Ionic Calcium Determination in Skim Milk with Molecular Probes and Front-Face Fluorescence Spectroscopy: Simple Linear Regression

R. R. Gangidi and L. E. Metzger¹

MN-SD Dairy Foods Research Center, Department of Food Science and Nutrition, University of Minnesota, St. Paul 55108

ABSTRACT

The purpose of this study was to determine if the ionic calcium content of skim milk could be determined using molecular probes and front-face fluorescence spectroscopy. Current methods for determining ionic calcium are not sensitive, overestimate ionic calcium, or require complex procedures. Molecular probes designed specifically for measuring ionic calcium could potentially be used to determine the ionic calcium content of skim milk. The goal of the current study was to develop foundation methods for future studies to determine ionic calcium directly in skim milk and other dairy products with molecular probes and fluorescence spectroscopy. In this study, the effect of pH on calcium-sensitive fluorescent probe (Rhod-5N and Fluo-5N) performance using various concentrations of skim milk was determined. The pH of diluted skim milk (1.9 to 8.9% skim milk), was adjusted to either 6.2 or 7.0, after which the samples were analyzed with fluorescent probes (1 μM) and front-face fluorescence spectroscopy. The ionic calcium content of each sample was also determined using a calcium ion-selective electrode. The results demonstrated that the ionic calcium content of each sample was highly correlated ($R^2 > 0.989$) with the fluorescence intensities of the probe-calcium adduct using simple linear regression. Higher than suggested ionic calcium contents of 1,207 and 1,973 μM were determined with the probes (Fluo-5N and Rhod-5N) in diluted skim milk with pH 7.0 and 6.2, respectively. The fluorescence intensity of the probe-calcium adduct decreased with a decrease in pH for the same ionic calcium concentration. This study demonstrates that Fluo-5N and Rhod-5N can be used to determine the ionic-calcium content of diluted milk with front-face fluorescence spectroscopy. Furthermore, these probes may also have the potential to determine the ionic calcium content of undiluted skim milk.

Key words: ionic calcium, fluorescent probe, fluorescence spectroscopy

INTRODUCTION

Ionic calcium concentration is known to influence the characteristics of skim milk. The ionic calcium concentration of skim milk varies with pH, storage temperature, and processing parameters (temperature and duration of the processing; Jenness and Patton, 1959; Fox and McSweeney, 1998). The storage of skim milk at low temperatures increases the ionic calcium concentration because of the solubilization of the calcium present as colloidal calcium phosphate (CCP; Davies and White, 1960), whereas heating skim milk reduces the ionic calcium content (Christianson et al., 1954; Baker and Gehrke, 1956; Tessier and Rose, 1958; DeMott, 1968; Muldoon and Liska, 1972). A decrease in pH also increases the concentration of ionic calcium because of the solubilization of the CCP (Rose, 1968); in contrast, an increase in pH decreases the ionic calcium because of the transfer of ionic calcium into the colloidal phase (Fox and McSweeney, 1998).

The other forms of calcium present in skim milk, in addition to the soluble ionic calcium, are the soluble calcium present as calcium citrate (complex), calcium phosphate, and the insoluble form associated with caseins as CCP. Approximately, 7 to 10% (2 to 3 mM) of the total calcium (30 mM) exists as ionic calcium (Smeets, 1955; Van Kreveld and Van Minnen, 1955; Fox and McSweeney, 1998). Another 18 to 19% (5 to 6 mM) of the calcium exists in a soluble form as calcium citrate; 3 to 4% exists as calcium phosphate (0.9 to 1.2 mM); and the bulk of the remaining 66% of the calcium (20 mM) is present as CCP (Smeets, 1955; Fox and McSweeney, 1998).

