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Volume 41 : Number 3
Coden: JFDSAZ 41(3) (475–727) 1976

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May-June 1976
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JOURNAL of FOOD SCIENCE

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THE CHEMISTRY AND CELL BIOLOGY OF THE VACUOLAR PROTEINS OF SEEDS

ABSTRACT

Ultrastructural, cytochemical and immunochemical evidence obtained using different plant families supports a suggested model for the biosynthesis of the storage proteins of seeds. Aleurins of the legumin type are proposed to be homologous. The proposed homologues are usually hexamers or tetramers of a disulfide bridged subunit of the A-S-S-B type. The large observed variations in amino acid composition of the proposed homologues imply they are liberal proteins; i.e., large variations in the amino acid sequence of the subunits do not cause lethal effects nor change the function of the protein. The list of proposed legumin homologues includes the legumins, glycinin, arachin, edestin and cocosin. Some unnamed cottonseed proteins also seem to qualify.

INTRODUCTION

PROTEIN CHEMISTS of the 18th and 19th centuries concentrated on the seed proteins. Osborne (1912) points out that Becarri described the isolation of "gluten" from wheat in 1747. Protein chemistry developed slowly during the 18th and 19th centuries. The research was designed to determine the multiplicity of proteins, the relationship between plant and animal proteins, and their chemical composition. Only during the last half of the 19th century was it clearly demonstrated that amino acids are components of the protein molecule. Seed protein chemistry of the early 20th century was dominated by T.B. Osborne. His review of the field (Osborne, 1924) is still considered a classic. Osborne felt that, except in closely related species, no two species contained proteins which cannot be readily distinguished chemically. He contributed greatly to the modern methodology for separating proteins.

A revolution in protein chemistry was started by James B. Sumner when he reported in 1926 "...I discovered on the 29th of April a means of obtaining from the jack bean a new protein which crystallizes beautifully and whose solutions possess to an extraordinary degree the ability to decompose urea into ammonium carbonate." This simple statement about jack bean urease implied that enzymes are proteins. Thenceforth, the research interests of protein chemists were largely transferred to these biocatalysts, and the seed proteins received a much smaller following than before. As a result of the shift of interest, the biochemistry of the seed proteins lags behind that for many other proteins. For example, so far as we know, the amino acid sequence cannot be written for a single major seed protein. The chemistry of seed proteins has advanced, however, as the general field of protein chemistry advanced (See Altschul et al., 1966).

ORIGIN OF ALEURIN DEPOSITS

DURING the last half of the 19th century, biologists looked at the seed proteins in another way. Hartig (1855) isolated proteinaceous granules from seeds by a nonaqueous technique. He later named the granules "aleuron grains" because the isolated fat-free material reminded him of flour. Pfeffer, in 1872, noted that dormant seeds of many species contain numerous intracellular protein granules. He preferred the term "protein granules" to "aleurone grains." This bias persists to this day among many botanists, who prefer the term "protein body" to "aleurone grain." Pfeffer (1872) observed that aleurone grains contain metal (magnesium) and phosphorous compounds in the form of spherical inclusions called "globoids." More recently Dieckert et al. (1962) and Sharma and Dieckert (1974) isolated aleurone grains and globoids from peanut seeds by a nonaqueous technique and demonstrated that essentially all of the phytic acid and a large fraction of the cellular potassium, magnesium, manganese and copper is located in the aleurone grains along with much of the protein of the seed. Lui and Altschul (1967) and Lott (1975) showed a similar composition for globoids from cotton and cucurbita, respectively. Clearly, aleurone grains are not simple protein bodies.

Early it was recognized that aleurone grains develop in vacuoles (see Guilliermond, 1941, for a review). This view is strengthened by the observation that mature aleurone grains of cotton (Engleman, 1966; Yatsu, 1965), shepherd's purse (Dieckert and Dieckert, 1972), and peanut (Dieckert and Dieckert, 1972) are enclosed in a characteristic single unit membrane similar to the tonoplast. A logical next step was to classify seed proteins on the basis of intracellular location. To this end Altschul et al. (1964) assigned the term "aleurin" as a class name for the vacuolar proteins of seeds. The following seed proteins are examples: arachin from peanuts (Altschul et al., 1964; Daussant et al., 1969); edestin from hemp seed (St. Angelo et al., 1968); vicilin and legumin from pea seeds (Varner and Schidlovsky, 1963); vicilin and legumin from *Vicia faba* (Graham and Gunning, 1970); and the 7s and 11s globulins in soybeans (Koshiyama, 1972).

Protein granules are also known to develop in vacuole-like structures in animal cells. A particularly well-studied case in animals is the formation of zymogen granules by the acinar cells of the pancreas (see Dieckert, 1971, for a review). In 1959, Palade pointed out that modulations in cell structure reflect changes in the biochemistry and physiology of the cell. He outlined the following hypothesis of zymogen granule

formation: the zymogens are synthesized by ribosomes attached to the cytoplasmic side of the endoplasmic reticulum (ER); the protein is then transported by way of the cavities of the ER to the Golgi bodies where the protein is concentrated and perhaps modified; the membrane bound protein droplets at the periphery of the Golgi body pinch off and coalesce with other such bodies to form the typical zymogen granule of the acinar cell; in response to certain stimuli the content of the zymogen granule is emptied into the acinus of the gland after the limiting membrane of the zymogen granule fuses with the plasma membrane interfacing with the acinus. Evidence cited in the present paper and elsewhere (Dieckert, 1969; 1971; Dieckert and Dieckert, 1972; 1975) suggests that a similar process leads to the synthesis and sequestration of the aleurins in the aleurone vacuoles of developing seeds. The classical aleurone grains then form upon the desiccation of the mature seed. In seeds that do not become desiccated at maturity, such as the coconut, the aleurins remain in a watery vacuole (Dieckert and Dieckert, 1975). The key to the problem is not the shape or consistency of the deposit of reserve protein in the mature seed but the process by which it is formed.

Some of the cytomorphological consequences of the hypothesis are shown in Figure 1. Cells of the embryo that are producing aleurins should show a highly developed rough endoplasmic reticulum (RER). Protein may or may not be found in the cisternae of the RER. Sacules of the dictyosomes (the plant homologues of the Golgi bodies of animal cells) should contain protein deposits. Vacuoles limited by a single unit membrane should be present and contain protein. Cells of the embryo that are not making aleurins should show the absence of this apparatus. The other usual cell organelles such as mitochondria, plastids, nuclei, etc. may be present in both cell types. Under this model the mature aleurone grain develops after the aleurone vacuoles are filled and condenses on desiccation of the seed. In seeds that do not become desiccated at maturity, watery aleurone vacuoles are expected. The shape of the aleurone vacuoles and the form of the protein deposits at seed maturity may be variable.

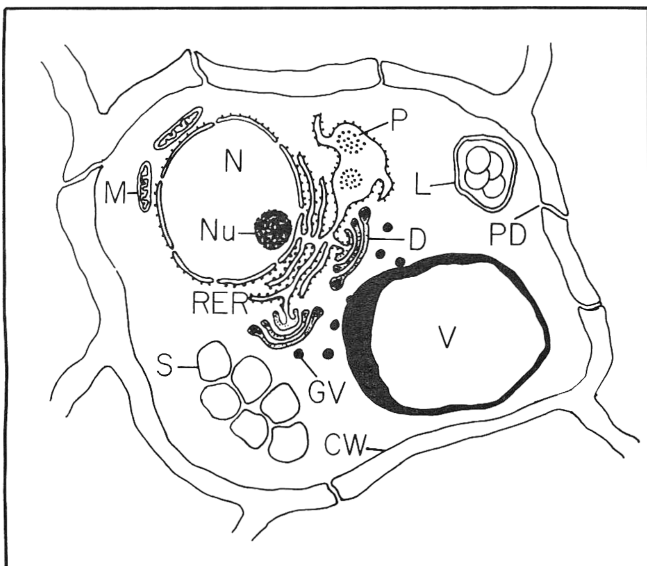

Fig. 1—Diagram of a plant cell. This plant cell is pictured as actively synthesizing, packaging and depositing protein in the vacuole (V) which will mature into an aleurone grain. The relationship between the dictyosomes (D) and rough endoplasmic reticulum (RER) is indicated, as the Golgi vesicles (GV) are formed and released into the cytoplasm. A coplanar diagram of the rough endoplasmic reticulum illustrates polyribosomes (P) in their spiral array. The cell is enclosed in a cell wall (CW) complete with plasmodesmata (PD) and contains the characteristic subcellular organelles such as mitochondria (M), spherosomes (S), leucoplasts (L) and the nucleus (N) and nucleolus (Nu).

The results of our early work with shepherd's purse were reported first in 1969 (Dieckert, 1969) and in more detail later (Dieckert and Dieckert, 1972). Briefly, the mature seed contains classical aleurone grains, each limited by a single unit membrane. In the early stages of development the expected apparatus is missing from the parenchymal cells of the cotyledons, and no protein deposits are observed in the vacuoles or dictyosomes. Later stages, when the mature seed is producing aleurins, show an abundant RER, dictyosomes with protein droplets, free-floating, small, membrane bound protein droplets in the cytoplasm, and small membrane bound protein droplets fused to large aleurone vacuoles partially filled with protein. Occasionally, protein deposits are observed in sacules of the RER.

The model for the synthesis of the aleurins demands polyribosomes arranged in spirals on the outer surface of the RER (Fig. 1). Cross sections of the RER show ribosomes attached to the outer surface of the ER but do not show the arrangement of the ribosomes on the mRNA to best advantage. A tangential section in the proper plane will show the characteristic spiral arrangement of the polyribosomes attached to the outer surface of the RER, as demonstrated for the cells of a developing peanut embryo producing arachin (Fig. 2a). A typical view of the polyribosomes of the RER in cross section is given in Figure 2b.

The same basic ultrastructural pattern was observed for peanut embryos as for shepherd's purse embryos producing aleurins. Unlike in shepherd's purse, the cisternae of the RER contained protein droplets (See Fig. 5.16 in Dieckert and Dieckert, 1972). In Figure 3a a dictyosome with protein droplets is shown. At higher magnification (Fig. 3b) protein droplets in various stages of being transferred to the aleurone vacuole can be seen. That the peanut embryos were actually producing arachin was verified by immunochemical experiments. The results of double diffusion tests (Fig. 5.2 in Dieckert and Dieckert, 1972) showed that arachin is present in peanut embryos that exhibit the highly developed RER-dictyosome-aleurone vacuole system and absent in those that do not.

The results with shepherd's purse and peanut embryos suggest that aleurone grains form the same way in at least two families of seed plants, the Cruciferae and the Leguminosae. Cotton embryos were examined as representative of the Malvaceae. The same pattern was observed: embryos of the barely rolled stage did not exhibit a highly developed RER, dictyosomes with protein content, nor protein-containing aleurone vacuoles (Fig. 5.8a in Dieckert and Dieckert, 1972). By the loose scroll stage a transformation occurred: highly developed RER, dictyosomes containing characteristic protein droplets, and aleurone vacuoles were present, and the vacuoles were filling with protein (See Fig. 5.8b and 5.9 in Dieckert and Dieckert, 1972). The electron stains normally used in biological electron microscopy are not specific for protein. Thus the question arises: what evidence is there that the electron dense material in the sacules and dilations of the dictyosomes, small protein droplets of the cytoplasm and the aleurone vacuoles is truly protein? To answer this question experiments were carried out with plant embryos actively sequestering aleurins. Glutaraledhyde fixed cotton embryos were used, as well as peanut embryos fixed in glutaraldehyde and post fixed in osmium tetroxide. Alternate serial sections of epoxy embedded tissue were digested with pronase, a mixture of proteolytic enzymes, and then stained as usual. The alternate sections were left untreated with pronase and simply stained. Each section was mounted separately for electron microscopy. Results of these experiments showed that cottonseed aleurins are

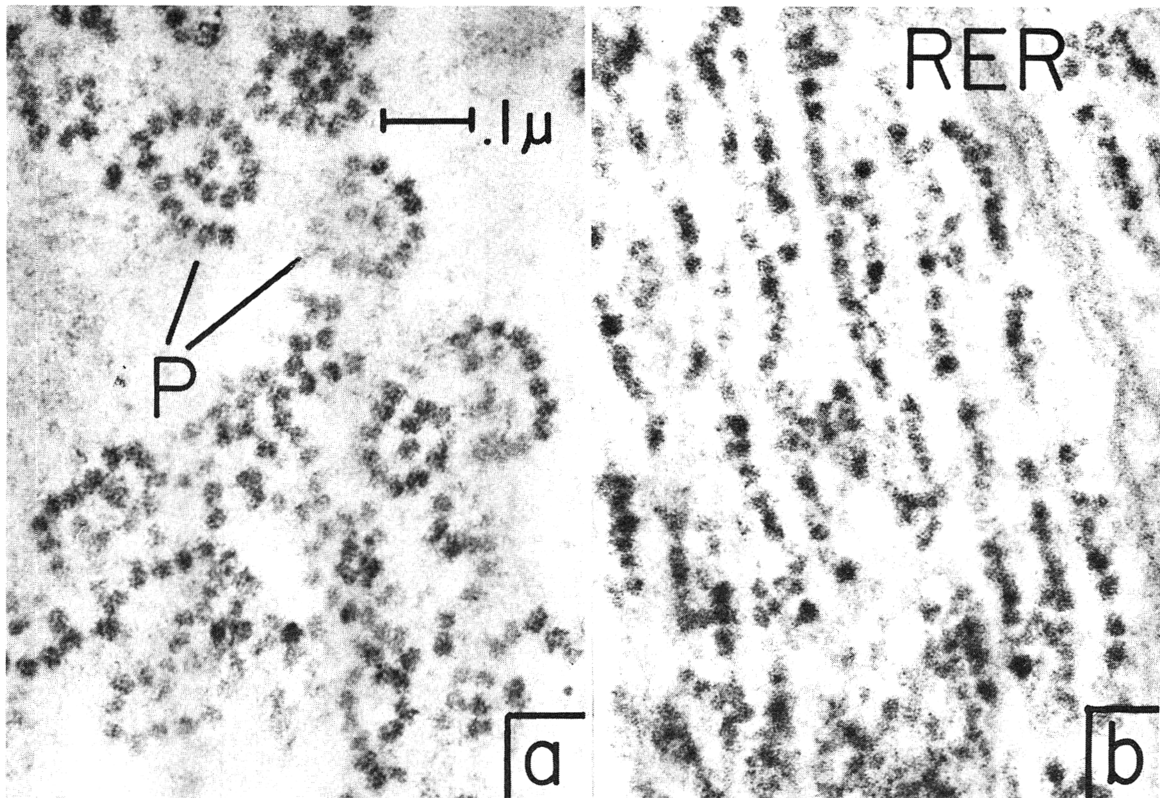

Fig. 2—Peanut embryo. (a) Section coplanar with the membrane of the rough endoplasmic reticulum showing the polyribosomes (P) in spiral array (X 119,000). (b) Cross section of the rough endoplasmic reticulum (RER) showing ribosomes attached to the outer cytoplasmic side of the flattened membraneous sacs of the ER system (X 119,000).

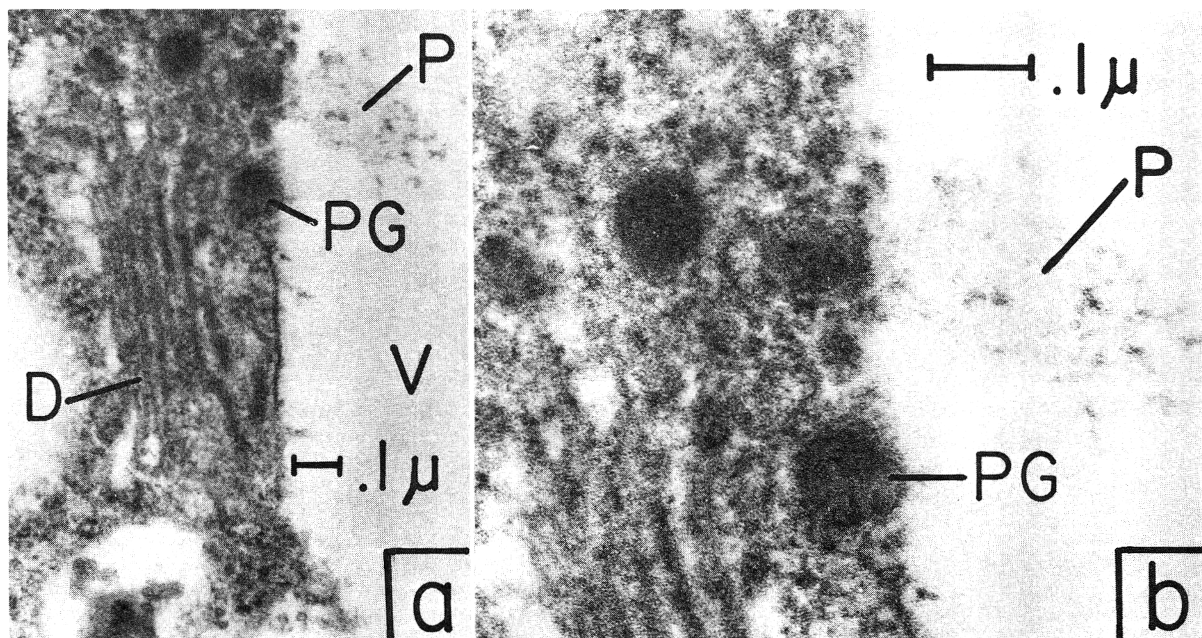

Fig. 3—Peanut embryo. (a) Cross section of a dictyosome (D) with characteristic protein granules (PG) (X 66,000). (b) At higher magnification the protein granules (PG) are seen in various stages of the transfer and release of protein (P) into the vacuole (V) (X133,000).

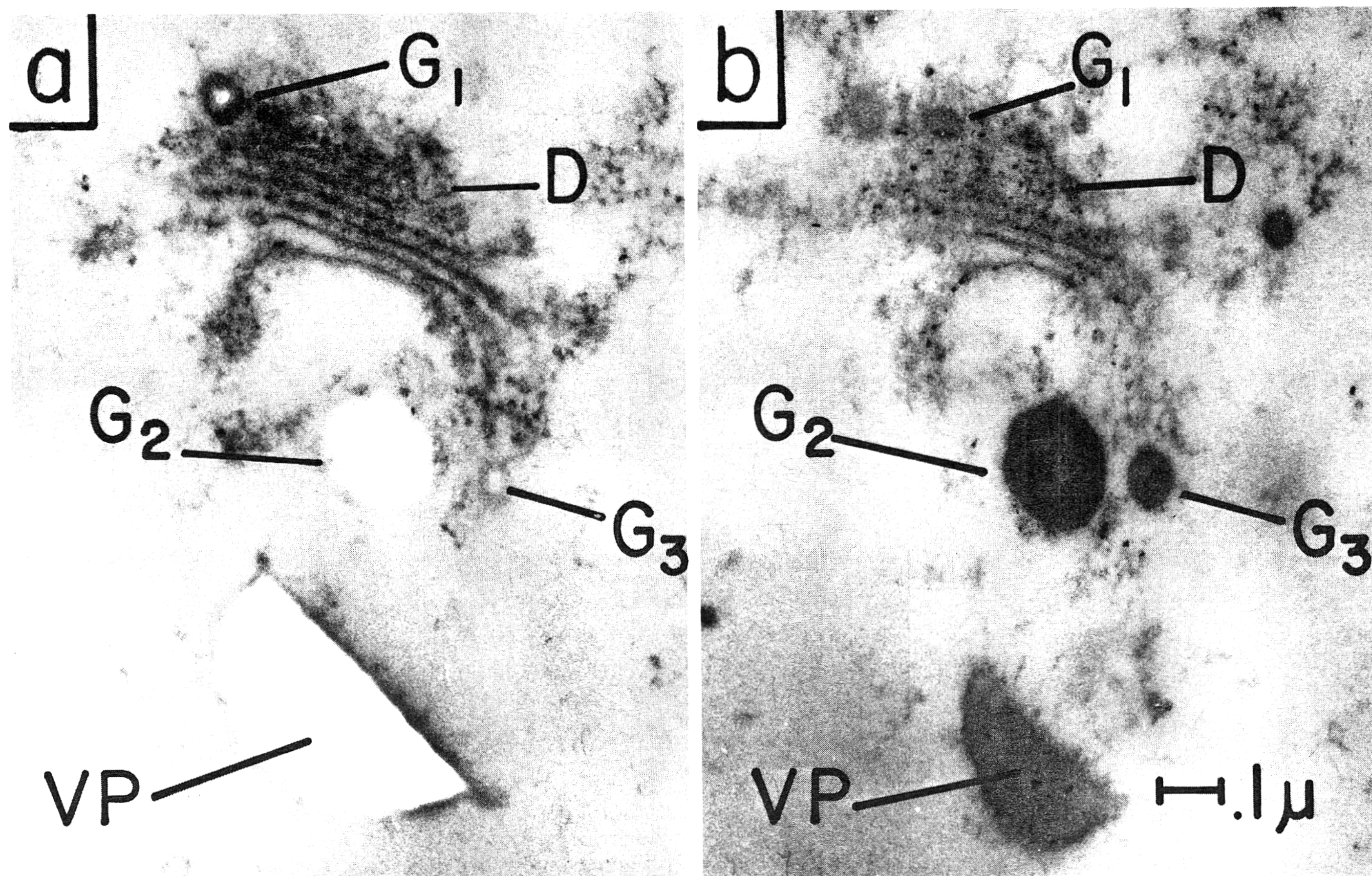

Fig. 4–Effect of pronase on cotton embryo cells at the loose scroll stage. Serial sections. (a) Pronase-treated section. Staining is abolished for dense granules (G_1, G_2, G_3), the dictyosome (D), and the vacuolar protein (VP) (X 82,500). (b) Untreated section of a cell containing an electron dense vacuolar protein deposit (VP), a dictyosome (D) and cytoplasmic dense granules (G_1, G_2, G_3) (X 82,500).

readily digested by pronase treatment. Regions of the section with aleurins are removed by the enzyme to leave electron transparent areas. The results (Figures 4a and 4b) clearly indicate that the dictyosomal cisternae and vesicles contain protein, as do the small cytoplasmic vesicles and large aleurone vacuoles. Experiments of the same type are depicted elsewhere (See Figs. 5.10, 5.11, 5.12 in Dieckert and Dieckert, 1972). A similar result was obtained with peanuts and coconuts (Dieckert and Dieckert, 1975). In coconut the aleurins form crystals. The digestion experiments leave a "cut-out" exactly omitting the digested crystal.

The above results provide good circumstantial evidence for the truth of the proposed model for aleurin synthesis and sequestration. However, important gaps exist in the data. Apparently, protein is located in the regions of the cell indicated, but what assurance is there that in each region the protein is a major aleurin? If the answer to the question is positive, then does each aleurin molecule of a major kind have to pass through the dictyosome on its way to the aleurone vacuole? There isn't much doubt that the proteins in the large aleurone vacuoles are indeed the major aleurins, but no definitive answer is yet available for the other regions of the cell. The important question of the direction of flow of protein in the system also remains to be determined.

SUSPECTED HOMOLOGIES

ONE OF THE MOST impressive features of an electron micrograph of a cell of a mature, dry seed is the reduced volume of cytoplasm and expanded volume of vacuolar proteins and other ergastic materials. For examples, see Figure 1 in Yatsu (1965) (cotton); Figure 5.6 in Dieckert and Dieckert (1972) (shepherd's purse); and Figure 13b in Dieckert and Dieckert (1975) (coconut). Typical cell organelles such as RER, dictyosomes, mitochondria and plastids are scarce in such a cell. The implication is that the protein of the cell organelles and cytoplasmic sap of the maturing tissue is absent or much reduced in quantity in the mature seed. Therefore, the population of proteins must change during seed maturation from one dominated by enzymatic and structural proteins of diverse kinds to one dominated by a few aleurins. Other less abundant proteins such as proteolytic enzymes and their inhibitors (Ryan, 1973) and the lectins (Sharon and Lis, 1972) may also be present in the aleurone vacuoles.

The aleurins can be looked at as products of gene action and genetic relationships between them sought. Neurath et al. (1967) use the term "analogous" to denote similarities in the function of proteins. Nolan and Margoliash (1968) go a step further and say that analogous proteins have similar functions but have different ancestral genes. Homologous proteins arise from genes evolving from a common ancestral gene (Nolan and Margoliash, 1968). These considerations, and the belief that evolutionary change is based on changes in the primary structure of the genes, led Neurath et al. (1967), Smith (1970), Nolan and Margoliash (1968) and others to compare amino acid sequences of functionally similar proteins in order to

study the genetic relatedness of proteins and the phylogenetic relationships between organisms. It is premature to bring these tools directly into the study of the reserve aleurins, since their amino acid sequences are unknown. However, some insight into the genetic relatedness of the principal aleurins can be obtained by comparing the amino acid compositions of highly purified subunits of the principal aleurins using the difference index of Metzger et al. (1968). Much of the available data on the chemistry of the principal aleurins can be rationalized on the assumption that the problem of nitrogen storage in the seed was solved in the evolutionary sense in only a few competitively effective ways, and that the solution was fixed in the germ plasm early in the evolution of seed plants. In terms of homology the hypothesis is made that the principal aleurins of a given species and of different species are the products of, at most, a few ancestral genes.

The best current indication of homology between proteins is a similarity in amino acid sequence (Nolan and Margoliash, 1968; Smith, 1970; Neurath et al., 1967). However, there are a variety of less satisfactory criteria for homology between proteins. Homologous proteins often yield similar peptide maps, are immunologically cross reactive, have similar amino acid compositions (Neurath et al., 1967), and exhibit similar functions. Peptide maps for the principal aleurins are scarce. Jackson et al. (1969) have provided tryptic maps for some of the aleurins of beans and peas. Unfortunately, the samples used contained more than one polypeptide and appear to be impure, leading to complex and confusing maps. Tryptic digestion was used to prepare the maps. Since arginine and lysine occur frequently in the legume aleurins, the number of peptides generated by the action of trypsin is large, adding further complexities to the interpretation of the tryptic maps. Possible intraspecific homologies were clouded by impure preparations. Comparative serological work on well-characterized seed proteins is also rare. Jensen (1973) reviewed the field for plant proteins in general, pointing out that the serological data on seed proteins is limited largely to phaseolin, legumin and vicilin. A more severe limitation of this approach to the determination of the relatedness of proteins may rest in the variable nature of the major aleurins. Antigenic cross reactivity is often lost early in the divergent evolution of proteins (London and Kline, 1973; Gasser and Gasser, 1971). For this reason, variable homologous proteins may not show serological cross reactivity even in species that are closely related phylogenetically.

The most extensive data presently available for comparing the principal aleurins are concerned with amino acid composition. To meet the problem of comparing the amino acid composition of two proteins we adopted the difference index (D.I.) of Metzger et al. (1968). Metzger noted that functionally unrelated proteins usually give D.I.'s of 15–40 and that homologous proteins often have low values for the difference index. The difference index is subject to misinterpretation. Porcine insulin:porcine proinsulin are clearly homologous proteins but have a D.I. of 19.3. The reason for the high value is that a section of the proinsulin polypeptide chain is excised to produce the insulin molecule, changing the amino acid composition drastically. Hen egg lysozyme and bovine lactalbumin are homologous proteins by comparison of their amino acid sequences but exhibit a value of 22.6 for the difference index. Divergent evolution to a new function seems to be the reason for the high D.I. The difference index for the corrected values of the amino acid composition for two separate determinations (four hydrolysis times each) for P_{67}, the principal covalently linked subunit of arachin, was 1.03. This provides some insight into the inherent variation to be expected between analyses of the same protein and a further source of confusion in the use of the difference index for discovering potential homologies.

A comparison of the amino acid composition of three batches of legumin from *V. faba* (Table 1) illustrates another problem associated with the use of the difference index in the search for homology between seed proteins. The difference indices show that the three preparations of legumin are not identical. Subunit analyses of the 1969 and 1974 isolates showed that the 1969 preparation had three subunits of different molecular weight, and the 1974 preparation had two subunits of different molecular weight. Wright and Boulter (1974) assigned the difference in subunit composition to a failure of the SDS gel electrophoresis method used in the earlier work. The large value of the difference index suggests otherwise. The often heard complaint that "the seed proteins are difficult to prepare in a pure state" seems correct in this case. Similar analyses of arachin preparations from various laboratories and within a given laboratory also show relatively large values of the difference index, even when data are obtained by equivalent methods. As in the case of legumin, it appears that arachin is difficult to purify.

Table 1—Comparison of the amino acid composition of "legumin(s)" from Vicia faba

System	D.I.
Legumin (1969)[a] : Legumin (1970)[b]	3.69
Legumin (1969)[a] : Legumin (1974)[c]	5.36
Legumin (1970)[b] : Legumin (1974)[c]	7.09

[a] Raw data from Jackson et al. (1969)
[b] Raw data from Bailey and Boulter (1970)
[c] Raw data from Wright and Boulter (1974)

Data of good quality are available from several sources on the amino acid composition of the subunits of individual aleurins. Recently, Weintraub and Tuen (1971) and Wright and Boulter (1974) isolated the reduced subunits of legumin from *V. sativa* and *V. faba* and determined their amino acid composition. Wright and Boulter (1974) did not report values for 1/2 cystine or tryptophan, as did Weintraub and Tuen (1971). Both groups reported values of the amino acids corrected for destruction and incomplete hydrolysis. Similar data are available from our studies for the subunits of aleurins from peanuts, coconut endosperm and cottonseed. The pairwise comparisons by difference index of the reduced subunits of aleurins from several sources are given in Table 2. The molecular weight assigned to each polypeptide is given in parentheses.

The pairwise comparison of the chains of legumin from *V. faba* and *V. sativa* with the difference index shows probable homologies (Table 2). The difference indices for β:A, α:B and α:C are small and suggest that these pairs are homologous polypeptides. The large values of the difference index for the other pairs from the same genus suggest that they are not homologous. On other grounds there seems little doubt that the legumins of *V. faba* and *V. sativa* are homologous proteins (Jackson et al., 1969). The important point here is that if reliable amino acid compositions are available for pure polypeptides, potential homologies between seed proteins are revealed by application of the Metzger difference index.

A comparison of the amino acid composition of the reduced subunits of other suspected legumin homologues (Table 2) shows two series that seem to be related. The first series consists of A (*V. sativa*), β (*V. faba*), P_{24} (*A. hypogaea*) and P_{20} (*C. nucifera*); and, the second series consists of B (*V. sativa*), C (*V. sativa*), α (*V. faba*), P_{43} (*A. hypogaea*) and P_{31} (*C. nucifera*). Of all sixteen possible pairings within each series only one (P_{43}:C) gives a difference index greater than 15 (D.I. = 15.54). The low values of the difference index suggest homology. Of all 20 possible pairings of one member from each of the two series only three exhibit a difference index less

than 15 ($P:P_{24}$, 13.81; $B:P_{20}$, 14.55; and, $P_{31}:P_{20}$, 10.27). The high values of the difference index suggest lack of homology. In the A series the order of increasing difference index is A (*V. sativa*):β (*V. faba*); A (*V. sativa*):P_{24} (*A. hypogaea*); β (*V. faba*):P_{24} (*A. hypogaea*); A (*V. sativa*):P_{20} (*C. nucifera*); β (*V. faba*):P_{20} (*C. nucifera*); and, P_{24} (*A. hypogaea*):P_{20} (*C. nucifera*). This series is the order of decreasing phylogenetic relatedness of the species. The members of the second series are more variable. Intrageneric but not intergeneric relationships are correctly reflected by the series of increasing difference indices. It should be noted that the molecular weights of the subunits vary more in the second series than in the first one. The rough parallel between the magnitude of the difference index and the phylogenetic relatedness of the species is expected for homologous proteins.

A MODEL FOR THE LEGUMIN HOMOLOGUES

IT IS GENERALLY RECOGNIZED that legumin in a "native" state consists of multiples of several different subunits.

Table 2—Comparison of the amino acid composition of the subunits of the disulfide bridged pairs of suspected legumin homologues

System		D.I.
A (24,300)	*V. sativa* : B (37,600) *V. sativa*[a]	16.72
A (24,300)	: C (32,000) *V. sativa*	21.63
A (24,300)	: β (23,800) *V. faba*[b]	3.70
A (24,300)	: α (37,000) *V. faba*	18.62
A (24,300)	: P_{24} (23,600) *A. hypogaea*[c]	11.12
A (24,300)	: P_{43} (43,100) *A. hypogaea*	24.74
A (24,300)	: P_{20} (20,300) *C. nucifera*[d]	13.37
A (24,300)	: P_{31} (30,900) *C. nucifera*	17.86
B (37,600)	*V. sativa* : C (32,000) *V. sativa*[a]	11.73
B (37,600)	: α (37,000) *V. faba*[b]	5.78
B (37,600)	: β (23,800) *V. faba*	19.10
B (37,600)	: P_{43} (43,100) *A. hypogaea*[c]	11.89
B (37,600)	: P_{24} (23,600) *A. hypogaea*	13.81
B (37,600)	: P_{31} (30,900) *C. nucifera*[d]	9.84
B (37,600)	: P_{20} (20,300) *C. nucifera*	14.55
C (32,000)	*V. sativa*[a]: α (37,000) *V. faba*[b]	7.63
C (32,000)	: β (23,800) *V. faba*	22.50
C (32,000)	: P_{43} (43,100) *A. hypogaea*[c]	15.54
C (32,000)	: P_{24} (23,600) *A. hypogaea*	18.87
C (32,000)	: P_{31} (30,900) *C. nucifera*[d]	13.22
C (32,000)	: P_{20} (20,300) *C. nucifera*	18.24
β (23,800)	*V. faba* : α (37,000) *V. faba*[b]	20.80
β (23,800)	: P_{24} (23,600) *A. hypogaea*[c]	12.70
β (23,800)	: P_{43} (43,100) *A. hypogaea*	25.33
β (23,800)	: P_{20} (20,300) *C. nucifera*[d]	13.41
β (23,800)	: P_{31} (30,900) *C. nucifera*	18.04
α (37,000)	*V. faba*[b] : P_{43} (43,100) *A. hypogaea*[c]	10.84
α (37,000)	: P_{24} (23,600) *A. hypogaea*	16.26
α (37,000)	: P_{31} (30,900) *C. nucifera*[d]	10.99
α (37,000)	: P_{20} (20,300) *C. nucifera*	15.90
P_{24} (23,600)	*A. hypogaea* : P_{43} (43,100) *A. hypogaea*[c]	20.85
P_{24} (23,600)	: P_{20} (20,300) *C. nucifera*[d]	13.67
P_{24} (23,600)	: P_{31} (30,900) *C. nucifera*	15.97
P_{43} (43,100)	*A. hypogaea*[c] : P_{31} (30,900) *C. nucifera*[d]	12.40
P_{43} (43,100)	: P_{20} (20,300) *C. nucifera*	21.05
P_{20} (20,300)	*C. nucifera* : P_{31} (30,900) *C. nucifera*[d]	10.27

[a] Raw data from Weintraub and Tuen (1971)
[b] Raw data from Wright and Boulter (1974)
[c] Raw data from Yu and Dieckert (Unpublished)
[d] Raw data from Wallace and Dieckert (Unpublished)

Weintraub and Tuen (1971) suggest that three different subunits (A, B and C) compose legumin from *V. sativa*, giving a stoichiometry of $A_6B_4C_2$. Wright and Boulter (1974) find two different subunits (α, β) for legumin from *V. faba* and propose the stoichiometry of $\alpha_6\beta_6$ for the native form. Arachin is also probably a homologue of legumin. The principal form of arachin seems to contain two subunits, P_{24} and P_{43} (Singh and Dieckert, 1973b). The stoichiometry of an arachin of this type is probably $[P_{24}]_6[P_{43}]_6$.

A structure of the type A-S-S-B is proposed for the legumin homologues. Usually six of these disulfide bridged subunits are held together by noncovalent forces in the so-called native protein. In support of the model are the following data: Singh and Dieckert (1973b) showed that a reducing agent is required to split P_{24} and P_{43} of arachin. More recently Yu and Dieckert (1975) found that the nonreduced but SDS-denatured principal subunit of arachin has a molecular weight equal to the sum of the molecular weights of the two different subunits—66,700. Disulfide interchange reactions were ruled out. A re-examination of the elegant experiments of Tombs and Lowe (1967) based on osmometry and the denaturants urea and guanidine · HCl is particularly illuminating. In 4M guanidine · HCl the number average molecular weight was found to be 66,840 ± 5000. In the presence of 4M guanidine · HCl and 0.1M sulfite the number average molecular weight dropped to 28,700 ± 400. These values compare favorably with our estimates of 66,700 for the disulfide bridged dimer and 33,300 for the average weight of the two monomers (see Singh and Dieckert, 1973a, for the data on the monomers). Tombs and Lowe (1967) concluded that "the intact molecule does not contain interchain disulfide bonds, but that there are interchain disulfide links in 8M urea is evident...." Tombs repeats the assertion in a later paper (Tombs, 1970). Recently Wright and Boulter (1974) suggested that the α and β subunits of legumin from *V. faba* are disulfide bridged. Their conclusions, as ours, are based on experiments with SDS gel electrophoresis with and without a reducing agent.

If the A-S-S-B model is correct, a question arises as to how the two subunits are joined "correctly." Otherwise, why are subunits of the type A-S-S-A and B-S-S-B not found? It is tempting to speculate that a post translational modification similar to the one for insulin synthesis is operative. If the principal aleurins are synthesized and processed as we suggest, a proinsulin-insulin type conversion might take place in the dictyosomes. At present there is no substantive evidence for or against this idea.

ADDITIONAL SUSPECTED HOMOLOGIES

UNDER THE PROPOSED model for the legumin homologues the legumin of *V. sativa* prepared by Weintraub and Tuen (1971) is probably a mixture of hybrids of the two subunits, A-S-S-B and A-S-S-C, in the overall molar ratio of 2 to 1. The legumin of *V. faba* prepared by Wright and Boulter (1974) is, as they indicate, $(\alpha\text{-S-S-}\beta)_6$, and the major form of arachin is $(P_{24}\text{-S-S-}P_{43})_6$. The model can be extended to glycinin of *Glycine max* based on the data of Catsimpoolas et al. (1971). No data was found to test the disulfide bridging concept for glycinin, however.

If the "legumin-type" proteins are multimers of a disulfide bridged pair, A-S-S-B, and if the amino acid composition and molecular weights are known for the subunits A and B, then the amino acid composition of the multimer can be calculated with the following formula:

$$AB_i = \frac{N_A}{N_A + N_B} A_i + \frac{N_B}{N_A + N_B} B_i$$

where AB_i is the mole % of the ith amino acid in A-S-S-B; A_i

and B_i are the observed mole % of the ith amino acid in A and B, respectively; and N_A and N_B are the numbers of amino acid residues in A and B, respectively. It is assumed that each subunit contains only amino acid residues.

The necessary data are available for the subunits of legumin of *V. sativa* (Weintraub and Tuen, 1971) and *V. faba* (Wright and Boulter, 1974). Similar data are available for the principal disulfide bridged subunits of arachin, P_{67} (Yu and Dieckert, 1975) and cocosin, P_{51} (Wallace and Dieckert, 1975). The subunit weights are available for glycinin (Catsimpoolas et al., 1971), but the data on the amino acid composition of the subunits are not satisfactory. Their analysis of the reduced and SDS-denatured glycinin shows two major molecular weight classes in what appears to be equimolar quantities. For the purposes of this paper we assume that the amino acid composition for the parent glycinin is representative of that of the proposed disulfide bridged monomer (MW 59,500) for glycinin. In building Table 3 amino acid composition of the structure shown was used if the data were available. Otherwise, the reconstructed values were used. The comparison of the amino acid compositions of covalently linked nonreduced subunits given in Table 3 includes two species from the same genus, three genera from the same family, and three families. All pairs of disulfide bridged subunits are compared. The difference indices are relatively small, suggesting that the subunits are homologous.

One might expect homologous functionally similar proteins from members of the same taxon to be more alike compositionally than those from different taxa. At the species level the *Vicia* legumins AB, AC and αB are compositionally more alike than are *Vicia* legumins and the suspected homologues in soybeans (glycinin), peanuts (P_{67}, arachin), coconut (P_{51}, cocosin) and cotton (P_{40}). Also, a pairwise comparison of representatives of any other two genera gives larger values of the

Table 3—Comparison of the amino acid composition of the disulfide bridged subunits of suspected homologues of legumin

System		D.I.
AB (61,900)	*V. sativa* : AC (56,300) *V. sativa*[a]	6.71
AB (61,900)	: αβ (58,600) *V. faba*[b]	4.58
AB (61,900)	: Glycine (59,500) *G. max*[c]	9.04
AB (61,900)	: P_{67} (66,700) *A. hypogaea*[d]	9.16
AB (61,900)	: P_{40} (39,800) *G. hirsutum*[e]	8.80
AB (61,900)	: P_{51} (51,100) *C. nucifera*[e]	10.03
AC (56,300)	*V. sativa*[a] : αβ (58,600) *V. faba*[b]	4.33
AC (56,300)	: Glycine (59,500) *G. max*[c]	6.86
AC (56,300)	: P_{67} (66,700) *A. hypogaea*[d]	11.22
AC (56,300)	: P_{40} (39,800) *G. hirsutum*[e]	9.38
AC (56,300)	: P_{51} (51,100) *C. nucifera*[e]	10.05
αβ (58,600)	*V. faba*[b] : Glycine (59,500) *G. max*[c]	7.57
αβ (58,600)	: P_{67} (66,700) *A. hypogaea*[d]	8.29
αβ (58,600)	: P_{40} (39,800) *G. hirsutum*[e]	10.27
αβ (58,600)	: P_{51} (51,500) *C. nucifera*[e]	9.04
Glycine (59,500)	*G. max*[c] : P_{67} (66,700) *A. hypogaea*[d]	9.47
Glycine (59,500)	: P_{40} (39,800) *G. hirsutum*[e]	8.99
Glycine (59,500)	: P_{51} (51,100) *C. nucifera*[e]	11.76
P_{67} (66,700)	*A. hypogaea*[d] : P_{40} (39,800) *G. hirsutum*[e]	6.45
P_{67} (66,700)	: P_{51} (51,100) *C. nucifera*[e]	12.45
P_{40} (39,800)	*G. hirsutum*[e] : P_{51} (51,100) *C. nucifera*[e]	9.68

[a] Raw data from Weintraub and Tuen (1971)
[b] Raw data from Wright and Boulter (1974)
[c] Raw data from Catsimpoolas et al. (1971)
[d] Raw data from Yu and Dieckert (Unpublished)
[e] Raw data from Wallace and Dieckert (Unpublished)

D.I. than was observed between the two species of *Vicia*. A comparison of suspected legumin homologues from different families gives equivocal results. The difference indices for pairs representing different genera or families cluster in the 8–12 region and show variable correlation with proposed phylogenetic relationships. Assuming the hypothesis of homology is correct, there must be some balance between the forces of divergence and convergence. Post translational modification may also have an effect.

If the A-S-S-B subunit is derived from a single polypeptide as insulin is produced from proinsulin, then each series represents the corresponding subsequence of a parent polypeptide. No solid evidence is available yet to test this hypothesis. If such a mechanism is operative, then under the homology concept one ancestral gene gave rise to the structural genes for the series of parent polypeptides. If such a mechanism is not operative, then apparently two ancestral genes are involved and the problem of how the subunits are paired remains.

There are other reasons for thinking that the legumin-type proteins discussed in this paper are homologous. Some of these are presented in an earlier paper (Dieckert and Dieckert, 1975). Except for cocosin they all seem to be hexamers of a disulfide bridged subunit. The molecular weights of the hexamers are similar. The estimated molecular weights are the following: legumin (*V. faba*), 320,000 (Bailey and Boulter, 1970); legumin (*V. sativa*), 360,000 (Weintraub and Tuen, 1971); glycinin (*G. max*), 363,000 (Wolf and Briggs, 1959); arachin (*A. hypogaea*), 330,000 (Johnson and Shooter, 1950). As pointed out by Dieckert and Dieckert (1975), cocosin and edestin also are probably homologues of legumin. These two proteins seem to be tetramers of a disulfide bridged subunit. The molecular weights of the native proteins are: cocosin, 208,000 (Sjogren and Spychalaski, 1930); and edestin, 212,000 (Svedberg and Stamm, 1929). Finally, from a biologist's point of view, the common function and cytological origins provide an additional argument for homology. What is needed now for a more secure decision is the amino acid sequence for a range of these interesting proteins.

There is evidence for charge density variants within the molecular weight classes of the reduced subunits of glycinin (Catsimpoolas et al., 1971). Thus there appear to be several genes coding for glycinin subunits within the soybean population sampled. The situation in other species is not clear.

RESERVE PROTEINS AS LIBERAL PROTEINS

SINCE the principal aleurins have no known enzymatic activity and disappear completely during germination, they apparently function as a store of organic nitrogen for the seedling. This is an old idea (Pfeffer, 1872) that has never been seriously challenged. Apparently, most seed plants provide a nitrogen reserve in the seed in the form of vacuolar protein deposits. The question naturally arises as to why such a complicated "solution" evolved to meet such a "simple" problem. And, it is fair to ask how many distinct solutions to the nitrogen reserve problem have survived the evolutionary selection process.

According to Dickerson and Geis (1969) each protein appears to have a characteristic period of time for one average acceptable amino acid change to occur per 100 residues. They point out that the reason for the apparent variability in the evolutionary rate of proteins is that the probability of a change in the amino acid sequence being lethal differs from one protein to another. Proteins like cytochrome C have strict structural requirements to function properly and, consequently, show little variation in amino acid sequence in sensitive regions of the proteins in phylogenetically diverse organisms. Proteins with strict requirements for the amino acid sequence are called conservative proteins. Fibrinopeptides, on the other

hand, show higher apparent rates of change in amino acid sequence. The functionality of the fibrinopeptides is, therefore, less sensitive to changes in the amino acid sequence than cytochrome C. Proteins that remain functional while sustaining numerous changes in amino acid sequence may be called "liberal proteins."

The major vacuolar proteins of seeds are likely to be liberal proteins (Dieckert and Dieckert, 1975). This concept seems plausible when hypothetical constraints for a successful reserve aleurin are considered. The principal aleurins appear to be nonenzymatic proteins. Therefore, the structural constraints for enzyme specificity are absent. Since the major aleurins are vacuolar proteins and are probably synthesized by the rough endoplasmic reticulum and packaged and/or modified by the dictyosomes, there may be significant restrictions imposed on their structure by the details of the synthesis and segregation processes. High molecular weight of the associated form of the principal aleurins is almost certainly a structural imperative. It was pointed out by Wetlaufer (1973) that the association of subunits to form multisubunit assemblies in proteins reduces the osmotic pressure a cell must bear, thus preventing the cell from rupturing when the weight concentration of a protein is high. In the maturing seeds we have examined, the aleurins are present in high concentration in the vacuoles; therefore, high particle weight seems like an important requirement. This requirement alone may explain why vacuolar proteins evolved to meet the problem of a nitrogen reserve in seeds. Finally, the principal aleurins should provide an adequate nitrogen source for the seedling. The capacity of plants to interconvert amino acids seems to minimize this source of constraint. In this connection the amino acid composition of the reserve proteins is biased toward a high content of the readily utilizable amino acids–arginine, glutamic acid (glutamine), or aspartic acid (asparagine).

The proposed model for the major aleurins offers a simple and unifying view of these complex, seemingly diverse proteins.

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Ms received 6/16/75; revised 10/9/75; accepted 10/15/75.

This research was supported by the following agencies: Texas Agricultural Experiment Station, Robert A. Welch Foundation Grant No. A-235, Cotton Incorporated Project No. 69-140, Cotton Incorporated Project No. 3156, USDA Grant No. 12-14-7988(34), National Science Foundation Grant No. GB-29 and U.S.A.I.D. Project No. csd-2804.

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APPLICATION OF MICROSCOPIC TECHNIQUES TO THE DESCRIPTION OF STRUCTURE OF DEHYDRATED FOOD SYSTEMS

ABSTRACT

A wide variety of optical microscopic techniques are available for elucidation of the structure of materials. Many can be applied by the food scientist for studying the structure of dehydrated food materials and the influence of the structure on physical properties of the food. Also recent developments in scanning electron microscopes and related instruments permit new approaches to the study of food structure. The application of some of these microscopic techniques for studying the structure of dehydrated food materials and of the relationship of the observed structure on some physical properties of the foods will be described by use of a number of selected examples.

INTRODUCTION

THE OPTICAL MICROSCOPE has for many years been a valuable tool for the description of solid systems in the fields of mineralogy, metallurgy, chemistry, biology and microbiology (Schaeffer, 1966; Bailey, 1967; Hartshorne and Stuart, 1970; Delly, 1973). Today, microscopic techniques are used routinely by analytical microscopists in the identification of almost all dust-size particles regardless of nature and origin. Besides the conventional brightfield microscope, variations like the darkfield, phasecontrast, fluorescence, polarizing and interference microscopes are commonly used for microscopic investigations.

Spiess (1969) used optical brightfield microscopy on freeze-dried food model systems to develop mathematical models describing heat and mass transfer properties of these systems. Darkfield microscopy is a variation in which the image of the sample is presented on a dark background so that small details in colorless samples appear to have greater contrast and their natural colors are enhanced.

The phasecontrast microscope is used to observe very transparent objects that would be nearly invisible in the conventional microscope. Its effect is to enhance the phase differences between light rays going through the object and the surrounding medium.

McGrath et al. (1973) used fluorescence microscopy to measure viability of HeLa cells subjected to various freezing and thawing rates, by observing uptake behavior by the cell of fluorescent dyes.

The polarizing microscope is a major tool in identifying and describing optical properties of crystalline materials (Schaeffer, 1966; Hartshorne and Stuart, 1970).

Boutelje (1972) used interference microscopy to determine changes in refractive index and volume of cellulosic plant cell walls with varying moisture contents.

Luyet (1960) has extended the microscopic technique to the evaluation of crystallization and freeze drying of biological systems. Flink et al. (1973) designed a freeze-drying microscopic stage capable of continuous observations at the high magnifications which were required for flavor retention studies.

Another valuable instrument is the scanning electron microscope (or SEM). Its major advantage lies in its ability to present three-dimensional high resolution views of a material's surface topology. Originally used for the study of conducting materials (metals, minerals, etc.) it is now recognized as a valuable tool for studying nonconductive systems. With nonconducting materials it is, however, necessary to first deposit a thin layer of a conducting material. A review of its application in biological research has been published by Hollenberg and Erickson (1973).

The electron microprobe, which is a technique based primarily upon detection of characteristic X-rays, has been extensively used for analysis of atomic composition of micron size areas of ores, alloys and surface coatings, (Labana and Wheeler, 1974). In recent years, biologists have found the electron microprobe to be useful for studying the distribution of elements in biological tissue on a cellular and subcellular scale (Robison, 1971).

Microscopy of foods and food related products has been conducted for many years, but it is only recently that the value of food microscopy has become more widely recognized. White and Shenton (1974) have recently initiated the publication of an extensive bibliography on food microscopy as an aid to food scientists.

The structure of individual food components, as well as of the finished product, plays an important role in determining appearance, flavor, rheological properties and keeping qualities. To study these interrelationships, the microscope in its various modes is being used to allow visualization of the different heterogeneities in the structure of food systems.

This paper reports on further demonstrations of the value of optical microscopy and scanning electron microscopy for the study of the microstructure of a variety of food systems.

MATERIALS & METHODS

Preparation of samples

Instant coffee powders. Spray-dried and freeze-dried instant coffee powders of commercial origin were used.

Freeze-dried milk powders. Freeze-dried samples of skim and whole milk were prepared. The milk samples were frozen at −20°C in 5 mm layers in petri dishes and freeze dried in a Virtis freeze dryer (Model 10-MRTR).

Freeze-dried oil-in-water emulsions. Freeze-dried oil-in-water emulsion systems were prepared from triolein (5%), water "soluble" solute [maltodextrin, or microcrystalline cellulose (Avicel)] (20%), and emulsifiers at a concentration of 9% of the oil phase (Tween 80 and Span 80 at a 2:1 ratio). The emulsions were prepared by blending the oil and Span 80 together and then adding this mixture to an aqueous solution or dispersion of the solid and Tween 80. The emulsification was carried out by high speed mixing in a Sorvall Omnimixer for 5–10 min.

Avicel dispersions were usually mixed in the Sorvall Omnimixer for 10 min before adding the oil phase to disintegrate clusters of Avicel. After emulsification the emulsions were transferred to trays and freeze dried in layers of about 5 mm.

Freeze-dried sucrose/corn syrup solids mixture. Recrystallization of freeze-dried mixtures of sucrose and corn syrup solids (1:6.3) was studied. The freeze-dried powders were stored at room temperature in desiccators of 0% relative humidity (RH) (over Drierite), and at 43% RH (over saturated K_2CO_3). The samples were removed from the desiccators and recrystallization observed by optical microscopy.

Freeze-dried gels. Observations of structure were made on gels, whose texture simulates fruits (Luh et al., 1976). The ingredients [sodium alginate (2.5%), pectin (2%), gelatin (1.5%), Avicel (0.25%) and sucrose (25%)] were homogenized with water and then refrigerated a few hours to set the gelatin gel. One (1) cm cubes, sliced from the gel, are cross-linked in a calcium lactate solution, osmotically pre-dried in a 50% aqueous sucrose solution for 2 hr and finally freeze dried.

Microscopic methods

Optical microscopy. Optical microscopy was conducted with an Olympus biological microscope, Model EH. While generally designed for transmitted brightfield illumination, by inserting crossed polarizers (and occasionally a quarter wavelength quartz plate) polarizing techniques for analysis of sample anisotropy could be conducted. An incident lighting unit can also be added, if desired.

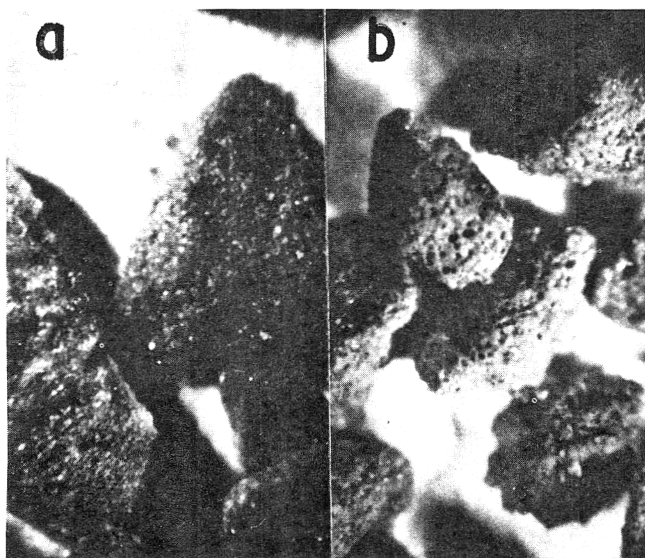

Fig. 1–Stereo microscopic views of freeze-dried coffee (25X): (a) fast frozen; (b) slow frozen.

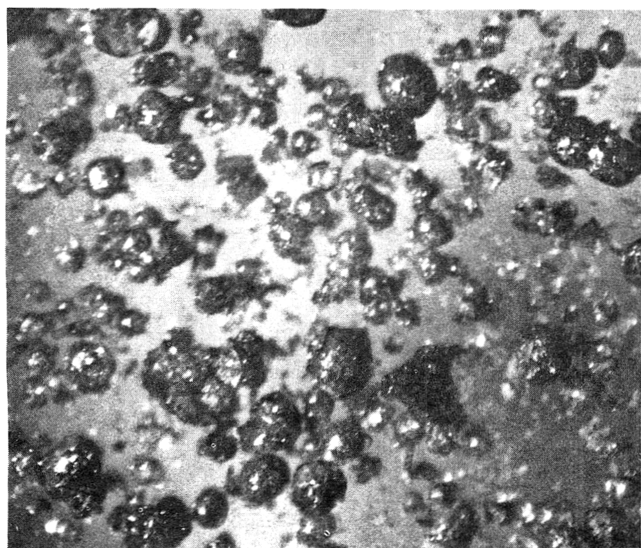

Fig. 2–Stereo microscopic view of spray-dried coffee (25X).

Dried samples were carefully ground and flakes transferred to a microscope slide using the tip of a spatula. The solid was mixed into a drop of immersion oil and a cover slip placed on the sample. In cases where samples were to be rehydrated (e.g., swelling studies of gels) water was used as the immersion medium. For low magnification studies, (i.e., less than 25×) an American Optical Stereomicroscope was used.

Staining to improve lipid phase clarity. In studies on freeze-dried multiphase system, it is generally desirable to improve the contrast between lipid and the other components. This contrast could be increased by incorporation of Fat Red 7B (Sigma Chemical Co.), a lipid soluble dye. The dye is dissolved in a minimal volume of hexanol and then mixed with the lipid prior to preparing the emulsion. The emulsified oil droplets have a distinct red color. Lipid films, however, are too thin to give any coloration.

Surface deposits of fat in the freeze-dried oil-in-water emulsions were visualized by staining with osmic acid. The dried fat-containing powder is placed on a slide inside a desiccator and a sealed ampoule containing a few crystals of osmic acid is broken and placed next to the slide. The desiccator is closed and the free fat turns dark due to reaction of unsaturated bonds with the osmic acid vapors. This method is adopted from Buchheim et al. (1974) who used osmic acid vapor for specific dyeing of surface fat in milk powder particles. The degree of darkening, which can be easily observed with the naked eye, varies from light brown to black, depending on exposure time (10–60 min) and surface oil concentration. This method is very specific for identifying surface deposits and free globules of oil. Inclusions of oil do not react with the osmic acid, due to the impermeable matrix.

Scanning electron microscopy. Small pieces of dry samples were attached with double sided adhesive tape to aluminum specimen holders called "stubs." The samples were then shadowed with a thin layer of aluminum or gold (approximately 300–600 Å) in a Bendix vacuum evaporator (model CVC-14). A JEOL JSM-U3 scanning electron microscope operating at 10–35 kv with an aperture of 200 μ was used. The detector was operated in its normal mode using secondary electrons for image formation.

Electron microprobe. The electron microprobe has been used for detecting surface oil of freeze-dried oil-in-water emulsions. The samples, which have been treated with osmic acid and coated with metal, are observed using the Mα osmium X-ray line which is emitted upon electron bombardment within the SEM column. The accelerating voltage was 15 kv and the beam diameter about 5μ. By scanning the sample surface, osmium rich areas appear as bright areas on a dark background.

Mapping of samples. Sequential studies with the optical and scanning electron microscopes are used in evaluating sample structure. The sample is firmly attached with double-sided adhesive tape to a glass slide. The glass slide is used in the optical microscope or mounted on the specimen stubs of the electron microscopes. Using markings on the slide as a reference point, a particular grain or flake can be easily located and exhibits the same orientation in all instruments.

The dry sample is first scanned in the optical microscope, then metal coated and observed in the scanning electron microscope, followed by the optical microscope and viewed in the dry state or in an immersion medium for more detailed study.

OBSERVATIONS & DISCUSSION

EACH of the various microscopic techniques supplies a particular bit of information about sample structure. In the following examples, using various dehydrated foods and food systems, it is shown how these bits of information complement each other and can be combined to provide a comprehensive view of the microstructure of food materials.

Food systems

Coffee powders can be used to demonstrate the range of information on the microstructure that can be obtained as higher magnifications are used. The naked eye shows only color and gross particle size. The stereo microscope permits the observation of porosity of individual particles, and also the presence of reflecting and glassy surfaces of the coffee solids. Figure 1a shows a freeze-dried coffee powder with particles of small pores and sharp corners and edges indicating the product was fast frozen without melting or collapse during subsequent freeze drying. Figure 1b shows a highly porous product. This could be due to slow freezing which results in larger ice crys-

tals or to gas incorporation in the coffee slurry to yield a porous dry product with lower bulk density. The presence of rounded edges suggests that some melting or collapse has occurred during drying. Spray-dried particles appear as individual spheres with highly reflecting surfaces, most likely resulting from a high air temperature causing the surface to partially melt (Fig. 2). At higher magnification (300–600x), individual flakes of the freeze dried matrix (produced by grinding) are seen to be quite complex, containing pigment bodies and air and liquid inclusions, and having a convoluted surface morphology (Fig. 3a). Figure 3b shows that intact spheres of spray-dried coffee solids envelop air bubbles, while broken pieces from larger spheres show similar characteristics as the freeze-dried coffee, i.e., liquid inclusions and pigment bodies.

Surface morphology is better visualized with the SEM where surface roughness and curvature is easily distinguished due to the larger depth of view. The freeze-dried coffee in Figure 4 shows a network of small, curved flakes. Large pores of 30–50μ diameter (as on right edge) come from primary ice dendrites, while smaller pores of 10μ or less originate from secondary or tertiary ice dendrites. In Figure 5 the intact spherical particle of spray-dried coffee shows folds and depressions on an otherwise smooth surface suggesting that some melting and contraction occurred during the drying step. This reinforces the observation in the stereomicroscope (Fig. 2). The small pieces scattered on the surface are from other broken spheres. In the upper right hand corner of Figure 5 is seen a broken edge showing the thin, dense wall which originally encapsulated an air bubble. Figures 1–5 also demonstrate the difference in microstructure between freeze-dried and spray-dried products, (i.e., parallel to cellularly arranged plates versus hollow spheres).

The examination of freeze-dried powders of whole and skim milk shows the differences in the microstructure which are related to the fat content. Commercial homogenized whole milk which contains about 3% (w/w) fat in the liquid state (about 20% fat in the dried material) shows a very heavy density of oil inclusions in the dried milk solids flakes (Fig. 6a). Skim milk, however, which is less than 0.1% (w/w) fat shows flakes with little or no oil globule inclusions (Fig. 6b). No evaluation of surface fat was made. The grain appearance is very much like the freeze-dried coffee described above. The oil inclusions are spherical and evenly dispersed in the flakes of nonfat milk solids. In the whole milk, droplets sit so close (and even in contact) that they have lost their individual spherical

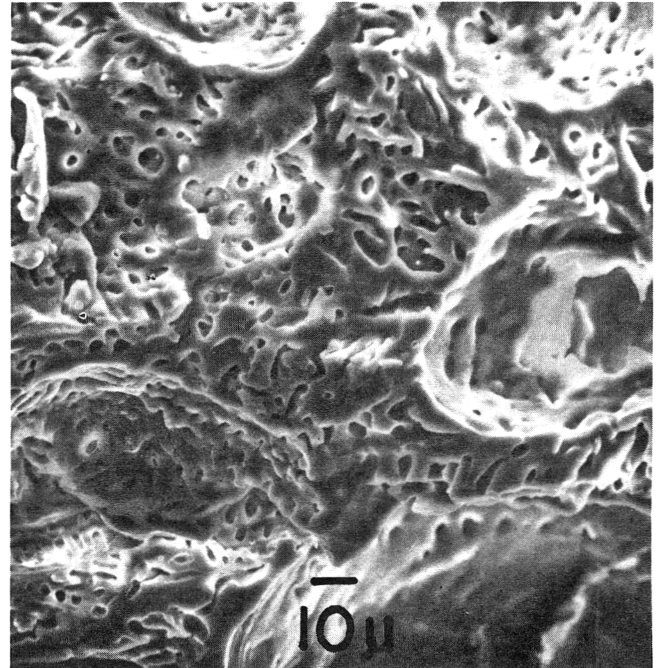

Fig. 4–Scanning electron microscope view of freeze-dried coffee (coated with aluminum) (600X).

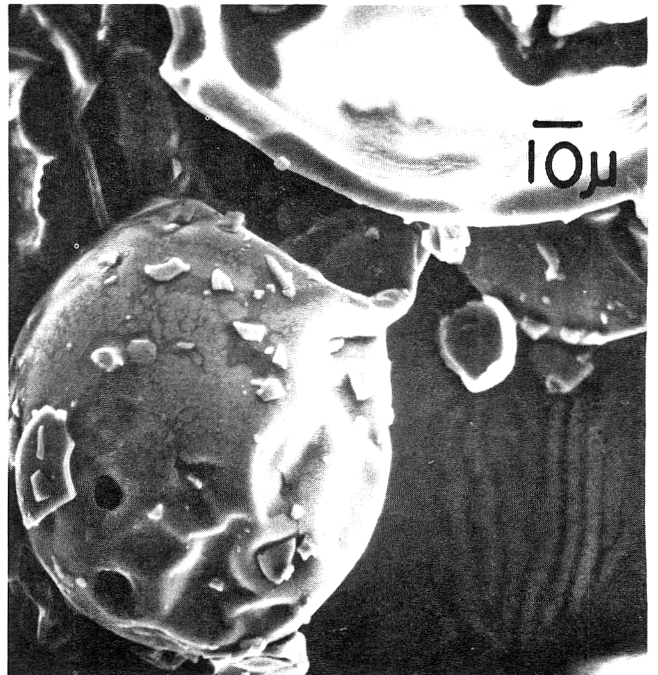

Fig. 5–Scanning electron microscope view of spray-dried coffee (coated with aluminum) (600X).

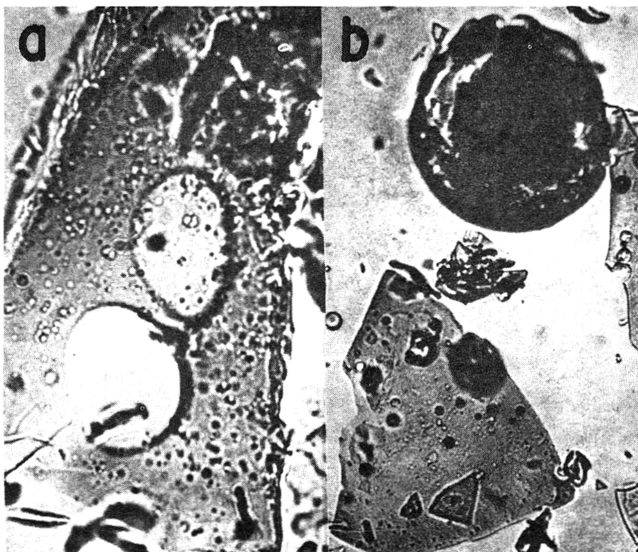

Fig. 3–Transmitted brightfield optical microscope views of dried coffee (in immersion oil): (a) freeze dried (600X); (b) spray dried (300X).

shape. This type of observation is valuable when considering the oxidative stability and solubility of milk powders. Fat globules sitting just under the surface can react with oxygen if the thin surface layer is broken. If other oil droplets are sitting in contact with this exposed oil droplet, oxygen will be able to diffuse down to the underlying oil and degrade the fat, leading to rancidity and off-odors. Oil globules leaking up to the surface will influence dispersibility due to their hydrophobic character. During freezing, the growing ice crystals can flatten and distort the spherical oil globules inside the concentrated solute phase, so that they eventually make contact. This would be expected to affect stability of the emulsion such that upon rehydration the oil globules in the dried matrix will be prone to agglomeration and/or coalescence, which can result in creaming.

Model food systems

The microstructure of freeze-dried oil-in-water emulsions containing dispersed or dissolved solids was observed. When fresh, maltodextrin-based emulsions which contained well dispersed, 1μ oil droplets were freeze dried, the oil was present in the dried matrix as spherical inclusions up to 4–5μ diameter. In some cases, however, the oil packing density can be so high that the oil inclusions are not observed as individual spherical droplets but rather as polygonal bodies sitting in contact.

Figure 7 shows an optical micrograph (150×) of a freeze-

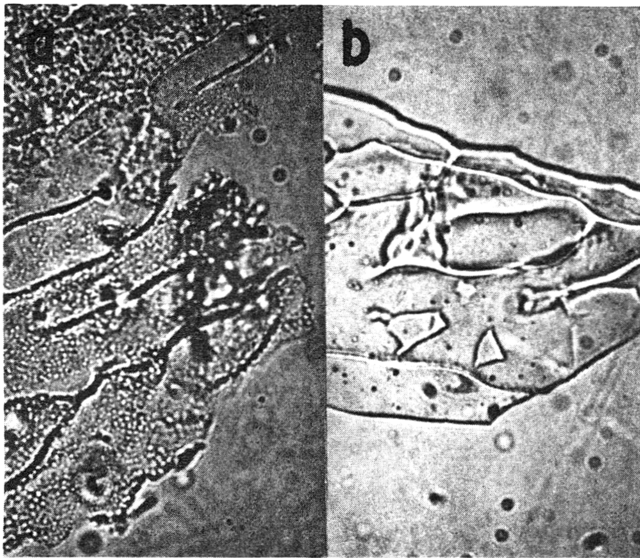

Fig. 6–Transmitted brightfield optical microscope views of freeze-dried milk (in immersion oil): (a) whole milk (600X), (b) skim milk (600X).

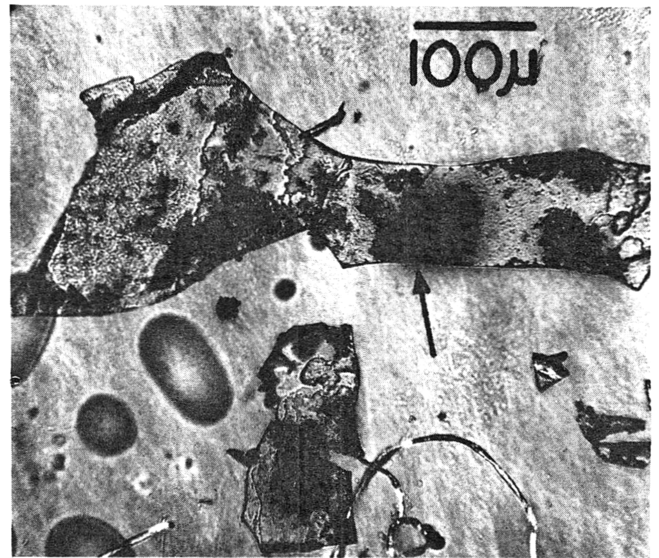

Fig. 8–Transmitted brightfield optical microscope view of freeze-dried oil-in-water maltodextrin emulsion after exposure to OsO_4 vapors (observed dry) (150X).

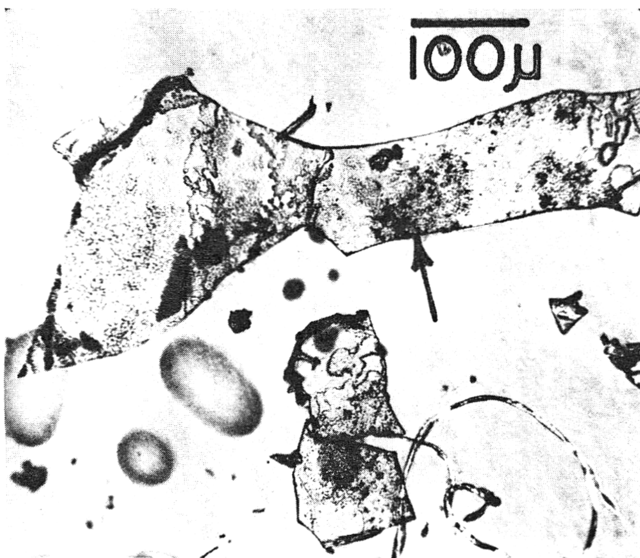

Fig. 7–Transmitted brightfield optical microscope view of freeze-dried oil-in-water maltodextrin emulsion (observed dry) (150X).

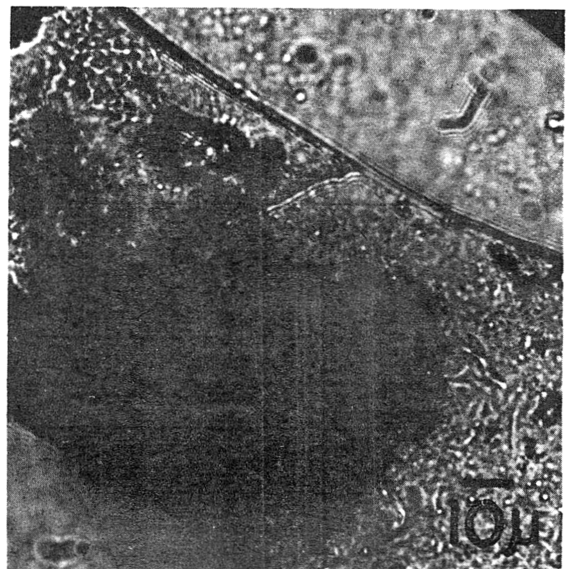

Fig. 9–Higher magnification (600X) view of center of Figure 8.

dried triolein-maltodextrin system. The boot-shaped flake shows dense oil inclusions (visible on left half) and some surface features which appear black due to the high difference in the refractive index for air and maltodextrin. The area pointed out by the arrow has many inclusions and somewhat rough surface. Reaction with osmic acid vapors gives dark (brown to black) areas in this region indicating the presence of surface oil (Fig. 8). On the left side of the grain there is practically no staining indicating good encapsulation. At a higher magnification (600x) of the area marked on Figure 8 the boundary of the stained area and the unstained encapsulated oil inclusions is clearly seen (Fig. 9). When this same grain was coated with gold and observed in the SEM (Fig. 10) a relatively smooth surface was noted. Figure 11 is the higher magnification (600x) SEM view of the same area as Figure 9. It shows some folds, cracks and bumps due to damage by the SEM electron beam, and more importantly, holes which occur exactly where the grain exhibited heavy staining with osmic acid vapors. These holes probably came from oil sitting at the ice/solute interface following freezing. The osmic acid vapors had good access to this oil. This can be the result of the oil in the holes leaking out on the surface, or by the osmic acid being able to diffuse down the holes to the encapsulated oil globules.

Sequential studies on a single sample demonstrate the value of utilizing both optical and scanning electron microscopy. While SEM allowed a good visualization of the overall surface morphology and matrix porosity, it was only in the optical microscope that the liquid oil inclusions could be observed.

In emulsions with Avicel (microcrystalline cellulose) as the solid support, the structural appearance is very different. In the liquid state, oil droplets ($1-10\mu$) and insoluble cellulose microcrystals are observed. In the dried state, no oil droplets can be observed (Fig. 12a). Reaction of the dried system with osmic acid shows that the oil droplets have spread to form a film coating the cellulose crystals. In the scanning electron microscope the cellulose crystals appear as rods with wrinkled surfaces (Fig. 12b). Small pieces, presumably shredded cellulose crystals, appear to have a bridging effect between the larger crystals resulting in a dense matrix structure. Surface oil again is not visible in the SEM.

To visualize surface fat deposits by electron optical methods, the electron microprobe was utilized. Since surface oil was reacted with osmic acid, the electron beam generates characteristic X-rays for osmium when hitting surface oil.

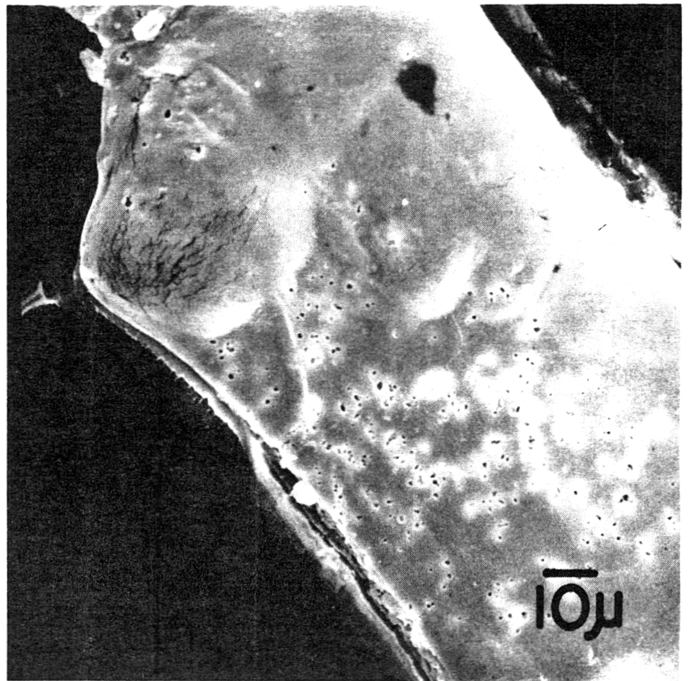

Fig. 11—Higher magnification (600X) view of center of Figure 10, same field as Figure 9.

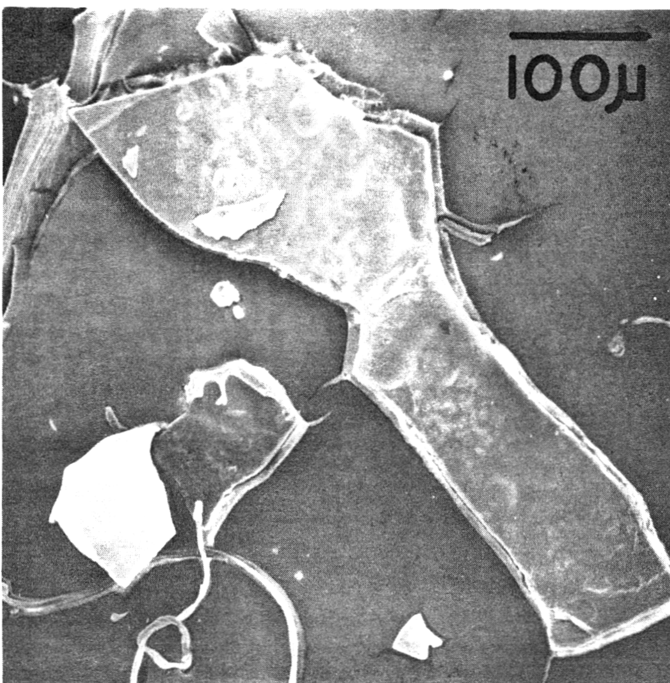

Fig. 10—Scanning electron microscope view of freeze-dried oil-in-water maltodextrin emulsion after exposure to OsO_4 vapors and coating with gold (150X).

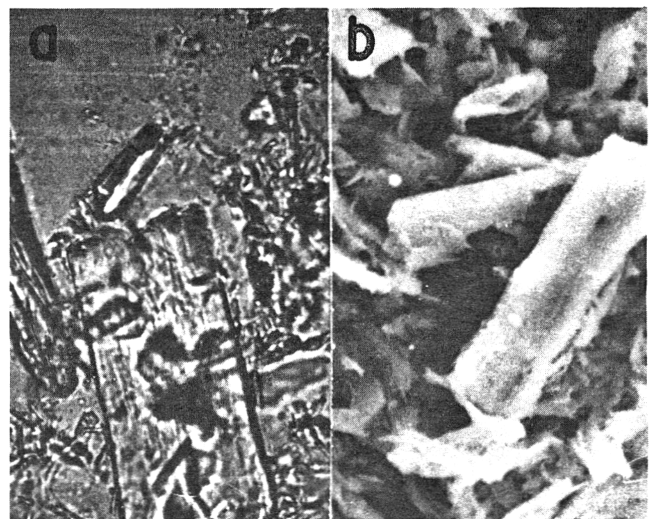

Fig. 12—Microscopic views of freeze-dried oil-in-water Avicel emulsion (not same fields): (a) transmitted brightfield optical microscope (600X); (b) scanning electron microscope (gold coated) (600X).

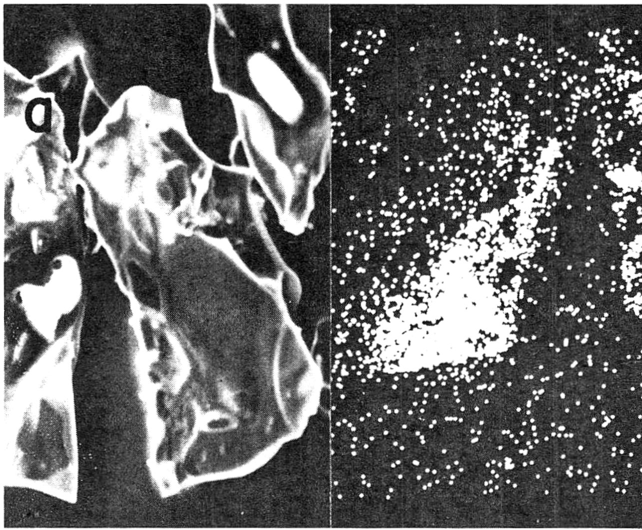

Fig. 13–Freeze-dried maltodextrin coated with triolein and exposed to OsO_4 vapors: (a) scanning electron microscope (aluminum coated) (100X); (b) electron microprobe, same field as (a) (100X).

These signals are recorded and pictures show very good agreement between dark stained areas seen in the optical microscope and the osmium X-ray intensity in the electron microprobe.

Grains of freeze-dried maltodextrin which were coated with triolein and exposed to osmic acid, showed no indication of surface fat in the SEM (Fig. 13a). In the electron microprobe, however, the same grains show osmium signals, indicating surface fat concentrations (Figure 13b).

Recrystallization phenomena, important in quality degradation of many foods, were observed using a freeze-dried system of corn syrup solids and sucrose. The aqueous solution of these solids was quickly frozen in liquid nitrogen, giving amorphous sucrose in the solid matrix after completion of freeze drying. The sucrose remains amorphous during subsequent storage at 0% RH. In Figure 14a, polarization microscopy shows an isotropic grain indicating the sucrose-corn syrup solids mixture is in the amorphous state. After 1 day at 43% RH the grain has collapsed due to absorption of water vapor, giving a highly viscous, but still amorphous mass of corn syrup solids and sucrose (Fig. 14b). After three additional days at 43% RH, small sucrose crystals have appeared within the amorphous phase (Fig. 14c).

The metastable condition between amorphous and crystalline states of sugars is of economic significance in many food products. By knowing crystallization patterns of sugars as functions of temperature and water activity, a product with optimal qualities, e.g., color, taste, hardness, can be obtained. For example, crystallization of sugars in candy can cause stickiness due to released water, and cause undesirable opaqueness on the surface (Andersen, 1968).

Polarization microscopy of flakes of freeze-dried gels composed of a complex mixture of alginate, Avicel, pectin, gelatin and sucrose show a heterogenous structure with isotropic, weak anisotropic, and strong anisotropic areas (Fig. 15a). The isotropic areas are presumed to be amorphous regions of alginate, pectin, gelatin and sucrose, while the highly birefringent rod-shaped areas are Avicel crystals [(1) in Fig. 15a]. The areas with low birefringence suggest another crystalline structure, [(2) in Fig. 15a] most probably due to highly ordered molecular arrangements of alginate chains held together by calcium ions.

That this is an ordered organization of alginate molecules is supported by the observation that addition of water causes both the gel to swell and the weakly anisotropic areas to disappear slowly (Fig. 15b). This is reversible as the anisotropy returns when the water evaporates. The Avicel crystals are not affected by the addition of water, since they are insoluble. These observations indicate that when water hydrates the gel, alginate chains outside the calcium junction regions are pushed apart and thus the alginate remains aligned only at calcium junction regions. The ordered arrangement and anisotropy are lost. As the water is removed, the chains come together again

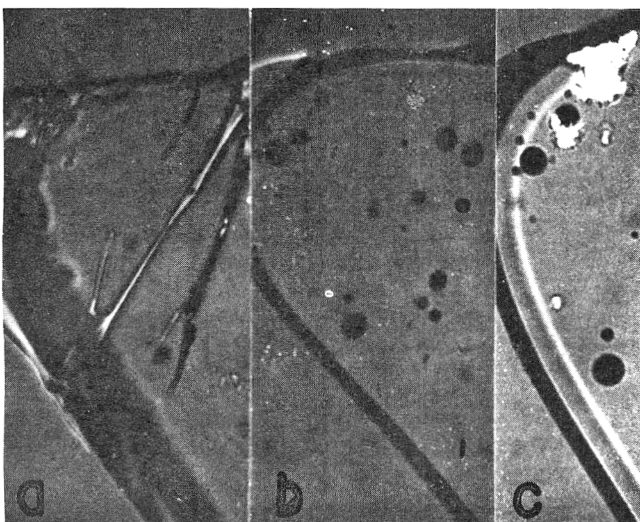

Fig. 14–Transmitted polarized light optical microscopy (with 1/4 wavelength quartz plate) of a freeze-dried corn syrup solids-sucrose mixture during humidification (observed in chloroform) (150X): (a) at 0% RH; (b) after 1 day at 43% RH; (c) after 4 days at 43% RH.

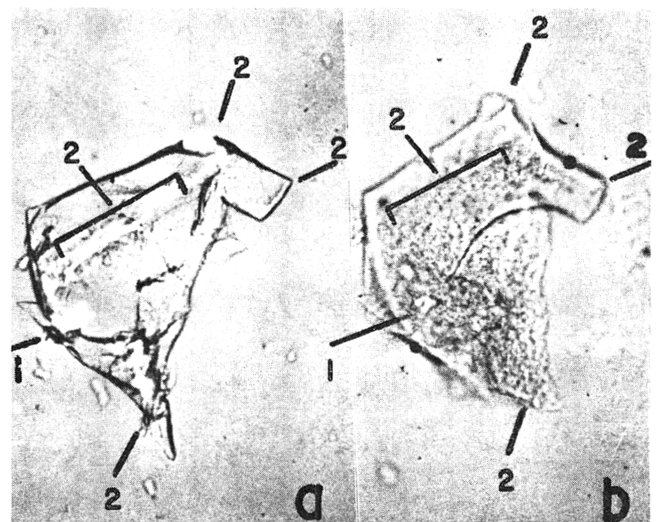

Fig. 15–Transmitted polarized light optical microscopy of a freeze-dried fruit simulating gel system (alginate-pectin-sucrose-Avicel-gelatin) (150X): (a) dry state (1) Avicel crystal, (2) anisotropic areas; (b) rehydrated state (1) Avicel crystal, (2) areas where anisotropy was lost.

and the ordered structure is essentially fully restored, causing anisotropy to return.

The observations on this particular gel system show not only the structure of the material, but demonstrate how the individual gel components interact, especially during hydration. Microscopy can similarly be used to observe the behavior of gels during temperature and pH changes, and during stress (which can be useful in textural studies).

SUMMARY

APPLICATION of microscopic techniques for studying microstructure of a variety of foods and food model systems is presented. The particular value of using optical and electron microscopic techniques can lead to an understanding of the spatial distribution of components of complex multiphase systems which is useful for understanding the physical and chemical properties of the complex material.

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Ms received 8/15/75; revised 10/20/75; accepted 10/28/75.

Presented at the 35th Annual Meeting of the Institute of Food Technologists, Chicago, Ill., June 8—12, 1975.

Contribution No. 2703 from the Dept. of Nutrition & Food Science.

The authors acknowledge the support of parts of this work by NASA/JSC (Contract No. NAS 9-12485) and HEW/PHS (Grant No. FD 00713-02). One of the authors (FGH) thanks Westreco, Inc. for financial support.

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THE PLASTEIN REACTION AND ITS APPLICATIONS: A REVIEW

ABSTRACT

Various methods for removing the bitterness arising from enzymatic hydrolysis of soybean and casein are reviewed. The plastein reaction is discussed in detail as well as its use in incorporating essential amino acids into the structure of soy protein plastein. Practical use of the β/α parameter in controlling the plastein reaction and in trying to enhance plastein productivity is reviewed along with alternate theories for plastein formation. Concludes that the plastein reaction has great potential (from a food processing point of view) provided the process can be controlled on an industrial scale; however, more intensive theoretical and practical investigations are needed before commercial utilization will be successful.

INTRODUCTION

ENZYMATIC HYDROLYSIS of soybean and casein is always accompanied by bitterness which arises from the presence of bitter peptides. Several reports have been published on methods of removing this bitterness.

Murray and Baker (1952) improved the taste of a commercial enzyme casein-hydrolyzate by carbon treatment and eluted an extremely bitter polypeptide from the carbon which had been used in the carbon treatment. Sato et al. (1969) applied leucine aminopeptidase to debittering of casein hydrolyzate and Fujimaki et al. (1970a) decreased the bitterness of diffusable bitter peptides in a peptic hydrolyzate of soybean protein considerably through a carboxy-peptidase A treatment. Carboxy-peptidase A was also found effective in debittering of a bitter tetracosapeptide isolated by Arai et al. (1970a).

The methods for debittering using exopeptidase, however, encounter certain limitations, since such enzymes produce significant amounts of free amino acids that may affect the food quality of the proteolyzates. To overcome this problem, Fujimaki et al. (1970b) studied the plastein reaction of soy protein, as well as other proteins.

THE PLASTEIN REACTION

WHEN A PROTEIN is hydrolyzed enzymatically and the hydrolyzate then incubated with certain proteolytic enzymes under appropriate conditions (high substrate concentration 30–50%, pH 3–7, temp 37–50°C and incubation time 24–72 hr), the hydrolysis is reversed and a high molecular weight protein-like substrate is formed whose properties are somewhat different from the original protein. The new protein-like substance is called 'plastein,' and may be regarded as a mixture of high molecular polypeptides (Fujimaki et al., 1971). In fact, Wieland et al. (1960) define plastein reaction as the formation of high molecular polypeptides.

At least three points are to be noted concerning the conditions under which the plastein reaction proceeds effectively. First, the concentration of substrate should be high. Second, the substrate should be of low molecular weight. Third, the pH for synthesis of the plastein is different than that for hydrolysis of the protein. The pH range for synthesis of plastein is narrower than the pH for hydrolysis (Yamashita et al., 1971).

When soy protein is partially hydrolyzed with an enzyme, e.g., pepsin, the hydrolyzate is bitter. On treatment of the hydrolyzate with α-chymotrypsin under suitable conditions a plastein is formed and the bitterness gradually disappears (Yamashita et al., 1970a). Fujimaki et al. (1970c) tested several enzymes for plastein production and found that two commercial proteinases (bioprase and prozyme) were as highly plastein productive as α-chymotrypsin.

In further studies on the modification of proteins in food, Yamashita et al. (1970b) found that the bitter peptides gly-leu and leu-phe play roles of plastein building blocks, constitute the plastein in cooperation with other peptides in the hydrolyzate, and are hence deprived of their bitterness. In the formation of the plastein the gly-leu participated mostly at its leucine terminal, with the result that the glycyl-leucyl residues are located at or near the N-terminus of the plastein chain. In the case of leu-phe, both termini participated in the plastein synthesis and consequently the leucyl-phenylalanyl residues seemed to be distributed almost uniformly in the plastein chains.

INCORPORATION OF ESSENTIAL AMINO ACIDS

IN OTHER COMMUNICATIONS, Yamashita et al. (1970c, 1971) reported experiments on using the plastein reaction as a means of incorporating methionine and cystine into the structure of soy protein plastein, thus producing a product of potentially greater nutritive value. Since the formation of a peptide bond by condensation between free amino acids is a strongly endogenic process (ΔF = 4 kcal/mole) and the free energy required to form a peptide bond from two peptides by condensation is far smaller (ΔF = 0.4 kcal/mole) the peptide chain growth during the plastein reaction is mostly attributable to the reaction among peptides. Therefore, it was necessary to prepare methionine and cystine containing peptides for the reaction. This experiment was quite successful for the incorporation of cystine in the molecule but not for incorporating methionine. In another experiment, a methionine ethyl ester was used. This combination was successful in introducing methionine in the new molecule to a level nearly six times that of the original soybean protein (Yamashita et al., 1971). Horowitz and Haurowitz (1959) had earlier reported on the use of amino acid ethyl esters for incorporation of a given amino acid into a plastein. The methionine occupied 33% and 85% on a molecular basis of the total N-terminal and C-terminal amino acids, respectively. Of the C-terminal methionine 14% remained in the ester form (Yamashita et al., 1972).

Other derivatives such as met-met and to a lesser extent N-acetyl-L-methionine (Ac-met) and L-methioninamide (met-NH_2) were also successful in incorporating methionine into the plastein. A method for preparing such a plastein on a practical scale has been devised by Arai et al. (1974). They further suggest that a 1:5 mixture of the met-plastein and soybean protein is best for optimum amino acid nutrition, since such a mixture resembles the ideal protein proposed by FAO/WHO (1965) with respect to the levels of sulfur containing amino acids. The described method was employed by Aso

et al. (1974) to improve zein with tryptophan, threonine and lysine. They used the ethyl esters of tryptophan and threonine and the ϵ-N-acetyl ethyl ester of lysine for best results. These results indicate the possibility of preparing plastein products having an amino acid composition similar to an ideal protein.

An amino acid with a hydrophobic side chain is effectively incorporated because such an amino acid can donate to plastein molecules a water insoluble property which is an important factor that makes the enzymatic reaction proceed toward the synthetic side (Tauber, 1951; Aso et al., 1973). However, from a food processing point of view, the production of a water soluble plastein is sometimes desirable.

Yamashita et al. (1974, 1975) incorporated glutamic acid into a soybean globulin fraction. The glutamic acid had to be made strongly hydrophobic by ethylating the α- and γ-carboxyl groups. After completion of the plastein reaction the ethyl ester linkages are hydrolyzed. This glu-plastein gave a clear solution in a 1% water solution, and no turbidity appeared during heat treatment of this solution at 100°C for 1 hr. This plastein, though nondialyzable, was of low molecular weight (avg mol wt, 6240), and it is suggested that this in conjunction with the high glu content (42%) is what causes its water solubility and heat stability. A circular dichroism (CD) study with the glu-plastein in solution gave a spectrum having a clear trough at 222 nm suggesting a partial formation of some helical structure probably consisting of an oligomeric glutamic acid sequence (Yamashita et al., 1975). This is in contrast to the control plastein which showed a CD spectrum almost similar to that of completely denatured soybean protein (Arai et al., 1970b). A plastein is usually considered to have no particular variable structure, partly due to the absence of any S-S bridge formation in the process (Aso et al., 1973). It was further noticed by Yamashita et al. (1974, 1975) that when this glu-plastein was hydrolyzed, the hydrolyzate had no bitter taste in contrast to the control plastein. It was, therefore, speculated that various glutamic acid bearing oligopeptides had a potent bitter-masking effect. The hydrolysis of glu-plastein with certain proteases (molsin and pronase) even gave a strong brothy taste to the hydrolyzates.

Recently Noguchi et al. (1975) published a report on the bitter masking activity of a glutamic acid-rich oligopeptide fraction in a glu-plastein hydrolyzate. The glu-plastein was prepared from a bitter fish protein concentrate (FPC) hydrolyzate. The glu-plastein hydrolyzate consisted of three peptide fractions: a slightly bitter basic fraction, a strongly bitter neutral fraction and a nonbitter acidic fraction. When the acidic fraction was combined with either the basic or the neutral fractions or both, the bitterness was completely masked. One of the main oligopeptides in the acidic fraction was glu-glu. Accordingly, its bitter masking activity was also evaluated. It was found that the acidic fraction and the glu-glu both were able to mask the bitterness, not only of the bitter hydrolyzate, but also of other bitter solutions such as: magnesium chloride, chlorogenic acid, caffeine, phenylthiocarbamide, brucine and bitter amino acids and dipeptides.

THE PARAMETER β/α

AS MENTIONED PREVIOUSLY, the precipitation of the plastein is one of the driving forces in the plastein reaction (Aso et al., 1973). Highly hydrophilic protein hydrolyzates are not effective substrates because the products are still soluble and eliminated with difficulty from the reaction system (Fujimaki et al., 1970c). On the other hand, if the hydrolyzate is highly hydrophobic the product easily precipitates before growing sufficiently in size (Aso et al., 1974). Both cases are, therefore, unfavorable when an optimum amount of plastein is intended.

Arai et al. (1975) have introduced a new term, β/α, which is related to plastein formation and may have practical use in controlling the plastein reaction and in trying to enhance plastein productivity. β is a measure of the insolubility of the plastein and α is a measure of the plastein production. Starting from a hydrophilic casein hydrolyzate, the plastein productivity (α) is 40% and the β/α value is 0.28. For zein, which is hydrophobic, the values are 57% and 0.87, respectively. In a 3:1 mixture of the two hydrolyzates values are: α = 88% and β/α = 0.47. Arai et al. (1975) propose that optimum plastein producing conditions are obtained when β/α approaches 0.5. The plastein yield from an ovalbumin hydrolyzate is more than 90% with β/α value of 0.49.Therefore, with a little knowledge of different hydrolyzates it is possible to find mixtures giving maximum plastein yield (90% or more).

In the presence of NaCl at concentrations of 0.1M and 0.8M the plastein yield of an ovalbumin hydrolyzate was increased by 10% (Tanimoto et al., 1975). At the lower concentration it was considered to be the result of increased enzyme activity and at the higher concentration a salting out effect of the products. v. Hofsten (1974) showed that by addition of NaCl in concentrations of 0.2M or more the plastein reaction proceeded faster.

ALTERNATE THEORIES FOR PLASTEIN FORMATION

DURING the plastein reaction a strong jelling very often occurs (Fujimaki et al., 1970c; Tsai et al., 1972; v. Hofsten, 1974). This was investigated in some detail by Tsai et al. (1972) who worked with substrate concentrations from 5 to 50% and found that the plastein reaction product in that range showed a rheological change from plasticity into elasticity. The jelly strength increased notably at substrate concentrations above 20% and the jelly was most developed in the product from a 35% starting substrate. At 40% the occurrence of solidification was rather prominent. The water-holding capacity of the gel reached 100% for substrate concentrations above 35%. v. Hofsten (1974) produced gels from whey protein hydrolyzates only after a few minutes incubation at 40–50°C. The gel was so rigid that the reaction beaker could be turned upside down. The gel was thixotropic.

These observations suggest that the jelling reaction belongs to the third of the three classes of sol-gel transformations reported by Heymann (1935). In this class belong the types of reactions where gels are formed by a process, where the dissolved compounds become insoluble, either by change in temperature, when one liquid is replaced by another, or when the gel is formed by a chemical reaction yielding an insoluble substance. In the sol the particles are hydrated. The hydration is decreased, before the gel formation takes place, providing the increase in entropy necessary to drive the endothermic structure forming reaction (Lauffer, 1975).

Fujimaki et al. (1971, 1973) defined plastein as the product insoluble in 10% TCA, 70% ethanol or acetone. For the detection of plastein, both TCA and dyes, especially amido black 10B, have been chosen, since they are known to have no affinity for low-molecular peptides and are used as a common reagent for the quantitative analysis of proteins. Tsai et al. (1974) estimated that amido black 10B could be employed for peptides with molecular weight above 1200 according to their experiments with acrylamide-gel electrophoresis.

According to v. Hofsten (1974) little attention has been directed to study the type of reactions capable of forming gels and insoluble products during the plastein reaction. The amount of product insoluble in 10% TCA increases during the reaction, but this is not primarily because of an increase in the chain length of the peptides, but more due to the fact that the product formed is insoluble. Aso et al. (1973) suggest from their results that hydrophobic bonding between peptides is largely responsible for the water insolubility or precipitation of the plastein produced from a soybean protein hydrolyzate

with pepsin. Tanimoto et al. (1972) produced a plastein with insoluble enzyme, which was removed from the reaction system after reaction. This rules out the possibility that the plastein might be formed from some chemical peptide-protein (enzyme) aggregation.

Kettman et al. (1966) in their study of thermal aggregation of poly-L-valyl-ribonuclease noticed that high NaCl concentrations permitted the aggregation to proceed faster and at a lower temperature. They suggest that the aggregation is due primarily to attractive nonpolar interactions, but that repulsive forces also are involved which can be diminished by higher ionic strength.

Yamashita et al. (1973) proposed that the plastein reaction is mainly a polycondensation process based on the fact that they demonstrated an actual peptidyl-enzyme intermediate. If plastein formation consisted merely of the formation of new peptide bonds, a decrease in the total number of free amino groups should take place. However, using three different methods for the determination of free amino groups, Horowitz and Haurowitz (1959) were unable to find any decrease during the plastein formation. The same observation was made by v. Hofsten (1974). These results and the observation that amino acid esters and not free amino acids are incorporated into plastein proved to them that plastein is formed, essentially by transpeptidation, i.e., by the transfer of amino acyl residues from donor peptides to acceptor peptides or amino acids.

Indications that both reactions may occur are in the report by Determann and Köhler (1965). They produced a condensation product from one pentapeptide and transpeptidation products from another pentapeptide. From a third pentapeptide Determann et al. (1965a) produced a plastein that was a homolog of the monomer. From a hexapeptide they were only able to produce the dimer. In another communication Determann et al. (1965b) reported on the specificity of pepsin in the plastein reaction. Using a pentapeptide containing leucine at the carboxyl end the condensation reaction proceeded. Substituting leucine with isoleucine or valine prevented the reaction and so did D-amino acids. In an earlier report Determann et al. (1963) reported on the active plastein productivity of hexa-, penta-, and tetrapeptides bearing aromatic amino acids on both termini. When alanine was substituted for the amino end tyrosine, the pentapeptides ability to form plastein was not affected, whereas the same substitution at the carboxy end caused inactivity.

Even though optimum conditions for the plastein reaction are in the pH range 3–7, v. Hofsten (1974) produced a gel from a whey protein hydrolyzate using esperase at an initial pH of 10.5. During the reaction the pH dropped to 8.4 due to proteolysis. The precipitated product was dissolved in 50% acetic acid and chromatographed on Sephadex G-50. The results show that the amount of low molecular peptides increased indicating that the gel formation is independent of an increase in peptide chain length. Furthermore, gels produced under optimal conditions were soluble in 8M urea or 6M guanidine at neutral pH which indicate that the gels are not held together by covalent bonds.

Apparently different reactions are involved in the plastein formation. If they are condensation reactions, a decrease of free amino groups would have to occur, which is not observed. Transpeptidation is hard to accept as the sole factor responsible for the formation of insoluble or gel-forming products. Another possibility is that hydrolysis of certain bonds could promote plastein formation (v. Hofsten, 1974). One possibility is that the enzyme's specificity could be affected by the extreme conditions of the reaction, i.e., high substrate concentration and a pH different than for optimal hydrolysis (v. Hofsten, 1974).

It seems most likely that the plastein reaction is an entropy-driven process, increase in entropy of water acting as the driving force (Lauffer, 1975) after an initial concentration of suitable peptides have been formed by either condensation or transpeptidation or both.

CONCLUSIONS

FROM A PRACTICAL point of view, it is possible to form plastein with most proteases aside from what the actual detailed mechanism might be. Fujimaki et al. (1973) describes a method for improving the quality of single-cell protein from n-paraffin-assimilating yeast cells by removing residual aliphatic hydrocarbons by hydrolysis, extraction of impurities followed by plastein synthesis.

From a food processing point of view, the plastein reaction has great potential if the process can be controlled on an industrial scale. However, the process has to be more fully investigated both theoretically and practically before any commercial utilization will be successful. The process could prove useful for the following applications, according to v. Hofsten (1974):

1. Removing bitterness of hydrolyzates;
2. Preparing gel-like products with excellent visco-elastic properties for incorporation in different types of foods;
3. Preparing products with improved amino acid composition using mixtures of hydrolyzates as substrates;
4. Preparing products with a very high level of a single amino acid which could be used as a dietary supplement to certain foods;
5. Preparing special types of soluble peptides having important flavor or other characteristics.

Aside from the possibility of unwanted microorganisms, nucleic acids and toxic substances being present in the hydrolyzates, special consideration must be given to the following problems:

1. The plastein will contain an enzyme. The content need not exceed that of cheese and it is possible to inactivate the enzyme before use of the plastein.
2. Theoretically, it is possible that the plastein reaction would produce toxic peptides under special conditions. The nutritive value of every plastein, therefore, has to be investigated in detail. So far, animal tests have indicated no toxicity.
3. Plastein must be considered a synthetic product and may not be readily accepted by the consumer.

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Ms received 8/22/75; revised 10/14/75; accepted 10/21/75.

Contribution No. 1073 of the Massachusetts Agricultural Experiment Station. This work was supported in part by Station Project No. 354.

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MASS TRANSFER AND KINETIC CHARACTERISTICS OF A COLLAGEN-β-GALACTOSIDASE (Aspergillus niger) REACTOR

ABSTRACT

As a prelude to an eventual pilot plant scale-up of the collagen-β-galactosidase system, mass transfer, kinetic, pH and temperature profiles were completed. The diffusional studies proved useful in elucidating the role of internal and external mass transfer in the collagen system. External mass transfer was negligible for a residence time in the range of 41.2 to 4.1×10^{-3} min or a flow rate of 48–495 ml/min. Internal transport influence increased with increasing film thickness. A thin film (0.4 mil or 10.16×10^{-3} mm) produced the highest activity per gram of complex. It is not advisable to use a film thinner than 0.4 mil with a bench-top reactor since the film will lack sufficient mechanical strength. Separate pH and temperature profiles were obtained with respective optima being 3.5 and 55°C. An activation energy of 10.7 kcal/mole was calculated by means of an Arrhenius plot. A kinetic study showed that the Michaelis constants for both the immobilized and soluble β-galactosidase were similar, 0.066 and 0.060M, respectively.

INTRODUCTION

THE IMMOBILIZATION of lactase to a solid support such as collagen makes the continuous hydrolysis of lactose possible over long periods of time and, in general, provides a more precise control over the hydrolysis of lactose. The recent success of glucose isomerase in the production of high fructose syrups (Mermelstein, 1975) may increase the commercial attractiveness of enzymatic hydrolysis of lactose to glucose and galactose. Such a system could help utilize the large quantity of acid whey which, as a source of a potential sweetener, remains untapped. The feasibility of hydrolysis by immobilized lactase has been demonstrated by several researchers (Olsen and Stanley, 1973; Stanley and Palter, 1973; Pitcher, 1974; Weetal et al., 1974a, b; Wierzbicki and Edwards, 1974; Barndt et al., 1975). However, when considering the scale-up of a reactor system, the investigator should remember that the attachment of an enzyme to a solid support may cause considerable changes in its kinetic properties. A thorough discussion of this subject is found in an article by Vieth and Venkatasubramanian (1974). In this article it is pointed out that factors such as a change in enzyme conformation, steric effects, microenvironmental effects, or external and internal mass transfer effects may change the kinetic behavior of the solid supported enzyme. Steric and conformational limitations are at best difficult to assess accurately. However, microenvironmental and transport or diffusional effects may be more readily determined.

The changes in the microenvironment are usually manifested in a shift of the pH and temperature profiles between the soluble and immobilized forms. Perhaps more important is the existance of a substrate concentration gradient across the solid support due to diffusional influence. A theoretical approach to the problem was first proposed by Thiele (1939). His concept of an effectiveness factor has since been readily adapted for a variety of situations including the use of immobilized enzymes (March et al., 1973). Bunting and Laidler (1972), Ford et al. (1972), Hamilton et al. (1974), Pitcher (1974) and Chu et al. (1975) have all mentioned the importance diffusional influence has in the systems which they have investigated. Recent articles by Lee and Tsao (1974) and Shukla (1975) discuss the subject at length.

The purpose of this paper is to present experimental data concerning the exploration of mass transfer, kinetic and microenvironmental characteristics of a collagen-β-galactosidase system.

MATERIALS & METHODS

Preparation of lactase-collagen complex

Microcut cattle hide collagen was obtained from the USDA Eastern Regional Research Center to be used as the enzyme support. β-galactosidase (Lactase LP) from *A. niger* was donated by the Wallerstein Company, Morton Grove, Ill. The enzyme was used without further purification in binding to the cattle hide collagen. The direct mixing technique was used in preparing the lactase-collagen complex (Barndt et al., 1975). The dried film was cross-linked in 0.6% glutaraldehyde for 6 min at 22°C. The glutaraldehyde solution was prepared in 0.1M, pH 7.0 sodium phosphate buffer. Following cross-linking, the film was washed thoroughly in distilled water and then allowed to air dry.

The dried collagen-lactase complex was used to construct biocatalytic reactors in the spiral reactor configuration (Vieth et al., 1972; Barndt et al., 1975).

Determination of lactase activity

Before any experiments were conducted, all biocatalytic reactors were brought to steady-state activity through continuous plug flow operation with 5% lactose dissolved in pH 4.6 sodium acetate buffer. All experiments were conducted at 37°C with the exception of the temperature profile. The experimental data were collected using a batch recirculation type of reactor. A 5% lactose solution was used as the substrate with the exception of the kinetic experiment. The enzyme assay was performed with Sigma glucostat reagents as outlined by Barndt et al. (1975). A unit of activity was defined as 1 μM glucose produced/min/g of complex at 37°C.

Mass transfer studies

To investigate the presence of external mass transfer, the flow rate of the bioreactor was varied while operating at a constant substrate concentration (5% lactose) in the first order region of the Michaelis-Menton kinetics. The flow rate varied from 48–495 ml/min (41.2 to 4.1×10^{-3} min residence time). Residence time was determined by dividing the reactor volume (ml) by the flow rate Q (ml/min). The substrate was recirculated for 30 min during which aliquots were removed to monitor enzyme activity. Duplicate runs were performed on the same reactor at each flow rate.

In examining for the presence of internal transport problems, one traditionally changes the particle size, or in this case, the collagen film thickness. Identical collagen-lactase complexes were prepared and cast at various thicknesses with the thickness of the dry film ranging from 0.4–6.5 mils (10.16×10^{-3} to 16.51×10^{-2} mm). Each film was operated at a constant flow rate of 200 ml/min for 30 min. Aliquots were removed during this time period to monitor enzyme activity.

Kinetic study

The kinetic constants K_m and V_{max} were obtained by operating the

steady-state reactor under the influence of increasing substrate concentrations. The substrate concentration ranged from 0.078–0.78M.

Microenvironmental studies

The activity of the immobilized β-galactosidase, as influenced by pH, was determined through a pH range of 3.5–6.0 while operating a nongradient recirculation-type reactor at 37°C. Each run lasted 30 min.

An identical system was used in obtaining a temperature profile through a range of 25–55°C. The pH of each substrate solution was maintained at 4.6 throughout the temperature study.

RESULTS & DISCUSSION

IN MAKING the transition from the soluble to solid supported phase, the enzyme must produce its catalysis in a heterogeneous surrounding. If the overall enzymatic reaction is described in terms of conventional heterogeneous catalysis, then at least five distinct steps can be identified. Steps 1 and 5, diffusion of the substrate from the bulk phase to the carrier surface, and diffusion of the product from the carrier surface back into the bulk phase, are influenced by external mass transfer. Steps 2 and 4, the transport of the substrate from the carrier surface to the domain of the enzyme, and transport of the product from the domain of the enzyme to the carrier surface, are controlled by internal mass transfer. The third step involves the enzymatic conversion of substrate to product.

The presence of external transport resistance results from a decrease in molecular diffusion across a boundary layer of fluid at the solid support surface. This boundary layer is known as the Nernst diffusional layer. Once the diffusional rate is decreased, the reaction rate will be slowed.

The testing for this situation was done with a nongradient recirculation reactor which operated with 5% lactose at various flow rates. If external mass transfer is limiting, then the observed reaction rate would increase as the flow rate, Q, increases since the diffusion rate is proportional to $Q^{1/3}$ If the diffusion rate is more rapid than the reaction rate, then the observed reaction rate should become independent of flow rate and be free of any external transport effects. In Figure 1, a recirculation flow profile is presented for the collagen-lactase system. The specific activity, R, divided by the maximum specific activity for that particular run, R_{max}, is plotted as a function of flow rate. The linearity produced from successive runs as opposed to exponential results presented by Ford et al. (1972) indicate that the system is virtually free of any external mass transfer effects within the limits of the experiment.

In examining for internal mass transfer, it was found that its role in the collagen-lactase system was of a more serious nature. As stated earlier, the presence of internal diffusion may be tested by following the experimental rate as the collagen film thickness is increased. The data presented in Figure 2 depict a situation where the experimental rate is diffusion controlled. In this case the experimental rate is inversely proportional to the thickness of the film. It is evident from these data that a higher specific activity is obtained by utilizing a thin membrane. The thinnest membrane used in this study was 0.4 mil (10.16×10^{-3} mm). The use of a film less than 0.4 mil with a bench-top scale reactor is inadvisable since the membrane will lack sufficient mechanical strength.

From these data it appears evident that the influence of internal mass transfer on the rate of lactase hydrolysis is more important than the effect of external mass transfer. The calculation of an effectiveness factor, η, can give an indication of how severe the internal diffusional problems are. The effectiveness factor is defined as the ratio of actual reaction rate in the matrix to the maximum rate obtainable without diffusional influences. A brief theoretical development of the Thiele modulus as it relates to internal diffusion is presented by Ford et al. (1972) where the first order Thiele modulus is defined as:

$$Q_1 = R\left[\frac{K_{cat}\,E_o}{K_m \quad Deff}\right]^{1/2}$$

where R = 1/2 thickness of the collagen film, 5.08×10^{-4} cm; K_{cat} = true catalytic rate constant for the immobilized enzyme, 15.87 sec^{-1}; K_m = Michaelis constant for the immobilized enzyme, 6.6×10^{-2} mole/liter; Deff = the effective dif-

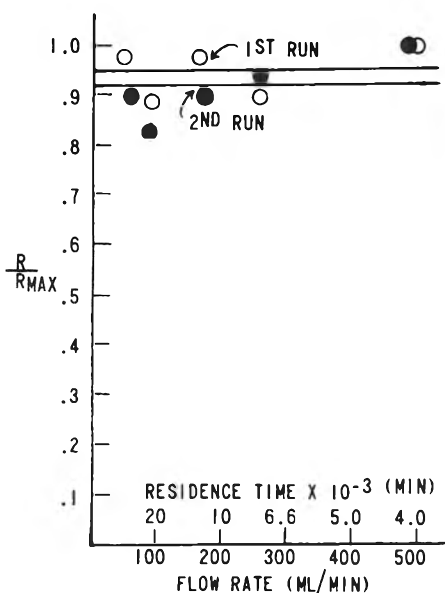

Fig. 1—Recirculation flow profile depicting fluctuation in specific activity as a function of increasing flow rate.

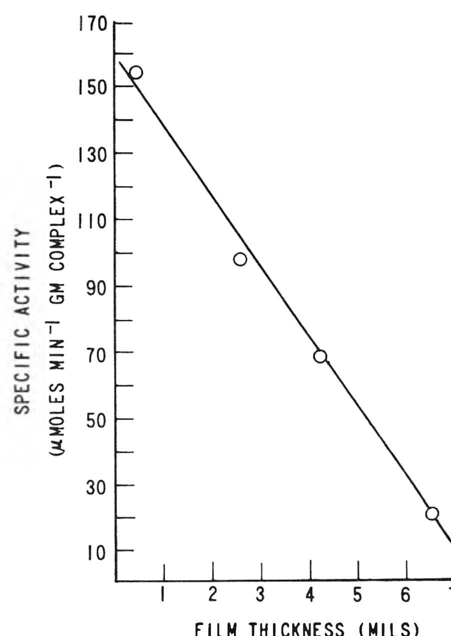

Fig. 2—Specific activity as a function of film thickness.

fusivity of the substrate within the collagen film, 5.7×10^{-8} cm^2/sec; E_o = active enzyme concentration within the porous matrix, 15×10^{-5} mole/liter.

In dealing with the collagen system, a flat surface rather than a spherical matrix is involved. This necessitates the use of 1/2 the collagen thickness rather than the radius of the porous matrix. In obtaining the true catalytic rate constant for the immobilized enzyme, K_{cat}, it was assumed that the attachment of the soluble enzyme to a solid support does not significantly alter the enzyme conformation. The similarity in the K_m values of the free and immobilized enzyme seem to bear this out. The true catalytic rate constant could have been obtained by reducing the collagen-lactase film into its smallest dimensions thereby eliminating internal mass transfer influence. A simple method of elimination could involve grinding the film into a powder form. The powder could then be used in a stirred tank reactor which is operated at a sufficiently high flow rate so that external mass transfer is absent. However, a more simplistic approach was used whereby in the first order region for the free enzyme, the first order rate constant can be obtained by dividing the V_{max} by the K_m of the soluble enzyme. The Michaelis constant for the immobilized enzyme may be affected by the film thickness as shown by the internal mass transfer data. The K_m for the immobilized enzyme, 0.066M, is for a film with a thickness of 0.4 mils. The diffusivity value for lactose was not readily available; however, a value for sucrose was. Due to the similarity in molecular structure between the two disaccharides, the diffusivity value for sucrose was used in the calculation. The active enzyme concentration was determined knowing the weight and steady state activity of the film, the activity of the enzyme in the soluble state and the molecular weight of the lactase.

The combination of experimental and estimated values with this equation resulted in a Thiele modulus of approximately 0.4, which is equivalent to an effectiveness factor of 1.0 (Lee and Tsao, 1974; Shukla, 1975).

Kinetic studies

The kinetic constants of K_m and V_{max} were obtained in usual fashion by plotting reciprocal velocity vs reciprocal substrate concentration (Fig. 3).

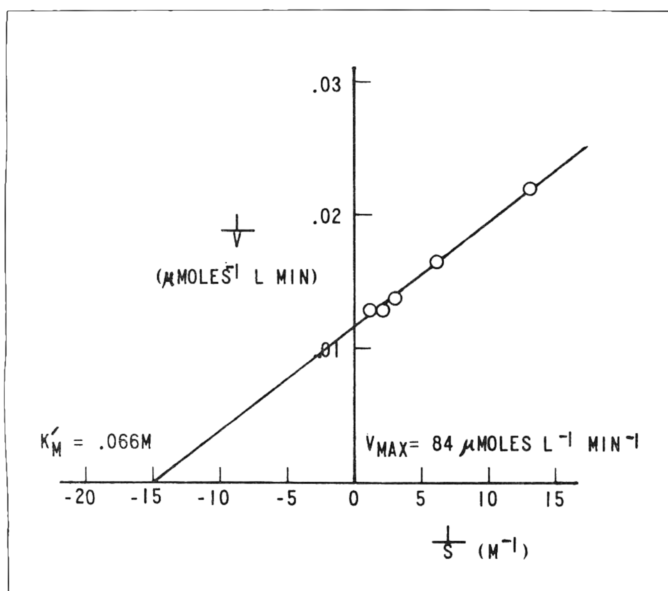

Fig. 3–Lineweaver-Burke type plot of immobilized β-galactosidase.

The reactor used for the kinetic studies was brought to steady-state activity prior to experimentation. It has been shown in previous work (Barndt et al., 1975) that the steady-state reactors remain stable over a number of repeated contacts and that activity losses are negligible throughout this period.

The K_m for the immobilized enzyme, 0.066M, is similar to the K_m of the soluble enzyme, 0.060M. The resemblance reflects the lack of conformational stress in the bound system manifesting itself in a similar affinity of enzyme for substrate in both phases.

Other researchers have reported similar K_m values for their fungal lactase preparations. Borglum and Sternberg (1972) reported the K_m of their fungal lactase as being 0.068; Weetal et al. (1974a, b) 0.07 for lactase W from Wallerstein; and Pitcher (1974) 0.052M for lactase LP. The V_{max} at 37°C was found to be 84 μmole/liter/min.

Microenvironmental studies

The microenvironment of the immobilized enzyme often becomes modified in such a way that the distribution of charged or uncharged substrates may differ from the bulk phase (Vieth et al., 1972). The uneven distribution of charged particles may account for a shift in the pH optimum between the soluble and immobilized enzyme. Several researchers have reported a slight shift in the pH optimum upon attachment of a fungal lactase to a solid support (Weetal et al., 1974a, b; Pitcher, 1974). The optima reported were from 4.0–4.5 for the soluble enzyme and 3.0–3.5 for the immobilized form. This is in agreement with the collagen-lactase system presented in Figure 4. The percent relative activity, as a function of pH, is presented for the soluble and immobilized form of lactase at 37°C. The data for the soluble enzyme curve were obtained from the Wallerstein Technical Bull. No. 710 for lactase. The pH profile of the immobilized enzyme exhibited maximum activity at pH 3.5, which is a shift of 0.5 pH units towards the acidic side. The immobilized form was not examined beyond pH 3.5 and 6.0 since it was desired to stay within the limits of the buffer system.

A comparison of the temperature profile of the immobilized and soluble forms of Lactase LP is presented in Figure 5. The data for the soluble enzyme were obtained from the Wallerstein Lactase LP Technical Bulletin. The immobilized system was evaluated in the range 25–55°C with the optimum temperature being 55°C. Other researchers have reported temperature optima for immobilized fungal lactase anywhere from 55–65°C. However, operation of a lactase reactor at its temperature optimum is not necessarily advantageous since researchers who have completed operational half-life studies report a marked decrease in stability at temperatures above 50–55°C (Woychik and Wondolowski, 1972; Olsen and Stanley, 1973). In the future, with the aid of information from operational half-life studies, hydrolysis may be conducted at temperatures higher than 37°C but less than 50°C, thereby enhancing the reactor rate.

The variation of reaction rate with temperature can be seen in an Arrhenius plot in Figure 6. The semilogarithmic plot features relative velocity as a function of reciprocal temperature ($°K^{-1}$). The slope of the linear plot allows the calculation of the activation energy which was found to be 10.7 kcal/mole. The calculated activation energy for immobilized lactase will be reduced substantially from the intrinsic value of free lactase if the system is completely controlled by mass transfer effects. Pitcher (1974) reports an activation energy of 12.0 kcal/g-mole (9.6–14.3 kcal/g-mole 95% confidence limits) for Lactase LP immobilized on glass beads in a system believed to be virtually free of internal transport effects. The calculated activation energy of the collagen system (10.7 kcal/mole) falls within the confidence limits reported by Pitcher (1974).

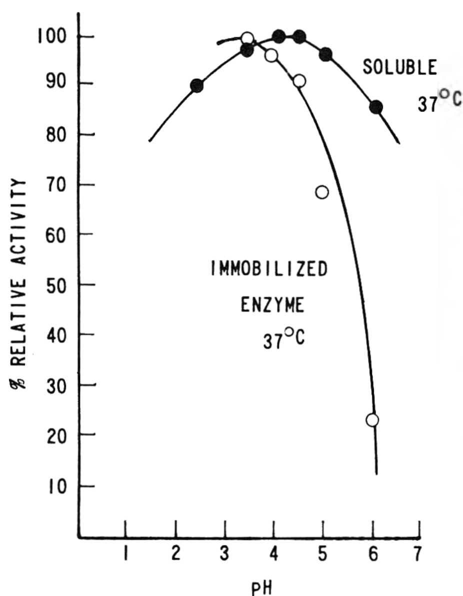

Fig. 4—Comparison of the pH profiles of the soluble and immobilized forms of β-galactosidase.

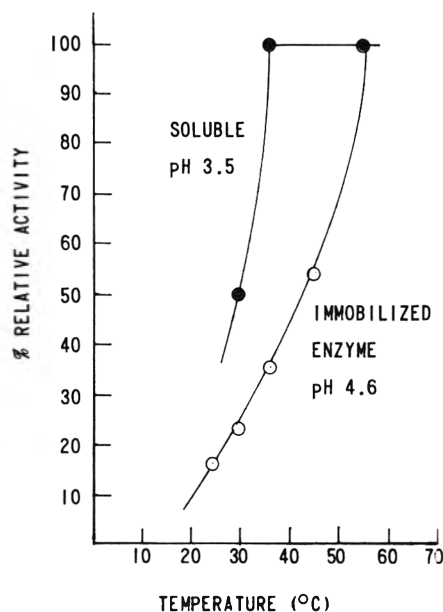

Fig. 5—A comparison of the temperature profiles for the soluble and immobilized form of β-galactosidase.

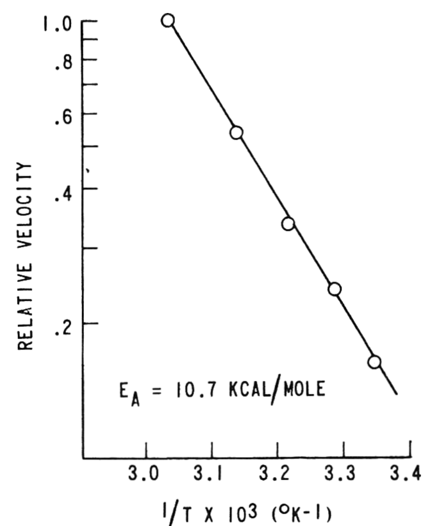

Fig. 6—Arrhenius plot of relative velocity of the immobilized β-galactosidase vs reciprocal temperature.

SUMMARY & CONCLUSIONS

THE STUDIES determining the external and internal mass transfer properties of the collagen-β-galactosidase system provided useful information. External transport effects were found to be negligible between a recirculation rate of 48–495 ml/min or a residence time of 41.2 to 4.1×10^{-3} min. Results of the internal mass transfer studies were most useful in determining that a thin film of approximately 0.4 mil (10.16×10^{-3} mm) produces the maximum activity per gram of complex. Considerable skill and patience is required to make a film less than 0.4 mil thick with good mechanical strength. In future pilot plant scale operation, it may be necessary to sacrifice a small degree of activity to obtain the required mechanical strength by increasing the film thickness to about 1.0 mil. The temperature and pH profiles are beneficial in selecting operating conditions. The pH optimum shifted from 4.0–3.5 for the transition between the soluble and immobilized forms. The optimum temperature was found to be 55°C. The temperature profile will become more beneficial once operational half-life studies are completed. The Michaelis constants for the soluble and immobilized β-galactosidase were similar, 0.060 and 0.066M, respectively.

The data presented were obtained by a model system of operation using 5% lactose solution as the substrate. These data may be altered when operating with another substrate such as acid whey.

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Ms received 7/31/75; revised 10/6/75; accepted 10/15/75.

Presented at the 35th Annual Meeting of the Institute of Food Technologists, Chicago, Ill., June 8–12, 1975.

Paper of the Journal Series, New Jersey Agricultural Experiment Station, Cook College, Rutgers—The State University of New Jersey, New Brunswick, NJ 08903

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FUNCTIONAL PROPERTIES OF ALFALFA LEAF PROTEIN: FOAMING

ABSTRACT

Alfalfa leaf proteins extracted with sodium chloride (1M), sodium hydroxide (0.05M) or Tris buffer (0.1M) had poor foaming properties compared to proteins obtained by direct expression of leaf juice. Extraction of lipids from all protein extracts markedly improved both foam formation and foam stability. Foam expansion of alfalfa leaf protein surpassed that of ovalbumin but the foam was less stable. The pH foam expansion profile resembled the solubility pattern of this protein. The delipidated protein preparations showed little pH effect being rather constant between pH 2–10. Foam stability of all preparations was markedly pH dependent showing minima in the isoelectric range around pH 3.5. Both sucrose and sodium chloride reduced the foaming of leaf proteins.

INTRODUCTION

IN ADDITION to developing new sources of proteins to meet expanding food needs there is a growing demand for more proteins, preferentially inexpensive, with requisite functional properties. Novel proteins with flexible functional properties should facilitate the fabrication of protein-enriched products for particular consumers and for a wide range of markets (Kinsella, 1976).

Foaming properties are important in proteins particularly for the domestic market. Thus, the capacity to form stiff stable foams is an important requirement for proteins to be used in angel food cakes, whipped toppings, desserts, and soufflé-like products. Foaming properties include 'whippability' and 'foamibility' and both are used interchangeably in the literature. 'Whippability' refers to the ability of a protein dispersion to form a foam when subjected to a high whipping/blending force usually employing a Hobart or household-type mixer (Lawhon et al., 1972a, b). 'Foamability' is measured under milder conditions by spurging air or a gas through, or by mixing/agitation of, a dilute dispersion of protein and measuring the volume of foam formed after a specified period (Yatsumatsu et al., 1972). The latter test requires less protein. Foam stability is measured by rate of breakdown, i.e., volume decrease of foam (Yatsumatsu et al., 1972; Lin et al., 1974) or by the rate of leakage of fluid from the foam (Richert et al., 1974; Lawhon et al., 1972a, b; Eldridge et al., 1963).

In addition to ovalbumin which is the standard for foaming proteins (Barmore, 1934; Henry and Barbour, 1933; Bailey, 1935) the whippability of several novel proteins have been studied. Watts (1937) first showed the excellent foaming capacity of aqueous extracts of soybean and Eldridge et al. (1963) carried out a systematic study of the factors (pH, salts, protein concentration, temperature, etc.) affecting foam expansion, i.e., volume increase, of soy protein foams. Yatsumatsu et al. (1972) related foaming to the soluble protein in soy protein preparations. Lawhon et al. (1972a, b) examined the foaming capacity and foam strength of the aqueous extracts of proteins from several samples of cottonseed, peanut, soybean, coconut, sesame and sunflower. They found that protein from liquid cyclone processed cottonseed and peanuts formed superior foams. Lin et al. (1974) showed that protein isolated from sunflower had excellent foaming properties and Huffman et al. (1975) described optimum conditions for foaming of sunflower proteins. McDonald and Pence (1961) examined the foaming potential of gliadin for food uses and recently Tybor et al. (1975) described the superior foaming properties of globin proteins in the pH range 5–6.

Several workers, Hansen and Black (1972), DeVilbiss et al. (1974) and Richert et al. (1974) demonstrated the excellent foaming properties of undenatured whey proteins. Richert et al. (1974) using an exemplary approach in terms of experimental design and statistical analysis for quantifying any protein functionality, studied all parameters influencing foaming to determine optimum conditions for the formation of a stable foam from whey protein.

Some of the physicochemical aspects of protein foam formation and stability have been addressed by Cumper (1953), DeVilbiss et al. (1974) and Buckingham (1970).

Buckingham (1970) studied the effects of protein concentration, pH and temperature on the strength of foams formed from cytoplasmic proteins isolated from clover leaves. Foam strength increased with protein concentration reaching a plateau at 120 mg/ml and maximum strength occurred within a narrow pH range slightly above the isoelectric point of the protein.

Several workers reported that partial hydrolysis (enzymatic or chemical) of relatively inert or denatured proteins, e.g., fish protein concentrate (Hermansson et al., 1971; Baldwin and Sinthalavai, 1974) soy proteins (Sair and Rathman, 1950) markedly improved their foaming properties. Recently, it has been demonstrated that succinylation improved the foaming capacity of proteins, i.e., fish proteins (Groninger and Miller, 1975) and soy proteins (Melnychyn and Stapley, 1973).

In this paper we report on foaming properties of proteins extracted from alfalfa leaves.

MATERIALS & METHODS

ALFALFA LEAF PROTEIN (ALP) samples were extracted, isolated and prepared as detailed previously (Wang and Kinsella, 1975; 1976). Separate batches of leaves from young alfalfa plants were macerated and the protein was extracted directly with the native juice; with water; sodium chloride (1M); sodium hydroxide (0.05M) and Tris buffer (0.1M, pH 7.4). The protein in each extract was precipitated at pH 3.7 using HCl and recovered by centrifugation. This protein was freeze-dried and half was stored in vacuum at 4°C; the remainder was exhaustively extracted with acetone, and freeze dried. The composition of these preparations was reported (Wang and Kinsella, 1975).

Modification of ALP by acid and alkali

Duplicate suspensions of protein (2g/100 ml) was adjusted to pH 1.5 or pH 11.0 with HCl (1.0M) or NaOH (1.0M) and incubated at 60°C for 24 hr. The hydrolyzed samples were dialyzed against water for 24 hr and freeze dried. Foaming properties were determined and described below.

Derivatization of ALP

Succinylation of ALP was carried out using the method of Hoagland (1966) by reacting 2–3g of ALP in sodium phosphate buffer (0.1M, pH 7) with 1–2g of succinic anhydride. The derivatized protein was recovered by freeze drying following dialysis for 24 hr against water to remove excess anhydride and acetic acid. A fluffy light colored protein was obtained.

Foaming properties

The foaming properties of proteins were performed on a shaker designed according to directions of Yatsumatsu et al. (1972). The shaker consists of a standard motor (1725 rpm) with a 2-in. pulley, connected to a 7-in. pulley which drives a basket on lubricated runners. The basket shakes horizontally with a 2-in. amplitude at 360 reciprocations per min. Alfalfa leaf protein samples (500 mg or approx 300 mg protein) were added to 50 ml of water in stoppered (100 ml) graduated cylinders. The protein was dispersed by sonication for 1 min and kept at 24°C for 30 min with occasional stirring. The cylinder plus contents was then shaken horizontally in the shaker basket for 1 min. A filter paper disc was placed on top of the foam, and it was gently pressed until resistance was encountered before each reading. The volume of the foam measured at 30 sec and 30 min following shaking was reported as foam expansion (FE) and foam stability (FS), respectively. The volume of foam was recorded at 5-min intervals up to 30 min and at 30-min intervals thereafter. All tests were done in triplicate and average values are reported.

When studying the effect of pH on foam expansion and foam stability, appropriate concentrations of HCl (4–20 mM) or NaOH (4–12 mM) solutions were used to obtain the desired pH values.

Water was replaced with NaCl (1M) or sucrose (50% w/v) solutions in studying the effect of additives. Egg albumin (Nutritional Biochemicals Corp.) at equal protein concentrations was used as a standard for comparison of foaming properties.

RESULTS

THE FOAMING PROPERTIES of the ALP extracted from leaves with different extractants were inferior. However, removal of the lipid components with acetone caused a significant improvement in both foam expansion (volume of foam at 30 sec) and foam stability (volume of foam after 30 min). Similar foaming properties were found in all acetone extracted ALP except the sample from sodium chloride extraction where a lower foam expansion but higher foam stability was consistently observed. The superior foaming properties of the acetone extracted ALP are consistent with the observation of Cooney et al. (1973) using whey proteins; the negative effect of fat on the foaming properties of soy proteins (Yatsumatsu et al., 1972), and the deleterious effects of bound lipids on foaming of soy concentrates (Eldridge et al., 1963).

The foaming properties of the proteins recovered from fresh alfalfa leaves, without using extractants showed good foaming properties particularly following extraction of the lipids (Fig. 2). The better foaming properties of this protein preparation, compared to those listed in Figure 1, is perhaps explained by their lower lipid content (50% lower) as reported by Wang and Kinsella (1975), though the component proteins may also have differed from those extracted using specific solvents. This protein was used in all the subsequent tests.

The mean values for foam expansion of ALP and acetone extracted ALP, respectively, between pH 2–10 are presented in Figure 2. Distinct differences in the foaming properties between the unextracted and acetone extracted ALP were obtained. The pH/foam expansion profile of acetone extracted ALP exhibited highest foam expansion at pH 2 but remained relatively constant between pH 3 and 10. This indicated that either there was an adequate amount of soluble protein to provide surface film for the amount of foam formed even at the lower solubility range for ALP (pH 3–5.5) or that the acetone extraction may have altered the conformation of some of the leaf proteins which facilitated their surface spreading and mutual interaction.

The pH-foam expansion profile of the unextracted ALP resembled that of pH-solubility profile, i.e., lower in isoelectric region (pH 3.5) and higher at pH values above or below this region.

The pH foam stability profiles of acetone extracted and unextracted ALP showed similar patterns but the former were higher at all pH levels. Minimum foam stability occurred in the vicinity of pH 3.5–4, i.e., isoelectric region for all ALP. This may be in keeping with the data of Buckingham (1970) who showed that while maximum foam strength of clover proteins occurred slightly above the isoelectric region, minimum strength was observed at isoelectric pH.

Foam stability was high at pH 2 and between pH 5 and 6.5.

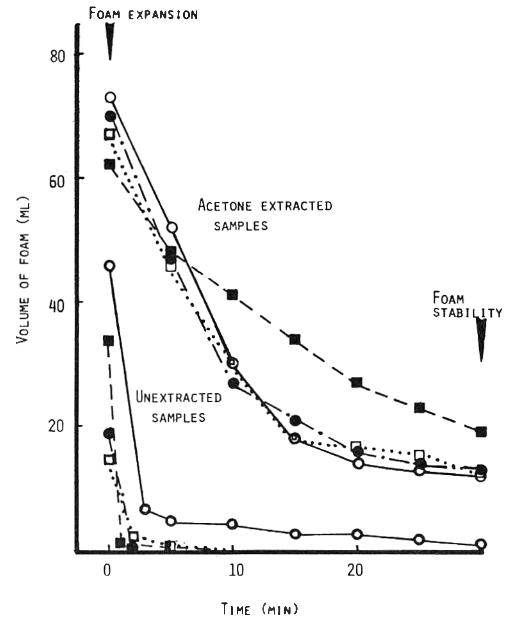

Fig. 1—Foaming properties of alfalfa leaf protein preparations extracted with different extractants. Acetone extracted samples denote those from which lipids were removed. [○ alfalfa leaf protein extracted with water; ■ alfalfa leaf protein extracted with NaCl (1M); □ alfalfa leaf protein extracted with NaOH (0.05M); ● alfalfa leaf protein extracted with Tris buffer.]

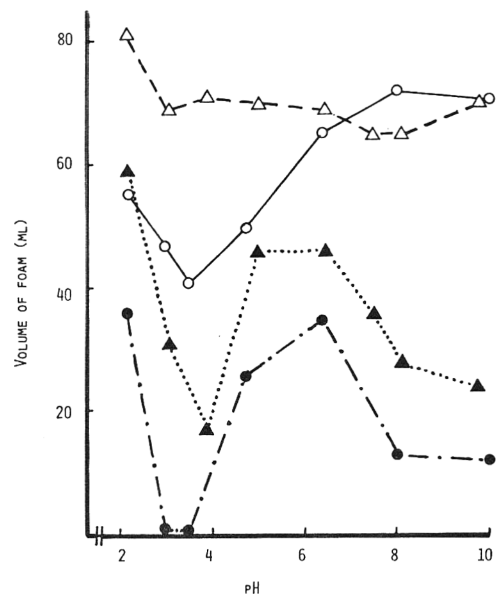

Fig. 2—Effect of pH on foam expansion and foam stability of alfalfa leaf protein. [○ foam expansion of alfalfa leaf protein; △ foam expansion of acetone extracted alfalfa leaf protein; ● foam stability of alfalfa leaf protein; ▲ foam stability of acetone extracted alfalfa leaf protein.]

The significance of higher foam stability and foam expansion at pH 2 is not clear at present. Since ALP is a mixture of proteins from alfalfa leaves, it may indicate that a particular protein solubilized at pH 2 has superior foaming properties. It is evident that pH values of 7 and above exerted an adverse effect on the stability of ALP film, since foam stability dropped sharply above pH 6.5. This may be attributed to the fact that between pH 5 and pH 6.5 foam stability is enhanced because cohesion between the peptides is maximum due to the balance in attractive charges on the polypeptides whereas above pH 7 excessive negative charges repelled neighboring peptides and destabilized the membrane.

A comparison of foaming properties of unextracted ALP with that of egg albumin was made. The egg albumin dispersion was adjusted to a protein level comparable to that of ALP, i.e., approx. 300 mg/ml. The results are shown in Table 1. The foaming properties of ALP at either pH 2 or pH 6.2 were comparable to those of the egg albumin solution despite the substantially higher amount of soluble protein present in the latter, i.e., > 90% compared to 10–20% for the alfalfa preparation. At pH 2, ALP showed a better stability than egg albumin up to 30 min though it was slightly lower thereafter. At pH 6.2, ALP showed a faster rate of reduction of foam volume initially which leveled off after 60 min.

Table 1–Comparison of foam expansion and foam stability of ovalbumin, alfalfa leaf protein and alfalfa leaf protein with and without salt or sugar

	Foam volume (ml)[a]						
		pH 6.2			pH 2		
Time (min)	Ovalbumin	ALP	ALP & NaCl	ALP & Sucrose	ALP	ALP & NaCl	ALP & Sucrose
0.5	44	55	51	40	56	41	36
5	37	48	44	30	48	14	23
10	36	46	43	28	45	9	15
15	36	43	39	26	43	9	5
20	35	41	37	24	41	6	3
25	34	38	33	23	39	5	2
30	33	31	30	22	37	5	1
60	29	20	16	16	29	5	–
90	25	18	12	15	20	4	–
120	25	14	12	14	15	4	–
150	24	13	10	13	13	4	–

[a] ALP = alfalfa leaf protein; mean values of triplicate tests

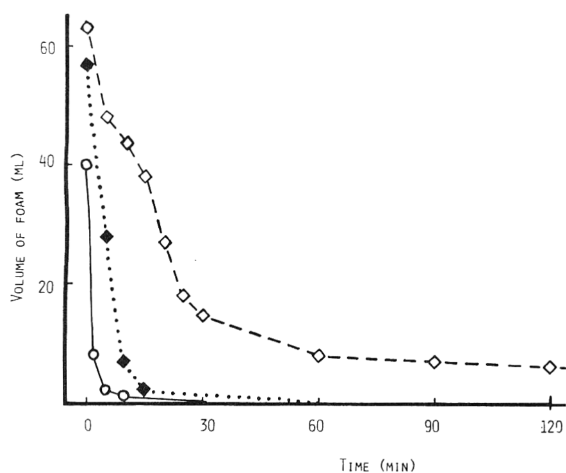

Fig. 3–Foaming properties of acid or alkaline modified alfalfa leaf proteins. [○ unmodified alfalfa leaf protein; ◇ acid modified alfalfa leaf protein; ◆ alkaline modified alfalfa leaf protein.]

The effect of NaCl and sucrose on the foaming properties of ALP was examined at pH 6.2 and pH 2 (Table 1). Sodium chloride had a little effect on foaming properties at pH 6 whereas sucrose reduced foam expansion and foam stability. At pH 2, foam stability was dramatically reduced by the presence of salt or sucrose.

Acid or alkaline hydrolysis did not increase foaming properties of ALP (Fig. 3). This was in contrast to other findings for fish protein concentrates (Hermansson et al., 1972) whose foaming capacity was enhanced following hydrolysis. It is probable that the dialysis of our ALP samples following hydrolysis removed solubilized proteins and peptides which are the functional foaming components. In this respect, DeVilbiss et al. (1974) concluded that nonprotein nitrogenous compounds were effective foaming components in whey protein concentrates and Jelen (1973) showed that nonprecipitable nitrogenous components of whey have excellent foaming properties. This possibility is under study for hydrolyzed ALP.

Succinylation, which enhanced aqueous solubility tenfold, also resulted in a marked increase in foaming. Foam expansion increased almost threefold and foam stability was improved over twentyfold (Franzen and Kinsella, 1976). These data, which are being further studied, are consistent with the fact that soluble protein is required for foam formation as shown by Eldridge et al. (1963), Yatsumatsu et al. (1972) and Lawhon et al. (1972a, b).

DISCUSSION

PROTEINS perform a number of functions in foaming. Surfactant proteins lower the surface (or interfacial) tension of the aqueous phase (and between the air/liquid interface); they facilitate spreading and permit the increase in surface area required for foaming, and impart viscosity to minimize leakage and breakdown. Coincidentally, with gas entrapment, (i.e., foam formation), soluble proteins orient, unfold, spread and by intermolecular association form elastic membranes around the gas droplets. By partial denaturation during the surface spreading involved in membrane formation (Bull, 1938; 1947) the proteins form a cohesive structural network imparting strength, elasticity and stability to the foam.

The ALP samples extracted from leaves with extractants contained 10–12% lipids whereas those squeezed from the leaves without solvent had about 5% (Wang and Kinsella, 1975). This may account for the superior foaming of the latter proteins. The lipids occur as lipoprotein complexes. These probably interfered with foam formation by impairing spreading of protein at the interface and weakening or disrupting the necessary cohesive forces between protein layers surrounding the air vacuoles. This resulted in rapid collapse of the foam. Eldridge et al. (1963) reported that removal of phospholipids from soy protein improved its foaming properties and the detrimental effect of oils and yolk lipids on the foaming of ovalbumin was demonstrated by Bailey (1935) and Henry and Barbour (1933).

The foaming behavior of native ALP at various pH values showed a pattern paralleling its pH solubility profile, i.e., minimum foam expansion occurred around the isoelectric range of minimum solubility (pH 3.5). This foaming pattern probably reflects the availability of soluble protein at these particular pH values since soluble protein(s) is necessary for foam formation (Eldridge et al., 1963; Buckingham, 1970; Lawhon et al., 1972a, b; Yatsumatsu et al., 1972; Hermansson et al., 1972). The minimum foaming at pH 3.5 probably reflects the strong ionic intermolecular attractions which impair unfolding and

spreading at this isoelectric pH. The foam expansion of the acetone extracted ALP showed little response to pH. Thus, as with fish protein concentrate (Hermansson et al., 1972), the acetone extracted ALP apparently contained sufficient soluble protein(s) over the entire pH range to form a foam and the denatured proteins stabilized this foam. In addition acetone induced changes in conformation of the protein may account for the absence of pH effect on foaming.

Foam stability varied with pH being minimum in the isoelectric range (pH 3–4); being maximum in narrow pH region above the isoelectric range where protein is slightly negatively charged; and showing a rapid decrease at alkaline pH values (pH 6). The latter effect may be explained by charge repulsion between proteins with resultant lack of adhesion, and also by some solubilization of the ALP proteins in this range (Lu and Kinsella, 1972; Betschart and Kinsella, 1974) thereby reducing the quantity of aggregated protein necessary to stabilize the foams. Thus, higher pH values not only caused lower foam stability (and emulsifying capacity) (Wang and Kinsella, 1975) of ALP, but it also resulted in undesirable grassy odor and yellowish color in both cases. The relatively high degree of foam stability of ALP at pH 5–6 is consistent with the findings of Buckingham (1970) who demonstrated that maximum foam strength of clover leaf cytoplasmic proteins occurred near to their isoelectric region when the proteins were still slightly electronegative. Eldridge et al. (1963) found that soybean protein dispersions showed minimum foam expansion and foam stability in their isoelectric region. The foam expansion of ALP was slightly greater than that obtained with ovalbumin though the stability of ALP foams, particularly at pH 6.2, was lower under the conditions of our test.

Several researchers (Eldridge et al., 1963; Barmore, 1934; DeVilbiss et al., 1974) observed that sucrose reduced foam expansion, but enhanced foam stability of resultant foams. Lawhon et al. (1972a, b) and DeVilbiss et al. (1974) showed that sugar markedly enhanced the viscosity of foams made from cottonseed flour extracts and whey protein, and it is generally conceded that higher surface viscosity in the foam membranes minimizes liquid leakage, and this is correlated with foam stability. The lack of a foam stabilizing effect of sucrose on ALP foams requires further investigation.

The marked increase in foaming properties following succinylation may indicate a practical approach to improving functional properties of leaf proteins. Similar improvements have been reported for succinylated fish proteins and soy proteins (Groninger and Miller, 1975; Chen et al., 1975; and Melnychyn and Stapley, 1973). The improvements in functionality following derivatization may also be useful in elucidating physicochemical basis of protein functionality.

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Ms received 7/26/75; revised 10/16/75; accepted 10/21/75.

Partial support of NSF grant no. 7517273 and the assistance of Kay Franzen are acknowledged.

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EFFECT OF PURIFIED AND NONPURIFIED PLANT FIBERS ON FECAL OUTPUT AND COMPOSITION

ABSTRACT

In recent years, much interest has been focused on the effects of dietary plant fibers on human nutrition. However, improper definition of the plant fiber under investigation and insufficient consideration of the interactions of plant fibers with other dietary components has resulted in some contradictory results. The pig-tailed monkey (*Macaca nemestrina*) is physiologically similar to humans and has been used in this study to investigate the effect of a purified plant fiber (PPF) and a nonpurified plant fiber (NPPF) on fecal output and composition. Fiber-free diets were used as a control. The experimental diets contained corn oil, coconut oil plus 0.1% cholesterol, or coconut oil plus 0.5% cholesterol, in addition to the plant fibers. The effect of the NPPF and PPF on fecal output and composition varied with the parameter studied, and additionally there appear to be interactions between the plant fibers and dietary lipids. This study points to the need for a more careful definition of plant fibers with regard to its purified or nonpurified state, and a recognition of the interactions between plant fibers and other macronutrients in the diet.

INTRODUCTION

IN RECENT YEARS, much interest has been focused on the possible beneficial effects of dietary plant fibers (PF) on human and other monogastric nutrition (Trowell, 1973; Cummings, 1973; Burkitt, 1975; Burkitt et al., 1974; Spiller and Amen, 1975a). Although the effects of dietary fiber on health are still equivocal (Hill, 1974), there is little doubt that the components of the plant cell wall will be shown to have a much greater effect on the human physiology than previously realized (Eastwood, 1973). Certainly, there now appears to be a general consensus among nutritionists that new and intense study of these effects is needed.

As more research is performed in this area, it is becoming apparent that some of the contradictions in the literature are a result of a poor choice of animal model, improper or a lack of definition of the fiber fraction in question, or a lack of sufficient consideration for the interaction of the cell wall components with other dietary ingredients.

Since microorganisms digest certain components of the plant cell wall, gut microflora considerations are extremely important in choosing an animal on which to perform dietary fiber studies. To avoid this problem the pig-tailed monkey (*Macaca nemestrina*) was chosen for this study. This monkey has a gastrointestinal tract morphologically and physiologically similar to humans, and has its microbial flora concentrated in the large bowel.

To avoid the problems of improper or ambiguous nomenclature in the literature, we have recently suggested (Spiller and Amen, 1974; 1975a, b) that any natural product containing the fibrous portion of the cell wall, plus all the associated factors, such as mineral matter, epicuticular waxes and proteinaceous material, be called *nonpurified plant fiber* or NPPF. Conversely, we suggested that any highly *purified* natural plant cell wall "fibrous" component such as pure cellulose, hemicellulose, pectin or lignin be called *purified plant fiber* or PPF.

Because of the binding and adsorption properties of plant fibers (Eastwood, 1973), they could be expected to interact with other nutrients and affect their absorption and excretion. That these interactions occur and are chemically important is evidenced by the many reports suggesting that plant fibers bind bile salts and induce a hypocholesteremia. This evidence has been reviewed by Trowell (1973), Eastwood (1973), Cummings (1973), Kritchevsky et al. (1975) and ourselves, (Spiller and Amen, 1975a).

This study was designed to determine the effect of a nonpurified plant fiber (NPPF), a purified plant fiber (PPF) and a fiber-free diet (FF) on fecal output and composition of pig-tailed monkeys in the presence of three dietary lipid patterns.

Table 1—Analysis of plant fiber fed at the 2% level

Plant fiber	Composition	
Solka-floc[a] [purified plant fiber (PPF)][b]	Cellulose	89.0%
	Hemicelluloses	10.0%
	Lignin	0.3%
	Ash	0.1%
	Lipids	traces
Slippery Elm Bark[c] [nonpurified plant fiber (NPPF)][b]	Cellulose	29.3%
	Hemicelluloses	28.5%
	Lignin	6.6%
	Ash	13.8%
	Lipids	2.1%

[a] Brown Company, Berlin, N.H.
[b] According to Spiller and Amen (1975a, b)
[c] Cell wall analysis by D.A.T. Southgate, Medical Research Council (England), London

MATERIALS & METHODS

Diets and experimental design

Three separate experiments were conducted using five adult male pig-tailed monkeys (*M. nemestrina*) in each experiment. Three different diets were fed in each experiment for a total of nine diets. One diet in each experiment contained 2% purified plant fiber (PPF), one diet contained 2% nonpurified plant fiber (NPPF) and one diet was fiber-free (FF). The analyses of the plant fibers are given in Table 1. The dietary lipid patterns were additionally varied in the three experiments: Diets 1, 2 and 3 (Exp 1) contained 2.76% corn oil; diets 4, 5 and 6 (Exp 2) contained 2.5% coconut oil (high in medium chain triglycerides and saturation), 0.25% corn oil (to insure enough essential fatty acids) and 0.1% cholesterol; diets 7, 8 and 9 (Exp 3) contained the same lipid systems as diets 4, 5 and 6, but the cholesterol level was raised to 0.5%. All diets had the same total amount of fat kcal. The other dietary components were held constant for all diets and supplied all the known requirements of the pig-tailed monkey (Spiller and Amen, 1975c). The dietary nitrogen was supplied by a protein hydrolysate known to have a biological value similar to egg white, and was fed at the level previously determined to maintain a 10 kg monkey in a positive nitrogen balance.

The composition of the vitamin and mineral mixes, details of the processing of the diets and general nutritional information have been previously published (Spiller and Amen, 1975c; Spiller et al., 1975). The carbohydrate was fed as corn syrup solids. The ingredients were emulsified and fed in a liquid form to supply approximately 1 kcal/ml. 700 ml of each diet were fed per day per animal for a period of 2 wk. After a 4-day adaptation period, feces were collected for 10 days before the morning feeding. The feces were acidified with acetic acid, defoamed with octanol, weighed and homogenized. This fecal homogenate was then analyzed for dry matter, nitrogen, ash and total lipids according to previous methodologies (Spiller et al., 1975).

Animals and housing

All animals were adult, male pig-tailed monkeys (*M. nemestrina*) that had lived in our colony and been adapted to a liquid diet regimen for at least 6 months. The animals were housed individually in metabolic cages containing two removable floors to allow for complete fecal collections. The construction of the upper floor allowed the feces to fall onto the lower one, preventing sample loss or coprophage. Diets were fed in a bottle supplied with a ball point feeding tube to prevent dripping. Water was available *ad libitum*.

The mean weight of the five monkeys used for Exp 1 (diets 1, 2 and 3), Exp 2 (diets 4, 5 and 6) and Exp 3 (diets 7, 8 and 9) was 10.2, 10.6 and 11.0 kg, respectively. Previous experiments with *M. nemestrina* showed that these minor differences in weight had no significant effect on fecal output and composition.

Statistics

All statistical tests were performed at the $P < 0.05$ level of significance. The values are reported as daily mean ± standard error of the mean (S.E.M.) for a 10-day experimental period for the five monkeys on the dietary regimen.

