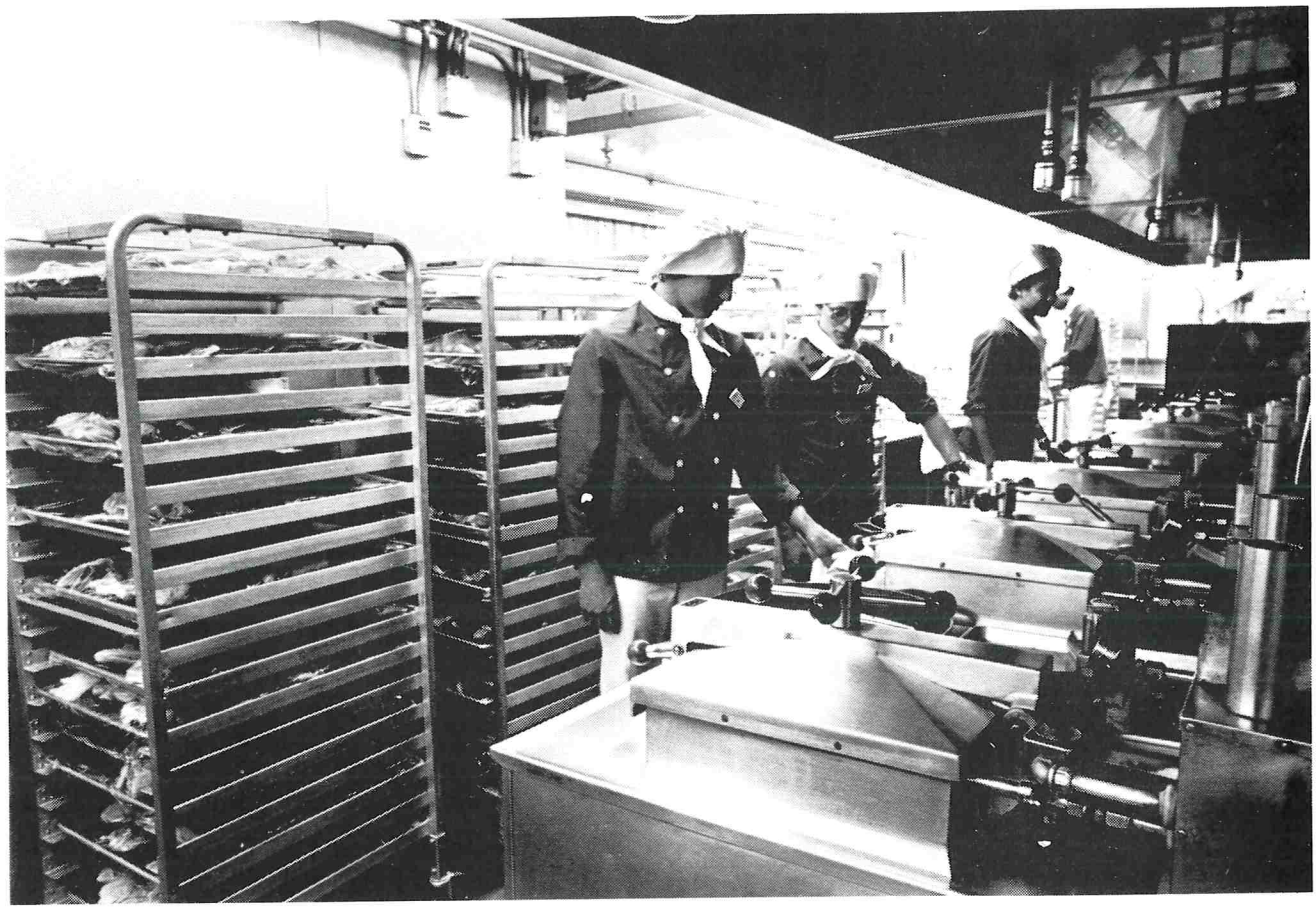
September, 1978, Vo. 41, No. 9:681-760

September, 1978
Vo. 41, No. 9
Pages 681-760
CODEN: JFPRDR 41(9):681-760(1978)
ISSN: 0362-028X

Journal of Food Protection™

An International Journal Concerned With An Adequate Food Supply That Is Safe, Wholesome, Nutritious, and Palatable

Published Monthly by the International Association of Milk, Food, and Environmental Sanitarians, Inc., Ames, Iowa, U.S.A.



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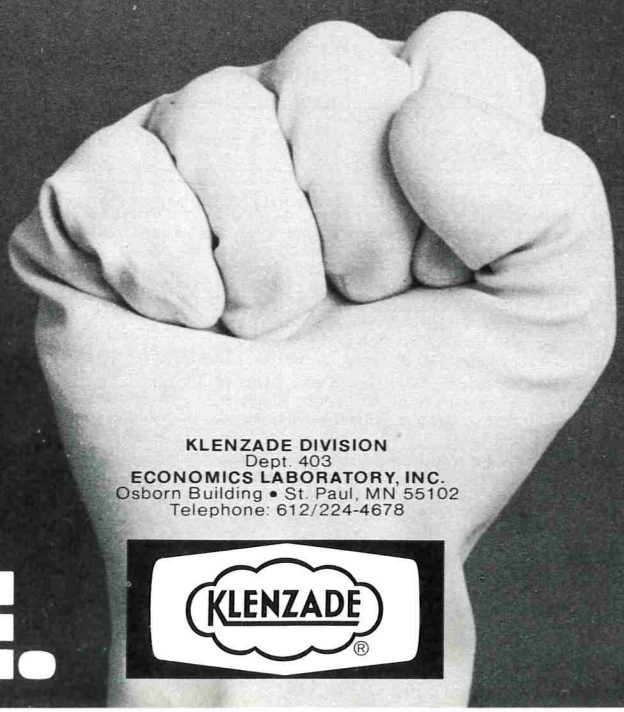

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2nd Class postage paid at Ames, Ia. 50010.

Editorial Offices: Dr. Elmer H. Marth, Dept. of Food Science, University of Wisconsin, Madison, Wis. 53706. Earl O. Wright, P.O. Box 701, Ames, Ia. 50010.

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Volumes on Microfilm are available from Xerox University Microfilms, 300 N. Zeeb Rd., Ann Arbor, MI 48106.

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Journal of Food Protection

(Formerly Journal of Milk and Food Technology)

Official Publication
International Association of Milk, Food, and Environmental Sanitarians, Inc., Reg. U.S. Pat. Off.

Vol. 41 Sept. 1978 No. 9

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Journal of Food Protection Vol. 41, No. 9. Pages 684-688 (September, 1978)

Efficacy of Chicken Pepsin as a Milk Clotting Enzyme[1]

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(Received for publication December 19, 1977)

ABSTRACT

Comparative laboratory tests of cheesemaking show similarity between chicken pepsin and calf rennet. Suitability of chicken pepsin for large-scale production of Emmental (Swiss) and Kashkaval-type cheeses was tested.

Use of calf rennet as a milk clotting enzyme in the manufacture of cheese has been predominant in the industry for centuries. Lately, a worldwide shortage of this enzyme has been predicted due to the increase in production and consumption of cheeses and the simultaneous decrease in the general availability of suckling calves' stomachs. Consequently, a great deal of interest has been generated in research for other effective and competitive rennets. However, only a few other animal proteases - such as pig and bovine pepsins - and some microbial rennet preparations have been found suitable as a rennin substitute and are presently used in cheesemaking (*4, 8*).

In Israel this problem is aggravated by religious requirements for the ritual slaughtering of calves, and the prohibition of certain clotting agents from animals such as pig pepsin. These reasons prompted local research efforts toward finding rennin substitutes, which in turn have led to production, development, and subsequent employment by the cheese industry of a pepsin of avian origin, i.e., chicken pepsin (*1, 5*). This paper describes a study of the various properties of this enzyme, such as milk clotting abilities and influence on ripening of cheeses.

MATERIALS AND METHODS

Commercial preparations (1:5000) of chicken pepsin (6.4 mg of protein/ml) (Enzyme Industries, Emek Hefer Israel) and calf rennet (1:10,000) (8.1 mg of protein/ml) (Frankental and Sons Ltd., Bene Beraq, Isreal) were employed. The strengths of the enzymes were determined by comparison with Hansen Standard Rennet powder (Chr. Hansen's Lab. A/S-Copenhagen, Denmark). For laboratory experiments purified enzymes were comparatively tested, with similar results.

[1]*Contribution from the Agricultural Research Organization, The Volcani Center, Bet Dagan, Israel. 1977 series, No 278-E.*

Enzyme activity assays

Enzyme activity was estimated by the milk clotting test done in test tubes periodically rotated in a thermostatic bath at 30 C, unless otherwise specified. To ensure reproducible results, a reconstituted skimmed milk known as "Berridge substrate" was employed. This substrate consists of 12 g of low-heat spray-dried skim milk powder (6 mg of whey protein/g index) dissolved in 100 ml of 0.01 M $CaCl_2$ solution. After adding the skim milk powder to the $CaCl_2$ solution, the mixture was stirred for 20 min and left to stand at room temperature for an additional hour. This preparation procedure was adopted since we noted that the time required for coagulation increased with the age of "Berridge substrate", particularly for freshly prepared milk solutions. Immediately afterwards, 10 ml of milk was heated at the assay temperature, 1 ml of enzyme solution was added, and the clotting time was determined.

The milk clotting time test was used to study the effects of the following parameters on the activity of both enzymes.

Enzyme dilutions. Both commercial enzymes were diluted to the range of 1:50 to 1:400 and their clotting times were tested.

Substrate concentration. Solutions containing 10, 11, 12, 13, or 14% low-heat, spray-dried skim milk powder in 0.01 M $CaCl_2$ were placed in tubes each containing 10 ml of solution. They were preincubated in a 30 C water bath for 5 min, after which 1 ml of enzyme solution was added. It is noted that the pH (6.36) was the same for all solutions employed in this test.

Substrate pH. In identical samples of reconstituted milk ("Berridge substrate"), the pH was adjusted over the range 5.1-6.7 with solutions of HCl or NaOH, 0.2 N. The final pH was measured after 20 min of stirring and 1 h of incubation at room temperature.

Calcium ion concentration. Twelve grams of low-heat spray-dried skim milk was dissolved in 100 ml of distilled water containing 0.0-0.1M $CaCl_2$ and was placed in tubes each containing 10 ml of solution in which the milk clotting tests were done. Although addition of $CaCl_2$ changed the pH of the milk from 6.60 (O M $CaCl_2$) to 5.61 (0.1 M $CaCl_2$), no pH correction was made. Attempts to correct the pH value by addition of acid or base yielded erratic results, most probably due to irreversible modifications of the micellar structure of the milk protein.

Reaction temperature. Tubes containing 10 ml of "Berridge substrate" were incubated in water baths, the temperatures of which were adjusted in the range of 25 to 55 C. After 5 min of incubation, 1 ml of each of the enzymes was added and the clotting time measured.

Proteolytic activity. The proteolytic activity of both enzymes was estimated by two sets of tests. (*a*) Aqueous solutions of casein (1.5%) adjusted to pH value of 5.49 were incubated at 30 C with chicken pepsin or calf rennet. At certain intervals samples were drawn from the solutions and TCA solution (6%) was added in the ratio 1:1. Samples were kept at 5 C for 30 min and then centrifuged. The nitrogen content of the supernatant fluid of each of the samples was determined. (*b*) To solutions of "Berridge substrate" incubated at 30 C, 1 ml of calf rennet of chicken pepsin (diluted 1:100) was added. The coagulum was knife-cut, dipped, and the nitrogen concentration in the whey was determined. A similar experimental procedure served to

estimate the amount of enzyme transferred to the whey. Thus the fully collected whey was lyophilized, the dry material left was dissolved in distilled water and the recovered activity of the coagulating enzyme was estimated by the milk clotting test, done in parallel to control tests with known amounts of enzyme which underwent the same treatment.

Cheese production

Emmental (Swiss) and Kashkaval-type cheeses were produced at the "Tnuva" Tel Yosef Dairy. Milk of identical origin, fat content and total solids was placed in two 5000-liter vats and coagulated with the calf rennin or chicken pepsin, respectively.

The Emmental-type cheese was produced from 3.1% fat standardized cows milk, HTST pasteurized (72 C for 16 sec) and cooled to 30 C. Starter, including thermoduric bacteria, 0.3 kg of $CaCl_2$/1000 liters and either water-diluted calf or chicken pepsin (adjusted to pH 4.0 with $NaHCO_3$), was added. After ca. 30 min the coagulum was ready for cutting. The cutting, cooking, whey expulsion, molding, brining and ripening were done as usual for Emmental cheese (*6*).

The Kashkaval-type cheeses was produced from 4.3-4.5% fat standardized sheep milk, preheated to 32 C. Starter and the renneting enzymes were added. After coagulation, the coagulum was cut into pea-size grains, heated gradually to 38 C followed by cutting and cooking at 85 C under kneading. The cheese was salted in a brine solution (24% NaCl) for 3 days and preripened in a curing room at 10 C for 3 weeks. The cheese blocks were waxed, packed in Saran sheets, and transferred to a cool (8 C) ripening room for 2 months.

This parallel production was repeated three times. The cheeses produced were sampled and analysed at two-week intervals during the ripening period, starting on the fifth day after production. Nitrogen analyses were made with the cheese solutions in sodium citrate (*9*). Total nitrogen and soluble nitrogen (unprecipitated at pH 4.7) were determined by Kjeldahl analysis. Free amino acids were determined by titration of the soluble nitrogen fraction with 0.1 N NaOH after coupling with formaldehyde. Ammonia was distilled from the cheese solution in the presence of $BaCl_2$, into 0.1 N HCl and the acid that remained was determined by back titration with standard NaOH solution. The moisture content was determined gravimetrically and fat content by the Gerber method (*6*). Organoleptic and texture tests were conducted by a taste panel.

RESULTS AND DISCUSSION

Enzyme characterization

To determine the suitability of chicken pepsin for replacing calf rennet, we compared their properties relevant to cheese production. The comparisons were made under identical experimental conditions in several laboratory tests.

Milk clotting activity. According to Holter (*2*), the relationship between clotting time (T) and enzyme concentration (C) is as follows:

$$T = \frac{K}{C} + t$$

where K and t are constants, depending upon the enzyme and milk substrate, respectively. The results of this test for chicken pepsin and calf rennet are shown in Fig. 1. A good inverse proportionality relation between the amount of enzyme and coagulation time was obtained for both enzymes.

Influence of substrate concentration on coagulation time. The results (Fig. 2) show an increase in coagulation time with higher skim milk powder concentrations, similar for both enzymes.

Influence of milk acidity on coagulation time. The acidity of milk has long been recognized as one of the critical parameters in cheese production. To determine the influence of this factor, the pH of otherwise identical samples of reconstituted milk was adjusted over the range of 5.1-6.7. The data obtained are in Fig. 3. In general, the greater the milk acidity, the shorter the

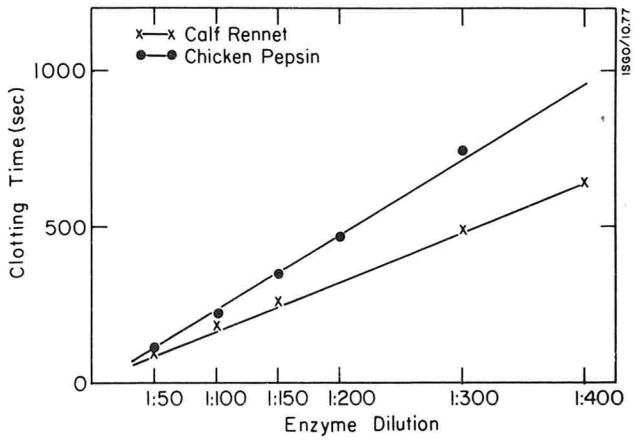

Fig. 1. *Effect of enzyme concentration on clotting time.*

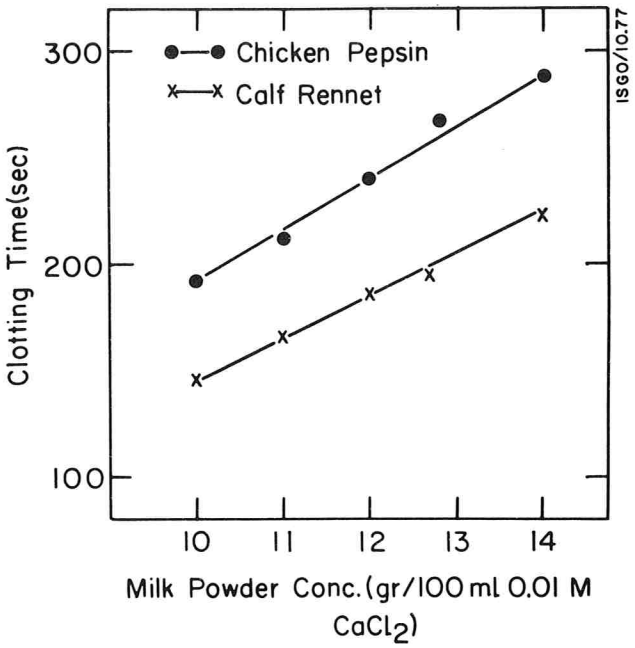

Fig. 2. *Effect of milk powder concentration on clotting time.*

clotting time became. The response of both enzymes to changes in pH was the same up to pH 6.2. Above this value the activity of chicken pepsin decreased drastically as compared with that of the calf rennet. It is noted that porcine pepsin is also inactive at pH values above 6 (*7*).

Dependence of coagulation time on concentration of Ca ions. Addition of $CaCl_2$ to pasteurized milk improves the clotting activity of the coagulating enzyme, and at the same time may regulate the water content of the cheese. The dependence of clotting time on concentration of Ca ions (Fig. 4) indeed verified the fact that increased amounts of $CaCl_2$ shorten the clotting time. The clotting activity of chicken pepsin at low concentrations of $CaCl_2$ was inhibited more than that of calf rennet. This is most probably due to the high pH values of the substrate at these concentrations of $CaCl_2$ (see Materials and Methods) which inhibited the activity of this enzyme.

Dependence of coagulation time on milk temperature. Since the enzymatic activity of proteases is temperature-dependent, this parameter is of primary importance in

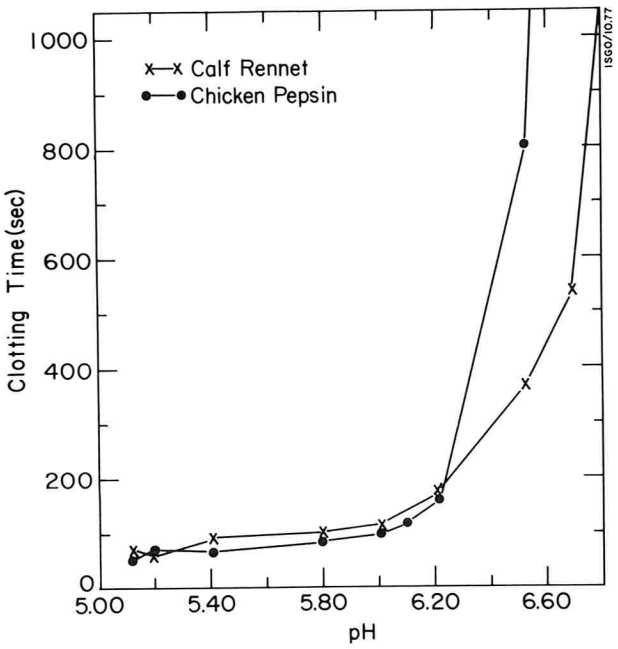

Fig. 3. *Dependence of coagulation time on milk acidity.*

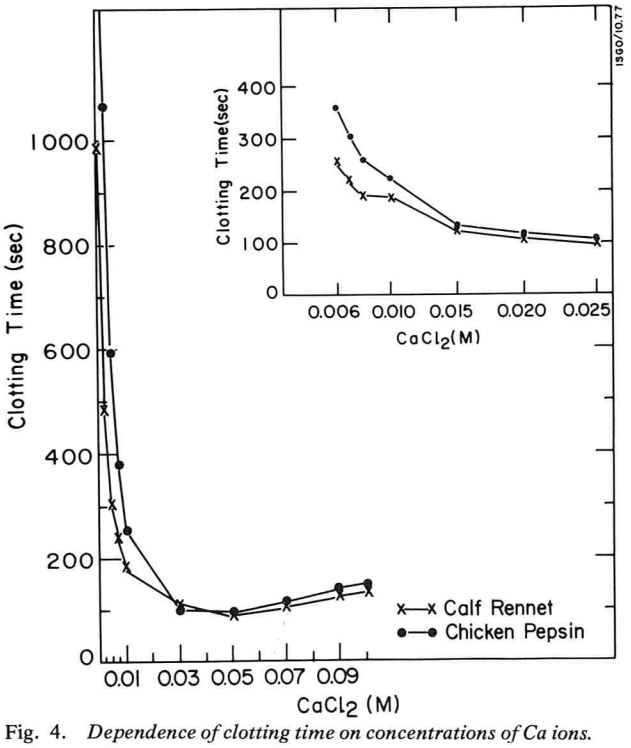

Fig. 4. *Dependence of clotting time on concentrations of Ca ions.*

cheese manufacturing. Obviously the gradient of temperature could be different for different enzymes. Results of these measurements (Fig. 5) indicates that the chicken pepsin was more temperature-sensitive over the range of 25-45 C.

Proteolytic activity. The proteolytic activity of a coagulating enzyme can be estimated by release of a small nitrogen-containing fraction (NPN) from the protein. As shown in Fig. 6, the proteolytic activity of both enzymes in the first 15 min of incubation was similar. Subsequently, in the chicken pepsin-treated solutions, an increase in the soluble nitrogen release was

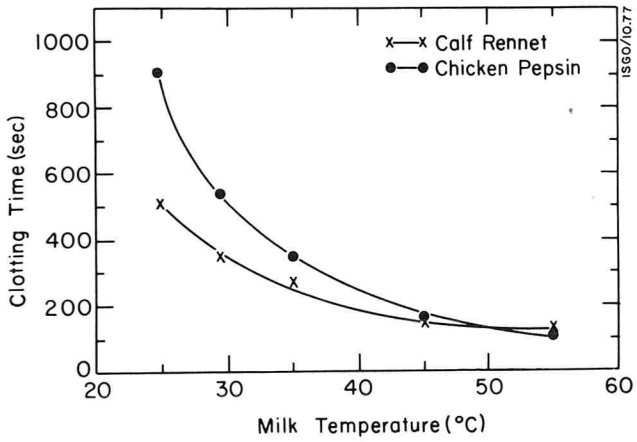

Fig. 5. *Dependence on coagulation time on milk temperature.*

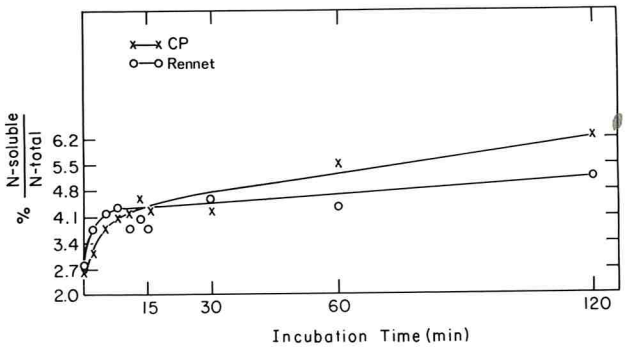

Fig. 6. *Effect of incubation time on NPN.*

observed, which indicated an enhanced proteolytic activity under these experimental conditions.

Nitrogen loss in whey. The proteolytic activity of a coagulant in production of cheese is also expressed by the nitrogen loss to the whey. This parameter is a primary indicator of the rate of utilization of milk proteins in the final product, i.e., cheese. Our results indicate that the nitrogen losses in whey which was separated from curd under identical experimental conditions for both enzymes, were virtually identical and unchanged with the incubation time (Fig. 7).

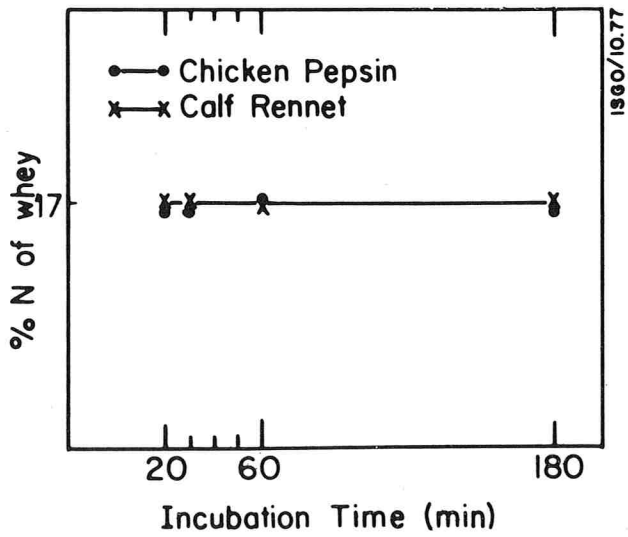

Fig. 7. *Nitrogen loss in whey.*

Transfer of enzyme to whey. Development of the body and flavor in cheese depends in part on the activity of the coagulant used. The residual coagulant in curd has an influence on the ripening process and on cheese quality. To estimate the amount of residual enzyme in curd, the amount of enzyme transferred to the whey was determined. The results showed that ca. 50% of the initial chicken pepsin and 30% of the initial calf rennet activity were found in the whey. We assume that the residual enzyme was retained in the curd.

Cheesemaking

The final criterion of performance for a rennet substitute is the quality of cheese made with it. Following laboratory experiments, it was evident that a substantial likelihood existed that chicken pepsin could replace calf rennet in cheese production. The suitability of chicken pepsin was examined by preparing two kinds of representative cheeses, Emmental and Kashkaval-type, for which several parameters were compared during the ripening period. Thus total solids, fat content, total nitrogen, soluble nitrogen, free amino acids and ammonia were monitored at different time intervals, from the fifth day until the ninth month of ripening. In the Emmental-type cheese no differences could be detected by these tests between cheese made with chicken pepsin or rennet. Most significant was the finding that no enhanced proteolytic decomposition was recorded for chicken pepsin, as reflected by comparison of soluble nitrogen, free amino acids and ammonia analyses (Table 1).

In Kashkaval-type cheese, the tendencies in changes of total solids (52-53%), fat content (45-46%), total nitrogen (8%), free amino acids and ammonia were also similar for the two enzymes (Table 2). However, the soluble nitrogen measurements indicated a more enhanced proteolytic cleavage for chicken pepsin. Specifically, this parameter varied between 0.89 and 2.83%, and 0.66 and 1.90% for cheese made with chicken pepsin or calf rennet, respectively. It is possible that this difference between the Kashkaval-type and Emmental-type cheese was due to the lower water content of the latter. However, organoleptic tests showed no definite difference in taste, flavor or body texture between the cheeses. A bitter taste was not detectable in any of the samples tested.

Attention to pH control and temperature, together with a slight modification in the technique of cheesemaking, have enabled satisfactory production of many types of cheeses in Israel with chicken pepsin. All unripened soft cheeses and some of the ripened ones (Emmental, Kashkaval, Edam, Danbo-type) have been manufactured successfully using this enzyme.

Cheddar cheese made with chicken pepsin has been reported (*3*) to be of poor quality, having soft body, weak flavor and intense off-flavors which indicate extensive proteolysis. The difference between this result and the present study could be due to the different processing conditions of the cheeses tested. Thus the processing of Emmental and Kashkaval-type cheeses requires high temperatures of 52 and 85 C, respectively, while during

TABLE 1. *Compositional analyses of Emmental-type cheese made with different clotting enzymes*[a].

Ripening time (days)	Rennet			Chicken pepsin		
	Soluble nitrogen	Free amino acids	Ammonia	Soluble nitrogen	Free amino acids	Ammonia
5	0.79	0.03	0.02	0.86	0.02	0.01
27	1.07	0.10	0.07	0.97	0.132	0.07
42	1.33	0.19	0.08	1.53	0.21	0.08
55	1.46	0.23		1.51	0.22	
76	1.60	0.31		1.76	0.31	
104	1.96	0.42		1.90	0.58	
160	2.10	0.55	0.10	1.97	0.59	0.10
270	2.55	0.56	2.10	2.67	0.59	1.92

[a]The percent of total solids which varied between 62-63% served as the basis for calculations. The amount of fat was unchanged at 51%.

TABLE 2. *Compositional analyses of Kashkaval-type cheese made with different clotting enzymes*[a].

Ripening time (days)	Rennet			Chicken pepsin		
	Soluble nitrogen	Free amino acids	Ammonia	Soluble nitrogen	Free amino acids	Ammonia
5	0.66	0.03	0	0.89	0.03	0
12	0.83	0.04	0.04	1.11	0.05	0.05
27	0.95	0.08		1.25	0.11	
55	0.95	0.15	0.05	1.29	0.11	0.06
76	1.07	0.27		1.50	0.31	
104	1.22	0.41	0.05	1.71	0.44	0.06
160	1.70	0.56		2.36	0.63	0.09
270	1.90	0.62	1.55	2.83	0.65	1.71

[a]Calculated as percent of total solids.

cheddaring the maximum temperature achieved is only 38 C. (*6*). The heat treatment of the curd might partially inactivate the enzyme, thus affecting the degree of proteolysis during ripening.

ACKNOWLEDGMENTS

The authors express their appreciation to Mr. H. Balaban and Mr. M. Frank of the Tnuva Tel-Yosef Dairy for their valuable help in the cheese produciton; and to Miss Christine Navrot and Mrs. Solange Bernstein for their technical assistance.

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Journal of Food Protection Vol. 41, No. 9, Pages 689-691 (September, 1978)

Effect of Temperature on Survival of Yeast in 45° and 65° Brix Orange Concentrate

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(Received for publication January 30, 1978)

ABSTRACT

A study was conducted to determine the effect of cold-temperature storage on yeast survival in 45° and 65° Brix orange concentrate, under simulated conditions of bulk storage. Suspensions were prepared from each of three strains of yeast which had been identified as to genus and species. Orange concentrates at 45° and 65° Brix were inoculated to contain approximately 1,000,000 organisms per ml. Inoculated samples were then stored at -17.8, -9.4, -1.1, and 4.4 C. Samples were analyzed for total viable yeast periodically during 15 months of storage. No yeast growth occured in either 45° or 65° Brix concentrate at temperatures below 4.4 C. Survival curves of one of the test organisms in both 45° and 65° Brix are presented. Yeast died faster in 45° Brix at -17.8 C than at -9.4 or -1.1 C, while in 65° Brix survival was greater at lower temperatures. Mold was detected in 45° Brix at -1.1 C and below after 7 or more months. In 65° Brix, mold was observed after 12 months at all temperatures above -17.8 C. Some samples showed a slight brown discoloration after extended periods of storage.

It has been the general practice in the citrus industry to store bulk product in 55-gal. drums at -17.8 to -23.3 C. This has involved the use of thousands upon thousands of drums each season. Product in drums requires a considerable amount of handling-filling, storage, when removed from the warehouse, thawing, and again when the product is blended back into the concentrate stream - not a very efficient operation. As consumption of frozen orange concentrate increased, it became apparent that a more practical method was needed to handle bulk product. As a result, large stainless steel tanks have been built, some of which have a capacity of over 100,000 gal. They are housed in refrigerated warehouses, the size of the buildings depending upon the number of tanks they will accommodate. The tanks are filled with orange concentrate, usually 65° Brix, at temperatures which may range at some plants from -9.4 C to as high as -1.1 C. Periods of storage could range from a few months to over a year. Little is known of what metabolic activity may take place under these storage conditions. Kitchel (*3*) investigated the survival of four different strains of osmophilic yeast in 60° Brix orange concentrate at − 15, − 6.7, and 4.4 C. All strains grew at 4.4 C but not at − 6.7, or − 15 C. Murdock and Dubois (*5*) investigated growth of four strains of osmophilic yeast in 58.5° Brix orange concentrate. They reported growth at 4.4 C. but not at − 9.4 and − 17.8 C. In 70° Brix concentrate no growth occurred at 4.4 C. Yeast have been reported to grow in other fruit products at temperatures below 0 C. Pederson et al. (*7*) found a *Candida* sp. to grow at − 2.2 C in grape juice. Berry and Magoon (*1*) reported *Torula* sp. to grow in berries in 40% sucrose at − 4 C.

This study was conducted to determine the effect of cold-temperature storage on yeast survival in 45° and 65° Brix orange concentrate, under simulated conditions of bulk storage.

EXPERIMENTAL PROCEDURE

Test organisms used in this investigation were three strains of yeast identified as *Zygosaccharomyces vini* (Y-35), *Z. rouxii* (Y-36), and *Hanseniaspora melligeri* (Y-10). According to Lodder (*4*) the first two strains have been classified as *Saccharomyces rouxii* and the third, *Hanseniaspora valbyensis,* being synonymous with *H. melligeri.* Suspensions of each strain (hereafter referred to as A, B, and C, respectively) were prepared by washing growth from Potato Dextrose Agar slants with sterile distilled water. The concentration of each suspension was determined by the agar plate method using Orange Serum Agar containing 5% sucrose. These suspensions were then used to inoculated 45° and 65° Brix orange concentrate so that each contained approximately 1,000,000 organisms per ml. The appropriate amount of strain A suspension was added to the concentrate and mixed in a Waring Blender for 2 min. Inoculated concentrate was then transferred to sterile test tubes (approximately 5 ml. per tube.) The same procedure was repeated with suspensions of yeast strains B and C. Approximately 40 replicate tubes of each variable were then placed in cold storage for each temperature investigated (-17.8, -9.4, -1.1, and 4.4 C). Duplicate samples of each variable were removed at various intervals over a 15-month period and analyzed for total viable count. Each sample was plated in duplicate, using Orange Serum Agar. Plates were counted after 48-72 h of incubation at 30 C.

The 65°Brix orange concentrate was prepared from 67°Brix evaporator pump-out by adding sufficient sterile water to obtain the desired Brix. Commercial product was used as the source of 45°Brix concentrate. Brix ÷ acid ratios for 45° and 65° Brix concentrate were 14.6 and 19.0.

RESULTS AND DISCUSSION

Survival of yeast strain A in 45° and 65° Brix concentrate at -17.8, -9.4, -1.1, and 4.4 C is shown in Fig. 1 and 2. Survival curves for strains B and C were quite similar to strain A, therefore they are not shown.

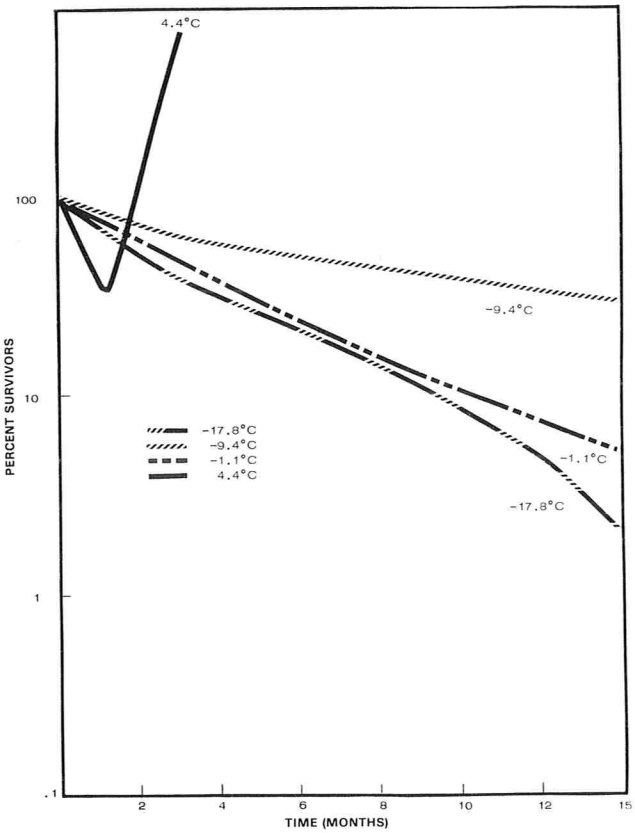

Figure 1. *Survival of yeast strain "A" in 45 ° Brix concentrate during bulk storage at –17.8, –9.4, –1.1, and 4.4 C.*

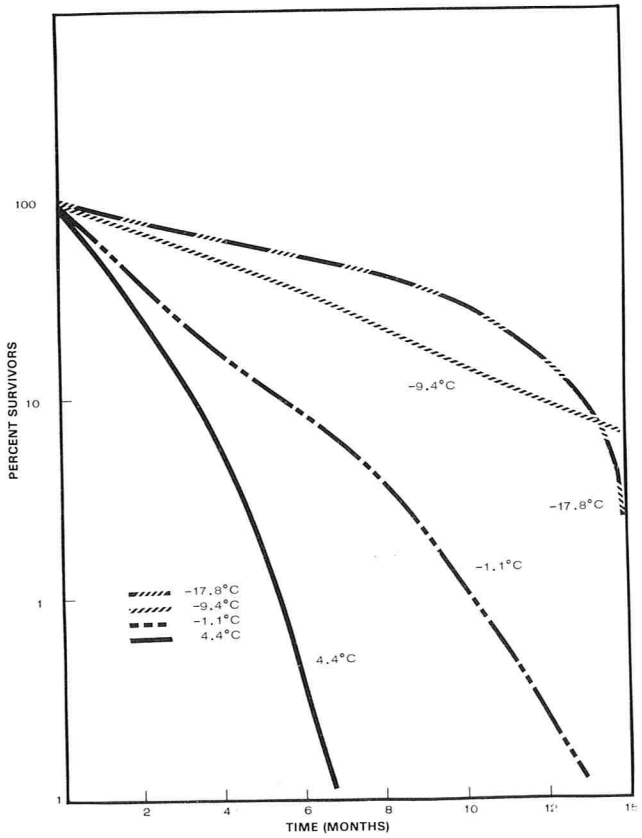

Figure 2. *Survival of yeast strain "A" in 65° Brix concentrate during bulk storage at–17.8, –9.4, –1.1 and 4.4 C.*

Yeast did not grow in 45° Brix concentrate at any of the temperatures investigated below 4.4 C. At this temperature, one strain grew in 2 months and the other two strains in 3 months. Mold was detected in 7 months at -1.1 C and 12 months at -17.8 and -9.4 C. A slight brown discoloration was noted at 7 months at -1.1 C but not at -17.8 and -9.4 C.

None of the yeast cultures grew in 65° Brix concentrate even when held for extended periods at 30 C. However, it is known that certain strains of osmophilic yeast will grow in 65° Brix concentrate. The Research Department of Continental Can Company, Inc. (*2*) investigated yeast spoilage in 65° Brix orange concentrate. Since our data showed no growth in 45° Brix concentrate at -1.1 C it is believed this would also be true in 65° Brix concentrate even if we had a yeast that would grow at this concentration. Mold growth was noted at 9 months at -1.1 C and 12 months at -9.4 and 4.4 C but not at -17.8 C. A slight discoloration was observed at -1.1 and 4.4 after 15 months, but not at -17.8 or -9.4 C.

The number of months required to reduce the yeast population by 90% is shown in Table 1. It is noteworthy that all three strains died faster in 65° Brix concentrate at 4.4 C than they did at the other temperatures investigated. Except for strain B, the yeast did not die as fast at -17.8 C as they did at the other three temperatures. Generally speaking, it appears that the lower the temperatures the slower the death rate. However, in 45° Brix concentrate this trend was not evident. At -9.4 C the yeast survived in larger numbers than they did at either -17.8 or -1.1 C.

Even though our data showed no microbial growth, metabolic activity still takes place in the viable cells, the end products of which could possibly produce off-flavors. Murdock and Brokaw (*6*) noted 6-oz. cans of 42° Brix orange concentrate held at 4.4 C swelled and sometimes burst when no increase in microbial population occurred. In this particular case, respiration of yeast apparently produced enough gas to cause cans to swell and sometimes burst. It is not known whether this evolution of gas could possibly be a problem after long periods of storage of bulk concentrate.

