the surface of the film if the wiping was not done.

Application of the immobilized pepsin for hydrolysing the leaf protein concentrate

Wax-pepsin mixture was also applied in the form of a film at the base of glass and stainless steel containers. The films were washed with 0.1N HCl thoroughly in order to make them free of any loosely bound pepsin. It was observed that these containers could be used at least 18 times for the hydrolysis of LPC when incubated in 0.1N HCl at 37°C for 48 hr. After the eighteenth use the surface was bruised slightly with a moist pad and washed thoroughly with 0.1N HCl. This operation improved the performance of the surface for the ninteenth and twentieth use. The films were active even after the twentieth use.

The binding of pepsin to paraffin wax appears to be a physical phenomenon. The embedded molecules on the surface of the mixture would be active if their active sites come in contact with or protrude into the covering aqueous medium.

The method described here for pepsin immobilization is relatively simple and less expensive than other methods (Rao et al., 1970; Line et al., 1971; Goldstein, 1973). The derivative obtained could be used repeatedly. It was observed in some experiments that if the wax-to-pepsin ratios were very high, hardly any pepsin escaped into the solution from the surface on the first contact with the aqueous medium. Hence it is also possible to immobilize all the pepsin and attain maximum ac-

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A Research Note

EVALUATION OF INJURED COMMERCIAL POTATO SAMPLES FOR TOTAL GLYCOALKALOID CONTENT

THOMAS J. FITZPATRICK, JANIS A. McDERMOTT and STANLEY F. OSMAN

ABSTRACT

Stress by physical damage is one of the most significant factors which may cause an increase in potato glycoalkaloids. A high percentage of potatoes available on the retail market are physically damaged and possibly are subject to an internal build-up of glycoalkaloids. To investigate this possibility, analyses for total glycoalkaloids were carried out on damaged potatoes purchased on the retail market. Four separate purchases of three different potato varieties were tested. From this limited sampling it cannot be concluded that no tubers containing a high level of glycoalkaloids due to physical stress reach the retail market. However, in the cross section of damaged potatoes analyzed, in no case was a dangerous level of glycoalkaloids observed.

INTRODUCTION

GLYCOALKALOIDS are constituents of potatoes that have been implicated as a causal factor in human poisoning when consumed in sufficient amounts (Bömer and Mattis, 1924; Gull, 1960; Nishie et al., 1971). Factors that affect the levels of glycoalkaloids in potatoes include exposure to light, types of soil, fertilization practices, climate, tuber size, maturity, and damage (Sinden and Webb, 1974). Several studies on the accumulation of glycoalkaloids due to stress by physical damage have appeared (Locci and Kuć, 1967; Wu and Salunkhe, 1976). In a recently completed study (Fitzpatrick et al., 1977), we found that the total glycoalkaloid (TGA) content increased markedly in potato slices incubated at room temperature for 4 days.

With these factors in mind, we observed the high percentage of potatoes on the retail market that have sustained at least some physical damage and thus are subject to internal build-up of glycoalkaloids. Therefore, we procured samples of several potato varieties that had sustained damage sometime between harvest and the time they were made available for sale and ran controlled studies on their total glycoalkaloid content. Since the only control for a study of TGA accumulation in damaged tissue would be undamaged tissue from the same potato, we selected those which had most of the damage confined to one end and used the opposite end for a control.

MATERIALS & METHODS

THREE CULTIVARS of potatoes, Katahdin, Russet Burbank and Red Pontiac were used in this study. Four samples (ca 3 lb) of each cultivar were used. Each sample was purchased from a different retail outlet, half being chain supermarkets and half smaller neighborhood stores.

The potatoes from each sample were scrubbed, dried and arranged in a row from the largest to the smallest. The selection of every third one for analysis gave a representative aliquot (ca 4–5 potatoes) of each sample. Having selected the damaged tubers so that the mechanical damage was confined to one end (but with no particular observation as to whether this was the bud or stem end), the undamaged end of each potato was used as a control in reference to the damaged tissue, while the analysis of the large portion from the middle of the potato when averaged with the values from the ends would give an indication of the overall TGA value. Therefore the tubers were cut into three sections and the entire section was included in the analysis. For the weights of each section relative to the other two see Table 1. These sections were designated: A for the damaged end; B for the center portion; and C for the undamaged end.

To prevent TGA build-up, the weighted sections were quickly diced, placed in appropriate jars and freeze dried overnight on a lyophilizer. It has been our observation (Fitzpatrick et al., 1977) that decreased temperatures lower metabolism to the point where no increase in TGA would be expected during the freeze drying period. This was evident in the final results. The dry weight compared to the fresh weight gave a close approximation of the percent solids contained in the potatoes. These dried samples were then ground to a fine powder in a Wiley Mill so that a homogenous representative aliquot could be taken for analysis, thereby insuring the proper ratio of cortex to pith. For extraction, an amount of the powder in grams equal to the percent solids (which represents 100g of fresh tuber) multiplied by 0.20 was used, thus making each sample equivalent to 20g of fresh tuber.

These samples were extracted and analyzed for total glycoalkaloids by the method of Fitzpatrick and Osman (1974), which consisted of extracting the tubers with a 2:1 methanol-chloroform bisolvent system

Table 1—Total glycoalkaloid analysis of bruised commercially available potatoes

Cultivar	Sample[a]	Fresh wt (g)	Dry powder (g)	% Solids	mg TGA/100g fresh wt
Katahdin	1-A	112.3	23.8	21	16.5
	1-B	147.7	29.5	19	6.3
	1-C	92.3	18.0	19	13.4
	2-A	65.5	13.6	20	14.5
	2-B	53.8	18.7	34	9.2
	2-C	94.0	11.7	12	8.4
	3-A	97.4	20.2	20	19.5
	3-B	129.8	25.0	19	15.0
	3-C	110.0	22.0	20	11.2
	4-A	61.1	12.4	20	19.3
	4-B	101.0	18.2	18	10.2
	4-C	110.0	20.2	18	10.0
Russet Burbank	5-A	113.0	28.0	24	5.8
	5-B	111.0	24.7	22	1.5
	5-C	110.0	22.0	20	3.1
	6-A	47.0	13.5	28	7.2
	6-B	57.0	13.6	23	1.5
	6-C	59.0	15.0	25	6.1
	7-A	198.5	49.0	24	7.6
	7-B	119.6	23.2	19	2.5
	7-C	88.1	20.0	22	3.3
	8-A	45.2	9.2	20	5.5
	8-B	80.0	16.6	20	1.8
	8-C	40.0	7.4	18	5.3
Red Pontiac	9-A	114.6	21.5	18	5.5
	9-B	123.6	21.5	17	2.3
	9-C	73.4	13.0	17	4.9
	10-A	122.0	21.0	17	6.9
	10-B	95.5	18.0	18	1.8
	10-C	87.5	14.0	16	2.1
	11-A	162.0	35.5	21	7.5
	11-B	133.0	26.0	19	4.6
	11-C	73.0	14.0	19	12.1
	12-A	79.5	14.0	17	6.07
	12-B	73.0	14.0	19	2.58
	12-C	51.0	10.0	19	6.26

[a] A = damaged end; B = center section; C = undamaged end.

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which was separated with 0.8% sodium sulfate. Grinding in a Waring Blendor for 5 min, filtering, then regrinding with fresh solvent for 2–3 min effectively extracted the glycoalkaloids. The procedure used is equally effective on both fresh and lyophilized tissue. An aliquot of the methanol layer was hydrolyzed with 2N H_2SO_4. The aglycones were then extracted into benzene which was taken to dryness, redissolved in methanol, and titrated with methanolic bromphenol blue solution.

RESULTS & DISCUSSION

THIS STUDY showed that at least 10% (and often higher) of loose white potatoes available on the retail market had sustained some degree of mechanical damage. The type of damage was primarily cracks and fissures, with some bruising but with no observable decay.

The results of the TGA analysis appear in Table 1. In all the samples but one (in the Red Pontiac Variety) the solids content of the damaged tissue was higher than the uninjured end because of local suberization. This solids difference was considered in the amount taken for analysis. Also, the TGA content of the damaged portion was higher than that of the undamaged end in all but two replicates of the Red Pontiac variety. The center sections of all but three replicates of the Katahdin variety had lower TGA values than either of their corresponding end sections. The maximum TGA value of every damaged end was below that which would be considered a health hazard. In addition, the weighted average of the TGA values of the damaged, center, and undamaged portions would effectively lower the TGA value to a point well below the level of excessive glycoalkaloid accumulation, which in most cases is considered to be above 20 mg/100g fresh weight.

From the limited number of samples tested one cannot conclude that physical damage may not abnormally elevate the glycoalkaloid content of some potatoes available on the retail market. However, in the representative sampling of damaged potatoes which we analyzed, in no case was a dangerous level of glycoalkaloids observed.

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Ms received 12/22/77; revised 4/3/78; accepted 4/10/78.

PEPSIN IMMOBILIZATION IN WAX . . . From page 1616

tivity on the surface of wax-pepsin mixture by selecting a proper wax-pepsin ratio.

The merits of immobilization in wax lie in the simplicity of the method, possibility of immobilizing all the enzyme added, ease in coating different solid supports, ability to work at a wider pH range, ease in cleaning active surface, possibility of immobilizing many enzymes in the same preparation by a single and simple method, longer active life of these preparations, and ease in storage. It should be possible to immobilize many other enzymes in this way. The wax immobilized preparations may play an important role in protein food technology.

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The authors thank Prof. K.B. Deshpande for facilities and the University Grants Commission for a fellowship for V.A.S.

A Research Note

COMPONENTS OF VITAMIN B_6 IN TURKEY BREAST MUSCLE

JANE A. BOWERS and JEAN CRAIG

ABSTRACT

Extracts of raw and cooked turkey breast muscle were chromatographically separated into pyridoxine, pyridoxal, and pyridoxamine fractions. Those fractions, in addition to an extract for total vitamin B_6, were assayed microbiologically. Pyridoxine and pyridoxal contents were higher in the raw than in the cooked muscle; the pyridoxamine level was higher in the cooked muscle. No significant differences were found in the sum of the three forms of vitamin B_6 or total vitamin B_6 between cooked and raw muscle.

INTRODUCTION

ALTHOUGH vitamin B_6 usually is classified as being heat stable, early research indicated considerable loss of that vitamin during cooking of meat. Henderson et al. (1941) and McIntire et al. (1944) observed that cooked meats retained only 14–50% of the amount in the raw muscle, depending on cooking method. Lushbough et al. (1959) reported 42–47% retention in muscle, with 1–13% of vitamin B_6 in the drip. Vitamin B_6 retention in oven-roasted beef loin was 72% and in oven-braised beef round, 49% (Meyer et al., 1969). Engler and Bowers (1975), who calculated values on a moisture- and fat-free basis, found that uncooked turkey thigh muscle had more vitamin B_6 than did muscle subjected to heat treatment.

It appears that the vitamin B_6 content of meat may be reduced during the heating process. However, the effect that cooking has on the three forms of vitamin B_6 in meat has not been investigated. The purpose of this study was to determine the pyridoxal, pyridoxine, and pyridoxamine content of raw and cooked turkey muscle.

EXPERIMENTAL

SIX FROZEN TURKEYS (10–12 lb), obtained locally, were halved and assigned randomly to one of two treatments: (1) raw or (2) cooked. At each of six periods, turkey halves were thawed at 3°C for 72 hr. One-half was roasted in a pyrex dish (37 × 23 × 5 cm) at 163°C to an internal (in pectoralis major muscle) temperature of 80°C. The other half remained uncooked. Pectoralis major muscles were removed and ground twice through a 1/8-in. plate in an electric grinder. Ground samples were analyzed for percentage of moisture and fat (AOAC, 1975) and for pyridoxal, pyridoxine, pyridoxamine, and total vitamin B_6 (Toepfer and Polansky, 1970). The three forms of vitamin B_6 were separated with a Dowex 50W-X8 column and the amount in each fraction determined by the growth response to *Saccharomyces Carlsbergenis* (ATCC 9080). In addition, total vitamin B_6 was determined for an unfractionated meat extract. The standard for the total vitamin B_6 determination was a mixture of the three forms of vitamin B_6.