Several methods have been developed for determination of the ionic calcium content of skim milk. Some examples are the use of cation exchange resins (Christianson et al., 1954) or various extraction methods to obtain skim milk serum, including permeate from ultrafiltration, diffusate from dialysis, serum from ultracentrifugation, and whey from rennet-induced coagulation (Davies and White, 1960). However, skim milk serum extraction techniques may not provide a true representation of the ionic calcium content of skim milk as some of the ions are not permeated or diffused (DeMan, 1962). The problem of extracting skim milk serum was solved

Received April 25, 2006.

Accepted May 28, 2006.

¹Corresponding author: lmetzger@umn.edu

with the advent of ion selective electrodes, which can be used directly in skim milk (Demott, 1968; Muldoon and Liska, 1969; Geerts et al., 1983). Early research demonstrated that there was no significant difference between the ionic calcium results obtained by the cation exchange resin method and the ion selective electrode (Muldoon and Liska, 1969).

An ion selective electrode measures the calcium ion activity instead of the actual ionic calcium concentration. The calcium ion activity is approximately 40% of the calcium ion concentration at a typical skim milk ionic strength of approximately 0.08 *M* (Van Kreveld and Van Minnen, 1955; Geerts et al., 1983). The typical commercial ion selective electrodes are designed for aqueous solutions, and the electrode has a membrane, usually made of polyvinyl chloride, containing a calcium selective ion exchanger. The electrode develops a potential across the membrane in the presence of calcium ions, and the potential difference is used in the determination of ionic activity, which can be used to determine the calcium ion concentration. Initially a calibration curve is developed with known concentrations of calcium standards, and the calibration equation is used to determine the calcium concentration in skim milk based on a semi logarithmic plot between electrode electron motive force (mV) and logarithmic ionic calcium concentration. The use of a logarithmic data transformation suggests that the determination of ionic calcium in skim milk may not be sensitive. In addition, the calcium ion sensitive membrane is prone to contamination and in some cases may affect the results, or may require regular cleaning or decontamination (May, 1995).

The previously described problems with the existing methods for measuring ionic calcium could be solved with the use of fluorescent molecular probes. These probes bind specifically to the calcium ion, and when excited with a specific wavelength of light, an emission spectrum at a higher wavelength is observed. The qualities of an ideal probe include a low energy excitation and emission (preferably in the higher regions of the visible spectrum), low emission of the probe when ionic calcium is absent, and a high dissociation constant (K_d ; Minta et al., 1989). A low energy excitation and an emission in the visible region are preferred over high energy excitation in the ultraviolet region, which can cause autofluorescence or degradation/photobleaching of the probe. Autofluorescence is due to the fluorescence of the inherent/intrinsic components present in the sample. The dissociation constant (K_d ; Units, μM) of a probe is the ratio of the equilibrium concentration of the calcium ions and probe, to the calcium ion-probe complex ($K_d = [Ca^{2+}][Probe]/[Ca^{2+} - Probe]$). Higher concentrations of ionic calcium can be determined with

probes that have a higher dissociation constant ($0.1 < K_d < 10$; Valeur, 1998). Calcium ion sensitive molecular probes currently available were designed for the cytosolic pH of 7.0 and the measurement of ionic calcium concentrations in the range of a few nanomoles to micromoles. However, the calcium ion concentrations of skim milk range between 2 and 3 *mM*, which is substantially higher than the probe was designed to measure. Additionally, skim milk has a pH of 6.6 to 6.7, which is slightly less than the suggested pH for the probes. It is generally agreed that performance of the probes is affected by pH because pH can result in structural changes in the probes. Fluo-5N and Rhod-5N are 2 available fluorescent probes that have high dissociation constants of 90 and 320 μM , respectively, and these probes could potentially be used to determine the ionic calcium content of skim milk (Molecular Probes, Eugene, OR). Fluo-5N and Rhod-5N are based on the structural modification of 1, 2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetra acetic acid, a calcium-specific chelator, and xanthene (Minta et al., 1989). Fluo-5N and Rhod-5N are analogues of the well-known fluorescent compounds, fluorescein and rhodamine.