Effect of temperature change on yeast population

It is the custom in the citrus industry to hold high-count product in the freezer until the total viable count reaches an acceptable level. Sometimes this may occur after a few weeks, several months, or it may never reach acceptable levels. Data presented herein show two of the three strains died slower in 65° Brix concentrate at -17.8 C than they did at the other three temperatures investigated. The effect of temperature

TABLE 1. *Number of months to reduce yeast population level 90%*

Temp. (C)	45° Brix Strain			65 Brix Strain		
	A	B	C	A	B	C
– 17.8	9	3	6	13	3	9
-9.4	> 15	8	12	11	2	7
– 1.1	10	4	5	5	6	6
4.4	G[a]	G[a]	G[a]	3	1	4

[a]G = Growth.

change on yeast population in 65° Brix orange concentrate was further investigated. One, designated as a laboratory study, involved inoculating 65° Brix orange concentrate with yeast strain A, placing the inoculated material into a series of duplicate test tubes, holding 1 set for 2, 4, and 6 days at -1.1 C and another group for the same length of time at 4.4 C, then placing tubes at -17.8 C for 1 week, after which the product was plated for total viable yeast count. The results in Table 2 show the greatest reduction in yeast population occurred when the product was held 2 days at either -1.1 or 4.4 C. Holding it for longer periods at these temperature did not appear to have any beneficial effect.

TABLE 2. *Effect of temperature change on yeast population in 65° Brix orange concentrate.*

Strain A
Laboratory Study
Products held 2, 4, 6 days at -1.1 and 4.4 C, then plated after 1 wk. at -17.8 C

	-1.1 C Org./ml x 10^3			4.4 C Org./ml x 10^3		
Days	Bef.	Aft.	% Dec.	Bef.	Aft.	% Dec.
2	630	260	59	480	280	42
4	550	290	47	550	460	16
6	450	320	29	460	310	33

In another investigation, 15 5 gal. drums of 58° Brix orange blend were removed from -17.8-C storage and held 2 days at room temperature (product temperature 10-15.6 C) and then 2 weeks at -17.8 C. Yeast population before treatment ranged from 240-3,000 per ml, and after, 12-825 - a sizable reduction. The results represent what might occur with a mixed yeast flora, as this was commercial product. Also, no flavor degradation was noted as a result of this treatment.

SUMMARY

In summary, our data indicated: (*a*) Yeast did not grow in 45° Brix concentrate at any temperature investigated below 4.4 C. At this temperature one strain grew in 2 months and the other two in 3 months. (*b*) Mold was detected in 45° Brix concentrate after 12 months at all temperatures investigated below 4.4 C. A slight brown discoloration was noted after 7 months at -1.1 C but was not evident in -9.4 or -17.8 C samples. (*c*) Yeast did not grow in 65° Brix concentrate at any temperature during extended storage. (*d*) Mold growth was noted in 65° Brix concentrate after 12 months at all temperatures investigated above -17.8 C. A slight discoloration was observed at -1.1 and 4.4 C after 15 months but not at -9.4 or -17.8 C.

ACKNOWLEDGMENTS

Presented at the 90th Annual Meeting of Florida State Horticultural Society, Lake Buena Vista, Florida, November 1-4, 1977. The authors thank J. F. Folinazzo of Continental Can Co., Inc., Chicago, Il., for the yeast cultures used in this investigation.

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Journal of Food Protection Vol. 41, No. 9, Pages 692-695 (September, 1978)

Survey of the Bacterial Populations of Bologna Products[1,2]

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(Received for publication January 30, 1978)

ABSTRACT

Bologna products most frequently are stored and consumed as refrigerated products. Thus bacteria that survive processing or those that contaminate the product subsequent to processing are not destroyed. Ten types of presliced, vacuum-packaged bologna products were purchased from a high-volume retail market and analyzed for total aerobic plate count (APC) and common foodborne pathogens. No *Salmonella* were isolated. Less than 1% of the 419 samples analyzed contained either *Clostridium perfringens* or *Escherichia coli*. *Staphylococcus aureus* was isolated from 4% of the samples, but only one sample contained more than 1000/g. Just over 5% of the samples contained coliform organisms. The manufacturer appeared to play an important role in bacterial quality of the finished items. An APC $< 5 \times 10^6$/g is a realistic criterion for bologna products at the time of delivery to retail markets.

Bologna products, particularly the presliced and vacuum-packaged items, are popular among consumers. In 1976, 880 million pounds of bologna products were produced in the United States (*3*). The formulation of these products varies among producers who often have proprietary recipes. Formulations are also manipulated by producers to take advantage of favorable price differentials. With the exception of specialty items such as all-beef bologna, formulations generally contain a minimum meat component of 40% beef or beef by-products; the other meat component is pork or pork by-products (*12*). The beef by-products commonly included are: cheek, weasand, heart and tripe; and the pork by-products are: tongue, stomach, fat and heart. Other ingredients frequently included are: salt, sugar, pepper, spices, skim milk, soy protein, sodium nitrite, sodium ascorbate, and various additives for specialty products such as pickles, pimentos, cheese, olives and special spices (*12*). These ingredients can provide sources of numerous bacterial species in the product before processing (*7,10,15*).

Processing of the different bologna products is generally quite similar. The basic ingredients are mixed thoroughly with water or ice depending on the temperature desired. This mixture is passed through a double-plated emulsifier or a conventional chopper until the emulsion paste reaches the desired texture. When specialty products such as pickle and pimento loaf, olive loaf etc. are prepared, the whole or chopped additives are mixed into the paste following emulsification and before stuffing it into casings. The casings are stuffed under considerable pressure; when stuffed each weighs about 18 lb. and has approximately a 15-inch circumference. They are transferred to the smokehouse where an internal temperature of 65-70 C or slightly higher is reached after 4 to 8 h. The product is then cooled by a cold water shower and held at 4 C until temperature equilibrium is reached. After the casing is stripped, the product is sliced and vacuum packaged and is then ready for commercial distribution (*5, 12, 16*).

In addition to fixing the emulsion, the heat treatment in the smokehouse is sufficient to reduce the bacterial population (*16*). The bacteriostatic effect of spices and additives, and relatively high NaCl brine concentration of the free fluids coupled with low storage temperatures further retard bacterial multiplication. Variation in formulations, the bacterial quality of the basic ingredients, and processing procedures, particularly the cooking temperature, are factors which influence the final bacterial population of the product. Slicing and packaging operations provide a source of contamination and a means of spreading existing bacterial foci.

This study was undertaken to determine the total and pathogenic bacterial populations of various presliced, vacuum packaged bologna type products available to consumers in the retail market.

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MATERIALS AND METHODS

Samples

Vacuum packages of 10 different types of bologna were selected weekly from the display case of a local high-volume retail outlet. Display case temperature was maintained at < 7 C. Production codes or expiration dates were recorded to eliminate duplication of lots. All samples were kept refrigerated at < 4 C for 5 days before testing.

Sample preparation

A 25-g portion of each sample was weighed into a sterile 1 l blender cup and 225 ml of sterile buffered water were added (*4*). The sample was blended for 3 min at high speed. Consecutive serial dilutions to 10^{-7} were prepared and the following analyses performed.

Total aerobic plate count

Duplicate plates, 10^{-1} through 10^{-7} were prepared, poured with 12-14 ml of Standard Methods agar, allowed to solidify and incubated at 32 C for 72 ± 2 h. Preparation, counting and reporting were done in accordance with the *Bacteriological Analytical Manual* (*BAM*)(*4*).

Total coliform and Escherichia coli plate count

Total coliform and *E. coli* plate counts were made in accordance with the procedures described in *Reference Methods for the Microbiological Examination of Foods* (*13*).

Total coliform and E. coli MPN count

Total coliform and *E. coli* MPN determinations were made by using the techniques described in the *BAM* (*4*).

Staphylococcus aureus MPN count

S. aureus MPN determinations were performed in accordance with the AOAC method (*9*) except that tellurite polymyxin egg yolk agar was substituted for Baird-Parker agar. The tube coagulase test (*9*) was done as needed.

Clostridium perfringens analyses

Duplicate pour plates containing 1 ml of the 10^{-1} homogenate and 12-14 ml of sulfadiazine-polymyxin sulfite (SPS) were prepared. The SPS plates were incubated anaerobically at 37 C for 48 ±2 h. Black colonies were verified as *C. perfringens* (*8*).

Salmonellae analyses

The procedure for heated, processed and dried meat products in *BAM* (*4*) was used to determine the presence of *Salmonellae.*

RESULTS AND DISCUSSION

The mean, range, and median for the aerobic plate counts (APCs) for each group of bologna products and 90% level based on APCs progressing from smallest to largest were calculated and are presented in Table 1. Information concerning isolation of indicator organisms and selected foodborne pathogens is also in Table 1. *Salmonella* species were not isolated from any of the 419 samples analyzed. *C. perfringens* was isolated from three samples, with counts of ⩽ 5/g. *S. aureus* was isolated by the MPN method from 15 samples; however, only one sample contained more than 1000/g. Only four samples contained *E. coli* as determined by the MPN method, and all contained less than 10/g. Twenty-three samples were positive for coliform organisms by the MPN method. Twenty-one of these samples contained less than 10/g, and the other 2 samples contained 7500 and 2400/g. Only one sample contained coliforms when analyzed by the plate method; this sample also had 2400 coli/g when analyzed by the MPN procedure.

The mean APC was lowest (2.9×10^5) for all-meat bologna and highest (1.8×10^8) for olive loaf. Because of extremely large APCs, i.e., 10^9 for some samples, the mean values were disproportionately high. The median APC was lowest (3.3×10^3) for pickle and pimento loaf and highest (5×10^7) for cheese loaf. Luncheon meat and olive loaf were the only other items to have a median APC in excess of 10^7/g. The seven other products had median

TABLE 1. *Basic statistics of aerobic plate count and significant results of differential analysis of selected bologna products.*

Item	N[a]	Aerobic plate count				Significant differential results		
		Mean	Range	Median	90% Level[b]	No. pos. samples	Determination	Count/g
All beef bologna	51	1.1×10^6	2.3×10^2 - 1.9×10^7	4.9×10^3	1.8×10^6	1	*C. perfringens*	⩽ 5
						1	*S. aureus* MPN	< 10
All meat bologna	56	2.9×10^5	1.1×10^2 - 1.5×10^8	5.5×10^3	1.4×10^6	3	Coli MPN	< 10
						1	*S. aureus* MPN	< 10
						1	*E. coli* MPN	< 10
Barbecue loaf	19	5.0×10^5	4.2×10^2- 3.0×10^6	4.0×10^4	3.0×10^6	None	None	None
Cheese loaf	50	1.3×10^8	4.2×10^3 - 9.3×10^8	5.0×10^7	4.1×10^8	1	*C. perfringens*	⩽ 5
						1	Coli MPN	7500
						1	*S. aureus* MPN	2900
Jalapeno peppered loaf	50	1.2×10^7	6.0×10 - 3.9×10^8	4.6×10^3	1.3×10^7	2	*S. aureus* MPN	< 10
Luncheon meat	50	1.1×10^7	8.0×10 - 7.0×10^8	2.2×10^7	3.4×10^8	1	Coli Plate	600
						2	Coli MPN	< 10
						1	Coli MPN	2400
Olive loaf	50	1.8×10^8	1.3×10^4 - 2.2×10^9	2.8×10^7	5.3×10^8	1	*C. perfringens*	⩽ 5
						3	Coli MPN	< 10
						1	*S. aureus* MPN	< 10
Peppered loaf	17	3.7×10^7	7.2×10^3 - 4.9×10^8	9.2×10^4	9.0×10^7	9	Coli MPN	< 10
						6	*S. aureus* MPN	< 100
						3	*E. coli* MPN	< 10
Pickle + pimento loaf	50	2.3×10^7	7.0×10 - 3.4×10^8	3.3×10^3	9.3×10^7	3	Coli MPN	< 10
						3	*S. aureus* MPN	< 10
Pizza loaf	26	4.6×10^5	5.8×10^2- 3.7×10^6	1.2×10^4	1.8×10^6	1	Coli MPN	< 100

[a]N signifies the number of samples analyzed.
[b]Percent based on aerobic plate count progressing from smallest to largest.

APCs of $< 10^5$/g.

The APC for the 90% level was $> 10^8$/g for three products, cheese loaf, luncheon meat, and olive loaf. The 90% level was just over an APC of 10^6/g for all-meat bologna, all-beef bologna, pizza loaf and barbecue loaf. The APC of the 90% level for the remaining three food products was between 10^7 and 10^8/g.

Table 2 shows the APC ($\log_{10}$) by processor with the cumulative percentage by count for each product. Processor C produced 50 (100%) of the samples of cheese loaf, 46 (92%) of the samples of olive loaf and 31 (62%) of the samples of luncheon meat, the three products with highest APC median and 90% level. Processor E produced the remaining 19 (38%) of the luncheon meat samples and had a 90% level APC of 10^7/g versus a 90% level of 5×10^8/g produced by Processor C. Only 24 samples of the other products analyzed were produced by Processor C, 20 of which were pickle and pimento loaf. The counts obtained for pickle and pimento loaf produced by Processor C were generally higher than those for Processor A, but APCs for C were lower than those found for Processor I.

Overall, the APC for 290 (69%) of the samples was $< 5 \times 10^6$. Processor C was responsible for 103 (25%) of the 129 samples that had APCs of $> 5 \times 10^6$. Of the 151 samples produced by C, 48 had APCs of $< 5 \times 10^6$ and 103 were $> 5 \times 10^6$. The three products with the highest APCs, cheese loaf, luncheon meat, and olive loaf, were produced primarily by Processor C. Ninety percent of the products from Processors A, B, and D through I had APCs of $< 5 \times 10^6$. It would appear that delivery to retail stores of bologna products with APCs of $< 5 \times 10^6$ is a realistic goal for producers.

This study was consumer-oriented; thus only samples available to the consuming public were obtained. Data of this type are the "bottom line," so to speak, for the consumer, but leave some very pertinent questions to be answered. Why are the differences in APCs obtained from the same products manufactured by different producers subject to such great variation? Why do producer's APCs vary so greatly between production lots? Speculation that the bacterial quality of the components reflects the final product quality has been refuted by Warnecke et al. (*16*), who concluded the initial

TABLE 2. *Aerobic plate count ($\log_{10}$) by processor for selected bologna products.*

Bologna products[a]	Processor[b]	N[c]	< 2.7	2.7-3.0	3.0-3.7	3.7-4.0	4.0-4.7	4.7-5.0	5.0-5.7	5.7-6.0	6.0-6.7	6.7 7.0	7.0-7.7	7.7-8.0	8.0-8.7	8.7-9.0	> 9.0
All beef bologna	A	37	8	4	12	10	0	0	1	0	1	0	1	0	0	0	0
	B	9	0	1	1	1	2	2	2	0	0	0	0	0	0	0	0
	C	3	0	0	0	0	0	0	0	0	2	1	0	0	0	0	0
	D	2	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0
	CP[d]	—	17.6	27.5	52.9	74.5	78.4	82.4	88.2	88.2	94.1	98.1	100.0	100.0	100.0	100.0	100.0
All meat bologna	A	32	0	0	15	12	3	0	1	0	1	0	0	0	0	0	0
	B	19	3	2	4	0	3	1	2	1	2	0	0	0	1	0	0
	C	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
	E	4	0	0	1	1	0	1	0	0	1	0	0	0	0	0	0
	CP	—	5.4	8.9	44.6	69.9	78.6	82.2	87.6	89.4	98.3	98.3	98.3	98.3	100.0	100.0	100.0
Barbecue loaf	F	19	1	3	1	1	5	3	0	2	5	0	0	0	0	0	0
	CP	—	5.3	21.1	26.3	31.6	57.9	73.7	73.7	84.2	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Cheese loaf	C	50	0	0	1	0	2	0	5	1	2	5	11	5	14	4	0
	CP	—	0.0	0.0	2.0	2.0	6.0	6.0	16.0	18.0	22.0	32.0	54.0	64.0	92.0	100.0	100.0
Jalapeno peppered loaf	G	50	18	3	4	3	6	1	1	2	4	1	5	0	2	0	0
	CP	—	36.0	42.0	50.0	56.0	68.0	70.0	72.0	76.0	84.0	86.0	96.0	96.0	100.0	100.0	100.0
Luncheon meat	C	31	0	0	0	0	0	0	0	0	1	1	5	10	11	3	0
	E	19	4	2	3	0	3	0	2	1	1	1	2	0	0	0	0
	CP	—	8.0	12.0	18.0	18.0	24.0	24.0	28.0	30.0	34.0	38.0	52.0	72.0	94.0	100.0	100.0
Olive loaf	C	46	0	0	1	0	4	3	3	2	4	3	7	8	5	3	3
	E	3	0	0	0	0	0	0	1	0	0	0	1	0	1	0	0
	H	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
	CP	—	0.0	0.0	2.0	2.0	10.0	16.0	24.0	28.0	36.0	42.0	60.0	76.0	88.0	94.0	100.0
Peppered loaf	A	17	0	0	0	2	3	4	2	0	3	0	1	1	1	0	0
	CP	—	0.0	0.0	0.0	11.8	29.4	52.9	64.7	64.7	82.4	82.4	88.2	94.1	100.0	100.0	100.0
Pickle + pimento loaf	A	22	15	3	3	0	1	0	0	0	0	0	0	0	0	0	0
	C	20	1	0	6	3	3	1	1	0	1	1	2	1	0	0	0
	I	8	0	0	0	0	0	0	0	1	1	1	0	1	4	0	0
	CP	—	32.0	38.0	56.0	62.0	70.0	72.0	74.0	76.0	80.0	84.0	88.0	92.0	100.0	100.0	100.0
Pizza loaf	E	26	0	1	7	2	5	2	5	0	4	0	0	0	0	0	0
	CP	—	0	3.8	30.8	38.5	57.5	65.4	84.7	84.7	100.0	100.0	100.0	100.0	100.0	100.0	100.0

[a]Bologna products listed in alphabetical order.
[b]Processor letter designation assigned for all products.
[c]N signifies number of samples analyzed.
[d]CP signifies cumulative percentage.

bacterial count had little effect on the count after cooking. High bacterial counts in the component ingredients did adversely affect texture, flavor and overall product desirability (*16*). The findings of Warnecke and co-workers were confirmed by other independent studies (*11,14*).

During processing the bacterial population in bologna products is reduced to $< 10^4$/g (*16*). The spreading of these surviving bacteria and additional post-processing contamination has been identified in the slicing and packaging procedures (*2,11*). Kempton and Bobier (*11*) found that good sanitary practices with more frequent cleanup had the greatest positive effect on the bacterial quality of prepackaged luncheon meats.

Probably the most significant reasons for high APCs in bologna products are the cumulative effects of time and temperature on bacterial multiplication. Failure to chill the product promptly after cooking can result in the production of several generations of bacteria. Duitschaever (*6*) speculated that temperature abuse at the retail level was the principal contributing factor to high bacterial counts. He also speculated that high APCs should not be ascribed solely to mishandling of products during retail marketing. Allen and Foster (*1*) reported APCs for vacuum packed sliced bologna remained below 10^4 for 30 days and increased to 10^7 after 60 days when stored at 1.1 C. However, when stored at 7.2 C the APCs reached 10^4 after 10 days and 10^7 after 30 days.

From the data presented in Tables 1 and 2 it appears that either the manufacturers or the different products play important roles in the bacterial quality. Some producers consistently have higher APCs than their competitors. Since only one retail market was involved and all products were held in a similar manner, variation in APCs cannot be attributed to the retailer.

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Journal of Food Protection Vol. 41, No. 9, Pages 696-698 (September 1978)

Microanalytical Quality of Unsweetened Chocolate

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(Received for publication January 30, 1978

ABSTRACT

A survey was made to determine the sanitary quality of unsweetened chocolate. The analytical data obtained were representative of unsweetened chocolate at the national retail level. The mean and range for each defect, based on a 100-g sample, were as follows: insect fragments, 23.69 (0-95); whole insects, 0.02 (0-4); feather fragments, 0.07 (0-9); and rodent hairs, 0.57 (0-6). Positive samples found for each defect were as follows: insect fragments 99.1%; whole insects 0.7%; feather fragments 4.6%; and rodent hairs 33.2%. The association between the defect pairs is shown in correlation coefficients. It was determined that each defect occurs independently of the others.

Food defect limits were established soon after passage of the 1906 Federal Food and Drug Act. Cacao was first regulated in 1931 through a Notice to the Trade that cited limits of 10% for insect infestation and/or mold contamination in cacao beans. In 1959 the Import Cocoa Bean Survey was conducted to evaluate the applicability of the 1931 defect limits for mold and insect contamination of cacao beans. As a result of this survey, the limits for insect and mold contamination in cacao beans were revised downward to 4% for insect infestation or mold or 6% combined total. For economic reasons, greater quantities of cacao beans were being processed abroad and offered for entry in the ground form. Limits for insect fragments were thus required for the regulation of products such as cocoa and chocolate.

In 1962 and 1965 investigations were initiated to determine the correlation between percentage of insect-infested cacao beans and insect fragment counts in the ground products. Both of these studies showed that whole bean insect infestation levels do not correlate with insect fragment counts in the ground products. In 1973 a retail market sampling and analysis program was conducted to develop data on insect, bird, and mammal contamination levels in unsweetened chocolate as it was offered to the consumer. The sampling and analytical details of that program are presented in this report.

MATERIALS AND METHODS

Samples were collected by Food and Drug Administration inspectors from 20 standard metropolitan statistical areas. These 20 areas were randomly chosen from the 276 areas defined by the bureau of the Census as integrated economic and social units with a recognized urban population nucleus of substantial size (*2*). The selection of the 20 areas was subject to the restriction that continental United States, Hawaii, and Puerto Rico be represented with statistical adequacy. In each metropolitan area, products in three chain stores and two independent stores were selected for sampling. Five retail units of unsweetened chocolate were collected at each of the sampling locations. Different brands of products were selected wherever possible in a given store; however, if brand duplication was necessary, different production codes were collected. The collection plan was designed to ensure random sampling that would provide adequate lot representation nationally.

Analyses were conducted by an independent laboratory under contract to the Food and Drug Administration. One hundred-gram samples were analyzed for insect fragments, whole or equivalent insects, feather fragments, and rodent hairs according to Method 44.006 of the Association of Official Analytical Chemists (*1*). Data were obtained on 435 samples.

RESULTS AND DISCUSSION

The defects found as a result of this survey were insect fragments, whole insects, feather fragments, and rodent hairs.

Table 1 presents a frequency distribution for insect fragments. The insect fragment contamination ranged from 0 to 95. Approximately 99% of the samples examined contained insect fragments and showed a broad distribution. The median insect fragment count was 21 and the mean was 23.7. Ninety-five percent of all samples had a count of less than 59. Insect fragments originate from a wide variety of field and storage insects that attack the cacao beans during processing in the country of origin or during handling and storage in the United States. Most insect contaminants are eliminated during shell removal; however, the few remaining are subsequently reduced to fragments during the grinding of the nibs into chocolate liquor.

TABLE 1. *Frequency distribution of insect fragments in 100 g samples of chocolate.*

No. of insect fragments	No. of samples	% samples	Cumulative %
0	4	0.9	0.9
1-10	107	24.6	25.5
11-20	101	23.2	48.7
21-30	103	23.7	72.4
31-40	51	11.7	84.1
41-50	28	6.4	90.5
51-60	24	5.5	96.0
61-70	9	2.1	98.1
71-80	4	0.9	99.0
81-90	2	0.5	99.5
91-95	2	0.5	100.0

Table 2 presents a frequency distribution for whole insects. The whole insect contamination varied from 0 to 4. Approximately 1% of the samples contained whole insects and showed a narrow distribution. The median whole insect count was 0 and the mean was 0.02. Ninety-five percent of the samples had a count of zero. The whole insect contamination represents either a rather infrequently occurring, post-milling, inplant contamination or infestation in the distribution or marketing channels.

TABLE 2. *Frequency distribution of whole insects in 100 g samples of chocolate.*

No. of whole insects	No. of samples	% samples	Cumulative %
0	432	99.3	99.3
2	1	0.2	99.5
4	2	0.5	100.0

Table 3 presents a frequency distribution for feather fragments. The feather fragment contamination ranged from 0 to 9. Approximately 5% of the samples contained feather fragments and showed a narrow distribution. The median feather fragment count was 0 and the mean was 0.07. Ninety-five percent of the samples had a count of zero. Feather contamination of cacao beans may occur during processing and handling before export from the producing country or during handling and storage in the United States. The presence of feather fragments in the chocolate suggests either whole bean contamination that has not been completely removed during shelling or airborne contamination of the semi-refined product.

TABLE 3. *Frequency distribution of feather fragments in 100 g samples of chocolate.*

No. of feather fragments	No. of samples	% samples	Cumulative %
0	415	95.4	95.4
1	16	3.7	99.1
2	3	0.7	99.8
9	1	0.2	100.0

Table 4 presents a frequency distribution for rodent hairs. The rodent hair contamination ranged from 0 to 6. Approximately 33% of the samples examined contained rodent hairs and showed a moderate distribution. The median rodent hair count was 0 and the mean was 0.57. Ninety-five percent of the samples had a count of less than 4. Rodent hairs found in chocolate can originate from a number of sources. Cacao beans are subject to contamination by a variety of rodents while being prepared for export in the producing country or during handling and storage in the United States.

TABLE 4. *Frequency distribution of rodent hairs in 100 g samples of chocolate.*

No. of rodent hairs	No. of samples	% samples	Cumulative %
0	290	66.8	66.8
1	81	18.6	85.4
2	40	9.2	94.6
3	15	3.4	98.0
4	5	1.1	99.1
5	3	0.7	99.8
6	1	0.2	100.0

Table 5 provides a statistical summary for each defect variable. Scatter diagrams and their associated correlation coefficients were used as a measure of association between defect variables. These diagrams did not reveal any significant association between the various combinations of defect variables; however, some association was shown in the following correlation coefficients: insect fragments vs. whole insects –0.06; insect fragments vs. rodent hairs 0.33; insect fragments vs. feather fragments 0.18; whole insects vs. rodent hairs –0.02; whole insects vs. feather fragments –0.01; and rodent hairs vs. feather fragments 0.09.

TABLE 5. *Statistical summary for defect variables in 100 g samples of chocolate.*

Statistic	Insect fragments	Whole insects	Feather fragments	Rodent hairs
Median	21	0	0	0
Mean	23.69	0.02	0.07	0.57
Standard Deviation[a]	17.30	0.29	0.50	0.99
Minimum value	0	0	0	0
Maximum value	95.00	4.00	9.00	6.00

[a]Assuming normal distribution.

The most significant positive associations occurred between insect fragments and rodent hairs and insect fragments and feather fragments. The correlation coefficient between insect fragments and rodent hairs was 0.33 and between insect fragments and feather fragments was 0.18. A perfect direct or inverse correlation is +1.0 and –1.0, respectively. For the 435 samples involved in this study, a correlation coefficient of +0.13 or greater would be expected to occur by chance alone only 1% of the time. Thus, the correlation coefficients of 0.33 and 0.18 give statistical evidence that some association exists between the variables. However, the fact that both values are closer to 0 than to 1 shows that these associations, though real, are weak.

The analytical data presented in this report represent a current data base representative of the sanitary quality of unsweetened chocolate on the retail market.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the work of the FDA investigators and inspectors who collected the samples for the study and the members of Microanalytical Branch, Division of Microbiology, who assisted in various aspects of this study.

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Journal of Food Protection Vol. 41, No. 9, Pages 699-702 (September 1978)

Inhibition of *Vibrio parahaemolyticus* by Sorbic Acid in Crab Meat and Flounder Homogenates

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ABSTRACT

The effect of sorbic acid on growth of three strains of *Vibrio parahaemolyticus* was studied using crab meat and flounder homogenate (pH 6.2). Addition of 0.05% sorbic acid resulted in delayed growth of all three strains of *V. parahaemolyticus* in crab meat and flounder homogenates. When 0.1% sorbic acid was incorporated into homogenates, no increase in numbers of the three strains occurred in the crab meat homogenate, and only slight increases occurred in the flounder homogenate.

Vibrio parahaemolyticus has been increasingly recognized as a cause of foodborne illness resulting from ingestion of contaminated seafood (*9,14*). Recent studies have reported the survival of *V. parahaemolyticus* in refrigerated and frozen seafood products (*2,3,6,8,15*). Rapid growth of *V. parahaemolyticus* in these products poses a potential health hazard if the product is subjected to temperature abuse.

Vanderzant and Nickelson (*9*) reported that *V. parahaemolyticus* can survive in a shrimp homogenate at pH values ranging from 6 to 10 without loss of viability. Robach and Hickey (*10*) demonstrated growth of three strains of *V. parahaemolyticus* in trypticase soy broth plue 2.5% NaCl at a pH of 5.5. Beuchat (*1*) demonstrated the growth of *V. parahaemolyticus* at pH 4.8 in trypticase soy broth plus 3.0% NaCl.

Chemical inhibition of growth of *V. parahaemolyticus* has focused mainly on the effect of NaCl concentration. Covert and Woodburn (*3*) demonstrated that NaCl has a protective effect on the viability of *V. parahaemolyticus* held at −18 C in Trypticase Soy broth. Gray and Muir (*6*) reported that *V. parahaemolyticus* survived in solutions of 0.5 M NaCl independent of temperature, but few organisms survived in a 0.01 M NaCl solution regardless of temperature. Emswiler and Pierson (*4*) found 100 mM potassium phosphate buffers (pH 6,7,8) without additional NaCl to be lethal to *V. parahaemolyticus*. Buffers containing 3% NaCl resulted in maximum survival.

Lee (*7*) reviewed Japanese work on the effect of 14 food preservatives against *V. parahaemolyticus* in a laboratory medium. He reported propylparaben to be effective at 0.05-0.1%. Robach et al. (*12*) reported that 50 ppm of butylated hydroxyanisole (BHA) inactivated *V. parahaemolyticus* 04:K11 in trypticase soy broth plus 2.5% NaCl, but 400 ppm of BHA were necessary to inactive the organism in a crab meat homogenate. Robach and Hickey (*10*) found that 0.2% potassium sorbate inhibited growth of three strains of *V. parahaemolyticus* in trypticase soy broth plus 2.5% NaCl at pH 6.0 and 0.05% potassium sorbate inhibited growth of the organisms at pH 5.5.

Sorbic acid and potassium sorbate, collectively known as the sorbates, have been used as antimicrobial agents in the food industry for over 30 years (*5*). Sorbates have been mainly used as antifungal agents in many foods and are GRAS (generally recognized as safe) food additives. Recently, sorbates have been found to exhibit antibacterial activity in fresh poultry (*11*) and in fish sausage (*16*). They also have been reported to inhibit growth of salmonellae in a cooked, uncured sausage (*13*) and in fresh poultry (*11*).

This study was designed to test the effectiveness of sorbic acid in inhibiting growth of three strains of *V. parahaemolyticus* in two different seafood homogenates incubated under conditions favorable to the rapid growth of the organism.

EXPERIMENTAL

Test organisms

Three strains of *V. parahaemolyticus* were used in this study: serotype 04:K11, ATCC 27519, and ATCC 17802. Stock cultures were transferred weekly by inoculating onto slants of Trypticase Soy agar (BBL) containing an additional 2.5% NaCl (TSAS; pH 7.0). Inoculum cultures were prepared by inoculating a 250-ml shake flask containing 50 ml of Trypticase Soy Broth with an additional 2.5% NaCl (TSBS) with a loopful of the slant culture and incubating for 15 h at 35 C.

Homogenate preparation

The crab meat and flounder homogenates were prepared by mixing 20 g of commercially pasteurized blue crab meat (*Callinectes sapidus*) for the crab meat homogenate and 20 g of fresh flounder fillet for

the flounder homogenate with 180 ml of distilled H_2O containing 3% NaCl. Mixtures were homogenized in a stomacher for 2 min at room temperature. Homogenates were then dispensed in 50-ml portions into 250-ml screw-capped Erlenmeyer flasks and autoclaved at 121 C for 15 min. After cooling to room temperature, appropriate amounts of sorbic acid were aseptically added to the homogenates and the pH was adjusted to 6.2 with 8 N NaOH.

Growth studies

The growth flasks containing the sterile, pH 6.2 homogenates were inoculated with a 15-h culture of the appropriate test strain to an initial inoculum of approximately 10^3 cells/ml. Flasks were incubated in a shaker water bath (American Optical, Buffalo, N.Y.) at 175 cycles per minutes and 35 C. Samples of the homogenates were withdrawn at selected intervals, and serial dilutions were made in sterile 0.1 M potassium phosphate buffer (pH 7.2) containing 3.0% NaCl before pour plating with TSAS. After the agar had solidified, plates were overlaid with TSAS to prevent spreading and assure accurate counts. Plates were incubated at 35 C and colonies were counted after 24 h.

RESULTS AND DISCUSSION

No growth of *V. parahaemolyticus* 04:K11 occurred for up to 48 h when 0.1% sorbic acid was added to the crab meat homogenate (Fig. 1). Addition of 0.05% sorbic acid to the crab meat resulted in a prolonged lag phase

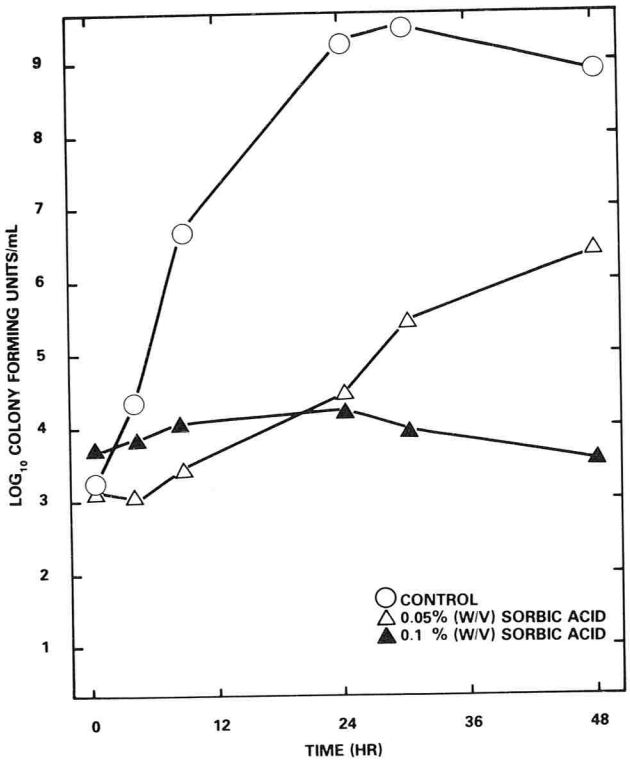

Figure 1. *Growth of* Vibrio parahaemolyticus *04:K11 at 35 C in crab meat homogenate containing 0, 0.05 and 0.1½ sorbic acid.*

and subsequent growth of strain 04:K11 was slow (Fig. 1). An initial decrease in viable cells of strain 04:K11 through 8 h and slow growth through 30 h was observed when the flounder homogenate contained 0.1% sorbic acid (Fig. 2). A prolonged lag phase of 8 h occurred when 0.05% sorbic acid was added to flounder homogenate after which normal growth was observed (Fig. 2).

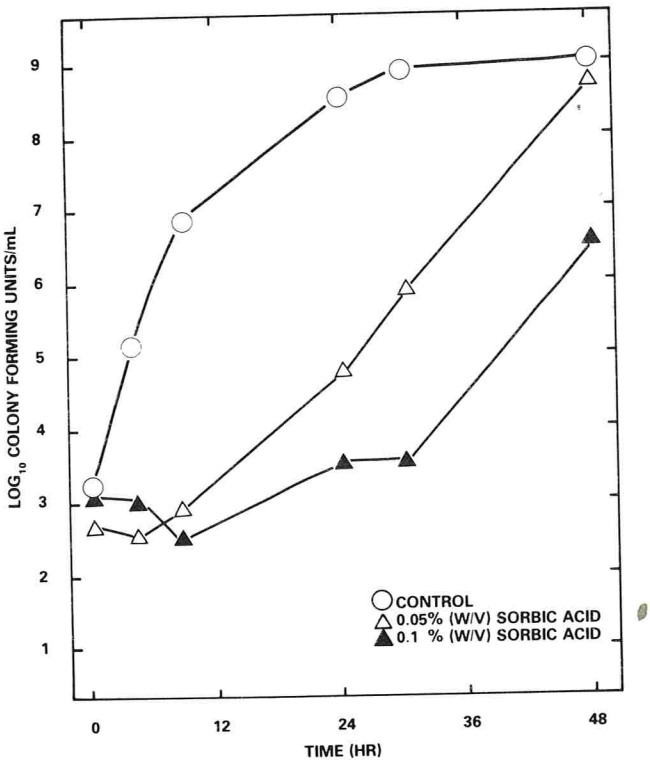

Figure 2. *Growth of* Vibrio parahaemolyticus *04:K11 at 35 C in a flounder homogenate containing 0, 0.05 and 0.1% sorbic acid.*

A sharp initial decrease in the number of viable *V. parahaemolyticus* 27519 was observed when 0.1% sorbic acid was incorporated into the crab meat homogenate (Fig. 3). Subsequent growth failed to reach the initial

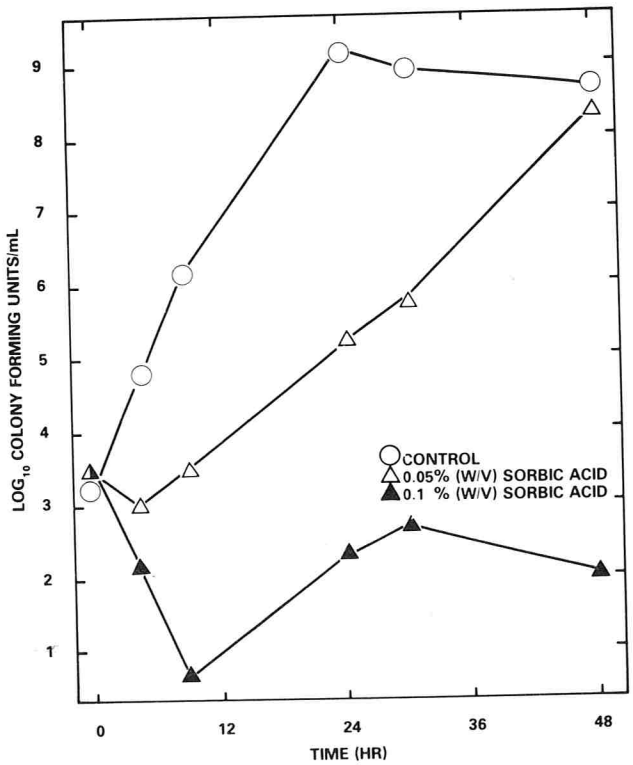

Figure 3. *Growth of* Vibrio parahaemolyticus *27519 at 35 C in a crab meat homogenate containing 0, 0.05 and 0.1% sorbic acid.*

inoculum level of 1.8×10^3 cells/ml through 48 h. Addition of 0.05% sorbic acid to the crab meat resulted in a slight decrease of viable cells of strain 27519, but after 48 h of incubation counts were approaching those of the controls (Fig. 3). When 0.1% sorbic acid was added to the flounder homogenate, a decrease in viable cells of strain 27519 was observed through 30 h of incubation (Fig. 4). A decrease in number of viable cells of strain 27519 was also observed when 0.05% sorbic acid was incorporated into the flounder homogenate but growth was initiated after 24 h of incubation (Fig. 4).

