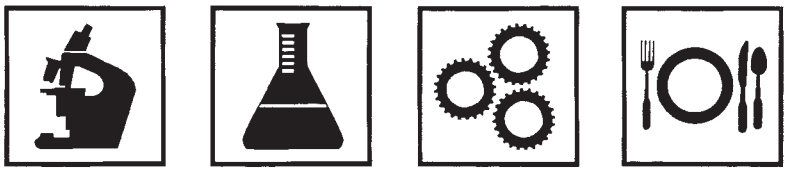
Journal of Food Protection



Evaluation of Alkaline Phosphatase Detection in Dairy Products Using a Modified Rapid Chemiluminescent Method and Official Methods

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Journal of Food Protection, Vol. 74, No. 7, 2011, Pages 1144–1154
doi:10.4315/0362-028X.JFP-10-422

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MS 10-422: Received 30 September 2010/Accepted 4 February 2011

ABSTRACT

Alkaline phosphatase is a ubiquitous milk enzyme that historically has been used to verify adequate pasteurization of milk for public health purposes. Current approved methods for detection of alkaline phosphatase in milk include the use of enzyme photoactivated substrates to give readings in milliunits per liter. The U.S. and European public health limit for alkaline phosphatase in pasteurized drinks is 350 mU/liter. A modified chemiluminescent method, fast alkaline phosphatase, was compared with the approved fluorometric and chemiluminescent alkaline phosphatase methods to determine whether the modified method was equivalent to the approved methods and suitable for detecting alkaline phosphatase in milk. Alkaline phosphatase concentrations in cow's, goat's, and sheep's milk and in flavored drinks and cream were determined by three methods. Evaluations in each matrix were conducted with pasteurized samples spiked with raw milk to produce alkaline phosphatase concentrations of 2 to 5,000 mU/liter. The tests were performed by the method developer and then reproduced at a laboratory at the National Center for Food Safety and Technology following the criteria for a single laboratory validation. The results indicated that the fast alkaline phosphatase method was not significantly different from the approved chemiluminescent method, with a limit of detection of 20 to 50 mU/liter in all the studied matrices. This modified chemiluminescent method detects alkaline phosphatase in the 350 mU/liter range with absolute differences from triplicate data that are lower and within the range of the allowed intralaboratory repeatability values published for the approved chemiluminescent method.

The importance of milk pasteurization is unquestionable in terms of public health safety. Regulatory agencies around the world take measures to prevent and control pathogenic bacterial contamination of milk products by enforcing pasteurization standards and conducting appropriate analyses with standardized and approved methods to determine pasteurization efficiency *(2, 4, 7–9, 13, 18, 19, 22)*.

Alkaline phosphatase is a heat-sensitive enzyme found in raw milk that is used as a marker for the efficacy of thermal pasteurization. The first biochemical assays used to detect alkaline phosphatase were colorimetric *(2, 10, 18)*. The unit of measurement for the colorimetric assays is micrograms of phenol per milliliter of milk, reflecting the amount of phenol reactant organically extracted from the ortho-phenyl phosphate substrate. Colorimetric assays and the phenol measurement method for alkaline phosphatase to indicate pasteurization effectiveness were adopted as public health standards for pasteurization in the 1950s through 1980s. The colorimetric methods have a limit of detection of approximately 0.05 to 0.2% residual or contaminated raw cow's milk *(3, 11, 13)*. Public health and regulatory agencies in many countries still use alkaline phosphatase reference levels of phenol as a measure of pasteurization efficacy.

More sensitive methods that eliminated the need for organic extraction of substrate were developed in the 1990s. These methods utilized either fluorometric or chemiluminescent substrates that are quenched by a covalently attached organophosphate molecule. Alkaline phosphatase enzyme hydrolyzes the organophosphate from the substrate and produces a photoactivated product that is detected by instruments *(11, 14, 17)*. The principle involved in these assays is use of an enzyme photoactivated substrate (EPAS) *(3)*. The unit of measurement in EPAS assays is expressed as milliunits of enzyme activity per liter of milk. A milliunit is defined as the amount of enzyme that catalyzes 1 ng of specific substrate hydrolyzed per minute per liter of solution. The validity of both colorimetric and fluorometric (EPAS) assays for cow's, goat's, and sheep's milk has been recently assessed with improved sensitivity and reproducibility using the EPAS principle *(5, 17)*. A study of the kinetics involved in alkaline phosphatase denaturation was conducted, and an alternative method for ensuring proper pasteurization was proposed *(12)* that utilizes an immunoassay, which is expensive and requires specialized training.