Data were analyzed statistically and significant differences determined by the T-test.

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0022-1147/78/0005-1619$02.25/0

Table 1—Mean[a] vitamin B_6 values (μg/g turkey) and percentage of moisture and ether extract for raw and cooked turkey breast muscle

	Raw		Cooked
Vitamin B_6 forms			
Pyridoxal			
Wet weight basis	1.86	**	0.57
MF-FF[b] basis	7.46	**	1.90
Pyridoxine			
Wet weight basis	0.87	**	0.42
MF-FF basis	3.51	**	1.39
Pyridoxamine			
Wet weight basis	1.94	**	4.12
MF-FF basis	7.70	*	13.35
Sum[c]			
Wet-weight basis	4.68	ns	5.11
MF-FF basis	18.68	ns	16.64
Total vitamin B_6			
Wet weight basis	5.98	ns	6.40
MF-FF basis	23.85	ns	20.84
Ether extract, %	0.72		1.25
Moisture, %	74.19		67.84

[a] Mean of six turkeys
[b] Moisture free – fat free
[c] Sum obtained by adding amounts reported for the three forms of vitamin B_6
* Statistically significant at 5%
** Statistically significant at 1% level

RESULTS & DISCUSSION

VALUES for the three forms of vitamin B_6 and total vitamin B_6 are reported on both a wet-weight basis (as consumed) and a moisture- and fat-free basis (Table 1). Amounts of pyridoxal and pyridoxine were less in cooked than in raw muscle; the amount of pyridoxamine was greater in the cooked muscle. The magnitude of the difference was greater when values were reported on a moisture- and fat-free basis, because the moisture content of the muscles differed. Hodson (1956), who observed that pyridoxamine content in milk products also increased with cooking (or heating), suggested that heat treatment in the presence of amino acids and protein converts part of the pyridoxal to pyridoxamine.

When the amounts of the three forms of vitamin B_6 were summed, no significant difference was found between the vitamin content of cooked and raw muscle. Total vitamin B_6 content determined from a meat extract was slightly greater in raw than in cooked muscle when values were calculated on a moisture- and fat-free basis but differences were not significant. Since vitamin B_6 is fairly heat stable any that was lost was probably leached from the muscle during heating.

Values obtained for total vitamin B_6 were slightly higher than those obtained by adding amounts for the 3 separated forms. For lima beans, Toepfer and Polansky (1970) reported slightly higher values for total vitamin B_6 than for the three forms added together; but for dried beef liver they reported lower values for total vitamin B_6 than for the sum of the three components. They attributed this difference to the high pyridoxamine content of liver.

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A Research Note

SEPARATION OF ALDOSES AND KETOSES BY ORGANIC RESINS HAVING PRIMARY AMINE MOIETIES

MASAMI YAMAGUCHI, TOSHIO ASANO, MINORU MAYAMA and ISAMU IWAMI

ABSTRACT

The organic resins having primary amine moieties, such as crosslinked polyvinylamine (PVAM), were found to adsorb aldose selectively. A satisfactory separation of glucose and fructose was achieved on the column of PVAM at a flow rate of SV = 0.5 hr^{-1} with amounts of sugars supplied of 0.4 g/g PVAM. These values indicate that the separation of sugars by the resins can be achieved in a shorter time with a larger amount of sugars in comparison with ion exchange resins usually used.

INTRODUCTION

THE USE OF organic resins for the separation of sugars has become increasingly popular in recent years. The organic resins hitherto employed have usually been ion exchange resins in the salt form. Anion-exchange resins in the bisulphite form and cation-exchange resins in the calcium form have been reported to be effective for the chromatographic separation of aldoses and ketoses (Larsson and Samuelson, 1965; Lindberg and Slessor, 1969; Lauer, 1969; Takasaki, 1972).

However, since the selective adsorptivity and the loading capacity of the ion exchange resins for sugars are not sufficient, a large amount of the resin should be used and a long time is necessary for the separation. We have found that organic resins having primary amine moieties, such as crosslinked polyvinylamine, are excellent in the selectivity and in the loading capacity.

This paper is concerned with the separation of aldoses and ketoses by the new organic resins having primary amine moieties.

EXPERIMENTAL

Synthesis of organic resins having primary amine moieties

Crosslinked polyaminostyrene (PAS) was prepared by polymerizing 80g of p-aminostyrene and 4g of divinylbenzene in 560 ml of water using 0.8g of azobisisobutyronitrile at 50°C for 24 hr.

Crosslinked polyvinylamine (PVAM) was prepared by Hofmann degradation of a copolymer of acrylamide and divinylbenzene (Tanaka and Senju, 1976), which was obtained by polymerizing 50g of acrylamide and 20g of divinylbenzene in 250 ml of dioxane using 0.4g of azobisisobutyronitrile at 70°C for 6 hr.

Crosslinked polyvinylbenzylamine (PBA) was prepared by Delepine reaction of a copolymer of chloromethylstyrene and divinylbenzene (Okawara et al., 1960), which was obtained by polymerizing 50g of chloromethylstyrene and 1g of divinylbenzene in 150 ml of water using 0.25g of azobisisobutyronitrile at 70°C for 7 hr.

Adsorption of sugars by the resins

10 ml of an aqueous solution dissolving 0.1g of a sugar was added to 0.5g of a resin in a test tube. The test tube was maintained at 70°C for 30 min, then the amounts of the sugar remained in the solution were determined to calculate the amounts of the sugar adsorbed on the resin.

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0022-1147/78/0005-1620$02.25/0

Table 1—Adsorption of sugars on the resins having primary amine moieties

Resin[a]	Type of amine moieties[b]	Sugar	Amt of sugar adsorbed on resin (mg/g resin)
PAS	A–⟨○⟩–NH_2	Glucose	11
PAS	A–⟨○⟩–NH_2	Fructose	0
PAS-HCl salt	A–⟨○⟩–$NH_2 \cdot HCl$	Glucose	82
PAS-HCl salt	A–⟨○⟩–$NH_2 \cdot HCl$	Fructose	0
PVBA	A–⟨○⟩–CH_2NH_2	Glucose	53
PVBA	A–⟨○⟩–CH_2NH_2	Fructose	1
PVAM	A – NH_2	Arabinose	121
PVAM	A – NH_2	Ribose	125
PVAM	A – NH_2	Xylose	120
PVAM	A – NH_2	Galactose	120
PVAM	A – NH_2	Glucose	118
PVAM	A – NH_2	Mannose	113
PVAM	A – NH_2	Maltose	88
PVAM	A – NH_2	Fructose	3
PVAM	A – NH_2	Sorbose	2
PVAM-HCl salt	A – $NH_2 \cdot HCl$	Glucose	34
PVAM-HCl salt	A – $NH_2 \cdot HCl$	Fructose	0

[a] HCl adsorption capacity of resin (meq/g) was as follows: PAS 6.2; PVBA 3.8; and PVAM 4.2.

[b] A means backbone chain mainly composed of $+CH_2-\underset{|}{CH}+_n$.

Separation of glucose and fructose on a column of PVAM

6 ml of a solution containing 1.9g of glucose and 1.9g of fructose was fed to a jacketed column having innerdiameter of 1.1 cm packed with 10g of PVAM resin. The column was maintained at 60°C and was washed with 120 ml of water and successively with 2N CH_3COOH at an eluting velocity of SV = 0.5 hr^{-1}.

RESULTS & DISCUSSION

AS A TEST of the adsorbing power of the resins having primary amine moieties, the resins were contacted with the solution of a sugar in a test tube. The amounts of the sugar adsorbed on the resin were determined and the results are shown in Table 1. It is seen that the resins having primary amine moieties can selectively adsorb aldoses including maltose, disaccharide of aldose type end group, as well as monosaccharides.

The selective adsorption of the resins for aldoses may be explained by the formation of aldosylamines with aldoses and the amine moieties of the resins. It is well known that aldosylamines are easily formed by the reaction of aldoses and lower molecular amines (Ellis and Honeyman, 1955).

However, the behavior of the resins having primary amine moieties was somewhat different from that of lower molecular amines. PAS in the free NH_2 form showed poorer adsorption for aldoses than that in the form of HCl salt, while aniline can easily react with aldoses. It seems that other kinds of affinity may be involved besides the affinity forming aldosylamines.

As a test of the resolving power of the column of the resins having primary amine moieties, an equimolar mixture of glu-

cose and fructose was fractionated on the column of PVAM. The elution pattern obtained is shown in Figure 1. It is seen that a satisfactory separation of glucose and fructose can be achieved on the column despite a high flow rate of SV = 0.5 hr^{-1} and a large amount of sugars supplied, 0.4 g/g PVAM. The recovery of the sugars was almost quantitative by eluting with water for fructose and with acidic aqueous solution for glucose.

The flow rate of SV = 0.25 hr^{-1} and the amount of sugar supplied of about 0.02 g/g resin was calculated from the report for the ion exchange resin in the bisulphite form (Takasaki, 1972). The flow rate of SV = 0.1 hr^{-1} and the amount of sugar supplied of about 0.03 g/g resin was also calculated for the ion exchange resin in the calcium form (Lauer, 1969).

The organic resins having primary amine moieties have advantages in selectivity, loading capacity and flow rate compared to ion exchange resins for the separation of aldoses and ketoses. They may be used to separate aldoses and ketoses in large scale as well as in laboratory operations.

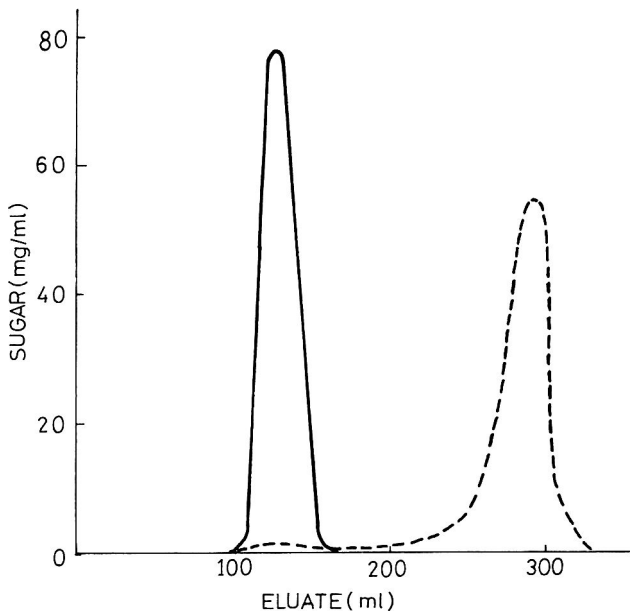

Fig. 1—Separation of glucose and fructose on PVAM column. The solid line is the elution diagram for fructose and the broken line for glucose.

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Ms received 1/16/78; revised 3/16/78; accepted 3/18/78.

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Ms received 1/16/78; revised 4/7/78; accepted 4/10/78.

Contribution No. 78-174-J, Department of Foods and Nutrition, Kansas Agricultural Experiment Station, Manhattan, KS 66506.

A Research Note

EFFECT OF MECHANICAL INJURY ON THE LIPID CONTENT AND QUALITY OF POTATOES

NELL I. MONDY and ROBERT L. KOCH

ABSTRACT

Bake King or Katahdin potatoes that were injured by bruising and/or wounding were compared with control tubers for crude lipid, phospholipid content and fatty acid composition. Both bruised and wounded tubers showed marked signs of discoloration at the time of analysis. The crude lipid content of both bruised and wounded tissue was significantly lower than the controls and the phospholipid content followed the same trend as the crude lipid. The fatty acid composition of the bruised tissue was similar to that of the control, but in the wounded cortical tissue the phospholipid showed an increase in saturated fatty acids and a decrease in unsaturated.