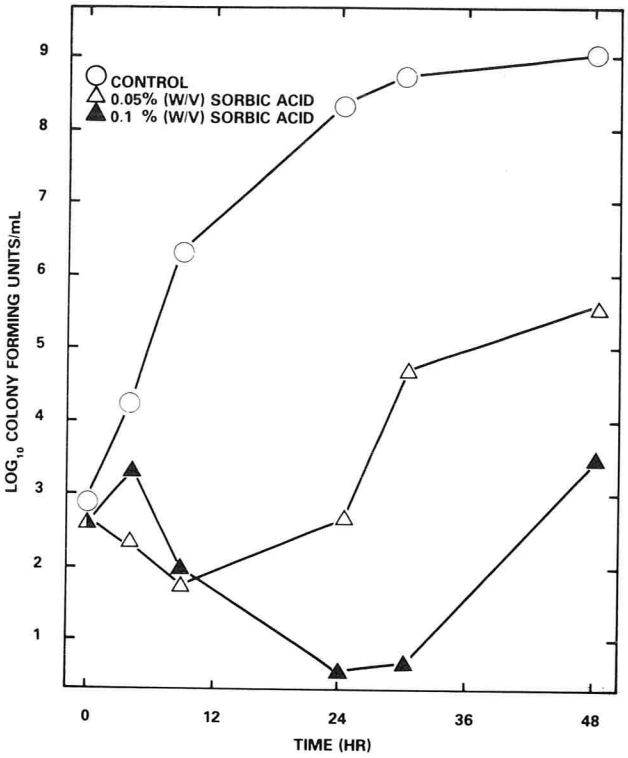

Figure 4. *Growth of* Vibrio parahaemolyticus *27519 at 35 C in a flounder homogenate containing 0, 0.05 and 0.1% sorbic acid.*

An initial 2-log cycle decrease in the number of viable cells and no subsequent growth of *V. parahaemolyticus* 17802 was observed when 0.1% sorbic acid was added to the crab meat homogenate (Fig. 5). Addition of 0.05% sorbic acid to the crab meat resulted in a slight increase in the lag time of growth of strain 17802, but later growth was relatively fast (Fig. 5). Addition of 0.1% sorbic acid to the flounder homogenate resulted in an initial 1.5-log cycle decrease in the number of viable cells of strain 17802 (Fig. 6). Growth was initiated after 8 h of incubation, but was slightly inhibited (Fig. 6). When 0.05% sorbic acid was added to the flounder homogenate, an extended lag phase for strain 17802 was observed (Fig. 6). Growth was initiated after 8 h of incubation but it also was slightly inhibited (Fig. 6).

Results obtained in this experiment indicate that sorbic acid has a definite antimicrobial effect against *V. parahaemolyticus*. Sorbic acid has been used for years as an effective antifungal agent, but only recently has its

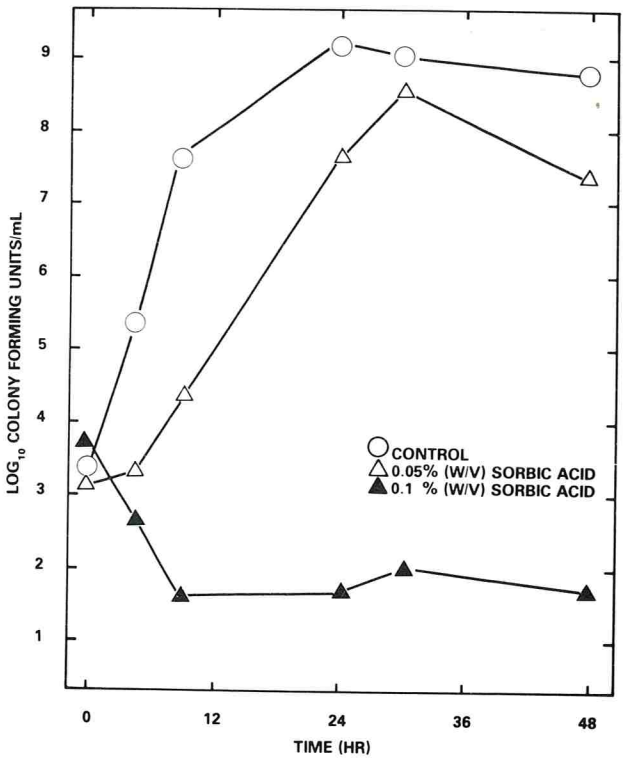

Figure 5. *Growth of* Vibrio parahaemolyticus *17802 at 35 C in a crab meat homogenate containing 0, 0.05 and 0.1% sorbic acid.*

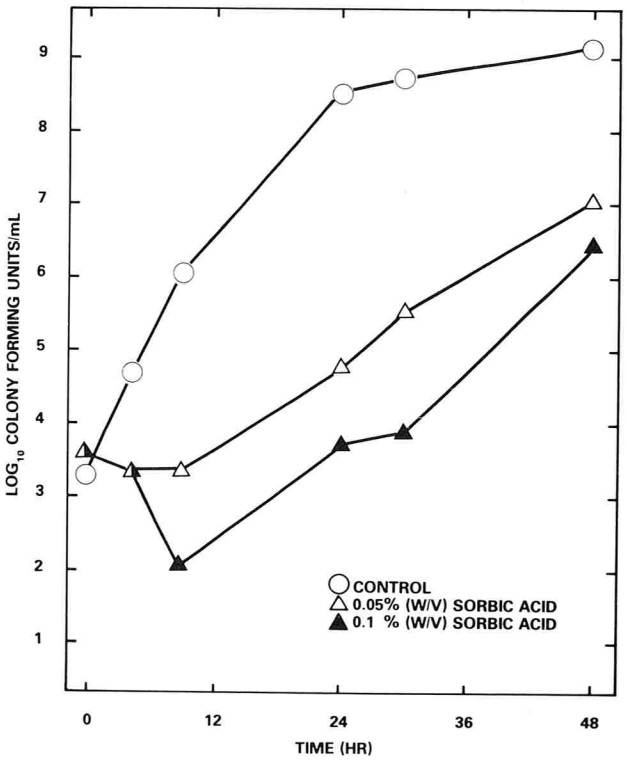

Figure 6. *Growth of* Vibrio parahaemolyticus *17802 at 35 C in a flounder homogenate containing 0. 0.05 and 0.1% sorbic acid.*

antibacterial properties been reported (*11,13,16*). Results obtained here and in other studies show that sorbic acid and/or potassium sorbate are effective antibacterial agents even in substrates with pH values in the 6.0-6.3 range. While this research focused on two model seafood systems, further studies involving different food substrates and other microorganisms are under way.

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Journal of Food Protection Vol. 41., No. 9. Pages 703-705 (September 1978)

Lactic Acid Bacteria as an Antispoilage and Safety Factor in Cooked, Mechanically Deboned Poultry Meat

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(Received for publication February 13, 1978)

ABSTRACT

Lactic acid starter cultures, *Pediococcus cerevisiae* ("Accel") and *Lactobacillus* ("Lactacel DS"), were inoculated in cooked, mechanically deboned poultry meat (MDPM) and used as repressors of three *Pseudomonas* species, *Salmonella typhimurium* and *Staphylococcus aureus*. A 50-50 mixture of the two starter cultures was the most effective treatment for delaying the time necessary for three *Pseudomonas* species to attain 10^7 cells/g, which was associated with the spoilage of MDPM. The mixture totally repressed growth of *S. typhimurium* and *S. aureus*. Changes in pH values during the storage period were too small to explain the repression observed.

Cooked, mechanically deboned poultry meat (MDPM) or MDPM obtained directly from cooked poultry meat may present a source of microbial contamination if improperly treated following cooking. Cooked products may be subjected to a great amount of handling during processing which increases the chances of recontamination with spoilage and pathogenic organisms (*10*).

Pseudomonas species are among the important bacteria dominating uncooked poultry meat at the time of spoilage (*1, 8*), and can be expected to recontaminate cooked poultry meat during refrigerated storage. *Salmonella typhimurium* and *Staphylococcus aureus* are often transmitted by meat. These organisms, respectively, were involved in 31.0 and 36.6% of the foodborne disease outbreaks in 1974 (*7*). Some strains of *S. typhimurium* are able to multiply at 10 C (*5*), a temperature which may arise with improper refrigeration. *S. aureus* is found on human skin (5-40% of the flora) and in the nose (*6*), which increases the chances for recontamination of a product with this pathogen. Growth of *S. aureus* in raw meat is repressed by the natural flora of the product. Thus, Baird-Parker (*2*) was able to show that staphylococcal poisoning outbreaks were usually associated with cooked meat.

One should remember that a cooked product is an ideal medium for growth of spoilage and pathogenic organisms because of the lack of competitors or repressors. Thus it could be of great importance, from both the economic and public health standpoints, to have an antispoilage and/or extra safety factor which will prevent or delay any significant alteration in the wholesomeness of food.

The lactic acid bacteria (LAB) are known for their ability to repress bacterial growth as means of acid production (*3, 14*), hydrogen peroxide formation (*11*), antibiotics (*4, 13*) and bacteriocin secretion (*9, 15*).

The objective of this work was to study the role of the LAB as an antispoilage and a safety factor in cooked MDPM, especially under conditions of probable recontamination and abuse using refrigerated temperatures.

MATERIALS AND METHODS

Pediococcus cerevisiae ("Accel") and *Lactobacillus plantarum* ("Lactaccel DS") were purchased from Merck & Co., Inc., Rahway, N.J. *Pseudomonas fluorescens*, and *Pseudomonas fragi* were obtained from the Microbiology Culture Collection, Cornell University. *S. aureus* FRI-100 was obtained from the Food Research Institute, University of Wisconsin. *Pseudomonas putrefaciens* (ATCC 8071) and *S. typhimurium* (ATCC 1311) were obtained from the American Type Culture Collection. The microorganisms were cultivated and prepared for the experimental work according to Raccach (*12*).

Pellets of either *P. cerevisiae* or *L. plantarum* were added to the cooked MDPM to attain a level of 10^9 cells/g. A 50-50 mixture of the two LAB (2×10^9/g) was also used.

The *Pseudomonas* species, *S. aureus* and *S. typhimurium* were added at the range of 10^3 - 10^4 cells/g MDPM.

P. cerevisiae and *L. plantarum* were enumerated using Rogosa SL Agar (Difco) (48 h, 35 and 30 C, respectively); the *Pseudomonas* species were assessed on Standard Methods Agar (BBL) (48 h, 25 C); *S. aureus* and *S. typhimurium* were enumerated on Baird-Parker medium (Difco) and Brilliant Green Agar (Difco), respectively (48 h, 35 C).

The MDPM was prepared from broiler backs and necks as previously described by Raccach (*12*). The MDPM was cooked at 121 C for 30 min and cooled to 4-5 C. The cooked meat was used on the day it was prepared.

A 10-g sample was blended with 90 ml of 0.1% Peptone (Difco) water for 2 min. Further dilutions, as required, were prepared with the same diluent. Repression of the test organisms was expressed as the logarithm (base 10) of the difference between the counts of an organism when grown in pure culture and in association with the LAB.

[1]*Present address: Microlife Technics, Box 3917, Sarasota, FL 33578.*

RESULTS AND DISCUSSION

During storage at 3 C, the mixture of the LAB had the greatest repressive action against the three *Pseudomonas* species (Fig. 1 and 2). Repression of *P. fluorescens, P. fragi* and *P. putrefaciens* was 2.5, 2.5 and 3.8 $\log_{10}$ cycles, respectively. The repressive action by pediococci was equal to that of the lactobacilli against *P. fragi* and *P. putrefaciens*, but the pediococci repressed growth of *P. fluorescens* about twice as much as did the lactobacilli. *P. putrefaciens* was the most sensitive (most easily repressed) of the three *Pseudomonas* species used.

A level of about 10^7 cells/g of *Pseudomonas* organisms in cooked MDPM was found to give an "off-odor" thus causing a rejection of the product. *P. fluorescens* in pure culture attained this level after 3 days but when grown in association with the lactobacilli and pediococci this level was attained only after 4 and 5 days, respectively, i.e., a delay of 1-2 days. The mixture of the LAB even after 5 days of storage repressed the growth of *P. fluorescens* to the extent that it did not attain 10^7 cells/g (Fig. 1A).

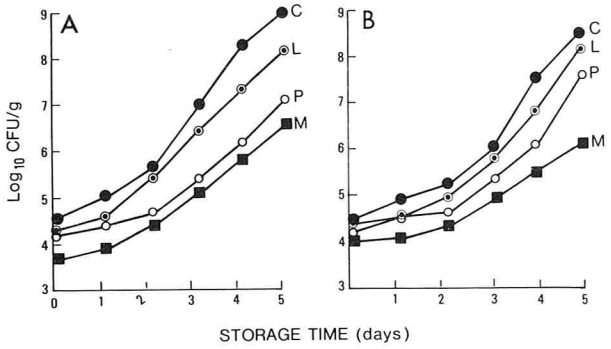

Figure 1. *Growth of* P. fluorescens *(a) and* P. fragi *(b) in pure culture (c) and in association with* L. plantarum *(L)* P. cerevisiae *(P) and their mixture (M) in cooked MDPM stored at 3 C. The LAB were added at a concentration of 10^9 cells/g.*

P. fragi in association with the pediococci attained a level of 10^7 cells/g 1 day later than the pure culture or the culture in association with the lactobacilli (Fig. 1B). The mixture of the LAB repressed the growth of the organism to the largest extent, resulting, after 5 days, in a cell density 2.5 $\log_{10}$ cycles smaller than the pure culture. *P. putrefaciens* in pure culture attained a level of 10^7 cell g after 4 days of storage, and after 5 days when grown in association with the lactobacilli but did not attain this level in the presence of either the pediococci or the mixture (Fig. 2) within the 5 days of the study. The mixture repressed the organism by 4 $\log_{10}$ cycles as compared to the pure culture. The LAB induced a lag phase on *P. putrefaciens* of 1, 2 and 3 days when grown in association with the lactobacilli, pediococci and the mixture, respectively. This was not observed with the other two *Pseudomonas* species.

The results with the *Pseudomonas* species show that the LAB, especially as a mixture, may serve as an antispoilage factor and as such they can minimize an economic loss due to bacterial action. This is extremely important in an era of ever increasing shortages of protein.

S. typhimurium was totally repressed when grown in association with the mixture of the LAB at 11 C (Fig. 3). The lactobacilli and the pediococci repressed growth of the pathogen by 1 and 2 $\log_{10}$ cycles, respectively. *S. aureus* in pure culture attained a density of 10^6 cells/g after less than 4 days at 15 C (Fig. 4). This level may be used as an indication of a potential food poisoning (*2*). Growth of the pathogen was totally repressed when grown in association with either one of the LAB or their mixture throughout the storage period of 7 days.

Results obtained with the two pathogens, *S. typhimurium* and *S. aureus,* show that the LAB (the mixture

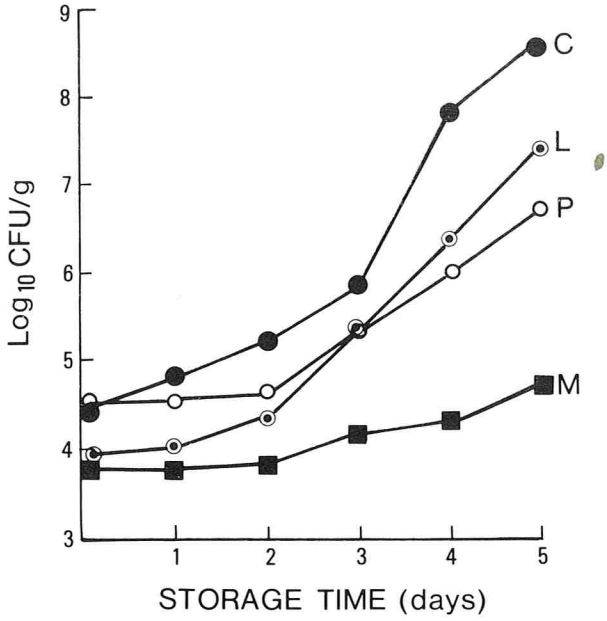

Figure 2. *Growth of* P. putrefaciens *in pure culture (C) and in association with* L. plantarum *(L),* P. cerevisiae *(P) and their mixture (M) in cooked MDPM stored at 3 C. The LAB were added at a concentration of 10^9cells/g.*

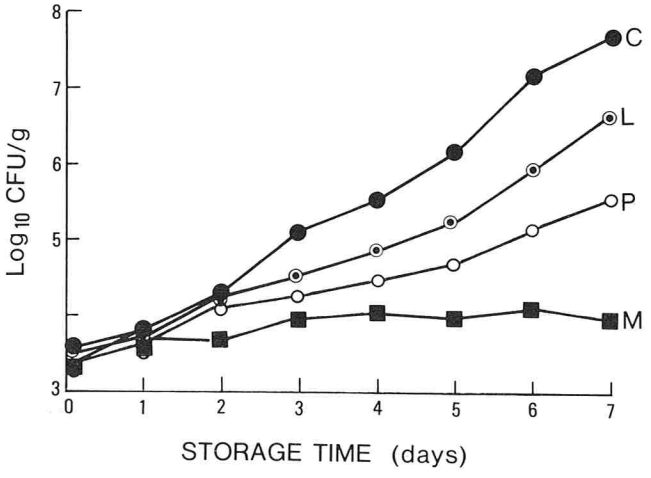

Figure 3. *Growth of* S. typhimurium *in pure culture (C) and in association with* L. plantarum *(L),* P. cerevisiae *(P) and their mixture (M) in cooked MDPM stored at 11 C. The LAB were added at a concentration of 10^9 cells/g.*

in the case of *S. typhimurium*) can be used as a safety factor for cooked products such as MDPM; they can prevent growth of the pathogens. The LAB were effective under conditions of refrigeration abuse (11 C and 15 C), which suggests their potential importance from a public health standpoint.

During the growth of all the test organisms, the pH value of the cooked MDPM changed by less than 0.2 - 0.3 unit. This is too small a change to explain the repression observed. The added LAB population did not increase throughout this study. Further work is necessary to characterize the repressive mechanism of the LAB.

In summary, it can be said that the use of LAB may in the future play an important role as an antispoilage and a public health safety factor for cooked MDPM.

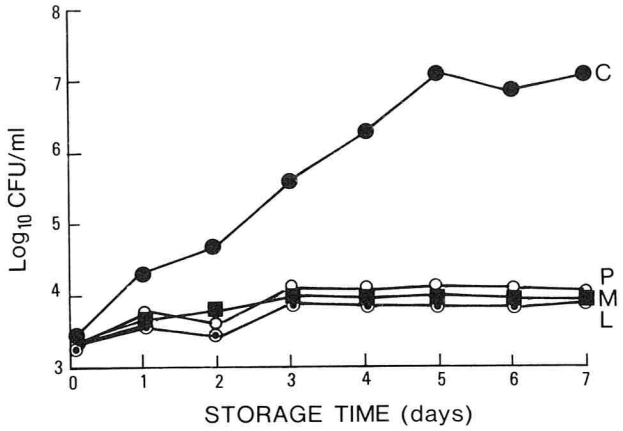

Figure 4. *Growth of* S. aureus *in pure culture (C) and in association with* L. plantarum *(L)* P. cerevisiae *(P), and the mixture (M) in cooked MDPM at 15 C. The LAB were added at a concentration of* 10^9 *cells/g.*

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Journal of Food Protection Vol. 41, No. 9, Pages 706-707 (September, 1978)

A Research Note

Persistence of Foot-and-Mouth Disease Virus in Dried Casein [1]

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(Received for publication February 17, 1978)

ABSTRACT

Dried casein produced from pasteurized milk of dairy cows infected with foot-and-mouth disease (FMD) virus retained infectivity for cattle in one of seven tests for 42 days of storage at 25 C. Thus, infectious FMD virus can persist after pasteurization of the milk at 72 C for 15 sec., acid precipitation and washing of casein, followed by drying of the casein in a hot air flow and conversion to sodium caseinate.

Results of a previous study (*3*) indicated that foot-and-mouth disease (FMD) virus could survive temperature and acid conditions used to produce casein and sodium caseinate with infective cow's milk. Limited data in that study also suggested that commercial casein drying procedures did not destroy FMD virus infectivity. The potential of such products to initiate an FMD epizootic in susceptible livestock is of particular concern in FMD-free countries. Therefore, in the following study, the persistence of infectious FMD virus in dried casein was assessed under controlled storage conditions.

MATERIALS AND METHODS

Details of milk and casein production, animal care, cell culture procedures, infectivity assays and viruses used in this study appear in a previous report (*3*). As near as possible, production and storage procedures were designed to simulate those used most often in the drug industries. Seven batches of dried casein were produced from skimmed milk of five dairy cows infected with FMD virus type A, subtype 3, strain Mecklenburg. All milk was obtained 1 day after infection, usually before clinical signs of FMD were evident.

Damp casein was dried in a stainless-steel vertical column drier. The casein was held on a fine-mesh brass screen tray vibrated by a eccentric cam on a motor-driven flexible drive shaft attached to the tray. Air (20-28 C) was blown upward through the column for 40 min. Then the input air was heated so that the tray was 65-68 C for 10 min. After drying, the casein contained 8-10% moisuture by dry-weight analysis.

Dried casein was coarsely ground with mortar and pestle, after which 2-g samples were gasket-sealed over approximately 2 ml of ambient air in screw-cap vials and stored away from direct light at room temperature (20-25 C) until used.

For infectivity testing, 2-g samples of dried casein were finely ground with mortar and pestle and dissolved in 18 ml of F-14 medium (Grand sland Biological Co., Grand Island, New York 14072). The mixture was stirred magnetically while pH was maintained between 7.0 and 8.0 by careful addition of 1 N NaOH. Dried casein slowly dissolved to sodium caseinate as the pH was raised to 8.0. In an effort to assure total solution, the slurry was repeatedly expelled through an 18-gauge needle.

After 1 day storage of dry casein, persistence of FMD virus in the derived sodium caseinate was tested in two steers. Six steers were used in all other sampling times. Each animal was inoculated with 2 ml of liquid sodium caseinate into 20 sites in the epithelium of the dorsal surface of the tongue and with 8 ml inoculated intramuscularly. Thus, each steer received 1 g of dried casein that was obtained from about 40 ml of skim milk. Six steers were inoculated at storage times of 21, 42, and 84 days. Steers were observed for 14 days postinoculation (DPI), and serum from non-reactors was assayed for FMD virus antibodies by virus neutralization procedures (*2*).

RESULTS AND DISCUSSION

Milk yield did not decrease nor were any other signs of disease seen in the dairy cows at 1 DPI other than fever in one of the five cows. However, the amount of FMDV recovered from their skim milk before pasteurization (72 C for 15 sec) ranged from 5.5 to 6.4 $\log_{10}$ plaque-forming units/ml (Table 1).

Four of the seven batches of dried casein contained FMD virus that was infectious for steers at 1 day of storage. However, only one (Batch No. 2, Table 1) of the four positive batches was infectious for steers after 21 days in storage. This batch remained infectious for steers through 42 days of storage at ambient temperature. Neutralizing antibody for FMD virus was not detected in 14-DPI serum from steers clinically negative for FMD.

Interestingly, only one of four batches of dried casein from the same pool of skim milk retained infectious FMD virus beyond 1 day of storage. This finding suggests that very minor deviations in techniques may affect survival of infectious FMD virus in the final product. In our studies, time, temperature, pH and moisture were carefully controlled to insure uniform conditions.

Observations in previous studies (*1, 3*) indicated a considerable difference in FMD virus-sensitivity between

[1] *Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the U.S. department of Agriculture and does not imply its approval to the exclusion of other products that may also be suitable.*

TABLE 1. *Infectivity of dried casein from foot-and-mouth disease virus-infected cows.*

Casein batch No.[a]	Raw skim milk titer[b]	Pasteurized skim milk titer[b]	Cattle results after days in dry storage[c,d]			
			1	21	42	84
1	6.4	<1.0	0/2	NT	NT	NT
2	6.4	<1.0	2/2	5/6	5/6	0/6
3	6.4	<1.0	0/2	NT	NT	NT
4	6.4	<1.0	0/2	NT	NT	NT
5	5.7	NT[d]	2/2	0/6	NT	NT
6	5.5	<1.0	1/2	0/6	NT	NT
7	6.3	1.6	1/2	0/6	NT	NT

[a]Casein batches 1 through 4 from the morning milking of one cow.
Casein batch 5 was from a mixture of morning and evening milk of the same cow (batches 1-4).
Casein batch 6 was from the pooled milk of three cows.
Casein batch 7 was from the milk of another cow.
[b]Log_{10} plaque forming units/ml.
[c]Two cattle used for 1-day storage assays; six cattle used thereafter; ratio=number of cattle positive/number inoculated.
[d]NT = not tested.

cell cultures and in vivo bovine tongue epithelium. Although cell cultures did not indicate infectious FMD virus in milk samples, Blackwell and Hyde (*1*) demonstrated infectious FMD virus when sample aliquots were inoculated into bovine tongue epithelium. Similarly, data shown in Table 1 (Batches No. 2 and 6) indicate a negative response in pasteurized skim milk assayed in cell cultures, yet dried casein produced from the same milk was infectious for steers.

Although responses shown in Table 1 indicate that the steers developed FMD, the disease was initiated by primary vesicles that developed at only one to five of the 20 sites inoculated on each tongue. These data suggest that casein thus dried and stored may contain a very small quantity of infectious FMD virus that is close to the threshold for detection in cattle.

ACKNOWLEDGMENTS

The authors are indebted to Mr. R. Trower and Mr. T. Sawicki for able technical assistance in this study.

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Journal of Food Protection Vol. 41, No. 9, Pages 708-711 (September, 1978)

Occurrence of *Staphylococcus aureus* in and the Moisture Content of Precooked Canned Bacon

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ABSTRACT

Staphylococcus aureus was found in 9.0% of 221 cans of precooked bacon. The count in 6.9% of the cans exceeded 1000/g and ranged as high as 1.7×10^5/g. Aerobic plate counts were greater than 10^5/g in 24% of the cans. The maximum moisture to salt ratio (percent moisture divided by percent salt) of 9.0, permitted by Federal Specifications, was exceeded in 73.0% of the cans and ranged from 5.97 to 21.44. This bacon production was rejected for military procurement.

Precooked Canned Bacon offers several advantages as a military subsistence item, not the least of which are stability without refrigeration and reduced weight. It is, however, a product which must be carefully processed and controlled, since it is not sterile and depends on low water activity for its stability.

Military Specifications (*12*) require a mean moisture-to-salt ratio (percent moisture divided by percent salt) of 9.0 or less to help assure the safety of Precooked Canned Bacon without refrigeration. This requirement was based on an empirical study of precooked canned bacon made by Whiting et al. (*13*) which showed that for military prefried canned bacon a brine ratio of approximately 9.0 corresponded to a moisture/salt × protein index of 0.400. This index (0.400) corresponded to a water activity below the point (0.9) at which *S. aureus* could grow anaerobically and would undoubtedly provide better assurance of microbiological safety than a moisture to salt ratio. However, because the cost of an additional protein analysis was considered to be prohibitive, a moisture to salt ratio requirement, only, was selected. Ultimately, a maximum water activity should be specified to assure microbiological safety of precooked canned bacon.

If water activity is not controlled, *S. aureus* is capable of growing and producing enterotoxin in bacon packed under vacuum (*3, 5, 6, 9, 10*) and will grow anaerobically at a water activity of 0.90 (*8, 13*). With sufficient time (30 days at 30C) the organism will also grow aerobically at a water activity as low as 0.86 (*8, 13*). Therefore, the water activity of precooked canned bacon should be strictly controlled to prevent growth of bacterial pathogens, particularly *S. aureus*, which may be introduced, since the bacon in question was hand-packed into cans after cooking and received no further heat treatment.

The following study of precooked canned bacon (*12*), produced for the United States Army, pointed out some potential problems. In this particular production, for example, the average moisture-to-salt ratio, lot value, ranged from 9.55 to as high as 18.7, for 12 lots tested by both a private laboratory and a Government Laboratory. The lot value must be 9.0 or less for a lot to be accepted. Since every lot tested failed to meet this requirement, the U. S. Army Research and Development Command (NARADCOM) agreed to analyze the bacon to confirm the findings of the other two laboratories and to determine if the product was microbiologically hazardous.

MATERIALS AND METHODS

Bacon

Sliced, precooked bacon, in No. 2½ cans was commercially produced for the United States Army in accordance with Military Specification MIL-B-35032C, as amended (*12*). Two hundred and twenty-one cans, comprised of 13 cans from each of 17 lots, each containing 22 oz. of bacon, were analyzed. The cans had been stored at ambient temperatures for 3 to 6 months before testing, and all appeared to be normal and in excellent condition. Vacuum of each can was determined with a Budenberg Vacuum Gauge (Broadheath, Nr. Manchester, England).

Chemical analyses

Moisture and salt determination. Procedures of the Association of Official Analytical Chemists (Chapter 24, Meat and Meat Products) were used for determination of percent moisture and percent salt (*2*).

Moisture-to-salt ratio. The moisture-to-salt ratio of each sample was determined by dividing the percent moisture by the percent salt. A lot of bacon was acceptable only when the 99% confidence interval of a single tailed "t" test fell below 9.0 for the lot average. This method was based upon 13 samples for each lot. The "t" factor for $T_{.99}$ and 12 degrees of freedom was determined from a Table of Percentiles of the "t" distribution and multiplied by the standard deviation of 13 samples. This product, added to the mean moisture-to-salt ratio of the 13 samples, must be below 9.0 for the lot to be acceptable when all other requirements are satisfied. Under these conditions no more than 1% of the lots accepted will have a M/S ratio greater than 9.0.

Water activity measurement. Sample jars containing 100 g of bacon were held at room temperature overnight to allow the bacon within the jar to come into moisture equilibrium with the head space atmosphere of the jar. Measurement of water activity (a_w) was then made, within a constant temperature chamber (24 C) with an EG&G Model 880 Dew Point Hygrometer (Environmental Equipment Division, Waltham, MA). Ambient (sample) temperature measurements were made by inserting a thermometer into the sample jar. Air was circulated within the closed system at the rate of 1.6 CFH and the hygrometer was allowed to come into equilibrium with the water vapor of the head space before dew point temperature readings were made. Dew point and ambient temperature readings were converted to corresponding water vapor from appropriate tables. Water activity of the bacon was calculated by dividing the dew point vapor pressure by the vapor pressure of pure water at the sample (ambient) temperature. The hygrometer was frequently checked against standard NaCl and $ZnSo_4$ solutions of known water activities with readings within +2% a_w of the solution value being the basis for acceptability.

Microbiological analyses

Sample preparation. Bacon was aseptically removed from each can and laid on a sterile surface inside of a class 100 laminar flow clean bench. A 50-g amount of bacon, obtained by cutting strips from the ends and the middle of the slab, was aseptically transferred to a sterile blender jar and blended in 450 ml of Butterfield's (*1, 11*) sterile buffered water (SBW) for 2 min. This slurry constituted a 1:10 dilution. Appropriate tenfold serial dilutions were made by transferring 10 ml into 90 ml of SBW.

Media. All media were purchased from Difco Laboratories, Detroit, Michigan.

Aerobic plate count. One milliliter of dilutions ranging from 10^{-2} to 10^{-4} was pipetted into duplicate petri plates, and poured with Plate Count Agar. Plates were incubated at 35C and counted after 48 h.

Yeast and mold count. One milliliter of 10^{-2} and 10^{-3} dilutions was pipetted into duplicate petri plates, and poured with potato dextrose agar acidified to pH 3.5. Plates were incubated at 23 C for 5 days before counting.

Staphylococcus aureus. A surface plating procedure (*1*) was used by distributing 1 ml of 1:100 dilution equitably (i.e.; 0.4 ml, 0.3 ml, 0.3 ml) over triplicate plates of Baird-Parker agar. The agar plates were previously prepared and dried by overnight incubation at 35 C. The inoculum was spread over the surface of the agar with sterile, bent glass streaking rods. Plates were incubated at 35 C and examined after 24 and 48 h for typical, black, shiny, convex colonies, surrounded by a clear zone (*1,2*). Typical colonies were tested for coagulase production (*1,2*).

RESULTS

Table 1 shows the distribution of microbial counts in 221 cans of precooked bacon analyzed. The aerobic plate count (APC) ranged from <100 (6.3%) to 3.5×10^7/g (1.4%). Seventy percent of the cans had APC's ranging from 10^2 to 10^5/g, but 24% had APCs greater than 10^5/g. *S. aureus* was found in 9.0% of the cans and counts in 6.9% of the cans were greater than 1000/g, ranging as high as 1.7×10^5/g. No yeasts or molds were detected at 1:100, or greater dilution.

TABLE 1. *Distribution of microbiological counts in Precooked Canned Bacon. (Percent of samples with various counts/grams[a].)*

Organism	100	101-1000	1001-10,000	10,0001 to 100,000	100,0001 to 1,000,000	1,000,001 to 10,000,000	> 10,000,000
Aerobic plate count	6.3	22.2	23.0	25.0	14.0	8.6	1.4[b]
S. aureus	90.9	2.3	3.2	2.3	1.4[c]	0	0
Yeast and mold	100	0	0	0	0	0	0

[a]Total number of cans sampled was 221.
[b]Highest APC obtained was 3.5×10^7/g.
[c]Highest *S. aureus* count was 1.7×10^5/g.

TABLE 2. *Bacterial counts, water activity (a_w), moisture/salt ratio and percent salt in cans containing coagulase positive staphylococci.*

Can No.	APC/g	Staphylococci/g	a_w	Moisture/[a] salt ratio	Percent salt
1	1.7×10^3	2×10^2	0.86	12.26	2.88
2	3.2×10^5	1×10^5	0.87	14.57	2.38
3	2.6×10^6	1.7×10^4	0.88	10.04	3.41
4	3.5×10^7	9.4×10^3	0.89	10.43	3.09
5	4.5×10^6	8.4×10^4	0.89	9.60	3.10
6	4×10^5	4×10^2	0.90	10.95	2.81
7	4.7×10^4	1.9×10^4	0.90	10.37	3.11
8	1.1×10^5	5.2×10^4	0.90	10.89	2.61
9	7×10^5	2.7×10^3	0.91	9.97	3.15
10	2.2×10^5	7×10^2	0.92	8.73	3.17
11	2.7×10^6	5×10^2	0.93	14.76	2.34
12	3.4×10^5	7.1×10^3	0.93	10.95	3.10
13	1.2×10^6	2.3×10^3	0.94	18.09	2.39
14	8.7×10^4	9.5×10^3	0.94	13.33	2.58
15	3.2×10^4	2×10^3	0.95	10.72	3.19
16	9×10^6	2.3×10^3	0.95	12.67	2.83
17	1×10^6	2.5×10^4	0.95	11.02	2.82
18	4×10^5	1.4×10^5	0.95	12.44	2.71
19	3×10^4	1×10^2	0.96	9.58	3.11
20	2.6×10^6	1.7×10^5	0.96	21.44	2.14

[a]Percent moisture divided by percent salt.

Table 2 shows the water activity, moisture/salt ratio, percent salt, and microbiological counts in the 20 cans in which *S. aureus* was found. No apparent relationship between any of these parameters was demonstrated since high counts were found in samples with both low (0.87) and high (0.96) water activity. Conversely, low counts were also found at both extremes of water activity. The water activity varied considerably in these and, indeed, in all cans produced and analyzed. There was great variation in the moisture/salt ratio in the 20 cans shown, which was typical of all cans tested, and which had no apparent relationship with bacterial counts, or with water activity. All of these cans, with one exception, and most (73.0%) of the 221 cans tested exceeded the maximum moisture/salt ratio of 9.0 permitted (*12*). Salt concentration varied between 2 and 3%. The vacuum pressure in 67% of the cans was 20 inches or greater as required by the specification and all cans had a vacuum.

DISCUSSION

Whiting et al. (*13*) suggested three criteria for predicting the spoilage potential of precooked canned bacon stored without refrigeration, which are listed as follows in order of importance: (*a*) a moisture divided by salt times protein index of 0.400, or less; (*b*) a brine ratio (moisture divided by salt) of 9.0, or less; and (*c*) a salt concentration of 1.7% or higher (*13*). Precooked canned bacon which met these three conditions was considered to have a water activity low enough (0.90 - 0.91) to inhibit growth of pathogenic bacteria, with the possible exception of *S. aureus* under aerobic conditions (*13*). Scott (*8*) reported that under aerobic conditions *S. aureus* had a limiting water activity for growth of 0.86 whereas under anaerobic conditions the limiting water activity was 0.90.

Failure of the bacon tested in this study to meet the second requirement; i.e., a moisture/salt ratio of 9.0, as required by Military Specification MIL-B-35032C (*12*), confirmed the findings of two other independent laboratories, and was the primary basis for rejection of the bacon by the Army. The poor microbiological quality (APC greater than 10^5/g) of nearly one-fourth of the cans tested (Table 1) and the presence of *S. aureus* in 9.0% of the cans, together with wide variations in both water activity and moisture/salt ratio, demonstrated the potentially hazardous nature of this product and supported the decision to reject the bacon. The *S. aureus* found could have been remnants of an even higher population which was destroyed by cooking and which could have produced enterotoxin before being cooked. They could also represent a growing population in the cans which would eventually reach sufficient numbers during storage, to produce enough enterotoxin to cause food poisoning symptoms. As reported by Thatcher (*9*), staphylococcal toxin is not completely destroyed unless the bacon is cooked to a crisp condition (205C for 12 min).

The fact that there was no physical indication of spoilage, or of bacterial growth in the canned bacon examined, was not sufficient assurance of its safety, since it has previously been reported (*9*) that under anaerobic conditions, bacon was acceptable as food even though staphylococci grew and produced enterotoxin. McCoy and Faber (*7*) alluded to this same phenomenon in other meats. Under aerobic conditions, however, the same bacon had a offensive odor and was obviously spoiled.

Although no case of food poisoning attributable to bacon has been reported in the literature (*4*), several investigators have reported both growth (*3, 5, 6, 9, 10*) and enterotoxin production (*9*) by staphylococci in uncooked Canadian and Wiltshire bacon under vacuum, and in anaerobic packs, at temperatures between 20 and 37 C. The likelihood of this happening in low salt (3% or less) precooked bacon is even greater since the spoilage, or indigenous microflora, will be destroyed, or partially reduced by cooking. Staphylococci introduced by human handlings, or from dirty equipment during the packing process, will have little or no competition and could grow quite well if the three conditions above are not met. It was shown that *S. aureus* was able to grow, and successfully compete with the indigenous microflora in uncooked bacon stored under anaerobic conditions at 25 C, and did so more rapidly, and attained greater numbers when the spoilage flora was first "decimated" by irradiation (*5, 6*).

This report and the literature cited clearly indicate the potentially hazardous nature of this nonsterile, precooked, vacuum-canned bacon, stored without refrigeration and further emphasizes the need for well-controlled manufacturing procedures during production. The presence of *S. aureus* usually indicates contamination from the skin, mouth, or nose of workers, or from dirty equipment. Presence of large numbers of staphylococci is generally a good indication of inadequate sanitation and temperature control. Since the bacon studied is hand-packed into cans after cooking and can easily become contaminated with staphylococci, it is extremely important to observe hygienic procedures, good manufacturing practices, cooking and low moisture-to-salt ratio, in particular, are met to produce a safe and stable product.

ACKNOWLEDGMENTS

The authors express their thanks and appreciation to Otto Stark and staff of the Analytical Chemistry Group, Food Sciences Laboratory, for providing the results of the moisture, salt, and water activity determinations. We also appreciate the efforts of George Walker and Justin Tuomy of the Food Engineering Laboratory who provided the bacon and coordinated the entire investigation.

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Journal of Food Protection Vol. 41, No. 9, Pages 712-716 (September 1978)

Organoleptic, Chemical and Bacterial Characteristics of Meat and Offals from Beef Cattle Fed Wet Poultry Excreta

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(Received for publication March 2, 1978)

ABSTRACT

Three groups of growing-finishing beef cattle were fed soybean meal, urea or acetic-propionic acid-treated wet-cage layer excreta as a protein supplement for 125 days. Rib-roasts from the cattle were evaluated for cooking loss, juiciness, flavor, tenderness and overall acceptability. Rib cuts were analyzed for moisture, fat and protein, and samples of the liver, heart, kidney, and longissimus muscle were analyzed for cadmium, lead, copper, iron, calcium and phosphorus. In addition, gall bladders and mesenteric lymph nodes obtained from the cattle at slaughter as well as layer excreta samples were cultured for salmonellae. No significant differences were found in any of the factors studied. Salmonellae were isolated from the cage layer excreta, but not from cattle tissues. Apparently, feeding organic acid-treated wet cage layer excreta to cattle would not affect the nutritive value, organoleptic quality or wholesomeness of beef.

