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Front-Face Fluorescence Spectroscopy Allows the Characterization of Mild Heat Treatments Applied to Milk. Relations with the Denaturation of Milk Proteins

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Emission and excitation spectra of intrinsic fluorophores present in milk were used to evaluate changes in milk following thermal treatments in the 57−72 °C temperature range from 0.5 min up to 30 min. Alternatively, the concentrations of native alkaline phosphatase, lactoferrin, immunoglobulin G, bovine serum albumin, β-lactoglobulin, and α-lactalbumin were determined in the same samples by enzymatic and immunochemical techniques. As principal component analysis applied to the normalized fluorescence spectra successfully discriminated different milk samples according to the temperature and time of thermal treatment, principal component regression was applied to predict the amounts of the native proteins investigated using fluorescence data. The results showed strong correlations between measured and predicted data for alkaline phosphatase and β-lactoglobulin. This study has demonstrated that front-face fluorescence spectroscopy has a promising potential to become a rapid and nondestructive analytical technique for the evaluation of physicochemical changes in milk induced by low thermal treatment.

KEYWORDS: Milk; heat treatment; radial immunodiffusion; front-face fluorescence; intrinsic fluorophores; multivariate analysis

INTRODUCTION

Heat treatment is the most common means used to extend shelf life of milk and also to eliminate pathogenic organisms of diseases associated with milk (*1*, *2*). However, heat treatment results in the physicochemical modification of many milk constituents. An important modification that has been studied extensively is whey protein denaturation. In general, the major milk proteins are sensitive to heat at temperatures above 60 °C, and this sensitivity is related to molecular structure, concentration, pH, and ionic strength. They undergo denaturation due to unfolding of their compact globular conformations and aggregation. At the same heating conditions, denaturation of individual proteins in milk occurs at different rates. Therefore, quantitative estimation of residual native proteins can be used to evaluate the extent and severity of the thermal treatment to which milk was subjected. Pasteurization, the most traditional thermal processing of milk, causes the total denaturation of alkaline phosphatase while casein micelles mainly remain stable.

Thermal behavior of milk proteins has been studied by means of different techniques including both physical and immunological methods. Alkaline phosphatase (ALP), a milk enzyme, has been found first to be a sensitive indicator to estimate heat treatment of milk. Its destruction requires slightly more severe conditions than for the destruction of the most heat resistant vegetative pathogens associated with raw milk (*3*, *4*). Immunological techniques are able to differentiate between native and heat-denatured ALP (*5*). Moreover, when this method is specific for bovine milk ALP, it can exclude microbial ALP, while physical techniques are not able to differentiate milk and microbial origins of ALP (*5*). Differential scanning calorimetry (DSC) study of water solutions of lactoferrin, an ion-binding red protein, has shown two transitional peaks (*2*). Mainly, the first peak (65 °C) was affected by pasteurization, while the second peak (92 °C) remained stable, showing that lactoferrin can also be used to assay heat treatment in milk. Thermal stability of immunoglobulins (IgG) in model systems has been studied over a temperature range of 62.7−80 °C. The results suggested that commercial pasteurization does not completely denature IgG, that is, 59−76% of the native protein can be detected after pasteurization (*6*). β-Lactoglobulin and α-lactalbumin represent the most thoroughly investigated proteins of milk. Thermal behavior of these proteins has been studied over a wide range of temperature (*4*, *7*−*13*). β-Lactoglobulin is normally regarded as more easily denatured than α-lactalbumin. Bovine serum albumin also undergoes conformational changes in the pasteurization range. Thus, heat treatment performed on

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milk can be assessed by monitoring the structural changes of individual proteins and enzymes present in milk.

The objective of this study was to investigate the potential of front-face fluorescence spectroscopy, a sensitive, rapid, and cheap technique, to estimate mild heating treatments applied to milk. The fluorescence data were successfully correlated with the amounts of native alkaline phosphatase and β-lactoglobulin measured on the same samples.