Fluorescence spectroscopy measures the excitation and emission of ultraviolet or visible light of an analyte, and hence can be used for quantification of analyte concentration based on the principles of spectroscopy. However, traditional right angle fluorescence spectroscopic techniques cannot be applied to thick substances, such as skim milk, due to large absorbance and scattering of light (Genot et al., 1992). Therefore, Parker (1968) developed a technique to reduce the scattering effect by changing the angle of incidence onto the sample from 90 to 56°. This technique is known as front-face fluorescence spectroscopy (FFFS), and can be used to measure fluorescence of turbid and concentrated samples, such as skim milk. Typically, multivariate statistical methods such as partial least squares regression are used to relate variations in spectra to the functional properties of foods (Martens and Naes, 1989). The FFFS technique has been used to determine the light-induced oxidation of the riboflavin in yogurt (Becker et al., 2003) and cheese (Wold et al., 2005); and to determine the Maillard reaction products, lactulose and furosine (Kulmyrzaev and Dufour, 2002), in heat-treated skim milk. Visco-elastic properties (Karoui and Dufour, 2003), molecular structure, ripening stages, and sensory properties of soft cheeses (Herbert et al., 2000) and hard or semihard cheeses (Dufour et al., 2000) were determined based on changes in the intrinsic fluorophores, vitamin A, and tryptophan, with the use of front-face fluorescence spectroscopy. In our laboratory, a study that utilized FFFS to predict process cheese meltability index, which is determined by dynamic

stress rheometry, was successfully completed (Gari-mella Purna et al., 2005). In a similar fashion, FFFS spectroscopy and the use of calcium ion sensitive fluorescent probes, such as Fluo-5N and Rhod-5N, could be used to measure the ionic calcium content of skim milk.

The objective of this study was to investigate the effect of pH on the fluorescence of calcium ion sensitive probes and to develop simple linear regression models to determine the calcium ion concentration of diluted skim milk utilizing ionic calcium sensitive molecular fluoroprobes with FFFS.

MATERIALS AND METHODS

Molecular Probes

Calcium binding molecular probes with a high K_d were obtained (Molecular Probes). The probes were Fluo-5N ($K_d = 90 \mu M$) and Rhod-5N ($K_d = 320 \mu M$). These probes can be excited at a single wavelength, and the emission spectra can be collected at a range of wavelengths in the visible region of the electromagnetic spectrum (Table 1). These probes show an increase in emission intensity with an increase in the formation of probe and calcium-ion adduct, until the probe is saturated.

Effect of pH on Fluorescent Properties of the Probes

The pH of distilled water was adjusted to 5.8 or 7.0 with 0.01 N NaOH or 0.01 N HCl, respectively. Subsequently, $CaCl_2$ was added to each pH-adjusted water to obtain an ionic calcium concentration of 125 μM . Each sample was prepared in duplicate, and one sample was spiked with 1 μM of Fluo-5N and another sample was spiked with 1 μM Rhod-5N. The sample-containing probe was excited, and the emission spectrum was collected with an Aminco-Bowman II Luminescence spectrometer (Thermo-Electron Corp., Madison, WI). The emission and excitation wavelengths of the probes were predetermined as suggested by manufacturer (Table 1). The voltage of the photo-multiplier tube (detector) was set at 700 V. The excitation band pass was 1 nm, and the emission band pass was 4 nm. The scan speed was set at 5 nm/s. Spectral data was collected in duplicate and then averaged. Each sample was analyzed with a right angle accessory (FP-111, Thermo-Electron Corp.). Preprocessing of the spectra was performed using the AB2 Luminescence spectrometer software version 5.5 (Thermo-Electron Corp.).