In an attempt to protect consumers, U.S. and European Union (EU) regulators have recently adopted more sensitive

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EPAS methods and phased out the older colorimetric methods for detection of alkaline phosphatase. Current U.S. and EU regulations specify that pasteurized milk and fluid dairy drinks must contain less than 350 mU/liter alkaline phosphatase *(4, 22)*. The current approved methods for detecting adequate grade A milk pasteurization include the U.S. Food and Drug Administration (FDA) approved and International Organization for Standardization standardized methods Fluorophos (Advanced Instruments, Norwell, MA) and Paslite (Charm Sciences, Inc, Lawrence, MA), which utilize fluorometric or chemiluminescent substrates that are photoactivated in the presence of alkaline phosphatase in milk *(7, 8)*. An International Dairy Federation (IDF) bulletin summarized the history of the chemiluminescent method Paslite. The Paslite method was incorporated into the U.S. Pasteurized Milk Ordinance in 1995 and subsequently was collaboratively validated in a variety of milk species to obtain ISO 22160 IDF 209 standardization in 2007 *(8, 17)*. The chemiluminescent method, Paslite, was significantly modified to simplify the procedure and calibration of the assay. The modified assay is branded as the fast alkaline phosphatase method (F-AP) *(16)*.

The primary objectives of this study were (i) to evaluate significant simplifications made to the Paslite chemiluminescent method (F-AP) and to compare F-AP with existing EPAS phosphatase methods for detection of alkaline phosphatase in six dairy matrices, (ii) to repeat the evaluation of F-AP at an independent laboratory and to compare with the data with those generated by the method developer, and (iii) to subject the F-AP to single laboratory validation and evaluation according to prior statistical parameters established for the chemiluminescent method *(17)*.

MATERIALS AND METHODS

Official alkaline phosphatase detection methods. Three approved methods for alkaline phosphatase analysis were performed as described in the FDA 2400 forms *(21)*: (i) the fluorescent method Fluorophos (Advanced Instruments) *(7, 14, 15, 20)*, the chemiluminescent method Paslite (Charm Sciences) using a NovaLUM with temperature compensation (Chemi-Lum) *(8, 17, 21)*, and (iii) the Paslite chemiluminescent method using a Charm II scintillation analyzer (Chemi-6600) with software calculations for milliunits per liter.

Modified chemiluminescent method. The modified chemiluminescent method, the F-AP assay, utilizes a NovaLUM analyzer with temperature compensation. Figure 1 shows the design of the F-AP method that uses the same chemiluminescent substrate and NovaLUM equipment as the Paslite method. The F-AP assay reduces manipulations of Paslite to addition of a single 100-μl milk sample to a vial containing 0.5 ml of predispensed chemiluminescent substrate in buffer. The contents in the vial are mixed for 5 s, attached to the NovaLUM adapter, and inserted in the upright NovaLUM analyzer. An F-AP channel specific to the matrix in the NovaLUM is activated. The F-AP channel has a built-in timer and temperature monitor to complete the analysis in 45 s for milk products or 90 s for cream. Flavored products, with added flavor ingredients such as chocolate or strawberry, are analyzed in 90 s but must first be prepared by centrifugation (minimum 1,200 × *g* for 3 min) to remove quenching solids. Programmed channels in the NovaLUM for white milks, flavored milks, and creams are calibrated with a single analysis of the same negative control and the 350 mU/liter calibrator used in the Paslite method *(8)*. This F-AP calibration is an additional simplification when compared with the Paslite method, which utilizes four calibrators averaged in triplicate.

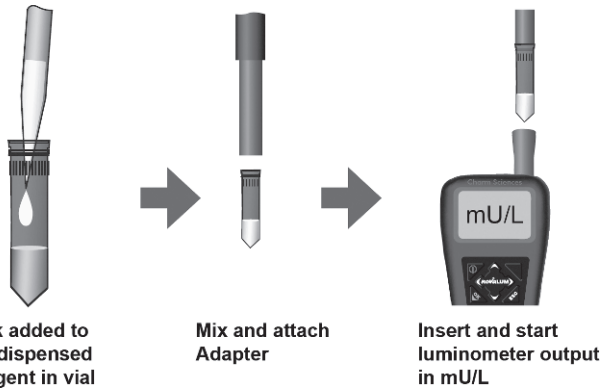

FIGURE 1. *Fast alkaline phosphatase method in a three-step sequence.*

Study samples. Because no milk reference standards for alkaline phosphatase were available, pasteurized milk samples were spiked with raw milk of the same species to obtain various levels of alkaline phosphatase. Locally purchased pasteurized cow's whole milk, skim milk, light cream (20% fat), and 2% fat chocolate milk (Hershey Co., Hershey, PA) were laboratory pasteurized to 95°C for 1 min and cooled on ice. Producer supplied pasteurized goat's whole milk (Jackson-Mitchell, Turlock, CA) was laboratory pasteurized to 95°C for 1 min and cooled on ice. Producer supplied sheep's milk (Old Chatham Sheepherding Co., Old Chatham, NY) was laboratory pasteurized at 63°C for 40 min and cooled on ice. Laboratory pasteurized samples were spiked with same species raw milk at 0.002 to 0.5% to cover 20 to 5,000 mU/liter alkaline phosphatase enzyme activity with the exception of raw goat's milk, which was spiked at 0.02 to 5.0% to create the study ranges. These ranges in milliunits per liter are within the published scope of the Paslite method *(8, 17)*. Each matrix preparation was tested in triplicate by the manufacturer using the F-AP method and the Paslite method with NovaLUM. Samples also were analyzed in duplicate by the fluorometric method. Positive and negative controls were included in each method.