MATERIALS AND METHODS

Milk Samples. Raw milk was obtained from the experimental dairy farm of INRA, Theix (France). Series of milk samples were prepared by heating the raw milk at 57, 60, 63, 66, 69, and 72 °C for 0.5, 1, 3, 10, and 30 min at each temperature considered. Ten milliliters of the raw milk was placed between two glass plates spaced by a plastic U frame of 2-mm thickness and immersed into a thermostatically controlled water bath (Polystat 44, Bioblock Scientific, Illkirch, France). Before counting specified heating time, the samples were allowed to reach the heating temperature. Then, heated samples were transferred into glass beakers using a syringe and immediately cooled to the room temperature in an ice water bath. The temperature during heating and cooling was controlled with accuracy of ±0.1 °C using thermic probe placed in the sample.

Purified Proteins. Immunoglobulins G_1 (IgG_1), β-lactoglobulin (β-lg), α-lactalbumin (α-la), and lactoferrin were purified from bovine colostrum or milk by a combination of gel permeation chromatography and ion exchange chromatography as previously described (*14*, *15*). Bovine serum albumin (BSA) was purchased from Sigma Aldrich Chimie (F-38297 St-Quentin Fallavier, France) and repurified by chromatography on Sephadex G-100 as described for β-lg.

Polyclonal Antibodies. Antibodies were produced on goats for IgG_1, on rabbits for β-lg, BSA, and lactoferrin, and on sheep for α-la. The proteins, emulsified in Freund's complete (first injection) or incomplete (booster injections) adjuvant (1:1, v/v), were administered at doses of 5 mg per goat and sheep or 1 mg per rabbit by multiple intradermal injections. Animals were bled 7−9 d after each monthly booster injection. The specificity of the antisera was assessed by double-immunodiffusion and agar gel immunoelectrophoresis. No cross-reactivity has been observed. Moreover, anti-β-lg reacts similarly with both A and B genetic variants in the single radial immunodiffusion (SRID) assay used to quantify the proteins.

Immunochemical Assay of Proteins. Concentrations of β-lg, α-la, IgG_1, BSA, and lactoferrin in milk samples were determined by SRID assay (*16*) using 1.85-mm-thick agar plates containing 12 g Noble agar/L in 0.05 M-veronal buffer, pH 7.2 containing 1 g sodium azide/L, and appropriate quantities of each specific antiserum. Circular wells (2-mm diameter) were punched out in the gel and filled with 3 μL portions of adequately diluted samples in the veronal buffer or 3 μL of purified proteins of known concentrations as standards. The purified proteins were dissolved in the veronal buffer containing 1 g human serum albumin/L and 1 g sodium azide/L. Plates were incubated in a moist box at 37 °C for 20−22 h (β-lg, α-la, IgG, BSA) or 48 h (lactoferrin), and the diameter of the ring-shaped precipitates was measured automatically using a magnifying video camera system (*14*). Standard curves were drawn by plotting the diameter of the precipitating ring versus the square root of the protein concentration. With the diffusion time used, a linear regression was obtained. Samples and controls were plated in duplicate. The CVs of the assays were 3−5%.

Alkaline Phosphatase Activity. Heated milk samples (0.02 mL) were added to 1 mL of a sodium carbonate buffer 30 mM, MgCl2 1 mM, pH 11, containing 600 mg para-nitrophenyl phosphate per liter, and incubated for 60 min in a light-protected water bath. The reaction was stopped by adding 1.8 mL saturated ammonium phosphate. After 10 min of light-protected incubation, the samples were centrifuged at 3000*g* for 10 min. For each sample, a duplicate reading at 405 nm was performed on 0.2-mL fractions of the supernatant, using a microplate reader (IEMS, Labsystem).

Fluorescence Spectroscopy. Fluorescence spectra of milk samples were recorded using a FluoroMax-2 spectrofluorometer (Instruments S.A., NJ) provided with a model 1962A single-position (56°) cell holder. Milk samples were placed in a 5-mL quartz cuvette and emission spectra of intrinsic fluorophores of milk were recorded at 280−480 nm (resolution: 1.0 nm; excitation: 250 nm) and 380−600 nm (resolution: 2.0 nm; excitation: 360 nm). Excitation spectra were also obtained between 290 and 490 nm (resolution: 2.0 nm) at emission wavelength set at 518 nm. The spectrum of each sample was recorded in triplicate using different aliquots.