Effect of Skim Milk pH and Dilution on Free Calcium Ion Content

A G-P combo w/RJ pH electrode (Corning, Corning, NY) connected to a pH/ion meter 340 (Corning) was

Table 1. Selected properties of calcium ion-specific molecular probes. (compiled from Molecular Probes¹ handbook)

Item	Probe	
	Fluo-5N	Rhod-5N
Molecular weight	958	900
Dissociation constant K_d (μM)	90	320
Excitation wavelength (nm)	470	549
Emission wavelength(s) (nm)	500 to 560	560 to 600
Ca^{2+} detection range (μM)	1 to 1,000	10 to 1,000

¹Molecular Probes, Eugene, OR.

used in adjusting the pH of undiluted skim milk and in the determination of the pH of the diluted skim milk. The pH of skim milk (from a local grocery store) was adjusted to 5.5 by adding 1 N HCl. Skim milk "as is" (pH = 6.6) was also used in the study. The skim milk was added to distilled water containing 1.96% of a 4 M KCl ionic strength adjustment buffer to obtain skim milk concentrations in the range of 1.9 to 8.9%. The range of skim milk concentrations were selected such that the concentration of the calcium ion present would fall within the probes' suggested detection range (~1 mM or 1,000 μM) at pH of approximately 7.0. The free calcium ion concentration of the diluted skim milk was determined using an epoxy-body combination-ion-selective electrode (Cole-Parmer, Niles, IL) connected to the Corning pH/ion meter 340. The reference solution was 4 M KCl solution (Cole-Parmer). The ion selective electrode was calibrated with a diluted 1,000-ppm (25-mM) calcium standard (Phoenix Electrodes, Houston, TX) in the range of 4 to 2,000 μM . This range was selected to reflect the Ca^{2+} concentrations in the pH adjusted diluted skim milk. Ionic strength adjustor, 4 M KCl (Phoenix Electrodes), was added (1.96%) to the diluted calcium standards. Calibration and slope checks on the ion selective electrode were performed regularly before the samples were analyzed.

FFFS

Probe Fluo-5N (50 μM) was dissolved in pH 7.0 skim milk, and similarly the probe (50 μM) was added to distilled water containing 1.96% of the 4 M KCl (ionic strength adjustor) to obtain a final 1 μM probe concentration in the skim milk and distilled water. The skim milk was then sequentially added to the distilled water present in a cuvette to obtain skim milk concentrations ranging from 1.9 to 8.9%. Using this approach, the probe concentration was maintained at 1 μM at all skim milk concentrations. Fluorescent spectra (excitation = 470 nm, and emission = 500 to 560 nm; Table 1) were collected at each skim milk dilution with a front face accessory (FP-113, Thermo-Electron Corp.). Duplicate

Figure 1. Fluorescence spectra of Fluo-5N (A) and Rhod-5N (B) at pH 5.8 and 7.0. All spectra were obtained with a probe concentration of $1 \mu\text{M}$ and an ionic calcium concentration of $125 \mu\text{M}$ in distilled water.

spectra were collected and then averaged for all the samples. A background fluorescent spectrum at all skim milk concentrations without probe was also collected. The corresponding background spectra were then subtracted from the spectra of diluted skim milk containing the probe to obtain the spectra of the Ca^{2+} -probe adduct and to remove the fluorescence due to intrinsic components of skim milk. To remove the spectral noise, the subtracted spectra were further smoothed with a single pass 5-point smoothing. Similarly, the procedure was repeated with the Fluo-5N probe added to pH 6.2 diluted skim milk, and Rhod-5N probe added to pH 6.2 and 7.0 diluted skim milk samples. Excitation and emission wavelengths for the 2 probes are shown in Table 1.

Figure 2. Change in pH upon addition of the pH 6.6 and pH 5.5 adjusted skim milk to water containing 2% 4 M KCl.

Simple Linear Regression Models to Determine Free Calcium Ion

The background subtracted and smoothed fluorescent intensity at 520 and 577 nm was obtained for each sample and was correlated to calcium ion concentration, determined by the ion selective electrode. Four separate linear regression models were developed for each of the probes (Fluo-5N and Rhod-5N) at each pH (6.2 and 7.0).