Recent research findings have shown that poultry wastes can be effectively utilized by ruminants as a source of protein (*7,26*) and minerals (*2,4,*). Other reports (*13,27*) have indicated that feeding poultry wastes to ruminants would not affect their health. However, the effect of feeding poultry wastes to ruminants on the palatability and wholesomeness of meat has not been adequately investigated. This study was designed to determine the effects of feeding organic acid-treated wet cage layer excreta (CLE) to cattle on the composition, organoleptic quality, microbial contamination and concentration of potentially toxic trace minerals in muscle and other tissues.

MATERIALS AND METHODS

A total of 90 Hereford growing-finishing steers initially averaging 210 kg were randomly assigned to three diets (the composition of which are shown in Table 1). The experimental diet contained 22% of wet-cage layer excreta obtained from hens fed drug- and additive-free diets. The average composition of the excreta is shown in Table 2. CLE was collected twice weekly, then treated with .5% (w/w) 80% propionic — 20% acetic acid mixture, and stockpiled for daily mixing into the basal diet and feeding.

At the end of the 125-day feeding period, steers were slaughtered at a commercial slaughter plant after a 24-h feed fast. Following a 24-h chill at 2 C, the percent edible meat in the carcasses was estimated; fat color and firmness, and lean color firmness, and texture were scored according to the classifications in Table 3.

TABLE 1. *Feed ingredient and nutrient composition of control and experimental diets.*

Feed ingredient	Diets		
	Control #1	Control #2	Experimental
			(%)[a]
Corn silage	96.0	92.0	68.0
High mositure corn	—	4.5	9.5
Shelled ground corn	—	2.25	—
Soybean meal (49%)	3.5	—	—
Urea (218)	—	.59	—
Cage layer excreta (CLE)	—	—	22.3
Limestone	.07	.08	—
Calcium phosphate dibasic	.05	.13	—
Potassium sulfate	.18	.25	—
Trace mineral salt	.20	.20	.20
Nutrient composition[b]	100%		
			se[c]
Crude protein (N × 6.25)(%)	12.0	12.6	12.7	.33
Fat (%)	2.5	2.9	2.8	.06
Calcium (%)	.36[f]	.36[f]	1.4[g]	.14
Phosphorus (%)	.34[f]	.36[f]	.72[g]	.05
Copper (mg/kg)	4.1[f]	3.9[f]	10.9[g]	.8
Lead (mg/kg)[d]	< .1	< .1	< .1	
Cadmium (mg/kg)[e]	< .1	< .1	<.12	
Arsenic (mg/kg)	.06	.08	.12	.02

[a] % as fed.
[b] % of dry matter.
[c] Standard error; n = 5 observations per mean.
[d] Lead detection limit = .11 mg/kg.
[e] Cadmium detection limit = .10 mg/kg.
[f,g] Means on the same line bearing different superscripts are different ($P < .05$).

The 9-11 rib cuts were obtained from the left side of each carcass and frozen at −20 C. The 30 cuts from each treatment group were later processed in lots of five as described below. The frozen cuts were ground twice through a 1.27-cm plate and twice through a .64-cm plate in a 24 HP meat grinder (Autio Co. Astoria, Oregon). Representative samples were freeze-dried and mixed with dry ice, then pulverized in a Waring blender and stored at 2 C for subsequent chemical analyses. Dry ice facilitated the grinding and prevented heating.

The heart, one kidney, the right lobe of the liver and longissimus muscle from the 5th rib were also taken at slaughter from 15 randomly selected steers from each treatment group. The tissues were freeze-dried, and the 15 samples within each treatment group were composited into three lots and stored at 2 C for subsequent mineral analyses.

TABLE 2. *Average composition of wet cage layer excreta.*

Component	Amount
Moisture, %	68.2 ± 1.9[a]
Composition of dry matter	
Crude protein (N × 6.25), %	31.9 ± 1.3
Fat, %	2.3 ± .6
Acid detergent fiber, %	16.2 ± 1.3
Neutral detergent fiber, %	27.6 ± 1.1
Ash, %	25.5 ± 1.4
Calcium, %	7.2 ± .5
Phosphorus, %	2.5 ± .4
Lead, mg/kg	Traces[b]
Cadmium, mg/kg	.52 ± .12
Arsenic, mg/kg	.35 ± .06
Copper, mg/kg	37.7 ± 1.8
Iron, mg/kg	357.8 ± 7.9

[a]Mean ± standard error; n = 5 observations per mean.
[b]Traces, i.e. below detection limit of .11 mg/kg.

Chemical Analyses

The 9-11 rib cut samples were analyzed for protein by the Kjeldahl procedure and were ether-extracted (*1*). The kidney, liver, heart and muscle samples were dry-ashed and analyzed for calcium, copper, iron, lead, arsenic and cadmium using the atomic absorption spectrophotometric procedure described by Buchanan-Smith et al. (*3*). Arsenic and cadmium levels were corrected for background interference. Phosphorus was determined by reduction of phosphomolybdic acid using an auto-analytical procedure (Technicon Ltd., Montreal, Quebec).

Bacteriological analyses

Samples of CLE taken fornightly during the feeding period as well as mesenteric lymph nodes and gall bladders obtained from excreta-fed steers at slaughter were cultured for salmonellae, using standard procedures (*18*). The objective was to determine the extent of transmission of this potential pathogen from CLE to animal tissues and subsequently to the consumer.

Organoleptic evaluation

Twelve 5-8 rib roasts were randomly selected from each treatment group at slaughter, vacuum-packed and stored at −20 C for subsequent cooking comparisons and organoleptic tests. At processing time, roasts were thawed to room temperature, trimmed to isolate the longissimus muscle which was then weighed and cooked at an oven temperature of 163 C to an internal temperature of 65 C. Temperatures were monitored using thermocouples and a recording thermometer. Cooked roasts were cooled to room temperature and weighed to determine cooking losses.

A 6.35-mm slice was then removed from each roast and divided into three equal samples after all outside surfaces had been removed. Six roasts (two per treatment group) were processed each day and evaluated for flavour, juiciness, tenderness and overall satisfaction by a six-member trained panel over a 6-day period, using the method of quantitative descriptive analysis (*29*). The order of presentation of the samples to each panel member was changed in a systematic manner each day.

Another slice of the roast, 5.4-mm thick was removed and three 25.4-mm diameter cores were obtained from 1/4, 1/2 and 3/4 distance along the longitudinal axis. These cores were then sheared in a recording shear apparatus, an adaptation of the Warner-Bratzler shear and Kramer press (*32*). The force required to shear each core was recorded in kg and the mean of the three values was calculated.

All data were analyzed by the least squares analysis of variance, and significant differences among means were determined by Tukey's *w* test (*28*).

RESULTS AND DISCUSSION

Carcass quality and quantity scores shown in Table 3 indicate that CLE did not adversely affect the overall carcass merit. The lean texture and firmness and color of both lean and fat were similar (P > .05) across treatments, indicating that retail cuts would be equally attractive to the consumer. Percent edible meat was about 57% for the three treatment groups. In other words, feeding CLE to cattle would not reduce the amount of retail yield obtained by the wholesale buyer. The gross chemical composition of rib cuts was similar (P > .05) across treatments. The chemical composition of rib cuts was determined as a simple measure of the influence of CLE on rumen function and nutrient metabolism. Apparently CLE did not modify nutrient digestion and metabolism to the extent of affecting the relative amounts of moisture, protein and fat produced by the animals, and consequently the nutritive value of meat.

TABLE 3. *Carcass quality and composition of rib cut from experimental and control steers.*

Component	Control #1	Control #2	Experimental	SE[a]
Carcass weight	192.9	192.7	198.6	2.6
Fat color[b]	1.8	1.8	1.8	.05
Fat firmness[c]	1.6	1.6	1.5	.06
Lean color[d]	2.2	2.1	2.1	.07
Lean firmness[e]	1.8	2.1	1.9	.09
Lean texture[f]	1.9	1.7	1.9	.07
% edible meat in carcass[g]	57.1	57.1	57.0	.21
Moisture content of rib cut (%)	49.9	50.1	48.8	.72
Fat content of rib cut (%)	27.3	27.5	27.7	.63
Protein content of rib cut (%)	16.9	17.0	15.4	.67

[a]Standard error; n = 6 observations per mean.
[b]1 = white, 2 = amber, 3 = pale yellow, 4 = yellow.
[c]1 = firm, 2 = slightly soft, 3 = soft.
[d]1 = light, 2 = bright, 3 = mid-dark, 4 = dark, 5 = very dark.
[e]1 = firm, 2 = soft, 3 = very soft.
[f]1 = fine, 2 = average, 3 = coarse.
[g]% edible meat = 53 - 3.9 (rib fat depth cm.) + .011 (longissimus muscle area sq. cm).

Several workers (*9,10,30*) reported that feeding high levels of various kinds of fat to cattle may result in changes in adipose tissue composition. In the present study as in several others (*7,11*), addition of CLE to the basal diets did not raise the fat content of test diet above that of the control diets. Furthermore, there is no evidence in the literature that fed as a protein supplement, poultry excreta would sufficiently modify rumen microflora as to affect lipid metabolism. In a study comparing soybean meal, urea or laying hen excreta as protein supplements for sheep, Evans (*11*) reported no significant dietary effects on the concentration of volatile fatty acids in the rumen. Other investigators (*5*) have also reported that feeding dried poultry waste to lactating cattle did not alter the fatty acid composition of milkfat. It is unlikely therefore that feeding CLE to beef cattle would alter the fatty acid composition of meat.

Significant correlations between total lipids and fatty acid composition of muscle on the one hand and its organoleptic qualities on the other have been reported in the literature (*8,33*). The observed similarity (P > .05) in both carcass fat firmness and meat organoleptic characteristics among the three treatment groups (Table

4) is further indication that CLE did not alter tissue and depot fat fatty acid composition.

TABLE 4. *Organoleptic quality of meat from control and experimental steers.*

Measurement Experimental	Control #1	Control #2	Experimental	SE[a]
Juiciness (mm)[b]	60.9	66.7	65.8	1.5
Tenderness (mm)[b]	51.8	63.8	59.8	1.7
Flavour (mm)[b]	54.8	60.9	61.1	1.2
Overall satisfaction (mm)[b]	51.2	59.3	58.6	1.3
Cooking loss (%)	22.0	19.4	20.4	2.6
Shear test[c]	6.9	6.8	6.4	1.7

[a]Standard error; n = 12 observations per mean.
[b]Scores on an unstructured line test as described by Stone et al. (*29*).
[c]Average force (kg) required to shear a 2.54 cm core of cooked muscle.

Results of the organoleptic evaluations of roasts are in Table 4. The roasts were cooked to an internal temperature of 65 C because this temperature corresponds to a medium-rare degree of doneness and approximates the temperature where changes in chemical and physical properties of beef occur relative to optimum expressions of palatability (*15,23*). No differences ($P > .05$) were observed among the roasts for cooking losses and shear values. The latter measurement suggests that the roasts were equally tender, and this was confirmed by the subjective taste panel evaluation. The panel also found the roasts similarly ($P > .05$) well flavored, juicy and satisfactory regardless of dietary treatments imposed on the steers. These results suggest that feeding CLE to beef cattle would not adversely affect the organoleptic qualities of meat. Other investigators (*7, 13*) have reported similar findings.

Poultry are potential carriers of several animal and human pathogens that have been recovered in excreta (*2*). A major concern over the feeding of CLE to cattle has been the potential risk of disease transmission from the excreta to cattle and eventually to humans consuming edible products from such cattle. Salmonellae which infect both animals and humans were monitored to evaluate the significance of microbial contamination of wet excreta fed to cattle. *Salmonella typhimurium* which owes its importance to its association with food poisoning in humans was found in an untreated CLE sample. However, the organism was not recovered in either the acid-treated CLE samples or cattle mesenteric lymph nodes and gall bladders. Apparently the unfavorable conditions created by organic acid treatment and storage in a cold barn effectively eliminated the organisms. Other processing methods that have successfully sterilized or pasteurized poultry wastes intended for animal feeding and hence prevented transmission of potential pathogens to cattle tissues include dry and moist heat treatment, ensiling with and without forage, and deep stacking (*2, 13*).

Various salmonellae serotypes have been isolated from feces of cattle fed wet-cage layer excreta (*27*). Carcasses may therefore be contaminated by alimentary contents at slaughter, resulting in infection in humans. The problem is, however, not peculiar to poultry excreta fed cattle, given the widespread presence of salmonellae in conventional animal feedstuffs (*6,14,25*). The most practical solution would be to maintain premises used for holding and slaughtering animals and for processing meat in good hygienic conditions.

CLE-fed cattle ingested higher ($P < .05$) amounts of minerals than the control animals (Table 1). Nevertheless, tissue mineral concentrations were similar ($P > .05$) across treatments (Table 5) and fall within normal ranges reported in the literature (*16,31*). The tissue concentration of such toxic minerals as cadmium, arsenic and lead falls within tolerance levels established for humans (*12,24*). There was a high degree of variation within treatments for some of the minerals, and this might partly explain the lack of significant differences. However, storage of excess dietary minerals in tissues is only one of the several mechanisms by which mineral homeostasis is maintained. According to Miller (*19*), other mechanisms include reduced absorption, increased urinary excretion and increased endogenous fecal losses. Evans (*11*) reported higher urinary and fecal excretion of both calcium and phosphorus in sheep fed poultry excreta than in controls fed soybean meal or urea. These routes might have been preferentially utilized, thus accounting for the similar tissue mineral concentrations across treatments.

It is noteworthy that although excreta-fed steers ingested higher levels of dietary minerals, their tissue mineral concentrations were in many instances lower, though not significantly, than those of the controls. This trend is attributed to the well-documented interactions among several elements such as cadmium-copper, and lead-calcium-phosphorus. Mills and Dalgarno (*20*) reported that increasing dietary cadmium level from .7 to 12.3 ppm decreased liver copper levels in sheep. Others (*21*) reported that dietary calcium and phosphorus in excess of requirements decreased intestinal absorption of lead, and that dietary calcium influenced the retention and metabolism of lead in bone and other tissues.

Another interesting finding, particularly from the consumer standpoint, was that the concentration of potentially toxic minerals such as lead, cadmium and copper were lower in the muscle than in other tissues such as the kidney. This finding confirms the conclusions of other investigators (*17,22*) that meat and milk, which constitute the major portion of human food sources from animals, are poor accumulators of heavy metals. Apparently through complex interactions as yet not unravelled, high levels of the less innocuous minerals such as calcium, phosphorus and iron present in CLE not only protected the CLE-fed cattle from the harmful effects of the more toxic ones such as copper, lead and cadmium which were also present in CLE, but also reduced the carryover of the latter into edible tissues.

The iron concentration in meat is nutritionally important, since meat is a principal source of iron for humans. Our data (Table 5) show that CLE diet did not affect the iron concentration in meat.

TABLE 5. *Mineral concentration in meat and organs from control and experimental steers.*[a]

Tissue	Mineral (ppm)	Control #1	Control #2	Experimental	SE[b]
Liver	Lead	Traces	Traces	Traces[c]	—
	Cadmium	.20	.31	.11	.07
	Calcium (%)	.08	.09	.09	.02
	Copper	33.6	27.0	21.8	7.6
	Phosphorus (%)	.38	.38	.38	.18
	Arsenic	.04	.03	.02	.006
Kidney	Lead	Traces	Traces	Traces[c]	—
	Cadmium	.94	.90	.76	.16
	Iron	170.3	169.0	148.1	7.0
	Copper	40.2	30.8	95.8	26.6
	Calcium	.14	.10	.09	.02
	Phosphorus	.65	.61	.59	.03
	Arsenic	.03	.03	.03	.006
Heart	Lead	Traces	Traces	Traces[c]	—
	Cadmium	.10	.23	.10	.04
	Iron	132.9	133.08	133.3	3.7
	Copper	57.0	22.9	46.3	19.2
	Calcium	.12	.09	.09	.02
	Phosphorus	.59	.54	.58	.02
	Arsenic	.03	.05	.02	.01
Longissimus muscle	Lead	Traces	Traces	Traces[c]	—
	Cadmium	.20	.10	.10	.06
	Iron	42.9	38.3	42.1	4.5
	Copper	Traces	Traces	Traces[d]	—
	Calcium	.11	.12	.12	.01
	Phosphorus	.50	.49	.47	.02
	Arsenic	.03	.03	.02	.006

[a]In dry tissue samples.
[b]Standard error; n = 3 observations per mean.
[c]Traces, i.e. below detection limit of .11 ppm.
[d]Traces, i.e. below detection limit of .04 ppm.

CONCLUSION

Results of this study indicate that no serious public health problems would result from feeding organic acid-treated wet-cage layer excreta to beef cattle. Beef from the experimental cattle was as palatable, nutritious and wholesome as beef from control cattle. Further studies using higher levels of CLE replacement, and more extensive chemical and microbial evaluation of the products from excreta fed cattle appear worthwhile.

ACKNOWLEDGMENTS

This project was funded by the Ontario Ministry of Agriculture and Food. The authors thank Mr. C. Haworth and Mrs. I. Fiser for their technical assistance and Dr. I. McMillan for statistical advice.

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Journal of Food Protection Vol. 41, No. 9, Pages 717-721 (September, 1978)

Identification of Yeasts Isolated from Bread Dough of Bakeries in Shiraz, Iran

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(Received for publication March 6, 1978)

ABSTRACT

Eighty-one yeast cultures isolated from 43 different bakeries in Shiraz, Iran, were studied for their morphological, sexual and physiological characteristics. Seventy-four of the cultures formed ascospores on 13 different media. They exhibited morphological and physiological characteristics similar to nine different species in the genus *Saccharomyces*. Thirty-four of these 74 yeast cultures were similar to *Saccharomyces cereviseae*, 18 to *Saccharomyces chevalieri*, nine to *Saccharomyces rosei*, four to *Saccharomyces ellipsoideus*, three to *Saccharomyces telluris*, two to *Saccharomyces pretoriensis*, two to *Saccharomyces kluyveri*, one to *Saccharomyces exiguus* and one to *Saccharomyces inconspicuus*.

Based on their morphological and physiological characteristics, four of the seven non-sporulating yeast cultures were similar to *Saccharomyces chevalieri*, two to *Saccharomyces cervisease* and one to *Saccharomyces rosei*. A comparative study was also made between the usual tube method of cycloheximide sensitivity testing of yeasts and the standard single disk technique with solid media as used for antibiotic susceptibility testing of bacteria. The disk method was applicable to yeasts and had advantages over the tube method.

Iranian breads are of five types: Sangak, Taftoon, Barbari, Lavish and village breads. The ingredients, compositions and preparation of these have been described by Kouhestani et al. (*3*).

Except for the village breads which sometimes may be unleavened, other Iranian breads are of the leavened type. Raising of bread dough is a result of fermentation of flour carbohydrates by active bakers' yeast (*Saccharomyces cereviseae*), but other types of yeasts as well as bacteria may also cause the dough to rise or to leaven.

Studies made on bread doughs used as leavenings in Shiraz bakeries, indicated that they contained variable mixtures of yeast strains. Eighty-one of the yeast cultures were characterized and found to be of 13 to 23 biotypes. Some cultures failed to sporulate, some did not seem to fit in the genus *Sacchromyces* and some were not suitable for baking at all (*7*).

The present report describes additional characteristics of these yeast cultures. The cultures were examined for their: (*a*) mode of vegetative reproduction and production of pseudomycelia, (*b*) ascospore forming ability; mainly, of the cultures which did not sporulate under the previous experimental conditions and (*c*) ability to ferment and assimilate sugars and to grow in the presence or absence of specific chemicals (*9*).

MATERIALS AND METHODS

Yeast strains

Eighty-one cultures of yeasts were isolated from 43 different dough samples (*7*). The cultures were stored on slants of Sabouraud Dextrose Agar (SDA) medium (Difco) and were subcultured once every 45 to 60 days.

A commercial "Red Star" baking yeast from the Universal Food Corporation, Milwaukee, Wisconsin, and a commercial baking yeast from Allinson, London, were used as standard strains for comparison throughout these experiments.

Characterization

Since vegetative reproduction of yeasts is used in identification, the cultures were studied for their mode of cell division in Malt Extract Broth (MEB) (Difco) as well as on Malt Extract Agar (MEA) (Difco) and for formation of pseudomycellia on Corn Meal Agar (CMA) (Difco). The Dalmau method of plate culture as described by Wickerham (*11*) was used to study production of pseudomycelia.

Non-sporualting cultures in previous experiments (*7*), were examined for their ability to sporulate on Starkey's ethanol and V8-Juice Agar as well as on Yeast-Extract-Malt Extract Agar (YEMEA) media (*9*). Sporulation ability of these cultures were also examined using strips of filter paper bearing the yeast cells suspended from the stopper in a 500-ml Erlenmeyer flask containing 100 ml of a 0.5% ethanol solution and incubated at 25 C as described by Miller (*5*). Smears were made from the cells collected from the filter paper, stained and examined daily for 20 days for the presence of ascospores.

The ability of the yeast cultures to ferment melibiose in Phenol Red Broth Base Medium (Difco) and to assimilate trehalose, melibiose, melizitose, alphamethyl-D-glucoside and DL-lactic acid, in Nitrogen Broth Base Medium (Difco) was examined. The ability of all the cultures to use potassium nitriate and ethylamine hydrochloride, to grow in the presence of 100 mcg of cycloheximide/ml and in vitamin-free medium and to split arbutin was also determined.

Additional characterization of selected cultures was determined by fermentation of carbohydrates such as trehalose, soluble starch, inulin; by assimilation of salicin, soluble starch, inulin, succinic acid, cellobiose, mannitol, glucitol, D-xylose; and by growth on 50% (w/w) Glucose Yeast Extract Agar (Difco). The latter series of carbohydrates

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R Upjohn Company, Kalamazoo, Michigan.

[1]Bioquest, Division of Becton, Dickenson and Co., Cockeysville, Maryland.

was selected for the final typing of some of the yeasts, in consultation with the keys to the identification of yeast genera (*4*) and species of *Saccharomyces* (*10*) by Lodder and van der Walt.

The detailed methods for detection of ascospore formation, fermentation and assimilation of the carbohydrates were reported previously (*7*). Bacto-Yeast Carbon Base Medium (Difco) supplemented with 0.078% (w/v) potassium nitrate was used for nitrate utilization and with 0.064% (w/v) of ethylamine hydrochloride for amino nitrogen utilization tests. Cycloheximide resistance of the yeasts was determined by addition of filter sterlized actidione^{R} to the Bacto-Yeast Nitrogen Base (YNB) (Difco) containing 1% glucose, to make a final concentration of 100 mcg/ml. The cycloheximide resistance of the yeasts was also determined by the methods of Turk et al. (*8*) and Bauer et al. (*2*). Each culture was grown in a Sabouraud Dextrose Broth (SDB) (Difco) and the cells were seeded by means of a swab on a SDA plate. A BBL[1] blank filter paper disk of 1/4-inch diameter was then impregnated with a solution containing 100 mcg of cycloheximide/ml, placed on the seeded plate (Fig. 1) and incubated at 25 C for 2 to 3 days. Absence of growth around the antibiotic disk (zone of inhibition) on the SDA medium was interpreted as susceptibility and the presence of growth as resistance to the antibiotic.

Splitting of arbutin was determined by growing the yeast cultures on a medium containing 10% yeast autolysate, 2% agar and 0.5% (w/v) arbutin. The yeast cultures were grown on Bacto-Vitamin Free Medium (Difco) to determine their vitamin requirements and on a medium composed of 50% glucose and 50 ml of 1% yeast autolysate which contained 3% (w/v) agar, to determine their ability to grow at high osmotic pressure. The procedures described by van der Walt (*9*) for preparation of media and inoculua, cell inoculation, temperature and length of incubation and evaluation of results were followed.

RESULTS

Asexual and sexual reproduction

Cell multiplication in MEB and MEA media was determined for all the cultures to be by multipolar budding. Ten of the cultures were also able to produce few to moderate number of pseudomycelia on the CMA.

Ten of the 17 cultures that had not previously demonstrated sporulation, produced ascospores after additional studies using Starkey's ethanol medium. Ascospores were not formed on V8-Juice Agar, MEYEA media and over a 0.5% solution of ethanol. Seventy-four (91%) of the 81 cultures formed ascospores. The sproulation abilities of all cultures on 13 different sporulating media are shown in Table 1. The table indicates that Starkey's ethanol and Gorodokowa Agar (*9*) were more suitable for sporulation of the yeasts than were carrot blocks, potato blocks, Acetate Agar, Corn Meal Agar, Yeast Extract Agar, Potato Agar and cucumber blocks. The remaining four sporulating media could not induce sporulation in any of the yeast cultures.

Seven of the 81 cultures were weakly resistant to cycloheximide by the tube test using YNB medium, but they were sensitive to this antibiotic when tested by the disk method on solid medium (Fig. 1). The remainder of the cultures gave similar results by the two methods.

The results of fermentation and assimilation of 33 sugars and other physiological reactions of yeast cultures are shown in Table 2. The fermentation and assimilation reactions obtained in previous (*7*) and present experiments are tabulated to allow full comparison of the result for the various groups of yeast. The cultures were

TABLE 1. *Sporulation ability, on various media, of yeasts isolated from bread doughs of bakeries in Shiraz, Iran.*

Sporulation media	# of cultures examined	# of cultures sporulated	% of cultures failed to sporulate
Starkey's ethanol medium	17	10	41
Gorodokowa agar	81	36	56
Carrot blocks	45	3	83
Potato blocks	81	9	89
Acetate agar	28	3	89
Corn meal agar	81	7	91
Yeast extract agar	81	5	94
Potato agar	35	1	97
Cucumber blocks	35	1	97
Gypsum blocks	81	0	100
Yeast extract-malt extract agar	17	0	100
V8 juice agar	17	0	100
0.5% solution of ethanol	17	0	100

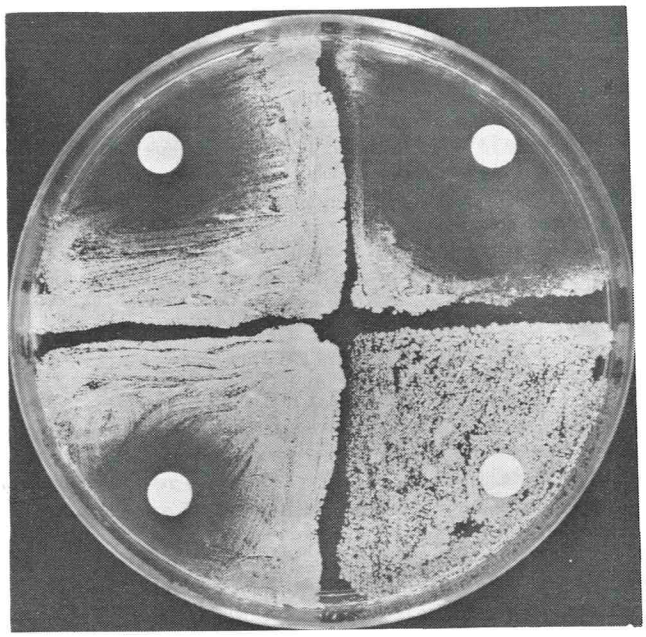

Figure 1. *Cycloheximide sensitivity test of four different yeast cultures on a SDA plate. Top right resistant and top left, bottom left and right show different degree of sensitivity, respectively.*

classified into 9 different groups, characteristics of which fit into the genus *Saccharomyces*. Identifed species and the key physiological characteristics for the 74 ascosporogenous yeast cultures in the nine groups are tabulated in Table 3. *S. cereviseae* (34 isolates in group 1) and *S. chevalieri* (18 isolates in group 2) were the predominant *Saccharomyces* species isolated from the bread dough samples. Nine of the cultures in group 3 were identifed as *S. rosei* and of the remaining 13 yeast cultures, four were *S. ellipsoideus* (group 4), three *S. telluris* (group 5), two *S. pretoriensis* (group 6), two *S. kluyveri* (group 7) and one each (groups 8 and 9 respectively) *S. exiguus* and *S. inconspicuus* (Table 3). In the group of yeasts designated *S. cereviseae*, nine cultures could utilize ethylamine hydrochloride. This characteristic varies the standard description for *S. cereviseae*. Of the 18 isolates

TABLE 2. *Biochemical characteristics of yeasts isolated from bread dough in Shiraz, Iran.*

No. of strains	Fermentation and assimilation reactions																		Growth			
	G.	Gl.	Su.	Ma.	Ra.	Tr.	Me.	Mz	In.	Ss.	La.	Mg.	So.	Sa.	S.A.	Et.	Ce.	Mn.	Dx	G. 50%	Vf.	Cy.	At 37 C
36	FA	FA	FA	FA	FA	FA	--	FA	FN	--	A	A	A	—	N	*	—	N	N	N	+	S	±
22	FA	FA	FA	-A	FA	FA	--	N*	-A	NN	A	—	A	—	*	*	N	N	N	N	+	S	+
10	FA	--	FA	-A	FA	NA	--	N-	FA	N-	A*	—	N	N	N	—	N	N	N	+	+	S	—
4	FA	FA	FA	--	FA	FA	FA	N-	FA	NN	A	—	A	N	A	A	N	*A	—	—	+	S	—
3	FA	--	--	--	--	--	--	--	NN	--	—	—	N	N	N	*	N	N	N	N	—	S	+
2	FA	-A	FA	FA	FA	-A	--	NA	FA	--	A*	A	N	—	N	A	—	N	N	N	+	S	±
2	FA	FA	FA	-A	FA	-A	FA	NA	FA	NN	A	*	N	A	N	A	N	N	N	N	+	S	+
1	FA	FA	FA	--	FA	*A	--	N-	--	NN	A	A*	*	N	—	A	N	N	N	—	—	S	—
1	FA	--	FA	--	-A	-A	--	N-	--	NN	A	—	*	N	N	A	N	N	N	+	+	S	—

# =Fermentation and assimilation of lactose, assimilation of nitrate and splitting of arbutin for all the yeasts were negative.
FA =Positive fermentation and assimilation. -- =Negative fermentation and assimilation.
FA =Some fermentation and assimilation positive and some negative.
* =Some cultures showed different reactions than those of standard descriptions.

G. =Glucose
Gl. =Galactose
Su. =Sucrose
Ma. =Maltose
Ra. =Raffinose
Tr. =Trehalose
Me. =Melibiose
Mz. =Melizitose
In. =Inulin
Ss. =Soluble starch
La. =Lactoc acid
Mg. =Alpha-methyl-D-glucoside
So. =Sorbitol
Sa. =Salicin
Mn. =Manitol
S.A. =Succinic acid
Et. =Ethylamine ClH
Ce. =Cellobiose
Dx. =D-xylose
G50% =50% glucose
Vf. =Vitamin free
Cy. =Cyclohemide
S. =Sensitive
N =Not tested.

identified as *S. chevalieri*, six had one and two had two assimilation reactions different from those of standard descriptions of *S. chevalieri*. These differences were observed in the assimilation of ethylamine hydrochloride, melizitose and succinic acid. Eleven of the remaining 22 cultures in other groups exhibited one and one culture exhibited three biochemical reactions different from those of the standard descriptions of their respective *Saccharomyces* species. The biochemical reactions which did not fit the standard descriptions of each species, were retested twice and remained the same.

Five of the pseudomycelium-producing cultures were identified as *S. cereviseae*, three *S. chevalieri*, one *S. telluris* and one *S. kluyveri*.

Based on their biochemical characteristics four of the seven non-ascospore forming cultures were identified as *S. chevalieri*, two *S. cereviseae* and one *S. rosei*. The standard strains of yeast examined were identified as *S. cereviseae*.

DISCUSSION

Preliminary studies preformed previously (*7*) on the 81 yeast cultures isolated from bread doughs of Shiraz bakeries indicated that only 38% of the cultures were similar to species of *Saccharomyces*. Additional studies on morphological, sexual and physiological characteristics demonstrated that 74 sporulating yeast cultures (91%) could be identified as *Saccharomyces*, because they all could reproduce by multipolar budding, were able to form ascospores and none were able to assimilate nitrate (*4*).

The key physiological characteristics (*10*) in Table 3 indicated that 34 (46%) of these yeast cultures were *S. cereviseae*. The remaining 40 cultures (54%) fit into other *Saccharomyces* species. Some differences in the biochemical reactions were observed in 29 of the yeast cultures when all the results obtained were compared with those of the respective standard descriptions of *Saccharomyces* species. Since these differences were not in the key biochemical reactions, they were not considered to be highly significant.

Thirteen different sporulating media were used [four in the present and nine in the previous (*7*) experiments] to induce production of ascospores in these yeast cultures (Table 1). Ninety-one percent of the cultures sporulated on nine of these media, yet seven of the cultures failed to form ascospores. Morphological and physiological characteristics of these yeasts were similar to some of the ascosporogenous cultures examined. This might suggest that still other inducing conditions for ascospore formation should be used to examine these yeasts. According to Barnett and Pankhurst (*1*) five possibilities may exist: (*a*) these yeasts may indeed belong to a genuinely anascosporogenous species; (*b*) only very few cells produced asci, and so the ascospores escaped notice; (*c*) they might be heterothallic haploid/yeasts; (*d*) unsuitable media were used to induce sporulation; or (*e*) the yeast might have lost its ability to form ascospores.

Methods used for cycloheximide susceptibility testing on solid medium (*2,8*) (Fig. 1) seemed to be more accurate and gave clearer results than those in YNB medium (*9*). It was possible to read the results of the disk test within 2 to 3 days. Sisler and Siegel (*6*) reported that yeasts growing in media containing cycloheximide usually develop resistance to the antibiotic. The possibility that yeast cells could develop resistance to the cycloheximide during the 4-week incubation period required for the test in the YNB medium should also be

TABLE 3. *Species and the key biochemical characteristics of ascosporogenous yeasts isolated from bread doughs of bakeries in Shriaz, Iran.*

Species	No. of cultures	Fermentation		Assimilation	
		Positive	Negative	Positive	Negative
S. cereviseae[a]	34	Ga. Su. Ma. Ra.	Me. Ss.	——	Sa. Ce.
S. chevalieri[b]	18	Ga. Su. Ra.	Ma.	Ma.	Me.
S. rosei[c]	9	Su. In.	Ga. Ma.	Ma.	Ma.
S. ellipsoideus	4	Ga. Su. Ra. Me.	Ma.	Tr. Me	Dx.
S. telluris[d]	3	——	Su. Ga. Ma. Me.	——	Tr.
S. pretoriensis[a]	2	Su. Ma. Ra.	Ga.	Ga.	Me. Ce. Sa.
S. kluyveri	2	Ga. Su. Ra. Me.	Ma.	Ma.	——
S. exigus	1	Ga. Su. Ra.	Ma. Me.	——	Ma. Me.
S. inconspicuus[e]	1	Su.	Ga. Ma. In.	La.	Ma.

[a] =Not able to split arbutin.
[b] =Sensitive to cycloheximide.
[c] =If maltose not assimilated, should be sensitive to cycloheximide and no growth at 37 C.
[d] =One or two ascospore per ascus.
[e] =Sensitive to cycloheximide, no growth at 37 C, and growth in vitamin free medium.

Ga. =Galactose Su =Sucrose Ma. =Maltose Ra. =Raffinose
Ss. =Soluble starch Ce. =Cellobiose Me. =Melibiose In. =Inulin
Tr. =Trehalose La. =DL-Lactic acid Sa. =Salicin Dx. =Dxylose

taken into consideration. For this reason the use of disk method for cycloheximide susceptibility testing of yeasts is preferable to the tube method (*9*).

Many different biotypes of yeasts are involved in bread doughs used as leavenings in Shiraz bakeries (Table 2 and 3). *S. cereviseae* was isolated from only 23 of the dough samples. This suggest that 20 out of 43 bread doughs were leavened by the action of yeasts belonging to other *Saccharomyces* species. Five cultures did not exhibit any baking strength (*7*). One isolate was *S. cereviseae,* one was *S. chevalieri* and the remaining three were *S. telluris.* These non-active yeasts were isolated from dough samples which also contained other active *Saccharomyces.* However, 59% of the yeasts with highest baking strength (*7*) (rising time between 58 to 120 min) were found to be in the group of *S. cereviseae.*

In recent years, two plants for production of "Red Star" baker's yeasts have been established in Iran; one in Tehran and the other in Tabriz. Yeasts produced by these plants, as well as imported baker's yeasts from other countries, are now available in some of the large stores in big cities in this country. Whether some of the species of *S. cereviseae* isolated from the dough samples have been derived from those plants is open to question. However, most of these samples contained from two to four different types of yeast species.

ACKNOWLEDGMENTS

Financial support of the Ministry of Science and Higher Education, Iran, is highly appreciated. Thanks are due Mrs. S. M. Eghtesadi and Mr. M. Sarvghad for providing technical assistance throughout these experiments. Also, reading of the manuscript by Dr. L. H. Lauerman and Dr. S. M. Morrison is appreciated.

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Journal of Food Protection Vol. 41, No. 9, Pages 722-725 (September, 1978)

Detection of Terpene Compounds from Hops in American Lager Beer[1]

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(Received for publication on March 7, 1978)

ABSTRACT

Analytical data for identification of three terpenoid compounds attributed to hops in American lager beer are presented. Glass capillary-column gas chromatography and computor analysis of mass spectrometric data were employed to facilitate identification of linalool, a-terpineol, and myrcene. Concentrations of each were estimated from gas chromatographic data, and this indicated that linalool should have the greatest influence of beer flavor since its concentration appeared to exceed that of its odor threshold of 6 ppb in water.

The flavor chemistry of beer has received much attention in recent years, the source of flavor compounds and their effect on taste continues to be an important part of this research. Hops contain abundant volatile flavor compounds, and in the past periodically have been considered substantial contributors to beer flavor. However, confusion has arisen because several reports in the literature have stated that essentially none of the many volatile flavor compounds found in hops survive the brewing process, especially wort boiling, and appear in the finished beer.

In the early 1960's Harold et al. (*8*) reported finding terpene compounds in Australian beer which they attributed to hops. Their identifications were based on gas chromatographic retention volumes in chromatograms showing only a few separated peaks. In light of more recent knowledge concerning the complexity of beer flavor profiles such identifications are doubtful. Subsequently, Likens and Nickerson (*9*) devised a distillation apparatus to specifically concentrate and isolate volatile hop flavor compounds in wort and beer. Several hop compounds were believed found in one commercial beer sample, and these were reported to be myrcene, methylbutyl isobutyrate, methyl octanoate, methyl deca-4-enoate, β-caryophyllene, methyl deca-4, 8-dienoate, humulene, and farnesene.

Later, Buttery et al. (*1,2*) used gas chromatography (GC) and mass spectrometry (MS) to separate and indentify more than 75 volatile hop oil components, and then followed this with a report of more than 90 compounds in the oxygenated fraction of hop oil alone (*7*). These investigators were unsuccessful in their attempts to isolate hop terpene compounds from finished beer. Two esters, ethyl dec-4-enoate and ethyl deca-4, 8-dienoate which were probably transesterified during brewing, were the only components found in beer which could be traced back to hops.

More recently Drawert and Tressl (*5*) have included a few terpene compounds as flavor components of European beer, but analytical data were not presented. Following this initial report, Tressl and Friese (*15*) reported that they found some natural oxygenated and oxidized terpenes in European beer, but did not observe any terpene hydrocarbons in these products. They attributed much of the loss of hop terpenes to adsorption to yeast cells rather than to losses in wort boiling. Sandra (*12*) and Sandra and Verzele (*13*) have also recently analyzed European beers, and have concluded that they could not routinely detect hop compounds. They further concluded that individual hop compounds could not be responsible for the hoppy aroma note of beer, but that collectively many trace hop compounds could have an influence on beer flavor.

This report gives analytical data from our laboratory for the isolation and indentification of linalool, *a*–terpineol and myrcene from an American lager which was lightly hopped beer. Linalool (3,7-methyl-6, 1-octadien-3-ol) is a tertairy alcohol while *a*-terpineol (p-menth-1-en-8-ol) is a monocyclic terpene alcohol. Myrcene (7-methyl-3-methylene-1,6-oct-adiene) is an acyclic terpene hydrocarbon which comprises the major portion of hop oil. These terpene compounds were encountered during the detailed analysis of volatile compounds in staling beer.