INTRODUCTION

POST HARVEST CHANGES in potatoes is a major problem that results in considerable loss of edible produce each year. Postharvest losses occur in both quantity and quality. Physical factors such as rough handling during harvesting, grading and packaging which result in mechanical bruising or cut injuries are responsible for a large share of these losses. Frequently the consumer is not aware of these two types of injuries at the time of the food purchase since bruised tissue is not visible until the tuber is peeled. Cut wounds may have healed over and may not be particularly visible.

Enzymatic darkening markedly reduces the acceptability of fresh potatoes and occurs following bruising or wounding of the tubers. This disorder not only results in the formation of undesirable dark tissue but may also reduce the nutritive value of the potato as well. Since potatoes purchased in today's market may exhibit black spot at the time of purchase, it is desirable to learn what alterations in the chemical composition have resulted from this damage.

Mechanical wounding causes a temporary, localized burst of respiration and cell division. Tubers form new protective tissues which serve to prevent desiccation and resist invasion by microorganisms. Priestley and Woffenden (1923) reported that the first process in the healing of a cut surface of a potato tuber is the deposition of a suberin layer composed of fatty acids. Kolattukudy and Dean (1974) analyzed suberin formed by wounded potato slices and found fatty acids (C_{16} to C_{26}) to be the major components. Lee and Chasson (1966) showed that the number of mitochondria and other components involving membranes increase during wound respiration. Willemot and Stumpf (1967) observed significant increases in fatty acid synthetase activity of potato tuber slices with resulting increases in linoleic and other long chain fatty acids. Reeve (1968) observed changes in the distribution of intracellular lipids in potato tissue exhibiting enzymatic darkening and suggested that lipid membranes may also be altered by mechanical disruption.

This study was undertaken in order to study the changes in the lipid composition of tuber following mechanical injury by either bruising or wounding, the injuries most likely to occur under storage conditions found in both industry and home.

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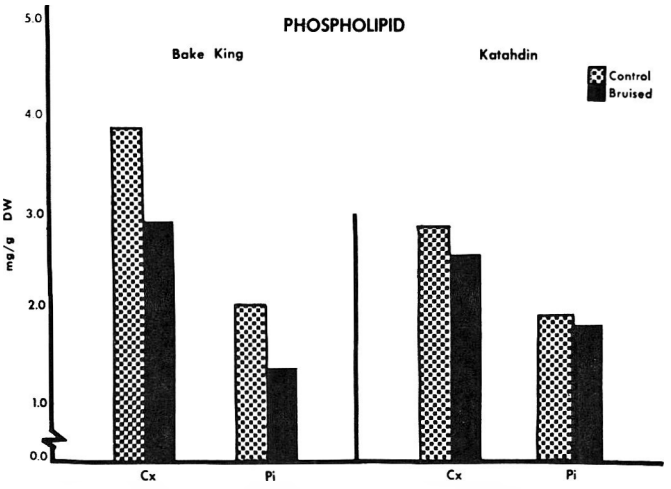

Fig. 1—Crude lipid content of cortex and pith tissue from Bake King and Katahdin potatoes.

MATERIALS & METHODS

POTATOES for this study were grown at the Cornell Vegetable Research Farm in Riverhead, Long Island during each of two growing seasons. The first year, Bake King potatoes were used and the second year, Katahdin potatoes were used. Following harvest, the potatoes were stored at 5°C until sampled.

Enzymatic discoloration accompanying bruising was mechanically induced in both varieties by dropping tubers from a stand 43.5 in. (110.5 cm) high onto a tiled concrete floor. Tubers were dropped 20 times and stored whole for 7 days at 5°C to allow discoloration to develop. Potatoes were sampled by cutting portions of the cortex longitudinally from bud to stem end.

In the study involving mechanical injury by wounding, tubers were cut into cortex and pith sections with a sterile stainless steel knife and both sections stored for 24 hr at 20°C in airtight containers in order to prevent desiccation. Analyses were then made following the 24 hr storage.

Determination of lipid content

The method used for the extraction of the crude lipid from potato powder has been described earlier by Mondy et al. (1963). Duplicate analyses were made for each of the treatments. Crude lipid was fractionated using the method previously described by Mondy et al. (1965). Fatty acid composition of the phospholipid portion of each sample was determined by gas chromatography in the manner described previously by Mondy and Mueller (1977).

Statistical analysis

The statistical significance of data was determined using a two-factor analysis of variance (Steel and Torrie, 1960).

RESULTS & DISCUSSION

CRUDE LIPID CONTENT in cortex and pith tissues of Bake King and Katahdin varieties was significantly reduced ($p < 0.05$) by bruising (Fig. 1). Phospholipid content followed the same trend as crude lipid (Fig. 2). Fatty acid composition was not significantly altered in the bruised tissues.

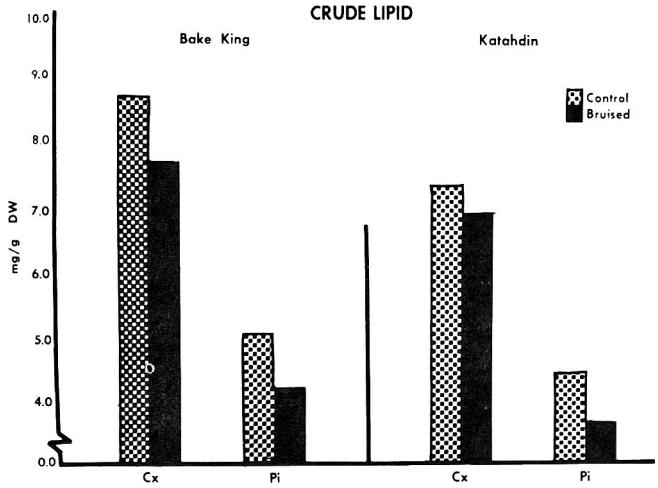

Fig. 2—Phospholipid content of cortex and pith tissue from Bake King and Katahdin potatoes.

The change in lipid content of cortex and pith tissues due to bruising is probably due to the action of the lipid-degrading enzyme, lipolytic acyl hydrolase (LAH) which occurs in many potato varieties (Galliard and Matthew, 1973; Galliard and Mercer, 1975). LAH liberates free fatty acids from lipoprotein membranes and its activity is manifested as soon as cells are disrupted (Galliard, 1970, 1973).

In the present study, perhaps bruising disrupted the membrane structures and allowed LAH, contained in the vacuoles, and the lipid substrates to interact. Crude lipid and phospholipid content was reduced but the fatty acid composition remained unchanged. Apparently LAH was equally active toward the different fatty acids, for although the phospholipid content was reduced the fatty acid composition remained unchanged.

Crude lipid and phospholipid contents of cortex and pith tissues of Katahdin potatoes were reduced significantly ($p < 0.05$) by wounding (Fig. 1). Wounding significantly lowered ($p < 0.05$) the relative amounts of linoleic (18:2) and linolenic (18:3) acids with respect to the levels of palmitic (16:0) and stearic (18:0) acids in cortex tissues. Similar trends were observed for fatty acid composition in pith tissues.

The change in lipid content of cortex and pith tissues following wounding is probably due to the action of LAH and an additional enzyme, lipoxygenase (LOX), an oxidizing enzyme which is also found at high levels in many potato varieties (Galliard and Matthew, 1973). LOX catalyzes the introduction of molecular oxygen into linoleic and linolenic acids to form their corresponding hydroperoxide derivatives (Galliard and Phillips, 1971) and as with LAH, its activity is manifested when cells are disrupted (Galliard, 1970). The activity of this enzyme has been reported to be increased in damaged tissue as compared to healthy tissue (Heinen and Brand, 1963; Lipetz, 1970). Galliard and Mercer (1975) found that lipoxygenase activity increased in wounded potato tubers along with phenol oxidases. Although the separate activities of these two enzymes was not determined, the oxygen dependent action of LOX was apparent in sliced, but not bruised, tissues as revealed by changes in the fatty acid composition. Apparently the oxidative mechanism in bruised tissues is different from that in cut or wounded tissue.

The lipid composition of tubers is altered significantly by mechanical injury either by bruising or cutting, and the destruction of lipid-containing membranes facilitates enzymatic darkening. Although lipids account for only a small portion of the total weight of tubers, they are important determinants of tuber quality.

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Ms received 12/23/77; revised 2/18/78; accepted 2/25/78.

The authors acknowledge the assistance of Drs. G.W. Selleck and P.A. Schippers, Dept. of Vegetable Crops and Dr. Robert Plaisted, Dept. of Plant Breeding for arrangements made in the growing, harvesting and transporting of the potatoes.

A Research Note

SENSORY EVALUATION OF BETANINE AND CONCENTRATED BEET JUICE

J. H. PASCH and J. H. von ELBE

ABSTRACT

Betalaine pigments extracted from red table beets (*Beta vulgaris L.*) have been employed as color additives frequently since the Federal Food and Drug Administration prohibited the use of FD&C Red No. 2 and No. 4. The contribution of betanine and beet juice concentrate to the flavor of buffered solutions and gelatin dessert was assessed by sensory tests. Results of triangle tests indicated betanine to be flavorless while concentrated beet juice had a flavor which can be successfully masked when added to flavored gelatin dessert powders.

INTRODUCTION

A FOOD COLORANT must not contribute an undesirable flavor to the product. Hence, the colorant must be either tasteless or its contributing flavors must be masked by the product's flavor. Presently used certified colors are tasteless, however many color additives which are exempt from certification, are crude extracts of plant material, and possess the characteristic flavor of their source. While the pigment itself may be tasteless, other cell constituents extracted with the pigment, can possess undesirable flavors. Because earthy flavors associated with beet juice concentrate are often not compatible with other food flavors the problem is especially acute.

The major contributor to the earthy flavor in beets is geosmin (*trans* 1,10-dimethyl-*trans*-9-decalol) (Acree et al., 1976; Murry et al., 1975). Using gas chromatography combined with mass spectrophotometry, Acree et al. (1977) identified geosmin and a potato-like odor which when combined with geosmin, produced an odor characteristic of the beet. They speculated that geosmin may be produced metabolically in the beet or absorbed from the soil where it is produced by microorganisms. In a later paper Acree et al. (1977) identified the potato-like aroma as coming from several 3-alkyl-2-methoxypyrazines.

Murry and Whitfield (1975) using gas chromatography investigated 3-isopropyl-, 3-*sec*-butyl-, and 3-isobutyl-2-methoxypyrazines in a number of vegetables and plants including beets and reasoned that the compounds must be of genetic origin and are quite distinct from other alkyl pyrazines which are recognized as being produced by cooking temperatures. Headspace analysis revealed a predominance of the sec-butyl species. They stated that these compounds could play a significant role in the aroma of cooked or processed beets. Parliment et al. (1977) identified 17 volatile components of cooked beets. These authors found 4 methyl pyridine and pyridine to constitute 60% of the total volatiles and also confirmed the presence of geosmin and 2-methoxy-3-sec-butyl pyrazine.

It was the purpose of this study to determine if betanine had a characteristic flavor, and if any flavor was detectable when beet juice concentrate was used as a colorant in gelatin desserts.

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0022-1147/78/0005-1624$02.25/0

Table 1—Sample composition and colorant used in sensory evaluation of betanine

Test code	Colorant source	Pigment[a] conc (ppm)	Sample composition
1	betanine	75 ± 5	0.005M citrate 0.01M phosphate
2	beet juice conc	74 ± 5	0.01M citrate 0.02M phosphate
3	beet juice conc	76 ± 5	strawberry flavor gelatin dessert

[a] Calculated as betanine

MATERIALS & METHODS

SENSORY EVALUATION by triangle test was used to determine the origin and effect of possible undesirable flavors caused by addition of beef juice concentrate to foods. Composition of the samples and colorant used for each test are given in Table 1.