Total fecal output

Analysis of the daily fecal output data from Table 2 shows that there is no significant difference between the induction of total fecal output (fecal bulk) by NPPF and PPF regardless of the lipid system fed (Exp 1, 2 and 3). The fiber-free diets, however, induced significantly less fecal output when the monkeys were fed the diets containing PUFA (Exp 1) and coconut oil with 0.1% cholesterol (Exp 2), than when the diets contained either fiber system ($P < 0.05$). When the cholesterol level was raised to 0.5% (Exp 3) the fecal output induced by the fiber-free diet was still slightly lower than for the two diets containing PF, but the difference was no longer significant at the $P < 0.05$ level. Perhaps this phenomenon is caused by an over-saturation with dietary cholesterol of the normal monkey gastrointestinal tract leading to an interference or alteration in function. This situation exemplified the effect of a PF:dietary component interaction overshadowing the "normal" effect of the plant fiber. Had this experiment (3) been performed without Exp 1 and 2, a misleading conclusion might have been drawn.

Fecal dry matter

A statistical analysis of the percentage of fecal dry matter, shown in Table 2 shows there is no significant difference on the effect either NPPF or PPF has on the percent fecal dry matter (and fecal moisture) in comparison to that induced by fiber-free diets in Exp 1 and 2. Both PF's induce a 45–46% fecal dry matter, compared to the 35–36% fecal dry matter induced by the fiber-free diets ($P < 0.05$). However, the presence of large amounts of dietary cholesterol (Exp 3) again causes different results. Here, there are statistically significant differences in percent fecal dry matter induced by all three diets. Unlike Exp 1 and 2, the NPPF fed in this diet induced a lower fecal dry matter ($P < 0.05$) than did the PPF.

Fecal nitrogen

Fecal nitrogen excretion is given in Table 2. The NPPF caused an increased excretion of nitrogen in the feces when compared to fiber-free or PPF diets ($P < 0.05$) in Exp 1 and 2. Since there was no difference in fecal nitrogen for either fiber-free or PPF diets, the increased nitrogen excretion induced by NPPF is probably not due to either cellulose or lignin, the two major components of the PPF used in these experiments. It may be hypothesized that the increased excretion of nitrogen induced by NPPF is due to (a) undigested cell wall-bound nitrogen products present in the NPPF; (b) adsorption by cell wall components of endogenous nitrogenous products from the gut; or (c) differences in fiber pattern in NPPF and PPF used. When cholesterol is present, there is no significant difference in fecal nitrogen excretion between any of the diets ($P > 0.05$). Moreover, the fecal nitrogen losses are higher for the PPF and FF diets. This again suggests a confounding of the complex interaction of dietary components as related to gastrointestinal physiology.

Fecal ash

The patterns of fecal ash excretion (Table 2) are similar for all three experiments. All diets containing NPPF (1, 4 and 7) always induce greater fecal ash ($P > 0.05$). This might be due to the larger content of mineral matter naturally present in the NPPF (13.8% ash) as compared to the PPF or FF diets. A strong interaction is evident between the type of dietary lipids fed and the subsequent fecal ash for the NPPF diets 1 and 4. The presence of PUFA seems to cause a much higher excretion of mineral matter than the more saturated medium chain triglycerides in the presence of the same PF.

Fecal lipids

The average daily fecal lipids data are presented in Table 2. The entire pattern of lipid-PF interaction is somewhat different than for the other fecal components studied. The fecal lipids induced by diets containing PUFA are lower than those induced by diets containing coconut oil and cholesterol. Moreover, for each dietary lipid system, the induced fecal lipids are lowest for the diets containing purified plant fibers. Statistical significance at the $P < 0.05$ level is found only in Experiment 1, but a somewhat lower level is noticed in all the experiments, sug-

Table 2—Effect of NPPF, PPF and FF diets on fecal output and composition in pig-tailed monkeys (M. nemestrina). (Means[a] ± S.E.M. for five monkeys[b] in each group)

	Experiment 1			Experiment 2			Experiment 3		
Diet	1 NPPF	2 PPF	3 FF	4 NPPF	5 PPF	6 FF	7 NPPF	8 PPF	9 FF
Total feces g/day	25.9a ±3.1	22.8a ±4.9	12.3b ±1.6	14.1a ±1.6	19.3a ±3.7	7.5b ±0.9	26.5a ±3.7	30.0a ±5.6	19.7a ±2.9
Fecal Dry Matter %	45.3a ±2.1	45.0a ±2.6	37.5b ±4.8	45.4%a ±1.8	45.2a ±1.6	37.5b ±3.6	40.0%a ±2.1	47.4b ±2.9	34.9c 3.8
Total nitrogen g/day	0.35a ±0.03	0.22b ±0.04	0.22b ±0.02	0.24a ±0.03	0.16b ±0.01	0.14b ±0.02	0.32a ±0.05	0.29a ±0.04	0.28a ±0.04
Total ash g/day	1.9a ±0.2	0.7b ±0.1	0.9b ±0.1	1.2a ±0.1	0.7b ±0.1	0.6b ±0.1	1.7a ±0.2	1.0b ±0.1	1.0b ±0.1
Total fecal lipids g/day	0.44a ±0.05	0.25b ±0.03	0.36a ±0.04	0.67a ±0.13	0.48a ±0.14	0.62a ±0.07	0.86aq ±0.24	0.76a ±0.12	0.82a ±0.09

[a] Results with similar letters or with no letters are not significantly different from each other at the $P < 0.05$ level. Results with different letters are significantly different from each other at the $P < 0.05$ level. All comparisons are made only within each experiment.
[b] Mean monkey weights: Exp 1, 10.2 kg; Exp 2, 10.6 kg; Exp 3, 11.0 kg.

gesting a common trend. However, no matter what dietary lipid system is fed, there is no difference in the induced excretion of fecal lipids between diets containing NPPF and diets that were fiber-free.

DISCUSSION

THIS EXPERIMENT was designed to determine if any significant physiological effects would occur when two fiber systems and varying *levels* of cholesterol and *type* of dietary triglycerides were fed for a short period of time. It was not intended to study the effects caused by a prolonged adaptation to either the presence of the fiber or the alteration in lipid system. This study suggests that plant fiber and dietary cholestrol *level* interactions, and plant fiber and *type* of dietary triglyceride interactions affect fecal excretion.

This preliminary investigation confirms that extreme caution is needed in attributing physiological and nutritional effects to "fiber" in general. Each plant fiber must be studied individually and results carefully analyzed. It appears that many contradictions in the literature might be due to the use of the terms "high-fiber" or "low-fiber" diet, and now we can also say to the lack of proper study of the correlation of plant fibers to other dietary components.

The pig-tailed monkey (*M. nemestrina*) appears to be an excellent model for plant fiber studies. Diets containing exactly the same amount of plant fiber each day can be fed to this animal for prolonged periods, while all other dietary components are carefully controlled.

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Ms received 7/31/75; revised 10/10/75; accepted 10/15/75.

Presented at the 35th Annual Meeting of the Institute of Food Technologists, Chicago, Ill. June 8–12, 1975.
The cooperation of Dr. D.E. Bidlack and Mr. R. Marks is hereby acknowledged.

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CHARACTERIZATION OF PRODUCTS FROM WET FRACTIONATION OF WHEAT BRAN

ABSTRACT

A study was conducted to determine the composition of a high protein flour (HPF) and a protein concentrate (WPC) obtained from the alkaline extraction of wheat bran. Baking characteristics of these fractions were determined using standardized techniques. Both HPF and WPC contain significant quantities of protein rich in lysine, and either can be used to fortify white bread with significant increases in protein and lysine contents. HPF can be added to a bread formulation in the largest amounts with least detrimental effect on bread qualities (loaf volume, crumb texture and color, crust appearance, aroma and flavor). WPC enhances protein content but rapidly lowers bread qualities; it can be added up to the 13% level and still yield an acceptable end product.

INTRODUCTION

A SERIOUS PROBLEM facing today's world is protein-calorie malnutrition. This problem is not due to shortage of food, but rather to poor utilization of existing food resources and the inability to economically transport food to locations of critical need. Since cereal grains are the primary source of carbohydrates and protein in the developing regions of the world, fortification and/or extension of cereal-based foods is one of many needed advancements that will contribute to an adequate food supply for the future.

Fog and Tinklin (1972), Tsen et al. (1973) and Marnett et al. (1973) investigated soy protein fortification of bread and cookie products respectively. Rooney et al. (1972) compared the baking qualities of the several oilseeds (cottonseed, peanut, sunflower, sesame). Rooney et al. stated that the oilseeds differ in their effects on dough mixing and loaf volume characteristics. Heating enhanced the breadmaking characteristics of cottonseed and sunflower proteins but was detrimental to those properties of peanut and sesame protein.

Wheat protein concentrate exists in two forms: a commercially produced low protein product (20% protein) obtained from the sifting of wheat bran, and a high protein concentrate (60–80% protein) obtained from the alkaline extraction of bran (Fellers et al., 1966; Woerman and Satterlee, 1974). The high protein wheat protein concentrate (WPC) compares favorably as a single protein source with soy protein in its nutritive value. WPC has a PER of 2.07 compared to 2.04 for soy protein (Woerman and Satterlee, 1974).

The purpose of this study was to determine the composition of a high protein flour and a protein concentrate obtained from the alkaline extraction of wheat bran. Secondly the baking characteristics of these fractions were determined using standardized techniques.

METHODS & MATERIALS

Wet fractionation of wheat bran

The fractionation of wheat bran was accomplished using a modification of the procedure described by Woerman and Satterlee (1974). The modification consisted of an initial bran wash with 10 volumes of water and a subsequent filtration through a 0.1 mm screen to separate suspended flour from bran. The high protein flour (HPF) was then spray dried in an Anhydro model spray dryer. The inlet temperature of the spray drier was 210°C with the outlet temperature at 90°C. The wet bran was finely ground with an equal volume of an aqueous NaOH solution (3.44g NaOH/liter) in a Waring Blendor using a Polytron Model BEW-5 rotor-stator head operating at 5000 rpm. Figure 1 illustrates the procedure for the recovery of a high protein flour (HPF) and wheat protein concentrate (WPC) from wheat bran.

In order to determine the yield of WPC from the modified extraction procedure, water washed bran was wet milled with the rotor-stator head for varying times and compared to dry bran which was pin milled up to six times. Reduction of particle size of the bran by wet milling and by dry milling were compared by measuring mean diameters of the particles with a microscope. Pin milling was performed on an Alpine Kolloplex Laboratory Mill 1602 pin mill operating at 14,000 rpm.

Compositional analyses

Proximate analyses were performed on breads which had either HPF or WPC incorporated. All proximate analyses were performed using AOAC procedures described for baked cereal products (AOAC, 1975). Crude protein was determined by the Kjeldahl method utilizing a factor of 5.7 for whole bran, HPF, whey and bran residue and 6.25 for WPC.

Amino acid analyses were conducted on all products using a standard 6N HCl, 24 hr vacuum hydrolysis at 110°C for all amino acids with the exception of tryptophan, which was released using a $Ba(OH)_2$ hydrolysis (Pataki, 1968). All hydrolyzates, both acid and alkaline, were analyzed on a Beckman 120°C amino acid analyzer.

Dough and baking characteristics of HPF and WPC

The dough mixing properties were determined at baking absorptions using a 10g mixograph as described by Finney and Shogren (1972). High protein flour (HPF) and wheat protein concentrate (WPC) were incorporated into the basic straing dough formulation of Marnett et al. (1973) at flour replacement levels of 5, 10, 15 and 25% for HPF, and 7, 13, 17 and 22% for WPC. Baking characteristics of heated WPC (121°C for 30 min) were also evaluated. All bread baking trials were performed in duplicate using the micro-baking technique of Van Scoyk (1939).

Baking quality of the bread was evaluated by measuring loaf volume as well as organoleptic characteristics. Sensory evaluation was performed using a 16-member panel to measure loaf texture, crust and crumb appearance, aroma and flavor. Analysis of the various loaves for significant sensory differences was performed using the Student Neuman Keul's test (Steel and Torrie, 1960).

RESULTS & DISCUSSION

THE TWO METHODS for the wet fractionation of wheat bran are illustrated in Figure 1. Washing of the bran prior to milling and alkaline extraction yields a high protein flour (HPF) which comprises over one-third of the original bran weight. Water washing removes much of the subaleurone cell material in the form of a HPF and significantly reduces the amount of carbohydrate which would have diluted the wheat protein concentrate (WPC) fraction. Table 1 gives the yield and protein content of all fractions obtained from the wet fractionation of the wheat bran. The two significant food products from this fraction, HPF from washed bran and WPC from unwashed bran, were further characterized.

The particulate size of the bran prior to alkaline extraction is a factor affecting WPC yield. Dry pin milling was the most

Table 1–Yield and protein content of components from wet fractionation of washed and unwashed bran[a]

	Water washed bran procedure		Unwashed bran procedure	
Component	Yield from water washed bran (%)	Protein content (%)	Yield from unwashed bran (%)	Protein content (%)
Whole bran	–	14.48	–	14.48
High protein flour (HPF)	35.92	17.35	–	–
Bran residue	58.61	10.08	79.20	9.54
Dry whey	7.12	31.64	14.10	27.97
Wheat protein conc (WPC)	0.67	45.29	8.30	70.59

[a] The various Kjeldahl conversion factors used were: 5.7 for HPF, bran residue and whey, and 6.25 for WPC and whole bran.

Table 2–The effect of wet and dry milling on the particle size of bran prior to extraction

Type of milling	Bran mean particle size (μ)
Unmilled bran	2600
Dry pin milling	
1 pass through mill	646
2 passes through mill	278
3 passes through mill	260
Wet-rotor stator milling	
1 min	858
2½ min	552
5 min	571
7½ min	487

Table 3–Amino acid composition of bran, high protein flour (HPF) and wheat protein concentrate (WPC) from washed and unwashed bran

	Gram of amino acid/100g protein			
			WPC from	
Amino acid	Whole bran	HPF	Unwashed bran	Washed bran
Aspartic acid	7.85	6.80	6.36	6.84
Threonine	3.09	2.89	3.42	3.26
Serine	4.17	3.81	4.84	3.98
Glutamic acid	20.52	24.08	22.43	20.76
Proline	5.39	6.01	6.69	5.70
Glycine	6.14	4.75	4.97	6.44
Alanine	5.57	4.80	4.28	5.09
½ Cystine	1.25	1.36	1.91	2.20
Valine	5.00	4.50	5.42	5.01
Methionine	1.08	1.01	1.67	1.36
Isoleucine	3.28	3.03	3.86	3.43
Leucine	6.08	5.67	6.96	6.77
Tyrosine	3.07	2.71	3.34	3.29
Phenylalanine	3.86	3.56	4.57	4.44
Lysine	4.10	3.62	3.79	4.46
Histidine	3.07	3.37	2.65	3.05
Ammonia	3.96	6.91	2.33	2.92
Arginine	7.51	6.14	7.32	8.14
Tryptophan	1.37	1.08	1.40	1.50

effective method to reduce the bran particle size, with two passes through the mill effectively reducing the particle size tenfold (Table 2). Additional pin millings were ineffective in further reducing particle size (Table 2). Wet milling washed bran with a rotor-stator attachment in a Waring Blendor required 2½ min to effectively reduce the bran particle to 1/5 original size. Additional wet milling time had little affect on particle size, but created excessive heat build-up (Table 2).

Table 3 gives the complete amino acid composition of the original bran, the HPF, and WPCs from both washed and unwashed bran. Lysine, which is the first limiting amino acid, was present in larger quantities in the HPF and WPCs, when compared to the lysine content of white flour (2.09g/100g protein).

Addition of HPF to the bread formulation replacing 5, 10, 15 and 25% of the hard red winter (HRW) wheat flour increased protein content from 8.15% up to 9.48% (Table 4). Addition of WPC to the formulation at flour replacement levels of 7, 13, 17 and 22% increased protein content from 8.15 to 18.76% (Table 4).

Fortification of the control white bread with HPF or WPC increased the lysine content of the final product. Addition of 25% HPF almost doubled the lysine content while increasing the protein content of the final product by 16%. Addition of WPC equal to 22% of the flour weight again nearly doubled the lysine content and increased the protein content by 130% in the final product (Tables 4 and 5).

Mixogram data shown in Figure 2 indicate that the presence of HPF and autoclaved WPC in the dough at all levels decreased mixing tolerance and increased mixing time slightly. The addition of unheated WPC had the greatest effect on dough properties. At all replacement levels of unheated WPC, there was an abnormal dough development. Although several baking absorptions were not quite optimum, as determined by mixogram characteristics, the curves gave uniform observations for each individual treatment.

The fortification with either HPF or WPC reduced the loaf

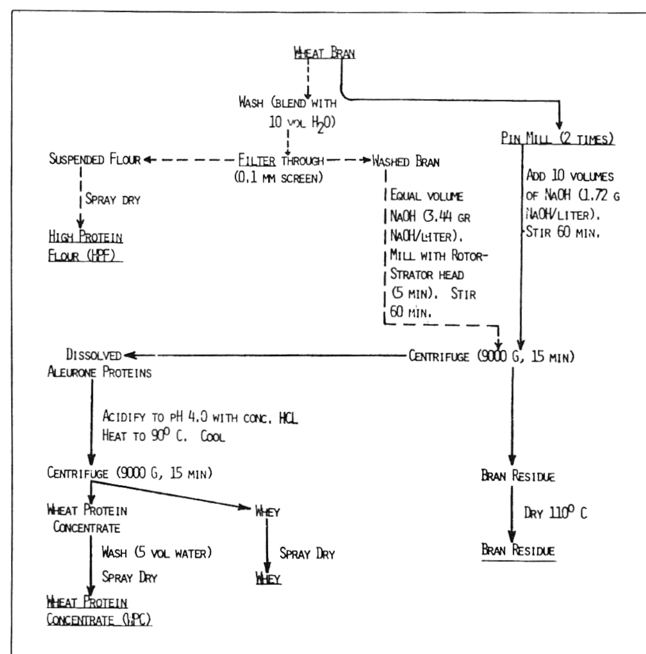

Fig. 1–The isolation of a high protein flour (HPF) and wheat protein concentrate (WPC) from wheat bran.

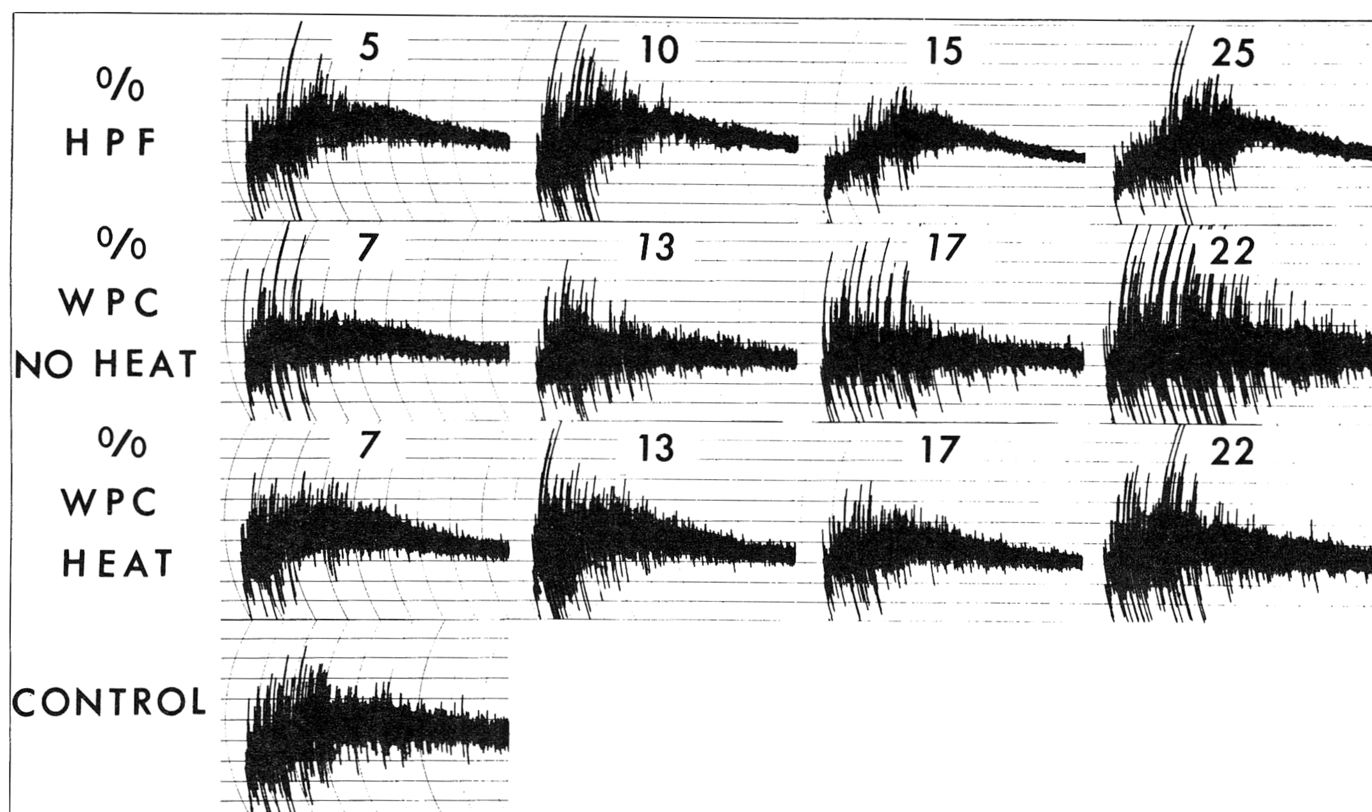

Fig. 2—Mixograms of flour containing the high protein flour (HPF), wheat protein concentrate (WPC) and autoclaved WPC at the replacement levels shown.

Table 4—Proximate composition of HPF and WPC fortified breads[a]

Bread sample	Fat (%)	Ash (%)	Protein (%)	Carbohydrates (%)
Control	3.52	2.31	8.15	56.03
HPF fortified[b]				
5%	3.18	1.58	8.37	52.11
10%	3.17	1.69	8.45	51.73
15%	3.32	1.94	8.99	52.54
25%	3.68	2.57	9.48	52.[illegible]2
WPC fortified[b]				
7%	4.25	1.41	11.30	53.04
13%	4.64	1.33	14.49	49.56
17%	3.80	1.37	15.91	48.92
22%	4.55	1.43	18.76	45.27

[a] Based on a 30% moisture content with Kjeldahl factor of 6.25 used for all protein values. Carbohydrate was determined by difference.

[b] Percent of HRW wheat flour replaced by HPF or WPC

Table 5—The amino acid composition of bread fortified with HPF and WPC from unwashed bran

		Grams amino acid/100g protein				
		HPF fortified[a]		WPC fortified[a]		
Amino acid	Control bread	10%	25%	7%	17%	22%
Aspartic acid	3.52	4.24	4.82	4.26	4.54	4.87
Threonine	2.39	2.38	2.66	2.54	2.41	2.32
Serine	4.13	4.02	3.89	4.34	3.72	4.90
Glutamic acid	33.77	31.16	30.81	31.48	29.65	29.51
Proline	9.59	8.41	7.58	8.46	8.22	7.21
Glycine	3.21	3.62	4.00	3.56	3.92	4.13
Alanine	2.68	3.17	3.67	3.12	3.57	3.91
½ Cysteine	1.37	1.45	1.23	1.85	1.17	1.16
Valine	3.87	3.99	4.18	4.24	4.46	4.48
Methionine	1.01	0.90	0.89	1.26	1.37	1.33
Isoleucine	3.19	3.14	3.36	3.22	3.55	3.34
Leucine	5.83	5.71	6.03	6.09	6.21	6.35
Tyrosine	3.47	2.82	3.25	2.12	3.03	3.08
Phenylalanine	4.11	3.94	4.21	4.21	4.28	4.14
Lysine	1.47	2.30	2.61	2.31	2.71	2.80
Histidine	1.48	2.35	2.42	1.96	2.32	2.26
Ammonia	6.92	6.01	4.97	4.52	4.53	4.58
Arginine	2.98	4.81	4.42	4.45	5.34	4.80
Tryptophan	1.08	0.75	1.78	0.85	1.04	1.16

[a] Fortified by replacing the HRW wheat flour with the % of HPF or WPC shown

volume of the final product. HPF, with its lower protein content, had the least detrimental effect on loaf volume (Fig. 3) and the best sensory panel acceptance (Table 6). Addition of HPF at 5–10% of the HRW wheat flour produced a loaf of exceptional quality. Higher levels of HPF lowered the texture, crumb color and flavor scores. No level of added HPF was considered objectionable by the panel even though the fortified loaves possessed a smaller loaf volume. HPF, which is golden-tan in color, imparts that color to the final loaf when used at levels ⩾ 15%.

Addition of unheated WPC greatly reduced loaf volume, whereas heating to 121°C for 15 min reduced its depressant effect on loaf volume. Because of the high protein content, WPC significantly altered loaf characteristics when used above the 7% level (Fig. 3, Table 6). Levels above 13% resulted in bread with the color, texture, aroma and flavor of whole wheat bread. This is illustrated in the sensory evaluation data shown in Table 6 and loaf volume data in Figure 3.

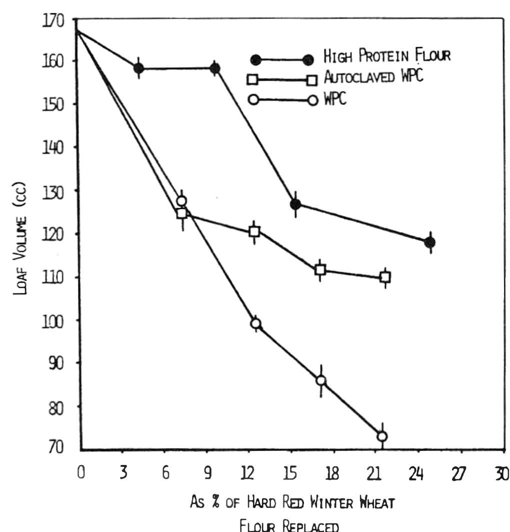

Fig. 3–The effect on loaf volume when hard red winter wheat flour (HRW) is replaced with high protein flour (HPC) and heated and unheated wheat protein concentrate (WPC). The verticle line through each data point represents the range in size of the loaves.

Table 6–Sensory evaluation of HPF and WPC fortified breads[a,b]

Bread sample	Crumb color	Crust appearance	Crumb texture	Aroma	Flavor	Overall eval
			HPF fortified			
Control	5.8a	5.7a	5.6a	5.2a	5.4a	5.4a
5%	5.5a	5.4a	5.4a	4.8a	5.3a	5.1a
10%	5.1a	5.3a	5.2a	4.6a	4.7a	4.9a
15%	3.8b	4.8a	4.1b	3.8a	3.4b	3.5b
25%	3.6b	4.4a	3.9b	3.6a	2.9b	3.3b
			WPC fortified			
Control	5.9a	6.4a	6.1a	5.6a	5.9a	6.1a
7%	5.3a	5.6a	5.3a	4.9a	5.2a	5.1a
13%	3.8b	3.5b	3.6b	3.4b	3.0b	3.0b
17%	2.9b	3.5b	3.3b	3.0b	2.0b	2.3b
22%	2.6b	3.3b	2.4b	2.9b	1.8b	1.9b

[a] A 1–7 hedonic scale was utilized with 7 being excellent, 1 being poor.

[b] Statistical analysis indicated that data for samples indicated by the letter a were not significantly different from the control loaf; characteristics of samples indicated by the letter b were significantly different from the control at the 0.05% level.

CONCLUSION

BRAN, a by-product of the dry milling of wheat can be further wet fractionated to yield a golden-tan high protein flour (HPF) or a tan colored wheat protein concentrate (WPC). A single wet fractionation procedure does not yield both products, but simple modifications in the procedure yield significant quantities of either protein product. Both the HPF and WPC contain significant quantities of protein rich in lysine. When either HPF or WPC are used to fortify white bread, the increases in protein content (16% for HPF and 130% for WPC fortification) and lysine content (almost doubled/100g protein for both) are significant.

Because the HPF is mainly a flour by nature, it can be added to a bread formulation in the largest amounts with the least detrimental effect on bread quality, i.e., loaf volume, crumb texture, etc. WPC, which is rich in protein, does an excellent job of enhancing the protein content of bread, but rapidly lowers the bread qualities mentioned above. WPC can be added up to the 13% level and still yield an acceptable end product.

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Ms received 8/4/75; revised 11/13/75; accepted 11/20/75.

Published as Paper Number 4047, Journal Series, Nebraska Agricultural Experiment Station. Research reported was conducted in part by a grant from the Nebraska Wheat Commission.

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PROTEIN CONCENTRATE FROM NORMAL AND HIGH-LYSINE CORNS BY ALKALINE EXTRACTION: PREPARATION

ABSTRACT

An alkaline extraction process was developed to produce protein concentrates and starch from ground normal and high-lysine corns. Optimum extraction of protein occurred at pH 11.7 in 0.1N sodium hydroxide with 150g corn per 900 ml solvent. The corn was extracted twice with sodium hydroxide solutions. After centrifugation, each alkaline extract was adjusted to pH 4.7 to recover the protein as a precipitate. Bran was removed from starch and protein by screening the second alkaline dispersion, and the protein and starch were separated by centifugation. The protein content (nitrogen × 6.25) of the concentrate varied between 63 and 71% and accounted for 52–63% of the total corn protein. High-lysine corn yielded more concentrate than normal corn, as well as more starch.

INTRODUCTION

ALTHOUGH PROTEIN from normal corn is deficient in lysine and tryptophan (Marias and Smuts, 1940), the endosperm mutant of *opaque-2* corn contained significantly greater amounts of lysine and tryptophan in its protein (Mertz et al., 1964; Nelson, 1969). The *opaque-2* gene changes both proportions of the protein fractions and lysine contents within the various proteins (Murphy and Dalby, 1971; Sodek and Wilson, 1971). Ethanol-soluble protein is sharply reduced in *opaque-2* corn proteins while the salt- and alkali-soluble proteins are increased.

A number of investigators have studied various aspects of corn proteins (Bressani and Mertz, 1958; Nielsen et al., 1970; Paulis et al., 1969; Robutti et al., 1974). Their results indicate that the corn kernel is bound by a matrix of disulfide cross-linked protein. In commercial wet milling, corn kernels are steeped in sulfurous acid from 28–48 hr to disrupt the matrix and permit separation of starch and protein (Anderson, 1970). Separation produces insoluble corn gluten and steep liquor which contains nitrogen consisting primarily of low molecular weight components (Christianson et al., 1965). Corn gluten accounts for 35 and 23% of the respective nitrogen in normal and high-lysine whole-kernel corn (Watson and Yahl, 1967). Reiners et al. (1973) eliminated lipid and water-soluble materials from commercial corn gluten to obtain a protein concentrate. Nielsen et al. (1973) prepared a protein isolate from corn germ.

It has long been established that alkali can solubilize much of the matrix protein of corn. Dimler et al. (1944) used an alkali process to prepare starch and protein from wheat flour, corn flour and other cereal flours. Since whole corn has a better amino acid composition and higher protein content than corn flour (endosperm), we used alkaline extraction to produce protein concentrates and by-products from ground normal and high-lysine corns. Our alkaline process provides more corn protein concentrate than the yield of gluten from standard wet milling as measured by Watson and Yahl (1967), especially from high-lysine corn.

EXPERIMENTAL

Corn

A normal corn, SX 48, and a high-lysine *opaque-2* corn, 50039, were supplied by Pfister Associated Growers, Aurora, Ill. The corns were ground twice in a hammer mill equipped with a screen containing 1/16-in. holes. Forty percent of the ground normal corn and 57% of the ground high-lysine corn passed through a 100 mesh screen. The normal corn has 11.6% protein (nitrogen × 6.25), dry basis, and the high-lysine corn, 11.5%. Some of the ground high-lysine corn was defatted by hexane.

Protein extraction

Ground corn (3–8g) was mixed with sodium hydroxide solution (0.025–0.1N) at a specified weight and volume ratio, stirred magnetically for 25 min and then centrifuged for 15 min in a Sorvall laboratory centrifuge at 3300 × G. A portion of the supernatant after centrifugation was analyzed for nitrogen by a micro-Kjeldahl method, and the remaining supernatant was freeze dried.

After 25 min of magnetic stirring, the effect on protein extraction was determined by additional mixing in a Waring Blendor (Model 700B, Waring Products Corp., Winsted, Conn.) for 1 min at low speed and for 1 min at high speed. The mixture was then centrifuged, and the supernatant was analyzed for nitrogen as described above.

Precipitation pH

The pH value, where the greatest precipitation of protein occurred, was determined by pipetting 6 ml of an alkaline extract of high-lysine corn into each of five centrifuge tubes and then adding hydrochloric acid solution dropwise to each tube until pH values ranged between 4.2–6.0. The mixture was stirred magnetically and then centrifuged at 3300 × G in a Sorvall laboratory centrifuge for 15 min. A portion of each supernatant after centifugation was analyzed for nitrogen by micro-Kjeldahl. Subtracting the protein in supernatant from that in the alkaline extract gave the amount of protein precipitated at each pH level.

Protein concentrate

To make protein concentrates and by-products by extracting protein at pH 10.7, 11.2 and 11.7, sodium hydroxide solutions of 0.05, 0.075 and 0.1N were needed. Ground corn (150g) and 900 ml of 0.1N sodium hydroxide were stirred for 25 min. The slurry at pH 11.7 was centrifuged at 3300 × G in a Lourdes centrifuge for 15 min, and the supernatant was decanted and adjusted to pH 4.7 with 6N hydrochloric acid to precipitate almost all the protein. The mixture was centrifuged at 3300 × G for 15 min to yield a precipitate and a supernatant, and they were freeze dried separately to get the first precipitate (protein concentrate) and supernatant (Fig. 1).

The alkaline residue from the first centrifugation was redispersed to original volume and pH by addition of water and sodium hydroxide solution (Fig. 1). This slurry was stirred magnetically for 25 min and passed through 100-mesh bolting cloth to remove bran. The slurry that passed through the cloth was centrifuged at 3300 × G for 15 min to obtain a supernatant, a starch layer and a layer above starch. The supernatant was adjusted to pH 4.7 by addition of 6N hydrochloric acid to precipitate most of the protein. The mixture was centrifuged at 3300 × G for 15 min to yield a precipitate and a supernatant, and they were freeze dried to obtain a second precipitate and supernatant. The starch, the layer above the starch and the bran that remained on the bolting cloth were each neutralized with 6N hydrochloric acid and freeze dried.

RESULTS & DISCUSSION

Solvent and stirring

The effects of pH and stirring on extraction of high-lysine corn protein at a ground corn-to-solvent ratio of 1:6 are shown in Table 1. The maximum percentage of corn protein extracted was at pH 11.6; the next highest was at pH 11.2. The

Table 1–Influence of pH and stirring on extraction of high-lysine corn protein (ground corn-to-solvent ratio, 1:6)

Sodium hydroxide normality	pH of slurry	Method of stirring	Protein extracted (%)	Protein in extract solids (%)
0.025	9.2	Magnetic	43	43
		Blending	43	40
0.037	9.8	Magnetic	45	43
0.045	10.4	Magnetic	52	46
0.048	10.6	Magnetic	61	49
		Blending	60	45
0.06	10.9	Magnetic	63	49
0.075	11.2	Magnetic	74	51
0.10	11.6	Magnetic	76	50

Table 2–Effect of corn-to-solvent ratio on extraction of high-lysine corn protein

Corn:solvent ratio	Protein extracted at pH (%)	
	10.5	11.6
1:3	Not determined	64
1:4	51	73
1:6	56	81
1:10	58	84

Table 3–Effect of precipitation pH on alkaline extract of high-lysine corn

pH	Protein precipitated (%)
4.2	68
4.7	70
5.1	69
5.5	68
6.0	64

protein content in extract solids (the dry alkaline extract without any addition of acid) changed from 40 to 51% in the pH range of 9.2–11.6. Since water, 0.5M sodium chloride and 0.1M acetic acid-0.5M sodium chloride extracted, respectively, 7, 17 and 13% of nitrogen from whole corn meal (Paulis and Wall, 1969), these solvents were not used.

The effect of stirring on extraction of corn protein was studied at pH 9.2 and 10.6 (Table 1). There was no increase in percentage of protein extracted but a small decrease in protein content of the extract solids when additional blending was done in a Waring Blendor, perhaps due to solubilization or occlusion of nonprotein material in the alkaline extracts. Only magnetic stirring was used for subsequent extractions, because it was simpler and gave better results than those with additional blending.

Corn-to-solvent ratio

Ground high-lysine corn was extracted with sodium hydroxide at various solid-to-solvent ratios from 1:3 to 1:10 at pH 10.5 and 11.6 (Table 2). At both pH values, the percentage of protein extracted increased more when corn-to-solvent ratio was increased from 1:4 to 1:6 than when corn-to-solvent ratio was increased from 1:6 to 1:10. At pH 11.6, the effect of corn-to-solvent ratio was more pronounced than that at pH 10.5. A corn-to-solvent ratio of 1:6 seemed to be a good compromise between the highest percentage of protein extracted and a minimum amount of extractant needed.

Precipitation pH

Five precipitation pH values ranging between 4.2 and 6.0 were evaluated, and the percentage protein precipitated from an alkaline extract of high-lysine corn varied between 64 and 70 (Table 3). The dependence of solubility on pH was not sharply defined in this range. Since the same starting alkaline extract of corn was used for each precipitation pH, the small difference in percentage protein precipitated was probably significant between pH 4.2 and 5.1. The maximum amount of protein precipitated was 70% at pH 4.7, and this pH value was used to precipitate corn proteins from subsequent alkaline extracts.

Products from corn

Alkaline extraction of ground corn gave seven fractions (Table 4). For high-lysine corn, the yield of protein concentrate increased substantially from 9 to 12% when pH was

Table 4–Yield and protein of products from aklaline extraction of high-lysine (HL) and normal (N) corns (solid-to-solvent ratio, 1:6 dry basis)[a]

Product	Yield (%)					Protein[b] of solid (%)					Total protein (%)				
	pH 10.7	pH 11.2		pH 11.7		pH 10.7	pH 11.2		pH 11.7		pH 10.7	pH 11.2		pH 11.7	
	HL	HL	N	HL	N	HL	HL	N	HL	N	HL	HL	N	HL	N
Protein conc (First ppt)	5	9	8	12	9	85	65	65	63	71	34	50	46	63	52
First supernatant	7	6	5	8	5	28	29	19	23	18	16	16	9	16	8
Second ppt	0.3	2	2	2	2	88	45	29	48	42	2	6	4	7	7
Second supernatant	1	1	1	2	1	30	26	16	18	14	3	3	1	4	1
Bran	38	14	45	16	31	7	8	8	4	8	22	10	31	6	21
Layer above starch	28	17	15	13	18	4	6	5	3	5	11	9	6	3	8
Starch	17	47	22	49	33	3	0.4	0.4	0.3	0.4	4	2	1	1	1
Total	96.3	96	98	102	99						92	96	98	100	98

[a] The ground corn was defatted by hexane for the pH 10.7 extraction but not defatted for the two others.
[b] Nitrogen X 6.25

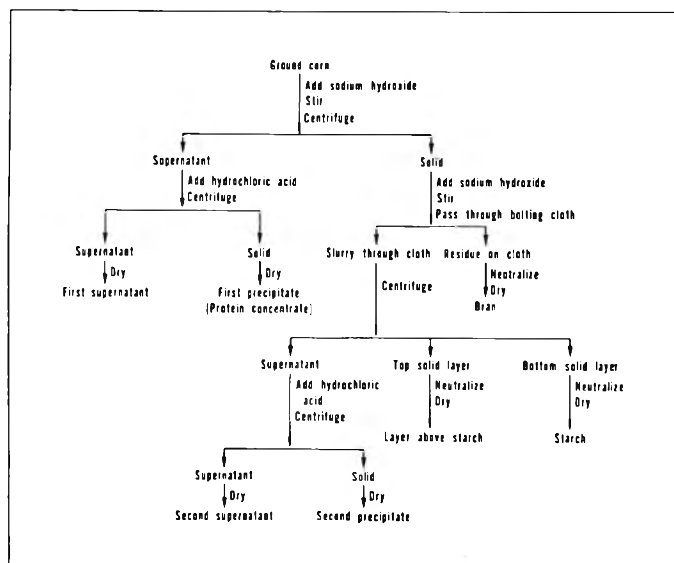

Fig. 1—Schematic diagram for preparing protein concentrate and by-products from ground corn by alkaline extraction.

changed from 11.2 to 11.7; whereas, the protein content of the concentrate remained about the same at 65 to 63%. The total protein accounted for by the protein concentrate increased from 50 to 63% as pH increased from 11.2 to 11.7. For normal corn, the yields of protein concentrate were about the same at pH 11.2 and 11.7, but protein contents increased from 65 to 71% when pH increased from 11.2 to 11.7. Also there was a large decrease in yield of bran and a large increase in yield of starch for normal corn at the higher pH.

Apparently variety of corn has an effect on extraction of protein at the same pH. The yields of the protein concentrate, first supernatant, second precipitate and supernatant (Table 4) were about the same at pH 11.2 for the two corns. The high-lysine corn also gave a much higher yield of starch and a much lower yield of bran compared with normal corn.

At pH 11.7, the yields of protein concentrate and first supernatant from high-lysine corn were higher than those from normal corn. The protein content of the protein concentrate was lower for the high-lysine corn compared with normal corn. The yields of bran and the layer above starch, as well as their protein contents, were lower for the high-lysine corn compared with normal corn.

An alkaline extraction of defatted high-lysine corn was carried out at pH 10.7 to see if good results can be obtained at the lower pH. The yields of protein concentrate and second precipitate and of starch were considerably lower than those at pH 11.2, whereas, yields of bran and the layer above starch were considerably higher. The defatted corn resulted in much higher protein content for protein concentrate and second precipitate compared with those from the same corn that was not defatted.

The high-lysine corn yielded more protein concentrate and more starch at pH 11.7 compared with normal corn. The improving yield (bushels per acre), the better nutritional value and the more favorable products obtained from alkaline extraction of high-lysine corn compared with normal corn may indicate a commercial potential for high-lysine corn protein concentrate prepared by alkaline wet milling. The corn protein concentrate may supply part of the total U.S. market potential for functional protein of approximately 3.1 billion lb annually (Hammonds and Call, 1972).

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Ms received 7/16/75; revised 11/17/75; accepted 11/24/75.

Presented at the 35th annual meeting of the Institute of Food Technologists, Chicago, Ill., June 8—12, 1975. The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

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PROTEIN CONCENTRATE FROM NORMAL AND HIGH-LYSINE CORNS BY ALKALINE EXTRACTION: COMPOSITION AND PROPERTIES

ABSTRACT

Protein concentrates and by-products produced by alkaline extraction from ground corn having normal and high contents of lysine were analyzed for amino acid composition, protein, starch, fat, ash, fiber and various neutral carbohydrates. A high-lysine concentrate contained 65% protein (nitrogen × 6.25) with 4.7g lysine and 4.0g total sulfur amino acids per 16g nitrogen recovered. The minimum nitrogen solubility of the concentrates was 3–5% near pH 5.5. The protein concentrates has good functionality relative to their emulsifying activity, emulsion stability and hydration capacity.

INTRODUCTION

AN ALKALINE PROCEDURE has been developed to produce protein concentrates and by-products from ground corn having normal and high contents of lysine (Wu and Sexson, 1976). During standard wet milling of corn, albumin and globulin proteins are lost although yields of zein and, possibly, glutelin are good. During the new alkaline process, which is predominantly based on soluble proteins, both yield and composition of the protein concentrates from high-lysine corn are better. The potential of these protein concentrates and by-products depends on their compositions as well as their functional properties. Consequently, we have determined the amino acid composition, protein, starch, fat, fiber, ash and total neutral carbohydrates of corn protein concentrates and by-products, as well as the nitrogen solubility, hydration capacity, emulsifying activity and emulsion stability of the concentrates.

EXPERIMENTAL

Materials

A normal corn, SX 48, and a high-lysine *opaque-2* corn, 50039, were ground twice in a hammer mill. Ground corn and 0.075N sodium hydroxide in a 1:6 ratio were stirred 25 min and then centrifuged at 3300 × G. The alkaline supernatant at pH 11.2 was adjusted to pH 4.7 and centrifuged to yield a precipitate and a supernatant, and they were freeze-dried separately to get the first precipitate (protein concentrate) and supernatant. The insoluble alkaline residue from the first centrifugation was restored to original volume and pH by addition of water and sodium hydroxide solution; the slurry was stirred and passed through bolting cloth to remove bran. When the slurry that passed through the cloth was centrifuged, a supernatant, a starch layer and a layer above starch resulted. The supernatant was again adjusted to pH 4.7 to precipitate most of the protein. This slurry was centrifuged to obtain a second precipitate and supernatant, and they were freeze dried. The starch layer, the layer above starch and the bran were neutralized and dried.

For making protein concentrate and by-products at pH 11.7, 0.1N sodium hydroxide solution was used instead of 0.075N. The preparation of protein concentrate and by-products has been reported in a companion paper in more detail (Wu and Sexson, 1976).

Methods

Protein content was calculated from duplicate micro-Kjeldahl analyses by multiplying percent nitrogen by 6.25 and correcting to dry basis. Gas-liquid chromatography (GLC) was used to determine total neutral carbohydrates of an acid-hydrolyzed sample (Sloneker, 1971). The cellulose fraction was analyzed by the same GLC procedure after other components were solubilized and removed (Sloneker, 1971). Fiber, ash and hydration capacity were determined by approved methods (AACC, 1971). Starch was measured by a polarimetric method (Garcia and Wolf, 1972). Fat was determined by GLC (Black et al., 1967) as well as by petroleum ether extraction (EE). Many experiments were carried out in duplicate, and the average value was reported. If the agreement between duplicate experiments was not within experimental error, duplicate experiments were usually repeated.

For amino acid analysis, each sample was hydrolyzed for 24 hr in refluxing 6N hydrochloric acid, evaporated to dryness and dissolved in citrate buffer at pH 2.2. A portion of the hydrolysate solution was analyzed in a Beckman Spinco Model 121 amino acid analyzer, and data were computed automatically (Cavins and Friedman, 1968).

Nitrogen solubility was determined by mixing 0.1g of protein concentrate with 10 ml of water, and either hydrochloric acid or sodium hydroxide solution was added dropwise to obtain the desired pH values. The mixture was stirred magnetically for 25 min, centrifuged at 1300 × G (or at 3300, 12100, or 27000 × G, if needed) for 20 min to separate solid and solution satisfactorily, and the supernatant was analyzed for nitrogen by micro-Kjeldahl. Emulsifying activity and emulsion stability were determined by the method of Yasumatsu et al. (1972) for a simple system, where the concentrate, soybean oil, and water were emulsified in a Virtis Homogenizer at 10,000 rpm and then centrifuged (for emulsifying activity) or heated and centrifuged (for emulsion stability).

RESULTS & DISCUSSION

Composition

The proximate analyses and starch contents of protein concentrates and by-products from normal and high-lysine corns are shown in Table 1. For extraction at pH 11.2 the protein concentrate from both normal and high-lysine corns had a protein content of 65% compared with less than 12% protein for the ground corns. Both concentrates had lower fiber, higher ash and higher fat compared with the ground corns. Ether extraction removes free lipid and part of the bound lipid, while GLC method removes all lipids. The lower fat value by EE compared with GLC for protein concentrate of high-lysine corn suggests that part of the bound lipid of the concentrate is not removed by EE. The second precipitates had lower protein (29 to 45%) and lower ash compared with the two protein concentrates.

The first and second supernatants from normal and high-lysine corns contained 17–29% protein, low fiber and high ash. These fractions contained albumins and globulins of corn, as well as salt and other water-soluble materials. The high ash contents of the two supernatants were partly a result of sodium chloride formed by neutralizing sodium hydroxide solution. Fat contents of the two supernatants from high-lysine corn were considerably lower than those from normal corn.

The bran had lower protein and fat, similar ash in general and higher fiber contents compared with the two ground corns. Starch content of the bran from high-lysine corn was considerably lower than that from normal corn. Because high-lysine corn is floury, separation of starch and fiber was better. The much higher fiber content of bran from high-lysine corn

Table 1—Composition of protein concentrates and by-products from high-lysine and normal corns (% dry basis)[a]

Fraction	Protein (N X 6.25)		Fat				Fiber		Ash		Starch	
			EE	GLC	EE	GLC						
	HL	N	HL		N		HL	N	HL	N	HL	N
					Extraction pH 11.2							
Ground corn	11.5	11.6	5.0	5.5	4.6	6.5	2.4	2.0	1.2	1.1	67.7	69.0
Protein conc (first ppt)	64.9	65.2	10.0	31.7	30.5	33.1	0.1	0.1	2.3	3.2	b	b
First supernatant	29.2	19.1	0.2	0.1	1.5	2.1	0.1	0.1	37.1	44.4	b	b
Second ppt	44.6	29.1	12.8	54.2	59.3	21.6	b	b	1.3	1.0	b	b
Second supernatant	25.9	16.5	0.6	b	2.5	9.7	b	b	47.6	43.0	b	b
Bran	8.2	8.1	1.8	1.7	1.1	1.3	9.3	3.2	1.9	1.0	42.8	75.7
Layer above starch	5.9	4.9	2.4	1.1	1.1	1.5	3.5	1.6	2.4	2.9	72.6	78.4
Starch	0.4	0.4	0.3	0.3	0	0.5	0.1	0.1	0.7	0.7	94.7	86.6
					Extraction pH 11.7[c]							
Protein conc (first ppt)	63.1	71.2	27.3		21.4				2.8	2.3	b	b
First supernatant	22.8	17.9	0.2		0.1				41.9	38.5	b	b
Second ppt	48.3	41.5	29.0		56.2				1.7	1.1	b	b
Second supernatant	18.0	13.9	0.3		0				40.7	57.9	b	b
Bran	4.3	7.9	1.3		1.4				2.0	1.5	54.8	69.8
Layer above starch	2.9	5.1	1.7		0.9				2.7	2.6	70.2	79.4
Starch	0.3	0.4	0.2		0.1				0.6	0.9	95.9	94.0

[a] Key to abbreviations: HL, high lysine; N, normal; EE, petroleum ether extraction; GLC, gas- iquid chromatography.
[b] Not determined
[c] Fat not determined by GLC and fiber not determined

compared with that from normal corn may be accounted for by the large difference in yields of the two fractions (14% for high-lysine and 45% for normal) reported in the companion paper (Wu and Sexson, 1976). The layer above starch from both corns had lower protein and fiber and higher starch and ash compared with bran. Both bran and the layer above starch contained starch, but the bran had less starch than the layer above starch. Starch from both corns was low in protein (0.4%).

Alkaline extraction at pH 11.2 was not too effective in breaking up the horny endosperm of normal corn. High-lysine corn being floury was readily extracted. A high lipid content in a protein concentrate may be detrimental during storage. Lipid can be removed by solvent extraction if desired.

At an extraction pH of 11.7, the protein concentrate from normal corn had higher protein content than the high-lysine equivalent or either protein concentrate extracted at pH 11.2. However, the higher yield of protein concentrate at pH 11.7 for high lysine more than compensated for the somewhat lower protein content of the concentrate from normal corn (Wu and Sexson, 1976). Apparently during extraction, protein and fat were concentrated in the protein concentrates for both normal and high-lysine corns at both pH 11.2 and 11 7. The second precipitates had protein contents of 42–48%, higher than the corresponding fractions extracted at pH 11.2

The first and second supernatants at pH 11.7 had somewhat lower protein contents than the corresponding supernatants extracted at pH 11.2. The first and second supernatants, as well as the second precipitate, from high-lysine corn had higher protein contents than those from normal corn.

Bran and the layer above starch from high-lysine corn were lower in protein than either normal corn extracted at pH 11.7 or high lysine extracted at pH 11.2. The starch from high-lysine corn contained only 0.3% protein.

Neutral carbohydrates

The amount and kind of neutral carbohydrates from acid hydrolysates of protein concentrates and by-products extracted at pH 11.2 from normal and high-lysine corns are listed in Table 2. The normal corn protein concentrate yielded no neutral carbohydrates, whereas the high-lysine concentrate produced 4% glucose. The first and second supernatants from the two corns yielded 11–21% neutral carbohydrates, which consisted mostly or exclusively of glucose. About half the sucrose will appear as glucose, and nearly all fructose is destroyed during acid hydrolysis (Sloneker, 1971). Since corn has about 2% sucrose and sucrose is likely to be in supernatants, part of the glucose from the supernatants is derived from sucrose.

Bran and the layer above starch from both corns yielded arabinose, xylose and a large amount of glucose; the two fractions from the high-lysine corn produced galactose in addition. Bran from high lysine corn yielded 7% cellulose. Table 2 lists 111% glucose for starch from high-lysine corn; glucose values above 100% were also observed for oat starches by the same method (Wu et al., 1973). Starch from both corns produced only glucose without any other neutral carbohydrate, and this absence, together with only small amounts of other materials, would indicate that the starch is quite pure.

Amino acid composition

The amino acid composition of protein concentrates and by-products from high-lysine and normal corns is listed in Table 3. The data were calculated to 100% nitrogen recovery and expressed in g amino acid per 16g nitrogen recovered. All the discussion on amino acids is based on g per 16g nitrogen recovered. The high-lysine corn had 4.8g lysine per 16g nitrogen in contrast to the lysine level of normal corn (2.8). The protein concentrate from high-lysine corn (extraction pH 11.2) had essentially the same high levels of lysine and total sulfur amino acids as the high-lysine corn, but the concentrate had significantly more leucine, tyrosine and phenylalanine and less aspartic acid than the corn. The second precipitate had a composition similar to the protein concentrate in general. Compared to the whole corn, the first supernatant had higher aspartic acid and proline but lower histidine, arginine, threonine, serine, alanine, half-cystine, valine, methionine, isoleucine, leucine, tyrosine and phenylalanine. Bran had higher

Table 2—Neutral carbohydrates from hydrolysates of protein concentrates and by-products from high-lysine (HL) and normal (N) corns (% dry basis, extraction pH 11.2)

	L-Arabinose		D-Xylose		D-Mannose		D-Galactose		D-Glucose	
Fraction	HL	N	HL	N	HL	N	HL	N	HL	N
Ground corn	1.1	1.2	1.8	1.6	0	0	0	0	92.0	76.0
Protein conc (first ppt)	0	0	0	0	0	0	0	0	4.3	0
First supernatant	0	0.8	0	0.2	0	1.3	0	0	20.7	17.9
Second ppt	0	0	0	0	0	0	0	0	Trace	5.0
Second supernatant	0	0.6	0	0	0	0	0	0	11.3	15.2
Bran	9.5	2.7	15.1	3.6	0	0	1.7	0	61.0[a]	79.3
Layer above starch	1.8	2.0	2.4	1.4	0	0	2.8	0	52.6	87.7
Starch	0	0	0	0	0	0	0	0	111	100.1

[a] Includes 7.0% cellulose

threonine, proline and leucine, but lower arginine, aspartic acid and half-cystine than ground corn. Protein concentrates from high-lysine corn extracted at pH 11.2 and 11.7 had similar amino acid composition. The small variation in lysine content of the high-lysine corn fractions suggests that zein (low in lysine) was proportionally distributed relative to the ground corn.

The lysine content of normal corn fractions was lower than the corresponding fractions from high-lysine corn. The lysine content of normal corn bran was considerably lower than in other fractions. Low lysine suggests that bran from normal corn has proportionally more zein, which is known to be low in lysine. Other fractions from ground normal corn had higher lysine than the ground corn. It appeared that more albumin, globulin and glutelin but less zein were proportionally extracted by alkali. The protein concentrates from ground normal corn extracted at pH 11.2 and 11.7 had similar amino acid composition, except the concentrate extracted at pH 11.7 had lower methionine.

Only the essential amino acids need to be considered for nutritional purposes. The essential amino acid composition of high-lysine protein concentrate meets or exceeds the FAO pattern (1965) for human consumption, while that of normal protein concentrate is deficient in lysine and slightly low in isoleucine.

Nitrogen solubility

The corn protein concentrates used for nitrogen solubility studies were extracted at pH 11.2. The percentages of nitrogen soluble at a number of pH values from 2–11 for normal and high-lysine corn protein concentrates are plotted in Figure 1. The high-lysine protein concentrate had a minimum solubility of 5% near pH 5.5. The corn protein concentrate was prepared by precipitation at pH 4.7 (minimum solubility), but the ionic strength of the solution due to salt present originally in corn and due to neutralization of alkali was higher than that used here. The difference in ionic strength likely accounts for the different pH values (4.7 and 5.5) for minimum solubility of protein concentrate. Also, the lower ionic strength used here makes the concentrate slightly soluble at pH 4.7 instead of insoluble. Solubility increased rapidly as pH increased beyond

Table 3—Amino acid composition of protein concentrates and by-products from high-lysine (HL) and normal (N) corns (g amino acid per 16g nitrogen recovered). (Extraction pH 11.2 unless otherwise specified)

			Protein concentrate (first precipitate)											
	Ground corn		pH 11.2		pH 11.7		First supernatant		Second precipitate		Second supernatant		Bran	
Amino acid	HL	N	HL	N	HL	N	HL	N	HL	N	HL	N	HL	N
Lysine	4.8	2.8	4.7	3.5	4.5	3.1	4.9	4.1	4.3	3.2	4.3	3.3	4.6	1.9
Histidine	3.5	2.9	3.5	3.5	3.5	3.3	2.9	3.7	3.6	3.5	3.4	4.2	3.5	2.5
Ammonia	2.1	2.7	1.7	2.6	2.0	3.3	3.5	5.3	1.9	3.1	3.6	4.5	2.1	4.1
Arginine	7.4	4.9	8.0	5.8	7.2	5.3	6.6	6.0	6.1	5.2	6.8	5.9	6.6	3.7
Aspartic acid	10.4	7.4	7.8	6.6	8.1	6.4	21.3	10.6	8.2	6.0	15.8	9.5	7.6	6.1
Threonine	4.0	4.0	4.0	3.8	4.2	3.7	3.0	3.3	4.3	3.6	3.1	3.5	4.9	3.6
Serine	4.9	5.3	5.3	4.9	5.3	5.0	3.3	3.6	5.4	4.6	3.6	3.8	5.3	4.8
Glutamic acid	18.4	22.0	18.4	19.1	18.4	19.7	19.5	15.8	19.0	18.8	17.7	16.5	17.0	21.5
Proline	7.4	9.6	6.9	8.8	7.2	7.8	9.6	7.3	8.3	14.0	9.8	9.5	8.6	8.6
Glycine	5.0	4.1	5.1	4.3	5.0	4.0	4.7	5.3	5.1	4.1	4.3	5.1	5.0	2.7
Alanine	6.6	8.4	6.9	7.5	7.0	7.4	4.7	4.6	7.1	6.6	4.8	4.9	7.3	8.3
Half-cystine	1.9	2.0	1.8	1.7	1.5	1.5	1.3	2.8	2.3	1.8	2.5	3.1	1.2	0.8
Valine	5.7	5.3	6.0	5.4	6.1	5.2	3.3	3.4	5.9	4.9	3.9	4.2	5.9	5.0
Methionine	2.2	2.4	2.2	2.7	2.4	2.2	1.0	1.4	2.4	2.4	1.4	1.6	2.3	2.1
Isoleucine	3.7	3.8	3.9	3.9	4.2	3.8	2.1	2.2	4.3	3.5	2.4	2.5	4.1	4.1
Leucine	9.0	13.1	10.0	12.3	10.5	12.5	3.0	3.7	10.9	10.5	4.8	5.5	11.3	15.0
Tyrosine	4.3	3.8	5.1	5.1	4.6	4.9	3.5	2.5	4.9	4.6	3.6	2.9	4.4	4.6
Phenylalanine	4.8	4.2	6.0	5.4	5.6	5.4	2.0	1.4	5.6	4.7	2.9	2.0	5.3	5.6

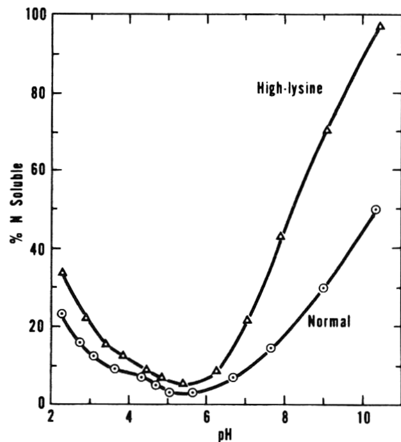

Fig. 1—Nitrogen solubility of corn protein concentrates (extracted at pH 11.2) at various pH values. Protein concentrate (0.1g) was stirred with 10 ml of water to which either hydrochloric acid (below pH 4) or sodium hydroxide solution (above pH 4.6) was added to arrive at the desired pH.

Table 4—Some functional properties of corn protein concentrates

Protein conc	Extraction pH	Hydration capacity	Emulsifying activity (%)	Emulsion stability (%)
High lysine	11.2	3.5	32	25
High lysine	11.7	4.2	55	54
Normal	11.2	3.6	49	24
Normal	11.7	4.2	22	21

6.5; almost all the nitrogen was soluble at pH 10.4. An increase in solubility was also observed below pH 5, and the solubility reached 34% at pH 2.3.

Protein concentrate from normal corn was less soluble than the high-lysine protein concentrate at all pH values in Figure 1, although the shape of the two curves are generally similar. Normal corn protein concentrate had a minimum nitrogen solubility of 3% also near pH 5.5. The increase in solubility was moderately rapid as pH increased beyond 7, and 50% of the nitrogen was soluble at pH 10.4. Solubility increased below pH 4 and 23% of the nitrogen was soluble at pH 2.3. Both protein concentrates are not very soluble in the pH 6–7 range where many food applications lie.

Hydration capacity

Hydration capacities (weight of sediment per weight of sample) of corn protein concentrates extracted either at pH 11.2 or 11.7 are shown in Table 4. The hydration capacity of high-lysine protein concentrate increased from 3.5 to 4.2 when extraction pH of the concentrate increased from 11.2 to 11.7. The same increase in hydration capacity of normal corn protein concentrate was observed when extraction pH of the concentrate increased. The difference in hydration capacity between 3.5 and 3.6 is probably not significant.

Emulsifying activity and emulsion stability

The emulsifying activity and emulsion stability of high-lysine protein concentrate increased significantly when extraction pH of the concentrate increased from 11.2 to 11.7 (Table 4). However, the emulsifying activity of normal corn protein concentrate decreased drastically while emulsion stability decreased slightly when extraction pH increased from pH 11.2 to 11.7. For concentrates extracted at pH 11.2, normal corn had considerably higher emulsifying activity than high-lysine corn. However, for concentrates extracted at pH 11.7, high lysine had much greater emulsifying activity and emulsion stability. A commercial soy protein isolate gave emulsifying activity and emulsion stability values of 45% under the same experimental conditions as the two corn protein concentrates (not in Table 4). Emulsifying activity and emulsion stability of high-lysine concentrate (extraction pH 11.7) and emulsifying activity of normal corn protein concentrate (extraction pH 11.2) were better than that of the commercial soy protein isolate.

Potential uses of protein concentrate and by-products

Corn protein concentrate may find application in foods as a protein ingredient. Since both ground corn meal and ground high-lysine corn meal were successfully extruded (Conway and Anderson, 1973), the residue after one protein extraction presumably can be extruded into either breakfast cereal or snack foods, or used as a starch source for fermentation. Pure starch can also be obtained as shown in the companion paper (Wu and Sexson, 1976). The protein concentrate from high-lysine corn has an attractive level of lysine and is also a nutritious product.

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Ms received 7/15/75; revised 11/17/75; accepted 11/24/75.

Presented at the 35th Annual Meeting of the Institute of Food Technologists, Chicago, Ill., June 8–11, 1975.

The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

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DEATH KINETICS OF PATHOGENS IN A PASTA PRODUCT

ABSTRACT

The death kinetics of *Salmonellae anatum* NF3 and *Staphylococcus aureus* 196E were determined as a function of temperature and water activity (a_w). Death kinetics were measured in a solid medium composed of a semolina-egg dough which was mixed at constant temperature in a Brabender Farinograph bowl. First order death kinetics were found at all conditions. The results showed that a maximum in resistance to heat occurred in the range of a_w of 0.75–0.80 for both organisms with a four- to tenfold increase in resistance respectively. These results show that to insure safety from pathogens, intermediate moisture food components may have to be pasteurized before mixing to the lower a_w.

INTRODUCTION

NUMEROUS STUDIES have been carried out to investigate the water relations of microorganisms (Scott, 1957; Troller, 1973). These studies were basically concerned with determining the minimum water activity (a_w) below which growth would not occur or determining the rate of growth as a function of a_w. These studies are important with respect to the storage stability and safety of foods. Of equal importance is the effect of a_w on the death rate of microorganisms as a function of temperature. This is an important parameter for the development of safe food processes because the time-temperature requirements in all pasteurization and sterilization processes are governed by the heat resistance of the indicator microorganisms.

With respect to bacterial spores, Murrell and Scott (1966) found that the greatest heat resistance was manifested at a_w values of about 0.2–0.4 when a_w was controlled from the vapor state. Angelotti et al. (1968) also reported that maximum heat resistance was observed within the same range of a_w. Alderton and Snell (1970) found that the optimal a_w for maximal heat resistance of bacterial spores was 0.28. Pace et al. (1972) showed continually decreasing survival of spores with increasing a_w from 0.5 to 0.9. All these results are consistent with those of Murrell and Scott (1966). This was further confirmed for bacterial spores by Harnulv and Snygg (1972) in studies where a_w was controlled by both vapor and aqueous solution systems.

With respect to vegetative cells, Goepfert et al. (1970) found that the heat resistance of salmonellae always increased as the a_w of the heating menstruum was reduced. Unfortunately, the range of a_w studied was only from 0.87–0.99 in sucrose solution due to the limitation of solubility and from 0.75–0.99 for aqueous glycerol solutions. Gibson (1973) showed that maximum heat resistance occurred in the range of 0.70–0.80 a_w for vegetative cells of bacteria and yeasts in aqueous sucrose or sucrose and glucose solutions. Elizondo-Ruiz (1973) and Hsieh et al. (1975) reported that heat resistance was enhanced in this same range of a_w for baker's yeast in glycerol-skim milk solutions and for *Staphylococcus* and *Salmonella* species in glycerol-Brain Heart Infusion solutions. Corry (1974) also found that maximum heat resistance of salmonellae in aqueous glycerol was at 0.6–0.8 a_w.

In general, therefore, spores exhibit maximum heat resistance around an a_w of 0.2–0.4, while vegetative cells are most heat resistant in the intermediate moisture food water activity range (0.65–0.90). The reasons for the changes in heat resistance with a_w and the apparent different responses between spores and vegetative cells have never been fully understood. It also should be pointed out that most of the cited studies only report either the percent of survival after a certain period of heating time or D-values at one temperature level. Moreover, these studies were generally conducted in a synthetic liquid medium. Thus, the results have had very little use for realistic food processes such as extrusion and drying in which both a_w and temperature can change simultaneously.

Of major interest among various food processes is the manufacture of macaroni products. Walsh (1972) has reviewed the bacteriological aspects, specifically the salmonella and staphylococcus problems, of pasta processing. Lee et al. (1975) reported that routine surveillance by the Food and Drug Administration showed *Staphylococcus aureus* contamination of some pasta products manufactured in the United States. The widespread consumption of these products and the increased number of seizures and recalls which involved microbiologically contaminated macaroni products (Walsh et al., 1974) suggest more extensive investigation is needed to determine the effect of pasta processing on death rate of microorganisms. The purpose of this study was to determine the effect of a_w on the heat resistance of two representative food pathogens over the ranges of a_w and temperature used in the drying of macaroni products. The temperatures of concern were 120–160°F (50–65°C) which is the range of most macaroni drying operations and a_w's of 0.93 to 0.6 which is the range that occurs in drying.

MATERIALS & METHODS

THE ORGANISMS used were *Staphylococcus aureus* 196E and *Salmonella anatum* NF_3. They were grown in TSYB (Trypticase soy broth (BBL) with 0.5% yeast extract (BBL)) at room temperature on a shaker for 24 hr. The final population was about 10^9–10^{10} organisms per ml.

The medium for the thermal destruction test was a typical extruded food–an egg macaroni product. For easier mixing and preparation, the raw materials–semolina (Como No. 1 Semolina, Capitol Durum Division, International Multifoods Corp., Mpls. MN) and whole egg solids (A.J. Pietrus & Sons Co., Sleepy Eye, MN)–were used instead of the finished product. The ratio of semolina to egg solids was determined first to meet the Standard of Identity (Anon., 1973) of 5.5% whole egg solids. Different amounts of water were then added to reach various water activities, which were measured by the vapor pressure manometric technique (Labuza, 1973). The isotherm of the solid medium is shown in Figure 1.

For thermal destruction tests in the solid medium, a water jacketed Brabender Farinograph bowl (C.W. Brabender Instruments, Inc., South Hackensack, N.J.) was used. This was connected to a constant temperature circulating water bath (Haake FK2, Haake Instruments, Inc., Saddle Brook, N.J.) and outfitted with stainless steel sheathed, subminature thermocouple probes (SCPSS-062G-6, Omega Engineering, Inc., Stanford, Conn.) to measure the medium temperature in the bowl. It was found that 5–6 min were necessary to achieve uniform distribution of microorganisms after addition of 1 ml inoculum while 12 to 13

minutes were needed to bring the medium temperature up to constant temperature. No temperature gradient was found within the medium after steady state was reached. The thermal destruction tests were conducted after uniform distribution of organisms in the medium was ensured and the medium temperature was at steady state. At preselected time intervals, approximately 1g of sample was taken out and weighed into a blender jar which contained 99 ml of 0.1% peptone water. The exact weight of the sample was recorded. The sample was then blended well and appropriate dilutions in peptone water were made and plated immediately by the surface spread technique on TSYA (Trypticase soy agar (BBL) with 0.5% yeast extract (BBL)). These plates were incubated at 37°C for 24 hr before enumeration.

RESULTS & DISCUSSION

ALL THE SURVIVOR CURVES were plotted as log N/N_o (where N_o is the number of vegetative cells at zero time and N is the number of survivors at various heating times). Some typical results are shown in Figure 2 for *S. aureus* 196E and Figure 3 for *S. anatum* NF_3. The decimal reduction time D was obtained from the linear portion of the survivor curve. The reciprocal of the decimal reduction time, times 2.3, gives the death rate constant k. The relationship between the heat resistance and water activity is shown in Figures 4 and 5 for *S. aureus* 196E and *S. anatum* NF_3, respectively.

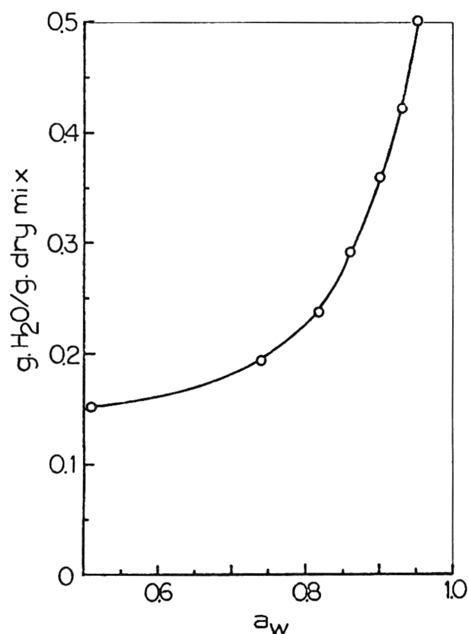

Fig. 1—Moisture isotherm of semolina-egg dough mix at 23°C.

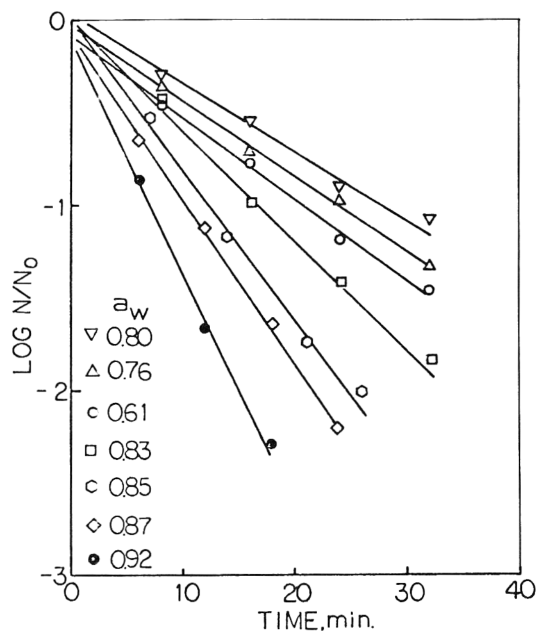

Fig. 3—Survivor curves of S. anatum NF_3 in semolina-egg dough at 54°C.

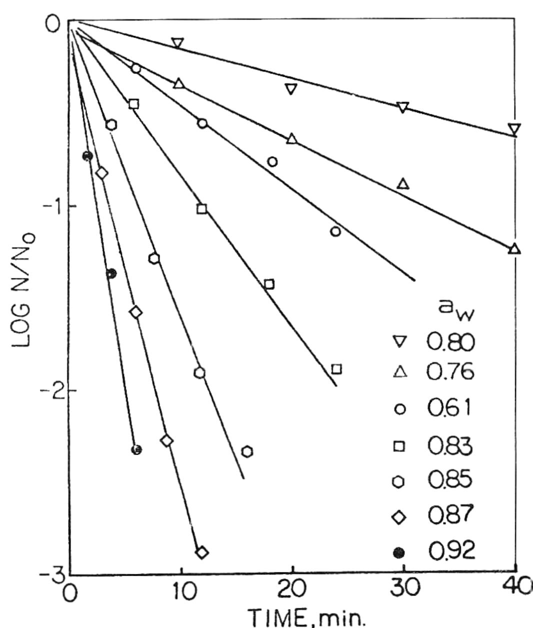

Fig. 2—Survivor curves of S. aureus 196E in semolina-egg-dough at 60°C.

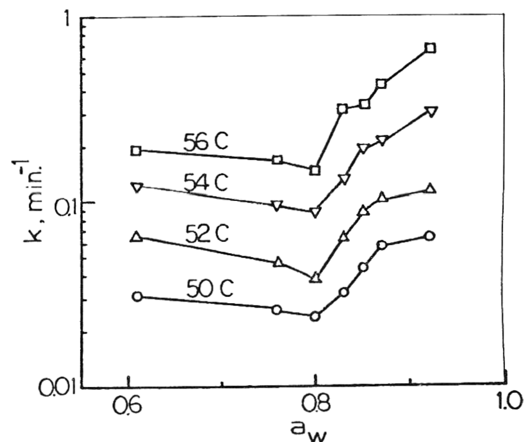

Fig. 4—Death rate constant (2.3/D) of S. anatum NF_3 as a function of a_w and temperature in semolina-egg dough.

Remarkable changes in the heat resistance of both *S. aureus* 196E and *S. anatum* NF_3 are observed when the a_w of the solid medium is lowered from 0.92 to that of the intermediate moisture food range. Since k is inversely proportional to D, the decimal reduction time, a decrease in k means a lower death rate. As seen in the range of 0.92 to 0.8 about a fourfold decrease in the death rate occurs for *S. anatum* NF_3 and a tenfold decrease in the death rate occurs for *S. aureus* 196E. Drastic changes in heat resistance of vegetative bacterial cells have been noted by Gibson (1973) and Corry (1974). Both of them found more than tenfold changes in heat resistance for salmonellae in this range of a_w's. It seems that both species and strains of organisms play a role in determining the changes of heat resistance with a_w. This was also noted by Goepfert et al. (1970).

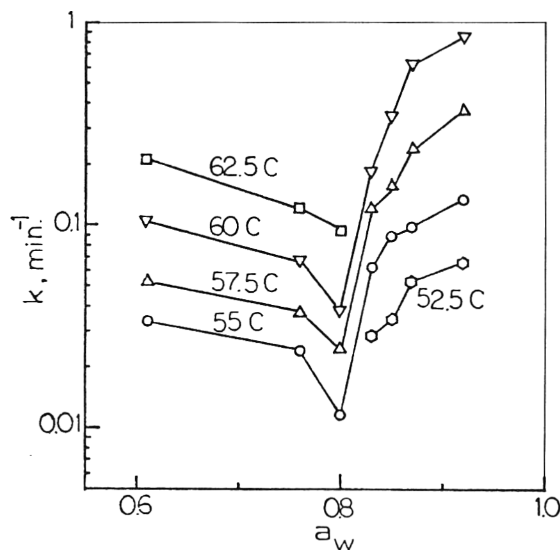

Fig. 5—Death rate constant (2.3/D) of S. aureus 196E as a function of a_w and temperature in semolina-egg dough.

It is also interesting to compare the results of this study with those of a previous study using a synthetic liquid medium, BHI-Glycerol (Hsieh et al., 1975). The k vs a_w values from both liquid and solid media are plotted at one temperature as shown in Figures 6 and 7 for *S. aureus* 196E and *S. anatum* NF_3, respectively. As seen, both pathogens show more heat resistance in solid media than in liquid media at the same a_w. In addition, both the a_w at which the maximum heat resistance occurs is slightly higher for solid medium than liquid medium. A small decrease in a_w of the solid medium is possible during the thermal destruction study because of water evaporation. However, this cannot fully explain the difference. The major reason is probably due to the nature of the medium. Presence of discontinuities (Matz, 1965) and formation of agglomerates (McDonough and Hargrove, 1968) or other factors which are not directly related to the measured a_w are also suggested.

The phenomenon that the heat resistance of vegetative cells increases when a_w is lowered from 1.0 to that of IMF range have been reported by many others (Riemann, 1968; Goepfert et al., 1970; Baird-Parker et al., 1970; Elizondo-Ruiz, 1973; Gibson, 1973; Corry, 1974; Hsieh et al., 1975). The results were similar whether a_w was controlled by vapor humidification of the cells or by adding a_w lowering agents, such as glycerol, sucrose, glucose or NaCl to the heating medium. The reason for this has been discussed by Gibson (1973), Elizondo-Ruiz (1973) and Hsieh et al. (1975) and reviewed by Hsieh (1975). Further research is still needed in this area to explain the reasons. However based on these results it can be seen that a maximum in heat resistance occurs in the IMF range.

CONCLUSION

THIS STUDY shows that the death rate of the vegetative cells of salmonellae and staphylococci is at a minimum in the intermediate moisture range of a_w around 0.8 at pasteurization temperatures (50–65°C) for solid medium. Although similar phenomenon was also shown for liquid medium, both the heat resistance and the rate of change in the heat resistance with a_w is different for different media. Thus, the heat resistance of microorganisms in foods cannot be inferred directly from data collected from tests in other liquid or synthetic media but must be experimentally determined in that particular food.

Because of an increase in the heat resistance when a_w is

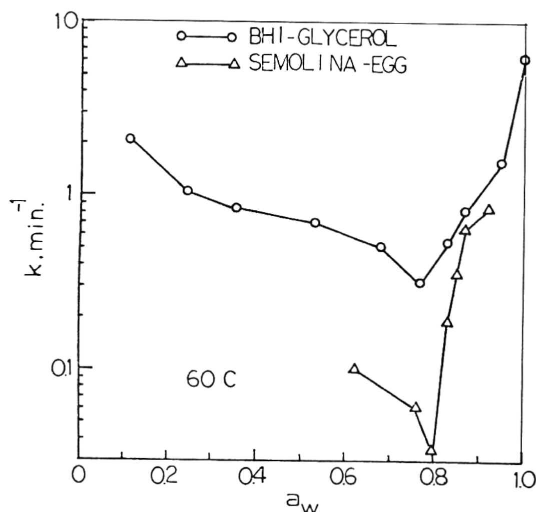

Fig. 6—Comparison of death rate constants for S. aureus 196E in brain-heart infusion – glycerol vs semolina-egg dough.

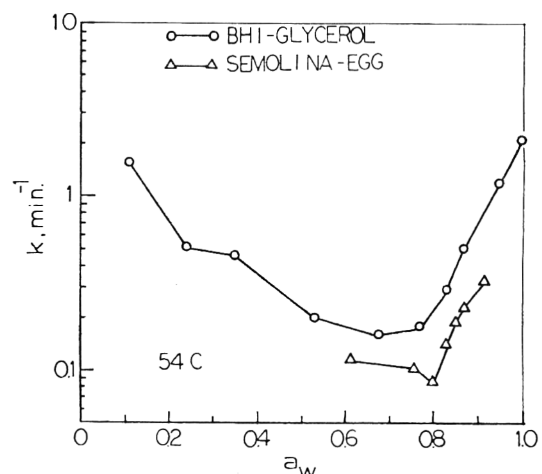

Fig. 7—Comparison of death rate constants for S. anatum NF_3 in brain-heart infusion – glycerol vs semolina-egg dough.

lowered, it seems advisable to pasteurize the food at high a_w whenever it is feasible. This will ensure adequate pasteurization with minimum cooking or processing. The data collected in this study are also very useful for prediction of the death of organisms during processing of macaroni products in which changes in a_w and temperature are known.

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Ms received 7/19/75; revised 10/3/75; accepted 10/6/75.

Presented at the 35th Annual Meeting of the Institute of Food Technologists, Chicago, Ill., June 8—12, 1975.

This is paper No. 9176 from the University of Minnesota Agric. Exp. Station. This study was supported in part by the University of Minnesota Agricultural Experiment Station Project No. 18-52M and Contract #NAS 9-12560, Lyndon Johnson Space Center, Houston, Texas.

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EFFECT OF GLYCOALKALOIDS AND PHENOLICS ON POTATO FLAVOR

ABSTRACT

The principles of the flavor profile method were used to establish the relationship between the flavor of potatoes and their total glycoalkaloid and total phenolic contents. Tuber tissues from the seven potato clones that had glycoalkaloid contents in excess of 14 mg/100g were rated as bitter by the taste panel. The correlation between glycoalkaloid contents and bitterness ratings for the 13 clones included in the study was highly significant (r = 0.93). Tissues that had glycoalkaloid contents in excess of 22 mg/100g also produced a mild to severe burning sensation in the mouth and throat. The intensity of the burning sensation was highly correlated (r = 0.97) with glycoalkaloid contents. The correlations between phenolic contents and either bitterness or burning ratings were not significant.

INTRODUCTION

ONE OF THE QUALITY factors sometimes noted as objectionable in cooked potatoes is an unpleasant, bitter flavor frequently associated with green potatoes. Potatoes that are exposed to light often develop elevated contents of glycoalkaloids, and the bitterness of green potatoes is believed to be caused by these abnormal levels.

According to Burr (1966), none of the four primary taste sensations of sour, salty, sweet, or bitter is ordinarily perceptible in normal cooked potatoes, although potatoes may become sweet if improperly stored or bitter if exposed to light for long periods of time. In addition to taste, the term "flavor" when applied to potatoes is also commonly used to denote a whole complex of sensations including aroma, mouth-feel, texture and even appearance (Burr, 1966).

Although the intensities of normal potato flavor and some types of objectionable off-flavors are probably varietal characteristics reflecting differences in chemical composition (Salaman, 1926), infection by certain pathogens (Burton, 1966) or soil treatments with pesticides such as benzene hexachloride (Potter et al., 1956) can also impart unpleasant flavors.

Bitterness is the most commonly studied component of taste in potatoes and the most frequently noted off-flavor (Baerug, 1962; Bomer and Mattis, 1924; Burr, 1966; Hilton, 1951; Lepper, 1949; Schwarze, 1962). In most of the reports on bitterness in potatoes no attempt has been made to relate the quantity of the suspected bitter compounds to even the most rudimentary organoleptic data. Baerug (1962) found a significant negative correlation between solanine content and flavor when freshly dug tubers were exposed to direct sunlight in the field for up to 72 hr. After only 6 hr of sunlight exposure, a deterioration in flavor was noted, while solanine content increased from 5 to 20 mg/100g fresh weight on a whole tuber basis. However, the tubers for organoleptic analysis were peeled before cooking, and these peeled tubers contained only 6 mg/100g of solanine even after 72 hr of sunlight exposure. Baerug did not specify the minimum level of solanine that adversely affected flavor or indicate whether bitterness was associated with the "deterioration in flavor" of the light exposed tubers. According to Bomer and Mattis (1924) and Hilton (1951), potatoes that are sunburned and have excessive solanine contents are very bitter and leave a persistent acrid or scratchy sensation in the pharynx. Potatoes that have not been exposed to light but have elevated solanine or glycoalkaloid contents due to unusual growing conditions (Bomer and Mattis, 1924; Hilton, 1951) or genetic background (Schwarze, 1962) can also be very bitter.

Gull and Isenberg (1960) found that a taste panel could not detect flavor differences in peeled, cooked tubers that had been exposed to 0 or 144 hr of flourescent light. However, solanine contents in the peeled tubers from the two treatments were also about equal. Mondy et al. (1971) suggested that bitterness and astringency in potatoes were related to their phenolic content. They found a significant correlation between phenolic content and bitterness and a highly significant correlation between phenolic content and astringency.

The purposes of this investigation were: to determine the relative importance of total phenolic and total glycoalkaloid contents in imparting bitterness and other off-flavors to potatoes; to determine the minimum levels at which these compounds affect flavor, and to define the taste and mouth feelings produced by elevated levels of these compound, in otherwise normal potato tissue.

MATERIALS & METHODS

Sample preparation

Potatoes for this study were grown at Presque Isle, Me., and Davis, W. Va. After screening more than 25 clones for glycoalkaloid and phenolic contents, a sample of 13 clones with a wide range of glycoalkaloid and phenolic contents was selected for flavor evaluations. Because both glycoalkaloids and phenolics are concentrated in the cortex tissues, the range of contents in the sample of 13 clones was extended still further by using only the cortex tissues of the tubers for chemical and flavor analyses.

Tubers were removed from storage at 4°C and peeled while still cold to minimize oxidation. Cortex tissues were carefully separated from the pith by slicing and then cutting around the vascular ring. Because it was difficult to remove all peel around eyes, the eyes were excised.

Samples for flavor evaluation were cooked in a steamer for 30 min and riced. Two 20-g samples of the cooked tissue were also analyzed for glycoalkaloid and phenolic content.

Chemical analyses

For glycoalkaloid and phenolic analyses the tissues were blended for 5 min in the extracting medium (95% ethanol, acidified with 25 ml glacial acetic acid per liter) and then extracted overnight in a Soxhlet apparatus (Sanford and Sinden, 1972). The extract was divided equally for glycoalkaloid and phenolic analyses. The method of Rosenblatt and Peluso (1941), with chlorogenic acid as the standard, was used to determine total phenolic contents; the method of Sanford and Sinden (1972), with α-solanine as the standard, was used for total glycoalkaloid contents.

Flavor evaluations

For flavor evaluations nine judges were trained to identify and rate the intensity of several important flavor components of potato using the principles of the flavor profile method. Caffeine at concentrations

of 0.05%, 0.10% and 0.20% (w/w) dissolved in normal, cooked and riced pith tissue (variety Irish Cobbler) was used as a bitterness standard to train the panel to distinguish degrees of bitterness. Bitterness responses of the judges were recorded every 30 sec for the first 2 min, since Neilson (1957) had shown that different types of bitter compounds could be differentiated by the rate at which the bitterness reached a peak intensity and then subsided. The maximum intensities reported by the judges were used to calculate the average bitterness ratings for the potato samples and for the caffeine standards.

Citric acid was dissolved in normal pith tissue at the same concentrations as caffeine to train the judges to detect sourness. During preliminary testing and training the panelists frequently noted the following additional flavors in moderate to high glycoalkaloid tissue samples: a warming sensation that often developed with time into a distinct burn throughout the mouth and throat; a spicy, hot-pepper effect; a "bite;" astringency; a "metallic" aftertaste; an "earthy" flavor; unusual dryness; and many other less consistent flavor impressions. All of the taste and mouth-feel impressions of the panelists were recorded, and the times when the impressions were reported.

We used the time-intensity method, developed by Neilson (1957) to measure bitterness in cough syrups, to measure both bitterness in potatoes and the distinctive burn response that was evoked by certain potato samples. All of the panelists' impressions of the sample were recorded over a 6 min interval; particular attention was given to the intensity of the bitterness, astringency and burn, by asking each panelist to judge the degree of each of these factors at specific intervals.

Before each flavor evaluation of a 10-g unknown sample, the panelist was given a 10-g sample of normal pith tissue (variety Irish Cobbler), in order to standardize the evaluations. Only rarely did this rather bland-tasting pith tissue evoke any response other than "a good potato flavor." In all cases the taste evaluations were conducted separately with individual panelists in an isolated setting, and panelists had no opportunity to compare their ratings or impressions. All samples were warmed (27–38°C) and presented to panelists 1–2 min later.

Because of the lingering nature of bitterness and burning sensations, a panelist reporting more than a slight degree of either sensation in an unknown sample was not asked to evaluate another unknown sample for more than 4 hr. Clones were chosen for taste evaluation and chemical analyses in a randomized order, and the whole experiment was repeated with different randomization.

Minimum levels

Because Baerug's results (Baerug, 1962) suggested that glycoalkaloid contents as low as 5 mg/100g in peeled tuber tissue could cause a deterioration in flavor, we attempted to determine a minimum level at which glycoalkaloid content begins to affect flavor. We prepared tissue samples with glycoalkaloid contents of 14, 11, 7.5 and 4.2 mg/100g by mixing tissues of clone 4 with tissues of clone 12. Panelists were asked to report any differences in flavor they detected, in paired-comparison difference tests (Larmond, 1967).

Additions of glycoalkaloids and phenolics

For determining the effects of pure glycoalkaloids on flavor and the minimum levels of glycoalkaloids that can affect potato flavor, pure α-solanine (K&K Labs.) and a purified mixture of α-solanine and α-chaconine from potato sprouts (Sinden et al., 1973) were added to normal, cooked and riced pith tissue (variety Irish Cobbler). The glycoalkaloids were added to the tissue at rates of 15, 20 and 60 mg/100g by dissolving in 1 ml of 0.001N HCl and mixing the solutions with the cooked potato tissue at a rate of 1 ml to 20g of tissue. Check tissues for this treatment were prepared using 0.001N HCl titrated to pH 5.3 and mixed with the tissue at the same rate.

Tyrosine and chlorogenic acid are the major phenolic compounds found in peeled tuber tissues (Schwimmer and Burr, 1959). To test the effects of high concentrations of these two compounds on potato flavor, each compound was dissolved separately in normal, cooked and riced pith tissue (variety Irish Cobbler) at a rate of 120 mg/100g of tissue.

RESULTS & DISCUSSION

Bitterness and burning

Cortex tissues of the seven clones with glycoalkaloid contents of more than 14 mg/100g were consistently rated as bitter by the taste panel (Table 1). All of the panelists detected some degree of bitterness in all samples of clones 1 and 2, ranging from slight bitterness (rating of 1) to very strong bitterness (rating of 4). Both of these clones, which had glycoalkaloid contents of over 50 mg/100g in the cortex tissues, had mean ratings of more than moderately bitter, based on 0.10% caffeine as the moderately bitter taste standard.

The six clones that had glycoalkaloid contents between 0.7 and 7.3 mg/100g in their cortex tissues (clones 8 through 13) all had mean bitterness ratings $\leqslant 0.2$ (1.0 equals slightly bitter). For two of these six clones (clones 8 and 13) there were no reports of bitterness from tasting the 36 samples. For the clone with the highest bitterness rating among these six (clone 9 with a mean rating of 0.2) there were only three reports of bitterness from the 18 samples that were tasted, and these three reports were of slight bitterness.

These results indicated that the cortex tissues of these six clones were similar in their bitterness to the pith tissue (variety Irish Cobbler) which was used as a standard for the taste tests. All nine panelists described the taste of this standard pith tissue with a glycoalkaloid content of 0.8 mg/100g and a phenolic content of 5.6 mg/100g as bland, with no bitterness, burning or astringency.

In contrast, cortex tissues of clone 7 with a glycoalkaloid content of 14.0 mg/100g had a mean bitterness rating of 0.8. There were nine reports of bitterness from tasting the 18 tissue samples of this clone, and two of the nine reports were of moderate (rating of two) to strong (rating of three) bitterness. Thus it appears likely that cortex tissues of clone 7 were more bitter than the pith tissue (variety Irish Cobbler) used as a standard for the taste tests.

A moderate to severe burn of the mouth and throat was also reported by panelists after tasting tissues with high glycoalkaloid contents. Usually the burning sensation started as a slight warmth on the tongue and roof of the mouth, about 1 min after the sample was tasted. The burning sensation then increased in intensity and spread to the back of the mouth and into the throat. Most panelists reported a sore or scratchy throat that often persisted for several hours after tasting samples with glycoalkaloid contents greater than 25 mg/100g. In addition to the term "burning," panelists also described the sensation as "warming," "glowing," "peppery," "spicy," or "numbing, as with a Novocain injection."

Bitterness and burning were highly correlated with glycoalkaloid contents. Both correlation coefficients were highly

Table 1–Effect of glycoalkaloid and phenolic contents on potato flavor

Clone[a]	Bitterness rating[b] (0–4 scale)	Burning rating[b] (0–4 scale)	Glycoalkaloid content (mg/100g)	Phenolic content (mg/100g)
1	2.4	3.4	58.0	29
2	2.2	3.2	51.0	43
3	1.8	2.0	25.0	17
4	0.9	1.7	23.0	23
5	1.3	1.7	22.0	41
6	1.9	1.7	22.0	33
7	0.8	0.6	14.0	59
8	0.0	0.1	7.3	27
9	0.2	0.2	5.9	30
10	0.1	0.0	4.4	31
11	0.1	0.1	2.0	29
12	0.1	0.0	0.9	24
13	0.0	0.0	0.7	21
LSD (0.05)	0.64	0.71	5.7	6.7

[a] Clones 1 through 8 are breeding lines; 9 through 13 are cultivars.
[b] Means of 18 evaluations; 0 = no bitterness or burning, 4 = very strong bitterness or burning.

significant (p = 0.01), 0.93 for bitterness, and 0.97 for burning and glycoalkaloid content.

Although bitterness and burning were highly correlated (r = 0.95), they were readily separated into two distinct flavor components by the time-intensity method (Neilson, 1957). In tissues that had moderate glycoalkaloid contents (clones 3–7), bitterness was usually reported in less than 30 sec and reached a peak intensity about 1 min after tasting the sample. The panelists did not usually notice warming or slight burning of the mouth until more than a minute had elapsed, and the sensation peaked in intensity at 3–5 min. With tissues containing very high glycoalkaloid levels (clones 1 and 2), warming or burning sometimes was reported within the first minute after tasting, but it was always preceeded by bitterness (Fig. 1); the burning sensation continued to increase between 1 and 3 min, while bitterness subsided during this period.

Astringency

The variation among panelists in their abilities to recognize and rate the intensity of astringency in the potato samples was much greater than for the bitterness and burning flavor components. Those panelists who did report astringency often had difficulty in separating this sensation from the burning sensation. Frequently the reports of astringency coincided with or were followed immediately by reports of a warming sensation on the tongue and throughout the mouth. Because of the variability among panelists in reporting astringency and the possibility that astringency was a part of the warming or burning sensation, the astringency ratings were not averaged or analyzed for correlations with glycoalkaloid and phenolic contents.

However, all 47 reports of astringency from the 234 samples were evoked by samples from clones 1 through 6 that had glycoalkaloid contents of at least 22 mg/100g. In all cases reports of burning always followed or coincided with the reports of astringency. Astringency was not reported in samples from clones 7 through 13 that had glycoalkaloid contents less than 22 mg/100g. Thus, it appears that astringency is also associated with high glycoalkaloid contents in potatoes. Other responses that were frequently evoked by moderate to high glycoalkaloid samples and that appeared to be associated with glycoalkaloid content were: "a metallic aftertaste," "a bite," "a coating of the teeth and mouth," and a nondescript "unpleasant aftertaste."

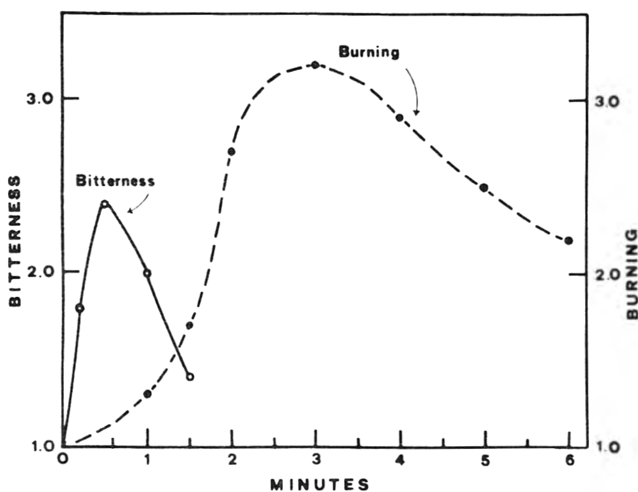

Fig. 1–Difference in rates of development of the intensities of bitterness and burning from tasting high glycoalkaloid (clone 2) potato tissue. Each point is the mean of 18 flavor evaluations.

Table 2–Effects on flavor from addition of solanine or a natural mixture of potato glycoalkaloids to normal potato tissue

Glycoalkaloid addition	Rate (mg/100g)	Bitterness[a] (0–4 scale)	Burning[a] (0–4 scale)
None[b]	–	0.0	0.0
Mixture[c]	15	0.4	0.2
Solanine	20	0.9	0.5
Mixture[c]	20	0.6	0.7
Solanine	60	2.7	2.5
Mixture[c]	60	3.1	2.8

[a] Means of nine evaluations; 0 = no bitterness or burning, 4 = very strong bitterness or burning.
[b] Pith tissue of Irish Cobbler with a glycoalkaloid content of 0.4 mg/100g was used as the starting material for glycoalkaloid additions.
[c] The mixture of glycoalkaloids contained 2/3 chaconine, 1/3 solanine.

Phenolic contents and flavor

In this sample of 13 potato clones, which included seven clones with moderate to high glycoalkaloid contents, phenolic content did not appear to be a factor in producing bitterness, burning, or astringency responses from the panelists. Although bitterness and phenolic content were positively correlated (r = 0.088), the correlation was not significant. Clone 7 had the highest phenolic content (59 mg/100g) and was rated as less than slightly bitter by the panel; while clone 3 had the lowest phenolic content (17 mg/100g) and was rated moderately bitter (Table 1). The burning sensation was likewise unrelated to phenolic contents. Our panel did not report even slight (rating of 1.0) mean bitterness or astringency in any of the low ($\leq$ 7.3 mg/100g) glycoalkaloid clones, even though phenolic contents in this group of six clones ranged from 21–31 mg/100g.

The panel did not report burning, bitterness, or astringency in tissues amended with either chlorogenic acid or tyrosine. However, four of the nine panelists reported a slight sourness in the tissues containing 120 mg/100g of added chlorogenic acid. No other off-flavors were reported in these amended samples. Since the concentrations of these two phenolic compounds in the amended tissue samples were much higher than those encountered in natural potato tissue, it seems unlikely that either tyrosine or chlorogenic acid is responsible for bitterness or other off-flavors in potatoes.

Tannic acid is known to be quite bitter and could be responsible for bitterness and astringency in foods such as peaches, where significant quantities are encountered. However, according to Schwimmer and Burr (1959), tannins and tannic acid are localized in the suberized tissue of the potato tuber, which was removed by peeling in this investigation. There are small quantities of several unidentified polyphenols in tuber tissue (Hunter et al., 1957) and perhaps one or more of these unidentified polyphenols is responsible for low levels of bitterness or astringency in low-glycoalkaloid tissues. There are probably many different organic compounds in potatoes that can contribute to bitterness, but results of this investigation showed that glycoalkaloid content was the major determinant of undesirable bitterness in cortex tissues of these 13 potato clones.

Glycoalkaloid additions

The panel detected low levels of bitterness in the samples amended with the solanine or the solanine/chaconine mixture, at addition rates greater than 20 mg/100g (Table 2). There were only small differences between solanine and the mixture of solanine and chaconine in producing bitterness and burning

responses. The amended samples also evoked flavor responses such as astringency, "a metallic aftertaste," "a bite," "a coating of the teeth and mouth," and "an unpleasant aftertaste," which are characteristic of samples naturally high in glycoalkaloid content.

The intensity of the bitterness response and, in particular, the intensity of the burning sensation produced by the amended samples were not as high as would be expected from the results we obtained with tissues that are naturally high in glycoalkaloid content (Table 1). Perhaps other compounds in clones 1 through 7 or synergistic relations among the glycoalkaloids and other compounds in these tissues enhanced the bitterness and burning sensations. Nevertheless, the tissues amended with solanine or the solanine/chaconine mixture did evoke the same types of flavor responses as tissues naturally high in glycoalkaloid content. These results strongly support earlier reports of an acrid bitterness in potatoes that have high solanine (glycoalkaloid) contents, whether due to light exposure or other causes (Bomer and Mattis, 1924; Burr, 1966; Hilton, 1951; Lepper, 1949; Schwarze, 1962).

In replicated paired-comparison difference tests (Larmond, 1967) for detecting a difference threshold, more than half of the panelists repeatedly identified the samples with glycoalkaloid contents of 11 or 14 mg/100g as more bitter than the samples with 4.2 mg/100g. Samples with a content of 7.5 mg/100g could not be differentiated from samples with a content of 4.2 mg/100g. Since no bitterness was reported in repeated tests of the low (4.2 mg/100g) glycoalkaloid tissue samples, these results suggest that glycoalkaloid levels below 7.5 mg/100g do not cause bitterness in potato tissues. When the glycoalkaloid levels of the tissues in these paired-comparison difference tests were 11 or 14 mg/100g panelists were able to detect a difference in bitterness, indicating that levels in excess of 11 mg/100g can cause bitterness in potato tissues.

Results of the taste tests of cortex tissues from the 13 clones (Table 1) also indicated that glycoalkaloid contents below 7.3 mg/100g do not cause bitterness in potatoes. All the cortex tissues that had glycoalkaloid contents $\geqslant$ 14 mg/100g (clones 1 through 7) evoked bitterness responses in 50% or more of the individual taste tests. Cortex tissues that had glycoalkaloid contents $\leqslant$ 7.3 mg/100g (clones 8 through 13) evoked low and inconsistent (less than 20% of the individual taste tests) bitterness responses.

Our nine panelists were not selected because of their sensitivity or lack of sensitivity to bitterness, so these results may be representative of the approximate minimum level of glycoalkaloids that can affect potato flavor for the public. The addition of salt, butter, or gravy to potatoes could of course affect the minimum level of glycoalkaloids that can cause bitterness in cooked potatoes.

Most normal potatoes have whole-tuber glycoalkaloid contents of less than 10 mg/100g fresh weight (Sinden and Webb, 1972). Peeled tubers have even lower contents, since up to 60% of the total glycoalkaloid in whole tubers is removed with the peel. Glycoalkaloid contents of more than 30 mg/100g are sometimes found under unfavorable growing conditions, when potatoes do not fully mature before harvest (Bomer and Mattis, 1924; Sinden and Webb, 1972). The results of this study suggest that these potatoes could have an undesirable bitter flavor and other off-flavors, and might even cause burning of the mouth and throat. Therefore, it would be desirable to grow potatoes that are inherently low glycoalkaloid producers and protect them from light and other inducers of glycoalkaloid synthesis in order to avoid the possibilities of bitterness and other off-flavors.

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Ms received 7/23/75; revised 10/6/75; accepted 10/15/75.

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CHANGES IN SOME MONOCARBONYL CLASSES DURING PROCESSING AND STORAGE OF SWEET POTATO FLAKES

ABSTRACT

Dehydrated sweet potato flakes were prepared in a pilot plant by (1) peeling and comminuting fresh sweet potatoes; (2) heating sweet potato puree to 75.5°C and holding at that temperature to allow naturally present amylases to convert a certain proportion of the starch into sugars; (3) heating puree to 105°C to inactivate enzymes; (4) atmospheric drum drying and flaking; and (5) packing flakes in cans under N_2 atmosphere. Total monocarbonyls in fresh sweet potatoes generally increased with time of storage of the roots. During processing of freshly dug sweet potatoes into dehydrated flakes, monocarbonyls increased as processing progressed. During processing of "cured," and of up to 4-months' stored sweet potatoes the content of monocarbonyls peaked during conversion of starch to sugars, and decreased after heating to 105°C prior to drum drying. Levels of saturated aldehydes and of methyl ketones were also highest during amylolysis. During storage of the packaged flakes at 27°C for 128 days, total monocarbonyl content remained rather constant, while saturated aldehydes increased and methyl ketones decreased. At 45°C storage temperature, total monocarbonyls and saturated aldehydes showed a marked increase, while ketones decreased. There was a slight net increase in CO_2 and O_2 and a slight decrease in N_2 in the gas present in cans of flakes stored for 1 yr at 24°C. After 12 months storage, 0.026% CO was found. In the dehydrated sweet potato flake product monocarbonyls, particularly aldehydes, are probably a factor in off-flavor development during storage.

INTRODUCTION

PRE-COOKED dehydrated flakes of sweet potato (*Ipomoea batatas*) is a relatively new introduction in the market. The research and development work done to the present on that product has stressed problems related to the manufacture, but little work has been done on basic aspects.

In food dehydration practice, it is generally recognized that when using high quality raw materials, processing them under good conditions to a finished product low in moisture and in oxygen, and storing the product at low temperatures, a highly acceptable product will result. Sometimes that is not true. Some food materials are in a dynamic state of chemical reactivity which may continue for some time in the finished product, even under optimum conditions of storage. Those conditions may apply to autoxidation of lipids in dehydrated sweet potato flakes.

Carbonyl compounds may develop in dehydrated sweet potato flakes following the scheme presented by Badings (1960). In that scheme, alkoxy radicals formed by hydroperoxide dismutation react forming an alkyl type radical and an aldehyde. Abstraction of a hydrogen atom from another molecule yields alcohols and new free racidals. The free radicals continue propagating the autoxidative chain reaction forming ketones and other nonradical end products.

Review of literature

Schwartz and Parks (1961) reported that trace amounts of carbonyls in solvents can be converted to their 2,4-dinitrophenylhydrazones when they are passed through a Celite column impregnated with 2,4-dinitrophenylhydrazine (DNPH), phosphoric acid, and water. Hornstein and Crowe (1962) found that solvents, such as hexane, that are badly contaminated with carbonyl compounds may be cleaned up effectively, rapidly, and continuously on a column prepared from Celite impregnated with concentrated sulfuric acid.

The use of 2,4-dinitrophenylhydrazine as a specific reagent for carbonyl compounds has been known for some time. Jones et al. (1956) found that the information afforded by the ultraviolet and visible spectra of the 2,4-dinitrophenylhydrazones in neutral solution and in basic solution presented a means of differentiating the type of parent carbonyl compound.

Separation of aliphatic monocarbonyls into classes is a valuable aid in the characterization of carbonyls obtained as a mixture at the micromole level. Schwartz et al. (1962) have described a method for separating the 2,4-dinitrophenylhydrazone derivatives of aliphatic monocarbonyls into classes on a magnesia-Celite column. The classes elute in the sequence: methyl ketones, saturated aldehydes, 2-enals, and 2,4-dienals. The short chain members of each class behave anomalously, moving close to, or into, the class immediately following. Characteristic colors for each class are displayed on the adsorbent and aid in their separation and identification. Methyl ketones are gray; saturated aldehydes are tan; 2-enals are rust-red; and 2,4-dienals are lavender.

The relationship between the carbonyl compounds produced by oxidation and the reversion flavor of soybean oil has been studied extensively. Mookherjee and Chang (1963) described a technique for the identification of the monocarbonyl compounds by column chromatography separation and liquid-liquid partition chromatography individual compound identification.

Another quantitative procedure for the direct isolation of carbonyl compounds is given by Schwartz et al. (1963). Carbonyl compounds in the fat are converted to their 2,4-dinitrophenylhydrazones, subsequently freed of fat, and fractionated by adsorption on activated magnesia and partially deactivated alumina. The fat-free monocarbonyl fraction is then separated into classes on magnesia and the members of each class are obtained by column partition chromatography and identified by supplementary techniques.

Boyd et al. (1965) conducted studies on the relationship between chocolate aroma and the monocarbonyl compounds in unroasted cocoa beans and chocolate liquor. The monocarbonyl compounds were converted to 2,4-dinitrophenylhydrazones and then separated into methyl ketone, saturated aldehyde, 2-enal, and 2,4-dienal fractions. Quantitative data showed that each carbonyl class is an important contributor to chocolate aroma.

MATERIALS & METHODS

Apparatus

(a) Beckman DU-2 Spectrophotometer

(b) Chromatographic columns. Columns used were similar to those reported in Boyd et al. (1965), except that dimensions of columns used

were as follows: Extraction: 2.5 cm i.d. by 80 cm long; Reaction: 2.5 cm i.d. by 30 cm long; Defatting: lower column, 1 cm i.d. by 15 cm long; upper 2.5 cm i.d. by 15 cm long; Alumina: lower, 0.75 cm by 15 cm long; upper, 2.5 cm i.d. by 15 cm long; Separation: lower, 1 cm i.d. by 15 cm long; upper, 2.5 cm i.d. by 15 cm long.

Reagents

Reagents used for the column chromatography separation of the different classes of compounds were those used by Boyd et al. (1965).

Processing of flakes

Centennial variety of sweet potatoes produced on the eastern shore of Virginia were used throughout this study. The size of the sweet potato roots ranged from 1½ to 3 in. in diameter. The processing of sweet potato flakes was based in principle on the method reported by Hoover (1967) with significant modifications in temperature and time conditions in most of the steps, as follows:

1. The sweet potato roots were washed in a reel-rod washer with water spray at 13°C.
2. The roots were then submerged in a water bath of 54°C for 30 min.
3. From the water bath the roots were submerged in a 14% solution of commercial grade caustic soda at 101°C for 5 min.
4. The roots were run through a vegetable brusher-washer with water sprays at a temperature of 13°C and conveyed into 18°C water baths where they were held for approximately 15 min until hand trimmed.
5. The peeled roots were conveyed into an 18°C water bath where they were held for approximately 15 min until hand trimmed and comminuted in a Rietz Disintegrator with a 0.032 in. mesh screen.
6. The total solids of the sweet potato were adjusted to 20% with 13°C tap water and heated to a temperature of 75°C by pumping it through a steam injection heater to activate the naturally present amylase enzymes. The heated puree was held in a starch-to-sugar conversion tank for a time sufficient to obtain an optimum ratio of starch to sugars.
7. The puree was pumped through another steam injection heater where the temperature of the puree was elevated to 105°C and held in a tank for 15 min at 93–99°C to inactivate the amylases before being pumped into a 12 in. diameter atmospheric double drum drier at 10 rpm and 80 psig.
8. Sweet potato flakes were packed in 211 × 301 plain tin plate cans, 163g in each can. Prior to double seaming, the cans were vacuumed three times under 26 in. vacuum and the vacuum broken with nitrogen gas, which resulted in a 95% N_2–5% O_2 atmosphere in the cans.

Analytical methods and experimental design

The procedures followed to isolate and quantify monocarbonyl compounds were based on those given by Boyd et al. (1965). The steps involve a series of extraction, reaction, defatting, alumina, and class separation columns. The classes of the chromatographic fractions were identified by their spectrophotometric absorption maxima. Data were collected on total monocarbonyls, methyl ketones, saturated aldehydes, 2-enals, and 2,4-dienals.

The work was divided into four major sections as follows.

Effect of storage time of raw sweet potatoes on content of monocarbonyls during processing. To determine the effect of storage time on raw sweet potatoes, analyses were made for carbonyls in freshly dug potatoes, cured potatoes, and in 2-months, and 4-months stored potatoes. Except for the freshly dug potatoes, all roots were "cured" at 27°C and 80% relative humidity for 7–10 days. They were then stored at 13°C and 80% relative humidity.

Samples were taken prior to and during the various unit operations in the processing of sweet potato flakes. Sampling was done at the following stages: (a) fresh sweet potatoes; (b) pureed raw sweet potatoes; (c) after enzymatic conversion of starch to sugars; and (d) during holding, after inactivation of amylases, just prior to drum drying.

Effect of temperature during amylolytic conversion of starch to sugars on monocarbonyl levels during processing of sweet potato flakes. Conversion temperatures of 66°C and 81°C were used. Conversion times were optimum for the condition of the raw product. Enzymes were inactivated by heating the product to 105°C for 5 min.

Effect of storage at different temperatures on monocarbonyls in sweet potato flakes. Flakes packed in cans were held in frozen storage for 90 days before being put in 27°C and 45°C storage. Samples were taken out of the 27°C storage at 0, 16, 32, 64 and 128 days, and out of the 45°C storage at 0, 4, 8, 16, 32, 64 and 128 days.

Changes in headspace gas composition during storage. Cans packed by the method described under Processing of flakes were stored at 24°C for 1 yr. Determinations of N_2, O_2, CO_2 and CO were made in duplicate cans every 3 months. A Model 29 Fisher Scientific Company Gas Partitioner was used. The gas partitioner was equipped with two columns, column 1 being filled with DEHS and column 2 with Molecular Sieve 13X. The carrier gas was helium, the sample size 500 μl, the flow 40 ml/min, and the chart speed 1 cm/min.

RESULTS & DISCUSSION

THE COLUMN CHROMATOGRAPHY methods used for separating monocarbonyls into classes did not make it possible to obtain a sharp separation and differentiation between classes. Consequently, the eluted dry weight of each of the carbonyl classes may have contained small amounts of other classes. This may explain some of the variability in the quantity of the different monocarbonyl classes within determinations of carbonyls among samples taken at a specific processing stage.

During mixing and grinding the carbonyls present were probably undergoing oxidation due to breakdown of tissues and subsequent exposure to large quantities of air and naturally present compounds liberated by lysing of the cells.

Effect of storage time of raw sweet potatoes on content of monocarbonyls during processing

Data in Table 1 indicate that the content of carbonyls of the fresh sweet potatoes generally increases during storage with time. The same data show that during processing of freshly dug sweet potatoes carbonyls increased as processing progressed, while in the cured, 2-months and 4-months stored sweet potatoes the content of carbonyls peaked during conversion of starch to sugars, and decreased after the 15-min holding prior to drum drying. As the storage time of different treatments of fresh sweet potatoes increased, the carbonyls content decreased after the 15-min holding period prior to drum drying. There was a marked increase in carbonyls during conversion of starch to sugars. The increase in carbonyls during starch to sugar conversion to a level above that found in fresh sweet potatoes may be caused by the higher temperature during conversion which increased the activity of the naturally present enzymes that catalyze the reactions producing carbonyls. The reduced carbonyl content found at the 93–99°C 15-min holding stage prior to drum drying may have caused binding of the carbonyls to other compounds, resulting in lower carbonyl levels at that stage of the process.

Traces of 2-enals and 2,4-dienals were detected in fresh sweet potatoes and at several stages of processing, but the data obtained showed no trends and were inconclusive. Therefore, no other results are reported on this phase of the work.

Figure 1 shows overall mathematical trend lines for total monocarbonyls content at different stages of processing freshly dug, cured, and stored sweet potatoes. A trend observed in the freshly dug sweet potatoes to increase in monocarbonyl content with longer storage periods–cured, 2-months storage, 4-months storage–was reversed to a decrease when the storage periods of the fresh sweet potatoes are considered in sequence, as shown in Figure 2. The lower levels of carbonyls observed in flakes prepared from sweet potatoes stored for longer periods prior to processing may indicate that length of storage prior to processing is a factor contributing to better flavor stability in the flake product.

Effect of temperature during amylolytic conversion of starch to sugars on monocarbonyl levels during processing of sweet potato flakes

The maximum content of total monocarbonyls was found during the starch conversion stage at both 66°C and 81°C, as shown by data in Table 2.

When the amylolysis took place at 66°C, the levels of saturated aldehydes and of methyl ketones were also highest at the starch-to-sugars conversion stage. When amylolysis took

Table 1—Changes in content of carbonyls during processing of sweet potato flakes, after different treatments of the fresh roots

	Fresh sweet potatoes				Pureed sweet potatoes			
	Freshly dug	"Cured"	Stored for two months	Stored for four months	Freshly dug	"Cured"	Stored for two months	Stored for four months
	(μM/100g)[a]				(μM/100g)[a]			
Total carbonyls	6.4	4.1	17.3	31.0	8.9	8.0	20.6	14.7
Methyl ketones	2.7	1.9	4.5	16.3	0.0	1.7	2.1	0.0
Saturated aldehydes	2.7	1.4	11.7	7.4	6.5	4.2	15.2	13.0

	After partial conversion of starch to sugars				Holding prior to drum drying			
	Freshly dug	"Cured"	Stored for two months	Stored for four months	Freshly dug	"Cured"	Stored for two months	Stored for four months
	(μM/100g)[a]				(μM/100g)[a]			
Total carbonyls	27.7	40.7	30.5	30.9	35.3	17.8	11.2	3.3
Methyl ketones	18.4	37.5	20.7	21.1	35.3	13.4	10.0	1.8
Saturated aldehydes	9.4	3.2	9.8	9.8	0.0	4.4	1.1	1.5

[a] Dry weight basis

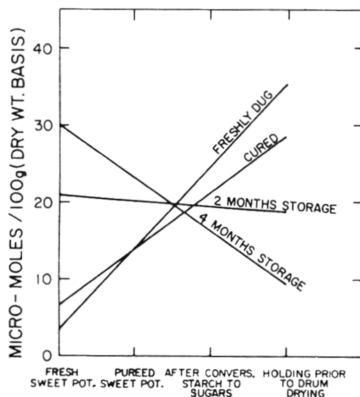

Fig. 1—Mathematical trend lines for total content of monocarbonyls at different stages of processing differently treated raw sweet potatoes.

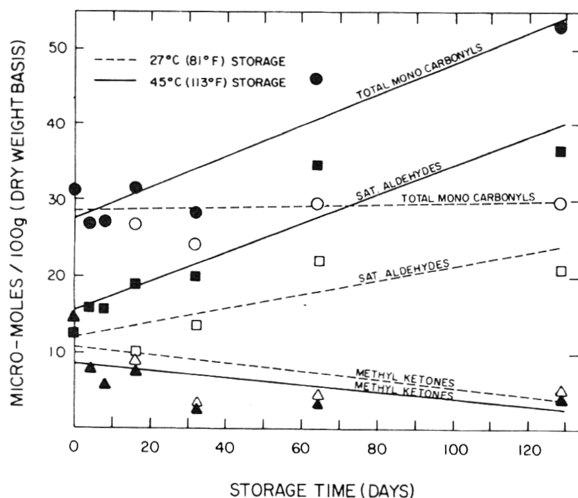

Fig. 2—Effect of storage temperature on monocarbonyls in sweet potato flakes.

place at 81°C, the level of ketones remained at a low, but rather constant level, while aldehydes which were at a rather high level in both the fresh sweet potatoes and during amylolysis, were reduced to almost zero after the holding period prior to drum drying. The decrease in total monocarbonyls and saturated aldehydes during holding prior to drum drying may be caused by loss through volatilization of those compounds.

Effect of storage temperature on carbonyls in sweet potato flakes

Figure 2 shows that during the storage period at 27°C total monocarbonyls remained rather constant, while saturated aldehydes increased and methyl ketones decreased. At the 45°C storage, total monocarbonyls and saturated aldehydes increased markedly, while ketones decreased. These observations indicate that saturated aldehydes as a class may be related to flavor changes during the storage of sweet potato flakes, while methyl ketones probably do not directly contribute to those changes.

Table 2—Changes in content of monocarbonyls during processing of "cured" sweet potatoes

	Fresh sweet potatoes	Pureed sweet potatoes	After partial conversion of starch to sugars	Holding prior to drum drying
	(μM/100g)[a]			
Total carbonyls	4.1	8.0	40.7	17.8
Methyl ketones	1.9	1.7	37.5	13.4
Saturated aldehydes	1.4	4.2	3.2	4.4

[a] Dry weight basis

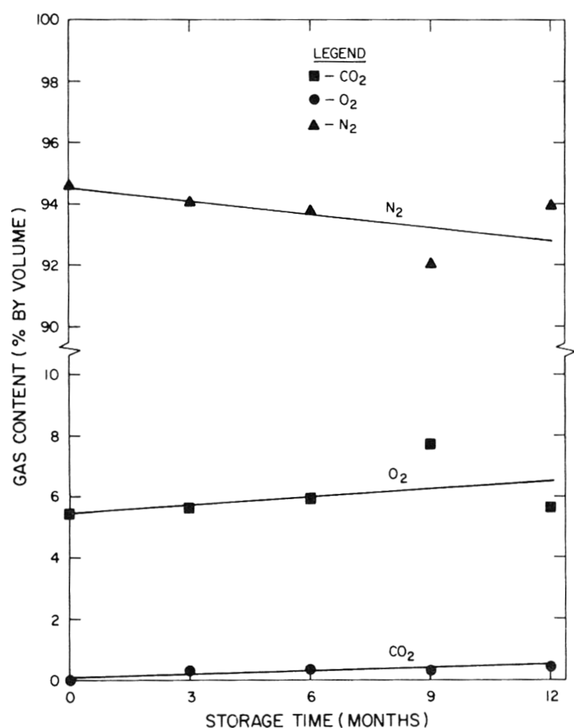

Fig. 3–Headspace gases in sweet potato flakes packed in cans and stored for 1 yr at 24°C.

Headspace gas composition during storage

Figure 3 shows the volumetric composition of the gas present in the can after 0, 3, 6, 9 and 12-months storage. The trends observed were of slight net increase in CO_2 and O_2, and slight decrease in N_2. CO was not detected until 12 months storage, when 0.026% was found. The results show that from a net end product and stoichiometric standpoints no significant reactions that consume oxygen took place during the 12-month storage period. The small quantities of CO_2 produced and small amount of CO detected, indicate the probability of decarboxylation reactions taking place at a slow rate during storage. Additionally some of the CO_2 produced might be dissolved and/or dispersed in the sweet potato material and thus not be present in the headspace gas. Volumetrically, the gas analysis accounted for between 99.80% and 100.05% of the gases present in each can at each storage period.

CONCLUSIONS

RESULTS in general show development of monocarbonyl compounds during the initial stages of processing of the sweet potato flakes, followed generally by a reduction in monocarbonyl content during the higher temperature heating process just before drum drying. Results also indicate that during storage of the dehydrated product there was an increase in total monocarbonyls and saturated aldehydes content, and a decrease in content of methyl ketones.

Considering the influence of autoxidation of lipids and development of carbonyl compounds on the flavor of other products, it is probable that in the dehydrated sweet potato flake product monocarbonyls, particularly aldehydes, are a factor in off-flavor development during storage. Monocarbonyls, however, do not accumulate during processing, but have a tendency to decrease due to exposure of the product during processing to rather high temperatures that probably produce a loss of carbonyls through volatilization. Monocarbonyls could also break-down at high temperature to CO_2 and other compounds, or be oxidized to dicarboxylic acids.

Although no trends were observed in the data collected on 2-enals and 2,4-dienals, the presence of these compounds was detected. It is possible that carbonyls of these classes may also contribute to off-flavor development during storage.

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Ms received 7/26/75; revised 10/18/75; accepted 10/21/75.

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EFFECT OF GLYCEROL ON NONENZYMATIC BROWNING IN A SOLID INTERMEDIATE MOISTURE MODEL FOOD SYSTEM

ABSTRACT

Maillard browning is one of the main chemical reactions causing deterioration and shortening shelf life of intermediate moisture food (IMF) systems. The purpose of this research was to study Maillard browning in an IMF model system containing casein, glucose and the liquid humectant glycerol. The kinetics of pigment production, glucose utilization and loss of DNP-available lysine were studied as a function of temperature, moisture content and water activity. It was found that the factors which control reactant (glucose and available lysine) utilization also control end-product (brown pigment) accumulation. The rate of the Maillard browning pigment production, after an initial induction period, follows zero order kinetics. The initial loss rate of both glucose and available lysine, however, follows first order kinetics. Exceedingly large nutritional (available lysine) losses occur before brown discoloration is appreciable. Slightly greater than one mole of glucose reacts per mole of lysine made unavailable. Based on this, nutritional losses may be relatively easily estimated by monitoring the loss of specific reducing sugars. The Maillard browning reaction proceeds 33 times faster at 45°C than at 25°C, with the maximum rate occurring at 0.4–0.5 a_w. This is an a_w range considerably lower than the 0.65–0.75 a_w range usually found for maximum browning in dehydrated foods. The downward shift in the a_w maximum for browning is because glycerol being liquid has water-like properties and increases reactant mobility and/or solubility at a_w's below which most water soluble reactions occur very slowly.

INTRODUCTION

FOOD STABILITY has been shown to be controlled by the moisture content or water activity (a_w) of foods (Bone, 1969; Labuza, 1970, 1975; Lea, 1958). Bacterial degradation of foods can usually be controlled by keeping the water activity of the food at less than a_w 0.9. Yeast and mold growth can be inhibited by maintaining the a_w at less than 0.8 (Bone, 1969). Chemical degradation reactions of foods can usually be controlled by keeping the water activity or moisture content of foods low. Maximum stability is usually thought to occur when the food's moisture content is near the BET (Brunauer et al., 1938) monomolecular moisture layer coverage of foods (Salwin, 1959). As the moisture content increases above the BET coverage, the rate of chemical degradation of foods usually increases. And as the moisture content decreases below the BET coverage, lipid oxidation can again increase to cause rapid degradation of foods.

Water controls the degradative reactions of foods by various means. Osmotic shock, insufficient availability of required nutrients, or a build-up of metabolic end-products that are toxic to the organism are thought to be some of the means by which low moisture contents or a_w limits or inhibits the growth of microorganisms. In addition, the chemical degradative reactions within foods are controlled through the effect of moisture content or a_w on reactant dissolution, mobility and concentration. An increase in moisture content or a_w can increase solute (reactant) solubility and/or mobility and thereby cause the rate of a chemical reaction to increase. Conversely, an increase in moisture content will tend to dilute the concentration of reactants and thereby decrease chemical reaction rate. If water is a product of the reaction of concern, an increase in moisture content will decrease the reaction rate by mass action, i.e., end-product inhibition. This increase may be small overall, but at the reaction site if diffusion is slow will have a great effect. One or a combination of the above factors may be rate limiting and thereby limit the rate of degradative reactions within a food as the food's moisture content or a_w is increased or decreased.

Maillard browning is a reaction wherein a reducing sugar, such as glucose, combines with a free amino group, such as the ϵ-amino group of available lysine and thereby decreases the biological availability of the protein. In food products where processing or storage conditions cause an accelerated rate or prolonged duration of the Maillard reaction, a significant loss of food quality may result.

It is generally observed that most foods exhibit a maximum rate of browning near a_w 0.65–0.75. The data of Loncin et al. (1968) shows that for milk powder which was humidified to a moisture content above the BET monolayer, both an undesirable accumulation of brown pigments and a loss of available lysine occurred when the milk powder was held at 40°C for only 10 days. It was observed that the maximum in pigment production occurred near a_w 0.65, an a_w of intermediate moisture foods, with a concommitant loss of 75% of the available lysine. A similar observation is found for most dehydrated foods humidified to an a_w common to intermediate moisture foods. Nonenzymatic browning (NEB) is one of the main degradative reactions that often occurs at a maximum rate in intermediate moisture foods which have an a_w range of 0.60–0.85 (Karel and Labuza, 1969; Labuza, 1970; Loncin et al., 1968).

Very little data are available on the extent of Maillard browning in IMF systems to which a liquid humectant has been added. A liquid humectant can increase the palatability of a food or lower the water activity of the food and thereby increase its microbial stability. Eichner and Karel (1972) studied the extent of Maillard browning in a liquid model system of glycine-glucose which contained the humectant glycerol. They found that under certain conditions the maximum in browning, as measured by pigment production, occurred near a_w 0.4. They concluded that glycerol, through its plasticizing effect, partially restores reactant mobility to increase the browning rate at low moisture contents.

Since solid IMF items, such as meal replacement items, are becoming more common in the marketplace, a study of the rate of the Maillard browning reaction as well as a determination of the loss of nutritional value within these IMF foods is necessary. This current study examines the rate of Maillard browning as it occurs during the storage of a solid IMF model system which contains the liquid humectant glycerol.

MATERIALS & METHODS

TO FACILITATE the collection of browning data, a model food system was used. The composition of the model system is shown in Table

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1. Potassium sorbate was used as an antimicrobial agent. Glucose is the sole source of reducing sugar for the Maillard reaction in this study. Glycerol is used as a liquid humectant to control the water activity and plasticity of the model system. Casein serves as the only source of free amino groups for the Maillard reaction. Apiezon B oil, which is liquid at room temperature, adds plasticity to the product. It is saturated and therefore should not participate in the Maillard reaction. Microcrystalline cellulose is inert to the Maillard reaction and serves as a solid support for the model system. Water is added in varying amounts so the rate of Maillard browning could thereby be studied as a function of water activity or moisture content. The average initial glucose/available lysine molar ratio was 2.9.

The components, as tabulated in Table 1, were mixed together in descending order of appearance in the Table. Water was added by two different methods such that the effect of method of water addition on the rate of Maillard browning could be studied. Direct Mix systems were brought to proper moisture content by mixing in a predetermined amount of liquid water to the nonaqueous ingredients. The Direct Mix samples were then held in vacuo over an appropriate saturated salt solution (Rockland, 1960) for 1 day at room temperature for final moisture equilibration. The Humidified Mix samples were humidified by storing the mixed nonaqueous components in vacuo over an appropriate saturated salt solution for 3–6 days at room temperature until the proper water activity was obtained. Moisture content was measured with a methanol extraction GC technique (Tjhio et al., 1969). Water activity was measured by a vapor pressure manometric technique (Karel and Labuza, 1967; Karel and Nickerson, 1964).

Following the formulation process, including the addition of water, the samples were transferred to 202 x 214 epoxy-lined cans. The cans were sealed and the newly sealed ends were dipped in glyptol to retard moisture loss should the can seal not be perfect. The canned samples were then incubated at 25, 35 or 45°C and periodically analyzed for extent of Maillard browning. If sample analysis could not be done on the desired day, the samples were held at −29°C until the analysis could be performed.

The Maillard browning reaction was monitored by measuring NEB (melanoidin) pigment production, glucose utilization, and loss of available lysine. NEB pigment concentration was measured by the trypsin digest, aqueous extract procedure of Choi et al. (1949) as modified by Labuza (1971). Glucose content was measured with a glucose oxidase Blood Sugar test kit [Bolhringer Mannheim Corp., New York, Cat. No. 15756. Method: adapted from Werner, W., Rey, H.G. and Wielinger, H. (1970). Z. Anal. Chem. 252: 224]. The FDNB method of Booth (1971) was used to measure available lysine content.

RESULTS & DISCUSSION

THE INCREASE of pigment concentration as a function of storage time, temperature and a_w is presented in Figure 1. After an initial induction period, the amount of NEB pigment increases linearly with time for each condition. During the initial induction period, predominantly colorless browning intermediates are being formed. After a sufficient amount of these intermediates have been formed, the rate of pigment production follows zero order kinetics.

Temperature controls both the length of the induction period and the rate of pigment production during the zero order period. At the higher temperature, the rate of NEB pigment production is increased. The average activation energy for pigment production is 32.9 kcal/mole, which gives a Q_{10} of about 6.1 between 25 and 35°C and 5.4 between 35 and 45°C. Thus, the reaction occurs 33 times faster at 45°C than at 25°C. Also, as is shown in Table 2, the induction time is less at the higher temperature.

The 35°C data of Figure 1 also show that browning rates are controlled by water activity. The influence of water activity on browning rate is further shown in Figure 2 for 35 and 45°C. The same pattern was also found at 25°C (Warmbier, 1975). As seen, the maximum rate of NEB pigment production occurs at a_w 0.45–0.55; this is unlike that found in most solid food systems which show a maximum rate near a_w 0.7–0.8. The rate maximum at 35°C occurs at a moisture content of 8.9g H_2O/100g solids. At 45°C, the maximum is at 10g H_2O/100g solids. The calculated BET monolayer for the systems of this study is 8.1g H_2O/100g solids. A dehydrated

Table 1—Model system composition

Component	Grams
K-sorbate	0.3
Glucose	10.0
Glycerol	20.0
Casein	30.0
Apiezon B oil	20.0
Microcrystalline cellulose	20.0
Water	Variable

Table 2—Percent loss of reactants (glucose and available lysine) during the induction time for melanoidin pigment production

Temp (°C)	Avg a_w	Browning induction time (Days)	Glucose % Loss	Lysine % Loss
25	0.86	100	10	60
25	0.72	100	21	65
25	0.67	100	26	55
35	0.87	20	13	20
35	0.51	20	33	60
35	0.32	20	18	12
45	0.84	7	29	37
45	0.39	7	33	69
45	0.14	7	17	71

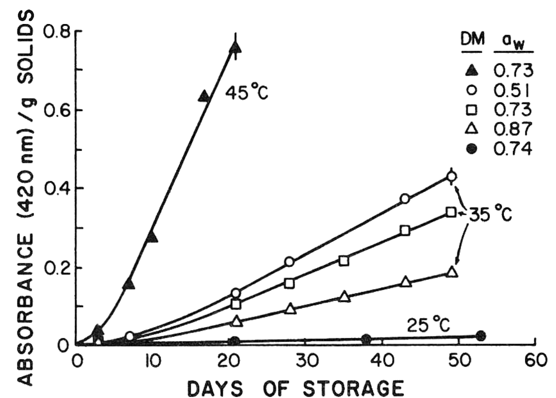

Fig. 1—Browning pigment production as a function of time in model systems as a function of a_w and temperature. DM—direct addition of water to system.

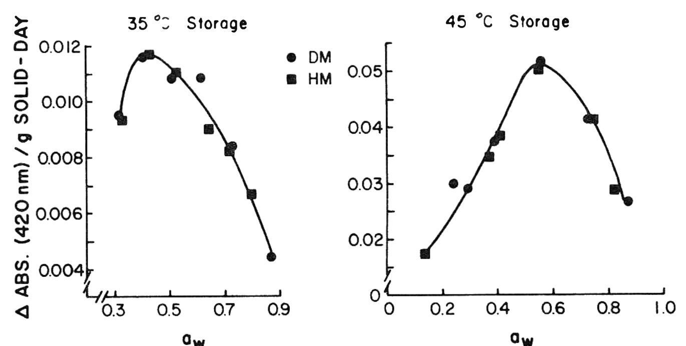

Fig. 2—Rate of browning as a function of a_w at 25 and 35°C. DM—water added directly as liquid; HM—water added by humidification.

food is usually considered to be most stable to chemical reactions if its moisture content is at or near the BET monolayer (Salwin, 1959). In this study, however, with a liquid glycol added a maximum in the rate of degradation occurs close to the BET value.

The above observation is similar to the findings of Eichner and Karel (1972). Their liquid model system containing glycerol had a maximum in browning at a_w 0.41 when stored at 37°C. They concluded that glycerol can increase the rate of browning at low a_w values by increasing reactant mobility and that as a_w increases, the water decreases the browning rate by a mass action effect. This should also be the mechanism in the solid food systems used in this current study. The results of Figure 2 thus indicate that glycerol could cause a decrease in browning if it is added to intermediate moisture foods.

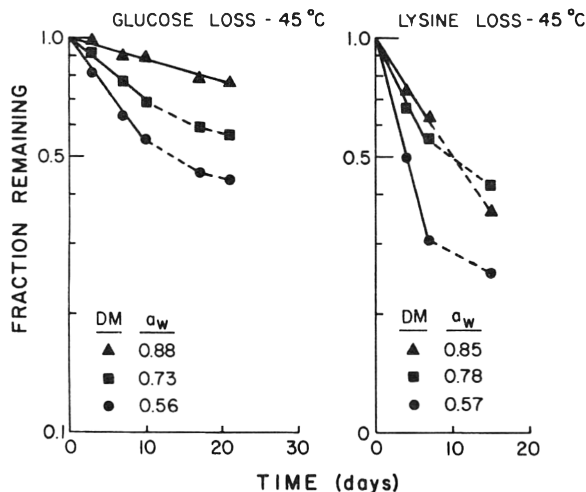

Fig. 3–Loss of glucose and lysine vs time as a function of a_w at 45°C. DM–water added directly as liquid.

One additional observation to be drawn from Figure 2 is that the method of water addition to the food samples does not appreciably influence the rate of NEB pigment production. At any given water activity or moisture content, samples to which liquid water was added directly (Direct Mix system) browned at the same rate as those samples to which water was added by a vapor humidification process (Humidified Mix system). This suggests that solubilization of the reactants is the same no matter what the method of addition of the water is.

As stated earlier, the induction period must occur before the rate of pigment production follows (constant) zero order kinetics. During this initial storage period when pigment production is not yet appreciable, reactants of the Maillard reaction are utilized to form colorless browning intermediates. Figure 3 shows the loss of both glucose, a reducing sugar, and available lysine at 45°C. The lysine serves as a source of free ε-amino groups for the formation of glycosylamines for the Maillard reaction. The data indicate that initially the destruction rates of both glucose and available lysine follow first order kinetics. A significant amount of glucose and available lysine is destroyed even before pigment production becomes appreciable. As is shown in Table 2, up to one-third of the glucose and, more importantly, as much as 70% of the available lysine is reacted before the rate of pigment production follows zero order kinetics and visual browning appears. Obviously this has serious nutritional implications. A food in which Maillard browning can occur may not have produced pigments at a sufficient rate such that the color of the food becomes objectionably brown during processing or storage. However, the protein nutritional loss within the food, as indicated by decreased available lysine content, may be significant.

As previously indicated in Figure 3, water influences the rate of glucose and available lysine loss. Figures 4 and 5 show the initial (first order) loss rate of glucose and available lysine at 35 and 45°C as a function of water activity. In general, the

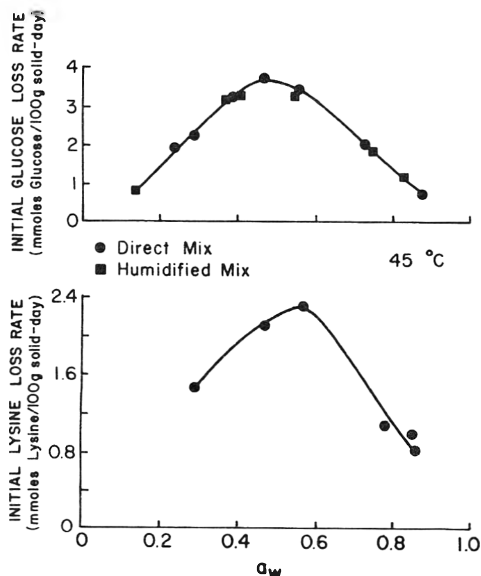

Fig. 4–Initial glucose and lysine loss rates as a function of a_w at 45°C.

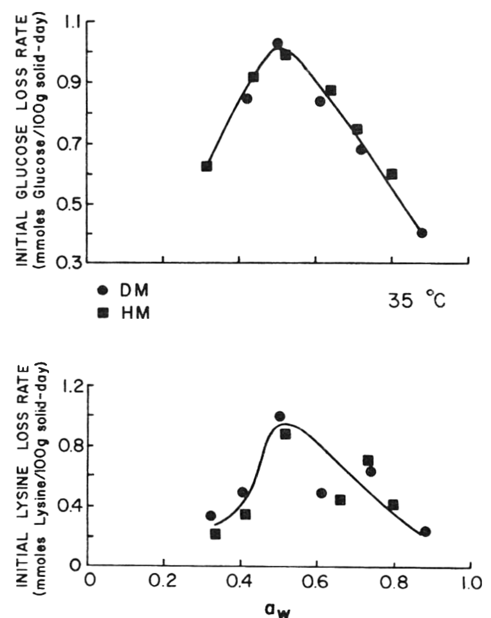

Fig. 5–Initial glucose and lysine loss rates as a function of a_w at 35°C. DM–water added as liquid; HM–water added by humidification.

loss rate of both glucose and available lysine are controlled in the same way by water activity. It can also be observed that the maximum loss rate of these reactants occurs near the same a_w as for the maximum rate of browning.

Loncin et al. (1968) showed that maximum lysine loss in dehydrated foods occurs at an a_w greater than approximately a_w 0.6. Their food systems were very similar to our model systems except a liquid humectant was not added to their systems. Therefore, the downward shift in the a_w maximum for lysine loss and glucose loss must be caused by glycerol in a manner similar to that which controls NEB pigment production as proposed by Eichner and Karel (1972). The practical significance of the addition of glycerol is thus obvious. Glycerol, or perhaps other liquid humectants, when added to meal bars, semi-moist pet foods, or other IMF's, can decrease the rate at which lysine, and perhaps other essential nutrients with free amino groups, becomes unavailable.

One further observation drawn from this study is that at 35 and 45°C, an average of 1.3 moles of glucose initially react for one mole of lysine that becomes unavailable. From a practical standpoint, the monitoring of the loss of glucose or other specific reducing sugars may be used as a relatively rapid and easy method for estimating the nutritional (available lysine) loss of foods suspected of being degraded by the Maillard nonenzymatic browning reaction. This type of method is much more simple than the FDNB procedure which requires much technical training and over 24 hr for the determination of available lysine.

SUMMARY

THREE SIGNIFICANT and practical conclusions can be found from this research. First, the addition of glycerol to intermediate moisture foods can cause the a_w maximum for the Maillard reaction to be shifted downward. Thus, IMF's which contain glycerol, or perhaps other liquid humectants, would have a browning rate that is less than would occur if a liquid humectant were not present. Second, the Maillard reaction can cause significant nutritional losses, e.g. of available lysine, to occur before the food has become appreciably or objectionably brown. Third, it is possible that the protein nutritional loss of foods susceptible to Maillard browning may be relatively easily and quickly estimated by following specific reducing sugar loss during storage.

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Ms received 7/11/75; revised 10/3/75; accepted 10/6/75.

This is paper No. 9157 from the University of Minnesota Agricultural Experiment Station. This study was supported in part by the Univ. of Minn. Agric. Expt. Stn. Project No. 18-72 and by Contract #NAS 9-12560, Lyndon B. Johnson Space Center, National Aeronautics & Space Administration, Houston, Texas.

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PREDICTION OF WATER ACTIVITY LOWERING ABILITY OF FOOD HUMECTANTS AT HIGH a_w

ABSTRACT

Equations for prediction of the a_w lowering effect of humectants in a complex IMF food system were investigated in the range 0.98–0.81 water activity. Three equations have previously been used to predict a_w lowering effect of simple solutes in solution, but have never been tested in an IMF system. In addition, two equations were derived, a linear slope method and a graphical procedure. Four commonly used food humectants were incorporated into a model IMF system and the resulting a_w's measured. The measured results were compared to the predicted values of these equations. The linear slope method was shown to be the most accurate of the five equations studied. It is simple and requires only the initial a_w and moisture content of the system along with a predetermined slope value for the moisture sorption isotherm for each humectant. The Ross derivation also provides a relatively accurate method of predetermining the final a_w in this range.

INTRODUCTION

MANY HYGROSCOPIC chemical compounds are employed by the food industry to bind water in food products. These water binding agents or humectants are incorporated into food systems to lower the water activity (a_w) of the food material in order to increase shelf stability (Labuza et al., 1974b; Karel, 1973).

These humectants are particularly important in the production of intermediate moisture foods (IMF) (Bone et al., 1974). A wide range of humectants including polyols, sugars and salts are incorporated into these foods to lower the a_w into the intermediate moisture range (Bone, 1973). This is usually between an a_w of 0.65–0.90.

When a humectant is incorporated into a food system, it is difficult to predetermine the final a_w of the product, as some humectants lower the a_w of the system to a greater extent than others depending on molecular weight, solubility and solute interactions (Sloan and Labuza, 1975a; Bone, 1969).

In order to minimize product development time for these products, it would be useful to have a prediction equation for humectant a_w lowering effect in a complex food system containing solids which do not go completely into solution.

Prediction equations available in the literature

Several investigators have derived equations for predicting the a_w lowering effect of simple solutes in solution. The majority of these equations were designed for use by the confectionery industry in order to predict the equilibrium relative humidity (ERH) of sugar solutions, syrups and confectionery products. The a_w (ERH ÷ 100) of a confectionery product determines whether loss or gain of moisture can occur during storage and whether the foodstuff will be susceptible to microbial deterioration during storage (Grover, 1947; Mansvelt, 1963; Mansvelt, 1973). These equations have not been used for most solid IMF foods possibly due to the nonideal behavior of the solutes and to food solids/humectant interactions. Even though correction factors have been established, no one has published results of the effectiveness of these equations.

The basic equation of a_w prediction, Raoult's Law, is seen in Eq (1):

$$a_w = \gamma \frac{N_1}{N_1 + N_2} = \frac{P}{P_o} \quad (1)$$

where: γ = activity coefficient; N_1 = moles water in system; N_2 = moles of theoretical solute in system; P = vapor pressure of water above system; and P_o = vapor pressure of pure water.

Raoult's Law usually cannot be applied due to the deviation in ideality of humectants beyond a minimum concentration; the inability to account for food solids/humectant interactions; and the inability to calculate a molecular weight for undissolved solids.

Hildebrand and Scott (1962) developed Eq (2) for the activity coefficient of binary regular solutions, as a function of solute concentration, employing Van Laar's approximations on molecular interactions (Toledo, 1973):

$$\ln \gamma_1 = KX_2^2 \quad (2)$$

where: γ_1 = activity coefficient of the solvent in the solution; K = a constant; and X_2 = mole fraction of the solute.

Norrish (1966) of Knecthel Laboratories Ltd. using similar reasoning as that of Hildebrand and Scott (1962) derived Eq (3):

$$\begin{aligned} \ln a_w &= \ln X_1 + \ln \gamma \\ &= \ln X_1 + K_2 X_2^2 \end{aligned} \quad (3)$$

where: a_w = final water activity; X_1 = mol fraction of water; γ = activity coefficient; K_2 = constant for specific humectant = slope of $\ln a_{w/x_1}$ vs $\ln X_2^2$; and X_2 = mole fraction of solute for predicting the final a_w lowering effect of a single sugar in solution, which he generalized to include multicomponent systems (Eq 4):

$$\ln a_w = \ln X_1 + ((-K_2)^{1/2} X_2 + (-K_3)^{1/2} X_3 + \ldots)^2 \quad (4)$$

where: a_w = final water activity; K_2, K_3 ... = binary coefficients of components 2 and 3 with water; and X_1, X_2, X_3 ... = mole fractions of water components 2 and 3 respectively. This equation does not account for the effect of food solids/humectant interactions, but does account for nonideality of the solute/water system. Norrish (1966) and Toledo (1973) presented activity coefficient factors for a wide range of solutes which are shown in Table 1.

Grover (1947), on an empirical basis, derived Eq (5) for the prediction of water activity of sugar-water solutions:

$$a_w = \frac{ERH}{100} = 104 - 10\,(E_s^o) + 0.45\,(E_s^o)^2 \quad (5)$$

where: $E_s = E_1 X + E_2 X_2 .....E_n X_n$ (6)

E_i = constant for specific component; and X_1 = g component in system/g H_2O in system. This formula predicts the final a_w based on the composition of the system and the weight ratios of each component to the amount of water in the food. Various empirical constants have been determined for several common food components and are listed in Table 2. Grover claims this equation to be effective between the a_w range 0.50–0.90 for confectionery items, but it is not considered valid at high concentrations.

Recently, Ross of the The Quaker Oats Co. derived Eq (7) based on the Gibbs-Duhem relationship (Ross, 1975):

$$a_f = a_i \cdot a_{H_1} \cdot a_{H_2} ..... a_{H_N} \quad (7)$$

where a_f = final a_w of system + humectant; a_i = initial a_w of food (no humectant added); a_{H_1} = a_w of specific humectant/H_2O solution based on total water content; and a_{H_2}, a_{H_3} = a_w of humectant/H_2O solution based on total water content for components 2 and 3, respectively. This equation assumed that in a food system, each a_w lowering component behaves independently. The final a_w is a product of each component a_w based on its being dissolved in all of the water in the system. Ross has discussed in detail the problems concerning his prediction equation and presents methods for coping with nonideality for simple solutions. However, these data have not been applied to most solid food systems.

In the Norrish and Ross prediction equations, a true sorption isotherm is needed to determine the a_w value of a humectant at a given moisture content.

MATERIALS & METHODS

Sorption experiments

Sorption experiments were conducted for the humectants listed in Table 3. True adsorption and desorption isotherms were measured for each of these humectants by static moisture gain from dry material using amorphous and/or crystalline material or by moisture loss from a high a_w liquid solution (0.95–0.97). A series of 10 saturated salt solution desiccators ranging from a_w = 0.11–0.97 (Rockland, 1960) were used for periods from 4–8 months at room temperature. In addition, desorption curves were prepared by measuring the a_w of humectant/water solutions by the vapor pressure manometric technique (Labuza, 1974a; Toledo, 1973). The humectant curves and procedures used are discussed in detail by Sloan and Labuza (1975). The curves may be obtained from the authors.

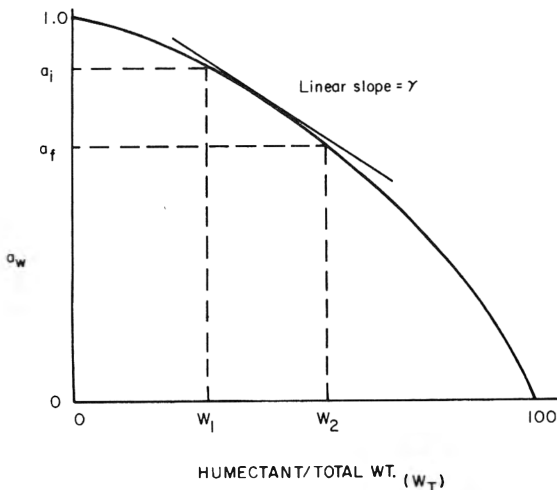

Fig. 1—Linear slope method derived by Sloan and Labuza for prediction of a_w. Graph is water activity plotted vs weight % (on 100 basis) of humectant/water solution. W_1 is initial % humectant, W_2 is final %, γ is slope in desired region.

In addition to our work, literature data on the water sorption properties of these humectants were also collected. Many other methods have been used as well.

New prediction equations derived

Due to the complexity of the previous prediction equations, two other prediction equations were derived. The first method, the linear slope method, assumes the food behaves as a humectant. As shown in Figure 1, the food at the initial a_w is assumed to have a theoretical

Table 1—Binary coefficients of solutes Norrish equation

Solute	K Value
Sucrose	−2.70
Corn syrup DE42	−2.31
Sorbitol	−0.85
Dextrose	−0.70
Fructose	−0.70
NaCl	−7.60
KCl	−4.70
Glycerol	−0.38
Propylene glycol	−0.20
1,3 Butylene glycol	−0.20

Table 2—Grover's food component conversion factors (E_i)

Sucrose, lactose	1.0
Invert sugar	1.3
Gelatin, casein	1.3
Confectioners glucose solids	0.8
Starch	0.8
Gums, pectin, etc.	0.8
Tartaric and citric acids and their salts	2.5
Glycerol and other glycols	4.0
Sodium chloride and other salts	9.0
Protein	1.3
Corn syrup DE42	0.8

Table 3—Humectants

Polyols	Supplier
Propylene glycol[a]	Dow Chemical
1,3 Butylene glycol	Celanese Chemical Co.
Glycerol	Mallinckrodt
Polyethylene glycol 400	Union Carbide Chemicals
Sorbitol (crystalline)[a]	ICI, United States
Sugars	
Fructose	J.T. Baker Chemical Co.
Sucrose[a]	Mallinckrodt
Glucose	J.T. Baker Chemical Co.
Lactose	Eastman Organic Chemicals
Corn syrup DE42	Clinton Corn Processing Co.
Salts	
KCL	Mallinckrodt
NaCl[a]	Mallinckrodt

[a] Humectants used for a_w prediction equation tests

humectant concentration at that a_w from the humectant isotherm. Water activity lowering is then based solely on the increase in weight of humectant above this concentration but corrected for the amount of water in the food itself. The predicted value can then be calculated from the slope of the curve, as shown by Eq (8):

$$a_f = a_i - \frac{\gamma z\,(1 - W_1)}{W_d} \quad (8)$$

where: a_f = final a_w; a_i = initial a_w; γ = slope of w/w of solution curve at initial a_w in (g humectant/g system)$^{-1}$; z = g humectant added per gram food; $1 - W_1$ = wet basis moisture content in g H_2O/g humectant system; and W_d = moisture content of system-wet basis in g H_2O/g food. Slope values are shown in Table 4 for all the humectants in the ranges between 0.90 and 0.85. The moisture content at a_w 0.90 is also shown. As with the Ross derivation, this method assumes that the food solids behave independently and only contribute to the initial a_w value and moisture content of the food. In this way the equations are similar.

