and wet sample uniformly. Place receiver containing 10 ml 0.02*N* H_2SO_4 in ice bath, with stem of condenser dipping in acid. Connect apparatus and let steam bubble through material. Adjust heating so that first drop of distillate drips into receiver 2–3 min after entry of steam. Collect 150 ml or more of distillate in 15 min. Conduct blank distillation with the reagents without sample. Titrate distillate against standard 0.02*N* NaOH, using methyl red as indicator. From difference in titer values, deduce arecoline content. 1 ml 0.02*N* NaOH is equivalent to 0.003108 g arecoline.

Arecoline Hydrobromide in Drugs

Dissolve about 1 g drug, accurately weighed, in 75 ml distilled water. Adjust pH to 9.0–9.1 with dilute alkali (0.02*N* NaOH), using a pH meter.[1] Dilute volume to 100 ml with distilled water. Pipet 5 ml solution into distillation flask, introduce steam, and proceed as above. 1 ml 0.02*N* NaOH is equivalent to 0.00427 g arecoline hydrobromide.

[1] For the analysis of pure arecoline HBr, addition of known amount of alkali may be enough, rather than adjustment with a pH meter. However, when arecoline HBr is mixed with other drugs, a known amount can not be added, and so alkali is added to adjust to a known pH.

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DAIRY PRODUCTS

Collaborative Study of a New Alkaline Phosphatase Assay System for Milk

By D. H. KLEYN and S. H. C. LIN (Department of Food Science, Rutgers—The State University, New Brunswick, N.J. 08903)

A new method in which phenolphthalein monophosphate is used as substrate has been developed for determining residual alkaline phosphatase: Upon hydrolysis, phenolphthalein is released and yields a red solution that is compared visually with a standard prepared from the same milk. Seven collaborators made 168 observations that were used in a statistical study of the results. Chi-square calculations and tests of independence indicated that the described method yields results on skim milk, whole milk, and light cream that are as precise and either as accurate or more accurate than those obtained with the Scharer I method. Based on these results, it is recommended that the method be adopted as official, first action.

Compared to the standard laboratory tests (1, 2), the Scharer I (rapid) method (2) for the determination of residual alkaline phosphatase in milk has gained wide acceptance in the United States because it is rapid and simple. This method does, however, possess some inherent weaknesses. There is a constant hazard of phenol contamination from reagents, glassware, and stoppers. The reagents and the colored compound formed by reaction of phenol with the dye are unstable. Emulsification frequently occurs during the extraction of the phenol with butanol, and the visual comparison of color is sometimes difficult, particularly with borderline cases.

For 20 years, the use of phosphate esters of indicators which produce a chromogen directly on hydrolysis has been recommended for determination of phosphatase activity in milk. Esters which possess such "built-in" indicators avoid the problem of non-specific color reactions. *p*-Nitrophenyl phosphate, which liberates yellow *p*-nitrophenol on hydrolysis, has been recommended, but it is very unstable and insensitive (requires a 2 hour incubation period for detection of contamination of pasteurized milk with 0.1% raw milk). Phenolphthalein diphosphate is more stable and releases phenolphthalein on hydrolysis. This substrate, however, lacks sensitivity because two phosphoryl radicals must be hydrolyzed. Recently, the Warner-Lambert Research Institute synthesized phenolphthalein monophosphate, a substrate, which is very stable and easily hydrolyzed by alkaline phosphatase to yield free phenolphthalein. They developed a simple method to determine residual alkaline phosphatase in milk, using this substrate (3).

This method was studied in our laboratory (4) before conducting the collaborative study. A preliminary comparison of the new method with the Scharer I (rapid) method was made by running the test on 30 samples of market milk, including homogenized whole milk, low-fat fortified milk, and skim milk. The results obtained by both methods were negative in all cases, indicating that the two methods were equally effective.