The laboratory at the National Center for Food Safety and Technology (Chicago, IL) prepared samples of the same milk types and dairy matrices and spiked the laboratory pasteurized samples at the same levels with raw milk of the same species as was done by the manufacturer. Sample preparations were tested in triplicate with Fluorophos, F-AP (Chemi-F-AP), Paslite with NovaLUM (Chemi-Lum), and Paslite with Charm II and software (Chemi-6600).

Analysis. Data were analyzed consistent with ISO-5725-2 by applying the maximum differences of the method averages and comparing these with published reproducibility values and applying the standard deviations (SDs) of determinations compared with repeatability values *(6)*.

RESULTS AND DISCUSSION

Milk samples with known concentrations of alkaline phosphatase (i.e., reference standards of alkaline phosphatase with defined activity in milk) are not currently available. Therefore, laboratory pasteurized samples were

prepared by pasteurizing at 95°C for 1 min or, for sheep's milk, at 63°C for 40 min to obtain samples with minimal alkaline phosphatase activity. Laboratory pasteurized samples were then spiked with various amounts of raw milk from the same species to obtain milk samples with target concentrations of 5,000, 500, 350, 100, 50, and 0 mU/liter alkaline phosphatase activity. The raw milk–spiked pasteurized samples were made to provide samples with alkaline phosphatase enzyme levels that have been previously evaluated in the Paslite collaborative study *(17)*. Results from measurement of alkaline phosphatase activity in raw milk–spiked whole milk and other fluid dairy drinks as determined by the manufacturer using the Paslite and F-AP methods are presented in Table 1. The values are the mean and SD of three determinations. The absolute differences of the Paslite mean and F-AP mean for each studied spike level are presented as m▲. The Paslite interlaboratory repeatability (*r*) and reproducibility (*R*) values from the previous collaborative study *(17)* are given in Table 1 for comparison.

In all cases, except the cream spiked at 0.0039% reflecting ~50 mU/liter alkaline phosphatase activity, the *R* values were larger than the differences between the method means, which indicates that the F-AP determinations are not significantly different from the Paslite results. This cream exception value was within 1 mU/liter. The difference between the maximum and the minimum F-AP and Paslite determination did not exceed the *R* value in the case of cow's whole milk, goat's milk, and skim milk at any of the alkaline phosphatase levels studied, indicating that F-AP method modification is not significantly different from Paslite method. The m▲ values for cream and sheep's milk at raw milk spiking levels of 0.0625% (~500 mU/liter alkaline phosphatase activity) and 0.0313% (~350 mU/liter alkaline phosphatase activity) did not exceed *R* but exceeded *R* at the lower concentrations 0.0078% (~100 mU/liter alkaline phosphatase activity) and 0.0039% (~50 mU/liter alkaline phosphatase activity), perhaps reflecting a higher F-AP background with these matrices. Chocolate milk was exceeded by 19 mU/liter at 0.0625% (~500 mU/liter) and exceeded by 3 mU/liter at the 0.0313% level (~350 mU/liter). The negative chocolate milk *R* value was exceeded by 5 mU/liter, perhaps reflecting higher background or calibration differences between the methods.

SDs of the Paslite and F-AP determinations compared with the *r* values determined by the Paslite collaborative study indicate that the values for these two methods are similar at the corresponding milliunit per liter concentrations. The SDs of the two methods were similar across the spiked concentrations, and the SDs were generally less than half of published repeatability values, which is consistent with the Paslite method *(17)*. With both F-AP and Paslite determinations, certain matrices such as chocolate milk and sheep's milk have higher background activity and thus greater variability at lower spiked levels. In the analysis of the chocolate milk samples with no added raw milk, i.e., with no alkaline phosphatase, the SD of both Paslite and F-AP results exceeded *r,* indicating a higher background level of activity. Overall, the data indicate that the F-AP method modification produced results that were not significantly different from those of the Paslite method, and values were within the published *r* and *R* values of the method.

The spiking level of 0.002 to 0.0039% raw milk most frequently simulates the Paslite method limit of detection of 20 mU/liter alkaline phosphatase activity. The F-AP method also detects enzyme activity distinguishable from negative controls at these low spiking levels, indicating a similar limit of detection. The 0.0313% level of raw milk spiking simulates milk samples containing 350 mU/liter alkaline phosphatase activity, which is the U.S. and EU regulatory action limit. This concentration is more than 10 times the spiking level used to simulate the limit of detection and indicates that both Paslite and F-AP have an appropriate level of quantitation at the action level and the results are linear. Thus, F-AP is a sensitive alternative measure of alkaline phosphatase activity suitable for use at the 350 mU/liter concentration in milk and fluid dairy drinks.

Under the National Conference of Interstate Milk Shipments (NCIMS) laboratory committee protocol, 25 data sets per matrix of a method modification in comparison to the reference or approved method may be submitted by the manufacturer to the FDA Laboratory Proficiency Evaluation Team for consideration for acceptance and incorporation into the Pasteurized Milk Ordinance (PMO) Evaluation of Milk laboratory documents *(20, 21)*. The manufacturer data support the hypothesis that the F-AP modifications result in a method that is equivalent to the chemiluminescent Paslite method. Independent single laboratory verification of the comparison data added support to this hypothesis. The National Center for Food Safety and Technology conducted the independent single laboratory verification and comparison studies.