The second method, a simplification of the slope method, is a graphical procedure; however, moisture content correction is not done. With this method, as shown in Eq (9), the predicted value can be read directly from a humectant isotherm assuming the food solids to be equivalent to humectant at the same a_w. For this method, only the initial a_w of the food and the humectant isotherm are necessary. By plugging into Eq (9), one gets M_2 and reads the final a_w from Figure (2) which is an isotherm for the humectant used:

$$M_2 = \frac{M_1}{100 + z} \times 100 \quad (9)$$

Where: M_1 = moisture content of humectant system in g H_2O/100g humectant at initial water activity (a_1) of food system; M_2 = moisture content at final water activity (a_2) in g H_2O/100g humectant solids. This is the predicted a_w of the system; and z = g humectant added per 100g of food system.

Table 4—Linear slope method

Humectant	Slope	(1 – W_1) at a_w 0.90
Glycerol	0.556	0.68
Propylene glycol	0.455	0.71
1,3 Butylene glycol	0.430	0.60
Polyethylene glycol 400	0.330	0.60
Sorbitol	0.343	0.57
Sucrose	0.182	0.415
Fructose	0.455	0.610
Lactose	0.882	0.030
Glucose	0.360	0.520
Corn syrup solids DE42	0.500	0.425
NaCl	0.960	0.975
KCl	0.526	0.825

Model system for testing a_w lowering

A meat-soy flour model intermediate moisture dog food system was employed in this study. The composition of this system is shown in Table 5. Increasing increments of four types of humectants most commonly used in IMF products (Table 3) were each dissolved in a predetermined amount of water, for example, the amount needed to reach an a_w of 0.90 without humectant, and blended for 5 min at high speed in a Brabender-Farinograph. The a_w's were measured by the vapor pressure manometric technique (Labuza, 1974a; Toledo, 1973) or the Fett-Vos isopiestic technique (Vos and Labuza, 1974) after 24 hr equilibration at 4°C. The final a_w's were calculated by each of the five equations previously discussed.

Table 5—Meat-soy flour model IMF system

	% by wt
Meat (lean ground hamburger)	19.5
Soy flour (PDI-20) (Cargill, Inc.)	78.2
Citric acid (J.T. Baker Chemical Co.)	2.0
Potassium sorbate (Anheuser-Busch, Inc.)	0.3

RESULTS & DISCUSSION

FIGURE 3 shows the isotherm measured for sorbitol. As shown, a hysteresis effect exists between the amorphous and anhydrous crystalline forms in which a different moisture content can occur above a given a_w value. Therefore, theoretically, the proper curve for a_w prediction must be used in terms of

Table 6—Model IMF dog food system

g added 100g system	Measured a_w	Ross	Linear	Graph	Norrish	Grover
		Amorphous sucrose				
4.69	0.94	0.94	0.94	0.95	0.96	0.91
9.38	0.93	0.93	0.93	0.95	0.96	0.90
14.06	0.92	0.92	0.92	0.95	0.96	0.89
20.31	0.91	0.91	0.91	0.94	0.95	0.89
25.00	0.90	0.90	0.89	0.94	0.95	0.88
		Crystalline sorbitol				
4.69	0.89	0.88	0.88	0.89	0.91	0.91
6.25	0.88	0.86	0.87	0.89	0.91	0.90
9.38	0.87	0.85	0.86	0.88	0.91	0.90
14.06	0.86	0.84	0.84	0.88	0.90	0.89
20.31	0.86	0.83	0.84	0.87	0.90	0.89
25.00	0.80	0.81	0.80	0.86	0.88	0.88

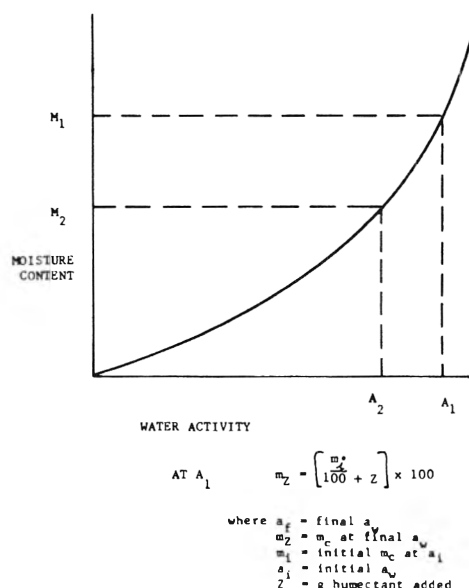

Fig. 2—Description of graphical procedure as derived by Sloan and Labuza. Moisture content of humectant/H_2O system in g/100g humectant vs water activity of system. M_1 is theoretical moisture content of food at initial a_w of a_1. M_2 is predicted moisture content after adding z grams humectant which gives a predicted a_w of a_2.

Table 7–Model IMF dog food system

g added 100g system	Measured a_w	Ross	Linear	Graph	Norrish	Grover
		Propylene glycol				
3.13	0.88	0.89	0.87	0.89	0.86	0.91
4.69	0.85	0.86	0.86	0.89	0.86	0.89
6.25	0.85	0.85	0.85	0.88	0.85	0.88
7.81	0.83	0.84	0.84	0.88	0.84	0.87
10.94	0.81	0.83	0.82	0.88	0.83	0.85
		Sodium chloride				
2.89	0.94	0.94	0.94	0.98	0.98	0.96
5.71	0.90	0.90	0.89	0.98	0.97	0.90
7.14	0.89	0.87	0.87	0.98	0.97	0.88
10.00	0.85	0.85	0.83	0.98	0.97	0.86

Table 8–Equation prediction ability

Method	Overall avg prediction variability	Range of deviation from predicted value
Ross	0.006	+0.03 to −0.02
Linear Slope	0.001	+0.01 to −0.02
Graphical	0.044	+0.13 to 0
Norrish	0.042	+0.12 to −0.2
Grover	0.020	+0.07 to −0.3

how the humectant was added to the food. However, as shown by Sloan and Labuza (1976), even if the humectant is added in a dry state, at least in the high a_w range, the amorphous solution curve can always be used for sugar-type material.

Table 6 shows the predicted a_w values and the actual measured values for amorphous sucrose and crystalline sorbitol. As seen, both the Ross method and the linear slope method predict the a_w values quite well, with the linear slope method predicting the a_w values within ± 0.01 a_w units and the Ross equation predicting final a_w values to within ± 0.02 units. This is certainly within the error of a_w measurement as described by Labuza et al. (1976). Both the graphical and the Norrish equation consistently predict much higher a_w values than those actually measured. Grover's predictions are also not as accurate as either the linear slope or Ross method.

The results obtained for propylene glycol and NaCl are shown in Table 7. Once again the linear slope method and the Ross derivation most accurately predict the final a_w. The other methods give consistently high a_w values as before.

The prediction variability and the overall a_w deviation obtained for each of the five methods employed are shown in Table 8. Both the linear slope method and the Ross equation are the best methods for predicting the final a_w.

Based on these results, the linear slope method has been shown to provide a very accurate means of predicting the final a_w value for a single humectant. It is simple and requires only the initial a_w and moisture content of the system along with a predetermined slope value for each humectant. The Ross derivation also provides a relatively accurate method of predetermining the final a_w in this range. The Grover equation, although not as accurate as the linear slope or Ross methods, is much more accurate than either the graphical or Norrish procedures but requires a long mathematical solution. It is expected that the other humectants for which sorption isotherms were determined will follow the same pattern.

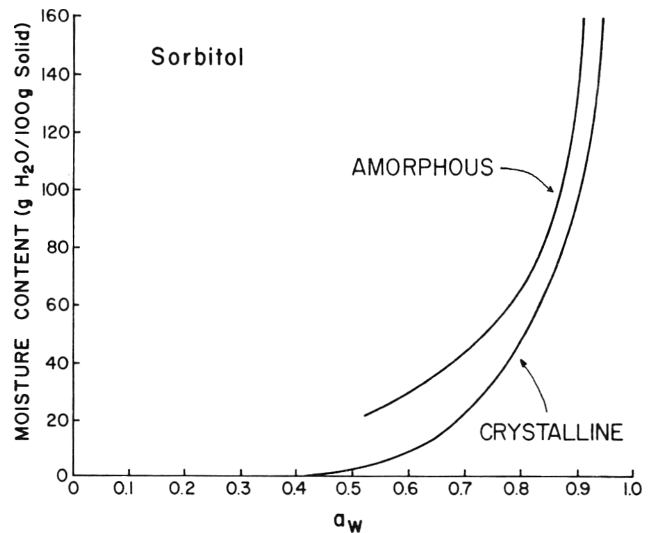

Fig. 3–Water sorption hysteresis showing amorphous vs crystalline material for sorbitol.

Prediction of the final a_w at higher humectant levels is being investigated. However, at higher levels the humectant would constitute such a major portion of the product that it would be organoleptically unacceptable.

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Ms received 7/11/75; revised 10/3/75; accepted 10/6/75.

Presented at the 35th Annual Meeting of the Institute of Food Technologists, Chicago, Ill., June 8–12, 1975.
Scientific journal series paper No. 9241, Minnesota Agricultural Experiment Station, St. Paul, MN 55108.
This project was supported in part by a grant from The Quaker Oats Company and NASA contract 12560, Lyndon Johnson Space Center, Houston, Texas.

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EFFECT OF ORDER-OF-MIXING ON a_w LOWERING ABILITY OF FOOD HUMECTANTS

ABSTRACT

The effect of the order-of-mixing on four humectants of major concern to the U.S. pet food industry was investigated using a meat-soy flour model intermediate moisture dog food system. Humectants were added dry or as a solution and the resultant water activities were measured. Both adsorption and desorption curves for pure humectant/water systems were prepared for each humectant. Even though the isotherms of some humectants exhibit a hysteresis effect, there was no significant difference in the final a_w achieved as a result of the order-of-mixing in the a_w range of intermediate moisture food products.

INTRODUCTION

THE SUCCESSFUL INTRODUCTION of intermediate moisture food (IMF) processing into the world pet food markets has resulted in an increased interest in this technology (Rhodes, 1975). Intermediate moisture food products have a moisture content of 15–40% water and a water activity (a_w) range of about 0.65–0.90. They require no rehydration and have a soft plastic texture. Intermediate moisture foods are shelf stable and can be formulated to meet specific nutrient needs (Smith and Norvell, 1975).

Various humectants, or water binding agents such as polyols, sugars and salts are incorporated into these products to lower the a_w into the intermediate moisture range (Bone, 1973; Bone et al., 1974). The increased shelf stability of these nonrefrigerated IMF products is based on the principle that added solutes lower the availability of water by binding it, thereby making it unavailable chemically and biologically. The solutes also increase the viscosity of the liquid phase, thereby lowering reactant diffusion rates (Heiss, 1967; Van Arsdel, 1963; Labuza, 1968, 1971, 1974a). In addition to their ability to bind water, some humectants also exhibit other desirable effects in a food system as a result of their antimicrobial properties (Labuza et al., 1974; Plitman, 1973; Celanese, 1971; Patsch, 1969), texturizing characteristics (Celanese S-26-10; Livengood, 1968), sweetening capacity and caloric value (Doherty, 1972).

Table 1—Typical composition of soft moist dog food

Ingredient	Per cent
Meat by-products	32.0
Soy flakes	33.0
Sugar	22.0
Skimmed dry milk	2.5
Calcium and phosphorous	3.3
Propylene glycol	2.0
Sorbitol	2.0
Animal fat	1.0
Emulsifier	1.0
Salt	0.6
Potassium sorbate	0.3
Minerals, vitamins and color	0.3

Semi-moist pet foods represent the majority of IMF food products now in the marketplace, accounting for close to 40% of the U.S. pet food market (Rhodes, 1975). A typical soft moist dog food formulation is shown in Table 1 (Bone, 1969). The humectants used in this particular formulation are sucrose, propylene glycol, sorbitol and salt. In this case sucrose is the main humectant, used at a level of 22% by weight. The other three humectants constitute a minor portion by weight of the product.

Humectants can be added into a food system either in a dry state or as a solution by previously dissolving the humectant in water. As a result of the method of addition of water into a food system, i.e., adsorption vs desorption, a hysteresis effect may occur (Rao, 1941; Taylor et al., 1961; Bettleheim and Ehrlich, 1963; Berlin et al., 1969; Mackenzie and Luyet, 1971). Wolf et al. (1972) also showed that a hysteresis effect can result in many foods especially those food products high in sugar. A hysteresis effect is one in which the amount of water which can be held in a food at a given a_w value differs depending on the method of addition of the water into the food, i.e., an adsorption vs a desorption procedure, with the desorption procedure holding more water at a given a_w.

The reasons for hysteresis have been discussed in detail by Labuza, 1968, 1974a; Labuza and Rutman, 1968; Gregg and Sing, 1967). The effect may be due to the structural effect of pores as described by Rao's theory (Rao, 1941) and shown by Labuza and Simon (1969). Two other factors which can contribute to hysteresis are supersaturation of solutes in the water of the food during desorption and the physical state of these solutes (Labuza, 1974b; Sloan and Labuza, 1975). Supersaturation can occur when the food is dehydrated. If the rate of water removal is rapid the viscosity may increase too rapidly for crystallization to take place. The resulting solution holds more water than the corresponding crystalline form. In addition the supersaturation state may form an amorphous glass (White and Cakebread, 1966; Parks et al., 1928). The third effect is related to this same phenomenon; namely, crystalline material absorbs less water per unit weight basis than does amorphous material because less hydrophilic sites are exposed to the vapor phase (Mankower and Dye, 1956). Thus, it is possible that there would be a smaller degree of a_w lowering by crystalline solutes if they are added dry to a food system (an adsorption process) as compared to adding the solutes by first dissolving them in water, which could be considered a desorption process.

This hysteresis or order-of-mixing phenomenon was investigated in a model semi-moist dog food system for the four humectants used in the dog food formulation (Table 1) as these are the humectants of major concern to the pet food industry.

MATERIALS & METHODS

THE AVAILABLE LITERATURE was surveyed to determine if a hysteresis effect occurred for pure humectant/water systems. In addi-

Table 2–Humectants investigated and suppliers

Humectant	Supplier
Propylene glycol	Dow Chemical, #156993, Midland, Mich.
Sucrose, (crystalline)	Mallinckrodt, #8360, St. Louis, Mo.
Amorphous sucrose	25% freeze-dried solution of crystalline sucrose (w/w)
Sorbitol (crystalline)	Atlas Chemical Div., ICI United States. #573', Lot 542D, Chicago, Ill.
Sodium chloride	Mallinckrodt, #54290, St. Louis, Mo.

tion both adsorption and desorption isotherms were measured for each humectant by static moisture gain from dry material using amorphous and/or crystalline material or by moisture loss from a liquid solution. A series of saturated salt solution desiccators were used for periods of from 4–8 months at room temperature. In addition, desorption curves were prepared by measuring the a_w of humectant/water solutions by the vapor pressure manometric technique (Labuza, 1974). The humectant curves and procedures are discussed in detail by Sloan and Labuza (1975). The actual data may be obtained from the authors. In all cases because of the confusion in the literature we used only our own sorption data.

The four humectants investigated in this study and their suppliers are listed in Table 2. The procedure used to investigate the order-of-mixing phenomenon for the four major humectants used in IMF pet foods is shown in Figure 1. A meat-soy flour model IMF dog food system was employed. The composition of this model system is listed in Figure 1. Lean ground beef, soy flour (20 PDI-Cargill, Inc., Minneapolis, MN) citric acid (T.J. Baker Chemical Co.) and potassium sorbate (Annheuser-Busch, Inc., St. Louis, MO) were ground in 50-g portions in an osterizer for 3 min at high speed. These portions were combined in a large stainless steel bowl, hand mixed and equilibrated in a covered 5-lb glass jar overnight. After equilibration the mixture was divided into two equal portions. In order to reach a given a_w, for example 0.90, a predetermined amount of water (14-ml per 50g solids) was mixed into one portion in a Brabender-Farinograph bowl, mixed at high speed for 5 min and re-equilibrated for 24 hr. The amount of water that was necessary to obtain a particular a_w was determined by preparing a moisture sorption isotherm of the meat-soy flour model system by adding increasing increments of water to the product and measuring the resultant a_w by the vapor pressure manometric technique (Labuza, 1974).

The remaining dry portion was subdivided into six equal portions. To each one of these six portions, the same amount of water necessary to reach the specific a_w for the wet-mix system was added. In this water, different amounts of the humectants shown in Table 2 were dissolved prior to their addition to the meat-soy flour system. This constituted the "wet-mix" system. All humectants were used as purchased. Amorphous sucrose was prepared by freeze drying a 25% w/w solution of crystalline sucrose for 48 hr. Each portion was subdivided into three equal parts and sealed in 202 × 214 cans. After an 18-hr equilibration period, one can per humectant concentration was removed and its a_w measured by the vapor pressure manometric technique. The remaining cans were stored at 4°C. The a_w was also measured after storage for various time periods.

After the initial re-equilibration period for the remaining premoistened initial mixture, increasing increments of humectants were added dry into the appropriate amount of system with an initial a_w of 0.90. This is the "dry mix" portion. The same procedure was followed for equilibration and measurement.

The Fett-Vos isopiestic technique for measurement of a_w was employed (Fett, 1973; Vos and Labuza, 1974) only for the sodium chloride system (run 1). The VPM was used for all other systems because of sample size limitations.

RESULTS & DISCUSSION

FIGURE 2 shows the sorption curve for propylene glycol as found in the literature. The references and methods used are listed in Table 3. Although these data show a hysteresis effect, the adsorption curves do not give a true indication of the water sorption properties, due to the occurrence of evaporation of the adsorption samples during equilibration in desiccators, at constant humidity. This does not occur for samples measured by the VPM for the desorption curve but would occur for desorption studies in desiccators (Sloan and Labuza, 1975). Because of this evaporation, a true equilibrium condition can never be reached. Secondly, in most studies of humec-

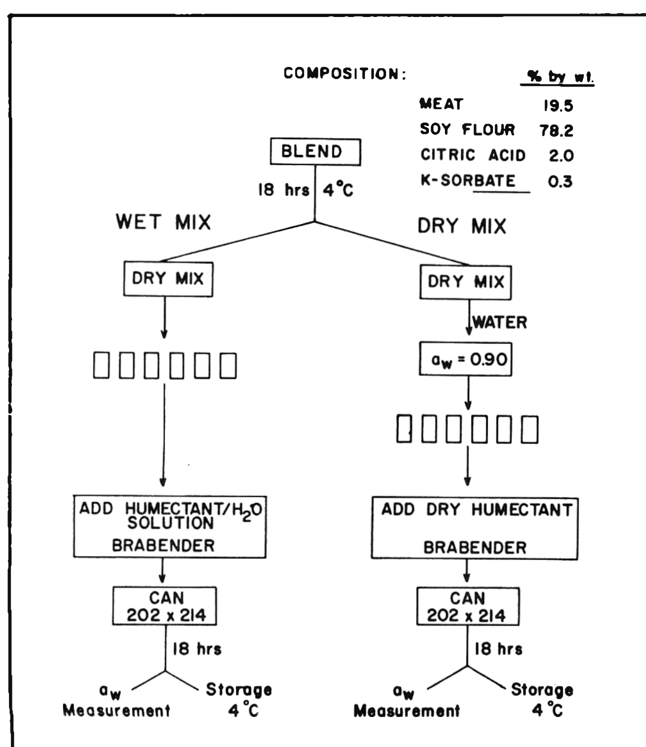

Fig. 1–Procedure used to investigate order-of-mixing phenomenon.

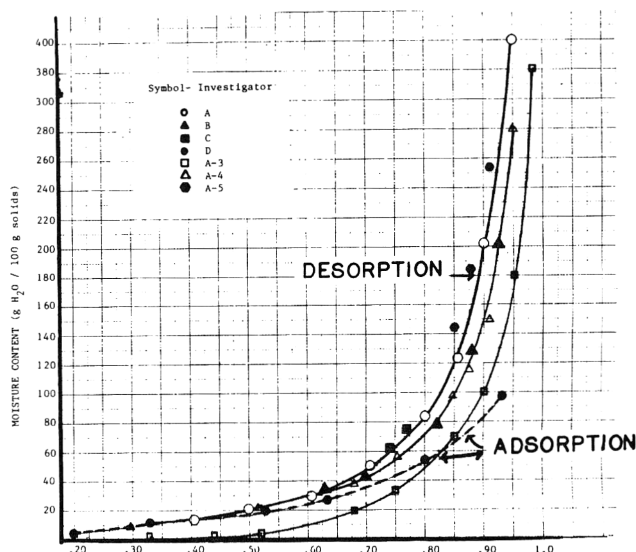

Fig. 2–Water sorption properties of propylene glycol.

tants using desiccators, not enough time was allowed for the samples to reach true equilibrium. As shown by our data for glycerol (Fig. 3) which was equilibrated for 9 months at 23°C, no hysteresis occurs when comparing adsorption, desorption and VPM data. Glycerol has a much lower vapor pressure and should not evaporate to any significant extent during equilibration. The same was found for polyethylene glycol 400. Thus, it can be presumed that liquid humectants such as propylene glycol should not show a hysteresis effect with water since there is no crystalline-amorphous transition.

The water sorption isotherm for sucrose is shown in Figure 4. A true sorption hysteresis for anhydrous-crystalline material occurs which is dependant on the initial physical form of the material. The lower curve is achieved from the dry state. The upper curve from the wet state. The same sorption curve however, is exhibited by amorphous sucrose for either adsorption of water by predried material, desorption from a solution or from a_w measurement of prepared liquid solutions. [For more detail on the sorption properties of humectants see Sloan and Labuza (1975).]

Table 3–Methods utilized for humectant isotherm determination

Letter[a]	Investigator	Method	Experimental procedure	Temp °C	Days to equilibration
A	Plitman (1970)	D	Electric hygrometer aqueous solution	25	–
B	Couvillion (1972)	D	Vapor pressure manometer	RT	–
C	International Critical Tables (1926)	D	Calculation from freezing point depression–aqueous solution	–	–
D	Celanese Chemical Co. (1975)	A	Equilibration chambers–Karl Fisher Analysis	23	7
H	Norrish (1966)	D	Hygrosensor in Dynamic System–Aq. Soln.	20	–
M	Heiss (1955)	A	Equilibration chambers–gravimetric analysis anhydrous sugar	20	60 (low) 12 (high)
Q	Dittmar (1935)	A	Equilibration chamber–gravimetric analysis anhydrous sugar	25	–
R	Whittier and Gould (1930)	D	Isoteniscope–aqueous solution	25	–
S	Browne (1922)	A	Equilibration chamber–gravimetric analysis anhydrous sugar	20	25
T	Nelson (1949)	A	Equilibration chambers–gravimetric analysis anhydrous sugar	20	NK
U	Mankower and Dye (1956)	A	Equilibration chamber–gravimetric analysis amorphous sugar	25	800
V	Money and Born (1951)	D	Dew point technique aoueous solution		–
Y	CPC International Inc.	A	Equilibration chambers–gravimetric analysis anhydrous sugars	20	9
A-3	Sloan and Labuza (1975)	A	Equilibration chambers–gravimetric analysis	23	5–9 mo
A-4	Sloan and Labuza (1975)	D	Vapor pressure manometer	RT	–
A-5	Sloan and Labuza (1975)	D	Equilibration chamber–GLC analysis	23	5 mo
A-6	Sloan and Labuza (1975)	D	Equilibration chamber–gravimetric analysis high a_w solutions	23	5 mo

[a] D = desorption; A = adsorption; RT = room temperature; NK = not known.

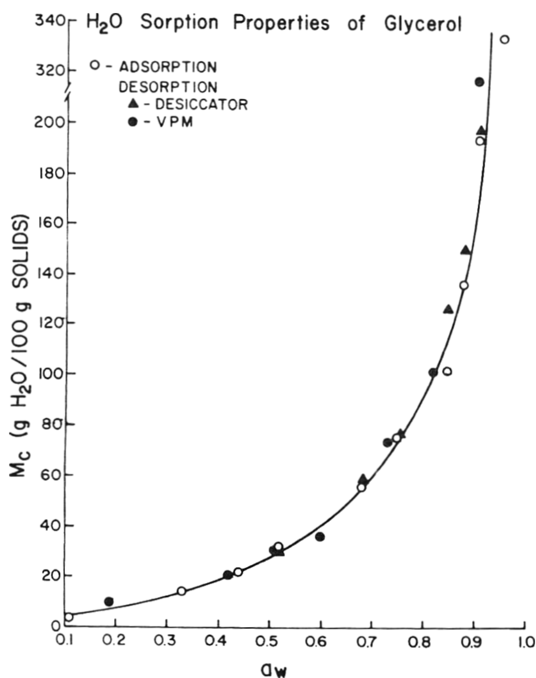

Fig. 3–Water sorption properties of glycerol (Sloan and Labuza, 1975) at 23°C.

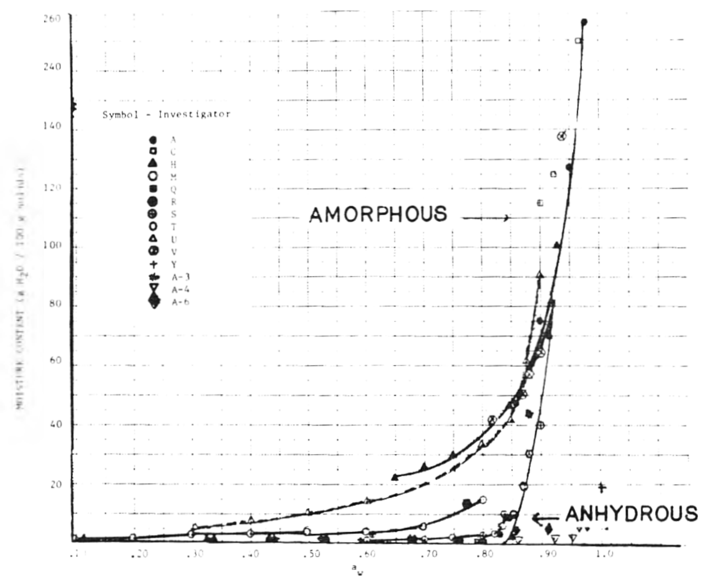

Fig. 4–Water sorption properties of sucrose.

The isotherms for sorbitol as well as other sugars and the two salts studied (NaCl and KCl) also exhibited the same effect. The hysteresis range for the crystalline materials used in this study was: (1) for sucrose 0.3–0.92; (2) for sorbitol 0.5–0.97; and (3) for sodium chloride 0.75–0.95. Thus it should be expected that if an order-of-mixing effect occurs, it would be manifested in the IMF range. In fact, based on this, if hystersis occurs, the crystalline humectants should show higher a_w's than the dissolved material below the upper closure point of the isotherm. This would be a value of 0.92 for sucrose and the maximum difference would exist at a_w 0.83. This is based on the equilibrium water activity condition. However, it is possible that kinetically the dry material dissolves first and then the crystalline state would be destroyed and equilibrium could not be reached.

Table 4 shows the resultant a_w's after the addition of propylene glycol to the meat-soy flour model system using both a wet and dry mix procedure. As expected, the order-of-mixing of this humectant has no effect on the degree of a_w lowering, since no true hysteresis was found for the water/humectant system. Thus, the method of addition of glycol in the food process should make no difference. In addition, there is little effect of storage.

The results obtained for crystalline and amorphous sucrose are shown in Table 5. It should be remembered that sorption hysteresis existed for crystalline sucrose between a_w = 0.30–0.92. As shown, the order-of-mixing of anhydrous crystalline sucrose does not have an effect on the resultant a_w even at high concentration. For example, at 20g sucrose added to the system in the crystalline state, the a_w if true hysteresis occurred should be about 0.91 since at the measured a_w of 0.83 crystalline sucrose holds almost no water. Thus in the dry mix system the sucrose must be rapidly dissolved in the available water and changed into a solution or amorphous form so that a_w is the same as in the wet mix system. As expected, as shown in Table 5, there is also no difference in the order of mixing for the amorphous sucrose. Although the humectant/water isotherm suggests that amorphous sucrose would lower the a_w of the system to a greater extent than does anhydrous crystalline sucrose, due to its higher degree of hydrogen binding sites, this is not the case in this model food system. There is no significant difference between the a_w lowering ability of crystalline vs amorphous sucrose as shown in Table 5. This is

Table 4—Effect of order-of-mixing of propylene glycol in a meat-soy flour model system

g Propylene glycol added per 50g system	Measured a_w					
	Wet mix			Dry mix		
	0 wk	3 wk	12 wk	0 wk	3 wk	12 wk
0	0.89	0.89	0.89	0.89	–	0.89
2	0.89	0.87	0.88	0.88	0.88	0.87
3	0.87	0.87	0.87	–	0.87	0.87
4	0.85	0.85	0.86	0.85	0.85	0.85
5	0.83	0.84	0.84	0.83	–	0.85
7	0.81	0.83	0.83	0.81	0.82	0.82

Table 5—Effect of order-of-mixing of sucrose in a meat-soy flour model system

Sucrose g added per 50g system	Sucrose Measured a_w			
	Crystalline sucrose		Amorphous sucrose	
	Wet	Dry	Wet	Dry
0	0.91	0.91	0.91	0.91
4	0.90	0.90	0.89	0.90
8	0.87	0.86	0.87	0.87
12	0.85	0.85	0.85	0.85
16	0.84	0.84	0.84	0.83
20	0.83	0.83	0.83	0.83

Table 6—Effect of order-of-mixing with NaCl in meat-soy flour model system

g Humectant added per 50g system	Dry		Wet	
Run 1:				
0	0.98		0.98	
2	0.94		0.94	
4	0.90		0.90	
5	0.89		0.89	
7	0.86		0.85	
	0 wk	3 wk	0 wk	3 wk
Run 2:				
0	0.90	0.89	0.90	0.90
2	0.87	0.87	0.88	0.88
4	0.84	0.84	0.84	0.84
5 partially crystalline	0.81	0.81	0.81	0.80
7 partially crystalline	0.76	0.76	0.76	0.76
8 partially crystalline	0.73	0.72	0.72	0.72

Table 7—Effect of order-of-mixing of crystalline sorbitol in a meat-soy flour system

g Sorbitol added per 50g system	Measured a_w			
	Dry mix		Wet mix	
	0 wk	10 wk	0 wk	10 wk
Run 1:				
0	0.90	0.90	0.90	0.89
3	0.90	0.87	0.87	0.87
6	0.88	0.88	0.87	–
8	0.86	0.86	0.86	0.86
10	0.86	0.86	0.85	0.86
12	0.84	0.84	0.83	0.83
Run 2:	0 wk	2 wk	0 wk	2 wk
0	0.91	0.91	0.91	0.90
4	0.86	0.86	0.87	0.87
8	0.86	0.86	0.86	0.85
12	0.83	0.82	0.82	0.83
16	0.81	0.80	0.81	0.81
20	0.79	0.79	0.79	0.79

Table 8—Effect of order-of-mixing with effect of storage time on a_w for crystalline sucrose

g Sucrose added per 50g system	Wet mix		Dry mix	
	0 wk	3 wk	0 time	3 wk
0	0.89	0.90	0.89	0.90
3	0.88	0.89	0.89	0.89
6	0.87	0.88	0.90	0.88
9	0.87	0.86	0.87	0.86
16	0.84	0.84	0.84	0.84

most likely due to the fact that crystalline sucrose dissolved rapidly as stated before and was changed into a solution or amorphous form.

The results obtained for sodium chloride are shown in Table 6 for two runs. In the second run as indicated not all the salt was able to be predissolved. Although crystalline NaCl showed true sorption hysteresis between a_w 0.75–0.95, as seen, no true order-of-mixing effect occurred. In addition, the storage conditions did not affect the water activities. In Table 7, no order-of-mixing effect was found for crystalline sorbitol. Thus, all crystalline materials dissolved rapidly enough in the pre-added water so that there was no effect or order-of-mixing in the range studied.

Additional storage results in Table 8 shows that after 3 wk time, with sucrose no significant change in water activity was found at 4°C.

In conclusion, even though the pure humectant water isotherm may exhibit a hysteresis effect, there is no significant difference in the final a_w achieved as a result of the order-of-mixing, in the a_w range of intermediate moisture food products. As a result of this investigation, it can be concluded that the method of addition of these humectants should make no difference in the resultant a_w value. Also, no significant difference in the a_w lowering ability of amorphous vs. anhydrous sugars was found in the IMF range. This is probably applicable to other materials. It is possible that at a lower a_w a difference may occur, but this would be of little interest for intermediate moisture foods.

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Ms received 7/25/75; revised 10/17/75; accepted 10/21/75.

Presented at the 35th Annual Meeting of the Institute of Food Technologists, Chicago, Ill., June 8—12, 1975.

Paper No. 9182 from the U. of Minnesota Agric. Expt. Station. Study supported in part by U.M. project 18-72 and Contract NAS 9-12560 from Lyndon Johnson Space Center, Texas.

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EVALUATION OF ANTIMICROBIAL AGENTS IN A MICROBIAL CHALLENGE STUDY FOR AN INTERMEDIATE MOISTURE DOG FOOD

ABSTRACT

Various microbial inhibitor systems were studied for their effectiveness in a semi-moist dog food formulation. The effectiveness was measured as inhibition of the natural contaminants *Aspergillus glaucus* and *Staphylococcus epidermidis* and of an inoculated challenge organism, *Aspergillus niger.* The study was carried out at a_w 0.85 and 0.88 and at pH 5.4 and 6.3 giving a total of 108 systems. It was found that very few compounds alone or in combination could prevent growth of all three organisms. The only FDA approved additive that was effective alone was propylene glycol.

INTRODUCTION

INTERMEDIATE MOISTURE FOOD (IMF) products and technology are among the newest in food science. Although semi-moist foods have been thoroughly researched, as indicated by many authors, the factors determining their stability have not been solved (Bone, 1973; Labuza, 1974; Haas et al., 1975). These IM foods are formulated to prevent most microbial growth. A relatively moist product is necessary for palatability but the water activity (a_w) must be below that allowing the growth of pathogenic bacteria, especially *Staphylococcus aureus* which can grow to a_w 0.84. To achieve this the microbes are usually inhibited by the addition of agents such as propylene glycol, potassium sorbate and sucrose. This study investigated several other antimicrobial agents for their effectiveness at two water activities against both natural and an added mold challenge.

The major microbes that occur naturally in semi-moist dog food are *Aspergillus glaucus* and *Staphylococcus epidermidis* (positive indentification was based on typical morphological characteristics and biochemical tests, U. of Minn. Dept. Veterinarian Medicine). The activity of these microbes is due to their ability to grow at low a_w's. *A. glaucus* can grow at a_w 0.73–0.75 (Christensen and Meronuck, 1974). In this study the *S. epidermidis* grew in the dog food as low as a_w 0.82. If it is prevented from growing, one can assume that *Staphylococcus aureus* will not grow (a_w minimum 0.84). The challenge organism was *Aspergillus niger.* Although not as osmotolerant as *A. glaucus,* this mold grows at the lowest a_w studied with the dog food. The minimum a_w for growth of *A. niger* in a chicken based IMF was 0.79 (Acott and Labuza, 1975). Use of these three organisms constitutes potential growth of both the natural contaminants and that of a ubiquitous air-borne mold that could inoculate the product and cause loss of acceptability. Haas et al. (1975) have also independently found these were the most important organisms to use in a challenge procedure for intermediate moisture foods.

MATERIALS & METHODS

Preparation of test systems

The semi-moist dog food used in this study was formulated at the Quaker Oats Co., Barrington, Ill., (36% meat by-products, 39% soy flour, 25% sucrose), excluding the antimicrobial system, phosphoric acid, propylene glycol and K-sorbate. After extrusion and canning, the dog food was held in frozen storage (−29°C) until used. After thawing and tempering to 23°C, the dog food for each study was taken aseptically from the cans and ground in a sanitized Hobart Silent Cutter (Hobart Manufacturing Corp., Troy, Ohio) to ensure homogeneity. An acidulant was added to give a pH of 5.4. Adipic, citric, fumaric, lactic and phosphoric acid were tested but none exhibited specific antimicrobial properties. Phosphoric acid, the least expensive one, was used in the subsequent studies. Figure 1 shows the scheme used to prepare the various test systems. After acidification, the batch was divided into two parts and sterile water was added to one-half giving an a_w of 0.88. The initial a_w of the other half of the dog food was 0.85. These systems were further divided and the inhibitors were added to give the desired levels. Following a 24-hr equilibration period at 23°C, each inhibitor system was divided into two equal parts (25g each). Sterile water (0.1 ml) was added to one and the other was inoculated with an *A. niger* spore suspension (0.1 ml). The water and suspension were mixed into the systems by blending in an Osterizer blender jar (1 cup size) for 1 min, stopping 2–3 times to scrape the material into the blades. The initial population of *A. niger* was 10^6 colony forming units (CFU)/100 grams of dog food. The inoculation procedures did not cause a change in the a_w of the systems. The 5-g samples were weighed into petri dishes (60 × 15 mm) and stored in closed chambers over saturated salt slurries of the appropriate a_w (Li_2SO_4 = 0.85 and $ZnSO_4$ = 0.88). The

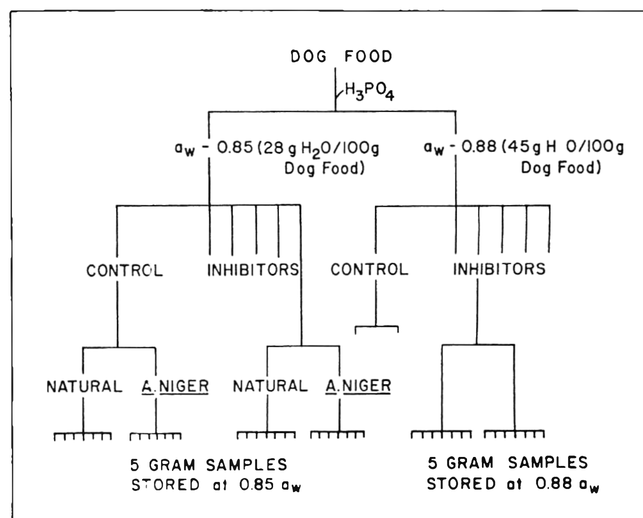

Fig. 1—Flow diagram for system preparation.

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Table 1–Chemical inhibitors in study

List of inhibitors tested	Levels tested g/100g dog food	Specification
	Metabolic inhibitors	
K-sorbate	0.3	Anheuser-Busch St. Louis, MO
Ca-propionate	0.3	Anheuser-Busch St. Louis, MO
Parabens:		
Methyl (USP)	0.3, 0.10	Tenneco Chemical
Propyl (USP)	0.01, 0.10	Heyden Div.,
Combined (2M:1P)	0.05, 0.10	New York, NY
Caproic acid[a]	0.1, 0.2, 0.3	
Pimaricin (Delvocid Instant)[a] (Natamycin)	0.002	Gist-Brocades, N.V. Delft-Holland
	Polyols	
Glycerol	1,2,3,8,10,13	Mallinckrodt #5092 St. Louis, MO
Propylene glycol	1,2,4,6,8,9	Mallinckrodt #5092 St. Louis, MO
Polyethylene glycol 400	16	Union Carbide Chem., New York, NY
1,3-Butylene glycol[a]	1,2,4,6,10	Celanese Corp., New York, NY
	Sugars	
Fructose	20	J.T. Baker Chem. Glen Ellyn, IL
Mannitol (USP)	1.0	Atlas, ICI America Wilmington, DE
Sorbitol (99% crystalline)	1,4,7,18	Atlas, ICI America Wilmington, DE

[a] Not FDA-approved

chamber lids were removed periodically to ensure adequate aerobiosis. The slurries were stirred as necessary for maintenance of the proper a_w.

Antimicrobial agents

The antimicrobial agents which were tested alone or in combination were of three types: metabolic inhibitors, polyhydric alcohols (polyols) and sugars. Each chemical and the FDA-approved levels tested are presented in Table 1. The chemicals which are not cleared for use in foods were tested in appropriate amounts.

The common food additives, K-sorbate and Ca-propionate, are called acid-type inhibitors as their effectiveness increases as pH decreases (Sauer, 1972). They are usually used in relatively acid foods. The parabens are esters of parahydroxybenzoic acid. This molecular configuration does not have the pH dependence for efficacy as do the acid-type inhibitors (Chichester and Tanner, 1968). Caproic acid has been recommended as a replacement for K-sorbate in IM dog food (Haas, 1973). The antiobiotic, pimaricin (Natamycin) is used in European countries in the production of sausage and cheese (Clark et al., 1964). It has selective inhibition of fungal but not bacterial growth.

The polyols are usually used in foods as humectants, plasticizers or emulsifiers. These chemicals also have some degree of antimicrobial action related to the location of the hydroxyl groups and chain length (Patsch and Hoehne, 1969). Some polyols, e.g., glycerol, are metabolized by certain microbes. When this situation exists that polyol will not function as an inhibitory agent. Currently propylene glycol is the most commonly used humectant-antimicrobial agent in semi-moist foods.

The sugars studied are usually employed as sweeteners in foods. They were tested here in their capacity as microbial inhibitors and humectants.

Preparation of inoculum

A. niger was grown for 5 days at 23°C on a 30 ml TSY (Trypticase Soy Agar + Yeast Extract, 0.5%, BBL Brand) agar slant in a cotton-plugged 150 ml prescription bottle. The spores were washed from the agar with 3–10 ml aliquots of sterile water and gentle shaking by hand. The population of the suspension was determined by plate counts on TSY agar. The stock suspension was diluted so that 0.1 ml in 25g of test system would give a population of 10^6 CFU/100g. Constant vortexing of the suspension was necessary while inoculating the systems to ensure consistency.

Analytical methods

The pH of the dog food was determined using the gran plot technique described by Acott and Labuza (1974). To 7-g samples of dog food, 0.5, 1.0, 3.0, 5.0 and 7.0g of water were mixed and equilibrated at 4°C for 18 hr after warming to room temperature. The pH of the slurries was determined using an Orion pH meter equipped with a semi-micro combination electrode (Orion #910200). The pH was plotted on gran plot paper (Orion Cat. No. 900093, 100% volume corrected) against the grams of water added to the dog food. Extrapolation to the pH at zero grams of water was taken as the pH of the semi-moist sample. The pH of the dog food with no acidulant added by the gran plot method was pH 6.3. This is different than from a direct reading taken on the semi-moist material (pH 5.9), or when a 1/100 dilution (AOAC, 1970) was made (pH 7.0). The gran plot method gives a theoretically more accurate estimation of pH.

The Fett-Vos Method (Vos and Labuza, 1974) was used to determine the a_w of the systems at the initiation of storage. After 6–8 wk of storage the a_w determinations were performed by the vapor pressure manometric (VPM) procedure (Labuza, 1974) on microbiologically stable 5-g samples.

The moisture content of the dog food was determined by vacuum drying oven at 29" Hg and 60°C for 24 hr. The analysis was done on dog food with and without the addition of water. Systems with inhibitors added were not used. The loss of polyols during drying would have resulted in erroneous values.

Microbial analysis

The microbial activity was monitored by blending 5-g samples with 45g of sterile phosphate buffer (0.125%), and spreading 0.1 ml of appropriate dilutions on prepoured TSY agar plates. The plates were inverted and incubated at 23°C for 5 days in humidified chambers (a_w = 1.0). Differential enumeration was based on the morphological differences of the colony forming units. *A. niger* produced typical black conidia over white spreading mycelia. *A. glaucus* formed compact masses of mycelia which failed to produce conidia but which were readily distinguishable from bacterial colonies due to the different reflectance of light. The *S. epidermidis* appeared as typical staph-like, buff-colored, smooth, round colonies. The growth of *A. niger* and *A. glaucus* on dog food produced typical colonies, black and green, respectively.

Effectiveness criteria

The effective antimicrobial agent was that which prevented the growth of both the molds and the bacteria at the given conditions. Inhibition of mold growth was determined by sample platings and visual observations. The bacterial growth was determined by sample platings. The antimicrobial agent was deemed unsuccessful when it allowed a two log cycle increase in *S. epidermidis* population and visible mold growth within 6 months of storage. Conversely, if the organism showed no positive growth the inhibitor was successful.

RESULTS & DISCUSSION

THE a_w DETERMINATIONS as shown in Table 2 indicated a change in a_w after 6–8 wk of storage in spite of the controlled environment. Part of the difference could be due to the use of different methods of a_w determination, as necessitated by the small sample size. Labuza et al. (1975) demonstrated that the Fett-Vos Method used for the initial determinations in this study gave values 0.02 units higher than the VPM Method when standardized against saturated salt slurries. However, this factor alone does not explain the decrease in a_w with storage time. Since the tested samples were microbiologically stable, the change may be due to reduced water-holding capacity caused by chemical reactions during storage. This decrease in a_w should act to make the food more stable to microbial deterioration, increasing effectiveness of the antimicrobial agents.

The compatability of the three species of microbes is demonstrated by the plate counts of the dog food without inhibitors as shown in Table 3, compared to the same system with added *A. niger.* As seen, addition of the challenge organ-

Table 2–a_w history for stored IMF dog food

Inhibitor	g/100g dog food	Initial[a] a_w	a_w of storage chamber	a_w After storage[b]
				2 mo
1,3-Butylene	4	0.88	0.88	0 82
glycol	6	0.88	0.88	0 86
Propylene	4	0.88	0.88	0 83
glycol	8	0.87	0.88	0 87
Glycerol	10	0.85	0.88	0.85
				3 wk
Sorbitol	4	0.83	0.85	0.82
	7	0.82	0.85	0.80
				6 wk
Sorbitol	4	0.94	0.95	0.79
	7	0.82	0.85	0.78

[a] Fett-Vos Method
[b] VPM Method

Table 4–Time for growth of microbes.[a] (Inoculated dog food with inhibitors pH 5.4)

Inhibitor	Storage conditions a_w = 0.85 9 mo storage	a_w = 0.88 6 mo storage
No Inhibitor added	*A. niger* – 2 wk	*A. niger* – 1 wk
	A. glaucus – 1 wk	*A. glaucus* – 1 wk
	S. epider. – 2 wk	*S. epider.* – ½ wk
K-sorbate (0.3%)	No mold	*A. niger* – 5 wk
	S. epider. – 25 wk	*S. epider.* – 3½ wk
Ca-propionate (0.3%)	*A. niger* – 25 wk	*A. glaucus* – 2 wk
	A. glaucus – 25 wk	*S. epider.* – 1½ wk
	S. epider. – 3½ wk	

[a] Mold – first visible sign; Bacteria – 2 log cycle increase

Table 3–Plate counts of dog food no inhibitors added[a]

Time	Unchallenged A. glaucus	Unchallenged S. epidermidis	Challenge A. niger	Challenge A. glaucus	Challenge S. epidermidis
0	12	9×10^4	5×10^5	12	9×10^4
4 days	12	8×10^4	4.3×10^5	12	8×10^4
2 wk	9.9×10^7	1.0×10^7	2.0×10^6	5.5×10^7	3.3×10^7

[a] Differential enumeration (CFU/100g dog food); pH 5.4 (H_3PO_4); a_w = 0.85

Table 5–Growth nomenclature for microbial inhibitor studies

NG(00)	– no growth in 00 weeks which was end of study
V00	– visible mold in 00 weeks
NT	– not tested
2L(00)	– increase of *S. epidermidis* in 00 weeks by two log cycles
D(00)	– decrease of *S. epidermidis* in 00 weeks by two log cycles

Successful change was prevention of 2 log cycles, increase in *S. eipdermidis* and no increase in mold as indicated visually and confirmed by plate counts.

Table 6–Metabolic inhibitors

Inhibitor	g/100g dog food	Initial a_w	pH	Inoculated A. glaucus	Inoculated S. epidermidis	Uninoculated A. niger	Uninoculated A. glaucus	Uninoculated S. epidermidis
K-sorbate	0.3	0.86*	5.4	NG(44)	2L(25)	NG(44)	NG(44)	2L(25)
	0.3	0.90**	5.4	NG(28)	2L(4)	V5	NG	2L(3)
Ca-propionate	0.3	0.87*	5.4	V25	2L(4)	V25	V25	2L(3)
	0.3	0.90**	5.4	V2	2L(2)	NG	V2	2L(1)
Caproic acid	0.1	0.85*	6.2	V3	NT	V3	V3	NT
	0.1	0.90**	5.4	V6	2L(1)	NG	V6	2L(1)
	0.2	0.90**	5.4	V(12)	2L(2)	NG	v(12)	2L(2)
	0.3	0.85*	6.2	NG(32)	2L(7)	NG(32)	NG(32)	2L(7)
	0.3	0.90**	5.4	NG(27)	2L(7)	NG(27)	NG(27)	2L(7)
Methyl paraben	0.03	0.87*	5.4	V4	2L(2)	V4	NG	2L(2)
	0.10	0.85*	5.4	V3	2L(1)	NG	V3	2L(1)
	0.10	0.90**	5.4	V1	2L(½)	V1	NG	2L(½)
Propyl paraben	0.01	0.87*	5.4	V4	2L(2)	V4	NG	2L(2)
	0.10	0.85*	5.4	V3	2L(2)	NG	V3	2L(2)
	0.10	0.90**	5.4	V1	2L(½)	V1	NG	2L(½)
Combination (2:1, Me:Pro)	0.05	0.86*	5.4	V4	2L(2)	V4	V4	2L(2)
	0.10	0.85*	5.4	V3	2L(3)	NG	V3	2L(2)
	0.10	0.90**	5.4	V1	2L(½)	V1	V½	2L(½)
Pimaricin	0.002	0.87*	5.4	V15	2L(2)	V4	NG	2L(2)
Controls	0	0.85*	6.2	V1	2L(2)	V2	V1	2L(2)
	0	0.87*	5.4	V1	2L(2)	V2	V1	2L(2)
	0	0.90**	5.4	V1	2L(½)	V1	V½-1	2L(½)

* stored at 0.85
** stored at 0.88

ism did not change the growth response of the other organisms.

The primary requirement for a microbiologically stable product is the control of the microbe that will initiate growth. Table 4 shows the effect of increased a_w on stability in control dog food and the results when K-sorbate and Ca-propionate were added. At the lower a_w all species had reached high populations in control dog food by 2 wk. At the higher a_w 0.88, the growth rate of *S. epidermidis* and *A. niger* was increased. When 0.3% K-sorbate was added to 0.85 a_w dog food, all mold was completely inhibited and the product appeared edible even after 9 months storage. The growth of *S. epidermidis* was slow but after 25 wk the population had increased by 2 log cycles. Although there are no "limits" on nonpathogenic staphylococci, the strict criterion of a 2 log cycle increase was used with *S. aureus* in mind. High populations of this organism when growing at a high enough a_w can produce enterotoxins and thus food poisoning (Troller and Stinson, 1975). Thus, using the 2 log cycle increase, this system failed challenge. At a_w 0.88, *A. niger* grew in the K-sorbate dog food but *A. glaucus* was controlled. Haas et al. (1975) found that a variety of this mold was particularly resistant to K-sorbate. The difference in the findings is most likely due to species variation and difference in the growth medium. This latter point is quite important and suggests one should be careful in extrapolating from one system to another.

Ca-propionate slowed the growth of the molds but after 25 wk both species were visible at the lower a_w 0.85. At 0.88 the growth rate of *A. glaucus* and *S. epidermidis* was significantly increased. The *A. glaucus* appeared within 2 wk. *A. niger* was completely inhibited. These data suggest that Ca-propionate should not be strictly substituted for K-sorbate.

Few of the antimicrobial agents were successful in preventing all growth when tested at FDA-approved levels. The overall

Table 7—Humectants—Sugars

Sugar	g/100g dog food	Initial a_w	pH	Microbiological response: Uninoculated		Microbiological response: Inoculated		
				A. glaucus	S. epidermitis	A. niger	A. glaucus	S. epidermitis
Mannitol	1.0	0.86**	5.4	NG	2L(3)	V4	NG	2L(3)
Fructose	20.0	0.81*	6.3	V3	2L(4)	V3	NG	5L(4)
Sorbitol	1.0	0.86**	5.4	V4	2L(4)	NG	V4	2L(4)
	4.0	0.83**	5.4	V4	2L(1)	NG	V4	2L(1)
	7.0	0.82**	5.4	V5	2L(2)	NG	V5	2L(2)
	4.0	0.89**	5.4	V1	6L(2)	V1	V1	2L(½)
	7.0	0.86**	5.4	V2	4L(½)	V2	V2	2L(½)
	18.0	0.80*	6.3	V3	NT	V9	NG	NT
Control	0	0.82*	6.3	V2	NT	V3	V2	NT
	0	0.85**	5.4	V1	2L(4)	V2	V1	2L(2)
	0	0.90***	5.4	V1	2L(½)	V1	V1	2L(½)

* stored at 0.82
** stored at 0.85
*** stored at 0.88

Table 8—Humectants—Polyols (Stored at a_w 0.85)

Polyol	g/100g dog food	Initial a_w	pH	Microbiological response: Uninoculated		Microbiological response: Inoculated		
				A. glaucus	S. epidermitis	A. niger	A. glaucus	S. epidermitis
1,3-Butylene glycol	1.0	0.84	6.3	V4	NT	V3	V4	NT
	2.0	0.86	5.4	NG(44)	2L(4)	NG(44)	NG(44)	2L(2)
	4.0	0.84	5.4	NG(27)	D(4)	NG(27)	NG(27)	D(4)
	4.0	0.84	5.9	NG(32)	NG(6)	NG(32)	NG(32)	NG(6)
	10.0	0.85*	6.3	NG(36)	D(4)	NG(36)	NG(36)	D(4)
Propylene glycol	1.0	0.86	6.3	V3	NT	V3	V3	NT
	2.0	0.86	5.4	V18	3L(4)	NG(44)	NG(44)	2L(4)
	5.0	0.84	5.4	NG(27)	D(6)	NG(27)	NG(27)	D(6)
	9.0	0.82	6.3	NG(36)	D(4)	NG(36)	NG(36)	D(4)
Glycerol	1.0	0.85	6.3	V2	NT	V2	V2	NT
	2.0	0.85	5.4	V4	1L(4)	V4	NG	1L(4)
	13.0	0.81*	6.3	V(3)	D4	V3	NG	D4
Polyethylene glycol 400	16.0	0.84*	6.3	V12	D12	V12	NG	D4
Control	0	0.86	6.3	V2	NT	V2	V2	NT
	0	0.87	5.4	V1	2L(4)	V2	V1	2L(2)
	0	0.88	6.3	V1	3L(½)	V1	V1	3L(½)
	0	0.82*	6.3	V2	NT	V3	V2	NT

* stored at 0.82

results are listed in Tables 6 through 9. The nomenclature used is shown in Table 5. In most cases the mold could be prevented but the *S. epidermidis* count increased rapidly. The usual metabolic inhibitors used in IMF, k-sorbate and Ca-propionate which were not effective alone, were tested with glycerol, propylene glycol, 1,3-butylene glycol, mannitol and sorbitol at levels from 1–10%. The results are similarly listed in Tables 10 and 11.

A summary of the very few successful antimicrobial systems found in this study is listed in Table 12. The advantage of using an acidulant is evident in the results for the 0.85 a_w systems. Only 4% 1,3-butylene glycol was needed at pH 5.4, while more than twice as much was necessary at pH 6.3. The same situation occurred with propylene glycol. The 4% sorbitol system with 0.3% K-sorbate added was also effective as an antimicrobial system. There may be some advantage to substituting some sucrose in a product with sorbitol. In addition, the results suggest that 5–7% propylene glycol in combination with K-sorbate at 0.85 as is presently used, is very effective since 5% propylene glycol alone is effective.

No inhibitor was effective alone at the higher a_w 0.88; however, by combining inhibitors the dog food system was

Table 9—Acidulants (a_w 0.85)

			Microbiological response				
			Uninoculated		Inoculated		
Acidulant	%	pH	A. glaucus	S. epidermidis	A. niger	A. glaucus	S. epidermidis
Adipic acid	3.6	4.8	V4	NG(4)	V4	V4	NG(4)
	1.0	5.2	V3	NG(4)	V3	V3	NG(4)
	0.6	5.5	V2	D(4)	V3	V2	D(4)
Citric acid	1.6	4.8	V4	NG(4)	V4	V4	NG(4)
	0.5	5.3	V2	NT	V2	V2	NT
Fumaric acid	1.2	4.9	V4	NG(4)	V4	V4	NG(4)
	0.5	5.7	V2	NT	V2	V2	NT
Lactic acid	2.0	4.8	V4	NG(4)	NG	V4	NG(4)
	0.8	5.3	V2	NT	V2	V2	NT
Phosphoric acid	0.55	4.8	V3	D(6)	NG	V3	NG(6)
	0.25	5.4	V1	2L(1)	V2	V1	2L(1)
Control	0	6.2	V1	None detected[a]	V2	V1	None detected[a]

[a] Mold count was very high; staph colonies not present on plates of highly diluted sample

Table 10—Humectants wtih 0.3% K-sorbate (pH 5.4, stored at a_w 0.88)

			Microbiological response				
			Uninoculated		Inoculated		
Humectant	g/100g dog food	Initial a_w	A. glaucus	S. epidermidis	A. niger	A. glaucus	S. epidermidis
Control	0	0.90	V1	2L(½)	V1	NG	2L(½)
Control w K-sorbate (0.3%)	0	0.90	NG(15)	2L(3)	V5	NG	2L(3)
Polyols							
1,3-butylene glycol	1	0.90	NT	NT	V6	NG(24)	2L(2)
	2	0.89	NG(24)	2L(5)	NG(24)	NG(24)	2L(5)
	4	0.88	NG(24)	D5	D5	NG(24)	D5
	6	0.88	NG(24)	D5	D5	NG(24)	D5
Glycerol	2	0.89	NT	NT	V4	NG	2L(3)
	4	0.88	NG(24)	2L(3)	V5	NG	2L(3)
	8	0.86	NG(24)	2L(5)	V10	NG	2L(5)
	10	0.85	NG(24)	2L(7)	NG(24)	NG(24)	2L(7)
Propylene glycol	2	0.90	NT	NT	V6	NG	2L(2)
	4	0.88	NG(24)	D3	D5	NG(24)	D5
	6	0.88	NG(24)	D3	D5	NG(24)	D5
	8	0.87	NG(24)	D3	D5	NG(24)	D5
Sugars							
Sorbitol	4	0.89	NG(22)	2L(2)	V19	NG	2L(2)
	7	0.86	NG(22)	2L(3)	NG(22)	NG(22)	2L(3)
	4	0.83*	NG(22)	NG(22)	NG(22)	NG(22)	NG(7)
	7	0.82	NG(22)	NG(7)	NG(22)	NG(22)	NG(7)
Mannitol	2	0.89	NG(22)	NG(5)	V4	NG	NG(5)
	4	0.89	NG(22)	NG(5)	V4	NG	NG(5)
	6	0.88	NG(22)	NG(5)	V4	NG	NG(5)

* stored at 0.85

Table 11–Humectants with 0.3% Ca-propionate (pH 5.4; stored at a_w 0.88)

			Microbiological response				
			Uninoculated		Inoculated		
Humectant	g/100g dog food	Initial a_w	A. glaucus	S. epidermidis	A. niger	A. glaucus	S. epidermidis
Control	0	0.90	V1	2L(½)	V1	NG	2L(½)
Control with Ca-propionate (0.3%)	0	0.90	V2	3L(3)	NG	V2	3L(3)
Polyols							
1,3-Butylene glycol	1	0.90	NT	NT	V6	NG	2L(2)
	2	0.89	NG(22)	2L(3)	NG(22)	NG(22)	2L(3)
	4	0.88	NG(22)	D3	NG(22)	NG(22)	NG(5)
	6	0.88	NG(22)	D3	D(5)	NG(22)	D(7)
Glycerol	2	0.89	NT	NT	V5	NG	2L(2)
	4	0.88	NT	NT	V5	NG(22)	2L(3)
	8	0.86	NT	NT	V6	NG(22)	2L(3)
	10	0.85	NT	NT	NG	V(28)	2L(4)
Propylene glycol	2	0.90	NT	NT	NG(22)	NG(22)	2L(2)
	4	0.88	NG(22)	NG(12)	D(5)	NG(22)	2L(6)
	6	0.87	NG(22)	D7	D(5)	NG(22)	D(7)
	8	0.87	NG(22)	D5	D(5)	NG(22)	D(5)
Sugar							
Mannitol	2	0.89	V3	NG(3)	V6	NG	2L(3)
	4	0.89	NT	NT	V6	NG	2L(3)
	6	0.88	V4	2L(4)	V6	NG	2L(2)

NT = not tested

made shelf stable. Propylene glycol and 1,3-butylene glycol (4 or 6%) used with K-sorbate or Ca-propionate (0.5%, maximum allowed by FDA) extended the microbiological shelf life of the dog food to 6 months. This suggests the use of 1,3-butylene glycol as a substitute for propylene glycol, although it is not yet cleared by FDA for such use. The results also indicate that Ca-propionate could be used instead of K-sorbate, contradicting the initial indications shown from Table 4 (i.e., K-sorbate effective against, but Ca-propionate not effective against *A. glaucus*). This point involves the complexity of the systems and our lack of understanding of the interactions occurring. Apparently there is a synergistic effect between the polyols and the metabolic inhibitors.

The antimicrobial systems required for stability in this product are more severe than those effective in a similar chicken based IMF system (Acott and Labuza, 1974). This illustrates the importance of product formulation on the degree of antimicrobial protection needed and the fact that it is hard to extrapolate from one system to another.

Table 12–% Levels of successful inhibitors

Inhibitor	pH 5.4	pH 6.3
at a_w 0.84–0.85		
1,3-Butylene glycol	4%	9%
Propylene glycol	5%	8.3%
Sorbitol with 0.3% K-sorbate	4%	—[a]
at a_w 0.88		
1,3-Butylene glycol with 0.3% K-sorbate	4%	
1,3-Butylene glycol with 0.3% Ca-propionate	4%	
Propylene glycol with 0.3% K-sorbate	4%	
Propylene glycol with 0.3% Ca-propionate	6%	

[a] — = not tested

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Ms received 9/16/75; revised 11/14/75; accepted 11/20/75.

Presented at the 35th Annual Meeting of the Institute of Food Technologists, Chicago, Ill., June 8–12, 1975.

This is paper No. 9218 from the Univ. of Minnesota Agric. Expt. Station. This study was supported in part by a grant from Quaker Oats Company, Barrington, Ill.; by Project No. 18-52 of the Univ. of Minn. Agr. Expt. Stn. and by NASA Contract NAS 9-12560, Lyndon Johnson Space Center, Houston, Texas.

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EFFECTS OF THERMAL STRESS AND REDUCED WATER ACTIVITY ON CONIDIA OF *Aspergillus parasiticus*

ABSTRACT

Conidia of *Aspergillus parasiticus* (an aflatoxin producer) were harvested from an agar medium, centrifuged, and resuspended in 0.05% Tween-80. The suspension was subjected to a thermal stress of 51°C for up to 4 hr. Viability and thermal injury were evaluatd by the use of two plating media, yeast extract agar and yeast extract agar + 10% NaCl. Evidence of injury was demonstrated by the difference in colony counts on the two media. The number of injured cells increased as the heating time continued. Colonies from injured conidia developed more slowly than colonies from unheated conidia. The effect of a reduced water activity (a_w) was evaluated by comparing the ability of unheated and heated conidia to grow in media of differing water activities. A most probable number technique was used to measure growth. Media of reduced a_w limited the ability of injured conidia to outgrow as compared to unheated conidia. Continued storage of injured conidia in media of reduced a_w eventually resulted in a loss of colony-forming ability. Solutes used to lower a_w were sodium chloride, glycerol, and sucrose.

INTRODUCTION

THE EFFECTS of sublethal thermal stress to gram positive and gram negative bacteria have been characterized. A partial list of these effects includes damage to the cytoplasmic membrane (Allwood and Russell, 1969; Iandolo and Ordal, 1966), degradation of ribonucleic acid (Miller and Ordal, 1972; Gray et al., 1973), alteration of enzymatic activity (Pierson and Ordal, 1971; Tomlins et al., 1971), alteration of nutrient and pH requirements (Clark and Ordal, 1969), increased sensitivity to inhibitors and selective agents (Tomlins and Ordal, 1971; Busta and Jezeski, 1963) and inducement of a prolonged lag phase. Of special concern to the food microbiologist is the increased sensitivity of heat injured cells to selective media. The use of such selective media may result in failure to detect injured organisms although uninjured cells are detected. This failure to detect injured organisms is critical in those instances where defined microbial limits exist because it is essential that all viable organisms be enumerated.

Although much research has been done in the area of sublethal thermal stress to bacteria (procaryotic cells), little work has been directed toward the effects of similar stresses on fungal (eucaryotic) cells. Studies by Nash and Sinclair (1968) and Nelson (1972) indicate that the effects of a sublethal thermal stress to yeast cells are similar to those that occur in sublethally injured bacterial cells.

The purpose of this study was to examine and characterize the effects of thermal stress and reduced water activity (a_W) on the conidia of *Aspergillus parasiticus.* This mold is of interest to the food industry because of its ability to grow under a wide variety of product storage conditions and because of its ability to produce carcinogenic secondary metabolites collectively known as aflatoxins.

EXPERIMENTAL

Thermal stress procedure

A. parasiticus NRRL 2999 was grown on Czapek-Dox agar at 25°C for 1 month and the mature culture was refrigerated until needed. In no case did the refrigerated storage exceed 50 days. Conidial suspensions were prepared immediately prior to use by gently washing the agar surface with water containing 0.05% Tween-80 (Nutritional Biochemicals Corp.), centrifuging the suspension, and suspending the pellet with additional 0.05% Tween-80. All suspensions were approximately 1 × 10^7 conidia/ml. Direct microscopic examination demonstrated that the suspensions were free of mycelial fragments. Direct microscopic counts of conidia in unheated suspensions closely approximated the viable plate counts. The incubation of all materials was at 25°C. Conidial suspensions were placed in a closed stainless steel vessel and heated in a circulating water bath at 51°C (El-Bisi and Ordal, 1956). To minimize settling, the suspension was agitated with a magnetic stirring bar. Unless otherwise indicated, a standard 4-hr heating period was used.

Assay procedure

A two-media method of demonstrating injury resulting from a thermal stress was employed. Samples were removed from the heating menstruum at various intervals, cooled, and serially diluted in distilled water. Yeast extract agar (YA) and yeast extract agar plus 10% NaCl (YSA) were surface inoculated in triplicate with 0.1 ml of a properly diluted sample. The counts obtained with YA represented the number of viable cells present in the sample, and the counts from YSA represented an evaluation of the number of the uninjured cells present. The difference in the two counts was therefore a measure of the injured cells. The composition of YA was 10.0g malt extract, 4.0g yeast extract, 4.0g dextrose, 15.0g Bacto-agar, and 1.0 liter distilled water. YSA contained these same ingredients plus 100.0g NaCl. The pH of both media was approximately 5.5.

Quantitation of cell leakage

Aliquots of a conidial suspension were collected at various intervals during a 4-hr heating period and centrifuged. The leakage of proteinaceous and nucleic acid materials from the conidia was detected by examining the supernatant liquid for absorbance at 280 nm and 260 nm. A Beckman DU monochromator with a modified Gilford Model 222 photometer was used to make all measurements.

Growth rate determinations

Rates of development of colonies from injured and unheated conidia were examined. Unheated conidia and conidia heated at 51°C for 4 hr were serially diluted using distilled water. YA plates were inoculated with 0.1 ml amounts of sample so that no more than 8 colonies appeared on a plate. There were 10 plates made from each sample. The number and diameter of colonies formed from injured and unheated conidia was measured at intervals over a 146-hr period. The increase in colony diameter was determined by averaging the diameters of the first group of observed colonies. A filar micrometer mounted on a stereoscope was used to measure colonies smaller than approximately 1 cm. Larger colonies were measured with a ruler and a stereoscope.

Water activity studies

The ability of injured conidia to outgrow in media of reduced water activity (a_w) was examined by a five tube most probable number (MPN) procedure. Injured and unheated conidia were enumerated in yeast extract broth (YB) with a_w levels of 0.99, 0.96, 0.93, 0.92, 0.90 and 0.88. The ability of injured and unheated cells to grow in the various media was compared. The 0.99 a_w YB which contained no added solute to reduce a_w was used as a reference for the comparisons. Three solutes were used to adjust the a_w of the YB media: sodium chloride, glycerol, and sucrose (Robinson and Stokes, 1968; Scott, 1957). The broth was prepared so that when 0.1 ml of sample was inoculated into a 20 mm × 150 mm screw top test tube containing 9.9 ml of broth, the mixture had the desired a_w level.

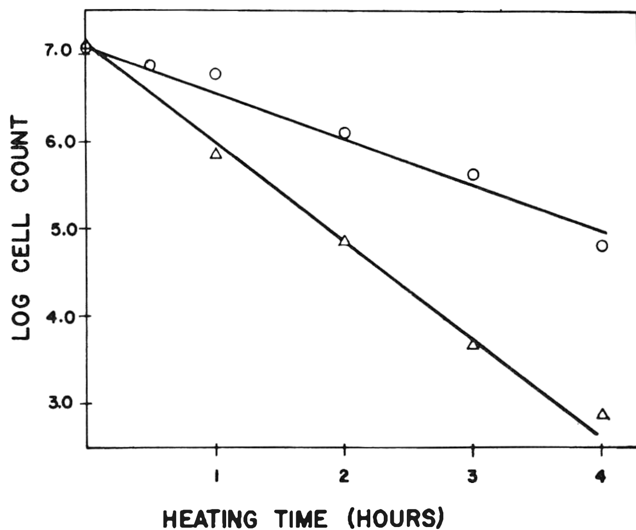

Fig. 1–Enumeration of thermally stressed conidia of A. parasiticus. Conidia were heated at 51°C for 4 hr and enumerated at intervals on: ○, YA; and △, YSA.

Storage of stressed conidia in a medium of reduced a_w

Cells heated at 51°C for 4 hr were serially diluted using distilled water. Screw top test tubes (20 mm × 150 mm) containing 9.9 ml of YB of 0.92 final a_w were inoculated with 0.1 ml quantities of proper dilutions and stored at 25°C. At intervals the viability of the stored cells was measured by an MPN technique. One milliliter amounts of the appropriately diluted cells stored at 0.92 a_w were inoculated into MPN tubes of YB with final a_w of 0.99.

RESULTS & DISCUSSION

THERMALLY INDUCED injury to conidia of *A. parasiticus* NRRL 2999 was demonstrated by a two-media plating technique (Fig. 1). Conidia plated before heating demonstrated equal ability to grow on each medium whereas heated conidia showed a reduced ability to grow on the medium with the added 10% NaCl. The sensitivity to the higher NaCl concentration increased with heating time. After 4 hr of heating nearly 99% fewer conidia were enumerated with YSA as compared to those enumerated with YA. Similar results have been observed with a variety of bacteria (Clark and Ordal, 1969; Clark et al., 1968; Miller and Ordal, 1972).

The measurement of material in the heating menstruum absorbing at 260 nm and 280 nm indirectly demonstrated that cytoplasmic membrane damage had occurred allowing the leakage of nucleic acid and proteinaceous materials into the heating menstruum (Fig. 2). As heating time increased, the levels of materials absorbing at 260 nm and 280 nm increased indicating increased cytoplasmic membrane damage. The unheated conidial suspensions had high levels of absorbing materials. This was attributed to materials washed from the surface of the conidia since repeated washings of the unheated cell suspensions lowered the initial levels.

The growth pattern of thermally injured conidia was found to be different from that of unheated conidia. Heated conidia required a longer incubation time for the first appearance of visible colonies and had increased variability in the appearance of visible colonies (Fig. 3). All of the colonies from the unheated suspensions were visible within 18 hr after the observance of the first colony. However, not only did the first appearance of a colony from a heated suspension lag 26 hr behind the first appearance of a colony from the unstressed suspension, but it also took 71 hr from the appearance of the first colony to the appearance of the last. The greater time span required for the appearance of all of the colonies from the heated suspension was an indication that the degree of injury varied among the individual conidia. Once the colonies from the injured or unheated conidia were visible, the increase in colony diameter with time was similar. This indicated that once colonies were visible, there was no morphological difference between growth patterns of colonies from injured or unheated conidia. The lag in the appearance of visible colonies from injured conidia was related to the time required for the conidia to recover from the thermal stress. Germination studies on heat injured conidia (unpublished data) show a comparable lag in first appearance of swelling and germ tube formation. Repair of thermally induced lesions in bacteria has

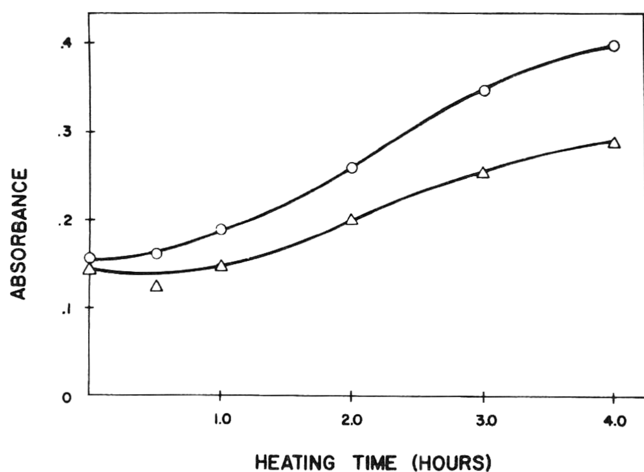

Fig. 2–Quantitation of materials in the heating menstruum absorbing at 260 nm and 280 nm. A conidial suspension of A. parasiticus was heated at 51°C. Samples of the heating menstruum were analyzed at intervals for absorbance at: ○, 260 nm; and △, 280 nm.

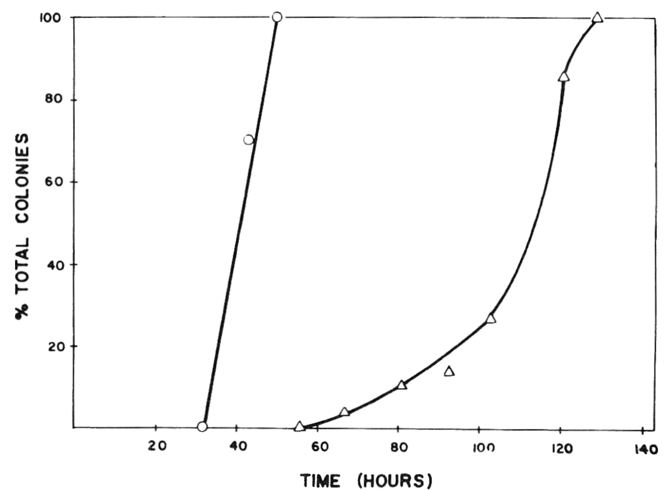

Fig. 3–The first appearance of colonies from heated and unheated conidia of A. parasiticus. Unheated cells (○) and cells heated at 51°C for 4 hr (△) were plated on YA. The first observation of colony presence was recorded and related to the total number of colonies eventually observed.

been found to occur during an extended lag phase which follows the heating of the bacterial cells (Iandolo and Ordal, 1966).

The results of the water activity studies demonstrated that unheated conidia were enumerated equally in YB with a_w levels as low as 0.90, when a_w was adjusted by addition of NaCl (Fig. 4). However, the enumeration of unheated cells with 0.88 a_w YB was drastically reduced. Although unheated cells were enumerated equally with media of a_w as low as 0.90, the growth rate as measured by visible cell mass was much slower in media of reduced a_w. Shih and Marth (1972) demonstrated that the growth rate of *A. parasiticus* (as measured by cell mass) was inhibited by increased NaCl concentration.

Media of reduced a_w limited the ability of thermally injured conidia to outgrow. The conidia demonstrated reduced ability to outgrow with increased heating time and decreased a_w (Fig. 4). After 4 hr of heating, the count on YB of 0.90 a_w was less than 0.01% of the count obtained with YB of 0.99 a_w.

When the solutes used to reduce the a_w of the enumeration broth (YB) were compared, the results were qualitatively similar but quantitatively different (Fig. 5). At the 0.96 a_w level, the number of conidia enumerated with the three solutes was nearly the same. However as the a_w was reduced to 0.90, the enumeration of conidia was found to vary with the solute used. The viability of the conidia was reduced the greatest by NaCl and least by sucrose. Overall, the effects of the three solutes were similar. Unheated conidia were enumerated equally in media of a_w as low as 0.90 regardless of the agent used to adjust a_w. Thermally injured conidia showed greater sensitivity to the media of reduced a_w which contained higher concentrations of the appropriate solute. Higher concentrations of all three solutes also reduced the growth rate as measured by cell mass.

The storage of heat injured conidia in media of 0.92 a_w with subsequent enumeration using a five-tube MPN YB (a_w 0.99) system showed that continued storage of heated conidia in media of reduced a_w resulted in a loss of colony-forming ability when enumerated in media of high a_w. Injured conidia exposed to a reduced a_w environment (0.92) demonstrated an initial drop in ability to form colonies. The count then remained constant through 4 days of storage after which the viable count progressively decreased as the storage time was extended. After 14 days of such storage the viable count was reduced by more than 99%.

The observations reported here have been attributed to injury to the conidia caused by a thermal stress. However, it was possible that other sources of stress were acting in combination with the thermal stress. One possible source of additional stress was the use of Tween-80 in the heating menstruum. Tween-80 has frequently been used as a wetting agent in preparing fungal cell suspensions (Charlang and Horowitz, 1971). A recent investigation (Scott and Alderson, 1974) indicated that Tween-80 may lower conidial resistance to stress by depletion of germination inhibitors. Likewise, use of mature cultures stored at refrigeration temperatures as a source of conidia for the experiments may also have altered the heat resistance properties of the conidia.

The data presented in this report provide preliminary evidence that a sublethal thermal stress to the conidia of *A. parasiticus* produces effects which are similar to those produced by a similar stress to bacterial cells. The release of cellular constituents into the heating menstruum implied cytoplasmic membrane damage. This was further substantiated by the increased sensitivity of the injured conidia to NaCl and to other solutes used to reduced the a_w of the conidial environment. The extended lag period following injury indicated that recovery or repair had to occur before germination and outgrowth could take place. This was substantiated by the fact that once outgrowth was detected the rate of colony size increase was comparable for colonies resulting from either unheated or thermally injured conidia. The observation that thermally injured conidia were unable to outgrow in media of reduced a_w whereas unheated conidia could outgrow under such conditions suggests that the combination of heat and reduced a_w could effectively control fungal food spoilage under a variety of food product conditions.

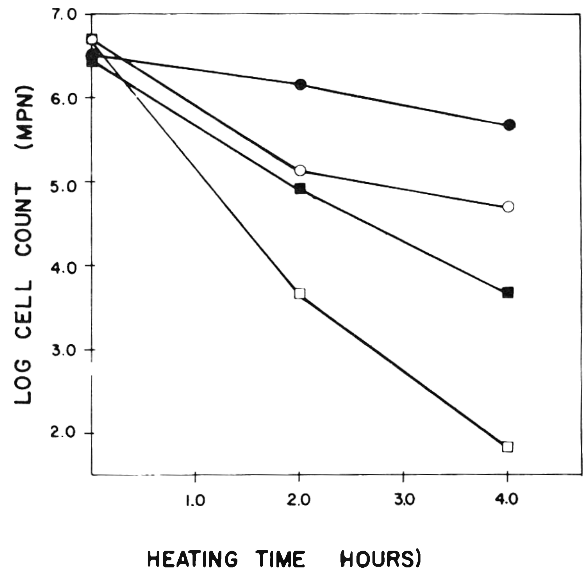

Fig. 4—Enumeration of thermally stressed conidia of A. parasiticus by a MPN procedure using media of various a_w. Conidia were heated at 51°C and enumerated at intervals by a MPN procedure using YB adjusted with NaCl to levels of: ●, 0.99 a_w; ○, 0.96 a_w; ■, 0.93 a_w; and □, 0.90 a_w.

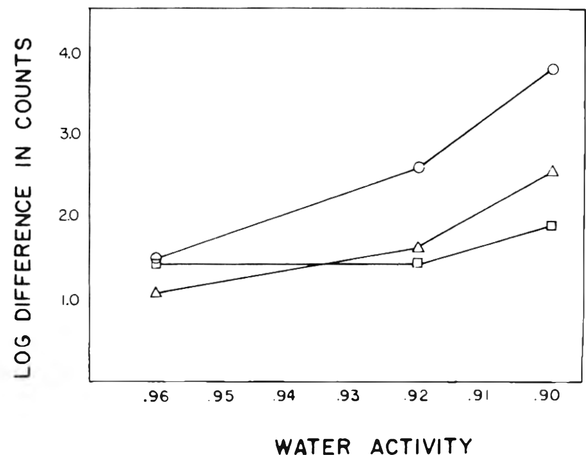

Fig. 5—A comparison of viable counts of thermally stressed A. parasiticus when enumerated by a MPN technique using a reference medium vs media of reduced a_w. Conidia heated at 51°C for 4 hr were enumerated by MPN technique. Counts obtained using YB of reduced a_w were compared to those obtained using YB (a_w 0.99) which had no solute added to lower a_w. Three solutes were compared: ○, NaCl; △, Glycerol; and □, sucrose.

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Ms received 9/13/75; revised 1/6/76; accepted 1/12/76.

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ISOLATION OF LIPOFUSCIN-LIKE FLUORESCENT PRODUCTS FROM RIPENING BANANA FRUIT

ABSTRACT

Ripening banana fruit contains water-insoluble pigments which have a molecular weight in excess of 4000 and a fluorescence spectrum identical to lipofuscin or age pigments. The pigments were not decomposed by irradiation with ultraviolet light. The flourescent substances increased linearly in the peel and quadratically in the pulp during the course of ripening. It is suggested that the fluorescent substances are products of membrane lipo-protein peroxidation like the lipofuscin pigments identified in animal tissues.

INTRODUCTION

AGE PIGMENT or lipofuscin has been implicated in cellular damage related to lipid peroxidation and aging (Porta and Hartroft, 1969; Sheldrake, 1974; Harman, 1972; Reichel, 1971; Tonna, 1975). It is commonly believed that age pigment accumulation is a result of cellular membrane phospholipid peroxidation via molecular oxygen or free radical reaction (Harman, 1972; Roubal and Tappel, 1966; Chio and Tappel, 1969a; Miquel et al., 1974). Lipofuscin accumulates with age and its accumulation is also a function of antioxidation nutrition or oxidant damage (Reddy et al., 1973; Chen, 1974; Barker and Brin, 1975). Lipofuscin has been isolated and characterized histologically and biochemically as complexed lipid and protein substances. The composition and character of lipofuscins indicate that they are derived by lipid peroxidation of polyunsaturated lipids of subcellular membranes (Hendley et al., 1963; Porta and Hartroft, 1969).

Biological membranes, especially those of subcellular organelles, are labile to lipid peroxidation because of their high content of polyunsaturated lipids. Among the products of oxidative deterioration of these unsaturated lipids are free radical intermediates, semistable peroxides and reactive carbonyls (Bidlack and Tappel, 1973; Pryor, 1970). The mechanisms by which cellular systems are damaged include free radical polymerization, aldehyde crosslinking, polymerization and disruption of membrane lipid integrity (Chio and Tappel, 1969a). Alteration in phospholipid structure also occurs during peroxidation of purified phospholipids and of complex phospholipids of membrane (Wills, 1971; Tam and McCay, 1970; May and McCay, 1968; Fong et al., 1973; Pederson and Aust, 1973).

The fluorescent property of age pigment has been investigated (Hyden and Lindstrom, 1950; Hendley et al., 1963). The pigments have characteristic fluorescence spectra with emission maxima in the range of 440–470 nm and excitation maxima in the range of 360–380 nm. Chio and Tappel (1969b) synthesized and characterized fluorescent products derived from malonaldehyde and amino acids. Malonaldehyde is one of the carbonyls produced during polyunsaturated fatty acid oxidation (Lillard and Day, 1964; Baker and Wilson, 1966). Malonaldehyde reacts bifunctionally with amino groups of proteins, enzymes and nucleic acids and results in inter- and intramolecular crosslinking. The Schiff base product, a conjugated imine system, RN=CH-CH=CH-NH-R, with a fairly strong electron donating group is the structure required for fluorescent production (Malshet and Tappel, 1973). Similar fluorescence spectra are observed when the membranes of the subcellular organelles are oxidized in vitro (Chio and Tappel, 1969a; Dillard and Tappel, 1971; Miquel et al., 1974), and in vivo as evidenced by the accumulation of fluorescent ceroid and lipofuscin pigments in various tissues (Porta and Hartroft, 1969; Reddy et al., 1973).

Age pigment granules were first reported to be present in mammals. More recently, lipofuscin has been found in protozoa (Rudzinska, 1961), nematodes (Epstein et al., 1972), houseflies (Sohal and Allison, 1971) and in fruit flies (Miquel et al., 1974). From these observations, age pigment deposition appears to be a universal phenomenon; however, there is no published information on the occurrence of age pigment in plant tissues.

Here we report experimental evidence indicating the presence of lipofuscin in ripening banana fruit. Quantitative study of lipofuscin, employing a fluorometric technique, showed an increase in lipofuscin as fruit ripened.

MATERIALS & METHODS

Materials

Green (No. 2), preclimacteric banana fruits were obtained from the United Fruit Company. Fruits were ripened at 20°C, 85–90% relative humidity in a ventilated incubator after ethylene (100 ppm) treatment.

Lipofuscin isolation

Lipofuscin (fluorescent pigments) were extracted by a procedure previously reported by Fletcher et al. (1973). Tissue (peel or pulp) was frozen in liquid nitrogen and dispersed to a fine powder with a blender (Haard and Hultin, 1968). The tissue (3–5g) was extracted with 20 ml of chloroform-methanol (2:1, v/v) for 3 min at 45°C. After extraction, the samples were separated into two equal portions. One portion was washed with an equal volume of distilled water and the other portion was not washed. The extracts were centrifuged (3000 × G) for 3 min at 10°C. A 2 ml aliquot of chloroform-rich layer was mixed with 0.1 ml of methanol in a fused quartz cuvette (10 × 10 × 46 mm, 5 ml total volume) and the excitation and emission spectra were recorded. In some instances, samples were exposed to UV light for 3 min at a distance of 3 cm above the cuvette prior to spectral analysis.

Fluorescence measurements

An Aminco-Bowman spectrophotofluorometer with a solid-state, blank-substract photomultiplier microphotometer was used to obtain the fluorescence spectra. The temperature of the cell jacket was controlled at 10°C. The slit arrangement of the spectrophotofluorometer was 3, 1 and 3 mm for the 3-, 4- and 6-slit positions. The machine was standardized with 0.1 μg/ml of quinine sulfate in 0.1N H_2SO_4. The standard had a relative fluorescence intensity of 1.9. The relative fluorescence units recorded were obtained by multiplying the meter multiplier value by the percent fluorescence divided by 100. The machine was blank adjusted and solvent subtracted. For quantitative comparisons, fluorescence of the standard was recorded just before recording the sample spectrum. Both the excitation and the emission spectra were obtained.

Molecular weight determination

Sephadex LH 20 (58 × 2.5 cm column) was used for estimation of the molecular weight of lipofuscin. Sephadex LH 20 (Pharmacia) was swollen in chloroform-hexane (65:35, v/v) solvent for 4 hr. Column flow was maintained by gravity feed at a rate of 12 ml/hr. The eluant was monitored for lipofuscin with a flow cell by measurement of fluorescence intensity at 360 nm excitation and 440 nm emission.

RESULTS

Fluorescence yield from fruit as a function of ripening

The excitation and emission spectra of lipofuscin from the pulp and peel of ripening banana fruit are shown in Figures 1 and 2. These spectra are comparable to those of lipofuscin isolated from mammalian tissues (Reddy et al., 1973). The chloroform-soluble fluorescent substance increased to a greater extent in peel tissue than in pulp tissue. The total chloroform soluble fluorescent materials extracted from ripening banana pulp and peel are shown in Figures 3 and 4. The water washing procedure may remove water soluble lipofuscin and also interfering substances which exhibit similar fluorescent properties, such as flavins and amines (Fletcher et al., 1973).

Total (unwashed) and chloroform-soluble (washed) fluorescent substances increased with ripening although the trends were quite erratic. Treatment of lipofuscin extracts from rat liver with UV light has been employed to remove retinol which possesses fluorescence at 470 nm emission (Fletcher et al., 1973). Treatment of banana extracts with UV light photodecomposed the carotenoids and chlorophyll (in the case of the unripe peel) present in the chloroform layer as evidenced by the rapid loss in yellow and green color. The influence of UV light treatment on fluorescence (440 nm emission) of banana extracts is shown in Figures 5 and 6. The data, notably from the pulp, were less erratic showing increased fluorescent material after day four of ripening. Statistical analyses of the pulp data showed a quadratic relationship when fluorescence intensity was analyzed as a function of ripening time. A linear relationship was observed for the peel data (Table 1).

Molecular weight

Chloroform-soluble and UV-treated fluorescent material isolated from postclimacteric banana fruit peel was eluted in the void volume of a column packed with sephadex LH-20, indicating a molecular weight in excess of 4000. The high molecular weight of this pigment is consonant with our view that it represents lipofuscin.

DISCUSSION

IN PLANT SYSTEMS, cellular aging, oxygen toxicity, freeze injury, and tissue damage promoted by photochemical smog, UV light and ionizing irradiation have been directly or indirectly linked to peroxidation of membrane lipids. Ozone phytotoxicity in green plants is manifest in cell permeability changes (Evans and Ting, 1973); and ozone-treated cells of the green alga *Chlorella* produce malonaldehyde arising from the oxidative breakdown of an ozonide of unsaturated fatty acid material (Frederick and Health, 1975). Membrane permeability changes during the fruit ripening process are well documented (Brady et al., 1970; Sacher, 1966; Hulme et al., 1968; de Swardt and Rousseau, 1973). In banana fruit, a three-fold decrease in methyl linoleate was observed as the pulp ripened, and a 40% decrease was observed as the peel ripened (Goldstein and Wick, 1969). Oxidative enzymes such as lipoxygenase, peroxidase, superoxide dismutase, catalase and hydroperoxide isomerase occur in plant tissue, and in many cases, are known to emerge with the onset of plant senescence (Wooltorton et al., 1965; Haard, 1973; Haydar and Hadziyev, 1973; Lumsden and Hall, 1974). As a consequence of oxidative enzyme activity during the ripening phenomenon, cellular membrane phospholipids may undergo peroxidation with a resulting accumulation of peroxidative damaged product. This accumulation may arise during fruit ripening because of an inability of tissue to metabolize the damaged product, more extensive peroxidation as the fruit ripens or because of a breakdown in the ability of the tissue to protect against oxidative stress.

The presence of fluorescent material in banana, which is

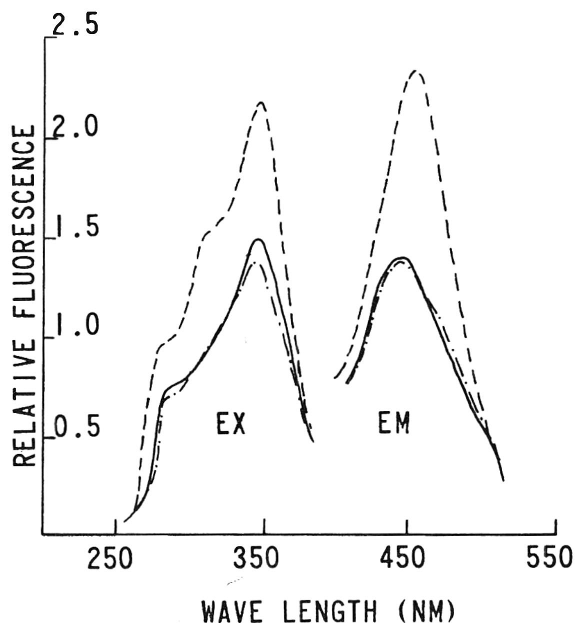

Fig. 1—Fluorescence spectra of chloroform-soluble pigments isolated from preclimacteric (——), climacteric (—•—), and postclimacteric (– – –) banana pulp tissue. Chloroform-methanol extracts were washed with an equal volume of distilled water. Data are for one experiment and are representative of three additional trials. Excitation at 280–290 nm was probably the result of flavin compounds not completely removed by water washing since additional washing resulted in decreased excitation of the UV end of the spectra.

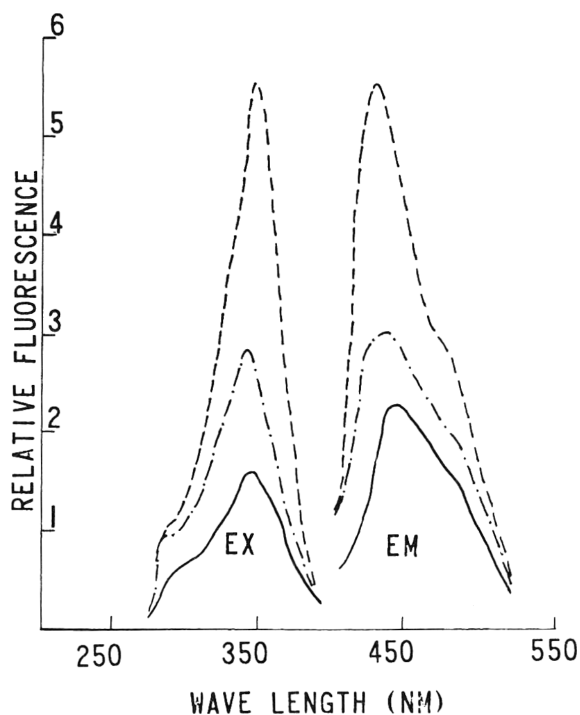

Fig. 2—Fluorescence spectra of chloroform-soluble pigments isolated from preclimacteric (——), climacteric (—•—), and postclimacteric (– – –) banana peel tissue. Chloroform methanol extracts were washed with an equal volume of distilled water. Data are from one experiment and are representative of three additional trials. Emission at 490 nm disappeared as a result of UV treatment and was probably contributed by carotenoids.

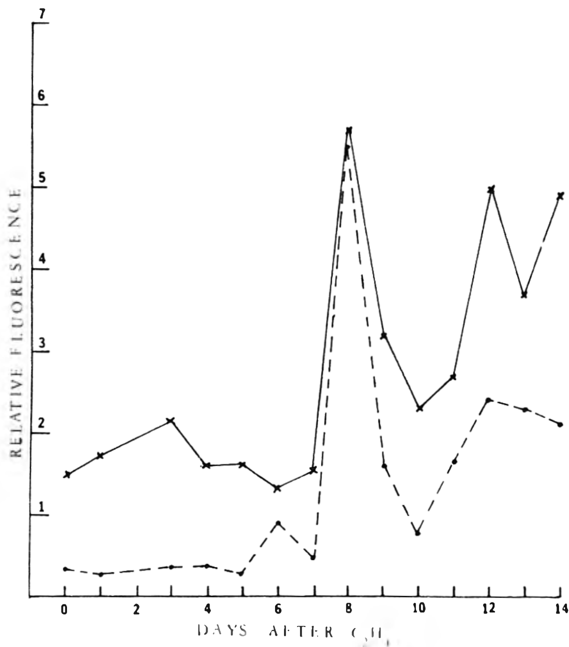

Fig. 3–Fluorescence yield from ripening banana pulp for total (not water washed) (——) and chloroform soluble (water washed twice with equal volumes of distilled water) (– – –) pigments. Data are representative of one additional experiment. The maximum fluorescence was 420–440 nm and 360 nm excitation for all extracts. Data represented are the mxaimum obtained for the respective samples.

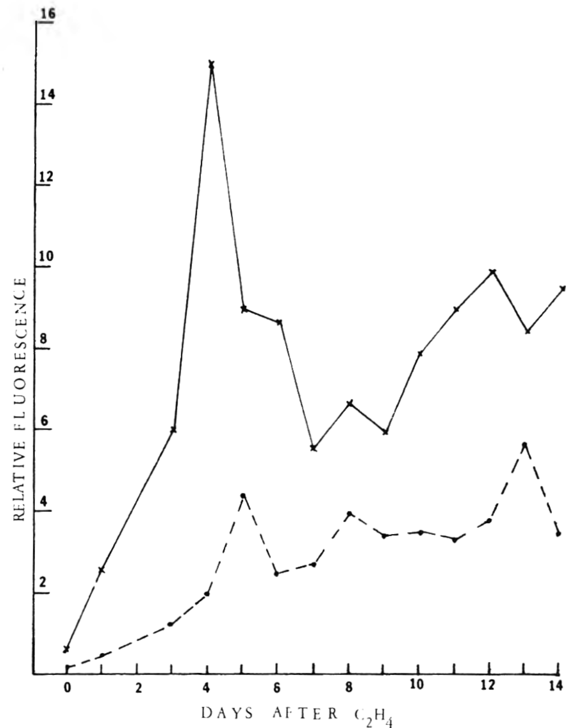

Fig. 4–Fluorescence yield from ripening banana peel for total (not water washed) (——) and chloroform soluble (water washed) (– – –) pigments. Data are representative of one additional experiment. The maximum fluorescence was 440 nm and maximum excitation was at 360 nm for all samples. An additional peak was observed at 680 nm in extracts from green peel. This peak disappeared when samples were treated with UV light and was presumable contributed by chlorophyll.

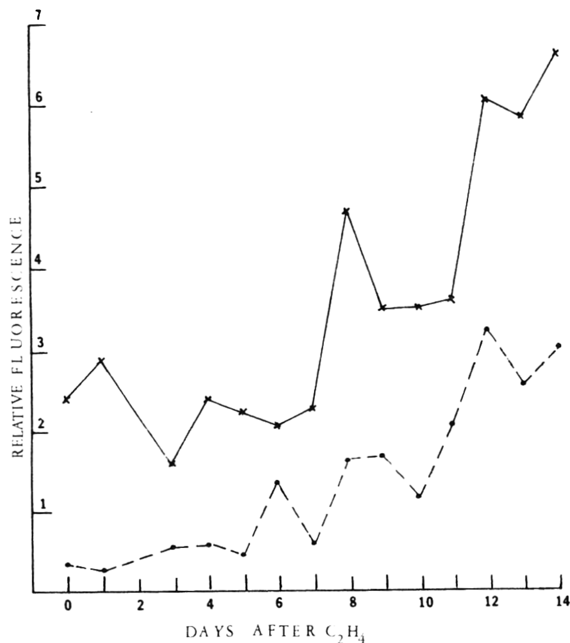

Fig. 5–Fluorescence yield from ripening banana pulp for total (not water washed) (——) and chloroform-soluble (water washed) (– – –) pigments after UV treatment as described in the text. The maximum fluorescence was identical to samples not treated with UV light and was the same for all samples. Data are representative of one additional experiment.

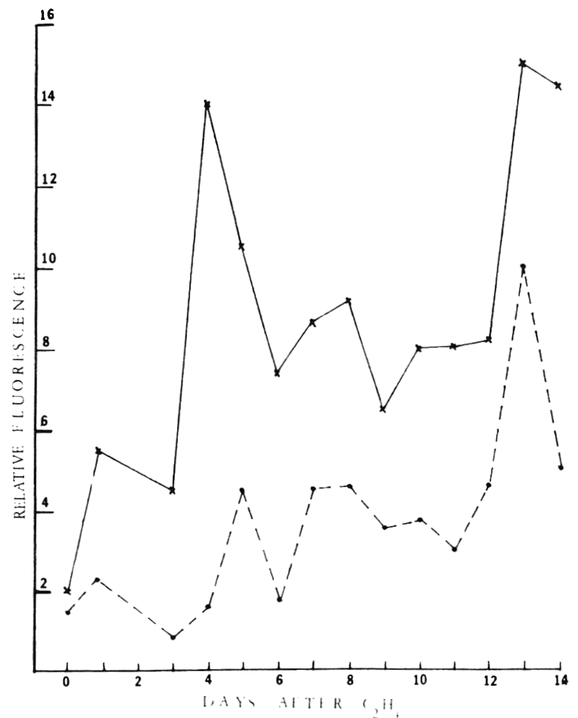

Fig. 6–Fluorescence yield from ripening banana peel for total (not water washed) (——) and chloroform soluble (water-washed) (– – –) pigments after UV treatment as described in the text. Fluorescence spectra were as described in the legend of Fig. 4. Data are representative of one additional experiment.

Table 1–Statistical analyses showing relation between fluorescence intensity as a function of stage of ripening[a]

Peel	R-square
Water washed	
F.I. = 0.84 + 0.28 days	0.66
Water washed, UV treated	
F.I. = 1.6 + 0.35 days	0.46
Not water washed	
F.I. = 3.16 + 0.50 days	0.63
Not water washed, UV treated	
F.I. = 3.49 + 0.64 days	0.65
Pulp	
Water washed	
F.I. = 0.27 – 0.008 days + (0.012 day)^2	
P 0.06	0.86
Water washed, UV treated	
F.I. = 0.36 – 0.01 day + (0.02 day)^2	
P 0.04	0.87
Not water washed	
F.I. = 2.00 – 0.23 day + (0.03 day)^2	
P 0.01	0.80
Not water washed, UV treated	
F.I. = 2.73 – 0.36 day + (0.05 day)^2	
P 0.00	0.93

[a] Data for day 4 is omitted from peel analyses and data for day 8 is omitted from pulp analyses. F.I. indicates fluorescence intensity. R-square represents the correlation coefficient. When all data were analyzed R-square for peel ranged from 0.40–0.67 and for pulp 0.29–0.84. The presence of erratic data points at these respective times were observed during other trials at approximately the same stage of ripening.

comparable to that identified from mammalian cells, is consistent with our view that membrane lipid peroxidation is a primary or secondary event of plant senescence. We have also observed similar increases in fluorescent substances during ripening of tomato and pear fruit (Maguire, 1975). The thesis that peroxidation of membrane lipids is causally related to the onset of fruit ripening is also compatible with the efficiency of current methods for delaying ripening (lowered temperature, oxygen and atmosphere pressure). Further studies to characterize the chemical makeup of plant lipofuscin are currently in progress in our laboratory.

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Ms received 8/15/75; revised 11/10/75; accepted 11/20/75.

Presented at the 35th Annual Meeting of the Institute of Food Technologists, Chicago, Ill., June 8–12, 1975.

Paper of the Journal Series, New Jersey Agricultural Experiment Station, Rutgers–The State University, New Brunswick, New Jersey 08903.

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IDENTIFICATION OF VACUUM STEAM-DISTILLED AROMA COMPOUNDS IN THE PRESS JUICE OF ARCTIC BRAMBLE, Rubus arcticus L.

ABSTRACT

The present study is concerned with the steam-distilled, volatile aroma compounds in the press juice of arctic bramble, *Rubus arcticus* L. More than 60 of over 200 components were identified by means of glass capillary gas chromatography-mass spectrometry systems. The total amount of the compounds identified in the press juice was 70–80 ppm. The main aroma component was 2,5-dimethyl-4-methoxy-2,3-dihydro-3-furanone. Smaller amounts of 2,5-dimethyl-4-hydroxy-2,3-dihydro-3-furanone were also found in the juice. The compounds identified, which cover about 90% of the total aroma concentrate, included further, 11 aliphatic alcohols, 8 aliphatic acids, 8 aliphatic esters, 12 aromatic compounds, 9 non aromatic terpenes, 9 aliphatic carbonyls (including acetoin, diacetyl and diacetone alcohol), 2 lactones and one nitrogen-containing compound, ethyl nicotinate. The most abundant compounds in the press juice were 2,5-dimethyl-4-methoxy-2,3-dihydro-3-furanone, acetoin, acetaldehyde, 2-heptanol, a pentenol (evidently trans-3-penten-1-ol), acetic acid, ethyl acetate, ethanol, linalool, 3-methyl-2-buten-1-ol and cis-3-hexen-1-ol. All these compounds exceeded 1 ppm in the fresh press juice. The aroma composition of cultivated berries was compared with that of wild berries from a different source.

INTRODUCTION

AMONG THE genus *Rubus*, the varieties of raspberry (*Rubus idaeus* L.) have been studied intensively. More than 100 aroma compounds of raspberry have been isolated and identified in several studies (Elze, 1929); Coppens and Hoejenbos, 1939; McGlumphy, 1951; Schinz and Seidel, 1957, 1961; Sundt and Winter, 1960, 1962; Winter and Sundt, 1962; Bohnsack, 1967a, b; Pisarnitskii et al., 1970; Duclos and Latrasse, 1971; Schmidlin-Meszaros, 1971; Winter and Enggist, 1971). Some investigations of aroma compounds in a blackberry (*Rubus ulmifolius inermis*) (Scanlan et al., 1970; Houchen et al., 1972) and black respberry (McGlumphy, 1951) have also been carried out.

The aroma compounds of some wild berries belonging to the genus *Rubus* have been investigated in Finland recently. An important object has been cloudberry (*Rubus chamaemorus* L.), of which over 70 aroma compounds have been reported in some preliminary studies (Honkanen, 1972; Honkanen et al., 1973).

Some data have also been published concerning the aroma composition of the hybrids between raspberry and arctic bramble (Hiirsalmi et al., 1974). A brief work on easily volatile carbonyl compounds (Kallio and Linko, 1973), and an analysis of the major flavor components of the arctic bramble (Kallio and Honkanen, 1975) are closely connected with the present study.

The object of this study, the arctic bramble (*Rubus arcticus* L.), occurs mainly circumpolarly, in the subarctic coniferous zone. The berries grow in Central Finland, too, and have some rare wild occurrences also in Northern Europe. The arctic bramble has been cultured in Finland (Ryynänen, 1973) and in Sweden (Larsson, 1970) for over 30 yr with promising results. Larsson (1970) also reports on the cultivation of cloudberry and *Rubus stellatus* Sm. (closely related to *Rubus arcticus* L.). Mäkinen and Oikarainen (1974) have done some preliminary work on the cultivation of cloudberry in Finland.

MATERIALS & METHODS

Berries

Most of the arctic brambles (*Rubus arcticus* L.) analyzed were from an experimental cultivation in central Finland (Agricultural Research Centre South Savo Experiment Station, Mikkeli, Finland. The geographical location of the station is 61°41'N lat., 27°14'E long.). Two wild strains of the plant, Mespi and Mesma (Ryynänen, 1973), planted in 1970 in alternating lines, were cultivated and studied as a mixed population. The samples used for the aroma studies were from the 1973 and 1974 harvests (Mi-73 and Mi-74). For reference purposes, the same two strains of the 1972 harvest from another experimental station in Eastern Finland (Agricultural Research Centre North Savo Experiment Station, Maaninka, Finland, 63°08'N lat. and 27°19'E long.) (Ma-72), were also investigated, and these were compared with wild, commercially available berries from Lumijoki (Western Finland), harvested in 1973 (Lu-73). The berries were picked optimally ripe, according to organoleptic estimations. The berries from the experimental stations were packed in polyethylene bags and frozen at −20°C immediately after harvesting, while the commercial berries were frozen after 2 days storage at +3°C. The storage temperature was in all cases −20°C. The samples were analyzed within 2 months from harvesting.

Isolation of aroma compounds

2 kg of partially thawed berries were macerated, and the juice caught by a hydraulic press was immediately distilled four times in a vacuum, using a climbing film evaporator (Jobling Lab. Div., Stone, Staffordshire, England), at a temperature of 22°C and a pressure of 10 torrs. The distillate was collected in a 2-liter receiving flask, chilled in a mixture of sodium chloride and crushed ice. The flask was connected to the vacuum pump by two cold traps chilled in liquid nitrogen.

After saturation with sodium chloride, the combined distillate was extracted for 24 hr with 200 ml pentane-diethyl ether (1:2, v/v), in a Kutscher-Steudel continuous extractor (Suomalainen and Nykänen, 1968). 5 ml dichloromethane was added to the pentane-ether mixture, in order to remove water from the receiving flask as azeotrope. The aroma extract was concentrated, using a Widmer column, at 35°C into the final volume of 250 μl (aroma concentrate). For the MS-analysis, the concentrating was however, continued to 100 μl, to enable the identification of the compounds with higher retention times.

Gas chromatographic analysis

The GLC-analyses of the concentrates were carried out on a Varian Aerograph, Model 2100-20 gas chromatograph, equipped with a flame ionization detector (Varian Instruments, Palo Alto, California), and the quantitation was done on a Hewlett-Packard 3373B integrator (Hewlett-Packard, Avondale, Pa.). The sensitivity of the integrator was chosen so that the noise did not exceed the minimum integration level at the temperature programming without injection. The column used was an FFAP-glass capillary one prepared in our laboratory (140m long and 0.32 mm i.d.). The capillary tubing was drawn by a Hupe & Busch apparatus (Grözingen, Karlsruhe, West Germany), from pyrex tubing 8.0 mm o.d. and 3.0 mm i.d. The column was made using a static coating procedure at 23°C after a dichloromethane pyrolysis treatment (Grob, 1968). The pyrolysis was carried out twice in an oven at 720°C for 30 sec. Pure dichloromethane was sucked into the carbonized column at a distance of about 3m before filling the column with the liquid film solution (0.5% (w/v) FFAP (Varian Aerograph), dissolved in dichloromethane), in order to prevent the phase from concentrating on the surface of the solution during sucking. The conditions of the analysis were as follows: Injector 245°C; detector 255°C; oven programme 60–230°C, 2°/min. The split ratio between the column and exit after the injection block was 1:100. The flowing rate of the carrier gas (nitrogen) was 0.7 ml/min. To optimize the detector response, the rate of the make-up gas to FID was 30 ml/min. The dead volumes were minimized both in the injector and the detector using glass capillary connections.

Mass spectrometry

The GLC-MS analyses were carried out on an LKB-9000 instrument (LKB-producter AB, Bromma, Sweden) by using the same capillary column (with helium as carrier gas) as in the GLC-analysis. No make-up gas was used. A Ryhage-type jet separator was employed. The energy used was 70 eV (in some cases also 20 eV), and the sweep time was 1.5 sec/decade.

Solvents and reference compounds

The solvents were all guaranteed reagents (E. Merck AG) and were always redistilled before use. The reference compounds were purest available commercial products, used without further purification. The following compounds were synthetized by Dr. Erkki Honkanen (Technical Research Centre of Finland, Lab for Food Research & Technology, Otaniemi, Finland): 2,5-dimethyl-4-methoxy-2,3-dihydro-3-furanone; 2,5-dimethyl-4-hydroxy-2,3-dihydro-3-furanone; acetoin acetate; and 3 methyl-3-butenoic acid.

Quantitative estimations

Estimations of the concentrations of the identified compounds in the press juice were carried out as follows: The total amount of the aroma compounds was determined by weighing the aroma concentrate, and the amounts of solvents left in the aroma concentrate were measured by gas chromatography. The relative gas chromatographic detection responses were determined using a mixture of the synthetic compounds (42 most abundant compounds identified, and 2,5-dimethyl-4-hydroxy-2,3-dihydro-3-furanone). The yield of the components in the press juice in the steam distillation was estimated by distilling a solution that simulated the juice (the above mixture of the 43 compounds dissolved in citrate buffer, pH 3.1). The integration data of the arctic bramble aroma compounds (percent of the total aroma concentrate) were first corrected for the detector responses. On the basis of these corrected values, it was possible to estimate the amounts of each compound in the weighed concentrate, and the content of each individual compound in 1 liter press juice was estimated according to the yield of each compound in the isolation procedure.

The blind test was identical with the analyses, except that distilled water was used in the distillation instead of the juice.

RESULTS

1 kg OF BERRIES gave about 800 ml press juice, pH 3.1. The yield of the aroma concentrate was on an average about 56 mg from 1 liter of press juice, but it varied considerably in berries from different sources. The compounds identifed in this study cover ca 90% (50 mg) of the concentrate. According to the corrections, the total quantitative amount of these compounds was calculated as ca 70–80 ppm in 1 kg press juice.

Almost 250 separate peaks can be seen in the capillary gas chromatogram of the aroma concentrate of the arctic bramble press juice given in Figure 1. Over 60 of them were identified using combined glass capillary gas chromatography–mass spectrometry. The blind test showed that none of the compounds identified was an artefact originating from the solvents used. When no spectral data were available or when the published spectra seemed to require confirmation, the spectra were analyzed using the reference compounds. For the identification to be positive it was required that, in addition to the mass spectral identification, the gas chromatographic characteristics were identical with the reference compounds. Some of the identifications are, however, tentative because of the lack of authentic compounds.

The compounds identified, listed in Table 1, are presented in the same order as they eluated in the GLC analysis, and the numberings in Figure 1 and Table 1 correspond to each other. Table 1 also shows percentages of the compounds in the aroma concentrate (integration values), the calculated amounts of the compounds in the press juice, the references used in the mass spectral identification, and a list of studies in which the same aroma compounds have been isolated or identified from other berries in the genus *Rubus.* To get a general view of the aroma, the compounds identified are divided in Table 2 into nine compound classes. Mesifurane and acetoin have also been taken as separate groups in Table 2 because of their significance for the aroma. The most abundant compounds in the juice were (content of each exceeding 1 ppm), in decreasing order, mesifurane, acetoin, acetaldehyde, 2-heptanol, trans-3-penten-1-ol, acetic acid, ethyl acetate, ethanol, linalool, 3-methyl-2-buten-1-ol, and cis-3-hexen-1-ol.

The most abundant of the classes in Table 2 are aliphatic alcohols (12–40 ppm in the juice). The anomalous large quantity of alcohol fraction in the commercial berries (Lu-73) is caused by the ethanol formed through post-harvest fermentation. Six of the eleven compounds exceeding the 1 ppm value in the juice were alcohols (cf. Table 1). In addition to these, methanol, 1-propanol, 1-butanol, 3-methyl-1-butanol, 1-hexanol, and trans-3-hexen-1-ol were found in smaller amounts.

The aliphatic carbonyl fraction is large because of the great amount of acetaldehyde. The other aliphatic carbonyl compounds are acetone, diacetyl, 2-heptanone, 2-methyl-2-butenal and 6-methyl-5-hepten-2-one. The amount of acetaldehyde was difficult to determine because of its great loss during the concentration.

The main component in all the cultured berry samples was compound No. 137, 2,5-dimethyl-4-methoxy-2,3-dihydro-3-furanone (compound II, Fig. 2). Because of its importance for the aroma of the arctic bramble (in Finnish mesimarja), the compound is hereafter called briefly mesifurane. The isolation and structural analysis of this compound has been reported earlier (Kallio and Honkanen, 1975). Compound No. 219 proved to be hydroxy compound, which corresponds to the mesifurane (2,5-dimethyl-4-hydroxy-2,3-dihydro-3-furanone, hydroxy mesifurane, compound I, Fig. 2). The mass spectrum of the compound, taken from the original aroma concentrate with the aid of capillary GLC-MS, was not very clear, but the structure of the compound was confirmed after preparatory GLC collection.

The two last peaks in the chromatogram given in Figure 1 (compounds No. 240 and 241) are two isomers, the mass spectra of which are presented in Fig. 3. The cracking pattern shows that they are probably formed through a dimerization of mesifurane via dehydrogenation. The fragmentation mechanism may be expressed according to that of mesifurane presented earlier (Kallio and Honkanen, 1975). This assumption has not yet been verified because of lack of synthetic reference compounds.

Sixteen aliphatic acids and esters were identified, with both classes existing in approximately equal amounts. Acetic acid (2–5 ppm) and ethyl acetate (3–9 ppm) were most abundant of these compounds in all the four berry samples studied. The other acids were 2-methylpropanoic (0.1–0.3 ppm), n-butanoic (0.05–0.2 ppm), 3-methylbutanoic (0.5–1 ppm), 3-methyl-2-butenoic (0.6–2.3 ppm), 3-methyl-3-butenoic (0.1–0.3 ppm), n-hexanoic (0.1–0.3 ppm) and propanoic acid (tr–0.2 ppm). The identified 3-methyl-3-butenoic acid is evidently very rare in fruits and berries. Its mass spectrum is presented in Figure 4 together with the spectrum of 3-methyl-2-butenoic acid. The molecule ion of the latter is clearly more stable than that of the former because of the conjugated double bonds. The amounts of esters varied over a wide range. The true concentrations in the juice were very difficult to determine because of the lability of the compounds. The amounts of additional esters, 3-methyl-2-butenyl acetate, 2-methyl-2-butenyl acetate, methyl pyruvate, acetoin acetate and ethyl formate were calculated to exist in the range 0.1–0.65 ppm in the juice. Also cis-3-hexenyl acetate existed in fairly small amounts. In addition to the esters in Table 1, the existence of 2-heptyl acetate is also evident, but was not verified, because its retention time was identical with that of trans-3-penten-1-ol.

The acetoine content varied to some extent in the different materials. The highest concentration (14 ppm) was found in Ma-72 berries. The acetoine acetate content, on the other

hand, was quite constant, ranging between 0.3 and 0.4 ppm. As far as we know, this ester is not so common in fruits in nature. The mass spectra (20 and 70 eV) of compound 93 from the distillate are presented in Figure 5. The results are identical with the spectra of the synthetized acetoin acetate. The fragmentation pattern of the ester is pronounced. The fragment m/e 43 ($C(=O)-CH_3^+$, base peak) may split from either end of the molecule. M^+ -43 (m/e 87) is only 6% and M^+ (m/e 130) 1% of the base peak when analyzed with 70 eV energy, and 23 and 5% (respectively) when analyzed with 20 eV.

The amount of terpene alcohols was 1–3 ppm in all juice samples, consisting mainly of linalool and α-terpineol Three different linalool oxides were found. Their total amounts in the different samples varied between 0.1 and 0.6 ppm. The content of aromatic alcohols was likewise noticeable (1 ppm). Benzyl alcohol and 2-phenylethanol are represented in considerable amounts, but their significance for the aroma of the arctic bramble is minute.

The aromatic esters found are ethyl benzoate, benzyl acetate, 2-phenylethyl acetate, dimethyl phthalate and diethyl phthalate and these cover together only about half percent of the aroma compounds. It is, however, questionable whether the two last mentioned compounds originate from arctic bramble.

The small amount of lactones is noteworthy. Only two of them were found, i.e., γ-butyrolactone and ε-caprolactone. The only nitrogen-containing compound found was ethyl nicotinate.

The sample had some further noticeable components, which remained, however, unidentified in spite of the useful mass spectra.

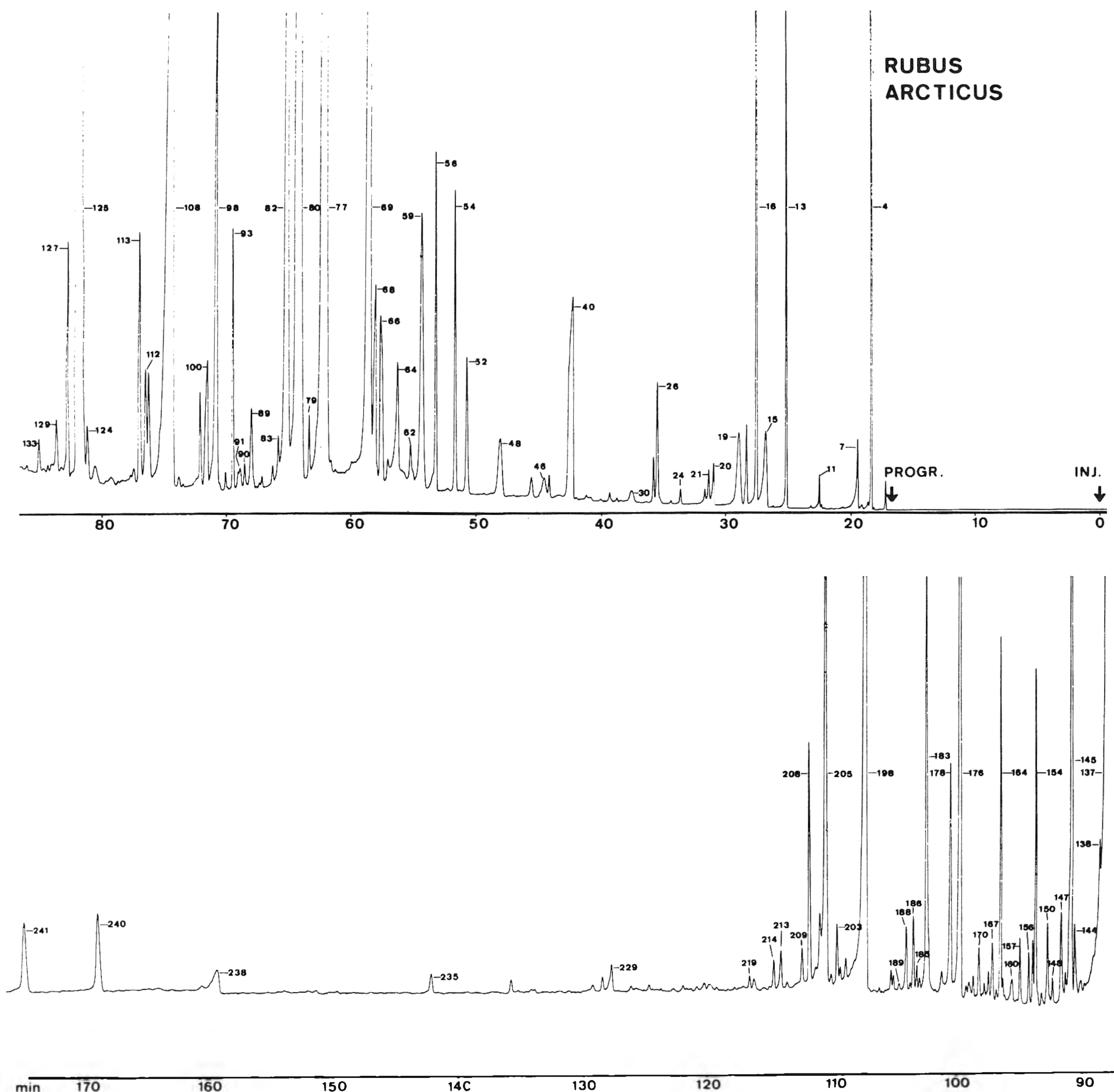

Fig. 1—Gas chromatogram of arctic bramble aroma concentrate, analyzed by FFAP-glass capillary column (Mi-73).

DISCUSSION

THE QUANTITATIVE estimation of the different compounds in the original press juice is a difficult problem. The use of several correction coefficients is not an accurate method for the calculation of the amounts of easily volatile compounds. As the detector responses of different compounds vary considerably, the use of the corrections makes the results, however, more reliable. For this reason, the results were also corrected according to the yield of different compounds in the isolation procedure. They varied usually between 50 and 98%, and it is evident that the loss in the concentration procedure is quite significant, because the yield of the compounds with shorter retention times in the chromatogram was only 50–65%. The recoveries of the most easily volatile compounds (acetaldehyde, acetone, and ethyl formate) were very low, 15–30%, which means that the accuracy of these results is not very high.

The sensitivity and efficiency of the gas chromatographic method used was sufficient for the separation of about 250 peaks in the aroma concentrate of arctic bramble from each other. In the mass spectrometric analysis the FFAP-column did not lose much of its effect in comparison with the GLC

Table 1–List of compounds identified from arctic bramble aroma concentrate (Mi-73). The values are given with two significant digits, and rounded to the nearest multiple of 0.05 ppm or %.

IUPAC-name	Common name	Compound number	% of the total aroma concentrate[a]	mg in 1 liter press juice[a]	Mass spectral identification[a] (ref)[b]	Compounds found in other berries in the genus Rubus references[b]			
						Raspberry	Blackberry	Black raspberry	Cloudberry
n-pentane		1	S		1				
diethyl ether	ether	4	S		1				
ethanal	acetaldehyde	7	0.20	9.0	1	6,7,8		12	
2-propanone	acetone	11	0.05	0.10	1	6,8		12	19
ethyl formate		11			1	9			
ethyl acetate		13	4.0	3.3	1	7,9–13	18	12	19
methanol		15	tr	tr	1	9,15			
dichloromethane		16	S		1				
ethanol		19	2.4	3.2	1	9–12,14	18	12	19
n-pentanal		20	0.10	0.10	1	8			
2,3-butanedione	diacetyl	21	0.20	0.60	1	6,7,11		12	
chloroform		24	S		1				
1-propanol		30	tr	tr	1				
1-butanol		48	0.25	0.20	1	14		12	
2-heptanone		52	0.25	0.15	1		18		
2-methyl-2-butenyl acetate		54	0.40	0.40	a				
2-methyl-2-butenal	tiglin aldehyde	56	0.25	0.20	a				
3-methyl-1-butanol		59	1.1	0.95	1	11,15			
methyl-2-oxo propanoate	methyl pyruvate	64	0.30	0.35	a				
3-methyl-2-butenyl acetate		66	0.65	0.65	a				
trans-3-penten-1-ol		69	7.1	5.2	t	15			
3-oxo-2-butanol	acetoin	77	7.8	9.7	1	6,15			
cis-3-hexenyl acetate		79	tr	tr	1	9,15			
2-heptanol		80	9.8	8.2	a		18		19
3-methyl-2-buten-1-ol		82	4.0	2.9	2	14			
6-methyl-5-hepten-2-one		83	0.15	0.10	3				19
1-hexanol		89	0.45	0.40	1	14,15	18		19
2-methyl-2-hydroxy-4-pentanone	diacetone alcohol	90	0.05	0.05	1				
trans-3-hexen-1-ol		91	0.10	0.10	1				19
3-oxo-2-butyl acetate	acetoin acetate	93	0.25	0.25	a				
cis-3-hexen-1-ol		98	1.3	1.0	1	9,14,15			19
acetic acid		108	4.0	5.1	1	7,9–13		12	
(-)-(2R),5R-trans-2-vinyl-2-methyl 5-(1'-hydroxy-1-methylethyl-) tetrahydrofuran	trans-linalool oxide	112	0.45	0.25	4				
(+)-(2R,5S)-cis-2-vinyl-2-methyl-5-(1'-hydroxy-1'-methylethyl-) tetrahydrofuran	cis-linalool oxide	113	0.50	0.30	4				
propanoic acid		124	0.20	0.15	1	13			19
3,7-dimethyl-1,6-octadien-3-ol	linalool	125	5.2	3.0	1	15	18		19
benzaldehyde		126	tr	tr	1	8,11		12	19
2-methylpropanoic acid		127	0.30	0.30	1	9,13			

analysis using a flame ionization detector. However, some of the separation power was lost through diffusion in the dead volume of the separator. Quantitative losses were noticeable in the easily volatile compounds in the separator. The most abundant compounds were pure enough for identification according to mass spectral data. The useful range of the concentration was wide; the difference between the largest and the smallest peaks identified was about 10^4-fold. However, in many cases the concentrations of the compounds were insufficient to give reliable spectra. The FFAP-liquid phase caused significant distortion of the spectra soon after the temperature exceeded 200°C, but the disadvantage could be avoided by subtracting the background from the spectra. Therefore, the corrected spectrum of e.g., the smallest peak identified, quaiacol, was very pure. It was thus possible to carry out the GLC-MS analysis of about 100 compounds, without any prefractionation of the aroma concentrate and over 60 of them were identified. A more detailed analysis will require either prefractionation of the mixture, or the use of several different columns, because the peaks of the largest compounds overlap a number of smaller compounds. A good example of such a case, where two compounds overlap each other, is the pair 2-phenylethyl acetate-n-hexanoic acid which form a similar, symmetrical peak in a ratio of 1:2.

It was found that mesifurane (compound II in Fig. 2) is the most important aroma compound in arctic bramble. This has been noticed in earlier studies as well, that adding this compound in a native concentration suffices to restore the native

Table 1—Continued

IUPAC-name	Common name	Compound number	% of the total aroma concentrate[a]	mg in 1 liter press juice[a]	Mass spectral identification[a] (ref)[b]	Compounds found in other berries in the genus Rubus references[b]: Raspberry	Blackberry	Black raspberry	Cloudberry
2,5-dimethyl-4-methoxy-2,3-dihydro-3-furanone	mesifurane	137	32	18	a				
n-butanoic acid		138	0.15	0.15	1				19
3-methylbutanoic acid		145	0.95	0.80	1	9,13			19
γ-butyrolactone		147	0.20	0.20	a				
1,4-butanedicarboxylic acid diethyl ester	diethyl succinate	148	0.10	0.05	a	9			
ethyl benzoate		150	0.20	0.05	1				19
1-methyl-4-isopropyl-1-cyclohexen-8-ol	α-terpineol	154	0.30	0.05	1		18		19
benzyl acetate		164	0.20	0.05	1				19
2-vinyl-2,6,6-trimethyl-5-hydroxytetrahydropyran	linalool oxide	167	0.10	0.05	4				
3-methyl-2-butenoic acid		176	0.90	0.55	a				
3-methyl-3-butenoic acid		178	0.20	0.10	a				
2-phenylethyl acetate		183	0.10	0.05	1				
n-hexanoic acid		183	0.20	0.10	1	9,11,13			19
3-pyridinecarboxylic acid ethyl ester	ethyl nicotinate	185	tr	tr	1				
4-(2,6,6-trimethyl-1-cyclohexen-1-yl)-2-butanone	β-dihydroionone	186	0.10	0.05	a	15			
2-cis-3,7-dimethyl-2,6-octadien-1-ol	nerol	187	tr	tr	1	15,16			
2-trans-3,7-dimethyl-2,6-octadien-1-ol	geraniol	188	0.05	tr	1	14,15,17			19
2-methoxyphenol	guaiacol	189	tr	tr	1				19
benzyl alcohol		198	1.9	0.70	1	9,11,15		12	
ε-caprolactone		203	0.05	tr	1				
2-phenylethanol		205	0.75	0.30	5	7,9–11			19
4-(2,6,6-trimethylcyclohex-1-yl)-2-butanone	β-tetrahydroionone	209	tr	tr	t				
phenol		213	tr	tr	1				19
1,2-dimethoxy-4-allylbenzene	eugenol methyl ether	214	tr	tr	1				
2,5-dimethyl-4-hydroxy-2,3-dihydro-3-furanone	hydroxy mesifurane	219	tr	tr	a				
4-allyl-2-methoxyphenol	eugenol	229	0.05	tr	1				
benzene-1,2-dicarboxylic acid dimethyl ester	dimethyl phthalate	235	tr	tr	1				19
benzene-1,2-dicarboxylic acid diethyl ester	diethyl phthalate	238	0.05	tr	1				

[a] S = solvent; tr = trace amounts, < 0.05% or < 0.05 ppm; a = mass spectra taken from the reference compounds by the author; t = tentative identification.

[b] References: (1) Stenhagen et al., 1969; (2) Willhalm and Thomas, 1968; (3) Thomas et al., 1969; (4) Felix et al., 1963; (5) Nibbering and de Boer, 1968; (6) Winter and Sundt, 1962; (7) Bohnsack, 1967b; (8) Pisarnitskii et al., 1970; (9) Bohnsack, 1967a; (10) Schinz and Seidel, 1957; (11) Coppens and Hoejenbos, 1939; (12) McGlumphy, 1951; (13) Palluy et al., 1963; (14) Sundt and Winter, 1962; (15) Winter and Enggist, 1971; (16) Elze, 1929; (17) Sundt and Winter, 1960; (18) Scanlan et al., 1970; (19) Honkanen, 1972.

aroma in a press juice of the arctic bramble, which has been distilled almost odorless. The mesifurane added may be either isolated from the berries or synthetic (Kallio and Honkanen, 1975). The same compound has been identified earlier in canned alphonso mango (Hunter et al., 1974).

The variation of mesifurane content in fresh berries seemed to correlate with their quality. The summer 1973 was warm and thus advantageous for the growth of the berries, while 1974 was cool and rainy. The berries in 1973 were of a very high quality and the harvest was rich in the whole country; the

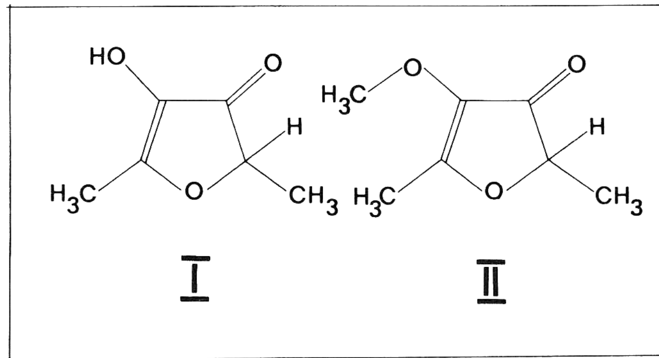

Fig. 2–Structures of 2,5-dimethyl-4-hydroxy-2,3-dihydro-3-furanone, compound I ("hydroxy mesifurane") and 2,5-dimethyl-4-methoxy-2,3-dihydro-3-furanone, compound II ("mesifurane").

Table 2–Compounds identified from arctic bramble divided into nine compound classes[a]

	ppm in press juice			
Compound class	**Ma-72**	**Lu-73**	**Mi-73**	**Mi-74**
aliphatic alcohols	12	40	22	32
aliphatic carbonyls	13	6	10	35
mesifurane	12	7	18	11
acetoin	14	4	10	4
aliphatic acids	9	9	7	5
aliphatic esters	6	13	6	7
terpene alcohols	1	3	3	3
aromatic alcohols	1	1	1	1
other compounds identified	< 1	< 1	1	1

[a] For explanation of abbreviations, see Materials & Methods.

Fig. 3–70 eV mass spectra of compounds No. 240(A) and 241(B).

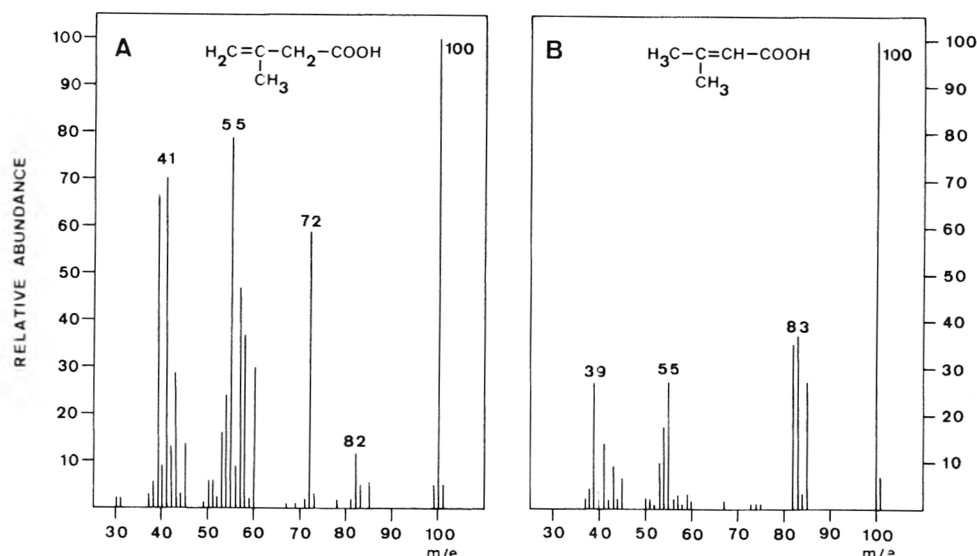

Fig. 4–70 eV mass spectra of 3-methyl-3-butenoic acid (A) and 3-methyl-2-butenoic acid (B).

content of mesifurane was 18 ppm (in the berries Mi-73, see Table 2). In 1974, when the harvest was scanty and of an average quality (Mi-74), the content of mesifurane was only 11 ppm. There was a still greater difference in the concentration of mesifurane between the cultivated and commercial berries. It is evident that the low mesifurane content in the latter was due to the post-harvest changes during storing, because there was a beginning fermentation noticeable in them. The changes in the aroma composition of the arctic bramble are very fast in unfavorable storage conditions (Ryynänen, 1973). The amount of another furanone derivative, hydroxy mesifurane (compound I in Fig. 2) is very small in the press juice, lower than 0.5 ppm. This compound is highly labile, and the exact content of it in the juice is very difficult to determine. Its threshold value is, however, low, and one can thus verify that these two furane derivatives form the basic odor of arctic bramble. The latter, hydroxy mesifurane, has been reported earlier in pineapple (Rodin et al., 1965), beef (Tonsbeek et al., 1968), popcorn (Walradt et al., 1970), heated maple syrup (Underwood, 1971), roasted filberts (Sheldon et al., 1972), strawberry (Büchi et al., 1973) and roasted almonds (Takei and Yamanishi, 1974). We can assume that this compound is metabolically consecutive to mesifurane in arctic bramble. They have evidently not been formed by a heat induced reaction, and the hypothesis that the hydroxy compound could be an intermediate in enzymatic biosynthesis of mesifurane may be allowed.

There are clear qualitative differences between the aroma compounds of the arctic bramble and the components identified in other berries of the same genus. Twenty-three of the compounds mentioned in Table 1 have not been found earlier in the other *Rubus* berries. Of these 23 compounds the following are worthy of note: mesifurane, hydroxy mesifurane, 2-methyl-2-butenyl acetate, 3-methyl-2-butenyl acetate, acetoin acetate, 2-phenylethyl acetate; 3-methyl-2-butenoic acid, 3-methyl-3-butenoic acid, eugenol and eugenol methyl ether.

There are also some substances which have been found only in some other *Rubus* berries. 2-Heptanone exists in blackberries (Scanlan et al., 1970), 6-methyl-5-hepten-2-one, ethyl benzoate and benzyl acetate only in cloudberry (Honkanen, 1972), and α-terpineol in blackberry (Scanlan et al., 1970) and in cloudberry (Honkanen, 1972).

The quantitative comparison of the aroma composition between the related berries is not easy, because such estimations have been reported in rare publications. An important difference between the aroma composition of cloudberry and arctic bramble is constituted by the rich aromatic fraction in the former (Honkanen, 1972). The main component of cloudberry, benzyl alcohol (30%), has a value of only 2–3% (0.7–1.1 ppm in the juice) in arctic bramble. The main class of

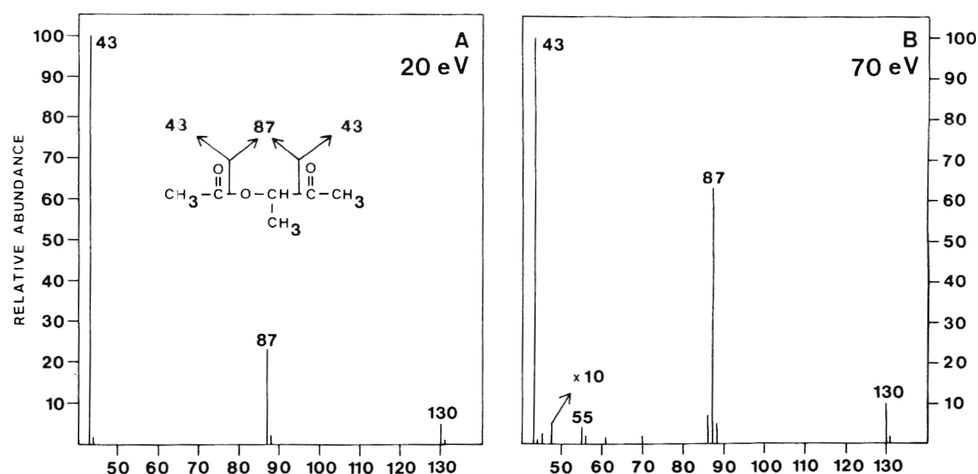

Fig. 5–Mass spectra of acetoin acetate.

aroma compounds in raspberry, according to many studies, is carbonyl compounds. Winter and Sundt (1962) reported that 14% of the distillate is carbonyls (estimated as acetaldehyde). Duclose and Latrasse (1971) studied the carbonyl compounds of several raspberry varieties spectrophotometrically as 2,4-dinitrophenylhydrazones. These compounds comprised 50% of the total volatile fraction. This compound class is quantitatively significant also in arctic bramble, but its importance for the aroma is assumed to be minute, because the fraction consists mainly of acetaldehyde. The alcohol fraction in arctic bramble is always much richer than the volatile acid fraction. According to Obretenov et al. (1972), the volatile acid content in different varieties of raspberry ranges between 40–60% of the total volatiles. This is an unusually high content.

Raspberries have several important flavor compounds which are missing in arctic bramble. One of them is p-hydroxyphenyl-3-butanone, the raspberry ketone (Schinz and Seidel, 1957, 1961; Schmidlin-Meszaros, 1971), which has not been found in other *Rubus* berries either. Nomura and Nozawa (1918) synthetized the compound as early as the beginning of the century, but they did not, however, realize its importance to the flavor of raspberry. The threshold value of the raspberry ketone is lower than 0.1 ppm (Schmidlin-Meszaros, 1971). α- and β-ionones are also typical raspberry compounds (Winter and Sundt, 1962; Bohnsack, 1967; Winter and Enggist, 1971). Neither of them has been found in the arctic bramble, blackberry or cloudberry, and according to McGlumphy (1951), black raspberries do not contain β-ionone. However, β-dihydroionone occurs in arctic bramble in trace amounts. It is an aroma component of raspberry, too (Winter and Enggist, 1971). We have also identified tetrahydroionone tentatively in arctic bramble (compound No. 209, Fig. 1), but we did not have any reference compound to confirm the identification.

Table 2 shows that the variation of different compound classes between the four harvests studied are appreciable. It is surprising that the differences of the same varieties (Mespi + Mesma) from the different cultivations and successive years are about as great as the differences between the cultivated and wild berries. It is, however, worth noting that the cultivated Mespi and Mesma varieties were chosen from wild cultures.

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Ms received 7/25/75; revised 10/18/75; accepted 10/21/75.

The author thanks Dr. Erkki Honkanen and Mr. Tapani Pyysalo, M.Sc., for their valuable help in the course of the whole study; Mr. Rainer Huopalahti, M.Sc., for technical assistance; Miss Annikki Ryynänen, D. Arg. Sc., and Mr. Onni Pohjanheimo, M.Sc., for giving the berries at his disposal, and Prof. Henrik Bruun and Mr. Rainer Ekman, M.Sc., for their help in the mass spectral analyses. Finally, the author thanks the Orion-yhtymä Research Foundation for financial help.

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DEVELOPMENT OF VOLATILE AROMA COMPOUNDS IN ARCTIC BRAMBLE, Rubus arcticus L.

ABSTRACT

The formation of volatile aroma compounds of a wild strain cultivated arctic bramble, *Rubus arcticus* L. was studied during the ripening of the berries in the field. The amount of 2,5-dimethyl-4-methoxy-2,3-dihydro-3-furanone, which is the most important aroma compound for the berry, increased from 0.2 up to over 20% of the volatiles. The total content of the most appreciable groups, alcohols, acids and esters, varied between 35–66%. The amount of the acids studied, acetic, 3-methyl-2-butenoic, 2-methylpropanoic, 3-methylbutanoic and 3-methyl-3-butenoic, increased up to ca 5% during the ripening. The relative amounts of 2-heptanol, benzylalcohol, cis-3-hexen-1-ol, 2-phenylethanol, 1-butanol and 1-hexanol decreased from unripe to over-ripe samples, whereas trans-3-penten-1-ol, 3-methyl-2-buten-1-ol, linalool, ethanol, α-terpineol and geraniol increased up to the ripe stage and began to decrease after that. The esters, ethyl acetate, methyl pyruvate, 2-methyl-2-butenyl acetate, benzyl acetate and cis-3-hexenyl acetate followed mainly the same pattern as the alcohols mentioned last. The three carbonyl compounds measured were acetone, 2-heptanone and 2-methyl-2-butenal, of which the last mentioned showed extremely rapid increase until the berries were ripe. The content of acetoin increased drastically during the ripening of the fruit.

INTRODUCTION

THE STUDY of the formation of biochemical metabolites such as aroma compounds in berries like arctic bramble is more difficult than, for instance, in apples, pears and bananas, since the berries are harvested fully ripe. This makes it impossible to investigate the native changes in the aroma composition of ripening berries after harvesting, as is customarily done with the before mentioned fruits (Romani and Ku, 1966; Tressl and Jennings, 1972; Drawert et al., 1972; Tressl and Drawert, 1973). In fruits, the precursors of the aroma compounds are usually formed during ripening from carbohydrates, synthesized by photosynthetic pathways in the plants. The flavor compounds are, however, formed after a postharvest respiratory climacterium. The formation of many volatiles in these fruits is a cyclic development, which can be traced, without destroying the fruit, using the technique developed by Tressl and Jennings (1972) for the study of flavor compounds. Whether the formation of the aroma compounds in arctic bramble has a similar cyclic mechanism is, however, a question that cannot be answered by the technique used in this study. As the changes in the quality of the arctic bramble berries are quick after harvesting (Ryynänen, 1973), the only proper method is to pick berries at different stages of ripeness and attempt to prevent the chemical and biochemical changes before and during the isolation of the aroma compounds. According to a study of the volatile carbonyl compounds of arctic bramble at various stages of ripeness (Kallio and Linko, 1973), the content of most carbonyls increases during the ripening, the largest changes occurring in the amounts of acetaldehyde, a pentenal, acetone, acetoin and diacetyl. The total amount of carbonyls is minute, except for acetaldehyde. Identification of over 60 volatile aroma compounds in arctic bramble by a GLC-MS analysis has been reported earlier by the present author (Kallio, 1976).

MATERIALS & METHODS

THE BERRIES (arctic bramble, *Rubus arcticus* L.) were cultivated in Central Finland (Agricultural Research Centre South Savo Experiment Station, Mikkeli, Finland). The two different clones (wild strains Mespi and Mesma) were planted in 1970 and grown in a mixed culture (Ryynänen, 1973). The berries were harvested in August, 1974 and assorted into four classes during the picking according to their stage of ripeness. These were: unripe, UR (small, green, hard), half ripe, HR (large, green or reddish, beginning to soften), ripe R (large, red, soft, optimal ripe), over-ripe, OR (red, very soft, beginning to wrinkle). The berries were frozen immediately and stored at −20°C. The samples were analyzed within 2 months after harvesting.

The press juice from 1 kg of the macerated berries was distilled at 22°C in the pressure of 3 torrs, and the aroma compounds of the distillate extracted with pentane-ether (1:2, v/v) in a continuous extractor. The extract was concentrated carefully with a Widmer column at 35°C to the final volume of 250 μl. The procedure is described in more detail in an earlier paper by the author (Kallio, 1975).

The quantitative analysis of the aroma compounds was carried out by glass capillary-GLC-system. The results are given in percent of the total amount of the compounds measured with an integrator. The capillary column was a self-made FFAP-column, 80m in length and 0.32 mm i.d. The identification of the aroma compounds was based on GLC-MS-analyses (Kallio, 1975).

The solvents were all guaranteed reagents (E. Merck AG) and were always redistilled before use. The reference compounds were purest commercially available products.

RESULTS & DISCUSSION

IN THE PRESENT STUDY, the development of the aroma spectrum in arctic bramble was examined in 1974 during the ripening. Cultivated plants were used instead of wild ones because it was required that the berries should be homogenous for comparison of the results from berries at different stages of ripeness. In spite of the efforts to classify and pick the berries of the four stages of ripeness, there was an appreciable amount of variation within the categories. The classification was based only on sensory estimations. Because of the large amount of single berries one can, however, assume that each of the four classes was homogenous enough in terms of averages. The changes in the contents of single aroma compounds were often 10- or even 100-fold during the ripening. The standard deviations were not computed because of small materials available. The variations of the yields of each compound ranged between 2–15% in parallel analyses of ripe berries (three separate determinations).

The yield of volatiles was about 60 mg of 1 kg of ripe berries, and only about 20 mg in unripe ones. During the development from unripe to half-ripe stage, the total amount of volatiles increased only slightly (some milligrams). In over-ripe berries the content of aroma compounds was at about the same level as in ripe ones.

The relative alterations of 31 aroma compounds typically occurring in arctic bramble were followed during the ripening of the berries from unripe to over-ripe stage. The compounds were classified in three different categories (Fig. 1–3) according to the modes of the formation curves. The results are presented in a logarithmic scale. Figure 1 shows the changes of the relative proportions of six alcohols, five esters and two carbonyls; there is a powerful increase during progress of the ripening. At the over-ripe stage these compounds diminish regularly. The amounts of the compounds reach their culmination point at about the same phase in the growth cycle, i.e., in the ripe berries. The most abundant ester in ripe berries was ethyl acetate (Fig. 1), the amount of which was about 6% of the isolated aroma concentrate. The relative amounts of the other important esters studied, methyl pyruvate, 2-methyl-2-butenyl acetate, benzyl acetate and cis-3-hexenyl acetate, were clearly lower. All of the alcohols (Fig. 1), trans-3-penten-1-ol, 3-methyl-2-buten-1-ol, linalool, ethanol, α-terpineol and geraniol, increased relatively two to 40-fold in the ripening phase. The decrease of these compounds is small but noticeable, however, after the berry has passed the optimally ripe stage. In general, the changes of the terpene alcohols and esters are not so sharp as in the aliphatic ones. The highest increases were found in 3-methyl-2-buten-1-ol, methyl pyruvate and trans-3-penten-1-ol. The two carbonyls belonging to the same category (Fig. 1) were acetone and 2-methyl-2-butenal. The increase of the latter compound was very sharp, from 0.02% in unripe berry to 2% in ripe berry.

Figure 2 shows the development of 11 volatile aroma compounds, characterized by an increase until the over-ripe stage. The most abundant compound is 2,5-dimethyl-4-methoxy-2,3-dihydro-3-furanone (Fig. 2, curve No. 14). This compound, called mesifurane, is the most important aroma component of arctic bramble (Kallio and Honkanen, 1975; Kallio, 1976), and its clear increase is directly connected with the ripeness of the berry, which can also be noticed organoleptically. The relative amount of mesifurane is about 0.2% in the aroma compounds isolated from green fruits. In ripe berries, its proportion is ca 20%, and in over-ripe ones, even 30%. The amount of this compound shows great variation in berries from different years and different sources. In the berries harvested optimally ripe, according to the same criteria, the amount of mesifurane was 32% in the same culture the year before. The ranges of variation are wide in other compounds, as well (Kallio, 1975).

The biosynthesis of acetoin is induced in greater amounts only towards the end of the ripening, and the increase progressed as the berry became over-ripe (Fig. 2, curve 15). Its content was as high as 14% in over-ripe berries, which is also a

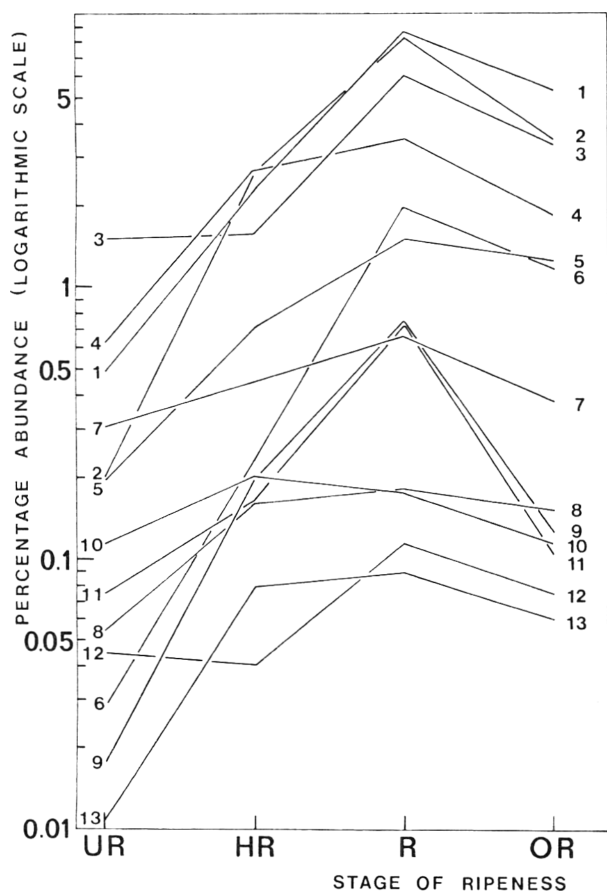

Fig. 1—Rates of formation of the aroma compounds in arctic bramble. The compounds which reach their maximum in the ripe stage. UR = unripe; HR = half-ripe; R = ripe; and OR = over-ripe. [(1) trans-3-penten-1-ol; (2) 3-methyl-2-buten-1-ol; (3) ethyl acetate; (4) linalool; (5) ethanol; (6) 2-methyl-2-butenal; (7) acetone; (8) α-terpineol; (9) methyl pyruvate; (10) geraniol; (11) 2-methyl-2-butenyl acetate; (12) benzyl acetate; (13) cis-3-hexenyl acetate.]