RESULTS AND DISCUSSION

Effect of pH on Probes

Figures 1a and 1b show the changes in the fluorescence intensities of Fluo-5N and Rhod-5N dissolved in water, at pH 5.8 and 7.0, with a constant calcium concentration of $125 \mu\text{M}$. Fluo-5N (Figure 1A) fluorescence

Table 2. Ionic calcium (μM) content in the pH 7.0 and 6.2 diluted skim milk (1.9 to 8.9%) as determined by the ion selective electrode

% Skim milk	Ionic calcium (μM)	
	pH 7.0	pH 6.2
1.9	511	511
2.9	654	770
3.8	739	944
4.7	835	1,112
5.6	907	1,258
6.4	984	1,422
7.3	1,068	1,675
8.1	1,112	1,818
8.9	1,207	1,973

Figure 3. Background fluorescence spectra (excitation = 470 nm, emission = 500 to 560 nm) of diluted skim milk (1.9 to 8.9%).

intensity decreased with decrease in pH. A similar trend was also observed for Rhod-5N probe (Figure 1B). The Ca^{2+} concentration, as verified by the ion selective electrode, was the same ($125 \mu\text{M}$) in all samples regardless of the pH. The results confirm that the pH has an effect on the fluorescent properties of each probe.

Effect of Skim Milk pH and Dilution on Free Calcium Ion Content

An increase in pH to approximately 7.0 was observed when (raw) skim milk at pH 6.6 was added to distilled water at levels of 1.9 to 8.9% (Figure 2). A similar increase to a pH of approximately 6.2 was also observed when skim milk with a pH of 5.5 was diluted in distilled water. This is due to the solubilization of the CCP and subsequent release of the hydroxyl ion from water upon dilution of the skim milk (Fox and McSweeney, 1998).

The ionic calcium concentrations of the diluted skim milk samples at pH 6.2 and 7.0 as measured with a selective ion electrode are shown in Table 2. As expected with a decrease in pH, an increase in relative free- Ca^{2+} in the corresponding diluted skim milk sample was observed. This is due to the solubilization of insoluble calcium associated with casein as CCP to form soluble calcium ions due to the reduction in pH. Additionally, dilution of the skim milk is known to further solubilize the CCP (Fox and McSweeney, 1998). The increase in free Ca^{2+} was nonlinear. For example, skim milk has a free Ca^{2+} concentration of approximately 2.5 mM , and 4.7% skim milk in water would be expected to have approximately $118 \mu\text{M}$ ionic calcium. However, the calcium ion selective electrode values for Ca^{2+} concentrations were 835 and $1,112 \mu\text{M}$ at 7.0 and 6.2, respectively

Figure 4. Raw fluorescence spectra (A) and background subtracted fluorescence spectra (B) of Fluo-5N probe ($1 \mu\text{M}$) in pH 7.0 diluted skim milk (1.9 to 8.9%). A one pass 5-point smoothing was performed on the background subtracted spectra. The ionic calcium concentration (μM) of each sample is indicated.

(Table 2). These values were approximately 7 to 9 times higher than the expected values based on simple linear dilution of the skim milk.

FFFS

Fluo-5N. The autofluorescence of the various concentrations of the milk is shown in Figure 3 and demonstrates that as the concentration of the milk increased the autofluorescence also increased. Consequently, the milk background fluorescence at each milk concentration needs to be subtracted from the corresponding diluted milk sample with the fluorescent probe added. The raw fluorescence spectrum of the diluted skim milk samples (adjusted to pH 7.0) containing $1 \mu\text{M}$ of Fluo-5N are shown in Figure 4A, and the corresponding back-

Figure 5. Raw fluorescence spectra (A) and background subtracted fluorescence spectra (B) of Fluo-5N probe ($1 \mu\text{M}$) in pH 6.2 diluted skim milk (1.9 to 8.9%). A one pass 5-point smoothing was performed on the background subtracted spectra. The ionic calcium concentration (μM) of each sample is indicated.