The sensitivity of the new method was compared to that of the Scharer I method for the detection of slight differences in residual phosphatase activity. Pairs of samples containing different levels of raw milk in pasteurized milk and cream with a ratio of 1 to 1.2 were prepared. In each pair, one sample contained 20% higher phosphatase activity than the other, as shown in Table 1. The differences in phosphatase activity were determined by both methods. The probability of correct differentiations is shown in Table 1, with 95% confidence intervals constructed. The new method achieved significantly higher probability of correct visual differentiation than the Scharer I method in detecting a 1:1.2 ratio of difference of residual phosphatase activity in either milk or cream (level of significance less than 0.001). The probabilities of detecting the same ratio of differences in milk and cream by the new method were the same, whereas those by the Scharer I method were slightly different (significant at $p < 0.25$). This observation indicates that fat content does influence the color of the butanol extract in the Scharer I method, while it has little or no effect on the new method.

A similar visual comparison was conducted to determine the ability of the new method to detect a 1:1.1 ratio of difference of raw milk in boiled milk. The probability of correct differentiation did decrease, but it was comparable to that of the Scharer I method in detecting a 1:1.2 ratio of differance of raw milk in boiled milk.

The new method is highly sensitive because of the ease of color comparison, the high rate of hydrolysis, the elimination of variations due to specific color reaction and extraction, and the slight contribution of yellow color of the milk fat to the pink color of phenolphthalein.

METHOD

Reagents

(a) *Phenolphthalein monophosphate soln (substrate concentrate).*—pH 10.15 at 25°. Dissolve 3.9 g dicyclohexylamine salt of phthln monophosphate and 73.2 g 2-amino-2-methyl-1-propanol in 21.9 ml HCl. Soln is stable indefinitely under refrigeration. (Available as Phosphastrate® Alkaline from General Diagnostics Div., Warner-Chilcott Laboratories, Morris Plains, N.J. 07950.)

(b) *Phenolphthalein-tartrazine soln (standard concentrate).*—0.01 and 0.04%, resp.; pH 10.15 at 25°. Dissolve 10 mg phthln, 40 mg tartrazine, and 73.2 g 2-amino-2-methyl-1-propanol in 21.9 ml HCl. Soln is stable indefinitely under refrigeration.

(c) *Color developer.*—2.5*N* NaOH.

Determination

Pipet 1 ml milk into each of two 15 × 100 mm test tubes and warm to 37°. To 1 tube add 1 drop (0.04 ml) substrate concentrate (pH of incubation mixt. 10.0) and to other add 1 drop std concentrate. Mix and incubate 30 min at 37°. Add 1 drop 2.5*N* NaOH to each tube, mix, and compare visually. If sample soln (in tube

Table 1. The probability of correct visual differentiation between two different levels of raw milk in pasteurized milk and cream

Sample	Method	Ratio of Difference[a]	No. of Pairs Observed	No. of Correct Observations	Fraction of Correct Observations	Confidence Limits at p = 0.95[b]
Milk	New	1:1.2	74	72	0.973	0.90–1.00
Milk	Scharer I	1:1.2	75	57	0.760	0.65–0.85
Milk	New	1:1.1	48	38	0.792	0.65–0.89
Cream	New	1:1.2	65	62	0.954	0.88–0.99
Cream	Scharer I	1:1.2	75	48	0.640	0.52–0.75

[a] 1:1.2 differences are 0.100:0.120%, 0.120:0.144%, 0.144:0.173%, 0.173:0.208%, and 0.208:0.250% raw milk; 1:1.1 differences are 0.100:0.110%, 0.110:0.120%, 0.120:0.132%, and 0.132:0.144% raw milk.

[b] Pearson, E. S., and Hartley, H. O., *Biometrika Tables for Statisticians*, Vol. 1, Cambridge University Press, 1954.

contg substrate concentrate) is less pink than std soln, milk has been pasteurized to extent equiv. to $< 0.1\%$ raw milk.

