# Comparison of Phosphatase Methods used for Milk Pasteurization Verification

S. M. Albillos[1], R. Reddy[2], and R. Salter[3]

[1]National Center for Food Safety and Technology, [2]U.S. Food and Drug Administration; 6502 S. Archer Rd., Summit-Argo, IL, [3]Charm Sciences Inc., Lawrence, MA

## Abstract

Chemiluminescent and Fluorometric methods are the recognized methods for detection of alkaline phosphatase, a heat susceptible enzyme used as a retrospective indicator for milk pasteurization. Proficiency test data collected from official state laboratories indicated that the chemiluminescent method may be more sensitive versus the fluorometric method. However, there are no phosphatase reference standards for comparison. Chemiluminescent, fluorometric and fast chemiluminescent assays were compared in this study. Cow whole, cow skim, goat, sheep and chocolate milks, and light cream were laboratory pasteurized and fortified with raw milk to attain phosphatase levels in the range of 20 to 5000mU/L. At 250-500 mU/L, levels that bracket the 350 mU/L action level, triplicate average results from the chemiluminescent methods were more positive than fluorometric method as listed: cow milk 2-10%; goat milk 11-77%; sheep milk 5-34%; skim milk 23-79%; chocolate milk 4-22%; and light cream 4-71%. Across the 20 to 5000 mU/L range, fast chemiluminescent method data was similar to the approved chemiluminescent method and linear correlation among the data from various methods was observed, except for chemiluminescent scintillation analyzer data which read at higher levels. The data can be used to develop conversion factors to make the mU/L readings of the various methods comparable.

## Introduction

Alkaline phosphatase is a heat sensitive enzyme in raw milk used as thermal marker for pasteurization. The FDA and ISO approved methods to detect presence of alkaline phosphatase utilize fluorometric or chemiluminescent substrates that are photo-activated in presence of alkaline phosphatase[1, 2]. The US and EU regulations specify that the pasteurized dairy products may contain less than 350 mU/L alkaline phosphatase[3, 4]. Preliminary work[1,2] as well as the data from the proficiency test from US official laboratories[5] indicate that the chemiluminescent method may have a positive bias relative to the fluorometric method at the action level of 350 mU/L. A faster chemiluminescent method that is easier to calibrate has also been developed that could further simplify alkaline phosphatase determination. The scope of the study is to evaluate the approved chemiluminescent and fluorometric methods in a number of dairy matrices and to compare a new chemiluminescent method (Fast Alkaline Phosphatase- FAP) to the existing methods.

## Materials and Methods

Laboratory controls for pasteurized milk products were prepared according to FDA 2400 series[6] Forms (heat treated at 95°C for 1 min) from whole milk, skim, chocolate, cream, sheep and goat milk. The pasteurized samples were spiked with the same species raw milk to attain various levels of residual alkaline phosphatase. Bovine and sheep milks were spiked with 0.5%, 0.065%, 0.03125%, 0.0155%, 0.0078%, 0.0039%, 0.002% and 0.001% of raw milk and goat milk was spiked with 5%, 0.65%, 0.3125%, 0.155%, 0.078%, 0.039%, 0.02% and 0.01% of raw goat milk. The added raw milk approximated a range of 20 – 7000 mU/L enzyme units for residual alkaline phosphatase activity in the spiked samples. The raw milk-spiked samples were analyzed by Fluorophos Test System (Advanced Instruments, Inc., Norwood, MA) and various Charm Sciences, Inc., (Lawrence, MA) chemiluminescent methods (PasLite™ with novaLum™, PasLite™ using Charm 6600/C2Soft© analyzer and FAP™ with novaLum™). Instrument calibrations and analysis of samples were performed according to FDA 2400 series and/or the manufacturer's instructions. The alkaline phosphatase activity at each level of spiking was compared by normalizing to the fluorometric value (Chemiluminescent mU/L / Fluorometric mU/L*100).

## Results

Tables 1-6 list the alkaline phosphatase activities detected by the various methods for the raw milk spiked milks and milk products. The normalized values to demonstrate the variance of the chemiluminescence methods compared to the fluorometric method are also presented. The normalized data show equivalence or a chemiluminescent positive bias at the spike levels that bracket the actionable level of 350 mU/L and higher. At concentrations less than 350 mU/L and greater than 50 mU/L results were generally ±40% which can be considered equivalent based on the published r and R values for the methods with the exception of some FAP values with skim milk that were biased more positively. The residual enzyme activities were dependent on the type of milk as well as the detection method.