ground subtracted spectra are shown in Figure 4B. An increase in fluorescence with an increase in skim milk concentration for the same Fluo-5N probe concentration was observed in both the raw and background subtracted spectra. As was the case with milk at pH 7.0, a similar increase in fluorescence was observed for the diluted pH 6.2 skim milk containing probe (Figure 5A) and the skim milk background subtracted fluorescence spectra (Figure 5B). However, the fluorescence of skim milk at pH 6.2 was lower than the fluorescence of skim milk at pH 7.0. For example, the Fluo-5N fluorescence intensity at 520 nm of the diluted skim milk at pH 7.0 (8.1% skim milk) and at pH 6.2 (4.7% skim milk) was

Figure 6. Background fluorescence spectra (excitation = 549 nm, and emission = 560 to 600 nm) of diluted skim milk (1.9 to 8.9%).

8.3 (Figure 4B) and 5.4 (Figure 5B), respectively, for the same ionic calcium concentration of $1,112 \mu\text{M}$. This confirms our earlier findings with right angle fluorescence spectroscopy (Figure 1A) where we observed a decrease in fluorescence with a decrease in pH. The decreased fluorescence intensity with a decrease in pH may be due to structural changes in the probe, such as protonation of the probe, which may result in fewer calcium binding sites on the probe.

Rhod-5N. The fluorescence of the diluted skim milk alone increased with increasing skim milk concentration (Figure 6), again suggesting autofluorescence in the 560 to 600 nm range. The raw fluorescence spectrum of the Rhod-5N probe in the pH 7.0 (Figure 7A) and pH 6.2 (Figure 8A) diluted milk increased with an increase in milk concentration. Additionally, the background subtracted fluorescence spectra also increased for the Rhod-5N containing diluted skim milk at pH 7.0 (Figure 7B) and pH 6.2 (Figure 8B). As was the case for Fluo-5N, fluorescence of the skim milk background subtracted spectra for the diluted skim milk at pH 7.0 was higher than the fluorescence of the diluted skim milk at pH 6.2. Fluorescences of 19.4 and 13.9 were observed for the pH 7.0 (8.1% skim milk; Figure 7B) and pH 6.2 (4.7% skim milk; Figure 8B), respectively, for the same ionic calcium content of $1,112 \mu\text{M}$.

Simple Linear Regression Models to Determine Free Calcium Ion Concentration

The Fluo-5N regression models, developed with diluted skim milk at pH 7.0 (Figure 9A) and 6.2 (Figure 9B), between fluorescence intensity (520 nm) and ionic calcium concentration showed high correlation ($R^2 >$

Figure 7. Raw fluorescence spectra (A) and background subtracted fluorescence spectra (B) of Rhod-5N probe (1 μM) in pH 7.0 diluted skim milk (1.9 to 8.9%). A one pass 5-point smoothing was performed on the background subtracted spectra. The ionic calcium concentration (μM) of each sample is indicated.

0.99). However, the slopes of models developed at pH 7.0 and 6.2 were 170 and 415, respectively. A higher slope represents a reduction in sensitivity because for a small change in fluorescence there is a large increase in ionic calcium content. This suggests that the probe is more sensitive at pH 7.0 than pH 6.2. A high correlation ($R^2 > 0.98$) was also observed for the Rhod-5N regression models developed with pH 7.0 (Figure 10A) and pH 6.2 (Figure 10B) diluted skim milk. The R^2 values were higher for Fluo-5N ($R^2 \geq 0.996$) when compared with the Rhod-5N models ($R^2 \leq 0.9893$). A small difference in R^2 values is significant, and based on this we can conclude that the Fluo-5N probe is slightly more accurate in determining the ionic calcium in skim milk. However, to determine ionic calcium concentrations in undiluted skim milk (2.5 to 3 mM), the fluorescent probes need to be tested at higher ionic calcium concen-

Figure 8. Raw fluorescence spectra (A) and background subtracted fluorescence spectra (B) of Rhod-5N (1 μM) in pH 6.2 diluted skim milk (1.9 to 8.9%). A one pass 5-point smoothing was performed on the background subtracted spectra. The ionic calcium concentration (μM) of each sample is indicated.

trations. In the current paper, we utilized simple regression models to determine ionic calcium at concentrations suggested by the probe manufacturers (1,000 μM at pH 7.0). Hence, further research needs to be conducted to establish the maximum ionic calcium content a probe can measure at a range of pH values. Additionally, the feasibility of developing multivariate regression models taking into consideration the fluorescence variation due to factors such as pH and temperature would be valuable.