Data from the independent laboratory study on Paslite and F-AP analyses of cow's whole milk, skim milk, chocolate milk, cream, goat's milk, and sheep's milk are presented in Table 2. The means ($n = 3$), SDs, and maximum difference between the two determinations are compared with the Paslite *R* and *r* values as was done with the manufacturer data in Table 1. The regulatory action limit of 350 mU/liter alkaline phosphatase activity was between the raw milk spiking levels of 0.0313 and 0.0625%. The F-AP results were similar to the manufacturer data; most of the individual value differences were within *r* and the means were within *R* of the Paslite method *(8, 17)*. With cow's whole milk, chocolate milk, and sheep's milk, for none of the studied levels did m▲ exceed *R*. For cream, there was a potential sample swapping error that may explain m▲ values that exceeded *R* at the spiking levels of 0.0156% (~50 mU/liter) and 0.0078% (~100 mU/liter). Skim milk values at 0.0313% (~350 mU/liter) and the 350 calibrator exceeded *R* by 27 and 49 mU/liter, respectively, which would explain the higher trend in the F-AP modification values in this data set. The skim milk 0.5% (~5,000 mU/liter) *R* was exceeded by 80 mU/liter, and the 0.0039% (~50 mU/liter) *R* was exceeded by 11 mU/liter, reflecting the higher F-AP values. Skim milk 0.0625% (~500 mU/liter), 0.0079% (~100 mU/liter), and positive control were in the range of *R* values. Goat's milk *R* values were in the range with the exception of the 0.625% (~500 mU/liter) and the positive control and the 350 mU/liter

calibrator, reflecting the slightly higher trend of F-AP modification values in comparison to Paslite values. The analysis duplicates the manufacturer data of Table 1 in that most values at various spiking levels and for all matrices as determined by F-AP modification were not significantly different from the Paslite values.

The independent laboratory also evaluated the same spiked samples with the Fluorophos method and the original Paslite method using Charm II analyzers. The means ($n = 3$) and SDs for all four methods are presented in Table 3. In most cases, the fluorometric method had an SD that was lower than or, in the case of chocolate milk, comparable to that of the chemiluminescent method, whereas the Charm II Paslite 6600 (Chemi-6600) method had the highest SD. The difference could be due to the fluorescent versus the photomultiplier chemiluminescent detection equipment, particularly the dual photomultiplier of the 6600 method, which creates a larger nonlinear signal. Although the larger SDs imply a greater uncertainty near the action level, these SDs are generally less than 10% of the 350 mU/liter intensity, which indicates an acceptable level of quantification and suitable determination of the efficacy of pasteurization because properly processed samples are normally below the method limit of detection of 20 mU/liter.

The most discrepant method of alkaline phosphatase analysis evaluated was the Chemi-6600 method, which reads luminescence by scintillation detection. Signals are converted with C2soft to milliunits per liter based on log-log linear regression analysis of three calibrators. Spiked samples near the 350 mU/liter concentration tended to have higher values but were still within 50% of the other determinations. Spiking levels above the highest calibrator concentration (350 mU/liter) extrapolated to higher values because of the nonlinear scintillation detection. Positive bias in milliunits per liter was not a concern from a public health perspective because the phosphatase activity was overestimated, and these levels are well above the public health action level. In the U.S. national proficiency testing studies, the Paslite Chemi-6600 produced results that were most divergent from those of the other methods *(23)*.

To compare the different methods, the means of the chemiluminescence methods were arbitrarily normalized to the fluorometric method means. These normalized data are shown in Table 4 to allow method comparison. In general, the alkaline phosphatase values obtained with the chemiluminescent methods were equal to or higher than the results obtained with the fluorometric method at the spiking levels that bracket the actionable level of 350 mU/liter. Results for concentrations lower than 350 mU/liter and higher than 50 mU/liter are generally equivalent within $\pm 40\%$, which can be considered equivalent to the Paslite Chemi-Lum based on the published R values and is consistent with other unpublished data comparing methods. Some Chemi-F-AP values for skim milk and goat's milk were higher than those obtained with the Paslite Chemi-Lum method. Chocolate milk values were in agreement between methods and were not lower than the F-AP values obtained by the manufacturer. Lack of similar data between laboratories may be explained by calibration differences between the F-AP and Paslite methods. The residual enzyme activities with all methods are dependent on the species of raw milk and the matrix and detection method.

The normalization data indicated that the Chemi-F-AP method was most similar to the Paslite Chemi-Lum method, as also indicated by the data in Tables 1 and 2. At the concentrations that bracket the 350 mU/liter action level, values obtained with both the Paslite and the F-AP methods were above the action level, with a 0 to 100% frequency compared with the fluorometric analysis, and the degree of the positive trend was matrix dependent. Cream had the most consistent 80% positive difference compared with the values obtained with the fluorometric method.