Collaborative Study

Ten samples each were distributed to 11 laboratories. One sample served as a temperature control. Milk samples X, Y, and Z were used to gain experience with the new method; the amounts of phosphatase activity and expected results were given to the collaborators (Table 2). The remaining six unknown samples consisted of two samples each of homogenized milk, light cream, and skim milk. The collaborators were asked to obtain satisfactory results on the samples of known phosphatase activity, and then to analyze the unknown samples in duplicate by the new method and, if possible, by either the Scharer I (rapid) method or another method currently used in their laboratories.

Table 2. The levels of alkaline phosphatase activity and expected results of its determination

Sample	Phosphatase Activity, % Raw Milk	Expected Result: New Method, Compared to Standard (S)	Expected Result: Scharer I, μg Phenol/ml
Values Known			
X (milk)	<0.05	<	<1
Y (milk)	0.10	=	1
Z (milk)	0.15	>	>1, <2
Values Unknown			
1 (milk)	0.12	>	>1, <2
2 (cream)	<0.05	<	<1
3 (skim)	0.12	>	>1, <2
4 (milk)	<0.05	<	<1
5 (cream)	0.12	>	>1, <2
6 (skim)	<0.05	<	<1

Collaborators were requested to report results obtained by the new method on the basis of color comparison with the standard solution, as less than standard ($< S$), equal to standard ($= S$), or greater than standard ($> S$). The standard solution contained 4 μg of phenolphthalein, equivalent on a molar basis to 1.18 μg of phenol. This is the amount of phenolphthalein liberated in 30 min by alkaline phosphatase present in 1 ml of pasteurized milk containing 0.1% raw milk. The levels of phosphatase activity selected for the unknown samples were slightly below and slightly above that of the standard (Table 2). The positive samples were prepared by heating the pasteurized milk, cream, or skim milk to 90°C for 2 min, cooling, and then adding the predetermined amount of raw milk. Negative samples were unmodified, pasteurized, commercial products. All reagents were supplied in small plastic bottles equipped with controlled dropper tips.

Results and Discussion

The data reported by the participating laboratories are presented in Table 3. Since Laboratories A–G determined phosphatase activity by both the new method and Scharer I method, only data from these laboratories have been used in the statistical study of the results. Laboratory H obtained the expected results by the new method on all samples except Sample 4 which should have been less than S rather than equal to S. The results obtained by this laboratory with the Sanders-Sager method agreed with those obtained by the new method with the

Table 3. Collaborative results of determinations of alkaline phosphatase activity[a]

	Values Known			Values Unknown					
Lab.	X (Milk)	Y (Milk)	Z (Milk)	1 (Milk)	2 (Cream)	3 (Skim)	4 (Milk)	5 (Cream)	6 (Skim)
A	<S, <S	=S, =S	>S, >S	=S, =S	<S, <S	>S, >S	<S, <S	>S, >S	<S, <S
	<1, <1	>1, >1	>1, >1	>2, >2	>1, >1	>2, >2	<1, <1	>2, >2	<1, <1
B	<S, <S	=S, =S	>S, >S	>S, >S	<S, <S	>S, >S	<S, <S	=S, =S	<S, <S
	<1, <1	1, 1	>1, >1	>1, >1	<1, <1	>2, >2	<1, <1	2, 2	<1, <1
C	<S, <S	=S, =S	>S, >S	=S, =S	<S, <S	>S, >S	<S, <S	=S, >S	<S, <S
	<1, <1	1, 1	2, 2	1, 1	<1, <1	>1, >1	<1, <1	1, 1	<1, <1
D	<S, <S	=S, =S	>S, >S	=S, =S	<S, <S	>S, >S	=S, <S	>S, >S	<S, <S
	<1, –	1, –	>1, –	1, 1	<1, 1	1, 1	<1, <1	1, 2	<1, <1
E	<S, <S	=S, =S	>S, >S	>S, >S	<S, <S	>S, >S	<S, <S	>S, >S	<S, <S
	<1, <1	1, 1	>1, >1	>1, >1	<1, <1	>1, >1	1, 1	>1, >1	<1, <1
F	=S, =S	>S, >S	>S, >S	>S, >S	<S, <S	>S, >S	<S, <S	<S, >S	<S, <S
	<1, <1	>1, >1	>1, >1	1, >1	<1, <1	>1, >1	<1, 1	<1, >1	<1, <1
G	<S, <S	=S, =S	>S, >S	>S, >S	<S, <S	>S, >S	<S, <S	>S, >S	<S, <S
	<1, <1	1, 1	>1, >1	1, 1	<1, <1	1, 1	<1, <1	>1, >1	<1, <1
H[b]	<S, <S	=S, =S	>S, >S	>S, >S	<S, <S	>S, >S	=S, =S	>S, >S	<S, <S
	P, P	B, B	NP, NP	NP, NP	P, P	NP, NP	P, P	NP, NP	P, P
I	<S, <S	=S, =S	>S, >S	=S, =S	<S, <S	>S, >S	<S, <S	>S, >S	<S, <S
	– –	– –	– –	– –	– –	– –	– –	– –	– –
J	<S, <S	=S, =S	>S, >S	>S, >S	<S, <S	>S, >S	<S, <S	>S, >S	<S, <S
	– –	– –	– –	– –	– –	– –	– –	– –	– –
K	Samples received in spoiled condition.								