Although we have obtained promising results in a diluted skim milk system, the application of this technique to other dairy products like whole milk or concentrated milk still needs to be investigated. Dairy products like whole milk or concentrated milk have a more complex sample matrix that may have an impact on the performance of the fluorescent probes. However, it

Figure 9. Linear regression analysis of ionic calcium content (μM) and the Fluo-5N fluorescence intensity (520 nm) of background subtracted fluorescence spectra of the pH 7.0 (A) and the pH 6.2 (B) diluted skim milk (1.9 to 8.9%).

is also possible that these sample matrix effects can be easily removed by performing a simple background subtraction prior to addition of the fluorescent probe, which was the case for diluted skim milk.

CONCLUSIONS

The simple linear regression models of Fluo-5N and Rhod-5N probes determined calcium ion concentrations up to 1,207 and 1,973 μM ionic calcium in diluted skim milk at pH 7.0 and 6.2, respectively. The fluorescence

Figure 10. Linear regression analysis of ionic calcium content (μM) and the Rhod-5N fluorescence intensity (577 nm) of background subtracted fluorescence spectra of the pH 7.0 (A) and the pH 6.2 (B) in diluted skim milk (1.9 to 8.9%).

intensity of the Fluo-5N and Rhod-5N probes decreased with the decrease in the pH for the same ionic calcium. The calcium ion concentration of diluted skim milk can be determined simply and rapidly with molecular probes, Fluo-5N and Rhod-5N, and FFFS. Additionally, the probes may have the potential to determine ionic calcium in undiluted skim milk and other dairy products if more complicated regression models are utilized.

ACKNOWLEDGMENTS

The project was supported by the USDA Cooperative State Research, Education and Extension Service, spe-

cial research grant number 2005-34328-16024. We are also grateful to Kraft Foods Inc. for supporting the research.