The tendency of chemiluminescent methods to generate higher alkaline phosphatase values in comparison to the fluorometric method may be due to an enzyme substrate specificity difference; the chemiluminescent methods use the same dioxetane substrate, whereas the fluorometric method uses a different compound. Because there is no reference standard for alkaline phosphatase and the definition of units is based on hydrolysis of substrate per minute by the enzyme, specificity of substrate hydrolysis in various hydrophobic and hydrophilic environments may explain the differences in the alkaline phosphatase values obtained. The data presented in this study could be used to establish correlations that would allow researchers to equate the differences between various methods for each matrix, allowing comparison of samples tested by different methods, e.g., in method comparison studies and interlaboratory studies such as proficiency testing programs. The manufacturers' method development data and single laboratory verification data comparing existing methods using raw milk for spiking milk and fluid dairy drinks of various species have been presented elsewhere *(1, 16)*. The data were submitted to the 2009 NCIMS for adoption into the PMO.

The alkaline phosphatase values obtained produced by independent laboratory testing of samples of milk and fluid dairy drinks spiked with raw milk were consistent with the data generated by the test manufacturer for similarly prepared milk samples. The 45-s or 90-s F-AP modification method and chemiluminescent method (Paslite) yield equivalent alkaline phosphatase values in milk and fluid dairy drinks. Alkaline phosphatase values obtained with the F-AP method were similar to those obtained with the Paslite NovaLUM method or were higher near the 350 mU/liter action level. Values obtained with both methods were correlated linearly with the amount of raw milk used for spiking, and limits of detection and quantitation in milk and fluid dairy drinks were similar. Determination by all methods of alkaline phosphatase in samples spiked with raw milk was dependent on the type of raw milk used (cow, goat, or sheep) the type of matrix (whole milk, skim milk, cream, or chocolate milk) being tested. Additional work is needed to equate some of the positive differences obtained using the chemiluminescent methods in relation to the fluorometric method. The F-AP modification method was proposed and accepted by the NCIMS at their 2009 conference as an alternative official method for detecting

TABLE 1. *Alkaline phosphatase values determined by the manufacturer using the F-AP and Paslite NovaLUM methods*[a]

	Cow's whole milk		Cow's skim milk		Cow's light cream		Sheep's whole milk		Goat's whole milk[b]		Cow's chocolate milk	
Mean % raw milk	F-AP (mU/liter)	Paslite (mU/liter)	F-AP (mU/liter)	Paslite (mU/liter)	F-AP (mU/liter)	Paslite (mU/liter)	F-AP (mU/liter)	Paslite (mU/liter)	F-AP (mU/liter)	Paslite (mU/liter)	F-AP (mU/liter)	Paslite (mU/liter)
0.5	5,494 ± 376	5,915 ± 69	4,568 ± 210	4,915 ± 200	6,804 ± 239	5,998 ± 38	7,082 ± 290	5,829 ± 64	7,832 ± 112	7,936 ± 241	3,701 ± 40	5,522 ± 170
Δ	421		347		806		1,253		104		1,821	
m▲	884		810		996		1,517		481		1,977	
r	846		505		351						915	
R	1,090		1,052		1,151						1,920	
0.0625	867 ± 20	715 ± 6	649 ± 11	648 ± 12	853 ± 31	852 ± 45	1,025 ± 28	864 ± 24	1,109 ± 44	1,090 ± 20	480 ± 13	699 ± 29
Δ	152		1		1		161		19		219	
m▲	180		23		89		218		88		263	
r	95		88		49				69		151	
R	195		128		179				172		244	
0.0313	418 ± 18	371 ± 21	336 ± 13	337 ± 10	436 ± 18	411 ± 9	506 ± 24	467 ± 8	592 ± 32	518 ± 10	246 ± 11	364 ± 5
Δ	47		1		25		39		74		118	
m▲	157		26		50		67		117		134	
r	126		41		44		76		57		50	
R	157		81		113		225		139		131	
0.0156	223 ± 33	218 ± 10	159 ± 5	169 ± 4	192 ± 3	213 ± 2	312 ± 17	226 ± 9	294 ± 18	303 ± 12	121 ± 10	184 ± 5
Δ	5		10		21		86		9		63	
m▲	63		20		75		113		35		75	
r							81					
R							194					
0.0078	109 ± 2	110 ± 2	81 ± 8	86 ± 6	82 ± 37	108 ± 2	160 ± 3	104 ± 7	153 ± 12	156 ± 5	43 ± 8	99 ± 3
Δ	1		5		26		56		3		56	
m▲	5		22		72		67		24		63	
r	38		12		23		28		23		21	
R	46		39		38		61		53		85	
0.0039	59 ± 3	59 ± 3	37 ± 4	47 ± 2	27 ± 23	55 ± 5	92 ± 7	43 ± 2	78 ± 5	74 ± 9	19 ± 0	44 ± 6
Δ	0		10		28		49		4		25	
m▲	6		15		60		57		17		31	
r	16		12		16		18		14		13	
R	40		22		27		51		26		51	
0.002	29 ± 3	29 ± 2	14 ± 1	22 ± 2	8 ± 9	23 ± 3	49 ± 6	18 ± 2	36 ± 5	39 ± 3	4 ± 10	11 ± 4
Δ	0		8		15		31		3		7	
m▲	6		10		26		40		10		19	