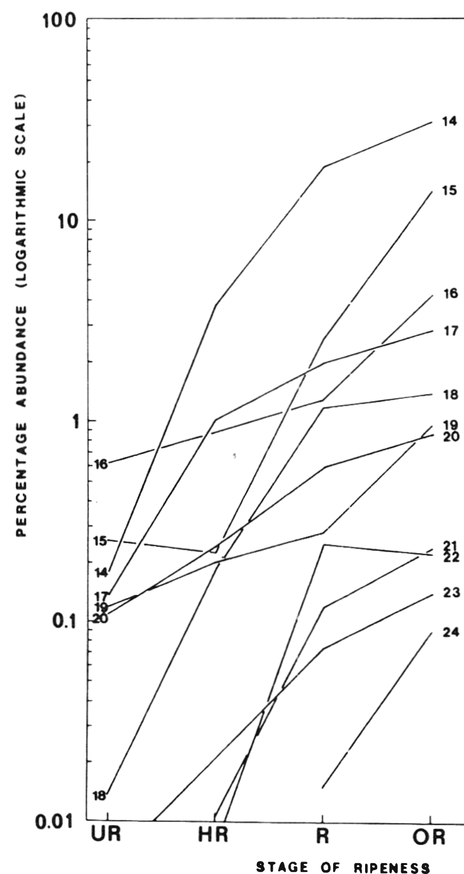

Fig. 2—Rates of formation of the aroma compounds in arctic bramble. The compounds, the relative amounts of which increase until over-ripe stage. [(14) 2,5-dimethyl-4-methoxy-2,3-dihydro-3-furanone (mesifurane); (15) acetoine; (16) 3-methyl-1-butanol; (17) acetic acid; (18) 3-methyl-2-butenoic acid; (19) 2-methylpropanoic acid; (20) 3-methylbutanoic acid; (21) acetoine acetate; (22) 3-methyl-3-butenoic acid; (23) diacetyl; (24) 1-propanol.]

typical feature of aged arctic brambles. The curve representing the formation of acetoin acetate showed identical shape (curve 21), and thus differed from the other esters studied. Drawert et al. (1969) supposed that the formation of acetoin shows the beginning of the second, glycolytic phase in apple.

Figure 2 also shows the development of five acids during the ripening. The increase of the relative amounts of these were noticeable. Four of the acids, acetic, 3-methyl-2-butenoic, 2-methylpropanoic and 3-methylbutanoic acid, showed an increasing tendency also in over-ripe berries, while the proportion of 3-methyl-3-butenoic acid was slightly decreasing in them. The relatively most rapid increase is seen in the case of 3-methyl-2-butenoic acid (curve 18), which increased from 0.01% in unripe to 1% in over-ripe berries. 3-Methyl-1-butanol, diacetyl and 1-propanol also follow mainly the same type of formation as the other compounds in Figure 2.

Figure 3 presents the changes in the relative amounts of the compounds which are fairly abundant in unripe arctic bramble, but decrease during the ripening. The most abundant volatile in unripe berries is 2-heptanol (curve 25), which comprises almost 50% (nearly 10 ppm) of the whole essence. The relative amount of 2-heptanol compared to the amount of the whole aroma concentrate decreases regularly and goes below the content of mesifurane (Fig. 2, curve 14) in the ripe berries. The content of 2-heptanol is about 8% in over-ripe berries. The other noticeable alcohols in this group are benzyl alcohol, cis-3-hexen-1-ol, 2-phenylethanol, 1-butanol and 1-hexanol. The curve of 2-heptanone shows an analogous decreasing form as 2-heptanol.

It must be noted, however, that the total amounts of volatiles vary in a manner different from the percentage curves (Fig. 1–3). The content of the volatiles in arctic bramble berries increases over threefold during the ripening. Thus e.g., the absolute contents of the compounds presented in Figure 3 are at about the same level in each stage of ripeness. Accordingly, it can also be estimated that the amount of mesifurane increases about 500-fold when the berry grows from green to over-ripe.

Figure 4 shows the summary curves of acids, alcohols and esters. Although the yields in isolation varied in different compounds, the shapes of the curves are correct, and they give a clear picture of the direction of the development. The alcohols were divided into two groups according to the type of variation. Curve number two in Figure 4 is summarized from the curves of the alcohols presented in Figure 3 (2-heptanol, benzyl alcohol, cis-3-hexen-1-ol, 2-phenylethanol, 1-butanol and 1-hexanol). In general the shape of each individual curve resembles that of the summarized curve. The total share of these alcohols decreased from about 50 to 10%. The only exception among these is 1-butanol, which exists at each stage at the level of 0.2%. The other alcohols shown in Figure 1, five carbon aliphatic, terpene alcohols and ethanol, are generated mainly during the ripening of the berry (Fig. 4, curve 3). Their total amount in unripe berries is 1–2%. At the first stage,

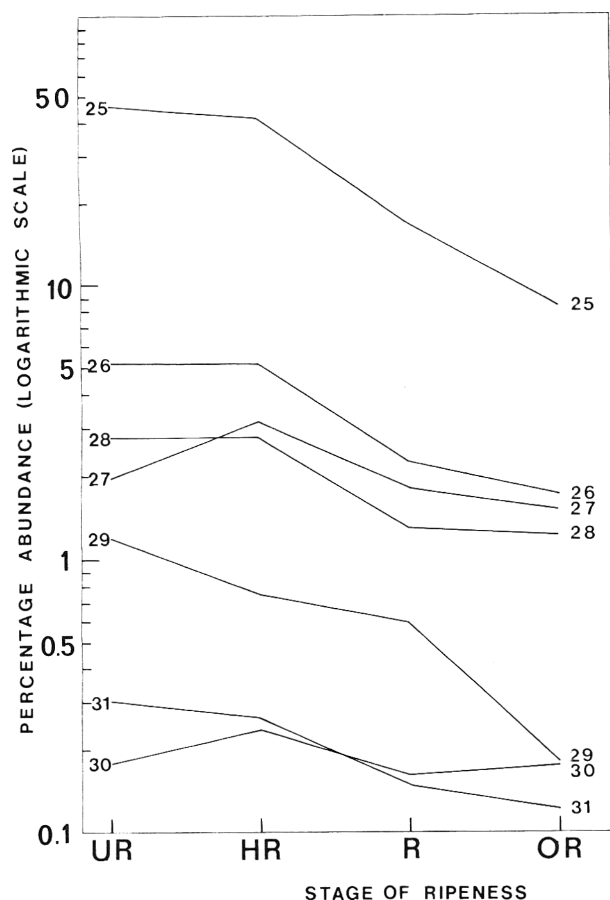

Fig. 3—Rates of decrease of some aroma compounds in arctic bramble. [(25) 2-heptanol; (26) benzyl alcohol; (27) cis-3-hexen-1-ol; (28) 2-phenylethanol; (29) 2-heptanone; (30) 1-butanol; (31) 1-hexanol.]

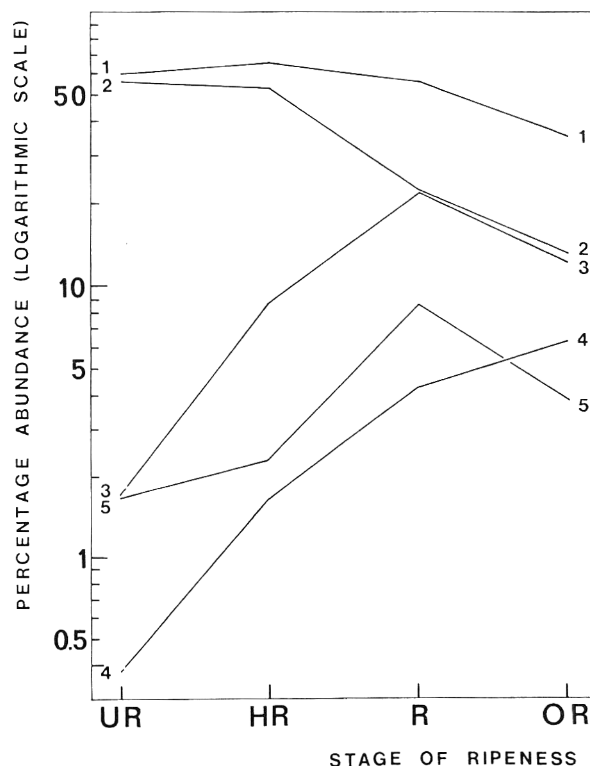

Fig. 4—Rates of variation of some aroma compound classes in arctic bramble. Curve 2 = alcohols from Fig. 3; curve 3 = alcohols from Fig. 1; curve 4 = acids from Fig. 2; curve 5 = esters from Fig. 1; curve 1 shows the proportional changes of sum of these alcohols, acids and esters in total aroma concentrate.

when the green unripe berries grow, and the reddish color begins to appear, the relative amount of the last-mentioned alcohols increase about sevenfold. The amounts of these alcohols increase very much during the whole ripening, and they reach a level of 20% in ripe berries, after which there is a rapid decrease by half (down to some 12%).

In the green unripe berries the volatile acids exist in smaller amounts (Fig. 4, curve 4). Their total amount is less than 0.5% in the isolated aroma fraction. The speed of formation follows roughly the rate of the alcohols in curve 3 in Figure 4, with the exception that they reach the level of 5% in ripe berries, and the increase continues up to 7% in over-ripe ones.

The main part of esters consisted of ethyl acetate at each stage. Other esters existed in amounts less than 1%. The total amount of esters increased fivefold during the ripening and reached the maximum (8%) in ripe berries (Fig. 4, curve 5). At the post-ripening stage their content decreased, like that of the alcohols. At the latter ripening phase (from half-ripe to ripe) the formation of the esters is slightly more rapid than that of the acids and alcohols. The summary curve 1 of the compound classes mentioned above (curves 2–5 in Fig. 4) shows that the percentage of these compounds decreased from over 60 to 35%. Their total amount was biggest in half-ripe berries, mainly because of the very high level of 2-heptanol.

The storage of the berries deserves proper care. As the changes in frozen storage cannot be fully avoided, the analyses were made as soon as possible. The aroma profile of frozen arctic bramble was followed half a year in the berries from the 1973 and 1974 harvests. The most noticeable change was decrease in most esters and 3-methyl-2-buten-1-ol, the losses of which were over 60% after half a year's storage. An exception among the esters was methyl pyruvate, the amount of which increased manyfold. The losses of trans-3-penten-1-ol, 2-heptanon and 3-methyl-1-butanol were also appreciable. 2,5-Dimethyl-4-hydroxy-2,3-dihydro-3-furanone disappeared from the distillate. Its yield in the isolation procedure was, however, so low, that the result can be only taken as an approximation. Mesifurane showed a slight decreasing tendency. The amount of cis-3-hexen-1-ol diminished, and the corresponding trans-configuration increased, which means that there is only isomerization involved. The highest change was noticed in the amount of propanol, which increased over 20-fold. Similarly, diacetyl and ethanol increased markedly. Some new compounds were also formed during frozen storage, but they have not been identified.

In general one can say, however, that the arctic bramble stands quite blameless in deep-frozen storage, which can be confirmed also organoleptically. In aroma research, however, when the aim is to resolve the native composition of the berry, long storages should be avoided.

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Ms received 7/26/75; revised 10/18/75; accepted 10/21/75.

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PSYCHOMETRIC ANALYSIS OF FOOD AROMAS BY PROFILING AND MULTIDIMENSIONAL SCALING

ABSTRACT

Panelists evaluated 20 commercial food aromas by two procedures: Profiling and estimation of overall qualitative dissimilarity. The scaling procedure of magnitude estimation was used for each task. Profiling produced unique signatures of attributes for each aroma, and illustrated variations in quality. Estimates of qualitative dissimilarity were treated as 'inter-stimulus' distances, and the method of multidimensional scaling was used to develop geometrical maps in which both aromas and descriptor terms were placed. These maps revealed that (a) aromas and descriptors (one's concept of an 'ideal' aroma) were fairly close to each other for fruit aromas, but often diverged for meats; (b) a pictorial representation of the qualitative dissimilarity (and similarity) of aromas and descriptors can be easily produced; and (c) individuals perceive qualitative differences in aromas in similar ways.

INTRODUCTION

THE DESCRIPTION of food aroma is complicated by the lack of any simple system for enumerating odor quality. Although many different systems have been proposed, ranging from four simple characteristics (fragrant, acid, burnt, caprylic; Crocker and Henderson, 1927) to more than 40 (Harper et al., 1968), none has proved entirely satisfactory for food analysis. Idiosyncracies in the food aromas evaluated for specific uses require tailored descriptor lists, and idiosyncracies in the way that panelists perceive and describe aromas lead to inflated within-panelist variability.

The present study concerns the description of food aromas by two procedures: profiling and dissimilarities estimation. Profiling methods require that the panelist evaluate the degree to which a quality inheres in an odorant, and is essentially a judgment about magnitude. Each odorant or food aroma generates its own unique 'signature', which can be represented by a bar graph. Differences among the signatures of various aromas can be assessed to see which characteristics differentiate the aromas perceptually. Dissimilarities estimation (also known as proximities estimation) require that the panelists rate how 'different' or perceptually dissimilar two aromas appear to be. The panelist is not instructed to attend specifically to one or a few salient features, but rather he is left free to select which attributes he wishes to use. In many instances the panelist may not even be asked about the attributes which he used. These dissimilarities ratings are then treated as distances between stimulus points in a geometrical space. Knowledge of the inter-stimulus distances allows the experimenter (through the use of 'canned programs') to reconstruct a 'map' of food aromas which might conceivably have been used by the panelist when he generated the estimates of dissimilarity.

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Proximities evaluation and the reconstruction of candidate perceptual maps have been previously used for the overall flavor evaluation of differences between pairs of equally sweet sugars (Moskowitz, 1972), and differences between the aromas of carrot root oil components and descriptor terms (Alabran et al., 1975).

EXPERIMENTAL

Procedure

The stimuli were commercial food aromas, each diluted to 1% of its starting concentration (as procured from flavor houses). The diluent was Nujol, a highly refined odorless paraffin oil. The odor stimuli were presented to the panelists in small scintillation bottles (1 oz). Inside each bottle was approximately 4 ml of the stimulus odorant, absorbed onto a cotton wick. This provided a large surface area from which the odorant could evaporate. When not in use the bottles were refrigerated at 4°C and stored airtight.

Thirteen panelists participated. All had had previous experience in the sensory analysis of aromas and flavors at the U.S. Army Natick Development Center, and were familiar with both the method of magnitude estimation, and with the procedures for estimation of qualitative dissimilarities. All of the panelists except one were housewives in the Natick-Framingham region. The thirteenth panelist was a 43-yr old male.

Methods profiling. The set of profile terms (see Table 1) were selected to fulfill the following criteria: (a) the terms would be general enough so that they would not apply to only one or two of the food aromas, or (b) the terms would be sufficiently familiar to capture one specific highlight of the aroma (viz. fruity citrus and cheeselike); and (c) the terms had to be meaningful to a naive panelist (even if only intuitively, such as 'flavorful,' 'foodlike,' 'distinctive').

The panelists were provided with the 20 bottles (see Table 1) and instructed to rate how strong each food aroma was on the attributes. Some were quite general (intensity, pleasantness), whereas some were quite specific (fruity citrus). The panelists rated each stimulus, in random order, a total of four times. The order of attributes shown in Table 1 was the same for all panelists and for all odorants, a method of experimentation which makes the task of profiling simpler for a panelist. Panelists were permitted to choose any numbers with which they felt comfortable, subject to the following constraints:

1. Ratios among numbers were to reflect ratios of sensory magnitude. If sample X was rated 5 on 'cheese-like,' and sample Y was rated 100 on 'cheese-like,' then sample X was to appear 20 times more 'cheese-like' than sample Y. This method of magnitude estimation is very useful as a procedure to obtain ratio-scale information on sensory qualities and intensities of tastes, aromas, textures, and food acceptabilities (Moskowitz, 1974).
2. No attempt was made to anchor the panelist's ratings to any physical exemplar, and every attempt was made to encourage the panelist to maintain a consistency in ratings across samples, across attributes and across replicates.
3. Pleasantness-unpleasantness was treated as a bi-polar continuum, with unpleasant smelling aromas to be rated negatively for unpleasantness magnitude (−), and pleasant smelling aromas to be rated positively. Zero was to reflect neutrality (neither pleasant nor unpleasant). Again the ratio rule applied: A +500 and a +250 meant that the former aroma

was twice as pleasing as the latter, and that both were pleasant. A +500 and a −250 meant that the former was pleasant, that the latter was unpleasant, and that the magnitude of the hedonic value (whether pleasant or unpleasant) was twice as much for the former as for the latter.

The arithmetic mean of all 52 ratings, across the 13 panelists provide a unique signature for each odorant. No effort was made to eliminate the naturally occurring variability, which might be expected on the basis of different scale values.

Qualitative differences judgments. On the second day the same panelists were provided with a booklet which instructed them to consider one odorant (or one odorant name) as a reference standard, and then to rate the overall dissimilarity between that reference standard and each of the remaining odorants and odor names. The booklet comprised 42 pages, one page devoted to each of the 20 odorants acting as a standard, and one page devoted to each of the 20 odorant names or descriptor terms acting as a standard. The 41st page was devoted to the concept of 'ideal familiarity' as a standard, and the 42nd page was devoted to the concept of 'ideal pleasantness' as a standard.

Panelists were instructed to rate overall qualitative dissimilarity by the method of magnitude estimation, with large numbers reflecting a large perceived qualitative dissimilarity between the reference and a comparison stimulus, and small numbers reflecting small perceived qualitative differences. Identity was reflected by a magnitude estimate of 0.

Because of the size of the task (completion of 42 × 42 matrix), the estimates required about 8 hr to complete. Panelists proceeded at their own pace, and did the task over several days.

The order of standards (references) was varied from one panelist to another, in order to counteract potential biases due to order. In addition, the order of comparisons of odorants and descriptor terms was randomized for each standard or reference, for each panelist. Between sniffs panelists were instructed to wait at least 10 seconds in order to forestall any carry-over of odor.

RESULTS

Profiling

Table 1 presents the profiles for each of the 20 foods on the different attributes, obtained by averaging together all of the replicates of the 13 panelists. Some estimated variabilities range from 0 (for descriptor terms such as cheese-like as applied to lemon oil) up to standard deviations around 30–70 for other attribute terms applied to various aromas. The following short paragraphs are précis of the various profiles:

Overall intensity. Most of the aromas were equally intense (between 300 and 400) with the exception of ginger (rating = 224), celery (rating = 202), and green bell pepper (rating = 233).

Pleasantness. Cheese, meats and vegetable aromas were the least pleasant (molasses odor was rated on the average as unpleasant), whereas the fruit aromas (cantaloupe, lemon, pineapple) were the most pleasant. Arithmetic means suggest about a 15:1 range in hedonic value (measured on a ratio scale). The spices (ginger, allspice, anise) were intermediate in hedonic tone.

Familiarity. This is an interesting descriptor, since all of the aromas here were initially assumed to have been household aromas, familiar to the housewives. The most familiar odorants were lemon, cantaloupe, strawberry and anise, and the least familiar were celery, bacon, beef and parmesan cheese. Based upon the ranking of the aromas a couple of conjectures can be made: (1) Fruit aromas tend to be the most salient (although for this to be testable by analysis-of-variance for statistical significance, one would have to control initially for intensity of the aroma). Panelists pay attention to fruit aromas, and probably more easily identify them. Perhaps aroma is the primary identifier of the fruits themselves. Anise and almond are also very familiar, perhaps because aroma is extremely important for them as well. (2) Meat aromas tend to be minimally familar, because the panelists use a variety of cues besides aromas to identify these products (e.g., color, taste, general appearance).

There is a 2:1 range in subjective estimates of familiarity. Lemon and cantaloupe are the most familiar, perhaps because these two are most often identified by their aroma. Lemon is a special example–products are sold commercially with advertising stating that the product has lemon freshness, lemon

Table 1–Odors, profile values

	Intensity	Pleasantness	Familiarity	Complexity	Fruity (non-citrus)	Fruity (citrus)	Sweet odor	Spicy	Meaty	Heavy	Cheese-like	Flavorful	Food-like	Stale, old	Distinctive
Lemon oil	335	387	415	80	21	489	245	77	0	67	0	407	341	14	457
Green bell pepper	233	48	241	129	17	0	6	39	6	100	9	96	108	167	210
Molasses	315	−21	208	186	10	0	142	82	59	193	32	114	146	110	241
Pineapple	299	361	349	83	354	79	270	59	0	88	4	336	366	18	364
Bacon	376	41	207	199	0	0	25	53	300	314	16	211	268	178	249
Tomato	336	148	314	163	114	10	51	80	82	208	108	217	325	118	342
Allspice	344	273	348	161	27	17	123	384	0	128	0	310	154	6	328
Chocolate	308	250	289	139	25	0	244	49	1	93	2	267	259	104	312
Raspberry	347	137	275	152	152	7	146	70	44	199	38	188	204	80	290
Apple	315	317	297	180	308	134	236	90	0	130	10	291	309	41	288
Ginger	224	248	251	164	13	46	134	229	32	119	0	222	206	9	280
Parmesan cheese	289	26	234	108	3	3	19	49	52	197	382	199	278	215	332
Celery	202	118	241	106	11	0	33	127	61	113	16	151	179	90	268
Beef	342	80	209	192	4	0	42	88	296	283	52	183	280	123	289
Cherry	316	364	344	133	387	48	268	60	3	86	0	372	359	33	382
Strawberry	337	367	403	89	445	30	276	36	0	95	0	399	415	15	440
Sausage	288	134	267	191	16	3	52	180	108	132	17	188	192	77	273
Anise	378	296	389	114	25	44	242	181	2	124	0	381	304	11	389
Almond	392	256	382	93	83	12	222	171	0	209	0	292	257	54	460
Cantaloupe	392	334	424	158	469	7	281	51	0	157	0	373	420	2	457

aroma, or a hint of lemon, which may predispose panelists to search for such attributes specifically.

Complexity. The meat aromas are the most complex, whereas the lemon, pineapple and fruit odors are the least complex. The ratio of ratings is slightly more than 2:1. Complexity seems to be inversely related to distinctiveness.

Fruity (noncitrus). This descriptor divides the group of aromas into three clusters: The highly fruity aromas (cantaloupe, strawberry, cherry, apple and pineapple), the intermediate fruity aromas (tomato, raspberry), and the remaining aromas, which are not fruity at all.

Fruity (citrus). The only aromas showing this attribute are lemon, and to a much lower degree pineapple and apple. This descriptor fruit thus effectively divides the aromas into citrus vs noncitrus aromas.

Sweet odor. As expected, the fruit aromas are the sweetest, whereas the meats, cheeses and vegetables are the least sweet. The presence of esters in fruit is correlated with the perception of olfactory 'sweetness.' Lemon, raspberry, cantaloupe, strawberry, etc., are very sweet, whereas green bell pepper, parmesan cheese, etc., are less sweet or not sweet at all. There appear to be three major clusters: The fruits and nuts, the spices (allspice, ginger and the aroma of molasses, which is not technically a spice), and the other aromas.

Spiciness. Most of the aromas tested were not spicy. Allspice is the spiciest, with ginger being considerably lower, and anise, almond and sausage being quite low. Strawberries, green bell pepper and parmesan cheese aroma are the least spicy. The overall range of spiciness is about 4:1.

Meatiness. Most of the aromas are not meaty. Beef and bacon are both the meatiest, with tomato and sausage aroma being about as third as meaty. Molasses, celery, parmesan cheese, raspberry and ginger are very slightly meaty. The attribute of meatiness divides the group of aromas into two (possibly three) distinct clusters. Note that this attribute was to refer to cooked meat aroma, not raw meat aroma.

Heaviness. Bacon, beef, tomato, anise and parmesan cheese are the heaviest aromas, whereas green bell pepper, cherry, pineapple, etc., are the least heavy. Probably heaviness correlates with the presence of meat-like or burnt notes in the aroma, and is inversely correlated with the presence of sweet smelling esters. The aromas form almost a continuously decreasing group in terms of the ratings—the term heaviness does not sharply cluster aromas into different groups. There is about a 3:1 range in terms of rated subjective heaviness of these aromas.

Cheese-like. This is an exceptionally discriminating descriptor, since virtually all aromas but that of parmesan cheese were rated around 0, wherease parmesan cheese was given a rating of 382.

Flavorful. This descriptor term is ambiguous, since all of the aromas here represented flavors. Lemon, strawberry, anise, cherry and cantaloupe are the most flavorful, whereas green bell pepper, molasses and celery are the least. Apparently, the connotative meaning of the term flavorful is one which evokes an image of something to eat. Since the aromas of fruit are salient, and since they have the greater number of character impacts, it is reasonable to find that the fruit aromas are most flavorful.

Foodlike. Again, this descriptor sorts out the aromas from high to low in terms of fruit aromas at the top, and meat, molasses and celery aroma to the bottom. Both flavorful and foodlike descriptors produce a continuum of responses so that the aromas fail to classify themselves into distinct clusters. It is interesting that raspberry aroma, a fruit, is not usually placed together with the other fruits, which suggests that the commercially prepared aroma of raspberry in this study is in a qualitatively different class than the aromas of the other fruits (lemon, cherry, cantaloupe, pineapple). Raspberry is also not a sweet smelling aroma compared to the aromas of the other fruits.

Stale, old. Most aromas were rated very low on this attribute (less than 100), with the exception of green bell pepper, molasses, bacon, tomato, parmesan cheese and beef. This descriptor distinguishes between the aromas of spice and fruit and the aromas of meat, cheese and vegetables.

Distinctiveness. The fruits were the most distinctive odors (i.e., they had more character impact), then did the vegetables and meats. This may be related to the fact that fruit aromas seem to be more easily identified as such than are the vegetable and meat aromas. Ginger, green bell pepper, molasses and bacon were the least distinctive. Part of the failure for these aromas to be distinct (in terms of subjective ratings) may result from a panelist's failure to label and identify them as quickly as the panelist can label and identify a fruit aroma. There is only a 2.5:1 ratio in distinctiveness, however, compared to the large 8:1 range in hedonics.

Multidimensional scaling

Since the panelists in this study provided magnitude estimates of the overall qualitative dissimilarity between pairs of aromas, between pairs of descriptor terms, and between heterogeneous pairs comparing one aroma and one descriptor term, it proved easy to perform a multidimensional scaling analysis (or proximities analysis). This analysis, by means of the M-D-SCAL 5M (Kruskal & Carmone, 1969) took the panelist's mean rating of overall dissimilarity for every entry in the dissimilarity matrix (42 X 42 symmetric matrix, 20 aromas, 22 descriptors X 20 aromas, 22 descriptors) and located the 42 points in geometrical spaces of 3, 2 and 1 dimensions, respectively. For each dimension, the computer was programmed to suggest four different potential arrangement of points, with each arrangement obtained by starting from a new, random initial configuration of points. The computer then computed the measure of 'stress,' which is akin to a residual sum of squares. That configuration minimizing the 'stress' value is, by definition, the one which most veridically reproduces the array of original estimates of qualitative dissimilarity. Mathematically, it is known that with higher dimensional spaces the stress becomes lower and lower, but also parsimony is reduced, and the experimenter always runs the risk of accounting in his geometrical space for noisy data.

In the present experiment, a two-dimentional space produces slightly higher stress than a three-dimensional space, and much lower stress than a 1 dimensional one. Hence, the two-dimensional geometrical representation was chosen on the combined basis of relatively low stress and relatively high parsimony. The parameters of the calculation were that the distances were to be represented by the standard Euclidean formula for distance, and that the recovered distances of points in the space were to be as closely related as possible to the original estimated dissimilarities by a simple polynomial (linear polynomial, no additive constant).

The configuration was developed after the averages from all 13 panelists were computed (4 replicates/panelist/pair). High dissimilarity values are reflected in Figure 1 by large interpoint distances, and low dissimilarity values are reflected by low inter-point distances. Note also that since panelists evaluated the dissimilarity between pairs of descriptor terms and between pairs comparising a descriptor term and an aroma, that these terms are embedded in the same space, and may be considered to be 'hypothetical' or 'ideal' aromas. The descriptor terms are underlined in Figure 1, whereas the actual aromas are not, to facilitate differentiation. Since the computer program produced geometrical maps in which interpoint distances *only* convey information (but not necessarily the orientation, nor the location of the map relative to the X-Y coordinate system), the actual X and Y values are themselves unimportant. Rather, the relative positions of the aromas and

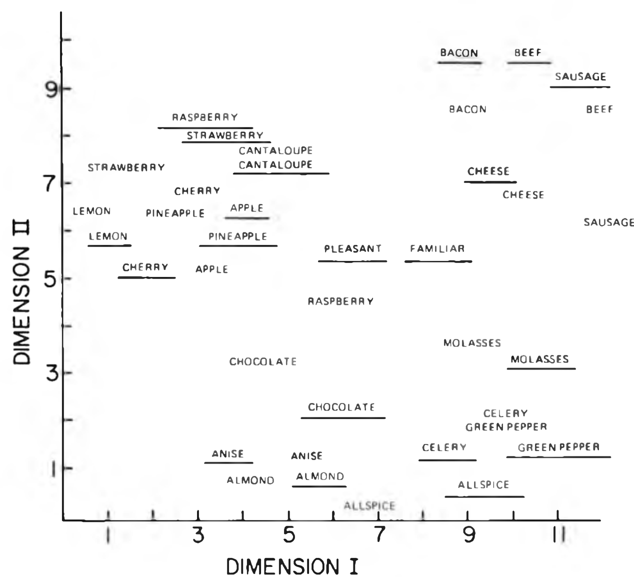

Fig. 1–Two dimensional geometrical space obtained by multidimensional scaling. The input to the MDSCAL program was the Symmetric 42 X 42 matrix of subjective dissimilarities between all pairs of aromas, descriptors, and heterogeneous pairs of aroma X descriptor. The map comprises two axes: The vertical (Y) axis appears to be the gradation between foodlike and nonfoodlike (viz. spicy). The horizontal (X) axis appears to be the gradation between meaty and fruity.

the descriptor terms convey information about the aroma quality.

The important results appearing in Figure 1 can be summarized as follows:

(1) Panelists do appear to be able to judge the qualitative dissimilarity between pairs of food aromas, between pairs of aroma descriptors, and between pairs in which one component is an aroma and the other is a descriptor of that aroma (or of another aroma). That these dissimilarity estimates seem to make sense can be seen from the arrangement of descriptor terms and aromas in natural-appearing clusters (with fruit aromas close to each other, and spice and meat aromas far away from the fruit aroma cluster). The ability of panelists to judge dissimilarity between a descriptor term (which represents an 'ideal' aroma in the panelist's conception) and the actual aroma is a powerful tool for determining how close together a stimulus and a concept lie.

(2) For most of the fruit aromas, the descriptor term and the aroma lie very close to each other. This implies that for fruits panelists may easily associate the descriptors with the aromas, and that the two are interchangeable. A panelist's conception of lemon aroma seems to be virtually identical with the sample of lemon aroma presented. Why fruits seem to be so similar to their descriptor terms is difficult to explain, except, perhaps, that the description of fruits may provoke a smell image more readily than the description of a meat, and vice versa.

(3) For meat aromas, the overlap between the descriptor terms and the actual aromas is close, but not nearly as close as for fruit. The biggest difference between the descriptor and the actual aroma arises for the sausage odor. Quite possibly we store concepts of meat aromas in a different way, and since meat (and perhaps other 'heavy' aromas) can vary so much, these aromas may not have an immediate character impact.

(4) Aromas cluster into several groups, and on two different axes. One group (far left) is the fruit group. These are the light, fruity, sweet smelling aromas. Most of the fruits, with the exception of raspberry aroma lie there. One group (far right) is the meat group, which are the heavier, sulfur-containing aromas. The X axis is possibly a fruity-meaty distinction. The aromas at the top of the Y axis are meats and fruits (food-like aromas), whereas those at the bottom of the Y axis are the spices, nuts, etc. The Y axis may be foodlike vs non-foodlike.

Individual differences scaling

The map shown in Figure 1 was obtained by averaging the results of the panelists. Individuals often differ in their perception of qualitative differences, especially with regard to odors of chemicals (Berglund et al., 1973). Although the average geometrical space for a collection of odorants may appear to be perfectly reasonable, the individual geometrical spaces may have no clear relation to the overall space (which would thus arise as an artifact from averaging). Berglund et al. (1973) suggested that we may live in quite different perceptual worlds when evaluating aroma, and that the average geometrical space to be obtained from multidimensional scaling probably does not reflect the space of any single individual. A similar conclusion was reached by Moskowitz (1975) in a re-analysis of dissimilarity judgments provided by 15 panelists, each of whom judged the overall qualitative dissimilarity of 30 stimuli (15 odorants, 15 descriptors, a design similar to the present one). Although the overall, average geometrical space was reasonable in light of the locations of the descriptor terms and the stimulus odorants (Moskowitz and Gerbers, 1974), the individual spaces varied quite considerably.

An analysis of the individual spaces, and how they relate to a single composite space, was undertaken with the aid of a computer program known as INDSCAL (Carroll and Chang, 1969). The basic premise of the INDSCAL approach is that each panelist possesses the same geometrical space as every other panelist, but that in addition each panelist brings to bear a set of weighting factors (stretching or shrinking). A simple example will bring out the approach. In the present study, were the first dimension (or axis) to be fruit vs meat, then all panelists would possess the same first dimension. Some panelists might attend quite closely to differences between aromas, using the dimension of fruity vs meaty. The weighting factors (or biases) of these panelists would be very high. Others might see the same differences in pairs of aromas, but might choose to ignore the differences entirely. Their weighting factors would be 0. The perception of the aroma would be the same, but the degree of importance given to differences on the dimension might vary from one individual to another.

The input to the appropriate INDSCAL program is a set of individual symmetric dissimilarity matrices, like the type obtained here. Since there were 13 panelists, the INDSCAL input here is a series of 13 matrices (size 42 X 42). The matrices were first made symmetric by averaging together the i, j and the j, i entries in each panelist's matrix, under the assumption that when the panelist judged the dissimilarity between stimulus i and stimulus j, then the order (i.e., which was the standard or reference, and which was the comparison) did not matter. This is itself an arguable assumption, but is necessary for the analysis. The output of the INDSCAL analysis is a grand space (like Fig. 1), as well as the individual weighting factors. Figure 2 shows the distribution of 13 such weighting factors. The factors are all positive for both dimension I (fruity vs meaty), and dimension II (food-like vs spice-like). This means that all panelists perceived the gradation of fruity vs meaty and foodlike vs nonfoodlike in the same way. However, there were some individual variations in stretching or shrinking of the dimensions. Panelists 9, 11 and 12 shrank dimension I relative to the other panelists. For dimension II

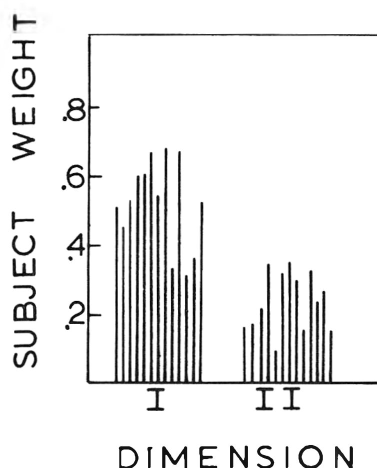

Fig. 2–The individual weights attached to dimensions I and II as obtained from the INDSCAL program. Note that all panelists show positive weights, implying that all panelists perceive the aroma space similarly, and vary only in the amount of shrinking or expansion they do on each attribute. Expansion (high subject weights) mean that the dimension is relatively critical in determining dissimilarity between two stimuli, whereas contraction (low subject weights) means that the panelist disregards differences on the dimension.

there was substantially more individual variation in the stretching vs shrinking, suggesting that the range of individual differences might be larger for this food attribute.

DISCUSSION & CONCLUSIONS

THE PRESENT FINDINGS address two major methodological areas of sensory evaluation: profile representation of flavor quality, and the assessment of individual differences in aroma perception by means of multidimensional scaling.

Profiling

No single number can capture the aroma of foods. Rather, aroma quality must be considered as a 'signature' which comprises a series of numbers, one number for each relevant attribute. Here 15 such attributes (if odor intensity is included) were used to evaluate 20 aromas. Actually, several hundred different attributes can be enumerated (Dravnieks, personal communication), and the literature of sensory evaluation for a variety of disciplines (e.g., perfumery, flavor, etc.) contains a host of such descriptors. The present approach is noteworthy methodologically, insofar as:

(a) It uses profiling procedures which embody magnitude estimation–a method of assigning numbers to stimuli in which ratios of numbers reflect ratios of perceived magnitude. Most approaches to sensory analysis, either with unidimensional continua (e.g., sweetness, acceptability) or with multi-attribute continua (different aspects of texture, etc.) use the traditional category scale, in which differences among categories reflect differences in sensory magnitude. Magnitude estimation permits the experimenter to state that the aroma intensity of aroma X is some number of times stronger or weaker than the aroma intensity of aroma Y, or that the sweetness-note of aroma A is five times (or how many times) stronger or weaker than, for example, its complexity. A more detailed description of magnitude estimation is proved by Moskowitz (1974).

(b) The present procedure dispenses with the use of external standards to represent magnitude, and utilizes, instead, the panelist's concept of what a 'moderate' intensity is. Traditionally, experiments in sensory evaluation have used explicit standards, with numbers attached to the standard to indicate the measure of intensity. The present procedure allows the panelists to compare all notes against an internal 'standard' which they call 'moderate,' and to which they assign the value of '30.' Variations in the ratings among panelists can be disentangled into variations resulting from experimental error (e.g., panelist variability and failure to replicate), and the more subtle variation due to what a panelist thinks is 'moderate,' 'intense,' etc., for that stimulus and class of aromas. Future work might consider explicit external stimuli as standards, which may not necessarily be assigned numbers, but would rather serve to anchor the panelist's judgments. With these explicit external standards, to which the panelist assigns magnitude estimates (with 30 or some other number always reflecting 'moderate') the advantages of mental references for number may be combined with the advantages of an invariant, unchanging reference stimulus.

Individual differences

Panelists differ in the way that they assign numbers to stimuli. In many olfactory experiments with simple chemicals which are not related to food the variation among panelists in assignments of numbers is so great that it casts suspicion upon the grand geometric map as a valid representation of overall qualitative dissimilarity. In a previous study (Moskowitz and von Sydow, 1975) with the profiling of a variety of fruit juices by different panelists, the INDSCAL approach was successfully used to show that all panelists rated reliably across days, but that they used different ways of assigning odor descriptors. In the present study, which uses the INDSCAL approach to analyze symmetric matrices of pairwise dissimilarities between stimuli, the finding is that panelists perceive overall qualitative differences in about the same way, although each panelist may bring to bear slight variations.

An overview

The multidimensional scaling technique provides an excellent way to obtain a pictorial representation of overall similarity (or dissimilarity) in product perception from different panelists. Such scaling ought to prove highly instructive in assessing the way trained panelists perceive qualitative differences. Here there is no *systematic* inter-individual variations in which dimensions (or axes) or the geometrical space are stressed, and which are eliminated. What might occur in the comparison of maps for trained vs untrained panelists is the next logical step in a program which utilizes the analytical and descriptive power of multidimensional scaling.

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Ms received 7/5/75; revised 10/18/75; accepted 10/21/75.

Presented at the 35th Annual Meeting of the Institute of Food Technologists, Chicago, Ill. June 8–12, 1975.

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A TEST OF THE MUTAGENIC EFFECTS OF SORBITOL ON *Drosophila melanogaster*

ABSTRACT

This investigation critically examines the genetic effects of sorbitol in *Drosophila melanogaster*. Tests for the induction of somatic mutations, sex-linked recessive lethals, and chromosome loss paralleled by appropriate positive and negative controls were performed. In all experiments, sorbitol proved to be nonmutagenic. In fact, the frequencies of exceptions in sorbitol-treated flies proved to be less than that in untreated controls.

INTRODUCTION

ALTHOUGH SORBITOL (D-glucitol) is "generally recognized as safe" by the Food & Drug Administration and is commonly added to confections and dietary foods, its use has been questioned. For example, Van Heyningen (1959) reported abnormally large amounts of sorbitol in the lenses of diabetic rats in the early stages of sugar cataract formation; Ward et al. (1972) demonstrated the accumulation of sorbitol and fructose in the nervous system in diabetic animals; and Drasnin (1973a, b) found that a high sorbitol diet promotes macroangiopathy, neuropathy and cataracts, and advocated stricter limitations on the amounts of sorbitol in foods and in individual diets.

A search of the literature uncovered no reports on the mutagenicity of sorbitol, a study we deemed to be advisable in light of its wide-spread use and pathological implications. Therefore, we have assayed the genetic effects of sorbitol in several experimental conditions using the vinegar fly *Drosophila melanogaster*. These tests were designed to cover a broad spectrum of genetic phenomena, providing maximum opportunity for the detection of deleterious effects. The results detailed below will verify that we found no evidence of harmful genetic effects of sorbitol.

Table 1—Synopsis of mutants and chromosomes used in this study.

Symbol	Location and remarks
asc	X – chromosome inversions marked with apricot eye color.
ec	X – 5.5, echinus eye. Fortuitously present in some of the stocks used.
Qn(1)w^i	X – 1.5, quintuplication of white region carrying 5 w^i mutants.
$sc^8 \cdot Y$	Y chromosome with duplication carrying y^+. Serves to identify the Y chromosome when in an otherwise yellow-bodied stock.
sn^3	X – 21, singed bristles. Used as contamination control.
v	X – 33, vermilion eye color. Used as contamination control.
w^i	X – 1.5, white-ivory eye color.
y	X – 0, yellow body. See note on $sc^8 \cdot Y$. Fortuitously present in other stocks.

EXPERIMENTAL

ALL *Drosophila* stocks were maintained at room temperature (21–25°C) on a standard medium of cornmeal, agar, brewer's yeast, and sucrose with propionic acid added as a mold inhibitor. The chromosomes and mutants used in these experiments are briefly described in Table 1. Additional information is available in Lindsley and Grell (1968). Particular crosses and stocks are described in connection with the experiments in which they were employed.

Each of the experiments reported here consisted of comparing the frequency of a genetic anomaly in flies or the offspring of flies treated with sorbitol to that observed in untreated controls. To assure that the experimental operations were in fact uncovering the types of events sought, positive controls, i.e., treatments known to induce the anomaly, were conducted simultaneously. In analyzing the data, the sorbitol-treated populations were compared to the untreated controls both by computing the appropriate fiducial limits and by comparison in a contingency table. In each experiment, the reliability of the conclusion was evaluated by testing the hypothesis that the actual frequencies of exceptions in the treated populations might be twice the spontaneous frequency, the low values observed being fortuitious deviations. Rejecting this very conservative hypothesis validates the adequacy of the population sizes.

RESULTS & DISCUSSION

BEFORE GENETIC TESTS were performed, flies were placed on various concentrations of sorbitol in order to observe the effect of the chemical on the flies and their imbibing behavior. Flies were placed in half-pint culture bottles with Kimwipes saturated with 1, 5, 10, 20 and 50% sorbitol solutions. After 132 hr, it was observed no flies had died, leading to the conclusion that large doses of sorbitol are not lethal. Feeding experiments with sorbitol solution colored with green food coloring led to the conclusions that flies use sorbitol to satisfy an energy requirement and that treatment of flies with concentrations of 3–5% sorbitol are equivalent.

According to Bowman (1969) the *white-ivory* mutant of *Drosophila melanogaster* is distinguished by its frequent reversion to wild type, not only in germinal cells but in somatic tissue as well. The eyes of the adult fly contain a total of about 1600 ommatidia and one can observe a one-cell reversion in the eye.

Since the frequency of mosaic spots in an eye is directly proportional to the number of w^i loci in the genome, a quintuplication of the *white* region carrying w^i in each segment, Qn(1)w^i, obtained from E.B. Lewis, was used to enhance the sensitivity of the test. The offspring of homozygous w^i females and *y* Qn(1)$w^i ec$ males were allowed to develop on medium containing 1% or 10% sorbitol. Flies raised on the standard media of cornmeal, agar, sucrose, yeast and propionic acid

served as a control. After eclosion, the eyes of female imagoes (w^i/y Qn(1)$w^i ec$) were scored for wild-type spots. Prior to scoring, the cultures were coded. Upon completion of the experiment, the code was revealed for the first time to the experimenter.

Mitomycin C, purchased from Sigma Chemical Company, was used as a positive control in this test. Eggs from the parental cross were collected over a 12-hr interval on Bakers' yeast. Three days later, the larvae were collected by washing the yeast through a 60-mesh sieve. The larvae were transferred to filter paper saturated with a mitomycin C solution (40 μg/ml in 1% sucrose). After 1 hr, the larvae were removed from the filter paper and washed. The entire MC treatment was carried out in the dark or in very dim light. The larvae were then placed in bottles of standard media and allowed to develop in the dark. After eclosion, the eyes of the imagoes were scored as before.

The results of the somatic mutation tests are given in Table 2. Fiducial limits, computed according to the tables of Stevens (1942), revealed the frequency of mosaics in the control group to be inseparable from that of the sorbitol-treated group. A contingency chi-square computed to test the homogeneity of the data suggests a 0.8 probability of observing the same or a greater difference than that actually observed between the treated and control populations. The hypothesis that the actual frequency of mosaics might be twice the spontaneous frequency can be rejected at the 0.005 level.

Germinal cell mutations are most readily determined as recessive lethals expressed in the generations following treatment. The sex-linked recessive lethal test developed by Muller (1942) using the *asc* chromosome scans the entire X chromosome for the occurrence of new lethal mutations. Homozygous *asc* females, which have apricot eyes, were mated to treated or to control wild-type males. The F_1 females, which need not be virgin and are heterozygous for the treated X and the balancer (*asc*) chromosome, were individually mated to their brothers or to *asc* males from the same stock from which the parental females were derived.

The F_2 generation was examined, without etherization, through the vial with the aid of the low power of a stereoscopic microscope. The cultures that did not contain wild-type-eyed males and did contain at least 20 *asc* males were considered lethals. If there were no wild-type-eyed males and fewer than 20 *asc* males, the F_3 was reared by mating *asc* males to F_2 wild-type females and scored until a total of 20 *asc* males were observed. If a wild-type-eyed male was observed, the chromosome was considered nonlethal.

Ethyl methanesulfonate (EMS) was used as a positive control in this test. The method for administering EMS was that of Lewis and Bacher (1968). Adult Canton-S males were collected as before and placed on Kimwipes saturated with an 0.02M solution of EMS in 1% sucrose. The crosses were performed and the flies scores as previously described.

The frequencies of induced sex-linked recessive lethals are presented in Table 3. Fiducial limits, computed as before, reveal the frequency of lethals observed in the control group to be inseparable from that of the sorbitol-treated group. A contingency chi-square was again computed to test the homogeneity of the data. The chi-square value computed suggests a 0.2–0.5 probability of observing the same or a greater difference than that actually observed. On the basis of the one-tailed test performed on the data, the hypothesis that the actual mutation rate is twice the observed control rate was rejected at the 0.005 level.

To determine if sorbitol induces chromosome loss, males of the genotype $y\ sn^3\ v/sc^8 \cdot Y$ were placed in half-pint culture bottles containing Kimwipes saturated with 5% sorbitol for four days and then mated to $y\ sn^3\ v$ virgins. F_1 males which fail to receive the Y chromosome from their father are readily recognized as yellow-bodied.

X-rays were used as a positive control in this experiment. P_1 males enclosed in a perforated gelatin capsule were given 3000r of X rays with a Seifert "Isovolt 150" operated at 140 kv and 12 ma with 1 mm Al filtration. The dose rate was 196r/min with the target to specimen distance being 30 cm. Approximately 65 males were placed in the gelatin capsule, and after irradiation, an equal number of females were mated to these males in half-pint milk bottles. The F_1 was scored as previously described.

Table 4 represents the data obtained from the chromosome loss test. Fiducial limits computed as before reveal the frequency of nondisjunction observed in the control group to be inseparable from that of the sorbitol-treated group. A contingency chi-square was again computed to test the homogeneity of the data. The chi-square value computed suggests a 0.5–0.8 probability of observing the same or a greater difference. On the basis of a one-tailed test performed on the data, the hypothesis that the actual mutation rate might be as large as twice that observed was again rejected at the 0.005 level.

Throughout our experiments we noted only one effect of sorbitol on *Drosophila*—a 2-day lag in eclosion. This may be trivial. The control media on which the larvae feed has been carefully balanced for rapid development. Any change in the standard recipe, such as adding significant quantities of sugar,

Table 2—The effects of sorbitol on the induction of w^i mosaics

Treatment	y Qn(1)w^i ec/w^i Flies scored	Mosaics	Mosaics/fly × 100
Control	4872	29	0.60
1% Sorbitol	4629	19	0.41
10% Sorbitol	6664	38	0.57
Mitomycin C	821	478	58.2

Table 3—Effects of sorbitol on the induction of recessive sex-linked lethality

Treatment	Number of chromosomes tested	Number of lethals	Percent lethals
Control	4233	4	0.09
Sorbitol	6097	4	0.07
Ethyl methanesulfonate	581	199	34.3

Table 4—Effects of sorbitol on the induction of chromosome loss

Treatment	Number of chromosomes tested	Number of y males	Percent loss of Y
Control	3956	7	0.18
Sorbitol	5842	9	0.15
X rays	419	23	5.49

would not enhance, but rather retard, development. There was no visible difference in larval activity on the two media. Beaumont et al. (1971) stated that sorbitol is not rapidly metabolized in most tissues, and this could be the cause for the retarded development. There is no evidence to suggest that sorbitol has any genetic effects on *Drosophila melanogaster.*

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Ms received 8/8/75; revised 10/9/75; accepted 10/15/75.

This research was supported in part by PHS Genetics Training Grant 1029.

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COMPARISON OF THE SWEETNESS OF GLUCOSE AND FRUCTOSE WITH THEIR RING-THIO ANALOGS

ABSTRACT

5-Thio-α-D-glucopyranose and 6-thio-β-D-fructopyranose are shown to be significantly sweeter than α-D-glucopyranose and β-D-fructopyranose, respectively, when tasted in crystalline form. In solution, only the thio analog of glucose is significantly sweeter than its parent sugar. The mutarotational formation of a nonsweet tautomer of thio-fructose in solution probably accounts for its fall in sweetness intensity. Reasons for the greater sweetness intensity of these thio analogs are proposed. 5-Thio-β-D-fructofuranose is shown to be essentially tasteless.

INTRODUCTION

QUANTITATIVE sugar sweetness intensity studies (e.g., Schutz and Pilgrim, 1958; Shallenberger, 1963; Moskowitz, 1971) have conclusively shown that β-D-fructopyranose is the sweetest naturally occurring sugar when tasted in crystalline form or as a freshly prepared solution. However, in solution its intensity falls with time to that approximating sucrose due to the mutarotational formation of such nonsweet isomers as β-D-fructofuranose (Shallenberger, 1971). Other sugars, such as D-glucose, may also undergo sweetness intensity shifts in solution. Freshly prepared solutions of α-D-glucopyranose are sweeter than mutarotated solutions, and a mutarotated solution of D-glucose is sweeter than freshly prepared β-D-glucopyranose (Pangborn and Gee, 1961).

Relationships between the molecular structure and intensity of response generated by sweet tasting compounds have also been extensively examined. The degree of intramolecular hydrogen bonding within sugar molecules satisfactorily explains much of their varying sweetness, but one structural feature whose role in sugar sweetness is as yet unclear is that of the ring oxygen atom. It has been suggested (Hodge et al., 1973) that the ring oxygen atom may act as an electronegative centre increasing sweetness and reducing bitterness. In view of this proposed action of the ring oxygen, it is of interest to examine the taste of some sulfur analogs in which a sulfur atom replaces the ring oxygen atom. We report here the detailed sensory evaluation of 5-thio-α-D-glucopyranose and 6-thio-β-D-fructopyranose (Fig. 1). The effect of a ring sulfur atom on sweetness is discussed. In addition, the qualitative evaluation of the taste of 5-thio-β-D-fructofuranose and its significance is reported.

MATERIALS & METHODS

5-THIO-α-D-GLUCOPYRANOSE, 6-thio-β-D-fructopyranose and 5-thio-β-D-fructofuranose were prepared by one of us (R.L.W.) using novel carbohydrate chemistry techniques (Rowell and Whistler, 1966; Chmielewski and Whistler, 1975; Whistler, unpublished data). The samples were chromatographically homogenous (TLC) and their conformations established by nuclear magnetic resonance.

Gas chromatography (GC) was carried out using a Varian Aerograph, Model 204, gas chromatographic unit. The vaporization chamber was maintained at 270°C to ensure complete volatilization. A stainless steel column (4 m × 3 mm o.d.) was used containing 3% (w/w) O.V-225 stationary phase on a 100/120 mesh support of Gas-Chrom Q. The column oven was maintained isothermally at 165°C. Nitrogen was used as the carrier gas, at a flow rate of 60 ml min^{-1}. The detector and collector temperatures were maintained at 270°C and 210°C, respectively. Trimethylsilation of sugars in aqueous solution followed the procedure of Bentley and Botlock (1967). A 10% aqueous solution of the sugar (30 μl) in pyridine (0.5 ml) was frozen in liquid nitrogen. Trimethylchlorosilane (1 ml) and hexamethyldisilazane (1 ml) were added and the mixture shaken gently for 5 min before standing at room temperature until silation was complete.

Eight experienced taste panelists were trained according to the recommendations of Spencer (1971). Relative sweetness intensity measurements were made on both crystalline and solution form. Tasting sessions were conducted in a specially equipped taste panel room with individual booths and rinsing facilities. Samples were presented in randomly coded (2 digit numbers) glass beakers at room temperature. When tasting solid material, panelists were requested to place a few milligrams on the tongue and to rate the intensity of response on an unnumbered ten-point scale. Sucrose was used as an internal standard. Equimolar (0.2M) solutions were evaluated by the same procedure, sample size being limited to 0.5 ml due to scarcity of material. The internal standard, sucrose, was also 0.2M. Difference testing was carried out by the paired comparison technique. Panelists were asked to identify the sweeter sample within a pair of test compounds, e.g., α-D-glucopyranose and 5-thio-α-D-glucopyranose, and to indicate the degree of difference within the pair. The results were subjected to statistical analysis (Byer and Abrams, 1953) and the significance of recorded differences within pairs calculated. All taste tests were carried out in duplicate with two pairs of test materials evaluated at each session.

The taste of 5-thio-β-D-fructofuranose was examined on a qualitative basis, panelists being asked to describe its taste according to one of the following descriptions: tasteless, trace sweet, sweet or intensely sweet.

RESULTS & DISCUSSION

CRYSTALS and solutions of the thio analogs give sweeter sensations than α-D-glycopyranose and β-D-fructopyranose as shown in Table 1. Results of paired comparison testing to assess the significance of differences in intensity between each sugar and its thio analog are given in Table 2. It should be noted that the values reported in Table 2 are not absolute sweetness scores, but merely describe the degree of the difference noted within an analogous pair of sugars. It is also important to be aware of the inherent imprecise nature of conducting taste panels using crystalline sugars. Amounts tasted will vary. Crystal size and rate of solution may cause confusion among panelists between actual sweetness intensity and impact time of the sensation (Birch et al., 1970). However, such tasting procedures were necessary for the complete sensory analysis of the test materials.

That the thio sugars are consistently sweeter than their carbohydrate analogs casts doubt on the suggestion that the ring oxygen atom acts as an electronegative centre to increase sweetness. Sulfur is less electronegative than oxygen, having essentially the same electronegativity as carbon (Pauling,

Table 1—Test sugar sweetness relative to sucrose

Sugar	Relative sweetness intensity	
	Crystalline form	In solution
6-Thio-β-D-fructopyranose	180	112
β-D-Fructopyranose	141	104
Sucrose	100	100
5-Thio-α-D-glucopyranose	77	65
α-D-Glycopyranose	60	51

Table 2—Sweetness scores of the thio analogs relative to their parent sugars

Sugar	Sweetness scores[a]	
	Crystalline form	In solution
6-Thio-β-D-fructopyranose	6.4**	5.1
β-D-Fructopyranose	5.3	4.8
5-Thio-α-D-glycopyranose	5.8*	5.0*
α-D-Glycopyranose	4.8	4.4

[a] Sweetness scores represent the average of the scores given by the panelists to indicate the degree of difference in intensity within a pair of test compounds. The significance of the differences is not based on these scores, but is calculated from the formula:

$$\sigma = (h - Np)/\sqrt{Npq}$$

where N = Total number of judgments; h = number of selections of the thio analog as sweeter; p = probability of selection of the thio analog by chance; q = probability of selection of the other sample within the pair by chance. The number of selections of the sweeter sample within a pair in excess of chance expectation is given in terms of standard deviations, or σ units. For significance at the 5% level, σ must be > 1.65 but < 2.33. For significance at the 1% level, σ must be > 2.33 but < 3.09 (Yule and Kendall, 1950).

* Significantly sweeter than the analog at the 5% level.

** Significantly sweeter than the analog at the 1% level.

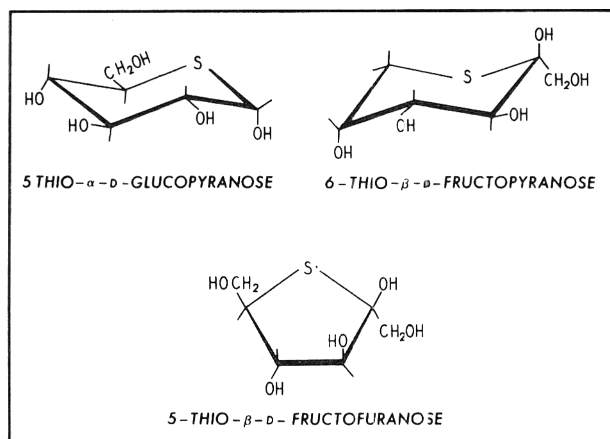

Fig. 1—Thio analogs of D-glucose and D-fructose.

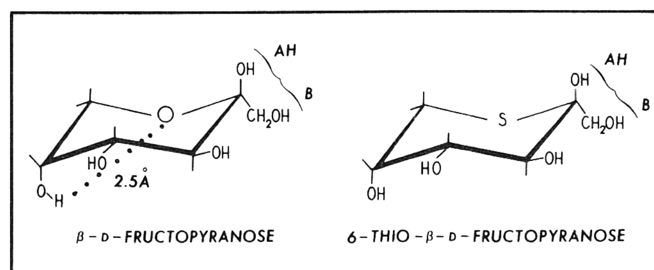

Fig. 2—The AH,B system of β-D-fructopyranose and 6-thio-β-D-fructopyranose.

1960) and cannot thus act as an electronegative center of consequence. Furthermore, if a third electronegative center were essential for enhanced sweetness, these thio sugars would elicit a less sweet response.

The major saporous unit of β-D-fructopyranose was proposed as being the anomeric and hydroxymethylene hydroxyl groups (Shallenberger and Acree, 1967). This assignment was confirmed by sensory and infrared spectral evaluation of structurally analogous compounds (Lindley and Birch, 1975) It was proposed that an intramolecular hydrogen bond between the C-5 hydroxyl group hydrogen atom and the ring oxygen atom prevents bonding between the anomeric hydroxyl hydrogen and ring oxygen atoms (Fig. 2). No such intramolecular hydrogen bonds are possible in 6-thio-β-D-fructopyranose because of the low electronegativity of the ring sulfur atom. Therefore there is an additional α-glycol group (C-4 and C-5 hydroxyl groups) capable of participating in the sensory response which could be responsible for its higher sweetness. However, the same argument cannot be applied to 5-thio-α-D-glucopyranose as the C-4 and C-3 hydroxyl groups have been shown to be of primary importance to the sweetness of D-glucopyranosyl structured (Birch et al., 1970).

There are two explanations which could account for the greater sweetness of both these thio sugars. The C-S-C bond angle is 105° ± 3°C and the C-O-C bond angle is 111° ± 3°C (Pauling, 1960). This change in bond angle causes a corresponding alteration of the interatomic distances within the molecule, possibly causing a better fit of the saporous unit and receptor site which results in higher sweetness intensity. A second possible reason for the enhanced sweetness of these thio sugars is that the sulfur atom increases access or binding of the molecule to the receptor site. It is interesting to note that 5-thio-D-glucopyranose and its 1-phosphate have K_i and K_m values for membrane transport or for enzyme reactions which indicate higher bonding strengths than the natural oxygen substrates (Whistler, unpublished results). If we consider odorous compounds of molecular formula H_2X, water (H_2O) is odorless, H_2S has a strong odor, and H_2Se and H_2Te possess still stronger odors (Moncrieff, 1967). As the atomic weight of the Group VI element (X) increases, so does the odor intensity, presumably due to increased access or binding to the receptor site. Such a phenomenon could also be apparent at the taste receptors. It would therefore be of interest to taste other ring analogs, such as selenium, phosphorous and nitrogen.

The results in Table 2 show that in crystalline form and in solution, 5-thio-α-D-glucopyranose is significantly sweeter than α-D-glucopyranose at the 5% level. On the other hand, 6-thio-β-D-fructopyranose is significantly sweeter than β-D-fructopyranose in crystalline form, but there is no significant differ-

ence in intensity in solution. It is known that the fall in sweetness intensity of β-D-fructopyranose in solution is due to the mutarotational formation of nonsweet β-D-fructofuranose and by analogy the mutarotational formation of a nonsweet tautomer of 6-thio-D-fructopyranose in water also probably accounts for its drop in subjective intensity. Although we were unable to detect mutarotation polarimetrically ($[\alpha]_D^{20}$ = −185.1; c. 4.0, H_2O), gas chromatographic analysis of an equilibrated aqueous solution showed, after prolonged trimethylsilation, the presence of two components, retention times 7.5 and 9.5 min, in the proportion 2:1. Silation of 6-thio-β-D-fructopyranose was very slow, the mixture taking up to 7 days for completion. Evidence for the complete silation of the filtered and evaporated mixture was obtained from its infrared spectrum which showed no sharp absorption band in the wavenumber range 3400–3600 cm^{-1}. Studies with diols have shown that sharp absorption bands in this range indicate free hydroxyl absorption (Brimacombe et al., 1958) Therefore there is strong presumptive evidence that 6-thio-β-D-fructopyranose undergoes mutarotation, although insufficient material was available for preparative GC to identify the tautomer so formed. This tautomer, as stated, accounts for approximately one-third of the mutarotated solution. Its lack of sweetness is proposed because of a corresponding fall in sweetness of 6-thio-β-D-fructopyranose when dissolved in water (Table 1). Interestingly, 5-thio-β-D-glucopyranose gave a single peak on GC after silation in aqueous solution (retention time 10.5 min) indicating that this compound did not mutarotate.

In evaluating the sensory properties of 5-thio-β-D-fructofuranose, all panelists reported a very slight trace of sweetness along with a slight contaminating sulfur taste. Despite the contaminating sulfur taste, this result supports speculation that β-D-fructofuranose should not taste sweet because of the sterically inappropriate disposition of hydroxyl groups (Shallenberger, 1971).

The substitution of a ring sulfur atom for a ring oxygen atom has no qualitative effect on the sensory properties of these compounds. Quantitatively, however, such substitution results in greater sweetness intensity when the test compounds are tested in crystalline form, i.e., the sensation is elicited immediately on dissolution in the saliva. The mutarotational formation of a nonsweet tautomer of 6-thio-β-D-fructopyranose is proposed to explain the nonsignificant difference in intensity between this compound and its parent sugar in equilibrated solution.

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Ms received 7/26/75; revised 10/28/75; accepted 11/5/75.

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ON THE STATISTICAL ANALYSIS OF INOCULATED PACKS

ABSTRACT

This presents new theoretical and practical results derived from the author's (1974) paper on calculating 12D from inoculated packs. The theoretical portion compares methods for estimating the number of surviving organisms and shows that the Halvorson-Ziegler formula is approximately derivable from the theory given previously. The more practical part shows several examples of what may hapen if the organism death kinetics are shifted—rather than simple exponential. A new test procedure is outlined that should lead to more reliable estimates of 12D than are now obtained.

INTRODUCTION

IN A RECENT publication (1974) the author described a new theory for analyzing data from inoculated pack experiments designed to determine the effectiveness of radiation as a means of food preservation. The purpose of the present paper is to describe several consequences, both theoretical and practical, of this new theory.

The main difficulty in analyzing the results of an inoculated pack is that the data do not provide direct estimates of the number of surviving organisms. One of the purposes of the present work is to make a systematic study of methods (both traditional and new) for estimating the number of surviving organisms in an inoculated pack. It is shown that the new theory gives nearly the same estimate as the Halvorson-Ziegler (1933) formula, previously used by Stumbo et al. (1950) and Anellis and Werkowski (1968) in thermal and radiation sterilization.

One of the themes of the preceding paper was that the simple exponential distribution may not always be the correct form of death kinetics for radiation sterilization. If the assumption of a simple exponential distribution is not tenable, we must ask what to use in its place. In the previous paper the Weibull and lognormal distributions were studied for this purpose. Here we shall show how to analyze the normal (Gaussian) and shifted-exponential distributions as well. Of these four, the shifted exponential is the simplest and the natural candidate to replace the simple exponential. Accordingly we give several examples showing what may be expected to happen with a shifted exponential, or, more generally, with any two-parameter distribution.

Summary of the method

In an inoculated pack experiment a number of replicate samples (cans) of food are inoculated with microorganisms, sealed and irradiated. After irradiation and suitable incubation the cans are examined for sterility according to some criterion, e.g., swelling, presence of toxin or viable botulinum.

We assume that under the conditions within the cans each individual microorganism possesses a minimum lethal dose, X. This lethal dose will be different for different individual microorganisms, and so we regard X as a random variable obeying the distribution function G(x), where x is the dose of the experiment. This means that

$G(x)$ = Probability that $X \leqslant x$
= Probability that minimum lethal dose $\leqslant x$
= Probability that organism is killed at dose x

and

$1 - G(x)$ = Probability that the organism survives at dose x

When n organisms are put in a can and irradiated, we define

$\phi(x)$ = Probability that the can is sterilized at dose x

If the death of each organism is unaffected by what happens to all the other organisms in the can (i.e., organisms die independently), it is known that

$\phi(x)$ = Probability that all organisms are killed at dose x.

$$\phi(x) = [G(x)]^n \tag{1}$$

or

$$G(x) = [\phi(x)]^{1/n} \tag{2}$$

If $1 - G(x) \ll 1$, then we obtain approximately from Eq (2)

$$(1/n)\,\ell n\,\phi = \ell n\,G = \ell n\,[1-(1-G)] \approx -(1-G) \tag{3}$$

$$G \approx 1 + n^{-1}\,\ell n\,\phi \tag{4}$$

$$n(1-G) \approx -\ell n\,\phi \tag{5}$$

$$\phi \approx e^{-n(1-G)} \tag{5a}$$

$$\ell n\,(1-G) \approx -\ell n\,n + \ell n\,(-\ell n\,\phi), \tag{6}$$

and the approximation is accurate when $n \gg 1$ and ϕ is not too near zero.

An inoculated pack experiment gives, at each partial spoilage dose, x, an estimated probability of can sterilization,

$$\hat{\phi}(x) = C(x)/N = C/N \tag{7}$$

where N is the number of cans tested at dose x and C(x) is the number of sterile cans at dose x. From this information we want to estimate the parameters of various possible forms of the distribution G(x) and also decide on the form, if possible. In this paper we consider five distribution forms, listed below:

Simple exponential

$$G(x) = 1 - e^{-x/\eta_e} \tag{8}$$

Shifted exponential

$$G(x) = 1 - e^{-(x-a)/\eta_s} \tag{9}$$

Normal

$$G(x) = F_g\left(\frac{x-b}{\sigma}\right) \tag{10}$$

where

$$F_g(z) = \frac{1}{\sqrt{2\pi}} \int_{-\infty}^{z} e^{-t^2/2}\, dt \qquad (11)$$

Lognormal

$$G(x) = F_g\left[\beta_L \ln (x/\eta_L)\right] \qquad (12)$$

Weibull

$$G(x) = 1 - e^{-(x/\eta_W)^{\beta_W}} \qquad (13)$$

The general method used in the analysis is that described by Ross (1974) for the lognormal and Weibull distributions. In essence it consists of testing the hypothesis that the distribution G(x) has a certain form (i.e., Weibull or lognormal, or shifted exponential, etc.). There are several ways in which this could be done, e.g., by using the Chi-Square test or Kolmogorov-Smirnov (K-S) test. Both these tests require more data than are likely to be available in inoculated packs, so we have chosen a different though still familiar way. First, the data are transformed so that its graph will be a straight line if it actually has a distribution of the assumed form. The transformed data are then fitted by a second degree polynomial using the least-squares criterion. The two parameters in the distributions are determined from the zero-th and first degree coefficients of the fitted polynomials, and the overall curvature is estimated from the second degree coefficient. The F-test is then used to decide whether the second degree coefficient is zero or not.

The transformations which linearize the data for Weibull and lognormal distributions were given by Ross (1974). The transformations applicable to the normal and shifted-exponential distributions are stated under formulae for various distributions. We do not use the general method described above in the case of the simple exponential, because only one parameter, η_e, needs to be found. Instead we simply estimate the value of η_e from the data at each dose, average the values to get an overall best estimate of η_e and use the variance, s_e^2 of this estimate as the measure of inaccuracy.

EXPERIMENTAL

Theoretical considerations

In this section we shall examine several different formulae used for estimating the number of surviving organisms in an inoculated pack. We imagine that we are analyzing an inoculated pack at dose x. It is assumed that each sample (i.e., can) is inoculated with n organisms prior to exposure and that N samples are exposed. After irradiation and incubation, it is found that C(x) cans are sterile, out of the N cans exposed.

In analyzing this data, a natural way of proceeding is to obtain an estimate of the total number of surviving organisms in all the cans, R(x). Ordinarily this cannot be measured directly but must be inferred from other data. We shall, therefore, see what Eq (1) through (7) imply about the total number of surviving organisms. To begin with we notice that

R(x)/N = (total number of survivors at dose x)/(total number of samples exposed)

n[1 – G(x)] = (initial number of organisms per sample) × (probability that an organism survives)

These two quantities are equal since they are merely different ways of expressing the average number of surviving organisms per sample at dose x. Hence

$$R(x)/N = n\left[1 - G(x)\right]. \qquad (14)$$

This simple and basic formula relates the number of survivors to the distribution function, G(x). It is familiar in the form which it takes for the exponential distribution,

$$R(x) = Nne^{-(x/\eta_e)}$$

but it is helpful to have its general form too.

We now combine formula (14) with Eq (2) and obtain

$$R(x) = Nn\left\{1 - \left[\phi(x)\right]^{1/n}\right\} \qquad (15)$$

This formula gives us a way of estimating R(x) if we use Eq (7) to calculate $\phi(x)$, i.e.,

$$R(x) = Nn\left\{1 - \left[C(x)/N\right]^{1/n}\right\}. \qquad (16)$$

We get a slightly different way of calculating R(x) if Eq (5), instead of Eq (2), is used with Eq (14). This leads to

$$R(x) = -N \ln \phi(x), \qquad (17)$$

and, using Eq (7) again, we find

$$R(x) = -N \ln\left[\frac{C(x)}{N}\right] \qquad (18)$$

or

$$R(x)/N = 2.303 \log_{10}\left[\frac{N}{C(x)}\right] \qquad (19)$$

This formula is the familiar Halvorson-Ziegler (1933) estimate of the most probable number of survivors, used by Stumbo et al. (1950) in calculating the number of survivors in thermal processing and by Anellis and Werkowski (1968) in radiation sterilization.

Finally we mention still a third way of estimating R(x), used by Schmidt and Nank (1960), namely

$$R(x) = N\left[1 - \phi(x)\right] = N - C(x) \qquad (20)$$

This amounts to assuming that exactly one organism survives in each can that is not spoiled. This very familiar formula has usually been used in conjunction with the assumption that G(x) is of simple exponential form, but it can be used without that assumption.

We have available now three different estimates of R(x), which we denote as

exact: $$R(x) = R_e(x) = Nn\left\{1 - \left[\phi(x)\right]^{1/n}\right\} \qquad (21)$$

approximate: $$R(x) = R_a(x) = -N \ln \phi(x) \qquad (22)$$

Schmidt-Nank: $$R(x) = R_s(x) = N\left[1 - \phi(x)\right] \qquad (23)$$

In comparing these, the dominant fact is that the correct formula is Eq (21), $R = R_e$. A well-known inequality about logarithms assures us that $R_a > R_s$, but both these are approximations which may or may not be accurate, depending on the circumstances.

In practice $R_a(x)$ usually provides a very good estimate of $R_e(x)$ because most of the time we are interested in the situation where $n \gg 1$ and $\phi(x)$ is not near zero. If we examine what happens to the three formulae as the dose, x, becomes large, and therefore $\phi = \phi(x) \rightarrow 1$, we

see that all three predict $R(x) \to 0$ as they should. Moreover we have from the binomial expansion

$$\phi^{1/n} = \left[1 - (1-\phi)\right]^{1/n} \approx 1 - (1-\phi)/n$$

$$R_e(x) = Nn\left[1 - \phi^{1/n}\right] \approx Nn\left\{1 - \left[1 - (1-\phi)/n\right]\right\}$$

$$R_e(x) \approx Nn(1-\phi)/n = N(1-\phi) = R_s(x) \quad (24)$$

Similarly, from the power series for the natural logarithm, we have

$$R_a(x) = -N\,\ell n\phi = -N\,\ell n\left[1-(1-\phi)\right] \approx -N\left[-(1-\phi)\right]$$

$$\approx N(1-\phi) = R_s$$

That is, all three estimates $R_e(x)$, $R_a(x)$ and $R_s(x)$ are nearly equal when $\phi(x)$ is close to 1. This agrees with the intuitive observation, made by Anellis and Werkowski (1968) and others, that, when almost all the cans in a pack are sterilized, the likeliest number of survivors in the cans not sterilized is 1.

The behavior of Eq (21), (22) and (23) as $x \to 0$, i.e., at very low doses, where $\phi(x) \to 0$, is also instructive. Eq (21) implies that

$$R_e(x) \to Nn, \quad (25)$$

i.e., the total number of survivors is the same as the initial number of organisms. This is clearly the correct prediction if radiation is the only cause of death. Eq (22) and (23) lead to erroneous results,

$$R_a(x)/N \to \infty \quad (26)$$

$$R_s(x) \to N \quad (27)$$

The reason why $R_a(x)$ gives a wrong prediction is that it is based on the approximate formula (5), which is inaccurate when $G(x) \to 0$ [and $\phi(x) \to 0$] as we see from the derivation, Eq (3). $R_s(x)$ is wrong because it is based on the assumption that exactly one organism survives in each unsterilized can, which is clearly dubious at very low doses.

Probably the most striking of these results is the observation that the Halvorson-Ziegler formula (19), is obtained as a consequence of formulas (14), (4) and (7). The Halvorson-Ziegler formula is not usually derived in that way. The fact that Eq (22), which is equivalent in practical terms to Eq (19), leads to the erroneous result Eq (26) should not be taken too seriously. Although it is a defect of the approximation leading to Eq (22), it is not very important in practice because we almost always use Eq (19) or Eq (22) when $\phi(x)$ is not near zero.

We should observe that in the foregoing derivations we have glossed over the distinction between random and deterministic quantities. A more careful treatment would show that R(x) and C(x) are random variables even if we surmise that n and x are deterministic (i.e., not random). Then Eq (16), (18) and (20) become relations between random variables. It is reasonable to assume that C(x) has a binomial distribution with

$$\text{Mean}\left[C(x)\right] = N\phi(x) \quad (28)$$

$$\text{Var}\left[C(x)\right] = N\phi(x)\left[1-\phi(x)\right] \quad (29)$$

The distributions of R(x) implied by Eq (16) and (18) are not easy to calculate in general, even with the help of the normal approximation to the binomial distribution.

Finally, we point out that nowhere in the derivations of this Section have we assumed the form of G(x). The conclusions therefore are not limited to one form of distribution (e.g., exponential). In particular the Halvorson-Ziegler estimate Eq (19) of the number of surviving organisms is applicable for any distributions and provides good though not perfect accuracy in most cases.

RESULTS

Formulae for various distributions

In applying the least-squares fitting procedure described earlier, we must have the formulae for transforming the data into a straight-line graph. Ross (1974) gave the appropriate formulae for the Weibull and lognormal distributions, and in this section we give the formulae for the normal, shifted-exponential and simple-exponential distributions.

First, for the normal distribution we obtain from Eq (4) and (10)

$$\frac{x-b}{\sigma} = F_g^{-1}\left(1 + \frac{1}{n}\,\ell n\,\phi\right).$$

Hence, if a linear least-squares fit is made of

$$y_n = F_g^{-1}\left(1 + \frac{1}{n}\ell n\,\hat{\phi}\right) = F_g^{-1}\left\{1 + \frac{1}{n}\,\ell n\left(\frac{C}{N}\right)\right\}$$

as a function of x, then σ = 1/slope; and b = –(y intercept)/slope.

The 12D dose for a normal spore-death distribution is given by

$$x_{cn} = b + 7.0345\,\sigma$$

Second, for the shifted-exponential distribution, Eq (6) and (9) lead to

$$\frac{x-a}{\eta_s} = \ell n\,n - \ell n\left(-\ell n\,\phi\right).$$

Hence, if a linear least-squares fit is made of

$$y_s \equiv \ell n\,n - \ell n\left(-\ell n\,\hat{\phi}\right) = \ell n\,n - \ell n\left\{-\ell n\left(\frac{C}{N}\right)\right\}$$

as a function of x, then η_s = 1/slope; a = –(y intercept)/slope and the 12D dose, x_{cs}, is $x_{cs} = a + 27.63\,\eta_s$.

Finally, for the simple-exponential distribution the estimate of η_e (or, equivalently, D, since $D = 2.303\,\eta_e$) is obtained by averaging the values (x/y_s) across all the data points. The 12D dose, x_{ce}, is then

$$x_{ce} = 12D = 27.63\,\eta_e,$$

and s_e is the estimated standard deviation in x/y_s.

The computer program described earlier, Ross (1974), was extended to include the above calculations. For each distribution (except the simple exponential) the F-value arising in the likelihood-ratio test of the hypothesis of zero curvature is calculated. For the simple exponential, s_e, which plays an analogous role to F, is computed. The program also provides the value of the logarithm of spore survival probability, i.e., $\ell n(1 - G)$, for each dose as obtained experimentally from Eq (6) and (7) as computed from the fitted parameters by the various distribution functions.

Examples

This section contains several examples where the preceding theory, Ross (1974), as extended in formulae for various distributions, is illustrated. The examples depict situations that are either typical in practice or are likely to appear bizarre when first encountered. Throughout this section we assume that the spore load (i.e., the number of spores in a can), n, satisfies $n \geq 10^6$ and all calculations are performed at doses, x, for which

$$\text{organism survival probability} = 1 - G(x) \geq 10^{-3}$$

Under these conditions the approximate formulae (5), (5a) and (6) are very accurate, and we shall use them instead of the exact formulae, (1) and (2).

We also assume throughout this section that G(x) has the shifted-exponential form, Eq (9). This choice was made for two reasons. First it is natural and traditional to plot graphs of $\log_{10}(1 - G)$ versus dose, x, and the shifted exponential is the most general distribution that gives straight lines on such graphs. On these graphs

$$\begin{aligned} \text{slope} &= -1/(2.303\,\eta_s) \\ \text{x intercept} &= a. \end{aligned}$$

Second, and more important, it has been customary to assume that G(x) is the simple exponential, Eq (8). There is some evidence (see references quoted by Ross, 1974), that in cans of food the distribution is not always of this form. If it is necessary to abandon the simple-exponential hypothesis, the next simplest distribution is the shifted exponential, and it is natural, therefore, to focus our attention upon that form. The examples will illustrate several consequences of changing our basic assumption from the simple to the shifted exponential.

The first example, distribution (I), has $a = 0.5$, $\eta_s = 0.1267$ and therefore $x_c = 12D = 4.0$. We may expect this to be typical of the results for a number of different foods. The survival-probability, or death-kinetic curve has a sharp shoulder at a dose of $a = 0.5$ megarads, and 12-D value about 4.0 megarads is commonly obtained. In contrast the second example, II, has $a = -2.5$, $\eta_s = 0.3076$ and $12D = 6.0$. This curve is in several ways not typical of what we expect in death kinetics. First, it has a negative shift (i.e., $a < 0$), which means that there is considerable death at zero dose. Second the 12D value is higher than usual for most foods. Nevertheless it is instructive to consider this distribution.

Information about these two distributions, labelled I and II, respectively, is graphed in two different ways in Figures 1a and 1b. The death-kinetic curves, i.e., graphs of $\log_{10}(1 - G)$ = logarithm of survival probability versus dose, x, in megarads, are shown in Figure 1a. These two curves are straight lines because of the assumed exponential form. Figure 1b shows for each distribution the theoretical can-sterilization probability, $\phi(x)$, based on the assumption that each can contains exactly 1×10^7 organisms. These curves are derived from the corresponding death-kinetic curves by using Formula (5a).

The theoretical partial spoilage ranges for the two distributions are also shown in Figures 1a and 1b. There is no unique way of defining what is meant by the theoretical partial-spoilage range, but in deducing these ranges, we have assumed that the theoretical partial-spoilage range consists of all doses x for which

$$0.05 \leq \phi(x) \leq 0.95.$$

The end points of the partial spoilage range can be found numerically by using Eq (6) with $n = 1 \times 10^7$ and taking ϕ = 0.05 and 0.95. For $\phi = 0.05$ we obtain from Eq (6)

$$\begin{aligned} \ln(1 - G) &= -\ln(1 \times 10^7) + \ln\{-\ln(0.05)\} \\ &= -15.02 \\ \log_{10}(1 - G) &= -15.02/2.303 = -6.52 \end{aligned}$$

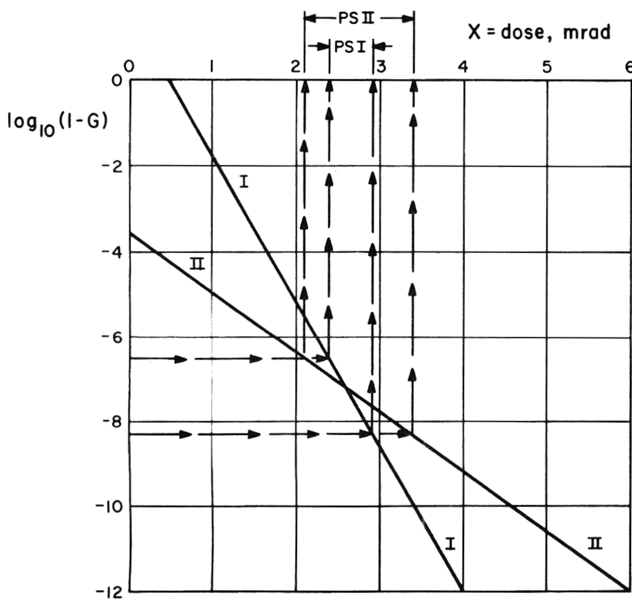

Fig. 1a—Death-kinetic graphs for distributions I and II. Arrows show construction lines for graphical determination of partial-spoilage ranges.

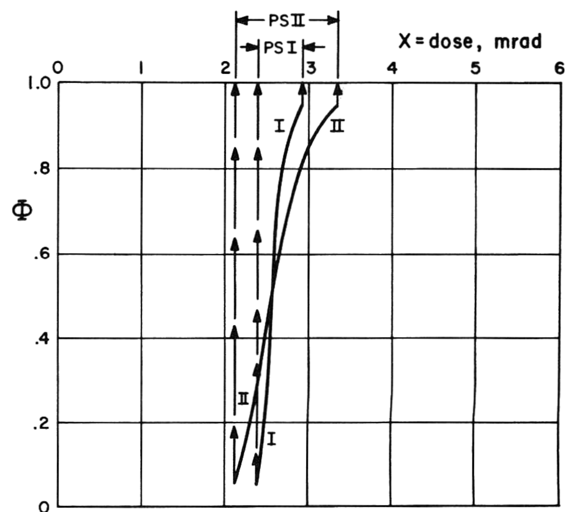

Fig. 1b—Theoretical can-sterilization probability, ϕ, as a function of dose, x, for distributions I and II.

and for $\phi = 0.95$ we get similarly

$$\ell n\ (1 - G) = -19.09$$
$$\log_{10}\ (1 - G) = -8.29$$

The end points of the partial spoilage range for each distribution are then found by solving Eq (9) for x,

$$x = a - \eta_s\ \ell n\ (1 - G)$$

For example, for distribution (I), the lower end point is at

$$x = 0.5 - \left[0.1267 \times (-15.02)\right] = 2.40$$

and the upper end point is

$$x = 0.5 - \left[0.1267 \times (-19.09)\right] = 2.92$$

Similarly, for distribution (II) the partial spoilage range is from $x = 2.12$ to $x = 3.37$. These end points can also be obtained graphically as in Figure 1a, drawing horizontal lines at the end-point values of $\log_{10}\ (1 - G)$, i.e., −6.52 and −8.29, finding the intersections with the straight lines I and II and drawing vertical lines from these intersections. The partial-spoilage end points are the x-values of these vertical lines. The partial-spoilage ranges are labelled PS I and PS II in Figures 1a and 1b.

Comparing these two partial-spoilage ranges, we see that, although they have roughly the same mid-point or LD 50, namely $x \approx 2.7$ megarads, distribution II has a much wider range than I. Indeed the range of I is so narrow, 2.4–2.9 megarads, that, if tests were conducted at dose increments of 0.4 megarads or greater, it is likely that only one dose would produce partial spoilage. This happened in inoculated packs for beef and ham, Anellis et al. (unpublished data), and the example of distribution I show that it is not an isolated occurrence. That is, if the distribution is anything like a shifted-exponential with moderate 12D value (say 4 megarads or less), the partial-spoilage range will always be so narrow that dose increments of 0.1 or 0.2 megarads are needed if we are to get two or three partial spoilage doses.

The practical implications of this observation are important. For, if we study the five distributions which are probably the most important, Eq (8) to (12), we see that all depend on two parameters (e.g., a and η_s for the shifted exponential, b and σ for the normal etc.), except the simple exponential, which depends only on η_e. No matter which distribution is assumed, if we are to find the 12D dose, we must estimate these parameters from the partial spoilage data. In order to estimate them we must have at least as many partial spoilage data points as the number of parameters, and ideally should have more.

This means that we must either choose very small dose increments (0.1 or 0.2 megarads), and hence a very large number of doses, or resort to some other test design. Two possibilities arise. First, we can carry out a two-stage test, in which the first stage is a coarse test designed only to locate the partial-spoilage range, and the second stage is the main pack, with very small dose increments covering a range slightly wider than the coarsely-estimated partial-spoilage range. The second alternative is to carry out tests with several different values of n, the spore density per can. This method was suggested by the author (1974) as a possible way of finding the general form of the distribution G(x), but it also improves the accuracy in estimating the parameters of G(x).

Returning now to some theoretical calculations, Figures 2 and 3 show the results of fitting all the five distributions, Eq (8) to (12), to partial-spoilage data derived from the distributions (I) and (II), respectively. The data were obtained by assuming $n = 1 \times 10^7$, using the G-functions of distributions (I) and (II) and then calculating ϕ by means of Eq (5a) for several doses in the respective partial-spoilage ranges. The doses were 2.4–3.0 megarads in increments of 0.1 for distribution (I) and 2.0–3.5 in increments of 0.3 for distribution (II). These data points were then fitted by all five distributions, using the method and computer program outlined in the previous section.

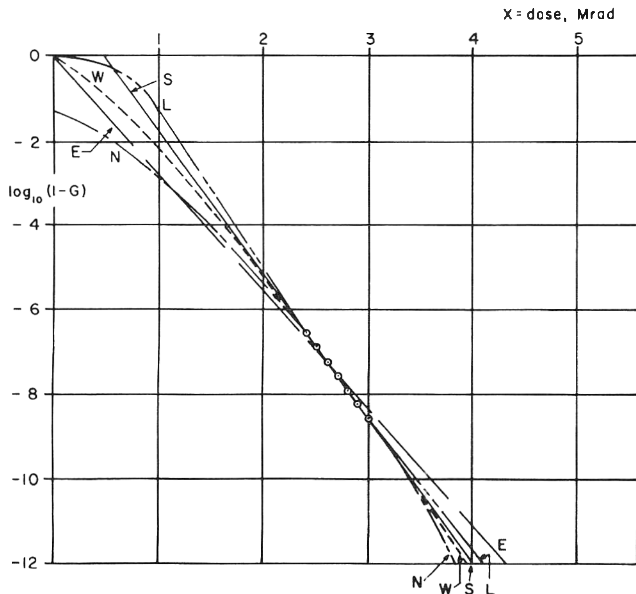

Fig. 2–The fitting of five distributions to data derived from distribution I.

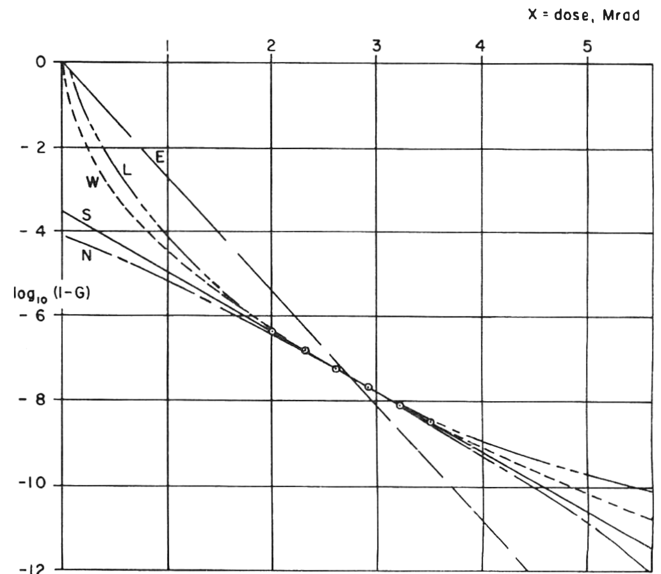

Fig. 3–The fitting of five distributions to data derived from distribution II.

The main conclusion that emerges from studying Figures 2 and 3 is that all the distributions fit the data very well except the simple exponential. This is very easy to see in Figure 3 and is still true, though less obvious, in Figure 2. Since all the distributions except the simple exponential have two parameters, we expect that they would provide a better fit than the simple exponential, and they do. However, none of the four two-parameter distributions fits appreciably better than the others, so we have no grounds for choosing, say, the shifted exponential (which fits the data exactly) over the others, which are only approximations. We should expect this also, because the writer (1974) has shown that tests at only one spore density are unlikely to distinguish clearly between two-parameter distributions. The present examples suggest that such tests can sometimes distinguish between one-parameter and two-parameter distributions but not usually between two-parameter distributions.

The estimated 12D doses in Figure 2 range from 3.84 Mrad for a normal fit to 4.11 for a lognormal fit and 4.31 for the simple exponential. None of these is far from the true value, 4.00. The scatter is much greater in Figure 3, where the true value is 6.00 Mrad and

estimated 12D =	4.41	for simple exponential
	5.59	for normal
	6.00	for shifted exponential
	6.96	for Weibull
	7.80	for lognormal.

These examples suggest that the normal gives the lowest estimate of 12D and the lognormal gives the highest among the two-parameter distributions.

In both Figure 2 and Figure 3 the true distribution is a shifted exponential. In Figure 2 all the distributions fit the data fairly well, though the Weibull and lognormal are probably the best. The normal is unacceptable, however, because it predicts more than 90% kill at zero dose, when in fact no kill occurs below a dose of 0.5 Mrad. In Figure 3, on the other hand, the normal is clearly the best-fitting distribution. This suggests that it may be difficult to single out any one of these four two-parameter distributions as universally the best.

Finally we consider two shifted-exponential distributions, labelled III and IV, with the following properties:

$$\text{III: } a = 2, \eta_{III} = 0.0724, x_{cIII} = 12D_{III} = 4$$
$$\text{IV: } a = -2, \eta_{IV} = 0.2534, x_{cIV} = 12D_{IV} = 5.$$

We assume that each can contains 1×10^6 organisms initially, and we examine the partial spoilage ranges for the two distributions, defined (as before) to be all doses, x, satisfying $0.05 \leqslant \phi(x) < 0.95$. Using Eq (6) and $n = 1 \times 10^6$, we find

$$\log_{10} (1 - G) = -5.52 \text{ and } -7.29$$

for $\phi = 0.05$ and 0.95, respectively. Figure 4 then shows that the partial spoilage ranges are 2.92–3.22 Mrad for III and 1.22–2.25 for IV. Thus the partial spoilage range of III, and hence its LD50 is entirely above that of IV. Ordinarily we might think, therefore, that the organism III is more resistant than IV. However, the 12D dose of III is lower than that of IV!

This example, in which the organism with the higher partial-spoilage range and LD50 has the lower 12D dose, illustrates a phenomenon that can occur for the shifted exponential (or, more generally, for most two-parameter distributions) that cannot happen with a simple exponential. Although this looks suspicious, it is a situation that could occasionally arise in a milder form than here. In this example we have deliberately chosen two rather "far out" distributions in

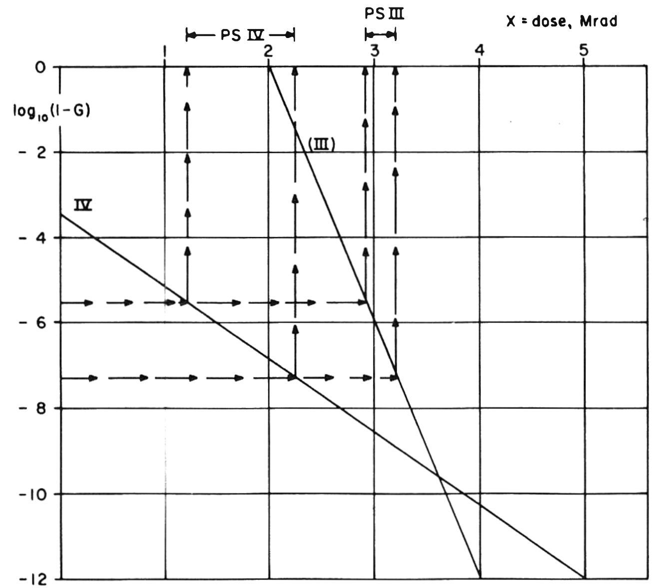

Fig. 4—Death-kinetic graphs for distributions III and IV, showing anomalous-appearing relation between 12D values and partial-spoilage ranges.

order to highlight the strangeness of the situation. Both distributions III and IV are peculiar, III because it has a wide shoulder and very steep death rate while IV has kill at zero dose and a much less steep death rate. It is unlikely that two such "extreme" distributions would arise in practice, but it is possible to have two organisms where one is adjudged the more resistant based on their LD50's and the other based on their 12D's. The final judgment should be based on the 12D-values in such a case. However, the main conclusion to be drawn from this example is that two-parameter distributions are much more flexible than one-parameter ones. In admitting two-parameter distributions, we are accepting behavior that may (infrequently) affront our intuition.

DISCUSSION & CONCLUSIONS

WE FIRST PRESENT a brief discussion of the results of theoretical considerations and propose an experiment that gives a test of certain aspects of the theory. This is followed by a discussion about estimating 12D from inoculated pack data and concludes with a description of a test procedure that should improve the reliability of this estimate.

The theory described in theoretical considerations leads to several formulae for estimating R(x), the total number of surviving organisms at dose x. Usually we are not as concerned about the values of R(x) in themselves as we are with the predictions of Eq (2), which can be viewed as the result of eliminating R(x) between Eq (14) and (15). However, R(x) as estimated by Eq (16) is somewhat interesting because it gives us a way to test the theory that does not depend on knowing G(x).

This experiment consists of irradiating test tubes containing the organism in a transparent medium. Survival counts would be made on some of the test tubes, and the rest would be tested merely for presence or absence of survivors by turbidity observation or some similar procedure. The survival counts give a direct estimate of R(x), and Formula (16) enables us to obtain an estimate of R(x) from the counts of test tubes having survivors. These two estimates should agree. For greatest sensitivity the spore density per test tube, n, should be

quite small, perhaps n = 20, the number of test tubes, N, should be fairly large, say N = 100, and the dose should be such that about 4–10 test tubes are sterilized. If these conditions can be attained, there will be a perceptible difference between $R_a(x)$ and $R_e(x)$ in Eq (16) and (18), and the survival counts ought to verify (16) and contradict (18).

Turning now to the general problem of estimating 12D from inoculated pack data, we perceive that there are two conceptually distinct steps in the process. First, the form of the death-kinetic distribution must be decided, and, second, the parameters of that distribution must be estimated. Both steps have to be taken in order to get an estimate of 12D. In the conventional treatment, which leads to the Schmidt-Nank formula, the form of the death-kinetic distribution is decided to be simple exponential regardless of the experimental data. This assumption simplifies the subsequent parameter estimation and 12D calculation so that they can all be accomplished by the familiar Schmidt-Nank formula (provided the additional assumption of one surviving organism per spoiled can is accurate).

There is evidence, cited earlier (Ross, 1974), that the death-kinetic distribution is not always a simple exponential. It makes sense, therefore, to admit other distribution forms as well. Many forms are possible, but the following, two-parameter distributions are commonly used: Weibull, shifted exponential (i.e., exponential with a shoulder), normal and lognormal. Each of these forms can be fitted to the data and the parameters estimated by the computer program described under formulae for various distributions. A 12-D estimate is obtained in this way for each of the four distribution forms. If there is sufficient data, the program can also provide information about the goodness-of-fit of each distribution form and confidence limits on the estimates of the parameters and of 12D.

In particular, since the simple exponential is a special case of both the shifted-exponential and the Weibull distributions, the graphs of the fitted distributions (e.g., Fig. 2 and 3) will tell us whether the distribution is a simple exponential or not. If the true distribution is a simple exponential, the Weibull and shifted-exponential lines will coincide with the simple-exponential line. In this case the data will have told us that the form is a simple exponential, and we shall be on much firmer ground in making a 12D estimate than if we had merely assumed that form, as is now done.

The use that we make of these graphs (e.g., Fig. 2 and 3) depends on several circumstances. In the case of Figure 2 we would probably conclude that any distribution except the normal was acceptable and gives a 12D of about 4.0. In a case like Figure 3 we would have to discard the normal and the shifted exponential (both give kill at zero dose) as well as the simple exponential which fits the data badly. There is little to choose between the Weibull and lognormal, so we might choose the lognormal with a 12D of 7.8 if we wanted to be ultra-safe.

Often there is a great deal of scatter in the empirical data of the graph. This will make the conclusions much less certain than in the noise-free cases of Figures 2 and 3. The best hope of overcoming this difficulty is to design the inoculated pack carefully. Two main statistical facts affect the design:

(a) The accuracy improves as the number of partial-spoilage data points increases.
(b) The accuracy improves as the dose-range of the data points becomes wider, even if the number of data points remains the same.

Taken together these imply that we want to get as many data points as possible and have them spread over as great a dose range as possible. The following test procedure, a translation into practical terms of these ideas, is an explicit and slightly refined version of the method sketched in the preceding paper (Ross, 1974) and combines the suggestions made under examples in this paper.

(1) Run a preliminary pack for the purpose of locating roughly the partial-spoilage ranges for spore loads of roughly 10^3, 10^5 and 10^7 organisms per can. This should involve fairly coarse dose increments, perhaps 0.5 megarad, and a small number of cans per dose, say 10. The quantal response of the cans would be based entirely on visual inspection for swelling after a few weeks of incubation.

(2) Having located the approximate partial spoilage ranges for spore loads of roughly 10^3, 10^5 and 10^7 organisms per can, now carry out the main pack at the same three spore loads, with doses at small enough increments so that 3–5 partial spoilage data points are obtained for each spore load. Typically 40–50 cans per dose could be exposed. Incubate these and assay them for toxin and viable cells in the usual fashion. Subject the viable and toxin data to the computations exemplified under examples.

The above two-stage procedure should provide enough data, spread over a wide enough dose range, so that the estimate of 12D will be fairly reliable. The total number of cans exposed in this procedure (about 1000–1300) is about the same as in conventional tests now being run. Of course, if there is prior knowledge about the partial spoilage ranges, it may be possible to curtail or dispense with the preliminary pack.

Many further refinements of the procedure are possible. For example, in the main pack smaller numbers of cans could be exposed at doses near the middle of the partial spoilage ranges and larger numbers near the ends, so as to get roughly uniform variance. In the computations a weighed least-squares analysis could be used, or we could take the least-squares results as a first approximation in an iterative scheme based on a maximum-likelihood criterion.

The main conclusion of this paper is that the above procedure should be tried. The dose increments and numbers of cans per dose can be varied if more or less accuracy is desired. A second conclusion is that, even if the conventional test design is retained, the computational scheme exemplified under examples (or some refinement of it) provides more reliable estimates of 12D than the usual method.

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Ms received 7/16/75; revised 10/31/75; accepted 11/5/75.

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THE FATE OF NITRITE: REACTION WITH PROTEIN

ABSTRACT

This paper presents results showing that one of the major pathways for loss of nitrite in cured meats may be through reaction with nonheme protein. Incorporation of the stable isotope ^{15}N from nitrite is shown to occur in both bovine serum albumin and the muscle protein myosin, at pH's below and at those found in cured meat. In bovine serum albumin solution at pH 5.5 and at 20°C, 60% loss of added nitrite (200 ppm) occurred within 1 wk and almost half of the lost nitrite nitrogen was recovered as ^{15}N chemically bound to the protein. Analysis of the reaction products of nitrite and myosin showed that, under the conditions used, 10–20% of the incorporated nitrogen was present as 3-nitrotyrosine. Several other products were found in acid hydrolysates of protein containing bound nitrite, but these did not appear to quantitatively account for the remainder of the incorporated nitrite

INTRODUCTION

THE FATE of added nitrite in cured foods is of great importance, especially in the context of inhibition of *Clostridium botulinum* outgrowth, cured pigment formation, flavor characteristics, and the production of nitrosamines. Studies of nitrite depletion have been carried out by a number of investigators including Greenwood (1940), Pivnick et al. (1967), Nordin (1969) and Herring (1973), who have looked at residual nitrite levels in either model systems or commercially processed products. In two aspects, their studies have had common factors—all have found nitrite levels fall with time, often rapidly, and all have failed to account for the majority of the nitrite lost.

Various end products have been proposed, apart from reaction with the muscle pigment, to describe the fate of the missing nitrite. Mirna and Hofmann (1969) found that nitrite and thiol groups disappeared equimolecularly from a minced meat system, and suggested that nitrosothiol formation was responsible. Olsman and Krol (1972) found that addition of a thiol alkylating agent to a chopped beef mix prior to nitrite addition did not greatly alter the extent of nitrite disappearance, showing that thiol compounds were not primarily responsible for nitrite loss. Kubberød et al. (1974) showed, by reaction of nitrite with the muscle protein myosin, that formation of nitrosothiols did not play an important part in nitrite loss, when using conditions simulating those in a cured product. Ando et al. (1971) have stated that most depletion occurs in a water soluble sarcoplasm extract. Fox and Nicholas (1974) have reported, in their meat systems, that of the compounds present in or added to meat, ascorbate was responsible for the greatest lowering of nitrite levels, followed by cysteine, histidine and to a smaller extent, reduced nicotinamide adenine dinucleotide ($NADH_a$). Many other natural constituents of meat did not cause any loss of nitrite from the meat systems. Tinbergen (1974) concluded that either an amino acid or low molecular weight peptide was responsible for an appreciable extent (in one case up to 70%) of the nitrite depletion in the model systems studied. None of these investigations has been able to identify the compounds causing the major loss of nitrite in cured meats.

Sebranek et al. (1973) analyzed samples of meat processed with ^{15}N-labelled sodium nitrite and found that in samples processed at 71°C, 8% of the added nitrite nitrogen (added sodium nitrite = 156 ppm) was bound to a hot water insoluble meat residue after 3 days incubation, increasing to 23% of the added nitrite after 20 days. The residue must be composed predominantly of protein.

That nitrite reacts with protein is substantiated by the number of uses to which nitrite has been put, modifying various amino acids in proteins (Philpot and Small, 1938; Means and Feeney, 1971). Recently, the possibility of protein reacting with nitrite in the stomach, at low pH, has been reported (Knowles et al., 1974).

It is the purpose of this paper to determine the importance of protein in the loss of nitrite in cured meat, and to examine the protein for ^{15}N incorporated on reaction with ^{15}N labelled nitrite. To initially reduce the number of variables, the model system employed was reduced to a buffered solution of protein and sodium nitrite.

MATERIALS & METHODS

PORCINE MYOSIN was isolated according to the method of Nauss et al. (1969) from longissimus dorsi muscle. A stock solution of myosin (1% w/v) was stored at 2°C until used. Bovine serum albumin was obtained from Sigma Chemical Co., and dissolved in buffer to produce solutions of the required concentrations. The buffers used were citrate-phosphate throughout (Barka and Anderson, 1963). Sodium nitrite labelled with ^{15}N (96.1% enrichment from Prochem, Lincoln Park, N.J.) in solution at various concentrations was added to the protein solutions and aliquots (1 ml) withdrawn after suitable periods for nitrite and ^{15}N analysis, and amino acid analysis.

Analysis for ^{15}N involved conversion of sample nitrogen first to ammonium sulfate and then to nitrogen gas. The nitrogen isotope ratio was measured on a Consolidated Nier isotope ratio mass spectrometer (Burris and Wilson, 1957). Conversion of nitrogen to ammonium sulfate was achieved either through a combination of the Devarda reduction method for nitrite, nitrate and ammonia (Bremner and Keeney, 1965), and the Kjeldahl method for organic nitrogen (AOAC, 1970), or through the modified Olsen method using acidified $KMnO_4$ and reduced iron (Bremmer and Shaw, 1958), or through the use of cupric selenite (catalyst) and concentrated sulphuric acid alone.

Three methods of protein isolation were used to determine whether they would have any effect on the nitrogen isotope recovery. Acetone precipitation was achieved by adding 20 volumes of acetone to 1 volume of protein solution, and after centrifugation, this process was repeated two times more. Dialysis of 1 ml samples was performed in No. 8 Visking tubing against three changes of 4000 ml distilled water in 36 hr at 2°. Using an Amicon ultrafiltration unit, 1 ml samples diluted to 50 ml with distilled water were filtered through Diaflo PM-10 ultrafiltration membranes, to 5 ml volume, and the dilution procedure followed two times more. After the proteins were washed sufficiently to remove residual nitrite, they were hydrolyzed under vacuum in 6N HCl at 110° for 22 hr, before application to a Phoenix amino acid analyser, equipped with a Mark Instrument MR-201 resin column.

Solutions were clarified with Carrez solutions I and II (Adriaanse and Robbers, 1969) to remove protein, and nitrite was determined by diazotization with sulfanilic acid and coupling with N-1-naphthylethyl-

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enediamine. The resulting chromophore was measured spectrophotometrically at 540 nm.

S-nitrosocysteine and glutathione-nitrosothiol were prepared by the method of Mirna and Hofmann (1969); 3-nitrotyrosine, L-tryptophan, 2,4-dinitrophenol, cysteine, imidazole, 1-nitroso-2-naphthol and glutathione were obtained from Sigma Chemical Company. 3,4-dihydroxyphenylalanine was obtained from Dr. F. Siegel, Kennedy Labs., University of Wisconsin-Madison. Chloromycetin (Sigma Chemical Co.), a broad spectrum antibiotic (at 10 mg/100 ml solution) and pentachlorophenol, (Pierce Chemical Co.), an antifungal agent, were added to the incubations to prevent growth of bacteria or molds.

Table 1–Residual nitrite in incubations[a] of bovine serum albumin[b] with sodium nitrite[c]

pH	Residual nitrite (ppm)
4.0	51
5.0	84
6.0	137

[a] Incubation carried out in 0.1M phosphate-citric acid buffer at 20° for 96 hr
[b] Bovine serum albumin 10% w/v
[c] $NaNO_2$ at 200 parts per million

Table 2–% Recovery of nitrogen from various compounds used to test the Kjeldahl reduction step

	Kjeldahl method CuSe + H_2SO_4	Modified Olsen method	Devarda reduction
2,4-dinitrophenol	66	73	72
imidazole	a	86	98
3-nitro-tyrosine	96	86	98
1-nitroso-2-naphthol	77	84	85
cysteine	87	93	92
S-nitroso cysteine	50	60	60
glutathione	90	70	88
glutathione NO thiol	50	68	63

[a] Not performed

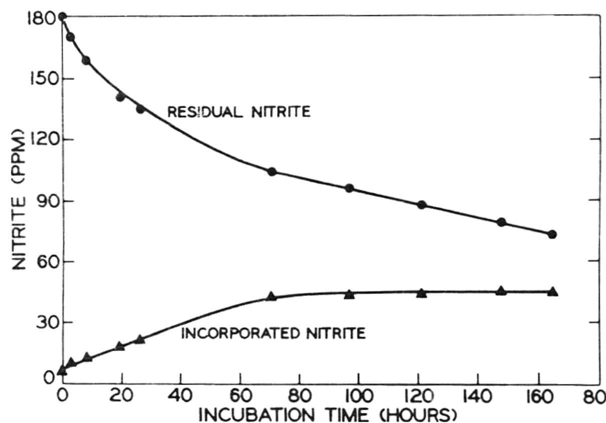

Fig. 1–Residual nitrite levels and incorporated nitrite in incubation of bovine serum albumin (10% w/v) and sodium nitrite (200 ppm) at pH 5.5 and 20°C, 0.1M phosphate-citric acid buffer. 36-hr dialysis used to isolate protein. Nitrogen content analyzed using CuSe and H_2SO_4 only.

RESULTS & DISCUSSION

THE RESULTS of the initial experiments showed that when sodium nitrite (200 ppm) was added to buffer solutions of bovine serum albumin (10% w/v) at several pH values at 20°C over 96 hr, the nitrite could not be quantitatively recovered. Table 1 shows the residual nitrite levels from a typical incubation of nitrite with the protein. Further experiments at pH 5.5 with analysis of the protein for ^{15}N, showed that as the residual nitrite level fell, the quantity of nitrite nitrogen chemically bound to the protein increased, as in Figure 1.

As the nitrite fell to 40% of its original value after 160 hr incubation, 30% of the missing nitrite nitrogen was found bound to the protein. Plotting the residual nitrite concentration on linear, logarithmic or to the second or 1/2 power on a logarithmic scale did not result in a linear relationship with time, indicating to us that the nitrite was combining with more than one reactant.

Of the 120 ppm of nitrite which was lost during 160 hr of incubation, only ∿ 45 ppm were found to be bound to the protein. This poor recovery prompted us to examine both the methods of protein separation and the efficiency of conversion of the Kjeldahl step.

Labile nitroso or nitro compounds could conceivably decompose during the 36 hr dialysis period, with release of the ^{15}N label. Consequently, bovine serum albumin solutions which had been incubated with labelled nitrite were filtered through Diaflo PM-10 ultrafilters, and ^{15}N measurements made on the retained protein. Samples of protein were also isolated by precipitation and centrifugation after addition of acetone. The results we obtained showed no difference between ^{15}N enrichment of protein isolated by dialysis, ultrafiltration or acetone precipitation. Acetone precipitation was used thereafter because it was the fastest of the three methods.

Modifications of the Kjeldahl step were then undertaken to see if this was causing the poor recovery of ^{15}N. Table 2 shows the recovery of nitrogen from various compounds, some related to what could possibly be found in the nitrite-bovine serum albumin incubations.

The results showed that no one method gave consistently higher recovery for all the compounds tested. The modified Olsen method and the Devarda method, however, generally gave higher values for the majority of the compounds. The Devarda reduction method was subsequently used for the remainder of the protein to ammonium sulfate conversions.

Subsequent analyses of nitrosated protein gave higher recoveries for ^{15}N incorporation values (80–90%). The labelled nitrogen still missing is ascribed to failure of quantitative Kjeldahl conversion, since nitrogen in the compounds tested was never completely recovered.

The protein was then analysed to see if it was possible to identify any amino acids which had reacted with the nitrite. At this point it was decided to use porcine myosin, the most abundant non-heme protein in meat, comprising approximately 5% of the wet weight of muscle. This was to enable correlation of the results of the amino acid analyses more closely to the situation in cured meats. Because the myosin solution was only 0.5%, nitrite concentrations were increased to 1000 ppm. Nitrite was also added to 10,000 ppm for ease of observation of new amino acid peaks.

Figure 2 shows the results of a typical amino acid analysis of myosin after incubation with 10,000 ppm $NaNO_2$ and at pH 3.0 at 20°C for 24 hr. The dotted peaks represent compounds not found in the amino acid analysis of myosin alone.

Table 3–^{15}N enrichment and 3-nitrotyrosine formation in mixtures of myosin and sodium nitrite[a]

Nitrite conc (ppm)	pH	Reaction time (hr)	Temp (°C)	Nitrite incorporation µg $NaNO_2$/mg myosin	Nitrite incorporated appears as nitrotyrosine	% Tyrosine as nitrotyrosine
10,000	3.0	24	20	11.0	10%	10%
10,000	5.0	24	20	9.4	8%	7%
1,000	5.0	24	20	1.5	–[b]	< 2%
1,000	5.0	2	70	2.2	–[b]	< 2%
1,000	5.0	240	20	8.1	20%	14%

[a] Myosin 0.5% w/v. 0.1M phosphate-citric acid buffer. 0.1M KCl. Protein precipitated with acetone, after reaction time.
[b] Could not be calculated owing to inaccuracy of % tyrosine as nitrotyrosine data.

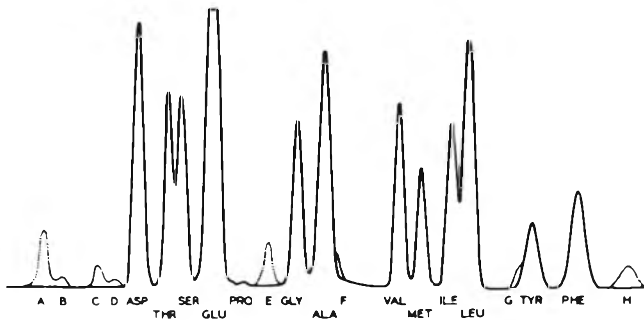

Fig. 2–Amino acid trace of nitrosated myosin. Dotted peaks (magnified) appear on nitrosation. Nitrite conc 10,000 ppm; myosin 0.5% w/v; 0.1M phosphate-citric acid buffer, pH 3.0; 0.1M KCl. Protein precipitated with acetone after 24 hr.

On the basis of retention time on the ion-exchange column and the chemistry of the possible nitrite reactions, we have identified G as 3,4-dihydroxyphenylalanine and H as 3-nitrotyrosine. Commercial samples of the above two compounds had identical retention times, when chromatographed singly or added as a spike to hydrolyzed protein samples.

3-nitrotyrosine is presumed to be the final product of nitrosation of tyrosine; the 3-nitroso derivative of tyrosine is formed initially, which is then oxidized by nitrous acid in the reaction mixture. An analogous reaction takes place in the nitrosation of phenols (Sykes, 1965). 3,4-dihydroxyphenylalanine is also formed from the nitrosation of tyrosine (Philpot and Small, 1938), and was observed both in amino acid analysis of hydrolyzed reacted protein, and from reaction of nitrite with free tyrosine. Peak F, appearing next to alanine, has the same retention time on the ion exchange column as the product we obtained from the reaction of nitrite and free tryptophan. Kurosky and Hofmann (1972) nitrosated N-acetyl tryptophan and obtained only one product, with spectrophotometric properties similar to the N-nitrosamine, (Agarwal et al., 1969).

3-nitrotyrosine appeared as a major reaction product of nitrite with myosin. Experiments were conducted to determine what proportion of the protein bound nitrite appeared as 3-nitrotyrosine under various reaction conditions (Table 3). Total nitrite incorporation increased with increasing nitrite concentration, increasing incubation time, and decreasing pH. Note that nitrite incorporation was similar for 240 hr reaction at 1,000 ppm as at 10,000 ppm for 24 hr. The nitrotyrosine accounted for only 10–20% of the total protein bound nitrite. Heating the reaction mixture did not cause significant nitrotyrosine formation.

The sites of nitrite binding on proteins do not appear to be totally accounted for in the new compounds found in acid hydrolysates. The hydrolysis conditions conventionally used (6N HCl, 110°C, 22 hr) may be severe enough to destroy some of the reaction products, particularly if nitrite is bound to any of the peptide bonds joining the amino acids. Another possibility is the side chains of tryptophan, an amino acid which should be potentially more reactive than tyrosine, and which is severely destroyed on acid hydrolysis. Different hydrolysis methodology will have to be employed to answer questions about the role of this amino acid in nitrite reactions in meats.

SUMMARY

THE FATE of nitrite in cured foods is of importance when considering the possible harmful effects of products of nitrite reactions. We have shown that bovine serum albumin and myosin, the major nonheme muscle protein, have the ability to bind appreciable amounts of nitrite, with resulting modifications of the protein. These include the production of 3-nitrotyrosine, 3,4-dihydroxyphenylalanine, and several other new compounds possibly including N-nitrosotryptophan. Reaction with proteins should then be considered one of the contributors to the fate of nitrite in cured foods.

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Ms received 7/5/75; revised 11/1/75; accepted 11/5/75.

Presented at the 35th Annual Meeting of the Institute of Food Technologists, Chicago, Ill., June 8–12, 1975.

Dept. of Meat and Animal Science, Muscle Biology Lab. Manuscript No. 91.

Research supported by the College of Agricultural and Life Sciences, Univ. of Wisconsin, Madison, and by the Food Research Institute.

J.G.S. supported by NCI Post-Doctoral Fellowship, Public Health Service Research Grant No. 6F22CA00414-01.

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DETECTION OF VARIOUS NONMEAT EXTENDERS IN MEAT PRODUCTS

ABSTRACT

The stacking sodium dodecylsulfate-acrylamide gel electrophoresis method was evaluated for the qualitative detection of various vegetable proteins in meat products. An additional study was also conducted to determine the influence of milk powder, casein, whey protein and egg white proteins on the previously reported electrophoretic procedure for the quantitation of meat and soybean protein content. Soybean, cottonseed flour, cottonseed protein concentrate and peanut protein concentrate exhibited their own unique electrophoretic patterns and were clearly identified when they are added to meat proteins singularly or in various combinations. It was also shown that meat and soybean protein can be successfully identified and quantitated in the presence of milk powder, casein, whey and egg white by employing the described electrophoretic method.

INTRODUCTION

UTILIZATION of vegetable proteins in various meat products has increased markedly over the last few years. There is, for example, a growing acceptance of ground beef-soy mixtures as evidenced by their prevalence in supermarkets. Soy protein is also used as an extender in sausages, frozen meat dinners, and many other convenience food items. At present, these textured vegetable proteins are derived entirely from soybeans, but it is very possible that cottonseed, peanuts, sunflower seed, safflower seed, and grains will be used as well in the near future. In view of the increasing interest in vegetable proteins by the food industry, the development of methodology for the detection and quantitative assessment of various vegetable proteins in meat products would be most helpful to regulatory agencies, consumers and manufacturers concerned with product quality.

Qualitative and quantitative identifications of soy protein added to meat products have been extensively investigated by several researchers (Bennett, 1948; Gils and Hidskes, 1973; Hofman and Penny, 1971; Parsons and Lawrie, 1972; Lee et al., 1975). No such research effort, however, has been reported for the detection and quantitative assessment of other vegetable proteins. Furthermore, many meat products may contain not only vegetable proteins but also other traditional nonmeat extenders such as nonfat milk powder, casein, whey protein and egg white protein. The presence of various nonmeat extenders would make it complicated to identify a specific vegetable protein.

The present paper describes the application of the stacking SDS-acrylamide gel electrophoresis for (1) qualitative identification of soybean, cottonseed and peanut proteins in various mixtures of beef-vegetable protein, and (2) detection of meat and soy protein in meat products containing nonfat milk powder, casein, whey protein and egg white protein.

EXPERIMENTAL

Preparation of samples

Fresh ground beef, textured soy protein, cottonseed flour, cottonseed protein concentrate, and peanut protein concentrate were obtained. A 5-g sample of each product was homogenized in 10 volumes of acetone. The homogenates were centrifuged at 18,000 × G for 15 min, the supernatants were discarded and residues were reblended in 10 volumes of acetone. The acetone treatment was repeated two additional times or until all the lipid was removed. The final pellets were dried at 60°C, ground and stored in a desiccator at room temperature. Nonfat milk powder, casein, whey protein and egg white protein were used directly without further lipid extraction.

A series of mixtures of dried meat powder and vegetable protein powder were prepared on a w/w basis: meat-soy protein (3:1), meat cottonseed protein (3:1), meat-peanut protein (3:1), meat-soy-cottonseed protein (3:1:1), meat-soy-peanut protein (3:1:1), meat-cottonseed-peanut protein (3:1:1), and meat-soy-cottonseed-peanut protein (3:1:1:1). Cottonseed protein used in the mixture could be either from cottonseed flour or from cottonseed protein concentrate, because both products showed exactly the same electrophoretic pattern. Another series of mixtures of dried meat powder, soy protein and animal origin nonmeat extenders were prepared on a w/w basis: meat-soy-nonfat milk powder (3:1:1), meat-soy-casein (3:1:1), meat-soy-whey protein (3:1:1), meat-soy-egg white (3:1:1) and meat-soy-casein-egg white (3:1:1:1).

Approximately 200 mg samples of each single protein preparation and the described mixture proteins were suspended in 15 ml of extraction solution containing 0.0625M Tris-HCl (pH 6.8), 3% SDS, and 1% β-mercaptoethanol. The suspensions were heated in a boiling water bath for 15 min and centrifuged at 20,000 × G for 15 min at room temperature. The supernatants were saved and the residues were resuspended in the extraction solution and extracted two more times. After final centrifugation, the supernatants were combined and the total volume was made up to 50 ml by adding extraction solution. The final protein concentration was approximately 4 mg/ml.

Stacking gel electrophoresis

The electrophoretic technique employed in this study was a slight modification of the method described by Laemmli (1970). The whole gel consisted of two portions; stacking gel (upper gel) and separating gel (lower gel). For a typical run of 12 gels, separating gels of 10% acrylamide were prepared by mixing 18 ml of acrylamide stock solution (22.2g acrylamide + 0.6g methylene bisacrylamide in a final volume of 100 ml), 10 ml of 1.5M Tris-HCl buffer containing 0.4% SDS (pH 8.8), 1 ml of ammonium persulfate (10 mg dissolved in 1 ml of water), 11 ml of water and 0.03 ml TEMED (N,N,N',N'-tetramethylethylenediamine). Ten cm gels were prepared in 15 cm glass tubes with an inside diameter of 6 mm. After complete polymerization of the separating gels, the stacking gels of 1 cm in length were formed on the top of the separating gels. The stacking gels (3% acrylamide) were prepared by mixing 0.66 ml acrylamide stock solution, 1.25 ml of 0.5M Tris-HCl buffer containing 0.4% SDS (pH 6.8), 0.1 ml of ammonium persulfate (10 mg per ml), 3 ml of water and 0.01 ml TEMED. After complete polymerization of the gels, 50–100 μg of the prepared samples were applied on top of the gel with 10 μl of bromphenol blue (0.05%) and 10 μl of 8M urea. The gel columns were then immersed in a bath buffer (pH 8.3) containing 0.025M Tris, 0.192M glycine and 0.1% SDS. Electrophoresis was carried out with a current of 1 mA per gel until the bromphenol blue marker completely migrated into the top of the separating gel (about 1.5 hr). The current was then changed to 2 mA per gel until the marker dye reached the bottom of the gel (about 4 hr). After electrophoresis, the gels were removed from the tubes, and stained overnight in test tubes at room temperature with 0.4% coomassie brilliant blue in 50% methanol and 9.2% acetic acid. The gels were rinsed and destained in a solution containing 50% methanol and 9.2% acetic acid for 8 hr. The gels were then transferred to a diffusion destainer (Hoefer Scientific Instrument, Inc., Calif.) filled with destaining solution of 7.5% acetic acid and 5% methanol. After complete destaining, photographs of the gels were taken.

RESULTS & DISCUSSION

Qualitative detection of vegetable proteins in meat-vegetable protein blends

Typical stacking SDS-acrylamide electrophoretic patterns for the protein extracts of beef, soy, glandless cottonseed flour, cottonseed protein concentrate, and peanut protein concentrate are shown in Figure 1. Protein extracts of ground beef showed 12 major bands and 4–5 minor weak bands. Soybean protein exhibited five major distinct bands, two of them were very strong and the remaining three were moderately strong. Protein extracts of cottonseed flour and cottonseed protein concentrate exhibited exactly the same electrophoretic patterns consisting of two strong bands, three moderately intense bands and two weak bands. On the other hand, peanut protein exhibited five strong bands and three to four minor weak bands.

The results clearly indicated that each vegetable protein studied showed a unique and characteristic electrophoretic pattern which can be distinquished easily from one another. No difference in the electrophoretic pattern between glandless cottonseed flour and cottonseed protein concentrate suggested that the electrophoretic method described could be applied for the detection of any form of cottonseed protein products. It was previously reported that the electrophoretic pattern of soybean protein was also consistent regardless of form or source of soy protein (Lee et al., 1975).

Various combinations of meat and vegetable proteins were electrophoresed to determine whether one vegetable protein could be detected in the presence of meat and other vegetable proteins, and the results are illustrated in Figures 2 and 3. Figure 3 is a schematic diagram of electrophoretic patterns shown in Figure 2. The electrophoretogram of meat protein only (Fig. 2a and 3a) served as the reference for other electrophoretograms. The two meat protein bands, denoted M1 and M2, served as the reference bands in describing the relative location of vegetable protein bands.

As seen in Figures 2b and 3b (soy-beef), four of the five soybean protein bands were easily distinguished from the meat protein bands and thus its detection in meat products was very

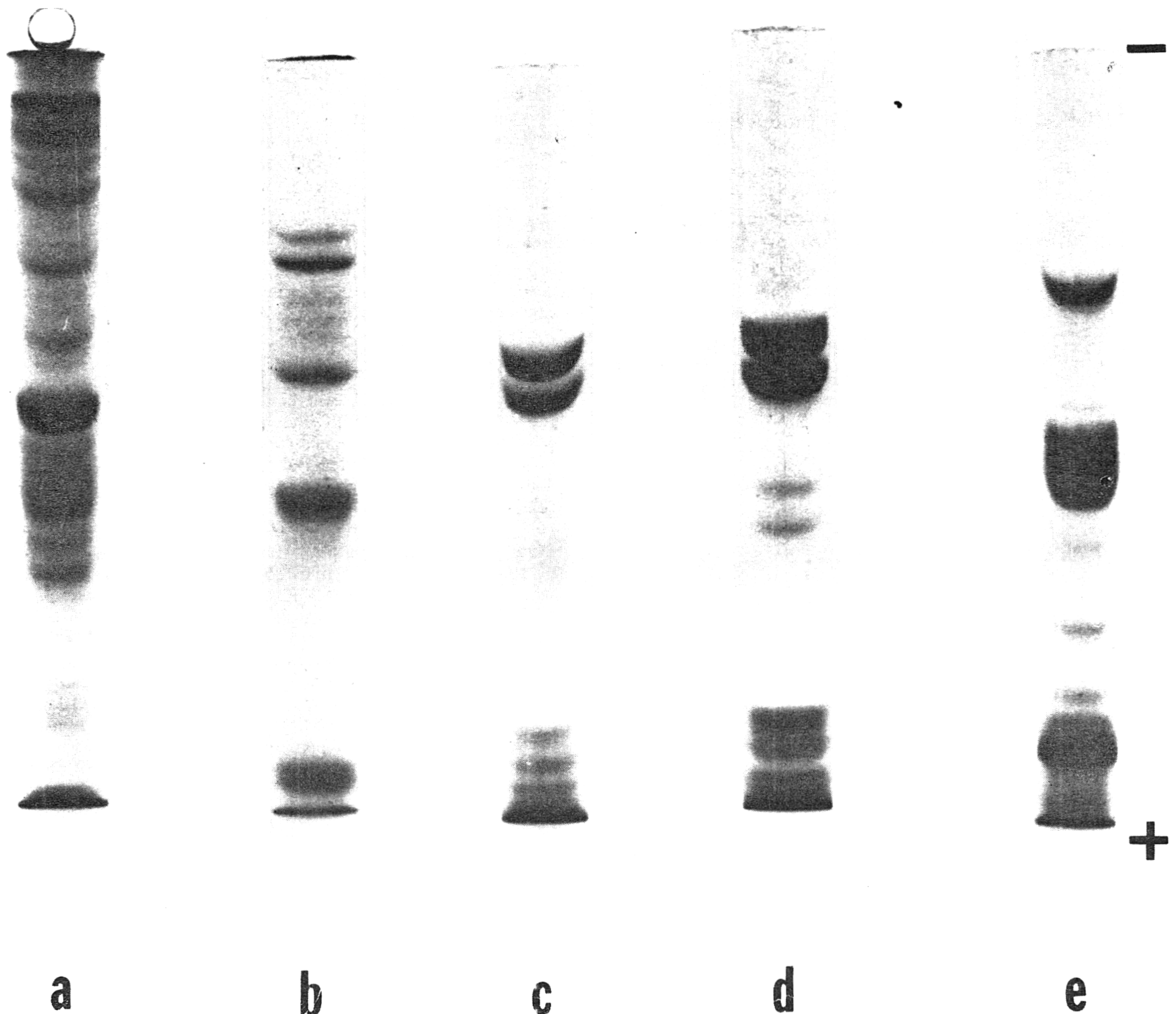

Fig. 1–SDS-acrylamide stacking gel electrophoretograms of (a) meat protein; (b) soy protein; (c) cottonseed flour; (d) cottonseed protein concentrate and (e) peanut protein concentrate.

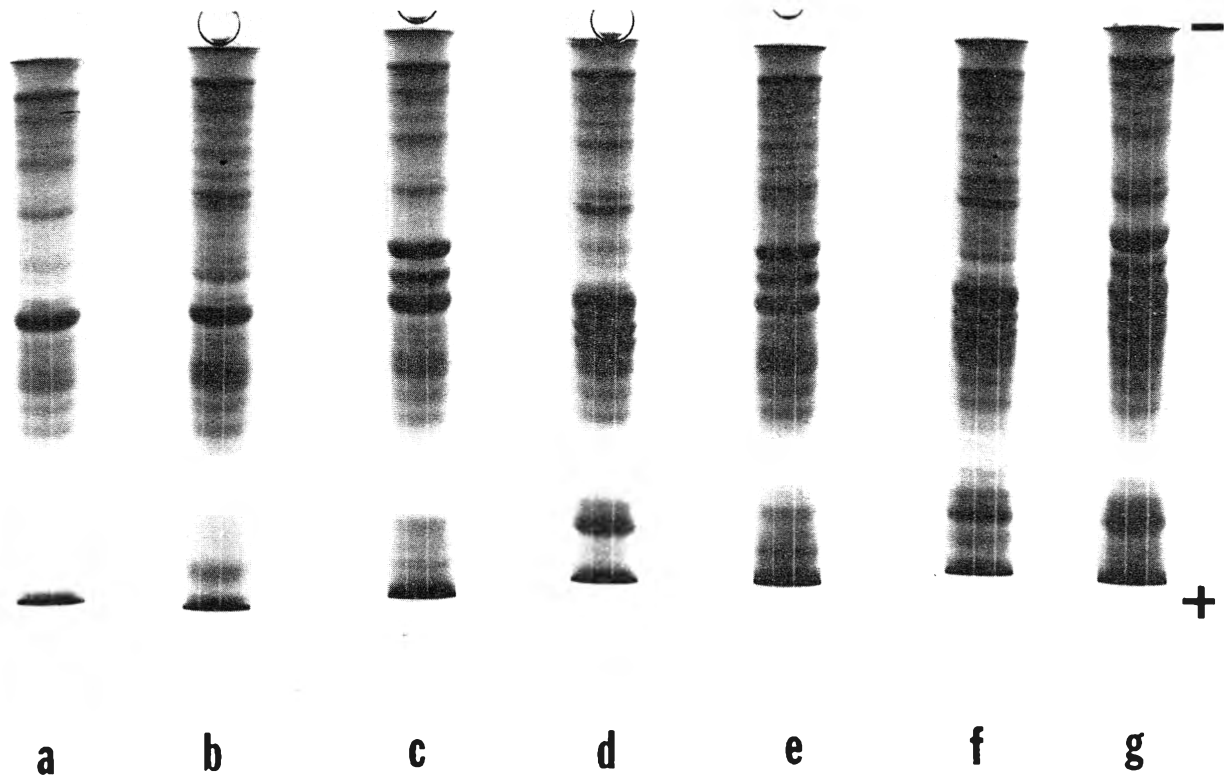

Fig. 2—SDS-acrylamide stacking gel electrophoretograms of (a) meat protein; (b) meat-soy; (c) meat-cottonseed; (d) meat-peanut; (e) meat-soy-cottonseed; (f) meat-soy-peanut; and (g) meat-cottonseed-peanut.

simple. The qualitative and quantitative identifications of soybean protein in meat products were extensively discussed previously (Lee et al., 1975).

Figures 2c and 3c illustrate the electrophoretograms obtained from beef and cottonseed protein mixture. The presence of two strong bands on top of the meat protein band M2 and 3 minor bands at the bottom of the gel were the characteristic indication of the presence of cottonseed protein in meat products.

The electrophoretogram for the beef and peanut protein mixture is illustrated in Figures 2d and 3d. Three of the five major peanut protein bands were separated and easily distinguished from meat protein bands; whereas, two partially overlapped by meat proteins. The presence of three strong bands immediately below band M2, one intermediate band below band M1 and one strong band at the bottom portion of the gel was a good index for the detection of peanut protein added to meat.

Figures 2e and 3e show the electrophoretograms of a three-way mixture of beef, soy, and cottonseed protein. Although some of the protein bands were further overlapped, three soy protein bands, denoted S, were still clearly separated and distinguished from both cottonseed and meat protein bands. The presence of two strong bands above band M2 was the best manifestation of the presence of cottonseed protein in the mixture.

The electrophoretogram obtained from a three way mixture of beef, soy, and peanut protein (Fig. 2f and 3f) revealed that four soy protein bands and four peanut protein bands were clearly distinquished from the rest of the protein bands, thus enabling the detection of respective vegetable protein in the mixture.

Similarly, the three-way mixture of beef, cottonseed and peanut proteins (Fig. 2g and 3g) again showed that one vegetable protein could be easily detected in the presence of other vegetable proteins contained in meat products.

Figure 4 presents the electrophoretogram of a four-way mixture of beef, soy, cottonseed and peanut protein. Two soybean protein bands, one cottonseed protein band and three peanut protein bands were not overlapped by either meat protein bands or reciprocal vegetable protein bands. This distinct separation of characteristic bands unique for each vegetable protein proved that the electrophoretic method described

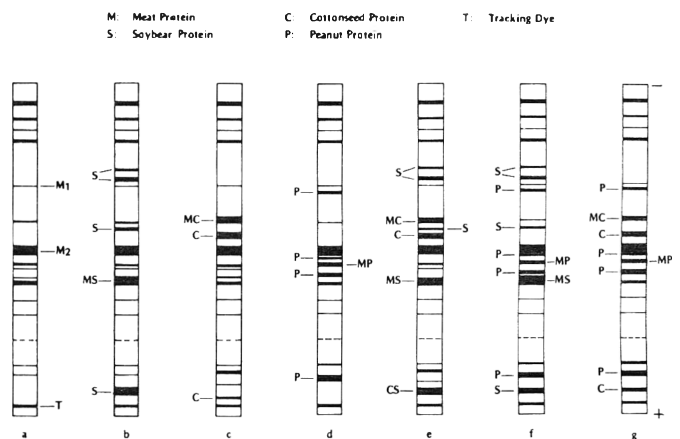

Fig. 3—Schematic diagram of electrophoretic patterns of (a) meat protein; (b) meat-soy; (c) meat-cottonseed; (d) meat-peanut; (e) meat-soy-cottonseed; (f) meat-soy-peanut; and (g) meat-cottonseed-peanut.

would be successfully utilized for the detection of various vegetable proteins added to meat products singularly or in combination with other vegetable proteins. It should be pointed out, however, that further research is needed to establish the lower limits of detection of these vegetable proteins.

Qualitative and quantitative identification of meat and soybean proteins in the presence of nonmeat extenders

A quantitative method to determine soybean protein content in the simple system of soy-beef blends was reported previously (Lee et al., 1975). Since many meat products contain animal origin nonmeat extenders such as nonfat milk powder, casein and egg albumin, an additional study was conducted to determine the influence of these protein sources on the published electrophoretic procedure (Lee et al., 1975) for the quantitation of soy and meat protein.

Typical electrophoretic patterns of nonfat milk powder, casein, whey protein and egg white protein are shown in Figure 5. Casein (either pure casein or Na caseinate) showed three major distinct bands and two weak bands. The weak band near the dye front of the gel was due to a contamination of whey protein. Whey protein exhibited one single band at the bottom of the gel. Nonfat milk powder which is simply a mixture of casein and whey proteins exhibited the characteristic three strong casein bands and one moderate whey protein band. Egg white protein showed one strong band and four weak bands. Though no attempt was made to identify the individual protein component, the strong band of egg white is believed to be ovalbumin (M.W. 45,000) which is the most abundant of the egg white proteins.

Figure 6 illustrates the electrophoretic patterns of three-way mixtures of beef + soy + one of the respective nonmeat extenders (a, b, c and d) and a four-way mixture of beef + soy + casein + egg white (e). The index band for meat protein (actin band, MA) was not overlapped by any nonmeat extender protein bands. One of the egg white proteins, ovalbumin, was very close to the actin band (M.W. 47,000) but did not overlap it. It is also clearly seen that three of five soybean protein bands were not overlapped by any of nonmeat extenders tested. Particularly the selected index band of soybean protein (SI) was clearly separated from all nonmeat extender

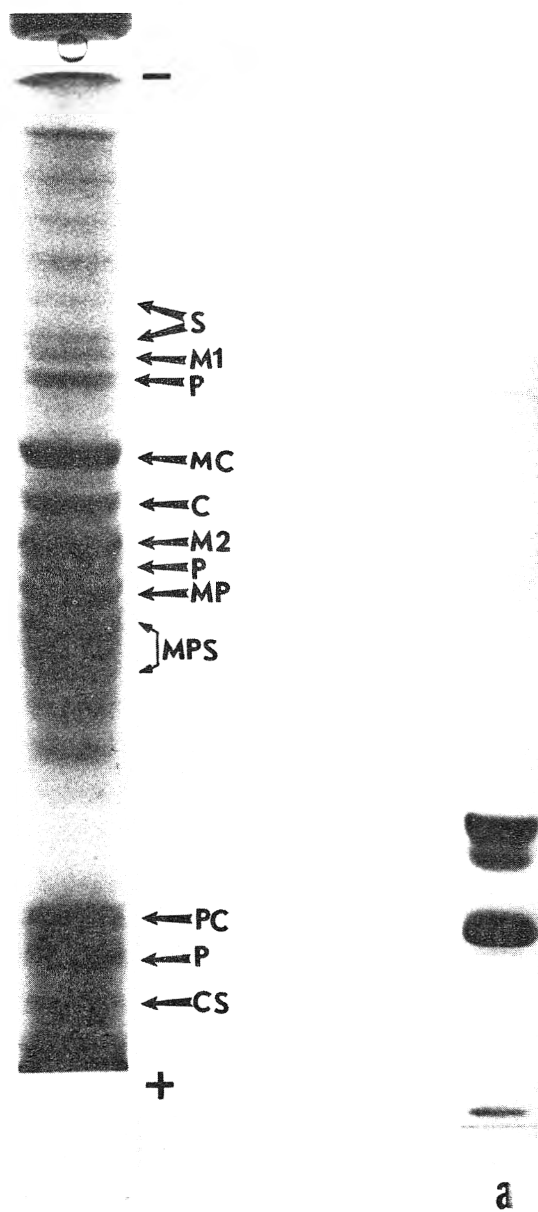

Fig. 4–Electrophoretogram of meat-soy-cottonseed-peanut protein mixture. The legends are same as in Figure 3.

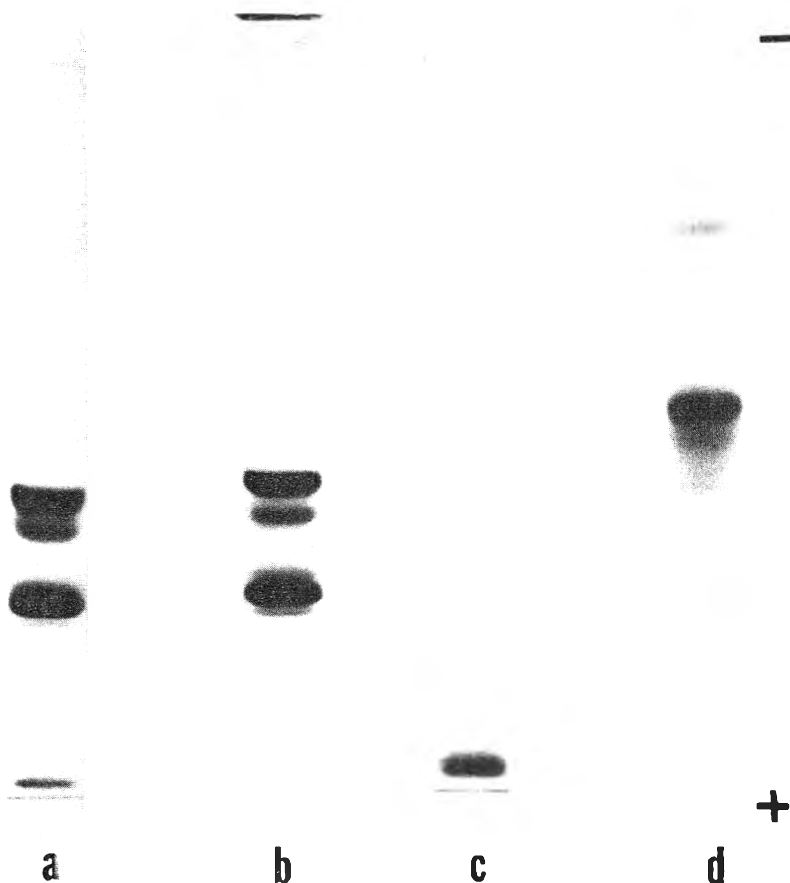

Fig. 5–SDS-acrylamide stacking gel electrophoretograms of (a) nonfat milk powder; (b) casein; (c) whey protein; and (d) egg white protein.

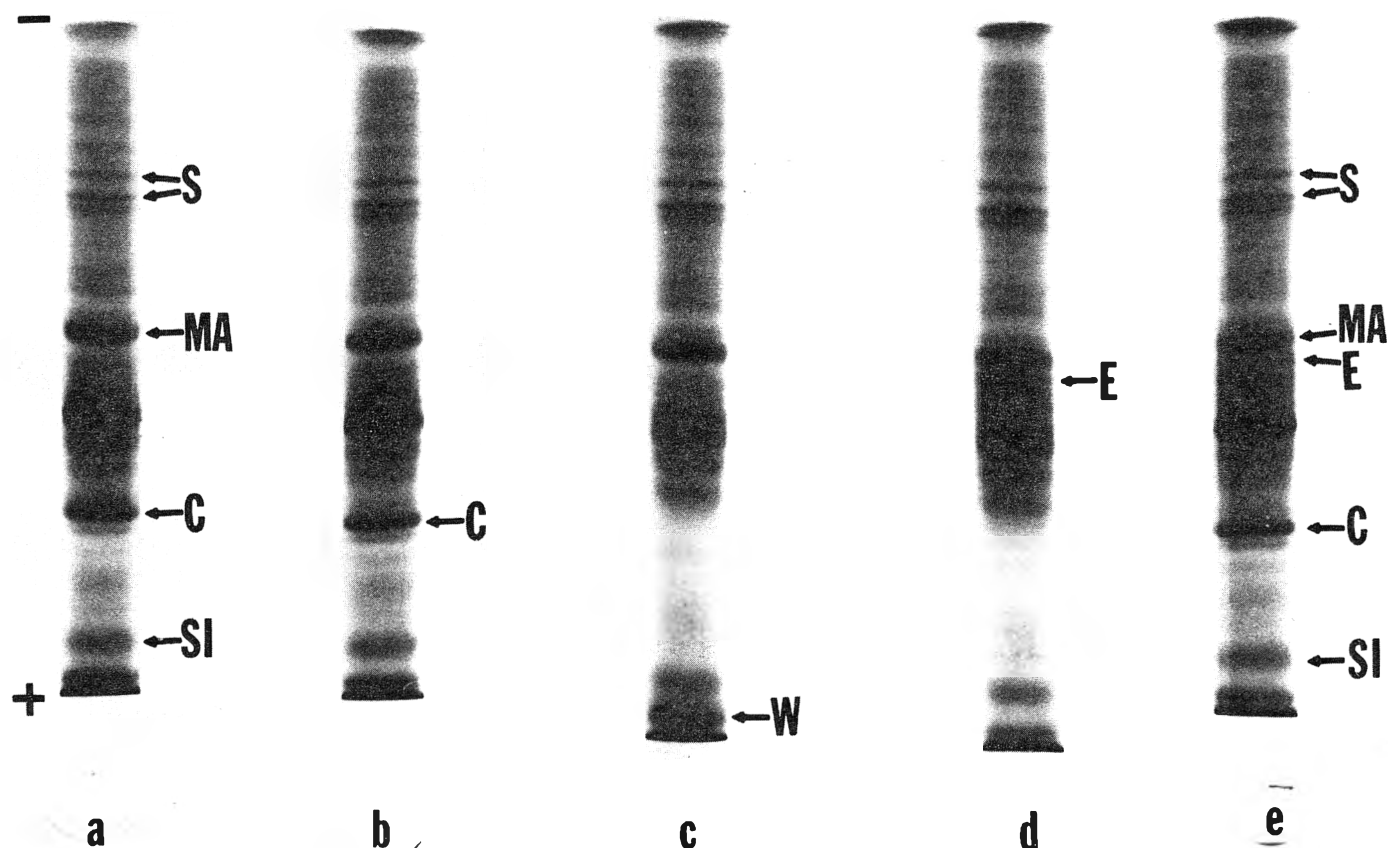

Fig. 6—SDS-acrylamide stacking gel electrophoretograms of (a) meat-soy-nonfat milk powder; (b) meat-soy-casein; (c) meat-soy-whey protein; (d) meat-soy-egg white; and (e) meat-soy-casein-egg white. MA-actin band of meat protein; S-soybean protein, SI-index band of soy protein; C-casein; W-whey protein; E-egg white protein.

bands; and thus, its optical density measurement was not interfered.

It is concluded from the foregoing results that it should be possible to quantitate both meat and soy protein in the presence of milk powder, casein, whey and egg white by the published method of Lee et al. (1975). Furthermore, at least one band of each of milk and egg proteins, was distinctly separated from the rest of the protein bands, thus making quantitative determination of these nonmeat extenders possible using the same method (Lee et al., 1975).

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Ms received 9/13/75; revised 11/8/75; accepted 11/12/75.

The authors expressed their appreciation to the Food Protein R&D Center, Texas A&M University, College Station, Texas for the generous gift of cottonseed protein concentrate and peanut protein concentrate.

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HYDROCARBON PRODUCTION FROM FREEZE-DRIED MEATS

ABSTRACT

The possibility of utilizing hydrocarbon measurement to follow lipid oxidation in freeze-dried foods was explored. In freeze-dried meats, TBA reactive substances (a common indicator of lipid oxidation) and hydrocarbons were analyzed during storage of control and phosphate treated meats. TBA numbers were initially much higher in control than in phosphate-treated meats, but decreased steadily during storage, presumably from reactions with other food components. Malonaldehyde added directly to meat also decreased. Unexpectedly, methane was the major hydrocarbon from both the control and the phosphate treated meats with significant amounts of ethane and propane and traces of butane and pentane. Further evidence is needed on the origin of methane and any relationship between its production and the overall quality of freeze-dried meats.

INTRODUCTION

THE IMPAIRMENT of quality of foods from heat applied during dehydration can be eliminated by freeze-drying techniques. However, lipids in freeze-dried meats are highly susceptible to oxidation because the porous nature of the products affords easy access to oxygen, and low moisture content of the products facilitates the rapid oxidation (Labuza et al., 1970). It was reported that the surface area of freeze-dried fish was 160–180 times larger than that of fish dried by a fan heater (Ohata et al., 1967). Since the purpose of freeze drying is to preserve the products without refrigeration, prevention of lipid oxidation is very important.

Methods of measuring the degree of oxidation of lipids are necessary in order to follow and control the rancidity of the freeze-dried meats during storage. There are several methods to follow lipid oxidation in moist meats and meat products. For freeze-dried meats, not many reliable methods are available presently. Chipault and Hawkins (1971) reported that the methods commonly used for the evaluation of rancidity of meats were not operative for freeze-dried meats for one reason or another. They concluded that the oxygen absorption measurement was the only method that could be used to follow the rancidity of the freeze-dried meats. Earlier reports by Martinez and Labuza (1968), Tuomy and Fitzmaurice (1971) and Heidelbaugh and Karel (1970) already indicated its suitability for the study of lipid oxidation in the freeze-dried foods despite the fact that protein oxidation also absorbs oxygen.

These facts may indicate that it would be of great value to find another method for the evaluation of lipid oxidation in freeze-dried meats.

Among the final products of lipid oxidation, hydrocarbons would be expected theoretically. Experimentally, hydrocarbon formation from lipid oxidation has been demonstrated by many workers. C_1-C_5 saturated hydrocarbons were separated but pentane made up more than 90% of the hydrocarbon fraction from autoxidized methyl linoleate (Horvat et al., 1964). Evans et al. (1967) also found that pentane was predominant in the hydrocarbons from thermal decomposition of linoleic acid. Pentane production from linoleic acid oxidation catalyzed by soybean lipogenase was also reported (Johns et al., 1973).

Hydrocarbons have also been separated from rancid foods: from autoxidized potato granules (Buttery, 1961) and soybean oil (Evans et al., 1961; Selke et al., 1970), short chain hydrocarbons were isolated. Scholz and Ptak (1966) reported that pentane concentration increased rapidly in rancid cottonseed oil and other vegetable oils with storage. Evans et al. (1969) and Jarvi et al. (1971) reported that the flavor scores were correlated with the amount of pentane released in vegetables oils. Warner et al. (1974) found significant linear correlations between the amount of pentane and the number of rancid descriptions obtained from potato chips and vegetable oil by an 18-member taste panel. From these reports it was assumed that hydrocarbon measurement might be a feasible method to evaluate the degree of lipid oxidation in freeze-dried meats.

Tripolyphosphate has been demonstrated to be a good antioxidant. Lehmann and Watts (1951) reported the antioxidant effect of tripolyphosphate in aqueous fat systems, and it has been used repeatedly to protect cooked meat and fish (Tim and Watts, 1958; Tarladgis et al., 1959; Zisper and Watts, 1961; Ramsey and Watts, 1963; Rao et al., 1975).

Malonaldehyde is one of the decomposition products of oxidized polyunsaturated fatty acids (Dahle et al., 1962). The TBA test for malonaldehyde has been used successfully for determining oxidation of lipids in numerous studies on meats and other food products. However, Patton (1974) has indicated that the test appears to be measuring malonaldehyde and other substances capable of yielding a closely related derivative of malonaldehyde during the test procedure. The literature contains much evidence of secondary reactions of malonaldehyde with other food constituents (Kwon et al., 1965; Chipault and Hawkins, 1965; Crawford et al., 1966; Green and Watts, 1966). In spite of the established disappearance of malonaldehyde, TBA values have in some cases distinguished between protected and control samples of freeze-dried meats (Kanamori et al., 1967; Kopecky, 1968).

The purpose of this study was to investigate the possibility of utilizing the hydrocarbon measurement and TBA test to follow lipid oxidation in freeze-dried meats.

EXPERIMENTAL

Preparation of meats and freeze-drying

Eye round beef was purchased from the local market. The meat was trimmed of excess fat, ground twice in Ward's electric chopper, Model Vgs-5169A, and then mixed by hand to obtain a homogeneous product. For the treated meats, granular sodium tripolyphosphate was dissolved in distilled water and added in concentration of 0.5% of the meat before cooking. After addition of sodium tripolyphosphate, the meat was packed into 307 × 113 C-enamel cans, sealed immediately with a hand seamer and processed in boiling water until the internal temperature reached 70°C, then cooled in running tap water. 30-g portions of cooked meat were weighed and put in an American Model L-3 Laboratory Freeze Drying Unit. The temperature of the heating plate was 37.7°C at gauge. The minimum vacuum was 200 mu Hg. It took about 20 hr for drying to a final weight of 9g.

For the malonaldehyde added samples, 6×10^{-3}M malonaldehyde

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solution was added in a concentration of 10% of the meat after cooking; this was to give the treated meat a TBA number approximately 43.2. The same proportion of distilled water was added to the control meat. A 6×10^{-3}M malonaldehyde solution was prepared by the acid hydrolysis of purifide malonaldehyde bis-(diethylacetal) (1,1,3,3-tetraethoxypropane) obtained from Kay-Fries Chemicals, New York (Kwon and Watts, 1963). No attempt was made to purify the prepared malonaldehyde solution. It is conceivable that the solution contains other impurities resulting from the release of ethoxy or ethyl groups.

Sample storage and analysis

9-g portions of dried meat were put into 125 ml Erlenmeyer flasks sealed with serum rubber caps at room temperature. Samples (4 ml) of head space gas were taken at intervals by a gas-tight syringe and introduced to the gas chromatograph for hydrocarbon analysis.

The TBA number of the meat was measured by the distillation methods of Tarladgis et al. (1960). Each determination was made in duplicate.

3-g of freeze-dried meat were further dried at 105°C for 4 hr for moisture determination. The moisture content of final products were 4.5% for controls and 4.6% for phosphate treated meats.