Sample preparation

Purified betanine used in Test 1 was isolated by methods described previously (Pasch and von Elbe, 1978). Beet juice concentrate used in Tests 2 and 3 was a commercial product. Deionized glass-distilled water and analytical grade reagents were used throughout the experiment. After preparation, each sample was analyzed spectrophotometrically for pigment content using Nilsson's method (Nilsson, 1970).

A possible flavor contribution by pigment alone was determined using buffered solutions of betanine (Test 1). Two liters of 0.01M Na_2HPO_4 and 0.005M citric acid at pH 5.00 ± 0.01 were prepared. Betanine crystals were added to 1 liter of the solution to yield a betanine concentration of about 75 ppm. The remaining solution was used as the uncolored sample. The pH of the betanine solution was then checked and crystalline Na_2HPO_4 added to attain the original pH. Approximately 10 ml of each sample was portioned into 3-oz red flint glass sampling cups coded with a three-digit random number. Samples were held at ambient temperature (21°C) for about 1 hr before evaluation.

The presence of a flavor in beet juice concentrate was determined by preparing 2 liters of 0.02M Na_2HPO_4 and 0.01M citric acid at pH 5.00 ± 0.01 (Test 2). Approximately one-half of the solution was removed and beet juice concentrate was added to the remainder to make a final betanine concentration of about 75 ppm and a final volume of 1 liter. The remaining solution was used as the uncolored sample. The pH was checked and sufficient crystalline Na_2HPO_4 added to attain the original pH. Approximately 10 ml of each sample was portioned into 3-oz red flint glass sampling cups coded with a three-digit random number. Samples were held at ambient temperature (21°C) for about 1 hr before evaluation.

The ability of other food flavors to mask that of betanine or beet juice was determined by preparing 2 liters of a solution consisting of 354g of uncolored strawberry-flavored gelatin dessert powder (Test 3). Approximately one-half of the solution was removed and beet juice concentrate added to make a final betanine concentration of about 75 ppm and a final volume of 1 liter. The remaining solution was used as the uncolored sample. After the pH was measured, approximately 10 ml of each solution were portioned into 3-oz red flint sampling cups coded with a three-digit random number and the gelatin hardened in the refrigerator (5°C) overnight.

Sample presentation

Sensory tests were held in the University of Wisconsin Sensory Evaluation Laboratory. To avoid the possibility of visually detecting the odd sample it was necessary to limit available light in the room where tests were done. Windows were covered with aluminum foil until

Table 2—Results of sensory evaluation of betanine by triangle difference tests

Sample code	Sample composition	Number of panelists	Correct judgments	Required[a] correct judgments
1	betanine in buffer	60	19	28
2	beet juice conc in buffer	28	24	15
3	beet juice conc in gelatin dessert	27	10	14

[a] Number of correct answers necessary to establish a significant differentiation at 5% level (Roessler et al., 1948).

the room was nearly "light tight." Individual booths were then illuminated by a single red-tinted 25 watt incandescent light bulb. The voltage was regulated so that in each booth, 0.1 ± 0.05 CP (Gossen Panlux Electronic Footcandle Meter, Berkey Marketing Co. Inc., Woodside, NY) was the minimum necessary for reading codes and marking ballots. Panelists were seated in individual booths equipped with running water for free juice mouth rinsing.

In Test 1 each panelist received two trays. One tray contained one uncolored and two colored samples, the other tray, the opposite. For each of the two combinations, 10 panelists received samples arranged on the tray in each of the three possible permutations. In the triangle tests to determine if beet juice concentrate could impart a flavor to a food (Tests 2 and 3) each panelist received 1 tray. Samples on trays were arranged in all possible combinations and permutations. When gelatin was evaluated, a red disposable plastic spoon was included on the tray, to avoid visual detection of the odd sample. A standard triangle difference ballot was used to determine which sample was unlike the other two samples (Ellis, 1961).

RESULTS & DISCUSSION

THE CONCENTRATION (75 ppm) of betanine used in these experiments was chosen because this amount results in maximum color intensity for most foods. In previous studies, maximum reported usage seldom exceeded 50 ppm (Pasch et al., 1975).

Betanine was isolated as the hydrochloride, and to avoid detection of the odd sample because of pH difference, a buffer was used for sensory evaluation. Buffer concentrations, however, were kept to a minimum to avoid masking any flavors. As a result of these low buffer concentrations, addition of the colorant caused a slight decrease in pH. The change was never more than 0.2 pH unit and the original pH was restored with a few crystals of Na_2HPO_4 before panelists received the samples. The use of a buffer in flavor testing was reported by Baldwin et al. (1973) who used a buffer composition identical to the one in this experiment to determine the flavor thresholds of fatty acids.

Results of the three sensory evaluation panels are given in Table 2. The data show that there was no significant contribution by betanine to the flavor of a buffer solution containing Na_2HPO_4 (0.01M) and citric acid (0.005M). The unavoidable use of a buffer as a result of the acidic nature of betanine might suggest the potential for masked flavors. However, lack of detectable flavor despite the high betanine concentration indicates that any flavor from this source would be of little or no consequence in food.

Results of the flavor evaluation when beet juice concentrate was compared to a buffer showed a statistically significant difference. Based on results of the previous test, the flavors detected in beet juice concentrate were caused by components of the concentrate other than betanine.

Data in Table 2 show that when beet juice concentrate was added to strawberry-flavored gelatin dessert no significant flavor difference existed between the colored sample and the control, despite a pH difference of 0.3 unit, i.e. the strawberry flavor in the gelatin masked any flavor the beet juice concentrate may have contributed. Strawberry flavor was chosen because it is a delicate flavor and therefore the nonsignificant results suggest that the flavor of concentrated beet juice is easily masked by gelatin dessert flavors.

From results of this study it is concluded that betanine does not have a detectable flavor when in buffered systems. Concentrated beet juice has a flavor which can be described as a typical beet flavor which can be successfully masked by gelatin desserts.

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Ms received 12/23/77; revised 3/25/78; accepted 4/5/78.

The assistance of the Sensory Evaluation Laboratory, Dept. of Food Science is acknowledged. Research supported by the college of Agricultural and Life Sciences, University of Wisconsin-Madison, and by funds from General Foods Corp., White Plains, NY.

A Research Note

PEELING AIDS AND THEIR APPLICATION TO CAUSTIC PEELING OF TOMATOES

H. J. NEUMANN, W. G. SCHULTZ, J. P. MORGAN and J. E. SCHADE

ABSTRACT

Tomato pulp recovered from acidified and screened caustic (sodium hydroxide) peelings obtained from rotating rubber disc peelers at a cannery in 1975 was found to be a potential food material. However, this pulp contained high levels (150–450 ppm) of peeling aids used in commercial caustic baths; these are not approved food additives. In the spring of 1976, a preliminary study evaluated 50 compounds and various procedures for their peeling aid efficiency. Compounds tested included carboxylic acids (C_2–C_{18}) and their esters and salts and other surfactants, emulsifiers, and proprietary mixes. The most effective and acceptable peeling aids were C_6–C_8 saturated fatty acids. For the processing variety, VF145B-7879, the most effective peeling was with a pretreatment of 0.5% octanoic acid at 65°C for about 1 min, followed by the usual caustic immersion. This was at least as effective as using the octanoic acid directly in the caustic bath.

INTRODUCTION

IN 1973 the rubber disc peeler was used successfully to peel tomatoes (Hart et al., 1974), producing peelings with a solids content similar to that of the original tomato. In 1974 a preliminary study indicated that the tomato pulp could be recovered from the caustic peelings for possible food use by screening out the skin and acidifying to pH 4.2 with food-grade hydrochloric acid (Ostertag and Robe, 1975). In 1975 tomato pulp with acceptable flavor, color, and minimal defects was recovered from caustic peelings of a commercial operation (Schultz et al., 1977). However, the peeling aids, which are not approved food additives, were in the recovered pulp at relatively high concentrations (150–450 ppm). In the spring of 1976, prior to pilot tests at a cannery in the summer, a preliminary study explored ways of lowering the peeling aid content in the pulp and/or to find peeling aids that would be more acceptable as additives. Some compounds, though toxic, were tested solely in a search for clues to the mechanism of peeling aid action. The peeling effectiveness of various peeling aids, modified caustic peeling practices, and precaustic bath treatments are summarized in this report.

EXPERIMENTAL

MOST OF THE PEELING TESTS used Walter and Tropic varieties (fresh market tomatoes); several lots of the processing variety VF145B-7879 and limited samples of Roma and VF145A-21-4 were also tested with the most promising procedures and compounds.

Experimentation compared the peeling efficiency of: (1) 11% NaOH (w/w) solution without peeling aid at 99°C for 10–30 sec as a standard treatment: (2) 11% caustic solution with a peeling aid added; and (3) a pretreatment bath containing a peeling aid, caustic or plain water, followed by the standard caustic bath. Immersion times and temperatures varied between 10–60 sec at 24–100°C. Holding periods of 15–30 sec were used after and between the various baths and treatments. Most pretreatments used 65°C.

For each peeling test, two tomatoes were weighed, submerged in 3–6 kg of test solution and agitated slowly, drained for 15 sec, slit about 1 in. across blossom and stem ends, placed on a rubber disc peeler for 15 sec to remove loosened peel, rinsed in cold water, drained and weighed. Tests were usually replicated once.

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Table 1—Pretreatments using VF145B-7879 tomatoes

Pretreatment			Sodium hydroxide				
Solution	Temp °C	Dip time sec	Conc % w/w	Temp °C	Dip time sec	Peeling wt loss %	Surface peeled[a]
None	–	–	11	99	20	6.6	+
None	–	–			30	9.7	+
Water	65	60	11	99	20	10.6	++
Octanoic	65	30	11	99	20	14.5	+++
acid		15			20	13.3	+++
(0.7% w/w)		15			10	9.2	++++
	77	15	11	99	10	9.4	++++
		15		88	10	5.1	++++
		30		71	30	7.1	++
		30			15	1.5	0
	65	60	5	99	15	14.1	+++
	77	60	5	99	30	18.5	+++
		30			15	12.2	+++
		30		88	30	13.2	+++
		30			15	0	0
		60		77	60	17.1	+++

[a] Code ++++ = ≥98% with ≤10% wt loss (≤0.5 in.² peel on each tomato); +++ = ≥98% with >10% wt loss; ++ = 90–97% (>0.5–2.0 in.² peel on each tomato); + = 50–89%; 0 = <50%.

Compounds tested included: (1) mono- and di-carboxylic acids (saturated, unsaturated, or ring) in the C_2–C_{18} range: (2) esters of carboxylic acids; (3) phosphate and fluoro-containing compounds; (4) chloroform; (5) sugar esters; (6) sodium lauryl sulfate, other surfactants and miscellaneous compounds.

Treated tomatoes were evaluated for the "% of surface peeled," which was based on a tomato diameter of 2½ in. For this size tomato, 0.5 in.² represents about 2% of surface. Peeling efficiency was rated as follows: (1) ++++ equals 98% or more of surface peeled with 10% or less weight loss; (2) +++ equals 98% or more of surface peeled with more than 10% weight loss; (3) ++ equals 90–97% surface peeled; (4) + equals 50–89% peeled; (5) 0 equals less than 50% surface peeled.

RESULTS & DISCUSSION

OF THE APPROXIMATELY 50 chemicals screened in these peeling tests, the most effective compounds were the saturated fatty acids with six or eight carbons. The most effective treatment for peeling variety VF145B-7879 used octanoic acid as a pretreatment, followed by an 11% caustic bath. Tomatoes were usually completely peeled (sometimes overpeeled) with a pretreatment of 0.7% octanoic acid at 65–77°C for 15–60 sec followed by either an 11% caustic solution at 71–99°C with immersion times of 10–30 sec, or a 5% (w/w) caustic solution at 77–99°C with dip times of 15–60 sec (Table 1). Dipping VF145B-7879 tomatoes in an 11% caustic solution at 99°C for 30 sec without pretreatment usually produced more variable and incomplete peeling.