REFERENCES

- Baker, J. M., and C. W. Gehrke. 1956. The ion-exchangeable calcium in raw and heated skim milk as determined by the "Resin Contact Time" method. *J. Dairy Sci.* 39:1221-1228.
- Becker, E. M., J. Christensen, C. S. Fredricksen, and V. K. Haugaard. 2003. Front-face fluorescence spectroscopy and chemometrics in analysis of yogurt: Rapid analysis of riboflavin. *J. Dairy Sci.* 86:2508-2515.
- Christianson, G., R. Jenness, and S. Coulter. 1954. Determination of ionized calcium and magnesium in milk. *Anal. Chem.* 26:1923-1927.
- Davies, D. T., and J. C. D. White. 1960. Use of ultrafiltration and dialysis in isolating the aqueous phase of milk and in determining the partition of milk constituents between aqueous and disperse phases. *J. Dairy Res.* 27:171-190.
- DeMan, J. M. 1962. Measurement of partition of some milk constituents between dissolved and colloidal phases. *J. Dairy Res.* 29:279-283.
- DeMott, B. J. 1968. Ionic calcium in milk and whey. *J. Dairy Sci.* 51:1008-1012.
- Dufour, E., G. Mazerolles, M. F. Devaux, G. Duboz, M. H. Duployer, and N. M. Riou. 2000. Phase transition of triglycerides during semi-hard cheese ripening. *Int. Dairy J.* 10:81-93.
- Fox, P. F., and P. L. H. McSweeney. 1998. Salts of milk. Pages 239-264 in *Dairy Chemistry and Biochemistry*. Blackie Academic & Professional, London, UK.
- Garimella Purna, S. K., L. A. Prow, and L. E. Metzger. 2005. Utilization of front-face fluorescence spectroscopy for analysis of process cheese functionality. *J. Dairy Sci.* 88:470-477.
- Geerts, J. P., J. J. Bekhof, and J. W. Scherjon. 1983. Determination of calcium ion activities in milk with an ion-selective electrode. A linear relationship between the logarithm of time, and the recovery of calcium ion activity after heat treatment. *Neth. Milk Dairy J.* 37:197-211.
- Genot, C., F. Tonetti, T. Montenay-Garestier, D. Marion, and R. Drapon. 1992. Front-face fluorescence applied to structural studies of proteins and lipid-protein interactions of visco-elastic food products. 2. Application of wheat gluten. *Sci. Aliments* 12:687-704.
- Herbert, S., N. M. Riou, M. F. Devaux, A. Riaublanc, B. Bouchet, D. J. Gallant, and E. Dufour. 2000. Monitoring the identity and the structure of soft cheeses by fluorescence spectroscopy. *Lait* 80:621-634.
- Jenness, R., and S. Patton. 1959. Pages 312-329 in *Principles of Dairy Chemistry*. John Wiley and Sons, New York, NY.
- Karoui, R., and E. Dufour. 2003. Dynamic testing rheology and fluorescence spectroscopy investigations of surface to center differences in ripened soft cheeses. *Int. Dairy J.* 13:973-985.
- Kulmyrzaev, A., and E. Dufour. 2002. Determination of lactulose and furosine in milk using front-face fluorescence spectroscopy. *Lait* 82:725-735.
- Martens, H., and T. Naes. 1989. *Multivariate Calibration*. John Wiley and Sons, London, UK.
- May, R. J. 1995. The shift of ionic calcium in milk systems. Master's Thesis, University of Minnesota, St. Paul, MN.
- Minta, A., J. P. Y. Kao, and R. Y. Tsien. 1989. Fluorescent indicators for cytosolic calcium based on rhodamine and fluorescein chromophores. *J. Biol. Chem.* 264:8171-8178.
- Muldoon, P. J., and B. J. Liska. 1969. Comparison of resin ion-exchange method and a liquid ion-exchange method for the determination of ionized calcium in milk. *J. Dairy Sci.* 52:460-464.
- Muldoon, P. J., and B. J. Liska. 1972. Effect of heat treatment and subsequent storage on the concentration of the ionized calcium in milk. *J. Dairy Sci.* 55:35-38.
- Parker, C. A. 1968. Apparatus and experimental methods. Pages 128-302 in *Photoluminescence of Solutions with Applications to Photochemistry and Analytical Chemistry*. C. A. Parker, ed. Elsevier, Amsterdam, The Netherlands.
- Rose, D. 1968. Relation between micellar and serum casein in bovine milk. *J. Dairy Sci.* 51:1897-1923.
- Smeets, W. G. M. 1955. The determination of the concentration of calcium ions in milk ultrafiltrates. *Neth. Milk Dairy J.* 9:249-252.
- Tessier, H., and D. Rose. 1958. Calcium ion concentration in milk. *J. Dairy Sci.* 41:351-359.
- Valeur, B. 1998. Principles of fluorescent probe design for ion recognition. Pages 21-48 in *Topics in Fluorescence Spectroscopy, Volume 4, Probe Design and Chemical Sensing*. J. R. Lackowicz, ed. J. R. Plenum Press, New York, NY.
- Van Kreveld, H., and B. Van Minnen. 1955. Calcium and magnesium ion activity in raw milk and processed milk. *Neth. Milk J.* 9:1-29.
- Wold, J. P., A. Veberg, A. Nilsen, V. Iani, P. Juzenas, and J. Moan. 2005. The role of naturally occurring chlorophyll and porphyrins in light-induced oxidation of dairy products. A study based on fluorescence spectroscopy and sensory analysis. *Int. Dairy J.* 15:343-353.