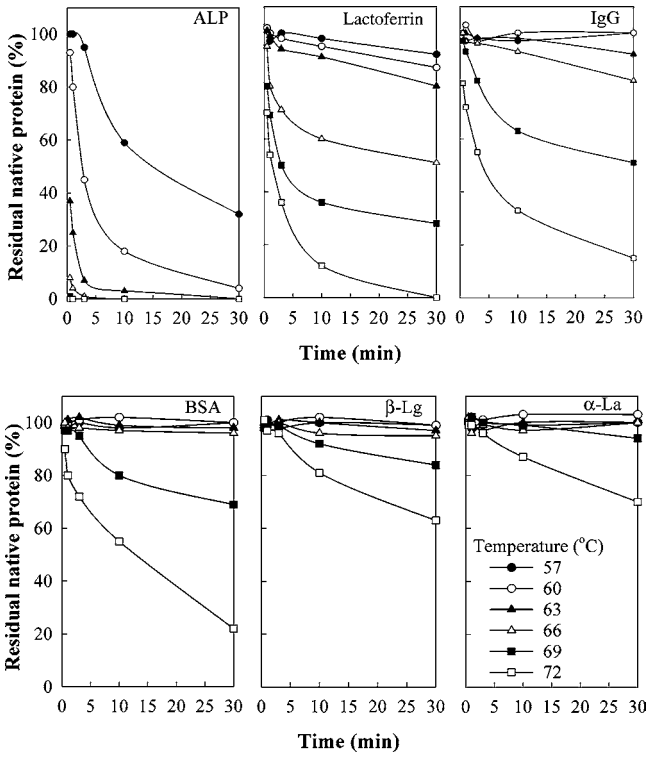

Figure 1. Thermal denaturation of selected milk proteins as determined by enzymatic and radial immunodiffusion assays. Residual native protein is expressed as percent of initial concentration.

To determine the origin of the fluorescence observed, fluorescence spectra of whole raw milk, skim milk, and washed cream were recorded in the same manner described above. To do so, the same batch of nonheated raw milk was centrifuged at 9000*g* for 10 min and creamed fat was removed from the skim. The cream was then washed three times with double-distilled water and centrifuged after each washing under the condition above.

Mathematical Analysis. To reduce the scattering effect, the spectra were normalized according to Bertrand and Scotter (*17*). Principal component analysis (PCA) was applied to the normalized data to obtain a map describing physical and chemical variations between the samples studied. PCA finds combinations of variables that describe major trends in the data. Mathematically, PCA relies upon an eigenvector decomposition of the covariance or correlation matrix of the process variables.

Prediction equations for the content of individual proteins analyzed were obtained using principal component regression (PCR) technique. PCR is a statistical technique to form regression models in systems where there is a good deal of covariance and independent or predictor variables. PCR conducts a PCA decomposition of the predictor variables and then regresses the PCA scores against the predicted variables.

RESULTS AND DISCUSSION

Determination of Residual Native Proteins and Alkaline Phophatase Activity. The concentrations of the native residual proteins measured in the milk samples are shown in **Figure 1**. ALP displayed the highest thermal sensitivity as a function of temperature and holding time among the proteins studied. Heating at 57 °C for 30 min has already caused considerable denaturation of this protein decreasing content of the native ALP

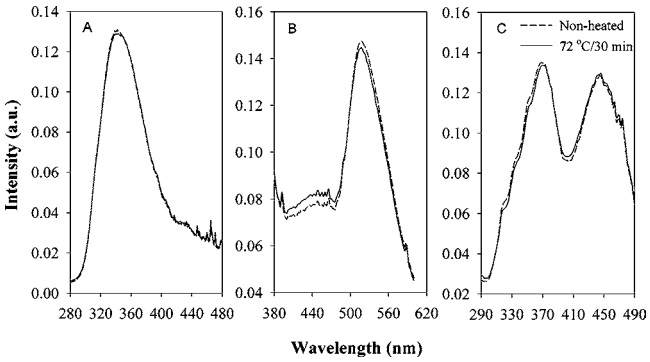

Figure 2. Fluorescence spectra of milk samples: (**A**) emission spectra of aromatic amino acids (excitation 250 nm); (**B**) emission spectra of NADH/FADH (excitation 360 nm); (**C**) excitation spectra of NADH/FADH (emission 518 nm).

to 32%. We observed complete destruction of ALP in the samples heated at 66 °C for more than 3 min. Vega−Warner and co-workers did not detect ALP activity in buffered solutions held at 70 °C for more than 30 s (*5*). However, the authors did not investigate the effect of heating below 70 °C. We found that heating at 63 °C for 30 min also completely suppresses ALP activity in milk. In fact, milk has to be heated at 62.8 °C for 30 min or 71.7 °C for 15 s to meet minimum requirements for adequate pasteurization (*5*).