[1]*Supported in part by the College of Agricultural and Life Sciences, University of Wisconsin-Madison, and a Research Grant from the Master Brewers Association of America.*
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MATERIALS AND METHODS

American lager beer containing hop extract was purchased locally, and was subjected to accelerated staling by holding 120 h at 40-45 C(*10*). The contents of a 12-oz. bottle of staled beer were poured into a separatory funnel without further treatment. Two successive 40-ml aliquots of chloroform were used to extract flavor compounds. This extract was dried over excess sodium sulfate, and the volume was brought to approximately 5 ml with a rotary evaporator under reduced pressure. Further evaporative concentration was achieved at room temperature by using a stream of nitrogen gas directed towards the surface of the extract. The sample was chromatographed on a 2.04-mm I.D. × 3.8-m packed stainless steel Carbowax 20 M column. The major portion of separated components by-passed the GC flame ionization detector through splitter arrangement, and were collected in glass capillary tubes submerged in liquid nitrogen. In this way the sample was divided into several fractions, and each sealed in a glass capillary tube.

The collected fractions containing the terpenoids were analyzed with a Varian 1740 GC equipped with 0.79-mm I.D. × 185-m glass capillary column coated with Carbowax 20 M which was interfaced to a Du Pont Model 21-491 spectrometer. Mass spectra were recorded, and background was automatically subtracted by computor.

RESULTS AND DISCUSSION

The mass spectrum of linalool obtained during the gas chromatographic separation is shown in Fig. 1. This spectrum agrees closely with those for authentic linalool reported by Dieckmann and Palamand (*4*) and Friedel et al. (*6*). The characteristic features of the spectrum are peaks at m/e 71 (100), 41 (87), 93 (77), 80 (38), 69 (36), and 55 (32). Linalool is typical of alcohols in that it does not give molecular ion peak (m/e 154), but it does show a peak at m/e 136 due to loss of the hydroxyl group plus a proton (M-18). The presence of small peaks at m/e 31, 59 and 73 also support the interpretation as an oxygenated compound.

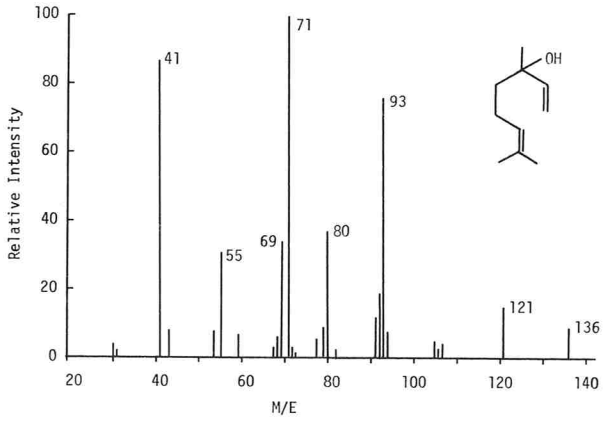

Figure 1. *Mass spectrum of linalool from American lager beer.*

Identification of linalool from the beer extract was further verified by its retention index on Carbowax 20 M using the method of Van Den Dool and Kratz (*16*). This method uses ethyl esters of n-aliphatic acids and is very convenient because most of the ethyl esters are present in the volatile compound profile of beer. An authentic sample of linalool gave a retention index of 8.97 while the chromatographed peak from beer was 8.95.

The experimental mass spectrum of *a*-terpineol is shown in Fig. 2, and agrees with those published by Dieckmann and Palamand (*4*) and others (*16*). The spectrum contains major peaks at m/e 59 (100), 93 (67), 121 (45), 43 (38), 81 (37) and 136 (25). *a*-Terpineol behaves similarly to linalool in not showing a molecular ion peak (m/e 154), but does show mass fragments for the loss of one hydroxyl group (m/e 136) and for the loss of a methyl group (m/e 121). The retention index of the experimental peak was 10.50 while an authentic *a*-terpineol sample had a value of 10.55.

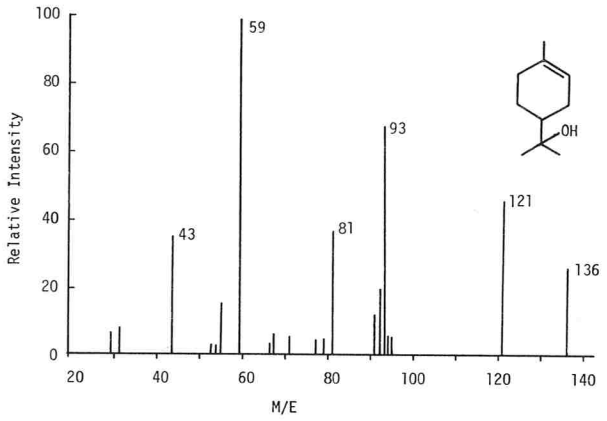

Figure 2. *Mass spectrum of a -terpeneol from American lager beer.*

The presence of myrcene was verified by its retention index of 5.30 which matched exactly that of an authentic sample. The experimental chromatographic peak was spectral pattern even after background had been subtracted. However, major peaks at m/e 41, 93, 69 and 39 were present in addition to smaller characteristic masses at m/e 77, 79, 80, 91, 92 and 94. The spectrum matched other published data (*14*) well enough to assure a positive identification. Myrcene does not give a significant molecular ion peak (m/e 136) because the double bond structure decreases molecular stability and results in ready fragmentation. The prominent ion of m/e 69 is characteristic of terminal isopentenyl cleavage in acyclic monoterpenoid compounds, such as myrcene and linalool.

While these terpeniods were identified during the analysis of stale beer, it is not implied that staling had any special significance. Based on the knowledge of the composition of hop oil (*7*), these compounds would be expected to derive from hops, and there was no indication that they were formed during staling. Initial studies of both fresh and stale beer using stainless steel 2.04-mm I.D. packed Carbowax 20 M columns did not reveal the presence of terpene compounds. The identifications were made possible by the increased inertness and higher resolution obtained through use of a glass, open-tubular capillary column which was used during more recent studies of stale beer.

Even with the use of a glass capillary column, linalool was not always separated completely from its neighboring peaks. It was sometimes observed as a shoulder on a much larger peak attributed to isobutyric acid. The separation was also further complicated by the presence of ethyl nonanoate which eluted close to this position on the chromatograph pattern. Alpha-terpineol was also only slightly separated from a gamma-lactone which preceded it by 0.03 retention index units.

Some physical properties of the three terpenoids are listed in Table 1. Based on apparent gas chromatographic data, linalool was considered most likely to have a potential effect on beer flavor of the three terpeniod compounds identified. This compound has a distinct odor which resembles the floral woodiness of rosewood, but also contains a spicy note. Buttery and Ling (*2*) have found that linalool comprises less than 1% of typical hop oil, but its odor threshold is relatively low at 6 ppb in water. Since it is an unsaturated alcohol, it exhibits a much lower threshold than its saturated counterparts (*11*). By contrast, myrcene which has been reportedly identified in a beer sample (*9*) comprises about 63% of hop oil and has an odor threshold of 13 ppb (*3, 7*).

TABLE 1. *Properties of terpene compounds isolated from beer.*

Property	Linalool	a-Terpineol	Myrcene
Molecular weight (amu)	154	154	136
Boiling point (C)	198	218	167
Odor threshold, ppb[1]	6	350	13
Retention Index[2]	8.97	10.55	5.30
Odor character	rosewood, spicy	terpentine, antiseptic	carrot tops, terpenoid

[1]In water.
[2]See text.

Superficially, it may seem unlikely that linalool, which is a component in low concentration in hop oil, should be found in beer while myrcene, the major component has not been routinely detected. Myrcene is a hydrocarbon that is less soluble in water and could be more completely distilled out of wort during boiling. On the other hand linalool is more water soluble, and coupled with its higher boiling point could be retained to a greater extent. Further, Dieckman and Palamand (*4*) have shown that the linalool concentration can increase through air oxidation of myrcene which is favored during storage of dried hops.

Although no specific effort was made to quantify linalool, it was estimated to be present in the low ppb range. This was based on the fact that the linalool peak was present at approximately 10% of the quantity of the 2-furfural peak (retention index, 8.22). McDougal et al. (*10*) reported the level of 2-furfural in beer punished in a similar manner to be about 340 ppb. From this basis, 34 ppb of linalool was estimated to be present in the current beer sample. Tressl and Friese (*15*) reported a level of 60 ppb of linalool in a European beer. An estimation of the amount of linalool added to wort for American lager beer through hops was calculated from the data of Guadagni et al. (*7*) and Buttery and Ling (*2*). Hops contained 1% of volatile oil which included 0.15 - 0.65% as linalool, and this would yield between 17 and 73 ppb of linalool if used at a common level of 1.12 g/L in American lager beer.

Meilgaard (*11*) and Sandra and Verzele (*13*) have reported a taste threshold of 80 and 100 ppb, respectively, for linalool when added to beer. The estimated 34 ppb level found in the experimental beer would be well below these reported thresholds, but would be present at a level well above its threshold of 6 ppb in water (*2*). Further, its role in possible subthreshold interactions with other flavor components is not known at this time. The possibility for exceeding a threshold level of linalool in beer is real considering the amount added to wort through hop flavoring. This is expecially true for European lagar beers which are brewed using up to three times the hopping rate of their American counterparts, or for ales which employ about eight times more hops in brewing.

The myrcene was estimated to be present at 7 ppb or about half its odor threshold value in water (*7*). This concentration is dramatically below the calculated amount of 7066 ppb that is available from hops used in American lager beers. This is the first fully documented report of myrcene in lager beer as Tressl and Friese (*15*) and Sandra and Verzele (*13*) have recently stated they were unable to detect any hop-derived terpene hydrocarbons in European beer.

Very little has been reported about *a*-terpineol in beer and hop literature. This compound was not indentified by Sandra and Verzele (*13*), Tressl and Friese (*15*), or during the extensive hop and beer flavor studies at the USDA Western Regional Research Laboratory (*1, 2, 7*). However, Dieckmann and Palamand have shown that in a model system myrcene cyclizes primarily to limonene which can then be oxidized by air to *a*-terpineol (*4*). Buttery et al. (*3*) has reported the odor threshold of *a*-terpineol in water to be 350 ppb while Meilgaard (*11*) found a taste threshold of 2,000 ppb when it was added to beer. The current beer sample contained approximately 20 ppb. This final concentration is greater than that found for myrcene, and probably reflects lower losses because of a higher boiling point and greater solubility in wort rather than a high concentration hop oil. In this respect it is similar to linalool. However, its effect on beer flavor would be expected to be much less because of its higher flavor threshold value.

This study has shown that sensitive techniques reveal the presence of ppb levels of terpenes in American lager beers. The actual amount of terpenes obtained from hops in beer logically depends upon the varity, the amount added to wort, the length of wort boiling, adsorption on yeasts, and the methods of wort filtration. Detailed research will be required to determine the ultimate role of the ppb levels of linalool and other hop-derived volatile constituents in the interactive flavor impressions of beer.

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Journal of Food Protection Vol. 41, No. 9, Pages 726-729 (September, 1978)

Geometrical Factors in Color Evaluation of Purees, Pastes, and Granular Food Specimens

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ABSTRACT

The standard measurement situation for color involves a specimen which is flat, uniform in color, and thin. For measurements of colors of many food products, instruments which give reliable and reproducible results when applied to the flat, uniform and thin traditional specimens are unreliable. This is because specimens of foodstuffs come in a variety of shapes, sizes and translucencies. The unreliability of instrument measurements can be shown to be related to instrument differences in geometry and to: (*a*) specimen nonflatness; (*b*) specimen variability in pattern, particle size, shape, compression, and/or humidity; and (*c*) specimen translucency.

IDEAL MEASUREMENT CONDITIONS

The standard model for color measurement by reflected light assumes that specimens are flat, uniform throughout, and opaque even in thin layers. The simple geometric models which are used to identify the two popular sets of geometric conditions of measurement are shown in Fig. 1. At the top, light is incident on the flat, opaque specimen at 45°; and the light reflected perpendicularly at 0° is then measured for color. Alternatively, at the bottom of Fig. 1 is shown the diffuse sphere technique by which all of the light reflected in all directions is collected for measurement. Here, the specimen is illuminated perpendicularly at 0° and light reflected in all directions is collected for measurement.

Thus the two popularly recognized conditions of illumination in view for color measurement by reflection are: (*a*) 45° illumination, 0° viewing; and (*b*) 0° illumination, diffuse viewing. There are so-called reciprocal conditions in which the angles for illumination and viewing are reversed, and, according to theory, usually identified by the name "Helmholtz Reciprocal Relation," the reciprocal conditions give results identical to the standard ones.

Actually, beams of light are not properly represented by vectors of single directions of projection, as is shown in Fig. 1. Instead, as is shown in Fig. 2, the directions of light in any given beam must always be identified by a cone of directions centering about the axial direction. In general, foodstuffs are relatively diffusing so angular

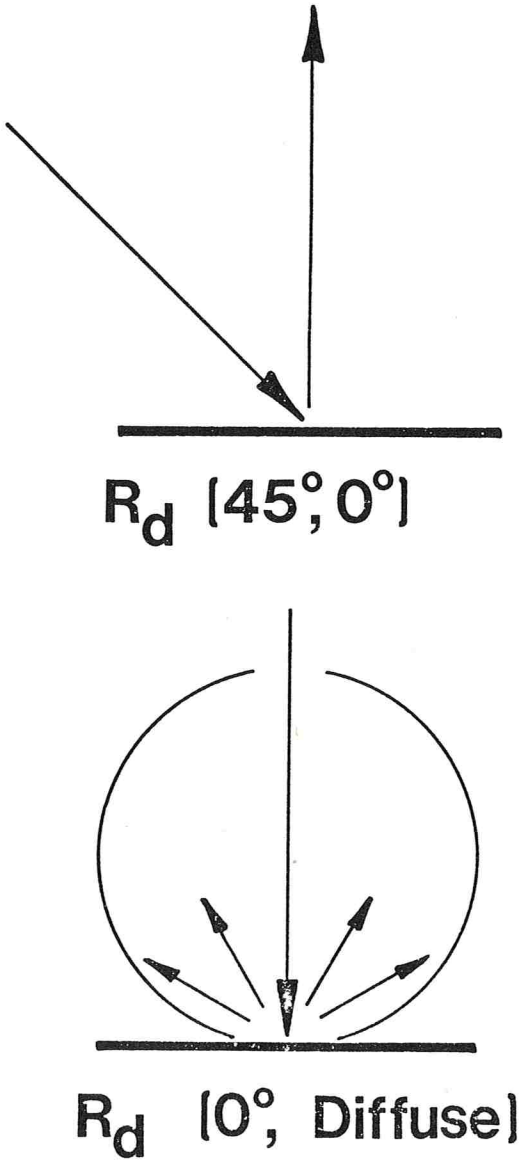

Figure 1. *Directions of incidence and view used for diffuse reflectance measurements.*

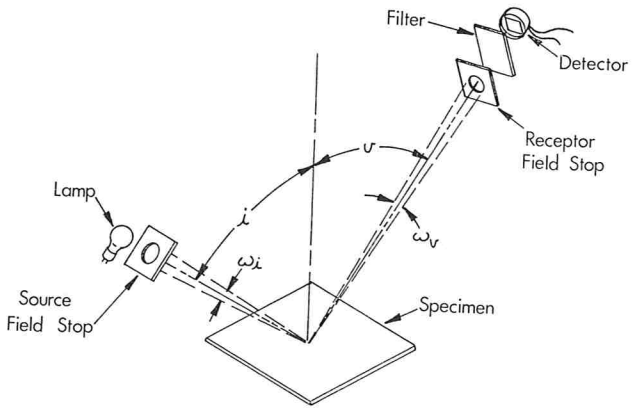

Figure 2. *Elements of a photometric instrument and the quantity it measures. Spectral specification:* $E_{\lambda(lamp)}\, xt_{\lambda(filter)}\, xs_{\lambda(photodetector)}$ *Geometric specification; i, v,* w_i, w_v

magnitudes of the cones of directions within a measuring instrument is seldom a significant factor.

However, what is important are the light-beam diameters and the edge-of-window clearances between beam diameters and specimen windows, as is shown in Fig. 3. This clearance between the diameter of the beam

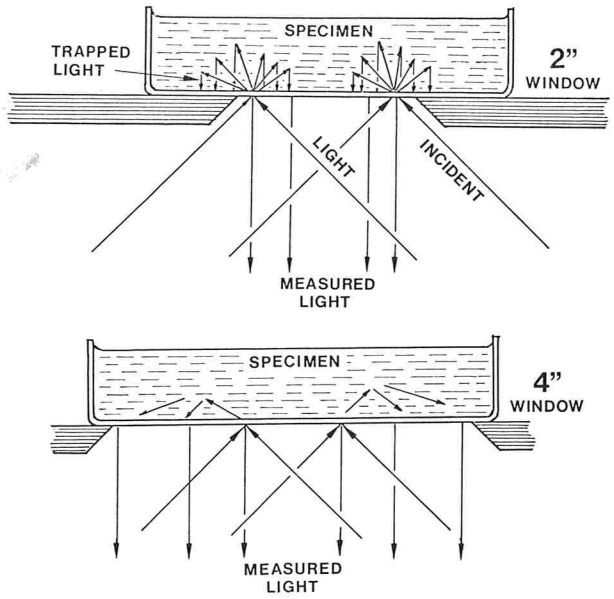

Figure 3. *Diagrams showing loss of light by trapping in translucent specimen (above), and use of wide window to avoid most of loss (below).*

of light incident upon the specimen and the window through which the specimen is exposed for measurement is, as will be shown below, a significant factor in the color measurement of both translucent foodstuffs and coarse, granular food materials. This viewing-window clearance is important wherever light penetrates the specimen.

FOOD INDUSTRY SPECIMENS

Where specimens are not flat, the light projected by reflection tends to decrease with surface roughness and with increasing granule size. Typical non-flat food-industry specimens are whole green peas, potato chips and French fries, and flakes of breakfast food.

Figure 4 is an impressive illustration of the part played by granule size in reflection and color. Here are four vials, all with the same green glass in them. The only variable is particle size and yet, as can be seen in the figure, the changes observed and the corresponding measured colors are very great. These differences are the result of contrasts between the optical phenomena of scattering and absorption. Light is reflected and diffused, making the powder look white, by repeated contacts of light with the particle surfaces. Light is colored green by the process of absorption while passing through the glass. The relative contributions of green absorption and white scattering differ greatly between the few large particles and the many small particles of the otherwise identical material.

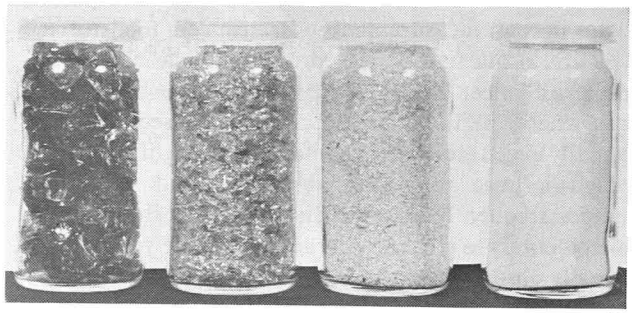

Figure 4. *Four vials containing the same green glass; large pieces on the left, fine powder on the right.*

The form of any given natural material will significantly affect its color, whether observed visually or measured by instrument. As an example, in Fig. 5 points are shown representing Hunter a,b color values of five forms of tomato product. There are whole fruit, cut fruit, tomato puree, concentrated solids, tomato paste, and freeze-dried tomato powder. Figure 5 is an a,b color diagram showing how greatly the colors of these different specimens differ from each other. Even within one of the specimens, a cut tomato, measurements within it show large variations. This is not surprising when one considers how white the interior of the tomato is near the stem end and how it varies in color from the central pulpy area to the edge.

However, with all such specimens as nonuniform as whole berries or meats, color appearance can be both measured and judged meaningfully. Success in measurement of these nonuniform specimens depends on adequate control and identification of the conditions of measurement.

THE TRANSLUCENCY ERROR

Attention was called above to the importance of window in a color-measuring instrument. Of primary importance is the clearance, shown in Fig. 3, between the diameter of the beam incident on the specimen and the edge of the window through which the specimen is

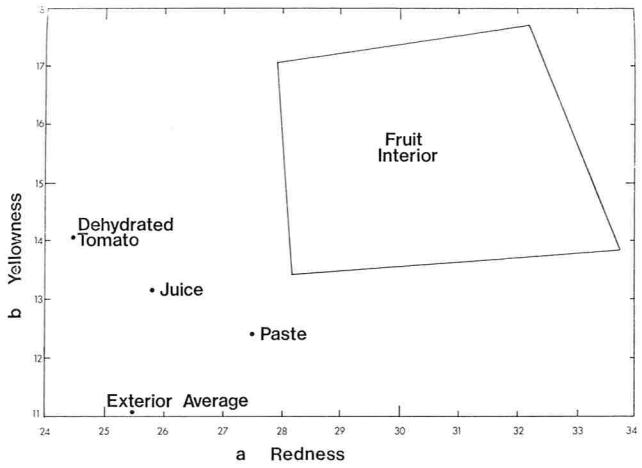

Figure 5. *Hunter a,b colors of five different forms of tomato product.*

illuminated and viewed. This clearance is an important factor in color measurements of translucent foodstuffs.

Figure 6 illustrates the problem in a sample of grapefruit juice. This grapefruit juice is in a colorless glass where, at the top, the material is seen as it is normally encountered in everyday living. Light enters the grapefruit juice and, after absorption and scattering, emerges frequently at some distance from the point at which it enters to give the characteristic pale yellow color normally observed. At the bottom of the glass, there has been added an opaque cardboard shield with color quite similar to that of juice. This cardboard color, however, is readily seen as being slightly darker than the juice at the top. If however, a retangular window is cut in the cardboard, the same juice seen through this window appears as dark or darker than the cardboard. This apparent darkening of the juice occurs because light going in through the window, is diffused by the translucent material to other areas of the sample. Relatively little of it comes back out the window through which it entered.

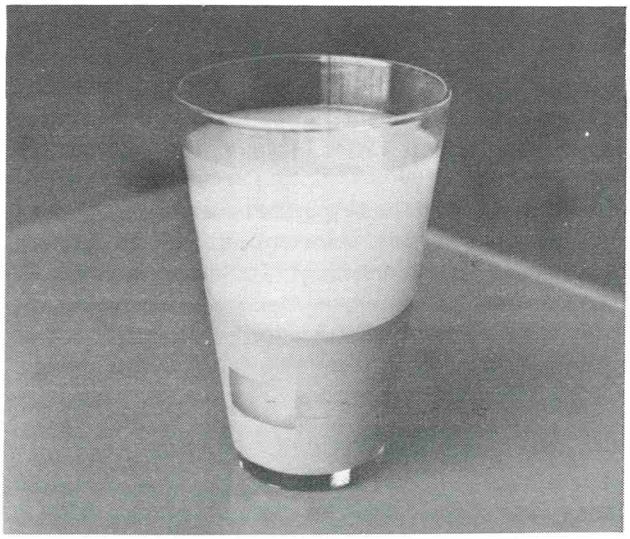

Figure 6. *Photograph of grapefruit juice. Note that juice is lighter above cardboard where light enters from all sides, but is darker below where light has to enter and exit through a restricted window.*

Trapping of light in translucent specimens by edges of window is illustrated in Fig. 3 and 6. The light which suffers most is that which strikes the specimen near the edge of the window. The dimension which is crucial is the average distance which rays travel before they encounter a window edge when they seek to emerge toward the observer or toward the observing light receptor. It can be appreciated that wavelengths, not readily absorbed by the product, will travel further within the translucent material and will therefore be more likely to be trapped than the wavelengths of light which are strongly absorbed and therefore do not travel very far. In other words, the red end of the spectrum, which is where the grapefruit juice transmits most strongly, is least absorbed and therefore is most likely to be trapped by the edge-of-window effect. The result is that the light seen in the window is not only darker than the grapefruit juice seen above, but is also grayer and perhaps somewhat greener.

Measurements were made of a number of foodstuffs, using for all the measurements, an incident beam of light about 1 inch in diameter on the center of the specimen. Three different conditions of observing the specimen were then used, and the changes in observed color (when compared with colors of completely opaque standards) were recorded. The smallest window was 1 1/4 inches in diameter, barely large enough to contain the 1-inch diameter light beam. The second window was 2 1/4 inches in diameter, thus giving a little over 1/2 inch leeway between incident beam and edge of the window. Finally a 4-inch window was used, giving an almost 1 1/2 inch opportunity for lateral travel of light before trapping.

Figure 7 shows the change of color, as the viewing window diameters were changed, of three samples of orange juice. The dimensions are Hunter L, *a* and *b*. It is noteworthy that the shifts of OJ score correspond to almost four full points. This is more than is regularly

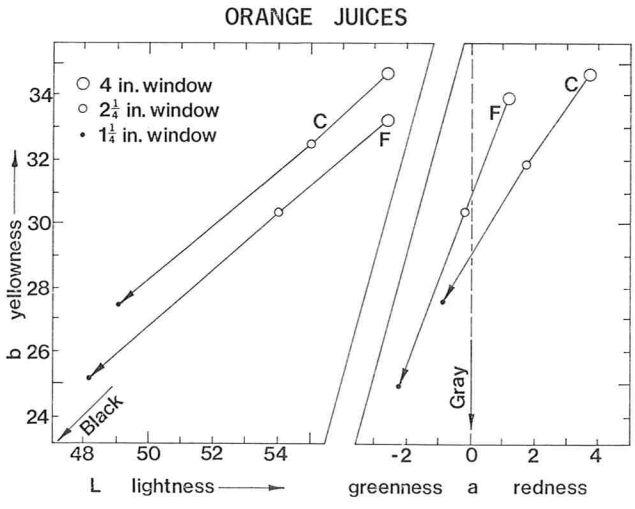

Figure 7. *Changes of measured color in Hunter, L,a,b of two specimens of orange juice, as instrument windows are decreased from 4 inches (best) to 1 1/4 inches in diameter.*

encountered in samples of the product going to market from any given region.

Figure 8 shows the same sort of changes in color of three samples of tomato juice. Here there is an L vs. *a* diagram at the top and a *b* vs. *a* diagram at the bottom. Note how these samples measure darker and less saturated in color as the window sizes become smaller.

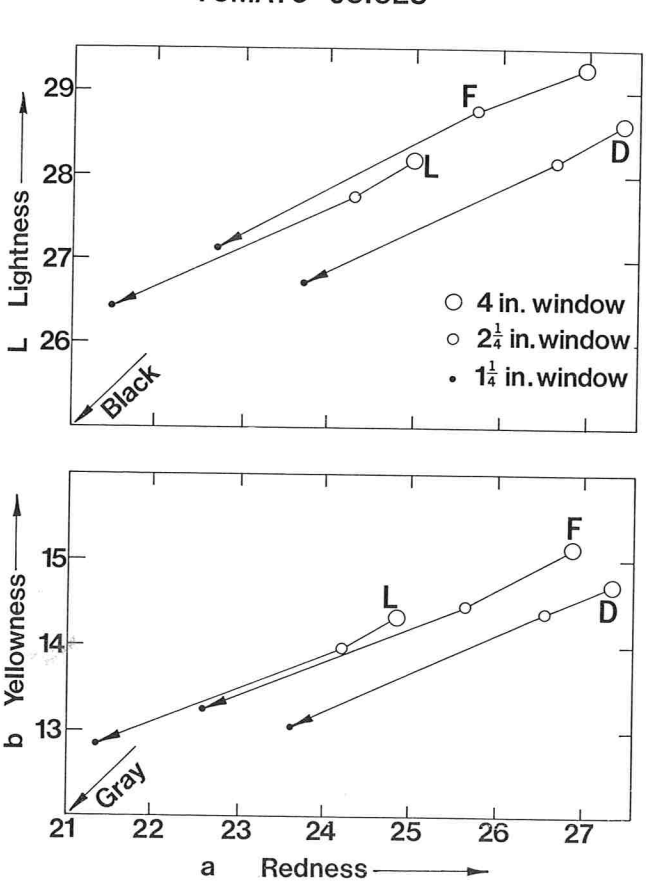

Figure 8. *Changes of measured color in Hunter L,a,b of three specimens of tomato juice, as instrument windows are decreased from 4 inches (best) to 1 1/4 inches in diameter.*

According to the tomato color formula developed by Yeatman and others, the smaller window improves the TC score, primarily because it makes the juice darker. Darkness rewards the scoring. By contrast, the orange juice color, shown in Fig. 7, improves in score as the windows became larger.

Figure 9 summarizes the measurements made of a number of different products, including peanut butter, steak, French dressing and others not mentioned above. The magnitude of change of measured color by adding 1 inch to the diameter of the window varies from less than 1 unit for peanut butter, which is quite opaque, to more than 8 units for orange juice, which is translucent. This translucency (or edge-of-window error) is potentially a significant problem in the measurement of the color of many different foodstuffs.

CHANGE OF MEASURED COLOR
BY ADDING ONE INCH TO DIAMETER OF WINDOW

SPECIMENS:	L a b UNITS	2	4	6	8
Peanut Butter	0.7	→			
Beef Steak	1.1	→			
Peas (frozen)	1.1	→			
French Dressing	1.6	→			
Tomato Juice	3.0	→			
Peach Preserves	4.5	→			
Orange Juice	8.3	→			

Figure 9. *Changes in measured color of seven foodstuffs from adding one inch to diameter of instrument window.*

ACKNOWLEDGMENT

Presented at a seminar, "Optical Measurements for Nondestructive Quality Evaluation of Horticultural Products," sponsored by the Horticultural Crops Laboratory of the Richard B. Russell Agricultural Research Center, Athens, Georgia, March 7-9, 1977.

Journal of Food Protection Vol. 41, No. 9, Pages 730-738 (September, 1978)

Comparative Review of the Thiochrome, Microbial and Protozoan Analyses of B-Vitamins

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ABSTRACT

A comparative review of the specificities of various B-vitamin analysis methods is presented. Methods of vitamin analysis employing protozoa, viz. *Tetrahymena* and *Ochromonas*, are compared to the commonly used methods of vitamin assay, including officially accepted methods. Since 1968 the protozoan techniques have routinely been used to evaluate clinical vitamin status. This is due to their more mammalian-like response to the various forms of the vitamins that occur in natural materials. Protozoa have more developed ingestive and digestive systems than bacteria and yeast, which allow them to respond to conjugated forms of the vitamins (e.g. thiamin pyrophosphate and folic acid polyglutamates). Also, fewer problems are encountered with non-specific stimulation (e.g. fatty acid stimulation of the *Lactobacillus casei* assay for riboflavin or the sparing of the vitamin B_{12} requirement of *Lactobacillus leichmannii* by deoxyribosides). Application of the protozoan methods to analysis of foods indicate significantly lower levels of biotin and vitamin B_{12} than official methods, while significantly higher levels of riboflavin, vitamin B_6, niacin and pantothenic acid are found in low-acid foods. This review also summarizes extraction methods for the B-vitamins from food samples and indicates the importance of enzymatic hydrolysis in extract preparation.

The relationship between nutrition and certain affections has been known but poorly understood until recently. For over 300 years, scurvy has been avoided by including fresh fruits or vegetables in the diet. Doses of cod liver oil, once given to children for almost any illness, helped to avert rickets. In 1897 Eijkman determined the relationship between beri-beri and diets of polished rice (*6*). Studies of nutritionally-related diseases revealed the presence of essential organic substances which were not carbohydrates, fat, or protein. Hopkins termed these substances "accessory food factors" (*17*). Funk (*7*) evolved this term to "vitamine", which changed to "vitamin" in 1913 (*1*).

The potential of microbiological assays for vitamin determinations was revealed by Williams in 1919 (*32*). Microbiological methods of vitamin determination are based on the observation that a given microorganism can reproduce only in the presence of a certain vitamin. When aliquots of the sample containing the vitamin being determined are added to the initially clear medium, followed by inoculation with the test organism, the organism multiplies in proportion to the vitamin content, which can be measured photometrically or the metabolic products can be measured. Over a defined concentration range, the measured response will be directly proportional to the amount of vitamin present, and, within this range, the sample solution and reference solution can be compared accurately. Simple and reliable procedures require the test organism to possess the following characteristics: specifically require the vitamin, be genetically constant during prolonged subculture, have an easily measured growth response, have a rapid growth cycle, possess nutritional requirements similar to those of man, and be non-pathogenic. As a group, the lactobacilli are most widely employed, although yeasts, molds, and protoza are used, their growth characteristics are usually less suitable. This review compares the available protozoan assay methods to commonly used and/or offical analytical methods for the B-vitamins. Specificities of the various organisms, procedures used for extraction, and problems encountered with the various methods are discussed.

VITAMIN ASSAY METHODS

The water soluble vitamins are a heterogenous group of low molecular compounds which act primarily as coenzymes in biochemical reactions. Animals and some microorganisms require minute quantities of these vitamins from their nutrient source. Microbiological assays are applicable when the physical and chemical determinations are neither specific nor sensitive enough.

Thiamin

Thiamin contains a pyrimidine moiety and a thiazole moiety joined by a methylene bridge. Thiamin solutions below pH 5 are stable to autoclaving and oxidation. At pH 7 or greater, thiamin is destroyed by boiling or storage at room temperature. Destruction presumably occurs because the thiazole ring opens and is rapidly oxidized, especially upon heating (*12*). Sulfite readily splits thiamin into its component moieties (*2*).

Thiamin is found in natural material in the free form, as the mono-, di-, and triphosphoric esters, and as the mono- and disulfide. The predominant form in animal tissues is the diphosphate (pyrophosphate, TPP, or cocarboxylase) which exists largely as a protein complex bound to the enzyme, carboxylase. Thiamin also occurs complexed with transketolase in a not readily dissociable form. The most abundant form in plant tissue is free thiamin. Derivatives found in plant extracts with high thiamin activity, but having open thiazole ring structures are allithiamine and its propyl analog (*12*).

Thiamin is usually extracted from natural materials by dilute acid hydrolysis which may be followed by phosphorolytic and/or proteolytic digestion. Generally, the sample is treated with 0.1 N sulfuric acid or hydrochloric acid on a steambath for 30 minutes or autoclaved 15 minutes at 121 C. After heating, the pH is adjusted to 4.5 and the enzymes are added. The mixture is then incubated either for 3 h at 45 to 50 C or overnight at 37 C (*25, 26*). Thiamin protein complexes are usually hydrolyzed by the acid treatment, while the thiamin phosphoric esters are enzymatically hydrolyzed. The amylolytic enzyme preparation Diastase®, which contains phosphatase, is commonly used (*29*). The amylolytic enzymes in Diastase® are useful in hydrolyzing the starches present in plant materials, which aids extract filtration and avoids problems of strach stimulating the test organism (*12*).

The first methods used to quantitate thiamin were animal assays. Animal assays are rarely used today because of their cost, time consumption, and lack of precision — even thought they are specific, measure the physiologically available thiamin content, and require no special extraction procedures. Table 1 indicates the comparative responses of the common thiamin assay methods.

The most widely used method for thiamin assay in natural materials is the thiochrome technique. Under certain conditions, this fluorometric technique is highly specific (*29*). This method depends on oxidation of thiamin to thiochrome by alkaline ferricyanide. Under ultraviolet light the thiochrome has an intense blue fluorescence. Other fluorescent substances can interfere with this determination and it also lacks sensitivity (*2*). Many of the interfering substances can be removed by column chromatography. After elution, thiamin is converted to thiochrome and extracted by isobutanol (*29*).

TABLE 1. *Responses induced by thiamin moieties relative to thiamin*[1].

Assay method	Thiamin	Pyrimidine	Thiazole	TPP
Saccharomyces cerevisiae	1.0	0.3	0.6	0.03
Kloeckera brevis[3]	1.0	0.0	0.0	0.7
Ochromonas danica[3]	+	—	—	+
Ochromonas malhamensis	1.0	0.0	0.0	1.0
Lactobacillus fermenti[4]	1.0	0.0[5]	0.0[5]	0.3
Lactobacillus viridescens	1.0	0.0	0.0	1.0
Thiochrome	1.0	0.0	0.0	0.0

[1]Reference: Saarivirta (*26*), Strohecker and Henning (*29*), and Pearson, et al. (*25*).
[2]"+" active, "—" inactive.
[3]Responds to thiamin orthophosphoric acid.
[4]Can synthesize thiamin if stock culture maintained on low thiamin level medium.
[5]Not active if incubated 18 h, responds about the same as intact thiamin after 48 h (requires presence of both pyrimidine and thiazole).

Thiamin-containing enzymes are very specific as to the form of the thiamin incorporated. Small alterations of the thiamin molecule not only will reduce the catalytic activity of the enzymes, but also may produce antimetabolites that will entirely block enzyme activity. Microorgamisms that require thiamin fall into five catagories: those that require intact thiamin, the pyrimidine moiety, the thiazole moiety, either the pyrimidine or thiazole moieties, or both the pyrimidine and thiazole moieties (*2*). Man and other animals require intact thiamin; hence this is a desired characteristic for the assay organism. Microbiological assays are used less for thiamin than for the other B-vitamins.

Lactobacillus viridescens requires intact thiamin for growth. Thiamin monophosphate (TMP) and thiamin diphosphate (TPP) are nearly as active as the free vitamin. The hydroxyethyl derivative is about 80% as active as free thiamin, although some researchers claim the activity of the hydroxyethyl derivative to be 160% as active since the dl form had been used in the previous determination (*25*). The differences in growth responses of the test organisms to various derivatives of a vitamin are often due to the amount of energy the cell must expend to transport the derivative across the cell membrane and to alter it to required forms. Thiamin disulfides are not fully active for *L. viridescens* unless large amounts of reducing substances are present during the autoclaving extraction step. *Lactobacillus fermenti* also requires intact thiamin. *L. viridescens* has been indicated to be more desirable than *L. fermenti* since it is not affected by pentoses, reducing agents, fructose, maltose, calcium, and glucose heat degradation products. The analytical data obtained with *L. viridescens* correspond better to the data from the thiochrome method (12). With the *L. fermenti* assay, the pyrimidine and thiazole moieties do not permit growth if the incubation period is limited to 18 h while TPP is about 30% more active than free thiamin. *L. fermenti* is capable of developing the ability to synthesize thiamin (*25*).

The methods using *Ochromonas danica* and *Kloeckera brevis* are the most specific. They respond only to the intact thiamin and its phosphoric acid esters. *O. danica* can be used to assay 200 pg thiamin per milliliter or gram sample (*31*). *O. danica* has a simple nutrition, whereas, the yeast, *K. brevis*, requires six B-vitamins for optimal growth. The *K. brevis* test is more sensitive than the *L. viridescens* assay, and TPP is about 70% as active as free thiamin (*25*). A thiamin requirement for *Tetrahymean pyriformis* was established by Lwoff and Lwoff in 1937 (*22*). Neither the pyrimidine nor the thiazole moieties suffice (*16*). *T. pyriformis* is not currently being used for thiamin analysis.

Riboflavin

The principle forms of riboflavin in nature are riboflavin-5'-phospahte (FMN) and flavin adenine dinucleotide (FAD). Both FMN and FAD occur protein bound (flavoproteins), with the latter occurring in higher proportion (60-90%) in natural products. Free riboflavin is rarely found in nature, being confined primarily to the milk of some species, urine, semen of certain bulls, fish retina, and the fish tapetum. The protein bound riboflavin is usually not analytically available without proteolytic digestion (*14*). Riboflavin solutions are rapidly destroyed by light. Alkaline solutions of riboflavin yield lumiflavin, while acid solutions yield lumichrome. Both lumiflavin and lumichrome are riboflavin antagonists (*2*).

Extraction with hot dilute acid (steam or water bath) suffices to split flavoproteins. The phosphate esters can only be broken by phosphatase treatment (Diastase®). Treatment with Diastase® is desirable in the examination of natural products because it simultaneously breaks down the starch present (*29*). More precise and higher riboflavin values have been obtained by extraction with dilute mineral acid for 15 min at 121 C. Riboflavin can also be liberated from natural substances by treatment with 5% trichloroacetic acid overnight at 37 C. Under these conditions FAD is split to FMN. Extractions employing only phosphorylytic and proteolytic enzyme digestions have been used (*26*).