[a] The top set of results for each laboratory was obtained by the new method; the bottom set was obtained by the Scharer I method.

[b] The bottom set of results was obtained by the Sanders-Sager method; P = pasteurized, B = borderline, and NP = not pasteurized.

exception of Sample 4; the reported result (pasteurized) was the "expected" one. Laboratory I obtained the expected result on all samples analyzed by the new method except for Sample 1, which was reported as equal to S rather than less than S. However, this result would still have led to the conclusion that the sample was "positive." Laboratory J reported the expected result on all six samples as analyzed by the new method.

A statistical analysis was made on the results obtained by Collaborators A–G to compare the new method and the Scharer I method. A simplified classification was adopted to process the data into two categories. One category contained all the correct observations, the other all the wrong observations. All reported borderline results, i.e., = S or 1 μg phenol/ml, were considered equivalent to positive tests. The data of the duplicate observations from all laboratories were pooled because the difference within duplicate observations and the variations among the laboratories were not found to be statistically significant (Table 4).

Chi-square (χ^2) was calculated and the test of independence of each sample-method combination was carried out. Expected values were computed under the assumption that the hypothesis of constant ratio of right and wrong observations in each sample-method combination is true within random sampling errors. The calculated χ^2_1 for all the data is 20.07 with 11 degrees of freedom and is significant at the 0.05 level. This indicates that the ratio of right and wrong observations is dependent on the sample-method combination. By eliminating two particular sets of data, namely, the results of negative milk and cream samples analyzed by the Scharer I Method, another χ^2, based upon the reduced data, was calculated. This χ^2_2 is 7.15 with 9 degrees of freedom and is not significant.

Based upon the above statistical evaluation, it is concluded that the new method is significantly more efficient than the Scharer

Table 4. Number of right and wrong observations obtained from seven collaborators (A–G)

	Positive Samples						Negative Samples						
	New Method			Scharer I Method			New Method			Scharer I Method			
	6 (Skim)	4 (Milk)	2 (Cream)	6 (Skim)	4 (Milk)	2 (Cream)	3 (Skim)	1 (Milk)	5 (Cream)	3 (Skim)	1 (Milk)	5 (Cream)	Total
Right	14	14	13	14	14	13	14	13	14	14	11	11	159
Wrong	0	0	1	0	0	1	0	1	0	0	3	3	9
χ^2_1	0.79	0.79	0.09	0.79	0.79	0.09	0.79	0.09	0.79	0.79	7.13	7.13	20.07
χ^2_2	0.31	0.31	1.67	0.31	0.31	1.67	0.31	1.67	0.31	0.31	–	–	7.15

I method in detecting negative samples of milk and cream; however, in detecting negative samples of skim milk and positive samples of skim milk, milk, or cream, the two methods are equally effective. These data further substantiate that fat affects results obtained by the Scharer I method. This factor is believed to have its greatest effect in samples with low levels of phosphatase activity.