Head space gases were analyzed with the 609 F&M Gas Chromatograph with hydrogen flame ionization detector. Aluminum columns, 6 ft long and ¼ in. diam were packed with about 28g of activated alumina as reported by List et al. (1965). Flow rate of helium, hydrogen and oxygen were 62.5 ml/min, 48 ml/min and 325 ml/min, respectively. The temperature of the injector port and the detector port was 200°C. Column temperature was programmed from 75–300°C, at a programming rate of 30°C per minute. The activated alumina, F-1, 60/80 mesh, was obtained from the Coast Engineering Laboratory. The column was conditioned by heating at 300°C for several hours, allowing the carrier gas to flow whenever the column response was dull. The amount of hydrocarbon in the head space was calculated by comparing the sample's peak height × retention time with that of the standard. Duplicate determinations were made and the average was reported.

Preparation of aqueous solution

Substrate compounds used in this study were dissolved in 0.05M phosphate buffer at pH 5.7 at a concentration of 0.01M. The buffering agents were dipotassium monohydrogen phosphate and potassium dihydrogen phosphate.

EDTA-ferrous complex was prepared by dissolving 1.2×10^{-3} mole of ethylenediaminetetraacetic acid dipotassium salt and 1.2×10^{-3} mole of ferrous sulfate in 100 ml distilled water separately. Equal parts of the two solutions were mixed before using to give 6×10^{-3}M EDTA-ferrous complex [Fe(II)-EDTA].

Preparation of fatty acid emulsion

The fatty acids, obtained from Hormel Institute, were emulsified according to Surrey (1964). Two ml of Tween 20 and 50 ml of 0.1M phosphate buffer (pH 5.7) were stirred and 0.001 mole of fatty acids was added drop by drop to form a homogeneous emulsion. Potassium hydroxide (IN) was added until the solution became clear. pH was adjusted to 5.7 by 6N hydrochloric acid and the solution made up to 100 ml with phosphate buffer. The concentration of the stock solution was 1×10^{-2}M.

Experimental procedures for aqueous systems

A 10-ml aliquot of 0.01M substrate solution was introduced into a 125 ml Erlenmeyer flask and 8 μl of 30% hydrogen peroxide was added to the solution followed by 3 ml of Fe(II)-EDTA. The flask was sealed immediately with a serum rubber cap and placed in a water bath at 30°C and shook. A 2-ml sample of head space gas was taken by a gas-tight syringe after incubation for 10 min, and injected into the gas chromatograph under the conditions described in the freeze-dried sample analysis. The final concentration of substrate and Fe(II)-EDTA were 7.7×10^{-3}M and 1.38×10^{-3}M, respectively. Flasks of 140 ml volume were carefully selected for uniformity of sampling.

Data were expressed based on results of duplicate tests throughout this study.

RESULTS & DISCUSSION

The TBA numbers in freeze-dried beef

The effect of added tripolyphosphate on TBA numbers of precooked freeze-dried beef was measured from 1 day to 5 months of storage (Table 1). Meat samples treated with 0.5% tripolyphosphate gave significantly lower TBA numbers than controls for the entire 5-month storage period. The TBA numbers of the control samples steadily decreased but those of the test samples increased during storage at room temperature. The increased TBA numbers of phosphate-treated meats suggested that phosphate protection may be limited to the cooking period, not extending to the storage of the dehydrated meat. It was already shown by other workers (Tim and Watts, 1958; Chipault and Hawkins, 1965) that the cooking process accelerated lipid oxidation and phosphate prevented this. The measured TBA number of meats immediately after cooking and again after freeze drying support the above assumption (Table 2). No increase in TBA number took place upon freeze drying the cooked meat.

In reference to the decreasing TBA numbers of control samples (Table 1), malonaldehyde produced from lipid oxidation may undergo secondary reactions. Such losses of malonaldehyde were demonstrated more clearly by adding malonaldehyde to the meats before freeze drying (Table 3). The initial TBA numbers, 4.3 for control and 35.1 for malonaldehyde-added meats, decreased to 2.4 and 25.7, respectively after 70 days storage.

The loss of added malonaldehyde may be due to polymerization of malonaldehyde (Kwon and Watts, 1964) and/or reactions with other food constituents as documented in the Introduction. It also could not rule out the possibility of re-

Table 1—The TBA numbers in precooked freeze-dried meat

Days of storage at room temp	TBA numbers mg/1000g	
	Control	0.5% phosphate treated
1	4.8	0.43
28	3.6	0.55
44	3.1	0.77
154	2.9	0.94

Table 2—The TBA number of meats after cooking and after cooking and freeze-drying

	TBA numbers mg/1000g	
	Control	0.5% phosphate treated
After cooking	7.1	2.8
After cooking, Freeze-drying	6.4	2.8

Table 3—Loss of malonaldehyde during storage at room temperature

Days of storage at room temp	TBA numbers mg/1000g	
	Control	Malonaldehyde added
Before freeze drying	7.4	42.1
After freeze drying	4.3	35.1
2	5.1	39.0
17	3.0	33.5
70	2.4	25.7

Table 4–Hydrocarbon production from freeze-dried meat during storage

Days of storage at room temp	Column response to 1 μl of methane[a] (cm X sec)	Methane (μl)		Ethane (μl)		Propane (μl)	
		Control	0.5% Phosphate treated	Control	0.5% Phosphate treated	Control	0.5% Phosphate treated
1		0	0	0	0	0	0
28	2840	0.66	0.46	0.40	0.21	0.29	0.15
44	2650	0.73	0.62	0.44	0.34	0.33	0.17
154	4520	1.19	0.70	0.51	0.51	0.19	0.19
266	6230	0.64	0.44	0.11	0.11	0.13	0.13
388	6560	0.84	0.83	0.40	0.40	0.31	0.31

[a] One μl of each gas is equivalent to 4.0×10^{-8} mole.

actions between malonaldehyde and the impurities of the prepared solution. Whatever the mechanism of its loss, some of the malonaldehyde and other TBA reactive substances bound in freeze-dried meats apparently is not easily released from its complex by the acid and heat treatment used in the distillation method for the TBA test.

The mechanism of the tripolyphosphate protection is not clear. Liu (1970) demonstrated a significant lipid oxidation catalyzed by nonheme iron in cooked meat. Therefore, it is quite reasonable to assume that the phosphate protects lipids by sequestering iron complexes.

Hydrocarbon analysis

Hydrocarbons in the head space gas were analyzed on the same samples used for the TBA numbers reported in Table 1. Methane was the major hydrocarbon from both control and phosphate-treated meats, with significant amounts of ethane and propane (Table 4). Traces of butane and pentane were also present. The chromatograms obtained from this experimental system were direct and simple for interpreting and identifying the component peaks. However, with different chromatographic columns and a specially designed apparatus for the isolation of volatile flavor compounds, more complicated chromatograms were obtained (Hirai et al., 1973). They reported that a total of 54 compounds were identified from the volatiles of boiled beef and very recently, Peterson et al. (1975) from the same laboratory found a total of 102 compounds in canned stew. In another report, Persson and Sydow (1973) identified 95 different compounds from the volatiles of canned beef.

The activated alumina column used in this study proved to be a useful and effective tool for hydrocarbon analysis because the column had adsorbed irreversibly all polar oxygen-containing compounds, allowing nonpolar compounds such as hydrocarbons to pass. This fact has already been reported by List et al. (1965). They indicated that the gas-solid chromatography with the activated alumina column offered a quantitative and direct method for hydrocarbon analysis in lipid oxidation systems.

Since column responses were different between columns and from day to day even with the same column (Table 4), the data were corrected according to changes in response of the column to known amounts of methane. The changes in the column responses might be due to the changes in the gas flow rate and/or differences in the degree of column deterioration. The repeated overnight column condition by heating at 300°C appeared to be causing the rapid column deterioration. Harris and Habgood (1960) indicated that loss of water by condensation of two surface hydroxyl groups was responsible for the alumina column deterioration and the process was only partially reversible. For understanding the longitudinal pattern of hydrocarbon production during storage, further studies are necessary.

Pentane rather than methane was the hydrocarbon expected from the freeze-dried meat based on the following facts. From the oxidation of linoleic acid, pentane was predominant (Table 5) and the major polyunsaturated fatty acid in meats is linoleic acid. Lipids average about 4.6% of lean beef and about 0.36% of the total lipid is linoleic acid (Hornstein et al., 1961). Chipault and Hawkins (1965) reported that 1.14% of the meat was linoleic acid in freeze-dried meat (3.3% moisture level). It was also based on the work reported by others as indicated in the introduction.

Methane source and possible relation to lipid oxidation are not clear. The fact that methane is obtained from ethanal (Table 5) suggests that ethanal may be at least one precursor of methane in freeze-dried meats. According to Gaddis et al. (1961), 5.9% and 1.2% of the carbonyls obtained from linoleate and linolenate oxidation, respectively, were ethanal. In general, carbonyls are superior to the respective parent fatty acids in production of hydrocarbons. Propanal is superior to linolenic acid in producing ethane and hexanal is far superior to linoleic acid in pentane production.

In addition to fatty acids as possible sources of ethanal in meats, alanine can give ethanal by a "Strecker degradation," i.e., an oxidative decarboxylation and deamination of alpha amino acids by carbonyls [-CO-(CH=CH)$_n$-CO-] to produce aldehydes having one carbon less than the original amino acid. Wasserman and Spinelli (1970) and Macy et al. (1970) reported that one of the major free amino acids in beef was alanine. Ethanol arising from alanine by such a reaction may degrade to methane in the presence of a free radical generating reaction, such as the oxidation of polyunsaturated fatty acids. Thus it is possible that methane production could be indicative both of amino acids and lipid degradation in dried meats. Further evidence is needed on the origin of methane from the

Table 5–Hydrocarbon production from various substrates[a]

	μl in 10 min				
	Methane	Ethane	Ethylene	Butane	Pentane
Ethanal	1.7	1.8			
Propanal	8.8[b]	3.0[b]	2.5[b]		
Hexanal					27.0[b]
Linoleic acid					1.9[b]
Linolenic acid		1.4			

[a] Substrate 7.7×10^{-3}M: Fe(II)-EDTA 1.38×10^{-3}M: H_2O_2 6.04 $\times 10^{-3}$M. Total volume of mixture, 13 ml. μl production from 13 ml reaction mixture.

[b] Average of two runs.

meats and conditions affecting its production as well as any relationship between methane production and the overall quality of freeze-dried meats.

The data on hydrocarbon production (Table 5) indicate that its production is very distinctive between the linoleic acid family ($CH_3CH_2CH_2CH_2CH_2CH$=) and linolenic acid family (CH_3CH_2CH=). The configurations of the methyl end (methyl carbon to the carbon which holds the first double bond) in fatty acids determine the major hydrocarbon produced.

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Ms received 7/5/75; revised 10/18/75; accepted 10/21/75.

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CHANGES IN PHOSPHOLIPIDS IN CHICKEN TISSUES DURING COOKING IN FRESH AND REUSED COOKING OIL, AND DURING FROZEN STORAGE

ABSTRACT

Chicken pieces were cooked in fresh corn oil and in corn oil previously heated up to 42 hr. Both raw and cooked chicken pieces were also frozen and stored for periods up to 6 months prior to analyses. Phospholipids were separated from muscle and skin, and identified primarily as phosphatidylethanolamine, phosphatidylserine, phosphatidylcholine, sphingomyelin and lysophosphatidylcholine. Total phosphorus content of phospholipids decreased during cooking in fresh corn oil by chemical reactions and/or by rendering fats from muscle. Phosphatidylcholine decreased the most. Use of reheated corn oil accentuated the changes in phospholipids. During frozen storage, phosphorus content of muscle decreased by an amount similar to that which occurred during cooking. Chicken skin contained less total phosphorus than muscle, and increased slightly during the cooking process.

INTRODUCTION

CHICKEN LIPIDS consist of neutral and phospholipids. The ratio between them is approximately 79 to 21 in muscle lipids, and 98 to 2 in skin lipids (Katz et al., 1966). Although phospholipids comprise a relatively small portion of total chicken lipids, Lee and Dawson (1973) showed that the phospholipids were more important in lipid deterioration based on the higher losses of fatty acids during cooking and during frozen storage than in the large portion of neutral lipids.

Further studies on the effect of cooking and frozen storage on phospholipids were made, including a classification of phospholipids from chicken lipids, and the quantitative changes in phospholipids during cooking and frozen storage.

PROCEDURE

Chicken processing and preparation

Chicken broilers, 7 wk of age, were obtained from a commercial farm and processed in the University poultry laboratory. After chilling 3 hr the birds were cut into portions identified as breasts, thighs, drumsticks and wings, packaged in Cryovac® bags and stored at −18°C. At appropriate time, chicken pieces were thawed at 3°C, breaded and cooked in fresh corn oil (Miesel brand, pure corn oil) or in corn oil previously heated to 200°C for 24 or 42 hr (Lee and Dawson, 1973). Some cooked and raw samples were stored up to 6 months at −18°C. Details of cooking procedures and preparation procedures were reported by Lee and Dawson (1973).

Isolation of phospholipids

Lipids from muscle tissue and skin tissue were extracted with chloroform-methanol-water 8:4:3 (v/v/v) according to method of Folch (Folch et al., 1957). Phospholipids were separated from neutral lipids by the procedures of Lee and Dawson, (1973).

Classification of phospholipids

Each sample, dissolved in chloroform containing 5–25 μg of phospholipids, was applied on a thin-layer chromatography plate along with standard materials obtained from Applied Science Laboratories, Inc. Each plate was developed in a chamber saturated with the solvent containing chloroform, methanol, acetic acid and water with the ratio of 25:25:4:2 by volume. After the development of the plate, the solvent was allowed to evaporate and the spots were made visible in iodine vapor.

The R_f value of each spot was obtained by dividing the distance moved by the solvent front into the distance moved by the compound (measured to the center of the spot). Both values were measured from the origin. Identification of each spot was made by comparing the R_f value to a standard.

Phosphorus content of chicken fats

Samples were dissolved in chloroform and applied on TLC plate containing approximately 200 μg phospholipids.

The plates were developed in a chamber saturated with a combination of chloroform, methanol, acetic acid and water with the ratio of 25:15:4:2 by volume. This solvent was allowed to rise to within 0.5 cm of the top of the adsorbent. Average running time was 1.5 hr.

After the plates were dried, they were exposed to iodine vapor, and each row of spots was immediately outlined. Each row of spots was scraped directly into a 30 ml Kjeldahl digestion flask. An adjacent area of blank silica gel corresponding in size and position to the areas containing phospholipid were also scraped into digestion flasks, in which 0.9 ml of perchloric acid and glass beads were added.

After complete digestion and cooling, 5 ml distilled water, 1 ml of 2.5% ammonia molybdate, 1 ml of 10% ascorbic acid, and 2 ml distilled water were added and the mixture was boiled for 5 min. Optical density of each solution at 820 mμ was measured using a Bausch & Lomb Spectronic 20, and phosphorus content was determined from a previously established and plotted standard curve.

RESULTS & DISCUSSION

Muscle phospholipids

The classes of chicken phospholipids were separated by TLC using a polar solvent system (Fig. 1), and identified by use of standards. They were phosphatidylethanolamine, phosphatidylserine, phosphatidylcholine, sphingomyelin, lysophosphatidylcholine and some minor phospholipids (Table 1).

Phosphatidylcholine, phosphatidylethanolamine and sphingomyelin were the predominant components of phospholipids from uncooked muscle fat and amounted to 85.2% of phospholipids. The high proportions of phosphatidylcholine and phosphatidylethanolamine is characteristic of chicken muscle fat (Table 2). These results are in agreement with those reported by Davidkova and Khan (1967) except for the slightly lower phosphatidyl serine and slightly higher sphingomyelin.

The total phosphorus content of phospholipids declined from 10.71 to 6.81 mg/g fat, or approximately 4 mg (Table 2) of phosphorus was lost during cooking in fresh corn oil. In other words, about 100 mg of phospholipids (4 mg × 25) were lost per 1 g of fat. The loss of phospholipids may be due to both the chemical deterioration and physical rendering of fats from muscle during cooking. The chemical deterioration of phospholipids may be characterized as autoxidation, hydrolytic decomposition, lipid "browning" reactions, and lipid-protein copolymerization reactions.

Each component of phospholipid declined during cooking, and phosphatidylcholine declined greatest in quantity, followed by sphingomyelin, lysophosphatidylcholine, phosphatidylserine and phosphatidylethanolamine (Table 2).

Greater total loss of phospholipids was found in the chicken cooked in corn oil previously heated for 42 hr than in fresh corn oil. The losses of phosphatidylethanolamine, sphingomyelin and phosphatidylcholine indicated that phosphatidyleth-

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anolamine and sphingomyelin are more sensitive to cooking in reused corn oil than in fresh corn oil. Since lysophosphatidylcholine is the hydrolytic product of phosphatidylcholine (Hanahan et al., 1954), its increase and the decrease in phosphatidylcholine strongly implied that hydrolysis occurred in muscle phospholipids during cooking in reused corn oil. More serious fat deterioration occurred when chicken was cooked in corn oil previously heated for 42 hr, than in fresh corn oil or that heated for 24 hr.

Fresh uncooked chicken muscle fats contained 9.71 mg of phosphorus per gram of fat. This decreased to 7.05 mg after 3 months storage, and to 6.30 mg after 6 months storage (Table 2). The mechanism of phospholipid deterioration during frozen storage is not clear. It is likely that several complicated reactions may be involved. They include oxidation, lipid "browning" reactions, lipid-protein co-polymerization reactions and lipolysis or enzymatic degradation. Changes in quantities of each component from muscle phospholipids, showed decreases of phosphatidylethanolamine, phosphatidylserine, phosphatidylcholine, sphingomyelin, lysophosphatidylcholine and minor phospholipids, but increases in the component which may include nonlipid phosphorus, peroxides and hydroxy compounds during the first three months, indicating oxidative deterioration. Phosphatidylcholine and lysophosphatidylcholine contined to decrease, whereas phosphatidylethanolamine and sphingomyelin did not decrease after 3 months of storage.

Lysophosphatidylcholine decreased during storage. This disagrees with the work of Davidkova and Khan (1967) who reported an increase. The samples studied by Davidkova were vacuum packed and oxidative degradation of the phospholipids in the samples was slow, with most deterioration due to lipolysis. However, the samples in this study were stored in polyethylene bags without vacuum sealing, and thus oxidative deterioration of phospholipids coupled with lipolysis were presumed to occur.

The total phosphorus in muscle fat from the birds cooked in fresh corn oil was 6.81 mg per gram, and decreased to 5.91 and 4.48 mg after 3 and 6 months storage respectively (Table 2). The changes in phospholipids during storage showed a loss of phosphatidylethanolamine during the first three months, and losses of phosphatidylethanolamine, phosphatidylcholine, sphingomyelin, and lysophosphatidylcholine during the second 3 months. Since phosphatidylethanolamine has been related to lipid browning deteriorations, its decrease may have been a consequence of involvement in the dark color in chicken found after storage. Thus it appears that the mechanisms for destruction of phospholipids in cooked chicken muscle during frozen storage are somewhat different from those of uncooked chicken.

A greater decrease in total phosphorus from the chicken cooked in reused corn oil than in fresh corn oil during storage, is shown in Table 2. Phosphatidylethanolamine, phosphatidylcholine, lysophosphatidylcholine decreased continuously through storage. This may indicate oxidative deterioration.

Skin phospholipids

The predominant components of phospholipids from uncooked skin fats were phosphatidylcholine, sphingomyelin and phosphatidylethanolamine. Skin fats had a relatively high proportion of phosphatidylcholine and a relatively higher proportion of sphingomyelin and a relatively lesser proportion of phosphatidylethanolamine found in muscle fats (Table 3).

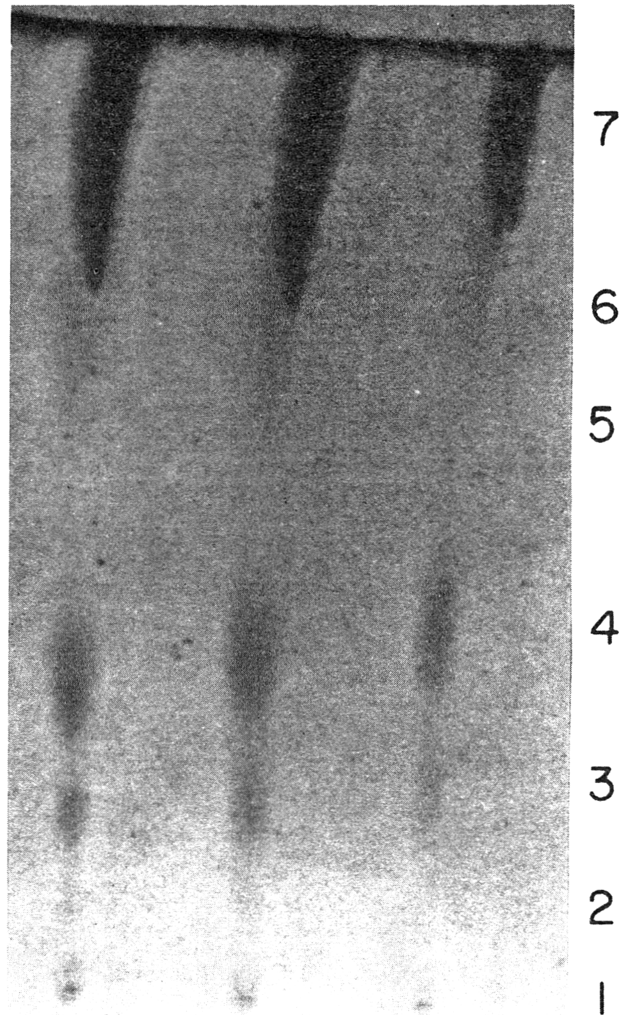

Fig. 1—Photograph of a thin-layer chromatogram showing separation of the classes of phospholipids: 1—Non lipid phosphorus; 2—Lysophosphatidylcholine; 3—Sphingomyelin; 4—Phosphatidylcholine; 5—Phosphatidylserine; 6—Phosphatidylethanolamine; 7—Neutral lipids and minor phospholipids.

Table 1—Identified classes of phospholipid

No. of spot	R_f of muscle phospholipid	R_f of skin phospholipid	R_f of standard materials	Classes of phospholipid
7	0.98	0.98		Neutral lipids & minor phospholipids
6	0.49	0.49	0.50	Phosphatidylethanolamine
5	0.47	0.47	0.46	Phosphatidylserine
4	0.26	0.26	0.26	Phosphatidylcholine
3	0.15	0.15	0.15	Sphingomyelin
2	0.08	0.08	0.07	Lysophosphatidylcholine
1	0	0		Everything below lysolecithin (nonlipid phosphorus, peroxides, hydroxy compounds)

Table 2–Changes in phospholipid content of chicken muscle lipids during cooking and frozen storage

	Storage time (mo.)											
	0				3				6			
Oil treatment[a]	Raw	A	B	C	Raw	A	B	C	Raw	A	B	C
Classes of phospholipids	Phosphorus content (mg p/g fat)											
Minor phospholipids	0.49	0.48	0.84	0.62	0.06	0.04	0.44	0.47	0.11	0.16	trace	trace
Phosphatidylethanolamine	2.68	2.51	2.27	1.85	2.08	1.69	1.54	1.31	2.08	1.37	1.17	1.17
Phosphatidylserine	0.36	trace	trace	trace	trace	–	–	–	–	–	–	–
Phosphatidylcholine	4.99	3.22	3.22	3.09	3.73	3.56	2.63	2.50	3.45	2.68	2.34	2.14
Sphingomyelin	1.46	0.36	0.12	trace	0.38	0.38	0.19	0.19	0.38	0.23	0.21	0.05
Lysophosphatidylcholine	0.73	0.24	0.36	0.74	0.36	0.24	0.18	0.22	0.16	0.02	trace	trace
Everything below lysolecithin (nonlipidphosphorus, peroxides, hydroxy compounds)	trace	trace	trace	trace	0.44	trace	0.23	0.27	0.12	0.02	0.02	trace
Total	10.71	6.81	6.81	6.30	7.05	5.91	5.21	4.96	6.30	4.48	3.74	3.36

[a] A–Fresh corn oil; B–Corn oil previously heated for 24 hr; C–Corn oil previously heated for 42 hr.

Table 3–Changes in phospholipid content of chicken skin lipids during cooking and frozen storage

	Storage time (mo.)											
	0				3				6			
Oil treatment[a]	Raw	A	B	C	Raw	A	B	C	Raw	A	B	C
Classes of phospholipids	Phosphorus content (mg p/g fat)											
Minor phospholipids	–	0.01	0.02	0.02	0.01	0.01	0.01	0.03	0.01	0.01	0.01	0.01
Phosphatidylethanolamine	0.09	0.12	0.13	0.13	0.09	0.13	0.10	0.09	0.07	0.13	0.10	0.10
Phosphatidylserine	trace	trace	–	–	–	–	–	–	–	–	–	–
Phosphatidylcholine	0.24	0.37	0.37	0.32	0.23	0.29	0.23	0.26	0.12	0.22	0.20	0.20
Sphingomyelin	0.14	0.10	0.11	0.10	0.10	0.09	0.06	0.07	0.06	0.07	0.06	0.07
Lysophosphatidylcholine	0.01	0.13	0.10	0.08	0.03	0.11	0.05	0.10	0.03	0.05	0.04	0.03
Everything below lysolecithin (nonlipid phosphorus, peroxides, hydroxy compounds)	0.02	–	0.01	0.05	0.02	0.02	0.04	0.03	trace	0.02	0.02	0.03
Total	0.50	0.73	0.74	0.70	0.48	0.65	0.49	0.58	0.29	0.30	0.43	0.44

[a] A–Fresh corn oil; B–Corn oil previously heated for 24 hr; C–Corn oil previously heated for 42 hr.

In contrast to the effect in muscle phospholipids, the total phosphorus of skin phospholipids increased from 0.50 to 0.73 mg/g fat after cooking in fresh corn oil (Table 3). Except for sphingomyelin, increases of other components were found. The apparent increase of phospholipid in skin lipids is presumed to be due to the loss of rendered fat, primarily neutral lipids.

Total phospholipid content of skin lipids decreased slightly in the chicken cooked in the corn oil previously heated for 42 hr (Table 3). Thus chemical reactions which occur in chicken cooked in fresh corn oil may vary with different tissues and oil treatments.

Fresh uncooked chicken skin fats contained 0.50 mg lipid phosphorus per gram. After 3 and 6 months storage, lipid phosphorus decreased to 0.48 and 0.29 mg respectively. The loss of phospholipids was due mainly to a loss of phosphatidylcholine and sphingomyelin, coupled with a minor loss of phosphatidylethanolamine. Since lysophosphatidylcholine is a hydrolytic product of phosphatidylcholine, it increased slightly and phosphatidylcholine decreased. This suggests that some degree of lipolysis occurred in skin phospholipids during storage (Table 3). The hydrolytic enzymes could be affected by many factors, such as the build up of substrates, oxidative reactions resulting in enzyme inhibition, and concentration of solutes during freezing and frozen storage. These factors may affect the mode of attack or the orientation of substrates, which in turn, affect the selectivity and rate of hydrolysis (Braddock and Dugan, 1972).

Chemical changes and physical rendering of lipids from chicken muscle and skin occurred during cooking and during frozen storage. The use of reheated cooking oil accentuated these changes, thus chicken cooked in reused corn oil was less stable during storage than that cooked in fresh corn oil.

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Ms received 7/13/74; revised 10/25/75; accepted 10/28/75.

Michigan Agricultural Experiment Station Journal Article No. 6870.

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SCANNING ELECTRON MICROSCOPY OF AGED FREE AND RESTRAINED BOVINE MUSCLE

ABSTRACT

Scanning electron microscopy was used to investigate changes in the membranous structures and in the myofibrils of free and restrained bovine muscle on aging. The various components of the sarcolemma were identified and the extensive ultrastructural changes in this complex membranous structure during postmortem aging are described. Possible contributions of the structural components of the sarcolemma, particularly the collagen fibrils and the amorphous layers, to tenderness are discussed. Progressive disruptions in the Z line and breaks at the Z-I junction occurred in both free and restrained myofibrils during aging.

INTRODUCTION

THE SCANNING ELECTRON MICROSCOPE (SEM) has only been used by a few investigators to study the ultrastructure of aged muscle (Schaller and Powrie, 1971; Stanley and Geissinger, 1972; Eino and Stanley, 1973; Stanley, 1974). Some of these studies concentrated on the surface topography of muscle at the fiber level (Stanley and Geissinger, 1972; Eino and Stanley, 1973) while others described the ultrastructure of myofibrils (Schaller and Powrie, 1971; Stanley, 1974). However, none of these studies presented a detailed description of the membranous complex surrounding the muscle fiber in at-death muscle or the ultrastructural changes in this complex on aging even though it is notably responsible for some of the mechanical properties of the muscle fibers (Franzini-Armstrong, 1973). Furthermore, a comparison of the surface ultrastructural changes in free and restrained muscles on aging has not been reported. Therefore, we felt that it would be valuable to present a description of the surface ultrastructural changes in the fiber membranous structures and in the myofibrils from free and restrained muscles on aging and to discuss the possible relationships between structural changes and tenderness.

MATERIALS & METHODS

DETAILS of the muscle treatments, the storage temperature and the storage times are given in Varriano-Marston et al. (1975a). A modification of the procedure by Schaller and Powrie (1971) was used to prepare bovine Longissimus dorsi samples for SEM. Samples approximately 1 cm^3 were cryofractured in liquid nitrogen and 3 mm fragments were transferred to a solution of 2% glutaraldehyde, 0.1M sodium cacodylate buffer (pH 7.0) held at 3°C. The fragments were stored in this solution for 1 hr at 3°C, rinsed 3 times with 0.25M sucrose and placed in 1% OsO_4, 0.125M sucrose overnight at 3°C. Specimens were dehydrated in a graded acetone series (25–100%), critical point dried with acetone-CO_2, mounted on aluminum stubs, and coated with carbon and gold in a vacuum evaporator. Scanning electron micrographs were taken on the Cambridge Stereoscan Mark III operated at 20 KV.

RESULTS

At-death muscle

Scanning electron micrographs of at-death muscle samples are shown in Figure 1. Severe contraction of the muscle fibers has resulted in the disruption of the membranous complex surrounding the muscle fiber (Fig. 1a and 1b). The nomenclature designating the components of this complex varies among authors. Franzini-Armstrong (1973) defines the multilayered complex as a sarcolemma, a term originally employed by light microscopists. She designates three components of the sarcolemma: the collagen fibrils, the basement membrane (amorphous layer) and the plasma membrane. In an earlier work, Mauro and Adams (1961) included an outer membrane as a fourth component of the sarcolemma. Other physiologists reserve the term "sarcolemma" for the plasma membrane only, and the terminology relating to the other membranous components surrounding the fiber is not well-defined (Bloom and Fawcett, 1975; Rhodin, 1974). This work will follow the three component designation of Franzini-Armstrong (1973).

Two components of the sarcolemma can be identified in the scanning electron micrographs shown in Figure 1. The endomysium has been pulled away from the muscle fibers in Figure 1a so that the individual muscle fibers are more clearly identified. The collagen fibrils described by Franzini-Armstrong (1973) are indicated in Figure 1a. A more careful examination of the muscle fibers showed the outer surface of the intact sarcolemma (Fig. 1b and 1c) and some remnants of what might be the amorphous layer (Fig. 1b). Removal of these sarcolemma components from the fiber reveals the myofibrillar surfaces shown in the micrographs of aged muscle in Figures 3a through 4e.

Postmortem changes in the sarcolemma

In general, the patterns of structural changes in the sarcolemma on aging were similar for both free and restrained muscles, so only a few micrographs are presented in Figure 2 to illustrate the progressive degradation of this membranous complex during the postmortem storage of muscles at 2°C. The outer surface of the sarcolemma that was puckered and disordered in the at-death samples (Fig. 1a) appears to be more organized and in a "relaxed" state after 24 hr postmortem (Fig. 2a). The underlying amorphous layer has undergone some structural disintegration so that the individual myofibrils are beginning to become distinguishable. After 4 days of postmortem storage, the components of the sarcolemma are severely degraded (Fig. 2b), and by 12 days postmortem (Fig. 2c) the remaining membranous structures have agglomerated into small masses on the surface of the myofibrils.

Postmortem changes in myofibrils

Micrographs of myofibrils from free and restrained muscles stored for various times at 2°C are presented in Figures 3 and 4, respectively. The most distinguishing structural characteristic in these micrographs is the transverse ridges delineating the sarcomeres. The interpretation of these transverse ridges seen at the level of the Z line in striated muscle remains in doubt. Sybers and Ashraf (1973) interpret these ridges in heart muscle as transverse tubules rather than protrusions of the Z line material. They based their conclusions on observations on transmission electron micrographs showing that the region of the Z line appeared to be slightly indented as compared to the

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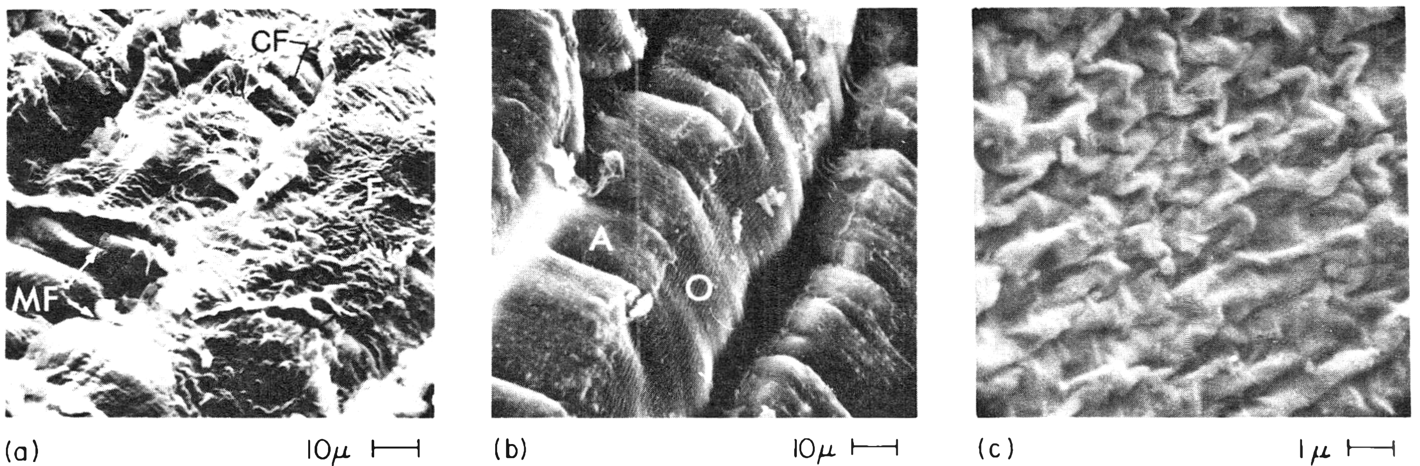

Fig. 1–At-death longissimus dorsi: (a) the contracted muscle reveals the muscle fibers, MF, the endomysium, E, and the collagen fibers, CF; (b) another area of the same sample as in (a), A, indicates the amorphous layer, O indicates outer surface of the sarcolemma; (c) higher magnification of the outer surface of the sarcolemma.

A band of the sarcomere. However, stereo pairs of aged muscle taken with the high voltage transmission electron microscope indicate that the reverse is true, i.e., the Z lines are positioned higher than the A bands (Varriano-Marston et al., 1975b). Since the 3-dimensional structure of muscle obtained with stereo pairs reveals more structural and spatial details than are observed in conventional electron micrographs, in the discussion presented below we assume that the transverse ridges are the Z line structures.

The 24 hr postmortem myofibrils shown in Figure 3a resemble the ultrastructure of cold-shortened muscle described by investigators using the transmission electron microscope (Stromer and Goll, 1967; Henderson et al., 1970). The myofibrils are severely contracted as is evident from the contraction bands at the Z line suggesting that the A band filaments were pushed against or through the Z line (Hoyle et al., 1965). However, the low resolving power of the SEM does not allow us to observe the myofilaments, so these structural changes in the banding patterns cannot be identified.

The free and restrained myofibrils stored for 2 days postmortem (Fig. 3b and 4a) are morphologically similar. Neither sample has the super-contracted appearance of the 24 hr myofibrils (Fig. 3a) but breaks and clumping at the Z line have begun to appear. The 2-day restrained myofibrils (Fig. 4a) exhibit the waves of rigor described by several investigators (Paul et al., 1944; Ramsbottom and Strandine, 1949; Lowe, 1948; Voyle, 1969). Voyle (1969) postulates that these waves of rigor are caused by the passive contraction of a group of fibers in response to the constraining influence of actively shortened fibers.

Additional disturbances in the myofibrillar structure occur with increased storage time at 2°C, but again, no differences between free and restrained muscles were observed. The loss of Z line material first noted after 2 days (Fig. 3b and 4a) is continuous during postmortem storage, reaching a maximum at 9 days postmortem (Fig. 3e and 4d) with no further changes at 12 days (Fig. 3f and 4e). Myofibrillar breakages are apparent in many of the postmortem samples and become progressively more frequent as storage time increases. The breakage pattern shown in the 4-day free (Fig. 3c) and the 6-day

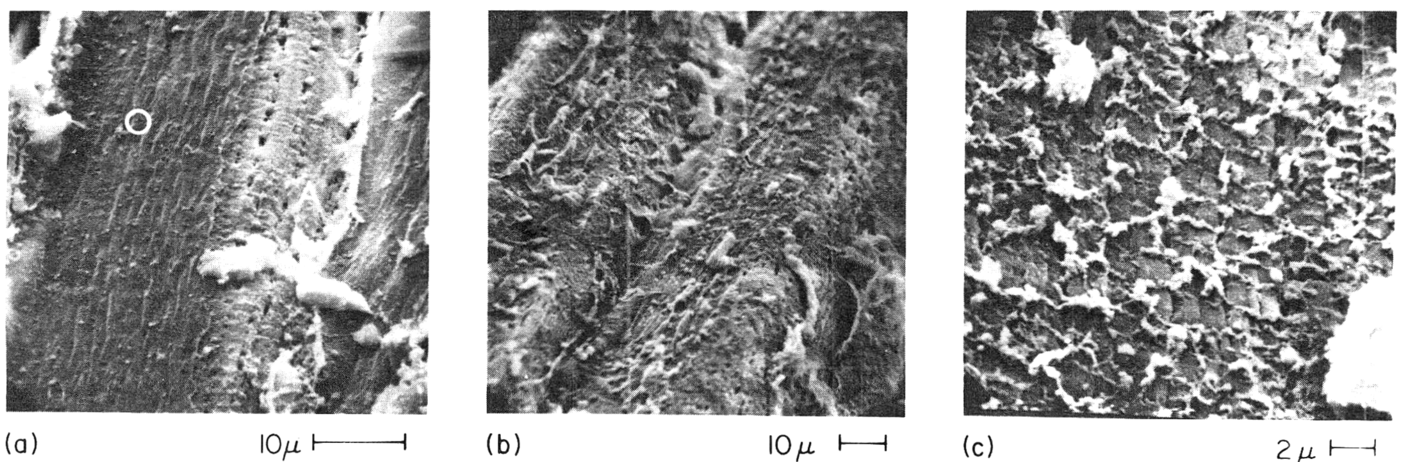

Fig. 2–Progressive degradation of the sarcolemma during postmortem (PM) aging: (a) 24 hr PM; O, outer surface of the sarcolemma; A, amorphous layer; (b) 4 days PM; (c) 12 days PM.

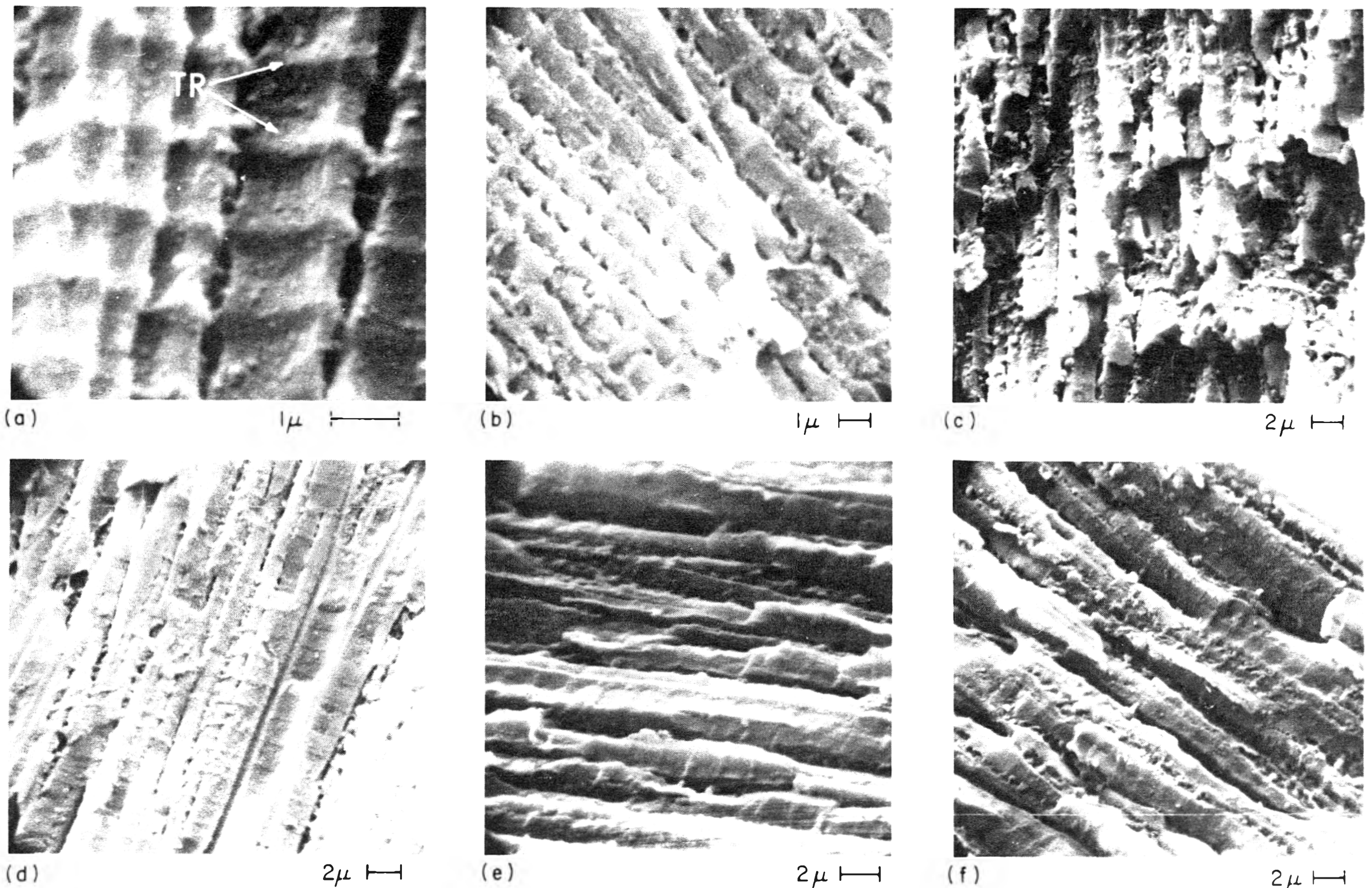

Fig. 3—Postmortem (PM) unrestrained longissimus dorsi: (a) 24 hr PM; TR, transverse ridges; (b) 2 days PM; (c) 4 days PM; (d) 6 days PM; (e) 9 days PM; (f) 12 days PM.

restrained (Fig. 4c) muscles was frequently observed at all postmortem storage times except for 24 hr (Fig. 3a). These breaks are a result of the cryofracture procedure and occur at the weakest points in the structure, presumably the Z-I junctions.

DISCUSSION

THE VIOLENT CONTRACTION and accompanying disruption of the structure in the at-death muscle samples may be a result of the pre-rigor excision of the muscle from the carcass. Muscles cut from the carcass immediately after death have been found to shorten up to 20–25% of their original length (Herring et al., 1964) and to develop irregular contraction bands (Cassens et al., 1963a). In addition, thaw-contracture initiated by the sample preparation procedure may also have contributed to the severe contraction of the muscle fibers. Several investigators, using light and transmission electron microscopes, have described the macro- and micro-structure of pre-rigor muscle undergoing thaw-contracture (Marsh and Thompson, 1958; Cassens et al., 1963a, b; Herring et al., 1964; Luyet et al., 1965; Luyet, 1966, 1968), but the surface ultrasturcture of thaw-rigor muscle has not been previously described. However, the muscle fiber structure shown in Figure 1b closely parallels the light microscopic observations of Luyet et al. (1965) on muscle fiber bundles subjected to thaw-contracture.

Considerable evidence indicates that meat tenderness is markedly influenced by changes in the myofibrillar contraction state pre-rigor (Newbold and Harris, 1972). However, researchers have generally neglected to consider the possible role of the sarcolemma in determining the tenderness of muscle. Studies on the mechanical properties of this complex membrane structure indicate that it has a high tensile strength (Casella, 1951; Mauro and Sten-Knudsen, 1952; Street and Ramsey, 1965; Fields, 1970) and that the principal rigidity of the sarcolemma is probably due to the collagen fibril layer (Fields, 1970). The amount of collagen in muscle does not significantly change during postmortem aging (Goll et al., 1970). However, changes in the molecular structure of collagen fibrils during aging as well as enzymatic degradation of the mucopolysaccharide-like substances of the amorphous layer in which the collagen is embedded (Dutson, 1974) undoubtedly contribute to the structural changes in the sarcolemma during postmortem storage. Therefore, it becomes apparent that tenderness or toughness in muscle is affected by the combined sarcolemma components rather than just the collagen fibrils.

Disruptions in the sarcolemma on aging may also influence tenderness by affecting the flow of ions and other substances into the cells. Once the sarcolemma is disrupted, the free diffusion of extracellular ions, i.e., Na, Cl, Ca, into the sarcoplasm changes the natural ionic environment surrounding the myofibrils. These extracellular ions in combination with the intracellular K, Mg and Ca may alter the myofibrillar structure through shifts in ion binding and changes in water-holding

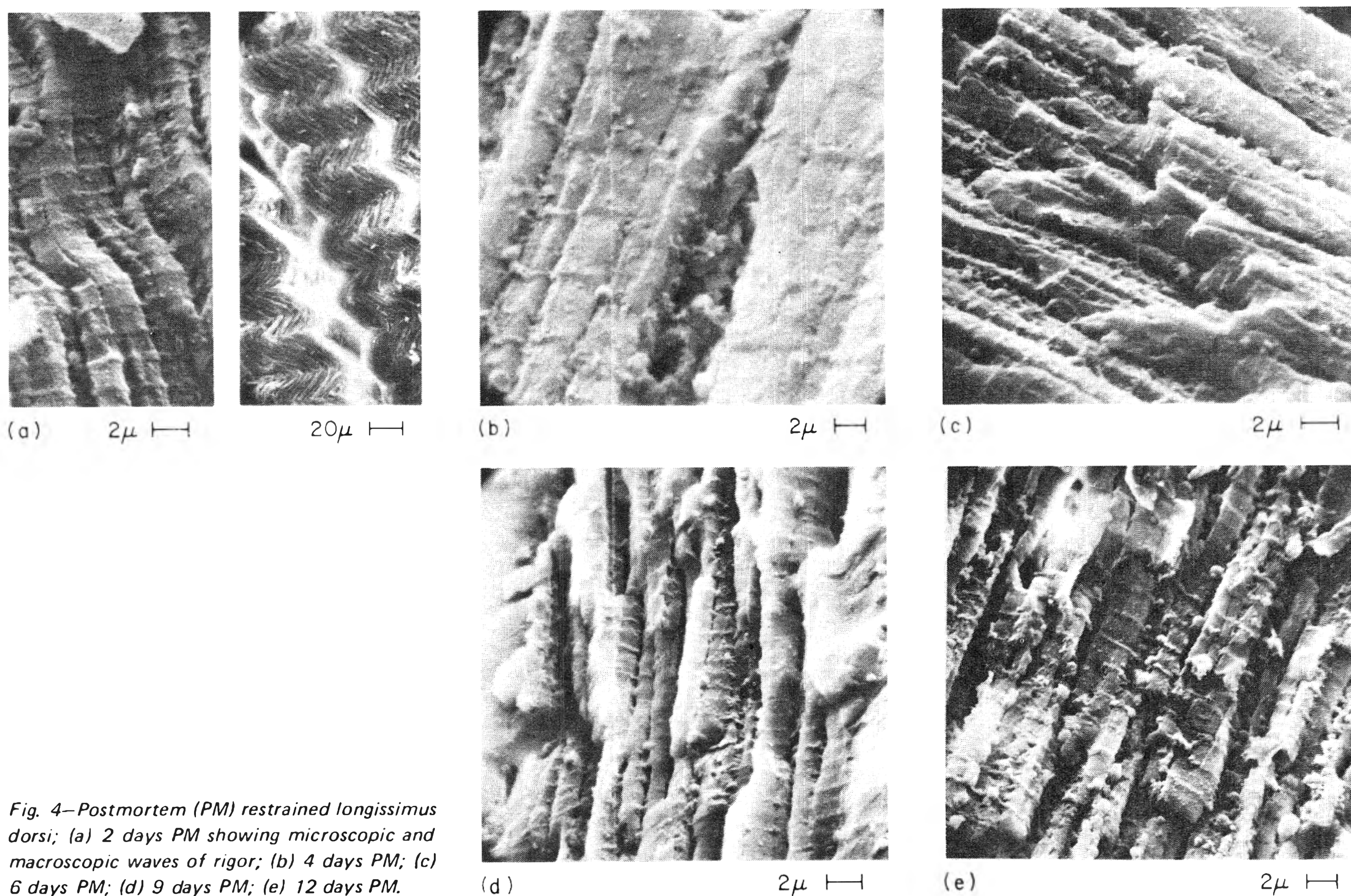

Fig. 4–Postmortem (PM) restrained longissimus dorsi; (a) 2 days PM showing microscopic and macroscopic waves of rigor; (b) 4 days PM; (c) 6 days PM; (d) 9 days PM; (e) 12 days PM.

capacity and such changes could ultimately affect tenderness (Hamm, 1960).

Degradation of the Z lines of the myofibrils was observed although in not as much detail as observed by transmission electron microscopy. However, it was not possible to distinguish differences in degradation patterns between free and restrained muscles. Differences in states of contraction were observed. Locker (1960), for example, has related contraction state to tenderness. On the other hand, Gothard et al. (1966) have indicated that contraction played a lesser role in tenderness. Furthermore, the contribution of the structural components of the sarcolemma, particularly the collagen fibril and amorphous layers, to tenderness cannot be ignored. Since the sarcolemma controls the ionic milieu surrounding the myofibrils, the necessity of X-ray microanalysis to determine the effect ionic concentration and movements have on the myofibrillar structure is further emphasized.

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Ms received 8/9/75; revised 11/24/75; accepted 12/2/75.

Paper No. 9189, Scientific Journal Series Article, Minnesota Agricultural Experiment Station, St. Paul, MN 55108.

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COMPOSITION AND PROPERTIES OF AN ANIMAL PROTEIN ISOLATE PREPARED FROM BONE RESIDUE

ABSTRACT

A protein isolate was prepared from the bone residue of mechanically deboned chicken necks and backs. Typically, the isolate contained 60–65% protein, 23–25% lipid, 5–10% ash and 4–6% moisture. Solubility and emulsifying capacity of the isolate were enhanced at elevated pH and ionic strength and in the presence of polyphosphates. The latter were effective in improving emulsifying capacity when used in combination with NaCl but not when used alone.

INTRODUCTION

NEW SOURCES of food protein are needed to bolster the world's supply. These new sources will no doubt include protein concentrates and isolates prepared from both plant and animal materials (Levin, 1959, 1970; Parkes and May, 1968; Lawhon and Cater, 1971; Satterlee et al., 1973; Tybor et al., 1973; Toledo, 1973; Young, 1975). One potentially important source of protein, bone residue from meat deboning processes, is lost from human food channels. This paper describes the preparation, composition and some of the properties of a protein isolate prepared from the bone residue from a poultry deboning machine.

METHODS & MATERIALS

Bone residue

The bone residue (BR) used in this study was recovered from a commercially operated Mark IV Princeworld deboner. The meat source was broiler necks and backs. The BR contained 40% solids of which 43% was protein, 32% fat and 25% ash (AOAC, 1970). The BR was stored at −30°C and was tempered for 24 hr at 0–2°C before use.

Preparation of protein isolate

The animal protein isolate (API) was prepared as shown in Figure 1. One part by weight BR was mixed with three parts by volume of 0.1M Na maleate, pH 7.0 containing sufficient NaCl to make the ionic strength of the solvent 0.5. No attempt was made to account for the contribution of BR minerals to ionic strength. The pH remained close to 7.0 after the BR was mixed with the buffer. The solvent was chilled to 2°C before use. The mixture was blended for 1 min in a 5000 ml Waring Blendor at maximum speed and then was allowed to set without mixing for 2 hr at 2°C. The mixture was filtered through two layers of cheesecloth to remove large particles and floating fat. The filtrate was diluted to ionic strength 0.2 and allowed to set for 2 more hours at 2°C. The supernatant was decanted. The precipitate was washed twice with cold water and then freeze dried. Plate temperature was 20°C. Final product temperature was 10°C. For purposes of this paper the dried product will be referred to as API.

Composition of the API

Proximate composition of the API was evaluated by AOAC (1970) methods. Protein was taken as Kjeldahl N × 6.25. Lipid was evaluated by extraction, ash by combustion and moisture by vacuum drying. Percent solids was obtained by dividing dry weight by total weight.

Protein solubility

Protein solubility was evaluated in a manner similar to that described by Tybor et al. (1973). One-half gram API was dispersed in 50 ml solvent and shaken mechanically for 2 hr. The supernatant was centrifuged to sediment the insoluble material and the soluble protein determined by the procedure of Lowry et al. (1951). Soluble protein was expressed as a percentage of the total protein in the sample.

Emulsifying capacity

The emulsifying capacity (EC) was evaluated in a manner similar to that of Swift et al. (1961). Briefly, the isolate was dispersed in 50 ml solvent for 1 min with an Omni-Mixer which was cooled in an ice bath. Corn oil was added at a rate of 15 ml/min until the emulsion "broke" as evidenced by a sudden decrease in viscosity. The EC was expressed as ml oil emulsified per total mg protein in the sample.

Experimental conditions

Four experimental conditions were evaluated: protein concentration, solvent pH, solvent ionic strength and the presence of polyphosphates.

Protein concentration. Duplicate protein suspensions were prepared to contain 1.6, 2.4, 3.2, 4.0 or 4.8 mg total protein per ml. The EC of these solutions were then determined.

Solvent pH. The effects of solvent pH on solubility and EC of the API were evaluated as follows: Buffers of 0.05M concentration were adjusted to pH 4.0, 5.0 (biphthalate), 6.0, 7.0, 8.0 (phosphate monobasic), 9.0 (borate), or 10.0 (carbonate-borate) and 0.5M NaCl. The protein solubility and EC of the API in these solvents were determined in duplicate.

Solvent ionic strength. The API was dispersed in water and desalted with an Amicon model 404 ultra-filter. The desalted dispersion was adjusted to 0.05M phosphate, pH 8.0. Duplicate aliquots were then adjusted to ionic strength 0.1, 0.2, 0.3, 0.4 or 0.5 with NaCl. Solubility and EC were evaluated in duplicate.

Polyphosphates. Solubility and EC of the API were evaluated in duplicate in the presence of 0.0, 0.2, 0.4 or 0.6% sodium pyrophosphate or sodium tripolyphosphate with and without 0.6M NaCl.

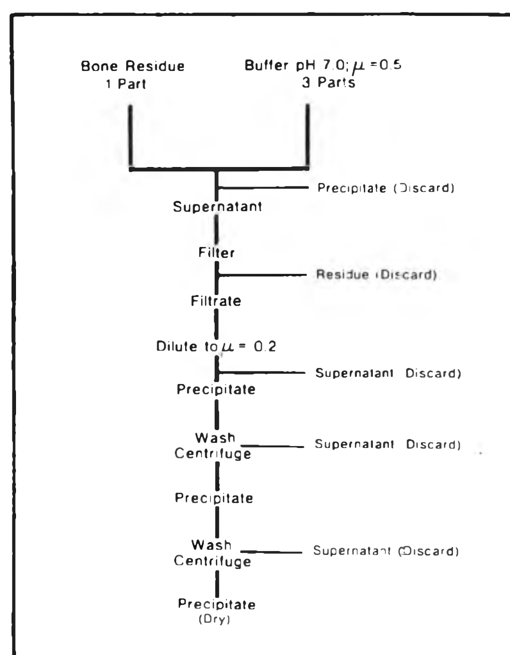

Fig. 1—Preparation of protein isolate from bone residue.

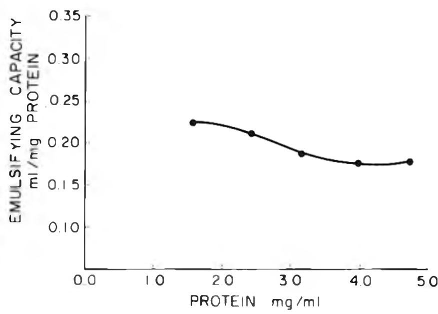

Fig. 2–Emulsifying capacity as a function of protein concentration.

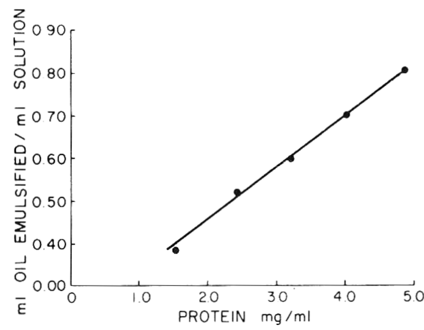

Fig. 3–Oil emulsified/ml solution as a function of protein concentration.

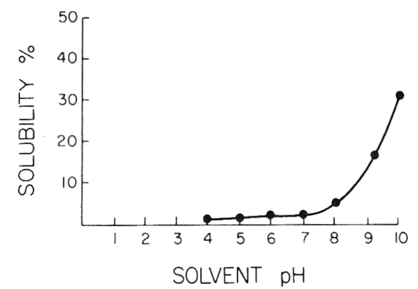

Fig. 4–Protein solubility as a function of solvent pH.

RESULTS & DISCUSSION

Composition of the API

A typical preparation of the API contained 4.8% moisture, 62.9% protein, 24.1% lipid and 8.8% ash, but composition tended to vary from lot to lot. This variation was attributed to differences in efficiency of fat removal. We are trying to find procedures to minimize the variation and quantity of fat in the API.

Yield of isolate also varied somewhat. Typical yields were 2–3g/100g of BR. We are trying to improve the total yield by recovering other isolates from the unextracted residue.

Protein concentration

As the protein concentration increased, the EC decreased slightly (Fig. 2). However, the total amount of oil emulsified per ml suspension increased with protein concentration (Fig. 3). These observations are consistent with those of Swift et al. (1961) and Ivey et al. (1970) who found that as protein concentration increased, EC decreased. Saffle (1968) attributed this decrease to "overloading" of the system, i.e., part of the emulsion remained on the side of the jar and there was imcomplete mixing as the end point was approached. More recently, Ivey et al. (1970) suggested that increasing the protein concentration caused thicker emulsifying agent (protein) layers to form on the fat droplets thus using more continuous phase per drop. As a result, they indicated that the EC is inversely related to protein concentration. Even though this relationship is inverse it is nonlinear (Saffle, 1968). Thus, the total amount of oil emulsified per ml solution should increase with protein concentration. For this reason, a constant dispersion of 1% API was used for all later EC tests. Calculation of the EC was based on the total amount of protein actually present in the sample.

Solvent pH

Solubility of the protein was only slightly below pH 7.5 but increased with pH above 7.5 (Fig. 4); pH had a similar effect on the EC (Fig. 5). The EC was relatively low at low pH but improved as pH increased. This relationship illustrates how a soluble protein is a more effective emulsifier than an insoluble one. Apparently when protein solubility and/or emulsifying capacity are important qualities, the API might be most effectively used in products with pH values greater than 7.5.

Solvent ionic strength

As the ionic strength of the solvent increased above 0.2, the solubility of the protein increased significantly at $p = 0.05$ (Fig. 6). This solubility pattern was expected since the protein was extracted from the bone residue with a high ionic strength solvent and precipitated at ionic strength 0.2.

The EC also increased with ionic strength (Fig. 7). These observations suggest that the API might most effectively be utilized in products with high pH and ionic strength.

Polyphosphates

The effects of sodium pyrophosphate and sodium tripolyphosphate on solubility of API with and without 0.6M NaCl are shown in Figure 8. Increasing the concentration of either polyphosphate increased the solubility and adding 0.6M NaCl did not appear to offer any added benefit. The EC (Fig. 9) did not follow this same pattern, however. Even though neither polyphosphate when used alone improved the EC, the improvement was marked in the presence of NaCl. This improvement may have been due to the general effects of ionic

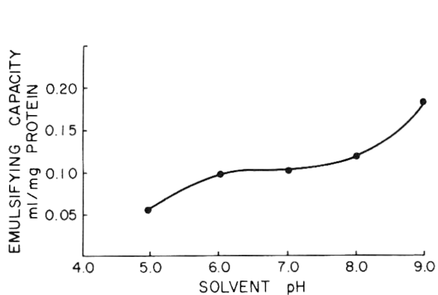

Fig. 5–Emulsifying capacity as a function of solvent pH.

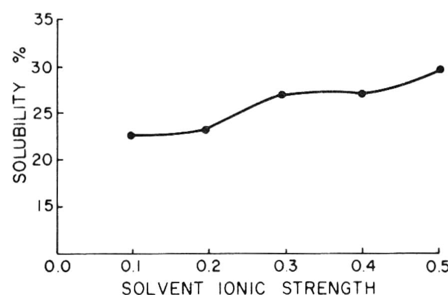

Fig. 6–Protein solubility as a function of solvent ionic strength.

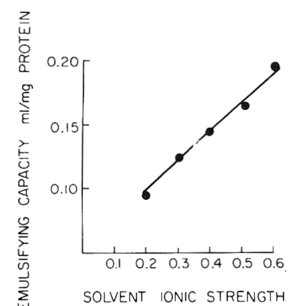

Fig. 7–Emulsifying capacity as a function of solvent ionic strength.

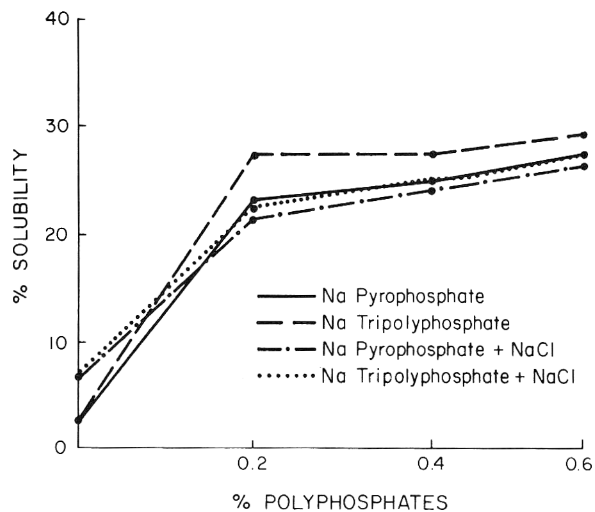

Fig. 8–Effect of sodium pyrophosphate and sodium tripolyphosphate on solubility of API with and without 0.6M NaCl.

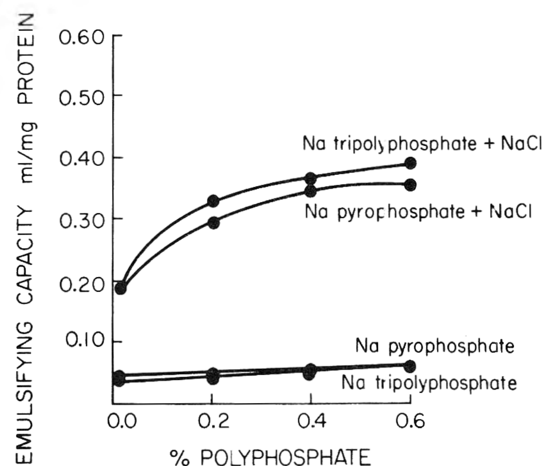

Fig. 9–Effect of sodium pyrophosphate and sodium tripolyphosphate on emulsifying capacity of API with and without 0.6M NaCl.

strength and pH or to a specific effect of polyphosphates on meat proteins (Hamm, 1971; Young, 1974). Further work will be necessary to evaluate these possibilities.

These experiments suggest that a protein isolate prepared from bone residue might have some use as a food ingredient. Elevated pH and ionic strength and the presence of polyphosphates improved the solubility and emulsification characteristics of the isolate. However, more data are necessary on such functional characteristics as gelling, foaming and heat stability.

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Ms received 8/30/75; revised 11/11/75; accepted 11/20/75.

The author thanks Mr. William Day for his assistance in this study.
Reference to company or product names does not imply approval or recommendation of the U.S. Department of Agriculture to the exclusion of others that may be suitable.

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OBJECTIVE EVALUATION OF TEXTURE OF MINCED BLACK ROCKFISH (Sebastes spp.) DURING FROZEN STORAGE

ABSTRACT

Objective measurements (shear values and drip loss) were made to characterize changes in texture of frozen binder-modified blocks of minced black rockfish as a part of a continuing study. Effect of variation in water content on sensory texture scores of washed-modified blocks was also determined. Objective measurements correlated with sensory evaluation of texture of fillets and minced products. Varying moisture content of the washed-modified product up to 4.5% above normal moisture content did not significantly affect texture, but lowering it to 1.6% below significantly reduced texture scores. Washing minced flesh before freezing into blocks resulted in higher shear values and cooked drip. Addition of a binder containing NaCl and sodium tripolyphosphate resulted in improved texture and lower shear values and cooked drip.

INTRODUCTION

IN A CONTINUING study of the variables affecting the quality of frozen minced blocks of black rockfish (*Sebastes* spp.). Miyauchi et al. (1975) reported that binder-modified blocks made with washed minced muscle were significantly better in color and flavor than those made with unwashed minced muscle during 12-month storage at −18°C. The results of objective measurements to characterize changes in texture of these blocks are reported here. Objective measurements included shear values and drip loss of the cooked flesh. The effect of variation in water content on the sensory texture scores of washed-modified blocks was also determined.

EXPERIMENTAL

Sample preparation

In two replicate experiments done in November and March, black rockfish commercially iced for 5 days in the round were used to prepare minced fish muscle as described by Miyauchi et al. (1975). The belt tension on the meat-bone separator (Bibun Model 15 with a drum perforated with 7-mm diameter holes) was set at light pressure.

Unmodified minced muscle (MM) blocks. Quantities (500g) of MM were packed into waxed frozen-food cartons (1-1/8 in. × 8-1/2 in. × 3 in.) and frozen in metal forms under pressure in a vertical plate freezer (about 2 hr at −40°C) into small blocks. The blocks were sealed in 2-mil polyethylene pouches and stored at −18°C on shelves with 3/4 in. clearance between packages. All of the blocks listed below were prepared, frozen and stored similarly.

Modified MM blocks. One hundred parts of MM were mixed with a fish-binder homogenate, which consisted of 2.5 parts fish muscle, 1 part NaCl, 0.15 parts sodium tripolyphosphate, 1 part sugar, 0.3 part monosodium glutamate, 1 part corn oil and 5 parts water.

Unmodified-washed blocks. The MM was washed (ratio of 5 parts by weight of water to 1 part by weight of MM), and the resultant fish-water slurry was dewatered by gravity draining.

Modified-washed MM blocks. The washed MM with the addition of fish binder was used.

Blocks of varying moisture content. MM blocks of varying moisture content were prepared by dewatering the washed minced muscle in a Bock centrifuge (Model 15RC) for intervals varying from 2–30 min: 2-min centrifuged muscle (84.0% moisture), 10-min centrifuged muscle (81.9% moisture) and 30-min centrifuged muscle (77.9% moisture). Unwashed MM was used as the control sample (79.5% moisture).

Fillets. Some rockfish from each of the various lots received at the laboratory were cut into skinless fillets. The two fillets from each individual fish were washed, drained, packed into 1-lb frozen-food cartons, and plate frozen. The cartons were inserted into 2-mil polyethylene pouches, sealed and stored at −18°C.

Reference samples. At each examination, modified-unwashed minced rockfish blocks were prepared from commercially caught, iced rockfish for use as a reference sample.

Sensory evaluation

The various minced rockfish blocks were evaluated at intervals during 12 months of storage at −18°C. They were cut into 5/8 in. × 1-1/8 in. × 3 in. sticks, battered and breaded, and deep-fat fried for 5 min at 177°C. The cooked sticks were presented as coded samples to a panel of 6 to 12 experienced judges and rated on a 5-point scale in comparison to the identified, freshly prepared reference sample, which was assigned the score of 5. The 5-point rating scale was: 5–very good; 4–good; 3–fair; 2–borderline; 1–poor. The differences in the mean texture scores among the various samples were analyzed by analysis of variance (Snedecor and Cochran, 1956).

Shear texture and percent cooked drip determination

Shear texture and percentage cooked drip were determined on 5/8 in. slices of the various blocks cooked from the frozen state. Slices were steamed in covered aluminum containers for 20 min, cooled to room temperature, and the drip was poured off and weighed. The cooked drip loss was expressed as a percentage of the starting frozen weight.

Shear texture was determined on the shear texture instrument of Dassow et al. (1962). Samples (15g) of cooled muscle were inserted in the jaws of the texture instrument, compressed, and sheared. The maximum hydraulic pressure recorded in the instrument gauge represented the sample shear texture. Ten to 12 determinations were made on each of two minced block samples at each 4-month examination period. Six determinations were made on each of 10–30 fillet block samples at various time intervals.

Moisture content determination

The moisture content was determined according to the method of Miyauchi et al. (1975).

RESULTS & DISCUSSION

Comparison of shear texture of fillets and MM blocks

Variation in shear texture for black rockfish blocks made from fillets, unmodified MM, and binder-modified MM during storage at −18°C up to 180 days is shown in Figure 1. The coefficient of variation of the plotted points ranged from about 4–17%. The higher coefficients of variation were related to either variance between individual fillets or the occasional presence of a bone in some of the MM samples. The shear texture values of fillets from individual fish varied widely at the initial examination but within 5–6 months reached values of over 40. Our sensory panel generally rated the samples unacceptably tough when shear values were greater than 30–35. Blocks made from the MM without binder were initially slightly lower in toughness than those made from individual fish fillets but had shear values over 30 within 4 months. However, blocks made from MM modified with binder were initially more tender than the most tender fillets and remained tender and in the acceptable range during frozen

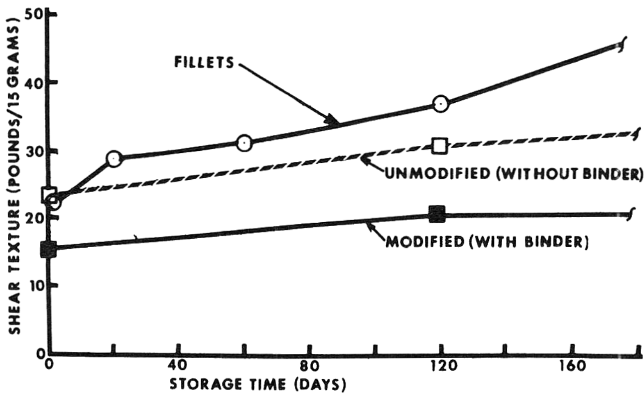

Fig. 1–Variation of shear texture in black rockfish blocks made from fillets, unmodified minced muscle, and binder-modified minced muscle, stored at −18°C. (○ represents 60–180 determinations; □ and ■ represent 20–24 determinations.)

storage (Fig. 1). Thus, to extend the useful frozen storage life of firm-textured species such as the black rockfish, the modified-block concept appears useful.

A word of caution about the use of binder may be appropriate. The salt-sodium tripolyphosphate used in the binder partially solubilizes the surface of the MM during mixing. Overmixing will affect the muscle particles to a greater extent than desirable and results in an overly elastic, rubbery texture. For softer-textured species whose muscle particles break up more readily during mixing, both a lower concentration of the salt-sodium tripolyphosphate and a shorter mixing time may be necessary to avoid developing a rubbery texture.

Comparison of shear texture of various MM blocks

Variation in shear texture in washed and unwashed modified and unmodified MM blocks stored at −18°C for 12 months is shown in Figure 2. The coefficients of variation of the plotted points ranged from about 4–16%. The unwashed-unmodified MM block after 4 months of frozen storage had shear values greater than 30 and sensory scores of good-to-fair. By 8 months the sensory texture scores declined to fair-to-borderline. The unwashed-modified block had the lowest shear values. The product was more tender and succulent and cooked drip loss was minimal. Texture was rated good after 8 and 12 months of frozen storage. The MM that was washed to remove the oxidation-prone sarcoplasmic fraction had the highest shear values, had very high cooked drip losses, and was quite tough. However, the washed-modified blocks had substantially reduced shear values (Fig. 2) and was more tender. Its texture was rated good after 8 and 12 months of frozen storage. Although washed and unwashed MM differed significantly in sensory texture, they became equally tender when modified with a binder.

Comparison of cooked drip in blocks

The cooked drip losses were substantially greater in the washed-unmodified block than in the unwashed-unmodified blocks (Fig. 3). Washing removes most of the sarcoplasmic protein fraction and more than replaces it with water. As a consequence, the moisture content of the washed muscle is usually some 3–4% higher than that of the unwashed muscle. On heating, this excess water is readily lost unless stabilized with the additives in the fish binder. Thus, the cooked drip losses of the modified blocks were substantially lower than that of the unmodified blocks made from either the unwashed or washed MM. The cooked drip values did not change significantly with storage time (Fig. 3). The coefficients of variation of the plotted points ranged from 2–20%.

Effect of variation in moisture content on texture

Varying the moisture content of washed-modified minced blocks from 4.5% above to 1.6% below that of unwashed-modified blocks did not seriously affect sensory texture during the 12-month storage period at −18°C (Fig. 4). The mean texture scores for any moisture level decreased but slightly during storage. The difference between the unwashed-modified

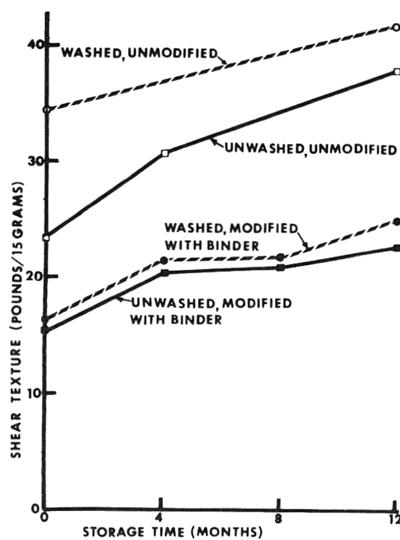

Fig. 2–Variation of shear texture in washed and unwashed, modified and unmodified black rockfish blocks stored at −18°C. (Each point represents 20–24 determinations.)

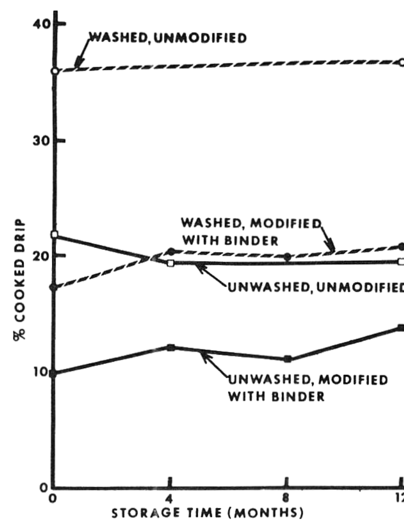

Fig. 3–Cooked drip in washed and unwashed, modified and unmodified black rockfish blocks stored at −18°C.

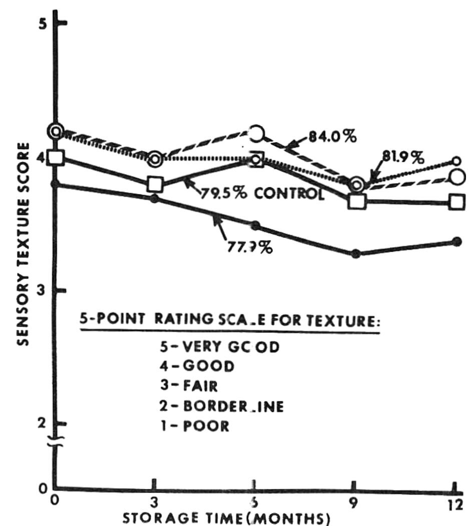

Fig. 4–Effect of variation in moisture content on texture scores of modified black rockfish blocks during 12 months storage at −18°C.

control (79.5%) and the washed-modified blocks with moisture contents above control (81.9% and 84%) was not statistically significant, but the difference between the scores of the control blocks and those of lowest moisture content (77.9%) was significant at the 1% level.

CONCLUSION

Objective measurements of shear values and cook drip correlated with sensory evaluations of texture of fillets and minced black rockfish products. Varying the moisture content of the washed-modified product up to 4.5% above the normal moisture content of the fish did not signficantly affect texture but lowering it to 1.6% below significantly reduced texture scores. Washing the minced flesh before freezing into blocks resulted in higher shear values and cooked drip. Addition of a binder containing NaCl and sodium tripolyphosphate to washed and unwashed minced flesh resulted in improved texture and lower shear values and cooked drip.

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Ms received 6/16/75; revised 12/5/75; accepted 12/8/75.

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APPLIED SCIENCE and ENGINEERING

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PROCESSING APPROACHES FOR LIMITED FREEZE DRYING

ABSTRACT

Methods are presented whereby "limited" freeze drying can be carried out to leave behind a predetermined and uniform moisture content (e.g., in the range 8–14%), so as to make the product suitable for compression. Experiments with mist-rewetting of fully freeze-dried products show a relatively uneven moisture content and, for beef, a tendency for browning. Desorption equilibrium data are presented for beef and turkey, including moisture contents and temperatures of interest for limited freeze drying. Two approaches for implementing limited freeze drying commercially were tested and evaluated. One involves modification and control of operating conditions of ordinary freeze dryers, while the other involves use of a hydrating salt as a humidity-regulating water-uptake medium in a circulating-gas apparatus. Both methods give a quite satisfactory product in small-scale tests. The hydrating-salt process should provide a simpler and more self-regulating situation for quality control of final moisture content, as well as giving more rapid drying rates.

INTRODUCTION

COMPRESSED freeze-dried foods are attractive from the standpoint of minimizing storage and transportation space. Volumetric reductions ranging from factors of 3–16 have been achieved with various meats, fruits and vegetables (Rahman et al., 1970a, b). Other likely advantages are less friability of the product and less severe packaging requirements because of the reduction in internal surface area and accessibility resulting from compression. Compressed foods generally require hot, rather than ambient-temperature water, for effective rehydration; however, for most applications hot water would be used anyhow. The quality of properly reconstituted compressed foods has usually been found to be equivalent or nearly equivalent to that of noncompressed freeze-dried foods (Rahman et al., 1970b, 1971; MacKenzie and Luyet, 1969, 1972).

Fully freeze-dried foods tend to fall apart upon compression. The most effective approach that has been found for giving foods the plasticity required for compression has been to achieve a certain predetermined, uniform moisture content after freeze drying and before compression. The optimal water content for compression has been found to be different for different foods (MacKenzie and Luyet, 1969, 1972); for beef the range 11 ± 3% moisture has been specified (Brockmann, 1973). There may also be other situations where it is desirable to produce a freeze-dried product having an appreciable, uniformly distributed moisture content; for example, for any foods which may have improved storage stability and/or appearance at higher moisture contents than would be achieved by conventional freeze drying. In most cases it is likely to be necessary for a compressed product to be further dried for storage stability.

The goals of the present work were to evaluate and test approaches whereby freeze drying can be accomplished economically to leave a uniform and predetermined moisture content in the product, typically selected to be somewhere in the range 8–14%. The necessary underlying desorption equilibrium data were also measured. Emphasis was given to methods which allow the desired moisture content to be reached at the end of the freeze-drying process itself ("limited" freeze drying), rather than those which require rewetting after full drying.

SPRAY REWETTING

MOST DEVELOPMENTAL WORK to date for compressed, freeze-dried foods has utilized full freeze drying in standard equipment, followed by rewetting to attain the desired moisture content for compression. The most common and convenient approach for small-scale tests has been to spray the food particles with a fine mist of water droplets until the desired average moisture content is achieved.

Procedure

In order to obtain quantitative data on the extent to which a uniform moisture content and good product quality can be obtained by spray rewetting, a series of experiments was carried out wherein 1.5-cm cubes of commercial freeze-dried cooked beef steaks were sprayed with distilled water using a fine mist from a spray bottle. This was done by placing 10 cubes in a container made of aluminum foil and then spraying them with a short burst of mist, shaking the pieces to expose new surface area and then spraying again, etc. This procedure was continued until an average moisture content of 12.8%, determined by weight increase, was achieved. Pieces were then analyzed individually for moisture content by weighing, drying in a vacuum oven at 60°C and less than 1 torr for 24 hr, and reweighing. Slices were also taken along a core cut through cubes in one direction and were analyzed for moisture content so as to determine local variations within a piece.

Results

Typical results are shown in Table 1, where it may be seen that the average moisture contents for individual pieces ranged from 5.1–17.5%. Also, the moisture profiles for individual pieces show a nonhomogeneous distribution of moisture, which was still detectible as long as 3 hr after spraying. Another difficulty, relating to product quality, was the appearance of browning on the surfaces of the cubes about 2 hr after spraying. This phenomenon was apparently related to

localized very high moisture contents following the impact of the droplets from the spray and was not noted in experiments where the same average moisture content was achieved by limited freeze drying. A more extensive description of these spray rewetting studies is given by Carn (1974).

Nonuniform rewetting and browning tendencies are both undesirable product-quality aspects of spray rewetting.

PROCESS ALTERNATIVES

ANOTHER POSSIBILITY for rewetting after full freeze drying is vapor-phase transfer of water, using either steam or humid air or water vapor in an otherwise evacuated chamber (Rahman et al., 1970a; MacKenzie and Luyet, 1969; Palwsorth and Hoge, 1973). This procedure involves slow rates, or can lead to the same problems of uneven rehydration and browning if the water-vapor partial pressure is made high enough to give more rapid rewetting.

From the standpoint of minimizing processing steps, it is preferable to carry out freeze drying to leave the desired uniform content at the end of drying, if possible, thereby avoiding the rewetting step altogether. The goal of leaving a uniform, substantial moisture content after freeze drying is complicated considerably, however, by the fact that freeze drying involves the inward retreat of an ice core, which is surrounded by a relatively dry layer (King, 1970). Therefore stopping ordinary freeze drying with an average residual moisture content of 8–14% will lead to a product with a small, but very wet and unstable core, with the outer regions being too dry to behave satisfactorily upon compression.

Various approaches can be conceived for attempting to carry out limited freeze drying to uniform residual moisture contents in the 8–14% range. For example, sufficiently rapid sublimation of the water in the form of ice crystals should leave behind bound water, defined as that portion of the moisture that exerts an equilibrium partial pressure less than that of ice. Subsequent equilibration of that bound water might then bring the product to the desired final moisture content. For meats, removal of bound water from fibers has been found to occur with time constants on the order of 30 min (Margaritis and King, 1971a, b); hence it does not appear to be possible to remove the ice sufficiently faster than the bound water to allow a substantial, uniform residual bound moisture content to be left at the end of freeze drying.

An alternative method is to carry out freeze drying in an environment such that the relative humidity at the surface and within the product does not fall below some predetermined value, which is high enough to ensure that the moisture content in any region will not drop below the prescribed value during drying. This approach offers the potential of reliable and close control of the product moisture content. Such a process was originally carried out by MacKenzie and Luyet (1969) who named the process "limited freeze drying." They found that extremely long drying times (25–100 hr) were required for 1-cm cubes of beef and for various vegetable products.

Accomplishing limited freeze drying through control of the environmental relative humidity does require that the temperature difference and water vapor-partial pressure difference driving forces for heat and mass transfer be less than in ordinary commercial freeze dryers, which necessitates longer drying times. However, the drying times can be reduced considerably below those encountered by MacKenzie and Luyet if proper steps are taken in the design of the process. In the experiments of MacKenzie and Luyet (1969) control of the environmental relative humidity was accomplished by keeping the specimen to be dried in an evacuated chamber in communication with a vial of ice. The temperatures of the vial of ice and the chamber walls were controlled so that the ratio of vapor pressures of water at the two temperatures corresponded to the desired relative humidity. There was a large radiant heat transfer resistance between the chamber walls and the surface of the specimen. Consequently the rate of heat transfer from the surroundings to the specimen was very low. It should be possible to accelerate the rate of freeze drying (1) if the heat transfer coefficient from the heat source to the surface of the product is increased; (2) if the temperature of the heat source is raised during the early part of drying so that the temperature of the product surface rises toward the point where the surface itself experiences the prescribed relative humidity; (3) if the temperature of the heating surface is increased while still keeping the temperature of the frozen core of the specimen below the upper limiting value; or (4) if control of environmental relative humidity is accomplished in some other way.

In the present work two methods were investigated for carrying out limited freeze drying through control of environmental relative humidity. The first involved modifying and controlling the operation of an ordinary, cabinet-and-tray freeze dryer, which in turn, required that the platen temperature and partial pressure of water vapor in the cabinet be closely controlled during drying. The second method involved using salt-hydrate reactions to accomplish control of the environmental relative humidity.

DESORPTION EQUILIBRIUM DATA

BOTH PROCESSING APPROACHES investigated require a knowledge of equilibrium moisture content of freeze-dried food as a function of relative humidity and temperature. Previous equilibrium data for meats are sparse and in poor agreement in the range of moisture contents and temperatures of interest; hence experimental measurements were carried out. Since measurements of water-sorption equilibria for foods frequently show a hysteresis between absorption and desorption data (Labuza 1968; King et al., 1968), it was felt important to obtain desorption equilibrium data.

Procedure

1-cm cubes of beef and turkey were used for the experiments. The beef was from sirloin tip roasts, purchased locally

Table 1—Typical results of experiments on spray rewetting of 1.5-cm beef cubes

Avg moisture contents of individual pieces

Piece no.	Wt % moisture (dry basis)
1	9.3
2	5.1
3	17.5
4	12.2
5	13.3

Typical moisture profiles within a core through a piece

Location	ℓ/L[a]	Wt % moisture (dry basis) Piece 1[b]	Piece 4[c]
Surface	0.07	18	15
	0.21	8	14
	0.36	8	13
Center	0.50	11	10
	0.64	6	10
	0.79	6	12
Surface	0.93	8	13

[a] ℓ/L = average distance from one face ÷ edge length of cube
[b] 15 min after spraying
[c] 2 hr after spraying

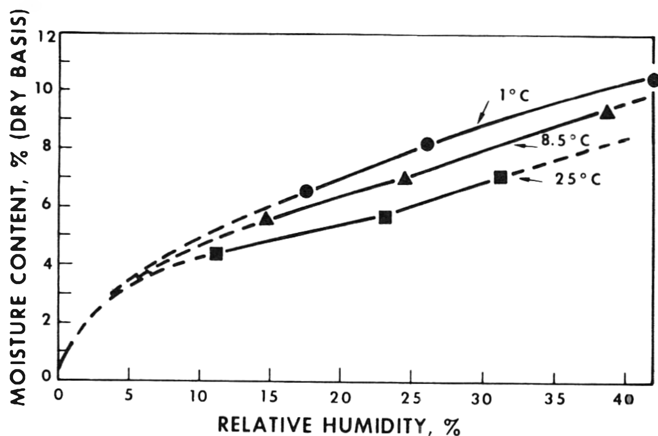


Fig. 1–Desorption isotherms for limited freeze-dried beef.

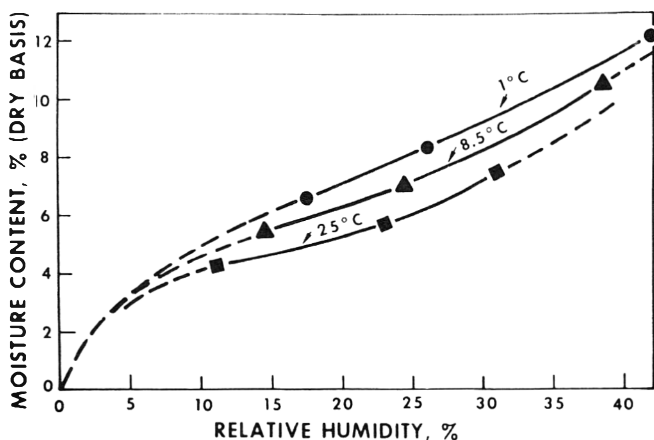


Fig. 2–Desorption isotherms for limited freeze-dried turkey.

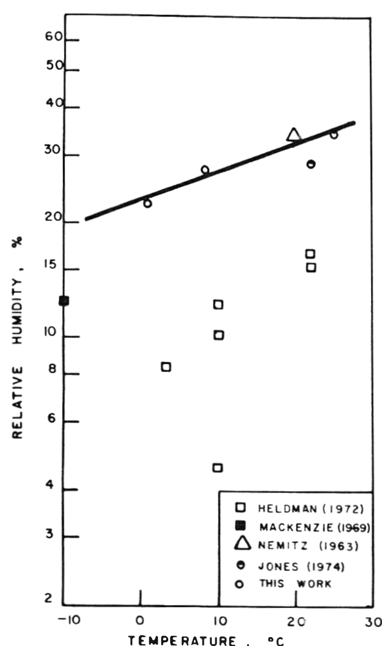


Fig. 3–Desorption-equilibrium relative humidity vs temperature at 7.5 wt % moisture content for freeze-dried beef.

and cooked at 175°C in an oven for 2½–3 hr, until the centers were above 77°C. Turkey from a commercial roast product was purchased locally and cooked at 218°C in an oven for 1.5 hr, until the center temperature rose above 79°C. Since desorption experiments were to be carried out, the pieces were initially processed by limited freeze drying at a relative humidity of approximately 22%, using the layered-bed, circulating-gas, salt-hydrate process described below. The pieces were then equilibrated in a desiccator at 50% RH and 5°C for 2 wk. At the end of this time the moisture contents averaged 12.7% and 18.3%, dry basis, for beef and turkey, respectively.

Individual groups of cubes were then equilibrated in thermostated desiccators for 2 wk, using mixtures of solid salt and saturated salt solution ($CaCl_2$, $KC_2H_3O_2$ and LiCl in descending order of humidity) to maintain selected relative humidities. So as to give desorption conditions, experiments with a group of pieces in a particular desiccator were carried out first at 1°C, then at 8.5°C, and finally at 25°C, all held within ±0.5°C. Moisture contents were determined by comparison of weights with a final dry weight, obtained after drying at 60°C and less than 1 torr for 24 hr in a vacuum oven. A more detailed experimental procedure is given by Carn (1974).

Results

Results of these experiments are reported in Figures 1 and 2. Each point in these figures represents the average of measurements for 10 different cubes. The average deviation of individual results from the points shown is 0.35% moisture for beef, and 0.25% moisture for turkey.

Interpolated results from the present work are compared in Figure 3 with interpolations from results for desorption-equilibrium measurements for cooked beef reported by Nemitz (1963), MacKenzie and Luyet (1969) and Heldman et al. (1972). Also included is a result obtained during the present study using a similar technique but with an equilibration time of 1 wk (Jones, 1975). Similar comparisons are obtained at 5.0 and 10.0% moisture contents (Carn, 1974). The agreement with the data of Nemitz (1963) is close while the data of MacKenzie and Luyet (1969) and of Heldman et al. (1972) correspond to substantially lower equilibrium relative humidity for a given moisture content.

In measurements of sorption equilibria it is essential to allow for very slow rates of equilibration of the bound moisture content (King, 1968). In desorption experiments, insufficient equilibration will result in reported equilibrium relative humidities that are lower than the actual values for a given moisture content. In the present work the change of moisture content during an equilibration period was monitored by weighing at intervals during the period. During the 2-day period prior to the end of the 2-wk equilibration periods for the 8.5°C and 25°C data, the average weight of a cube changed by about 0.0005g, which was 5% of the change in moisture content for pieces in a desiccator when the temperature was changed. Similarly, for the 1°C data the weight change during the second week was 13% of the total weight change over the 2-wk period. These results suggest that some equilibration could still have been occuring at the end of the 2-wk period, even though the equilibration time is, for the most part, longer than those used in the other studies reported in Figure 3. Care must be taken in deriving such a conclusion, however, since weight changes late in the equilibration period could also be attributable to aging of the meat or attrition of the samples during handling.

The form of plotting used in Figure 3 should give a nearly straight line if the enthalpy change upon desorption is insensitive to temperature. From the present results a desorption enthalpy of −23,400 BTU/lb mole can be derived for 7.5% moisture, representing an increase of 21% over the latent heat of vaporization of pure water.

MODIFICATION OF CONVENTIONAL FREEZE-DRYING

FOR FREEZE DRYING in conventional equipment, the gradients of temperature and water-vapor partial pressure in the void spaces are such that the surface of a food piece is necessarily subjected to a lower relative humidity than is any region within the piece; hence the surface will also be the location of the lowest local moisture content. Accomplishing limited freeze-drying through control of relative humidity, without local overdrying, therefore devolves to a problem of controlling the relative humidity at the piece surface and allowing all portions of the product to equilibrate toward that relative humidity as the drying process is completed. Controlling the relative humidity at the piece surface in conventional freeze-drying implies simultaneous control of the temperature of the piece surface and of the water-vapor partial pressure at the piece surface, at a combination of values corresponding to the desired relative humidity.

It is impossible to monitor surface temperatures for all the many pieces in a freeze dryer. Hence a more practical and conservative procedure is to control the temperature of the heating-platen surface at the same value as the desired final piece-surface temperature, taking advantage of the fact that the piece-surface temperature will rise from an initial value close to the frozen-core temperature to a final value approaching the platen temperature. Similarly, the water-vapor partial pressure within the drying chamber may be held at the value desired for the piece surface, with the knowledge that the water-vapor partial pressure at the piece surface will approximate that value and be no lower. Under these conditions, the relative humidity at the piece surface will be no lower than the prescribed value, and will be at a higher value during the early portion of the drying process. This will give a slower drying rate, but should still lead to reliable limited freeze drying to a predetermined product moisture content.

The water-vapor partial pressure in the drying chamber can be sensed with a hygrometer or other means and can be regulated either through adjustment of the condenser temperature (i.e., vaporization pressure of the refrigerant), or through adjustment of a large valve in the vapor line leading from the chamber to the condenser, if an external condenser is used.

Procedure

In the present work the approach of regulating a throttle valve in the vapor line to an external condenser was utilized, with the water-vapor partial pressure in the drying chamber being sensed through the temperature of a thermocouple placed inside a sublimating piece of ice within the chamber. Sublimation of the ice is dominated by radiative heat transfer; hence the thermocouple reads the frost point of the water vapor in the chamber.

Experiments were carried out on two scales of operation using both the laboratory freeze dryer described by Bellows and King (1973) and a 25 cu ft Buflovak Model 6389 pilot-scale freeze dryer. In the laboratory freeze dryer the platen temperature was maintained by feedback control of the power input to an electrical heater designed to give a uniform platen temperature. In the pilot-scale freeze dryer the platen temperature was maintained by circulating a stream of chilled water from an external reservoir at a high flow rate, about 2 cu ft/min. Details of apparatus and procedure, and discussion and interpretation of drying rates are reported elsewhere (Carn and King, 1975; Carn, 1974). Product quality results are reported here.

Moisture contents were measured gravimetrically, following the same procedure as in the desorption equilibrium experiments. Comparisons of moisture contents of outer layers with the center layer of a piece were made by sectioning pieces vertically into three layers of approximately equal size. Shrinkage was measured by computing the volume from linear dimensions measured by calipers before and after freeze drying. The degree of rehydration achievable was measured by weighing a sample after immersion in water for 20–30 min at ambient temperature, and then blotting. This weight was then compared to the weight of the same sample before freeze drying. Compressibility was judged qualitatively by observing the tendency to compress well or fall apart under a heavy weight.

Results

Typical results for product quality obtained by freeze drying 1-cm cubes at 2.0 torr chamber water-vapor pressure and 5°C platen temperature are shown in Table 2, for both the laboratory-scale dryer and the pilot-scale dryer. The total pressure in the chamber, as measured by a thermocouple gauge, was found to be essentially equal to the measured water-vapor partial pressure. The drying times are those found to be necessary for sufficiently complete limited freeze drying (Carn and King, 1975). It can be seen that the product moisture contents are somewhat higher than 8.5%, which is indicated by Figure 1 to be in equilibrium with 30.6% relative humidity, corresponding to the platen temperature and chamber pressure employed. This is not surprising in view of the long times required for equilibration in the desorption-equilibrium measurements. However, except for the one piece in the laboratory-scale freeze dryer which had a remaining frozen core, the product moisture contents and uniformity fall well within the stated range for good product quality. Re-

Table 2—Typical product-quality characteristics for limited freeze drying in modified conventional freeze dryers

Chamber pressure: 2.0 torr (constant)
Platen temperature: 5°C (constant)
Ultimate relative humidity (chamber pressure/vapor pressure) of water at platen temp: 30.6%
Meat samples: 1-cm cubes of cooked beef

	Lab-scale dryer	Pilot-scale dryer
Time in freeze dryer (hr)	13.0	12.0
Final moisture contents (dry basis)		
Avg moisture content	9.9%	10.8%
Piece-to-piece variation		
Number of pieces	11	24
Avg abs dev (moisture content)	0.48%[a]	0.99%
Comparison of moisture content of outer layers with that of the center of the same piece		
Number of pieces measured	3	6
Avg moisture content of outer layers (± abs avg dev)	10.0 ± 0.8%	9.6 ± 0.9%
Avg moisture content of center (± abs avg dev)	10.0 ± 1.0%	9.9 ± 1.5%
Shrinkage upon freeze drying		
Number of pieces	4	6
Loss of vol during freeze drying/frozen vol, ± abs avg dev	0.21 ± 0.03	0.10 ± 0.06
Rehydration characteristics		
Number of pieces	4	6
Wt after reconstitution ÷ wt before freeze drying, ± abs avg dev	0.88 ± 0.06	0.90 ± 0.05
Compressibility of product	Fair to Good	Good

[a] Does not include one piece with a visible frozen core remaining and an average moisture content of 17.3%.

hydration and compression characteristics are also acceptably good.

The experiment with the pilot-scale dryer reported in Table 2 was made with three groups of beef cubes placed in different locations on a tray–front, center and back. The rest of the dryer was filled with pieces of ice on trays which were partly insulated from the platens, so as to give a more realistic vapor load for chamber-pressure control. There was no discernible trend of final moisture content with respect to position on the tray. This is an encouraging result; however, it would still be desirable for more extensive tests to be carried out with a large-scale freeze dryer so as to determine the extent to which the platen temperature can be kept uniform. Variations in platen temperature should directly affect the local relative humidity, and would thereby cause variations in the final moisture contents reached by pieces in different locations. Calculations based upon a theoretical model (Carn and King, 1975; Carn, 1974) indicate that a platen temperature uniformity of approximately ±2.5°C is required to control the product moisture content within a range of ±1% (i.e., from 10–12% moisture).

The much lower temperature-difference driving forces available for heat transfer in limited freeze drying serve to make the drying times substantially longer than for conventional freeze drying. The drying time of 12–13 hr required to achieve a uniform product moisture content in the range of 10% may be compared with a drying time of 4 hr required to reach a 2.5% moisture content by ordinary freeze drying at 40° platen temperature and 0.4 torr chamber pressure (Carn and King, 1975).

For the drying conditions reported in Table 2 the surface temperatures of the beef cubes were found to be well below the platen temperature for most of the run (Carn and King, 1975). Proper programming of the platen temperature and/or improvement of the heat transfer from the platens to the piece surfaces should enable a shorter drying time, with the relative humidity at the piece surfaces still not falling below the prescribed value. Work exploring ways of accelerating limited freeze drying through modified operation of conventional freeze dryers is continuing in our laboratory.

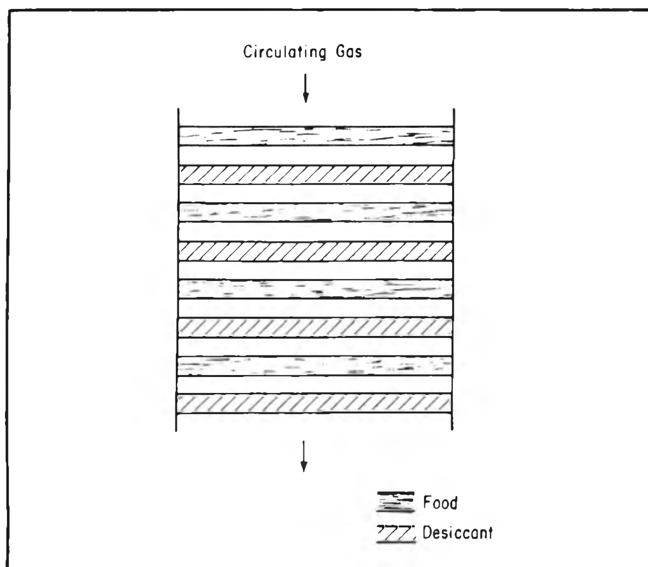


Fig. 4–Layered-bed, circulating-gas approach for freeze drying.

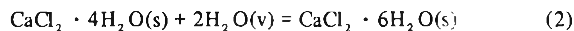
USE OF SALT HYDRATES

FOR SELF-REGULATING control of the environmental relative humidity during limited freeze drying, an ideal approach would be to utilize a water-uptake medium which maintains a particular, predetermined relative humidity, independent of temperature or of the amount of moisture taken up.

This property is possessed by hydrating salts, for which the reaction

$$A(s) + nH_2O(v) = A \cdot nH_2O(s) \quad (1)$$

corresponds to a fixed equilibrium partial pressure of water vapor at a given temperature, no matter what the extent of conversion. The reacting salt, A, may be anhydrous, or may itself be a lower hydrate. Furthermore, for most salt hydrate formation reactions the trend of the reaction equilibrium constant with temperature is such that the equilibrium relative humidity maintained during a particular hydrate transition is only weakly dependent upon temperature. Thus for



$$CaCl_2 \cdot 4H_2O(s) + 2H_2O(v) = CaCl_2 \cdot 6H_2O(s) \quad (2)$$

The equilibrium relative humidity is about 22% for a wide range of temperatures around ambient (Hougen et al., 1954). From Figures 1 and 2, this would correspond to an equilibrium moisture content of about 7.5% for either beef or turkey. Operationally, since a drying process would not allow full equilibration, the residual moisture content of meat would be expected to be somewhat higher if it were freeze dried using the reaction of Equation 2 for water uptake at the temperature of the meat.

Ways of implementing solid desiccants in freeze drying processes have been considered by King and Clark (1968), by Clark and King (1971) and by King (1970). For the present work the layered-bed, circulating-gas approach was chosen, as shown schematically in Figure 4. Air, or a more inert gas, at moderate absolute pressure (5–50 mm Hg, abs) circulates through alternating layers of food and of desiccant, serving to convey moisture from the freeze-drying food layers to the desiccant layers and to convey heat from the desiccant layers to the food layers. Heat is released by water uptake on the desiccant and is required for sublimation of water from the freeze-drying food. The process operates batchwise.

Calcium chloride undergoing the transition to the hexahydrate was chosen as the water-uptake medium in the present work. In order to increase the water-uptake capacity, experimental runs were usually started with the calcium chloride in the monohydrate state, rather than the next lower, tetrahydrate state. This allows for the use of only about 1.1 lb of $CaCl_2 \cdot H_2O$ per lb initial frozen meat in a completely closed system, if an average hydrate level of $CaCl_2 \cdot 5.7H_2O$ is reached at the conclusion of the freeze-drying process. Starting with the monohydrate level can mean that the equilibrium relative humidity drops below the desired final value early in a run. Another factor which may alter the equilibrium relative humidity is the fact that the heat release and consumption effects can make the desiccant operate warmer than the surfaces of the freeze-drying food pieces. This would provide an off-setting effect of increasing the equilibrium relative humidity at the surfaces of the food pieces early in a run. Both effects should diminish toward the end of freeze drying, thereby allowing the predetermined ultimate relative humidity to be reached in a reliable fashion.

Procedure

An apparatus was built for carrying out limited freeze drying by this approach on a pilot scale, and a procedure was developed for making calcium-chloride pellets with sufficiently accessible interior regions (porosity = 50–65%, based upon

calcium chloride in the anhydrous form). The calcium chloride pellets measured 1.5–2.5 cm long and 1 cm in diameter, and could be regenerated repeatedly by vaporization of water in a vacuum chamber.

The experimental apparatus and procedure, measured drying rates and their interpretation in terms of freeze-drying theory are all presented elsewhere (Jones and King, 1975; Jones, 1975). Quality results are presented here, and were measured in the same way as reported for runs with modified conventional freeze drying.

Results

Product-quality results from a typical run drying with circulating air at 30 mm Hg abs and a velocity of 90 ft/sec at a food-layer loading of 1.0 lb/ft^2 are shown in Table 3. Calcium chloride was charged as the monohydrate, in a loading ratio of 1.1 lb/lb of frozen meat. Under these conditions it was found that approximately 8.5 hr are required for reliable limited freeze drying and adequate residual-moisture equilibration of 1-cm cubes of beef and turkey. The average final moisture content achieved is 11.0% for turkey and 9.5% for beef, with average deviations for five pieces of 0.3% and 0.8% moisture for turkey and beef respectively. Even though the final moisture contents still exceed the equilibrium value of about 7.5% for turkey or beef at 22% relative humidity, the moisture distribution is very uniform. Moisture distributions within a piece also fall well within the desired range, and shrinkage, rehydration and compressibility characteristics are all good.

Table 3—Typical product-quality characteristics for limited freeze drying using hydrating calcium chloride

Circulating gas: Air
Pressure: 30 mm Hg abs
Gas velocity: 90 ft/sec
Food loading: 1.0 lb/ft^2
Drying time: 8.5 hr
Piece sizes: 1-cm cubes

		Turkey	Beef
Final moisture contents (dry basis)			
Piece-to-piece variation		11.2%	11.3%
		11.2%	9.1%
		11.8%	8.2%
		10.7%	9.4%
		10.1%	9.3%
Comparison of moisture content of outer layers with that of the center of the same piece:			
Piece No. 1 –	Outer layers	9.8%	8.7%
	Center core	10.4%	10.6%
Piece No. 2 –	Outer layers	10.4%	10.2%
	Center core	11.6%	14.4%
Shrinkage upon freeze drying			
(Loss of vol during freeze drying/ frozen vol)			
Piece A		0.19	0.13
Piece B		0.14	0.23
Rehydration characteristics			
(Wt after reconstitution ÷ wt before freeze drying)			
Piece A		1.06	0.90
Piece B		1.12	0.93
Compressibility of product		Good	Good

From these and other results, as well as from taste tests (Jones, 1975), product quality from these experiments is felt to be excellent.

In various runs conversions of calcium chloride to average hydrate levels as high as 5.9 have been achieved without difficulty. Even though the next degree of hydration beyond the hexahydrate is saturated solution, the desiccant has proven to have sufficient internal accessibility so that no evidence of saturated-solution formation has been found at the surface of desiccant pieces or elsewhere.

DISCUSSION

The same equipment may be used for full freeze drying as well as limited freeze drying, if a molecular sieve is used as the desiccant rather than a hydrating salt (King and Clark, 1968; Clark and King, 1971). A greater loading ratio of molecular sieve to food than of hydrating salt to food is required, but the freeze-drying time can be considerably shorter, leading to offsetting effects on the amount of chamber volume required to process a given amount of food per unit time. Freeze drying of beef and turkey cubes under gas-velocity and loading conditions similar to those described in Table 3 was found to require about 3.5 hr.

Advantages of the layered-bed, circulating-gas approach include elimination of the elaborate system of heating platens required for conventional freeze drying, and reduction of the refrigeration requirements for the drying process itself, probably by a factor of 5–10 compared to conventional freeze drying. Energy expenses can therefore be considerably less, even allowing for the need of regenerating the desiccant. With a salt hydrate-to-food ratio of the order of 1.0 or 1.5:1, the chamber volume should also be comparable to that for conventional freeze drying, because of the elimination of platens. Rates of freeze drying should also be faster than for limited freeze drying carried out in conventional freeze dryers, based upon results so far with both approaches. Implementation of the process does require capital investment for new equipment, and requires that some facility be provided for regeneration of the salt hydrate.

CONCLUSION

LIMITED FREEZE DRYING offers advantages of greater product moisture uniformity and fewer processing steps over conventional freeze drying, followed by partial rehydration, as a method for preparing freeze-dried foods for subsequent compression or for other applications where an intermediate moisture content is desired. Equilibrium sorption isotherms at various temperatures provide the wherewithal of judging the environmental relative humidity during freeze drying required to reach a particular final moisture content, although product moisture contents from limited freeze drying will necessarily exceed the equilibrium value for the relative humidity used.

Very good moisture distribution and product quality have been achieved by limited freeze drying, both with modified conventional freeze dryers and with a novel process using a hydrating salt for water uptake. The use of modified conventional freeze dryers minimizes equipment expense for processors with existing freeze dryers, at the expense of reduced drying rate. The hydrating salt process offers less energy expense for freeze drying, closer and simpler control of the product moisture content, and the probability of more rapid drying and more uniform moisture content from piece to piece.

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Ms received 6/3/75; revised 10/31/75; accepted 11/5/75.

Presented at the 35th Annual Meeting of the Institute of Food Technologists, Chicago, Ill., June 8–12, 1975.

This research was supported through a grant from the U.S. Army Natick Laboratories to the USDA Western Regional Research Center, working cooperatively with the University of California, Berkeley. The authors are grateful for advice and helpful discussions from Maxwell C. Brockmann, Robert P. Graham and William C. Rockwell.

Reference to a product name does not imply endorsement by the U.S. Department of Agriculture to the exclusion of others that may be suitable. Patent rights for the hydrating salt-limited freeze-drying approach are assigned to the Regents of the University of California.

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COTTAGE CHEESE FROM ULTRAFILTERED SKIM MILK

ABSTRACT

Ultrafiltration of skim milk was conducted at 4.4, 21.0 and 49.0°C to provide retentates for cottage cheese trials. Fractionation at 21.0 and 49.0°C improved initial permeation flux rate (PR) by 50 and 300%, respectively, over that observed at 4.4°C. Rates of decline of PR decreased with increasing temperature. Retentate ratios of undenatured whey protein nitrogen:casein nitrogen (CN) and proteose-peptone:CN were unchanged during ultrafiltration whereas nonprotein nitrogen:CN declined at each temperature. Calcium concentration of permeate was 1–2% less at 49.0 than 4.4°C because of increased calcium concentration of retentate at higher temperature. Large curd, creamed cottage cheese was prepared successfully from each retentate, cooking temperature and/or time being reduced because of initially firmer curd. Intermediate temperature (21.0°C) processing induced rapid microbial proliferation after > 2 hr. The utility of ultrafiltration as a preconcentration step for continuous cottage cheese production is discussed.

INTRODUCTION

NUMEROUS REVIEWS of the applicability of membrane processing to cheese wheys have appeared, based largely on such pilot scale studies as those of Fenton-May et al. (1971) and McDonough and Mattingly (1970). Commercial scale processing of cottage cheese whey is now feasible (Horton, 1973).

Less work has been published about membrane processing of skim milk. Fenton-May et al. (1972) stated on the basis of their work that concentration of skim milk by reverse osmosis was possible to 22% total solids (TS) while fractionation to provide 50–80% protein (dry weight basis) could be achieved by ultrafiltration. They suggested that such skim milk retentates could be used in cheese making. Peri et al. (1973) studied process optimization of protein recovery from skim milk by ultrafiltration. They determined that for an extensive purification (up to 90% protein in dry matter), a two-step process was desirable, removing 40% of the initial skim milk volume directly, followed by constant addition of water to the retentate while fractionating to the required degree. Higher permeate flux rates (PR) were thereby maintained, with reduced processing times. Pompei et al. (1973) concluded that ultrafiltration of skim milk was more economical at 50°C than at 5°C, PR being four- to fivefold higher. Most bacterial growth was inhibited at both temperatures. They observed only minor changes in rejection performance of their membranes and were not able to detect any changes in protein quality.

Work to date has been mainly concerned with characterizing and optimizing the process. However, Maubois and Mocquot (1971) utilized ultrafiltration to prepare a 27% TS skim milk retentate, which, when combined with cream of appropriate fat content, gave a fluid of the same composition as a soft cheese. Addition of starter and rennin, followed by ripening, gave a cheese of good organoleptic quality. Cheese yield was improved by incorporation of solids which would normally have been expelled in whey.

Cottage cheese production

Production of cottage cheese curd in the U.S.A. was 3.66 × 10^8 kg in 1972 (USDA, 1973). The quantity of cottage cheese whey resulting from this production was approximately 2.75 × 10^9 kg. The associated pollution problem has been well documented (Groves and Graf, 1965; Nielsen, 1970).

Cottege cheese is generally made by a batch process using lactic starter culture. A continuous method (Ernstrom, 1967) was developed but has not been adopted commercially. Skim milk at 2–5°C was acidified directly in-line and pumped through a vertical, tubular heat exchanger where coagulation occurred. The system performed optimally when milk was preconcentrated (to less than 15% TS) but thermal evaporation was too costly to permit competitive overall operation.

Purpose of this study

Ultrafiltration was investigated for pre-concentrating milk for cottage cheese production. Removal of half the initial milk volume as a lactose-rich, acid-free permeate would in turn reduce the weight of acid whey resulting if cheese were made from the skim milk retentate. Waste BOD would be substantially reduced and the increased protein content of the milk would increase production capacity of a cottage cheese operation.

Permeate removed during ultrafiltration was reserved, as were the acid wheys obtained from making cottage cheeses from the skim milk retentates. These fluids were blended for total solids fortification of beverage skim milk, reported separately (Matthews et al., 1976).

METHODS & MATERIALS

Ultrafiltration (UF)

Raw skim milk was obtained from the University of Wisconsin dairy plant, pasteurized at 62.8°C for 30 min and cooled to the required processing temperature. Milk was held in a jacketed, 750 liter stainless steel vat which served as feed well to the ultrafiltration unit. Processing temperatures utilized were 4.4, 21 and 49°C ± 0.5°C. Milk was recycled continuously to the feed well from the UF unit until the desired degree of fractionation was obtained, as determined by cumulative weight of permeate removed.

The membrane unit used throughout was a Havens "Osmotik" (Universal Oil Products, San Diego, CA) with five horizontally mounted "215" tubular modules (with displacement rods) arranged in series. Total membrane surface area was 8.26m^2 obtained from 18 1.27-cm diam tubes, each 2.44m long, per module. For each run a constant applied back pressure of 7.03 kg cm^{-2} was maintained. Permeate flux rates (liters m^{-2} day^{-1}, LMD), inlet pressure (kg cm^{-2}), and temperature (°C) were monitored throughout.

After each run, water of the same temperature as the milk processed was used to flush the unit for 15 min. "Ultraclean" (Abcor, Inc., Cambridge, Mass.) was used to clean the membranes. This detergent plus proteolytic enzyme formula was passed through the system for 30–40 min at 50°C, followed by a water rinse at 33°C for 15 min. Sanitizing was with Antibac "B" (Wyandotte Chem. Corp., Wyandotte, Mich.) to provide 100 ppm available chlorine for 15 min. Cold water (14°C) was used to flush the unit of chlorine. Restoration of a water flux of 625 LMD at 14°C and 7.03 kg cm^{-2} applied back pressure was routinely achieved.

Cottage cheese manufacture

Approximately 130 kg of skim milk retentate at 21°C was cultured with 10 ml concentrated lactic starter culture (Marschall Div., Miles Labs., Madison, WI) and 0.2 ml single strength rennet. The curd was cut in 0.95 cm cubes when it reached pH values 4.62–4.68 (approx 16 hr).

After 5 min, the curd was cooked by raising the temperature to 50.5°C over 1.5 hr. Whey was drained and the curd washed with water at 27°C, followed by a cold wash at 2°C. Curd was drained for 1 hr and creamed to give 4% butterfat in the final product. After 1 day's storage at 2°C to permit cream adsorption by the curd, evaluation of flavor and texture was conducted by a 25-member panel, using a preference-reference ballot with a long-set, large curd cottage cheese made by the University dairy plant as reference. Data were analyzed by computation of least significant differences among flavor and texture score means (Larmond, 1970). The complete processing and evaluation sequence is depicted in Figure 1. Two preliminary trials were conducted prior to the trials reported in this paper. Results presented are representative of all trials.

Analyses

Samples of skim milk retentates and permeates were analyzed as follows:

- Total nitrogen (N) by a semi-micro Kjeldahl method (Bradstreet, 1965);
- Total solids (TS) by a gravimetric method (AOAC, 1970);
- Undenatured whey protein N (UDWPN), nonprotein N (NPN, soluble in 12% trichloracetic acid), proteose-peptone N (PPN), and casein N (CN) according to Wyeth (1972);
- Total calcium according to Kamel (1960);
- Standard microbial plate count (SPC), coliforms on violet red bile agar, and yeasts and molds according to methods of the Amer. Public Health Assoc. (1967).
- Wheys were analyzed for TS, N, calcium, coliforms and yeasts and molds by the same methods.
- Cottage cheeses were analyzed for TS (pre-creaming) and coliforms.
- Solids contents were estimated by crushing curd particles and drying approximately 0.5g to constant weight at 102°C.

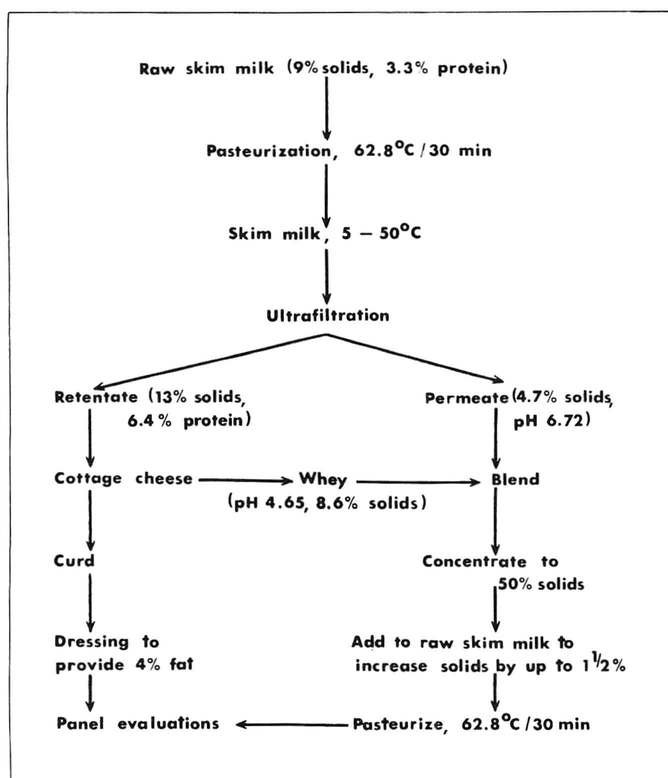

Fig. 1—Processing sequence for production of cottage cheese from retentates prepared by ultrafiltration of skim milk and for fortification of beverage skim milk with a condensed permeate/whey blend.

RESULTS & DISCUSSION

Ultrafiltration of skim milk

Permeate flux rates (PR) are depicted with respect to total solids of skim milk retentates at three processing temperatures in Figure 2. This pattern has now been well established (Fenton-May et al., 1972; Pompei et al., 1973) and illustrates the advantage of higher temperature processing wherever possible within equipment and fluid stability limitations. Initial flux rates were improved by processing at 21 and 49°C by approximately 50 and 300%, respectively, over that achieved at 4.4°C. Flux declines while increasing retentate solids from 9 to 12% were 16, 15 and 7-1/2% at 4.4, 21 and 49°C, respectively, indicating a further advantage of higher temperature processing in that less decline in PR occurred for any degree of fractionation.

Preliminary experiments in which milk volume was halved by ultrafiltration yielded retentates which, in trial cottage cheese production, were found to give unmanageably large amounts of curd per unit volume. Agitation while cooking was difficult, leading to localized over-heating with commensurate curd particle matting. Fractionation to volumetric concentration ratios (VCR, or initial milk weight divided by final retentate weight) of < 2 was therefore necessary.

Cottage cheese production

Data collected during production of each batch of cottage cheese are summarized in Table 1, trials 1, 2 and 3 being

Table 1—Production data from manufacture of cottage cheese from skim milk retentates produced by ultrafiltration (UF)

Characteristic				Trial 1	Trial 2	Trial 3
UF temperature		°C	–	4.4	21.0	49.0
Analyses						
Skim milk	Solids[a]	%	9.06	9.06	9.06	9.06
	Nitrogen[b]	%	5.70	5.70	5.70	5.70
	Calcium	g/liter	1.25	1.25	1.25	1.25
	Weight	kg	229	229	234	252
Retentate	Solids[a]	%	–	12.20	12.91	13.12
	Nitrogen[b]	%	–	7.27	7.62	7.69
	Calcium	g/liter	–	1.77	1.94	2.05
	Weight	kg	–	132	127	136
	VCR[c]		1.0	1.74	1.84	1.86
Culturing						
Setting temperature		°C	21	21	21	21
Starter volume		ml	20	10	10	10
Rennet volume		ml	0.5	0.25	0.25	0.25
pH after setting			4.65	4.62	4.68	4.62
Setting time		hr	15	16	20	20
Cooking						
Curd size		cm	0.95	0.95	0.95	0.95
Time		hr	1.5	1.5	1.5	1.5
Final temperature		°C	58.0	50.5	50.5	50.5
Final curd solids		%	20.1	21.0	22.2	22.5
Yields						
Weight of dry curd		kg	30.4	30.0	29.5	31.9
Curd wt/milk wt		%	13.3	13.1	12.6	12.6
Curd wt/wt of retentate		%	–	22.7	23.2	23.4
Coliforms		g^{-1}	<1	<1	<1	<1
Whey analyses						
Solids[a]		%	6.53	8.09	8.52	8.66
Nitrogen[b]		%	1.94	2.72	2.89	2.96

[a] Total solids, percent, wet weight basis
[b] Total nitrogen in dry matter, percent
[c] Volumetric concentration ratio

prepared from retentates obtained by ultrafiltration of skim milk at 4.4, 21.0 and 49.0°C, respectively. No difficulty was encountered in any case in terms of curd formation with the exception of slow acid production in two trials. This was attributed to the lactic starter culture rather than the milk as earlier trial runs indicated no problem in this regard. The coagulum was considerably firmer after reaching cutting pH (4.62–4.68) than that of a skim milk control and, after cutting, was initially more difficult to agitate.

Titratable acidity was not used as a measure of acid development because of the higher initial protein content of the retentates (Table 1). The associated increased buffering capacity of the milk raised titratable acidity values which necessitated using pH as a measure of sufficient acid production.

Snyeresis was even and a firm, cooked curd was obtained with less heating than was required for regular cottage cheese production. The University dairy plant heated curd to 58°C but it was found with skim milk retentates that this degree of cooking produced a tough curd of undesirable texture. Cooking to 50.5°C produced a curd, pre-creaming, of 21–23% TS so that even less cooking would be required.

However, as heating the curd to temperatures greater than 52°C is necessary to destroy psychrotrophic and starter bacteria, it is doubtful whether this is an advantage. Further work is required to determine optimum cooking conditions which would give uniformly textured curd of 20% TS and low bacterial count. Subsequent to this work, it was pointed out that cutting the curd at lower pH might be advantageous in that settled curd particles would have less tendency to mat and would require longer cooking.

Yields of cottage cheese were calculated as percentages of initial weights of skim milk used and of weights of cultured retentates. Control yield was 13.3% of initial skim milk weight, which is also achieved by the University dairy plant in its commercial scale operation. Similarly, expressed yields for trials 1, 2 and 3 were 13.1, 12.6 and 12.6%, respectively. Commercially produced cottage cheese curd usually contains 20% TS whereas curd TS values in this series were higher (Table 1). When corrected to 20% TS, yield values were directly comparable. When expressed as percentages of weights of cultured retentates, respective yields were 22.7, 23.2 and 23.4% for trials 1, 2 and 3 (Table 1). One of the major advantages to pre-fractionating skim milk is this greatly improved yield of curd per batch. Yield improvement per vat is dictated by extent of fractionation and is limited essentially by how much curd can be handled in the vat without matting during cooking.

The whey which is obtained from such an operation has higher total solids than that of a control on account of whey protein concentration during ultrafiltration. Total solids for wheys from trials 1, 2 and 3 were 8.09, 8.52 and 8.66%, respectively, corresponding to VCR's of 1.74, 1.84 and 1.86. An improved yield of cottage cheese, expressed as a percentage of the weights of skim milk used, would therefore be expected because of the retention of whey solids in the curd. The scale of these experiments was small, however, and random errors in yield determinations would have tended to mask any marginal improvements. On a larger scale, yield improvement would be detectable to an extent dictated by the degree of fractionation. The latter would be limited, however, by the increased difficulty of cooking a thicker coagulum.

The BOD of whey which results from the production of cottage cheese from skim milk retentate would be higher than that of regular acid whey (6.5% TS). The second half of this study will report the use of this more concentrated whey, combined with the sweet (pH 6.72) permeate removed during ultrafiltration, as a fortification material for beverage skim milk.

Flavor panel evaluation

Flavor and texture scores for each cottage cheese are depicted in Figure 3. No significant differences were found among flavors and that of a good quality reference, indicating that no undesirable odors or flavors were transmitted to the milk during processing and that microbially-induced off-flavors were absent. The panel also ranked samples from trial 1 which had been stored at 2°C for 2 wk. The flavor score is indicated in Figure 3 and shows that product keeping quality was acceptable.

Textures of some samples were judged somewhat tough, especially that from Trial 1 which absorbed cream strongly. However, this was not considered serious as texture can be

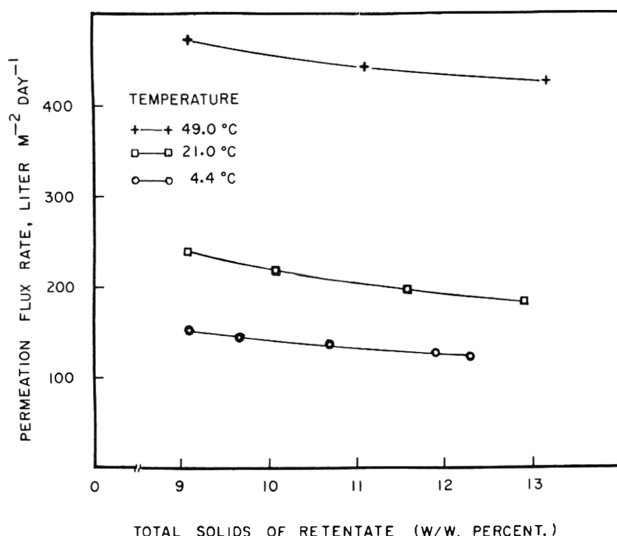

Fig. 2–Change in permeation flux rates with retentate solids concentration during ultrafiltration of skim milk at three temperatures.

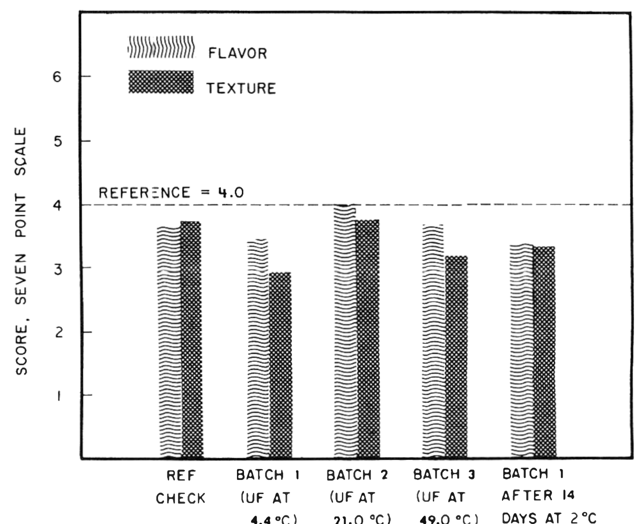

Fig. 3–Flavor and texture scores of cottage cheeses prepared from retentates obtained by ultrafiltration of skim milk.

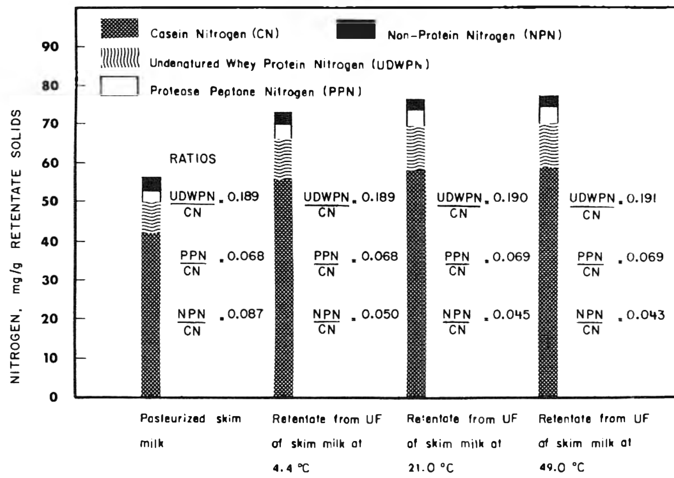

Fig. 4–Concentrations of principle nitrogenous fractions of unfractionated skim milk and of retentates obtained by ultrafiltration of skim milk.

readily improved by adjusting the cottage cheesemaking operation.

Analyses

Relative amounts of nitrogen fractions in skim milk at 0 time and in retentates after processing at 4.4, 21 and 49°C to VCR's of 1.74, 1.84 and 1.86, respectively, are shown in Figure 4. Results at 0 time were within published ranges (Jenness and Patton, 1959). Retentate analyses indicate that of the major N fractions, only nonprotein N (12% TCA soluble) was free to pass through the membrane. Ratios of undenatured whey protein N and proteose peptone N to casein N remain comparatively unchanged whereas NPN declined, as a ratio of casein N, from 0.087 at 0 time to 0.043 after processing at 49°C.

Permeate analyses indicated only minor changes in permeate composition during ultrafiltration. Total solids increased slightly, reflecting increased TS of retentates. Nitrogen compounds in permeates were soluble in 12% TCA at each temperature.

Calcium determinations indicated that transmembrane transport of this ion was limited, as expected because of its colloidal association with casein which is 100% retained. Permeate from the 49°C operation was detectably lower in calcium than permeate obtained during UF at 4.4°C (0.35 vs 0.41 g/liter) while retentates, at any given TS, had higher calcium contents with increasing temperature (Fig. 5). Calcium, being less soluble at higher temperature in its ionic form, would tend to associate more strongly with colloidal milk protein. Increased calcium retention would therefore be expected.

Bacteriological examinations of retentates and permeates

Low temperature (2–5°C) membrane processing inhibits microbial growth but causes low flux rates. Temperatures greater than 60°C also inhibit growth but reduce membrane lifetimes by accelerating cellulose acetate hydrolysis (Pompei et al., 1973). Heat denaturation of proteins may also occur. Commercial-scale fractionation of whey at 50–55°C has been reported (Horton, 1973).

Microbiological analyses in this study indicated that processing in the range 20–25°C was undesirable. Flux rate improvement over that achieved at 4.4°C was not large (approx

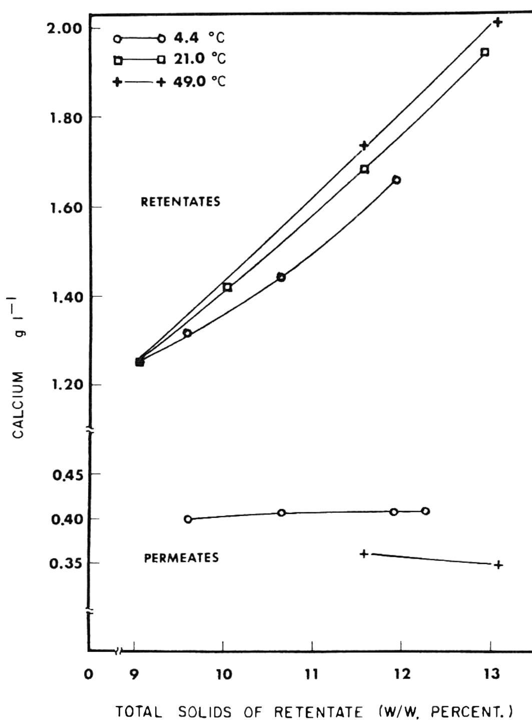

Fig. 5–Changes in calcium concentrations of retentates and permeates with retentate solids during ultrafiltration of skim milk at three temperatures.

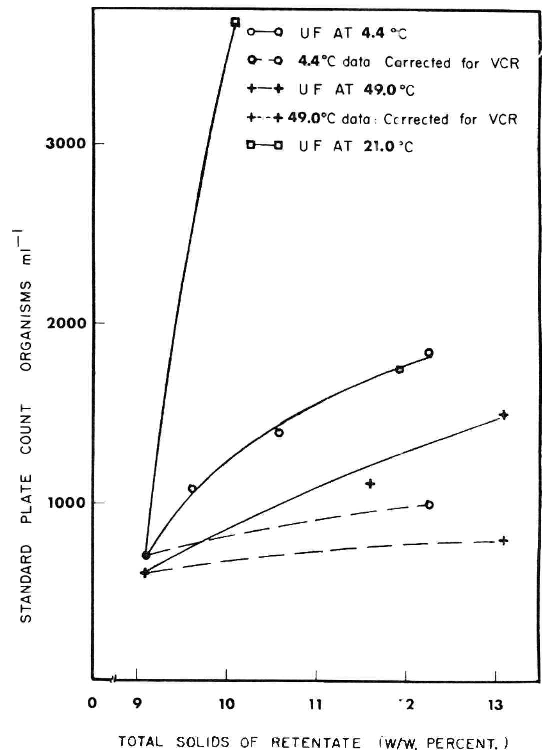

Fig. 6–Changes in microbiological standard plate counts with retentate solids concentration during ultrafiltration of skim milk at three temperatures.

Table 2—Microbiological analyses of retentates and permeates during ultrafiltration of skim milk at three temperatures

		Ultrafiltration temperature, °C					
		4.4		21.0		49.0	
Sample	Time hr	Coliforms[a] (ml)	Y&M[b] (ml)	Coliforms (ml)	Y&M (ml)	Coliforms (ml)	Y&M (ml)
Retentate	0	<1	<1	<1	<1	<1	<1
	1	6	1	>200	100	22	<1
	2	2	1	>200	>200	55	2
	3	8	1	>200	>200		
	4	14	<1				
Permeate	1	5	<1	>200	<1	72	<1
	2	2	<1	>200	<1	55	<1
	3	1	<1	>200	<1		
Water rinses[c]							
Retentate exit		<1	<1	<1	<1	<1	<1
Permeate exit		<1	<1	<1	<1	<1	<1

[a] Coliforms on violet red bile agar
[b] Yeasts and molds on acidified potato dextrose agar
[c] Rinse samples taken before processing at temperature indicated

50% higher) whereas at 49°C PR was 300% higher and curd forming properties were not imparied. Standard plate counts for retentates are depicted in Figure 6 and have been corrected for volumetric concentration. Results at 4.4 and 49°C are acceptable whereas 21°C samples showed rapid proliferation of bacteria and also yeasts and molds (Table 2).

Sanitizing the unit did not appear to be totally effective when manufacturer's recommended procedures were followed. Higher temperature processing, in particular at 21°C, appeared to flush bacteria from the permeate side of the membranes. Water at the same temperature and faster flow rates failed to dislodge organisms, SPC's, coliforms, and yeasts and molds being consistently < 1/ml in water rinse samples.

McDonough and Hargrove (1972) studied sanitation procedures for an ultrafiltration unit identical to that used in this study. They found that chlorine sanitizers could not be used for long periods (over several days) at levels to provide greater than 50 ppm available chlorine because of irreversible damaging effects on cellulose acetate membranes. For complete sanitation, they recommended that modules be vertically mounted to permit complete flooding with an iodine sanitizing solution. Horizontally mounted systems were found difficult to sanitize.

The infected state of the (horizontally mounted) membrane modules used in this study was therefore presumably perpetuated by an ineffective sanitizing procedure.

CONCLUSIONS

ULTRAFILTRATION can be used as a pre-fractionation step to increase the production capacity of a cottage cheese operation. High temperature (50–55°C) processing gives high permeate flux rates (> 400 LMD) which decline by < 10% in achieving a VCR of 1.86. Bacterial growth is inhibited in this temperature range although precautions would be necessary to avoid accumulation of thermophilic species.

It is proposed that ultrafiltration of skim milk, without resorting to high degrees of fractionation which would reduce PR and increase sanitary problems, could serve as an efficient, nondamaging means of pre-treatment for the continuous, direct acidification process. Curd-forming properties are not impaired.

As fractionation effects an increase in whey solids while increasing the concentration of casein, an improved yield of cottage cheese solids, expressed as a percentage of weight of skim milk used, would be expected.

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Ms received 7/31/75; revised 11/21/75; accepted 11/24/75.

Research supported by the College of Agricultural and Life Sciences, University of Wisconsin, Madison and by Dairy Research, Inc.
The authors thank the Fluid Sciences Division of Universal Oil Products for making available ultrafiltration membranes and equipment.

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SKIM MILK FORTIFICATION USING BLENDS OF SKIM MILK ULTRAFILTRATE AND COTTAGE CHEESE WHEY

ABSTRACT

Permeate (pH 6.71) from ultrafiltration of skim milk was blended 1:1 and 2:1 (w/w) with whey (pH 4.68) resulting from production of cottage cheese from skim milk retentate. Blends were condensed to 50% solids and used to increase solids concentration of skim milk by up to 2%. With increasing solids addition, skim milk pH declined and ash, lactose, and protein concentrates (wet weight basis) increased. The ratio of undenatured whey protein nitrogen:casein nitrogen increased by 50% in milk fortified with 2% added solids provided by condensed 2:1 permeate:whey blend. Bacteriological quality of fortified samples was satisfactory. Sensory evaluations indicated that skim milk fortification limits using 1:1 and 2:1 permeate:whey blends were 0.5 and 1.0% added solids, respectively.

INTRODUCTION

FLUID SKIM MILK marketed commercially is commonly fortified with added nonfat milk solids (SNF) to provide a more desirable consistency. It has also become common to use lactose to achieve the same effect or a combination of lactose and SNF. Emulsifiers and stabilizers have been used to lower the weight of added solids required to achieve a given viscosity although such practice has been criticized from a nutritional standpoint (Anon., 1972). Typical addition levels in skim milks commercially available in Madison, Wis., range from 0.5% lactose to 1.5% SNF. One brand is fortified with 0.5% SNF plus 0.5% lactose.

Quantitative estimations of sensory discrimination of fats and SNF in milk by taste panels were obtained by Pangborn and Dunkley (1964a, b; 1966). For milks containing 0, 2.0, 4.0 and 6.0% fat at SNF levels of 8.5 and 10.0%, the initial levels of fat and SNF did not significantly affect detection of added SNF, with 0.5% addition detected 67% of the time by a trained panel. They noted, however, that if skim milk were subjected to less heating than is required for legal pasteurization and vacuum treated to remove interfering odors, added SNF was slightly easier to detect, correct responses at 0.5 and 1.0% addition being 73 and 90%, respectively. Added SNF was more readily detected in skim milk than in 2.0 and 3.5% fat milks which had also been subjected to less heating and vacuum treated. Criteria most used by the judges in assessing added SNF were sweetness, flavor, tactile response, aftertaste and saltiness.

More specific information about the contribution of individual components of skim milk to the latter's tactile properties was obtained by the same authors (Pangborn and Dunkley, 1966). They determined that milk salts (from milk ultrafiltrate) and lactose, when added to skim milk and to milks containing 2.0 and 3.5% fat, were detected at lower concentrations than added nondialyzable (mostly protein) components (NDC). In skim milk, added milk salts were detected by a trained panel at the 0.03% level with 67% regularity and added lactose was detected with the same regularity at the 0.33% level. Detection sensitivity was lower in 2.0 and 3.5% fat milks. Added NDC was detected with 67% regularity at the 2.5% level, indicating that of the principle components of nonfat milk solids, protein was the least effective in detectably altering organoleptic properties of skim milk. Added salts were found to increase the saltiness impression and to alter tactile properties. Added lactose increased sweetness and aftertaste. The authors concluded that proteins and colloidal salts were relatively unimportant in contributing to the palatability of milk. Such information has provided the basis for commercial fortification of skim milk using lactose or SNF.

Ultrafiltration of skim milk

Several studies have been published describing membrane processing of skim milk (Glover, 1971; Fenton-May et al., 1972; Peri et al., 1973; Pompei et al., 1973). Comparatively little has been reported on how to utilize skim milk retentates and permeates produced by ultrafiltration although Maubois and Mocquot (1971) described production of soft cheeses from skim milk retentates containing 27% solids.

Purpose of this study

Permeate resulting from ultrafiltration of skim milk or whey is dilute (< 6% solids) but has a high chemical oxygen demand (deFilippi and Goldsmith, 1970). If proteins only are recovered, the pollution load of the waste stream remains high. Permeate from ultrafiltration of skim milk has a high lactose concentration (over 85% in dry matter) and is not acid (pH approximately 6.7).

Fractionating skim milk by ultrafiltration reduces the volume of acid whey if cottage cheese is prepared from retentate. Such whey is more concentrated than regular acid whey (Matthews et al., 1976).

For this study, it was proposed that the permeate and acid whey streams resulting from the ultrafiltration of skim milk and production of cottage cheese from retentate be blended, condensed to 50% solids, and used to fortify beverage skim milk to provide up to 2% added solids.

METHODS & MATERIALS

PERMEATE from the ultrafiltration (UF) of skim milk was collected during preparation of retentates for cottage cheese making (Matthews et al., 1976) and stored at 2°C. Whey drained from the cottage cheese vats was allowed to stand overnight at 2°C to permit insoluble casein curd particles to settle. Permeate and whey were blended at different ratios to give 300 kg fluid which was condensed to approximately 50% total solids (TS) in a falling film, single effect evaporator (Arthur Harris & Co., Chicago, Ill.). Recycling was continued until the desired concentration was achieved, using an operating vacuum of 40 cm Hg and a calibrated refractometer (Zeiss No. 133695) to determine TS.

Unpasteurized skim milk was obtained from the University of Wisconsin–Madison dairy plant and cooled immediately to 2°C. From the TS of the condensed permeate/acid whey blend, the weights required to fortify skim milk by 0.5, 1.0, 1.5 and 2.0% solids were calculated. The cooled skim milk was fortified immediately after evaporation was completed to avoid lactose crystallization in the condensed fluid and pasteurized at 62.8°C for 30 min. Storage was at 2°C.

Sensory evaluation was conducted by a 30-member panel after 1 and 8 days' sample storage using a preference-reference ballot. A commercial skim milk which had been fortified with 0.5% lactose plus 0.5% SNF was used as reference. An unfortified sample was also evaluated.

Data were analyzed by analysis of variance and computation of least significant differences between flavor score means (Larmond, 1970). Samples were served at 21°C.

The processing and evaluation sequence, including those steps described in the first part of this work (Matthews et al., 1976) are summarized in Figure 1.

Analyses of skim milk permeate, cottage cheese whey, whey: permeate blends, unfortified and fortified skim milks were conducted as follows:

- Total solids by a gravimetric method (AOAC, 1970);
- Total nitrogen by a semi-micro Kjeldahl method (Bradstreet, 1965);
- Undenatured whey protein nitrogen (UDWPN), proteose-peptone N (PPN), and nonprotein N (NPN) soluble in 12% trichloracetic acid, according to Wyeth (1972);
- Titratable acidity (TA) as percentage lactic acid by titration with n/10 NaOH with phenolphthalein indicator, and pH (glass electrode, Leeds & Northrup, Philadelphia, PA);
- Microbiological analyses [standard plate counts (SPC), coliforms, and yeasts and molds] according to methods of the American Public Health Assoc. (1967);
- Total incombustible matter according to AOAC (1970);
- Ether extractable lipid by the Mojonnier method (AOAC, 1970).

RESULTS & DISCUSSION

Compositions of permeate, whey and permeate/whey blends

Results of permeate analyses are summarized in Table 1 and show that this fluid was a dilute (4.53% solids), slightly acid (pH 6.71) material containing approximately 88% lactose, dry weight basis (DWB). Ash content was 9.27%, DWB. As protein content was negligible (nitrogenous compounds present were soluble in 12% TCA), buffering capacity of permeate was low. Titratable acidity was 0.06% (percent lactic acid).

Whey resulting from production of cottage cheese from skim milk retentate was more concentrated (8.66% solids) than normal cottage cheese whey (6.5% solids). This increase, however, resulted from concentration of whey proteins during ultrafiltration of skim milk. The protein concentration of whey used in this study was 18.9% (DWB, N percent × 6.38) whereas regular whey solids contain approximately 13% protein. The nutritional quality of whey protein has been well documented (Wingerd et al., 1970; Wingerd, 1971) so that potential value of the whey solids was enhanced by effecting a partial fractionation during ultrafiltration. Ash content of this whey was high (11.20%, DWB). Titratable acidity was 0.69% (as lactic acid).

Permeate and whey were blended in two separate trials to provide two parts permeate to one part whey (w/w) and one part permeate to one part whey. Results from analyses of these blends are included in Table 1. Each blend had a high ash concentration (over 10%, DWB). The high titratable acidity of the whey was reduced after blending with permeate. Values were 0.37% and 0.27% for the 1:1 and 2:1 blends of permeate:whey, respectively. Corresponding pH values were 5.00 and 5.48. Total solids were 6.08 and 5.92% for the 1:1 and 2:1 blends, respectively, so that solids levels were slightly less than that of normal cottage cheese whey.

Compositions of fortified skim milks

Results from analyses of fortified skim milks are summarized in Table 2. Ash and lactose levels, (%, wet basis) increased with increasing levels of fortification. Acidity also increased, pH declining from 6.74 (control, unfortified) to 6.41 (2% addition using 2:1 permeate:whey blend).

Nitrogen contents (%, dry basis) declined with increasing levels of fortification. When expressed on a wet weight basis, "protein" (% total N × 6.38) did not change appreciably because of the high levels of non-protein N soluble in 12% TCA (NPN), proteose-peptone N (PPN), and undenatured whey protein N (UDWPN) present in the blends. The nitrogenous fractions were therefore determined separately. The concentration of casein declined and that of UDWPN increased with increased level of fortification.

The effect of a 1:1 permeate:whey blend was more marked, with changes in pH, ash, and lactose being more pronounced than those observed when using a 2:1 blend for equivalent levels of fortification.

Microbiological examinations of permeate, whey and fortified skim milks

Coliforms and yeasts and mold counts in permeate and whey were < 1 per ml. Good quality, fresh skim milk was used and after fortification and pasteurization (62.8°C for 30 min), SPC was no greater than 500 per ml per sample. No coliforms

Table 1—Analyses of permeate from the ultrafiltration of skim milk and of whey resulting from production of cottage cheese from skim milk retentate

Characteristic		Sample			
				Permeate/whey blends	
		Permeate	Whey	1:1	2:1
Total solids	%	4.53	8.66	6.08	5.92
Total nitrogen[a]	%	0.55	2.96	1.80	1.38
Ash[a]	%	9.27	11.20	10.20	10.30
pH		6.71	4.68	5.00	5.48
Titratable acidity[b]		0.06	0.69	0.37	0.27
Conc after evaporation	%	–	–	53	54

[a] As percent of dry matter
[b] As percent lactic acid

Table 2—Analyses of skim milks fortified with blends of permeate from the ultrafiltration of skim milk and whey resulting from the production of cottage cheese from skim milk retentate

Blend (Vol permeate/ vol whey)	Characteristic		Added solids, percent (Approx)				
			0	1/2	1	1-1/2	2
2:1	Total solids	%	9.04	9.71	10.06	10.48	11.08
	Solids added	%	–	0.67	1.02	1.44	2.04
	Lactose[a]	%	4.76	5.36	5.64	5.98	6.50
	Ash[a]	%	0.72	0.77	0.82	0.89	0.94
	Lipid[a]	%	0.08	0.08	0.09	0.09	0.09
	Protein[a]	%	3.47	3.49	3.51	3.52	3.53
	Nitrogen[b]	%	6.02	5.63	5.47	5.27	5.00
	UDWPN[c]	%	0.75		0.75		0.83
	PPN[d]	%	0.24		0.20		0.20
	NPN[e]	%	0.42		0.49		0.48
	CN[f]	%	4.61		4.03		3.49
	(UDWPN/CN)		0.16		0.19		0.24
	pH		6.74	6.62	6.58	6.49	6.41
1:1	Total solids	%	9.20	9.84	10.17		
	Solids added	%	–	0.64	0.97		
	Protein[a]	%	3.41	3.54	3.55		
	Nitrogen[b]	%	5.92	5.64	5.44		
	pH		6.75	6.54	6.48		

[a] Percent wet basis
[b] Percent dry basis
[c] Undenatured whey protein nitrogen
[d] Proteose peptone nitrogen
[e] Nonprotein nitrogen
[f] Casein nitrogen

or yeasts and molds were detected in any samples evaluated by the flavor panel. There should therefore have been no off-flavors attributable to microbiological growth in the samples.

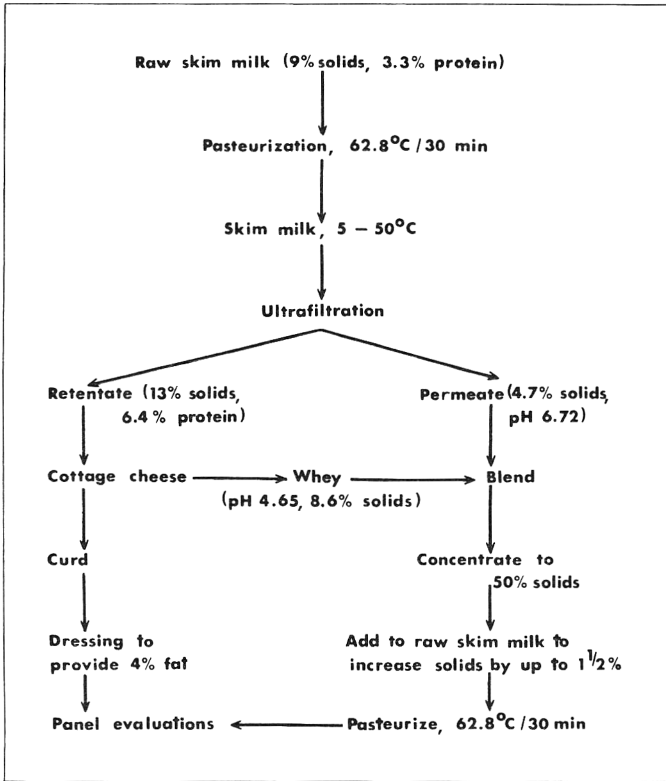

Fig. 1—Processing sequence for production of cottage cheese from retentates prepared by ultrafiltration of skim milk and for fortification of beverage skim milk with a condensed permeate/whey blend.

Flavor panel evaluation of fortified skim milks

From Table 2, all percentage additions of ash and lactose used in this study would have exceeded levels stated by Pangborn and Dunkley (1966) to be significant in altering organoleptic qualities of skim milk. Most panelists had no difficulty in detecting changes in skim milks fortified with permeate/whey blends. Panel evaluation data of skim milks fortified with a 2:1 permeate:whey blend to contain 0.67, 1.02, 1.44 and 2.04% added solids are presented in Figure 2. Data from evaluation of skim milks fortified with a 1:1 permeate:whey blend to contain 0.64 and 0.97% added solids are presented in Figure 3.

No significant differences ($P < 0.01$) were detected between reference and skim milks containing 0.67 and 1.02% added solids. Reference score was arbitrarily set at 4.0 and when included as a coded sample, the reference average score ($n = 30$) was 3.93, well within the 1% confidence level. Unfortified skim milk had a lower score and most panelists described this sample as "dilute."

Beyond 1% solids addition, scores declined and deviations from the reference score were significantly greater than the 1% confidence interval. Panelists' responses to these samples were "salty," "sour," and "sweet-sour," indicating that the high salt, acid and lactose concentrations in the added solids were readily apparent at these levels of fortification.

After 8 days storage at 2°C, samples were compared to a fresh reference. A downward displacement of the flavor score curve was noted (Fig. 2). This deterioration was not attributed to the added solids, however, as flavor score of the unfortified sample also declined.

From these data, fortification to provide up to 1% added solids using a condensed 2:1 blend of permeate from ultrafiltration of skim milk with acid whey resulting from production of cottage cheese from skim milk retentate appears feasible.