TABLE 1. *Continued*

	Cow's whole milk		Cow's skim milk		Cow's light cream		Sheep's whole milk		Goat's whole milk[b]		Cow's chocolate milk	
Mean % raw milk	F-AP (mU/liter)	Paslite (mU/liter)	F-AP (mU/liter)	Paslite (mU/liter)	F-AP (mU/liter)	Paslite (mU/liter)	F-AP (mU/liter)	Paslite (mU/liter)	F-AP (mU/liter)	Paslite (mU/liter)	F-AP (mU/liter)	Paslite (mU/liter)
0.0	5 ± 2	0 ± 0	0 ± 0	0 ± 0	0 ± 0	1 ± 1	0 ± 0	0 ± 0	0 ± 0	0 ± 0	6 ± 9	12 ± 3
Δ	5		0		1		0		0		6	
m▲	8		0		2		0		0		16	
r	14		3		5		1		6		2	
R	40		3		13		7		19		11	
350	363 ± 13	342 ± 13	319 ± 39	291 ± 15	370 ± 15	376 ± 13	361 ± 8	335 ± 12	391 ± 24	356 ± 9	357 ± 15	375 ± 32
Δ	21		28		6		26		35		18	
m▲	42		69		37		44		66		66	
r	126		41		44		81		57		50	
R	157		81		113		194		139		131	
Positive control	403 ± 5	421 ± 11	395 ± 27	421 ± 13	531 ± 68	430 ± 4	458 ± 4	420 ± 4	478 ± 14	482 ± 14	350 ± 7	410 ± 18
Δ	18		26		101		38		4		60	
m▲	34		69		149		62		30		89	
r	95		88		49		76		69		151	
R	195		128		179		225		172		244	

[a] Values are mean ± SD of three measurements. Mean difference (Δ) and max difference (m▲) are compared with similar collaboratively studied concentrations using reproducibility (*R*) and repeatability (*r*) values.

[b] Spiking levels for goat's milk are 10 times the listed levels. For example, for the listed 0.5% spiking level, goat milk samples were spiked with 5.0% raw goat's milk.

TABLE 2. *Alkaline phosphatase values determined by an independent laboratory using the F-AP and Paslite NovaLUM methods*[a]

	Cow's whole milk		Cow's skim milk		Cow's light cream		Sheep's whole milk		Goat's whole milk[b]		Cow's chocolate milk	
Mean % raw milk	F-AP (mU/liter)	Paslite (mU/liter)	F-AP (mU/liter)	Paslite (mU/liter)	F-AP (mU/liter)	Paslite (mU/liter)	F-AP (mU/liter)	Paslite (mU/liter)	F-AP (mU/liter)	Paslite (mU/liter)	F-AP (mU/liter)	Paslite (mU/liter)
0.5	3,647 ± 53	4,384 ± 241	5,446 ± 69	4,566 ± 243	5,635 ± 139	5,526 ± 432	9,987 ± 243	10,108 ± 62	6,138 ± 191	4,010 ± 150	4,071 ± 129	3,427 ± 116
Δ	737		880		109		121		2,128		644	
m▲	999		1,132		800		478		2,501		855	
r	846		505		351						915	
R	1,090		1,052		1,151						1,920	
0.0625	518 ± 39	562 ± 24	675 ± 6	605 ± 20	753 ± 27	756 ± 42	1284 ± 24	1299 ± 7	748 ± 29	519 ± 25	578 ± 10	423 ± 7
Δ	44		70		3		15		229		155	
m▲	99		96		83		48		278		172	
r	95		88		49				69		151	
R	195		128		179				172		244	
0.0313	255 ± 19	263 ± 15	359 ± 9	275 ± 16	312 ± 14	332 ± 29	614 ± 1	668 ± 26	373 ± 24	266 ± 10	231 ± 14	188 ± 14
Δ	8		84		20		54		107		43	
m▲	33		108		65		74		138		62	
r	126		41		44		76		57		50	
R	157		81		113		225		139		131	
0.0156	116 ± 7	127 ± 4	200 ± 15	146 ± 9	175 ± 7	125 ± 8	284 ± 15	343 ± 13	171 ± 13	132 ± 6	128 ± 8	84 ± 8
Δ	11		54		50		59		39		44	
m▲	23		76		66		91		57		62	
r							81					
R							194					
0.0078	57 ± 5	67 ± 1	100 ± 4	75 ± 1	69 ± 10	54 ± 2	142 ± 2	171 ± 11	98 ± 14	64 ± 3	67 ± 3	41 ± 8
Δ	10		25		15		29		34		26	
m▲	16		29		28		41		45		62	
r	38		12		23		28		23		21	
R	46		39		38		61		53		85	
0.0039	39 ± 2	42 ± 2	50 ± 1	39 ± 2	31 ± 10	11 ± 2	58 ± 4	84 ± 6	43 ± 3	30 ± 1	33 ± 2	72 ± 10
Δ	3		11		20		26		13		39	
m▲	7		33		29		37		17		53	
r	16		12		16		18		14		13	
R	40		22		27		51		26		51	
0.002	19 ± 1	20 ± 2	23 ± 3	18 ± 1	0 ± 0	0 ± 0	18 ± 6	44 ± 6	9 ± 2	16 ± 1	16 ± 4	11 ± 14
Δ	1		6		0		26		7		5	
m▲	4		8		0		34		10		26	