Other 1% solutions of carboxylic acids and esters used as pretreatments at 65°C for 60 sec improved caustic peeling. Particularly effective were hexanoic acid and 10-undecenoic acid, and to a lesser extent, oleic and sorbic acids and ethyl hexanoate (Table 2). Caustic peeling was somewhat improved, although more variable, when benzoic acid, succinic acid, or ethyl oleate were used as pretreatments prior to the same standard caustic bath treatment. Other carboxyl acids and

Table 2—Peeling fresh market tomatoes

Peeling aid	Peeling aid in caustic (11% w/w, 99°C)				Peeling aid as pretreatment to caustic (11%, 99°C)			
	Peeling aid conc % (w/w)	Dip time sec	Peeling wt loss %	Surface peeled[a]	Peeling aid conc % (w/w)	Caustic dip time sec	Peeling wt loss %	Surface peeled[a]
Octanoic acid	0.5	10	5.6	++++	1[b]	10	10.1	+++
Octanoic acid	0.7	20	10.5	+++	0.7[c]	10	10.6	+++
Hexanoic acid	–	–	–	–	1[b]	10	10.6	+++
C_6–C_9 fatty acid mix[d] (Faspeel)	0.2	30	11.7	++	–	–	–	–
10-Undecenoic acid	–	–	–	–	1[b]	15	12.4	+++
Sodium 2-ethylhexyl sulfate	0.2	30	12.4	++	0.2[e]	10	11.1	++
Sodium 2-ethylhexyl sulfate	–	–	–	–	0.2[e,f]	10	11.8	+++
Trimethyl nonanol[g] (Tergitol TMN-6)	–	–	–	–	0.1[b]	10	13.1	+++
Sodium mono- and di-methyl naphthalene sulfonates	–	–	–	–	0.2[b]	10	10.6	++
Sodium oleate	0.3	15	8.3	++	–	–	–	–
Phosphate washing mix[d] (Sanel)	–	–	–	–	0.3[b]	20	14.4	++
Polyglycol ester[h] (Santone 3-1-S)	–	–	–	–	1[b]	30	14.2	++
Ammonium hydroxide	–	–	–	–	5[b]	30	14.4	++
Sorbic acid	–	–	–	–	1[b]	30	13.0	++
Ethyl hexanoate	–	–	–	–	1[b]	30	12.6	++
Oleic acid	–	–	–	–	1[b]	10	10.9	++
Chloroform	–	–	–	–	100[i]	10	6.1	++
Water only	–	–	–	–	0[b]	30	10.2	+

[a] Code: ++++ = ≥98% with ≤10% wt loss (≤0.5 in.^{2} peel on each tomato); +++ = ≥98% with >10% wt loss; ++ = 90–97% (>0.5–2.0 in.^{2} peel on each tomato); + = 50–89%; 0 = <50%

[b] Pretreatment, 65°C, 60 sec

[c] Pretreatment, 65°C, 15 sec

[d] BASF Wyandotte Corp., Wyandotte, MI

[e] Pretreatment 99°C, 10–15 sec

[f] In 5% caustic

[g] Union Carbide Corp., New York, NY

[h] Durkee Industrial Foods Group, SCM Corp. Cleveland, OH

[i] Pretreatment 21°C, 60 sec

esters that were no more effective than water only as a pretreatment included fumaric acid, malic acid, tartaric acid, ethyl octanoate, methyl 10-undecenoate, methyl laurate, ethyl laurate, and ethyl levulinate.

Pretreatments using the proprietary phosphate mix, Sanel, resulted in some peeling aid (Table 2). Other phosphate compounds (trisodium-, tripoly-, triethyl-, and sodium pyro-) used in pretreatments (1%, 65°C, 30 sec) showed no improvement in peeling compared to a water pretreatment.

Trimethyl nonanol (0.1%) was also very effective as a pretreatment (Table 2). Compounds currently used by processors in caustic solutions for peeling tomatoes (sodium 2-ethylhexyl sulfate, sodium mono- and dimethyl naphthalene sulfonates, and short-chain fatty acids) were fair to good peeling aids when used in pretreatments. Sodium lauryl sulfate (0.5%), sorbitan monostearate (1%), fluoro-containing surfactants (0.05%) and various sugar esters (1%) used in pretreatment solutions at 65°C for 60 sec prior to a standard caustic bath for 10–15 sec, showed little or no better peeling than water only.

Immersing tomatoes in chloroform prior to a caustic treatment also improved peeling and illustrates the benefit of rapidly penetrating or removing the waxy surface. Commercial use of chloroform is not suggested, however, because of its toxicity and residues. A double caustic dip with fresh market tomatoes using a pretreatment of 5% caustic solution at 99°C for 15 sec, holding 10 sec, followed by a 10 sec dip in 11% caustic at 99°C produced distinctly better peeling than a pretreatment with water alone.

These studies indicate that some surfactants do more than modify interfacial tension between the waxy tomato surface and the caustic solution. Occasionally some tomatoes were peeled using an aqueous solution of short-chain fatty acids at 65°C, and this suggests that these chemicals act as peeling aids by permeating the cuticle and outer cell layers and disrupting the subsurface cells.

In summary, evaluation of various chemicals and procedures for ease of peeling with optimum quality indicated that a pretreatment with octanoic acid was most promising. Pilot plant studies at a cannery later in 1976 showed that 0.15% octanoic acid in a pretreatment solution for 30 sec at 65°C rather than in the caustic bath was an effective peeling procedure and also minimized the amount of peeling aid in the recovered pulp (Schultz et al., 1977). Octanoic acid is effective, convenient to use, and commercially available in food grade at competitive prices. Currently it has FDA approval as a synthetic flavor emulsifier, lubricant, and similar use in food and as a peeling aid (FDA, 1977).

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Ms received 1/6/78; revised 3/21/78; accepted 4/5/78.

A Research Note

STAPHYLOCOCCAL ENTEROTOXIN AND NUCLEASE PRODUCTION IN FOAMS

M. J. WOODBURN and T. N. MORITA

ABSTRACT

Air was incorporated into culture media by the addition of cellulose or nonabsorbent cotton pads to cornstarch pastes and by foam formation in an agar gel. Production of staphylococcal enterotoxin types A, B and C during incubation at 37°C was increased both on a per ml basis and per 10^6 CFU by such air incorporation but not in deaerated controls. Nuclease production followed the same trend. Cell numbers were less affected. Even limited inclusion of air within a food system thus increases the rate of staphylococcal production of enterotoxin.

INTRODUCTION

THE PRODUCTION of enterotoxins by *Staphylococcus aureus* appears to be very dependent on optimum levels of oxygen (Baird-Parker, 1971; Jarvis et al., 1973). This could lead one to conclude that enterotoxin production in foods is primarily a surface phenomena. However, foods often have air entrapped at the interface with the container, in voids between pieces of solids, or in foams. The effect of this limited amount of air has not been investigated despite a number of studies on the effectiveness of rates of continuous aeration in a fermenter or in shaker flasks. Therefore, test systems were developed to examine the influence of air trapped in the depth of a culture medium.

MATERIALS & METHODS

FOR THE INTRODUCTION of air, cellulose or cotton sponges (5 × 5 × 1 cm and weighing 2.5g) were added just after inoculation of the sterile starch-thickened paste. A 4% starch paste (Woodburn et al., 1973) was made with 3% NZ Amine-NAK (Humko Sheffield Chemical, Lyndhurst, NJ) and 3% PHP (a Mead Johnson casein hydrolysate no longer available). The paste was dispersed in aliquots of 300 ml into 12 cm tall 400 ml beakers (Fig. 1) and sterilized at 121°C for 15 min. Controls were similar systems in which foams were in place before autoclaving.

A third foam system was an aerated agar gel. A 0.75% agar medium using Bacto-agar (Difco Lab., Detroit, MI) in 3% NAK and 3% PHP, with thiamine and niacin added and the pH adjusted to pH 6.8, was dispensed in 100 ml aliquots into bottles and sterilized. After being cooled to 45°C, the agar was inoculated, poured into a beaker, set in an ice bath, and beaten with a hand electric mixer at low speed under aseptic conditions for 3 min to incorporate air. Volume expansion was 15%. The foam was then poured into the beaker. A control beaker contained inoculated agar gel with no incorporated air bubbles. A double beaker arrangement (Fig. 1) was devised with the bottom portion of the foam or gel being sealed with 10 ml of sterile light mineral oil as well as with a layer of 30 ml uninoculated agar. The control was an agar gel which had not been whipped.

Staphylococcus aureus strains S-6 (producer of enterotoxin B mainly) and 361 (enterotoxin C_2 producer) were obtained from M.S. Bergdoll (Univ. of Wisconsin-Madison) and 265-1 (enterotoxin A producer) from R.W. Bennett (Food and Drug Administration, Washington, DC).

Inoculum was 0.5 ml of a dilution of a 24 hr broth culture of one strain of *S. aureus* to give 10^3 organisms per ml.

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0022-1147/78/0005-1628$02.25/0

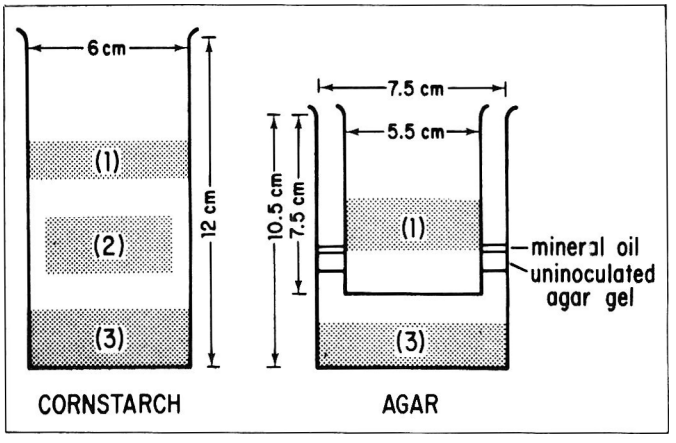

Fig. 1—Diagram of experimental beakers for cornstarch and agar systems. Location of samples shown by number and shading.

Sampling times during 37°C incubation were 8 hr in selected trials and 24 hr. The three samples obtained from the cornstarch medium were: (1) surface area, 30g; (2) middle area, 50g; and (3) bottom area, 50g which included sponge or cotton (Fig. 1). The two samples obtained from each beaker of agar gel medium were: (1) surface area from the 150 ml beaker, 30g; and (3) the bottom area of the 400 ml beaker, 50g (shaded areas in Fig. 1).

Enterotoxin was quantitated by the Oudin single gel-diffusion method as described by Hall et al. (1963) after concentrating the supernatant from centrifuged samples (10,000 × G for 10 min) 7 to 10 times with Aquacide (Calbiochem, Los Angeles, CA). When the toxin concentration was insufficient for quantitation by this method, the microslide gel-double diffusion technique as adapted by Fung et al. (1976) was used. Purified enterotoxins A and B used for these assays were provided by E.J. Schantz (University of Wisconsin-Madison) and enterotoxin C and antiserum for C by M.S. Bergdoll.

Nuclease assays were done immediately using the turbidimetric assay method as modified by Erickson and Deibel (1973).

Colony forming units (CFU) were determined by plating appropriate dilutions of samples on plate count agar (Difco).

Two to five replications were done for each strain. Results are reported as means.

RESULTS & DISCUSSION

ENTEROTOXIN VALUES were low in these nonshaken media (Table 1). However, in the cornstarch medium, differences were detectable at 8 hr between the amounts of enterotoxin produced at different levels in the medium and between controls and samples with air incorporated (foams). At 8 and 24 hr for strain 265-1, enterotoxin A was detectable only in surface samples of control beakers; in the aerated beakers, enterotoxin was found at all three locations in the order of magnitude of surface (1) > middle (2) < bottom (3). In medium inoculated with strain S-6, enterotoxin B was detectable at 8 hr in the bottom samples aerated with cotton as well as all surface samples. By 24 hr, all replications for strains S-6 and 361 showed the (1) > (2) > (3) order of magnitude for controls and (1) > (2) < (3) order for foams.