Based on data presented in Figure 3, a 1:1 permeate:whey blend could not be used at levels to provide more than 0.7% added solids without causing a significant drop in flavor score. Panelists described the sample containing 0.97% added solids as "sour" and "salty-sour." The sour character was therefore more apparent at lower percentage additions than was observed using 2:1 permeate:whey blend. The limit to percentage

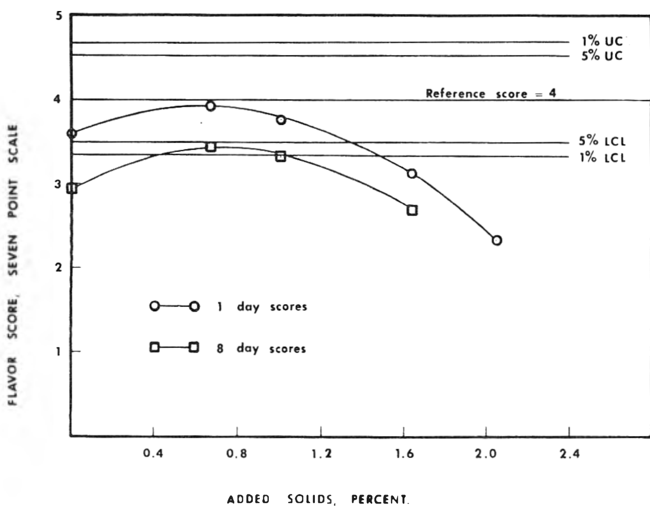

Fig. 2—Flavor panel evaluations of beverage skim milks fortified with a condensed 2:1 blend of permeate from ultrafiltration of skim milk with cottage cheese whey.

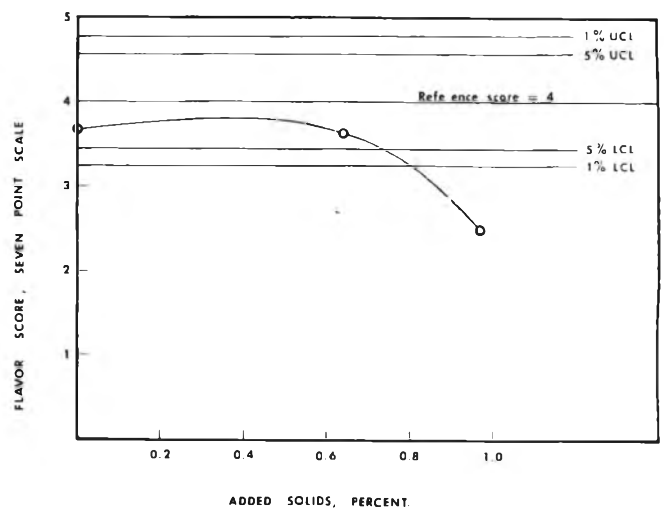

Fig. 3—Flavor panel evaluations of beverage skim milks fortified with a condensed 1:1 blend of permeate from ultrafiltration of skim milk with cottage cheese whey.

fortification using the 1:1 blend was felt to be 0.5% compared to 1% when using the 2:1 blend.

It is suggested that fortification of skim milk using these materials is feasible as a means of utilizing what may otherwise have been considered to be waste streams from ultrafiltration and cottage cheese production.

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Ms received 7/31/75; revised 11/21/75; accepted 11/24/75.

Research supported by the College of Agricultural and Life Sciences, University of Wisconsin, Madison and by the Stauffer Chemical Company.

The authors thank the Fluid Sciences Division of Universal Oil Products for making available ultrafiltration membranes and equipment. Also research assistant S.E. So for his assistance.

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PALATABILITY AND VISUAL ACCEPTANCE OF DARK, NORMAL AND PALE COLORED PORCINE M. LONGISSIMUS

ABSTRACT

Pork loins from carcasses weighing 68–75 kg were compared for quality characteristics. A total of 120 loins, with equal numbers of pale and watery, normal and dark colored loins, were evaluated. Pale chops had a significantly higher cooking loss than normal or dark colored chops. The consumer panel scored the pale chops significantly lower in organoleptic acceptability than normal or dark chops. The trained panel gave a similar rating for the organoleptic evaluation. When the consumer panel selected pork chops from a retail display case, the normal colored chops received the highest rating and the pale, watery chops the lowest. The pale chops were the most unstable and developed a greenish-gray cast after 2–3 days' storage. The normal colored chops had significantly more intramuscular fat and less protein than either pale or dark chops.

INTRODUCTION

COLOR CHARACTERISTICS vary considerably between and within pork cuts. The color variation has stimulated some industrial groups to discuss the need for grading retail pork cuts to improve uniformity and quality of pork in the retail market. However, no consumer evaluation or acceptance studies for pale, watery versus normal or dark colored pork chops have been reported.

Divergent research results have been published on the organoleptic acceptability of pale and watery, normal or dark colored pork chops when evaluated by a trained panel of 6 to 8 members. According to Judge et al. (1960) tenderness of broiled pork chops increased as the muscle pH and firmness decreased. Bennett et al. (1973) reported that pale chops were more tender and less juicy than normal or dark colored chops, whereas Searcy et al. (1969) found no significant organoleptic differences among the three classes of muscle color. Conversely, Lewis et al. (1962) and Sayre et al. (1964) reported that cooking loss increased and tenderness decreased as pH and water-holding capacity decreased. Those latter two reports suggested that dark colored pork was more tender than pale, watery pork. This project was designed to evaluate the acceptability of pale, normal and dark pork chops by both trained and consumer panels.

EXPERIMENTAL

WOMEN in the Des Moines, Iowa, metropolitan area were randomly selected from the Des Moines directory to participate in the consumer preference evaluation. The 150 participants represented a wide variety of demographic characteristics, shopping, food preparation and meat consumption habits.

Forty of each of the three types of loins were compared: (1) pale and watery; (2) normal colored; and (3) dark colored loins from pork carcasses weighing 68–75 kg. At the Iowa State Meat Laboratory, the center portion of each loin was cut into chops 1.25 cm thick and packaged for the following evaluations: one chop each of dark, normal and pale pork was placed in a plastic retail meat tray, covered with a stretch wrapped transparent plastic film and placed in a 2°C cooler for 3 days. The next day the chops were placed in a retail display case at 8–9°C and evaluated by a consumer panel. Individual panel members evaluated the chops during scheduled times from 9:00 AM to 9:00 PM. Participants were asked to visually evaluate and rank the three types of chops from most to least desirable.

The consumer panel made two organoleptic comparisons of the loins at a central location in Des Moines. Two chops from each of 120 loins were pan broiled to 74°C internal temperature and a 1.25 cm square section representing each of the three color comparisons was served warm to each of the 150 panelists. They scored the chops for overall acceptability on a 9-point hedonic scale. Panelists made the second organoleptic comparison in their homes. They compared pale, watery chops with either normal or dark colored chops or normal colored with dark. Each participant made only one of three comparisons. Chops for these specific comparisons were packaged and given to the consumers after the chops had been stored in the dark for 3 days at 2°C at the Iowa State University Meat Lab. Each panelist was told to refrigerate the chops until they were cooked and evaluate the chops within 2 days after distribution.

Each panelist was told to cook the chops by the method she most commonly used to prepare pork chops, then to taste and indicate her preference. All chops were identified and evaluated only by code.

At Iowa State University a seven-member trained panel evaluated two chops from each of the 120 loins used in the consumer test. The chops had been frozen for about 1 month before evaluation. The chops were thawed and pan broiled to 74°C internal temperature, cut into 1.25 cm squares and served to panelists. They scored the samples for flavor, juiciness, tenderness and overall acceptability on a 9-point hedonic scale. Cooking loss was determined by weight loss.

One chop from each loin was used for analyses. For the M. longissimus, pH, expressible juice and percent light reflectance were determined by the methods reported by Matsushima and Topel (1969). The reflectance values were used to classify the loins into the three color groups before they were packaged at the meat laboratory. Protein, fat and moisture were determined on the M. longissimus by AOAC (1965) procedures. A least squares analysis of covariance (Harvey, 1960) was used to adjust the data for differences in cooking time and temperature. Differences of the adjusted means for the three muscle groups were tested for significance by the following orthogonal contrasts: (1) normal vs. pale and dark and (2) pale vs. dark.

RESULTS & DISCUSSION

DIFFERENCES were highly significant ($P < 0.01$) between the pale and dark colored chops for percent reflectance, pH and expressible juice (Table 1). When these characteristics for the pale and dark colored chops were averaged, reflectance and pH did not differ from values for normal chops. This comparison indicated that the normal chops were intermediate (between the extremes) to the pale and dark colored chops. Expressible juice, however, was significantly more from pale than from normal chops, and was least from the dark chops. Pale and dark colored chops had significantly ($P < 0.01$) less intramuscular fat and more protein than normal chops. Percents of moisture, fat and protein were not significantly different between pale and dark chops.

The consumers' visual appraisal of pork chops showed that normal chops were preferred over the pale or dark colored chops (Table 2). Panelists who selected the pale chops over the

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Table 1—Least square means and standard errors for characteristics of M. longissimus

Muscle group	% Reflectance[a]	pH	Expressible juice[b]	Percent Moisture	Percent Fat (wet wt)	Percent Protein (wet wt)
Pale, soft muscle	29.6[d**]	5.44[d**]	2.88[d**]	73.41	5.33	21.24
Normal colored muscle	22.1	5.80	2.06[c**]	73.28	6.92[c**]	10.75[c**]
Dark, firm muscle	16.5[d**]	6.20[d**]	1.93[d**]	73.83	4.66	21.48
Standard error	0.43	0.04	0.06	0.23	0.85	0.16

[a] The higher the value, the lighter the meat color. A value of 22 is considered normal pork color.
[b] The higher the value, the greater amount of expressible juice. As expressible juice increased, the water-holding capacity decreased.
[c] Indicates the comparison of pale and dark vs. normal muscle color groups.
* ($P < 0.05$)
** ($P < 0.01$)

normal or dark chops made their evaluations during the first 3–4 hr of the 12-hr retail display period. During those hours the pale chops were brightest in color as compared with normal or dark chops and most of the consumers selected the pale chops as most desirable. After 5 to 6 hr, however, the pale chops started to develop a gray-greenish cast; but the normal or dark chops showed no major color change. As the gray-green cast developed on the pale chops, consumer preference decreased greatly and most panelists ranked the pale chops as least desirable. It was concluded that under our test conditions pale, watery chops were not as color stable as normal or dark chops. The consumers were very sensitive to abnormal color change in the selection of pork chops.

Bembers and Satterlee (1975) reported significantly greater instability of the myoglobin from pale, watery muscle than normal pork muscle. Myoglobin from pale, watery muscle had a more rapid rate of autoxidation, greater heat instability of both the met and oxy forms and a varying isoelectric point. Flynn and Bramblett (1975) also reported that PSE muscle developed rancidity at a faster rate than normal or dark colored pork when frozen for 9 months.

Each consumer was asked to taste and indicate preference between two chops cooked at home. These chops were either a pale and a normal chop, normal and a dark chop or a pale and a dark chop. When normal chops were compared with pale or dark chops, 67.9% of the panel members preferred normal over pale chops and 61.7% ranked normal over dark chops (Table 3). When pale and dark chops were compared, 66.7% of the panel members selected the dark chops. These results confirmed that pale chops were rated less desirable than normal and dark chops.

Results of sensory evaluation by the consumer panel of chops prepared and served by the methods reported for the trained panel are shown in Table 4. The consumers were asked to rank the three types of chops for overall acceptance on the same scale used by the trained panel. Normal colored chops were scored highest in acceptability and the pale chops, lowest.

Sensory evaluations and rankings by the trained panel were similar to those by the untrained consumer panel for overall acceptance (Table 4). The trained panel scored the chops slightly but not significantly lower than the consumer panel. The trained panel, however, ranked the normal chops significantly ($P < 0.01$) higher for overall acceptance and flavor. Dark and normal colored chops were significantly more tender than the pale chops and had a lower cooking loss. These results agree with those reported by Sayre et al. (1964) and Kauffman et al. (1964). However, possibly due to differences in cooking technique, Deethardt and Tuma (1971), Merkel (1971) and

Table 2—Order of preference by consumer for retail-displayed pork chops[a]

Types of chops and order by visual preference: Best	Middle	Worst	% of participants who ranked the chops
Normal	Pale	Dark	34.7
Normal	Dark	Pale	33.5
Pale	Normal	Dark	15.9
Pale	Dark	Normal	5.9
Dark	Normal	Pale	7.6
Dark	Pale	Normal	2.4

[a] Based on 150 participants

Table 3—Taste preference by consumer panel from pork chops cooked at their household

	Pale chops					
	Normal	vs Pale	Normal	vs Dark	Pale	vs Dark
Percentage preference	67.9	32.1	61.7	38.3	33.3	66.7

Table 4—Least square means and standard error for M. longissimus flavor, tenderness, juiciness, cooking loss and overall acceptability scores[a]

Pork chops	Flavor	Tenderness	Juiciness	Cooking loss, %	Acceptance: Trained panel	Acceptance: Consumer panel
Pale & soft	5.95	5.37	5.68	18.31	5.57	6.09
Normal colored	6.03[b*]	6.12[d**]	6.07	15.88[d*]	6.04[b**]	6.64[b**]
Dark & firm	5.70	6.19[c**]	5.97	14.38[c**]	5.87	6.46
Standard error	0.08	0.16	0.11	0.26	0.10	0.11

[a] Nine-point hedonic scale
[b] Indicates the comparison of pale and dark vs. normal muscle color group
[c] Indicates the comparison of pale vs. dark muscle color group
[d] Indicates the comparison of normal vs. pale colored chops
* ($P < 0.05$)
** ($P < 0.01$)

Table 5—Visual preference vs. taste preference of normal and pale pork chops by the consumer panel

Taste evaluation	Visual preference of chops[a]	
	% pale, watery chops chosen over normal	% normal chops chosen over pale, watery
Preference for pale, watery chops after tasting	57.2	28.3
Preference for normal colored chops after tasting	42.8	71.7

[a] Visual preference was based on rankings of independent samples at an independent site where the housewife was asked to choose between normal, dark and pale.

Table 6—Visual preference vs. taste preference of normal and dark colored pork chops by the consumer panel

Taste evaluation	Visual preference of chops[a]	
	% normal chops chosen over dark	% dark chops chosen over normal
Preference for dark chops after tasting	42.9	0.00
Preference for normal colored chops after tasting	57.1	100.0

[a] Visual preference was based on rankings of independent samples at an independent site where the housewife was asked to choose between normal, dark and pale colored chops.

Table 7—Visual preference vs. taste preference of pale, watery and dark colored pork chops by the consumer panel

Taste evaluation	Visual preference of chops[a]	
	% pale, watery chosen over dark	% dark chosen over pale, watery
Preference for pale, watery chops after tasting	31.8	41.6
Preference for dark chops after tasting	68.2	58.3

[a] Visual preference was based on rankings of independent samples at an independent site where the housewife was asked to choose between normal, dark and pale colored chops.

Bennett et al. (1973) found pale, soft muscle and chops to be more tender than normal or dark colored chops.

The consumer panel members' visual preferences are compared with their taste preferences of cooked chops in Tables 5, 6 and 7. Most panelists who selected normal chops by visual evaluation also preferred normal chops by taste evaluation. Panelists who visually selected pale over dark chops showed an opposite response for the taste preference. The variation in most comparisons indicated that consumers did not accurately associate their criteria for visual selection with the actual taste evaluations of chops. Visual selection was, therefore, not related to their taste for pork chops. This observation supports the hypothesis for statistical independence of taste from visual preference in each table.

Results of this study indicate that consumers discriminate against pale pork chops. Pale chops have a high cooking loss, which is important to the consumer, and also have a short shelf life which is important to the retailer and ultimately to the consumer and possibly to the entire pork industry.

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Ms received 6/26/75; revised 9/29/75; accepted 10/6/75.

Journal Paper No. 8065 of the Iowa Agriculture and Home Economics Experiment Station, Ames, Iowa. Project 2008. This work was supported in part through a cooperative agreement with the U.S. Department of Agriculture, Agricultural Research Service, Agricultural Marketing Research Institute, Meat Science Research Laboratory. The authors thank Linda Overfield for her assistance with the taste panel portion of the study.

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USE OF DIAMIDE IN DETERMINING THE SHELF LIFE OF REFRIGERATED CHICKEN

ABSTRACT

A study was done to determine if a relationship exists between plate counts determined on trypticase soy agar containing diamide and the shelf life of refrigerated chicken carcasses. Results show that determining plate counts with TSA containing 0.05% diamide appears useful as a means of predicting shelf life of raw chicken and possibly other fresh meats stored under refrigeration.

INTRODUCTION

PREDICTING the shelf life of fresh meats has been attempted with various physical, biological and chemical methods, but none has been entirely satisfactory. Spoilage of refrigerated fresh meats normally results from contamination and subsequent growth of species of *Pseudomonas* and *Alcaligenes* (*Achromobacter*). Walker and Ayres (1956) found species of these genera to be primarily responsible for the spoilage of poultry at 4.4°C in polyethylene bags, though Ayres et al. (1950) had isolated from fresh and defrosted cut-up chickens a large variety of microorganisms belonging to 18 different genera. Thornley (1960) and Barnes and Impey (1968) also found species of *Pseudomonas* predominate in refrigerated poultry meat. Barnes and Impey (1968) considered that the genus *Acinetobacter* fits more appropriately some spoilage bacteria previously classified as *Achromobacter*.

Diamide (diazenedicarboxylic acid bisdimethylamide) is a thiol-oxidizing agent (Kosower et al., 1969; Wax et al., 1970) whose bactericidal effects are not fully understood (Rose et al., 1971). Rose et al. (1971) found substituted diazenes, incorporated into trypticase soy broth, to permit growth of some pseudomonads but to inhibit several bacteria including *Escherichia coli, Enterobacter aerogenes*, and species of *Shigella, Proteus*, and *Salmonella*. Verbovsky and Collins (1973) studied the influence of diamide (the only available substituted diazene) on several bacteria and found that 0.05% added to trypticase soy agar inhibited some species of *Pseudomonas* and *Alcaligenes* in addition to two species of *Lactobacillus* three of *Streptococcus, E. aerogenes*, and *E. coli*. Nevertheless, it did not inhibit *Pseudomonas fluorescens*, an organism that is particularly important in poultry products (Board, 1966; Barnes and Impey, 1868).

This study was done to determine if there is a relationship between plate counts determined on trypticase soy agar containing diamide and the shelf life of refrigerated chicken carcasses.

EXPERIMENTAL

THE TISSUE-SAMPLING PROCEDURE of Avens and Miller (1970) was used for sampling the skin of chicken carcasses, purchased from local supermarkets and stored at 4.4°C in polyethylene bags. Skin samples totaling 10 cm^2 were removed aseptically with a sharp cork-borer (diameter, 1.1 cm) from under the wings and near the vent of a chicken carcass on a table under ultraviolet light in the media-pouring room. The skin was placed in a dilution blank containing 99 ml sterile physiological saline (0.9% NaCl) and glass beads and shaken 50 times prior to making additional dilutions. The plating medium was trypticase soy agar (TSA; BBL) with and without 0.05% diamide (Calbiochemical Co., Los Angeles, CA). A solution of diamide (1%) was sterilized by filtration, and appropriate amounts were added to the TSA after it was sterilized and cooled to 50°C. Plates, subsequent to the initial experiment, were incubated 3 days at 22°C. Except as stated otherwise, procedures were according to *Standard Methods for the Examination of Dairy Products* (APHA, 1972).

RESULTS & DISCUSSION

Optimal temperature and time for plate counts

Five sets of duplicate plates (poured with TSA containing 0.05% diamide) were prepared with skin from a chicken carcass that had been stored at 4.4°C until off-odor was detected; one set was incubated at each of the following temperatures: 5, 22, 26, 30 and 35°C. The plates at each temperature were observed daily for the development of colonies, and the plate counts reported in Figure 1 were determined.

Results showed that the optimal temperature for incubating the plates was 22°C. An incubation time of 3 days at this temperature was selected for additional experiments, though slightly higher counts were found after plates had been incubated at this temperature for 4 or 5 days. Colonies developed at 35°C, but the numbers were comparatively small (1.0 × $10^2/cm^2$ after incubation for 4 days) and not plotted in Figure 1.

Identification of microflora

A chicken was stored at 4.4°C until off-odor was detected, skin was plated at 22°C on TSA with 0.05% diamide, and several colonies were isolated and studied macroscopically and microscopically. Tests included Gram stain, spore stain, the oxidase test of Klinge (1960), and testing for fluorescent pigment production on Flo agar (BBL), the medium of King et al. (1954).

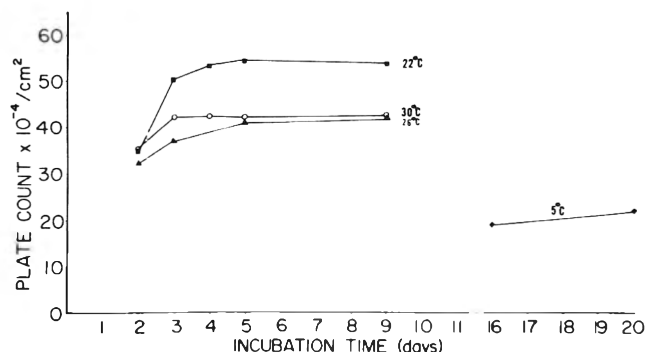

Fig. 1—Influence of temperature and time on plate count of skin of a chicken carcass that had been stored at 4.4°C until off-odor was detected. The plating medium was trypticase soy agar containing 0.05% diamide

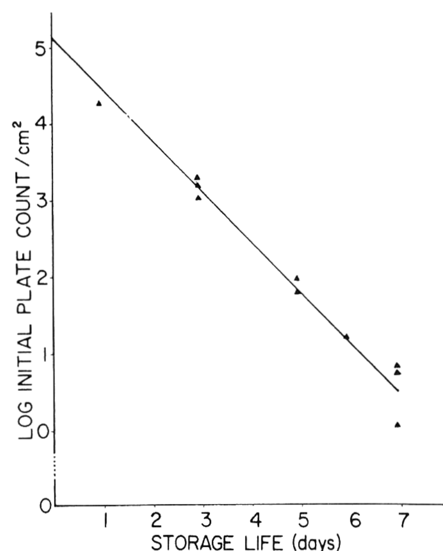

Fig. 2–Relation of initial plate count to shelf life of chicken carcasses stored at 4.4°C. The plating medium was trypticase soy agar containing 0.05% diamide.

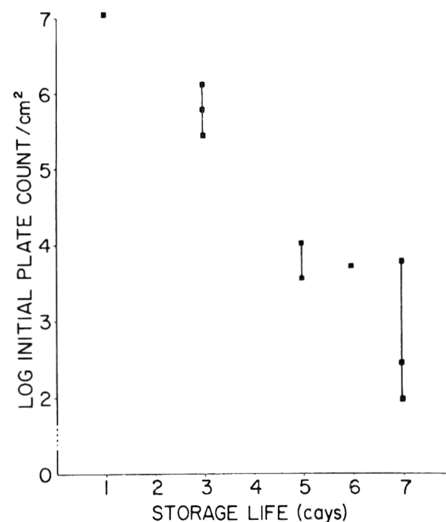

Fig. 3–Relation of initial plate count to shelf life of chicken carcasses stored at 4.4°C. The plating medium was trypticase soy agar.

The colonies that developed were similar in morphology (off-white, glossy, slimy, smooth surfaces, entire borders, low convex cross-sections), and the plates had putrid odor. Six isolates that were studied in greater detail were Gram negative, unipolarly flagellated, nonsporeforming, short rods. All were oxidase positive and three produced fluorescent pigment. Bacteria present on the chicken skin and able to grow in the presence of diamide thus were substantiated as being species of *Pseudomonas,* and obviously species of *Pseudomonas* other than *fluorescens* formed colonies on TSA containing 0.05% diamide, since only half of the six isolates studied in detail produced fluorescent pigment.

Relation of initial count to shelf life

Skin samples from 10 different chicken carcasses were plated on TSA with and without diamide. Subsequently, the chickens were stored at 4.4°C in the original polyethylene bags and smelt twice per day to determine the occurrence of spoilage. Logarithms of the initial counts determined with and without diamide were plotted against observed shelf life.

With (Fig. 2) or without (Fig. 3) diamide in the medium, low initial plate counts were indicative of longer storage before spoilage, but with diamide in the medium there were fewer variations in the relationship between initial count and storage life of the chickens. It was apparent that the higher initial counts on TSA without diamide were influenced by contaminants that were not later involved in spoilage of the refrigerated chicken carcasses and that adding diamide to the medium permitted selective enumeration of those microorganisms, primarily pseudomonads, that were related to spoilage of the chicken carcasses at 4.4°C. Determining plate counts with TSA containing 0.05% diamide thus appears useful as a means of predicting the shelf life of raw chicken and possibly other fresh meats that are to be stored under refrigeration.

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Ms received 8/23/75; revised 10/14/75; accepted 10/21/75.

This work was supported in part by the Scholarship and Research Foundation of the Pacific Egg and Poultry Association.

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THE INFLUENCE OF TWO KINDS OF PROTECTED LIPID SUPPLEMENT ON THE FLAVOR OF LAMB

ABSTRACT

Lambs were fed for 6 wk on diets containing formaldehyde-protected sunflower seed-casein or safflower oil-casein. The flavor of these and conventional lamb were compared by laboratory taste panel and GLC-olfactory assessment of the flavor volatiles. Both protected lipid diets significantly decreased conventional lamb odor and flavor intensity and imparted 'oily' odor and flavor characteristics attributed to the presence of abnormally high levels of deca-2,4-dienal in the cooked meat. Meat from lambs fed the protected sunflower seed-casein but not the protected safflower oil-casein possessed a characteristic 'sweet' odor and flavor, attributed to increased amounts of cis-γ-dodec-6-enolactone in the lipid portions.

INTRODUCTION

FORD et al. (1975) examined the flavor of lamb and yearling sheep meats containing nearly 20% of linoleic acid in the fatty acids of subcutaneous fat obtained by supplementing the diet of the animals with a formaldehyde-treated sunflower seed-casein preparation, described by Scott et al. (1972). Laboratory taste panels found that the cooked 'high-linoleic' sheep meats possessed a more 'sweet' and 'oily' aroma and flavor than meat from other sheep pastured on alfalfa or fed a normal feedlot ration. The fatty portions of these cooked high-linoleic sheep meats were found (Park et al., 1974) to contain elevated levels of 4-hydroxydodec-cis,6-enoic acid lactone (cis-γ-dodec-6-enolactone) and trans,trans-deca-2,4-dienal in the cold-finger fraction obtained by high-vacuum degassing. These components were believed to contribute to the 'sweet' and 'oily' aroma and flavor characteristics, respectively in the cooked meat, following consideration of their concentration in these fractions and their odor or flavor thresholds (Park et al., 1975).

The 'sweet' aroma was considered likely to prove undesirable to consumers and was detectable in and around the kitchen after cooking. In small-scale (Scott, 1974) experiments a formaldehyde-treated safflower oil-casein-preparation (Scott et al., 1971) was fed to sheep and produced 20% linoleic acid mutton or lamb which elicited no comments from consumers on the presence of a 'sweet' aroma or flavor in the cooked state. This prompted the present study, which reports a comparison of the flavor of lambs fed a conventional feedlot ration (basal diet), alone or supplemented with either formaldehyde-treated safflower oil-casein (basal plus protected safflower oil-casein) or formaldehyde-treated sunflower seed-casein (basal plus protected sunflower seed-casein).

EXPERIMENTAL

Animals and feed

36 Merino wether lambs, approximately 7 months old and of mean liveweight 25.8 kg were divided into three equal groups on a stratified random basis to distribute liveweights evenly. Each group was assigned to one of three dietary regimes and held outdoors in pens at the CSIRO Meat Research Laboratory. The diets used were: (i) basal; (ii) basal plus protected sunflower seed-casein both described previously (Ford et al., 1975); and (iii) basal plus protected safflower oil-casein as fed to sheep by Scott et al. (1971). The two supplemented diets contained approximately equal amounts of linoleic acid, by design. The amount of feed offered daily was gradually increased from 0.4 kg to 1.5 kg/head. Six wk after feeding commenced all lambs were removed from the diets and slaughtered at a nearby abattoir. The lamb carcasses were chilled to about 1°C and after 24 hr sectioned into the desired portions and held in polypropylene bags at −30°C until required, when they were thawed at 5°C for 24–48 hr. Oxidative deterioration was insignificant during this period of frozen storage, as assessed by measuring changes in the peroxide value of the subcutaneous fat.

Taste panel evaluations

Laboratory taste panel evaluations were carried out within 5 wk of slaughter. To allow for valid comparisons of data from taste panel and flavor volatile chemical examinations, the meat for taste panel examinations was prepared from boiled, ground meat (served hot) as in the method of Park et al. (1972). This cooking technique while unusual, has been found to give results comparable with those obtained from more conventional cooking procedures (Ford et al., 1975). A panel of 20 members was selected in a manner similar to that described by Ford et al. (1975) and comprised 14 men (24–45 yr) and six women (22–42 yr). Samples were presented three at a time for ten sessions from weight rank-matched lambs, one from each of the dietary treatment groups. The two lambs showing minimum weight gain from each group of 12 were excluded.

Panel members scored the samples for meat flavor properties as described by Park et al. (1972) i.e., intensity of meat aroma, 'different' aroma, meat flavor, 'different' flavor and flavor acceptability. An intensity rating scale of 0 = zero to 8 = very strong was used for the first four of these properties and a hedonic scale of 0 = very poor to 8 = very good used for rating of flavor acceptability. Panel members entered scores on computer cards (Gipps and Casimir, 1973). These data were analyzed for differences between dietary treatments for each flavor property.

Taste panel members were also asked to provide comments describing any flavor or aroma characteristics peculiar to any of the samples being tasted. The panel members were provided with a modified version of the descriptive terms collated by Harper et al. (1968) to assist them in describing such qualities.

Fatty acid analysis

Subcutaneous fat tissue was excised from each animal carcass near the tail, 24 hr after slaughter. 1-g samples of these were taken, the lipid was extracted and trans-esterified and the resultant methyl esters separated by GC. The percentage of individual fatty acids was determined, as described earlier (Park et al., 1975).

Examination of volatile flavor components

Of the carcass portions unused for taste panel assessment the meat and fat from four animals from each treatment group were individually ground and cooked and the volatiles distilled under reduced pressure using the methods described earlier (Park et al., 1975). In this way the volatiles from the steam-distillable portion, the more volatile (cold trap) and less volatile (cold-finger) fractions from high-vacuum degassing of the fat were concentrated into volumes of 1 ml or less. These fractions were subsequently examined in a Packard 7300 gas chromatograph, using a 30.5m stainless steel SCOT column coated with Carbowax 20M (Perkin Elmer Products Corporation) as described previously (Park et

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al., 1975) and fitted for olfactory examination of eluting components. The quantitative composition of fractions was estimated by the measurement of peak areas obtained for individual components on recorder chart traces and summing these. Components of interest had been previously identified as constituents of similar fractions by GC-MS techniques.

RESULTS & DISCUSSION

Animal feeding

The three groups of lambs exhibited similar weight gains over the 6-wk experimental feeding period and no significant differences in weights of the carcasses. The mean levels of linoleic acid in subcutaneous (tail) lipids were found to be 2.6, 20.5 and 21.7% from the basal, basal plus protected sunflower seed-casein and basal plus protected safflower oil-casein diets, respectively.

Taste panel evaluations

Mean taste-panel responses and related least significant differences ($P = 0.05, 0.01, 0.001$) resulting from comparison of the diets and adjusted for missing values are given in Table 1. From these data it can be seen that two of the three diets induced significant differences in flavor and aroma properties when compared to the third diet. The meat from lambs fed the basal diet exhibited significantly lower ($P < 0.001$) 'different' aroma and flavor scores than that from lambs fed either protected lipid supplement. The meat from lambs fed the protected sunflower seed-casein supplement also gave significantly lower 'different' aroma ($P < 0.001$) and 'different' flavor ($P < 0.01$) scores than that from lambs fed the protected safflower oil-casein. These results confirm and extend the initial findings (Ford et al., 1975) that protected lipid supplements, in raising linoleic acid levels to about 20% of total fatty acids, produce at the same time marked flavor changes in the cooked meat which lowered its flavor acceptability to a majority of panel members. The mean rating of flavor acceptability of the basal diet lamb meat was normal for ground, boiled lamb.

Meat from lambs fed the protected safflower oil-casein elicited more comments from panellists on the presence of an 'oily' or 'paint-like' flavor than that from lambs fed the protected sunflower seed-casein or basal diets (18:8:1 and 9:0:0, respectively). In contrast meat from lambs fed the protected sunflower seed-casein drew more comments on the presence of a 'sweet' aroma and flavor than that from lambs fed the other diets (7:4:4, respectively).

Examination of volatile constituents

The dietary treatments markedly influenced the yield of the individual components of cold-finger fractions from high-vacuum degassing of lipid portions from the cooked meat. This is illustrated in Figure 1 which shows GC traces from fractions representative of each of the dietary treatments, obtained on a 30.5m Carbowax 20M-coated SCOT column. The most significant, between-diet differences noted amongst these fractions were:

(1) Fractions from the basal diet treatment, unlike the others, consistently contained only small amounts of trans,trans-deca-2,4-dienal;

(2) Fractions from the protected sunflower seed-casein treatment contained both the decadienal and cis-γ-dodec-6-enolactone as major components i.e., at least 10% of the total;

(3) Fractions from the protected safflower oil-casein treatment contained the decadienal as a major component and the unsaturated lactone as a prominent (1–5% of total) component. However, whereas there was at least twice as much decadienal as occurred in fractions from the protected sunflower seed-casein treatment, there was only 1/4–1/5 as much of the unsaturated lactone. The level of both lactones was almost twice that obtained from the basal diet treatment;

(4) Decadienal levels in fractions from the safflower oil-casein treatment were twice that found in corresponding fractions from the sunflower seed-casein treatment.

The finding of elevated levels of trans,trans-deca-2,4-dienal in high-linoleic beef (Ford et al., 1976) as well as in the high-linoleic sheep meats from the two different protected lipids here lends support to the report of Patton et al. (1959) that linoleic acid-rich lipids generate this aldehyde on heating in the presence of water. In addition it appears that some elevation in levels of both the saturated and unsaturated γ-dodecalactones is also a consequence of generating the linoleic-rich lipids in meat. However, the large differences obtained in yields of cis-γ-dodec-6-enolactone through the use of the two different

Table 1—Mean taste panel ratings and least significant differences in flavor properties of lamb from basal diet or basal diet supplemented with one of two protected lipids

Feeding treatment	Meat aroma	Meat flavor	Different aroma	Different flavor	Flavor acceptability
			Mean taste panel ratings[a]		
Basal diet	3.79	4.18	0.94	0.71	4.70
Basal diet plus protected sunflower seed-casein	3.37	3.55	1.66	1.87	3.56
Basal diet plus protected safflower oil-casein	3.06	3.11	2.75	2.94	3.02
			Least significant differences		
Level of significance					
Prob = 0.05	0.20	0.42	0.24	0.59	0.57
Prob = 0.01	0.28	0.58	0.33	0.80	0.79
Prob = 0.001	0.38	0.78	0.46	1.09	1.07

[a] Intensity rating scale: 0, zero; 2, slight; 4, moderate; 6, strong, and 8, very strong intensity. Hedonic scaling of acceptability: 0, very poor; 2, poor; 4, moderate; 6, good and 8, very good acceptability.

protected lipid supplements are considered likely to be of considerable consequence to flavor properties of the meat. The concentration of this lactone in cold-finger fractions from the protected sunflower seed-casein treatment (1–2 ppm in lipid portions of meat) was similar to those reported earlier (Park et al., 1975) and, for the reasons more fully discussed then, is considered sufficient to contribute significantly to the aroma properties of the meat. In contrast, the yield in corresponding cold-finger fractions derived from lambs consuming the protected safflower oil-casein diet (0.1–0.2 ppm in the lipid portions) is considered to be insufficient to make a significant contribution to the aroma or flavor of the meat.

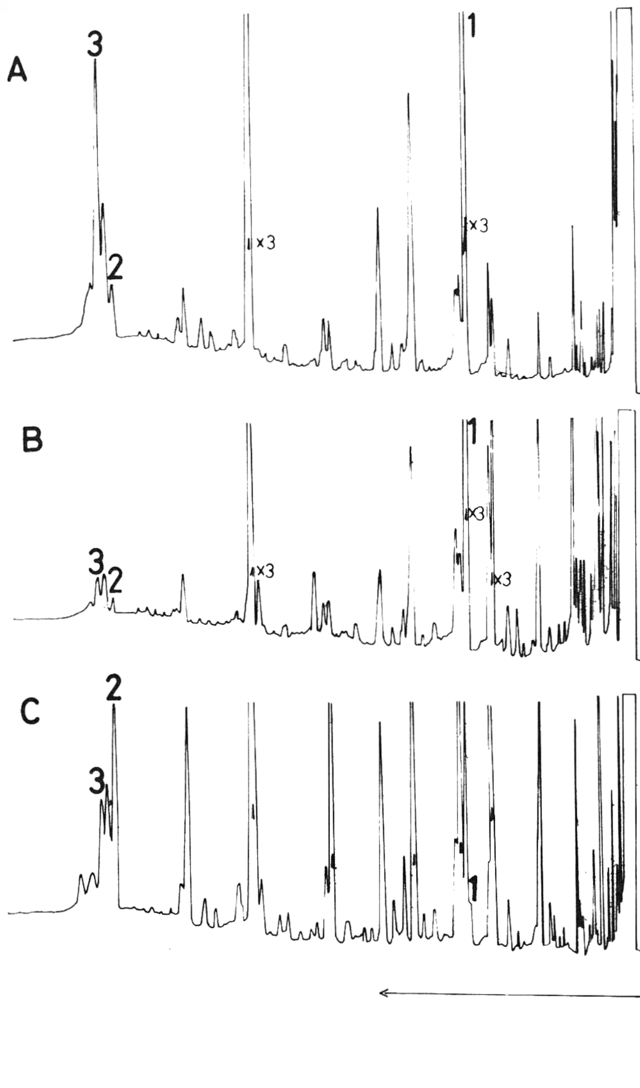

Fig. 1—Gas chromatograms of 0.1 μℓ samples of cold-finger fractions from vacuum degassing of lipid from cooked lamb fed prior to slaughter: A, formaldehyde-protected sunflower seed-casein; B, formaldehyde-protected safflower oil-casein; or C, basal diet. Peaks 1, 2 and 3 are attributed respectively to trans,trans deca-2,4-dienal, γ-dodecalactone and cis γ-dodec-6-enolactone. Gas chromatographic conditions: 100 ft Carbowax 20M SCOT stainless steel column, N_2 Flow rate 4 ml per min, column oven temperature 120°C for 5 min then 1° rise per minute to 170°C, flame ionization detector sensitivity, 3×10^{-11} amp full scale deflection.

The steam-volatile fractions obtained by distillation of the meat from the two protected lipid diets were found qualitatively identical and contained greater quantities of n-hexanal, hept-2-enal, oct-2-enal n-pentanol and n-butanol, than corresponding fractions from the basal diet. These components are common products of the oxidation of linoleic acid in lipid systems. Since the meat generating these components showed no signs of autoxidative deterioration prior to cooking, it must be assumed that they were generated during cooking. We are not able to ascribe any consequence to flavor properties of the meat from such differences as they were recovered in insufficient yield from high-linoleic meat to indicate likely individual contributions to aroma or flavor of the meat.

No consistent differences were found between protected lipid diets in the qualitative or quantitative composition of the more volatile fractions (Park et al., 1975) obtained by vacuum degassing of lipid portions from the cooked meat.

The observed effect of protected lipid type on levels of cis-γ-dodec-6-enolactone in lipid portions of cooked lamb remains unexplained. This question, because of its relevance to the flavor of cooked high-linoleic lamb, is the subject of current investigations by us and related groups in CSIRO.

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Ms received 7/11/75; revised 9/16/75; accepted 9/23/75.

These experiments were funded in part by grants from Alta Lipids (Aust.) Pty. Ltd. and the Australian Meat Research Committee. The animals used in these experiments were obtained from the flocks of the CSIRO Div. of Animal Physiology Pastoral Research Laboratory at Armidale, New South Wales. The protected sunflower seed-casein supplements was provided by the CSIRO Div. of Animal Physiology and the protected safflower oil-casein supplement by the Victorian Dept. of Agriculture. The technical skills of T. Steginga, P. Hancock, I. Griffiths, Phillipa Hancock and Judith Darwen and the participation of laboratory staff as taste panel members are gratefully acknowledged.

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QUALITY CHARACTERISTICS OF PORTION CONTROLLED TENDERLOIN STEAKS

ABSTRACT

Steaks from mechanically pressed tenderloins and steaks from paired, unpressed control tenderloins from Good and Choice grade steers were studied. Pressing did not adversely affect overall quality of the tenderloin steaks; however, fibers of the pressed steaks were kinked and compacted when compared to control steaks. No differences in fiber breakage or in sarcomere length were noted. Fat, moisture, lean color, marbling texture, marbling amount, lean texture, lean firmness and moisture on the steak surface were similar in pressed and unpressed steaks. Panel scores for tenderness, juiciness and flavor and Warner-Bratzler shear values were also similar for pressed and unpressed steaks. Pressed steaks had greater cooking loss, but they were more uniform in size and shape than control steaks.

INTRODUCTION

INTRODUCTION of the meat press has greatly simplified the purveyor's task of meeting size, weight and trim specifications by mechanically shaping subprimal cuts, such as tenderloins, prior to portioning into steaks. However, studies on quality characteristics before and after pressing are limited (Mahoney, 1973; D.L. Huffman, personal communication).

In this study we determined some histological changes which occurred in pressed beef tenderloins (psoas major muscle and attached psoas minor muscle) and we compared steaks from pressed and unpressed tenderloins for quality. Cooked steaks were evaluated for appearance, cooking loss and palatability.

MATERIALS & METHODS

20 PAIRED TENDERLOINS from 10 Good and Choice steer carcasses ranging in weight from 307–341 kg, were used for comparisons. The steers, all from the same feedlot, were slaughtered by a commercial packer and the carcasses aged for 48 hr at 1°C. The paired tenderloins were then removed, trimmed and frozen at –40°C for 24 hr. After tempering at –2°C for 48 hr, the control tenderloin of the pair was cleaved into 3.8 cm thick steaks of varying weights. The other tenderloin was shaped into a uniform "log" by a Bettcher Press and then cleaved into 3.8 cm thick steaks weighing approximately 250g each.

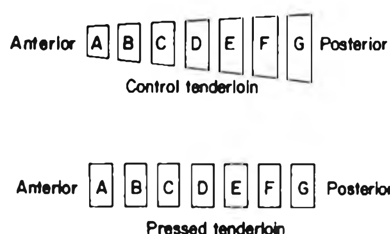

Fig. 1—Anatomical identification of tenderloin steak pairs.

After portioning, the steaks were vacuum packaged with a DuPont Bivac packaging system which formed a tight ionomer film around the contour of the meat. Steaks from corresponding anatomical locations on the tenderloins were letter designated, with A being steaks nearest the anterior ends and succeeding letters proceeding toward the posterior ends of the control and pressed tenderloin pairs (Fig. 1). Upon receipt at the University of Wyoming the packaged steaks were stored at –10°C and evaluated within 90 days.

Three experienced judges scored color, marbling texture, marbling amount, moisture, lean texture and firmness of lean for individual steaks from locations B, C, D, F and G (Fig. 1). Paired steaks were removed from the freezer and tempered at 2°C for 18 hr. One hr before scoring, the steaks were unwrapped and placed in a 21°C room. Each judge individually scored quality traits according to USDA (1965) standards.

Steaks D and F (Fig. 1) from each tenderloin were broiled to 60°C internal temperature. Cooking time was recorded and the cooked steaks were weighed for cooking loss computation immediately after removal from the oven. Separation of psoas major and psoas minor muscles during cooking was subjectively scored from one to five, with one being "holds together" and five being "separates completely." Cooked steaks from locations B and F (Fig. 1) were used for sensory panel testing. A 10-member specialized panel was served wedge-shaped untrimmed portions from the psoas major muscle. The outside fat was left untrimmed to approximate consumer servings. Each day, panel members were served samples from two control and two pressed steaks. Each member scored tenderness, juiciness and flavor using a hedonic scale of one to nine, where one was "dislike extremely," five was "neither like nor dislike," and nine was "like extremely." Panel members were instructed to score the most tender steaks the highest on the one to nine hedonic scale. Steaks C and E (Fig. 1) were equilibrated to room temperature for 2 hr after cooking. Two 2.54-cm cores from each steak were sheared four times each with a Warner-Bratzler shear.

A 1-cm thick slice from the face of frozen steaks D and G (Fig. 1) was removed with a band saw for histological study. Two longitudinal and two transverse frozen samples, each from different quadrants of the slice, were carefully mounted on microtome chucks so that orientation of the fibers were not disturbed. After equilibration to room temperature, the samples were quick-frozen in liquid nitrogen, and 10μ thick tissue slices were made on a Slee Cold Microtome. Two tissue samples from each chuck were mounted on cover slips, stained with eosin for 1 min to highlight fiber characteristics, and mounted on slides with glycerine gel. Thus, four longitudinal and four transverse slides were prepared for each steak. Two workers each estimated fiber angle in four random fields of each of the transverse slide preparations. Ninety degrees was a perfectly transverse fiber, and zero degrees a perfectly longitudinal fiber. The greatest fiber diameter of 16 random fibers was also measured. Since each sample was carefully removed and mounted in the same manner, greatest fiber diameter was a measure of the squamous angle of the muscle fibers in each steak. The greater the fiber diameter, the greater was the distortion of the transverse fiber. Slides of longitudinal sections were used for study of mechanical kinking and breaking of fibers. Obvious breaks were counted in four random 450× fields by two workers. The procedure of Smith et al. (1969) was used to determine sarcomere length. Myofibril fragility was scored by counting sarcomeres on 25 random myofibril segments based on procedures outlined by Takahashi et al. (1967) and Goll (1968). Fiber diameter determination was as outlined by Smith et al. (1969). Homogenized psoas major muscle samples from steaks D and G were used for fat and moisture determination following AOAC (1970) procedures.

RESULTS & DISCUSSION

AVERAGE QUALITY SCORES for color, marbling texture and marbling amount were similar for pressed and control steaks (Table 1). Marbling amount tended to be greater in steaks cut from the centers of the tenderloins (moderate amount) than in steaks cut from the ends (modest amount). Frozen pressed steaks tended to appear firmer and to display less surface moisture than frozen unpressed control steaks when both groups of steaks were thawed. Conversely, Mahoney (1973) reported penetrometer values to be higher (less firmness) for thawed pressed steaks when compared to steaks which were not frozen or pressed.

Cooking losses were significantly greater ($P < 0.05$) for pressed steaks when compared to controls (Table 2). The greater cooking loss, which has also been observed in commercial practice, could be a result of reduction of intercellular spaces within the muscle protein structure during pressing. Hamm (1964) pointed out that unbound water is normally trapped within the intercellular spaces causing increased water-holding capacity. Reduction of the spaces by pressing could cause water normally caught and held there to be lost during cooking.

Control steaks displayed greater ($P < 0.05$) separation of psoas major and psoas minor muscles than pressed steaks making the control steaks appear less attractive when cooked. This finding is contrary to that often observed in commercial practice where pressed steaks are sometimes criticized for having greater muscle separation than unpressed steaks. Differences between our findings and those observed in practice may be due to such factors as frozen storage time, processing variations in tempering, freezing or pressing, or to the different conditions under which meat is thawed and cooked.

Steak location did not affect cooking loss or muscle separation. Cooking times were similar for steak locations B and F (Table 2) and for control versus pressed steaks. Sensory panels found no differences in tenderness, juiciness or flavor of steaks, either by treatment or by location. Warner-Bratzler shear values for control and pressed steaks were also similar.

Measurement of fiber angle and greatest fiber diameter in pressed and control steaks showed significant ($P < 0.05$) fiber distortion as a result of pressing (Table 3). However, no differences in muscle fiber angle between anatomical location D or G on the tenderloins were evident. No muscle fiber breakage due to pressing occurred as measured by breaks per field or by sarcomeres per myofibril. Since sarcomere length was the same and fiber diameters were similar for pressed and control steaks, *no passive shortening* of fibers occurred during pressing. If passive shortening of fibers had occurred, the pressed steaks would have had shorter sarcomeres (Herring et al., 1967; Takahashi et al., 1967). Shorter ($P < 0.05$) sarcomeres at the G location (posterior) with accompanying greater fiber diameters indicate that greater contraction occurred at the G location than at the D location (center) of the tenderloins. Number of fiber breaks in eight 450X fields show that no fiber breakage

Table 1—Least-squares means and standard errors for subjective quality scores of steaks by treatment and location[a]

Variable	Treatment[b] Control (N = 50)	Pressed (N = 49)	S.E.	Location (N = 20 steaks) B	C	D	F	G[c]	S.E.
Color[d]	4.2	4.2	0.16	4.1	3.9	4.3	4.1	4.6	0.25
Marbling texture[e]	3.3	3.2	0.13	3.2	3.3	3.5	3.0	3.3	0.20
Marbling amount[f]	18.9	19.0	0.55	18.4	19.9	20.8	18.0	17.7	0.89
Lean texture[e]	4.1	4.1	0.23						
Lean firmness[g]	4.6	3.8	0.39						
Moisture[h]	6.4	5.7	0.39						

[a] Greatest standard error in unequal treatments is given. None of the least-squares means on the same line was significantly different for treatment or location ($P < 0.05$).
[b] Steaks in each treatment from combined locations B, C, D, F and G
[c] Location "G," n = 19.
[d] 4 = Avg A maturity; 5 = A+ maturity.
[e] 3 = slightly medium; 4 = medium.
[f] 18 = modest +; 19 = moderate –; 20 = moderate.
[g] 3 = moderately firm; 4 = slightly firm; 5 = slightly soft.
[h] 5 = moderately moist; 6 = moist; 7 = very moist.

Table 2—Least squares means and standard errors for cooked steaks by treatment and location (N = 20)

Variable	Treatment[a] Control	Pressed	S.E.	Location B	F	S.E.
Cooking loss (%)	26.7[c]	29.8[d]	0.63	27.8	28.7	0.63
Muscle separation[b]	3.0[c]	2.2[d]	0.26	2.6	2.6	0.26
Cooking time (min)	25.4	26.3	0.84	25.5	26.2	0.84
Panel tenderness	7.3	7.1	0.13	7.2	7.2	0.13
Panel juiciness	7.0	6.8	0.09	6.9	6.9	0.09
Panel flavor	6.9	6.9	0.08	7.0	6.9	0.08
W-B shear (kg)	4.7	4.4	0.31			

[a] Steaks in each treatment from combined locations B and F except W-B shear where steaks C and E were evaluated.
[b] 1 = holds together; 5 = falls apart.
[c,d] Different superscripts indicate means are significantly different ($P < 0.05$) between control and pressed steaks.

Table 3—Least squares means and standard errors for histological and chemical values by treatment and location

Variable	Treatment[a] Control (n = 20)	Pressed (n = 19)	Location D (n = 20)	G (n = 19)
Angle, degrees	83 ± 2.68[b]	72 ± 2.75[c]	79 ± 2.68	77 ± 2.75
Greater fiber diameter (μ)	60.3 ± 5.01[b]	76.4 ± 5.15[c]	68.6 ± 5.01	68.0 ± 5.15
Fiber diameter (μ)	49.7 ± 1.25	51.3 ± 1.29	48.9 ± 1.25	52.1 ± 1.29
Breaks, per fields	40.6 ± 3.78	33.3 ± 3.78	42.4 ± 3.68	31.5 ± 3.88
Sarcomeres per myofibril	12.5 ± 0.48	12.2 ± 0.49	11.8 ± 0.48	13.0 ± 0.49
Sarcomere length (μ)	2.9 ± 0.11	2.9 ± 0.11	3.1 ± 0.11[d]	2.8 ± 0.11[e]
Percent fat	7.4 ± 0.65	6.9 ± 0.67	6.9 ± 0.65	7.4 ± 0.67
Percent moisture	69.8 ± 0.69	70.4 ± 0.71		

[a] A total of 38 special and 31 clawmeat samples were tested fresh. After 7 days storage at 4°C, 24 special and 21 clawmeat samples were retested. About 18% of the special and 22% of the clawmeat fresh samples were positive for coliforms (0.2g sample). Counts represent average and their ranges.
[b] Vp: presumptive V. parahaemolyticus

occurred as a result of pressing. The decreased number of fiber breaks evident in pressed steaks when compared to control steaks indicates that pressing compaction probably obscured some breaks which were present. The large standard errors for fiber breaks within control or pressed steaks explains why means for breaks per field were not significantly different. Number of sarcomeres per fragmented myofibril further support the finding that no fiber breakage occurred as a result of pressing. If pressing had caused fracturing, it would have decreased the average number of sarcomeres per myofibril fragment of the pressed steaks (Davey and Gilbert, 1967; Takahashi et al., 1967).

Visual evidence in longitudinal slides showed that pressing caused kinking of fibers in the pressed steaks. The normally straight or slightly wavy fibers were often kinked into v-shaped rows.

Percent ether extraction and percent moisture in control and pressed steaks were not significantly different. Since the paired tenderloins were stored for the same length of time and processed under the same conditions this would be expected. It is evident that no moisture loss occurred as a result of pressing.

The main effects of pressing on tenderloin steaks were kinking and compaction of fibers and increases in cooking losses. Neither was detrimental to palatability of the pressed product. The uniformity and eye appeal of the pressed tenderloin steaks over control steaks, coupled with lack of quality differences, gives the pressed steaks a definite advantage over unpressed steaks. The results found in this study with an inherently tender muscle group may not be applicable to tougher muscle groups. Additional study is needed to determine effects of pressing on quality of cuts other than tenderloins.

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Ms received 8/8/75; revised 9/25/75; accepted 9/30/75.

University of Wyoming Agricultural Experiment Station Journal Article No. 647.

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EVALUATION OF A CALCIUM ALGINATE COATING AND A PROTECTIVE PLASTIC WRAPPING FOR THE CONTROL OF LAMB CARCASS SHRINKAGE

ABSTRACT

Ninety lamb carcasses were evaluated for shrink loss, microbial growth and temperature reduction following treatment with: (1) an edible calcium alginate coating, Flavor-Tex®; (2) plastic wrap; or, (3) no treatment (control). Lambs (n = 30) were slaughtered on three consecutive days with 10 carcasses/day being randomly assigned to each treatment. Carcasses receiving the edible coating were significantly ($P < 0.05$) lower in 24-hr shrinkage loss (1.55%) than the controls (2.77%); however, those in plastic wrap had the least amount of shrinkage (1.20%), and maintained this advantage through 7 days postmortem. Total surface microbial counts from the sirloin area indicated a significant ($P < 0.05$) reduction at day 5 and 7 for the alginate coated carcasses, with the plastic wrap carcasses having the highest counts on all days. Internal leg temperature reduction (chilling) was essentially uniform at the end of a 24-hr chill at 2°C regardless of treatment. No significant differences were observed between treatments for cooking loss, flavor, juiciness, off-odor or overall acceptability.

INTRODUCTION

THE LOSS OF WEIGHT, or shrinkage, associated with lamb carcasses during the post-slaughter chilling, storage and distribution periods is of major concern to the meat packing industry. In addition to weight loss, shrinkage may be accompanied by surface desiccation and color deterioration detrimental to shelf life and consumer acceptability (Smith and Carpenter, 1973). Several workers have shown that carcass shrinkage can be controlled by the maintenance of low temperature conditions accompanied by high relative humidity and minimal air circulation (Fleming and Earle, 1968; Smith and Carpenter, 1973). However, control of shrinkage and surface microbial growth appear to be interrelated in that moist carcass surfaces are more conducive to bacterial growth than dry surfaces.

Attempts to protect the surface of fresh meats by the application of an edible film coating have been limited to poultry, fish and retail red meat cuts. Allen et al. (1963) evaluated beef steaks, pork chops and poultry pieces by first dipping them into either sodium alginate or alginate-cornstarch solutions at 87.7°C followed by dipping into 5M $CaCl_2$ for 1–2 sec. In general, the alginate-cornstarch mixture retarded moisture loss more than plain alginate, while both coating solutions effectively reduced moisture loss when compared to untreated controls. Coated samples were significantly better in juiciness, texture and color; however, a bitter off-flavor was detected and attributed to the flavor imparted by the $CaCl_2$ solution. Earle (1968a) concluded that the bitter taste could be eliminated if the calcium chloride solution was below 0.5M and if the viscosity of the gelling solution was controlled. Earle (1968b) described a calcium alginate gel coating to be used for the protection of raw fish, meat and poultry. This edible coating, known commercially as Flavor-Tex®, involves the formation of a film around the food product by gelling the malto-dextran sodium alginate coating with a calcium chloride-carboxymethylcellulose solution. This process is presently used on seafoods and certain extruded products; however, its application on meat carcasses and cuts has not been evaluated.

The purpose of this study was to compare shrinkage loss, chill rate, surface microbial growth, carcass appearance and palatability characteristics of lamb carcasses which had been (1) coated immediately post-slaughter with Flavor-Tex, (2) wrapped in a low moisture permeability plastic film, applied for the initial 24-hr postmortem period or (3) left unprotected (controls).

MATERIALS & METHODS

Processing of lamb carcasses

A total of 90 lambs were slaughtered on three consecutive days (30/day). Each lamb was randomly assigned to a day of slaughter and to one of three shrinkage treatments. Immediately following slaughter and washing, the carcasses were moved by overhead rail into an adjacent room (10°C) where an initial microbial count, internal temperature from the thick area of the legs, hot carcass weight and shrinkage treatment were conducted. Lamb carcass weights ranged from 17.8–28.8 kg and fat thickness measurements were between 3.8–8.9 mm.

Microbial determinations

Surface microbial samples were collected from the sirloin and belly (flank-plate juncture) areas of the carcass. A 6.46 cm^2 area was swabbed using the standard moist-swab technique (APHA, 1972). Serial dilutions were prepared using Butterfield's phosphate diluent and plates were poured using Standard Plate Count Agar (Difco) for aerobic counts. Incubation of the plates was for 5 days at 20°C. Samples were collected at 0, 2, 5 and 7 days postmortem.

Carcass temperature

Carcass temperature was determined by averaging the internal temperature from both hind legs. Temperatures were collected at 0, 6, 24 and 48 hr postmortem using a pyrometer equipped with a 3-in. probe (PYRO Surface Pyrometer, Pyrometer Instrument Co.).

Carcass weight

Carcass weights were determined at 0, 1, 2, 3, 5 and 7 days postmortem using a Toledo model 2071 scale with 0.1 lb gradations. Shrinkage at each day postmortem was based on the initial hot carcass weight taken after washing the carcass.

Shrinkage treatments

The edible calcium alginate film coating, Flavor-Tex (U.S. patent No. 3,395,024, Food Research Inc., Tampa, Fla.) consists of two parts: (1) a malto-dextran sodium alginate solution 142 g/liter water), and (2) a $CaCl_2$-carboxymethylcellulose solution. Both solutions are prepared at room temperature and can be stored at 4°C for at least 2 wk. Solution 1 was sprayed directly onto all surfaces of the carcass, followed immediately by the spraying of solution 2 over solution 1. Interaction of these two solutions by cooperative association causes formation of a clear homogeneous film over the entire carcass. Both solutions were applied using a Binks Model 33–112 compressed air system fitted with dual spray guns (Binks Mfg. Co., Chicago, Ill.).

Carcasses which received the plastic wrap (Borden Resinite-90, a low moisture, high oxygen transfer wrap) were wrapped while hanging from the overhead rail. At the end of 24 hr the plastic wrap was removed.

Control carcasses received no external covering. All carcasses were

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placed in a 2°C cooler with a relative humidity of 80% and a wind velocity of approximately 24 km/hr.

Sensory evaluation

Following the 7-day shrinkage experiment, six control and six Flavor-Tex-coated carcasses were retained for sensory evaluation. To evaluate the effect of the 7-day experiment as well as the effect of recoating of the primal cut with Flavor-Tex, six legs from the initial control group were coated with Flavor-Tex and assigned to the control-Flavor-Tex group (C/F). The remaining six legs from the original control treatment were assigned to the control-control group (C/C). Six legs from the initial Flavor-Tex treatment were recoated (F/F), while the six remaining legs were assigned to the Flavor-Tex-control group (F/C). All legs were frozen until evaluation (4 wk). Lamb legs were thawed at 4°C overnight and cooked in individual shallow pans to an internal temperature of 75°C in a 177°C oven. Percent cooking loss was calculated by comparing cooked weight with the weight after thawing. A trained eight member sensory panel evaluated the medial sections of the semimembranosus and biceps femoris muscle samples for flavor, juiciness, off-odor and overall acceptability.

All data were analyzed using the Statistical Analysis System (SAS) employing Duncan's New Multiple Range Test (Barr and Goodnight, 1972).

RESULTS & DISCUSSION

MEAN VALUES for postmortem shrinkage of lamb carcasses analyzed by shrinkage treatment and by day of slaughter are presented in Table 1. At all time periods postmortem, the carcasses coated with Flavor-Tex or wrapped in plastic film had significantly ($P < 0.05$) lower shrinkage than the control carcasses. In addition, the plastic wrapped carcasses had significantly less shrinkage than the Flavor-Tex-treated carcasses.

Smith and Carpenter (1973) reported that 75% of the 72 hr weight loss of lamb carcasses was incurred during the initial 24-hr post-slaughter period. These workers attributed this large initial shrinkage to loss of water added during the washing procedure. Subsequent weight loss was attributed to moisture loss, in the form of evaporation from the carcass tissue.

In the present study, the plastic wrap impeded both evaporation of moisture and heat transfer from the carcass during the first 24 hr. The 1.20% shrinkage recorded for this treatment group should primarily reflect loss of accumulated wash water. Accumulated moisture on the inside portion of the wrap and very moist carcass surfaces were noted for this group. These conditions would be expected to influence both microbial growth and initial chill rate.

The alginate coating does not form a moisture vapor barrier as does the plastic wrap and therefore retards shrinkage via a different mechanism. Commercial alginates are largely copolymers of polymannuronic acid, and/or polyguluronic acid (Anonymous, 1973). Although the exact mechanism of gel formation is not known, it has been proposed that calcium is substituted for the monovalent cation (mostly sodium) and inter- and intramolecular crosslinks between the copolymer strands of alginate are formed (Anonymous, 1973). The calcium alginate coating process in the present study resulted in the addition of about 270g to the weight of the hot carcass. Approximately 90% of this added weight is water which is lost through evaporation prior to the evaporation of the carcass tissue moisture. Therefore, the alginate coating acts as a sacrificing agent rather than a moisture barrier. The 1.55% shrinkage value at 24 hr postmortem for the alginate-treated group (Table 1) reflects loss of accumulated wash water and loss of gel moisture, but little tissue desiccation. As moisture is removed from the coating, the latter becomes thinner, tougher and more intimately bound to the carcass.

Visual evaluation of carcass appearance revealed treatment differences. Those carcasses receiving the plastic wrap treatment had moist surfaces and a softer, whiter subcutaneous fat covering than did the other carcasses. The alginate coated carcasses had a glossy pseudo-moist appearance and the surface fat was slightly darker than the fat on the control carcasses.

Table 1–Mean values[a] for postmortem shrinkage (% ar[illegible] interna[illegible] leg temperature (°C) of lamb carcasses by shrinkage treatmen[illegible] and da[illegible] of slaughter

Day postmortem	Shrinkage treatment			Day of slaughte[illegible]		
	Control	Ca-alginate	Plastic wrap	1	[illegible]	3
	Shrinkage loss (%)					
1	2.77a	1.55b	1.20c	2.22a	1.5[illegible]b	1.6[illegible]b
2	3.25a	2.22b	1.88c	2.93a	2.0[illegible]b	2.35b
3	3.80a	2.96b	2.49c	3.49a	2.6[illegible]b	3.0[illegible]b
5	4.71a	4.01b	3.43c	4.72a	3.6[illegible]b	3.75b
7	5.36a	4.81b	4.19c	5.59a	4.3[illegible]b	4.40b
Hour postmortem	Internal temperature (°C)					
0	38.7a	38.2a	38.4a	36.4a	[illegible]9.[illegible]	39.8[illegible]
6	10.3a	10.0a	14.3b	10.3a	[illegible]1.5[illegible]	12.7[illegible]
24	4.1a	4.4a	5.0a	3.3a	4.7[illegible]	5.4b
48	5.3a	5.4a	5.4a	4.7a	[illegible].C[illegible]	5.5b

[a] Means on the same horizontal line bearing different letters differ significantly ($P < 0.05$)

Little variation in lean color was observed between the treatment groups.

The carcasses from the first day's slaughter group had significantly ($P < 0.05$) greater shrinkage values on all subsequent days than did those carcasses slaughtered on following days (Table 1). Addition of 30 hot carcasses/day to the chill cooler presumably reduced the efficiency of the cooler. However, a significant interaction between shrinkage treatment and day of slaughter was not observed.

In order to measure the effect of shrinkage treatment on chill rate, internal leg temperatures were collected (Table 1). At 6 hr postmortem the plastic wrap had retained significantly ($P < 0.05$) more carcass heat, but after 24 hr, no difference was observed. Again, the addition of 30 hot carcasses/day to the cooler significantly affected ($P < 0.05$) the chilling rate of the carcasses; however, no interaction between shrinkage treatment and day slaughtered occurred, indicating the loss of carcass heat was uniform across the treatments regardless of the effects of day slaughtered.

Shrinkage treatment effects of surface microbial growth were monitored by swabbing the sirloin and belly (flank-plate juncture) areas of each carcass at 0, 2, 5 and 7 days postmortem. These areas were selected to represent areas which are predominantly covered with fat (sirloin) or with lean (belly). Mean $\log_{10}$ values for total microbial count/6.46 cm^2 from both sampling areas are presented in Table 2. In the sirloin area, no significant differences were observed between treatment groups immediately post-slaughter. At 2 days postmortem, those carcasses which had been wrapped with the plastic wrap had significantly higher ($P < 0.05$) microbial counts ($\log_{10} = 3.65$) than did the control ($\log_{10} =$ [illegible].04) or alginate treated ($\log_{10} = 2.87$) carcasses. This difference was maintained through day 5 and day 7 with the day [illegible] plastic wrap carcasses having a significantly higher microbial count than either control or alginate-treated carcasses. Elevated microbial counts from the plastic wrap carcasses were due to the reduction in surface evaporation thereby maintaining a more favorable water activity (a_w) for growth. The calcium alginate-coated carcass tended to have lower surface microbial counts from the sirloin area at all time periods, although not always significant. The ionic effect of the calcium chloride would be expected to influence microbial growth on the carcass surface.

Microbial counts from the belly area were not significantly different among treatments at all intervals evaluated (Table 2). The high counts and lack of significant differences were probably due to cross contamination that occurred during the weighing of the carcasses.

Table 2—Mean $\log_{10}$ values[a] for total microbial count/6.46 cm^2 from sirloin and belly areas of control and treated lamb carcasses

Postmortem	Shrinkage treatment		
	Control	Ca-alginate	Plastic wrap
		Sirloin area	
0	3.64a	3.75a	3.86a
2	3.04a	2.87a	3.64b
5	3.47a	2.80b	3.82c
7	3.45ab	3.11b	4.14a
		Belly area	
0	3.90a	3.90a	4.03a
2	3.84a	3.99a	4.09a
5	4.24a	4.34a	4.31a
7	4.47a	4.46a	4.01a

[a] Means on the same horizontal line bearing different letters differ significantly ($P < 0.05$).

Table 3—Mean values for cooking loss and sensory panel scores of control and Flavor-Tex-coated lamb legs

	Treatment[a]			
	C/C	C/FT	FT/C	FT/FT
Cooking loss(%)	25.20	25.52	26.77	25.71
Flavor[b]	6.25	6.13	5.68	5.98
Juiciness[b]	5.56	5.46	5.45	5.33
Off-odor[c]	1.04	1.08	1.15	1.10
Overall acceptability[b]	6.03	5.72	5.55	5.67

[a] Treatment designated as follows: C/C = control/control; C/FT = control/Flavor-Tex; FT/C = Flavor-Tex/control; FT/FT = Flavor-Tex/Flavor-Tex.
[b] Based on an 8-point scale in which 8 = like extremely and 1 = dislike extremely.
[c] Based on a 3-point scale in which 3 = definite off-odor detected and 1 = no detectable off-odor.

The effects of coating lamb legs with calcium alginate on sensory panel scores were evaluated. Legs from six control and six calcium alginate-coated carcasses were removed following the conclusion of the shrinkage trial (7 days postmortem) and paired legs were coated with calcium alginate or left for evaluation of the original treatment. Prior to cooking, the coated legs could be visually distinguished from the control legs by the presence of the film. This difference was especially evident for the legs recoated following the shrinkage trial. After cooking, no differences in appearance were evident because the calcium alginate gel dissolves upon heating.

Percent cooking loss and sensory panel scores are summarized in Table 3. Treatment groups were found to not differ significantly ($P > 0.05$) for cooking loss or for any of the sensory panel evaluations. All samples were rated acceptable in flavor, juiciness and overall acceptability. No off-odors were detected.

The loss of moisture from lamb carcasses during chilling, shipping and holding periods is of physiological and economic importance. The results of this study indicate that shrinkage loss can be reduced by using a calcium alginate gel or plastic film. Consideration of carcass chill rate and surface microbial growth would favor the use of the calcium alginate coating because the plastic-wrapped carcasses chilled more slowly and had higher microbial counts than the coated carcasses. Economically, the monetary return from the weight saved by the application of the calcium-alginate coating more than compensates for the cost of materials and application. Mechanization of application techniques would further increase the savings.

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Ms received 8/11/75; revised 10/17/75; accepted 10/21/75.

Florida Agricultural Experiment Stations Journal Series No. 6042.

We thank D.H. McKee Inc., Tampa, Fla. for permitting the authors to study the Flavor-Tex process on lamb carcasses.

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TOASTING AND HEXANE:ETHANOL EXTRACTION OF DEFATTED SOY FLAKES
Flavor of Flours, Concentrates and Isolates

ABSTRACT

Flours and protein concentrates, prepared from defatted soybean flakes steamed up to 20 min before or after extraction with hexane:ethanol azeotrope 82/18 v/v, were presented to a 15-member trained taste panel. Flavors and odors were described and rated for intensity on a scale of 1 to 10 where 1 is strong and 10 is bland. Azeotropic extraction for 6 hr by itself significantly affected flavor of flours and of concentrates so that they scored 7.4 and 6.8, respectively, compared to 4.0 for raw, hexane-defatted, soy flour. Toasting after azeotropic extraction raised flavor scores of flours and protein concentrates to 7.9, a value which compares favorably with 8.1 for wheat flour. Toasting is also necessary to inactivate trypsin inhibitors and other antinutritional factors in azeotropic-extracted soybean flakes. A protein isolate from toasted, azeotropic-extracted flakes scored 7.3 compared to 8.0 for sodium caseinate. Yields of protein isolates are good if the heat-processed flakes are extracted with hot water at 74°C and pH 7.2.

INTRODUCTION

AS DEMAND INCREASES for soy protein products in a wide variety of food systems, the search continues for methods to remove more completely incompatible flavors and their precursors from soy flours, protein concentrates and isolates. To expand the use of soy proteins in foods greatly, objectionable odors and flavors must be eliminated without sacrificing functional and nutritional qualities of the proteins (Hammonds and Call, 1972).

An organoleptic evaluation by Kalbrener et al. (1971) of commercial soy flours, concentrates and isolates showed that they were not bland, that some beany and bitter flavors of mature whole soybeans remained and that other derived flavors were formed.

Raw soybeans contain trypsin inhibitors and other substances that cause differing biological and physiological responses in various species of animals and perhaps in man (Rackis, 1974). Hexane:ethanol azeotropic extraction alone does not inactivate trypsin inhibitors; nutritive value of the azeotrope-extracted full-fat and defatted soy flakes is low and pancreatic hypertrophy occurs when they are fed to rats (Rackis et al., 1975).

This paper reports combined effects of hexane:ethanol azeotropic extraction and toasting on the organoleptic qualities of defatted soy flakes and of protein concentrates and isolates prepared from them. Flavor and odor scores of these products and flavor intensity values (FIV) of their individual flavor descriptions were recorded by a trained taste panel. Decreases in protein solubility resulting from azeotrope extraction and toasting are reflected in decreased yields of extractable protein. Studies were carried out with hot water and dilute calcium hydroxide extraction processes to evaluate their effect on protein yields and flavors of the resulting products. Also investigated was liquid CO_2 extraction for removing flavor components from raw, defatted and azeotrope-extracted flakes.

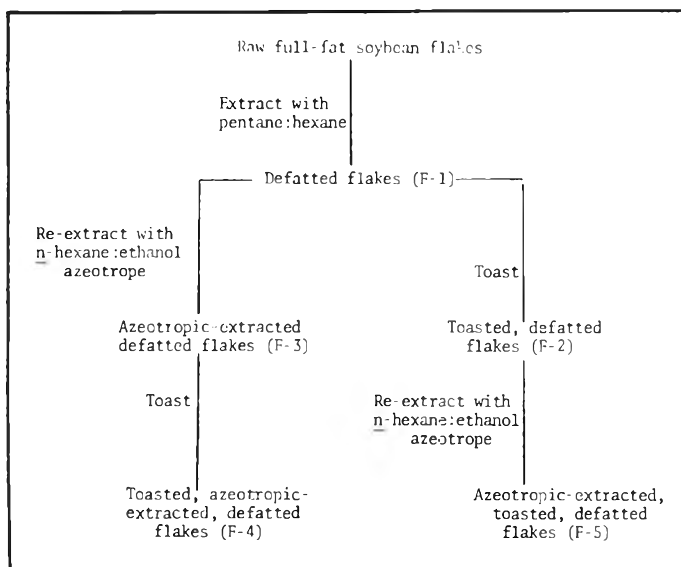

Fig. 1—Preparation of toasted, hexane:ethanol azeotropic-extracted, pentane:hexane defatted soy flakes. A 6-hr extraction, toasting with live steam at 100°C for 10 min.

MATERIALS & METHODS

Soy flakes, concentrates and isolates

Dehulled, full-fat and pentane-hexane defatted flakes were prepared from certified seed-grade soybeans (Amsoy, 1971 crop) according to previously described procedures (Sessa et al., 1969). The defatted flakes were further processed with combinations of toasting and hexane: ethanol azeotropic extraction (Fig. 1). Toasting involved live steam treatment for 10 min at 100°C in a preheated sterilizer autoclave. Extraction with hexane:ethanol (82:18 v/v, b.p. 59°C) was carried out in a Soxhlet apparatus for 6 hr. The solvent:meal ratio was 5:1. Defatted flakes were also produced by direct azeotropic extraction (Fig. 2) of full-fat flakes. After the processed flakes were air dried at room temperature for 24 hr, that portion used for flavor evaluation was ground to a flour in a Wiley mill equipped with a 40-mesh screen.

Protein concentrates were prepared by extraction with dilute hydrochloric acid (pH 4.5) at room temperature, as described by Sair (1959), from pentane:hexane defatted and hexane:ethanol azeotropic-extracted defatted flakes. After the extraction mixture was centrifuged and the supernatant was discarded, the residue was neutralized to pH 7.2 with 0.2N sodium hydroxide and freeze dried.

Protein isolates were prepared by extraction with water at room temperature, according to the procedure of Eldridge et al. (1971) from pentane:hexane defatted flakes and azeotropic-extracted defatted flakes. From these same flakes protein isolates were also extracted with hot water (70–74°C) at pH 7.2, as described by Circle et al. (1959), and with 0.05N calcium hydroxide at pH 10.6 (Mizrahi et al., 1967). After centrifugation of the extraction mixture, the supernatants were acidified to pH 4.4 with hydrochloric acid. The precipitates were then centrifuged, washed three times with water, dispersed by stirring in water, neutralized to pH 7.2 with sodium hydroxide and freeze dried. All products were stored at 4°C.

Liquid CO_2 extraction

Samples of pentane:hexane defatted flakes (F-1, Fig. 1) and hexane: ethanol azeotropic-extracted defatted flakes (F-3) were further treated with liquid CO_2 by the procedure of Schultz et al. (1974). About 450g of flakes was extracted with 4 liters CO_2 for 72 hr at 71°F and 69 atm. The extractions were carried out by W. G. Schultz at the Western Regional Research Lab., Berkeley, Calif.

Odor/flavor evaluation

Odor and flavor scores of the soy protein products, based on a 10-point scoring system (Kalbrener et al., 1974) where 1 is strong and 10 is bland, were determined by 15 experienced tasters. Panel members also described and rated the intensity of odors and flavors. The flavor intensity value (FIV) of individual descriptions was then calculated as reported by Rackis et al. (1972), based on a value of 1 for weak, 2 for moderate and 3 for strong. All samples were tested as 2% dispersions in charcoal-filtered tap water at room temperature. Usually three samples were presented in random order at each tasting to prevent sample interaction, and samples with the least odor were tasted first.

At preliminary sessions, raw defatted flakes (F-1, Fig. 1) and a protein isolate prepared from them were presented so as to familiarize the tasters with the odors and flavors of raw soybeans, particularly the grassy/beany and bitter characteristics. Flavor scores averaged 4.0 and 5.2 for the raw defatted flakes and isolate, respectively. Wheat flour served as a reference standard for a bland flour and sodium caseinate (Carnation Co. Van Nuys, Calif.), as a proteinate standard.

Samples were examined for flavor at two or more taste panel sessions, unless otherwise noted. All samples of soy flakes were compared directly against toasted, pentane:hexane defatted flakes (F-2, Fig. 1). Statistical differences between samples were determined by analysis of variance based on overall standard deviations within trials or on variations in the mean score for the same material in different trials. Least significant difference (LSD) values were based on a 95% confidence level.

Analytical procedures

Nitrogen solubility index (NSI) was determined by the procedure of Smith et al. (1966). Protein content was based on Kjeldahl nitrogen values as-is × 6.25.

Vapor phase analysis of residual solvents in liquid CO_2 extracted meals was a modification of the direct determination method described by Dupuy et al. (1971). Meal samples of 30-mg each were placed in a short precolumn between glass wool and septums. The precolumn was inserted into the inlet block of a Packard 7409 gas chromatograph, then connected to carrier gas and a 3-ft 3-mm i.d. glass column packed with Porapak P or Q. Chromatographic conditions were: Inlet, 150°C; detector, 220°C; oven programmed from 30–180°C at 3°/min; helium flow, 30 ml/min; hydrogen flow, 30 ml/min; air, 350 ml/min. A flame-ionization detector was used. Quantities and identification were determined from peak areas and retention times of known standards.

RESULTS

Flavor evaluation of soy flours

Combined effects of toasting and hexane:ethanol azeotropic extraction on odor and flavor scores of defatted soy flour are summarized in Table 1. Flour sample F-1 prepared from raw defatted flakes scored low for odor and flavor.

Toasting raw defatted flakes for 10 min (sample F-2) significantly increased odor and flavor scores. Azeotrope extraction was a more effective process in improving the flavor of defatted flakes since the score of 7.4 for azeotropic-extracted flakes (F-3) was significantly higher than a score of 6.6 for toasted defatted flakes (F-2). Maximum flavor scores (7.8) were obtained with defatted flakes that were extracted with hexane:ethanol azeotrope and then toasted (sample F-4). A score of 7.4 was obtained with defatted flakes that were toasted before azeotrope extraction (F-5, Fig. 1).

In a second series of tests, flours comparable to sample F-4 (Fig. 1) were prepared to evaluate the effect of time of azeotrope extraction and toasting on flavor scores.

Flakes extracted for 1 hr and toasted 10 min had flavor scores of 7.4 compared to scores of 7.6 for 3- and 6-hr azeotropic-extracted flours. No significant differences in flavor scores were obtained with azeotropic-extracted samples that were toasted for 10 or 20 min.

Undesirable flavors can form from oxidation of unsaturated fatty acid components of lipids during the preparation of raw, pentane:hexane defatted soy flakes as described in Figure 1. Toasting and, to some extent, hexane:ethanol azeotropic extraction inactivate certain oxidative enzymes (Rackis et al., 1975). These processes may also affect flavor by removing flavor components or their precursors. Therefore, a modified procedure (Fig. 2) was developed to prepare toasted, azeotropic-extracted, defatted flakes. In this process cracked, dehulled beans were immediately toasted for 10 min, flaked and then defatted by a direct 3-hr azeotrope extraction.

Defatted soy flour (F-7, Fig. 2) prepared by the direct azeotropic extraction of toasted full-fat flakes had a flavor score of 7.7 comparable to the score for flour sample F-4 shown in Table 1. Therefore, toasting of the cracked soybeans before processing into defatted flakes had no significant effect on flavor score. Whether there is little lipoxygenase activity after cracking or the effects of such lipoxygenase activity are all removed by azeotropic extraction and toasting requires further study.

The flavor score of 7.3 for flour sample F-6 (Fig. 2), prepared by direct 6-hr azeotrope extraction of raw full-fat flakes, is comparable to that for azeotrope-extracted defatted flakes (F-3, Table 1).

Odor and flavor intensity values

Odor and flavor descriptions noted by the panelists were similar for various samples of processed soy flakes (Table 2).

Table 1—Odor and flavor scores of toasted azeotropic-extracted defatted soy flours

Flour sample[a]	Scores[b]	
	Odor	Flavor
F-1	5.2	4.0
F-2	7.4	6.6
F-3	7.8	7.4
F-4	8.1	7.8
F-5	8.5	7.4
Wheat flour	8.3	8.1
LSD[c]	–	0.67

[a] See Fig. 1 for details of sample preparation.
[b] Strong = 1, bland = 10.
[c] LSD = Least significant difference at the 95% confidence level.

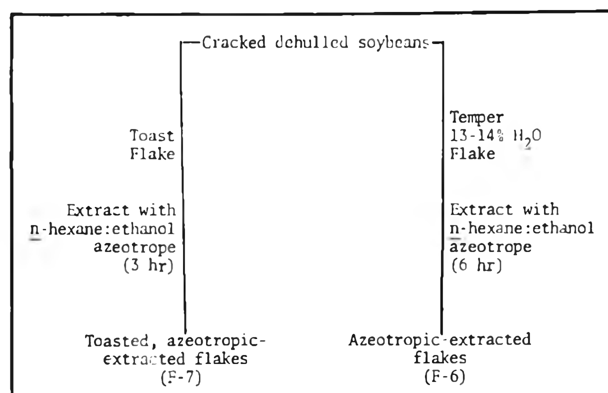

Fig. 2—Preparation of defatted soy flakes by direct hexane:ethanol azeotropic extraction of full-fat flakes. Toasting: live steam, 100°C for 10 min.

Based on FIV for individual flavor components, the high overall scores (Table 1) for the azeotropic-extracted flours F-3 and F-4 when compared to initial, raw, defatted flakes (F-1) can be attributed primarily to the reduction in intensity of grassy/beany, bitter and astringent components. The odor intensity value for grassy/beany was reduced tenfold and the grassy/beany FIV was reduced nearly sevenfold. Both bitter and astringent FIV's were decreased more than 50% after toasting and azeotrope extraction. On the other hand, the musty and cereal-grain FIV's were higher in azeotropic-extracted flours. Presumably, these flavor components were masked by the intensity of the predominant grassy/beany and bitter flavors in the original raw flakes or were formed during and after processing.

Flavor evaluation of concentrates

Protein concentrates prepared from raw defatted flakes (sample A) have low odor and flavor scores (Table 3). However, odor and flavor scores of the concentrates can be greatly improved by toasting (sample B) or by preparing them from hexane:ethanol azeotropic-extracted flakes (sample C). A combination of toasting and azeotrope extraction increased the odor and flavor scores of concentrates (samples D and E) even more. The improvement in flavor scores of concentrates prepared by this combined treatment was statistically significant at the 95% confidence level. On the basis of FIV's given in Table 3, the marked increase in flavor scores correlated with the greatest reduction in intensity of grassy/beany and bitter flavor components.

Flavor evaluation of soy isolates

Since a combination of azeotrope extraction and toasting improved the flavor of soy flours and concentrates, isolates prepared from toasted azeotropic-extracted flakes were also evaluated for flavor (Table 4).

Isolates II prepared from toasted defatted flakes received a flavor score of 6.2, which was significantly higher than that for isolate I. Further improvement in flavor score for isolate III from azeotrope-extracted flakes was also statistically significant. Although isolate IV prepared from toasted azeotrope-extracted flakes was tasted only once, apparently this combined treatment improves flavor no further. The odor score for isolate III was comparable to that for the standard protein, sodium caseinate, while the flavor score was lower.

As with the defatted flours and concentrates, azeotrope extraction of the flakes greatly reduced the grassy/beany FIV in the isolates; however, azeotrope extraction was much less effective in reducing the bitter FIV of the isolates. These data indicate that additional processing will be required to improve the sensory characteristics of soy protein isolates further.

Table 2—Odor and flavor intensity values of soy flour

	Descriptions				
Sample[a]	Grassy/ beany	Bitter	Astrin-gent	Cereal/ grain	Musty/ stale
F-1					
Odor	2.0	0.0	0.0	0.1	0.1
Flavor	2.9	1.0	0.9	0.1	0.0
F-2					
Odor	0.2	0.0	0.0	0.6	0.4
Flavor	0.6	0.4	0.2	0.9	0.1
F-3					
Odor	0.2	0.0	0.0	0.4	0.1
Flavor	0.4	0.4	0.3	0.6	0.2
F-4					
Odor	0.2	0.0	0.0	0.4	0.3
Flavor	0.4	0.3	0.1	0.4	0.0

[a] See Fig. 1 for details of sample preparation.

Table 4—Odor and flavor of soy protein isolates[a]

		Scores[c]		Flavor intensity value		
Sample	Starting flakes[b]	Odor	Flavor	Grassy/ beany	Bitter	Astringent
I	Raw defatted F-1	6.9	5.2	1.8	0.9	0.8
II	Toasted defatted F-2	7.5	6.2	0.6	0.8	0.6
III	Azeotrope-extracted F-3	8.2	7.2	0.2	0.6	0.2
IV	Azeotrope-extracted F-4	8.1	7.3	0.3	0.6	0.2
Sodium caseinate		8.3	8.0	0.1	0.2	0.1
LSD[d]		–	0.67			

[a] Extracted at pH 7.2 and room temperature
[b] See Fig. 1 for details.
[c] Sample IV tasted once; other scores mean of two or more tastings
[d] Least significant difference at the 95% level

Table 3—Odor and flavor of soy protein concentrates

		Scores		Flavor intensity values	
Sample	Starting flakes[a]	Odor	Flavor	Grassy/beany	Bitter
A	Raw defatted F-1	6.2	5.0	2.0	0.9
B[b]	Raw defatted F-1	7.8	7.0	0.7	0.2
C	Azeotrope-extracted F-3	7.8	6.8	0.6	0.5
D[c]	Azeotrope-extracted F-3	8.3	7.9	0.2	0.4
E	Toasted, azeotrope-extracted F-5	8.3	7.7	0.4	0.4
LSD[d]		–	0.67		

[a] See Fig. 1 for details.
[b] Concentrate A toasted 10 min after preparation
[c] Concentrate C toasted 10 min after preparation
[d] LSD = Least significant difference at the 95% confidence level.

Table 5—NSI values and protein yields from azeotrope-extracted soy flakes

		Soy concentrate		Isolate	
Starting flakes[a]	NSI	Protein (%)	Protein yield[b] (%)	Protein (%)	Protein yield[b,c] (%)
F-1	89	66	78	94	49
F-2	36	–	–	97	14
F-3	78	66	79	100	26
F-4	24	–	–	99	6
F-5	–	73	89	–	–
F-7	32	–	–	95	11

[a] See Fig. 1 and 2 for details.
[b] As $\frac{\text{g protein isolated}}{\text{g protein in starting flakes}} \times 100$.
[c] Water extraction at room temperature and pH 7.2

Protein solubility and yields of concentrates and isolates

Effects of toasting and azeotrope extraction on NSI of flakes and yields of protein concentrates and isolates are shown in Table 5. With azeotropic-extracted defatted flakes, NSI values dropped to 78, and with toasted, azeotropic-extracted defatted flakes, to 24. Decreases in NSI were reflected in increased yields of protein concentrate with a higher protein content, whereas yields of protein isolate were greatly reduced. When isolates were prepared from toasted, azeotropic-extracted defatted flakes, yields were only 6% of the protein in the starting material. If yield can be improved, preferably isolates might well be prepared from F-3 or F-4 flakes, since these isolates had the highest flavor scores (Table 4); in addition, the residues could be useful food additives. The residues contain more than 55% protein, are free of oligosaccharides that cause flatulence and have flavor scores of about 8.0.

Different extraction techniques improved yields of protein isolate prepared from low NSI flakes (Table 6). When azeotrope-extracted flakes (F-3) were extracted with 0.05N $Ca(OH)_2$ instead of the normal extraction process with water at room temperature, protein yield increased from 26 to 41%. This latter value is closer to the yield of 49% from raw undenatured defatted flakes (F-1) (NSI = 89). Hot water extraction of toasted flakes was almost as effective as $Ca(OH)_2$ extraction in improving protein yields, which increased from 6 to 36%, although flavor scores were lower than those of isolates prepared by extraction with water at room temperature (see Table 6).

Liquid CO_2 extraction

Liquid CO_2 extraction has successfully improved removal of solvent residues from defatted nuts and extracted flavor essences from fruits, spices and other food products (Schultz et al., 1974). The feasibility of applying such a technique to extract residual solvents and undesirable flavor constituents from soybeans was investigated (Table 7).

As a result of liquid CO_2 extraction, flavor scores of raw defatted flakes and of the isolate from such flakes increased, but the increase was less than that for toasting or hexane: ethanol azeotropic extraction. Liquid CO_2 extraction did not increase flavor scores of azeotrope-extracted flakes or of the corresponding isolate. However, it was quite effective in extracting residual solvents in pentane:hexane defatted and hexane:ethanol azeotropic-extracted flakes. Vapor-phase analysis indicated a reduction in ethanol content from 1400 to 180 γ/g and of hexane from 1290 to 220 γ/g for the azeotrope-extracted flakes, whereas the amount of pentane:hexane was reduced from 200 to 70 γ/g for defatted flakes. The CO_2 extract of F-1 flakes had a strong, beany, rancid odor, whereas the extract from azeotrope-extracted flakes had a weaker beany odor and no rancidity.

Table 6—Effect of calcium hydroxide or hot water extraction on yields and flavor scores of soy protein isolates

Sample	Starting flake[a]	Score[b] Odor	Score[b] Flavor	Flavor intensity value Grassy/beany	Flavor intensity value Bitter	Protein yield[c] (%)
V[d]	F-3	7.8	6.4	0.25	0.3	41
VI[e]	F-5	8.3	6.8	0.30	0.9	36

[a] See Fig. 1 for details; azeotrope extractions are for 6 hr
[b] Single determinations
[c] $\frac{\text{g protein isolated}}{\text{g protein in starting flakes}} \times 100$
[d] 0.05N $Ca(OH)_2$ extraction at pH 10.5 and RT
[e] Extraction at pH 7.2 and 72–74° C

Table 7—Flavor scores of liquid CO_2 extracted soy flakes and of their protein isolates

Starting flake[a]	Flavor score[b]	Flavor intensity value Green/beany	Flavor intensity value Bitter
F-1	5.1	2.4	0.8
F-3	6.3	0.7	0.3
F-1 proteinate[c]	6.0	1.2	0.7
F-3 proteinate[c]	6.2	0.3	0.4

[a] See Fig. 1 for details
[b] Single determinations
[c] Extracted at room temperature and pH 7.2

DISCUSSION

ELDRIDGE et al. (1971) reported that flavor scores of hexane:ethanol azeotropic-extracted flakes and proteinates prepared from them were significantly higher than those prepared commercially. Besides confirming previous results, our studies have been extended to determine processing conditions of azeotrope extraction and toasting needed to improve further the flavor scores of soy protein products. Hexane:ethanol azeotrope extraction plus toasting is more effective than either process by itself in producing bland defatted soy flours and concentrates. Flavor scores approached those of wheat flour for soy flours and concentrates prepared from toasted, azeotropic-extracted flakes. Toasting not only improved flavor scores of flours and concentrates but also destroyed antinutritional factors in hexane:ethanol azeotrope-extracted flakes. When the azeotrope-extracted flakes were toasted, protein efficiency ratio of the flakes increased from about 1.2 to 2.2 on a basis of a value = 2.5 for casein (Rackis et al., 1975).

Improved flavor scores of toasted, azeotropic-extracted flours and concentrates are associated with reductions in FIV's for grassy/beany, bitter and astringent flavor components. Moser et al. (1967) reported that with only 3 min steaming, taste panel members noted the presence of a nutty-toasted flavor in pentane:hexane defatted flakes and that increasing steaming time increased the intensity of these derived flavors. Toasted and nutty flavors are frequently present in commercial products as well (Kalbrener et al., 1971). When hexane:ethanol azeotropic-extracted flakes were toasted for as long as 20 min, the taste panel did not report the presence of nutty-toasted flavors. The absence of intense flavors should extend the use of soy products into bland food systems, such as dairy-type products.

Soy protein isolates, prepared from azeotrope-extracted flakes, toasted or untoasted, have low intensity values for the grassy/beany component; however, they still retain a distinctly bitter taste. This taste indicates that further improvements in the toasting-azeotrope extraction process are needed to remove more effectively the bitterness in soy isolates.

Toasting flakes, together with azeotrope extraction, lowers NSI and thereby increases protein content and yield of the concentrates, while increasing their flavor scores. On the other hand, since toasting lowers NSI of azeotrope-extracted flakes, yields of protein isolate are lower with no further increase in isolate flavor score.

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Ms received 7/24/75; revised 10/28/75; accepted 11/5/75.

Presented at the 35th Annual Meeting of the Institute of Food Technologists, Chicago, Ill., June 8–12, 1975. The mention of company names or trade products does not imply that they are endorsed or recommended by the U.S. Dept. of Agriculture over other firms or similar products not mentioned.

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SOYBEAN EXTRUDED PRODUCT: A RESPONSE SURFACE ANALYSIS

ABSTRACT

Response Surface Analysis (RSA) was used to study the effect of three variables, process temperature (120, 145 and 170°C), feed moisture content (20, 30 and 40%) and screw speed (800, 900 and 1000 rpm) on extrudate characteristics. Unheated soybean flakes extruded under different conditions were subjected to Warner-Bratzler shear (WBS), water absorption (WA) measurements and residual trypsin inhibitor activity (TIA) assays. Low feed moisture contents induced higher internal heat production causing residual TIA in the product to be lower than expected, WA to increase and WBS to be independent of process temperature (at constant screw speed of 900 rpm). Variation of screw speed permitted the attainment of desired values of product characteristics when moisture and temperature had to be fixed.

INTRODUCTION

COOKING EXTRUDERS for new foods have found industrial applications due to their particular abilities. Smith (1974) cites among them moisture entrainment, gelatinization of starches, protein denaturation, heat labile growth inhibitor control and restructuring and retexturing of process material.

These changes are attained by feeding protein mixtures, mainly vegetable flours, water and supplementary optional additives into the cooker barrel and raising pressures and temperatures. The resulting plastic mass is extruded through a die to atmospheric pressure causing the release of steam and the expansion of the material. After cooling the product sets into a structure with completely different characteristics than those of the starting material.

Textured protein products may provide about 10% of the U.S. meat equivalent consumption by 1985 (Hammonds and Call, 1970). Extruded products made from flours (50–55% protein) are currently being used as meat extenders while higher cost spun product manufacture uses isolates (90% protein) resulting in improved texturization properties that allow them to be "engineered" alone or in certain combinations with extruded protein to simulate ham, breakfast sausage, chipped beef, fried chicken, bacon and other meatlike products.

The bulk of the patent literature related to these type of products is almost two decades old (Gutcho, 1973) but the experience borrowed from the plastics and textile industry presently indicates both are at a stage of "art." Specifically in the case of food extrusion, Harper and Harmann (1973) discussed some of the current research needs in the food area while pointing out that most of them no longer constitute a problem in plastics manufacture. This is in part due to the complexity and nonhomogenity of food materials.

Tadmor and Klein (1970) introduced Response Surface Analysis (RSA) as a useful statistical tool for analyzing experimental data from plasticating extrusion by studying the effect of six variables on the extrudate temperature. This methodology was deemed appropriate for the evaluation of the nutritional improvement of unheated defatted soybean flakes known to be dependent on temperature, moisture and time (Liener, 1972). Functional properties of the extruded product have also been reported as being principally affected by the same three variables (Conway et al., 1968, Conway, 1971b) and thus suitable to RSA.

From all the troublesome substances found in raw soybeans that must be inactivated during heat processing to improve their nutritional value and storage capabilities, trypsin inhibitors (TI) are known to be the least heat labile (Baker and Mustakas, 1973). Hence, thermal treatments need to be calculated in such a way as to provide the necessary TI inactivation and simultaneously avoid any overheating of the soybeans that has proven to be destructive of several amino acids and vitamins (Smith, 1971).

The objective of this study was to explore RSA as a tool for a better understanding of the relationship between extrusion conditions and product characteristics and as a means for optimizing the process through the simulanteous analysis of temperature, feed moisture content and screw speed.

MATERIALS & METHODS

Response surface analysis (RSA)

RSA is based on the assumption that when k factors (or independent variables) are being studied in an experiment, the response (or dependent variable) will be a function of the levels at which these factors are combined (x_k). Thus,

$$y = \phi (x_1, x_2, ...x_k) \quad (1)$$

where y is the Response Function, (Davies, 1954).

Careful selection of the number of variables to be studied is worthwhile since with more than three x-variables, geometrical representation can be used only partially. In this case, a canonical transformation of the surface equation simplifies it allowing its interpretation. The polynomial to be used depends on the accuracy needed and on the contribution of extra terms to the overall fitting as measured, for instance, by an analysis of variance.

For the case of three variables, it was proven satisfactory to fit the data with a quadratic polynomial of the type:

$$y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{11}x_1^2 + b_{22}x_2^2 + b_{33}x_3^2 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3 \quad (2)$$

that takes into account variations due to linear and quadratic order effects as well as those due to interactions. Surface contours are obtained by making one variable equal to a constant value and then solving the fitted equation as a quadratic in the other two. By varying the value of the fixed variable a set of planes with contour surfaces can be built up into a 3-dimensional representation.

A presentation of the method and its potential application in the food industry can be found in a work by Henika (1972).

Experimental design and data analysis

A three-variable, three-level fractional factorial design with three replicates at the center point was used. The center point of the design was chosen based on the extruder manufacturer's (Wenger, Mfg., Sabetha, Kans.) suggestions for the production of a texturized soybean product and on previous experience at our laboratory. The range of the three variables is presented, Table 1. It allowed for the development of several different product characteristics while keeping the experiment confined to a sufficiently narrow area.

The selection of temperature, feed moisture and screw speed as the most critical independent variables of the process was based on findings

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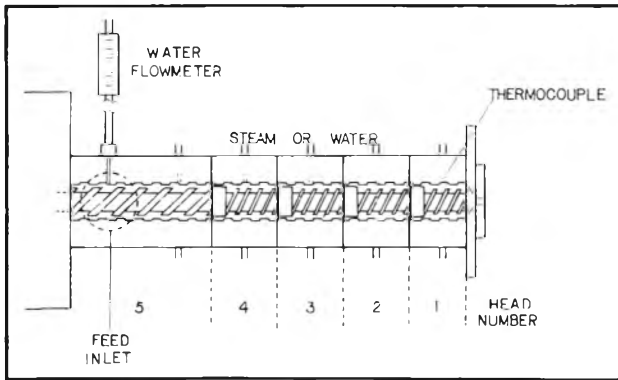

Fig. 1–Main components of the extruder.

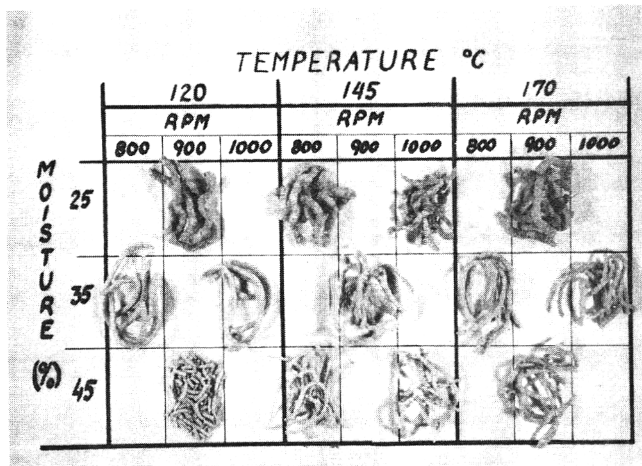

Fig. 2–Extruded products obtained under the experimental pattern.

Table 1 – Factor levels

Factor	Level –1	Level 0	Level 1	Unit
1. Process temperature (C)	120	145	170	25
2. Feed moisture content (%)	25	35	45	10
3. Screw speed (rpm)	800	900	1000	100

Table 2–Experimental pattern and response values of Trypsin inhibitor activity (TIA) TUI/mg (TIA of soybean grits 72.5 TUI/mg)

		Process temperature (°C) 120			145			170		
		rpm			rpm			rpm		
		800	900	1000	800	900	1000	800	900	1000
Feed	25		3.7		3.9		3.7		2.0	
Moisture	35	10.0		6.0		3.5 4.5 6.4		4.5		3.3
Content (%)	45		33.4		31.0		21.0		4.4	

reported by Conway et al. (1968, 1971a, 1971b) working with corn and sorghum grits and soybeans, Mustakas et al. (1970) for full-fat soybeans, Lawton et al. (1972) for corn starch, Lorenz et al. (1974) for triticale.

Responses under observation were: residual trypsin inhibitor activity, Warner-Bratzler shear and water absorption capacity.

A Hewlett-Packard Model 9820A calculator was fed with the experimental data and equations taken from Davies (1954) to perform the calculations. The output consisted of ten regression coefficients which were used to obtain contour maps at predetermined conditions with a Hewlett-Packard 9862 Plotter. The analysis of variance was also performed by using the output from the computer.

Experimental procedure

Runs were performed randomly in a 5-head Wenger X-5 extruder using a 1/8 in. die (Fig. 1). Tap water was circulated through the jacket in heads 4 and 5. Head 1 was closely maintained at the temperature reported herein as process temperature by running steam through the jacket. It is in this section of the extruder where cooking is done. The two intermediate heads were kept between 0 and 20°C below the process temperature as more precise control on them was not possible.

Defatted, dehulled, unheated soybean flakes, Soyabits white (Central Soya Co., Inc., Chicago, Ill.) with a moisture content of 8% were fed to the extruder after thorough mixing in the hopper and with feed rate maintained constant.

Water was added at the entrance point in the extruder and its rate was measured with a previously calibrated flowmeter. Extruder screw speeds were determined with a hand tachometer.

Extruded samples were collected, placed in glass jars and kept refrigerated.

Methods of analysis

All 15 samples were analyzed for moisture content (AOAC, 1960), density, water absorption capacity, Warner-Bratzler shear and trypsin inhibitor activity.

Water absorption (WA) capacity was evaluated by soaking 5g of extruded product for 15 min in water at 25°C. After draining, excess water was blotted on brown paper and the sample reweighed. Values are reported as grams of water absorbed/gram dry weight of product.

The Warner-Bratzler shear instrument (WBS) consists of a 1-mm thick metal blade by which a certain amount of force is applied to shear the sample. The force was determined by using an Instron Universal Testing Machine (Instron Corp., Canton, Mass.) as a sensing and recording device. Three strands were sheared simultaneously with at least five replicates per sample. Shear forces are usually reported on a cross-sectional area basis. According to Cumming et al. (1972), it is more suitable to use density as a dimensionless divisor for Warner-Bratzler shear values on porous extruded products, a system that has been adopted here. WBS values are expressed in kg.

Trypsin inhibitor activity (TIA) was measured by the improved method of Kakade et al. (1974) and Rackis et al. (1974). Results are reported as trypsin units inhibited per milligram dry weight of sample, TUI/mg.

RESULTS & DISCUSSION

THE WIDE VARIETY of physical forms of extruded product obtained under the experimental pattern is shown, Figure 2. Tables 2, 3 and 4 summarize the experimental results obtained by the different combination of variables for trypsin inhibitor activity, Warner-Bratzler shear and water absorption respectively. Regression coefficients as calculated by the computer are listed in Table 5. For TIA the best fit was found when log y instead of y was used in the regression equation. WBS and WA were fitted by using Eq (2).

The analysis of variance is shown in Table 6. Although second order terms in the case of TIA are not significant, their inclusion in the equation contribute to a better fitness of the data at high values of the response. In general, results from the analysis of variance have to be critically evaluated in the light of accuracy required, agreement to theoretical background and magnitude of residual errors in critical zones of the surface.

Typical contours for the three responses at a screw speed of 900 rpm are observed in Figures 3 to 5. Water absorption presents a minimum at about 145°C and 40% moisture and the highest values are found at high temperatures and low moistures with a strong dependence between both variables (Fig.

3) A satisfactory resemblance is observed when product rehydration data from Cumming et al. (1972) as a function of process temperature is compared to the water absorption vs. temperature curve obtained by passing a plane through a constant moisture value. The existence of a point of minimum product rehydration is demonstrated in both cases. At this point, RSA comes into play. Should by any reason the process temperature be maintained within a narrow range about this point, Figure 3 indicates that by varying the moisture content of the feed, improved water absorption properties can be obtained. The operation point has then to be selected according to the requirements set for texture (e.g.: as measured by WBS), nutritional considerations (e.g.: TIA value), or any others deemed of importance.

WBS values decrease as feed moisture content increases, (Fig. 4). This effect is more pronounced at moisture levels below 35% and practically disappears above this value. As opposed to the WA case, this time the dependency between the property and processed temperature is practically nil.

Trypsin inhibitor activity values required a more detailed analysis. Rackis (1972) reported that higher temperatures were required to inactivate trypsin inhibitors in whole soybeans at lower moisture content, a point apparently in contradiction with the present findings. Results obtained in extrusion cooking of full-fat soy flour show an inversion in this trend only at high temperatures (150°C) and short residence times (or high screw speeds) of 30 sec (Mustakas et al., 1970). The time that particles spend inside the extruder follows a certain residence time distribution (Zuilichem et al., 1973). Determination made with the aid of a dye at 1000 rpm and 45% moisture revealed that the product spent between 15 and 35 sec in the barrel section.

Additional runs made using a different untoasted soybean product, Soyafluff 200W (Central Soya, Chicago, Ill.), confirmed the reported findings at moisture values of 15% and duplicated values well inside the experimental range. Runs performed without a die restriction (open discharge) showed much higher residual TIA than would be predicted from the regression equation indicating, as it was expected, that the die restriction generates significant internal heating of the product with the consequent increase in stock temperature.

The amount of heat supplied to the product depends upon the sign and magnitude of the conducted heat portion (through the barrel) and the frictional heat portion, the latter being directly proportional to the viscosity (Tadmor and Klein, 1972). Harper et al. (1971) working with cereal doughs in the moisture range between 15–30% reported an exponential increase of viscosity as moisture content decreases. Thus, lower residual trypsin inhibitor activity in materials extruded at low moisture contents can be explained because of the higher internal heat production.

Additional support to this interpretation comes from the fact that thermoplastic extrusion of foods obtains most of the heat needed for cooking from viscous dissipation. This is why product temperature must not be confused with barrel temperature, described here as process temperature, since this is a controllable variable as opposed to product temperature that depends not only on barrel temperature, but also on screw speed and moisture content. Moreover, since cooking is performed in the last turns of the screw, a precise control of barrel temperature at that head is more critical.

The distortion in the pattern of TIA curves, as low moisture contents are approached, cannot be explained on scientific grounds. Careful inspection and elimination of terms in the regression equation that contribute to the anomaly did solve this problem. However, in that area, the experimental error is comparable in magnitude to the value of the response (similar to the case in Fig. 4, above 35% moisture) so any improved shape the pattern may take is of reduced practical value. Moreover, Rackis and McGhee (1973) demonstrated the absence of

Table 3—Experimental pattern and response values (Warner-Bratzler shear force, kg)

		Process temperature (°C)								
		120			145			170		
		rpm			rpm			rpm		
		800	900	1000	800	900	1000	800	900	1000
Feed	25		5.1		6.9		3.5		4.3	
Moisture	35	1.9		1.3		1.3 1.7 1.5		1.9		1.4
Content (%)	45		0.2		0.9		1.4		1.5	

Table 4—Experimental pattern and response values (Water absorption capacity, g water/g dry weight)

		Process temperature (°C)								
		120			145			170		
		rpm			rpm			rpm		
		800	900	1000	800	900	1000	800	900	1000
Feed	25		1.8		2.0		1.4		2.4	
Moisture	35	1.8		1.6		1.5 1.5 1.5		1.7		1.4
Content (%)	45		1.6		1.5		1.6		1.7	

Table 5—Regression coefficients

Coefficient[a]	TIA	WBS	WA
b_0	0.668	1.5	1.5
b_1	−0.199	0.075	0.05
b_2	0.349	−1.975	−0.15
b_3	−0.069	−0.50	−0.125
b_{11}	−0.062	−0.1375	0.1875
b_{22}	0.194	1.4125	0.1875
b_{33}	0.132	0.2625	−0.0625
b_{12}	−0.194	0.525	−0.125
b_{13}	0.022	0.025	−0.025
b_{23}	−0.037	0.975	0.175

[a] 1 = Process temperature; 2 = Feed moisture content; 3 = Screw speed.

Table 6—Analysis of variance

		TIA	WBS	WA
	d.f.	M.S.	M.S.	M.S.
First order terms	3	0.442 (27.6*)	11.08 (277*)	0.108[a]
Second order terms	6	0.064 (4.0)	2.1 (105*)	0.08[a]
Lack of fit	3	0.028 (1.7)	0.18 (9)	0.016[a]
Error	2	0.016	0.02	0.00
R^2		0.94	0.99	0.99

[a] Since the experimental error is zero, significance test is meaningless

* Significant at 5% level

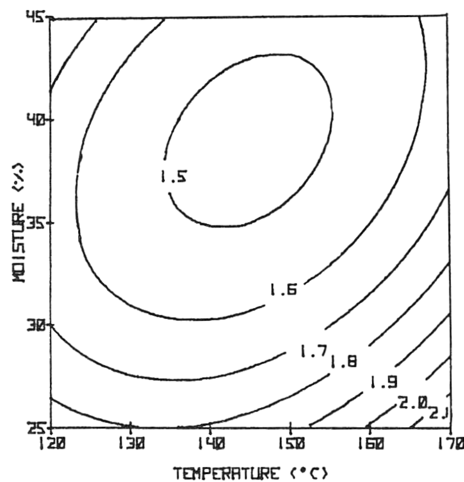

Fig. 3—Water absorption (g/g) contours as a function of process temperature and feed moisture content. Screw speed 900 rpm.

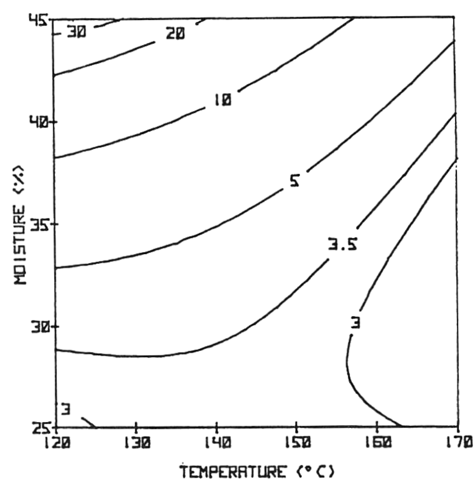

Fig. 5—Trypsin inhibitor activity (TUI/mg) contours as a function of process temperature and feed moisture content. Screw speed 900 rpm.

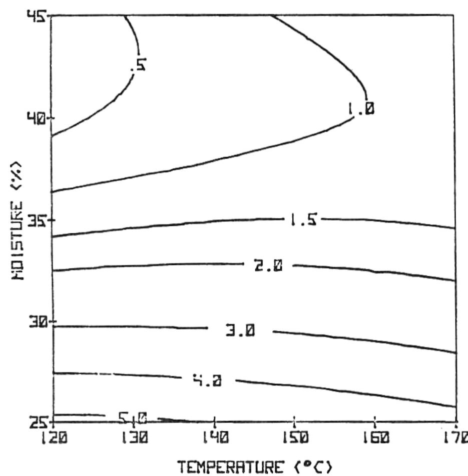

Fig. 4—Warner-Bratzler shear force (kg) contours as a function of process temperature and feed moisture content. Screw speed 900 rpm.

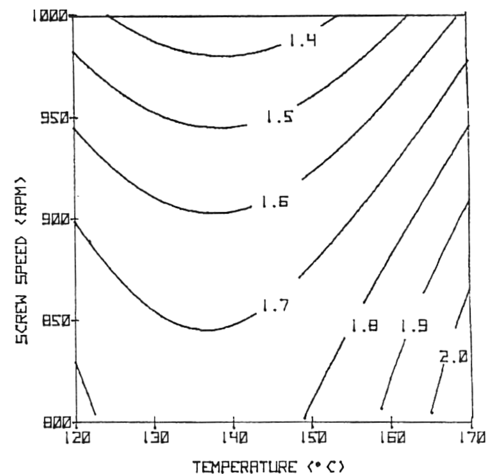

Fig. 6—Water absorption (g/g) contours as a function of process temperature and screw speed. Feed moisture content 30%.

antinutritional effects in products containing below around 12 TUI/mg. This means that adequate fitness between data and the regression equation is required at high moisture contents and low process temperature values (where this critical TIA value is reached), and this is provided by the function used.

Response surfaces similar to the ones presented can be obtained showing the effect of temperature and screw speed or the effect of moisture and screw speed. Figure 6 illustrates this point by displaying the marked effect of screw speed on WA at high temperatures when feed moisture content is 30%.

In summary, Response Surface Analysis is shown to provide the food extrusion process with control and flexibility necessary to develop products with specific functional properties by an adequate choice of the levels of controllable variables. Thus, the same basic ingredients can provide a product with utilization functions as a gruel, meat extender or a ground nutritous mixture. Results reported here are particular of the equipment used but the methodology can be applied to commercial scale operations.

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Ms received 6/16/75; revised 9/30/75; accepted 10/6/75.

The authors acknowledge the assistance provided by Mr. George Houghton in reviewing the statistical aspects of RSA.

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ENZYMATIC AND ULTRASONIC TECHNIQUES FOR SOLUBILIZATION OF PROTEIN FROM HEAT-TREATED COTTONSEED PRODUCTS

ABSTRACT

Extraction of protein from cottonseed meal was fourfold more efficient when the meal was treated with trypsin than when treated with ultrasonic energy. Combination of the two techniques caused efficient protein extraction with low expenditures of time, enzyme and energy. The extracted proteins bound > 3.5 ml oil/g concentrate and > 2.4g water/g concentrate and emulsified > 325 ml oil/g concentrate.

INTRODUCTION

PROTEIN can be readily extracted from a variety of substrates if no heat has been applied to the material. If heat denaturation has occurred, extractability is markedly reduced (Lu and Kinsella, 1972; Martinez et al., 1970).

Two generalized techniques have been found successful in increasing the extractability of protein from heat denatured substrates. Molina and Lachance (1973) noted that the use of proteolytic enzymes to partially hydrolyze coconut meal prior to chemical extraction with dilute alkali increased the efficiency of protein extraction. This technique has been modified and extended to screw-expressed cottonseed meal by Childs (1975) where a three-to fivefold increase in protein solubilization was noted. Another successful approach has been the use of ultrasonic energy to aid protein extraction from toasted soybean flakes (Wang, 1975). With this technique, a marked increase in protein extractability was noted. The ultrasonic method, if as efficient, would have several advantages over the proteolytic enzyme-chemical method including elimination of costly enzymes and reduced processing times.

This experimental study was undertaken to (a) compare the proteolytic enzyme-chemical and ultrasonic techniques for extraction of protein from cottonseed products; (b) develop a superior protein extraction technique which combines the desirable characteristics of each extraction approach; and (c) define selected characteristics of concentrates prepared from the solubilized proteins.

MATERIALS & METHODS

Samples

Glandless cottonseed flour, a gift of the National Cottonseed Products Association, was stored in sealed bags at room temperature until experimentation. Autoclaved cottonseed flour samples were prepared by autoclaving the material 30 min at 250°C and 15 psig. Cottonseed meal samples were purchased at a local feed mill and held in sealed bags at room temperature until experimentation.

Proteolytic enzyme-chemical extraction technique

The technique used was a modification of earlier proteolytic enzyme-chemical procedures (Childs, 1975). 25.0g of cottonseed product (either meal or flour) and 0.10g trypsin (1:300 hog pancreas, Nutritional Biochemicals Co.) were suspended in 100 ml of 0.02M sodium phosphate buffer (pH 8.1) which brought the pH of the reaction system to 6.75. This suspension was stirred for 60 min and then rapidly filtered through cheesecloth to separate the trypsin and extracted protein (filtrate) from the remaining cottonseed product (filter cake). The filtrate was then centrifuged at 10,000 × G for 20 min to yield a clarified protein suspension. The supernatant was saved for Kjeldahl analysis and referred to as the "enzyme fraction." The filter cake from the cheesecloth filtration was resuspended in 100 ml of 0.075% NaOH and stirred for an additional 60 min at 60°C. This mixture was then filtered and centrifuged as described above. The supernatant after centrifugation (10,000 × G for 20 min) was labeled the NaOH Fraction. The residual filter cake was also saved for Kjeldahl analysis and labeled the "residue fraction." Data were calculated as a percentage of total Kjeldahl protein which was extracted.

Sonication technique

The technique used for ultrasonic extraction of cottonseed product was essentially that of Wang (1975). 5g of cottonseed product were suspended in 50 ml of either water or 0.075% NaOH in a 100 ml beaker. The mixture was sonified for 8 min (Sonic 300 Dismembrator, Artek Inc.). The sonic probe was inserted 1 in. into the mixture and operated at 210 acoustical watts of output power. Following sonication, the slurry was rapidly filtered through cheesecloth to separate the extracted protein (filtrate) from residue (filter cake). The filtrate was centrifuged at 10,000 × G for 20 min and the protein content of the supernatant determined by Kjeldahl analysis. The filter cake and centrifuge pellet were pooled for Kjeldahl analysis and referred to as the "residue fraction." Data were calculated as percentage of protein which was extracted.

Combined sonication, proteolytic enzyme-chemical extraction technique

5g of sample and 0.02g trypsin (1:300 hog pancreas, Nutritional Biochemicals) were suspended in 50 ml of 0.02M buffer at pH 8.1. This system was sonicated for 8 min as described above, filtered through cheesecloth to separate the enzyme and solubilized protein from the substrate, and the filtrate centrifuged at 10,000 × G for 20 min. The supernatant was reserved for Kjeldahl analysis. The filter cake was resuspended in 50 ml of 0.075% NaOH and sonicated an additional 8 min, filtered and centrifuged as above, and the supernatant from the centrifugation (NaOH fraction) and the filter cake (residue fraction) held for Kjeldahl analysis. Data were calculated as percentage of protein which was extracted.

Parameters affecting combined technique efficiency. Experiments were performed to ascertain (a) the effect of time of sonication of the enzyme-substrate mixture on extraction efficiency utilizing times of 15 and 30 sec, 1, 2, 4, 8 and 16 min coupled to an NaOH sonication time of 8 min and using 0.02g trypsin/5g sample; (b) the effects of trypsin concentration on the efficiency of extraction utilizing 0.001, 0.005, 0.010, 0.020, and 0.030g trypsin per 5g of sample coupled to 1 min enzyme and 8 min NaOH sonication times; and (c) the effect of NaOH sonication time was also examined utilizing a 1 min sonication for the trypsin incubation (0.01g trypsin per 5g sample) and NaOH sonication times of 1, 2, 4 and 8 min.

Functionality of solubilized protein

Preparation of concentrates. Solubilized proteins were selectively removed from the extracts by pH induced precipitation. Preliminary tests indicated maximum protein precipitation at pH 3.5–4.0. All extracts were adjusted to pH 3.75 with HCl and allowed to stand for 1 hr. This system was centrifuged at 3000 × G for 20 min to recover the precipitated protein and the supernatant was decanted and discarded. The pellet was freeze dried and used as the protein sample for subsequent investigations.

Low molecular weight nitrogen. Rather than measure the amount of nitrogen precipitated by trichloroacetic acid, an analysis of the % nitrogen in molecular forms with a formula weight < 1000 daltons was determined. 0.20g of protein was suspended in 20 ml of water or 0.075% NaOH and pipetted into an ultrafiltration cell. The membrane

filter had a nominal molecular weight cut-off of 1000 daltons. The volume of the protein solution was reduced to 5 ml. This was rediluted with liquid and concentrated two times to assure that all low molecular weight nitrogen had passed the ultrafilter. Kjeldahl analysis was performed on both the filtrate and the nonfiltered material. The % nitrogen in molecular forms < 1000 daltons was calculated from these data.

Solubility. 0.02g of protein concentrate and either 3 ml of 0.01M sodium phosphate buffer (in 0.5 pH increments from pH 2–11) or 3 ml of 0.10M NaCl, 0.01M sodium phosphate buffer in 0.5 pH increments were placed in a 15 ml glass centrifuge tube. The tube was agitated on a Vortex mixer for 0.5 min, shaken on a wrist shaker for 5 min and allowed to stand an additional 5 min. The sample was then centrifuged 5 min at 1000 × G. The supernatant was decanted and its protein content (amount of soluble protein) ascertained by the Biuret reaction (Snow, 1950).

Water-holding capacity. 0.1g of concentrate and 5.0 ml of 0.02M citrate buffer (pH 3.5) were added to a weighed 15 ml glass centrifuge tube. The tube was agitated on a Vortex mixer for 1 min and then centrifuged 15 min at 1000 × G. The supernatant was decanted and discarded. After the tube was allowed to drain, the pellet was weighed and the weight of water bound per gram protein concentrate was calculated.

Oil-holding capacity. Oil-holding capacity was determined in the same manner as water-holding capacity except 5 ml of cottonseed oil was substituted for the 5 ml of citrate buffer.

Emulsifying capacity. Emulsifying capacity was determined by a slight modification of methods described by Webb et al. (1970). 0.05g of protein concentrate were suspended in 30 ml of 5% NaCl and blended at 12000 rpm for 30 sec. 25 ml of cottonseed oil were added and blended with the saline-protein solution. An ohmmeter was then connected to the system. This mixture was blended at 12000 rpm with more oil being added. The breaking point of the emulsion was judged to have been reached when the ohmmeter recorded infinite resistance. The emulsifying capacity was calculated as ml of oil emulsified/g protein concentrate.

RESULTS & DISCUSSION

Enzyme-chemical vs ultrasonic technique

The trypsin-chemical method was some fourfold more efficient for solubilization of cottonseed protein from screw-expressed meal than the ultrasonic method (Table 1). In fact, ultrasonic energy in either water or dilute alkali caused no marked increase in protein solubilization over simple water extraction in previous studies (Childs, 1975).

Initially these data seemed to contradict those of Wang (1975) who found application of ultrasonic energy increased the efficiency of protein extraction from autoclaved soybean flakes. To ascertain the basis for this possible divergence in data, experiments were performed to ascertain the efficiency of the ultrasonic technique on cottonseed flour, autoclaved cottonseed flour, and screw-expressed cottonseed meal. Sonication caused solubilization of > 90% of protein from both native and autoclaved cottonseed flour (Table 2). However, less than 20% of cottonseed meal protein was solubilized.

Because of the distinct advantages associated with use of the ultrasonic technique (e.g., cavitation of sample to create new sites for enzyme activity, increased mixing efficiency, decreased reaction times, and reduced energy expenditures), experimental studies were performed to determine the feasibility of combining the proteolytic enzyme-chemical technique with the ultrasonic technique. The combined enzymatic-chemical-ultrasonic technique increased the efficiency of total protein extraction from cottonseed meal some fourfold but caused no increase in extraction efficiency from native or autoclaved flours (Table 3). Since this technique combined the technical advantages of the ultrasonic technique (Wang, 1975) and the efficiency of the proteolytic enzyme-chemical technique (Childs, 1975), experimental studies were performed to describe parameters affecting the efficiency of the combined technique with screw-expressed cottonseed meal. Because the ultrasonic technique alone was very effective in extracting protein from native and autoclaved flour, no further work with this cottonseed product was performed.

Factors affecting efficiency of combined technique

There were no significant differences in the percentage protein extracted for sonication times varying from 1–16 min of the trypsin-cottonseed meal mixture (Table 4). These data suggested that sonication times of 1 min were adequate. Evaluation of other parameters utilized a 1.0 min sonication of the trypsin-cottonseed product mixture.

When the amount of trypsin used in the extraction was varied, no significant increase in extraction efficiency was noted at concentrations > 0.010g trypsin/5g cottonseed meal (Table 5). This was 50% of the amount of trypsin required for the proteolytic enzyme-chemical technique originally described by Childs (1975). 0.010g trypsin/5g meal was utilized in subsequent studies of the length of time required for NaOH sonication.

With a sonication time of 1 min for the enzyme-meal mixture and a trypsin concentration of 0.01g/5 cottonseed meal, it was found that > 2 min of sonication of the NaOH-cottonseed meal suspension caused no significant increase in efficiency of protein solubilization (Table 6).

Therefore, the combined enzyme-chemical-ultrasonic tech-

Table 1—Solubilization of cottonseed meal protein by enzymatic-chemical or sonication techniques. (All data are the mean of three replicates ± standard deviation)

Technique	Extraction medium	% Protein extracted[a]
Enzyme-chemical	pH 8.1 buffer, 0.075% NaOH	65.66 ± 4.98a
Sonication	HOH	14.81 ± 2.88b
Sonication	0.075% NaOH	17.91 ± 1.30b

[a] Values for percent protein extracted followed by different letters were significantly different ($P < 0.01$).

Table 2—Solubilization of cottonseed product protein by the ultrasonic technique. (All data are the mean of three replicates ± standard deviation)

Sample	% Protein extracted[a]
Raw cottonseed flour	93.01 ± 0.70a
Autoclaved cottonseed flour	92.34 ± 2.05a
Cottonseed meal	17.34 ± 2.51b

[a] Values for percent protein extracted followed by different letters were significantly different ($P < 0.01$).

Table 3—Effect of trypsin addition on ultrasonic aided solubilization of protein from native cottonseed flour, autoclaved cottonseed flour, and screw-expressed cottonseed meal. (All values are the mean of three replicates ± standard deviation)

Sample type	Trypsin	% Protein Extracted[a]
Native cottonseed flour	+	93.24 ± 2.04a
Native cottonseed flour	–	93.01 ± 0.70a
Autoclaved cottonseed flour	+	93.73 ± 0.93a
Autoclaved cottonseed flour	–	92.34 ± 2.03a
Screw-expressec cottonseed flour	+	72.99 ± 3.18b
Screw-expressec cottonseed meal	–	17.34 ± 2.51c

[a] Values for percent protein extracted followed by different letters were significantly different ($P < 0.01$).

nique can increase the efficiency of protein solubilization from cottonseed meal some fourfold with a sonication time for the enzyme reaction of 1 min, utilizing 0.01g/5g trypsin product, and a sonication time of 2 min for the NaOH extraction. These operating conditions reflect savings of time, enzyme and energy over previous techniques.

Characteristics of solubilized cottonseed meal protein

Three protein concentrate fractions from cottonseed meal were prepared and investigated: (a) the enzyme fraction isolated by the enzymatic-chemical technique; (b) the NaOH fraction isolated by the enzymatic-chemical technique; and (c) the NaOH fraction isolated by the combined technique. There was no acid precipitable protein in the enzyme fraction in the combined technique samples. This suggests that in the presence of ultrasonic energy little or no protein hydrolysis took place as no hydrolyzed protein could be precipitated. Trypsin has previously been shown to have a variety of activities (reviewed by Whitaker, 1972).

Only the enzyme fraction of meal extracted by the enzymatic-chemical technique contained a large amount of (37.9%) of nitrogen in low molecular weight forms (Table 7). The NaOH fraction from the combined technique had the lowest amount of low molecular weight nitrogen again suggesting that the specificity of trypsin is altered in an ultrasonic field from protein hydrolysis to reactions which make the protein more susceptible to NaOH extraction.

The solubility of the protein concentrates was typical of solubility of cottonseed protein as a function of pH. Solubility was minimal at acidic pH's and increased in basic pH's (Crenwelge et al., 1974). There were no marked differences in solubility in 0.10M NaCl vs water. This suggests little or no interaction between pH and ionic strength.

There were no significant differences in the water- or oil-holding capacities of the samples (Table 8). However, the emulsifying capacity of the NaOH fraction produced by the combined technique was significantly greater than that of other fraction concentrates. It has often been suggested that emulsifying capacity is a function of molecular radius (Carpenter and Saffle, 1965) and these data tend to confirm that observation since the highest emulsification capacity was noted with the concentrate having the lowest amount of low molecular weight nitrogen.

Comment

These data indicated that combining ultrasonic techniques (Wang, 1975) and proteolytic enzyme-chemical techniques (Childs, 1975) resulted in an efficient technique for solubilization of protein from screw-expressed cottonseed meal. The solubilized protein was easily separated by pH precipitation and was functional as measured by water-holding capcity, oil-holding capcity, and emulsifying capacity.

Attempts have been made in this laboratory to immobilize trypsin for use in these procedures. That technique is not feasi-

Table 4—Effect of sonication time of the enzyme-cottonseed matrix on solubilization of cottonseed meal protein. (All values are the mean of three replicates ± standard deviation)

Time of enzyme sonification (min)	% Protein extracted with trypsin
1	70.00 ± 1.23[a]
2	67.50 ± 1.78
4	67.52 ± 3.41
5	73.22 ± 6.08
8	69.04 ± 4.08
10	77.43 ± 6.59
12	61.25 ± 9.02
16	71.62 ± 7.83

[a] No values in this column were significantly different from any other value.

Table 5—Effect of trypsin level on solubilization of protein from cottonseed meal. (All values are the mean of three replicates ± standard deviation)

g Trypsin/5g meal	% Protein extracted[a]
0.001	46.33 ± 2.63a
0.005	56.23 ± 5.32b
0.010	62.70 ± 2.82c
0.020	64.77 ± 3.33c
0.030	63.45 ± 5.87c

[a] Values for percent protein extracted followed by different letters were significantly different ($P < 0.01$).

Table 6—Effect of NaOH sonication time on solubilization of protein from cottonseed meal. (All values are the mean of three replicates ± standard deviation)

Sonication time (min)	% Protein extracted[a]
1	56.22 ± 6.54a
2	61.23 ± 7.23b
4	64.32 ± 6.86b
8	68.50 ± 4.17b

[a] Values for percent protein extracted followed by different letters were significantly different ($P < 0.01$).

Table 7—Amount of solubilized N with a molecular weight < 1000 daltons. (All values are the mean of three replicates)

Sample	% N < 1000 daltons
Meal-enzyme extract (Stir)	39.2
Meal-NaOH extract (Stir)	4.78
Meal-combined technique NaOH extract	0.00

Table 8—Functionality of cottonseed meal protein isolates prepared by the protelytic enzyme-chemical technique (PC) and the combined ultrasonic-enzymatic (UE) technique. (All data are the mean of three replicates ± standard deviation)

Sample	Oil-holding capacity (ml/g)[a]	Water-holding capacity (ml/g)[a]	Emulsifying capacity (ml/g)[a]
Enzyme fraction (PC)	4.03 ± 1.10a	2.43 ± 0.86a	333 ± 4.0a
NaOH fraction (PC)	3.53 ± 0.32a	2.80 ± 0.26a	443 ± 4.0a
NaOH fraction (UE)	3.66 ± 0.15a	2.73 ± 0.15a	586 ± 2.5b

[a] Values in the same column followed by different letters were significantly different ($P < 0.01$).

ble in combination with the ultrasonic technique because the enzyme and its carrier are separated in an ultrasonic field. Current investigations are centered on the feasibility of elimination of trypsin from the extraction system. Preliminary data have indicated that unique combinations of particle size and meal/solvent ratio for substrates of varying Nitrogen Solubility Index might eliminate the need for trypsin. Those studies are continuing and will be reported at a later date.

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Ms received 8/14/75; revised 10/14/75; accepted 10/21/75.

Presented at the 35th Annual Meeting of the Institute of Food Technologists, Chicago, Ill., June 8—12, 1975.

This research was supported through project H-387 "Utility of Enzymatic-Chemical Extraction Procedures for Protein Recovery from Selected Crops and Food Processing Wastes" of the University of Tennessee Agriculture Experiment Station. Gratitude is expressed to A. Abajian, K. Park and C. Kupiec for technical assistance in this project.

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PROTEIN FORTIFICATION OF CORN TORTILLAS WITH OILSEED FLOURS

ABSTRACT

In work performed at the Food Protein R&D Center, three oilseed flours–deglanded liquid cyclone process (LCP) cottonseed flour, glandless cottonseed flour, and a high nitrogen solubility soy flour were used to fortify corn tortillas. Tortillas containing each of these flours blended with a corn tortilla flour to give 11%, 13%, and 15% protein in the blends were statistically compared in sensory tests for flavor, texture, and overall acceptability. An analysis of variance performed on panel scores indicated that the panel had no preference among the corn control tortillas and tortillas fortified with LCP and glandless cottonseed flours. The panel did, however, show a significant preference for each of these tortillas over soy-fortified tortillas. In color comparisons made with a Hunter Color-Difference meter, tortillas fortified with deglanded LCP cottonseed flour were increased in color over that of tortillas fortified with the other flours at the 11% protein level. As the amount of oilseed flours increased above the 11% blend level, the color of tortillas fortified with glandless and LCP increased noticeably. However, the color of soy-fortified tortillas essentially remained the same. Fortification of tortillas at the 11%, 13% and 15% blend levels increased the total protein by 18%, 40% and 61%, respectively. These tests indicated that protein-enriched tortillas can be prepared using oilseed flours.

INTRODUCTION

TORTILLAS are widely consumed in the South and Southwest where corn is a staple in the diet. They are considered to be the universal food in Mexico being consumed by all social classes and usually eaten at most meals where they serve as a bread substitute. The importance of tortillas in Mexico may be appreciated by noting that average annual per capita consumption is approximately 120 kilos (Del Valle and Villasenor, 1974). The Mexican government has encouraged the use of soy-fortified tortillas as a means of upgrading the nutritional level of its people (Anon, 1974).

Although tortillas are a good calorie source, their protein quantity is low (9% on a dry basis) and of poor quality, having a Protein Efficiency Ratio (PER) of 1.5 (Cravioto et al., 1950).

In very poor families, tortillas become a principal component in the diet, often to the exclusion of other protein sources. When this happens, protein malnutrition can and often does occur. This fact is supported by a recent nutrition survey sponsored by the Department of Health, Education, and Welfare. This survey revealed a prevalence of protein deficiency among low income Mexican-Americans (Ten-State Nutrition Survey, 1970).

There are two basic types of tortillas–flour and corn. The latter is utilized more extensively in traditional Mexican dishes of enchiladas, tamales, tacos and tortilla chips. Approximately twice as many corn tortillas are marketed commercially as flour tortillas (Nelson, 1975).

A number of investigations have been carried out attempting to increase the amount and quality of protein in corn tortillas: (1) Enrichment with soy flour (Cravioto et al., 1950; Cravioto and Cervantes, 1965); (2) Amino acid supplementation (Bressani, 1972); (3) Development of Opaque II corn (Bressani, 1972); and Enrichment with whole soybeans (Del Valle and Villasenor, 1974).

In the work reported here, three oilseed flours–glandless cottonseed flour, deglanded liquid cyclone process (LPC) cottonseed flour, and a high nitrogen solubility soy flour (Soy HS) were used to protein-enrich corn tortillas. Each oilseed flour was mixed with corn tortilla flour to produce blends having protein levels of 11%, 13% and 15%. Tortillas made with these blends were statistically compared with each other and with a corn tortilla control in sensory tests for flavor, texture, and overall acceptability (Fig. 1).

EXPERIMENTAL

Oilseed flours

The glandless cottonseed flour was made from the Rogers GL-7 variety of glandless cottonseed by direct hexane extraction. Low temperatures (ca 71°C) were maintained during desolventization.

Deglanded LCP cottonseed flour was from the USDA Southern Regional Research Center, New Orleans, La. and prepared by a widely-reported process (Gastrock, 1968).

The soy flour was a commercial product purchased from Central Soya Chemurgy Div., Chicago, Ill. This flour, Soyafluff 200W, had a high nitrogen solubility (NSI-65) and was designated in this experiment as soy (HS).

Although corn is an oilseed, it is not referred to as an oilseed in this paper. References to corn tortilla flour pertain to the flour used for the control product. The three flours used for fortification purposes are referred to as oilseed flours.

Tortilla preparation

In the traditional method of preparing corn tortillas described by Cravioto et al. (1945), one part whole-kernel white corn is submerged in two parts limewater and cooked for 20–50 min (or until the outer hull of the kernel will rub off readily) at a temperature of 80°C using a lime dose approximately 1% of the weight of the dry mixture. The cooked mixture is allowed to stand (12 hr), after which the corn is carefully washed two times with water. At this point, the corn can either be ground in a conventional stone mill into a dough which is then ready to be shaped into pancake-like tortillas, or it can be flash dried and ground into a corn flour which will reconstitute with water on a 2:1 ratio to make a dough.

The control flour used for this study was a commercial corn tortilla flour. The product, as marketed, is fortified with niacin, iron, thiamine and riboflavin.

Formulas used to prepare the control and fortified tortillas appear in Table 1. Weights of oilseed flours required to produce a particular protein level varied because the flours differed in protein content. The quantity of water used in doughs was kept constant.

Doughs were made by combining corn tortilla flour and oilseed flour blends with water after which the mixture was kneaded manually for 10 min before feeding into the tortilla oven. Tortillas were prepared on a Model TO-1100 oven manufactured by J.C. Ford Mfg. Co., Monterey Park, Calif. This unit cuts and bakes the thin dough in a self-contained, conveyorized, gas-fired oven.

Analytical measurements

Total nitrogen was determined by the micro-Kjeldahl method (AOAC, 1965). Carbohydrates, in terms of glucose, were measured colorimetrically by a phenol-sulfuric acid method (Dubois et al., 1956). Moisture, oil, and crude fiber were determined according to AOCS (1971) methods.

Color measurements were made on the tortillas with a Hunterlab Digital Color and Color Difference Meter, Model D25D.

Amino acid analyses of the cooked tortillas were quantitatively determined by the procedure developed by Spackman et al. (1958).

Fig. 1—Corn tortillas containing three oilseed flours in blends with corn tortilla flour at three blend levels, plus a 100% corn flour (control) tortilla.

Samples were hydrolyzed for determination of all amino acids in constant boiling hydrochloric acid for 24 hr under a nitrogen flush.

Sensory evaluation and statistical design

Samples were submitted for sensory evaluation to an untrained ten-judge taste panel. The panel was composed of five Mexican-Americans and five Anglo-Americans, all of whom were employed in food-related occupations. They were purposely chosen to represent a cross-section of tortilla consumers.

Taste panel members rated samples for overall acceptability, flavor and texture on a 1 to 8 scale which associated the following terms with the numbers:

8 – Like extremely	4 – Dislike slightly
7 – Like very much	3 – Dislike moderately
6 – Like moderately	2 – Dislike very much
5 – Like slightly	1 – Dislike extremely

Samples were randomly served to the judges in accordance with a randomized complete-block design which employed a factorial treatment arrangement. A block of four tortilla samples (the control flour plus each of the oilseed flours blended to a common protein level) was served to each judge at each of nine judging sessions. The identity of each sample was obscured by coding. Samples containing a particular protein level were scored in three different sessions. The protein level used on a given day was randomly chosen. One judging session was held per day.

An Analysis of Variance was first performed on means of the ten scores assigned each sample by the ten judge panel. Subsequently, means of the scores assigned by each of two five-judge panels (that is, scores from the five Mexican-American judges and from the five Anglo-American judges) were similarly analyzed. Those sources of variation shown to be significant by Analysis of Variance were further tested using Duncan's New Multiple Range Test. Analysis of Variance calcu-

Table 1—Formulas used with control and fortified tortillas

		Glandless cottonseed			LCP cottonseed			Soy (HS)		
		Protein in blend			Protein in blend			Protein in blend		
Ingredients	Corn (control)	11%	13%	15%	11%	13%	15%	11%	13%	15%
Corn flour	600g	519.54	555.12	530.76	582.3	561.24	540.24	577.32	550.32	523.32
Oilseed flour	0g	20.46	44.88	69.24	17.7	38.76	59.76	22.68	49.68	76.68
Water	580ml	580	580	580	580	580	580	580	580	580

Table 2—Analytical data on corn flour and other oilseed flours used to fortify corn tortillas

Type of flour	Moisture (%)	Oil (%)	Nitrogen (%)	Protein[a] (%)	Crude fiber (%)	Total sugar (%)
	dry weight basis					
Glandless cottonseed	6.9	1.18	10.3	64.3	2.33	15.6
LCP cottonseed	4.7	1.36	11.1	69.5	2.08	12.5
Soy (HS)	7.3	0.80	9.29	58.1	2.62	14.8
Corn (control)	9.44	3.9	1.65	10.3	1.8	5.2

[a] Protein calculated as follows: Oilseed nitrogen X 6.25

lations were made following a method derived by Addelman (1974) for use with experiments involving qualitative factors and zero amount of quantitative factors.

RESULTS & DISCUSSION

ANALYTICAL DATA on corn and oilseed flours used in the experiments appear in Table 2. Table 3 contains analytical and color data on corn tortillas. The increase in protein is apparent.

Tortillas containing LCP cottonseed flour were the darkest tortillas, followed by tortillas with glandless cottonseed flour, and then those with soy. As the amount of oilseed flour increased above the 11% blend level, the color of the tortillas fortified with glandless and LCP increased noticeably. However, the color of soy-fortified tortillas essentially remained the same. All fortified samples were darker in color than the corn flour control. Since cottonseed-fortified samples had the same appearance (i.e., darker yellow) as tortillas containing higher concentrations of lime (i.e., an intensified yellow color) panel members did not find them to be less desirable because of their color. Color is largely an aesthetic value.

The amino acid composition of both control and fortified tortillas is presented in Table 4. Lysine, the first limiting amino acid of corn was substantially increased. Other changes in the amino acid profile resulting from fortification are apparent in the table.

Cravioto has already demonstrated that the addition of soy flour to corn masa at a level of 10% increases the protein efficiency ratio from 1.45 to 2.45 (Cravioto and Cervantes, 1965).

Although preliminary laboratory work with the tortillas indicated that optimum amounts of water vary with the different oilseed flours used, it was beyond the scope of this paper to establish optimum amounts. Thus, a constant amount of water was used.

Sensory evaluation

Ten-judge panel results. At the 5% level of significance, the panel showed no preference among the corn control tortillas and tortillas fortified with LCP and glandless cottonseed flours. They did, however, show a significant preference for each of these tortillas over soy-fortified tortillas as indicated by the overall acceptability and texture mean scores given in Table 5.

The flavor scores also indicated that the panel found the corn control and tortillas fortified with LCP and glandless cottonseed flours equally acceptable. In this instance, the panel significantly preferred only the corn control and LCP cottonseed fortified tortillas over those fortified with soy flour. Scores assigned soy flour and glandless cottonseed flour fortified tortillas could not be statistically separated.

As illustrated in Table 6, panel results demonstrated that judges had no significant preference for one blend level over another as related to either overall acceptability, texture, or flavor. However, there was a significant interaction between flours and protein levels for each of the categories evaluated (i.e., blend protein levels in combination with flours did influence panel scores).

Five-judge Mexican-American results. Mexican-American scores were, on the whole, higher than Anglo-American scores which may be attributed to the fact that these panel members were more accustomed to eating tortillas as they were served for taste panel purposes (i.e., warmed on a grill), whereas the Anglo-American judges more often consume tortillas as an ingredient in traditional Mexican dishes as previously stated.

When data from Mexican-American taste panel members were analyzed separately for overall acceptability, the results (see Table 6) indicated a preference for LCP cottonseed-fortified tortillas at the 13% blend level over soy at all blend levels. They did not, however, indicate a significant preference for LCP at 11% and 15% blend levels over soy at any level.

Considering their rating of tortillas by flours (i.e., without regard to protein levels), this subpanel very definitely pre-

Table 3—Analytical data on corn tortillas containing blends of corn flour and other oilseed flours

Protein in blend	Flour in blend	Moisture (%)	Oil (%)	Nitrogen (%)	Protein[a] (%)	Color reading (L. scale)
			dry weight basis			
11%	Glandless cottonseed	42.6	1.28	2.02	12.63	63.7
	LCP cottonseed	44.2	1.00	2.01	12.56	60.2
	Soy (HS)	44.3	1.08	1.97	12.31	61.6
13%	Glandless cottonseed	40.9	0.83	2.39	14.94	60.2
	LCP cottonseed	44.3	0.94	2.30	14.38	57.4
	Soy (HS)	47.1	0.98	2.34	14.63	61.7
15%	Glandless cottonseed	44.9	1.05	2.76	17.25	59.3
	LCP cottonseed	38.6	0.68	2.65	16.56	54.9
	Soy (HS)	44.6	1.02	2.65	16.56	62.8
9.3%	Corn (control)	43.5	1.68	1.77	11.06	62.6

[a] Protein calculated as follows: Oilseed nitrogen X 6.25

Table 4—Amino acid composition of corn tortillas fortified with oilseed flours (g/16g N)

	11%			13%			15%			
Amino acids	Glandless cottonseed	LCP cottonseed	Soy (HS)	Glandless cottonseed	LCP cottonseed	Soy (HS)	Glandless cottonseed	LCP cottonseed	Soy (HS)	Corn (control)
Lysine	1.49	1.52	1.77	1.75	1.78	1.90	1.79	2.00	2.34	1.35
Histidine	1.43	1.48	1.59	1.61	1.51	1.43	1.48	1.70	1.50	1.48
Ammonia	1.29	1.34	1.36	1.25	1.19	1.12	1.26	1.29	1.18	1.34
Arginine	2.97	2.97	2.77	3.82	3.38	2.83	3.74	4.51	3.15	2.36
Aspartic acid	3.55	3.61	4.02	3.92	3.71	3.89	3.69	4.37	4.68	3.26
Threonine	1.78	1.87	1.95	1.92	1.82	1.83	1.75	2.00	2.00	1.81
Serine	2.34	2.53	2.60	2.48	2.37	2.30	2.18	2.72	2.54	2.38
Glutamic acid	11.44	12.02	12.02	12.72	11.65	10.87	12.11	13.64	11.65	11.30
Proline	4.45	4.17	4.83	4.51	4.31	4.05	3.68	4.32	4.25	5.04
Glycine	2.31	2.03	2.11	2.14	2.02	1.91	1.94	2.27	2.12	2.13
Alanine	3.72	3.92	3.97	3.63	3.51	3.24	3.21	3.59	3.38	4.02
Valine	2.45	2.72	2.62	2.59	2.42	2.30	2.40	2.83	2.53	2.41
Methionine	1.15	1.09	1.09	1.04	0.98	0.95	1.15	1.15	1.03	1.19
Isoleucine	1.88	1.90	2.03	1.90	1.81	1.87	1.81	2.11	2.17	1.82
Leucine	6.44	6.54	6.80	6.14	5.95	5.64	5.41	6.12	5.91	6.84
Tryosine	2.23	2.25	2.31	2.19	2.09	2.04	1.98	2.25	2.16	2.22
Phenylalanine	2.75	2.73	2.78	2.95	2.70	2.49	2.76	3.14	2.74	2.55

Table 5—Comparison of flour mean scores assigned to fortified tortillas by the ten-judge sensory panel and each of the five-judge subpanels

	Overall acceptability means[a]			Flavor means[a]			Texture means[a]		
Oilseed flour in blend	10-Judge panel	5-Judge Mexican-American	5-Judge Anglo-American	10-Judge panel	5-Judge Mexican-American	5-Judge Anglo-American	10-Judge panel	5-Judge Mexican-American	5-Judge Anglo-American
Glandless cottonseed	5.98^{a}	6.51^{a}	5.44^{b}	6.13^{ab}	6.44^{a}	5.73^{a}	5.90^{a}	6.69^{a}	5.13^{b}
LCP cottonseed	6.09^{a}	6.60^{a}	5.64^{b}	6.33^{a}	6.67^{a}	5.89^{a}	5.83^{a}	6.42^{a}	5.47^{ab}
Soy (HS)	5.57^{b}	5.69^{b}	5.44^{b}	5.90^{b}	6.07^{a}	5.73^{a}	5.32^{b}	5.56^{b}	5.09^{b}
Corn (control)	6.33^{a}	6.51^{a}	6.16^{a}	6.36^{a}	6.56^{a}	6.16^{a}	6.20^{a}	6.56^{a}	5.64^{a}

[a] Means shown to be significantly different by Duncan's Multiple Range test at the 5% level will not have a common alphabetical superscript. Values are means for 30 scores.

ferred corn flour tortillas and tortillas containing the cottonseed flours to those containing soy flour.

Relative to flavor, these judges expressed no preference on the basis of flours or protein levels taken separately or in combination.

This panel decidedly preferred the corn control along with glandless and LCP cottonseed-fortified tortillas to the soy tortillas for textural qualities.

Five-judge Anglo-American results. When rating overall acceptability, the Anglo-American subpanel significantly preferred the control to any of the three oilseed flour tortillas. They expressed no preference among oilseed flours as did the Mexican-American five judge panel.

The Anglo-Americans, like the Mexican-Americans, showed no flavor preference for a given protein level or flour. The panel further found no preference among protein levels or flours when rating tortillas for texture.

CONCLUSION

TORTILLAS prepared with corn flour blended with oilseed flours to give 11%, 13% and 15% protein in the blends gave a substantial protein increase in the tortillas of 18%, 40% and

Table 6—Comparison of mean scores assigned to tortillas fortified to different protein levels by the ten judge sensory panel

		Taste panel scores		
Oilseed flour in blend	Protein in blend (%)	Overall acceptability	Flavor	Texture
		Means[a]		
Glandless cottonseed	11	5.93^{abc}	6.17^{ab}	5.79^{abc}
	13	6.07^{ab}	6.17^{ab}	5.97^{ab}
	15	5.90^{abc}	6.07^{ab}	5.97^{ab}
LCP cottonseed	11	6.03^{abc}	6.30^{ab}	5.83^{abc}
	13	6.40^{a}	6.60^{a}	6.27^{a}
	15	5.83^{abc}	6.10^{ab}	5.40^{bc}
Soy (HS)	11	5.77^{abc}	5.93^{b}	5.50^{bc}
	13	5.60^{bc}	6.03^{ab}	5.30^{bc}
	15	5.33^{c}	5.73^{b}	5.17^{c}

[a] Means shown to be significantly different by Duncan's Multiple Range test at the 5% level will not have a common alphabetical superscript. Values are means for 30 scores.

61%, respectively. Since a blend of corn flour and oilseed flour can easily be further enriched with vitamins and minerals, it should be considered an ideal vehicle for upgrading the nutritional level of the Mexican-American population. Even more important, no basic change in eating habits is involved.

Furthermore, since the presence of such oilseed flours as glandless cottonseed and LCP cottonseed were considered by taste panel members to be equally as acceptable as the corn flour control, a food manufacturer could incorporate a desirable fortification level without loss of consumer acceptance.

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Ms received 6/16/75; revised 10/3/75; accepted 10/6/75.

Presented at the 35th Annual Meeting of the Institute of Food Technologists, Chicago, Ill., June 6–12, 1975.

This research was funded in part by the Natural Fibers and Food Protein Committee of Texas.

The authors thank Donna Mullinnix, Jose Montemayor, and Bacilio Becerra for technical assistance.

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HEAT TREATMENT: A PROCESS TO CONTROL THE DEVELOPMENT OF THE HARD-TO-COOK PHENOMENON IN BLACK BEANS (Phaseolus vulgaris)

ABSTRACT

Whole black beans were heat treated for 2, 5 and 10 min at 121°C and 10, 20 and 30 min under steam (98°C). Heat treatments did not affect the physical appearance of the grains but significantly ($P < 0.05$) decreased the development of the hard-to-cook phenomenon in seeds stored at 25°C and 70% RH for 9 months. After 9 months of storage no significant difference ($P < 0.05$) was found between hardness of cooked beans subjected to the shortest heat treatments and control samples kept at 4°C. Nitrogen solubility in 0.01N NaOH increased significantly ($P < 0.05$) in all samples as well as solubility in 5% KCl except in samples subjected to the longer heat treatments. Water absorption of the beans was favored by the heat treatments evaluated. A high correlation ($r = 0.91$) was found between the cooked beans hardness value and the lignified protein content of the cotyledon.

INTRODUCTION

IT IS KNOWN that beans as well as other legume seeds, constitute traditional foods in the diet of populations of tropical and sub-tropical areas, providing significant amounts of protein and calories for both rural and urban populations (INCAP, 1969; Molina et al., 1975).