TABLE 2. *Continued*

	Cow's whole milk		Cow's skim milk		Cow's light cream		Sheep's whole milk		Goat's whole milk[b]		Cow's chocolate milk	
Mean % raw milk	F-AP (mU/liter)	Paslite (mU/liter)	F-AP (mU/liter)	Paslite (mU/liter)	F-AP (mU/liter)	Paslite (mU/liter)	F-AP (mU/liter)	Paslite (mU/liter)	F-AP (mU/liter)	Paslite (mU/liter)	F-AP (mU/liter)	Paslite (mU/liter)
0.0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 1	9 ± 1	0 ± 0	0 ± 0	1 ± 2	0 ± 0
Δ	0		0		0		9		0		1	
m▲	0		0		0		10		0		4	
r	14		3		5		1		6		2	
R	40		3		13		7		19		11	
350	370 ± 22	333 ± 10	422 ± 22	327 ± 13	351 ± 13	405 ± 11	366 ± 17	414 ± 4	492 ± 14	365 ± 17	411 ± 24	353 ± 7
Δ	37		95		54		48		127		58	
m▲	65		130		70		70		157		93	
r	126		41		44		81		57		50	
R	157		81		113		194		139		131	
Positive control	409 ± 13	399 ± 21	512 ± 10	444 ± 19	372 ± 6	478 ± 11	431 ± 23	487 ± 2	553 ± 40	401 ± 21	419 ± 16	402 ± 21
Δ	10		68		106		56		152		17	
m▲	41		98		120		83		200		56	
r	95		88		49		76		69		151	
R	195		128		179		225		172		244	

[a] Values are mean ± SD of three measurements. Mean difference (Δ) and max difference (m▲) are compared with similar collaboratively studied concentrations using reproducibility (*R*) and repeatability (*r*) values.

[b] Spiking levels for goat's milk are 10 times the listed levels. For example, for the listed 0.5% spiking level, goat milk samples were spiked with 5.0% raw goat's milk.

TABLE 3. *Alkaline phosphatase values obtained by four methods for samples spiked with raw milk*

	Chemi-F-AP (mU/liter)		Paslite Chemi-Lum (mU/liter)		Paslite Chemi-6600 (mU/liter)		Fluorophos (mU/liter)	
Mean % raw milk	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Cow's whole milk								
0.5	3,647	53	4,384	241	8,817	298	3,908	71
0.0625	518	39	562	24	722	126	510	14
0.03125	255	19	263	15	276	23	250	4
0.0156	116	7	127	4	117	20	129	2
0.0078	57	1	67	5	49	12	68	0
0.0039	39	2	42	2	31	6	44	1
0.002	19	1	20	2	31	6	23	1
Negative	0	0	0	0	31	6	<10	
Cow's skim milk								
0.5	5,446	69	4,566	243	13,570	1,034	3,829	44
0.0625	675	6	605	20	859	101	481	6
0.03125	359	9	275	16	289	39	223	3
0.0156	200	15	146	9	140	13	111	3
0.0078	100	4	75	1	57	7	53	4
0.0039	50	1	39	2	48	8	22	2
0.002	23	3	18	1	27	8	<10	
Negative	0	0	0	0	19	0	<10	
Cow's light cream								
0.5	5,635	432	5,526	139	19,619	700	3,179	166
0.0625	753	27	756	42	461	47	443	29
0.03125	312	14	332	29	334	23	212	2
0.0156	175	7	125	8	107	12	133	4
0.0078	69	10	54	2	58	11	93	5
0.0039	31	10	11	2	39	12	55	2
0.002	0	0	0	0	18	12	44	5
Negative	0	0	0	0	18	12	33	2
Sheep's whole milk								
0.5	9,987	243	10,108	62	87,112	4,983	6,727	158
0.0625	1284	24	1299	7	2999	149	949	6
0.03125	614	1	668	26	904	60	500	9
0.0156	284	15	343	13	340	18	268	9
0.0078	142	2	171	11	184	11	152	5
0.0039	58	6	84	6	98	14	94	3
0.002	18	3	44	5	28	7	68	2
Negative	0	1	9	1	36	0	35	1
Goat's whole milk								
5.0	6,138	191	4,010	150	9,348	686	3,026	32
0.625	748	29	519	26	456	85	408	13
0.3125	373	24	266	10	211	19	224	3
0.156	171	13	132	6	108	16	127	4
0.078	98	14	64	3	63	5	74	3
0.039	43	3	30	1	30	10	49	1
0.02	9	1	16	2	23	6	34	1
Negative	0	0	0	0	23	6	<10	
Cow's chocolate milk								
0.5	4,071	129	3,427	116	8,858	236	3,339	30
0.0625	578	10	423	7	528	48	408	13
0.03125	231	14	188	13	177	12	204	6
0.0156	128	8	84	8	117	13	100	2
0.0078	67	3	41	8	55	4	55	1
0.0039	33	2	72	10	44	31	30	1
0.002	16	4	11	14	38	33	<10	
Negative	1	2	0	0	46	41	<10	