The most heat resistant protein was α-lactalbumin: 70% of this protein remained stable in the milk samples upon heating at 72 °C for 30 min. α-Lactalbumin normally denatures at a lower temperature than *β*-lactoglobulin, but its thermal unfolding is reversible under most milk processing conditions (*18*). Therefore, the order of increasing thermal stability of the proteins taking into account irreversible changes was ALP < lactoferrin < IgG < BSA < *β*-Lg < α-La.

Fluorescent Properties of Milk. Milk proteins contain three aromatic amino acid residues, namely, tryptophan, tyrosine, and phenylalanine, contributing to their fluorescence. Coenzymes nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FADH) are also fluorophores naturally occurring in milk. Individual fluorescence properties of these molecules were taken into account when settings for measurements of fluorescence of the heated milk samples were established.

The emission spectra of the intrinsic fluorophores of milk excited at 250 and 360 nm, and the excitation spectra obtained by emission at 518 nm are shown in **Figure 2A**, **B**, and **C**, respectively. Upon excitation at 250 nm, we expected that all aromatic amino acids become excited. The emission spectra recorded between 280 and 480 nm (exc. 250 nm) showed a maximum fluorescence of proteins at 342 nm (**Figure 2A**), which is most likely to be attributed to the tryptophan residue exhibiting maximum fluorescence in water at 348 nm. The emission maxima of tyrosine and phenylalanine in water occur at 304 and 282 nm, respectively (*19*). The quantum yield of phenylalanine in proteins is small, so the emission from this amino acid is rarely observed. Although milk proteins contain the number of tyrosine residues often equal (or greater in some cases) to that of the tryptophan residues, the tyrosine emission of most proteins is small and undetectable (*19*). Therefore, absorption of proteins at 250−280 nm is supposed to be predominantly that of the tryptophan residue.

The maximum tryptophan emission wavelength shifted from 342 nm for the raw milk to 343 nm for the heated milk, which is known as red-shift of emission spectra. The observed shift of the maximum emission wavelength to the longer wavelength range can be explained by the heat denaturation of milk proteins and exposing more tryptophan residues to the aqueous phase of milk. At the same time, the exposed tryptophan residues are shielded from the aqueous phase by other protein molecules as a result of protein−protein interaction, the rate of which increases with protein unfolding. The shielding of tryptophan residues by protein aggregation has greater impact on the fluorescence intensity compared to heat-induced protein unfolding, which causes more tryptophan residues to become exposed to the aqueous phase of milk.

Intrinsic fluorophores of the milk samples were also excited at 360 nm and emission spectra in the wavelength range of 380−600 nm were recorded (**Figure 2B**). NADH is highly fluorescent, with absorption and emission maxima at 340 and 470 nm, respectively, in water (*19*), while fluorescence of FADH is characterized by absorption and emission maxima at 445 and 520 nm, respectively (*20*). Thus, fluorescence of these two coenzymes found in milk was supposed to be observed in this measurement. Analyzing the spectra, one can distinguish a small spectral component near 460 nm, which is suggested to be due to NADH. Another peak at 518 nm is sharp and distinct, and it can confidently be attributed to FADH. Indeed, this emission maximum for FADH is in agreement with the data reported by ref 21.

To confirm our suggestions on the responsibility of the coenzymes for the fluorescence shown in **Figure 2B**, excitation spectra of the milk samples between 290 and 490 nm were recorded at emission wavelength set at 518 nm (**Figure 2C**). Two peaks located at 370 and 445 nm were observed on the excitation spectrum. An excitation maximum at 370 could probably be assigned to NADH, which is characterized by excitation maximum at 340 nm when dissolved in water. The shift of the excitation peak of NADH in milk relative to that of NADH in water to longer wavelength, so-called red-shift can be interpreted as being due to the influence of other proteins in milk. It is well known that the fluorescent properties of fluorophores strongly depend on their environment (*19*). The second peak located at 445 nm on the excitation spectrum is related to FADH, which is approximately the same as the excitation maximum of FADH in water, that is, 450 nm. Consequently, the data shown in **Figure 2C** confirmed that a weak emission observed at 460 nm and dominating emission at 518 nm on the spectra shown in **Figure 2B** were due to NADH and FADH, respectively.