Previous studies have shown that black beans (*Phaseolus vulgaris*), as well as other legume seeds, develop a hard-shell condition upon storage (Ruiloba, 1973). This condition has been defined as that in which the seed fails to imbibe water within a reasonable time when it is moistened (Bourne, 1967). Hard-shell seeds are a problem to seedmen because they do not sprout, and to food processors because they do not soften during cooking. Therefore, the development of a hard-shell condition of the beans upon storage is a limitation in increasing their production, and thus, their availability. The nutritional implications of a low availability of legume seeds are evident, considering that such grains have been accepted as a natural protein complement of cereals providing the lysine in which the latter are deficient (Bressani et al., 1962).

The use of a low storage temperature (4°C) or the practice of storing beans with a low moisture content (around 8–10%) in a relatively low humidity environment has been shown to minimize the development of a hard-shell condition in legume seeds, including black beans (Burr et al., 1968; Kon, 1968; Morris and Wood, 1956; Muneta, 1964; Ruiloba, 1973). Both conditions suggest that the development of hard shells can be the product of a chemical or enzymatic process in the seeds.

Based on the previous considerations, the present work was undertaken to study the possibility of minimizing the development of the hard-to-cook phenomenon in the black bean through a short heat treatment prior to their storage. If successful, this possibility could enable the development of a short thermal-treatment process for black beans prior to storage, which could probably be less costly than storaging them under controlled environmental conditions.

The above mentioned possibility is partially supported by the findings of previous authors (Gloyer, 1932; Morris et al., 1950; Steinkraus et al., 1964) who have reported a favorable effect of a heat treatment on the water absorption of beans, thus minimizing the hard-shell development in the grain. However, other authors (Burr et al., 1968) have reported that beans that rehydrate as quickly as normal beans usually need a prolonged cooking time, thus indicating no correlation between water absorption capacity and cooking time. These findings reveal that, at least in some bean varieties, a higher water absorption capacity (lower hard shell) is not necessarily correlated with a shorter cooking time.

MATERIALS & METHODS

THE BLACK BEAN (*Phaseolus vulgaris*), variety S-19-N, used in this study was grown at INCAP's experimental farm (San Antonio Pachalí, Guatemala) at an altitude of 1,480m above sea level and corresponded to the 1974 crop.

The whole, recently harvested beans, were separated in six lots of approximately 400g each. Each lot was placed in separate glass jars, taking care that all the beans formed one layer at the bottom of the jar. Three lots were heated in the retort at 15 psig (121°C) for 2, 5 and 10 min, respectively. The other three lots were heated under steam (98°C) without pressure, for 10, 20 and 30 min, respectively. After treatment, the beans were allowed to cool under ambient conditions. Each lot was then divided in two samples which were placed in cloth bags. One of the two samples from each treatment evaluated was stored under constant temperature (25°C) and relative humidity (70%), while the other was stored under refrigeration (4°C). A sample of untreated whole black beans, placed in a similar cloth bag, was included as a standard in each of the storage conditions used. The storage times evaluated were 0, 3, 6 and 9 months.

The germinating capacity of the seeds was measured according to the method described by the USDA (1965). Moisture, ether extract, crude fiber, nitrogen and ash were determined according to the AOAC (1970). Protein was calculated using the customary nitrogen conversion factor of 6.25. Available lysine was determined following the method described by Conkerton and Frampton (1959). Methionine and cystine were determined microbiologically following the procedure described by Elías et al. (1964), and the lignified protein fraction according to Van Soest and Wine (1968). The alcohol insoluble fraction (AIS) and the oxalate-soluble pectin fraction were established using the technique described by Ahmed and Scott (1957).

The water absorption capacity of the untreated and heat-treated whole beans was measured by immersing the dry beans in distilled water for different periods of time using a bean:water ratio of 30:100 at ambient temperature (about 20°C). After each period of time tested the beans were removed from the water as quickly as possible, drained and weighed. The water absorption capacity was calculated as percentage of the weight of the dry beans.

The nitrogen solubility of the untreated and heat-treated beans in 5% KCl and 0.01N NaOH was determined in a whole bean flour prepared by passing a bean sample through a hammer mill, equipped with a 40-mesh screen. The conditions used for the nitrogen solubility tests were the same as those applied by Molina et al. (1975).

The color measurements were performed using a Lovibond Tintometer type D (The Tintometer Ltd., Salisbury, England).

All determinations were carried out in triplicate. The bean's hard-

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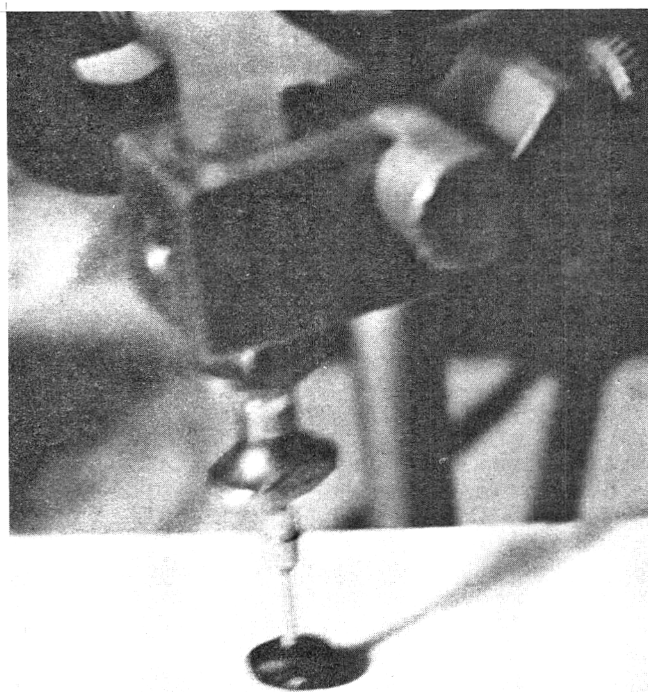

Fig. 1–Cooked bean set in wooden block in readiness for puncture test.

Table 1–Influence of the different heat treatments evaluated on the germinating capacity of the bean seed[a]

Treatment	Germination capacity (%)
None	99.3
10 min steaming (98°C)	88.7
20 min steaming	47.3
30 min steaming	0.0
2 min in the retort (15 psi, 121°C)	90.0
5 min in the retort	74.7
10 min in the retort	60.0

[a] This determination was carried out only at zero time of the storage period.

ness was measured by the puncture test. Prior to this test, both the untreated and the heat-treated whole dry beans were soaked in distilled water at ambient temperature (20°C) for 18 hr and then cooked in boiling distilled water for exactly 20 min. The puncture test was done in a locally built texture-testing machine (Ruiloba, 1973; Gómez-Brenes et al., 1974), equipped with an automatic digital readout which registers the electromagnetic force applied to the sample. The principle of the test in the machine used is similar to that of the Instron Universal Testing Machine when adapted to the puncture test (Bourne, 1972). A circular, flat-faced steel punch 0.216 cm diameter was attached to the inverted load cell of the instrument. Centered directly beneath was a small wooden block that had a 0.300 cm diameter-hole drilled through it. A countersink on the upper side helped to center the cooked bean and hold it in place (Fig. 1).

The puncture force was expressed in grams according to the calibration of the apparatus (Gómez-Brenes et al., 1974). For this test, a minimum of 20 replicas were run per sample.

RESULTS & DISCUSSION

THE EFFECT that the different heat treatments evaluated had on the germinating capacity of the seeds is shown in Table 1. As the data reveal, the germinating capacity of the grains decreased as the thermal treatment increased. This finding is indicative that the thermal treatments, either in the retort or through steaming, had a progressive inactivating effect on the enzymatic or enzymatic-chemical pathways of the grain as measured through the germinating capacity. This effect was found to be statistically significant ($P < 0.05$). No further evaluation of this parameter was carried out during the storage period.

The proximate composition and the methionine, cystine and available lysine content of both the untreated and heat-treated beans is presented in Table 2. As can be appreciated, none of the heat treatments evaluated had any appreciable effect on the chemical composition of the seeds. Furthermore, the general appearance of both the untreated and heat-treated beans was very similar, indicating that the heat treatments studied did not alter the physical appearance of the grains. In general, the proximate composition of the samples remained practically unchanged during the 9 months of storage. Although the methionine and available lysine contents of the raw beans tended to increase with storage, the change observed was not statistically significant ($P < 0.05$).

Since previous studies (Ruiloba, 1973; Molina et al., 1975) have shown that during storage of black beans under ambient conditions, the concentrations of the nitrogen fractions soluble in KCl and NaOH solutions increase, it was thought of interest to follow the concentration of such nitrogen fractions during the storage of both the untreated and heat-treated bean

Table 2–Percent composition and methionine, cystine and available lysine content of untreated and heat-treated beans[a]

	Untreated beans	Heat treatment					
		Retort (15 psi, 121°C)			Steaming (98°C)		
		2 min	5 min	10 min	10 min	20 min	30 min
Moisture	12.4	12.6	12.8	12.5	13.2	13.6	12.9
Ether extract	1.8	2.0	1.9	2.2	1.9	1.9	2.0
Crude fiber	2.8	3.0	3.0	3.0	3.0	2.7	2.8
Protein (N X 6.25)	19.6	20.2	20.1	20.2	19.4	21.2	19.9
Ash	3.2	4.1	3.8	3.9	3.6	3.6	3.3
Nitrogen-free extract	60.2	58.1	58.4	58.2	58.9	57.0	59.1
Methionine[b]	1.1	1.1	1.1	1.0	1.1	1.1	1.1
Cystine[b]	0.5	0.5	0.6	0.5	0.6	0.5	0.6
Available lysine[b]	5.1	5.0	5.1	5.1	5.3	5.0	5.2

[a] The results presented in this table correspond to those obtained at the beginning of the storage period. The values obtained for the same samples at 3, 6 and 9 months of storage did not differ significantly.
[b] As g/16g N

Table 3—Changes in the concentration of the nitrogen fraction soluble in 0.01N NaOH of untreated and heat-treated beans stored at 25°C and 70% RH[a] (Expressed as % of total nitrogen)

Storage time (months)	Untreated beans	Heat treatment					
		Retort (15 psi, 121°C)			Steaming (98°C)		
		2 min	5 min	10 min	10 min	20 min	30 min
0	93.5	95.8	95.2	95.2	93.3	93.8	95.2
3	98.3	98.1	98.0	98.0	98.3	98.2	98.0
6	99.4	99.3	99.2	98.9	99.5	99.0	98.8
9	99.9	99.9	99.4	99.5	99.8	99.3	99.2

[a] The percent of this fraction in the control samples stored at 4°C remained constant at 93.7 ± 1.6% during the 9 months of storage.

samples evaluated in this study. The results on nitrogen solubility in 0.01N NaOH and 5% KCl during the storage periods studied are presented in Tables 3 and 4, respectively. As the data show, there is an increase in the nitrogen fraction soluble in 0.01N NaOH through storage at 25°C and 70% RH, of both the untreated and heat-treated black bean samples (Table 3). Analysis of the data indicated that such an increase was statistically significant ($P < 0.05$). In the case of the untreated and heat-treated bean samples stored at 4°C no change was observed in the concentration of this nitrogen fraction during the storage period evaluated (9 months). In this case, the concentration of the nitrogen fraction soluble in 0.01N NaOH remained practically constant (93.7 ± 1.6%) for all samples. These findings indicate that some protein metabolic changes still occur in the seed even after being subjected to the heat treatments evaluated.

The nitrogen fraction soluble in 5% KCl (Table 4) of the beans stored at 25°C and 70% relative humidity also increased significantly ($P < 0.05$) in all cases, except in those beans treated for 30 min under steam (98°C) or for 10 min in the retort (15 psi, 121°C). Here again, the value of this nitrogen fraction of the untreated and heat-treated samples stored at 4°C remained practically unchanged during the 9-month storage period (70.3 ± 2.1%). The significance of the increase in this N fraction as related to the bean's protein digestibility (Molina et al., 1975) still remains to be determined. We cannot offer at present any explanation as to why the increase observed is larger in those beans treated for 10 or 20 min under steam, than in the untreated beans or in those treated for 2 or 5 min in the retort.

The effect of the heat treatments evaluated on the development of the hard-to-cook phenomenon in the beans, as measured by the puncture test, is shown in Figures 2 and 3. As the data reveal, the shortest heat treatments evaluated (either 2 min in the retort or 10 min under steam) were the most effective in preventing the development of the hard-to-cook phenomenon in the black beans tested. Furthermore, only those samples which received the shortest heat treatments did not show a significant ($P < 0.05$) increase in their puncture force value after 9 months of storage at 25°C and 70% RH. However, in all cases the heat-treated black beans showed a significantly lower ($P < 0.05$) puncture force figure than the untreated beans. The hardness-puncture force value of all samples stored at 4°C remained practically constant and, therefore, their pooled values were plotted as the control sample value in Figures 2 and 3. This value was found to be statistically equal to that of the samples which received the shortest heat treatments evaluated, at any storage period.

In order to explain, partly at least, the changes observed in the development of the hard-to-cook phenomenon in the untreated and heat-treated whole bean samples, several tests were carried out in the 9-month stored samples.

Since pectic fractions have been found to have a relation with the rates of water imbibition of dry beans (Hamad and Powers, 1965) and this phenomenon in turn is related to the hard-shell development of the grains (Bourne, 1967), the AIS and oxalate-soluble pectin fraction were determined in all bean samples stored for 9 months. Here, however, no appreciable change was found in the concentration of these two components in all samples, either stored at 25°C or 4°C. The concentration of the AIS was found to be 96.2 ± 3.1% (dry basis) and that of the oxalate-soluble pectin fraction, 6.5 ± 1.2% (dry basis) for all the dry unheated and heat-treated bean samples.

Table 4—Changes in the concentration of the nitrogen fraction soluble in 5% KCl of untreated and heat-treated beans stored at 25°C and 70% RH[a] (Expressed as % of total nitrogen)

Storage time (months)	Untreated beans	Heat treatment					
		Retort (15 psi, 121°C)			Steaming (98°C)		
		2 min	5 min	10 min	10 min	20 min	30 min
0	69.7	69.7	70.9	69.7	69.7	69.0	69.7
3	70.5	70.4	71.1	70.8	70.6	69.7	69.1
6	72.1	73.6	72.1	70.7	77.0	76.7	69.9
9	73.2	73.9	73.6	71.0	78.5	77.3	69.9

[a] The percent of this fraction in the control samples stored at 4°C remained constant at 70.3 ± 2.1% during the 9 months of storage.

Table 5—Lignified protein content of the cotyledon and seed coat in cooked (18 hr soaking, 20 min boiling), untreated and heat-treated beans stored at 25°C and 70% RH for 9 months[a] (Expressed as % of total nitrogen)

Treatment	Lignified protein	
	Cotyledon	Seed coat
None	17.2	44.2
10 min steaming (98°C)	13.0	44.2
20 min steaming	14.1	44.2
30 min steaming	15.7	45.3
2 min in the retort (15 psi, 121°C)	12.7	43.2
5 min in the retort	14.3	44.2
10 min in the retort	14.7	45.3

[a] The mean value of the lignified protein content in the cotyledon and seed coat of the bean samples stored at 4°C were 9.2 ± 2.3% and 29.5 ± 3.´%, respectively.

The water absorption capacity rates of the dry bean samples at different times are presented in Figures 4 and 5. It is evident that all heat treatments evaluated exerted a beneficial effect on the water absorption capacity of the beans at any period of time studied. This effect was found to be statistically significant ($P < 0.05$). However, no correlation was found at any time between the water absorption capacity values and the hardness-puncture force values obtained in the same samples after 9 months of storage (Fig. 2 and 3). Such results are in accordance with those of Burr et al. (1968) who reported no correlation between water absorption values and cooking time in dry beans.

The lignified protein content of both the cotyledon and the seed coat of the untreated and heat-treated beans stored for 9 months at 25°C is given in Table 5. This determination was carried out after submitting the dry beans to the cooking process used for the hardness measurements (18-hr soaking, 20-min boiling). Likewise, the separation of the seed coat was done after the cooking process. All samples were air dried at room temperature (around 25°C) prior to the test. As the data show, the lignified protein fraction of the cotyledon of the beans stored at 25°C tends to increase as the hardness value increases (Fig. 2 and 3). In fact, a high correlation ($r = 0.91$) was found between both parameters. The lignified protein fraction in the seed coat was found to remain practically the same in all samples. Similarly, the value of this protein fraction was practically the same (both in the cotyledon and in the seed coat) for all samples stored at 4°C. In addition, these samples presented the lowest lignified protein content.

It was of interest to observe that the color of the cotyledons from the cooked bean samples differed significantly. It

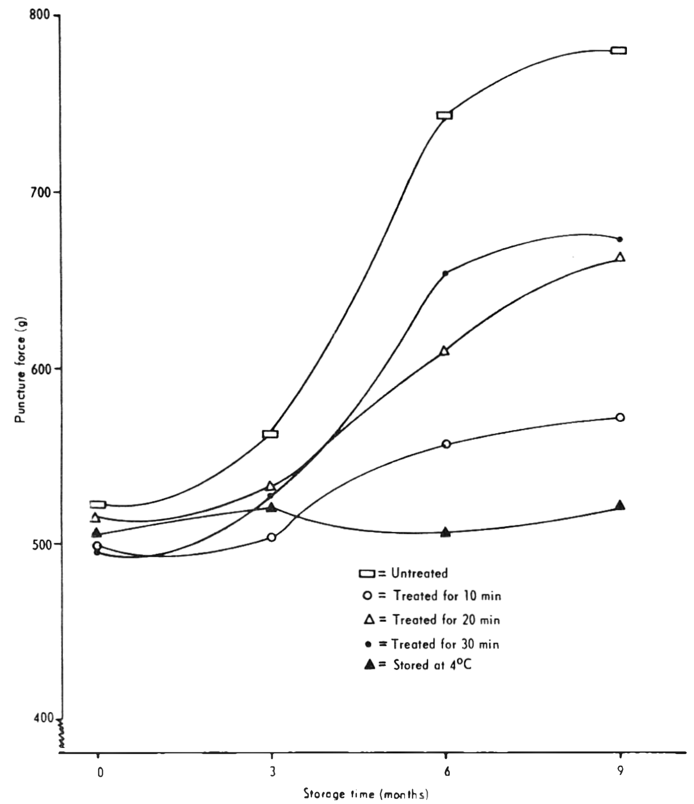

Fig. 3—Effect of storage (25°C, 70% RH) time on the hardness of cooked (18 hr soaking, 20 min boiling), untreated and steam-treated (98°C) black beans.

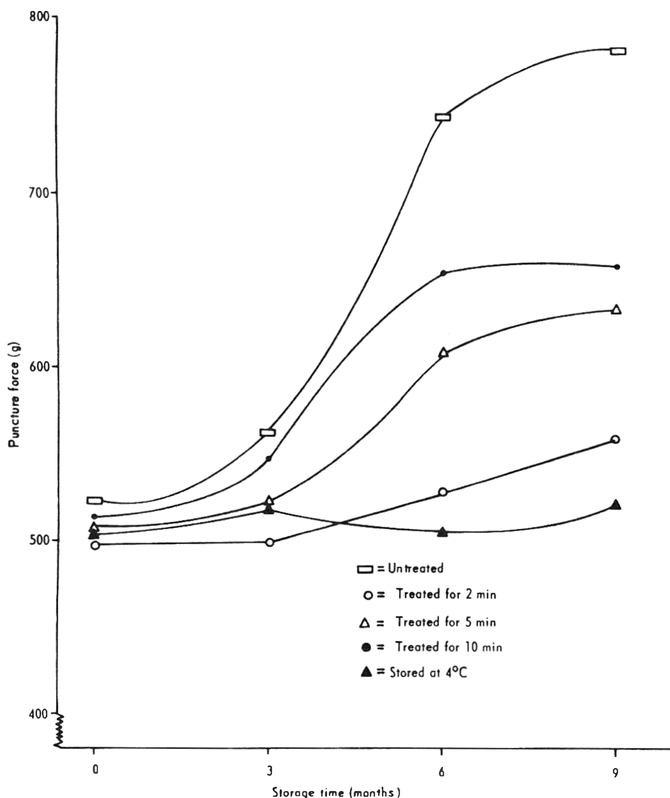

Fig. 2—Effect of storage (25°C, 70% RH) time on the hardness of cooked (18 hr soaking, 20 min boiling), untreated and retort-treated (15 psi, 121°C) black beans.

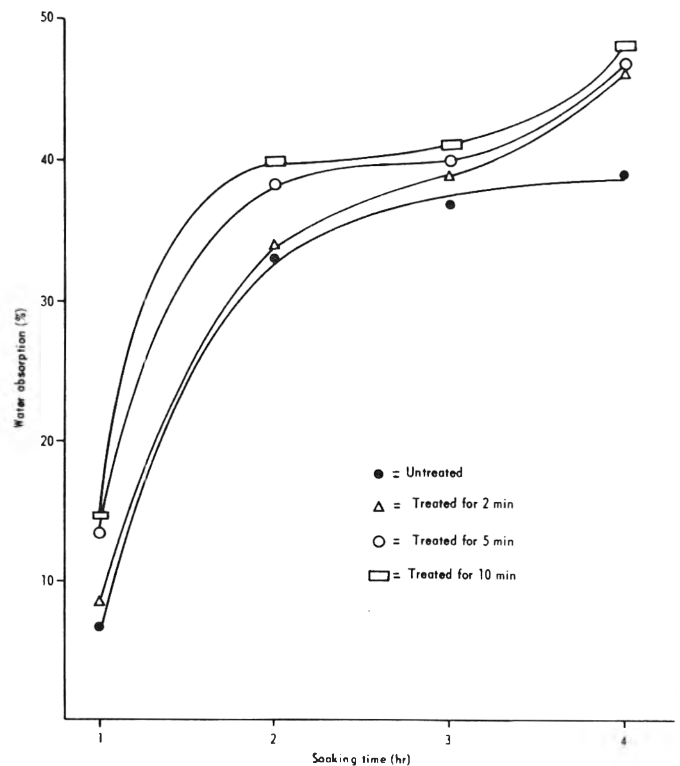

Fig. 4—Percent water absorption at different soaking times of untreated and retort-treated (15 psi, 121°C) black beans stored for 9 months at 25°C and 70% RH.

Table 6—Interpretation of the visual color of the cotyledon in cooked (18 hr soaking and 20 min boiling), untreated and heat-treated beans stored at 25°C and 70% RH for 9 months[a]

Thermal treatment	Interpretation of visual color							
	Brightness	Neutral tint	Red	Orange	Yellow	Green	Blue	Violet
None	–	0.5	–	0.3	–	–	–	–
10 min steaming (98°C)	–	0.2	–	–	–	0.4	0.5	–
20 min steaming	–	0.6	–	–	0.1	0.1	–	–
30 min steaming	–	0.5	–	–	0.1	0.1	–	–
2 min in the retort (15 psi, 121°C)	–	0.3	–	–	–	0.3	0.6	–
5 min in the retort	–	0.7	–	–	–	0.1	0.1	–
10 min in the retort	–	0.6	–	–	0.1	0.1	–	–

[a] All samples stored at 4°C had a visual color interpretation, similar to that obtained in beans treated in the retort for 2 min or under steam for 10 min.

was evident that the samples with the lowest hardness value (Fig. 2 and 3) had cotyledons with a darker-bluish color, indicative that in these samples the pigments contained in the seed coat were being diffused more readily. Such a characteristics is typical of recently harvested cooked black beans.

The interpretation of the visual color read in the Lovibond Tintometer using the cotyledons from the cooked (18-hr soaking, 20-min boiling) beans stored for 9 months at 25°C is presented in Table 6. As the data clearly reveal, the intensity of the blue and green colors significantly decreases as the hardness value increases (Fig. 2 and 3). At present we are trying to establish whether the lignified protein fraction of the cotyledon affects the diffusion of the seed coat pigments, and if so, how does it affect it.

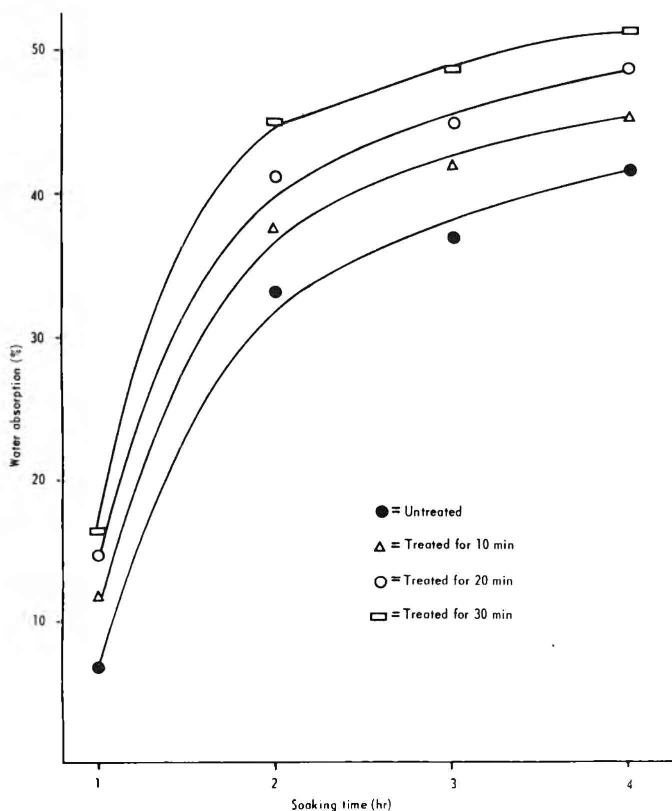

Fig. 5—Percent water absorption at different soaking times of untreated and steam-treated (98°C) black beans stored for 9 months at 25°C and 70% RH.

We are also investigating the possibility of effecting the short-time heat treatment to prevent the hardness development through the use of dry heat. This possibility is being investigated in order to provide the black bean producer with a low-cost technology that will enable him to preserve his product in its "normal" raw (not precooked) state for a longer time, with the desirable characteristics needed by the consumer. If such a low-cost technology would be available, it could easily result in an increase in production and thus in a greater availability of black beans.

Concomitantly, we are interested in defining the basic mechanism of action of the short-time heat treatment in preventing the development of the hard-to-cook phenomenon in black beans. This is pursued in the search for new and, possibly, cheaper processes that could have the same effect. The possible effect of a short-time heat treatment in preventing or diminishing the degree of either a rancidity type of deterioration or a biodeterioration of the bean during storage is also being investigated.

Since our population at present is not yet in the habit of consuming precooked food products, we believe that a simple technology as the one described herein, whereby the product is improved in storage quality but with an appearance similar to the raw seed, is of great value.

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Ms received 8/18/75; revised 10/18/75; accepted 10/21/75.

Presented at the 35th Annual Meeting of the Institute of Food Technologists, Chicago, Ill., June 8–12, 1975.

This research was carried out with partial support from the Research Corporation, New York, N.Y. (Grant-in-aid INCAP No. 740).

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AFLATOXIN CONTENT AND SKIN REMOVAL OF SPANISH PEANUTS AS AFFECTED BY TREATMENTS WITH CHEMICALS, WATER SPRAY, HEATED AIR, AND LIQUID NITROGEN

ABSTRACT

Unshelled Spanish peanuts contaminated with aflatoxin were shelled and treated with forced heated air, liquid nitrogen, H_2O_2, HCl, sodium oleate, and water spray. After passing through a whole nut blancher the aflatoxin content and percentages of blanched (skin removed) and whole kernels were determined. Blanching percentages were significantly higher for sodium oleate, water spray, liquid nitrogen, and H_2O_2 than for HCl or heat treatments. The heat treatment produced the highest percentage of whole kernels. In most tests, the unblanched kernels contained higher levels of aflatoxin than those that blanched fully. The treatments which were most effective in producing low levels of aflatoxin in the blanched kernels were H_2O_2, water spray and HCl.

INTRODUCTION

SINCE THE DISCOVERY of aflatoxin in the early 1960's, widespread efforts have been made to detect and reduce aflatoxin in peanut lots suspected to be contaminated. In a suspect lot, only a small fraction of the peanuts may actually contain aflatoxin. Cucullu et al. (1966) reported that assays of sub-samples varied widely due to nonuniform distribution of aflatoxin within individual peanut kernels and to uneven distribution of a few highly contaminated kernels among noncontaminated kernels. Whitaker and Wiser (1969) reported that in a population of shelled peanuts less than 0.5% may actually contain aflatoxin, but concentrations in those kernels may be as high as 1,000,000 ppb. A method to remove those few highly contaminated peanuts would be desirable.

The objectives of this research were to determine: (1) the effect of treatments with liquid nitrogen, hydrochloric acid, hydrogen peroxide, sodium oleate, heated air, and water spray on percentages of blanching and of whole kernels for aflatoxin contaminated Spanish peanuts (blanching in this context refers only to removal of the skin (testa) from the peanut kernel.); (2) whether such treatments reduced the aflatoxin content of the peanut samples; (3) whether separation of treated peanuts into blanched and unblanched groups would effectively remove highly contaminated kernels.

EXPERIMENTAL

THE PROCEDURE (Fig. 1) basically consisted of obtaining segregation 3 naturally contaminated pods, shelling the peanuts, applying various treatments, blanching the kernels, separating the blanched from the unblanched and then determining the aflatoxin content of these two groups. (USDA defines segregation 3 peanuts as those which contain visible *Aspergillus flavus* mold. *A. flavus* mold is a necessary but not sufficient condition for the presence of aflatoxin.) Major interest was in those treatments producing blanched kernels with acceptably low aflatoxin levels. The experimental design was a randomized complete block with six treatments and five replications. The treatments used were selected because they yielded relatively high blanching percentages of noncontaminated Spanish peanut kernels without producing objectionable odor or flavor (Shackelford et al., 1972, 1973).

A lot of approximately 900 kg of unshelled segregation 3 peanut pods were purchased, shelled and sized. Only those whole kernels retained on a 0.6 cm (15/64 in.) official USDA grading screen were used and any without fully intact skins were discarded. Of the remaining 60 kg of kernels, five samples of approxiamtely 5 kg each were randomly drawn, hygroscopically conditioned to 8% moisture content (MC) and held at 4°C until tested. (All moisture contents were computed as percent wet basis unless otherwise noted.)

Immediately prior to testing, each of the five samples representing replications was divided into six sub-groups, one for each of the six treatments. One 50-g pre-treatment sample was randomly drawn from each of the 30 sub-groups for assays of initial aflatoxin contamination.

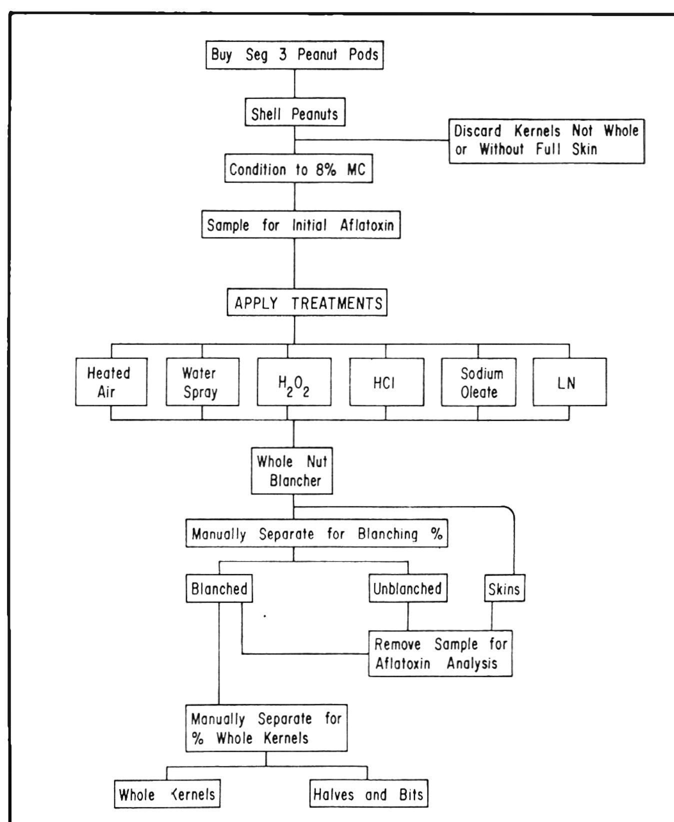

Fig. 1—Experimental procedure flow chart.

Results of 18 randomly selected aflatoxin assays indicated concentrations of individual samples initially ranged from not detectable (ND) to 2300 parts per billion (ppb) with an average of 217 ppb. The samples were assayed for aflatoxin B_1, B_2, G_1 and G_2 by the thin layer chromatographic method developed at the USDA Southern Regional Research Laboratory (Pons, 1966).

For all heat, water spray, hydrogen peroxide (H_2O_2), sodium oleate, and hydrochloric acid (HCl) treatments, 650-g samples at 8% MC were used. After each treatment the samples were dried to 5% MC in a drying tray with a screen bottom. The kernels were dried in a laboratory-constructed heated forced air dryer which maintained drying air constantly at 71°C. After drying, the kernels were cooled for about 2 hr to room temperature before blanching.

For heat treatment, individual kernels were conveyed through a skin slitter which placed a longitudinal slit in the skin on each side of the kernel. The 650-g samples were then dried with heated forced air at 71°C from 8 to 5% MC and cooled.

In the water spray treatment, the peanut skins were slit. The peanut samples were placed in a large screen-meshed bottom tray which accommodated a layer of kernels about 2.5 cm deep. The tray of kernels was enclosed in a chamber and sprayed at 275 × 10^3 pascal (40 psig) with water at 38°C for 16 min. Then the kernels were removed, placed on dry paper towels for 5 min to remove excess moisture, forced air dried and cooled.

For the H_2O_2 treatment the kernels were placed into a closed screen-meshed container and immersed for 5 min in 5% H_2O_2 at 21°C.

In the sodium oleate treatment, the samples were immersed for 5 min in 0.3% sodium oleate at 66°C. Then, the samples were removed and neutralized in 0.5% calcium chloride for 2 min.

For the HCl treatment, the samples were immersed for 1 min in 0.1% HCl at 32°C. Kernels were removed and neutralized in 0.5% sodium bicarbonate for 2 min.

After these procedures for H_2O_2, sodium oleate, and HCl treatments, the samples were rinsed thoroughly with tap water for 2 min, placed on dry paper towels for 5 min, forced air dried, and cooled.

For treatment with liquid nitrogen (LN_2), 300g of 8% MC kernels were placed on a screen mesh bottom tray and conveyed through a stream of LN_2. A variable speed conveyor inside the insulated LN_2 chamber was designed to expose the thin layer of kernels to the LN_2 spray at −196°C for a specified time. Previous blanching tests (Shackelford, 1974) for noncontaminated kernels indicated that a 2-min treatment would give a relatively high blanching percentage. For a single layer of kernels, the 20 × 30 cm tray limited samples to 300g.

After the samples were given the particular treatment they were passed through a blancher constructed similar to the Ashton whole nut blancher. It separated the skins from the kernels. The percentage of blanched kernels was found by hand separating each peanut kernel sample into two groups: (1) the fully blanched group with all of the visible skins removed; and (2) the unblanched with some or all of the skin remaining on the kernel. Blanching percentage was 100 times the weight ratio of the fully blanched group to the sum of fully blanched plus unblanched kernels.

After treating and blanching, aflatoxin assay samples were collected from the following three groups: (1) 100g from the fully blanched kernels, (2) 50g from the unblanched kernels, and (3) 5g from the skins removed by the whole nut blancher.

The blanched kernels were then used for determining the percent whole kernels. Whole kernels were hand-separated from halves, bits and

Table 1—Effect of treatments on the percentages of moisture, blanched and whole kernels for alfatoxin contaminated peanuts

Treatment	% Moisture		% Kernels blanched		% Whole kernels	
	Before treatment	Before blanch	Sample	Treatment mean	Sample	Treatment mean
Cryogenic	8.1	–	91.4		1.5	
2 min	8.1	–	87.2		2.5	
	8.1	–	90.7	89.2	1.1	3.7
	8.3	–	88.0		7.8	
	8.1	–	88.5		5.4	
Heat	8.2	5.0	79.7		59.3	
71° C	7.5	5.3	75.3		66.5	
	8.4	5.5	81.5	80.3	72.3	66.4
	8.1	5.5	83.8		68.9	
	8.2	5.7	81.1		65.1	
Hydrogen peroxide	8.6	5.0	86.6		54.7	
5%, 21° C	9.0	5.1	90.4		59.5	
5 min	8.1	5.4	88.6	88.9	66.4	62.4
	8.3	5.3	89.8		69.6	
	8.3	5.4	89.2		61.7	
Sodium oleate	8.4	5.5	90.9		29.4	
0.3%, 66° C	9.1	5.2	89.9		27.4	
5 min	8.1	5.2	89.9	90.1	28.9	30.1
	8.2	5.2	90.9		34.0	
	8.3	5.3	88.7		30.9	
Hydrochloric acid	8.7	5.2	84.1		46.4	
0.1%, 32° C	9.0	5.4	83.9		41.1	
1 min	8.2	5.2	89.6	86.2	46.8	45.9
	8.1	6.0	87.5		46.8	
	8.1	5.4	85.7		48.6	
Water spray	8.9	5.2	91.8		23.8	
38° C	8.6	5.1	86.0		40.2	
16 min	8.7	5.1	92.2	89.5	26.4	34.0
	8.0	5.2	88.1		45.2	
	8.2	5.4	89.4		34.5	

Table 2—Effect of treatments on the aflatoxin content[a] of the three peanut components

Treatment	Initial sample[b]	Skin, ppb		Unblanched kernels, ppb		Fully blanched kernels, ppb			
		Sample	Mean	Sample	Mean	Assay 1	Assay 2	Assay avg	Mean
Cryogenic	30	11		6		21	13	17.0	
2 min	517	40		304		Trace	<5	3.0	
		ND	12.8	452	162	20	112	66.0	36.3
		13		50		Trace	137	69.5	
	2	ND		ND		ND	52	26.0	
Heat	ND	21		333		39	9	24.0	
71° C	170	44		1580		40	279	159.5	
	8	94	36.8	660	666	ND	103	51.5	195.5
		12		39		37	<5	20.5	
		13		716		1440	<5	722.0	
Hydrogen peroxide	ND	21		9730		<5	ND	2.0	
5%, 21° C		9		461		Trace	ND	1.0	
5 min		69	25.0	1230	2400	ND	ND	0.0	8.0
		26		479		74	ND	37.0	
	470	ND		97		ND	ND	0.0	
Sodium oleate	12	ND		336		420	ND	210.0	
0.3%, 66° C	11	Trace		61		23	23	23.0	
5 min	ND	ND	4.4	42	417	Trace	Trace	2.0	70.7
		18		608		Trace	119	60.5	
	ND	Trace		1040		Trace	114	58.0	
Hydrochloric acid	218	19		5		18	82	50.0	
0.1%, 33° C		Trace		233		ND	Trace	1.0	
1 min		8	46.0	516	193	51	Trace	26.5	17.6
		192		206		17	Trace	9.5	
	44	9		5		Trace	ND	1.0	
Water spray	2300	17		8500		<5	25	14.5	
38° C		50		938		Trace	Trace	2.0	
16 min	ND	Trace	17.0	1610	2331	16	16	16.0	12.6
	133	Trace		65		40	Trace	21.0	
	Trace	14		542		Trace	17	9.5	

[a] Total ppb of B_1, B_2, G_1, G_2
[b] For statistical purposes, ND = 0 ppb, trace = 2. ppb, < 5 = 4 ppb.

pieces. Percent whole kernels was 100 times the weight ratio of whole kernels to the sum of whole kernels plus halves, bits and pieces.

RESULTS & DISCUSSION

THE PERCENT MOISTURE, blanched and whole kernels for the various treatments are presented in Table 1. Analysis of variance indicated that treatments significantly affected percent blanched. Based on a least significant difference (LSD) of 2.61 ($\alpha = 0.05$), sodium oleate, water spray, liquid nitrogen and H_2O_2 all obtained significantly higher mean percent blanched than HCl or heat treatment. Mean percent blanched kernels for the heat treatment was significantly lower than for the other treatments.

The percent whole kernels was highest with the heat treatment, followed by H_2O_2, HCl, water spray, sodium oleate, and liquid nitrogen (Table 1). Analysis of variance indicated that treatments had a significant effect on the percent whole kernel. Based on LSD of 5.64, a significant difference existed between the mean percent whole kernels of all treatments except between heat and H_2O_2 and between water spray and sodium oleate.

Effects of heat, liquid nitrogen, water spray, and chemical treatments on the aflatoxin content of the three peanut components are shown in Table 2. Due to the large variation in aflatoxin content between replicated samples and the tendency for a large number of samples to contain small or nondetectable amounts of aflatoxin, a plot of frequency versus aflatoxin content did not follow a normal distribution curve. A Kolmogorov-Smirnov one-tailed nonparametric statistical test (Siegel, 1956; Goodman, 1954) was used to determine significant differences between treatments and the three peanut components.

Screenivasamurthy et al. (1967) reported that heating aflatoxin contaminated peanut meal in a solution of H_2O_2 at pH 9.5 for 30 min at 80°C reduced aflatoxin by 97%. Table 2 indicates that for the H_2O_2 treatment, the unblanched kernels had the highest mean contamination level, 2400 ppb. However, the H_2O_2 treated blanched kernels had the lowest mean contamination level and in nine out of ten assays showed less than 20 ppb (Table 3 summarizes data from Table 2). Kolmogorov-Smirnov tests indicated that the H_2O_2 treated blanched kernels had significantly lower contamination than unblanched samples (Fig. 2). The aflatoxin content of blanched samples was significantly lower in those treated with H_2O_2 than with heat, LN_2, sodium oleate, or water spray. Thus, hydrogen peroxide appears to be an effective treatment in producing blanched kernels with low aflatoxin levels.

Peanuts from the water spray treatment had the second highest mean contamination level, 2331 ppb, among the un-

Table 3–Effect of treatments on relative distribution of aflatoxin concentration

	Number of samples within each aflatoxin range								
	Skins, ppb			Unblanched kernels, ppb			Blanched kernels, ppb		
Treatments[a]	0–20	21–200	>200	0–20	21–200	>200	0–20	21–200	>200
Sodium Oleate	5	0	0	0	2	3	5	4	1
Water Spray	4	1	0	0	1	4	8	2	0
Cyrogenic	3	2	0	2	1	2	6	4	0
H_2O_2	2	3	0	0	1	4	9	1	0
HCL	4	1	0	2	0	3	8	2	0
Heat	2	3	0	0	1	4	4	4	2

[a] Treatments are ranked from top to bottom in order of decreasing blanching percentage.

blanched groups and the second lowest mean contamination level among blanched groups, 12.6 (Table 2). Eight out of ten blanched samples from the water spray treatment contained less than 20 ppb (Table 3). The other samples contained less than 50 ppb. Goldblatt (1971) noted that because high concentrations of aflatoxin are deeply embedded in individual kernels, washing of whole peanuts with water probably would not remove aflatoxin. Aflatoxin levels in blanched kernels were not significantly lower from water spray than other treatments. But, Kolmogorov-Smirnov tests indicated that water spray treated samples had mean levels of contamination significantly lower for blanched than for unblanched samples (Fig. 2). Treatment with water spray alone did not produce blanched kernels with aflatoxin levels as low as when the water spray was followed by heating and blanching.

Treatment of highly contaminated peanut meal with HCl has been found (Feuell, 1966) to reduce aflatoxin content. In our study, blanched kernels from the HCl treatment had the third lowest aflatoxin level. Eight out of ten samples contained less than 20 ppb. In blanched samples, aflatoxin levels were not significantly lower from HCl than for other treatments.

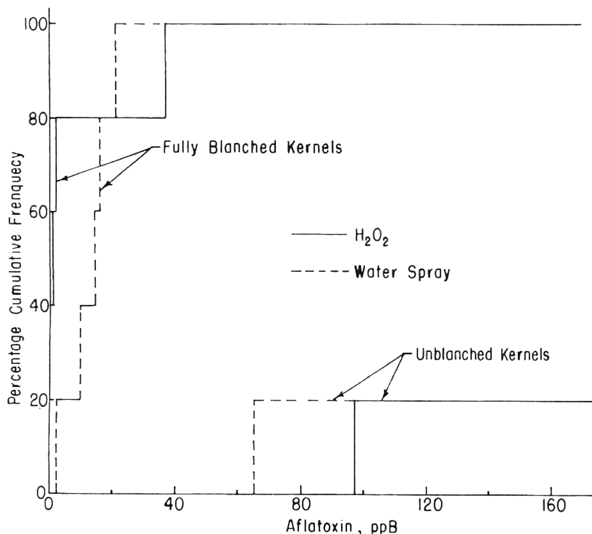

Fig. 2–Kolmogorov-Smirnov tests showing a significant difference (at α = 0.05 level and N = 5) between blanched and unblanched kernels when cumulative frequency of aflatoxin content differs by a least 80%.

Mann et al. (1967) found that aflatoxin content of oilseed meals was reduced only slightly by heating at 60–80°C. However, when contaminated peanut meal at 20% moisture content was heated at 100°C for 2 hr aflatoxin was reduced 34%. The heat treated blanched peanuts in our tests had the highest mean contamination level. The 196 ppb aflatoxin level after heat treatment approaches the average initial level of 217 ppb for the lot. Of ten heat treated blanched samples, four had less than 20 ppb (Table 3). Apparently, heat treatment at 71°C did not reduce aflatoxin content.

A Kolmogorov-Smirnov test at the 0.05 level of significance indicated that skins from samples treated with sodium oleate had an aflatoxin level significantly lower than skins from any other treatment. The mean contamination level was less for skins than for unblanched kernels for all treatments. Before the experiment it was hypothesized that if there was a difference, the skins would have greater amounts of aflatoxin than the unblanched kernels. Compared to the aflatoxin levels of the blanched kernels the skins' aflatoxin level was lower than was expected.

There is strong evidence to believe that kernels which completely blanch contain lower levels of aflatoxin than kernels which do not blanch completely. In 25 of 30 tests, unblanched kernels contained more aflatoxin than blanched kernels or skins. Kolmogorov-Smirnov tests indicated that, for H_2O_2 and water spray treatments, mean levels of contamination were significantly higher in unblanched kernels than in skins (Table 2). These data support the hypothesis that removing unblanched kernels after the blanching process reduces the average aflatoxin level.

SUMMARY & CONCLUSION

PERCENT BLANCHING did not differ significantly (0.05 level) among contaminated kernels that had been treated with sodium oleate, water spray, liquid nitrogen and H_2O_2. Heat treatment gave the highest mean percentage of whole kernels.

With H_2O_2 and water spray treatments, mean aflatoxin contents were significantly lower in fully blanched than in unblanched kernels. For blanched samples aflatoxin contents were significantly lower for H_2O_2 treated samples than for samples treated with heat, liquid nitrogen, sodium oleate or water spray. With H_2O_2 and water spray treatments, mean contamination level was highest in unblanched and lowest in blanched samples. These results show that H_2O_2, followed by water spray and HCl were the most effective treatments in producing blanched kernels with low aflatoxin levels.

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Ms received 7/31/75; revised 10/6/75; accepted 10/15/75.

Presented at the 35th Annual Meeting of the Institute of Food Technologists, Chicago, Ill., June 8—12, 1975.

Journal Article No. 2908, of the Agricultural Experiment Station, Oklahoma State University, Stillwater, Okla. This research was supported in part by contract 12-14-100-10995 of the USDA, ARS Southern Regional Research Center.

The authors acknowledge the assistance of Ms. Iris Wilson in conducting aflatoxin analyses.

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REVERSE OSMOSIS SEPARATIONS AND CONCENTRATIONS OF FOOD SUGARS

ABSTRACT

The physicochemical criteria approach offers a means of predicting quantitatively reverse osmosis separations of D-glucose, D-fructose, sucrose, maltose and lactose in aqueous solutions using porous cellulose acetate membranes. The presence of a small quantity of pectin in aqueous sucrose solution increases its viscosity and decreases its mass transfer coefficient during reverse osmosis. Reverse osmosis data with aqueous sucrose feed solutions containing small quantities of $CaCl_2$ show that the latter does not exist as an independent entity in such solutions. A set of process design calculations is illustrated for reverse osmosis concentration of food sugars in aqueous solutions using a typical cellulose acetate membrane.

INTRODUCTION

THE PREFERENTIAL sorption-capillary flow mechanism for reverse osmosis (Sourirajan, 1970a) has led to a fundamental physicochemical criteria approach to reverse osmosis separations (Matsuura et al., 1975b, 1976). Referring specifically to organic solutes and reverse osmosis systems involving preferential sorption of water at the membrane-solution interface, it has been shown that when the solute molecule is nonionized, and its molecular structure does not contain more than three straight chain carbon atoms not associated with a polar functional group, solute separation in reverse osmosis is governed by polar and steric effects only. These effects can be expressed quantitatively in terms of appropriate free energy parameter for each solute molecule, and the steric parameter for each solute-membrane system, so that solute separations in reverse osmosis can be predicted quantitatively for any membrane just from data on membrane specifications given in terms of a reference solute such as sodium chloride. The application of this approach for the quantitative prediction of reverse osmosis separation and recovery of apple flavor components has been illustrated (Matsuura et al., 1975a) with particular reference to aromatic polyamide membranes. This paper gives a similar illustration of the application of the above approach for quantitative predictions of reverse osmosis separations of D-glucose, D-fructose, sucrose, maltose and lactose in aqueous solutions using porous cellulose acetate membranes. This work also includes some studies on the reverse osmosis treatment of aqueous sucrose solutions containing small quantities of pectin, or pectin and calcium chloride. On the basis of the experimental and analytical results obtained, a set of numerical process design calculations is illustrated for reverse osmosis concentrations of sugars in aqueous solutions using a typical cellulose acetate membranes.

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EXPERIMENTAL

REAGENT GRADE solute substances and batch 316(10/30)-type cellulose acetate membranes made in the laboratory (Pageau and Sourirajan, 1972) were used. Unless otherwise stated, the flow-cell apparatus, and the experimental procedure used were the same as those reported earlier (Sourirajan, 1964; 1970a). The operating pressure used was 1000 psig in all cases. The specifications (Sourirajan, 1970b) of the membranes used are given in Table 1 in terms of pure water permeability constant A and solute transport parameter $D_{AM}/K\delta$ (defined as solute flux/difference in solute concentration on either side of membrane) for sodium chloride. Table 1 also includes solute separation and product rate data for the membranes used with 5000 ppm NaCl-H_2O feed solutions at feed flow rates corresponding to mass transfer coefficients (k) in the range 15.5×10^{-4} to 33.5×10^{-4} cm/sec on the high pressure side of the membrane.

The solute concentration in the feed solutions used was in the range 1000 ppm to about 40% by weight as specified in each case. All experiments were of the short-run type, and carried out at the laboratory temperature (23–25°C). The reported product rates are those corrected to 25°C using the relative viscosity and density data for pure water. In

Table 1—Specifications of the cellulose acetate membranes used and some performance data[a]

Film no.	1	2	3	4	5	6	7	8
Operating pressure, 1000 psig								
Pure water permeability constant								
A, $\frac{\text{g mole } H_2O}{\text{cm}^2 \text{ sec atm}} \times 10^6$	1.10	2.43	3.47	4.01	0.96	1.50	1.96	1.74
Solute transport parameter								
$(D_{AM}/K\delta)_{NaCl}$, (cm/sec) $\times 10^5$	1.21	21.37	60.62	64.50	1.93	30.83	71.46	108.4
$\ln(D_{AM}/K\delta)_{NaCl}$	−11.24	−8.43	−7.58	−7.25	−10.85	−8.08	−7.24	−6.83
$\ln C^*_{NaCl}$	−12.61	−9.80	−8.95	−8.62	−12.22	−9.45	−8.61	−8.20
$\ln \Delta^*$	0	−0.63	−0.92	−0.92	0	−0.92	−0.92	−0.92
$(\delta^* \Sigma E_s)/(\delta^* \Sigma E_s)_{lim}$	1.00	0.850	0.732	0.695	1.00	0.799	0.694	0.659
Solute separation, %	97.7	81.2	65.9	53.3	96.5	75.4	61.1	50.4
Product rate, g/hr	55.74	122.91	177.71	182.72	49.74	81.00	106.5	96.2

[a] Area of film surface: 13.2 cm^2; Feed conc: 5000 ppm of NaCl; Feed flow rate: 400 cm^3/min

all experiments, the terms "product" and "product rate" refer to membrane permeated solutions. The fraction solute separation f in each case was obtained from the relation:

$$f = \frac{\text{solute molality in feed} - \text{solute molality in product}}{\text{solute molality in feed}}$$

In each experiment, the pure water permeation rate (PWP) and product rate (PR) in grams per hour per given area of film surface (13.2 cm^2 in all cases in this work), and percent solute separation were determined at the operating conditions.

A Beckman total carbon analyzer, Model 915, was used to measure the concentrations of sugars present in low concentrations (1000 ppm or less). Higher concentrations of sugars were determined by refractive index measurements using a Bausch and Lomb refractometer. The concentrations of sodium chloride and calcium chloride in aqueous solutions were measured by using a conductivity bridge, and the concentrations of calcium chloride in sugar solutions were determined by the atomic absorption method. The viscosities of sucrose solutions were measured using a Ubbelohde viscometer.

RESULTS & DISCUSSION

Basic equations

Data needed to predict reverse osmosis separations. Following the Kimura-Sourirajan equations for reverse osmosis transport, solute separations in reverse osmosis can be predicted if applicable data on pure water permeability constant A, solute transport parameter $D_{AM}/K\delta$, and mass transfer coefficient k on the high pressure side of the membrane are available, or can be calculated. This prediction technique has been extensively illustrated and discussed (Sourirajan, 1970b). For a given membrane under a given set of operating conditions, the value of A [which can be calculated from the (PWP) data] is independent of any solute under consideration, whereas the values of $D_{AM}/K\delta$ and k depend on the nature of the solute. For dilute feed solutions, the applicable values of $D_{AM}/K\delta$ and k for a given sugar solute can be calculated from the corresponding values obtained from experimental reverse osmosis data for a reference solute such as sodium chloride (Matsuura et al., 1975a).

Calculation of $D_{AM}/K\delta$ values for sugar solutes in dilute solutions. All the sugars with which this report is concerned exist essentially as nonionized molecules in aqueous solutions; further, at the cellulose acetate membrane-aqueous sugar solution interface, water is preferentially sorbed (Matsuura and Sourirajan, 1971a, 1973b). Hence the following relation is applicable:

$$\ell n(D_{AM}/K\delta) = \ell n C^*_{NaCl} + \ell n\Delta^* + \left(-\frac{\Delta\Delta G}{RT}\right) + \delta^*\Sigma E_s \quad (1)$$

All the symbols used are defined at the end of the paper. The basis of Eq. (1), and the meaning of the four quantities on the right side of Eq. (1) have been discussed (Matsuura et al., 1975a, 1976).

The quantity $\ell n C^*_{NaCl}$ can be calculated from data on $D_{AM}/K\delta$ for sodium chloride [represented by the symbol $(D_{AM}/K\delta)_{NaCl}$] using the relation:

$$\ell n C^*_{NaCl} = \ell n(D_{AM}/K\delta)_{NaCl} - \left\{\left(-\frac{\Delta\Delta G}{RT}\right)_{Na^+} + \left(-\frac{\Delta\Delta G}{RT}\right)_{Cl^-}\right\} \quad (2)$$

The numerical values of the free energy parameters $(-\Delta\Delta G/RT)_{Na^+}$ and $(-\Delta\Delta G/RT)_{Cl^-}$ at 25°C are 5.79 and −4.42, respectively, from previous work (Matsuura et al., 1975b) for the cellulose acetate membranes used.

The quantity $\ell n\Delta^*$ for the membrane can be obtained from the correlation of $\ell n C^*_{NaCl}$ versus $\ell n\Delta^*$ established earlier (Matsuura et al., 1976) as given in Figure 1.

The quantity $(-\Delta\Delta G/RT)$ in Eq. (1) representing the polar free energy parameter for the sugar solute under consideration can be calculated using the relations (Matsuura et al., 1975b, 1976):

$$\Delta\Delta G = \Delta G_I - \Delta G_B \quad (3)$$

$$\Delta G_B = \Sigma\gamma_B \text{ (structural group)} + \gamma_{B,O} \quad (4)$$

$$\Delta G_I = \Sigma\gamma_I \text{ (structural group)} + \gamma_{I,O} \quad (5)$$

The available data on the numerical values of γ_B (structural group), $\gamma_{B,O}$, γ_I (structural group), and $\gamma_{I,O}$ are listed in Table 2.

Table 2—Structural group contributions for ΔG_B and ΔG_I at 25°C

Structural groups[a]	$\gamma_{B,O} = -12.04$	Cellulose acetate $\gamma_{I,O} = -41.21$
	γ_B	γ_I
$-CH_3$	11.07	24.23
$>CH_2$	0.17	0.24
$>CH'_2$	–	−0.24
$\rightarrow CH$	−10.62	−23.65
$-\overset{|}{\underset{|}{C}}-$	−21.50	−47.39
cyclic, five-membered	20.60	46.99
cyclic, six-membered	20.49	47.12
−OH	3.99	17.04
−COO−	−5.14	–
>O	−4.03	−4.59
−CN	5.19	–
>C=O	−5.80	−6.32
−CHO	5.80	18.84

[a] $>CH_2$ refers to methylene groups which are not separated from a polar functional group by more than two carbon atoms in a straight chain; $>CH'_2$ refers to methylene groups which are separated from a polar functional group by three or more carbon atoms in a straight chain.

Table 3—Structural group contributions for $(\delta^*\Sigma E_s)_{lim}$ of solutes with polyfunctional groups at 25°C

Structural groups[a]	Cellulose acetate $\phi_O = -4.83$
	ϕ
$-CH_3$	2.90
$>CH_2$	−0.02
$>CH'_2$	−0.01
$\rightarrow CH$	−3.19
$-\overset{|}{\underset{|}{C}}-$	−6.88
cyclic, five-membered, single ring	5.22
cyclic, six-membered, single ring	4.70
cyclic, five-membered, two rings	5.99
cyclic, six-membered, two rings	5.47
−OH	2.01
>O	0.63
>C=O	−1.15
−CHO	2.14

[a] See footnote to Table 2

The quantity $\delta^*\Sigma E_s$ in Eq. (1) may be treated as a single quantity for the purpose of this discussion. This quantity for the particular membrane-solute system can be calculated by multiplying the ratio $\delta^*\Sigma E_s/(\delta^*\Sigma E_s)_{lim}$ by the quantity $(\delta^*\Sigma E_s)_{lim}$. The correlation of $\ell n C^*_{NaCl}$ versus the above ratio for cellulose acetate membranes has been established as given in Figure 2 (Matsuura et al., 1976). The quantity $(\delta^*\Sigma E_s)_{lim}$ can be calculated from the relation (Matsuura et al., 1976):

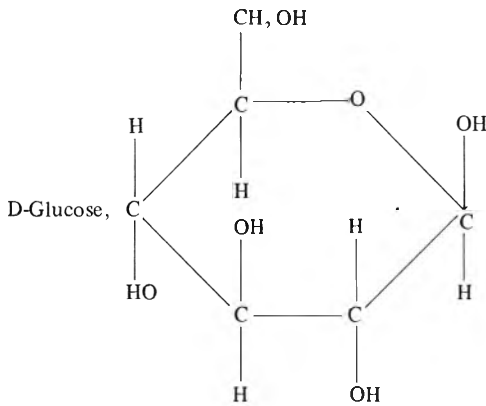
$$(\delta^*\Sigma E_s)_{lim} = \Sigma\phi\ (\text{structural group}) + \phi_0 \quad (6)$$

The available data on the numerical values of ϕ (structural group) and ϕ_0 are given in Table 3.

Thus Eq. (1) enables one to calculate $D_{AM}/K\delta$ values for a sugar solute for cellulose acetate membranes of different surface porosities (as represented by different values of $\ell n C^*_{NaCl}$) just from applicable data on $(D_{AM}/K\delta)_{NaCl}$.

Sample calculations

The numerical values of $(-\Delta\Delta G/RT)$ and $(\delta^*\Sigma E_s)_{lim}$ for use in Eq. (1) with respect to glucose and sucrose solutes are calculated below for illustration, using Eq. (3) to (6) and the data from Tables 2 and 3.



Since 99% of glucose, under equilibrium conditions, is of the cyclic form (Bordwell, 1963), the above cyclic structure is chosen for calculation of free energy and steric parameters for glucose.

$\Delta G_B = \gamma_B(CH_2) + 5\,\gamma_B(CH) + 5\,\gamma_B(OH) + \gamma_B(\text{cyclic, six-membered}) + \gamma_B(O) + \gamma_{B,O} = -28.56$

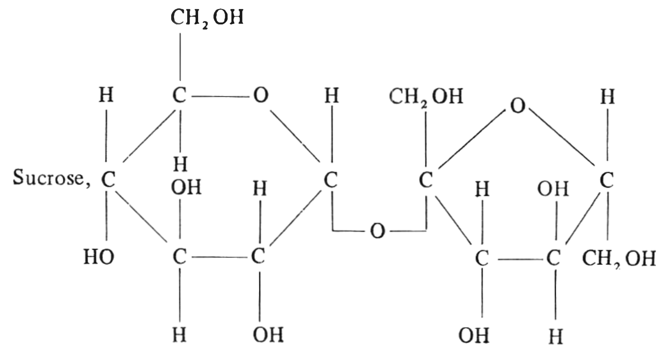
$\Delta G_I = \gamma_I(CH_2) + 5\,\gamma_I(CH) + 5\,\gamma_I(OH) + \gamma_I(\text{cyclic, six-membered}) + \gamma_I(O) + \gamma_{I,O} = -31.49$

$\Delta\Delta G = (-31.49) - (-28.56) = -2.93$

$(-\Delta\Delta G/RT) = -(-2.93/0.5925) = 4.95$

$(\delta^*\Sigma E_s)_{lim} = \phi(CH_2) + 5\,\phi(CH) + 5\,\phi(OH) + \phi(\text{cyclic, six-membered, single ring}) + \phi_0 = -5.42.$

Similar calculations show that the values of $(-\Delta\Delta G/RT)$ and $(\delta^*\Sigma E_s)_{lim}$ for D-fructose are the same as those for D-glucose.



$\Delta G_{B'} = 3\,\gamma_B(CH_2) + 8\,\gamma_B(CH) + \gamma_B(C) + \gamma_B(\text{cyclic, six-membered}) + \gamma_B(\text{cyclic, five-membered}) + 8\,\gamma_B(OH) + 3\,\gamma_B(O) + \gamma_{B,O} = -57.07$

$\Delta G_I = 3\,\gamma_I(CH_2) + 8\,\gamma_I(CH) + \gamma_I(C) + \gamma_I(\text{cyclic, six-membered}) + \gamma_I(\text{cyclic, five-membered}) + 8\,\gamma_I(OH) + 3\,\gamma_I(O) + \gamma_{I,O} = -60.42$

$\Delta\Delta G = (-60.42) - (-57.07) = -3.35$

$(-\Delta\Delta G/RT) = -(-3.35/0.5925) = 5.65$

$(\delta^*\Sigma E_s)_{lim} = 3\,\phi(CH_2) + 8\,\phi(CH) + \phi(C) + \phi(\text{cyclic, six-membered, two-rings}) + \phi(\text{cyclic, five-membered, two-rings}) + 8\,\phi(OH) + 3\,\phi(O) + \phi_0 = -7.86$

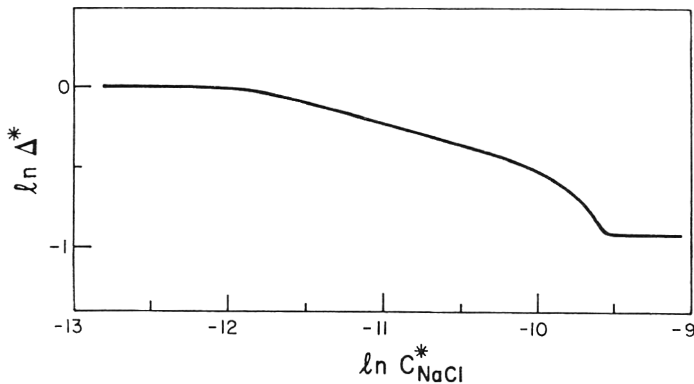


Fig. 1—Correlation of $\ell n\ \Delta^*$ vs $\ell n\ C^*_{NaCl}$ for batch 316(10/30)-type cellulose acetate membranes.

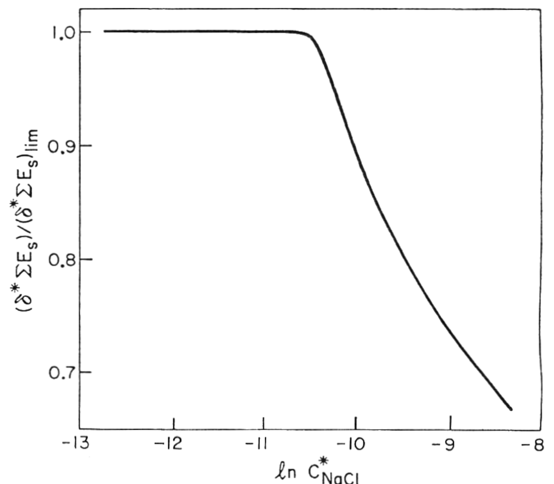


Fig. 2—Correlation of $(\delta^*\Sigma E_s)/(\delta^*\Sigma E_s)_{lim}$ vs $\ell n\ C^*_{NaCl}$ for batch 316(10/30)-type cellulose acetate membranes.

Table 4—Calculated and experimental values of solute transport parameter and solute separation for some food sugars[a]

Solutes		D-Glucose	D-Fructose	Sucrose	Maltose	Lactose
$-\Delta\Delta G/RT$		4.95	4.95	5.65	5.65	5.65
$(\delta^*\Sigma E_s)_{lim}$		−5.42	−5.42	−7.86	−7.86	−7.86
				Film 1		
$k \times 10^4$, cm/sec		9.6	9.5	7.4	7.4	7.4
$\ln(D_{AM}/K\delta)$	calc	−13.08	−13.08	−14.82	−14.82	−14.82
	exp	−13.29	−13.30	—	—	—
Separation, %	calc	99.4	99.4	99.8	99.8	99.8
	exp	99.5	99.5	99.3	99.6	99.8
				Film 2		
$k \times 10^4$, cm/sec		17.9	17.7	13.7	13.7	13.7
$\ln(D_{AM}/K\delta)$	calc	−10.09	−10.09	−11.46	−11.46	−11.46
	exp	−10.09	−10.00	−11.46	−11.41	−11.61
Separation, %	calc	93.3	93.2	97.1	97.1	97.1
	exp	93.3	92.6	97.1	97.0	97.5
				Film 3		
$k \times 10^4$, cm/sec		20.5	20.3	15.7	15.7	15.7
$\ln(D_{AM}/K\delta)$	calc	−8.89	−8.89	−9.97	−9.97	−9.97
	exp	−8.72	−8.68	−10.13	−9.82	−9.85
Separation, %	calc	80.5	80.2	86.8	87.3	87.0
	exp	77.7	76.6	87.5	85.5	85.6
				Film 4		
$k \times 10^4$, cm/sec		20.8	20.6	15.9	15.9	15.9
$\ln(D_{AM}/K\delta)$	calc	−8.36	−8.36	−9.35	−9.35	−9.35
	exp	−8.31	−8.41	−9.53	−9.11	−9.28
Separation, %	calc	69.3	68.7	75.1	76.8	76.1
	exp	68.3	69.8	75.6	72.3	74.8

[a] Operating pressure: 1000 psig; Solute conc in feed: 1000 ppm

Similar calculations show that the values of $(\Delta\Delta G/RT)$ and $(\delta^*\Sigma E_s)_{lim}$ for maltose and lactose are the same as those for sucrose.

Comparison of calculated and experimental solute separation data for sugars

Using the $D_{AM}/K\delta$ values predicted by Eq. (1), the fraction solute separation f was calculated from the following relationship derived earlier (Matsuura and Sourirajan, 1973b) for dilute feed solutions:

$$D_{AM}/K\delta = \frac{(PR)}{3600S\rho} \cdot \frac{1-f}{f}\left[\exp\left\{\frac{(PR)}{3600Sk\rho}\right\}\right]^{-1} \quad (7)$$

The quantity (PR) may be assumed to be essentially the same as (PWP) for dilute feed solutions whose osmotic pressures are negligible compared to the operating pressure. The (PWP) for each film can be calculated from data on A given in Table 1. The applicable values of k were determined by the method described earlier (Matsuura et al., 1974b).

A set of reverse osmosis experiments on separation of food sugars was conducted. Four membranes of different surface porosities (films 1 to 4 specified in Table 1) were used. The solute concentration in the feed solution was 1000 ppm in these experiments. The results, given in Table 4, showed excellent agreement between calculated and experimental data on solute separation and $(D_{AM}/K\delta)$.

Studies on aqueous sucrose solutions

The values of $D_{AM}/K\delta$ calculated by Eq. (1) above are applicable only for dilute feed solutions. However, it has been shown (Matsuura and Sourirajan, 1971b) that with respect to D-glucose, maltose and lactose, $D_{AM}/K\delta$ is independent of solute concentration; it was found in this work that this was also the case with respect to D-fructose. Therefore, with respect to the above four sugars, the values of $D_{AM}/K\delta$ obtained from Eq. (1) are also applicable for concentrated solutions.

With respect to sucrose, it has been shown (Kimura and Sourirajan, 1968; Sourirajan, 1970b) that $D_{AM}/K\delta$ decreases with increase in sucrose concentration in the boundary solution as given by the expression:

$$(D_{AM}/K\delta)_{sucrose} = (D_{AM}/K\delta)^*_{sucrose}\exp(-\epsilon X_{A2}) \quad (8)$$

with $\epsilon = 33$ for batch 18-type cellulose acetate membranes at 1000 psig.

Natural sugar solutions, such as cane juices, contain small quantities of pectin which may be expected to increase solution viscosity and hence decrease the mass transfer coefficient significantly in reverse osmosis. Hence it was of practical interest to study the effect of sucrose and pectin concentration on mass transfer coefficient.

Reverse osmosis experiments (runs 1 to 6) were carried out at different feed flow rates using four membranes (films 1 to

4) of different surface porosities using the following feed solution compositions:

run 1: 0.0032 molal sucrose (∿1000 ppm)
run 2: 0.61 molal sucrose
run 3: 1.25 molal sucrose
run 4: 0.774 molal sucrose + pectin
run 5: 1.31 molal sucrose + pectin
run 6: 1.94 molal sucrose + pectin

The weight ratio of pectin/sucrose was 0.00075 in runs 4, 5 and 6. The reverse osmosis data obtained were then analyzed to determine the values of X_{A2}, $D_{AM}/K\delta$, and k with respect to sucrose. Since the solute concentration in run 1 was very dilute, Eq. (7) was used to determine $D_{AM}/K\delta$. The general Kimura-Sourirajan equations (Sourirajan, 1970b) were used to analyze the data for the other runs. The results obtained are plotted in Figures 3 to 6.

Figure 3 gives the semilog plots of X_{A2} versus $D_{AM}/K\delta$ for sucrose for two films; the correlations for the other two films were similar. The straight lines shown correspond to the correlation represented by Eq. (8) with ϵ = 26. The effect of variation of k with X_{A1} (mole fraction of sucrose in feed solu-

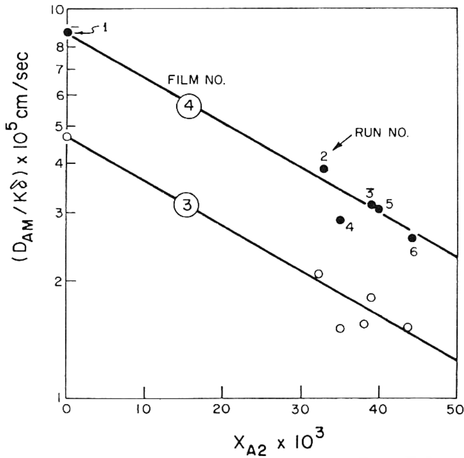


Fig. 3–Correlation of $D_{AM}/K\delta$ vs X_{A2} for aqueous sucrose solution with and without addition of pectin. Film, batch 316(10/30)-type cellulose acetate membranes; feed sucrose molality, 0.0032∿1.94; weight ratio of pectin/sucrose, 0.00075 in runs 4, 5 and 6; feed rate, 400 cc/min; operating pressure, 1000 psig.

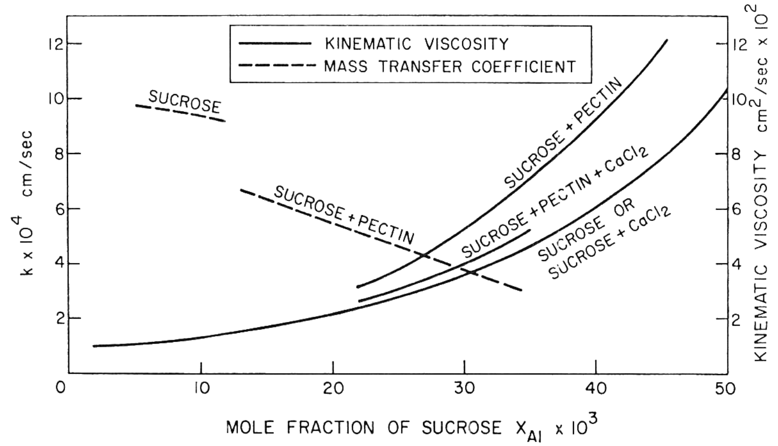


Fig. 4–Mass transfer coefficient k and kinematic viscosity change with sucrose concentration X_{A1}. k values for film, batch 316(10/30)-type cellulose acetate membranes; feed sucrose molality, 0.33∿1.94; weight ratio of pectin/sucrose, 0.00075; feed rate, 400 cc/min; operating pressure, 1000 psig.

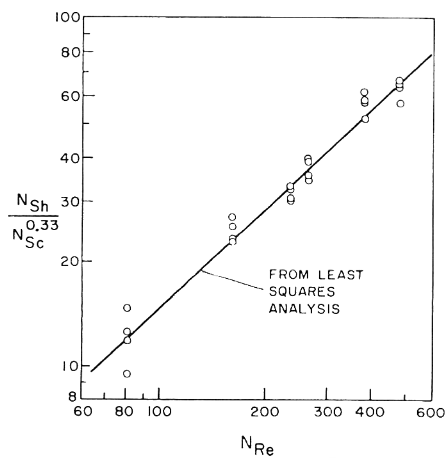


Fig. 5–Correlation of $N_{Sh}/N_{Sc}^{0.33}$ versus N_{Re} for aqueous sucrose solution with and without addition of pectin. Film, batch 316(10/30)-type cellulose acetate membranes; feed sucrose molality, 0.33∿1.94; weight ratio of pectin/sucrose, 0.00075 when pectin was added; feed flow rate, 400 cc/min; operating pressure, 1000 psig.

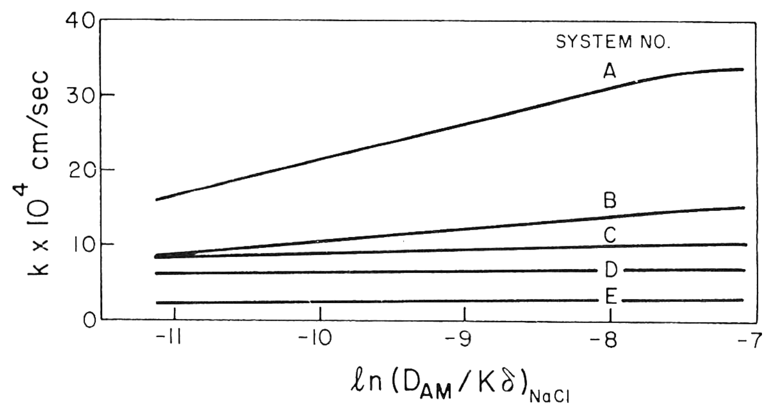


Fig. 6–Mass transfer coefficient k vs $\ell n(D_{AM}/K\delta)$. Film, batch 316(10/30)-type cellulose acetate membranes; feed, aqueous solutions of (A) sodium chloride 5000 ppm; (B) sucrose molality 0.0032; (C) sucrose molality 0.33; (D) sucrose molality 0.77; (E) sucrose molality 1.94. Weight ratio of pectin/sucrose, 0.00075 in D and E; feed rate, 400 cc/min; operating pressure, 1000 psig.

Table 5–Calcium chloride separations from the sucrose-pectin-calcium chloride feed solutions[a]

Run no.	Molality of sucrose	Film no.	Separation of $CaCl_2$, %: Calc at $k = \infty$	Exp
9	0.816	5	96.4	98.8
		6	65.1	–
		7	48.4	72.3
		8	37.2	61.6
10	1.35	5	92.1	98.6
		6	43.4	77.2
		7	27.8	64.0
		8	20.1	52.8
11	2.00	5	64.6	95.1
		6	15.7	55.3
		7	11.0	43.1
		8	8.1	33.7
12	1.35	5	92.1	98.3
		6	43.4	76.4
		7	27.8	62.0
		8	20.1	–

[a] Concentration of $CaCl_2$ = 0.0025 molal in all cases; pectin/sucrose wt ratio = 0.00075 in runs 9, 10 and 11; operating pressure = 1000 psig

tion) is shown in Figure 4 which also gives experimental data on the viscosity of aqueous sucrose solutions with and without pectin. Figure 5 gives a log-log plot of N_{Re} versus $N_{Sh}/N_{Sc}^{0.33}$. Figure 6 shows the effect of change in average pore size on the membrane surface [as represented by $\ln (D_{AM}/K\delta)$ for NaCl] on k.

While the value of k always tends to increase with an increase in average pore size on the membrane surface, this tendency is practically insignificant with viscous solutions of high sucrose content with or without pectin for which the values of k are low because of the viscosity effect. Consequently, one can conclude that the linear correlation shown in Figure 5 can be assumed to be practically independent of pore size on the membrane surface for viscous sucrose feed solutions. The straight line correlations given in Figure 5 may be expressed by either one of the following equivalent relations:

$$\log(N_{Sh}/N_{Sc}^{0.33}) = 0.946 \log N_{Re} - 0.719 \qquad (9)$$

or

$$k = 0.191 \frac{1}{d} \left(\frac{Q}{h}\right)^{0.946} \frac{D_{AB}^{0.67}}{\nu^{0.62}} \qquad (10)$$

While Eq. (9) and (10) are applicable only for the particular apparatus used in this work, similar expressions can be established for any other apparatus one may use for the reverse osmosis treatment of viscous sucrose solutions.

Effect of presence of $CaCl_2$ in aqueous sucrose solutions with or without pectin

Natural sucrose solutions, such as cane juices, contain small quantities of inorganic salts in addition to pectin. It is often desirable to separate these inorganic salts in the concentration of such natural sugar solutions. Hence the separation characteristics of inorganic salts present in aqueous sucrose or (sucrose + pectin) feed solutions are of practical interest.

The separation characteristics of calcium chloride (chosen as model inorganic salt) present in aqueous sucrose feed solutions with or without pectin, were briefly studied in this work. The concentration of $CaCl_2$ used was 0.0025 molal in all cases. When the feed solution contained pectin also, the concentration of the latter was such that the weight ratio of pectin/sucrose was 0.00075.

A set of experiments was carried out on viscosity measurements. The results (Figure 4) showed that whereas the viscosities of aqueous sucrose solutions were not affected by the presence of $CaCl_2$, the viscosities of sucrose solutions containing pectin were significantly reduced indicating a change in the structure of the sucrose-pectin solution brought about by the presence of calcium chloride.

A set of reverse osmosis experiments was carried out with films 5 to 8 (specified in Table 1) using the following feed solutions:

run 7: sucrose (0.0032 molal)–water
run 8: $CaCl_2$ (0.0025 molal)–water
run 9: [sucrose(0.816 molal) + pectin + $CaCl_2$] –water
run 10: [sucrose(1.35 molal) + pectin + $CaCl_2$] –water
run 11: [sucrose(2.0 molal) + pectin + $CaCl_2$] –water
run 12: [sucrose(1.35 molal) + $CaCl_2$] –water

The experimental data on sucrose separation and product rate are given in Figure 7 which also shows (solid lines) the corresponding data calculated from the Kimura-Sourirajan transport equations together with Eq. (8). The agreement between the experimental and calculated results is very good.

The calculated values of $(D_{AM}/K\delta)^*_{sucrose} \times 10^5$ (in cm/sec) for the films 5, 6, 7 and 8 were 0.05, 0.55, 1.23, and 2.0, respectively. Assuming $\epsilon = 0$ [i.e., $(D_{AM}/K\delta)_{sucrose}$ is independent of X_{A2}], a set of calculations on solute separations and product rates was made for film 6. The results of these calculations, given in Figure 7 by dotted lines, showed that there was practically no difference between the above results and those obtained for the case $\epsilon = 26$. This means that when the value of $(D_{AM}/K\delta)_{sucrose}$ is sufficiently small, the value of ϵ may be taken to be zero for purposes of practical calculations.

The experimental data on solute separation with respect to $CaCl_2$ obtained with the four membranes tested are given in Table 5. These data show that the extent of $CaCl_2$ separation decreased significantly with increase in sucrose concentration in the feed solution.

An attempt was made to predict separations of $CaCl_2$ present in aqueous sucrose feed solutions following the method identical to that used earlier (Matsuura et al., 1974b) for predicting separations of t-butyl alcohol present in aqueous sucrose feed solutions. This method involves the use of Eq. (7) and the experimental (or calculated) data on $(D_{AM}/K\delta)$ for $CaCl_2$ obtained in run 8, and the (PR) data obtained for aqueous sucrose or (sucrose + pectin) feed solutions. For using Eq. (7) to calculate f, the data on applicable values of k for $CaCl_2$ are needed for the calculation of which data on diffusivities of $CaCl_2$ in aqueous sucrose or (sucrose + pectin) solutions are needed. No precise data on such diffusivities are available in the literature. Therefore solute separations were calculated for the limiting case of $k = \infty$. The results obtained are given in Table 5. These results represent the maximum limiting values for $CaCl_2$ separation according to Eq. (7); the actual values should be considerably lower. However the experimental results showed the opposite tendency; the actual $CaCl_2$ separations were considerably higher with all the films tested in both aqueous sucrose and aqueous (sucrose + pectin) feed solutions. This result indicates that in aqueous sucrose solutions, $CaCl_2$ does not exist as an independent entity. Probably, $CaCl_2$ is bound to the sucrose molecule to form some complex compound.

Improved mass transfer coefficients in the reverse osmosis treatment of viscous sugar solutions

Low mass transfer coefficients on the high pressure side of the membrane constitute a general problem in the reverse osmosis treatment of viscous food sugar solutions (Matsuura et al., 1973a; 1974a). Recently a new laboratory apparatus has been developed (Thayer et al., 1975) for improving mass transfer coefficients in confined liquid reverse osmosis operations which can be used in food processing applications. In this apparatus, the mass transfer coefficient on the high pressure side of the membrane is increased by sweeping the membrane surface at a predetermined frequency by a back-and-forth movement of the feed liquid itself through an adjustable narrow channel in the cell.

A few reverse osmosis experiments were carried out with (sucrose + pectin) feed solutions using the above apparatus, setting the channel width at 0.015 cm, differential pressure at 30 psi and sweeping frequency at 60 min^{-1}. The concentrations of sucrose in the feed solutions used were in the range 20–40 wt %; and, the weight ratio of pectin/sucrose was 0.00075 in all cases. The values of k obtained in the above reverse osmosis experiments were compared with those obtained in a nonflow type reverse osmosis cell provided with a mechanical stirrer (Matsuura et al., 1974a) using aqueous sucrose and (sucrose + pectin) feed solutions under the same experimental conditions of operating pressure and range of solute concentration in feed solutions. The results obtained are given in Figure 8 which shows a threefold improvement in the values of k with the new apparatus, in the entire range of sucrose concentrations studied.

Application of physicochemical criteria approach for process design calculations – an illustration

The object of this section is to give a practical illustration of some results of process design calculations relevant to batchwise concentration of food sugars. Film 1 was chosen for this illustration. From data on specifications for film 1 given in Table 1, the values of $(D_{AM}/K\delta) \times 10^5$ (in cm/sec) for the film with respect to D-glucose, D-fructose, sucrose, maltose, and lactose can be calculated to be 2.1, 2.1, 0.4, 0.4 and 0.4, respectively. The above values were considered to be independent of feed concentration for the purpose of these calculations.

Two concentration problems are considered in this illustration. In the first problem, the object is to concentrate aqueous sugar solutions from 1–10 wt %; all the sugar solutes considered are included in this problem. In the second problem,

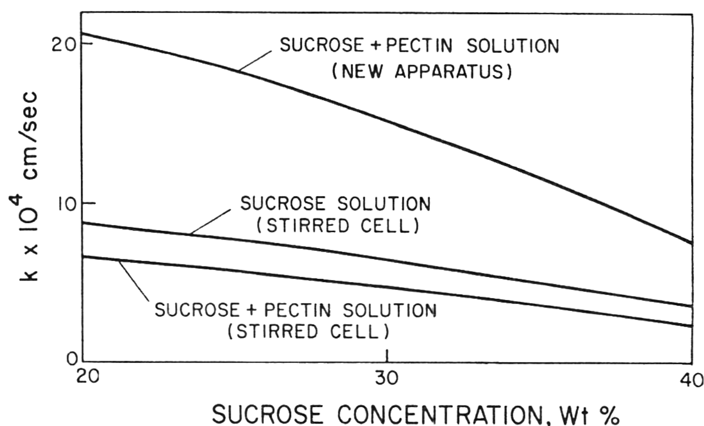


Fig. 8–Mass transfer coefficient k vs feed concentration. Film, batch 316(10/30)-type cellulose acetate membranes; feed sucrose solution, 20∿40%; weight ratio of pectin to sucrose, 0.00075 when pectin was added; operating pressure, 1000 psig.

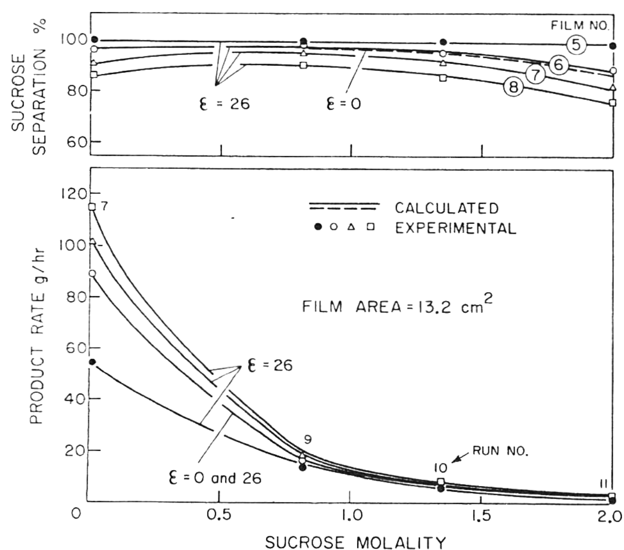


Fig. 7–Sucrose separation and product rate data from reverse osmosis experiments with sucrose or sucrose + pectin + $CaCl_2$ solutions. Film, batch 316(10/30)-type cellulose acetate membranes; feed sucrose molality, 0.0032∿2.0; weight ratio of pectin to sucrose, 0.00075 in runs 9, 10 and 11; $CaCl_2$ molality, 0.0025; feed rate, 400 cc/min; operating pressure, 1000 psig.

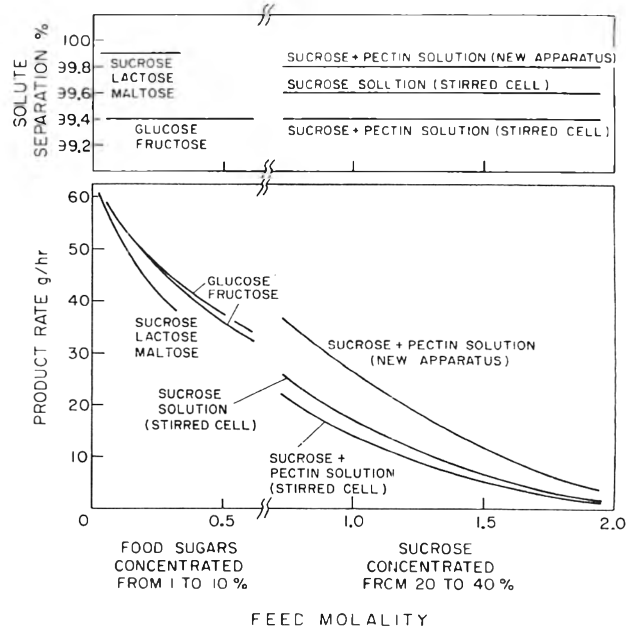


Fig. 9–Separation of food sugars and product rate data. Film, batch 316(10/30)-type cellulose acetate membranes; weight ratio of pectin to sucrose, 0.00075 when pectin was added; operating pressure, 1000 psig.

the object is to concentrate aqueous sucrose solutions with or without pectin from 20–40 wt % sucrose. In order to predict membrane performance in the above range of sugar concentrations, the applicable values of k are needed. These values were based on data given in Figures 6 and 8.

Figure 6 shows that for film 1, the values of k are essentially the same (7.4×10^{-4} cm/sec) for aqueous sucrose feed solutions in the concentration range 1 to 10 wt %. Following the method given earlier (Matsuura et al., 1974b) the corresponding values of $k \times 10^4$ for D-glucose, D-fructose, maltose, and lactose are 9.5, 9.5, 7.4 and 7.4, respectively. These values were used in the solute concentration range 1–10 wt %. For aqueous sucrose solutions with or without pectin in the sucrose concentration range 20 to 40 wt %, the data given in Figure 8 were used.

Using the $D_{AM}/K\delta$ and k values given above for the various solutes, and the values of A for film 1 given in Table 1, solute separation and product rate data were calculated in the concentration range 1–10 wt % for each of the aqueous sugar solutions, and 20–40 wt % for aqueous sucrose solutions by the method described in the literature (Sourirajan, 1970c). The results obtained are given in Figure 9 which shows that product rates decrease with increase in feed concentration, and solute separations remain essentially constant in the entire range of concentrations studied.

Using the results given in Figure 9, the volume change during concentration, $(V)_i/(V)_f$, fraction solute recovery, $\bar{F} = (W_s)_f/(W_s)_i$, and processing capacity of the membrane, during a batchwise concentration process were calculated using the relations derived in the literature (Sourirajan, 1970c). The results obtained are shown in Figures 10 and 11.

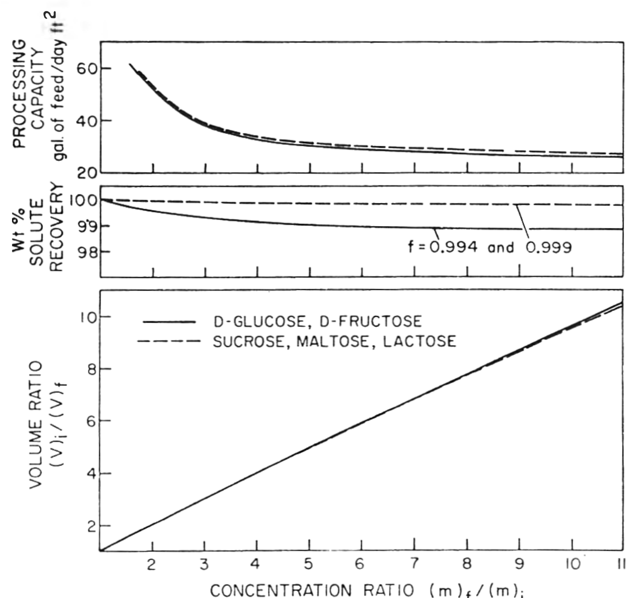


Fig. 10—Results of reverse osmosis system analysis for the concentration of some food sugars from 1∿10%. Film, batch 316(10/30)-type cellulose acetate membranes.

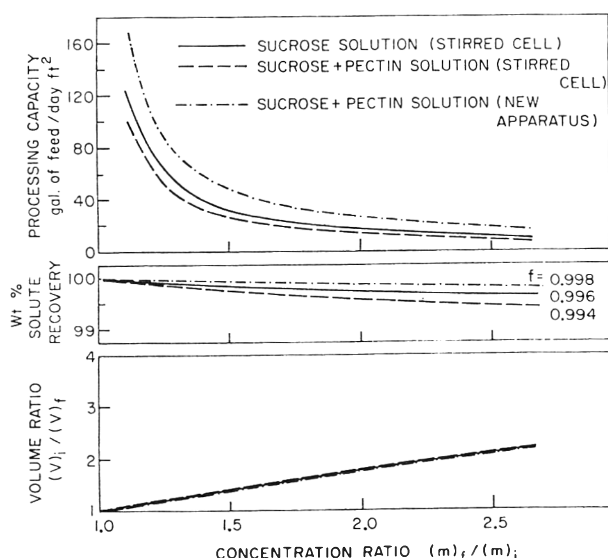


Fig. 11—Results of reverse osmosis system analysis for the concentration of sucrose from 20–40%. Film, batch 316(10/30)-type cellulose acetate membranes. Weight ratio of pectin to sucrose, 0.00075 when pectin was added.

CONCLUSIONS

THIS PAPER illustrates the application of a fundamental physicochemical criteria approach to the problem of reverse osmosis concentration of food sugar solutes using porous cellulose acetate membranes. With appropriate additional research, this approach can be extended to include a wide variety of such solutes and membranes. With reference to the problem considered in this paper, if the object is to recover to a specific extent any particular sugar solute by reverse osmosis, the above approach is capable of predicting what membrane specifications and operating conditions can accomplish the desired objective.

LIST OF SYMBOLS

A	=	pure water permeability constant, g mole water/cm² sec atm
c	=	molar density of solution, g mole/cm³
d	=	diameter of flat membrane used, cm
D_{AB}	=	diffusivity of solute in water, cm²/sec
$D_{AM}/K\delta$	=	solute transport parameter (treated as a single quantity), $= N_A/(c_2X_{A2} - c_3X_{A3})$, cm/sec.
$(D_{AM}/K\delta)^*_{sucrose}$	=	limiting value of $(D_{AM}/K\delta)$ for sucrose at $X_{A2} = 0$, cm/sec
f	=	fraction solute separation
$\bar{F}$	=	fraction solute recovery in the batchwise concentration process
ΔG	=	free energy of hydration, kcal/g mole
$(-\Delta\Delta G/RT)$	=	polar free energy parameter
h	=	depth of cell (= 1.43 cm in this work)
k	=	mass transfer coefficient on high pressure side of membrane, cm/sec
$\ln C^*_{NaCl}$	=	constant representing the average pore size on membrane surface
$\ln\Delta^*$	=	adjustment parameter to cancel the overlapping effect of pore size on the quantities $\ln C^*_{NaCl}$ and $\delta^*\Sigma E_s$
m	=	solute molality
N_A	=	solute flux, g mole/cm² sec
N_{Re}	=	Reynold's number = $Q/h\nu$
N_{Sc}	=	Schmidt number = ν/D_{AB}
N_{Sh}	=	Sherwood number = kd/D_{AB}
(PR)	=	product rate, grams/hour per given area of membrane surface
(PWP)	=	pure water permeation rate, grams/hour per given area of membrane surface
Q	=	feed flow rate, cm³/sec

R = gas constant
S = effective area of membrane surface, cm^2
T = absolute temperature, °K
V = volume of solution on the high pressure side of membrane during batchwise concentration process, cm^3
W_s = wt of solute in the above solution, grams
X_A = mole fraction of solute

Greek letters

γ(structural group) = incremental free energy of hydration for the structural group concerned, kcal/g mole
γ_o = characteristic constant applicable to all structural groups, kcal/g mole
$\delta^*\Sigma E_s$ = contribution of molecular steric parameter to the quantity $\ell n\ (D_{AM}/K\delta)$
$(\delta^*\Sigma E_s)_{lim}$ = limiting value of the quantity $\delta^*\Sigma E_s$
ϵ = constant defined by Eq. (8)
ν = kinematic viscosity of solution (based on data in Fig. 4), cm^2/sec
ρ = density of solution, gram/cm^3
ϕ(structural group) = incremental quantity of steric parameter for the structural group concerned
ϕ_o = characteristic constant applicable to all structural groups

Subscripts

B = bulk solution phase
I = membrane-solution interface
1 = bulk solution on the high pressure side of the membrane
2 = concentrated boundary solution on the high pressure side of the membrane
3 = membrane permeated product solution on the atmospheric pressure side of the membrane

Suffixes

i = initial state of feed solution
f = final state of feed solution

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Ms received 6/25/75; revised 10/28/75; accepted 11/5/75.

Issued as NRC No. 15218.

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EFFECT OF NEODIOSMIN ON THRESHOLD AND BITTERNESS OF LIMONIN IN WATER AND ORANGE JUICE

ABSTRACT

The thresholds of limonin and naringin in water and limonin in orange juice were determined in the presence of varying amounts of neodiosmin (NEO), the flavone analog of the bitter flavanone neohesperidin. Up to 40 ppm of NEO in water solution was essentially tasteless, but as little as 10 ppm in water increased the threshold of naringin and limonin 3.5- and 4.0-fold, respectively. Regression analysis showed the relation between limonin and naringin threshold and NEO concentration in water was best described by a power function with similar slope exponents for naringin and limonin. This indicated that increasing concentrations of NEO had a similar bitter suppressive effect for both compounds. The data for limonin threshold and NEO concentration in orange juice was also described best by a power function but with a much smaller slope exponent. Nevertheless, NEO was effective in significantly reducing bitterness in orange juice at threshold and suprathreshold levels of limonin. The addition of 60–100 ppm of NEO to orange juice containing 10 ppm of added limonin reduced limonin bitterness to the equivalent of 4–5 ppm of limonin in orange juice.

INTRODUCTION

THE THRESHOLDS of limonin and naringin, and the effect of various sweeteners on the threshold of these bitter constituents have been reported (Guadagni et al., 1973, 1974a). Subthreshold interactions between limonin and naringin and these bitter materials and various sweetening agents have also been studied (Guadagni et al., 1974b). These studies indicate that the thresholds of limonin and naringin were increased by addition of sucrose, dihydrochalcones and other sweeteners, and that the suprathreshold bitterness of these substances was significantly reduced by some of these sweeteners. Earlier Horowitz and Gentili (Horowitz, 1964) found that a solution of naringin containing a large amount of rhoifolin (the flavone analog of naringin) is less bitter than naringin alone. They suggested that rhoifolin competes with naringin for sites on the taste receptors even though it is a tasteless compound. This observation suggested that other tasteless citrus constituents which have structural similarities to naringin or limonin might also have the ability to suppress bitterness.

This paper deals with the effect of the citrus constituent neodiosmin (NEO) on the threshold and bitterness of naringin and limonin in water and on limonin in orange juice. Neodiosmin is the tasteless flavone analog of the bitter flavanone neohesperidin (Horowitz, 1964).

MATERIALS & METHODS

ALL SOLUTIONS of limonin, naringin and NEO were made by weighing the dry crystals to the nearest 0.1 mg and dissolving in the required amount of triple distilled water. The orange juice was reconstituted with triple distilled water from a uniform lot of commercially prepared frozen concentrate. The added limonin and NEO were dissolved in the water used for reconstituting the juice.

Sensory evaluation

Thresholds of limonin and naringin were determined exactly as described previously (Guadagni et al., 1973). The effect of NEO on threshold of the bitter substances was determined by adding specified amounts of NEO to both control and limonin-containing sample and then determining threshold of limonin in the usual way. Briefly, the panelists were presented a series of sample pairs in which one sample contained distilled water and NEO or orange juice and NEO, and the other sample contained the same constituents plus increasing amounts of the bitter substance. The task was to determine which sample in each pair contained the bitter material. The data were corrected for chance by subtracting 50 from percentage correct responses above chance and multiplying the result by 2. This figure was plotted against concentration on log-probability paper, and the threshold was taken at 50% detection. This corresponds to the familiar LD_{50} commonly used in dose-response experiments (Litchfield and Wilcoxon, 1949). The thresholds were determined with 20–25 trained panelists who evaluated five successive concentrations with three replications each giving a minimum of 60 judgments for each concentration.

The effect of NEO on suprathreshold bitterness was determined by adding suprathreshold amounts of limonin to orange juice. This juice was paired with another sample of the same juice containing a specified amount of NEO. The pair of samples was presented to the panel which was instructed to check the code of the more bitter sample. In some cases one sample in the pair contained orange juice without limonin or NEO and the other sample contained a constant amount of limonin and varying amounts of NEO. As above, the task was to determine which sample in each pair was more bitter. Significance of the results was determined by the sign test (Gacula et al., 1971).

RESULTS & DISCUSSION

Taste of added compounds

In previous work, it was found that bitterness of limonin and naringin in water and orange juice was reduced by additives which possessed a characteristic taste. Sucrose and dihydrochalcone sweeteners were effective in increasing the threshold of limonin and naringin and in reducing the apparent bitterness of limonin or naringin solutions. However, these effects were only achieved at sweetener levels well above the thresholds of the sweeteners indicating the characteristic sweet taste may have interfered with the perception of bitterness. Relatively large amounts of citric acid also increased the threshold of limonin and naringin, but this was probably due to the "noise" effect of the intense sourness of the mixture. Obviously excessive sourness is not a practical suppressant for bitterness because it is equally objectionable, but a certain amount of sweetening is not only tolerable but in some cases desirable. Ideally, a bitterness suppressor should have no taste of its own and not interfere with the characteristic taste of orange juice except to reduce limonin bitterness. Neodiosmin was essentially tasteless to practically all of the panel members. A few panelists occasionally described the taste of 40 ppm solutions in water as bitter; some described the taste as sweet and others as astringent. Attempts to determine the threshold of NEO in water are shown in Table 1. It is readily apparent that up to 40 ppm of this compound in distilled water did not have a taste distinguishable from water. Furthermore, the sensory data do not show any trend of increasing

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correct responses with increasing concentration over the range 1.25–40 ppm. This suggests that the threshold is well beyond 40 ppm.

Effect of NEO on threshold of limonin and naringin

The effect of increasing concentrations of NEO on the thresholds of limonin and naringin in water and of limonin in orange juice is shown in Figure 1. Regression analysis for the values obtained from NEO concentration and limonin threshold indicated the best fit for the data was given by a power rather than a linear or exponential function. The exponents in the equations for the power functions indicate very similar slopes for both limonin and naringin thresholds in water solution. This indicates that increasing amounts of NEO reduced the panel's sensitivity to limonin and naringin bitterness at a similar rate as is apparent by the essential parallelism between the lines for limonin and naringin thresholds in water. Neodiosmin increased the threshold of limonin from 1.0 to 4.0 ppm and that of naringin from 20 to 65 ppm giving a 3.5- to 4-fold increase in threshold with only 10 ppm of NEO. This suggests that the kinetics of the bitter suppression reaction or process is similar for both bitter substances.

Table 1–Detectability of neodiosmin in water solution (Taste threshold)

Neodiosmin (ppm)	N	Judgments indicating more bitter sample was: Neodiosmin	Water
1.25	20	10	10
2.5	61	27	34
5	61	33	28
10	61	27	34
20	20	11	9
40	20	9	11

Table 2–Effect of neodiosmin on threshold of limonin in water and orange juice

Neodiosmin (ppm)	Medium	Limonin threshold (ppm)	95% confidence limits
0	water	1.0	0.8–1.3
10	water	4.0	3.4–4.8
0	juice	3.4	2.7–4.2
10	juice	5.2	4.0–6.8

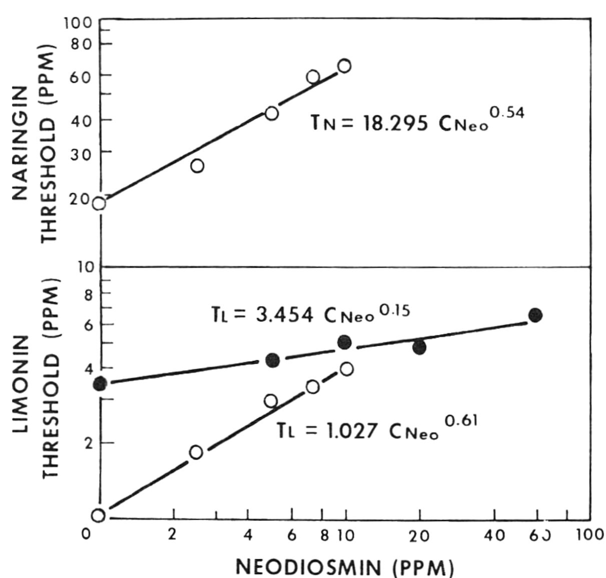

Fig. 1–Relation between limonin and naringin threshold and neodiosmin (NEO) concentration in water and in orange juice. ○ = water, ● = orange juice, T_N = threshold of naringin, T_L = threshold of limonin, C_{NEO} = concentration of NEO.

As shown in Table 1, a concentration of 10 ppm is far below the threshold of NEO in water, so that its effectiveness in suppressing bitterness is not associated with a taste of its own such as may occur with sweetening agents. At a level of 10 ppm, NEO is far more effective than the dihydrochalcones in increasing the thresholds of limonin and naringin in water solution (Guadagni et al., 1974a). This suggests the possibility that NEO may prevent bitterness perception by some type of chemical or physical reaction or association with the bitter receptor sites. The relative degree of perceived bitterness, as measured by threshold concentrations, would depend on the competition between NEO and the bitter substances for the receptor sites and on the kinetics of the process.

Within the range of concentrations studied for NEO in water, the intensity of bitterness remains at the threshold level with increasing concentration of limonin provided the concentration of NEO and limonin are within the limits described by: $T_L = 1.027\ C_{NEO}^{0.61}$ where T_L = threshold concentration of limonin and C_{NEO} = concentration of NEO. For example, 4.2 ppm of limonin in water containing 10 ppm NEO would be just perceptibly and equally bitter to a solution containing 2.5 ppm NEO and 1.8 ppm limonin.

In orange juice, the situation is considerably more complicated with many more known and unknown constituents exerting an effect on bitterness perception (Guadagni et al., 1973) The relation between limonin threshold and NEO concentration is still described best by a power function. but the slope exponent of 0.15 is only about 1/4 of the exponent obtained for water. This indicates that bitterness suppression in terms of increased limonin threshold per unit concentration of NEO is much less in orange juice than in water (Fig. 1) Approximately 60 ppm of NEO was required to double the threshold of limonin in orange juice, whereas only 10 ppm of the same compound increased limonin threshold in water 4-fold. Nevertheless, limonin threshold steadily increased from 3.4 to 6.8 ppm over a range of 1–60 ppm of added NEO. This represents a significant increase in bitterness suppression in an important range of limonin concentration. Table 2 compares limonin thresholds in water and orange juice with and without 10 ppm of NEO. Even this small amount of additive significantly increased the limonin threshold over the control sample. As mentioned above, the increase in water was much higher than in orange juice. The limonin concentration in the juice was raised 1.8 ppm without increasing apparent bitterness. This amounts to an increase of approximately 53% based on the limonin threshold of the control juice. Therefore, it is apparent that NEO was effective in reducing limonin bitterness in orange juice as well as in water solutions.

The results so far have dealt with effects of NEO on the threshold concentration of limonin or the amount that 50% of the panel can distinguish from normal control juice. This is an important concept because obviously bitterness does not represent a problem if it is not perceived. However, there are situations where bitterness is present above threshold levels for many consumers and potential consumers of citrus juices. Under these conditions it would be useful to know if NEO could reduce bitterness at suprathreshold levels of limonin in citrus juices.

Effectiveness of NEO in reducing limonin bitterness at suprathreshold levels

Figure 2 shows the relation between relative bitterness and NEO concentration in orange juice containing 6 ppm added limonin. This amount of limonin is almost twice the threshold level, and hence 85% of the responses indicated the sample containing 6 ppm limonin and no NEO was more bitter than the control juice without additives. As the concentration of NEO was increased in the sample containing 6 ppm limonin, the percentage of correct responses (indicating sample with 6 ppm as more bitter) steadily decreased from 85 to 50% over the range of 0–40 ppm NEO. With 40 ppm NEO the sample containing 6 ppm limonin was undistinguishable from the control, indicating that NEO had neutralized the bitter effect of limonin. This is essentially the same value for limonin threshold predictable from the equation: $T_L = 3.45\ C_{NEO}^{0.15}$ where T_L = threshold of limonin and C = 40 ppm NEO (Fig. 1). In one case (threshold) 6 ppm limonin in the presence of 40 ppm NEO was required for significant bitterness perception, and in the other 40 ppm NEO was required to neutralize the bitterness of juice containing 6 ppm of limonin. The confirmation of the effectiveness of NEO by the two different sensory procedures adds validity to the relation shown in Figure 1.

Table 3—Relative bitterness of orange juice containing varying amounts of added limonin and neodiosmin

Comparison	N	Judgments indicating more bitter sample was: Limonin alone	Limonin plus neodiosmin
4 ppm limonin vs: 4 ppm limonin plus 10 ppm neodiosmin	40	16	24
6 ppm limonin vs: 6 ppm limonin plus 10 ppm neodiosmin	20	8	12
8 ppm limonin vs: 8 ppm limonin plus 10 ppm neodiosmin	38	15	23
10 ppm limonin vs: 10 ppm limonin plus 60 ppm neodiosmin	40	26	14
10 ppm limonin vs: 10 ppm limonin plus 100 ppm neodiosmin	40	30	10[a]

[a] Significant at $P \leq 0.01$

Table 3 shows the effect of NEO in reducing bitterness in orange juice containing various amounts of added limonin. It will be noted that both samples in each comparison contained the same amount of added limonin, but varying amounts of NEO were added to one of the samples in each pair. This is a rather difficult test in that both samples are above the bitterness threshold, and the panelist must distinguish between two relatively bitter samples rather than a nonbitter control sample and one that is just perceptibly bitter as in the threshold test. Figure 3 shows that the limonin difference threshold for juices with varying amounts of added limonin varies linearly over the range 2.4–20.4 ppm limonin. Thus for a range of 4–10 ppm of limonin in juice, the limonin difference threshold would vary from about 4.9 to 5.3 ppm. This means that the NEO must reduce bitterness by an amount equivalent to that of 4.9 to 5.3 ppm of limonin for the panel to significantly distinguish between samples with and without NEO. For example, about 5.3 ppm of limonin would have to be neutralized in a juice containing 10 ppm for the panel to find a statistically significant reduction in bitterness. Table 3 shows the bitterness of juices with 4–8 ppm of limonin was not significantly affected by 10 ppm of NEO. With 10 ppm of added limonin, 60–100 ppm NEO were required to cause a statistically significant reduction in limonin bitterness. As mentioned above, this represents a bitterness reduction roughly equivalent to 5–6 ppm of added limonin.

Neodiosmin was effective in reducing limonin bitterness in water and orange juice under all the sensory conditions used to

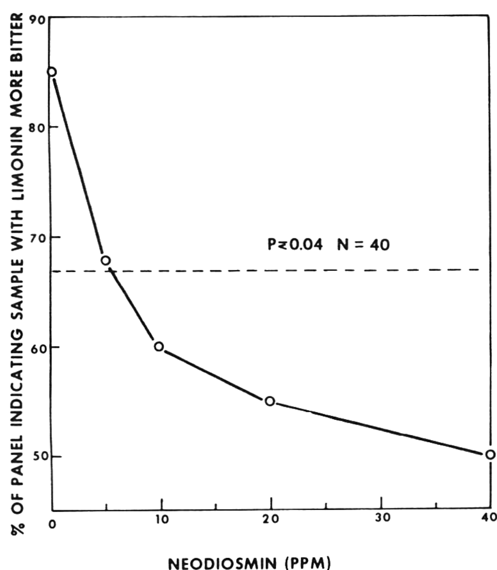

Fig. 2—Effect of neodiosmin on relative bitterness of orange juice containing 6 ppm of added limonin.

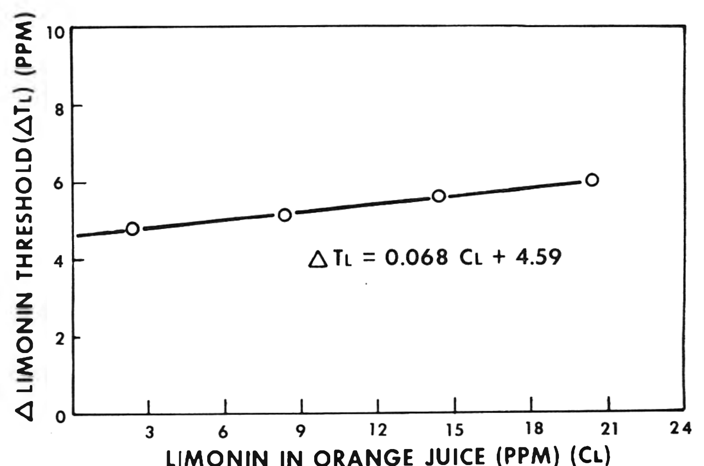

Fig. 3—Effect of limonin concentration in orange juice on the difference threshold for limonin. ΔT_L = difference threshold for limonin in orange juice. C_L = concentration of limonin in orange juice.

measure changes in bitterness. It was effective in increasing limonin threshold in water and orange juice; it was effective in reducing bitterness of suprathreshold levels of limonin when compared against control juices; and it was effective in reducing bitterness of high limonin juices when both samples compared contained high levels of limonin. By its ability to reduce bitterness under all of these conditions, it is probable that NEO could significantly increase the acceptability of juices containing relatively high levels of limonin, especially for consumers whose limonin thresholds are lower than average.

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Ms received 8/25/75; revised 10/23/75; accepted 10/28/75.

We thank R.M. Horowitz and Bruno Gentili of the Fruit and Vegetable Lab., Pasadena, Calif., for supplying the crystalline neodiosmin.

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ESTIMATION OF THE CENTRAL TEMPERATURE OF THERMALLY CONDUCTIVE FOOD IN CYLINDRICAL AND RECTANGULAR CANS DURING HEAT PROCESSING

ABSTRACT

The author proposes a classification of cylindrical and rectangular cans in can-shape-groups in order to reduce the number of temperature response curves necessary to carry out an accurate estimation of the central temperature of thermally conductive foods.

INTRODUCTION

THE IMPORTANCE of the accurate calculation of the central temperature in a canned food during heat processing has been pointed out in several papers. In this respect, theoretical equations were applied by Gillespy (1953), Hicks (1951) and Riedel (1947).

Newmann (1936) stated that, by means of the multiplication rule applied to equations valid for infinite bodies, it is possible to obtain solutions for finite bodies. As a consequence, from the graphic solutions proposed by Gurney and Lurie (1923) for infinite cylinder and infinite slab, it is possible to obtain temperature response charts for cylindrical and rectangular cans. Hayakawa (1969) proposed temperature response charts for finite cylinders, presenting a curve for each can shape factor, in order to estimate the central temperature of thermally conductive food. Alles and Cowel (1971) pointed out a theoretical equation for the evaluation of the central temperature in rectangular food containers. We did not find any graphical solution for the calculation of such temperature.

The main purpose of this paper is to propose an adequate definition of can-shape-groups to enable representing each group by a unique temperature response curve for the evaluation of the central temperature.

PROCEDURE TO DEFINE CAN-SHAPE-GROUPS

Cylindrical cans

The application of the multiplication rule to the equations proposed by Charm (1971) permits evaluating the temperature in a given point of a finite cylinder (Eq. 1):

$$u = 4 \sum_{i=1}^{\infty} \sum_{j=1}^{\infty} \frac{\sin\lambda_i \cdot \cos(2\lambda_i x/l)}{\lambda_i + \sin\lambda_i \cos\lambda_i} \cdot \frac{J_0(2\beta_j r/d)}{[1 + (\beta_j/m)^2]\beta_j J_1(\beta_j)} \cdot \exp[-4\lambda_i^2 (\alpha\theta/S_{ij})] \quad (1)$$

where

$$S_{ij} = \frac{d^2}{(d/l)^2 + (\beta_j/\lambda_i)^2} \quad (2)$$

(Definitions for symbols used are given under Nomenclature.)

In order to define a given Fourier number (L) for all the terms of the series (Eq. 1) and to define a parameter S that will be a function only of can dimensions, we have to define a new parameter R_{ij}, so that:

$$u = 4 \sum_{i=1}^{\infty} \sum_{j=1}^{\infty} \frac{\sin\lambda_i \cos(2\lambda_i x/l)}{\lambda_i + \sin\lambda_i \cdot \cos\lambda_i} \cdot \frac{J_0(2\beta_j r/d)}{[1 + (\beta_j/m)^2]\beta_j J_1(\beta_j)} \cdot \exp(-4\lambda_i^2 R_{ij} L) \quad (3)$$

where:

$$R_{ij} = \frac{(d/l)^2 + (\beta_j/\lambda_i)^2}{(d/l)^2 + (\beta_1/\lambda_1)^2} \quad (4)$$

$$L = \frac{\alpha\theta}{S} \quad (5)$$

$$S = \frac{d^2}{(d/l)^2 + (\beta_1/\lambda_1)^2} \quad (6)$$

Since the value of u is mainly influenced by the first term of the series (Eq. 1), the $S = S_{11}$ definition is the best one.

Assuming $m = \infty$ (Hayakawa, 1969; Hayakawa and Ball, 1969), the central temperature can be estimated as follows:

$$u = 4 \sum_{i=1}^{\infty} \sum_{j=1}^{\infty} \frac{\sin\lambda_i}{\lambda_i \beta_j J_1(\beta_j)} \cdot \exp(-4\lambda_i^2 R_{ij} L) \quad (7)$$

where:

$$S = \frac{d^2}{(d/l)^2 + 2.344} \quad (8)$$

Comparing the results given by Eq. (7) with the results given by the equations for the infinite cylinder and infinite slab (Ball and Olson, 1957), we can easily verify that:

(a) When the shape factor is $d/h < 0.25$, the equation valid for an infinite cylinder permits estimating the central temperature.
(b) When the shape factor is $d/h > 4.00$, the equation valid for an infinite slab can be used to estimate the central temperature.

Then, Eq. (7) will be applied when $0.25 \leq d/h \leq 4.00$

Computing the central temperature by Eq. (7) and assuming that a difference smaller than 2°C between two estimated temperatures for different values of the can shape factor is negligible, 11 can-shape-groups were defined when T_0 = 50°C and T_1 = 121°C by means of a suitable computer program. Table 1 shows the temperature history for the smallest and the largest shape factor of each can-shape-group. The limit of error occurs when the value of L is small (≤ 0.11) and, in this range, the observed temperature differences do not influence markedly the lethal rate values. In fact, assuming z = 13°C, the maximum temperature difference observed in the can-shape-group No. 2 (Table 1), leads to a lethal rate value variation from 0.0006607–0.0004669.

If we define an average value of R_{ij} ($\bar{R}_{ij}$) for each can-shape-group the temperatures differences obtained will be smaller than 1°C. Since the error obtained occurs for low temperatures (smaller than 80°C), the assumed upper limit of temperature error (<2°C) is a proper one, because in this range the error obtained in the lethal rates is negligible.

Table 1—Temperature history for the smallest and the largest shape factor of each can-shape-group (T_1 = 121°C and T_0 = 50°C)

Can-shape-group No.	(d/1)	Temperature (°C)									
		L = 0.01	L = 0.03	L = 0.05	L = 0.07	L = 0.09	L = 0.11	L = 0.13	L = 0.17	L = 0.21	L = 0.25
1	0.25	38.23[a]	45.23	52.39	62.22	71.92	80.44	87.62	98.44	105.73	110.65
	0.42	38.85	47.18	53.91	62.97	71.80	79.77	86.59	97.17	104.57	109.72
2	0.43	38.89	47.28	53.96	62.84	71.72	79.66[a]	86.48	97.07	104.44	109.65
	0.58	39.39	48.54	54.08	61.79	69.99	77.70	84.58	95.62	103.52	109.06
3	0.59	39.42	48.60	54.05	61.69	69.86[a]	77.57	84.46	95.54	103.48	109.03
	0.75	39.72	49.24	53.37	60.05	67.03	75.78	82.97	94.67	103.02	108.80
4	0.76	39.73[a]	49.26	53.32	59.95	67.83	75.70	82.90	94.63	103.01	108.80
	1.45	37.79	48.97	53.26	59.54	67.24	75.11	82.41	94.37	102.88	108.74
5	1.46	37.73	48.95	53.30	59.59[a]	67.28	75.14	82.44	94.37	102.88	108.74
	1.76	36.06	48.48	54.74	61.58	69.04	76.46	83.35	94.76	103.03	108.80
6	1.77	36.00	48.46	54.79	61.65[a]	69.11	76.52	83.39	94.77	103.04	108.80
	2.06	34.35	47.75	56.03	63.63	71.03	78.07	84.53	95.32	103.28	108.90
7	2.07	34.29	47.72	56.07	63.70	71.09[a]	78.12	84.58	95.35	103.29	108.91
	2.39	32.61	46.68	56.95	65.53	73.08	79.87	85.97	96.12	103.67	109.09
8	2.40	32.56	46.65	56.97	65.58	73.13[a]	79.92	86.02	96.14	103.69	109.09
	2.80	30.76	45.12	57.32	67.11	75.10	81.86	87.71	97.22	104.30	109.42
9	2.81	30.72	45.08[a]	57.32	67.14	75.14	81.90	87.75	97.25	104.32	109.43
	3.31	28.92	43.10	56.90	67.98	76.66	83.67	89.48	98.56	105.16	109.94
10	3.32	28.89	43.06[a]	56.89	67.99	76.69	83.69	89.50	98.58	105.18	109.95
	3.86	27.42	41.09	55.88	67.97	77.37	84.77	90.74	99.72	106.03	110.52
11	3.87	27.40	41.06[a]	55.86	67.96	77.37	84.78	90.75	99.74	106.05	110.53
	4.00	27.11	40.62	55.58	67.87	77.42	84.93	90.95	99.96	106.23	110.66

[a] Maximum temperature difference obtained applying Eq. (7).

The applications of Eq. (7) to a given can-shape-group is carried out replacing R_{ij} by the average value $\bar{R}_{ij}$, calculated by Eq. (9):

$$\bar{R}_{ij} = \frac{1}{x_2 - x_1} \cdot \left\{ \left[x - \sqrt{\beta_1/\lambda_1} \text{ arc tg } (x\sqrt{\lambda_1/\beta_1})\right] \Big|_{x_1}^{x_2} + \sqrt{\beta_j/\lambda_i} \left[\sqrt{\lambda_1/\beta_1} \cdot \text{arc tg } (x\sqrt{\lambda_1/\beta_1})\right] \Big|_{x_1}^{x_2} \right\} \quad (9)$$

where $x = d/1$; $x_1 = (d/1)_1$ = the smallest shape factor of the can-shape-group; $x_2 = (d/1)_2$ = the largest shape factor of the can-shape-group.

From the calculated values of $\bar{R}_{ij}$ (Table 2), it is possible to prepare the temperature response curves for the estimation of the central temperature in cylindrical cans. Figure 1 is an example of the curves obtained.

Table 1 shows that when L ⩾ 0.25 the temperature differences are smaller than 2°C, whatever will be the can shape factor. It then seems possible to define a critical value $L_c = 0.25$ for all the can shape factors, when T_1 = 121°C and T_0 = 50°C. Since L is a dimensionless number, in this case, when L ⩾ 0.25, Eq. (10) can be applied whatever the can shape factor value:

$$u = 2.040 \exp(-\pi^2 L) \quad (10)$$

Table 2—Average values of R_{ij} for the 11 can-shape-groups of cylindrical cans (T_1 = 121°C and T_0 = 50°C)

Can-shape-group No.	$(d/1)_1$	$(d/1)_2$	Average values of R_{ij}							
			$\bar{R}_{12}$	$\bar{R}_{13}$	$\bar{R}_{21}$	$\bar{R}_{22}$	$\bar{R}_{23}$	$\bar{R}_{31}$	$\bar{R}_{32}$	$\bar{R}_{33}$
1	0.25	0.42	5.070	12.392	0.152	0.604	1.418	0.084	0.247	0.540
2	0.43	0.58	4.848	11.770	0.198	0.626	1.395	0.134	0.288	0.565
3	0.59	0.75	4.581	11.024	0.254	0.652	1.367	0.194	0.337	0.595
4	0.76	1.45	3.817	8.885	0.413	0.726	1.289	0.366	0.479	0.681
5	1.46	1.76	3.030	6.683	0.577	0.802	1.208	0.543	0.624	0.770
6	1.77	2.06	2.667	5.666	0.652	0.838	1.171	0.625	0.691	0.811
7	2.07	2.39	2.370	4.835	0.714	0.866	1.140	0.691	0.746	0.845
8	2.40	2.80	2.102	4.085	0.770	0.892	1.113	0.752	0.796	0.875
9	2.81	3.31	1.858	3.401	0.820	0.916	1.088	0.807	0.841	0.903
10	3.32	3.86	1.659	2.845	0.862	0.935	1.067	0.851	0.878	0.925
11	3.87	4.00	1.561	2.571	0.883	0.945	0.057	0.873	0.896	0.936

$(d/1)_1$ = the smallest shape factor of can-shape-group; $(d/1)_2$ = the largest shape factor of can-shape-group; R_{11} = 1.0 for all can shape factors

Rectangular cans

In a similar way, we can state:

$$u = 8 \sum_{i=1}^{\infty} \sum_{j=1}^{\infty} \sum_{k=1}^{\infty} \frac{\sin\lambda_{ai} \cdot \cos(2\lambda_{ai}x/a)}{\lambda_{ai} + \sin\lambda_{ai}\cos\lambda_{ai}} \cdot \frac{\sin\lambda_{bj} \cdot \cos(2\lambda_{bj}y/b)}{\lambda_{bj} + \sin\lambda_{bj}\cos\lambda_{bj}} \cdot \frac{\sin\lambda_{ck}\cos(2\lambda_{ck}z/c)}{\lambda_{ck} + \sin\lambda_{ck}\cos\lambda_{ck}} \cdot \exp(-R_{ijk}L) \qquad (11)$$

where:

$$R_{ijk} = \frac{\lambda_{ai}^2 b^2 c^2 + \lambda_{bj}^2 a^2 c^2 + \lambda_{ck}^2 a^2 b^2}{\lambda_{a1}^2 b^2 c^2 + \lambda_{b1}^2 a^2 c^2 + \lambda_{c1}^2 a^2 b^2} \qquad (12)$$

$$L = \frac{\alpha\theta}{S} \qquad (13)$$

$$S = \frac{a^2 b^2 c^2}{\lambda_{a1}^2 b^2 c^2 + \lambda_{b1}^2 a^2 c^2 + \lambda_{c1}^2 a^2 b^2} \qquad (14)$$

Assuming $m = \infty$ and $(x/a) = (y/b) = (z/c) = 0$:

$$u = 8 \sum_{i=1}^{\infty} \sum_{j=1}^{\infty} \sum_{k=1}^{\infty} \frac{\sin\lambda_{ai}\sin\lambda_{bj}\sin\lambda_{ci}}{\lambda_{ai}\lambda_{bj}\lambda_{ck}} \cdot \exp(-\pi^2 R_{ijk} L) \qquad (15)$$

where:

$$S = \frac{a^2 b^2 c^2}{b^2 c^2 + a^2 c^2 + a^2 b^2} \qquad (16)$$

By analysis of Eq. (15) we can easily verify that:

(a) the same equation is valid for the cans (a,b,c); (a,c,b); (b,a,c); (b,c,a); (c,a,b) and (c,b,a);

(b) the value of R_{ijk} (Eq. 12) for the can (na,nb,nc) is independent of the value of n(n > 0).

Then, we can define a generic can collection based on their relative dimensions:

$$A = a/a = 1;\ B = b/a;\ \text{and}\ C = c/a$$

where *a* is the smallest dimension.

Assuming again that a difference smaller than 2°C between two estimated temperatures for different values of the dimensions A, B and C is negligible, 11 can-shape-groups were defined when T_0 = 50°C and T_1 = 121°C, by means of another suitable computer program (Table 3). Table 4 shows the history temperature for the first and the last can of each can-shape-group. Again the results obtained show that the assumed upper limit of temperature error (<2°C) is a proper one.

Defining an average value R_{ijk} ($\bar{R}_{ijk}$) for each can-shape-group, we can state that the error is smaller than 1°C. The application of Eq. (15) to a given can-shape-group is carried out replacing R_{ijk} by $\bar{R}_{ijk}$ calculated by the arithmetic mean of R_{ijk} for each can of the considered can-shape-group. Table 5 gives the values $\bar{R}_{ijk}$ for each can-shape-group.

As stated in the case of cylindrical cans, when L is larger than L_c = 0.25, Eq. (17) can be applied whatever the can dimensions are:

$$u = \frac{64}{\pi^3} \exp(-\pi^2 L) \qquad (17)$$

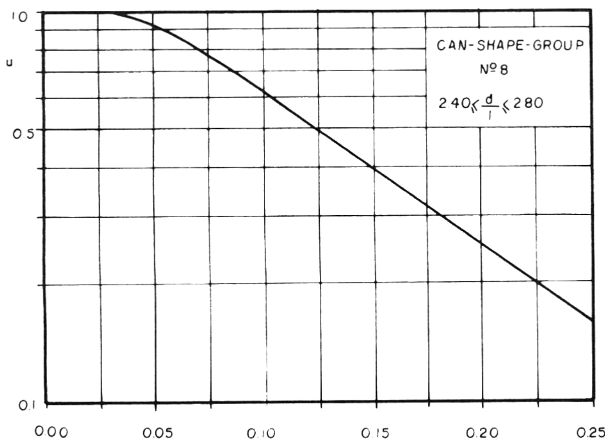

Fig. 1—Temperature response chart for the can-shape-group No. 8 (2.40 ≤ d/1 ≤ 2.80) of cylindrical cans.

COMPARISON WITH EXPERIMENTALS RESULTS

FIGURE 2 shows the experimental and the calculated central temperature of a 300 X 406 can of potato puree. In this

Table 3—Definition of the rectangular can-shape-groups (T_1 = 121°C and T_0 = 50°C)

B	C	Can-shape-group No.
1.0	1.0 – 1.5	1
	1.6 – 1.9	2
	2.0 – 2.7	3
	2.8 – 4.0	4
1.1	1.1 – 1.5	1
	1.6 – 2.0	2
	2.1 – 2.8	3
	2.9 – 4.0	4
1.2	1.2 – 1.5	1
	1.6 – 2.0	2
	2.1 – 2.8	3
	2.9 – 4.0	4
1.3	1.3 – 1.5	1
	1.6 – 2.0	2
	2.1 – 2.9	3
	3.0 – 4.0	4
1.4	1.4	1
	1.5	2
	2.0 – 2.2	5
	2.3 – 2.8	3
	2.9 – 3.6	6
	3.7 – 4.0	4
1.5	1.5 – 1.9	2
	2.0 – 2.5	5
	2.6 – 4.0	6
1.6	1.6 – 1.8	2
	1.9 – 2.4	5
	2.5	7
	2.6 – 4.0	6
1.7	1.7	2
	1.8 – 2.3	5
	2.4 – 2.9	7
	3.0 – 4.0	6
1.8	1.8 – 2.1	5
	2.2 – 3.0	7
	3.1 – 4.0	8
1.9	1.9 – 2.0	5
	2.1 – 2.8	7
	2.9 – 4.0	8
2.0	2.0 – 2.7	7
	2.8 – 4.0	8
2.1	2.1 – 2.4	7
	2.5 – 3.9	8
	3.9 – 4.0	9
2.2	2.2 – 2.3	7
	2.4 – 3.5	8
	3.6 – 4.0	9
2.3	2.3 – 2.4	8
	2.5 – 3.8	9
	3.9 – 4.0	10
2.4	2.4 – 3.5	9
	3.6 – 4.0	10
2.5	2.5 – 3.3	9
	3.4 – 4.0	10
2.6	2.6 – 3.0	9
	3.1 – 4.0	10
2.7	2.7 – 2.9	9
	3.0 – 4.0	10
2.8	2.8	9
	2.9 – 4.0	10
2.9	2.9 – 4.0	10
3.0	3.0 – 4.0	10
3.1	3.1 – 3.9	10
	4.0	11
3.2	3.2 – 4.0	11
3.3	3.3 – 4.0	11
3.4	3.4 – 4.0	11
3.5	3.5 – 4.0	11
3.6	3.6 – 4.0	11
3.7	3.7 – 4.0	11
3.8	3.8 – 4.0	11
3.9	3.9 – 4.0	11
4.0	4.0	11

A = a/a; B = b/a and C = c/a; where a, b and c are the rectangular can dimensions

Table 4—Temperature history for the first and last can of each can-shape-group (T_1 = 121°C and T_0 = 50°C)

Can-shape-group No.	B	C	Temperature °C									
			L = 0.01	L = 0.03	L = 0.05	L = 0.07	L = 0.09	L = 0.11	L = 0.13	L = 0.17	L = 0.21	L = 0.25
1	1.0	1.0	43.88	49.89	52.59	58.43[a]	66.15	74.19	81.69	93.93	102.63	108.58
	1.4	1.4	43.29	49.93	53.89	60.11	67.64	75.30	82.45	94.25	102.74	108.62
2	1.0	1.6	43.10	49.38	53.85	60.67[a]	68.74	76.16	83.20	94.70	102.97	108.73
	1.7	1.7	42.42	49.96	55.60	62.39	69.72	76.93	83.63	94.68	102.96	108.71
3	1.0	2.0	42.51	48.61	54.22	61.98	70.18[a]	77.86	84.69	95.66	103.52	109.03
	1.4	2.8	41.53	48.23	55.27	63.69	72.03	79.61	86.23	96.72	104.20	109.44
4	1.0	2.8	41.73[a]	47.13	53.75	62.69	71.66	79.70	86.59	97.24	104.64	109.77
	1.4	4.0	40.85	46.82	54.34	63.53	72.53	80.55	87.41	97.97	105.25	110.23
5	1.4	2.0	42.41	49.47	55.18	62.36[a]	70.01	77.38	84.10	95.14	103.18	108.83
	1.9	2.0	41.65	49.87	56.90	64.25	71.55	78.47	84.81	95.40	103.25	108.84
6	1.4	2.9	41.45	48.08	55.21	63.75[a]	72.16	79.77	86.41	96.88	104.32	109.52
	1.7	4.0	40.28	47.53	55.99	65.10	73.66	81.26	87.81	98.02	105.18	110.14
7	1.6	2.5	41.50	49.11	56.31	64.25[a]	72.04	79.23	85.65	96.11	103.73	109.13
	2.2	2.3	40.75	49.60	58.10	66.17	73.56	80.23	86.28	96.25	103.71	109.07
8	1.8	3.1	40.59	48.62	57.06	65.69[a]	73.71	80.87	87.13	97.17	104.42	109.55
	2.3	2.4	40.48	49.48	58.40	66.71	74.16	80.83	86.76	96.56	103.88	109.16
9	2.1	3.9	39.55	48.04	57.76[a]	67.05	75.28	82.43	88.43	98.27	105.19	110.08
	2.8	2.8	39.37	48.79	59.25	68.53	76.36	83.01	88.73	97.92	104.72	109.65
10	2.3	3.9	39.21	48.06	58.33	67.82[a]	76.02	83.04	89.04	98.50	105.30	110.12
	3.3	3.4	38.32	47.83	59.45	69.63	77.97	84.82	90.52	99.38	105.74	110.31
11	3.1	4.0	38.12	47.51	59.23	69.56	78.04	85.00[a]	90.77	99.68	106.01	110.13
	4.0	4.0	37.43	46.77	59.12	70.00	78.84	85.98	91.31	100.60	106.72	110.53

[a] Maximum temperature difference obtained applying Eq. (15).

Table 5—Average values of R_{ijk} for the 11 can-shape-groups of rectangular cans (T_1 = 121°C and T_0 = 50°C)

Average values of R_{ijk}	Can-Shape-Group										
	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6	No. 7	No. 8	No. 9	No. 10	No. 11
$\bar{R}_{112}$	2.986	2.364	1.718	1.370	2.147	1.495	1.933	1.592	1.689	1.544	1.483
$\bar{R}_{113}$	6.957	5.093	3.153	2.109	4.442	2.485	3.800	2.776	3.067	2.631	2.449
$\bar{R}_{121}$	3.644	3.525	4.040	4.271	2.899	3.199	2.558	2.475	2.091	1.855	1.573
$\bar{R}_{122}$	5.630	4.890	4.758	4.641	4.046	3.694	3.492	3.067	2.780	2.399	2.056
$\bar{R}_{123}$	9.601	7.618	6.193	5.380	6.341	5.684	5.358	4.251	4.158	3.486	3.022
$\bar{R}_{131}$	8.933	8.576	10.120	10.814	6.697	7.598	5.675	5.425	4.273	3.566	2.718
$\bar{R}_{132}$	10.918	9.940	10.837	11.184	7.844	8.093	6.608	6.017	4.962	4.109	3.201
$\bar{R}_{133}$	14.890	12.669	12.273	11.923	10.139	9.082	8.475	7.201	6.340	5.196	4.167
$\bar{R}_{211}$	3.370	5.110	5.242	5.359	5.954	6.306	6.508	6.933	7.220	7.601	7.944
$\bar{R}_{212}$	6.356	6.475	5.960	5.729	7.101	6.801	7.442	7.525	7.909	8.145	8.427
$\bar{R}_{213}$	10.327	9.203	7.395	6.468	9.396	7.790	9.308	8.709	9.287	9.232	9.393
$\bar{R}_{221}$	7.014	7.636	8.282	8.630	7.853	8.505	8.067	8.408	8.311	8.456	8.517
$\bar{R}_{223}$	12.971	11.728	10.435	9.739	11.295	9.990	10.866	10.184	10.378	10.087	9.966
$\bar{R}_{231}$	12.303	12.686	14.362	15.173	11.650	12.904	11.183	11.358	10.493	10.167	9.663
$\bar{R}_{232}$	14.288	14.051	15.080	15.543	12.798	13.399	12.117	11.950	11.182	10.710	10.147
$\bar{R}_{233}$	18.260	16.779	16.515	16.282	15.093	14.388	13.983	13.134	12.560	11.796	11.111
$\bar{R}_{311}$	11.110	13.331	13.727	14.077	15.861	16.917	17.525	18.799	19.660	20.804	21.833
$\bar{R}_{312}$	13.096	14.696	14.445	14.447	17.008	17.412	18.458	19.391	20.349	21.347	22.316
$\bar{R}_{313}$	17.067	17.424	15.880	15.186	19.303	18.402	20.325	20.575	21.727	22.434	23.282
$\bar{R}_{321}$	13.754	15.857	16.767	17.349	17.760	19.117	19.084	20.274	20.751	21.659	22.406
$\bar{R}_{322}$	15.740	17.221	17.485	17.718	18.907	19.612	20.017	20.866	21.440	22.202	22.889
$\bar{R}_{323}$	19.711	19.949	18.920	18.457	21.202	20.601	21.883	22.050	22.818	23.290	23.854
$\bar{R}_{331}$	19.043	20.907	22.847	23.891	21.558	23.515	22.200	23.224	22.933	23.369	23.551
$\bar{R}_{332}$	21.029	22.271	23.565	24.261	22.705	24.010	23.133	23.816	23.622	23.913	24.034

$\bar{R}_{111}$ = 1, 0; $\bar{R}_{222}$ = 9 and $\bar{R}_{333}$ = 25, whatever the can-shape group is

experiment the puree initial temperature was 27.5°C, the retort temperature was 121.1°C and the cooling water temperature was 25.0°C. Figure 3 shows the experimental and calculated central temperature of an 80 mm X 94 mm X 168 mm can of potato puree. In this experiment the puree initial temperature was 20.0°C, the retort temperature was 121.1°C and the cooling water temperature was 25.0°C.

To calculate the theoretical temperature and to represent the experimental temperature in Figures 2 and 3, the effect of the retort come-up time (2 min) was corrected according to Ball and Olson (1957). The average thermal diffusivity of the puree, calculated by the method proposed by Hayakawa (1969), was 0.092 cm^2/min.

The temperature during the cooling phase was calculated by means of Eq. (18), proposed by Hayakawa (1969) and Hayakawa and Ball (1969):

$$\frac{T - T_w}{T_1 - T_0} = \frac{T_1 - T_w}{T_1 - T_0} \cdot u(L) - u(L + L_b) \quad (18)$$

where: $L = \alpha\theta_b/S$; θ_b = corrected length of time for heating phase.

Figure 4 presents the results obtained when the 300 X 406 can was heated in a nonagitated water bath at 96.6°C. In this case, m cannot be assumed to be ∞; good agreement between experimental and estimated temperatures was obtained assuming m = 20. The average thermal diffusivity of the puree was, in this test, 0.106 cm^2/min.

CONCLUSIONS

HAYAKAWA (1969) proposed temperature response curves to estimate the central temperature in cylindrical cans for 20 can-shape factors in the range $0.2 \leqslant (d/l) \leqslant 4.00$. If we consider a shape factor different from those presented by Hayakawa (1969), the accuracy of the results will be certainly affected. In this paper the proposed classification of cylindrical or rectangular cans in can-shape-groups considerably reduce the number of temperature response curves (11 in each case), and the definition of average values of R_{ij} and R_{ijk} leads to better agreement between the estimated and the experimental central temperature of thermally conductive food.

When the value of L is larger than a critical value, only one curve, in each case, is sufficient to evaluate the central temperature whatever the can dimensions are.

NOMENCLATURE

a.b,c	= rectangular can dimensions (m)
d	= cylindrical can diameter (m)
h	= individual heat-transfer coefficient (kcal/h · m^2 · °C)
$J_0(x)$	= zeroth order Bessel function of first kind
$J_1(x)$	= first order Bessel function of first kind
k	= thermal conductivity (kcal/h · m· °C)
l	= cylindrical can height (m)
m	= dimensionless resistence ($m = hr_m/k$)
r	= cylindrical can radius (m)
r_m	= infinite cylinder and infinite slab characteristic dimension (m)
T	= temperature at the center of conductive food in cylindrical and rectangular cans (°C)

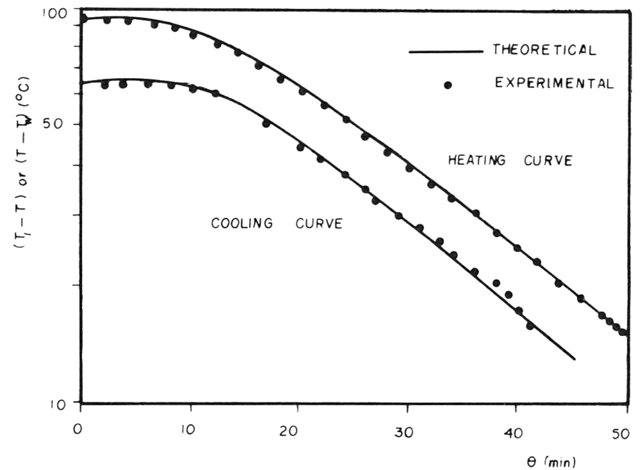

Fig. 3–Experimental and calculated central temperatures of potato puree in a 80 mm X 94 mm X 168 mm rectangular can. T_1 = 121.1°C; T_0 = 20°C and T_w = 25.0°C.

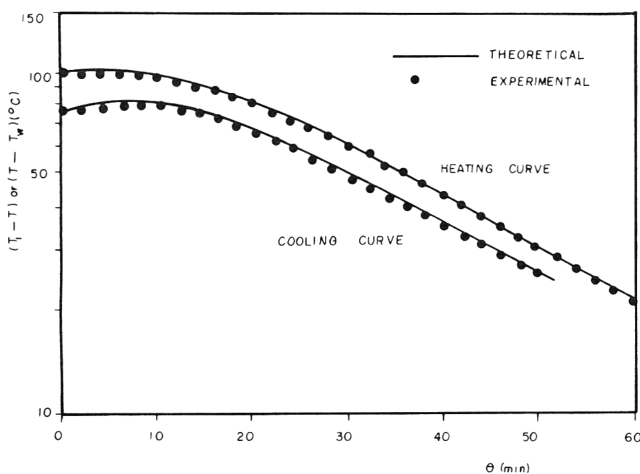

Fig. 2–Experimental and calculated central temperatures of potato puree in a 300 X 400 can. T_1 = 121.1°C; T_0 = 27.5°C and T_w = 25.0°C.

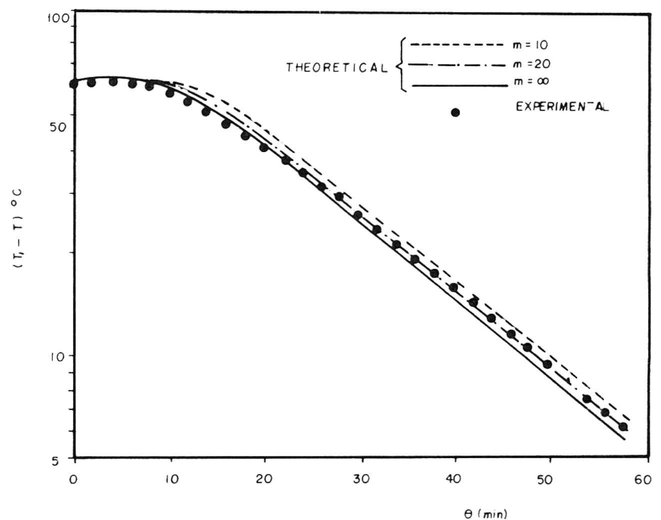

Fig. 4–Experimental and calculated central temperatures of potato puree in a 300 X 406 can heated in a nonagitated water bath at 96.6°C. T_0 = 33.0°C.

T_0	= temperature of food at zero time of heating (°C)
T_1	= holding temperature of heating medium. If the heat treatment is carried out in a retort, T_1 is the retort temperature (°C).
T_w	= temperature of cooling water (°C)
u	= dimensionless temperature of conductive food $[u = (T_1 - T)/(T_1 - T_0)]$
u(L)	= same with u
$u(L + L_b)$	= Formula obtained by entering $L = L + L_b$ in Eq. (7) and (15).
x,y,z	= rectangular coordinates. Directions of heat flux.
α	= thermal diffusivity (m^2/h)
β_j	= Jth positive root of equation: $\beta J_1(\beta) = mJ_0(\beta)$
λ_{ai}	= ith positive root of equation: $\lambda_a = (ha/2k)\cot\lambda_a$
λ_{bj}	= Jth positive root of equation: $\lambda_b = (hb/2k)\cot\lambda_b$
λ_{ck}	= kth positive root of equation: $\lambda_c = (hc/2k)\cot\lambda_c$
θ	= time variable (h)

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Ms received 5/19/75; revised 9/23/75; accepted 9/30/75.

The author acknowledges the valuable suggestions of Prof. Walter Borzani.

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ESTIMATION OF THE AVERAGE TEMPERATURE OF THERMALLY CONDUCTIVE FOOD IN CYLINDRICAL AND RECTANGULAR CANS DURING HEAT PROCESSING

ABSTRACT

A classification of cylindrical and rectangular cans in can-shape-groups is proposed in order to reduce the number of temperature response curves necessary to carry out an accurate estimation of the average temperature of thermally conductive foods.

INTRODUCTION

IN A PREVIOUS PAPER (Leonhardt, 1976) a classification of cylindrical and rectangular cans in can-shape-groups is proposed in order to be able to represent each family by a unique temperature response curve for the evaluation of the central temperature of a conductive canned food. Similar results can be obtained if we are interested in calculating the average temperature.

The average temperature of conductive food in cylindrical cans was calculated by Ball and Olson (1957), Charm (1961) and Stumbo (1964). Newmann (1936) stated that, applying the multiplication rule to the equations valid for infinite bodies, it is possible to obtain solutions for finite bodies. As a consequence, from the temperature response curves proposed by Jakob (1962) and McCabe and Smith (1967) for an infinite cylinder and infinite slab, it is possible to obtain graphic solutions for cylindrical and rectangular cans. Hayakawa and Ball (1969) proposed temperature response charts that permit obtaining a dimensionless parameter curve for any can shape factor, in order to estimate the average temperature of thermally conductive food. We did not find temperature response curves for the evaluation of the average temperature of food in rectangular cans.

The main purpose of this paper is to propose an adequate definition of can-shape-groups to enable representing each group by a unique temperature response curve for the evaluation of the average temperature.

PROCEDURE TO DEFINE CAN-SHAPE-GROUPS

Cylindrical cans

The application of the multiplication rule to the equations proposed by Carslaw and Jaeger (1959) permits evaluating the average temperature of a finite cylinder (Eq. 1):

$$\bar{u} = 8 \sum_{i=1}^{\infty} \sum_{j=1}^{\infty} \frac{1}{\lambda_i^2 \beta_j^2} \cdot \exp\left[-4\lambda_i^2 (\alpha\theta / S_{ij})\right] \tag{1}$$

where:

$$S_{ij} = \frac{d^2}{(d/l)^2 + (\beta_j/\lambda_i)^2} \tag{2}$$

(Definitions for symbols used are given under Nomenclature.)

Eq. (1) is valid for $m = \infty$ (Hayakawa, 1969; Hayakawa and Ball, 1969). In order to define a given Fourier number (L) for all the terms of the series (Eq. 1) and to define a parameter S that will be a function only of the can dimensions, we have to define a new parameter R_{ij} (Leonhardt, 1976), so that:

$$\bar{u} = 8 \sum_{i=1}^{\infty} \sum_{j=1}^{\infty} \frac{1}{\lambda_i^2 \beta_j^2} \cdot \exp(-4\lambda_i^2 R_{ij} L) \tag{3}$$

where:

$$R_{ij} = \frac{(d/l)^2 + (\beta_j/\lambda_i)^2}{(d/l)^2 + 2.344} \tag{4}$$

$$L = \frac{\alpha\theta}{S} \tag{5}$$

$$S = \frac{d^2}{(d/l)^2 + 2.344} \tag{6}$$

Comparing the results given by Eq. (3) with the results given by the equations for the infinite cylinder and infinite slab (Carslaw and Jaeger, 1959), we can easily verify that:

(a) When the shape factor is $d/l < 0.25$, the equation for an infinite cylinder permits estimating the average temperature.
(b) When the shape factor is $d/l > 4.00$, the equation for an infinite slab can be used to estimate the average temperature.

Eq. (3) then will be applied when $0.25 \leq d/l \leq 4.00$.

Computing the average temperature by Eq. (3) and assuming that a difference smaller than 2°C between two estimated temperatures for different values of the can shape factor is negligible, two can-shape-groups were defined when $T_0 = 50°C$ and $T_1 = 121°C$, by means of a computer program. Table 1 shows the temperature history for the smallest and the largest shape factor of each can-shape-group. The results obtained show that the assumed upper limit of temperature error (< 2°C) is a proper one.

If we define an average value of R_{ij} ($\bar{R}_{ij}$) for each can-shape-group

Table 1—Temperature history for the smallest and largest can shape factor of each can-shape-group (T_1 = 121°C and T_0 = 50°C)

Can-shape-group No.	(d/l)	Temperature (°C)					
		L = 0.01	L = 0.03	L = 0.05	L = 0.07	L = 0.09	L = 0.11
1	0.25	73.43[a]	83.35	92.85	98.35	102.63	106.05
	3.28	73.48	85.13	92.75	98.43	102.84	106.33
2	3.29	73.48	85.12[a]	92.74	98.42	102.84	106.33
	4.00	73.32	84.65	92.18	97.89	102.37	105.94

[a] Value of L where maximum temperature difference was found

the temperature differences will be smaller. The application of Eq. (3) to a given can-shape-group is carried out replacing R_{ij} by the average value $\bar{R}_{ij}$, calculated by Eq. (7).

$$\bar{R}_{ij} = \frac{1}{x_2 - x_1}\left\{[x - \sqrt{\beta_1/\lambda_1}\ \text{arc tg}\,(x\sqrt{\lambda_1/\beta_1}\,)]\Big|_{x_1}^{x_2} + \sqrt{\beta_j/\lambda_i}\ [\sqrt{\lambda_1/\beta_1}\ \text{arc tg}\,(x\sqrt{\lambda_1/\beta_1}\,)]\Big|_{x_1}^{x_2}\right\} \quad (7)$$

where: $x = d/l$; $x_1 = (d/l)_1$ = the smallest shape factor of the can-shape-group; $x_2 = (d/l)_2$ = the largest shape factor of the can-shape-group.

From the calculated average values of R_{ij} (Table 2) it is possible to prepare the temperature response curves for the estimation of the average temperature in cylindrical cans (Fig. 1 and 2).