In the agar gel medium, enterotoxin was found at both surface and bottom locations at 24 hr for all three strains (Table 1). In the control system, enterotoxin levels of the

Table 1—Comparison of enterotoxin production and CFU for three strains of S. aureus after 8 and 24 hr incubation at 37° C in aerated cornstarch and agar foam media (Data are means of 3–6 replications)

Sample		8 hr				24 hr					
		265-1		S-6		265-1		S-6		361	
Type	Location[a]	Toxin μg/ml	CFU X10^6	Toxin μg/ml	CFU X10^6	Toxin μg/ml	CFU X10^7	Toxin μg/ml	CFU X10^7	Toxin μg/ml	CFU X10^7
Control,	(1)	0.01	96	0.02	50	2.0	63	5.8	140	3.9	78
starch only	(2)	b	9.3	b	29	b	20	0.11	11	0.38	16
	(3)	b	1.5	b	0.07	b	1.3	0.01	2.7	0.01	4.2
Control,	(1)	0.03	17	0.06	49	2.1	35	5.0	71	3.8	41
sponge or	(2)	b	4.5	b	4.9	b	20	1.9	35	1.2	22
cotton	(3)	b	1.6	b	4.1	b	8.7	0.12	99	0.03	9.4
Cornstarch	(1)	0.35	12	0.06	14	1.5	57	4.9	41	3.5	15
with aerated	(2)	0.01	1.4	b	1.9	0.04	13	0.67	29	0.23	13
sponge	(3)	0.06	6.0	b	12	0.97	27	1.5	25	3.7	26
Cornstarch	(1)	0.14	43	0.02	10	2.4	27	5.3	65	3.7	39
with aerated	(2)	0.01	1.7	b	1.5	0.38	15	1.1	23	0.35	19
cotton	(3)	0.14	17	0.04	24	2.0	33	2.4	40	2.8	29
Control,	(1)	c	c	c	c	4.2	190	3.9	130	1.3	220
Agar gel	(3)	c	c	c	c	0.8	69	0.17	42	0.3	38
Agar foam	(1)	c	c	c	c	3.5	130	3.9	160	2.1	140
	(3)	c	c	c	c	3.5	140	4.2	55	2.5	61

[a] Location of sample: (1), surface; (2), middle; (3), bottom
[b] Negative with samples concentrated 7–10 times using microslide assay technique
[c] Not determined

surface samples were 4 to 19 times higher than those in the bottom samples. In the foam system with air bubbles incorporated in the agar, the bottom samples in all replications exhibited toxin levels approximately equal to those in surface samples.

Nuclease activity showed a similar response to the presence of air.

For all three strains, differences in CFU of 24 hr samples between controls and foams were found. For the cornstarch medium, CFU values of samples from surface (1), middle (2), and bottom (3) areas in the control were in the order of descending magnitude; the CFU at the surface was 3 to 10 times greater than in the middle and 20 to 50 times greater than the bottom area. A similar pattern of descending order of magnitude was also found for samples from beakers in which cotton and sponge foam had been autoclaved with the media. However, in the beakers with air incorporated from the sponge or cotton foam, the CFU level was in the order of descending magnitude of surface, bottom and middle or bottom, surface, and middle. The 8 hr CFU showed the same differences in order of descending magnitude between controls and foams for strains 265-1 and S-6, the 2 strains studied at 8 hr.

In the agar system, only surface (smaller beaker) and bottom (larger beaker) area samples were taken (Fig. 1). The surface CFU for the control agar for strain 265-1 at 24 hr was 3 times that of the bottom sample but there was little difference for samples of gel foam. Differences between samples from control agar gel and foam were not evident for the other two strains.

In characterizing microbial ecosystems, Marshall (1976) has summarized possible differences at interfaces as including altered gas availability, pH, nutrient concentrations, and adsorption effects on macromolecules. Controls were included in this series for cotton and sponge foams. Since the materials had little effect if deaerated by being autoclaved in the medium, the introduction of air appears to be the stimulus for increased production of enterotoxin and nuclease. The frequent implication of such salads as potato and chicken in foodborne illness may be related to the air which is incorporated throughout such mixtures as well as the absence of microbial competition. The observations of Crisley et al. (1964) that a synthetic cream pie filling that did not support staphylococcal growth alone did so when put into a pie shell may have been partially explained by the effect of the layer of adsorbed air between the shell and the filling. Thus, the characteristics of the food environment which must be considered include the internal air which may be in voids between solid particles, foam, or adsorbed to solids.

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Ms received 12/24/77; revised 3/26/78; accepted 4/5/78.

Technical Paper No. 4683 of the Oregon Agricultural Experiment Station.

A Research Note

ENRICHMENT OF HOME–PREPARED TORTILLAS MADE FROM GERMINATED CORN

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ABSTRACT

The RNV as determined with *Tetrahymena pyriformis* of corn meal made from nongerminated corn increased from 65% to 95% for corn meal made from germinated corn. The lysine and tryptophan contents (measured microbiologically) of corn meal made from nongerminated corn were 23 mg/gN and 3 mg/gN, respectively; but increased to 68 mg/gN and 26 mg/gN after germination. With laboratory panels, there was no difference in mean scores for color, texture and flavor of tortillas made with lime-treated corn and tortillas made with a 50:50 mixture of lime-treated corn and germinated corn. The mean scores for tenderness in the tortillas were significantly lower in tortillas made from the 50:50 mixture when compared to the tortillas made from lime-treated corn. Results of consumer panels indicated that the tortillas made from lime-treated corn were preferred over tortillas made from a 50:50 mixture of lime-treated and germinated corn. The RNV of tortillas made from lime-treated corn was 69% whereas the tortillas made with a 50:50 mixture was 76%.

INTRODUCTION

CORN TORTILLAS are a popular, staple food in Central and South America. Tortillas contribute protein and carbohydrates to the diet of poorer people of this area. The most important factor of improving nutrition is to improve the food that is consumed daily such as tortillas. In certain geographical areas where corn is a part of the diet, techniques to improve the amino acid balance in corn are highly desirable.

Corn is deficient in the amino acids lysine and tryptophan. Recent studies were made on methods to improve the nutritional quality of tortillas by adding soybeans (Franze, 1975), by adding cottonseed flour (McPherson and Ou, 1976), by adding cottonseed or soy flour (Green et al., 1976) or by using a cooked corn-soybean mixture (Valle et al., 1976). These additives relieve the amino acid deficiencies but the homemaker must have the additives on hand or must be able to purchase them prior to making the tortillas.

In this research, corn meal made from germinated corn was used to replace some of the traditional lime-treated corn to make tortillas. Laboratory and consumer panels were used to ascertain the desirability of the tortillas. Nutritional quality of the tortillas was measured by *Tetrahymena pyriformis* W and by measuring lysine and tryptophan microbiologically.

MATERIALS & METHODS

CORN was soaked 6–18 hr in tap water. The soaked corn was spread to form a thin layer in a shallow, wooden tray with a metal screen as a bottom. To maintain a high moisture level, cheese cloth was placed on the seeds and then covered with a metal tray. The germinated seeds were washed 2–3 times a day with tap water.

Seventy to 100% of the seeds germinated after 4 days at 30°C. They were then dried overnight at 50°C. The dried corn was ground in a Wiley mill using a 1 mm screen. Duplicate batches of corn meal were prepared for % RNV and amino acid analyses. Another batch of germinated corn was prepared for comparison with lime-treated corn meal (also one batch) in the laboratory panels. A separate batch of germinated corn was prepared for the consumer taste panel. A total of four separate batches of germinated corn were prepared in this research.

Corn meal for making tortillas was prepared by the method described by Franze (1975) with some modifications. Water and lime (1.5% of the weight of the corn) were brought to a boil. The corn was added and heat turned off. The corn was allowed to stand in the lime water overnight. The next morning, the lime water was poured off and the corn was washed several times with water to remove the excess alkali. The corn was dried at 55–65°C under flowing air overnight. The dried, lime-treated corn was ground in a Wiley mill.

The tortillas were made following the formula of Kennedy (1972). Each tortilla was 5 inches in diameter. The tortillas were cooked in a Sunbeam skillet (Model RC, Sunbeam Corp., Chicago, IL). The temperature was set at 340°F (171°C). The control tortillas were made of 100% of the lime-treated corn, whereas the test tortillas were made by substituting 50% of the lime-treated corn (made into a meal) with corn meal prepared from germinated corn seeds. After cooking, the tortillas were cut into 8 equal pieces for taste panel samples. In the laboratory panels the tortillas were made about ½ to 1 hr before serving. In the consumer panels, the tortillas were prepared 1 day before the panels were conducted and were stored in sealed plastic bags at room temperature.

Seven judges (faculty and graduate students) comprised the laboratory panel. Three replicate panels (N = 21) were performed on three consecutive days. Samples were coded with 3-digit random numbers according to a table of random numbers (Snedecor, 1950). Samples were randomly served according to a table of random permutations of 9. A descriptive score sheet was used to judge color (golden brown to pale brown), tenderness (crisp to soggy), texture (fine to coarse) and flavor (desirable to undesirable). For analysis, categories on the score sheets were assigned numbers from 1 to 5. The data were studied using analysis of variance (Snedecor, 1950).

The consumer panel consisted of 50 college students (N = 50). The students were asked to check one of the descriptive phrases (like very much, like, neither like nor dislike, dislike, or dislike very much) after tasting the tortillas. The data were analysed by the method of analysis of variance.

The micro-Kjeldal method of the AOAC (1975) was used to determine the nitrogen content of the ground seeds. By adjusting the sample size the nitrogen content was used to adjust the samples to the same nitrogen content for determining the relative nutritive value of the meals.

The relative nutritive value (RNV) of the meals was determined using *Tetrahymena pyriformis* W. (ATCC 10542) according to Stott and Smith (1963). The RNV was calculated according to the equation of Helms and Rølle (1970).

After samples containing 100 mg N were predigested with 1 ml of 2% (w/v) papain solution for 3 hr at 56°C, as described by Ford (1964), the microbiological assay method of Difco (1971) was employed for the determination of available lysine and tryptophan. *Pediococcus cerevisiae* ATCC 8042 was the test organism for the lysine assays. *Lactobacillus plantarum* ATCC 8014 was used for the tryptophan assay. Reagent-grade L (+)-lysine monohydrochloride and DL-tryptophan (both of Eastman Kodak Co.) were used in preparing standards.

RESULTS & DISCUSSION

THE MEAN RNV of duplicate batches of corn meals produced from germinated and nongerminated whole kernel corn was 65% for the nongerminated and 95% for the germinated corn. These data indicate that germination increased the nutritive value of the protein.

This improvement in RNV was expected because of increases in the lysine and tryptophan contents of corn meal made from germinated corn. The lysine content increased from 23 mg/gN to 68 mg/gN after germination, which is about

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0022-1147/78/0005-1630$02.25/0

Table 1—Mean scores of tortillas by laboratory panels

Treatment	Mean scores[a]			
	Color	Tenderness	Texture	Flavor
Control[b]	4.09	3.95[c]	3.81	3.76
Substituted corn[d]	4.05	2.71	3.71	3.52

[a] N = 21; 5-point hedonic scales from 1 to 5 with 5 denoting the most desirable attribute. Color, 1 = pale yellow; 5 = golden yellow. Tenderness, 1 = soggy; 5 = crisp. Texture, 1 = coarse; 5 = fine. Flavor, 1 = undesirable, 5 = desirable.
[b] Corn meal made from lime-treated corn
[c] Significant ($P < 0.05$) difference between means for that attribute
[d] 50% of the lime-treated corn substituted with corn meal prepared from germinated corn

Table 2—Relative nutritive value (RNV) of corn meals and tortillas[a]

Product	%N	%RNV[b]
Corn meal made from whole kernel corn (control)	1.22	67
Corn meal made from lime-treated corn (control)	1.26	66
A 50:50 mixture of meals[c]	1.26	78
Tortillas made from lime-treated corn (control)	1.21	69
Tortillas with 50% corn meal made from germinated corn	1.28	76

[a] Mean of duplicate analysis
[b] All products (except the corn meal made from whole kernel corn) were dried before assay. RNV of casein is 100%.
[c] A 50:50 mixture of corn meal made from lime-treated corn and corn meal made from germinated corn

a threefold increase. The increase in tryptophan content was more impressive (3 mg/gN to 26 mg/gN or about an eightfold increase). The data in this study are in agreement with the data of Tsai et al. (1975) who found that there was an increase in both lysine and tryptophan during germination of maize seeds.