Several photometric and fluorometric methods are available for riboflavin analysis. The particular method selected depends on the nature of the sample and the expected riboflavin content (*29*). Only a few microorganisms require an external source of riboflavin, and most of these are lactobacilli. The classic microbiological riboflavin assay with *Lactobacillus casei* is still used. *Leuconostoc mesenteroides* is 50 times more sensitive than *L. casei* (*24*). A more recently developed technique is that described by Baker and Frank (*2*) using the protozoan, *T. pyriformis*. Neither the *L. mesenteroides* nor the *T. pyriformis* procedures have seen wide use (*24*). Free riboflavin, FAD, and FMN have significantly different growth promoting activities for *L. casei*, with free riboflavin having the highest activity. This is usually of only academic interest, because the acid extraction procedures normally hydrolyze FAD to FMN and then to free riboflavin. Fatty acids have a growth stimulating effect on *L. casei*; thus, when high fat materials are being assayed (e.g., cheese), it is advised to carry out an ether extraction. Values determined by *L. casei* have shown good agreement with the fluorometric and rat method values in a wide variety of samples (*24*). The *L. casei* test can be affected by starch, glycogen, free fatty acids, and certain protein compounds. Therefore, sample extraction must eliminate these substances. In 1944, Hall noted that *T. pyriformis* required riboflavin (*10*). The absolute requirement was established in 1945 by Kidder and Dewey (*20*). *T. pyriformis* responds equally on a molar basis to riboflavin, FMN, and FAD. Addition of amino acids, vitamins, lecithin, diacetyl, tartaric esters of tallow glycerides (TEM), triolein, cholesterol, lanosterol, beta-sitosterol, mevalonic acid lactone, farnesol, and squalene at 1-10 mg/ml do not relieve galactoflavin inhibition or spare riboflavin for this organism (*2*). Other lipids and fatty acids do not act as growth stimulants as they do for *L. casei*. Several flavins can replace riboflavin in the metabolism of some microorganisms, but only riboflavin can be used in mammalian and protozoan metobolism (*2*). Enzymatic digestion with Clarase® or Diastase® is still recommended to facilitate extraction. *T. pryiformis* analysis of riboflavin levels than the *L.. casei* method (*30*).

Vitamin B_6

Vitamin B_6 occurs in nature in three basic forms: pyridoxol (pyridoxine), pyridoxal, and pyridoxamine. Pyridoxal and pyridoxamine phosphates are the predominant forms of vitamin B_6 which occur in animal tissue. In plants, pyridoxol occurs in higher proportion (*26*). The functional form is pyridoxal phosphate. The three vitamin B_6 isomers are stable to heating in 5 N sulfuric acid or hydrochloric acid. In strong alkali, pyridoxamine is stable but pyridoxal and pyridoxol are destroyed. The isomers are stable to oxidation by atmospheric oxygen; they are all light-sensitive, being rapidly destroyed by strong light in neutral and alkaline solution (*2*). Keeping properties can be adversely affected by impurities in the solution (*29*). In natural products, vitamin B_6 partially exists complexed with proteins and must be liberated from these complexes before analysis by enzymatic or acid hydrolysis (*26*). Vitamin B_6 occurs in natural materials as the three basic isomers and their phosphorylated derivatives, pyridoxamine-5-phosphate and pyridoxal-5-phosphate. For feed and food enrichment only pyridoxol is used. All of these vitamin B_6 forms have the same biological activity in man. This same characteristic is desired in the assay organism. Most of the literature values have been determined by the *Saccharomyces uvarum* method (*11*). *S. uvarum* was called *Saccharomyces carlsbergensis* in earlier citings.

Chemical and physical methods have only been used to a limited extent for assaying vitamin B_6 in natural materials (*29*). Microbiological assays are available that can quantitate the separate isomers or measure their total activity. Table 2 lists the comparative responses of various assay methods to pyridoxol and only the *S. uvarum* and *T. pyriformis* tests give relatively equal responses to equal molar amounts of the three basic isomers. There is disagreement in the literature as to the true response of the *S. uvarum* test; experiments show little if any difference in relative activities of the vitamin B_6 isomers for *S. uvarum* (*26*). High levels of d-alanine affect the *L. casei* and *Streptococcus faecalis* assays; however such concentrations are unlikely in natural materials (*29*). A source of pyridoxol or pyridoxamine was determined to be essential for growth of *T. pyriformis* by Kidder and Dewey in 1949 (*21*). "Pyridoxine mutants" not able to utilize this isomer have been reported (*16*). *T. pyriformis* has a specific

TABLE 2. *Responses induced by Vitamin B_6 derivatives relative to pyridoxol.*[1].

Assay method	Pyri-doxol	Pyri-doxal	Pyri-doxa-	Pyri-doxal-phos-phate	Pyri-doxa-mine-phosphate
Saccharomyces cerevisiae	1.0	0.8	0.4	* [2]	*
Saccharomyces uvarum	1.0	1.1	0.6	1.0	1.0
Neurospora sitophila[4]	1.0	1.4	1.4	+	+
Streptococcus faecalis[3]	—	1.0	1.2	+	0.5
Streptococcus faecium	—	1.0	1.1	*	*
Lactobacillus casei[3]	—	1.0	—	*	*
Tetrahymena pyriformis[5]	1.0	1.2	1.2	+	+
White rat	1.0	1.2	1.6	+	+

[1]References Saarivirta (*26*), Strohecker and Henning (*29*), Baker and Frank (*3*).
[2]"+" active, "—" inactive, "* " no data.
[3]Responds to the alanine level in the assay medium.
[4]Assay medium with excess thiamin.
[5]Pyridoxal and pyridoxamine have less potency when added together than when added separately.

requirement for vitamin B_6 that can not be spared by amino acids, and the organism has been used successfully in the assay of biological fluids (*2*). This test organism has the potential of replacing the indirect methods (xanthurenic acid, kynurenine, transaminase) for determination of vitamin B_6 in biological fluids. Analysis of the vitamin B_6 content of foods using *T. pyriformis* as the test organism have indicated higher levels of vitamin B_6 than the *S. uvarum* method (*30*).

Vitamin B_{12}

Cobalamin is the name of a group of compounds which contain a porphyrin-like corrin nucleus with a central cobalt atom. The compound known as vitamin B_{12} (cyanocobalamin) is more appropriately named alpha-(5-6-dimethylbenzimididazoyl) cobamide cyanide, though, the trivial names vitamin B_{12} and cyanocobalamin are still used. Some other cobalamins are: alpha-(5-6-dimethylbenzimididazoyl) cobamide cyamide, alpha-hydroxo-cobamide (aquocobalamin and hydroxocobalamin), alpha-(2-methyladenyl)-cobamide cyanide (Factor A), and alpha-(adenyl) cobamide (pseudovitamin B_{12}). The coenzyme forms have an organic ligand attached to the central cobalt atom and are called cobamides or cobalamines; for example, the coenzyme derived from cyanocobalamin is 5'-deoxyadenoxylcobalamin or alpha-(5-6-dimethylbenzimidazoyl)-Co-5'-deoxyadenoxylcobamide (*26*). The cyano group attached to the cobalt atom can be replaced by other ions or groups to yield other cobalamins, such as hydroxocobalamin, chlorocobalamin, nitrocobalamin, and thiocyanatocobalamin. All are readily converted back to cyanocobalamin with cyanide. Another series of analogs can be produced by varying the nucleotide moiety. Besides 5,6-dimethyl-benzimidizole, adenine-, 2-methyladenine-, and quanine containing cobalamins are found in nature; another, factor B, contains no nucleotide base. Cyanocobalamin is a neutral molecule with a net charge of zero. For optimum stability, solutions should be adjusted to pH 4 to 4.5. Alkaline hydrolysis, strong mineral acids, and ultraviolet or strong visible light rapidly decompose and inactivate vitamin B_{12} (*2*).

It is well known that vitamin B_{12} is synthesized by a number of microorganisms. Higher animals cannot synthesize this vitamin and must obtain it directly or indirectly from bacterial sources. Thus, vitamin B_{12} passes through the food chain. Quantities of the vitamin are produced in ruminants by rumen bacteria in the presence of adequate cobalt. Vitamin B_{12} is not synthesized by higher plants, and the small amounts that may be present are assumed to be due to bacterial action or contamination (*28*). Cyano-and hydro-oxocobalamin have been isolated from natural products and were thought to be artifacts produced by chemical decomposition (effects of light, heat, cyanide) during isolation (*26*). Pseudocobalamins, which differ from "true" cobalamins in that the 5,6-dimethyl-benzimidazole group is replaced by other groups, such as adenine, do not possess animal activity. They have not been found in animal tissue and fluids. Pseudocobalamin B_{12}, although known to be clinically inactive, is a coenzyme in glutamate metabolism in some microbial systems (*26*). The cobalamin content of an unknown sample determined by analysis represents its potential vitamin B_{12} activity. This may be a mixture of free, conjugated and bound cobalamins. For example, the major portion of cobalamin in liver is in the form of 5,6-dimethylbenzimidazole cobalamin, which on extraction gives hydroxocobalamin. Hydroxocobalamin is an ionizable heat labile complex that has a protein binding affinity exceeding that of cyanocobalamin, but is readily converted to cyanocobalamin by cyanide. Cyanocobalamin is non-dissociable and is relatively heat-stable. The stability of cyanocobalamin has resulted in its being the most widely isolated and known cobalamin. It is the form used to prepare standards for comparison of vitamin B_{12} activity. Recent evidence indicates differences in the physiological activity of cyanocobalamin and other cobalamins. By definition, cyanocobalamin is vitamin B_{12} and hydroxocobalamin is vitamin B_{12a}. The biological activity is assumed to be a function of the cobalamin moiety, regardless of the ligand attached. "Vitamin B_{12} activity" is thus used to designate the total cobalamins (*28*).

In natural materials, the vitamin B_{12} group exists largely in coenzyme forms in which an adenosyl moiety linked from carbon atom 5' to reduced cobalt replaces cyanide and other ions (*26*). These coenzyme forms are normally bound to cellular protein, which must be hydrolyzed before quantitative extraction. The non-cyanocobalamines are converted to cyanocobalamins to increase their stability. The best extraction procedure depends on the material being studied. The extraction medium has to contain a reducing agent such as metabisulfite, ascorbic acid, thioglycollic acid, or thiomalic acid, and, in addition, sodium cyanide which converts the labile hydroxocobalamin to the stable cyanocobalamin. Heating in an autoclave liberates the protein-bound cobalamins. Enzymatic digestion with papain is recommended for animal tissues (*26*). The picogram vitamin B_{12} levels present in foods are usually assayed by microbial techniques. The interfering

substances (e.g., deoxyribosides, methionine, and sodium metabisulfite) that may invalidate an assay must be considered (*13*). The AOAC indicates that the maximum tolerance of the *Lactobacillus leichmanni* assay to sodium metabisulfite is 0.03 mg/ml (*18*).

The microbiological and isotopic cobalamin analysis methods are most suited for determinations in natural materials. Probably all the reported vitamin B_{12} contents of foods were determined by the microbial methods. The responses of various assay cultures to different cobalamins are summarized in Table 3. The choice of method depends mainly upon the sample composition. Owing to their generally lower activity, the effect of interfering impurities is usually eliminated by dilution. For example, the deoxyribosides are 4000 times less active than vitamin B_{12}. However, it is still advisable to carry out a series of comparative tests for each new type of material being analyzed; that is, different test organisms may be used, or the same test orgamism used on extracts prepared by different methods. To avoid a loss of vitamin B_{12} activity during analysis, it is advised to add 1 mg of potassium cyanide to each liter of working-strength test medium (*29*). Inclusion of both sodium cyanide and methionine in the *O. malhamensis* basal medium increases the sensitivity of this method 10-fold (*28*).

Early reports on the vitamin B_{12} contents of food are complicated by the fact that comparative validity checks were often not made. Comparative studies of vitamin B_{12} contents of foods using *L. leichmannii* and *O. malhamensis* have shown differences with some foods, especially fruits and vegetables (*30*). The deoxyriboside interference of the *L. leichmannii* test becomes important only with materials high in nucleic acids. *O. malhamensis* is the most specific of the assay organisms. It responds only to the clinically active forms of cobalamin, but has the disadvantage of requiring several days for incubation. *O. malhamensis* may yield slightly higher results than other assay organisms (*20*). *Euglena gracilis* responds to the clincally active forms, to some pseudoanalogues, but not to the noncobalamin analogs devoid of nucleotide. Serum and blood promote the growth of *Euglena* (*2*). *L. leichmannii* resembles the *Euglena* test in both sensitivity and response but will respond to deoxyribosides. The *E. coli* test is even less specific than *E. gracilis* and is suitable only for pharmaceutical analyses (*26*). Methionine will spare the *E. coli* vitamin B_{12} requirement (*26*). *O. malhamensis* responds to the "true" vitamin B_{12} content; whereas *E. gracilis* responds to the "total" vitamin B_{12} content. It has been explained that beta-methylaspartate may by-pass the vitamin B_{12} requirement of *O. malhamensis*; however, under the usual assay conditions neither β-methylaspartate nor methylmalonate interfere to a serious extent (*13*).

Biotin

Biotin is present in natural materials as free biotin, biocytin (biotinyllysine), and biotin sulphoxide (*26*). Only the d (+) biotin isomer is biologically active. Aqueous solutions of biotin can be obtained by neutralizing the free form with the equivalent amount of alkali. Biotin solutions are stable to boiling from pH 4 to 9 (*2*).

In animals and microorganisms, biotin is present in conjugated and/or protein-bound forms. In plants it is partially present in the free state. The methods used to extract biotin from natural materials vary from heating in water to autoclaving in 6 N sulfuric acid. Enzymatic digestions are also used (*26*).

The common biotin assay techniques for natural materials employ microorganisms (*29*). Table 4 lists the

TABLE 3. *Relative response of organisms to cobalamins and other substances.*[1]

Compound[2]	*Escherichia coli*	*Lactobacillus leichmannii*	*Euglena gracilis*	*Ochromonas malhamensis*[3]	Clinical Activity
Cyanocobalamin	1.0	1.0	1.0	1.0	+[4]
Pseudovitamin B_{12}	1.0	0.7	1.0	—	—
Factor A	1.0	0.7	0.7	—	—
Factor B	1.0	—	—	—	—
Factor C	1.0	0.3	0.3	—	—
Factor D	—	—	—	—	—
Factor E	1.0	—	*	—	—
Factor F	1.0	*	*	—	—
Factor G	1.0	0.3	*	— or 0.3	—
Factor H	1.0	0.3	*	—	—
Factor I or Factor III	1.0	0.7	*	0.7	+
B_{12}-benzimidazole	1.0	1.0	*	1.0	+
B_{12}-5-methyl-benzimidazole	1.0	1.0	1.0	1.0	+
B_{12}-naphthimidazole	1.0	1.0	*	1.0	+
Deoxyribosides	—	1.0	—	—	—
Intact DNA	—	0.3	—	—	—
Methionine	1.0	—	—	—[3]	—

[1]References: Baker and Frank (*2*) and Strohecker and Henning (*29*).

[2]Base of the nucleotide (cobalamine: base of the nucleotide): cyanocobalamin: 5,6-dimethylbenzimidazole, pseudovitamin B_{12}; adenine, Factor A: 2-methyladenenine, Factor B: no nucleotide, Factor C: guanine (guanosine diphosphate), Factor D: not known, Factor E: not known, Factor F: 2-methyimercaptoadenine, Factor G: hyposanthine, Factor H: 2-methylhypoxanthine, Factor I: 5-hydroxybenzimidazole, B_{12}-benzimidazole: benzimidazole, B_{12}-5-methylbenzimidazole: 5-methylbenzimidazole, B_{12}-naphthimidazole: naphthimidizole.

[3]Responds to large amounts of methionine.

[4]"+" active, "—" inactive, "* " no data.

comparative responses of several assay methods to biotin and its derivatives. Biotin estimation by microbiological assay has two major limitations: the occurrence of chemical compounds with non-specific biotin activity and the difficulty in completely extracting the bound biotin (*9*). The clinically inactive biotin-related compounds include oxybiotin, desthiobiotin, biotin sulfone, and biotin sulfoxide. However, they do not occur in significant amounts in foods. The biotin oxidation products, the sulfoxide and sulfone, may be formed during the heat-treatment employed during extraction (*29*). *Lactobacillus plantarum* is probably the most widely used test organism. *Allescheria boydii* has a high specificity toward biotin, although the biotin analogs oxybiotin and desthiobiotin can partly replace biotin in this test. Aspartic acid, cysteine, glutathione, pimelic acid, oleic acid and Tween 80 do not interfere with this test (*29*). Desthiobiotin and biocytin have no growth promoting action on *L. plantarum*, while oxybiotin and probably biotin sulfone and-sulfoxide, oleic acid, elaidic acid, linoleic acid, linolenic acid, and some other lipids do have biotin activity (*9,29*). The toxic material in raw eggwhite is the glycoprotein, avidin. Avidin combines with biotin to form a biologically inactive complex. This complex is stable to stomach digestion but is readily split by heating. *O. danica* will not grow on the avidin-biotin complex. Pimelic acid, asparatic acid, and Tween 80 do not stimulate *O. danica.* Desthiobiotin, the sulfur free biotin analog, competitively inhibits growth of *O. danica.* Because *O. danica* is phagotrophic, it can ingest and digest many of the low molecular forms of biotin, for example, biocytin (*2*). *O. danica* analyses of the biotin content in foods have indicated significantly lower levels of biotin than the *L. plantarum* method (*30*). Biotin is probably necessary for growth of *T. pyriformis*, although an absolute requirement has not been demonstrated. Neither avidin nor desthiobiotin is inhibitory. some *T. pyriformix* strains have been isolated from the Pacific Ocean regions that require biotin (*16*).

Niacin

Niacin (nicotinic acid) exists in natural materials chiefly in the nicotinamide form as a constituent of three important conenzymes: nicotinamide adenine dinucleotide (NAD), formerly designated as coenzyme I or diphosphopyridine nucleotide (DPN): nicotinamide adenine dinucleotide phosphate (NADP), formerly designated as coenzyme II or triphosphopyridine nucleotide (TPN); and, nicotinamide mononucleotide phosphate (NMNP or coenzyme III). Niacin and its amide are unaffected by light, pH, boiling, or oxidation agents (*2*).

Niacin and nicotinamide are widely distributed in foods. Animal tissues and milk contain nearly all of this vitamin as nicotinamide, while plants contain much less and a more variable amount of the total activity as nicotinamide. Nicotinic acid has been extracted from natural materials by autoclaving the sample with water, sulfuric acid, or hydrocholoric acid. Equal results have been obtained using only enzymatic digestions (*26*).

Numerous microorganisms require an external source of niacin (*8*). *L. plantarum, L. mesenteroides,* and *T. pyriformis* have been used to quantitate niacin. *L. plantarum* responds equally well on a molar basis to nicotinic acid, nicotinamide, nicotinuric acid, and NAD. *L. mesenteroides* responds only to nicotinic acid. Picolinic acid, isonicotinic acid, quinolinic acid, torzonelline, niocotinic acid diethyamide, and arecoline are all inactive for the *L. plantarum* and *L. mesenteroides* tests (*29*). *T. pyriformis* utilizes nicotinic acid and nicotinamide, but when they are added together at the same concentration the growth increment may not be equal to the sum of the increments when the two forms are added separately (*2*). *T. pyriformis* has a wider sensitivity range than does *L. plantarum* (1-100 ng/ml versus 0.5-2.0 ng/ml) (*31*). Several natural occurring nicotinic acid derivatives that have been tested for their niacin-like activity with *T. pyriformis* are listed in Table 5. Of these, only nicotinic acid and nicotinamide permitted full growth; nicotinmethylamide and ethylamide showed

TABLE 4. *Responses induced by biotin derivatives relative to biotin.*[1]

Assay Method	Biotin	Biocytin	Biotinyl-glycine[2]	Biotinyl-d-sulfoxide[2]	Oxybiotin
Lactobacillus plantarum	1.0	—[3]	1.0	±	1.0
Lactobacillus casei[4]	1.0	1.0	+	—	0.4
Saccharomyces cerevisiae[5]	1.0	+	*	1.0	0.2
Neurospora crassa	+	+	*	*	*
Ochromonas danica[6]	+	+	*	*	*
Micrococcus sodonensis	+	++	*	*	*
Allescheria boydii[7]	1.0	1.0	*	*	*
White rat	1.0	1.0	*	—	0.05

[1]References Saarivirta (*26*), Baker and Frank (*2*), Gyorgy and Langer (*9*).
[2]Not known if compound occurs in nature.
[3]"+" active, "—" inactive, "±" literature disagrees, "* " no data.
[4]Responds to biotinamide and biotinyl-beta-alanine.
[5]Biotin spared by aspartic and oleic acid and pimelic acid. Responds to desthiobiotin.
[6]Desthiobiotin inhibits.
[7]Pathogenic fungus. Responds to desthiobiotin.

some activity; and, compounds having other modifications of the carboxyl groups did not result in growth. The chief urinary products do not promote a response by either *T. pyriformis* or *L. mesenteroides*, while *L. plantarum* does respond (*2*). High levels of tryptophan do not spare the niacin requirement for *T. pyriformis* (*16*). Niacin levels in low-acid foods determined by *T. pyriformis* are consistently higher than those given by *L. plantarum* (*30*).

Pantothenic acid

Pantothenic acid is composed of two moieties, pantoic acid and beta-alanine, joined by a peptide linkage. Pantothenic acid is stable in neutral solutions, but not in hot acid or alkaline solutions. Under these conditions it decomposes to pantoic acid and beta-alanine (*2*). Pantothenic acid is physiologically active as part of coenzyme A (CoA) and is part of acyl-carrier protein (ACP).

Pantothenic acid is extracted from natural materials with a neutral aqueous medium and/or by enzymatic digestion. The following enzymes have been used: Clarase® Takadiastase®, Mylase®, papain, prostrate monophosphatase, intestinal phosphatase, and CoA pyrophosphatase (*26*). Significate amounts of bound pantothenic acid other than CoA have been found in microorganisms, animal tissues, and milk (*23*).

Many microorganisms require pantothenic acid, but only a few have been used as assay cultures (*26*). The comparative responses of several assay cultures towards pantothenic acid and its derivatives are given in Table 6. The descrepancy between microbiological and chick assays for pantothenic acid is due to the ineffective release of bound panthothenate, such as pantothenyl phosphate and CoA complexes. Sequential digestions with the liver enzymes alkaline phosphatase and acid phosphatases has solved this problem (*4*. *L. plantarum* has replaced *L. casei* and *S. carlsbergensis* as the primary assay organism for pantothenic acid since it has a simpler nutrition (*4*). With the development of defined media, it was found that *T. pyriformis* has a specific requirement for this vitamin. *T. pyriformis* shows maximal response to free pantothenic acid followed by pantetheine, and pantetheine phosphate, but cannot readily use CoA (*16*). *T. pyriformis* is recommended when non-specific contamination from natural materials becomes a problem (*2*). Pantothenate levels in foods determined by *T. pyriformis* are consistently higher than those given by *L. plantarum* (*30*).

Folic acid

Folic acid (folacin) has several synonomous names given in the literature. "Vitamin M" was the term given to a factor found essential for monkeys. An anemia in chickens was said to respond to a "vitamin B." Other workers found an essential factor for *L. casei* and called it the "*L casei* factor." Mitchell et al. (*23*) isolated a factor from spinach and called it "folic acid," from the Latin

TABLE 5. *Growth response of* T. pyriformis *to niacin and its derivatives as measured by absorbance.*[1]

Concentration (ng/ml)	Compound[2]									
	A	B	C	D	E	F	G	H	I	J
Control	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
0.1	0.2	0.22	0.26	0.24	0.22	0.22	0.22	0.2	0.2	0.2
0.3	0.2	0.24	0.3	0.24	0.26	0.22	0.22	0.24	0.2	0.2
1.0	0.22	0.24	0.3	0.24	0.26	0.24	0.22	0.24	0.2	0.2
3.0	0.24	0.24	0.32	0.24	0.26	0.24	0.22	0.24	0.2	0.2
10	0.32	0.36	0.48	0.24	0.34	0.24	0.24	0.24	0.20	0.2
30	0.48	0.7	0.88	0.30	0.44	0.26	0.26	0.24	0.22	0.2
100	0.94	1.18	1.22	0.36	0.76	0.26	0.48	0.24	0.22	0.2
300	1.2	1.3	1.34	0.4	0.98	0.4	0.7	0.24	0.22	0.22
1000	1.22	1.3	1.34	0.4	1.2	0.44	0.9	0.24	0.22	0.22

[1]Reference: Baker and Frank (*2*).
[2]A=nicotinic acid; B=nicotinamide; C=nicotinic acid plus nicotinamide (equal concentrations of each, total is twice the value given in the first column); D = nicotinuric acid; E = nicotinmethylamide; F = nicotindimethylamide; G = nicotinethylamide; H = nicotindiethylamide; I = trigonelline (N'-methyl nicotinic acid); J = N'-methylnicotinamide.

TABLE 6. *Responses induced by pantothenic acid derivatives relative to pantothenic acid.*[1]

Assay culture	Pantothenic acid	Phospha-pantothenic acid	Pantetheine	Phospho-pantetheine	Dephospho-CoA	CoA
Lactobacillus bulgaricus[3]	±[2]	—	+	*	*	—
Lactobacillus casei (ATCC 7469)	1	—	1	*	—	—
Lactobacillus helvetica (ATCC 10246)	0.01	0.01	1.0	1.0	*	—
Lactobacillus plantarum[3,4]	1	—	1	—	—	—
Acetobacter suborydans	—	*	*	+	*	+
Saccharomyces carlsbergensis	+	—	—	—	—	—
Tetrahymena pyriformis[4]	+	*	+	+	*	—

[1]References: Saarivirta (*26*) and Baker and Frank (*2*).
[2]"+" active, "—" inactive, "±" active if present in high concentration, "* " no data.
[3]Medium supplemented with 10 μg/ml cysteine, if medium cysteine-free pantetheine has 0.4 activity of pantothenic acid.
[4]Pantothenol and pantoyl lactone (with and without beta-alanine) are inactive.

word *folium* meaning leaf. These and several other factors were later shown to be pteroylglutamic acid compounds (*1*).

Folic acid or folate refers to a group of related compounds based on pteroic acid (PA), and it is the trivial name for pteroylmonoglutamic acid (PGA). PA is composed of a pteridine ring and para-aminobenzoic acid. The simplest folate member, PGA, has one glutamic acid residue bound to the para-aminobenzoic acid of PA. This glutamic acid may be conjugated to at least five more glutamic acid residues. The folates may exist as reduced di- or tetra-hydroderivatives (THF). The enzymatically active forms are reduction products of the THF linked in the N^5, N^{10}, or N^5-N^{10} positions with formyl, hyroxymethyl, methyl, or formimino groups. Folate functions as a carrier of these groups in the metabolism of purines, pyrimidies, and certain amino acids (e.g., methionine, serine, valine, and histidine) (*26*).

PGA is rapidly inactivated by light and boiling at pH 1, but as the pH increases its stability increases. PGA is insoluble at less than pH 5, sparingly soluble in water as the free acid, and highly soluble in water as the sodium salt. THF derivatives are readily degraded on exposure to air when in alkali or in solutions with pH greater than 6. Only THF derivatives substituted in the 5-position are oxygen stable; for example, N^5-formyl-THF (leucovorin, citrovorum factor, or folinic acid) and N^5-methyl-THF. The N^5-formyl-THF is the most stable of the reduced folates (*26*).

The naturally occurring folates are the various active coenzyme forms, which are tetrahydrofolates, and usually exist as conjugates with more than one molecule of glutamate. There is no evidence that PGA is found in nature. Various amounts of the vitamin are lost by oxidation and decomposition during extraction and analysis. Of the various one-carbon adducts of THF, only the N^5-formyl and perhaps the N^5-methyl-THF appear to be stable enough to resist most isolation procedures (*15*). Present evidence indicates that the predominant folates in natural materials are the reduced folates (*26*). Since folates are destroyed by acid and alkali, enzymatic digestion must be used for extraction. Reduced folates, except for N^5-formyl-THF are oxygen labile and must be protected with ascorbic acid during extraction. This avoids oxidative cleavage at the 9-10 position. Another problem that may be encountered is the isomerization of N^{10}-formyl-THF to N^5-formyl-THF. The polyglutamate conjugates containing more than three glutamate residues must either be assayed using *T. pyriformis* or undergo enzymatic treatment to reduce the number of glutamate residues to three or less, depending again on the test organism (*16*). Some samples, such as liver, kidney, serum and plasma, can be prepared for assay by autolyss in phosphate-ascorbate buffer. To reduce the number of glumate residues, two types of conjugases are commonly used, one from chicken pancreas with a pH optimum of 7.5 that splits polyglutamates to the diglutamate level, and the other from hog kidney with a pH optimum of 4.5 that splits polyglumates to monoglutamates. The chicken pancreas enzyme is active on a wider diversity of materials, and is therefore usually preferred. Sometimes both conjugases are used together (*26*).

Folic acid is widely distributed in natural materials, but occurs in such low concentrations that chemical assays are difficult; although indirect methods such as measuring urinary formiminoglutamic acid (FIGLU) levels, for determining folate status in man are common. FIGLU determinations may be misleading if vitamin B_{12}, methionine, or glycine are ingested, since FIGLU levels would be normal even though folate was still deficient. Microbiological methods are the most important quantitative methods for folate in natural products. The *L. casei* test is the most common. The comparative responses of various assay cultures to folic acid and its derivatives are given in Table 7. The responses of the various test organisms vary greatly. PA, N^{10}-formyl-PA, and N^5-formyl-tetrahydro-PA are active for *S. faecalis* but not for *L. casei, Pediococcus cerevisiae,* or man. These three compounds are all inactive for *T. pyriformis*. The responses of *S. faecalis* to the various folate forms varies in degree, it may be high (PA, PGA, rhizopterin), reduced (pteroyldiglutamic acid), or completely absent (pteroylheptaglutamic acid) (*29*). The *S. faecalis* test is not appropriate for foods, since it does not respond to all the N^5-methyl-pteroylglumates and does respond to PA. These compounds may occur in fairly high quantity in foodstuffs. *L. casei* is the only test organism that responds well to all the N^5-methyl-pteroylglutamates, which appear to be the main folates in milk, human

TABLE 7. *Responses induced by folic acid derivatives relative to folic acid.*[1]

Folate [2]	*Streptococcus faecalis*	*Lactobacillus casei*	*Pediococcus cerevisiae*	*Tetrahymena pyriformis*
PA	+[3]	—	—	—
N^{10}-CHO-PA	+	—	—	—
N^5-CHO-PA-H_4	+	—	—	—
PGA	+	+	—	+
N^5-CH_2OH-PGA	+	+	*	*
N^{10}-CHO-PGA	+	+	—	*
N^5-CH_3-PGA	—	+	—	—
PGA-H_2	+	+	+	*
N^{10}-CHO-PGA-H_2	+	+	—	*
N^5-CH_3-PGA-H_2	+	+	—	*
PGA-H_4	+	+	+	+
N^5-CHO-PGA-H_4	+	+	+	+
N^5-CHNH-PGA-H_4	+	+	+	*
N^5-CH_2OH-PGA-H_4	+	+	+	*
N^5N^{10} = CH-PGA-H_4	+	+	+	*
N^5-CH_3-PGA-H_4	—	+	—	—
PDGA	+	+	*	+
N^5-CHO-PDGA-H_4	±	+	+	*
PTGA	—	+	*	+
N^5-CHO-PTGA-H_4	—	—	—	*
PHGA	—	—	—	*

[1]References: Saarivirta (*26*) and Strohecker and Henning (*29*).
[2]Abbreviations: PA = pteroic acid, PGA = pteroylglutamic acid; PDGA = pteroyldiglutamic acid, PTGA = pteroyltriglutamic acid, PHGA = pteroylheptaglutamic acid, PGA-H_2 = dihydro PGA, PGA-H_4 = tetrahydro PGA.
[3]"+" active, "—" inactive, "±" active if present in high concentrations, "*" no data.

serum, and liver. Some studies have shown that the *L. casei* folate activity of foods extracted without conjugase treatment is a color approximation of the food folate available for absorption by man (*15*). Assay with *S. faecalis* gives the total reduced mono- and poly-pteroylglutamates and assay with *L. casei* yields the total folate activity (oxidized and reduced mono-and poly-pterylglutamates). The ability of *L. casei* to utilize polyglutamate folates (up to three glutamate residues) makes *L. casei* the superior test organism (*2*). Since each of the test organisms measure a group of folates, results should be expressed as units of folate activity for the specific test organism, and not as folic acid. That is, "each gram of this material contains 100 ng of folate activity for *L. casei*" (*15*).

No protozoan assays are currently being used for folate determinations, although *T. pyriformis* has the potential. Studies by Kidder in 1946. (*19*) showed that folate was essential for the growth of *T. pyriformis*. The mono-and poly-glutamates have equal activity, since this organism produces conjugase enzymes. *Tetrahymena* does not possess the enzymes necessary for joining the pteridine and para-aminobenzoic acid moieties or for joining PA to glumatic acid residues, thus it cannot utilize folic acid fragments. Some substituted pyrimidines, such as thymidine, can spare the folate requirement of *Tetrahymena*, however, *S. faecalis, L. casei,* and possibly man follow this same response (*2,16*).

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Journal of Food Protection Vol. 41, No. 9, Pages 739-742 (September, 1978)

A Field Topic

Comparison of the Quality of Two Types of Milk at Two Sources in the Belo Horizonte, Brazil Market

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(Received for publication February 11, 1978)

ABSTRACT

Type B and type C milk samples were collected at the processing plant and at random points of retail sales. Coliform counts (MPN) were significantly higher in comparable milk sampled at retail than when sampled at the processing plant. Standard plate counts were higher at retail than at the processing plant. Thirty one of 53 samples of type C milk contained in excess of the 150,000/ml maximum for bacteria specified by code when tested at retail compared to 14 of 63 when tested at the creamery. Tests to predict shelf life by incubating specially prepared samples at 20 C indicated a sharp decline in quality between the industry and consumption. This sharp decline in quality between industry and consumption appeared to be a major problem that must be resolved in the present milk market. Type B milk was found to be a much superior product when examined by quality tests of this study. Flavor scores for the type B milk were consistently higher than the comparable type C samples. Type B milk was more commonly criticized for feed flavor while type C milk was more commonly critized as coarse/high acid.

Fresh milk being offered to the consumer in Belo Horizonte, the capital of the state of Minas Gerais, Brazil is sold in 1-liter plastic bags. Each bag as filled is imprinted with a code date for the next day. Due to the shortage of milk in the area, very little milk is ever returned. Usually the milk supply for any given day is sold by the end of the day.

This fresh milk supply is of two types and sold at two prices; a liter of type C milk presently sells for four cruzeiros while a liter of type B milk is selling for eight. The Ministerio da Agricultura, (governmental regulating agency) presently requires the dairy cooperative that serves the estimated 1.6 million inhabitants of Belo Horizonte, to supply a minimum of 400,000 liters of fluid milk daily before it can divert any of the incoming milk to the more lucrative manufactured products. All but a small part of this milk is type C. The present consumption of type B milk is 4,000 liters daily.

The two types of milk differ in the way they are collected, handled and controlled. Unlike the type C dairy, the type B dairy has each cow identified by photograph and each is certified to be free of tuberculosis and brucellosis by a licensed veterinarian. The milking facilities in type B dairies are modern, the working areas are of concrete and sanitation requirements are stringent. Most of the type C milk is still being obtained by hand milking and from cows kept on open range. It is also handled without refrigeration on the farm as opposed to the required refrigeration for the type B dairies. Total bacteria counts after pasteurization are not to exceed 40,000 per ml for type B milk and not to exceed 150,000 per ml for type C milk.

The difficulties facing the control of milk quality were aptly covered by Nogueira (*6*) in his presentation during the 1974 National Dairy Meetings in Juiz de Fora, Brazil. The problems as he saw them were two-fold: first, to maintain the quality of the milk supply and second, to not lose quantity or production. Brunelli (*3*) voiced the concern of nutritionists at the low per capita daily consumption of milk in Brazil which averages 200 ml.

That the quality of the milk produced in the state of Minas Gerais is improving is evident from the work of Dias (*4*) who reported that while 3% of the milk was rejected on the platform in 1962, only 1% was rejected in 1972. This improvement in quality is further borne out in the report of the Departemento Inspecao de Produtos de Origem Animal (DIPOA) which indicates that the amount rejected decreased further to 0.6% in 1976 (*1*).

Type B milk was introduced into the Belo Horizonte market in July 1977 with 4,000 liters daily. Sales of type B milk for this market, based on the introduction of similar quality milk into the Rio de Janeiro and Sao Paulo markets 2 years ago, for some reason are far short of industry projections.

The purposes of this study were to check the quality of the milk consumed in Belo Horizonte at both the processing plant and the market place, and to compare the two types of milk presently being offered to the consumer.

MATERIALS AND METHODS

Sampling

Milk samples packaged in liter plastic bags were collected from September 1, 1977 to October 26, 1977. One set of samples was collected directly from the filler at the processing plant by the personnel of DIPOA

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and stored under refrigeration at 5 C until analyzed. The daily supply of type B milk was processed from one large tank and two samples per run were collected for the day. The daily supply of type C milk was processed from eight tanks and four samples per run were collected at random each day.

On alternate days, samples corresponding to the previous day's production were collected in the market place by the staff of the Department of Public Health. Two samples of type B milk and four samples of type C milk were collected from bakeries, markets, grocery stores and supermarkets selected at random. The plastic bags of milk were packed in a STYROFOAM® box for transfer to the laboratory.

At the laboratory the plastic bags of milk were shaken vigorously to mix the milk thoroughly and then divided into two subsamples. One set was stored at 5 C for shelf life determination, the other set was used for the various other tests.

Microbiological analysis

Using the procedures specified in *Standard Methods for the Examination of Dairy Products* (*10*), each of the six daily milk samples were tested for total bacterial count using the Standard Plate Count (SPC) method.

Each day one sample of type B and one sample of type C milk were selected at random and examined for coliforms by the most probable number (MPN) method.

Chemical tests

The fat content for each milk sample was determined using the Gerber test. Total solids were estimated with the lactometer. The presence of peroxidase in each sample was determined using the method specified by Overby (*8*). The alkaline phosphatase method specified in *Standard Methods* (*10*) was used for detection of improper pasteurization.

Shelf life tests

A modification of the method of Broitman et al. (*2*) was used to estimate shelf life. Sodium lauryl sulfate was substituted, gram for gram, for the Nacconol used by Broitman et al. and the buffer solution was warmed to 40 C to prevent crystallization before to use. One ml of the dye-detergent-buffer solution was added to 10 ml of the milk being tested. The mixture was shaken thoroughly and was incubated at 20 C until color developed. A distinct pink color was considered to be the endpoint.

Organoleptic analysis

Each milk sample was warmed to 20 C and scored for flavor by a qualified judge according to procedures outlined in the USDA Publication No. 2111 (*5*). Subsamples of the first 50 samples that were studied were stored at 5 C and tasted daily until such time as they were considered to be unacceptable for sale.

RESULTS AND DISCUSSION

Estimated coliform counts per ml of milk are shown in Table 1. The probability of finding coliforms was much higher in the milk that was collected at market outlets than it was for milk collected at the production plant. In 29 of the 63 samples tested, no coliforms were detected, in eight samples under 100 per ml were present, and in 26 of the samples the count was estimated at more than 100 per ml. Coliform counts as measured by MPN were high during the same time period that the presence of alkaline phosphatase was detected in some of the milk samples. It was during this period that one type B and three type C milk samples were found to have developed gas in all 15 tubes used for the test. This would indicate an excess of 24,000 coliforms per ml of milk.