Recommendation

The results of this collaborative study show that the new alkaline phosphatase assay system, based upon the hydrolysis of phenolphthalein monophosphate, yields results that are as precise and either as accurate as or more accurate than those obtained with the Scharer I method. In addition, the new method is much simpler, has eliminated the need for positive and negative controls, and utilizes reagents that are relatively stable. It is recommended that the method as presented and studied be adopted as official, first action.

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The following analysts participated in this collaborative study:

Eleanor Baldwin and Margaret Poinsett, New Jersey State Department of Health, Trenton, N.J.

Dale Nickerson, Colorado Department of Agriculture, Denver, Colo.

Marjorie S. Neff, England Laboratories, Frederick, Md.

Florence Smith, Indiana State Board of Health, Indianapolis, Ind.

Dean A. Glatt, Kansas State Board of Agriculture, Topeka, Kan.

Margarethe Oakley and S. Bounds, Maryland State Department of Health, Baltimore, Md.

Homer K. Hansen, Iowa State Department of Agriculture, Des Moines, Iowa

Harold W. Woodson and Carlton O. Stansberry, Illinois Department of Public Health, Chicago, Ill.

This report of the Associate Referee, D. H. Kleyn, was presented at the 81st Annual Meeting of the Association of Official Analytical Chemists, Oct. 9–12, 1967, at Washington, D.C.

The recommendation of the Associate Referee was approved by the General Referee and by Subcommittee C, and was adopted by the Association. See *This Journal* **51**, 396 (1968).

Arthur G. Rand, Jr., University of Rhode Island, Kingston, R.I.

Felix A. Burrows, Jr., University of Maryland, College Park, Md.

A. H. Duthie, University of Vermont, Burlington, Vt.

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Paper of the Journal Series, New Jersey Agricultural Experiment Station, Rutgers—The State University, Department of Food Science, New Brunswick, N.J.

Collaborative Study of a Method for the Determination of Weight Per Unit Volume of Packaged Ice Cream

By LARRY E. HABEGER (Food and Drug Administration, 240 Hennepin Ave., Minneapolis, Minn. 55401)

A new method and the official AOAC method (15.181) for the determination of weight per unit volume of ice cream were compared in a collaborative study by twelve collaborators in six laboratories. In the official method, hard frozen ice cream is immersed in a specially constructed overflow can filled with deodorized kerosene and the volume of the ice cream is determined by measuring the volume of kerosene displaced. In the new method, hard frozen ice cream is immersed in a container of fixed volume filled with water and the volume of water displaced is determined by weight loss. This study shows that the new method is more precise than the old one. It is therefore recommended that the new method be adopted as official, first action.

Problems encountered with the official AOAC method (1) for determining weight per unit volume of frozen desserts have led to the development of a new method for packaged ice cream. In the new method, the volume of ice cream is calculated from the weight of water displaced from a container of fixed volume.

Ice cream is defined in the Federal Food Standards, sec. 20, par. 20.1, as a product having a total solid content of not less than 1.6 lb/gal., a milk fat content of not less than 10% (vanilla ice cream), and a minimum weight of 4.5 lb/gal.

The AOAC methods for determining fat and moisture (**15.184**, **15.186**) give reliable results and present no problem. The AOAC method for determining the weight per unit volume, **15.181–15.182**, does not always give reproducible results. In this method, a hard frozen block of ice cream is immersed in a specially constructed overflow can filled with deodorized kerosene, and the displaced kerosene is weighed. The weight of kerosene is divided by its density to obtain the volume of the immersed sample.

This official method (1) has several shortcomings. The density of kerosene changes considerably with temperature and must be determined for each new lot; also, it takes some time for the kerosene to stop running from the overflow can. The hard frozen block of ice cream has a tendency to melt along the edges, thereby changing its shape and volume. Special deodorized kerosene is necessary for the determination, and it cannot be easily recovered for future determinations; it is sometimes hard to obtain, and it is not a convenient immersing fluid to work with.

In 1939 the Canadian government laboratories adopted a procedure (2) using water