The fluorescence properties of natural fluorophores present in milk are sensitive to environmental conditions and changes in the three-dimensional structures of the proteins. The emission spectra of the aromatic amino acids overlap at all heating times and temperatures applied to the milk samples (**Figure 2A**). The only notable difference found in these spectra is a slight decrease in the emission maximum that can be seen in the spectrum of the milk subjected to the highest heating conditions (72 °C for 30 min) relative to the emission maximum of the nonheated raw milk. This assumes that the extent of heating applied in the present study was not as high as to induce considerable conformational changes that could significantly modify fluorescence properties of milk. As it can be seen in **Figure 1**, only a part of milk proteins undergoes denaturation upon heating at 57−72 °C. In our earlier work, we have demonstrated that ultrahigh temperature treatment leads to broadening of the emission spectra of tryptophan in milk and maximum emission decreases as heating temperature increases (*22*). A similar trend was also observed when the influence of a combination of

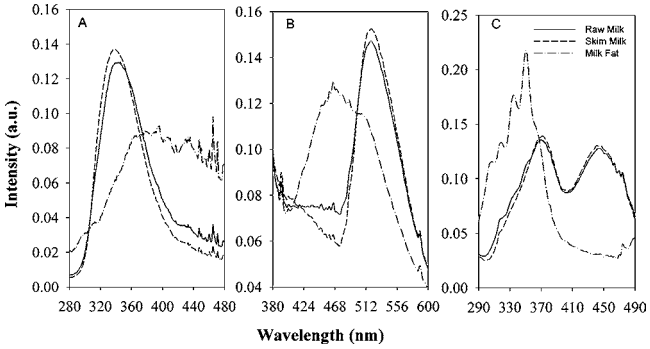

Figure 3. Fluorescence spectra of raw milk, skim, and fat: (**A**) emission spectra of aromatic amino acids (excitation 250 nm); (**B**) emission spectra of NADH/FADH (excitation 360 nm); (**C**) excitation spectra of NADH/FADH (emission 518 nm).

thermal treatment and homogenization on the fluorescence of milk was examined (*23*).

Unlike aromatic amino acid emission spectra, the fluorescence spectra of NADH and FADH had some patterns differentiating them in accordance with the extent of heat treatment. For instance, a maximum emission at 518 nm appeared to be dependent on heating temperature, that is, the emission intensity at this point decreased as temperature increased up to 72 °C. On the contrary, the intensity of the weak emission near 460 nm increases as temperature increases.

A series of emission and excitation spectra of raw milk, skim milk, and washed cream were recorded applying the same measurement settings used for heated milk samples (**Figure 3A**, **B**, **C**). Analysis of the spectra indicated that all the fluorophores detected were originated from the proteins dispersed in the aqueous phase of milk. Comparing respective spectra in **Figures 2** and **3**, one can easily note similarity of the shapes and characteristic wavelengths such as maximum emission and excitation wavelengths. Some modification of the spectra can also be seen. For instance, the emission intensity of tryptophan in skim milk is higher compared to that of the raw milk and the maximum emission wavelength is shifted from 342 to 338 nm. Our measurements showed that proteins at the surface of fat globules in milk can also contribute to the tryptophan emission spectra. A modification of the emission and excitation spectra of NADH and FADH was also observed for milk, skim milk, and cream, except the emission maximum of the coenzymes remained constant (**Figure 3B** and **C**). The excitation spectrum of the milk fat with a maximum at 352 nm (**Figure 3C**) is defined as to be due to NADH, which can be found as a coenzyme of some proteins of the fat globule membrane. In addition, three shoulders located at 308, 318, and 336 nm (**Figure 3C**) were observed. They may originate from the fluorescence of vitamin A (*23*). Thus, the fluorescence spectra presented in **Figure 3** mainly originated from the intrinsic fluorophores dispersed in the aqueous phase of milk, that is, tryptophan and coenzymes (NADH and FADH).