Estimates of total bacteria by SPC are shown in Table 1. The samples of type B milk collected at the dairy plant had very much lower total bacterial counts than did the type C milk. The average count for type B milk was 10,000 per ml as compared to 124,000 per ml for type C milk. Of the 29 type B milk samples taken at the dairy plant, and subsequently plated, 23 samples (80%) had less than 10,000 bacteria per ml. Only four of the 29 samples (14%), taken over the 63-day period had more than the 40,000 bacteria allowed by law. In contrast, of the 29 samples of type B milk from market outlets that were plated, only 16 (55%) were within the 40,000 bacteria per ml legal limit. This represented a marked loss in quality between the time the milk left the plant and the time it was available for sale. Apparently this represents one of the major areas for more work for those concerned with improving the quality of milk offered to the consumer.

In 1966 Santos (*9*) tested the efficiency of pasteurization as practiced by the two large creameries then selling the major part of the 138,000 liters of type C milk being consumed daily in Belo Horizonte. At that time the milk from one plant, which represented approximately half of the total milk, contained active alkaline phosphatase, milk from the other plant did not. In the present study, 11 years later, only 16 of the 213 samples tested contained active alkaline phosphatase. The cause for the contamination was traced to a faulty gasket in one of the plate heat exchangers. Active peroxidase was present in all 213 samples indicating that none had been heated excessively.

A comparison between the tests reported by Broitman et al. in 1958 and those of the present study done

TABLE 1. *Comparison of type B and type C milk for coliform counts and bacterial counts at the processing plant and at the point of sale to the consumer.*

Milk	Coloform count (Most probable number)		
	Zero	Zero to 100	More than 100
Type C milk			
Industry[a]	10	3	4
Consumption[b]	4	3	9
Type B milk			
Industry	9	0	6
Consumption	6	2	7

Milk	Standard Plate Count			
	Zero to 10,000	10,000 to 40,000	40,000 to 150,000	More than 150,000
Type C Milk				
Industry	3	10	26	14
Consumption	0	1	31	31
Type B milk				
Industry	23	2	4	0
Consumption	9	7	8	5

[a]Samples taken from the filler at the processing plant.
[b]Samples taken at random from market places.

at Universidade Federal de Minas Gerais (UFMG) is shown in Fig. 1. The slope of the line for the work performed at UFMG is steeper than the slope of the line reported by Broitman et al. for the milk in the East Lansing area of the United States.

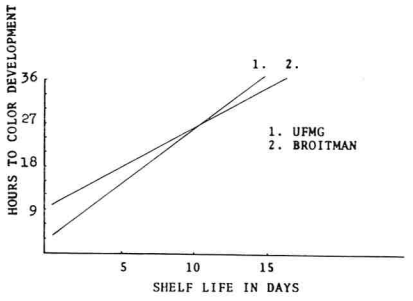

Figure 1. *A comparison of color development time with shelf life for milk samples as reported by Broitman et al. and as observed by workers at Minas Gerais Brasil.*

In the present study comparative samples of the same milk were taste-tested to determine actual spoilage time as well as being tested by the dye-detergent-buffer technique. Those samples with color development in 20 h or less had less than 4 days of shelf life. Those samples that required 30 h or more to develop color, were still sweet and salable after 10 days of storage at the specified temperature.

Type B milk samples had a milkfat content range from 3.2 to 3.7% with a mode of 3.6%, a mean of 3.5%, with a standard error of .01 as shown in Table 2. These samples had a solids-not-fat content ranging from 8.27 to 9.36% with a mode of 8.69%, a mean of 8.67%, with a standard error of .05.

Type C milk samples had a milkfat content range from 1.9 to 2.3% with a mode of 2.1%, a mean of 2.1% with a standard error of .01. These samples had a solids-not-fat content range from 8.04 to 9.11% with a mode of 8.90%, mean of 8.86% with a standard error of .04.

When type B milk samples from the dairy plants were compared with those from the markets, a shift in predicted shelf life was evident, as noted in Fig. 2. Since the predicted time for the two should vary only by 1 day, but did in fact vary by several days, a conclusion may be drawn that storage and handling conditions were not optimal. Although the type B milk had a prediction of longer shelf life time than did the type C milk as noted in Fig. 3, it had a similar loss in quality between samples from industry and market. Two samples of type B milk collected from retail markets were sour when opened and contained a core of frozen milk, indicating storage at below 0 C. The day of the week, the code date for that day was correct on the package, but a strong probability existed that the samples were from milk meant to be sold on the comparable day of the previous week.

Flavor scores were slightly lower for the milk samples from the

TABLE 2. *Milkfat and solids-not-fat content of two types of milk.*

Milk	Number of samples	Solids-not-fat		Milkfat	
		Mean	S.E.	Mean	S.E.
Type B	66	8.67	0.05	3.5%	0.01
Type C	136	8.86	0.04	2.1%	0.01

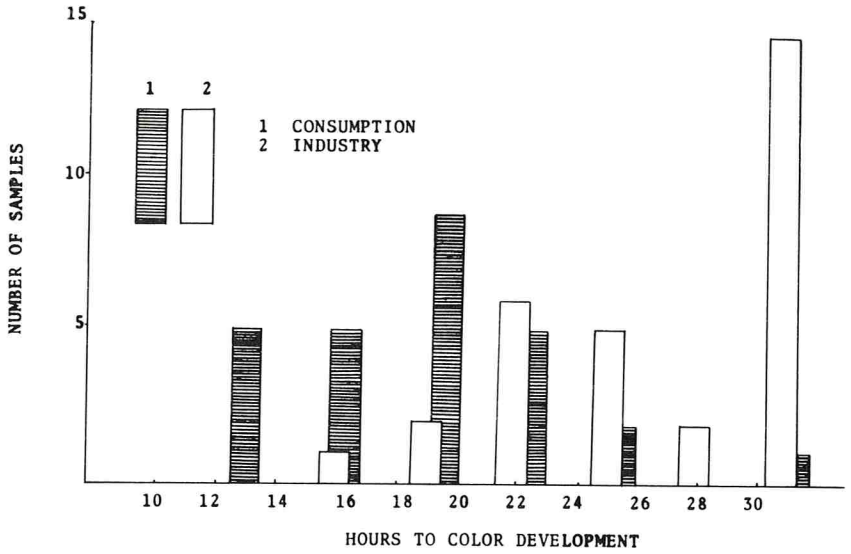

Figure 2. *A comparison of hours required for color development of type B milk from two sources incubated at 20 C with buffer sodium lauryl sulfate tetrazolium.*

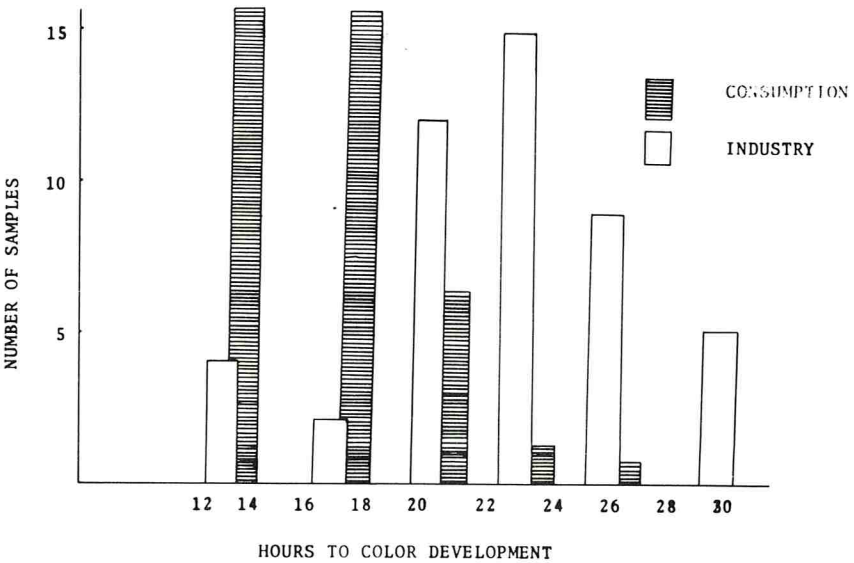

Figure 3. *A comparison of hours required for color development of type C milk from two sources and incubated at 20 C with buffer sodium lauryl sulfate tetrazolium.*

market than from the plant. The type C milk had a flavor score average for plant samples of 34.4 compared to 33.8 for market samples. For the type B milk the flavor score average was 36.5 for plant samples and 35.0 for market samples. A comparison of the types of flavor criticisms may be found in Table 3. In type B milk the most common cause for criticism of flavor was feed. The most common criticism in type C milk was acid or coarse. Another common criticism was a "foreign" taste, where an unusual taste of a soap-like nature could be detected. This criticism was four times more common in milk from markets than from dairy plants, indicating that the undesirable flavor became more pronounced with time.

Although only three of the 37 type B samples taken at the plant were scored with no criticism, this in itself is a giant step in the right direction. These three samples were equal to the best quality milk offered anywhere in the world and indicates a potential to continue to produce milk of this desired quality.

TABLE 3. *Comparison of the numbers of each flavor criticism found in milk samples collected at two points and of two types of fluid milk.*

Defect	Type C milk		Type B milk	
	Industry	Consumption	Industry	Consumption
Bitter	1	1	0	0
Cooked	2	1	1	0
Coarse (high acid)	47	28	1	2
Foreign	5	21	0	1
Rancid (lipase)	7	3	0	0
Cowy (barny)	11	0	0	0
Feed	8	7	32	24
Flat (watery)	0	1	0	3
No criticism	0	0	3	0

ACKNOWLEDGMENTS

The taking of samples for this study was arranged by the staff of the federal inspection service (DIPOA) and the staff of the Belo Horizonte Department of Public Health. Their cooperation was very valuable and is deeply appreciated.

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Journal of Food Protection Vol. 41, No. 9, Pages 743-754 (September, 1978)

Detection and Occurrence of Enteric Viruses in Shellfish: A Review

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(Received for publication March 20, 1978)

ABSTRACT

During feeding, bivalve mollusks (oysters, mussels and clams) can accumulate pathogenic human enteric viruses when present in sewage-polluted seawater. It has been well established that infectious hepatitis virus is transmitted by consumption of raw or inadequately cooked shellfish. But because of the lack of epidemiologic techniques, transmission of other enteric viruses by shellfish has not been established. Other enteric viruses, such as polio, echo, coxsackie and reo, have been detected in shellfish. Enteroviruses have been detected in shellfish taken from both "open" and "closed" areas, based on bacteriological standards used at present in the United States. Field and laboratory studies have indicated that enteric viruses can survive for long periods in seawater and in shellfish. Recent advances in methodology have led to development of more rapid and less expensive methods for detection of a greater number of enteric viruses in shellfish.

Edible bivalve mollusks of the class Pelecypoda (oysters, mussels, clams) are characterized by two shell valves hinged together at one end and closed by a large adductor muscle to the valves near the other end. These commercially harvested marine organisms are one of the few shellfish which are commonly eaten raw. In view of a great protein shortage in the world, shellfish are assuming a greater nutritive importance. The total world catch of aquatic animals and plants was 656×10^8 metric tons in 1972 (*94*), of which 8% were shellfish. The United States presently leads the world in the catch of bivalve mollusks (*64*).

Bivalve mollusks are filter-feeding organisms, i.e., they sieve out suspended food particles from a current of water passing through the shell cavity. In its quest for food, an oyster may filter as much as 396 gal. (1500 liters) of water per day (*2*). The amount of water screened by quahaugs varies between 0.05 and 0.015 liters per hour per gram of meat (*81*). The feeding rates, however, depend upon such variable factors as salinity, temperature, particulate matter and availability of suitable food. If the water in which shellfish are feeding contains pathogenic bacteria or viruses, they may become entrapped on the mucous membranes and transferred to the digestive tract. Since the entire shellfish is usually consumed along with the gastrointestinal tract, shellfish may act as passive carriers of human pathogenic microorganisms. The effective control of enteric bacterial disease spread by shellfish has resulted from establishment of bacteriological standards using the coliform and fecal coliform index as the basis for a certification program. Lesser documented and studied are problems associated with transmission of viral disease by shellfish, because of the lack of sensitive techniques for their study. The purpose of this report is to review information on the occurrence of enteric viruses in shellfish and shellfish-growing areas and recent progress in methodology for their detection.

More than 100 types of viruses are present in human feces and may find their way into domestic sewage (*32*). These include the enteroviruses (polio, coxsackie, echo), reoviruses, adenoviruses, infectious hepatitis, and rotavirus. These viruses cause such illnesses as fever, paralysis, meningitis, respiratory disease, diarrhea, etc. Although in advanced countries water purification processes have reduced the number of waterborne disease outbreaks, viruses are still present even if coliforms are reduced to acceptable levels. Viruses may remain infectious for several weeks or longer after discharge into receiving waters, allowing sufficient time for them to be transported to coastal shellfish-growing areas. Once inside a shellfish, their survival appears to be further prolonged (*67*).

DISEASE OUTBREAKS ATTRIBUTED TO SHELLFISH

It has been well established that infectious hepatitis virus is transmitted by consumption of raw or inadequately cooked clams and oysters. The first documented outbreak of infectious hepatitis traced to consumption of raw oysters occurred in Sweden in 1955 (*54,82*). A number of well-documented outbreaks involving over 2,000 cases have been described since then. These outbreaks have been reviewed by Mosley (*75*) and more recently by Goldfield (*33*). In Table 1 are listed outbreaks which have been documented since the latter report.

Even in nonepidemic times a relationship between

consumption of shellfish and occurrence of hospitalized cases of infectious hepatitis is known to exist (*48*), indicating that the true incidence of shellfish-associated viral disease is probably greatly underestimated (*33*). In addition, residents of coastal states rather than inland states seem to have a higher occurrence of infectious hepatitis (*13*). The predominantly occult nature of other waterborne viruses has made it difficult to document their transmission by shellfish, but their occurrence in shellfish is well documented (*29,71,91*) and the potential for disease always exists.

Outbreaks of hepatitis have been attributed to the European oyster (*Ostrea edulis*) (*45,83*), eastern and Gulf coast oysters of North America (*Crassostrea virginica*) (*43*), northern quahaug clam (*Mercenaria mercenaria*) (*6,38*), the sand clam (*Mya arenaria*) (*36*), and mussels (*Mytilus edulis*) (*16*). Several recent outbreaks are of particular interest because of new light shed on survival of hepatitis viruses in shellfish.

Recently, a large outbreak of hepatitis A in Houston (270 persons with a history of consuming raw oysters at one of ten Houston restaurants) and a smaller outbreak in Calhoun, Georgia (15 of 225 persons attending one of two seafood dinners sponsored by a Calhoun social club) occurred simultaneously during the fall of 1973. These outbreaks were oyster-associated and oysters incriminated in each outbreak originated from Louisiana. Considered together, these cases represented the second largest recorded outbreak of oyster-associated hepatitis (*61*).

As a result of intensive investigation, a peak in oyster-associated cases of hepatitis A in Louisiana (37 of 137 reported cases) was shown to coincide with those in Houston and Calhoun. Several theories were put forth to account for the source of contamination in Louisiana oysters and all were discarded. The possibility which gained support from many investigators was that due to flooding in the Mississippi River during April and August 1973 the oyster beds experienced the greatest degree of freshwater intrusion and fecal pollution. Following discovery of a high coliform index, the Louisiana Health Department closed the area for shellfish harvesting in early May; this restriction was lifted on August 20, 1973, following receding of the Mississippi River and falling of coliform counts. The oysters implicated in the outbreaks, however, were not sold until September 16, which meant that at the height of flooding, oysters concentrated virus in their tissues to a level in excess of that existing in the surrounding waters. This indicated that hepatitis virus remained viable for 1-2 months after the receding of the Mississippi River water from the area and at levels sufficient to cause disease.

Dienstag et al. (*16*) incriminated incompletely cooked mussels (*Mytilus edulis*) as the vehicle of acute viral hepatitis in seven members of a family of 14. The role of mussels was confirmed epidemiologically as well as serologically by demonstration of rises in titers of serum antibodies to hepatitis A antigen as determined by immune adherence hemagglutination.

Koff et al. (*48*) conducted a prospective, controlled epidemiologic study to determine the modes of transmission of nonepidemic infectious hepatitis cases among patients in 10 Boston Hospitals. Many cases of infectious hepatitis thought to be sporadic fell into epidemic patterns. Ingestion of raw shellfish was significantly more frequent (34/185) in infectious hepatitis patients than in controls (10/185). Only four of the 74 patients who had eaten raw shellfish had a history of contact with jaundiced persons. Also, ingestion of steamed clams (13/104) was more common in patients than in matched controls (2/104); only 3 of the 13 patients had been exposed to jaundiced persons. When considered together, ingestion of steamed clams or raw shellfish was as frequent a potential exposure to hepatitis as was contact with jaundiced persons.

There may also be sporadic cases of infectious hepatitis attributed to ingestion of shellfish. Koff and Connelly (46) reported sporadic cases in New England and Stille et al. (*90*) in Germany. The study in Germany

TABLE 1. *Recent documented outbreaks of viral disease due to shellfish consumption.*

Year	Source	Place	Number of cases	Remarks	Reference no.
1963-1966	shellfish	Boston, Mass.	46	prospective study	*48*
1968-1971	shellfish	Frankfurt, Germany	34	—	*90*
1971	raw and steamed clams	Rhode Island	3	clams were dug on the Rhode Island shore	*28*
1973	oysters from Louisiana	Houston, Tex. Calhoun, Ga. Louisiana	263 15 ~37	—	*79*
1973	raw clams	Minneapolis, Minn.	1	suspected?	*37*
1976	incompletely cooked mussels	Victoria, Australia	7	confirmed serologically and epidemiologically	*16*
1976	cockles	Southampton and Chelmsford, U.K.	797	food poisoning due to small, round, virus-like particles	*3*

indicated that consumption of contaminated mollusks accounted for an estimated 19% of hepatitis type A cases in Frankfurt. The German cases were chiefly attributed to eating oysters and mussels from the Mediterranean lithoral, especially southern France and Italy. Even so-called sporadic cases may not be truly sporadic, because a common source in two or more cases is often not suspected and goes unnoticed.

In addition to infectious hepatitis, shellfish may also be responsible for outbreaks of nonbacterial gastroenteritis (*25,33,63,80*). In a series of 33 outbreaks, 797 people suffered from food poisoning during one year in Southampton and Chelmsford, U.K. (*3*). Epidemiologically, all of the outbreaks were traced to consumption of cockles. Cockles were gathered from waters known to be polluted by sewage and at a time when gastroenteritis was apparently widespread in a nearby area. Small, round, virus-like particles were detected by electron microscopy in a high proportion of fecal specimens from three of the outbreaks. On the basis of morphology, these particles were thought to be the agent of winter vomiting disease but proved serologically distinctive. Again, few investigations have been concerned with outbreaks of nonbacterial gastroenteritis because of the lack of methodology. Recent advances in detection of rotaviruses, a leading cause of diarrhea in infants and apparently adults (*27*), will undoubtedly make such studies more feasible in the future.

ISOLATION OF VIRUSES FROM SHELLFISH

Several studies on the occurrence of enteric viruses in oysters have been made, and their results are summarized in Table 2. The occurrence of enteric viruses has been reported in *M. edulis* (*24*), *C. virginica* (*20,87*), and *M. mercenaria* (*57*). Viruses have most commonly been isolated from heavily polluted areas closed to shellfish harvesting, but have also been detected in areas approved for shellfish harvesting (*29,35*). Echoviruses have been isolated from mussels obtained from an Italian market (*7*). Denis (*15*) reported recovery of coxsackie A viruses in market samples taken in France, and Fugate et al. (*29*) reported isolation of poliovirus in oysters imported into the United States from Japan. Thus it appears that enteroviruses may be present in marketed oysters even in developed countries and further investigation appears warranted.

Unfortunately, field studies in the past have been handicapped by the lack of dependable quantitative techniques for detection of enteric viruses and only limited numbers of shellfish could be sampled. Recent developments for detection of enteric viruses in large volumes of water and in shellfish should prove to be an impetus for studies to provide additional insight into occurrence of these pathogens in the marine environment.

Metcalf (*66,68a*) did the first field studies on occurrence of enteroviruses in shellfish and shellfish-harvesting waters. These studies resulted in the realization that examination of only the water in which the shellfish were growing could not be used as an indication of the presence of viruses in shellfish taken from the same water. This results from the fluctuating nature of water quality within the estuarine environment. The discontinuous patterns of polluted waters are influenced greatly by both tidal and freshwater inflow into the estuary. Thus viruses could be retained by shellfish even after sewage pollution was no longer evident in the area. Human enteric viruses were detected in shellfish beds 7 to 8 miles from the nearest sewage outfall (*70a*).

In more recent studies, Metcalf et al. (*71*) carried out parallel examinations of oysters and overlying seawater in Galveston Bay, Texas, for 3 months for the presence of fecal coliforms and enteroviruses. On seven occasions, water samples (25 to 105 gal.) were processed but no virus was isolated. In simultaneously collected oyster samples, however, polioviruses were found on two occasions. Some of the poliovirus type 1 isolates possessed virulent markers, which is significant from a public health viewpoint.

Earlier studies depended on use of "grab" samples or the placement of "gauze pads" in the water being tested for isolation of viruses and the direct assay of crude shellfish homogenates, but more recent developments in methodology allow for quantitative enumeration of enteric viruses in hundreds of gallons of marine water (*78*) and for the concentration of viruses from large pools of shellfish (*89*).

Using these new techniques, Goyal et al. (*35*) conducted a 1-year study on occurrence of enteroviruses in Galveston Bay, along the upper Texas coast. On several occasions, viruses were isolated from 100-gal. (378-liter) water samples and from oysters which were taken from areas open to commercial shellfish harvesting.

In a recent study of shellfish growing waters of Great South Bay, Long Island, New York, enteroviruses were recovered 37.5% of the time in water and oyster samples taken from areas open to shellfish harvesting (*90a*).

Mahoney et al. (*62*) found hepatitis B virus, the major cause of serum hepatitis, in hard-shelled clams harvested from a polluted bed on the coast of Maine, where a hospital was discharging sewage nearby. In closed system aquariums, these clams were able to filter and concentrate hepatitis B virus. No replication was demonstrated. Kater et al. (*44*) detected hepatitis B virus in one of four soft-shell clams taken from an estuary known to be bacteriologically polluted. Koff and Connelly (*46*), on the other hand, were unable to detect hepatitis B antigen in approved clam beds (both hard-and soft-shell) in Maine, Massachusetts and Rhode Island. Thus, hepatitis B virus would only appear to be a concern in those areas where untreated or poorly treated hospital wastes are a major source of pollution.

It has been assumed that shellfish act only as passive carriers of virus because no virus multiplication has ever

been demonstrated in them. Convincing proof was provided by Chang et al. (*11*) who inoculated proflavine-labeled, light-sensitive virus into quahaugs and examined them subsequently for light-resistant viral progeny. Failure to find such progeny was convincing proof that viruses are only concentrated and do not multiply in shellfish.

ADEQUACY OF BACTERIOLOGICAL STANDARDS

It is usually difficult and time-consuming to routinely determine the presence of pathogenic bacteria and viruses in wastewater and shellfish. The fecal coliform index is therefore used as an indicator of sanitary quality because they are normal inhabitants of the gastrointestinal tract of warmblooded animals and are excreted in feces in large numbers. Presence of fecal coliforms in a certain sample is then considered as evidence of recent fecal pollution of that sample. Standards exist in the United States for shellfish and shellfish-growing waters which state that "Most probable numbers of coliforms in water should not exceed 70 per 100 ml and no more than 10% of samples should exceed 230 coliforms per 100 ml" (*95*). Shellfish meat in itself is required to contain no more than 230 fecal coliforms per 100 g (*95*). On the basis of these standards, oyster beds have been classified into three separate categories, i.e., closed, approved and conditional.

Recent studies, however, indicate that these standards

TABLE 2. *Virus isolation studies on shellfish.*

Reference no.	Species of shellfish	Source	Virus type isolated	% positive	Remarks
66	*C. virginica*	Little and Great Bay, New Hampshire, known to be polluted with raw sewage	echo 9, coxsackie B4	6 of 10 pools of 10 oysters each	virus was found in oysters 4 miles from the source of pollution
77	mussels	Genoa Harbor, Italy	polio 3	—	—
7	mussels	Italian markets of Bari and Parma	echo 3, 9 and 13	50 samples of 3 mussels each	echo 13 isolated in monkey kidney; echo 3 and 9 in human amnion
68	*C. virginica*	New Hampshire estuaries (for 4 years)	polio 1,2,3; echo 9; reo 1; coxsackie B2, B3, B4	114 of 459 pools	
60	*Mer. mercenaria* *C. virginica*	Atlantic coast of U.S.	?	33% of clams, 55% of oysters	area was known to be polluted
44	*Mya arenaria*	area known to be bacteriologically polluted	hepatitis type B antigen	1 of 4	—
8	*Mytilus galloprovincialis*	Leghorn coast of Italy	echo 5,6,8,12; coxsackie A18	5 of 68 pools of 10 mussels each	grown or temporarily maintained in polluted water
70	*C. virginica*	estuarine inlet of North Atlantic ocean	1 enterovirus, 30 coliphages	59 of 130 pools positive either for coliphage or enterovirus	treated effluent discharged into the estuary
15	(*a*) oysters (*b*) mussels	Poitiers, France	coxsackie A18 predominant	(*a*) 7 of 70 pools (*b*) 2 of 10 pools	12 oysters or mussels in each pool
71	*C. virginica*	Galveston Bay, Texas	polio 1,2	28.6%	—
62	*Mer. mercenaria*	Atlantic coast of Maine	hepatitis type B antigen	100% samples positive from this site but none from 20 other sites	beds were polluted as a result of discharge of untreated sewage from a hospital
29	*C. virginica*	(*a*) Texas Gulf coast (*b*) Louisiana Gulf coast (*c*) frozen and shucked oyster imported from Japan	echo 1, polio 1 polio 3 polio 1	2 of 17 samples 1 of 24 samples 1 of 1 sample	oysters taken from approved waters were positive on occasion
74	*Mer. californiacus*	collected from stations remote from sewage outfalls; placed in cages and suspended at various depths below buoys located offshore near outfall diffusers	?	18 of 39 samples	—
91	*C. virginica*	Great Bay, New Hampshire	(*a*) coliphages (*b*) enterovirus	80 of 158 12 of 158	pools of 10-12 oysters each
39	*C. virginica, Mer. mercenaria, Mya arenaria*	Chesapeake or Chincoteague Bay, Maryland	chlamydia, rickettsia, mycoplasma and their phages		—
35	*C. virginica*	Galveston Bay, Texas	polio 2	—	isolated from beds open for shellfish harvesting
90a	clams and oysters	Great South Bay and Oyster Bay, Long Island, N.Y.	polio 1,2 echo 2,15,20,23 coxsackie B3	3 of 22 in open waters; 5 of 24 in closed waters	isolated from beds open for shellfish harvesting

cannot be relied upon because coliform bacteria are more sensitive to treatment processes or natural inactivation factors than some of the more resistant enteroviruses (*85*). Morris et al. (*74*) calculated that enteric viruses could survive in mussel tissue three to six times longer than coliform bacteria. In several studies, enteroviruses have been isolated from shellfish otherwise having a satisfactory coliform index. Thus, Fugate et al. (*29*), Vaughn and Landry (*90a*), and Goyal et al. (*35*) detected enteroviruses in oysters taken from approved waters. In the second largest outbreak of shellfish-associated hepatitis ever reported, the implicated shellfish were harvested from a bay where the water met national sanitation standards for shellfish growing and was certified for oyster harvesting (*61*).

Similarly, Metcalf et al. (*71*) described a study carried out in Galveston Bay in which poliovirus types 1 and 2 were isolated from two of seven pools of oysters tested; the density of fecal coliforms in these two instances was only 7 and 9 per 100 ml of water and 20 and 79 per 100 g of oyster meat, respectively.

In a 2-year study on occurrence of enteric viruses, bacteriological indicators and pathogens along the Texas coast, no significant statistical relationship was demonstrated between virus concentration, bacteriological indicators and pathogens, and a number of various physicochemical characteristics of the water, except pH (*34*). A strong correlation, however, was found between virus concentration and the presumptive total coliforms in sediments.

Considerable interest has recently been shown toward the possible use of coliphages as an indicator of enteric virus status of shellfish and seawater (*52,53*). Indicators are acceptable only if they constantly and accurately reflect the status of the organism concerned. Vaughn and Metcalf (*91*) described results of parallel examinations of sewage effluent, mud, shellfish and water for coliphages and enteroviruses in a polluted estuarine environment over 3 years. With the exception of mud samples, coliphages occurred more often than enteroviruses in all samples. They doubted the usefulness of the coliphage indicator system for viral presence in estuarine water and shellfish for the following reasons: (*a*) coliphages were consistently present in raw sewage samples which yielded inconsistent enterovirus isolations; (*b*) treated effluents were coliphage-positive but enterovirus-negative; (*c*) many (63%) enterovirus isolations occurred without any phage isolation; (*d*) replication of phages in estuarine water during the summer months (if proper bacterial hosts are present) makes them unsuitable for use as an indicator system; and (*e*) in a controlled experiment on comparative uptake of coliphages and enteroviruses by oysters, uptake of coliphage was found to be five- and 30-fold higher than that of enteroviruses (*70*). They argued that a determination of microbial acceptability of shellfish for human consumption should include assessment of the viral content (*70*).

Recent developments in virus concentration and rapid techniques for detection of viruses, such as radioimmunoassay, should make monitoring of shellfish for presence of viruses more feasible on a routine basis in the future and could serve as an adjunct to regular bacteriological monitoring programs.

ACCUMULATION OF VIRUS BY SHELLFISH

Numerous studies (*9,11,14,40,59,66,73*) have shown that several common species of shellfish rapidly accumulate virus if present in polluted waters. Uptake has been demonstrated in Mediterranean mussels, *Mytilus edulis* (*24*) and *M. galloprovincialis* (*72*); European oysters, *O. edulis* (*92*); eastern and Gulf coast oysters, *C. virginica* (*20,93*); hard clams or northern quahaug, *Mer. mercenaria* (*55*); New Zealand marine blue mussel, *M. edulis aoteanus* (*60*); Pacific oysters, *C. gigas* (*42*); Olympia oysters, *O. lurida* (*42*); and Manila clams, *Tapes japonica* (*42*). Maximum accumulation takes place within a few hours, and the level is maintained as long as sufficient virus is present in the surrounding water. It has been demonstrated that shellfish can concentrate virus in their tissues at a level much higher than the surrounding water (*38,57,73*). As the concentration of virus decreases in the water, the viral content in shellfish also starts decreasing. This process is called "depuration" or "purging" (*5,40,66*).

Table 3 shows the results of various experimental studies done on accumulation and subsequent depuration of virus by different species of shellfish. In these experimental systems, shellfish are exposed to known concentrations of virus for a period in aquariums with static or flow-through water supplies. Most workers have demonstrated that maximum concentration of virus occurs in the digestive system of shellfish (*20,54,56,57,63, 66*). Transport and uptake rates of virus by shellfish are dependent on a number of factors including turbidity and particulate matter. Hoff and Becker (*42*) reported that the uptake rate is dependent on the state in which virus exists in natural waters, i.e., whether it is present as free particles or attached to larger particulate matter. They found that the accumulation factor for crude poliovirus was from 10- to 900-fold, whereas for clarified virus preparations, it was only 0.4- to 3.6-fold. Uptake rate is higher in a flow-through than in a static system (*59*). DiGirolamo et al. (*20*) demonstrated interspecies differences in the uptake rate when they found that more than 86% of poliovirus present in seawater was concentrated within 12 h by Olympia oysters (*O. lurida*); for the same degree of concentration, Pacific oystes (*C. gigas*) took 48 h. The effect of turbidity on accumulation was demonstrated by Hamblett et al. (*38*), who showed that the accumulation factor of poliovirus in *C. virginica* was 9.5- to 18.1-fold under conditions of low turbidity (8-24 ppm), whereas it was only 4.2- to 4.6-fold in high turbidity (54-80 ppm). Metcalf and Stiles (*68*) found that oysters in polluted water at temperatures below 7 C did not accumulate virus. At temperatures above 7 C,

pumping and feeding activity are initiated and viruses are accumulated.

The Florida conch and sea hare (*Aplysia dactylomela*) also accumulate viruses when placed for short periods in water contaminated with poliovirus (*86*). Conches, a mollusk commercially harvested for food in the Caribbean, accumulate large amounts of virus in their digestive gland and intestine. Viruses could also be detected in the hemolymph. Virus was found to persist at least 8 days after a brief 2-h exposure to contaminated seawater.

Besides mollusks, crustaceans also accumulate virus. DiGirolamo et al. (*22*) showed that Pacific coast shore crabs (*Pachygrapsus* sp. and *Hemigrapsus* sp.) could accumulate high titers of poliovirus when placed in artificially contaminated seawater. When allowed to feed on virus-contaminated mussels (*Mytilus californianus*), the crabs accumulated 74-94% of the virus present in shellfish.

The same authors (*23*) demonstrated that the edible west coast crabs — the Dungeness crabs (*Cancer magister*) and "Red Rock" crabs (*C. antennarius*) — could accumulate coliphage T4 in high titers if residing in artificially contaminated seawater. They also showed that virus in crabs withstood boiling, which is a normal method of preparing crabs before consumption. The survival rate varied between 2.5 and 20%, depending on

TABLE 3. *Virus accumulation and depuration studies on shellfish*

Reference no.	Virus type	Species of shellfish	Uptake		Virus accumulation factor	Depuration	
			System used	Time		System used	Time
14	polio 2	mussel (*Mer. edulis*)	—	—	—	intermittent flow	48 h
40	polio 3 (Saukett)	European oyster (*O. edulis*)	circulating water	2 h; maintained for 6 days at 23 C	1-fold	flow-through	> 100 h
66	polio 1 (Chat), coxsackie B3 (Nancy)	Eastern oyster (*C. virginica*)	circulating water	72-96 h; maintained for 28 days at 5 C	1-fold	static	—
56	polio 1 (LSc)	hard clam (*Mer. mercenaria*)	static	4-48 h	30-fold in 6 days	—	—
57	polio 1 (LSc)	*Mer. mercenaria*	flow-through	—	3- to 9-fold	—	—
43	phage	*C. gigas*	—	—	10-fold	—	—
73	polio 1 (LSc)	*C. virginica*	flow-through	1 h	10- to 27-fold	flowing	95% in 8 h; 99.9% in 24 h; 100% in 48 h
79	staphylococcus phage	*C. virginica, Mer. mercenaria*	—	2-3 h	—	—	incomplete in 100 h
59	polio 1 (LSc-2ab)	hard clam	—	4 h; maintained for 3 days	—	static flowing	2-4 days 2-4 days
58	polio 1 (LSc-2ab), polio 3 (Leon-12ab), coxsackie B4 (POW)	hard clam	running water	94% in 73 h	—	flowing	72-96 h
26	polio 3 (Saukett), coxsackie A8 (NHI-A805)	blue rock mussel (*Myt. edulis aoteanus*)	—	18-36 h	—	—	—
38	polio 1 (LSc)	*C. virginica*	flowing	24 h	9.5- to 18.1-fold at low turbidity; 4.2-to 4.6-fold at high turbidity	—	48 h
83	polio 1 (LSc)	hard clam	—	—	10- to 100-fold	—	48-72 h
42	polio 1 (LSc)	*O. lurida, C. gigas, T. japonica*	flowing	—	10- to 900-fold for crude virus; 0.4- to 3.6-fold for clarified	flowing	48 h for clarified virus; 72-96 h for crude virus
9	coliphage S-13	*Mer. mercenaria*	flowing	—	2- to 1100-fold	—	several days to weeks if low levels of virus are accumulated
22	polio 1 (LSc)	west coast shore crab (Pachygrapsus sp., *Hemigrapsus nudus*)	—	25% in 12 h 63% in 48 h	—	—	—
23	coliphage T4	west coast crabs (*C. magister, C. antennarius*)	—	58% in 24 h; 73% in 48 h	—	—	—
69	coxsackie B3	*C. virginica*	—	—	—	—	at 9-13 C, 7 days; at 21-22 C, 3 days
20	polio 1 (LSc)	*C. gigas*	static	46% in 12 h; 88% in 48 h	—	static	21% remained after 120 h
20	polio 1 (LSc)	Olympia oyster (*O. lurida*)	—	86% in 12 h; 94% in 24 h	—	static flowing	16% remained after 120 h 24% remained after 24 h; 1% remained after 72 h

the time of boiling. After boiling for 10 and 15 min, the virus survival was 15 and 10%, respectively. It was also postulated that since crabs are not sedentary like bivalve mollusks, they may enter a sewage polluted area either in search of food or with tidal changes and may come back to clean water, where they subsequently act as vectors of viral disease (*23*).

MECHANISMS OF ACCUMULATION

Using aquariums and static seawater, Metcalf and Stiles (*67*) showed that most viruses were accumulated in the digestive diverticulum of American oysters. Using northern quahaugs and poliovirus type 1, Liu et al. (*56*) confirmed this observation and were also able to detect virus in the stomach, hemolymph and mantle cavity fluids. The digestive tract, though, is the major site of virus accumulation. Up to 15% of the total virus accumulated, depending on the species of shellfish, may diffuse into the body of the oyster within a 48-h period (*20*). This finding is important in considering depuration processes, since depuration times used must be long enough to permit the shellfish to cleanse themselves not only of virus in the digestive area but of residual virus in the body as well.

Results of binding-release studies have shown that viruses become attached to the secreted mucus and then are ingested by shellfish during feeding (*21*). Attachment of virus to mucus was due to ionic binding and involved the binding of viral particles to sulfate radicals on the mucopolysaccharide moiety of shellfish mucus.

DEPURATION

Depuration, or purification, is a mechanical process effected by the physiological functioning of shellfish in clean water (*4*). Natural purification is also called relaying. When shellfish are relayed on the seabeds in areas where seawater is unpolluted, they unload their fecal contaminants.

Artificial purification or cleansing of shellfish from polluted areas is accomplished by placing them in tanks or basins which are filled with sterilized water. Sterilization of water may be done by filtration, chlorination or ozonation. Shellfish, however, are extremely sensitive to chlorine; even small amounts cause slowing down or complete inhibition of shellfish physiological processes.

The factors affecting the process of purification (depuration) are: (*a*) Maximum depuration is possible only when environmental conditions are such that the feeding activity of shellfish is optimized. (*b*) Liu et al. (*58*) showed that the reduction of salinity to 75% of the original did not have any significant effect on the depuration process, whereas a further reduction in salinity to 50-60% of the original resulted in stoppage of depuration. (*c*) Efficiency of depuration is a function of temperature (*58,59,69*). At 5 C, 72-96 h were required for depuration but at 20 C depuration was complete in 24-72 h (*58*). (*d*) Depuration occurs more rapidly in a free-flow system than in a static one (*20,55,58,73*). DiGirolamo et al. (*20*) observed that after 120 h of depuration in a stationary system, *C. gigas* still retained 21% of the virus accumulated, while *O. lurida* retained 16%. Under free-flow conditions, however, depuration was rapid and efficient. Only 1% of the accumulated virus remained in the oysters after 72 h.

The observed unloading of contaminants by shellfish upon being placed in a clean environment has been exploited commercially; oysters harvested from polluted areas are put in fresh, uncontaminated water to allow shellfish to purge themselves of viral contaminants (*95*). This self-cleaning mechanism appears to be uniformly efficient, but considerable study is still required to define the optimum conditions. The problem encountered in commercial purification is the lack of a source of clean water. For economic reasons, the source of clean water should be close to the polluted oyster-harvesting areas, which is not always possible. Even if a source of clean water is found, it is susceptible to sporadic contamination by overland runoff and by wild and aquatic birds. The remedy may lie in the use of sterilized water. Sterilization by chlorination is feasible but is not optimal because of the reasons mentioned above. Chlorination followed by dechlorination may be a practical means toward this goal.

Use of chlorination has also been advocated to disinfect shellfish. However, it was shown by *Galtsoff* (*30*) that the shell movement and the flow of water through the gills was inhibited in the presence of chlorine. Thus, chlorination may cause only external disinfection. Also, chlorine is not a reliable disinfectant for certain viruses (*12,84*) at concentrations often used. Hedstrom and Lyke (*40*) observed inactivation of virus in oysters by 0.7-20 ppm of chlorine, indicating that the viruses were retained in the tissues in such a way that no disinfection was possible.