Thus we observe (Table 1) that when L is larger than a critical value (L_c), only the first term of the series (Eq. 3) affects the value of $\bar{u}$. When $T_0 = 50°C$ and $T_1 = 121°C$, the critical value of L is $L_c = 0.09$. In this case, when $L \geqslant 0.09$, Eq. (8) can be applied whatever will be the shape factor value:

$$\bar{u} = 0.561 \exp(-\pi^2 L) \quad (8)$$

Rectangular cans

In a similar way, we can state:

$$\bar{u} = 8 \sum_{i=1}^{\infty} \sum_{j=1}^{\infty} \sum_{k=1}^{\infty} \frac{1}{\lambda_i^2 \lambda_j^2 \lambda_k^2} \cdot \exp(-\pi^2 R_{ijk} L) \quad (9)$$

where:

$$R_{ijk} = \frac{4(\lambda_i^2 b^2 c^2 + \lambda_j^2 a^2 c^2 + \lambda_k^2 a^2 b^2)}{\pi^2 (b^2 c^2 + a^2 c^2 + a^2 c^2)} \quad (10)$$

$$L = \frac{\alpha\theta}{S} \quad (11)$$

$$S = \frac{a^2 b^2 c^2}{b^2 c^2 + a^2 c^2 + a^2 b^2} \quad (12)$$

Eq. (9) is valid for $m = \infty$.

By analysis of Eq. (9) we can easily verify that:

(a) The same equation is valid for the cans (a,b,c); (a,c,b); (b,a,c); (b,c,a); (c,a,b) and (c,b,a).

Table 2—Average values of R_{ij} for the two can-shape-groups of cylindrical cans ($T_1 = 121°C$ and $T_0 = 50°C$)

Can-shape-group No.	$(d/l)_1$	$(d/l)_2$	Average values of R_{ij}							
			$\bar{R}_{12}$	$\bar{R}_{13}$	$\bar{R}_{21}$	$\bar{R}_{22}$	$\bar{R}_{23}$	$\bar{R}_{31}$	$\bar{R}_{32}$	$\bar{R}_{33}$
1	0.25	3.28	3.029	6.689	0.563	0.796	1.215	0.528	0.612	0.763
2	3.29	4.00	1.644	2.803	0.865	0.937	1.066	0.855	0.880	0.927

$(d/l)_1$ = the smallest can shape factor of the can-shape-group
$(d/l)_2$ = the largest can shape factor of the can-shape-group
$\bar{R}_{11}$ = 1.0 whatever the can shape factor is

Table 3—Definition of the rectangular can-shape-groups ($T_1 = 121°C$ and $T_0 = 50°C$)

B	C	Can-shape-group No.	B	C	Can-shape-group No.	B	C	Can-shape-group No.	B	C	Can-shape-group No.
1.0	1.0–4.0	1	1.8	1.8–4.0	1	2.5	2.5–2.9	1	3.2	3.2–4.0	2
							3.0–4.0	2			
1.1	1.1–4.0	1	1.9	1.9–4.0	1				3.3	3.3–4.0	2
						2.6	2.6–2.8	1			
							2.9–4.0	2			
1.2	1.2–4.0	1	2.0	2.0–3.7	1				3.4	3.4–4.0	2
				3.8–4.0	2						
						2.7	2.7	1			
1.3	1.3–4.0	1					2.8–4.0	2	3.5	3.5–4.0	2
			2.1	2.1–3.5	1						
				3.6–4.0							
1.4	1.4–4.0	1				2.8	2.8–4.0	2	3.6	3.6–4.0	2
			2.2	2.2–3.3	1						
1.5	1.5–4.0	1		3.4–4.0	2	2.9	2.9–4.0	2	3.7	3.7–4.0	2
1.6	1.6–4.0	1	2.3	2.3–3.2	1	3.0	3.0–4.0	2	3.8	3.8–4.0	2
				3.3–4.0	2						
1.7	1.7–4.0	1				3.1	3.1–4.0	2	3.9	3.9–4.0	2
			2.4	2.4–3.0	1						
				3.1–4.0	2						
									4.0	4.0	2

A = a/a = 1; B = b/a and C = c/a; where a, b and c are the rectangular can dimensions

(b) The values of R_{ijk} (Eq. 8) for the can (na,nb,nc) is independent of the values of n (n > 0).

Then we can define a generic can collection based on their relative dimensions:

$$A = a/a = 1;\ B = b/a;\ \text{and}\ C = c/a$$

where *a* is the smallest dimension.

Assuming again that a difference smaller than 2°C between two estimated temperatures for different values of the dimensions A, B and C is negligible, two can-shape-groups were defined when T_0 = 50°C and T_1 = 121°C, by means of another computer program (Table 3). Table 4 shows the history temperature for the first and the last can of each can-shape-group. Again the results obtained show that the assumed upper limit of temperature error (< 2°C) is a proper one.

Defining an average value of R_{ijk} ($\bar{R}_{ijk}$) for each can-shape-group, we can state that the error is smaller than 1°C. The application of Eq. (9) to a given can-shape-group is carried out replacing R_{ijk} by $\bar{R}_{ijk}$ calculated by the arithmetic mean of R_{ijk} for each can of the considered can-shape-group. Table 5 gives the values of $\bar{R}_{ijk}$ for each can-shape-group. Temperature response curves for the evaluation of the average temperature in rectangular cans are given in Figures 3 and 4.

As stated in the case of cylindrical cans, when L is larger than L_c, only the first term of the series (Eq. 9) affects the value of $\bar{u}$. When T_0 = 50°C and T_1 = 121°C, the critical value of L is L_c = 0.11. In this case, when L ⩾ 0.11, Eq. (13) can be applied whatever the shape factor is:

$$\bar{u} = 0.533 \exp(-\pi^2 L) \qquad (13)$$

During the cooling phase the dimensionless temperature [($\bar{T}$ –

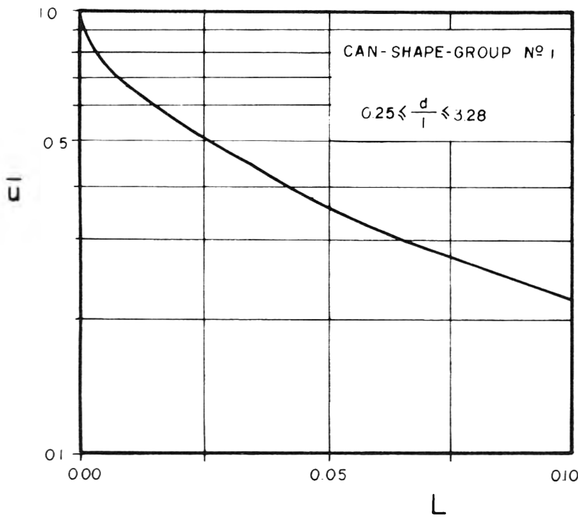

Fig. 1—Temperature response chart for can-shape-group No. 1 (0.25 ⩽ d/h ⩽ 3.28) of cylindrical cans.

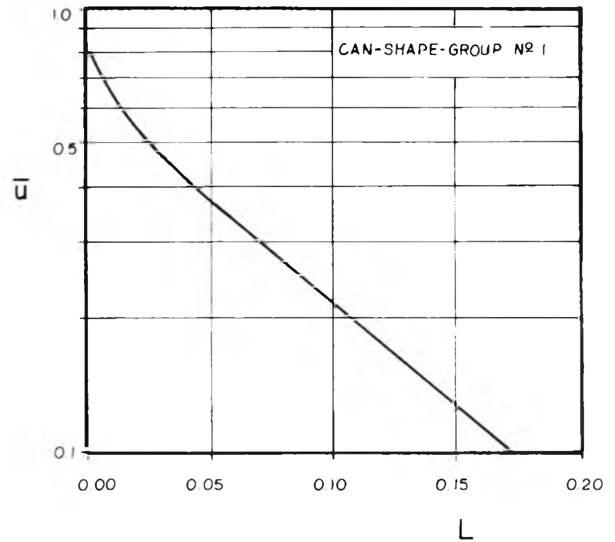

Fig. 3—Temperature response chart for the can-shape-group No. 1 of rectangular cans

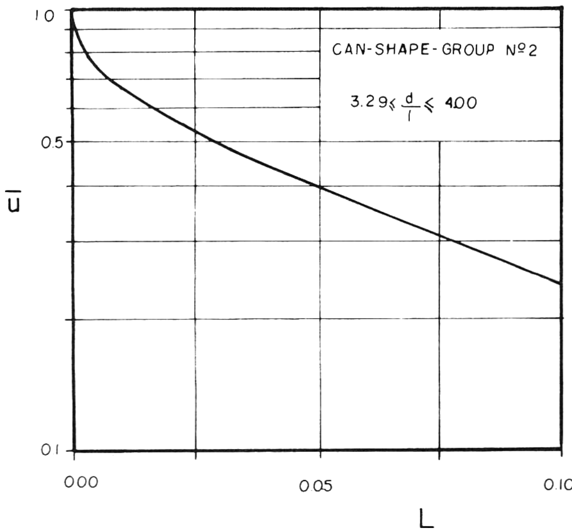

Fig. 2—Temperature response chart for the can-shape-group No. 2 (3.29 ⩽ d/h ⩽ 4.00) of cylindrical cans.

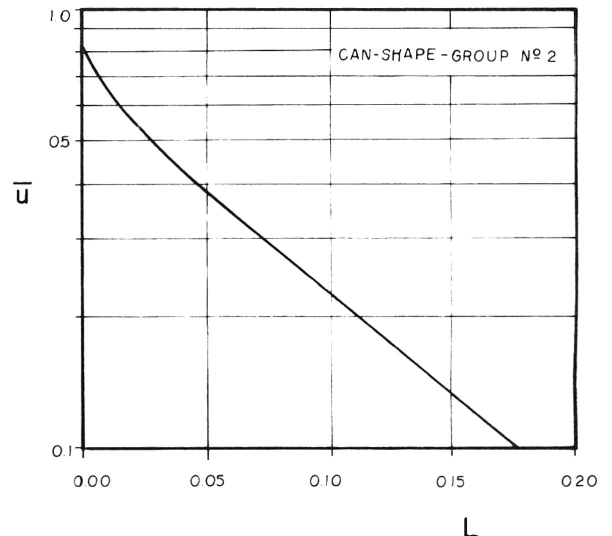

Fig. 4—Temperature response chart for the can-shape-group No. 2 of rectangular cans.

$T_w)/(T - T_0)]$ can be estimated by Eq. (14), whatever the can dimensions are (Hayakawa and Ball, 1969).

$$\frac{T - T_w}{T_1 - T_0} = \frac{T_1 - T_w}{T_1 - T_0} \cdot \bar{u}\,(L - L_b) - \bar{u}\,(L) \qquad (14)$$

Where L_b is the L value corresponding to the end of the heating phase.

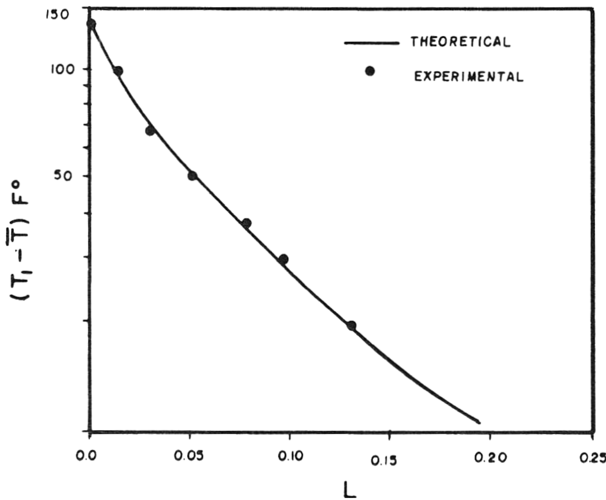

Fig. 5–Experimental and calculated average temperatures of 9.09% bentonite aqueous suspension in a 414 × 307 cans.

Table 4–Temperature history for the first and the last can of each can-shape-group (T_1 = 121°C and T_0 = 50°C)

Can-shape-group No.	B	C	L = 0.01	L = 0.03	L = 0.05	L = 0.07	L = 0.09	L = 0.11
			Temperature (°C)					
1	1.0	1.0	74.92	88.02	95.71[a]	101.00	104.94	107.98
	2.7	2.7	74.08	86.05	93.73	99.33	103.64	107.01
2	2.0	3.8	74.13	86.10[a]	93.70	99.25	103.52	109.56
	4.0	4.0	73.66	84.93	92.44	98.15	102.60	108.98

[a] Value of L where the maximum temperature difference was found

COMPARISON WITH EXPERIMENTALS RESULTS

FIGURE 5 shows the experimental values obtained by Hayakawa and Ball (1969) in tests carried out with a 9.09% bentonite aqueous suspension in a 414 × 307 can, and the average temperatures calculated by the method proposed in this paper.

We did not find, in the case of rectangular cans, experimental data to compare with the calculated values.

CONCLUSIONS

TO EVALUATE the average temperature in cylindrical cans by the temperature response charts proposed by Hayakawa and Ball (1969), we have to obtain a dimensionless parameter curve for the considered can shape factor. In this paper, the proposed classifications of cylindrical or rectangular cans into can-shape-groups considerably reduces the number of temperature response curves (two in each case), and the definition of average values of R_{ij} and R_{ijk} leads to an accurate estimation of the average temperature of thermally conductive food.

When the value of L is larger than a critical value, only one curve, in each case, is sufficient to evaluate the average temperature whatever the can dimensions are.

NOMENCLATURE

a,b,c	=	rectangular can dimensions (m)
d	=	cylindrical can diameter (m)
h	=	individual heat-transfer coefficient (kcl/h · m² ·°C)
J_o	=	zeroth order Bessel function of first kind
k	=	thermal conductivity (kcal/h · m · °C)
l	=	cylindrical can height (m)
m	=	dimensionless resistence ($m = hr_m/k$)
r_m	=	infinite slab characteristic dimensions, according to the heat flux direction (m)
$\bar{T}$	=	average temperature of conductive food in cylindrical and rectangular cans (°C)
T_c	=	temperature of food at zero time of heating (°C)
T_1	=	holding temperature of heating medium. If the heat treatment is carried out in a retort, T_1 is the retort temperature (°C)
T_w	=	temperature of cooling water (°C)
$\bar{u}$	=	dimensionless temperature of conductive food $[\bar{u} = (T_1 - \bar{T})/(T_1 - T_0)]$
$\bar{u}(L)$	=	same with $\bar{u}$

Table 5–Average values of R_{ijk} for the two can-shape-groups of rectangular cans (T_1 = 121°C and T_0 = 50°C)

Can-shape-group No.	$\bar{R}_{112}$	$\bar{R}_{113}$	$\bar{R}_{121}$	$\bar{R}_{122}$	$\bar{R}_{123}$	$\bar{R}_{131}$	$\bar{R}_{132}$	$\bar{R}_{133}$	$\bar{R}_{211}$	$\bar{R}_{212}$	$\bar{R}_{213}$	$\bar{R}_{221}$
1	1.816	3.447	3.224	4.040	5.671	7.672	8.488	10.119	5.960	6.776	8.407	8.184
2	1.528	2.583	1.859	2.387	3.442	3.577	4.105	5.160	7.613	8.141	9.196	8.472

Can-shape-group No.	$\bar{R}_{223}$	$\bar{R}_{231}$	$\bar{R}_{232}$	$\bar{R}_{233}$	$\bar{R}_{311}$	$\bar{R}_{312}$	$\bar{R}_{313}$	$\bar{R}_{321}$	$\bar{R}_{322}$	$\bar{R}_{323}$	$\bar{R}_{331}$	$\bar{R}_{332}$
1	10.631	12.632	13.448	15.079	15.871	16.696	18.328	18.105	18.920	20.522	22.553	23.368
2	10.055	10.190	10.718	11.773	20.840	21.367	22.423	21.697	22.226	23.282	24.417	23.944

R_{111} = 1; R_{222} = 9 and R_{333} = 25 whatever will be the family

$u(L - L_b)$ = formula obtained by entering $L = L - L_b$ in Eq. (3) and (9)

α = thermal diffusivity (m^2/h)

β_j = jth positive root of equation: $J_0(\beta) = 0$

$\lambda_i, \lambda_j, \lambda_k$ = ith, jth and kth positive root of equation cot $\lambda = 0$

θ = time variable (h)

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Ms received 5/19/75; revised 9/23/75; accepted 9/30/75.

The author acknowledges the valuable suggestions of Prof. Walter Borzani.

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SECONDARY DRYING OF DRUM-DRIED THERMOPLASTIC FOODS

ABSTRACT

An investigation was initiated to obtain data on basic drying rates for design of secondary driers which can handle fruit products between drum drying and final finishing. It was found that by using the staged-drying principle, it is possible to successfully drum dry foods that are low melting, thermoplastic, or heat sensitive. Rate data obtained in this investigation may now be used as a rational basis for the design of secondary driers to supplement drum-drying operations.

INTRODUCTION

DRUM-DRIED FOODS are well known–cereals, milk, starch, instant mashed potatoes, soup mixes, yeasts, and so on. Fruits are not drum dried because of serious drying problems that are encountered with materials whose solids are predominantly low-melting sugars. Such products become thermoplastic, if not molten masses, upon the drying surfaces and the doctor blades. The temperature attained by the product on the drums depends upon the heat transfer rate, the evaporation rate, and the concentration of the solubles that elevate the boiling point (or the temperature of evaporation). As the film dries on the drums, concentrations increase rapidly to saturation and product temperatures approach that of the drum surface. Thermal decompositions accelerate, as do Maillard-type reactions between amino acids and sugars (Gee et al., 1967), producing darkened color and modified flavor. In a drying time of only 15–30 sec on the drums, a sort of race develops between the drying and the deteriorative actions, which has resisted rationalization.

Earlier research (Lazar and Morgan, 1966; Lazar and Miers, 1971; Bolin et al., 1973) has shown that, in drum drying fruits, a high-velocity airstream used on the lower half of the drum surfaces, sweeping countercurrently across the drying film of product, accelerated drying, improved product peeling, and reduced heat damage. The recommended procedure of early product takeoff (at 5–8% moisture) and finishing drying in bins or conveyors with through-flow warm air was valid for many products, but with others, the still-damp sheet lacked friability to be flaked or ground for finishing drying. The patent literature describes some types of equipment for handling products leaving the drum drier (Eolkin et al., 1964; Lorant et al., 1961) but it is questionable if such equipment could perform well with thermoplastic fruit products. Little or no data on basic drying rates are available for design of secondary driers which can handle fruit products between drum drying and final finishing. An objective of this investigation was to obtain such data for drying these difficult-to-dry foods without the use of additives or drying aids, to facilitate rational design of secondary driers and other auxiliaries that might be required in post-drum-drying operations.

EXPERIMENTAL

THE FEED MATERIALS used in these tests were commercially-available pure concentrates (approx 30% solids content) of tomato, apricot, peach and pear. For ease in handling, all concentrates were diluted to 20% solids content before drying; feed was metered to the drum drier using a positive delivery feed pump with a variable speed drive. The drum drier was operated at a constant temperature in the range 290–300°F and was essentially as described in a previous publication (Lazar and Miers, 1971), except that the dehumidified collection zone was omitted in favor of a secondary drier (Fig. 1) for collecting data on drying rates of drum dried sheets of product. Drying-rate data were obtained from samples taken along the travel path of the sheet of product. Movement of the product through the unit was synchronized with the output of the drum drier take-off rolls. Temperatures and air in the unit were generally held at about 250°F. To obtain significantly high drying rates in the secondary stage, without creating additional zones of potential heat damage to the product, temperatures must be restricted to the range 240–260°F for the products tested. Air velocities were held below values that would make the product airborne, usually between 50–100 fpm, depending on sheet thicknesses. Dehumidified air was optional in the cooling zone. Drying was not continued below the level of moisture content necessary to obtain friability for crushing into flakes to finish-dry as previously described (Lazar and Miers, 1971).

Moisture analyses were run by a 16 hr, 65°C vacuum oven method. Values obtained by this method are comparable to those obtained on these products by the AOAC method (6 hr, 70°). Heat damage of products was evaluated by the water-soluble color method of Miers et al. (1971).

RESULTS & DISCUSSION

A DRUM DRIER (Fig. 2) consists of two internally-heated drums turning toward each other (at the top), to dip into the pool of feed puree contained between two close-fitting end plates. The gap G between the drums is adjustable. Under steady state operation, the deposit on the drums divides at the gap into two films, one on each drum, and each with initial thickness 0.5G. As the drums with diameter D rotate at R rpm, the films on each drum may shrink or puff as they dry, depending on the product, accounted for by a constant S. At the doctor blades, the product a thermoplastic mass, is removed by take-off rolls with diameter d, which in our work turn at r rpm, a surface speed considerably slower than the surface speed of the drum-drier rolls, allowing a substantial

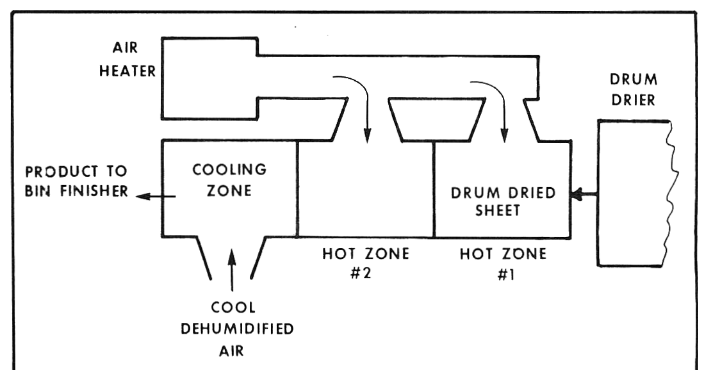

Fig. 1—Secondary drier used for measuring rate of drying of drum-dried sheets (schematic–plan view).

thickening of the sheet. In some cases, take-off rolls can be eliminated and the product can be transferred directly to the next stage drier; in this case, belt velocity can be selected to regulate sheet thickness as desired, providing sufficiently

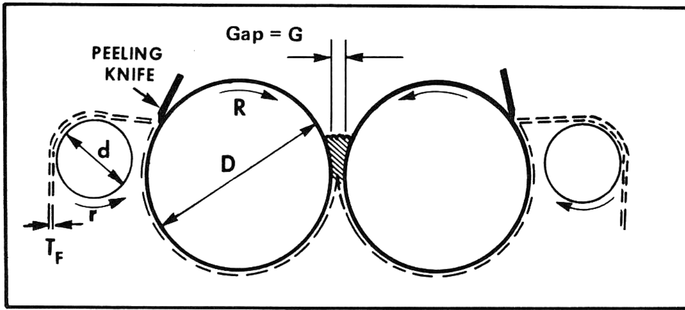

Fig. 2—Drum drier characteristics (schematic).

Table 1—Drying rates of drum dried sheet (Drum gap = 0.008 in.; finishing air temp 225–235°F)

Product	D.D. temp (°F)	Col. A Take-off rpm ratio[a]	Col. B Thick-ness ratio[b]	Col. C Drying rate % M/min	Col. D Function of moist. evap rate[c]
Tomato	290	3.15	7.2	0.78	5.62
Tomato	295	2.35	5.4	0.99	5.34
Tomato	290	2.00	4.6	1.23	5.66
Apricot	295	3.80	8.7	0.35	3.04
Apricot	295	2.80	6.4	0.33	2.11
Apricot	295	2.22	5.1	0.35	1.38
Peach	295	3.77	8.6	0.55	4.73
Pear[d]	299	1.32	3.0	0.82	2.46

[a] Ratio R/r
[b] Ratio of thickness of sheet after and before take-off (Col. A X 2.28) where D/d = 2.28
[c] Col. B X Col. C
[d] Required chilled air at doctor blades for suitable sheet formation (Lazar and Morgan, 1966)

strong and intact sheets produced by the drum drier. The final sheet thickness, T_F, is proportional to the ratio of the peripheral speeds of the drums and the take-off rolls, according to the equation $T_F = 0.5\ G\ S\ RD/rd$. For a given operation, the values G, S, D and d are constant, but the rotational speeds R and r may be varied independently to give the take-off ratio and final sheet thickness as desired, limited only by the inherent sheet-forming properties of the product dried. This procedure yields sheets *thickened* by as much as eightfold or more (Table 1), differing from practices of *stretching* the sheets on take-off (Lorant et al., 1961). Because of the tendency of thermoplastic products to show waves of compression and relaxation during take-off, these sheets show a rippled or crepe-paper-like structure which provides instant wettability during reconstitution. An additional benefit is that the flaked product has a higher bulk density than when prepared by the "stretch" procedure.

A typical drying curve in the secondary drier at 228°F is shown in Figure 3 for a sheet of tomato that was drum dried at 290°F, 3 rpm, take-off rpm ratio = 2.35 (sheet thickness 5.4X). The cooling zone was operated with ambient air It can be seen that the drying rate is about 1% moisture per min, but the pickup of moisture by the hygroscopic product in the cooling zone at ambient % RH, is almost as rapid. If dehumidified air, at 10% RH or less, was used in the cooling zone, moisture pickup was retarded sufficiently so that the dried product could be cooled and become friable for flaking for the final drying operation in fluid beds, without significant moisture pickup. When producing hygroscopic final products in locations where ambient relative humidity is excessive, it becomes obvious that cooling the final product with dehumidified air becomes mandatory.

The slopes (the drying rates) of the secondary drier drying curves are listed in Table 1 for tomato, apricot, peach and pear. Also shown are the ratios of increase of sheet thickness by variation of the take-off rpm ratio, and the (function of) evaporation rate. Tomato has increased drying rates as sheets become thinner, but absolute moisture removal rate remains fairly constant (column D); apricot exhibits relatively constant drying rates as sheet thickness varies, but the absolute moisture removal rate (column D) increases over twofold from the thinnest to the thickest sheets. This indicates the capacity advantage in operating secondary driers with thick films of

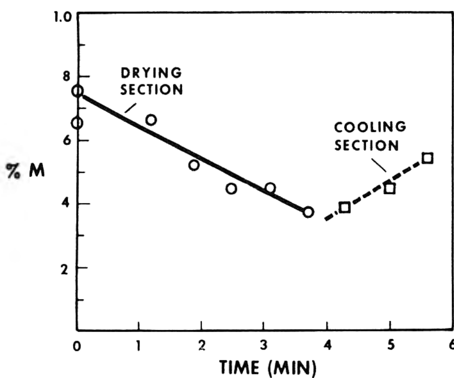

Fig. 3—Typical drying curve (% moisture vs drying time) for sheet of tomato in secondary drier, and moisture reabsorption during cooling. (Drum dried at 3 rpm, 290°F, gap 0.008 in.; secondary drier at 228°F.)

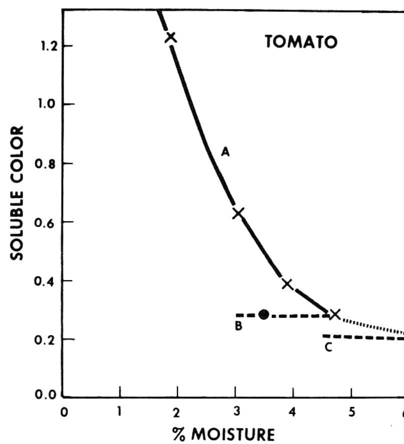

Fig. 4—Soluble (scorch) color development—tomato. (A. Drum dried only; B. Damp drum dried, plus secondary drier; C. Original feed paste, control.)

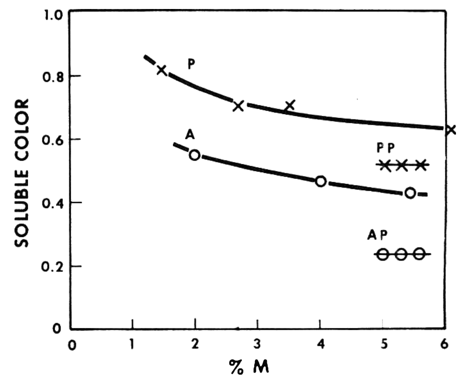

Fig. 5—Soluble (scorch) color development vs % moisture—peach and apricot. (P—peach; PP—peach puree, control; A—apricot; AP—apricot puree, control.)

apricot, but no similar advantage is found with tomato. Peach shows about 60% greater evaporative capacity than apricot at the same sheet thickness. Pear is also shown in the Table, but pear required chilled air at the doctor blade take-off for suitable sheet take-off (Lazar and Morgan, 1966).

The extractable color (an indication of heat damage) is shown for tomato in Figure 4 as a function of moisture content, indicating the desirability of halting the drying on the drums at a moisture content where little or no heat damage has developed, and continuing the drying operation by milder methods. Drum drying down to 3% M can increase color to 350% of the original feed paste value, whereas drum drying to about 5% M, a value of 150% of original is obtained. Drying from 5% M down to 3–3.5% M can be performed in a secondary drier without raising the soluble color value, and after flaking the product, it can be finish-dried in fluid beds as previously proposed, without heat damage problems.

Figure 5 shows similar extractable color values for apricot and peach, using the same color method as for tomato. Increases in color values are not as dramatic, even when drying down as low as 2%. Perhaps it is significant that the protein/sugar ratio for tomato is 2.5 times that for apricot or peach, enhancing Maillard-type reactions. Subjective examination indicates that undesirable heat damage has occurred in drying apricot or peach totally on the drum drier, more than indicated by the color value, changes that are avoided when the intermediate drier is used as described. Preliminary chromatographic tests on these dried products show promise for evaluating heat damage, which will be the subject of a later report.

In conclusion, we have found that by using the staged-drying principle, it is possible to successfully drum dry foods that are low melting, thermoplastic, or heat sensitive. Rate data obtained in this investigation may now be used as a rational basis for design of secondary driers to supplement drum-drying operations. By extrapolation, we would anticipate that similar benefits would be obtained using staged drying of starchy materials such as rice cereals, which have been drum dried but only with great difficulty, or in drying other foods that are easily heat-damaged, such as highly pigmented or delicately flavored foods.

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Ms received 8/20/75; revised 11/24/75; accepted 12/2/75.

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MICROWAVE HEATING OF FOOD MATERIALS AT VARIOUS ALTITUDES

ABSTRACT

Temperature differences, moisture losses and viscosity changes of food products prepared in a microwave oven at different elevations were determined. Experiments with apples, potatoes, squash, ground meat, scrambled eggs, custard sauce and soups were carried out at atmospheric pressures equivalent to sea level, 2500 ft, 5000 ft, and 7500 ft of elevation. Results indicated that altitude affects final temperatures and weight losses of foods heated in a microwave oven and has to be considered when microwave heating conditions are established for people living in the high altitude region of the U.S.

INTRODUCTION

FOOD PREPARATION with microwave energy is becoming increasingly more popular in homes, institutions and foodservice establishments. This method of cooking and baking offers conservation of time and energy as well as convenience to the user.

Differences in atmospheric pressure affect the sensory aspect of foods (Maga and Lorenz, 1972), rheological characteristics (Lorenz, 1973, 1974; Morrow and Lorenz, 1974) and overall quality of food products prepared by conventional food preparation procedures (Kulas, 1950; Lorenz et al., 1971).

Microwave food preparation appears to be similarly affected by varying atmospheric pressures. Bowman et al. (1971) determined the effects of cooking fresh and frozen vegetables in a microwave oven at 5000 ft of elevation on the color, flavor, tenderness, texture and overall acceptability. Microwave cooking times needed to be extended over those recommended by the manufacturer of the appliance for sea level application.

Meaningful altitude conversion factors, however, cannot be calculated because of differences in performance of microwave ovens from different manufacturers, the effect of oven load, type and shape of the food and the quality and maturity of the product at the time of cooking, all of which affect microwave heating times.

The difference in cooking times at different elevations is due to a linear relationship between atmospheric pressure and the temperature at which water boils. The boiling point of water decreases by about 1.8°F for every 1000 ft increase in elevation making it necessary to cook most food products for a longer time at higher altitudes to obtain the same tenderness, texture, and overall food acceptability which can be achieved in a shorter time under sea level conditions.

More than one-third of the United States is located in the high-altitude region (above 2500 ft of elevation) and although these areas are sparsely populated, more than 15 million people make their home there (Lorenz et al., 1971). A study was, therefore, conducted to determine temperature differences, moisture losses and viscosity changes of food products prepared in a microwave oven at different elevations.

MATERIALS & METHODS

Altitude chamber

The investigation was carried out in a special laboratory consisting of a steel cylinder 7 ft in diameter and 9 ft high. This laboratory can be ventilated, and temperature and humidity controlled. The atmospheric pressure can be adjusted and maintained to simulate conditions between sea level and 12000 ft of elevation. The experiments were carried out at atmospheric pressures equivalent to sea level, 2500 ft, 5000 ft and 7500 ft of elevation. The temperature and relative humidity inside the laboratory were maintained at 22°C and 48–50%, respectively.

Microwave oven

An Amana Radarange, Model RR-4D operating at 2450 mHz with a cavity 9 in. high, 14-1/2 in. long and 13 3/4 in. deep was used.

Food products

Foods were chosen that would be representative of those generally used in microwave food preparation. Foods varying considerably in moisture content were selected since moisture content of the food affects microwave cooking time to a much greater extent than conventional cooking time.

The following foods were prepared in the microwave oven at different elevations: (a) soup (split pea); (b) ground meat (extra lean); (c) apples (Rome); (d) potatoes (Idaho Russets); (e) squash (acorn); (f) eggs (scrambled); and (g) a custard sauce. A large enough supply of each food product was purchased at a time to conduct experiments at all elevations, which assured product uniformity. The apples, potatoes, and the squash were selected to be reasonably uniform in size and weight. The food products were kept at the same refrigeration temperature prior to testing in the microwave oven to assure the same initial temperature.

The following procedures, which were developed in preliminary experiments, were used.

Apples (Rome), potatoes (Idaho Russets), squash (acorn), ground meat (extra lean). Apples (avg wt 197g) were placed in a pint-size pyrex dish and heated for 4 min. Potatoes (avg wt 289g) were heated for 7 min. A whole acorn squash (avg wt 553g) was placed into a pyrex dish and heated for 12 min. Ground meat (125g) was placed into a 400 ml beaker and heated for 2 min 45 sec. The temperature of all food products were recorded before and after microwave heating to determine temperature differences due to elevation. Weight losses due to heating at varying atmospheric pressures were determined by weighing all food products before and after microwave cooking.

Soup (split pea). A can of split pea soup and one can of water were blended according to label directions, and 200g of this soup were poured into a 250 ml beaker. The temperature of the soup was recorded and initial consistency determined with a Brookfield viscosimeter at 20 rpm using spindle #2. The weight of the soup was adjusted to 200g, compensating for losses during consistency determinations, and heated in the microwave oven for 1 min 30 sec. The temperature of the soup was recorded immediately upon removal from the microwave oven. Weight loss was determined by reweighing the beaker containing the soup after microwave heating. Consistency of the soup was measured as soon as the soup had cooled to 120°F.

Eggs and sauces. Scrambled eggs were prepared by beating 2 eggs, 30 ml of milk and 1g salt with a hand rotary beater for 15 sec at medium speed. The temperature of the scrambled eggs was recorded and 100g were poured into a 250 ml beaker and heated for 2 min 15 sec. Immediately upon removal from the microwave oven the temperature of the scrambled eggs was recorded and weight loss determined by reweighing the eggs.

A custard sauce was prepared by blending 4 eggs with 500 ml of milk, 50g of sugar and 1g of salt using a hand rotary beater for 30 sec at medium speed. The temperature was recorded and 175g of the sauce poured into a 250 ml beaker and heated in the microwave oven for 3 min 45 sec. Immediately upon removal from the microwave oven the temperature was measured and weight loss determined by reweighing the sauce.

Table 1–Effect of elevation on temperature difference and weight loss of foods prepared with microwave energy[a]

	Elevation (ft)	Apples	Potatoes	Squash	Ground meat	Eggs	Custard sauce
Avg temp difference	sea level	134.5	132.1	138.7	151.3	136.1	150.5
(initial-final temp)	2500	133.2	132.9	135.2	144.9	132.0	143.8
(°F)	5000	126.0	123.2	126.1	137.1	126.1	135.8
	7500	131.0	124.2	128.6	138.3	129.6	132.3
LSD – (°F)	–	1.82	2.44	3.19	2.76	2.55	2.69
Avg wt loss	sea level	30.0	50.6	106.5	18.1	15.6	19.2
(grams)	2500	28.6	48.1	103.9	17.7	14.4	20.6
	5000	25.0	52.9	104.0	19.7	10.3	22.5
	7500	22.5	52.8	106.4	20.0	16.0	24.8
LSD – (grams)	–	1.53	2.68	–	0.60	0.71	1.37
Avg wt loss	sea level	16.2	20.1	19.8	14.5	15.6	11.0
(%)	2500	15.0	16.0	18.6	14.2	15.4	11.8
	5000	10.9	17.3	18.8	15.7	10.3	12.9
	7500	12.3	17.8	18.9	16.0	16.0	14.2

[a] Results represent the averages of 10 separate trials at each elevation.

Experimental design

Ten trials were conducted with each food product at each elevation (sea level, 2500 ft, 5000 ft and 7500 ft) to obtain sufficient data for statistical analyses of the results.

The 10 replications with each food product at each elevation were not conducted at one time with pressure set at one level, but rather in a random fashion over a 12-month period to demonstrate with these 10 measurements the repeatability of the experimental results.

Statistical evaluation of data

The experimental data were analyzed by one-way analyses of variance. Whenever significant F values were obtained, LSD values were calculated and multiple comparison plots constructed as described by Carmer and Swanson (1973).

RESULTS

Temperature differences and weight losses

The effects of elevation on temperature differences of foods heated in the microwave oven are shown in Table 1. The temperatures of foods, heated for a fixed period of time, decreased as elevation increased from sea level to 5000 ft. Product temperature then increased again as the elevation reached 7500 ft. Custard was the only exception showing a steady decrease in temperature as elevation increased.

Weight losses, mainly due to loss of moisture, are shown in Table 1. The trends were similar as observed for temperatures. The losses decreased as elevation increased up to 2500 or 5000 ft depending on the food under consideration and then increased again at higher elevations. Only the custard showed a steady increase in weight loss as elevation increased from sea level to 7500 ft.

The results of preparing soups in the microwave oven at different elevations are summarized in Table 2. The average temperature difference between the initial and final product temperature after heating in the microwave oven for a given length of time and the weight loss decreased with increasing elevation up to 5000 ft as observed with apples and scrambled eggs, but then increased at 7500 ft. Final soup consistency values reflect moisture losses during microwave heating. Consistency values (cps) at 20 rpm decreased up to an elevation of 5000 ft and then increased at higher elevations.

Statistical evaluations

The statistical evaluations of the experimental data are presented in the form of multiple comparison plots in Figures 1 and 2. Figure 1 shows temperature difference confidence intervals while Figure 2 shows weight loss confidence intervals. In these plots two half confidence intervals must not overlap to be significant at the alpha level.

The ground meat exhibited significant differences ($\alpha = 0.05$) in temperatures and in weight losses due to heating at different elevations as determined by one-way analyses of variance. The relationship between altitude and temperature was linear. There was a linear, but also a slight cubic relationship between altitude and weight loss.

Since it was impossible to obtain potatoes which were exactly the same weight, an analyses of the effects of potato weight on product temperatures and weight losses was necessary. This analysis indicated that the observed differences were independent of the weight of the potatoes used. Differences in weight losses and product temperatures due to elevation were significant ($\alpha = 0.05$). The relationship between weight loss or temperature of the food product and altitude was predominantly linear. The plot, however, also indicated a slight cubic relationship.

The difference in the weights of the apples used has no effect on the results of these experiments. Temperature differences and weight losses due to altitude were significant statistically at the 5% level. The relationship between weight loss and elevation was linear. Both a linear and a slight cubic relationship was indicated between temperature and altitude.

The temperature differences and weight losses observed for acorn squash heated in the microwave oven at different elevations were independent of the weight of the squash used. The

Table 2–Effect of elevation on the final temperature, weight loss and consistency of soup heated in a microwave oven[a]

Elevation (ft)	Avg final temp (°F)	Temp difference (initial-final, °F)	Avg wt (%) loss	Avg wt (grams)	Avg final consistency (CPS) 20 rpm
Sea level	154.0	81.5	1.49	2.98	470
2500	147.0	73.8	1.40	2.81	402
5000	142.5	66.7	1.06	2.13	359
7500	144.7	71.5	1.16	2.32	383

[a] Results represent the averages of 10 separate trials at each elevation. LSD – Temperature (°F) = 4,14; LSD – Weight Loss (grams) = 0.37

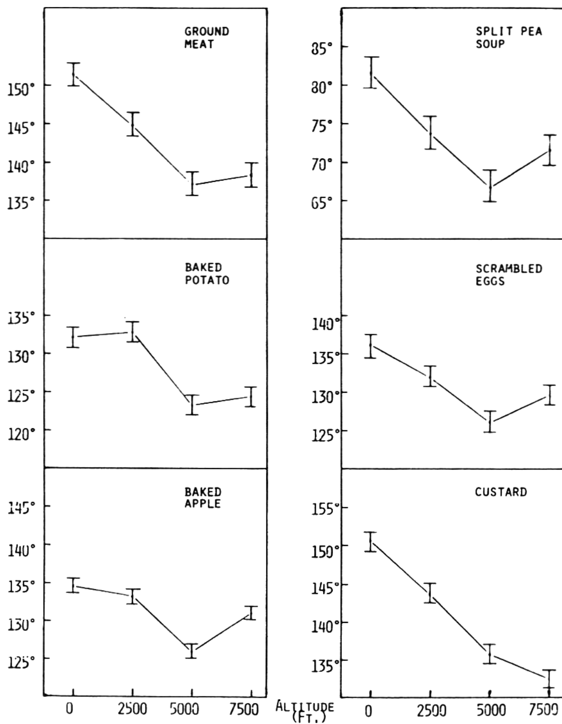

Fig. 1—Multiple comparison plots of temperature differences (°F) of microwave heated foods (α = 0.05).

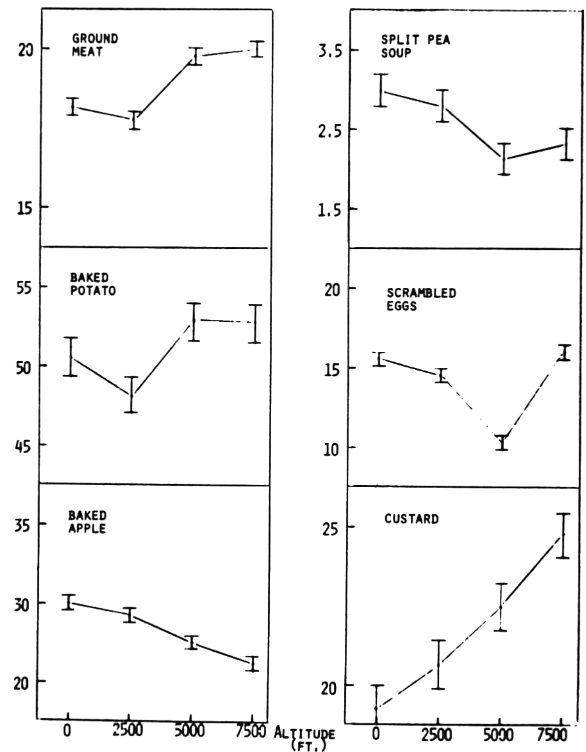

Fig. 2—Multiple comparison plots of weight losses (grams) of microwave heated food (α = 0.05).

multiple comparison plot, which is not shown in Figure 1, indicated a linear relationship between temperature and altitude. The differences were significant at the 5% level. Losses in weight due to heating at different elevations, however, were not significant. This is really not surprising considering that a whole squash was heated each time. The tough skin of the squash prevented large enough differences in moisture loss which might have been significant statistically.

Custard showed significant differences in temperature and weight loss due to elevation. These relationships were linear.

Elevation produced significant differences in temperatures and weight losses heating scrambled eggs in the microwave oven. While the relationship between altitude and temperature was linear, that between altitude and weight loss was cubic as the multiple comparison plot indicates.

The differences in temperatures and weight losses of split pea soup due to elevation were significant ($\alpha = 0.05$), but not the differences in consistency. The consistency values, however, show a decreasing trend with higher elevations indicating a more viscous product. The relationship between temperature and elevation was linear.

Attempts to find a relatively simple mathematical equation, which would express all the observed changes due to elevation failed. This is really not surprising considering the differences in food products, initial moisture content, size, weight and heating time in the microwave oven of the food evaluated.

DISCUSSION

IT IS REALIZED that microwave heating does not proceed by conduction as heating by conventional methods but rather as the results of molecular excitation of charged particles, especially water. Since microwaves have great penetrating power, a food product being heated has hardly any heat gradient, although measurements of the temperature distribution in an item in a microwave oven revealed that the surface was slightly cooler than the area below the surface (Anon., 1970). This is caused by radiation of heat from the food surface to the cooler surroundings of the microwave oven. A heat equilibrium, however, is reached in a similar way by microwave heating as by conventional heating. Conventional heating of food is affected by altitude and one might expect that this is also true for microwave cooking even though cooking times are much shorter.

Elevation has been shown to affect the temperature which is reached when a food product is heated in a microwave oven for a fixed period of time. The average difference between the initial and final product temperature after heating in the microwave oven for a given length of time decreased as elevation increased from sea level to 5000 ft of elevation and then decreased at 7500 ft of elevation. The lower temperature differences at reduced atmospheric pressures are due to the lower boiling point of water and the faster rates of evaporation as elevation increases.

At reduced pressures, evaporation takes place faster provided the temperature is maintained constant, because of the reduced obstruction offered by the air molecules. Investigations on distilled water at a constant temperature of 90.9°C, evaporating into a room whose relative humidity was 38% ± 2% at various altitudes, showed that the rate of evaporation increased 15% at 5000 ft and 29% at 8000 ft above that at sea level, although the boiling point was never reached (Vaillant, 1910).

The increased speed of evaporation at high altitudes causes the temperature in foods being baked or cooked to drop, because not enough heat can be supplied to maintain that rate

(Barmore, 1936). In microwave cooking this reduction continues until the temperature has been lowered to a point where the microwave energy absorbed by water evaporating again equals the microwave energy transferred to the food product. Therefore, other things being equal, the higher the altitude the lower the temperature of an evaporating solution, or food products, even though the boiling point is not reached.

With microwave heating this is true up to an elevation of 5000 ft. At 7500 ft the rate of evaporation, which is influenced by both temperature and atmospheric pressure becomes the predominant factor, counteracting the decrease in temperature of microwave heated products with altitude and causing a greater moisture loss which coincides with an increase in temperature.

Moisture has a direct bearing on the microwave heating rate of a food product. For example, a food product of a fixed size and weight containing 50% water will take less time to heat to a specific temperature than one containing 75% water, or if they are both heated for the same length of time, the former will reach a higher temperature than the latter (Anon., 1970). Since more moisture was lost as elevation increased to 7500 ft, the lower moisture content of the food will absorb all of the microwave energy permitting the temperature to increase again at the greater altitude.

The food products evaluated in this study behaved slightly differently when heated in the microwave oven at different elevations. This, however, should not be surprising considering the differences in initial moisture content, size, weight, and heating time in the microwave oven.

CONCLUSIONS

ALTITUDE has been shown to affect final temperatures and weight losses of foods heated in the microwave oven and has to be considered when microwave heating times are established for the people living in the high altitude region of the U.S.

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Ms received 8/18/75; revised 11/21/75; accepted 11/24/75.

Scientific Paper No. 2090 of the Colorado State University Experiment Station.

The authors thank Dr. T. Boardman of the CSU Statistical Laboratory and Dr. A. Frey of the Dept. of Agricultural Engineering for the statistical evaluation of the data.

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INHIBITORY CAPACITY OF SODIUM BENZOATE FOR NITRATE-INDUCED CORROSION OF TIN PLATE

ABSTRACT

The efficiency of adding three levels of sodium benzoate (100, 1000, 2500 ppm) in inhibiting corrosion of tin by different organic acid solutions at pH = 3.5 containing 125 ppm nitrate, was tested in canned model packs experiments. Model pH = 3.5 solutions consisting of 1% citric acid, or 1% malic acid, or a mixture of 0.8% citric acid + 0.1% malic acid + 0.1% oxalic acid, and containing 15% sucrose, 500 ppm ascorbic acid and 125 ppm NO_3^-, were compared with and without the addition of sodium benzoate. The decrease in the nitrate concentration and the increase in the concentration of tin and iron were followed in the canned model solutions throughout suitable storage periods at room temperature. The amounts of dissolved tin and iron were taken as corrosion criteria. Inhibitory efficiency was expressed as the ratio of corrosion rates in solutions with and without the addition of an inhibitor. Results showed that sodium benzoate gave incomplete protection against corrosion of tin by nitrate. Addition of sodium benzoate, e.g., at 1000 ppm, showed that it is most inhibitive in citric acid, less so in malic acid, and least in the 1% citric-malic-oxalic acid mixture.

INTRODUCTION

THE ACCELERATING EFFECT of nitrates on the dissolution of tin observed in tin plate cans of acid products has become a major cause of concern in the last 20 yr (Evans, 1971; Farrow et al., 1969; Horio et al., 1964/65, 1966, 1968; Strodtz and Henry, 1954).

The seriousness of the economic and toxicological consequences of the problem led to much research all over the world in an effort to find methods of reducing the effect of nitrate and to define its reaction mechanism (Board and Holland, 1969; Farrow et al., 1970, 1971; Sherlock and Britton, 1973).

When considering a natural medium like food, two possible reactions must be emphasized: by the presence of reducible compounds which would increase the cathodic kinetics, thus increasing corrosion rates; and by substances with inhibitory action which, in polarizing the anodic reaction, would decrease corrosion rates.

The present investigation is part of a study (Albu-Yaron and Semel, 1976) to determine the influence of each of the natural chemical components in a particular food, e.g., organic acids, oxidizing or reducing components as well as the permitted chemical substances added to preserve foods, on the corrosion induced by nitrates, as a preliminary to a search for inhibitors.

Benzoic acid and its sodium or potassium salts are widely used as chemical preservatives in the food preparation industry at the maximum permitted level of 0.1% (Borgstrom, 1968). Benzoic acid also occurs naturally in cranberries, logan berries, some blackberries, European plums and green gage plums, and sometimes in quantities exceeding the maximum limit (0.1%) permitted in the canning industry. Only the undissociated benzoic acid molecules are active in the germicidal action; the sodium salt, being more soluble than the acid form in water, is preferred for industrial use. Its optimum pH range varies between 2.5 and 4.0. Although the effects of sodium benzoate in inhibiting the corrosion of iron (Davies and Slaiman, 1971, 1973) or steel (Brasher and Mercer, 1968) have been widely investigated, its effectiveness in inhibiting tin plate corrosion has not yet been studied.

In this experiment the effect of the addition of three different levels of sodium benzoate (100, 1000 and 2500 ppm) on the dissolution of tin in plain tin plate cans containing various organic acid solutions (1%) with added sucrose (15%) and ascorbic acid (500 ppm) at pH = 3.5 and 125 ppm NO_3^- level, has been investigated.

EXPERIMENTAL

Materials

Solutions were prepared from chemicals of analytical reagent quality and deionized water (< 0.22 ppm NO_3^-). To solutions of 15% sucrose, additions of organic acids, ascorbic acid, sodium benzoate and sodium nitrate were made as required. Adjustment of the pH to 3.5 was accomplished by the addition of small amounts of 0.1M sodium citrate or sodium malate before making up to the mark with deionized water. The amounts of the acid salts required to bring the solutions to this pH value were not significant.

The organic acids chosen for the experiment were citric acid (0.15N), malic acid (0.15N), and a mixture of 0.12N citric acid, 0.015N malic acid and 0.022N oxalic acid (total acid concentration of 1%). Ascorbic acid was added to give a 500 ppm concentration. Sodium nitrate was used at a concentration of 2×10^{-3}M. The solutions investigated were 100, 500, 1000 and 2500 ppm sodium benzoate. Control packs lacking sodium benzoate were prepared similarly. Table 1 gives the exact compositions of the model solutions used.

Table 1—The composition of pH = 3.5 model solutions with added sucrose (15%) and ascorbic acid (500 ppm) at 125 ppm nitrate level

Organic acids			
Citric (%)	Malic (%)	Oxalic (%)	Sodium benzoate (ppm)
1	–	–	–
1	–	–	100
1	–	–	500
1	–	–	1000
–	1	–	–
–	1	–	100
–	1	–	1000
–	1	–	2500
0.8	0.1	0.1	–
0.8	0.1	0.1	100
0.8	0.1	0.1	1000
0.8	0.1	0.1	2500

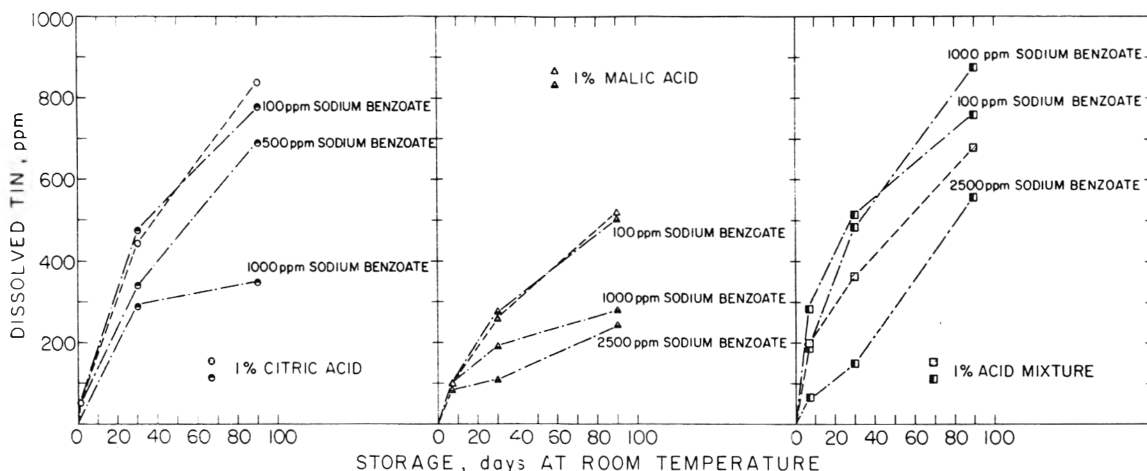

Fig. 1–Effect of sodium benzoate in organic-acid solutions (pH 3.5) containing 125 ppm NO_3^-, 15% sucrose and 500 ppm ascorbic acid on tin plate can dissolution, as a function of storage. (Shaded points indicate zero level sodium benzoate).

Experiments were made with 100g, 202 × 202 plain cans of electrolytic tin plate having a free-tin coating weight of 0.82 lb/bb, an alloy coating weight of 0.11 lb/bb, and an ATC value of 0.11 $\mu A/cm^2$, manufactured by the Israel Can Company.

Analytical methods

Nitrite and nitrate were determined by the method previously described by Green (1970) and Fudge and Truman (1973). The method uses sulfanilamide and N-(1-Naphthyl)ethylene diamine and involves the formation of a colored complex with nitrite and its subsequent extinction measurement at 540 nm. The nitrate determination required a preliminary reduction step to nitrite using a cadmium column. The same method has been developed for use with the Technicon Auto-Analyzer (Henriksen and Selmer-Olsen, 1970) and lower concentrations of nitrate and nitrite were determined by means of this instrument. Standards of nitrate and nitrite were prepared in solutions of the same composition as model solutions. All analyses were carried out in duplicate. The minimum detectable amounts were 0.05 ± 0.02 ppm nitrate and 0.03 ± 0.02 ppm nitrite.

Tin and iron were determined by atomic absorption analysis performed on a Pye Unicam model SP-90 atomic absorption spectrometer using an air-acetylene flame with an air-flow rate of 5 liter/min and acetylene flow rates of 1.4 liter/min and 1.7 liter/min for iron and tin, respectively. The iron and tin responses were measured at wavelengths of 248.3 nm and 286.3 nm, respectively. In order to avoid errors resulted from interferences of various ions composing model solutions, [Roos and Price (1971) reported that citrate ions suppress the absorbance of iron in atomic absorption spectrometry with air-acetylene flame], standards of iron and tin ions were prepared in solutions of the same composition as the model solutions used throughout the work. All analyses were carried out in duplicate. The minimum detectable amount of iron was 1 ± 0.1 ppm and of tin 25 ± 5 ppm.

Tin coating thickness was determined by the coulometric method of Kunze and Willey (1952). ATC determinations were made by a standard instrument constructed in accordance with ASTM A623 (1973).

Procedure

Cans were filled with model solutions at 90°C. After filling (with minimum head space) the cans were closed and processed for 10 min in water at 82–85°C. After water cooling, the cans were stored at room temperature. At 1-, 7-, 30-, 90- and 180-day examinations, two cans from each treatment were opened and the contents analyzed for dissolved tin and iron, nitrate, nitrite, sucrose and pH. Vacuum was determined before each opening. The presence or absence of corrosion was noted after opening and the residual internal tin-coating weight was determined by the coulometric method. All determinations were carried out in duplicate.

RESULTS & DISCUSSION

THE EFFECT of sodium benzoate in reducing the contribution of nitrate to corrosion of tin was explored at different concentrations and for different solutions at pH = 3.5, on a total of 150 samples, over a storage interval of 3 months.

Results are presented in Figures 1 and 2 for room temperature storage. Results are expressed as dissolved ppm tin (Fig. 1) and as change in nitrate concentration (Fig. 2) during storage.

The shape of the curves obtained for the three levels of

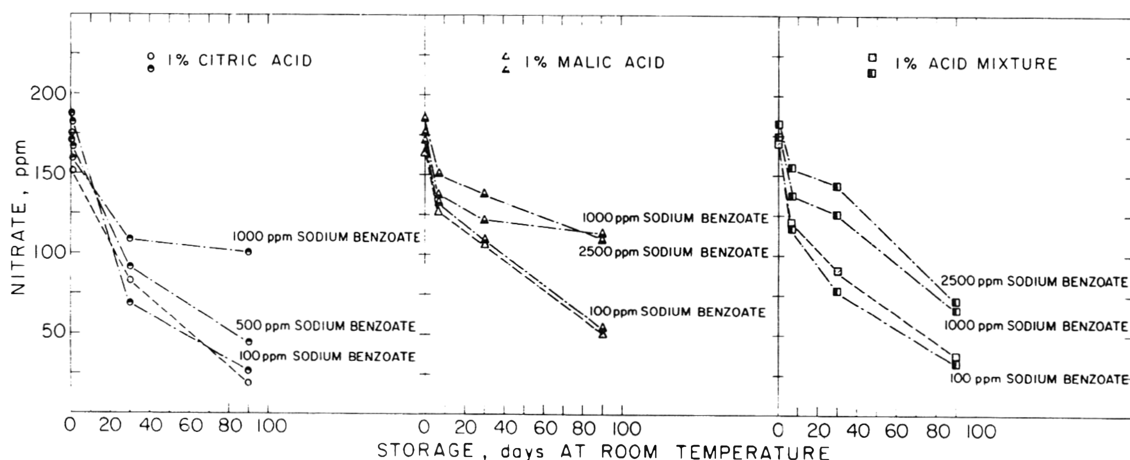

Fig. 2–Decrease of NO_3^- from an initial 125 ppm level during detinning in organic acid solutions (pH 3.5) containing 15% sucrose, 500 ppm ascorbic acid and three levels of sodium benzoate, as a function of storage. (Shaded points indicate zero level sodium benzoate.)

Table 2—Degree of inhibition of nitrate-induced corrosion of tin in acid solutions (pH = 3.5) at three levels of sodium benzoate

Model solutions containing[a]	K^b		
	Sodium benzoate (ppm)		
	100	1000	2500
Citric acid	1.02	2.3	–
Malic acid	1.04	1.73	1.85
Citric acid + malic acid + oxalic acid	1.89	0.77	1.17

[a] See Table 1 for the exact composition
[b] The ratio of corrosion rates in solution without and with sodium benzoate addition.

sodium benzoate with citric acid and malic acid showed an initial rapid detinning reaction up to 1 month, followed by a slower rate during later storage. By increasing the level of sodium benzoate, its inhibitory action was improved: up to 500 ppm, the shape of the curves was similar to that obtained with solutions without sodium benzoate, but 1000 ppm and 2500 ppm changed its shape. The shape of curves obtained in 1% acid mixture showed an increasing rate of detinning over the 3-month period of storage, for all three levels of sodium benzoate.

Measurements of corrosion in 0.15N citric acid at pH = 3.5 containing 125 ppm NO_3^- showed inhibition to be 2.5% for 100 ppm, 13.7% for 500 ppm, and 56.2% for 1000 ppm additions. In 0.15N malic acid at the same nitrate level (125 ppm), inhibition was 4% for 100 ppm, 44% for 1000 ppm, and 48% for 2500 ppm additions. In the 1% acids mixture (0.125N citric acid, 0.015N malic acid, 0.022N oxalic acid) containing 125 ppm NO_3^-, sodium benzoate at levels up to 1000 ppm appeared to stimulate corrosion, but 2500 ppm gave moderate inhibition (15%).

In Table 2, the values calculated for the degree of inhibition on nitrate-induced tin plate corrosion of sodium benzoate expressed as the ratio of corrosion rates in solution without and with inhibitor addition are shown for the three model solutions studied at pH 3.5. Although the 1% malic acid containing 500 ppm ascorbic acid and 15% sucrose appeared to be 35% less corrosive than the 1% citric acid containing the same additives and at the same nitrate level (125 ppm NO_3^-), addition of sodium benzoate, e.g., at 1000 ppm level, showed that it is most inhibitory in citric acid, less so in malic acid and least so in the 1% citric-malic-oxalic acid mixture.

The relevant corrosion curves (Fig. 1) show too that sodium benzoate gave incomplete protection against corrosion of tin by nitrate; even at the 2500 ppm level, more than 250 ppm dissolved tin was found after only 3 months' storage in all three model solutions.

Nitrate concentrations (Fig. 2) changed at the same rate as the tin corrosion rate; thus, changes in nitrate concentration were faster in cans with low benzoate levels than in those with high levels.

The quantity of dissolved iron did not vary markedly between treatments. Variations were from 1–4.5 ppm, showing that at this nitrate level the acid solutions containing sodium benzoate dissolve mainly tin. In 1% malic acid solution the corrosion of iron increased with increasing sodium benzoate concentration, e.g., at concentrations that inhibited nitrate-induced detinning. In 1% citric acid solution and in the 1% acid mixture, there were no differences in the rate of iron corrosion with increasing levels of sodium benzoate.

Nitrite was not detected in any of the cans. Can vacuum did not change appreciably during storage. Examination of the internal appearance of the cans showed progressive detinning.

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Ms received 6/23/75; revised 10/9/75; accepted 10/15/75.

Contribution from the Agricultural Research Organization, The Volcani Center, Bet Dagan, Israel. 1975 Series, No. 180 E. This research was financed in part by a grant from the National Council for Research and Development, Jerusalem, Israel.

Our appreciation is expressed to Mrs. Aviva Berzin for her assistance in the laboratory work.

RESEARCH NOTES

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A Research Note
PRODUCTION OF LIMONOATE DEHYDROGENASE BY Pseudomonas

ABSTRACT

The growth conditions of *Pseudomonas* sp. 321-18 were studied to maximize yields of limonoate dehydrogenase, which prevents the development of limonin bitterness in citrus juices. The organism grew well on 0.5% or less limonoate media, but it did not grow on media containing 1.0% or more limonoate. Galactose and fructose were excellent substrates for its growth, but sucrose and glucose were found to be poor. The organism grew best at 25°C and pH 7–7.5. Cells grown on substrates other than those containing limonoids did not produce limonoate dehydrogenase, but the enzyme was induced with low concentrations of limonoids. Enzyme activity was increased five- to sixfold by use of the induction method on galactose grown cells.

INTRODUCTION

DESPITE the abundance of high-quality navel oranges each year, very little unblended juice of navel orange is consumed. This low consumption is due to the fact that the juice from the fruit becomes bitter soon after its extraction. This phenomenon is generally referred to as delayed bitterness (Kefford, 1959; Joslyn and Pilnik, 1961). It has been shown that development of the bitter principle in the juice is due to the conversion of nonbitter limonoate A-ring lactone to intensely bitter limonin (Maier and Beverly, 1968; Maier and Margileth, 1969).

In dealing with this bitterness problem, we have attempted, by one approach, to develop an enzymic debittering process or an enzymic process which prevents the formation of the bitter principle in the juice and other processed products. The search for microbial enzymes has resulted in the isolation of four limonoid-metabolizing enzymes (Hasegawa et al., 1972; 1974a, b). Among them, two limonoate dehydrogenases from *Pseudomonas* and *Arthrobacter* have been found to prevent limonin formation by converting limonoate A-ring lactone to nonbitter 17-dehydrolimonoate A-ring lactone, thereby preventing delayed bitterness.

The effectiveness of the two enzymes in debittering navel orange juices (fresh and processed), citrus seed meal, and citrus peels has been demonstrated (Hasegawa et al., 1973; Brewster et al., 1975). The limonoate dehydrogenase of *Pseudomonas* sp. 321-18 is of particular interest since it functions better in the low pH range of the natural juice than does that of *Arthrobacter globiformis* (Brewster et al., 1975).

We report a study of the production of limonoate dehydrogenase by *Pseudomonas* sp. 321-18 under various conditions.

EXPERIMENTAL

Assay methods

Limonoate dehydrogenase activity was assayed by following the increase in optical density due to the formation of NADPH at 340 nm. Activity was assayed in 1 ml of a reaction mixture consisting of 0.1M Tris buffer (pH 8.0), 1×10^{-2}M limonate, 5×10^{-4}M $NADP^+$, 1×10^{-3}M KCN and an appropriate amount of enzyme. Changes in optical density at 340 nm were measured at 23°C in a standard silica cuvette of 1-cm light path with a Varian Techtron 635 spectrophotometer. Under the above conditions, one unit of enzyme activity was defined as "that amount which catalyzes the formation of 1 mμ mole of NADPH per minute."

Growth of cells

The growth substrate, 200 ml of a mineral salt medium (Saunders et al., 1948) and the carbon source of interest, was placed in a 2-l Erlenmyer flask and inoculated with 3 ml of a 48–72 hr culture of *Pseudomonas* sp. 321-18. Incubation was at 25°C on a shaker, and growth was followed by measuring the increase in optical density of the medium at 400 nm. After incubation, cells were harvested by centrifugation at 20,000 × G for 10 min, washed with cold 0.1M phosphate buffer (pH 7.0), and frozen until used for enzyme analyses.

Preparation of enzyme

All operations were carried out in an ice bath. Frozen cells obtained from 200 ml of medium were suspended in 50 ml of 0.1M phosphate buffer (pH 7.0) containing 10^{-3}M dithiothreitol, and disrupted in a Rosset flask with a Branson sonifier, J-22. The supernatant from centrifugation at 20,000 × G for 10 min was brought to 0.9 saturation with $(NH_4)_2SO_4$ by the addition of the solid salt with continuous stirring. The mixture was centrifuged at 20,000 × G for 15 min, and the precipitate dissolved in a measured amount (20–25 ml) of 0.01M phosphate buffer (pH 7.0) and used as an enzyme source for the estimation of the activity.

Preparation of grapefruit seed meal extract

Dried seeds were finely ground with a Comminuting Machine, Model D, Fitz Mill, and the meal was extracted by refluxing with petroleum ether. The residue was suspended in water (10%, w/w) and autoclaved for 15 min at 250°F. The mixture was filtered, and the filtrate which contained about 400 ppm of limonoate and a few ppm of other limonoids mainly nomilin and obacunone, was used as the "grapefruit seed meal extract" (GSME).

RESULTS & DISCUSSION

Growth on limonoids

The strain of *Pseudomonas* sp. 321-18 isolated from soil grew well on a limonoate or limonin medium, and produced limonoate dehydrogenase intracellularly (Hasegawa et al., 1974a). When grown on 0.1, 0.3, 0.5 and 1.0% of limonoate, the organism grew best on a 0.3% medium; optical density of the medium reached 2.30 after 78 hr. On a 0.5% medium, the lag phase was 25–30 hr longer than it was on a 0.1 or 0.3% medium, but the growth eventually reached the maximal level equivalent to or slightly less than that of 0.3% medium after 120 hr of incubation. The organism did not grow at all on media containing 1.0% or more limonoate. The reason for this is not clear. The mineral salt medium used contained nutrients sufficient to support greater growth. For instance, when the

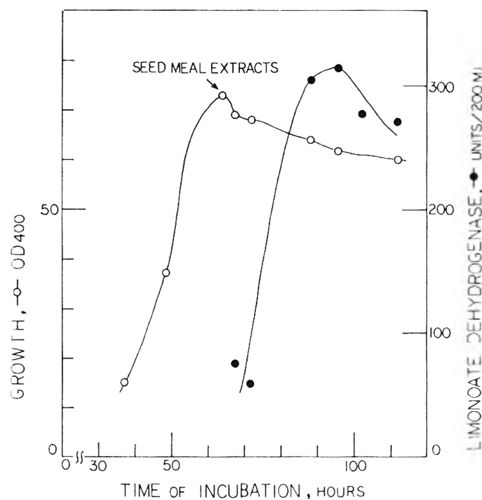

Fig. 1—Induction of limonoate dehydrogenase of Pseudomonas sp. 321-18 with grapefruit seed meal extracts. The organism was grown on a 4% galactose medium under the conditions described in the text. After 64 hr of incubation, water extracts of grapefruit seed meal (0.75%) were added to induce enzyme activity. Activity was assayed by standard procedures.

organism was grown on a 7.0% galactose medium, its optical density at 400 nm reached 70. Thus, limonoate might have had an inhibitory effect on the organism. *Arthrobacter globiformis*, however, grew well on relatively high concentrations of limonoate (Hasegawa et al., 1972).

Growth on other carbon sources

Because the organism did not grow well on media containing high concentrations of limonoate, a search was made for a substitute carbon-source. Tests of various sugars revealed that galactose and fructose were excellent substrates. The organism grew moderately well on xylose, but very poorly on sucrose and glucose. The organism was able to grow on media containing up to 10% galactose, and the cell yield reached as much as 30 times that from a medium containing limonoate as a sole carbon source. Thus, galactose was used as a carbon source for the growth of the organism throughout this investigation, unless otherwise stated.

The organism was also capable of growing on GSME. A medium which contained 0.5% GSME soluble solids and 4% galactose increased cell yields by 50% and reduced incubation time by 30 hr as compared with 4% galactose alone.

Temperature and pH effects on growth

When incubated on 0.3% limonoate at 8, 20, 25 and 30°C, the organism grew best at 25°C. Growth was slightly less at 20°C, and at 30°C the growth rate was one-half that at 25°C. The organism did not grow well at 8°C. The organism grew best at pH 7–7.5.

Induction and production of limonoate dehydrogenase

Cells grown on a medium containing either 0.5% limonoate or GSME plus 0.1% limonoate had about 200–300 units of limonoate dehydrogenase per 200 ml culture. Cells grown on substrates other than those containing limonoids did not produce the enzyme activity. However, we found that the enzyme activity could be induced with relatively low levels of limonoate supplied either as added pure limonoate or as limonoids present in GSME.

Two methods to induce enzyme activity were studied. By the first, the organism was grown on a galactose medium until growth was considerable; limonoate or the GSME was then added to induce the enzyme. Typical results from such experiments are shown in Table 1 and Figure 1. Activity appeared a few hours after addition of the GSME, and reached its maximal level after 24 hr. Afterwards, activity decreased gradually (Fig. 1). Activity was maximal, 300 units per 200 ml culture, after addition of extract at the 0.5–0.75% level (soluble solids/total, w/v). The addition of extracts at levels higher than 1.0% did not increase activity, but the addition of limonoate at the 0.1% level resulted in the production of up to 700 units of enzyme activity per 200 ml culture. The addition of as little as 10 ppm limonoate induced enzyme activity, but activity was low.

By the second method for the induction of enzyme activity, cells were incubated on a limonoate medium immediately after harvest from galactose media on which they had grown maximally. The results of the experiment are shown in Table 2. The original growth medium contained 4% galactose and 0.25% GSME. The cells were collected by centrifugation, washed with 0.1M phosphate buffer (pH 7.0), suspended in mineral salt media of five different concentrations of limonoate, and incubated under the standard conditions. Generally, limonoate dehydrogenase activity varied directly with limonoate concentration in the medium. With 0.5% limonoate, 1550 units per 200 ml culture were obtained. This value was

Table 1—Induction of limonoate dehydrogenase of Pseudomonas sp. 321-18 with grapefruit seed meal extracts (GSME)

Incubation after GSME added	Amount of GSME added		
(hr)	0.25%	0.5%	0.75%
	Enzyme activity[a]		
4.5	51.6	77.2	77.2
7.5	64.4	77.3	58.0
24.0	142.0	303.0	303.0
27.0	70.8	213.0	312.0
31.5	169.0	247.0	276.0
48.0	88.0	251.0	270.0

[a] Activity: units per 200 ml culture. The organism was grown on a 4% galactose-mineral salt medium. When growth was maximal, GSME was added to the medium to induce enzyme activity. The growth conditions and enzyme assay method are described in the text.

Table 2—Induction of limonoate dehydrogenase of Pseudomonas sp. 321-18 with various concentrations of limonoate

Length of incubation	Concentration of limonoate				
(hr)	0.01%	0.05%	0.10%	0.20%	0.50%
	Enzyme activity[a]				
16	892	795	623	1060	–
20	–	617	833	1130	–
24	–	604	832	1020	1550

[a] Activity: units per 200 ml of culture. Average of duplicate analyses. Cells were grown on a medium containing 4% galactose and 0.25% GSME. At maximum growth, the organism was collected by centrifugation, washed with cold 0.1M phosphate buffer (pH 7.0), suspended in mineral salt media of five different concentrations of limonoate, and incubated under the conditions described in the text. Enzyme activity was assayed by standard procedures.

five to six times greater than that obtained from cells which were grown on a limonoate medium.

Several repeated experiments clearly showed that the second method produced higher enzyme activity than the first method. In the first method, residual galactose or metabolites of galactose present in the medium apparently interfered with induction of the enzyme by limonoate. This was demonstrated directly. Cells were grown on galactose medium, harvested, and washed. Those subsequently treated with limonoate-galactose developed about 50% less activity than those treated with limonoate alone.

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Ms received 7/26/75; revised 11/13/75; accepted 11/20/75.

Reference to a company or product name does not imply approval or recommendation of the product by the U.S. Dept. of Agriculture to the exclusion of others that may be suitable. This investigation was supported, in part, by the Citrus Products Technical Committee, Los Angeles, Calif.

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A Research Note
A POTENTIAL MICROBIOLOGICAL ASSAY OF FRUIT CONTENT IN ORANGE JUICE PRODUCTS

ABSTRACT

A microbiological assay has been developed to estimate the content of orange juice in a product. A bacterium was found which under standard assay conditions grew in proportion to the amount of orange juice in the mixture. Imitation orange beverages did not support growth. Commercial concentrates from various sources were assayed by the microbiological procedure, and the variability of the results was about the same as or slightly lower than that of many of the other constituents used to estimate juice content. Preliminary tests suggest that the organism is a homofermentative *Lacobacillus* of the subgenus *Streptobacterium.*

INTRODUCTION

THE PROBLEM of detecting adulteration in orange juice or of determining juice content in an orange juice product has attracted a great deal of interest over the years. Many constituents have been proposed as indices of authenticity. Some indices have been based on single compounds such as nicotinic acid (Sawyer, 1963), betaine (Lewis, 1966), and on mineral content (Koch and Hess, 1971). Likewise, various classes of compounds such as amino acids, phenolics and reducing substances have been proposed by others (Vandercook et al., 1975; Benk, 1958). Various combinations of constituents have been tried to improve the sensitivity of adulteration detection ranging from simple linear relationships (Zamorani et al., 1973) to highly complex statistical distributions (Lifshitz, 1974). Each proposal has certain advantages and limitations in terms of analysis time, instrumentation, ability to detect gross adulterations, and sensitivity to small dilutions.

In spite of the wide variety of assay procedures in the literature, no one seems to have used microbiological assay procedures to measure the content of orange juice in orange drinks. We noted bacterial growth in a sample of orange juice buffered to pH 6.9. Growth resulted when additional sterile orange juice was inoculated with the contaminated juice. This led us to investigate the possibilities of a microbiological assay for orange juice content where the growth limiting factor(s) are component(s) of natural orange juice.

MATERIALS & METHODS

Stock culture

The test bacterium (NRRL B-4306, USDA, ARS, Northern Regional Research Lab., Peoria, IL 61604) was streaked on plates of Bacto Micro Assay Culture agar (Difco Laboratories, Inc.) prepared with orange juice serum instead of water. The orange juice serum was prepared from commercial orange juice concentrate diluted to single strength with water and centrifuged at 17,000 × G for 15 min. The pH of 90 ml of the supernatant was adjusted with 2N NaOH to 6.9, and the volume was adjusted to 100 ml. After incubation at 35–37°C for 48 hr, the plates were stored in the refrigerator (4°C). Transfers were made monthly.

Inoculum

The inoculum for assays was prepared by subculturing from a stock culture into a tube containing Bacto-Micro Inoculum Broth (Difco Laboratories, Inc.) prepared with pH 6.9 orange juice serum instead of water. After incubation at 35–37°C for 24 hr, the cells were centrifuged, under aseptic conditions, and the supernatant decanted. The cells were washed twice in sterile saline (0.85% NaCl) and centrifuged. A suspension of the cells in 10 ml sterile saline was diluted 1:10 with additional sterile saline. One drop of the inoculum was used to inoculate each assay tube.

Sample preparation

Single strength juice or a concentrate diluted to single strength was centrifuged at 17,000 × G for 15 min to give a clear sample. The pH of 20 ml of the juice was adjusted to 6.9 with NaOH and the volume was adjusted to 100 ml.

Assay

Five replicate culture tubes (25 × 150 mm) were prepared with 10 ml of each sample and autoclaved at 15 lb pressure for 15 min. Two tubes were held as a control, and three tubes were inoculated with the organism. All tubes were incubated for 30 hr at 35–37°C. Bacterial growth (as turbidity) was determined by measuring absorbance at 550 nm of the inoculated sample against the uninoculated control. The values were expressed as absorbance.

RESULTS & DISCUSSION

MICROBIOLOGICAL ASSAYS are widely used for specific constituents such as vitamins and amino acids. In this investigation the concept of a microbiological assay based on a growth-limiting nutrient has been applied to a whole agricultural commodity in a processed food product. Presumably, one or more of the minor nutrients in the juice become growth limiting for the organism.

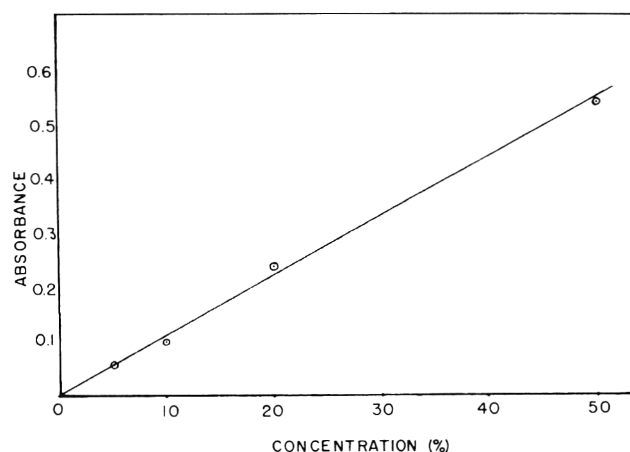

Fig. 1—Bacterial growth (absorbance at 550 nm) in a sample of orange juice as a function juice concentration.

[1] USDA Northern Regional Research Lab., Peoria, Ill.

Table 1—Bacterial growth (absorbance) under standard assay conditions in 28 commercial orange juices

	Absorbance[a]	
California	0.22, 0.30, 0.21, 0.21, 0.17, 0.25, 0.30	0.24
Florida	0.23, 0.16, 0.13, 0.16, 0.15, 0.32, 0.22	0.20
Texas	0.38, 0.47, 0.25, 0.18, 0.23, 0.22	0.29
Israel	0.21, 0.21, 0.18, 0.21	0.20
Mexico	0.30, 0.27	0.29
Spain	0.21, 0.17	0.19
	All samples	0.24 ± 0.07

[a] Average of three replicates

Early in the investigation we found that growth of the organism was proportional to the concentration of orange juice in the assay mixture. Figure 1 shows the results of a typical experiment with different levels of a single orange juice sample. A 20% juice concentration was selected as a reasonable level for routine determinations on the basis of ease of sample preparation, sensitivity and absorbance values.

The effects of pH, temperature, inoculum concentration and time on the growth of the bacterium were investigated, although not all possible combinations of the parameters were considered. Growth of the bacterium in orange juice was substantial after 20–30 hr at 37°C, but was essentially absent at room temperature (21–23°C) and 45°C. Inoculum dilutions greater than 1:10 required longer than 30 hr for the growth to reach its maximum level. The growth response to pH was nearly flat from 5.5–7.0; growth was less above and below that range. A pH of 6.9 was selected for the assay.

Commercial orange concentrates from various sources were assayed by the microbiological procedure, and the results are listed in Table 1. The overall mean absorbance and standard deviation were 0.24 ± 0.07, respectively. The variability of the results was less than that reported for total amino acids, total acidity, phenolics and some of the individual amino acids used to estimate juice content (Vandercook et al., 1975). There was no growth, under the standard assay conditions, on a commercial orange flavored instant breakfast drink or on orange soda (degassed under vacuum).

Preliminary tests established that the assay organism was a Gram-positive, nonmotile rod. It was both catalase and oxidase negative. Nitrate reduction was not evident even after 12 days. The organism produced acid but no gas from glucose, but generated both acid and gas from gluconate. Thin-layer chromatography on silica plates (ethanol:ammonium hydroxide: water 80:4:16) of an ether extract of the medium after bacterial growth produced a spot corresponding to lactic acid. A test by the method of Barker and Summerson (1941) also indicated that lactic acid was produced. Finally, the organism grew well at 37°C but not at 45°C. These results indicated that the organism is a homofermentative *Lactobacillus* belonging to the subgenus *Streptobacterium.*

Results of these experiments indicate that a microbiological assay for the juice content in orange juice products is possible. For whole juice products, the variability is comparable with previously reported chemical methods.

Work is continuing on the identification of the organism. Known species of *Lactobacillus* are also being tested for their usefulness in this type of assay on citrus and other juices.

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Ms received 9/22/75; revised 11/11/75; accepted 11/20/75.

Presented at the 35th Annual Meeting of the Institute of Food Technologists, Chicago, Ill., June 8–12, 1975.

The mention of a product does not imply endorsement of the U.S. Dept. of Agriculture over similar products that may be suitable.

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A Research Note
NONENZYMIC BROWNING IN A SYNTHETIC ORANGE-FLAVORED DRINK

ABSTRACT

Storage resistance of a powdered synthetic orange-flavored drink (OFD) to nonenzymic browning as measured by formation of three browning products at 38°C was compared with that for natural instant orange juice (IOJ). OFD was freeze dried to afford a homogenous OFD mixture comparable to IOJ and its storage stability was compared with that of IOJ. Although OFD was more resistant than IOJ to nonenzymic browning when OFD had a lower moisture level than IOJ, at the same moisture level, OFD was no more stable. This indicated that the greater storage stability of OFD is due to its lower moisture content, rather than to its discrete separation of components which might retard sugar-acid reactions.

INTRODUCTION

AS DEHYDRATED FOODS become increasingly available, more fundamental information on nonenzymic browning and other reactions that produce off-flavor in these foods is needed. Synthetic powdered orange-flavored drinks (OFD) have greater storage stability to off-flavor development at ambient temperatures than natural powdered instant orange juice (IOJ) (Berry et al., 1972) but the reason for this difference in flavor stability has not been determined. Elucidation of this difference may help provide guidelines for improving storage stability of dried food products.

Previous studies showed that nonenzymic browning is responsible for off-flavor development in IOJ (Tatum et al., 1967; Shaw et al., 1970). In these studies, natural homogeneous mixtures of components were employed, whereas most synthetic powdered drinks are nonhomogeneous mixtures of discrete solid components dry-blended together. Such nonhomogenous mixtures might be less susceptible to sugar-acid degradation reactions during storage than would homogenous mixtures of the same moisture content where the sugars and acids are in more intimate contact. Since moisture content affects the rate of nonenzymic browning in dehydrated foods (Reynolds, 1963), any differences in moisture levels between samples could account for differences in storage stability.

The current study was undertaken to determine the importance of discrete component separation and moisture content to resistance to nonenzymic browning in OFD and in IOJ.

EXPERIMENTAL

Drink samples

Synthetic powdered orange-flavored drink containing sucrose, citric acid, potassium citrate, and ascorbic acid was purchased from a local food market. A 175-g portion of this powdered drink was dissolved in 1182 ml of distilled water and placed in trays having 1680 sq cm surface area to approximately 1 cm depth. Samples were frozen at −68°C, placed in a Stokes Model 12-F freeze drier with platen temperature of 4°C, and dried for ca 7 hr. Instant orange juice was prepared by dehydration of 58° brix concentrated orange juice using the foam-mat drying process (Berry et al., 1972). Samples were stored at 38°C and checked weekly by thin-layer chromatography (TLC) for the presence of browning compounds. Reference samples were stored at −20°C.

Moisture determinations and adjustments

Moisture content of OFD and IOJ samples was determined by using a modification of the Karl-Fisher method (McComb and Wright, 1954). Portions (98.93g and 95.54g) of OFD containing 0.18% moisture were shaken vigorously as 1.07g and 4.46g of distilled water was added dropwise to increase the moisture content to 1.25% and 4.64%, respectively. Vigorous shaking was continued for several minutes to evenly distribute the water throughout each sample. Moisture was allowed to equilibrate throughout the samples during storage.

Extraction procedures

Small extractions for TLC were carried out by shaking 1g of powdered sample in 1 ml of water with 2 ml of acetone and spotting 15-μℓ portions of the clear, upper layer on TLC plates.

Preparative TLC or gas chromatographic (GC) samples were isolated from powder (100g) that was slurried with 150 ml of 1:1 water-acetone solution extracted with three 150-ml portions of ether as described previously (Tatum et al., 1967). The combined ether extracts were dried over sodium sulfate and concentrated to small volume (ca 5 ml) prior to TLC or GC separation.

Thin-layer chromatography

Samples were spotted on Silica Gel HF_{254} plates and developed to 10 cm in a solvent system by the upper layer from equilibrated benzene (200), ethanol (47), water (15), and acetic acid (1 part) (Berry and Tatum, 1965). Preparative TLC was carried out by spotting samples on 20 × 20 cm plates of Silica Gel HF_{254} 1 mm thick and developing them twice in the same solvent system. Individual ultraviolet-absorbing bands were scraped off and components eluted with ether.

Gas chromatography

GC separations were performed on a Hewlett-Packard Model 7620A instrument equipped with a 0.20 in. × 9 ft column packed with 20% Carbowax 20M on 60–80 mesh Gas Chrom P with He flow of 100 ml per min. Temperature programming was 100°C initially, then raised at 4°C per min up to 225°C. The thermal conductivity detector temperature was 275°C and on-column injection was employed.

Spectral analyses

Infrared (IR) spectra were determined as thin films or chloroform solutions on a Perkin-Elmer Model 137 infracord spectrometer. For solution spectra, a 4 power beam condenser was employed. Ultraviolet (UV) spectra were determined in ethanol solution on a Cary Model 14 spectrophotometer.

Authentic comparison samples

Hydroxymethylfurfural was purchased from ICN Life Sciences Group, Plainview, New York. Hydroxyacetylfuran (Tatum et al., 1967) and maltol hydrate (Shaw et al., 1971) were prepared as described previously.

RESULTS & DISCUSSION

THE OFD was found to be more stable to nonenzymic browning than was IOJ as demonstrated by formation of three indicator compounds during storage at 38°C (Table 1). The three compounds, hydroxymethylfurfural (HMF), hydroxyacetylfuran (HAF), and maltol hydrate (MH) (2,3-dihydro-3,5-dihydroxy-6-methyl-4*H*-pyran-4-one) were detected by using a previously published TLC procedure (Berry and Tatum, 1965). These three compounds were shown to be major nonenzymic browning products in IOJ (Tatum et al., 1967) and in a fructose-acid model system (Shaw et al., 1967) and thus, are good indicators of nonenzymic browning in the present systems.

Table 1—Stability of dehydrated synthetic orange drink (OFD) and natural orange juice (IOJ) to nonenzymic browning during storage at 38°C

Powdered drink	Moisture content (%)	No. wk at 38°C	Browning components detected and detection method		
			HMF	HAF	MH
OFD	0.18	20	N[a]	N	N
OFD	0.18	98[b]	TLC,GC,IR,UV[c]	N	N
Freeze-dried OFD	4.64	3	TLC,GC,IR	TLC,GC,IR	TLC
OFD & H_2O	4.64	3	TLC,IR	TLC,IR	N
OFD & H_2O	1.25	3	TLC,IR	TLC,IR	N
IOJ	1.25	3	TLC,GC	TLC,GC	TLC,GC

[a] N = not detected
[b] Stored at ambient temperature
[c] λ_{max}280 nm (ϵ 5300)

HMF was first detected in IOJ after 3 wk, but it was not detectable in OFD at the end of 20 wk. Although none of the three browning indicators in Table 1 was detected in OFD after 20 wk storage at 38°C, HMF was detected in a sample of OFD that had been stored for 98 wk at ambient temperature (range 18–30°C), but the other browning products were not detected.

Since OFD was a blend of discrete components rather than a homogenous mixture as found in IOJ, a solution of OFD was prepared and freeze dried to obtain a homogenous, dry mixture of components comparable to that found in IOJ. As indicated in Table 1, the freeze-dried OFD sample was no more stable to nonenzymic browning than was IOJ, since they both developed detectable levels of HMF, HAF and MH after 3 wk storage at 38°C.

Moisture content is known to be a critical factor in rate of nonenzymic browning in dehydrated foods and in model systems (Reynolds, 1963). Since the moisture content of commercial OFD was significantly lower than that of IOJ or of freeze-dried OFD, this lesser moisture content in commercial OFD could be a reason for its greater storage stability. In order to explore this possibility, samples of OFD were prepared with a moisture content equal to that of IOJ (1.25%) and that of freeze-dried OFD (4.64%). Results in Table 1 indicate OFD containing 1.25 or 4.64% moisture to be no more stable to nonenzymic browning as measured by HMF and HAF formation than either IOJ or freeze-dried OFD at the same moisture levels. The third major indicator of nonenzymic browning in IOJ and in freeze-dried OFD, MH, was not detected in the other OFD samples studied.

Moisture content in IOJ has been shown to affect storage stability. Thus, when IOJ with a significantly lower moisture content (0.88%) than that usually produced (ca. 1.25%) was evaluated by a taste panel as described previously (Berry et al., 1972), it was stable twice as long (6 wk) at 38°C as was a sample containing 1.25% moisture (3 wk) (Wagner, 1975).

This study indicates that low moisture content is of much greater importance in achieving greater storage stability to nonenzymic browning in high sugar-acid dry foods than is the discrete separation of components through dry-blending of individual crystals.

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Ms received 12/10/75; accepted 1/12/76.

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A Research Note
FUNCTIONAL PROPERTIES OF ACYLATED GLANDLESS COTTONSEED FLOUR

ABSTRACT
Glandless cottonseed flour was acylated with succinic and acetic anhydride. Acylation caused an increase in the specific viscosity of the flour and an increase in its functionality. Water-holding, oil-holding, emulsifying, and foam capacities were increased from 1.2 to tenfold over nonacylated flour.

INTRODUCTION

THE DEVELOPMENT of glandless cotton varieties and liquid cyclone processing techniques has allowed production of cottonseed flours that have very low or zero concentrations of gossypol (Harper and Smith, 1968; Pons and Eaves, 1967). These products have been characterized both functionally and nutritionally as a partial replacement for wheat flour in yeast breads (Rooney et al., 1972; Matthews et al., 1970. Tsen et al., 1971; Harden and Yang, 1975).

Extension of cottonseed flour utilization to other types of food products might be feasible if the flour itself or a chemically modified flour were found to have high functionality. A technique which has been successfully utilized with fish protein concentrates, eggs and wheat to alter functionality is acylation of the protein with acidic anhydrides (Groninger and Miller, 1975; Groninger, 1973; Gandhi et al., 1968; Grant, 1973). Acylation of proteins has been noted in some cases to cause an increase in the electrostatic repulsion forces in the protein resulting in an expansion of the molecule (Habeeb et al., 1958). Carpenter and Saffle (1965) showed a high correlation ($r = 0.975$) between the radius of proteins (as quantified by the limiting viscosity number) and the emulsifying capacity of proteins.

It, therefore, seemed feasible to define experimentally the effects of acylation of whole cottonseed flour on the functionality of the flour. Functional characteristics which were investigated were: (a) water-holding capacity, (b) oil-holding capacity, (c) emulsifying capacity, and (d) foaming properties. In addition, the specific viscosity of the flours was defined.

MATERIALS & METHODS

GLANDLESS COTTONSEED FLOUR samples were provided by the National Cottonseed Products Association, Memphis, Tenn. and stored in sealed moisture-vapor proof plastic bags at room temperature until experimentation.

Glandless cottonseed flour was acylated by reaction with either succinic or acetic anhydride. 5g of flour were suspended in 150 ml of distilled water. 25g of succinic or acetic anhydride were added over a period of 60 min with constant stirring. The reaction mixture was held in the general range pH 7.5–8.5 by addition of 4N NaOH and the temperature was held at 0°C by immersion in an ice bath. The acylated material was dialyzed overnight at 4°C against distilled water and freeze dried.

For water-holding capacity determinations 0.1g of cottonseed flour and 5.0 ml of 0.02M citrate buffer (pH 3.5) were added to a weighed 15 ml glass centrifuge tube. The tube was agitated on a vortex mixer for 2 min and then centrifuged for 15 min at 500 × G. The clear supernatant was decanted and discarded. The pellet was weighed and the weight of water bound per gram flour was calculated as water-holding capacity.

Oil-holding capacity was determined in the same manner as water-holding capacity except 5 ml of cottonseed oil was substituted for the 5 ml of citrate buffer.

Emulsifying capacity was determined by a slight modification of methods described by Webb et al., (1970). 0.10g of cottonseed flour product was suspended in 45 ml of 5% NaCl, 0.02M sodium carbonate solution and blended at 12,000 rpm with more oil being added. The breaking point of the emulsion was judged to have been reached when the ohmmeter showed a rapid and large increase in resistance. The emulsifying capacity was calculated as ml of oil emulsified/g cottonseed flour product.

For foam capacity determinations 1.0g of cottonseed product and 100 ml of water were placed in a beaker and mixed to allow dispersion of the cottonseed product. The mixture was then whipped at 14,000 rpm for 10 min and the volume was determined. The foam forming unit was defined as ml foam/g product. The foam was then transferred to an 11 mm funnel supported on a 100 ml graduate cylinder and the liquid drip from the foam was quantified after 15 min.

Specific viscosity was determined in Ostwald viscometers at 27 ± 0.05°C. Varying amounts of cottonseed flour product (0.01, 0.03, 0.05, 0.07 and 0.09g) were dissolved in 10 ml of water and the relative viscosity (N_r) of the product solution defined as N_S/N_O where N_S is product solution viscosity and N_O is water viscosity. The specific viscosity was calculated as $N_r - 1.0$.

RESULTS & DISCUSSION

ACYLATION of glandless cottonseed flour increased its water-holding capacity greatly. Succinylated cottonseed flour bound 1.5 times more water than unmodified glandless flour and acetylated flour bound greater than 2.0 times more water (Table 1).

Oil-holding capacity of cottonseed flour was markedly increased by acylation (Table 1). Acetylated cottonseed flour bound three times more oil than normal cottonseed flour and succinylated flour bound seven times more oil.

The emulsifying capacity of glandless cottonseed flour was significantly increased by succinylation but not by acetylation (Table 1). Succinylation increased the emulsifying capacity twofold and to the same general range as skeletal muscle on a dry weight basis (Carpenter and Saffle, 1965).

Table 1—Functionality of acylated cottonseed products[a]

	Glandless cottonseed flour (ml/g)	Acetylated cottonseed flour (ml/g)	Succinylated cottonseed flour (ml/g)
Water-holding capacity	3.50a	7.87c	5.53b
Oil-holding capacity	2.60j	8.97k	19.60L
Emulsifying capacity	456.67m	416.67m	776.67n
Foam capacity	126.67x	250.00z	150.00y

[a] All data are the mean of three replicates. Methodology is defined in the text. Data in the same horizontal row with different letters are significantly different at the 0.05 level.

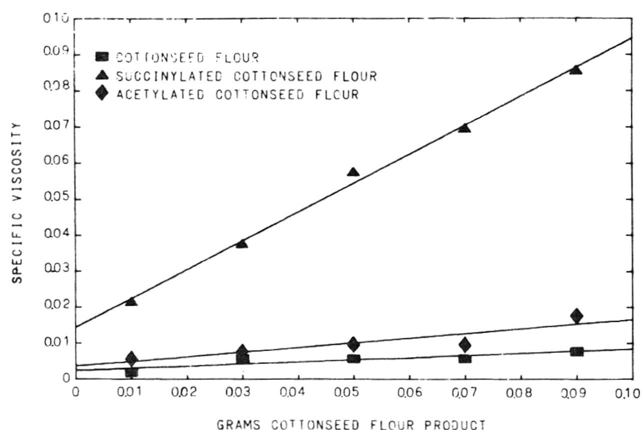

Fig. 1—Specific viscosity of cottonseed flour products as a function of grams of cottonseed flour product/10 ml water. All data are the mean of three replicate determinations. In no case was the standard error of the mean greater than the size of the symbol used to denote the data point. Relative viscosity (N_r) was defined as the quotient of the viscosity of the cottonseed flour product solution, N_s, and the viscosity of water, N_o, specific viscosity was defined as $N_r - 1.0$.

Neither glandless cottonseed flour nor its acylated derivatives produced large volumes of foam (Table 1). Cottonseed flour produced approximately 25% the foam volume of soybean flour (Groninger and Miller, 1975). However, acylation of glandless cottonseed flour did increase its foam capacity. In addition, the cottonseed foam was markedly unstable with > 90% drip within 15 min of foam production.

Figure 1 indicates the succinylated product had the greatest viscosity, followed by the acetylated product, and native cottonseed flour. It is generally recognized that the viscosity of a polymeric solute is a function of the volume of the polymer molecule in solution and further that instrinsic viscosity is a function of molecular weight for a series of linear homologous polymers (Karata et al., 1972). Because of the heterogeneous composition of these products, the data can only be interpreted as indicating an increase in volume of a significant number of the polymer molecules.

Since Carpenter and Saffle (1965) have noted a relationship between the molecular radius of proteins and their emulsifying capacity, the superior performance of acylated flours as emulsifying agents may be related to increased molecular volume.

These data indicate the water-holding, oil-holding, emulsifying and foam capacity of glandless cottonseed meal are increased significantly by acylation reactions. Acetylation selectively increased water-holding and foaming capacities while succinylation selectively increased oil-holding and emulsifying capacities. Pilot plant studies are being undertaken to define (a) optimum conditions for production of acylated cottonseed flours and (b) the extent of acylation necessary to produce desired functional characteristics. Production of adequate amounts of acylated product to allow product development work is also underway.

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This research was supported through Project H-387 "Utility of Enzymatic-Chemical Extraction Procedures for Protein Recovery from Selected Crops and Food Processing Wastes" of the University of Tennessee Agriculture Experiment Station. Gratitude is expressed to J. Forté for her technical advice and counsel.

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A Research Note

MONO- AND OLIGOSACCHARIDE COMPOSITION OF GROUNDNUT (Arachis hypogea)

ABSTRACT

The mono- and oligosaccharide composition of two different varieties of groundnuts has been determined. 70% alcohol was used for the extraction of mono- and oligosaccharides. The total monosaccharides constituted 5.08 and 5.00%, and were identified as D-glucose (2.89 and 2.88%) and D-fructose (2.19 and 2.11%); while the total oligosaccharides amounted only to 3.23 and 3.38% of the flour solids, respectively for the two varieties, and were identified as sucrose, 0.91 and 0.95%; raffinose, 0.94 and 1.06%; stachyose, 1.04 and 0.51%; and verbascose, 0.2 and 0.38%. Final identification of the sugars was achieved by gas liquid chromatographic analysis as their trimethylsilyl derivatives. In addition, one of the varieties "Nambyquarae" (hybrid peanut) also contained ajugose, 0.48%. These groundnuts are unusual in that they contain a high proportion of free monosaccharides.

INTRODUCTION

IN A PREVIOUS publication from this laboratory (Tharanathan et al., 1975) the total carbohydrate make-up of defatted edible groundnut flour, both processed and unprocessed has been reported. It was shown that out of 38% total carbohydrates in the unprocessed flour, mono- and oligosaccharides account for 18%, with sucrose, the major oligosaccharide, amounting to 14%; while glucose and fructose represent 0.80 and 0.41%, respectively. As part of studies on oilseed carbohydrates, the free sugar and general composition of two different varieties of groundnuts are reported in the present communication.

MATERIALS & METHODS

TWO DIFFERENT VARIETIES of groundnuts were used in the present investigation. Both the varieties belong to runner types. One of them (presumably HG-4 variety) is cultivated in Akola, Maharashtra State, bears medium-sized pods containing 3–4 oval red seeds (30–35 seeds/10g). It was obtained in Oct. 1974. The other is "Nambyquarae" variety obtained from the National Chemical Lab. Garden, Poona, Maharashtra State. It bears large-sized pods containing 1–2 slightly flat but big seeds (10–12 seeds/10g) with white streaks on the red testa, comparatively thicker shells. It was grown in a local garden and was obtained in Oct. 1974 (Fig. 1).

Triplicate 10-g batches of defatted groundnut flour were used for the isolation of mono, di- and oligosaccharides; the analytical methods, procedure for visualization and identification of sugars have been described previously (Tharanathan et al., 1975). Thin-layer chromatography was done on silica gel G plates (0.2 mm) using butanol-ethanol-water (2:1:1) and ethylacetate-isopropanol-water (11:4:2) solvent systems. High voltage electrophoresis was conducted using Whatman No. 3 MM papers at a voltage of 40 V/cm for 2 hr using borate buffers pH 9.2 and 10.0.

Purification and silylation of sugars

The fractionation of sugars was achieved by the method of Whistler and Durso (1950). Ethanol extract (1.0g in 5 ml water) was placed on a charcoal-Celite (Koch Light Co.) column (2:1 by weight, 31 × 4.0 cm). The column was eluted with 1.6 liters of water for the isolation of monosaccharides. The sugars remaining in the column were then desorped by eluting successively with water containing increasing proportions of alcohol (2–25%, v/v). The purification and characterization of sugars were done as described previously (Tharanathan et al., 1975). The sugars along with myo-inositol were silylated to their trimethylsilylated (TMS) derivatives with 1 ml of TriSil (Pierce Chemical Co.), shaken, and let stand at room temperature for a few minutes while oligosaccharides required incubation at 65°C for 1 hr. 1 μl of the clear solution was injected into the gas chromatograph.

Gas chromatography

A Varian Aerograph gas chromatograph Series 1400 with a flame ionization detector was used. The 5 ft × 1/8 in. o.d. stainless steel column was packed with 3% OV-1 on Chromosorb W (HP) 80/100. The column was operated isothermally at 300°C. Flow rates employed were carrier N_2, 30 ml/min; H_2, 17.5 ml/min; and air, 300 ml/min. The detector and injector temperatures were 280°C and 260°C, respectively.

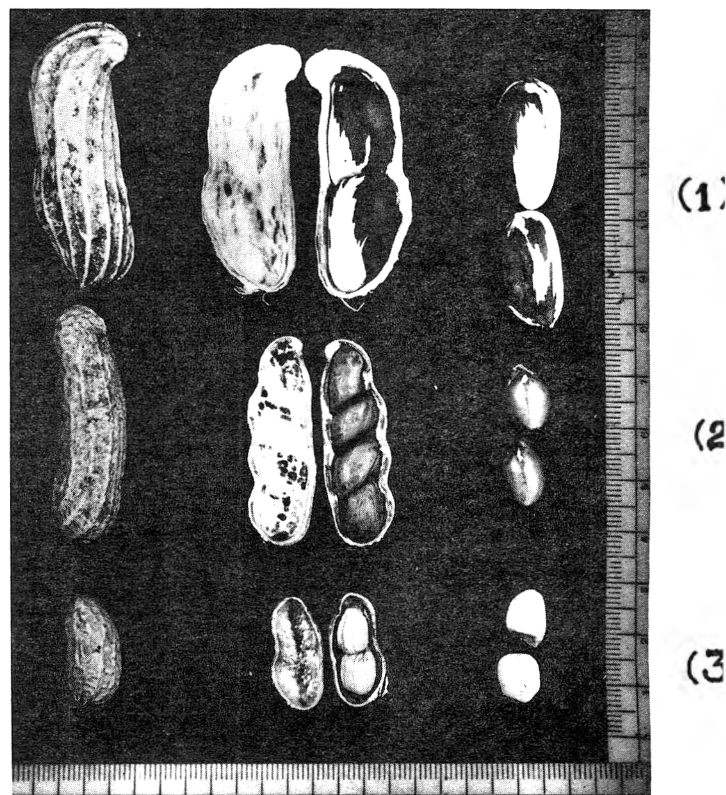

Fig. 1—Different varieties of groundnuts (Arachis hypogea) showing shape, size of the pods and seeds: (1) Nambyquarae; (2) HG-4; (3) red netal.

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Table 1—Chemical composition[a] of ethanol-soluble and ethanol-insoluble materials from defatted groundnut flour

	HG-4 groundnut		Nambyquarae groundnut	
	Soluble material	Insoluble material	Soluble material	Insoluble material
Yield[b]	9.88	89.66	9.98	90.00
Total sugar[c]	89.20	24.00	90.00	25.50
Protein (N X 6.25)[c]	3.06	61.25	2.19	62.50
Moisture[c]	6.26	6.65	4.26	4.68
Ash[c]	0.31	5.26	0.20	4.88
Sugars detected[d]	Glu, 2.89	GalA, 1.70	Glu, 2.88	GalA, 1.88
	Fru, 2.19	Gal, 3.04	Fru, 2.11	Gal, 3.26
	Suc, 0.91	Glu, 8.60	Suc, 0.95	Glu, 5.20
	Raf, 0.94	Ara, 4.30	Raf, 1.06	Ara, 6.17
	Verb, 0.20	Xyl, 2.51	Sta, 0.51	Xyl, 4.29
	Unk, 0.14		Verb, 0.38	
			Aju, 0.48	

[a] Figures are averages of results of triplicate experiments in all cases.
[b] Percentage of dry solids extracted from dry defatted groundnut flour.
[c] Figures represent the percentage composition of ethanol-soluble and ethanol-insoluble material.
[d] Glu, Glucose; Gal, Galactose; Fru, Fructose; Ara, Arabinose; Xyl, Xylose; Suc, Sucrose; Raf, Raffinoise; Sta, Stachyose; Verb, Verbascose; Aju, Ajugose; GalA, Galacturonic acid; Unk, unidentified. [The figures represent the percentages (on moisture-free basis) of defatted groundnut flour, average values in triplicate].

RESULTS & DISCUSSION

THE NAMBYQUARAE VARIETY contained a low percentage of oil (41.3%) compared to HG-4 variety (50%); the protein contents were 33.4% and 26.5%, respectively. Preliminary examination of the extracts on paper and thin-layer chromatograms revealed the presence of glucose, fructose, sucrose, raffinose, stachyose and verbascose. Final identification of sugars was achieved by GLC. A preparative scale fractionation was effected by charcoal-Celite chromatography. It should at the very outset be stated that the two varieties were not grown under similar conditions and hence the results may not be comparable. However, the results represent triplicate analytical values. The results (Table 1) indicate that the total monosaccharides consituted 5.1 and 5.0% and L-fructose 2.2 and 2.1%, while the total oligosaccharides amounted to only 3.2 and 3.4% of the defatted flour and were identified as sucrose, 0.90 and 0.91%; raffinose 0.90 and 1.0%; stachyose 1.0 and 0.50%; and verbascose, 0.20 and 0.40% in the HG-4 and Nambyquarae varieties, respectively.

In addition, the Nambyquarae variety also contained a higher oligosaccharide to the extent of 0.48%. This fraction had a very low mobility on paper and thin-layer chromatograms, and gave a characteristic blue color when sprayed with urea-spray reagent (Dedonder, 1952). Its optical rotation, +164° (Ca 0.5 in water) was almost identical with that of ajugose (+163°). On partial hydrolysis with 0.5N H_2SO_4 at 100°C for 6 hr, verbascose (trace), raffinose, glucose, galactose and fructose were noticed on chromatograms. The formation of verbascose confirms the fraction to be ajugose. Further confirmation was achieved by enzymic hydrolysis. Thus, incubation with α-galactosidase (contaminated with invertase), isolated from *Agave vera cruz*, in acetate buffer, pH 5.6 at 37°C for 12 hr revealed the presence of verbascose, stachyose, raffinose and sucrose (faint spots) along with galactose. From these results it is beyond doubt that the new fraction isolated is definitely ajugose. This is the first report of the occurrence of higher oligosaccharide (DP, 6) in groundnuts.

It is of significant interest to note the high proportion of free monosaccharides (5%) and sucrose (0.90%) reported in the present study is quite different from the earlier quantitative reports (Tharanathan et al., 1975; Aylward and Nichols, 1961). Similar to these groundnuts, sesame (*Sesame indicum*) has been shown to contain a high proportion of monosaccharides (7.4%) and only 1.7% of oligosaccharides (Wankhede and Tharanathan, 1975). Mikolajezak et al. (1970) reported that sunflower meal contained 9.70% of di- and oligosaccharides, mainly sucrose, trehalose and raffinose; on the other hand, Sabir et al. (1975) reported that "mono- and oligosaccharides in ethanol extract of sunflower and head constituted 9.80 and 6.2%, respectively, of the flour solids."

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Ms received 9/27; revised 12/18/75; accepted 12/25/75.

One of the authors (R.N.T.) thanks to C.S.I.R., New Delhi (India), for a position in the Scientists' Pool. Thanks are also due to Shri M.N. Satyanarayana for the gift of α-galactosidase.

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A Research Note

THE SENSITIVITY OF FOOD SPOILAGE YEASTS TO ACETALDEHYDE VAPORS

ABSTRACT

Acetaldehyde (Aa) vapors inhibited development in vitro of the eight species of yeasts tested, fungicidal action being a function of concentration and length of exposure. The nonfermentative species *Crytococcus difluens* was the most sensitive species, 99% of its cell population being inactivated by exposure to 0.5% for 5 hr. Exposure to 0.5% for 10 hr was lethal to almost all species tested. A similar result was obtained by exposing the yeasts to a higher Aa concentration (3%) for a shorter period of time (3 hr). A concentration of 1% Aa, given for 1, 3 and 5 hr, was not lethal to the species tested but reduced the survival percentage of all yeast populations and usually caused a prolongation of the incubation period in culture. Colonization of *Schizosaccharomyces pombe,* the main factor responsible for the spoilage of natural concentrated citrus juices, was markedly inhibited by Aa vapors.

INTRODUCTION

ACETALDEHYDE (Aa) is a natural volatile compound of different plant organs, including fruit tissue (Nursten, 1970). Recently, Aa was reported to have fungicidal and bactericidal properties against various microorganisms causing postharvest decay of fruits and vegetables (Aharoni and Stadelbacher, 1973; Aharoni and Barkai-Golan, 1973; Prasad and Stadelbacher, 1973; Stadelbacher and Prasad, 1974). This paper reports the effect of Aa vapors on the survival of yeast species, most of which are responsible for postharvest decay of fruits or spoilage of natural and concentrated fruit juices. The present study was undertaken to provide a basis for the possibility of using this natural chemical, which is known as a flavoring agent (Hayes, 1963), also as a fumigant in the processes of preservation of fruit and juice.

MATERIALS & METHODS

THE EFFECT of Aa was tested on the following yeast species: *Cryptococcus diffluens* (Zach.) Lodder et Lodder, isolated from stored grapes; *Candida rugosa* (Anderson) Diddens et Lodder, isolated from slices of grapefruit; *C. utiles* (Henneb.) Lodder et Lodder (unknown origin); *C. tropicales* (Cast.) Berkh. (unknown origin); *Schizosaccharomyces pombe* Lindner, isolated from orange juice; *Saccharomyces cerevisiae* Hansen, isolated from grape must; *S. bayanus* Sacc., isolated from stored grapes; and *S. bailii* Lindner, isolated from concentrated orange juice.

Pure cultures were grown on potato-dextrose agar (PDA) for 24 hr at 23°C. Aliquots of 0.5 ml of yeast suspended in distilled water at 10^5 cells/ml were dispersed in 5 × 5 cm petri dishes and exposed to Aa vapors in 8-liter desiccators. Aliquots of liquid Aa were injected into the desiccator in order to attain the desired vapor concentration as described previously (Aharoni and Barkai-Golan, 1973). The exposure of yeast cells to Aa was done at room temperature (20–23°C). Vapor concentration was expressed as percentage of the atmosphere by volume.

Aa concentration and length of exposure for each species are given in Table 1. After treatment, cell suspensions were diluted 1:10, after which aliquots of 0.25 and 0.5 ml were spread onto the surface of PDA in 9-cm petri dishes and colonization ability at 23°C was recorded daily for 6 days. Nine replicates were made of each treatment.

RESULTS

THE DIFFERENT SPECIES of yeasts tested were found to have different degrees of sensitivity to given concentration of Aa vapors (Table 1). The most sensitive species was *Cryptococcus difluens,* which failed to grow when exposed to 1% Aa for 5 hr and only 1% of the original population survived 0.5% Aa for 5 hr. Exposure to 0.5% Aa for 10 hr was found to be

Table 1—Effect of various acetaldehyde (Aa) vapor concentrations and exposures on survival of eight yeast species

		% Killed[a]							
% Aa	Exposure (hr)	Cryptococcus difluens	Schizosaccharomyces pombe	Candida rugosa	Candida utilis	Candida tropicalis	Saccharomyces bailii	Saccharomyces bayanus	Saccharomyces cerevisiae
0.5	5	99	91	54	30	0	44	0	0
	10	100	100	100	100	100	100	100	86
1.0	1	18	0	68	18	0	13	0	0
	3	95	80	69	55	0	37	4	0
	5	100	87	80	55	13	53	22	0
3.0	1	100	0	69	61	0	12	25	27
	3	100	93	100	100	100	72	100	100

[a] Numbers represent percent of yeast cells that failed to grow after 6 days on PDA at 23°C.

lethal to all yeast species tested except *Saccharomyces cerevisiae*, of which about 15% of the original cell population survived such treatment. Increasing the time of exposure of the lowest concentration tested (0.5%) from 5 to 10 hr, proved to be more effective than increasing the chemical concentration from 0.5% to 1% applied for the former exposure period (5 hr) (Table 1). Lethal effects were also obtained by exposing the yeasts to 3% Aa for a shorter period of time (3 hr). Concentrations of 1% Aa given for 1, 3 and 5 hr did not kill the species tested (except *C. difluens*) but reduced the survival percentage of yeast populations (Table 1) and usually prolonged their incubation period in culture.

DISCUSSION

SIMILAR to what has been reported for fungal species (Aharoni and Stadelbacher, 1973; Prasad and Stadelbacher, 1973; Aharoni and Barkai-Golan, 1973; Stadelbacher and Prasad, 1974), Aa vapors proved to have fungicidal properties against yeast species, the extent depending upon both concentration and length of exposure. The reaction of the majority of the species tested is in good agreement with that of *Alternaria tenuis* and *Stemphylium botryosum* spore populations, in which 99% mortality was caused by exposure to 0.5% Aa concentration for 10 hr (Aharoni and Barkai-Golan, 1973). However, exposure to 0.5% Aa for only 2 hr was sufficient to cause a total inactivation of *Panicillium expansum* spore suspensions (Aharoni and Stadelbacher, 1973).

The yeast species *Cryptococcus difluens* and *Candida rugosa*, which showed the highest sensitivity to Aa vapors in the present study, belong to the nonfermentative group of yeasts. Their high sensitivity to the fungicide may suggest the possibility of using Aa vapors for removing unwanted nonfermentative yeasts during a controlled process of fermentation.

One of the yeasts responsible for serious damage in natural concentrated juices belongs to the fermentative genus *Schizosaccharomyces*, which is capable of developing at high concentrations of sugar. This yeast is highly resistant to SO_2, which is commonly used for preservation in the citrus juice industry. Its sensitivity to Aa, which was proved in the present study, may lead to the use of this chemical during industrial processes to avoid fermentation of citrus juices.

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Ms received 10/29/75; accepted 12/2/75.

Contribution from the Agricultural Research Organization, The Volcani Center, 1975 Series, No. 146-E.

Thanks are extended to Rachel Karadavid and Azica Copel for their technical assistance.

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A Research Note
NUTRITIVE VALUE OF SPROUTED WHEAT FLOUR

ABSTRACT

Proximate analyses and determinations of ascorbic acid, thiamine and riboflavin were made on four flours prepared from sprouted Wanser wheat and on a control flour prepared from unsprouted wheat. The four sprouted flours were prepared from 1/4 and 1 in. sprouts dried by two methods: convection or vacuum oven. Sprouted flours were found to have significantly higher crude protein, crude fat, thiamine and riboflavin concentrations than the control flour. For both sprout lengths higher thiamine concentrations were obtained by drying under vacuum.

INTRODUCTION

SPROUTED GRAINS are frequently believed to have miraculous nutritive values. Although sprouts have been used for centuries, particularly in the Orient, they have only recently become popular in the United States. Sprouted wheat, for example, is being promoted not only for traditional use as a fresh vegetable but also as a flour. Wheat is estimated to provide 20% of the total world food calories and is the staple food in about 45 countries (USDA, 1970). Therefore, treatments which might improve the nutritive quality of whole wheat flour should be of interest to the consumer and to the flour industry.

Studies using electrophoretic techniques indicate that protein fractions of wheat undergo changes during germination, with the major differences being qualitative rather than quantitative (Coulson and Sim, 1965; Daussant and Abbott, 1969). The apparent synthesis of α-amylase during wheat germination is of particular interest from a baking standpoint. Although α-amylase has improving effects when present in low levels, in higher levels α-amylase decreases bread volume and produces a sticky crumb texture.

Dapron et al. (1969) demonstrated an increase of lipolytic activity during germination of wheat. Therefore, changes in the composition of lipid fractions during wheat germination would also be expected to occur. Although Elwood (1964), in a natural foods book, purports that the sprouting of wheat has been found to improve protein quality and increase levels of ascorbic acid, thiamine, riboflavin, niacin, pantothenic acid, pyridoxine, biotin and folic acid, there seems to be little published research data to substantiate these claims.

EXPERIMENTAL

TWO VARIABLES expected to affect the composition of sprouted wheat flour were tested: degree of germination of wheat kernels and drying method. Germination was carried out in a commercially available sprouting kit consisting of a dome-covered plastic pan containing a replaceable foam pad. The germination method was designed to closely approximate household preparation of sprouted wheat. 90g of Wanser wheat was weighed into the pan, 75g of water was added and the pan placed in a 30°C incubator. The wheat kernels were germinated for 1–3 days to sprout lengths of 1/4 in. and 1 in. The germinated kernels were dried in a convection oven at 50°C or in a vacuum oven maintained at 27 in. Hg at 37°C. The four germinated samples and the ungerminated wheat kernels were milled in a Hobart mill.

A proximate analysis was conducted using approved American Association of Cereal Chemists methods (AACC, 1972). Moisture was determined at 100°C in a vacuum oven maintained at 27 in. Hg Crude fat was extracted with ethyl ether in a Goldfisch apparatus. Crude protein was determined by multiplying micro-Kjeldahl nitrogen by 5.7. The Association of Official Analytical Chemists microfluorometric method was used to quantitatively determine ascorbic acid (AOAC, 1970). AACC (1972) fluorometric methods were followed for thiamine and riboflavin determinations. All analyses were made in triplicate. Results are reported on the 14% moisture basis conventionally used for cereal products. Differences among the treatment means were tested at the 5% significance level by Duncan's new multiple range test (Steel and Torrie, 1960).

RESULTS & DISCUSSION

THE PROXIMATE composition, thiamine and riboflavin concentrations of the four germinated flours and the control flour are given in Table 1. Crude fiber quantity did not change significantly among the five treatments. Differences in ash concentration were not statistically significant, ranging from a low of 1.18% for the control to 1.38% for the 1-in. sprouts dried in the vacuum oven.

The most notable differences in the proximate composition of sprouted and control flours were the significantly greater crude fat and crude protein concentrations determined for all four sprouted flours. Protein was 12.30% for the control sample and 12.83% and 13.13% respectively for 1/4- and 1-in. sprouts dried in the convection oven. Vacuum oven samples showed similar trends. The crude protein concentration of the control flours was significantly lower than that for any of the sprouted flours. The wheat kernels were sprouted in water

Table 1—Nutrient analyses of unsprouted and sprouted wheat flours[a]

		1/4-in. sprout		1-in. sprout	
		Drying conditions		Drying conditions	
Component	Control	convection	vacuum	convection	vacuum
Thiamine (mg/100g)	0.56	0.58	0.63	0.59	0.67
Riboflavin (mg/100g)	0.11	0.15	0.15	0.16	0.16
Moisture (%)	8.23	6.21	6.86	6.94	6.43
Fat (%)	0.94	1.54	1.64	1.57	1.71
Protein (%) (N X 5.7)	12.30	12.83	12.98	13.13	13.01
Ash (%)	1.18	1.25	1.32	1.34	1.38
Crude fiber (%)	2.24	2.18	2.21	2.07	2.15
Total carbohydrate (by dif) (%)	71.58	70.38	70.06	69.96	69.90

[a] Thiamine, riboflavin, fat, protein, ash, crude fiber, and total carbohydrate concentrations corrected to a 14% moisture basis.

with no intentionally added nutrients. Therefore, the apparent differences in protein concentration may reflect a loss of carbohydrate material during respiration or an alteration of the nitrogenous substances present, rather than an actual increase of protein.

The crude fat concentration of the control flour was also significantly lower than values for any of the sprouted flours. Further investigations are necessary to determine whether this difference is due to an actual increase in lipids in the flour prepared from sprouted grain or whether this difference is due to a change in the type of lipid components. Preliminary data from gas chromatographic determination of fatty acid methyl esters indicates no notable differences between the fatty acid patterns of the control and sprouted flour samples.

No measurable ascorbic acid was found in any of the flour samples. Wheat flour does not generally contain measurable ascorbic acid, although Kent-Jones and Amos (1967) report that germinated wheat may contain low amounts of this vitamin. The thiamine concentration of flour was significantly affected by both sprouting and drying method. The sprouted flours had a mean thiamine concentration of 0.62 mg/100g as opposed to 0.56 mg/100g for the control flour. The thiamine concentration of all the sprouted flours significantly exceeded that of the control. Differences due to drying method were smaller, but statistically significant. For both the 1/4-in. sprouts and the 1-in. sprouts, higher thiamine concentrations were obtained by drying under a vacuum. This is not surprising, since the heat lability of thiamine is well documented. To take full advantage of the higher thiamine concentration of sprouted grain it appears essential to use low drying temperatures. Since cereal grains are an important source of thiamine, retention of this vitamin during processing is of great importance in a whole wheat flour which will not be enriched.

The riboflavin concentration of all the sprouted flours significantly exceeded the mean riboflavin concentration of the control flour. The mean value for the sprouted flours was 0.16 mg/100g as opposed to 0.11 mg/100g for the control. No significant differences were found among sprouted flours.

On the basis of these preliminary studies it appears that the natural nutritive value of whole wheat flour may be improved by sprouting. The increase in thiamine and riboflavin concentration of flour due to sprouting is of nutritional importance to persons who prefer natural enhancement of nutrients to enrichment. However, the nutritional benefits to be accrued from baking with sprouted flour will be lessened by the necessity to blend sprouted flour with unsprouted flour in order to obtain a good quality bread. Use of 100% sprouted wheat flour yielded low loaf volume in baking tests conducted by the Western Wheat Quality Laboratory (1974). However, home baking tests indicate that good loaf volume and crumb texture are obtained by blending sprouted wheat flour with unsprouted whole wheat flour in the ratio of 1:4 by volume (Murray, 1975).

Further studies on protein and lipid fractions as well as further vitamin assays are needed to further elucidate the nutritional significance of compositional changes occurring during wheat germination.

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Ms received 11/8/75; revised 12/23/75; accepted 12/29/75.

Presented at the 35th Annual Meeting of the Institute of Food Technologists, Chicago, Ill., June 8–12, 1975.

Scientific Paper No. 4455, College of Agriculture Research Center, Pullman, WA 99163, Project 0216.

This investigation was supported by the Washington Agricultural Experiment Station. We are grateful to Murray Hill Enterprises, Inc., Pendleton, Oregon for supplying the wheat and sprouting kits used in this study.

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A Research Note

TEXTURE PROFILE ANALYSIS PARAMETERS OBTAINED BY AN INSTRON UNIVERSAL TESTING MACHINE

ABSTRACT

A method for the interpretation of the force-deformation curves obtained by the Instron Universal Testing Machine is suggested. The method provides a more specific physical meaning to the experimental parameters and treats separately the mechanical aspects of the recoverable and nonrecoverable deformations. The method also provides for the possibility of the introduction of new mechanical parameters and terms which might represent textural properties.

INTRODUCTION

THE TEXTURE PROFILE ANALYSIS (TPA) originally developed for the General Foods Texturometer (Szczesniak, 1963; Szczesniak et al., 1963) has been a useful method for the evaluation of textural parameters for a wide range of foods.

Bourne (1968) derived a technique by which the texture profile parameters could be evaluated from the force-deformation curves obtained by the Instron Universal Testing Machine.

Though the magnitude of these parameters is influenced by experimental variants (such as the deformation rate) they can provide objective and comparative information on the textural properties if obtained under standardized conditions.

The simplicity of carrying out the test procedure by the Instron and other universal testing machines made this TPA procedure widely used. A critical review of the applications of TPA and the experimental conditions under which it has been carried out can be found in a recent work published by Breene (1975).

INSTRON-TPA TERMINOLOGY

A TYPICAL force deformation curve of the first and second bite which are obtained by the Instron machine are shown in Figure 1. Some of the textural parameters which can be derived from the curves are originally defined as follows (Bourne, 1968):

Cohesiveness $= (A_1/A_2)$
Gumminess $=$ (Hardness) $\times$ (Cohesiveness)
$=$ (Hardness) $\times$ (A_1/A_2)
Chewiness $=$ (Gumminess) $\times$ (Springiness)
$=$ (Hardness) $\times$ (A_1/A_2) $\times$ (Springiness)

where A_1 and A_2 are the areas under the force-deformation curves of the first and second bites, respectively; "hardness" is the force measurement at the second major peak of the first bite (at the end of the first downstroke); and "springiness" the width (deformation units) of the second bite curve.

EXPERIMENTAL CURVES AND THE MEANING OF SOME OF THEIR PARTS

THE STANDARD PROCEDURE for TPA performance includes the setting of a predetermined deformation (LF in Fig. 1). Once this deformation is reached (in both bites) the crosshead is returned up to its initial position. The part of the curves which are marked by ΔA_1 and ΔA_2 in Figure 1 represent this return stage. It is quite obvious that at this stage the deformation as well as the force are both decreasing. The true representation of the force-deformation curves is shown in Figure 2 where the correct directions of the deformations are taken into account. (The apparent error in Fig. 1-type curves is due to the fact that the chart direction was not reversed during the outstroke and therefore, the abcissa to the right of the point LF are in fact time coordinates and not deformation.)

It should also be noted, that if the return speed of the crosshead during the upstroke is much higher than the speed during the downstroke and the chart speed is kept constant, the area or the width of the ΔA's in Figure 1 will appear very small and might lead to their neglection in the calculation of the TPA parameters (Ahmed and Dennison, 1971). If, however, the return speed is the same as that of the downstroke or the adjustment for the chart crosshead speed ratio is done by calculation, the areas of the ΔA's in Figure 1 might be considerable and for certain kinds of materials could reach the level of several tenths of the original areas A_1 and A_2. In such cases, the effect of the ΔA's on the evaluated TPA parameters would be quite considerable (Olkku and Rha, 1975).

Furthermore, it is quite obvious that since the specimen which is subjected to the second bite is the same specimen at

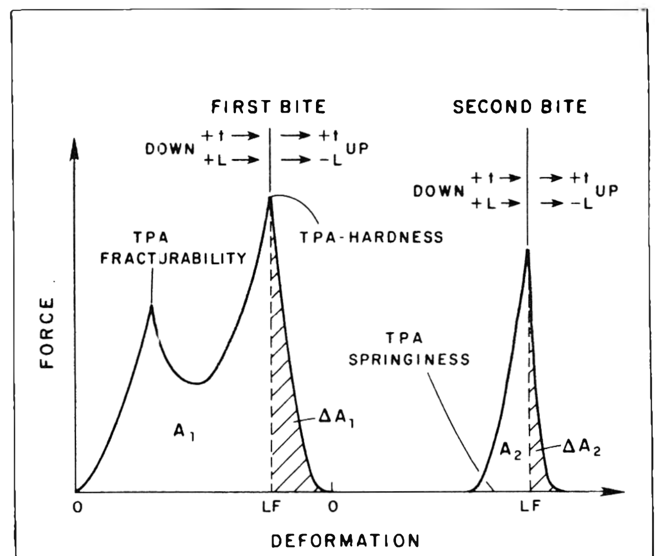

Fig. 1—Typical shape of a TPA curve obtained by the Instron Universal Testing Machine. (LF is the predetermined maximum deformation from which the upstrokes start.)

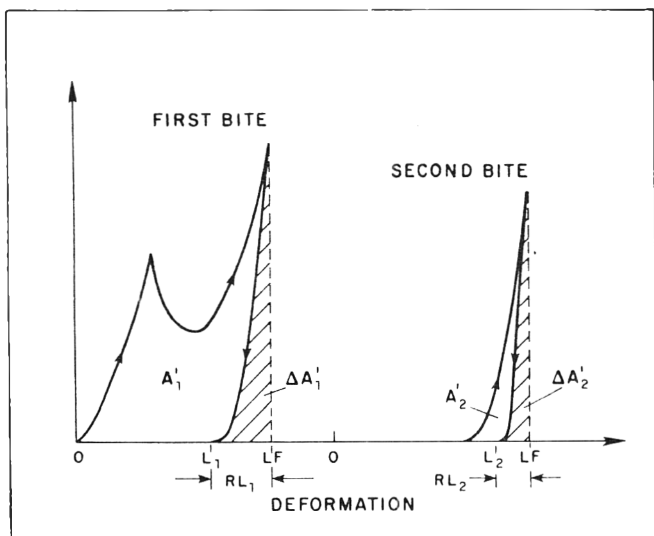

Fig. 2—The corrected shape of a TPA curve obtained by the Instron Universal Testing Machine. (LF is the predetermined maximum deformation; L_1 and L_2 are the retained deformations after the removal of the force after the first and second bites; RL_1 and RL_2 are the recovered deformations after the first and second bite.)

the end of the first bite, its length is the sum of the residual length after the predetermined deformation and the recovered deformation after the first bite. In other words, the "springiness" as shown in Figure 1 is approximately the recovered deformation (RL_1) of the first bite in Figure 2. (It would be theoretically equal to this value if the recovery of the deformation would be ideal elastic. However, since some retarded recovery might be expected due to the viscoelastic properties of most food materials, the value of the "springiness" might be slightly bigger, theoretically, and even dependent on the time elapsed between the bites.)

THE CORRECTED CURVE

IF CORRECTED CURVES as seen in Figure 2 are plotted, the corrected A_1 and A_2 areas have a specific physical meaning. They now represent the work invested in the nonrecoverable deformations at the first and second bite. The ΔA_1 and ΔA_2 now represent the recoverable work after the deformation of the specimen to the predetermined length. The corrected values of the TPA parameters (cohesiveness, gumminess and chewiness) can now be derived from the new parameters and their values will differ significantly from the old values, especially due to the much greater effect of the correction on the magnitude of the area of the second bite (A_2).

The corrected curves can also open the way for the introduction of additional textural parameters. An example might be the derivations of the ratios $\Delta A/A'$ which represent the ratio between recoverable and nonrecoverable work necessary for the deformation of a specimen. The recoverable deformations RL_1 and RL_2 and their derivative combinations might also be correlated with textural properties. However, as in other cases of textural evaluation by mechanical means, evidence should be provided that these mechanical parameters really and generally represent the sensory textural properties.

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Ms received 11/18/75; accepted 1/8/76.

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A Research Note
TRIMETHYLAMINE-SPECIFIC ELECTRODE FOR FISH QUALITY CONTROL

ABSTRACT

Trimethylamine (TMA) is one of the major components of the smell of spoiled marine fish. An increased TMA level is so characteristic of spoilage that the TMA levels have been used as an objective index of fish quality. A specific electrode was developed in order to simplify the measurement of TMA. The Orion ammonia electrode was made specific for TMA by replacing the inner filling solution with 0.01M TMA · HCl in 0.04M KCl and by adding enough formaldehyde to the sample solution to obtain a concentration of 0.22% (0.075M). The electrode is suitable for the measurement of TMA in aqueous solutions and in homogenates of fish muscle. The use of this electrode is much simpler than the methods now used for TMA analysis and it may be applicable in commercial practice.

INTRODUCTION

THE TRIMETHYLAMINE (TMA) LEVEL in fish is an important factor in the subjective evaluation of fish quality because of its close association with fish spoilage and its low odor detection threshold. Measurement of the TMA level provides an objective measurement of fish spoilage.

A simpler method of TMA determination is needed for use in fish quality control. The widely used colorimetric method involves several steps, including an extraction of TMA with toluene (Dyer, 1945). The method is tedious and the toluene is toxic and expensive. Recently, gas chromatography has been used (Keay and Hardy, 1972; Ritskes, 1975). The chromatographic method is rapid and accurate, but the preliminary sample handling is tedious and the equipment requires a high initial investment, constant maintenance, and a cumbersome gas supply.

Ion-specific electrodes have greatly simplified many analytical problems. These electrodes have the advantages of being rapid, accurate, and simple to use. Many of them can be used with an ordinary pH meter. Therefore, a TMA-specific electrode was developed for use in fish quality control.

The starting point for the design of a TMA-specific electrode was a commercial gas-sensing ammonia electrode (Orion Research Inc., Cambridge, Mass.). This electrode consists of a glass (pH) electrode and an AgCl reference electrode bathed in an internal filling solution of NH_4Cl, neutral salt(s), and a dye. The internal solution is separated from the sample by a gas-permeable, ion-impermeable membrane. Dissolved ammonia from the alkalinized sample solution diffuses through the membrane and raises the pH of the internal solution. At equilibrium, the pH of the internal solution is an accurate reflection of the ammonia concentration of the sample.

EXPERIMENTAL

TMA-specific electrode

The inner filling solution of the Orion ammonia electrode was replaced by a solution containing 0.01M TMA hydrochloride and 0.04M KCl. The electrode was connected to a Beckman Expandomatic pH meter model SS-2. The electrode was mounted 20° from the vertical to prevent trapping bubbles of air between the sample and the gas-permeable membrane.

TMA was measured by adding either 0.5 ml of fish extract or a small amount of a standard TMA solution to about 8 ml of water in a 10-ml volumetric flask. This was followed by 0.05 ml of 37% formaldehyde and 0.10 ml of 10M NaOH. Water was added to bring the volume to 10.0 ml and then the flask was stoppered and inverted five or six times to mix the contents. The stoppered flask was then left at room temperature to allow the formaldehyde to complex with ammonia in the sample. After 30 min the contents of the flask were poured into a 7 dram (25 × 50 mm) shell vial. Then the electrode was inserted and the sample was stirred with a magnetic stirrer. The electrode potential was measured after it had stopped drifting. This took a few seconds with concentrated samples and a couple of minutes with samples containing millimolar concentrations of TMA. The electrode potentials were translated into TMA concentrations with a standard curve.

Preparation of fish extracts

Fresh fish were purchased at local markets. 100g of fish were homogenized with 200 ml of deionized water and 3.5 ml of 37% formaldehyde in a Waring Blendor. The homogenized fish was then vacuum filtered through filter paper and the filtrate was used for TMA analysis. Filtration was unnecessary for the electrode analyses, but was important for the picric acid procedure.

Determination of TMA with picric acid

The picric acid procedure (Dyer, 1945; Castell et al., 1974) was adopted for use with the equipment in our laboratory. A sample of fish extract or standard TMA solution was added to a 20 ml (28 × 58 mm) scintillation vial, and enough water was added to bring the total volume to 2.0 ml. One-half ml of 3.7% formaldehyde was added, followed by 5 ml of toluene and 1.5 ml of 5M KOH. The vials were then capped and shaken at 250 rpm for 25 min in a gyratory shaker (New Brunswick Model G2). After separation of aqueous and organic phases, about 4 ml of the organic phase was then transferred into another scintillation vial which contained about 1/3g of anhydrous Na_2SO_4. The vial was capped and shaken at 250 rpm for 10 min. After the Na_2SO_4 had settled out, 2 ml of the toluene solution was removed carefully to a 18 × 150 mm test tube containing 2 ml of 0.02% picric acid in toluene. After mixing, the 410 nm absorbance of the solution was read.

RESULTS & DISCUSSION

FORMALDEHYDE was added to the sample solution to reduce the response of the electrode to ammonia. Without formaldehyde, the Orion electrode responds equally well to ammonia and TMA. The concentration of formaldehyde chosen was sufficient to prevent interference from ammonia but low enough not to release irritating fumes.

Once formaldehyde was added to sample solutions, it became necessary to modify the internal filling solution. The electrode potentials obtained with the standard Orion internal filling solution were erratic and not reproducible. The internal filling solution described in the experimental section was found to be suitable for samples containing 0.10–10 mM TMA, the range of concentrations expected in fish.

The response and selectivity of the TMA electrode was first tested in aqueous solutions of amines. The results are summarized in Figure 1. The slope of the TMA curve is 57 mV/decade, which is very close to the theoretical 59 mV/decade predicted by the Nernst equation. The selectivity of TMA over ammonia at a concentration of 1 mM varied between 10 and 500 depending upon the condition of the electrode. The elec-

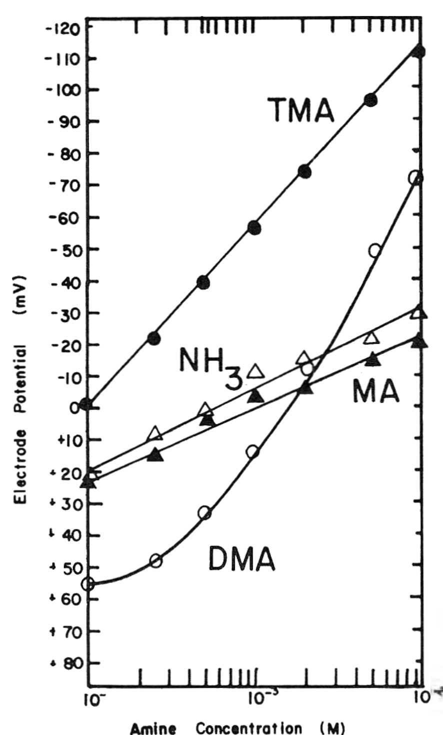

Fig. 1–Response of the TMA electrode to trimethylamine (TMA), dimethylamine (DMA), methylamine (MA) and ammonia.

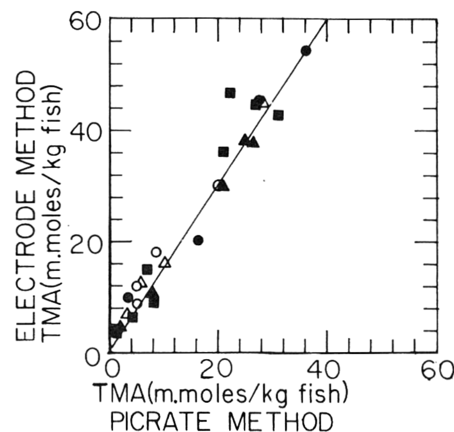

Fig. 2–Measurement of TMA in extracts of fish after storage for various times at 5°C. Correlation between determinations made with the electrode and those made with picric acid method. The fish used were the West Coast varieties of English sole (Parophrys vertulus, ○), ocean perch (Sebastodes alutus, ●), ling cod (Ophiodon elongatus, △), sand dab (Citharichthys stigmaeus, ▲), and red snapper (Sebastodes miniatus, ■).

trode response to dimethylamine (DMA) was not linear, presumably because DMA (pK_a = 10.70) is much more basic than the TMA (pK_a = 9.80). When DMA was used in the internal filling solution, a linear response was obtained for DMA, but the TMA response curved downward. It may be possible to exploit these differences to design a paired-electrode system which will yield both the TMA and the DMA concentrations of fish extracts. The DMA content appears to be an important indication of quality in frozen fish (Castell et al., 1974).

TMA measurements made on aqueous solutions by the electrode agreed very well with those made by the picric acid procedure. A series of samples of TMA at concentrations ranging from 0.1–2.0 mM gave a correlation curve of TMA (by electrode) = 0.944 TMA (by picric acid) + 0.0484 mM with a correlation coefficient of 0.962.

When the electrode and the picric acid methods were compared using fish extracts, the agreement was somewhat poorer (Fig. 2). The data were fitted by the line TMA (by electrode) = 1.452 TMA (by picric acid) + 1.107 mmoles/kg fish flesh. The correlation coefficient was 0.971. The poorer agreement is probably attributable to the fact that the electrode is not quite as selective for TMA over ammonia as the picric acid method is. Whereas the electrode had selectivity factors ranging from 10–500, the picric acid method consistently had selectivities of about 100.

Despite its modest selectivity, the electrode offers many advantages for fish quality control. It is fast, accurate, and economical to use. TMA determinations can be done with only a fraction of the materials, apparatus, sample handling, and time required for the conventional picric acid procedure. The electrode is much simpler and demands much less laboratory skill than the conventional method. The electrode's small size, simple operation, and simple instrumentation requirements recommend it for field work.

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Ms received 6/16/75; revised 12/19/75; accepted 12/25/75.

Based in part on a paper presented at the 35th Annual Meeting of the Institute of Food Technologists, Chicago, Ill., June 8–12, 1975.

This work was supported in part by the California Agricultural Experiment Station and University of California Sea Grant #R/F-23. We thank Mr. Paul Schneeman for performing the statistical analyses and Professor T.G. Barnes for help in identifying the fish.

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A Research Note
ZOOPLANKTON AND PHYTOPLANKTON FROM GALVESTON BAY: TAXONOMIC DISTRIBUTION AND COEXISTENCE WITH Vibrio parahaemolyticus

ABSTRACT

Taxonomic distribution and *Vibrio parahaemolyticus* count of 36 samples of zooplankton and four samples of phytoplankton are presented. In nearly all samples, immature forms of zooplankton accounted for more than 50% of the zooplankton population; *Nitzschia, Coscinodiscus, Navicula* and *Thalassiosira* species were present in each of the four phytoplankton samples. Mean *V. parahaemolyticus* counts (MPN/g) of 7,500 and 670 were detected in zooplankton and phytoplankton, respectively.

INTRODUCTION

Vibrio parahaemolyticus has been recognized as a potential pathogen associated with marine species and their environments. Consumption of seafoods in which this organism has been allowed to reach high viable populations can cause gastroenteritis in man. Most of the confirmed outbreaks in the United States have been associated with seafoods which were mishandled involving cross-contamination and temperature abuse. Little information is available on the presence of *V. parahaemolyticus* in organisms (e.g., plankton) utilized by various marine species. As oysters are filter feeders and are often consumed raw, *V. parahaemolyticus* concentrations in water and plankton are of interest. Low levels of *V. parahaemolyticus* (< 10/ml) are frequently present in waters of Galveston Bay including those approved for oyster harvesting (Thompson et al., 1976). Another area of association between *V. parahaemolyticus* and plankton deals with the annual cycle of this bacterium for the Rhode River subestuary of Chesapeake Bay (Kaneko and Colwell, 1973). Organisms surviving in the sediment in the winter were released from the bottom communities and became attached to the zooplankton in late spring to early summer. They proliferated as the temperature increased and became detectable in the water column as the water temperature reached 19–20°C. This note reports the *V. parahaemolyticus* count and generic distribution of plankton from Galveston Bay.

MATERIALS & METHODS

36 PLANKTON SAMPLES were collected from oyster harvesting areas in Galveston Bay during Aug–Sept, 1974 by personnel of the Dept. of Marine Science, Texas A&M University at Galveston. Locations of sampling sites are presented in a previous paper (Thompson et al., 1976). Each sample, consisting of 250 gal (945 liters) of seawater, was poured (in 2.5-gal aliquots) through a number 25 plankton net (mesh size 65 μm). The plankton (primarily zooplankton and some phytoplankton) retained by this net was collected in a liter glass jar attached to the bottom of the net. This fraction was designated as the zooplankton sample. During the collection of four of the samples, an effort was made to obtain a fraction that contained primarily phytoplankton. To accomplish the separation, the first five aliquots (12.5 gal) of water poured through the net were collected in a large washtub and stored in large glass jars. The filtrate was designated as the phytoplankton sample. From each of the two samples, 30 ml were withdrawn, fixed with 1% formaldehyde and examined for numbers and types of plankton.

The remainder of the samples (containing the unfixed plankton) were then filtered through a 0.45 μm Millipore filter within 6–8 hr after collection. Prior to filtration the samples were held at 7–12°C. After filtration the wet weight of the plankton was determined. Appropriate dilutions (10^{-1}–10^{-4}) of the plankton then were prepared with Glucose Salt Teepol Broth (GSTB). Isolation, confirmation and enumeration (MPN) of *V. parahaemolyticus* from plankton and water was by enrichment in GSTB and subsequent plating on thiosulfate citrate bile salts sucrose agar (TCBS) as described for oysters, water and sediment (Thompson et al., 1976).

RESULTS & DISCUSSION

DATA ON *V. parahaemolyticus* count, taxonomic description and distribution of 36 samples of zooplankton are presented in Table 1. The concentration of *V. parahaemolyticus* ranged from 0–110,000/g, with a mean of 7,500/g. *V. parahaemolyticus* counts in the 26 positive samples were distributed as follows: 1–10/g, (11.5%); 11–100/g, (11.5%); 101–1,000/g, (15.4%); 1,001–10,000/g, (34.6%) and > 10,000/g, (26.9%). In 26 samples, immature copepods (naupliar and copepodid stages) accounted for at least 50% of the zooplankton population. Immature forms accounted for more than 50% of the zooplankton in 32 samples. The *V. parahaemolyticus* count of the phytoplankton samples (Table 2) ranged from 9.2–2,400/g, with a mean of 670/g. The phytoplankton population in the samples consisted entirely of diatoms. The more fragile forms such as flagellates and dinoflagellates were probably disrupted by the preservative. Examination of the zooplankton samples showed that some phytoplankton was retained by the net, the filtrate consisted primarily of phytoplankton. In general the distribution of genera of phytoplankton in both samples was the same. No relationship could be established between relative abundance of zooplankton or phytoplankton types and *V. parahaemolyticus* counts.

Several reports (Kaneko and Colwell, 1973, 1975a, b; Simidu et al., 1971) indicate a close association in the marine environment between the occurrence of *Vibrio* sp. and plankton. According to Simidu et al. (1971) about 70–80% of the heterotrophic bacteria isolated from zoo- or phytoplankton samples collected off Nishiura Bay, Japan were *Vibrio* sp. The percentage occurrence of *Vibrio* in seawater of this area was about 40%. Kaneko and Colwell (1973) reported that in midsummer 100% of the total viable bacterial count of plankton from the Rhode River area of Chesapeake Bay consisted of *Vibrio* sp. Organisms closely related to *V. parahaemolyticus* comprised 6.5% of the *Vibrio* sp. count. Only 9.5% of these organisms were identified as *V. parahaemolyticus*. More than 80% of *V. parahaemolyticus* as well as organisms related to *V. parahaemolyticus* and *Vibrio* sp. were associated with plankton, plankton detritus or other particulate matter trapped dur-

Table 1–Taxonomic distribution and Vibrio parahaemolyticus counts of 36 zooplankton samples collected in Galveston Bay in Aug–Sept, 1974

Description	Number of samples and range of distribution of zooplankton types						Mean
	<1	1–20	21–40	41–60	61–80	81–100%	%
Adults–Arthropoda							
Order Copepoda							
Suborder Calanoida		27[a]	8[a]			1[a]	17[a]
Cyclopoida	11	21	2	2			6
Harpacticoida	25	11					<1
Immature stages							
Order Copepoda	1	1	5	9	15	5	58
Larvae							
Class Polychaeta	3	26	6	1			11
Pelecypoda	12	23	1				4
Cirripedia	20	16					1
Miscellaneous[b]		1					
Protozoa							
Fam. Tintinnidae	26	7	3				3
V. parahaemolyticus (MPN/g)[c]	<1	1–10	11–100	101–1,000	1,001–10,000	>10,000	7,500/g
	10	3	3	4	9	7	

[a] In 27 of 36 samples Calanoida constituted 1–20% of the zooplankton types; in eight samples 21–40%; in one sample 81–100%; with a mean of 17%.
[b] Other zooplankton immature stages (crab etc.).
[c] Per gram (wet wt) of plankton.

ing plankton hauls. The phenomenon of association between *Vibrio* and plankton was further strengthened when Kaneko and Colwell (1975a) showed adsorption of *V. parahaemolyticus* onto chitin and copepods. Efficiency of adsorption was related to salinity, pH and ions present in seawater. Samples from the Chesapeake Bay area (Kaneko and Colwell, 1973; 1975a, b) were collected with a number 20 plankton net (mesh size, 77 μm) and therefore relate primarily to zooplankton. *V. parahaemolyticus* counts of zooplankton from Chesapeake Bay (Kaneko and Colwell, 1973) ranged from a low of 0 in April–May to 10^7/g in July; in Galveston Bay (Aug–Sept) from zero to 1.1×10^5/g. In general, the *V. parahaemolyticus* counts of zooplankton collected during summer months in Galveston Bay were somewhat lower than those of samples from comparable months in the Rhode River subestuary.

Table 2–Generic distribution and Vibrio parahaemolyticus count of four samples of phytoplankton collected in Galveston Bay in Aug–Sept, 1974

Generic description	Generic distribution[a]			
	1	2	3	4
Coscinodiscus	180	120	180	240
Chaetoceros			120	
Ditylum	60			
Melosira			240	360
Navicula	120	120	180	540
Nitzschia sp.		1020	420	1200
Nitzschia seriata	1140			
Nitzschia closterium				300
Rhizosolenia			60	180
Synedra	60			
Thalassiosira	120	120	420	120
V. parahaemolyticus (MPN/g)[b]	9.2	92	190	2400

[a] Number of organisms per 1 ml of each four samples
[b] Per gram (wet wt) of plankton

Many factors including differences in characteristics of the marine environment such as salinity, temperature and pH, as well as differences in the isolation procedure for *V. parahaemolyticus* may account for this observation. Although the types of zooplankton in the samples collected from different areas in Galveston Bay usually were similar, large differences existed in the *V. parahaemolyticus* concentration ($0-1.1 \times 10^5$/g). It is possible that differences in salinity, pH and ion composition of the waters influenced the adsorption capacity of the copepods for *V. parahaemolyticus*. Increases in salinity or pH and ions such as Mg^{2+} or K^+ reduced adsorption (Kaneko and Colwell, 1975a). The lower mean *V. parahaemolyticus* count of phytoplankton as compared with zooplankton samples may reflect the limited number of phytoplankton samples examined (4 vs 36). However, the adsorption capacity of different plankton types for *Vibrio* sp. may differ because of differences in surface characteristics. The surfaces of copepods, diatoms and dinoflagellates and flagellates consist primarily of chitin, silicon and pectins, and cellulose, respectively. Hence, differences in surface structure of copepods and diatoms may be responsible for differences in count.

A comparison of the *V. parahaemolyticus* concentration of whole water samples with that of the plankton samples from the water indicates that 20–100% of the *V. parahaemolyticus* was associated with the plankton. The accuracy of these figures is somewhat limited because of the limitations in sample size and in the enumeration techniques (MPN-procedure). Although comparable figures reported by Kaneko and Colwell (1973) usually exceeded 80%, they also reported a case of lower degree of association between *V. parahaemolyticus* and plankton.

Kaneko and Colwell (1973) reported a seasonal distribution of adult and immature copepods among zooplankton samples from the Rhode River subestuary. In the winter, the population was composed primarily of adult copepods with low levels of juveniles and eggs. In mid-summer, samples contained primarily juveniles. In most of the samples collected in Galveston Bay, with water temperatures of 29–31°C, more than 50% of the zooplankton population consisted of immature forms with few adult copepods present.

Since oysters are filter feeders, feeding mostly on phytoplankton and organic detritus, *V. parahaemolyticus* concentrations in water and phytoplankton are of interest from a public health standpoint. Although oysters may ingest some zooplankton, such organisms do not constitute a significant food source. Thus the numbers of *V. parahaemolyticus* in zooplankton may be of less interest from a public health standpoint that those in phytoplankton or detritus. A previous study (Thompson et al., 1976) did not show a seasonal distribution of *V. parahaemolyticus* in Galveston Bay. This is most likely related to the relatively high water temperature during the winter months (> 10°C) as compared with the Chesapeake Bay area.

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Ms received 11/8/75; revised 12/17/75; accepted 12/25/75.

This study was supported by Public Health Service Grant FD-00582 from the Food and Drug Administration.

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