## Conclusions

1. Chemiluminescent assays showed consistent positive bias when the residual alkaline phosphatase activities were compared in various types of milk (at levels bracketing the action level) by fluorometric and chemiluminescent methods.
2. Based on the data, a coefficient could be developed for equivalent comparisons of residual alkaline phosphatase activity in pasteurized milk products at the actionable levels of 350 mU/L.
3. The new FAP chemiluminescent method compares similarly to the PasLite™ with novaLum™ chemiluminescent method across the range of matrices and spike levels.

**Table 1.** Alkaline Phosphatase measurements of raw cow milk spiked into whole milk using fluorometric and chemiluminescent methods and the normalized difference of the chemiluminescent method using the fluorometric value.

| Alkaline Phosphatase Analysis of Samples by Method | | | | | Normalized Value-Chemiluminescent /Fluorescent | | |
|---|---|---|---|---|---|---|---|
| % Raw Milk in Whole Milk | Fluoro-metric | Chemi-Lum | Chemi-FAP | Chemi-6600 | Chemi-Lum | Chemi-FAP | Chemi-6600 |
| 0.5 | 3908 | 4384 | 3647 | 8817 | 12.2% | -6.7% | 125.6% |
| 0.0625 | 510 | 562 | 518 | 722 | 10.2% | 1.5% | 41.5% |
| 0.03125 | 250 | 263 | 255 | 276 | 5.4% | 2.2% | 10.6% |
| 0.0156 | 129 | 127 | 116 | 117 | -1.6% | -10.6% | -9.8% |
| 0.0078 | 68 | 67 | 57 | 49 | -1.4% | -16.2% | -28.0% |
| 0.0039 | 44 | 42 | 39 | 31 | -2.8% | -10.5% | -28.1% |
| 0.002 | 23 | 20 | 19 | 31 | -13.5% | -16.4% | 35.4% |
| Negative | <10 | 0 | 0 | 31 | - | - | - |

**Table 2.** Alkaline Phosphatase measurements of raw cow milk spiked into skim milk using fluorometric and chemiluminescent methods the normalized difference of the chemiluminescent method using the fluorometric value.

| Alkaline Phosphatase Analysis of Samples by Method | | | | | Normalized Value-Chemiluminescent /Fluorescent | | |
|---|---|---|---|---|---|---|---|
| % Raw Milk in Skim Milk | Fluoro-metric | Chemi-Lum | Chemi-FAP | Chemi-6600 | Chemi-Lum | Chemi-FAP | Chemi-6600 |
| 0.5 | 3829 | 4566 | 5446 | 13570 | 19.2% | 42.2% | 254.4% |
| 0.0625 | 481 | 605 | 675 | 859 | 26.0% | 40.5% | 78.8% |
| 0.03125 | 223 | 275 | 359 | 289 | 23.4% | 60.9% | 29.5% |
| 0.0156 | 111 | 146 | 200 | 140 | 31.8% | 80.7% | 26.4% |
| 0.0078 | 53 | 75 | 100 | 57 | 40.0% | 86.9% | 6.9% |
| 0.0039 | 22 | 39 | 50 | 48 | 78.2% | 125.1% | 119.0% |
| 0.002 | <10 | 18 | 23 | 27 | - | - | - |
| Negative | <10 | 0 | 0 | 19 | - | - | - |

**Table 3.** Alkaline Phosphatase measurements of raw goat milk spiked into whole goat milk using fluorometric and chemiluminescent methods the normalized difference of the chemiluminescent method using the fluorometric value.

| Alkaline Phosphatase Analysis of Samples by Method | | | | | Normalized Value-Chemiluminescent /Fluorescent | | |
|---|---|---|---|---|---|---|---|
| % Raw Goat Milk in Whole Goat Milk | Fluoro-metric | Chemi-Lum | Chemi-FAP | Chemi-6600 | Chemi-Lum | Chemi-FAP | Chemi-6600 |
| 5.0 | 3026 | 4010 | 6138 | 9348 | 32.5% | 102.8% | 208.9% |
| 0.625 | 408 | 519 | 748 | 456 | 27.1% | 83.3% | 11.6% |
| 0.3125 | 224 | 266 | 373 | 211 | 18.6% | 66.5% | -6.0% |
| 0.156 | 127 | 132 | 171 | 108 | 4.3% | 34.8% | -14.9% |
| 0.078 | 74 | 64 | 98 | 63 | -13.3% | 32.1% | -14.6% |
| 0.039 | 49 | 30 | 43 | 30 | -39.2% | -11.3% | -39.2% |
| 0.02 | 34 | 16 | 9 | 23 | -53.4% | -73.8% | -32.0% |
| Negative | <10 | 0 | 0 | 23 | - | - | - |

**Table 4.** Alkaline Phosphatase measurements of raw sheep milk spiked into whole sheep milk using fluorometric and chemiluminescent methods and the normalized difference of the chemiluminescent method using the fluorometric value.