The RNV, like other methods of estimating the nutritive value of proteins, is only an estimate. Rølle (1975) stated that the amino acid requirements of *T. pyriformis* W are in reasonable agreement with requirements of a human being. Kidder and Dewey (1961) found that the amino acid requirements of *T. pyriformis* W were similar to the rat. More recently, Evancho et al. (1977) reported that RNV was correlated with rat PER bioassays ($r = 0.9$, $P < 0.01$).

The mean panel score for all attributes for tortillas in laboratory panels indicate that both the control and the substituted treatment rated above the average score (2.5), as shown in Table 1. The control tortilla had a significantly ($P < 0.05$) higher mean score for tenderness than the mean score for tortillas made from 50% germinated corn. Even though tortillas made with 50% germinated corn scored 2.71 (a mean score of 21 tests), the panel score for tenderness was still about 2.5 middle score. The initiating factor for utilizing germinated corn to improve the nutritive value of the tortillas appeared to be tenderness. This factor obviously needs more research to improve the product.

Results of the 5-point hedonic tests by 50 consumers (College students) indicated that the tortillas made with corn meal from germinated corn were in the dislike category. When numerical values were assigned to each class (like very much = 5 and dislike very much = 1), an analysis of variance indicated that difference between the mean scores of 3.06 (control) and 2.10 (50–50% substitution) was significantly different ($P < 0.01$).

The % N in each of the meals and tortillas, as well as RNV, are given in Table 2. The % N is adjusted on each sample prior to RNV assay. The RNV values indicate that the amino acid balance was increased by using a mixture of 50% corn meal made from germinated corn and 50% corn meal made from lime-treated corn. The limiting factor of using more corn meal made from germinated corn was the adhesive qualities of the lime-treated corn which holds the tortillas together. If this problem can be overcome, corn meals with higher RNV can be used to produce tortillas.

The use of corn meal from germinated corn to enrich tortillas offers another valid alternative to improve the nutrition of rural poor in Central and South America where resources and funds are limited.

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Ms received 1/16/78; revised 3/27/78; accepted 4/5/78.

Contribution from the Missouri Agricultural Experiment Station, Journal Series No 7906.

A Research Note

SOME COMMENTS ON THE ESTIMATION OF SENSORY THRESHOLD BY KAERBER AND SCOVILLE METHODS

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ABSTRACT

Though poor precision is inherent in many organoleptic methods, the widely reported discrepancies of the Scoville results encountered in both within- and between-laboratories are partly attributable to the "3 out of 5 rule," which is not consistent with the standard definition of sensory threshold. Greatly improved precision is shown in this paper by the application of the Kaerber mean and standard deviation to the response data, generated by algebraic combinations and obtained experimentally. Further improvement can be made with the increased number of dilutions presented. Since, finally, the response magnitude seems better correlated to the logarithm of concentration, it is suggested that the present dilution schedule of the Scoville method be revised to logarithmic scale.

SOME COMMENTS ON THE ESTIMATION OF SENSORY THRESHOLD BY KAERBER AND SCOVILLE METHODS

LET THE STIMULUS LEVELS, x_i, be ordered from weak to strong, e.g. $x_1 < x_2 < \ldots\ldots < x_k$. And let R_i and r_i be the number of responses for x_i and the number of cumulative responses for x_i, respectively. If, in particular, $R_1 = 0$ and $r_k = 5$ (in the case of a 5-subject panel) and if $2 \leqslant R_i \leqslant 4$ for $2 \leqslant i \leqslant k - 1$, x_1 and x_k cover the entire range of critical levels in the population.

The Scoville heat method (ASTA, 1968) requires that x_i's covering the range of the critical levels be presented to the 5-subject panel and the lowest x_i for which $r_i = 3$ be called the threshold value, or Scoville heat units. Given k dilutions, any one of the k dilutions can be the threshold except the first dilution, x_1. Now, let us see in how many ways, C_i, x_i can be threshold in the Scoville method, where $r_k = 5$. By definition, x_i is threshold when $r_i \geqslant 3$ and $r_j < 3$ for all $j < i$. Then, C_i is the sum of the number of ways x_i is threshold with $r_i = 3$ and the number of ways x_i is threshold with $r_i = 4$. When $r_i = 4$, $r_j \leqslant 2$ for all $j < i$. Since $R_2 \geqslant 1$, and $R_i \geqslant 2$, $r_i = 4$ in $i - 1$ ways. When $r_i = 3$, $r_j = 1$ or 2 for $j < i$ and $r_1 = 3$ or 4 for $i < 1 < k - 1$, and similary, $r_i = 3$ in $(i - 1)(k - i)$ ways. Hence,

$$C_i = (i - 1) + (i - 1)(k - i)$$

For k dilutions, the total possible way of obtaining threshold, ΣC_i, is

$$\sum_{i=1}^{k} (i - 1)(k - i + 1) = \binom{k+1}{3}$$

which can be proved by inductive method.

Thus, for example, if the stimulus levels covered are 10, 12, 14 and 16 ppm, 12 ppm can be the threshold in 3 different ways, 14 ppm in 4 different ways, and 16 ppm in 3 different ways, or a total of 10 ways in which 3 of the 4 dilutions can be considered the threshold (The Scoville heat method expresses the concentration in number of parts water per part sample, but ppm is adopted in this paper for the sake of convenience). As the number of the critical levels presented, k, increases, so does C_i. The panel results of this nature lend themselves to the Kaerber method of analysis of sensitivity testing (Natrella, 1963). Or, instead of resorting to the "3 out of 5 rule," we can calculate the mean and standard deviation. For 5-subject results, the mean, m, and variance, s^2, may be obtained from the following equations:

Table 1—Comparison of Scoville units and Kaerber means for 56 possible panel results of 7 dilution presentation

Cumulative panel response for (ppm)								Kaerber	
10	11	12	13	14	15	16	Scov.	m	s
0	1	1	1	1	1	5	16	14.50	1.98
0	1	1	1	1	2	5	16	14.30	1.92
0	1	1	1	2	2	5	16	14.10	1.94
0	1	1	1	1	3	5	15	14.10	1.83
0	1	1	2	2	2	5	16	13.90	2.04
0	1	1	1	1	4	5	15	13.90	1.72
0	1	1	1	2	3	5	15	13.90	1.83
0	1	2	2	2	2	5	16	13.70	2.21
0	1	1	1	2	4	5	15	13.70	1.70
0	1	1	2	2	3	5	15	13.70	1.92
0	1	1	1	3	3	5	14	13.70	1.81
0	2	2	2	2	2	5	16	13.50	2.43
0	1	1	2	2	4	5	15	13.50	1.77
0	1	2	2	2	3	5	15	13.50	2.08
0	1	1	1	3	4	5	14	13.50	1.65
0	1	1	2	3	3	5	14	13.50	1.88
0	1	2	2	2	4	5	15	13.30	1.92
0	2	2	2	2	3	5	15	13.30	2.30
0	1	1	1	4	4	5	14	13.30	1.57
0	1	1	2	3	4	5	14	13.30	1.70
0	1	2	2	3	3	5	14	13.30	2.02
0	1	1	3	3	3	5	13	13.30	1.92
0	2	2	2	2	4	5	15	13.10	2.13
0	1	1	2	4	4	5	14	13.10	1.60
0	1	2	2	3	4	5	14	13.10	1.83
0	2	2	2	3	3	5	14	13.10	2.23
0	1	1	3	3	4	5	13	13.10	1.72
0	1	2	3	3	3	5	13	13.10	2.04
0	2	2	2	3	4	5	14	12.90	2.04
0	1	2	2	4	4	5	14	12.90	1.72
0	2	2	3	3	3	5	13	12.90	2.23
0	1	2	3	3	4	5	13	12.90	1.83
0	1	1	3	4	4	5	13	12.90	1.60
0	1	3	3	3	3	5	12	12.90	2.13
0	2	2	2	4	4	5	14	12.70	1.92
0	2	2	3	3	4	5	13	12.70	2.02
0	1	2	3	4	4	5	13	12.70	1.70
0	1	1	4	4	4	5	13	12.70	1.57
0	2	3	3	3	3	5	12	12.70	2.30
0	1	3	3	3	4	5	12	12.70	1.92
0	2	2	3	4	4	5	13	12.50	1.88
0	1	2	4	4	4	5	13	12.50	1.65
0	2	3	3	3	4	5	12	12.50	2.08
0	1	3	3	4	4	5	12	12.50	1.77
0	3	3	3	3	3	5	11	12.50	2.43
0	2	2	4	4	4	5	13	12.30	1.81
0	2	3	3	4	4	5	12	12.30	1.92
0	1	3	4	4	4	5	12	12.30	1.70
0	3	3	3	3	4	5	11	12.30	2.21
0	2	3	4	4	4	5	12	12.10	1.83
0	1	4	4	4	4	5	12	12.10	1.72
0	3	3	3	4	4	5	11	12.10	2.04
0	2	4	4	4	4	5	12	11.90	1.83
0	3	3	4	4	4	5	11	11.90	1.94
0	3	4	4	4	4	5	11	11.70	1.92
0	4	4	4	4	4	5	11	11.50	1.98

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0022-1147/78/0005-1632$02.25/0

Table 2—Experimental data for "40,000 Scoville" cayenne

Scov. dil'n →	34,000	37,000	40,000	45,000	50,000	Threshold in ppm (in Scoville units)				
in ppm →	29.41	27.03	25.00	22.22	20.00	Scoville		Kaerber m		Kaerber s
Run #1	0	1	2	2	5	20.00	(50,000)	23.51	(42,535)	2.96
Run #2	0	1	3	3	5	25.00	(40,000)	24.49	(40,832)	2.81
Run #3	0	3	4	4	5	27.03	(37,000)	26.36	(37,936)	2.68
						m	(42,333)		(40,434)	
						s	(6,807)		(2,325)	
						s/m	0.16		0.06	

$$m = \frac{1}{10}\sum (r_{i+1} - r_i)(x_{i+1} + x_i)$$

$$s^2 = \frac{1}{100}\left[5\sum (r_{i+1} - r_i)(x_{i+1} + x_i)^2 - \left\{(r_{i+1} - r_i)(x_{i+1} + x_i)\right\}^2\right] - \frac{1}{60}\sum (r_{i+1} - r_i)(x_{i+1} - x_i)^2$$

The purpose of this paper is to compare the threshold values obtained by the Scoville method of computation with those obtained by the Kaerber method of analysis and to show that the latter provides the mean threshold value that may be said to better represent the data, that its run-to-run variation (precision) is considerably smaller, and that the standard deviation it yields enables us to make further statistical inferences. Its computations, to be sure, are not so straightforward as the 3 out of 5 rule but with any of the many programmable desk-top calculators that are in wide use these days it is nearly as simple. (A limited supply of the programs for computing the Kaerber mean and standard deviation for up to 9 dilutions using a Hewlett-Packard 97 calculator is available from the author.)