AQUACULTURE

Some imaginative approaches have been studied for use of sewage effluents as a nutrient source for production of marine phytoplankton to be used for the aquaculture of marine animals on a commercial scale (*65*). However, use of sewage effluents in the system indicates the possibility of potential public health risks in the form of human virus transmission. To examine the potential of such a risk, Metcalf (*65*) did a study of enteroviruses in an aquaculture system developed at the Woods Hole Oceanographic Institute and raised serious questions about its suitability either for tertiary treatment of sewage or culture of shellfish. Despite a 99% reduction in virus titer, the system was judged unacceptable because as many as 1×10^4 viruses survived inactivation and appeared daily in discharge waters. Physiologically active juvenile oysters grown in sewage-seawater mixtures were shown to accumulate enteric viruses more or less routinely.

In laboratory experiments it was demonstrated that in the hypothetical aquaculture system under study viruses

could be transmitted through the food chain as follows: shellfish → pseudofeces → sandworms → detrital feeding fin fish. It was also found that lobsters fed virus-contaminated mussels accumulated virus in their alimentary tract and hepatopancreatic tissues. These results indicated that almost complete elimination of virus from sewage effluents would be necessary for their use in the aquaculture of shellfish, if the shellfish are to be marketed for human consumption.

SURVIVAL OF VIRUSES IN MARINE WATER AND SHELLFISH

Field and laboratory studies have indicated that enteric viruses can survive from a few days to over 130 days in marine water. Survival is dependent on a number of factors which include temperature, salinity, type of virus, bacterial antagonism, suspended solids, and pollution. Survival of viruses becomes greatly prolonged once they become associated with sediments (*88*), and their concentration may be many fold greater in sediments than in the overlaying water (*31*). Generally, viruses survive longer at lower temperature, at low salinity, and in sewage-polluted waters. Several good reviews on virus survival in marine water are available (*1*).

Metcalf and Stiles (*67*) have investigated the period of viral retention in the natural environment. The American oyster, poliovirus type 1, a coxsackievirus and an echovirus were used. The experimentally polluted oysters were maintained in a New Hampshire bay for 3 months and samples of shellfish were taken periodically for assay. No reduction of virus titer occurred for 30 days during the winter when the water temperature was about 1 C. As the water temperature rose above 8 C, viruses in the shellfish were rapidly reduced. It was postulated that the shellfish remained dormant until the water temperature reached a critical level. At that point, the oysters began to feed and through depurative activity freed themselves of virus. Temperatures of 4 C or less favored virus retention. Liu et al. (*55*) suggested that this may serve as an explanation as to why most hepatitis outbreaks occur during the winter and early spring.

Several studies have shown that viruses can survive in shellfish for long periods. Under conditions of dry storage at 5 C, coxsackie and polioviruses were found viable even after 28 days (*66*). Depending on storage temperature, poliovirus survived in refrigerated oysters for 30 to 90 days (*18*). In chilled Olympia oysters, poliovirus was reduced by only 10% after 5 days of storage and by 60% after 15 days. Even after 30 days of storage at 5 C, 13% of virus remained viable although the oysters were badly decomposed. In frozen Pacific oysters held at −17.5 C, 91% of added poliovirus was viable after 2 weeks of storage, 40% at 6 weeks, and 10% after 12 weeks. The tendency of the virus to aggregate and its incorporation into shellfish mucus by ionic binding were speculated to be the means by which the virus was able to survive in chilled oysters. It was further shown that not only fresh but also cooked oysters could serve as vectors of viral disease. Between 7 and 10% of poliovirus was still viable after baking, frying, stewing and steaming (*18*).

DiGirolamo and Daley (*17*) reported that 29 and 40% of coliphage T4 was recoverable even after 120 days at 8 C from unprocessed and processed (boiled) west coast crabs *C. magister* and *C. antennarius*, respectively. When kept at −20 C for 30 days, 35 and 17% of virus survived in unprocessed and processed crabs, respectively. Canzonier (*9*) demonstrated that 50-90% of the accumulated coliphage S-13 in oysters was retained during a 2-month period of observation in a free-flowing aquarium, whereas bacteria were eliminated rapidly within 24 h. Metcalf and Stiles (*68*) stored contaminated oysters at 5 C in estuarine water and found that coxsackievirus B3 and echovirus 5 survived for 4 months under these conditions.

Koff and Sear (*47*) reported that the internal temperature of soft-shell clams (*M. arenaria*) did not reach 100 C until 4-6 min of boiling/steaming, whereas their shells were open in only 60 sec. Clams are normally consumed as soon as the shells are opened, not because of hygienic reasons but because of palatability. DiGirolamo et al. (*18*) showed that 7-13% of added poliovirus survived in oysters even after four commonly used processing methods, i.e., steaming, frying, baking and stewing. It was proved by heat penetration studies that the internal temperature was not sufficient to inactivate all virus present. An outbreak of infectious hepatitis attributed to steamed clams was reported from Massachusetts in 1972 (*47*). Mosley and Galainbos (*76*) have advocated the use of boiling temperature for at least 20 min.

Use of ionizing radiation has been proposed recently to eliminate microorganisms from foods. In a preliminary study, DiGirolamo et al. (*19*) demonstrated that poliovirus 1 in oysters was able to survive relatively high doses of gamma radiation. The rate of survival varied between 7.3 and 87% depending on the dose of radiation and the nature of the sample (whether whole or shucked). To inactivate more than 90% of the virus present, a dose of 400 krad was required, which also resulted in undesirable organoleptic changes.

METHODS FOR VIRUS DETECTION IN SHELLFISH

A number of methods for recovering enteric viruses from natural or laboratory-contaminated oysters, clams and mussels have been described (*38,41,66,73*). All of these methods begin with homogenization of the whole shucked shellfish or part of it. In earlier methods this homogenate, with or without addition of diluent, was centrifuged to remove solids. The supernatant fluid was then assayed directly or further clarified by treatment with fluorocarbon extraction (*73*) or ethyl ether extraction (*38,66,73*). These methods have several shortcomings, which include: (*a*) lack of significant information on the degree of efficiency of the methods; (*b*) too large a volume to feasibly assay when attempting

to detect small numbers of viruses; and (*c*) toxicity to the animal cells used to assay the virus. Cytotoxic components present in the homogenate often destroy the cell culture before viruses can be quantified. This appears to be especially true with homogenates obtained from clams.

Metcalf et al. (*65*) homogenized pools of 10 oysters followed by low-speed centrifugation. The supernatant fluid was centrifuged at high speed (160,000 × *g*) for 2 h and the resulting pellet was resuspended in a small volume of Hank's balanced salt solution. Bendinelli and Ruschi (*8*) homogenized one part of mussels with an equal amount of ethyl ether and let it stand at 4 C for 18 h, followed by low-speed centrifugation. The lower aqueous phase was again centrifuged at 104,000 × *g* for 2 h, and the pellet resuspended in 3 ml of Earle's solution.

Metcalf and Stiles (*66*) blended pools of 10 oysters for 90 sec; the mixture was centrifuged at low speed, and the supernatant fluid was mixed with an equal volume of ethyl ether. This mixture was stored overnight at 5 C and then centrifuged. The resulting supernatant fluid was again centrifuged at 125,000 rpm for 2.5 h and the pellet was resuspended in 4 ml of Hank's balanced salt solution.

Morris et al. (*74*) homogenized the digestive glands (∼ 20 g) from 2 kg of mussels. This was followed by washing with pH 5.5 buffer at low salt concentration to remove substances toxic to the cells. After low-speed centrifugation, the sediment was adjusted to pH 3.7 with 0.7% saline buffer to elute virus. The supernatant fluid recovered after recentrifugation was mixed with fetal calf serum (FCS) to make a final concentration of 10% FCS. The efficiency of recovery with poliovirus type 3 was 32-38%.

Konowalchuk and Spiers (*49*) found that the toxicity associated with the shellfish extracts became an important factor in isolation of small numbers of enteroviruses. They found that even when oyster extract were diluted to a point where no visible cytotoxicity occurred, plaque counts were reduced to 50% of the controls. Much higher toxicities were found for clams and mussels. Because of the cytotoxicity, the maximum volume of extract that could be assayed at one time was 1.1 ml for oysters, 0.015 ml for mussels, and 0.006 ml for clams. Acid precipitation and ether treatment of shellfish extracts were found to reduce, but not eliminate, problems with cytotoxicity. In addition, acid precipitation was found to be effective only with freshly homogenized shellfish, and at least 50% of the virus remained in the acid precipitate.

Kostenbader and Cliver (*50,51*) described a procedure for detection of enteric viruses from shellfish and other foods; the procedure involved clarification of the shellfish homogenate by addition of a polyelectrolyte (Cat-Floc). This caused flocculation of oyster solids which were then removed by filtration. The volume of the filtrate was then reduced by either ultracentrifugation or ultrafiltration before assay. When ∼ 70 PFU (plaque-forming units) of poliovirus type 1 were added to oysters, efficiencies of 86-96% were achieved (*50*). However, problems with cytotoxicity still remained. Severe cytotoxicity was encountered with extracts of clams (*51*). These problems necessitated additional treatments to reduce these effects and blind passages in the tissue culture assay system.

Sobsey et al. (*89*) reported a method which eliminated the problems of dealing with large volumes of extracts and of cytotoxicity. They found that by controlling the pH and salt concentration of the initial oyster homogenate, viruses could be easily and rapidly separated from the homogenized oyster tissues by a procedure involving two basic steps. In the first step viruses are adsorbed to the oyster solids at pH 5.5 and a low salt concentration. When this homogenate is centrifuged at low speed, nearly all of the viruses sediment with the oyster solids so that the first supernatant fluid can be discarded. The adsorbed viruses are then eluted from the sedimented oyster solids by resuspending the solids in pH 3.5 glycine-buffered saline, and the virus-free solids are then removed by low-speed centrifugation. The virus-containing supernatant fluid is relatively low in turbidity and dissolved and colloidal organic matter and is easily filtered through a membrane filter to remove bacteria, molds and other particulate matter without removing viruses.

The viruses in the resulting filtrate are efficiently concentrated to a volume of a few milliliters by ultrafiltration. Because the final concentrate is of small volume, the viruses obtained from the entire initial oyster pool can be inoculated into a small number of cell cultures.

When this procedure was tested with relatively small amounts (∼ 30 PFU) of four different enteroviruses in experimentally contaminated pools of three oysters (20-65 g per pool), virus recovery efficiency averaged about 63%.

Sobsey et al. (*88a*) recently reported a modification of the earlier method (*10*) in which elution from solids was accomplished with glycine-saline at pH 7.5 rather than 3.5 and concentration of viruses by acid precipitation. When this method was tested with oysters and clams experimentally contaminated with polioviruses, reoviruses and adenoviruses, recovery efficiencies averaged from 25-50% depending on the type of virus.

ACKNOWLEDGMENTS

This work is a result of a research program sponsored in part by the Texas A&M University Sea Grant College Program, supported by the National Oceanic and Atmospheric Administration Office of Sea Grant, Department of Commerce, under grant #04-6-158-44108.

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News and Events

Jan Gauger Richards has accepted the position of Assistant Executive Secretary of the International Association of Milk, Food, and Environmental Sanitarians (IAMFES) and Associate Editor of the Journal of Food Protection, announced Earl O. Wright, Executive Secretary for IAMFES.

Mrs. Richards, a native of Ames, received her B.S. degree from Iowa State University in 1977 with majors in journalism and home economics. She is a member of Phi Upsilon Omicron, professional honorary, and Sigma Delta Chi, professional society. She was communications director for the Dairy Council of Greater Kansas City, Kansas City, Mo., before coming to IAMFES.

Mrs. Richards' responsibilities will include production supervision of the Journal of Food Protection, work with state and national affiliate groups of IAMFES, coordination of workshops and shortcourses sponsored by the Association, and organization of student affiliate groups throughout the United States.

Affiliate Meetings

Sept. 14 and 15. The Minnesota Sanitarians' conference will be held at the Sheraton Inn, Northwest, Brooklyn Park, Minnesota.

Sept. 13 and 14. The Wisconsin Sanitarians will hold their annual conference at the Holiday Inn, Fond Du Loc.

Sept. 20-22. The New York Sanitarians Association will hold their annual conference at Stevensville Country Club, Swan Lake, N.Y.

Sept. 26, 27 and 28. The Indiana Sanitarians' annual conference will be held at Merrillville, Indiana.

Crumbine Award to Arlington Co., Va. Health Bureau

The Environmental Health Bureau of Arlington County, Virginia, which is responsible for ensuring food service sanitation for 170,000 county residents in an area of nearly 30 square miles directly across the Potomac from the nation's capital, has been chosen the winner of the 1978 Samuel J. Crumbine Consumer Protection Award.

This year's award was given for outstanding achievement in a total food and beverage sanitation program embracing such specific elements as effective planning and management, innovative evaluation methods, excellent information and education activities, and sustained program growth and improvement.

The Crumbine Award given to the Arlington County agency consists of a bronze medal and an engraved plate mounted on a walnut plaque. In addition, a bronze medallion is presented to individual agency officials who are directly responsible for the winning program.

Coming Events

University of Maryland dairy products conferences and ice cream short course for 1978-79 are as follows:

November 8, 1978. University of Maryland 34th Annual Dairy Technology Conference, Center of Adult Education, University of Maryland, College Park, Maryland 20742.

March 19-28, 1979. University of Maryland 28th Annual Ice Cream Short Course, Department of Dairy Science, Animal Sciences Center, University of Maryland, College Park, Maryland 20742.

March 29, 1979. University of Maryland 28th Annual Ice Cream Conference, Center of Adult Education, University of Maryland, College Park, Maryland 20742.

For information contact Dr. Joseph F. Mattick, Department of Dairy Science, Animal Sciences Center, University of Maryland, College Park, Maryland 20742. (301) 454-3928.

The National Sanitation Foundation has planned a series of special regional workshops for foodservice equipment designers and manufacturers.

Sept. 12, 1978	Boston, MA
Sept. 14, 1978	Philadelphia, PA
Sept. 25, 1978	Chicago, IL
Oct. 16, 1978	Los Angeles, CA
Oct. 20, 1978	St. Louis, MO
Dec. 5, 1978	Atlanta, GA

The University of Wisconsin-Eau Claire will offer Community Hygiene I and II during the 1978-79 school year. The first semester will be held at Wisconsin Center, 702 Langdon St., Madison, Wis., starting Sept. 1, 1978. The second semester will be held there, also, and will start Jan. 19, 1978. For more information write University of Wisconsin, Eau Claire, Wis.

Holders of 3-A Symbol Council Authorizations on August 20, 1978

Questions or statements concerning any of the holders of authorizations listed below, or the equipment fabricated, should be addressed to Earl O. Wright, Sec'y.-Treas., P.O. Box 701, Ames, Iowa 50010.

01-06 Storage Tanks for Milk and Milk Products

28 Cherry-Burrell Corporation (10/ 3/56)
575 E. Mill St.
Little Falls, New York 13365

102 Chester-Jensen Company, Inc. (6/ 6/58)
5th & Tilgham Streets
Chester, Pennsylvania 19013

2 CREPACO, Inc. (5/ 1/56)
100 C.P. Avenue
Lake Mills, Wisconsin 53551

117 Dairy Craft, Inc. (10/28/59)
St. Cloud Industrial Park
St. Cloud, Minnesota 56301

76 Damrow Company (10/31/57)
196 Western Avenue
Fond du Lac, Wisconsin 54935

115 DeLaval Company, Ltd. (9/28 59)
113 Park Street South
Peterborough, Ontario, Canada
(not available in USA)

109 Girton Manufacturing Company (9/30/58)
State Street
Millville, Pennsylvania 17846

114 C. E. Howard Corporation (9/21/59)
P.O. Box 2507
City of Industry, California 91746

127 Paul Mueller Company (6/29/60)
P.O. Box 828
Springfield, Missouri 65801

31 Walker Stainless Equipment Co. (10/ 4/56)
Elroy, Wisconsin 53929

02-06 Pumps for Milk and Milk Products

214R Ben H. Anderson Manufacturers (5/20/70)
Morrisonville, Wisconsin 53571

212R Babson Bros. Co. (2/20/70)
2100 S. York Rd.
Oak Brook, Illinois 60621

29R Cherry-Burrell Corporation (10/ 3/56)
2400 Sixth St., Southwest
Cedar Rapids, Iowa 52406

63R CREPACO, Inc. (4/29/57)
100 CP Avenue
Lake Mills, Wisconsin 53551

205R Dairy Equipment Company (5/22/69)
1919 South Stoughton Road
Madison, Wisconsin 53716

65R G & H Products, Inc. (5/22/57)
5718 52nd Street
Kenosha, Wisconsin 53140

145R ITT Jabsco, Incorporated (11/20/63)
1485 Dale Way
Costa Mesa, California 92626

26R Ladish Co., Tri-Clover Division (9/29/56)
9201 Wilmot Road
Kenosha, Wisconsin 53140

303 E. R. Mitchell Co., Inc. (3/ 8/78)
576 Haddon Ave.
Collingswood, NJ 08108

236 Megator Corporation (5/ 2/72)
125 Gamma Drive
Pittsburgh, Pennsylvania 15238

241 Puriti S. A. (9/12/72)
Alfredo Noble #39, Industrial Pte. de Vigas
Tlalnepantla, Mexico
(not available in USA)

148 Robbins & Myers, Inc. (4/22/64)
Moyno Pump Division
1345 Lagonda Avenue
Springfield, Ohio 45501

306 Stamp Corp. (5/ 2/78)
1309 Culmen St.
Madison, WI 53713

72R L. C. Thomsen & Sons, Inc. (8/15/57)
1303 43rd Street
Kenosha, Wisconsin 53140

219 Tri-Canada Cherry-Burrell Ltd. (2/15/71)
6500 Northwest Drive
Mississauga, Ontario, Canada
(not available in USA)

175R Universal Milking Machine Div. (10/26/65)
National Cooperatives, Inc.
First Avenue at College
Albert Lea, Minnesota 56007

52R Viking Pump Div. (12/31/56)
Houdaille Industries, Inc.
406 State Street
Cedar Falls, Iowa 50613

5R Waukesha Foundry Company (7/ 6/56)
Waukesha, Wisconsin 53186

04-03 Homogenizers and High Pressure Pumps of the Plunger Type

247 Bran and Lubbe, Inc. (4/14/73)
1241 Rand Rd.
Des, Plaines, IL 60016

87 Cherry-Burrell Company (12/20/57)
2400 Sixth Street, Southwest
Cedar Rapids, Iowa 52404

37 CREPACO, Inc. (10/19/56)
100 CP Avenue
Lake Mills, Wisconsin 53538

75 Gaulin, Inc. (9/26/57)
44 Garden Street
Everett, Massachusetts 02149

237 Graco Inc. (6/ 3/72)
P.O. Box 1441
Minneapolis, Minnesota 55440

309 General Dairy Equipment (7/19/78)
434 Stinson Boulevard
Minneapolis, Minnesota 55413

256 Hercules, Inc. (1/23/74)
2285 University Avenue
St. Paul, Minnesota 55114

282 Knudsen Corporation (11/ 8/76)
715 N. Divisadero Street
Visalia, California 93277

05-13 Stainless Steel Automotive Milk Transportation Tanks for Bulk Delivery and/or Farm Pick-up Service

131R Almont Welding Works, Inc. (9/ 3/60)
4091 Van Dyke Road
Almont, Michigan 48003

70R Brenner Tank, Inc. (8/ 5/57)
450 Arlington,
Fond du Lac, Wisconsin 54935

40 Butler Manufacturing Co. (10/20/56)
900 Sixth Ave., Southeast
Minneapolis, Minnesota 55114

66 Dairy Equipment Company (5/29/57)
1919 South Stoughton Road
Madison, Wisconsin 53716

45 The Heil Company (10/26/56)
3000 W. Montana Street
Milwaukee, Wisconsin 53235

297 Indiana Tank Co., Inc. (8/29/77)
P. O. Box 366
Simmitt, Indiana 46070

305 Light Industrial Design Co. (3/23/78)
3726 Halverstick Rd.
Sumas, WA 98295

201 Paul Krohnert Mfg., Ltd. (4/ 1/68)
811 Steeles Avenue
Milton, Ontario, Canada L9T 2Y3
(not available in USA)

85 Polar Manufacturing Company (12/20/57)
Holdingford, Minnesota 56340

121 Technova Inc. Gosselin Division (12/ 9/59)
1450 Hebert c.p. 758
Drummondville, Quebec, Canada
(not available in USA)

189 A. & L. Tougas, Ltee (10/ 3/66)
1 Tougas St.
Iberville, Quebec, Canada
(not available in USA)

47 Trailmobile, Div. of Pullman, Inc. (11/ 2/56)
701 East 16th Avenue
North Kansas City, Missouri 64116

25 Walker Stainless Equipment Co. (9/28/56)
New Lisbon, Wisconsin 53950

08-17 Fittings Used on Milk and Milk Products Equipment and Used on Sanitary Lines Conducting Milk and Milk Products

291 Accurate Metering Systems, Inc. (6/22/77)
1731 Carmen Drive
Elk Grove Village, IL 60007

79R Alloy Products Corporation (11/23/57)
1045 Perkins Avenue
Waukesha, Wisconsin 53186

245 Babson Brothers Company (2/12/73)
2100 South York Road
Oak Brook, Illinois 60521

284 Bristol Engineering Company (11/18/76)
210 Beaver Street
Yorkville, Illinois 60560

301 Brown Equip. Co., Inc. (12/ 6/77)
9955-9¼ Ave.
Hanford, California 93230

82R Cherry-Burrell Company (12/11/57)
2400 Sixth Street, Southwest
Cedar Rapids, Iowa 52406

260 CREPACO, Inc. (5/22/74)
100 CP Avenue
Lake Mills, Wisconsin 53551

304 EGMO Ltd-Isreal (3/16/78)
(Martin Silver P.E.)
406 Kinderamack Rd.
River Edge, NJ 07661

271 The Foxboro Company (3/ 8/76)
Neponset Street
Foxboro, Massachusetts 02035

67R G & H Products, Inc. (6/10/57)
5718 52nd Street,
Kenosha, Wisconsin 53140

203R ITT-Grinnell Company, Inc. (11/ 7/68)
DIA-FLO Div
33 Centerville Rd.
Lancaster, Pennsylvania 17603

34R Ladish Co., Tri-Clover Division (10/15/56)
9201 Wilmot Road
Kenosha, Wisconsin 53140

287 Koltek OY (1/14/77)
Kotinummentieiz
SF-00700 Helsinki 70
Finland
(not available in USA)

239 LUMACO (6/30/72)
Box 688,
Teaneck, New Jersey 07666

200R Paul Mueller Co. (3/ 5/68)
P.O. Box 828
Springfield, Missouri 65801

295 Precision Stainless Products (8/11/77)
5636 Shull St.
Bell Gardens, CA 90201

242 Puriti, S. A. (9/12/72)
Alfredo Nobel #39 Industrial Pte. de Vigas
Tlalnepantla, Mexico
(not available in USA)

149R Q Controls (5/18/64)
Occidental, California 95465

73R L. C. Thomsen & Sons, Inc. (8/31/57)
1303 43rd Street
Kenosha, Wisconsin 53140

300 Superior Stainless, Inc. (11/22/77)
211 Sugar Creek Rd.
P.O. Box 622
Delavan, Wisconsin 53115

191R Tri-Canada Cherry-Burrell, Ltd. (11/23/66)
6500 Northwest Drive
Mississauga, Ontario, Canda L4V 1K4
(not available in USA)

250 Universal Milking Machine (6/11/73)
Div. of Universal Cooperatives
407 First Ave, So.
Albert lea, Minnesota 56007

278 Valex Products (8/30/76)
9421 Winnetka
Chatsworth, California 91311

86R Waukesha Specialty Company, Inc. (12/20/57)
Darien, Wisconsin 53114

Inlet and Outlet Leak Protector Plug Valves for Batch Pasteurizers

09-07 Instrument Fittings and Connections Used on Milk and Milk Products Equipment

269 Babson Bros. Company (1/23/76)
2100 South York Road
Oak Brook, Illinois 60521

206 The Foxboro Company (8/11/69)
Neponset Avenue
Foxboro, Massachusetts 02035

285 Tank Mate Company (12/ 7/76)
1815 Eleanor
St. Paul, Minnesota 55116

32 Taylor Instrument Process Control (10/ 4/56)
Div. Sybron Corporation
95 Ames Street
Rochester, New York 14601

246 United Electric Controls (3/24/73)
85 School Street
Watertown, Massachusetts 02172

10-00 Milk and Milk Products Filters Using Disposable Filter Media, As Amended

35 Ladish Co., Tri-Clover Division (10/15/56)
9201 Wilmot Road
Kenosha, Wisconsin 53140

296 L. C. Thomsen & Sons, Inc. (8/15/77)
1303 43rd St.
Kenosha, Wisconcin 53140

11-03 Plate-type Heat Exchangers for Milk and Milk Products

20 A.P.V. Company, Inc. (9/ 4/56)
395 Fillmore Avenue
Tonawanda, New York 14150

30 Cherry-Burrell Corporation (10/ 1/56)
2400 Sixth Street, Southwest
Cedar Rapids, Iowa 52404

14 Chester-Jensen Co., Inc. (8/15/56)
5th & Tilgham Streets
Chester, Pennsylvania 19013

38 CREPACO, Inc. (10/19/56)
100 CP Avenue
Lake Mills, Wisconsin 53551

267 De Danske Mejeriers Maskinfabrik (10/15/75)
The Danish Dairies' Machine Factory
P.O. Box 66, 6000 Kolding, Denmark
(not available in USA)

120 DeLaval Company, Ltd. (12/ 3/59)
113 Park Street
South Peterborough, Ontario, Canada
(not available in USA)

279 The Schluter Co. (8/29/76)
112 E. Centerway
Janesville, WI 53545

17 The DeLaval Separator Company (8/30/56)
350 Dutchess Turnpike
Poughkeepsie, New York 12602

15 Kusel Dairy Equipment Company (8/15/56)
820 West Street
Watertown, Wisconsin 53094

12-04 Internal Return Tubular Heat Exchangers, for Milk and Milk Products

248 Allegheny Bradford Corporation (4/16/73)
P.O. Box 264
Bradford, Pennsylvania 16701

243 Babson Brothers Company (10/31/72)
2100 S. York Road
Oak Brook, Illinois 60521

103 Chester-Jensen Company, Inc. (6/ 6/58)
5th & Tilgham Street
Chester, Pennsylvania 19013

307 G&H Products, Inc. (5/ 2/78)
5718-52nd St.
Kenosha, WI 53141

217 Girton Manufacturing Co. (1/23/71)
Millville, Pennsylvania 17846

252 Ernest Laffranchi (12/27/73)
P.O. Box 455
Ferndale, California 95536

238 Paul Mueller Company (6/28/72)
P.O. Box 828
Springfield, Missouri 65801

96 C. E. Rogers Company (3/31/64)
P.O. Box 188
Mora, Minnesota 55051

298 Sanitary Processing Equip. Corp. (11/ 3/77)
Butternut Drive
East Syracuse, New York 13213

13-06 Farm Milk Cooling and Holding Tanks

240 Babson Brothers Company (9/ 5/72)
2100 S. York Road
Oak Brook, Illinois 60521

11R CREPACO, Inc. (7/25/56)
100 CP Ave.
Lake Mills, Wisconsin 53551

119R Dairy Craft, Inc. (10/28/59)
St. Cloud Industrial Park
St. Cloud, Minnesota 56301

4R Dairy Equipment Company (6/15/56)
1919 South Stoughton Road
Madison, Wisconsin 53716

92R DeLaval Company, Ltd. (12/27/57)
113 Park Street
South Peterborough, Ontario, Canada
(not available in USA)

49R The DeLaval Separator Company (12/ 5/56)
Dutchess Turnpike
Poughkeepsie, New York 12602

10R Girton Manufacturing Company (7/25/56)
Millville, Pennsylvania 17846

95R Globe Fabricators, Inc. (3/14/58)
3350 North Gilman Rd.
El Monte, California 91732

179R Heavy Duty Products (Preston), Ltd. (3/ 8/66)
1261 Industrial Road
Preston, Ontario, Canada
(not available in USA)

12R Paul Mueller Company (7/31/56)
P.O. Box 828
Springfield, Missouri 65801

249 Sunset Equipment Co. (4/16/73)
3765 North Dunlap Street
St. Paul, Minnesota 55112

42R VanVetter, Inc. (10/22/56)
4 South Idaho Street
Seattle, Washington 98134

16R Zero Manufacturing Company (8/27/56)
Washington, Missouri 63090

16-04 Evaporators and Vacuum Pans for Milk and Milk Products

164R Anderson IBEC (4/25/65)
19609 Progress Drive
Strongsville, Ohio 44136

254 Anhydro, Inc. (1/ 7/74)
165 John Dietsch Square
Attleboro Falls, Massachusetts 02763

132R A.P.V. Company, Inc. (10/26/60)
137 Arthur Street
Buffalo, New York 14207

263 C. E. Howard Corporation (12/21/74)
240 N. Orange Avenue
City of Industry, California 91746

107R C. E. Rogers Company (8/ 1/58)
P.O. Box 118
Mora, Minnesota 55051

277 ConTherm Corp. (8/19/76)
P.O. Box 352
Newbury Port, MA 01950

294 DeLaval Separator Co. (7/19/77)
1600 County Rd. F.
Hudson, WI 54016

186R Marriott Walker Corporation (9/ 6/66)
925 East Maple Road
Birmingham, Michigan 48010

273 Niro Atomizer Inc. (5/20/76)
9165 Rumsey Road
Columbia, Maryland 21044

299 Stork-Bowen Engr. Co. (11/16/77)
P.O. Box 898
Somerville, New Jersey 08876

17-04 Fillers and Sealers of Single Service Containers For Milk and Milk Products

192 Cherry-Burrell Corporation (1/ 3/67)
a unit of AMCA International Corp.
2400 Sixth St., Southwest
Cedar Rapids, IA 52404

137 Ex-Cell-O Corporation (10/17/62)
2855 Coolidge,
Troy, Michigan 48084

220 Hercules, Inc., Package Equipment Div. (4/24/71)
2285 University Ave.
St. Paul, Minnesota 55114

281 Purity Packaging Corporation (11/ 8/76)
4190 Fisher Road
Columbus, Ohio 43228

211 Steel & Cohen (Twin-Pak, Inc.) (2/ 4/70)
745 Fifth Avenue
New York, New York 10022

19-02 Batch and Continuous Freezers, For Ice Cream, Ices and Similarly Frozen Dairy Foods, As Amended

286 Alfa-Hoyer (12/ 8/76)
Soren Nymarksvei 13
DK-8270 Hojbjerb, Denmark
(not available in USA)

146 Cherry-Burrell Company (12/10/63)
2400 Sixth Street, Southwest
Cedar Rapids, Iowa 52404

141 CREPACO, Inc. (4/15/63)
100 CP Avenue
Lake Mills, Wisconsin 53551

22-04 Silo-Type Storage Tanks for Milk and Milk Products

168 Cherry-Burrell Corporation (6/16/65)
575 E. Mill St.
Little Falls, New York 13365

154 CREPACO, Inc. (2/10/65)
100 CP Avenue
Lake Mills, Wisconsin 53551

160 Dairy Craft, Inc. (4/ 5/65)
St. Cloud Industrial Park
St. Cloud, Minnesota 56301

181 Damrow Company, Division of DEC (5/18/66)
International, Inc., 196 Western Ave.
Fond du Lac, Wisconsin 54935

262 DeLaval Company Limited (11/11/74)
113 Park Street
South, Peterborough, Ontario, Canada

156 C. E. Howard Corporation (3/ 9/65)
240 N. Orange Ave., Box 2507
City of Industry, CA 91746

155 Paul Mueller Co. (2/10/65)
P.O. Box 828
Springfield, Missouri 65801

165 Walker Stainless Equipment Co. (4/26/65)
Elroy, Wisconsin 53929

23-01 Equipment for Packaging Frozen Desserts, Cottage Cheese and Milk Products Similar to Cottage Cheese in Single Service Containers

174 Anderson Bros. Mfg. Co. (9/28/65)
1303 Samuelson Road
Rockford, Illinois 61109

209 Doboy Packaging Machinery (7/23/69)
Domain Industries, Inc., 869 S. Knowles Ave.
New Richmond, Wisconsin 54017

302 Eskimo Pie Corp. (1/27/78)
530 E. Main St.
Richmond, Virginia 23219

258 Hercules, Inc. (2/ 8/74)
2285 University Ave.
St. Paul, Minnesota 55114

24-00 Non-Coil Type Batch Pasteurizers

161 Cherry-Burrell Corporation (4/ 5/65)
575 E. Mill St.
Little Falls, New York 13365

158 CREPACO, Inc. (3/24/65)
100 CP Avenue
Lake Mills, Wisconsin 53551

187 Dairy Craft, Inc. (9/26/66)
St. Cloud Industrial Park
St. Cloud, Minnesota 56301

177 Girton Manufacturing Co. (2/18/66)
Millville, Pennsylvania 17846

166 Paul Mueller Co. (4/26/65)
P.O. Box 828
Springfield, Missouri 65601

25-00 Non-Coil Type Batch Processors for Milk and Milk Products

275 Bepex Corporation (7/12/76)
150 Todd Road
Santa Rosa, California 95402

162 Cherry-Burrell Corporation (4/ 5/65)
575 E. Mill St.
Little Falls, New York 13365

159 CREPACO, Inc. (3/24/65)
100 CP Avenue
Lake Mills, Wisconsin 53551

188 Dairy Craft, Inc. (9/26/66)
St. Cloud Industrial Park
St. Cloud, Minnesota 56301

167 Paul Mueller Co. (4/26/65)
Box 828
Springfield, Missouri 65801

202 Walker Stainless Equipment Co. (9/24/68)
New Lisbon, Wisconsin 53950

26-00 Sifters for Dry Milk and Dry Milk Products

228 Day Mixing, Div. LeBlond, Inc. (2/28/72)
4932 Beech Street
Cincinnati, Ohio 45202

229 Russell Finex Inc. (3/15/72)
156 W. Sandford Boulevard
Mt. Vernon, New York 10550

173 B. F. Gump Division (9/20/65)
Blaw-Knox Food & Chem. Equip. Inc.
750 E. Ferry St.. P.O. Box 1041
Buffalo, NY 14211

185 Rotex, Inc. (8/10/66)
1230 Knowlton St.
Cincinnati, Ohio 45223

176 Koppers Company, Inc. (1/ 4/66)
Metal Products Division
Sprout-Waldron Operation
Munsy, Pennsylvania 17756

172 SWECO, Inc. (9/ 1/65)
6033 E. Bandini Blvd.
Los Angeles, California 90051

28-00 Flow Meters for Milk and Liquid Milk Products

272 Accurate Metering Systems, Inc. (4/ 2/76)
1731 Carmen Drive
Elk Grove Village, Illinois 60007

253 Badger Meter, Inc. (1/ 2/74)
4545 W. Brown Deer Road
Milwaukee, Wisconsin 53223

223 C-E IN-VAL-CO, Division of Combustion (11/15/71)
Engineering, Inc.
P.O. Box 556, 3102 Charles Page Blvd.
Tulsa, Oklahoma 74101

265 Electronic Flo-Meters, Inc. (3/10/75)
P.O. Box 38269
Dallas, Texas 75238

226 Fischer & Porter Company (12/ 9/71)
County Line Road
Warminster, Pennsylvania 18974

224 The Foxboro Company (11/16/71)
Neponset Avenue
Foxboro, Massachusetts 02035

270 Taylor Instrument Process Control (2/ 9/76)
Sybron Corporation, 95 Ames Street
Rochester, New York 14601

29-00 Air Eliminators for Milk and Fluid Milk Products

30-00 Farm Milk Storage Tanks

257 Babson Bros. Co. (2/ 7/74)
2100 S. York Road
Oak Brook, Illinois 60521

31-00 Scraped Surface Heat Exchangers

274 Contherm Corporation (6/25/76)
P.O. Box 352
Newburyport, Massachusetts 01950

290 CREPACO, Inc. (6/15/77)
100 So. CP Ave.
Lake Mills, WI 53551

32-00 Uninsulated Tanks for Milk and Milk Products

264 Cherry-Burrell Company, (1/27/75)
a unit of AMCA International Corp.
575 E. Mill St.
Little Falls, NY 13365

268 Dairy Craft, Inc. (11/21/75)
P.O. Box 1227
St. Cloud, Minnesota 56301

33-00 Polished Metal Tubing for Dairy Products

310 Allegheny Bradford Corporation (7/19/78)
P.O. Box 264
Bradford, PA 16701

289 Ladish Co., Tri-Clover Division (1/21/77)
9201 Wilmot Road
Kenosha, Wisconsin 53140

308 Rath Mfg. Co. Inc. (6/15/77)
2505 Foster Ave.
Janesville, WI 53545

35-00 Continuous Blenders

292 Waukesha Foundry Div. ABEX Corp. (8/24/77)
1300 Lincoln Ave.
Waukesha, WI 53186

36-00 Colloid Mills

293 Waukesha Foundry Div., ABEX Corp. (8/24/77)
1300 Lincoln Ave.
Waukesha, WI 53186

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The manual costs $12.50. There is also a $1.50 charge for postage and handling. For further information or to order the manual, contact: AOAC, Box 540, Benjamin Franklin Station, Washington, D.C. 20044.

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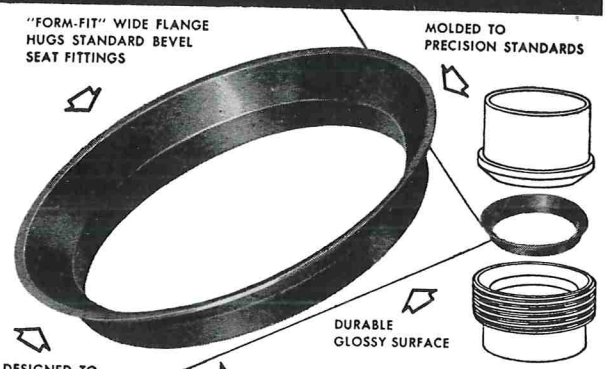

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Two Easy Steps to More Milk and Healthy Udders

By Dr. L.J. Bush
Professor, Department of Animal Sciences and Industry
Oklahoma State University—Stillwater

Significant advances have been made in recent years in the design and development of milking equipment. Now the challenge is for the dairyman to take full advantage of what they can do for him. By using this new equipment in accordance with suggested milking procedures, a dairyman can help keep his cows healthy and increase profits from his herd. Two important steps in getting the most from equipment are proper adjustment of milking units and prompt removal of the units as milking is completed.

Proper Adjustment Avoids Injury and Improves Milk Flow

To do the best job of milking cows by machine, it is important that the teat cups remain in the correct position on the teats. If the teat cups are allowed to creep up on the teats as milking progresses, the passageway for milk is partially blocked due to compression of the tissue by the teat cup liners. Injury to the tissue at the base of the udder may occur. This can be prevented by providing a slight downward pull on the teat cups as the cow is milked out. With "claw" type units this usually can be accomplished only by holding down on the unit as milking nears completion, whereas suspended units can be positioned to provide the tension needed. The mere fact that the milking unit is suspended does not insure proper downward tension on the teat cups, however. It must be adjusted properly to get the job done right. Higher milk yields will result due to more complete milking, and less machine stripping time is required.

Remove Teat Cups When Milk Flow Stops

Another important step in milking is to remove the milking unit as soon as the flow of milk has stopped. Removing teat cups too early will rob you of milk. On the other hand, there is evidence that overmilking causes severe injury to teats of some cows. Hemorrhage and inflammation of the membrane lining the teat cavity often occurs, and, in some cases, the streak canal is injured. These injuries lower resistance to bacterial invasion and mastitis may result.

Cows vary in milk yield and rate of milk flow. Individual quarters of the same cow vary in the same way. Therefore, the ideal situation is for individual teat cups to be removed from the quarters as each one is milked out. Milking units designed for automatic removal of individual teat cups accomplish this with precision, if serviced regularly and used in the recommended way. It should be noted though that the same results can be achieved with conventional milking units by careful attention to good milking procedures.