TABLE 4. *Alkaline phosphatase values obtained with fluorometric and chemiluminescent methods and the normalized difference for milk samples spiked with raw milk*[a]

Type of milk	Mean % raw milk[b]	Alkaline phosphatase activity (mU/liter)[c]				Normalized chemiluminescence values (%)[d]		
		Fluorophos	Chemi-Lum	Chemi-F-AP	Chemi-6600	Chemi-Lum	Chemi-F-AP	Chemi-6600
Cow's whole milk	0.5	3,908	4,384	3,647	8,817	12.20	−6.70	125.60
	0.0625	**510**	**562**	**518**	**722**	**10.20**	**1.50**	**41.50**
	0.03125	**250**	**263**	**255**	**276**	**5.40**	**2.20**	**10.60**
	0.0156	129	127	116	117	−1.60	−10.60	−9.80
	0.0078	68	67	57	49	−1.40	−16.20	−28.00
	0.0039	44	42	39	31	−2.80	−10.50	−28.10
	0.002	23	20	19	31	−13.50	−16.40	35.40
	Negative	<10	0	0	31			
Cow's skim milk	0.5	3,829	4,566	5,446	13,570	19.20	42.20	254.40
	0.0625	**481**	**605**	**675**	**859**	**26.00**	**40.50**	**78.80**
	0.03125	**223**	**275**	**359**	**289**	**23.40**	**60.90**	**29.50**
	0.0156	111	146	200	140	31.80	80.70	26.40
	0.0078	53	75	100	57	40.00	86.90	6.90
	0.0039	22	39	50	48	78.20	125.10	119.00
	0.002	<10	18	23	27			
	Negative	<10	0	0	19			
Cow's chocolate milk	0.5	3,339	3,427	4,071	8,858	2.60	21.90	165.30
	0.0625	**408**	**423**	**578**	**528**	**3.70**	**41.50**	**29.30**
	0.03125	**204**	**188**	**231**	**177**	**−7.80**	**13.20**	**−13.20**
	0.0156	100	84	128	117	−16.50	28.10	17.10
	0.0078	55	41	67	55	−25.80	22.80	−0.30
	0.0039	30	72	33	44	138.70	9.40	45.90
	0.002	<10	11	16	38			
	Negative	<10	0	1	46			
Cow's light cream	0.5	3,179	5,526	5,635	19,619	73.80	77.30	517.20
	0.0625	**443**	**756**	**753**	**461**	**70.50**	**69.80**	**4.10**
	0.03125	**212**	**332**	**312**	**334**	**56.50**	**47.10**	**57.40**
	0.0156	133	125	175	107	−5.90	31.70	−19.20
	0.0078	93	54	69	58	−41.60	−26.20	−37.30
	0.0039	55	11	31	39	−80.10	−43.80	−30.00
	0.002	44	0	0	18			
	Negative	33	0	0	18			
Goat's whole milk	5.0	3,026	4,010	6,138	9,348	32.50	102.80	208.90
	0.625	**408**	**519**	**748**	**456**	**27.10**	**83.30**	**11.60**
	0.3125	**224**	**266**	**373**	**211**	**18.60**	**66.50**	**−6.00**
	0.156	127	132	171	108	4.30	34.80	−14.90
	0.078	74	64	98	63	−13.30	32.10	−14.60
	0.039	49	30	43	30	−39.20	−11.30	−39.20
	0.02	34	16	9	23	−53.40	−73.80	−32.00
	Negative	<10	0	0	23			
Sheep's whole milk	0.5	6,727	10,108	9,987	87,112	50.30	48.50	1,195.00
	0.0625	949	1,299	1,284	2,999	36.90	35.40	216.20
	0.03125	**500**	**668**	**614**	**904**	**33.70**	**22.90**	**80.70**
	0.0156	**268**	**343**	**284**	**340**	**28.00**	**5.80**	**26.80**
	0.0078	152	171	142	184	11.90	−6.60	20.90
	0.0039	94	84	58	98	−10.30	−37.70	4.70
	0.002	68	44	18	28	−34.90	−73.60	−58.90
	Negative	35	9	0	36			

[a] Bold values bracket the alkaline phosphatase action level of 350 mU/liter.
[b] Samples were spiked with raw milk of the same species.
[c] Values are the mean of three replicates.
[d] Means of the chemiluminescence methods were arbitrarily normalized to the fluorometric method means: % = (chemiluminescent/fluorescent − 1) × 100.

alkaline phosphatase in grade A milk products at the U.S. regulatory action level of 350 mU/liter as specified in the PMO *(24)*.

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