While a general approach would be desirable from the standpoint of mathematical rigor, a few specific examples may be more useful for our purpose and yet without loss of generality since the Scoville method limits the panel members to 5 and since the number of dilutions in actual practice is often as few as 3, seldom more than 5. Thus, let us assume that the preliminary test by the Scoville method shows the threshold to lie somewhere between 10 and 16 ppm, and further assume that the dilutions subsequently presented to the panel consist of 10, 11, 12, 13, 14, 15 and 16 ppm. Then, there are 56 different ways of obtaining the panel results as enumerated in Table 1, where the Scoville value along with the Kaerber mean and standard deviation is listed for each set of the panel responses. This standard deviation may be useful in characterizing the panel when necessary.

That the Kaerber means are less widely scattered than the Scoville units may not be too apparent from the examination of the Table. For this reason, let us consider the mean and its standard deviation of the means. The mean of the Kaerber means in Table 1 is 13.00 with standard deviation of 0.68, and that of the Scoville values, 13.50 with standard deviation of 1.51. The comparison of the 2 means by Student "t" test indicates a highly significant difference. It can be shown similarly that the mean of the Kaerber means for the 4 dilution (10, 12, 14 and 16 ppm) presentation, 13.00 with standard deviation of 0.73, is again significantly different from the mean of Scoville means, 14.00 with standard deviation of 1.63. The same can be said about the 3 dilution (10, 13 and 16 ppm) presentation for which the Kaerber mean is 13.00 with standard deviation of 0.77 and the Scoville mean, 14.50 with standard deviation of 1.73. Note that while the mean for the Scoville units varies with the number of dilutions, the Kaerber mean at 13.00 ppm remains the same so long as the no response level, 10 ppm, and all response level, 16 ppm, are fixed. Also, the Kaerber standard deviation, in terms of coefficient of variability (ratio of standard deviation to mean), ranging from 5.2–5.9% are much smaller than the Scoville standard deviation varying from 11.1–11.9%. The Scoville mean approaches the Kaerber mean as the number of dilutions increases, and the standard deviation becomes smaller for both Kaerber and Scoville methods as the number of dilutions increases.

We now consider a set of actual experimental data obtained on "40,000 Scoville" cayenne. Three repeat runs gave the results shown in Table 2. We note that there are 20 different ways of threshold by a formula given earlier. The 5 dilutions are a segment from the dilution schedule provided in the Scoville method. The Scoville heat units by 3 out of 5 rule of 50,000, 40,000 and 37,000 (expressed in Scoville for those who are more accustomed to this mode than ppm) for run #1, 2 and 3, respectively, show much greater variations than the gradations adopted by the trade for such products. The same panel results treated by the Kaerber method are much closer together with a coefficient of variability of 6%, as compared with 16% for the former. Though poor precision is inherent in many organoleptic methods, the widely reported discrepancies (Rhyu, 1972; Suzuki, 1957) of the Scoville results encountered in both within- and between-laboratories are partly attributable to the 3 out of 5 rule.

Finally, since the response magnitude seems better correlated to the logarithm of concentration as, for example, Beidler equation (Teranishi, 1971) shows, the present dilution schedule of the Scoville method may be revised to logarithmic scale. That is, if we wish to have n dilutions for each decade, starting with x_1 (i = 1, first dilution), the i^{th} dilution, x_i, is antilog $(\log x_1 + \frac{i-1}{n})$, so that each decade is repeated in multiples of 10. It is worth noting also in this connection that closer spacing of the dilutions is desirable, especially if the number of panel members is to remain at only 5.

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Ms received 10/25/77; revised 2/18/78; accepted 4/5/78.

A Research Note

EFFECT OF PH ON THE GROWTH OF Clostridium botulinum IN CANNED FIGS

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ABSTRACT

Fresh figs were pureed, the puree mixed with one of three syrup variables (water, light or heavy syrup), the pH adjusted at 0.1 pH intervals from 4.6 to 5.4, and the tubes inoculated with a composite of 5 type A and 5 type B. *C. botulinum* spores at 2 inoculum levels (10^2 or 10^6 spores/tube). Anaerobic incubation was at 30°C for almost 1 yr. The results of our experiments show that maintenance of pH 4.9 or below in this product will prevent the outgrowth of *C. botulinum* spores. An inoculated pack utilizing commercial procedures confirmed these results.

INTRODUCTION

Meyer and Eddie (1965) in their review of human botulism in the United States and Canada cite 12 outbreaks of botulism from home canned figs. In these home canning incidents, the product apparently received a process insufficient to destroy *C. botulinum* spores. The home canner is reluctant to give a severe process to the product because such a process would cause the figs to lose their character. The commercial canner has realized the detrimental effect of attempting to process figs sufficiently to destroy *C. botulinum* spores and instead has utilized the addition of acid to prevent the growth of *C. botulinum* spores in the product. The Standard of Identity for canned figs (1977) recognizes this as an acceptable procedure, and requires the addition of lemon juice, concentrated lemon juice or organic acid, when necessary, to reduce the pH to 4.9 or below. Townsend et al. (1955) using figs in heavy syrup showed that maintenance of pH 4.9 or below would prevent the outgrowth of *C. botulinum* spores. No commercially canned figs have ever been involved in a botulism incident.

It was the purpose of this study to determine the pH levels necessary to inhibit the growth of *C. botulinum* spores in figs packed in water, light, and heavy syrup.

MATERIALS & METHODS

Spore suspensions

C. botulinum strains 33A, 62A, 73A, 78A, 32B, 113B, 169B, and 213B (from K.F. Meyer, G.W. Hooper Foundation for Medical Research, San Francisco, Calif.) and strains 74B3-44A, 12033B, and 13983B (from National Food Processors Association, Berkeley, CA) were used.

The spores of *C. botulinum* 62A and 213B were prepared in polypeptone broth (5%) and were washed and harvested by the methods of Tsuji and Perkins (1962). The spores of the remaining strains were prepared in Wheaton beef heart-casein broth and were harvested by the methods of Denny et al. (1958).

Prior to subsequent use, equal numbers from each of the spore suspensions were mixed together to form a single composite spore suspension.

Studies in fig puree and figs

Whole figs, obtained from trees (*Ficus carica*) in California orchards, were utilized upon receipt and within 48 hr of picking.

Cleaned, trimmed figs were blanched for 5 min in flowing steam at 93.3°C. The blanched figs were cooled, thoroughly comminuted using a Waring Blendor, placed in beakers and sterilized for 20 min, at 121.1°C. Then the figs were mixed with either water, light syrup or heavy syrup at the ratio of approximately 35% added liquid to 65% pureed figs. The pH of the liquid-pureed figs mixture was adjusted, using either concentrated lemon juice or sodium hydroxide, to a range of pH values starting with about 4.6 and rising at approximately 0.1 pH unit intervals to pH 5.4.

Each of the samples was transferred to 12 culture tubes (16 × 150 mm). The air was exhausted from the product, and 8 of the tubes inoculated with 0.1 ml of spore inoculum, previously heat shocked in sterile distilled water at 82.2°C for 10 min. Two inoculum levels (10^2 and 10^6 spores/tube), were divided among the inoculated tubes. The remaining tubes were used as uninoculated controls and inoculated with 0.1 ml of sterile distilled water. All tubes were overlaid with sterile vaseline and incubated at 30°C.

An inoculated pack was performed using whole figs. The figs were washed, blanched and hand packed into size 303 × 406 cans (fill wt 11 oz). One fig, inoculated with 0.1 ml of the spore suspension (1×10^6 spores) into the fig tissue, was placed in each can. The cans were topped with the appropriate water or syrup-acid combination so as to have a figs-to-liquid ratio of about 65 to 35. The containers were then sealed and the containers given a commercial process. A total of 15 containers (10 inoculated and 5 uninoculated controls) was prepared for each variable tested. The processed figs were incubated at 30°C.

Determination of the growth of C. botulinum

Outgrowth of spores was determined by gas formation and microscopic examination of the product. Tubes in which gas formation was observed were tested for toxicity. All inoculated tubes, at the level below the lowest pH at which gas formation was observed, were opened, examined microscopically for growth of microorganisms and a portion of the tube's contents tested for presence of toxin. The presence of toxin was determined by the method suggested by Kautter and Lynt (1976).

RESULTS & DISCUSSION

THE RESULTS obtained from inoculated fig puree are given in Table 1. The pH, as measured at the time of product inoculation, changed slightly during incubation. This pH change was determined by measuring the pH of the uninoculated control tube at the time that gas formation was first observed in the inoculated tube or the pH level at which no growth was observed, at the termination of the experiment.

At all three syrup variables, growth occurred in tubes with a large inoculum. In the water pack, growth occurred at pH 5.6, but not at pH 5.35. The toxin present was a type B. In the light syrup pack, growth was observed at pH 5.6 and 5.45. The toxin present was a type A. No growth was observed at pH 5.22. In the heavy syrup pack growth was observed at pH 5.7 and 5.45 with no growth at pH 5.1. Type B toxin was present at pH 5.7 and type A at pH 5.45. No growth was observed in tubes with the small inoculum except in the heavy syrup pack where growth was observed at pH 5.7, but no growth was observed at 5.45.

The inoculated pack consisted of figs packed in three syrup levels (water, 17° Brix and 35° Brix) and three pH variables (no additives, pH 4.6; acid added, pH 4.5; base added, pH 5.0).

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0022-1147/78/0005-1634$02.25/0

Table 1—Outgrowth of C. botulinum spores in pureed figs[a]

Type of pack	Adjusted pH	pH at time of growth	Spore inoculum level (per tube)	Growth-Gas formation	No. of positive tubes	Days of incubation first found gas formation	Toxin
Water[b]	5.35	5.1	10^2	–	0/4	–	–
			10^6	–	0/4	–	–
	5.6	5.58	10^2	–	0/4	–	–
			10^6	+	4/4	9	+
Light syrup[c]	5.22	4.83	10^2	–	0/4	–	–
			10^6	–	0/4	–	–
	5.45	5.45	10^2	–	0/4	–	–
			10^6	+	3/4	8	+
	5.6	5.55	10^2	–	0/4	–	–
			10^6	+	4/4	9	+
Heavy syrup[d]	5.1	4.83	10^2	–	0/4	–	–
			10^6	–	0/4	–	–
	5.45	5.45	10^2	–	0/4	–	–
			10^6	+	4/4	8	+
	5.7	5.58	10^2	+	1/4	23	+
			10^6	+	4/4	7	+

[a] pH of original products (beforeadjustment) was 4.65; water pack was incubated for 375 days at 30°C; light and heavy syrups, 372 days
[b] No growth below pH 5.58
[c] 17° Brix solution (put-on); no growth below pH 5.45
[d] 33.8° Brix (put-on); no growth below pH 5.45

All containers were incubated for at least 360 days before the experiment was terminated. No growth occurred in any of the containers in the inoculated pack variables studied.

The results of our studies are in agreement with those of Townsend et al. (1955) who utilized only heavy syrups in their experiments. Our results indicate that syrup concentration, in the range utilized, does not appreciably affect the outgrowth of *C. botulinum* spores in figs.

The results obtained in our studies show that the maintenance of pH 4.9 or less for figs packed in water, light or extra heavy syrup will prevent the outgrowth of *C. botulinum* spores.

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Ms received 1/30/78; accepted 4/10/78.

We thank Mr. Robert S. Bowen and Mr. Roderic R. Frost for providing research materials.

ERRATA NOTICES

J. Food Sci. 43(2): 634–635 (1978). Effect of plant fiber on lipase, trypsin and chymotrypsin activity by Barbara Olds Schneeman. On page 634, in Table 1 under the column entitled Trypsin, the last two values should be switched: the value for rice bran should be 92.1 ± 4.9 and the value for safflower meal should be 56.3 ± 3.9*. Please change accordingly.

J. Food Sci. 43(4): 1056–1058 (1978). Recovery of betalaines from red beets by a diffusion-extraction procedure by Robert C. Wiley and Ya-Nien Lee. On page 1056, Introduction, correct line five to read ". . . and others," and line eight to read "can contain varying proportions of betacyanines and betaxan-." Under Figure 1, line three, change "although" to read "Even though." Page 1058, Results and Discussion, line 22, change to read "Olsen (1962) in his studies found a large number of dif-."