| Alkaline Phosphatase Analysis of Samples by Method | | | | | Normalized Value-Chemiluminescent /Fluorescent | | |
|---|---|---|---|---|---|---|---|
| % Raw Sheep Milk in Whole Sheep Milk | Fluoro-metric | Chemi-Lum | Chemi-FAP | Chemi-6600 | Chemi-Lum | Chemi-FAP | Chemi-6600 |
| 0.5 | 6727 | 10108 | 9987 | 87112 | 50.3% | 48.5% | 1195.0% |
| 0.0625 | 949 | 1299 | 1284 | 2999 | 36.9% | 35.4% | 216.2% |
| 0.03125 | 500 | 668 | 614 | 904 | 33.7% | 22.9% | 80.7% |
| 0.0156 | 268 | 343 | 284 | 340 | 28.0% | 5.8% | 26.8% |
| 0.0078 | 152 | 171 | 142 | 184 | 11.9% | -6.6% | 20.9% |
| 0.0039 | 94 | 84 | 58 | 98 | -10.3% | -37.7% | 4.7% |
| 0.002 | 68 | 44 | 18 | 28 | -34.9% | -73.6% | -58.9% |
| Negative | 35 | 9 | 0 | 36 | - | - | - |

**Table 5.** Alkaline Phosphatase measurements of raw cow milk spiked into 2% chocolate milk using fluorometric and chemiluminescent methods and the normalized difference of the chemiluminescent method using the fluorometric value.

| Alkaline Phosphatase Analysis of Samples by Method | | | | | Normalized Value-Chemiluminescent /Fluorescent | | |
|---|---|---|---|---|---|---|---|
| % Raw Milk in Cboc. Milk | Fluoro-metric | Chemi-Lum | Chemi-FAP | Chemi-6600 | Chemi-Lum | Chemi-FAP | Chemi-6600 |
| 0.5 | 3339 | 3427 | 4071 | 8858 | 2.6% | 21.9% | 165.3% |
| 0.0625 | 408 | 423 | 578 | 528 | 3.7% | 41.5% | 29.3% |
| 0.03125 | 204 | 188 | 231 | 177 | -7.8% | 13.2% | -13.2% |
| 0.0156 | 100 | 84 | 128 | 117 | -16.5% | 28.1% | 17.1% |
| 0.0078 | 55 | 41 | 67 | 55 | -25.8% | 22.8% | -0.3% |
| 0.0039 | 30 | 72 | 33 | 44 | 138.7% | 9.4% | 45.9% |
| 0.002 | <10 | 11 | 16 | 38 | - | - | - |
| Negative | <10 | 0 | 1 | 46 | - | - | - |

**Table 6.** Alkaline Phosphatase measurements of raw cow milk spiked into light cream using fluorometric and chemiluminescent methods and the normalized difference of the chemiluminescent method using the fluorometric value.

| Alkaline Phosphatase Analysis of Samples by Method | | | | | Normalized Value-Chemiluminescent /Fluorescent | | |
|---|---|---|---|---|---|---|---|
| % Raw Milk in Light Cream | Fluoro-metric | Chemi-Lum | Chemi-FAP | Chemi-6600 | Chemi-Lum | Chemi-FAP | Chemi-6600 |
| 0.5 | 3179 | 5526 | 5635 | 19619 | 73.8% | 77.3% | 517.2% |
| 0.0625 | 443 | 756 | 753 | 461 | 70.5% | 69.8% | 4.1% |
| 0.03125 | 212 | 332 | 312 | 334 | 56.5% | 47.1% | 57.4% |
| 0.0156 | 133 | 125 | 175 | 107 | -5.9% | 31.7% | -19.2% |
| 0.0078 | 93 | 54 | 69 | 58 | -41.6% | -26.2% | -37.3% |
| 0.0039 | 55 | 11 | 31 | 39 | -80.1% | -43.8% | -30.0% |
| 0.002 | 44 | 0 | 0 | 18 | - | - | - |
| Negative | 33 | 0 | 0 | 18 | - | - | - |

Shaded samples bracket the action level of 350 mU/L

## References

1. International Organization for Standardization (ISO), 11816-1 IDF 155-1; 1997 (E).
2. International Organization for Standardization (ISO), 22160 IDF 209; 2007 (E).
3. US Department of Health and Human Services, (2005) Public Health Service Food and Drug Administration, Publication 229, Pasteurized Milk Ordinance.
4. Commission Regulation (EC) No. 1664/2006, Official J of the European Union
5. Food & Drug Administration. CFSAN-Laboratory Proficiency & Evaluation Team. 2006 – 2007 Milk Split Samples Reports.
6. US Department of Health and Human Services, (2007) Public Health Service Food and Drug Administration, Form 2400