As milk contains a large number of intrinsic probes fluorescent properties of which can be widely modified depending upon the extent of heating, it is difficult to accurately discriminate fluorescence of each individual probe in the overall emission and excitation spectra of milk. However, our measurements showed that the resulting fluorescent spectra of milk are sensitive to heat treatment, and valuable information on the physicochemical changes in the milk constituents, particularly on heat-induced protein denaturation, can be derived using these spectra.

Multivariate Analysis of Milk Fluorescence Spectra. Principal component analysis (PCA) was applied to extract

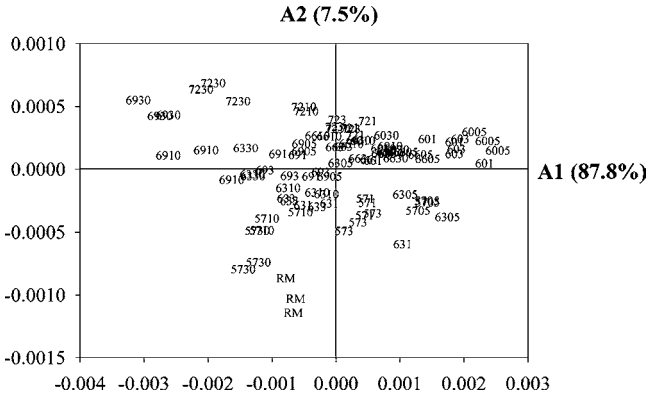

Figure 4. PCA similarity map for NADH/FADH excitation spectral data.

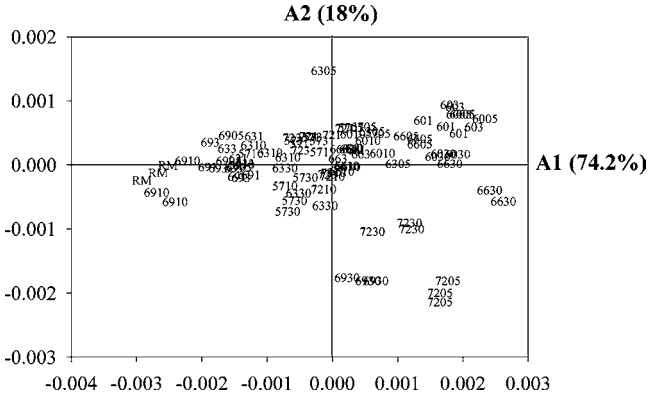

Figure 5. PCA similarity map for NADH/FADH emission spectral data.

information on the conformational changes occurring in milk proteins because of thermal treatments. Using the PCA tool, the emission and excitation spectra of the milk samples subjected to heat treatment with varied temperature and holding time can easily be compared and differentiated into groups with similar physicochemical properties.

Better differentiation of the milk samples by PCA was obtained using the emission and excitation spectra of NADH/FADH, while differentiation of the samples on the basis of the emission spectra of tryptophan was relatively poor. Samples shown on the PCA similarity maps (**Figures 4** and **5**) were coded according to applied heating temperature and time. The first two numbers of the sample name mean heating temperature in °C, and the next two numbers mean heating time in min. Raw milk was coded as RM. The similarity map for the NADH/FADH excitation spectra is shown in **Figure 4**. The plots of the first versus second PC scores, which describe 95.3% of the variance, exhibited the clearest discrimination of the spectra according to heating temperature. The raw milk, which was not subjected to heat treatment, was found at the lowest position with negative scores, whereas the samples heated at the highest temperature (72 °C) for the longest time (30 min) were grouped at the top being scored positively. Other samples were separated into groups in relation to the corresponding heating temperatures and were placed within these two limits.

The best discrimination was obtained for the NADH/FADH emission spectra (**Figure 5**). Principal components A1 and A2 described 74.2% and 18%, respectively, of the variance in the spectral data. There was a discrimination of the samples according to heating temperature according to the principal component A1. Moreover, the spectral patterns corresponding to the principal components provide information about the characteristic peaks which are the most discriminating for the samples observed on the map. The spectral patterns correspond-

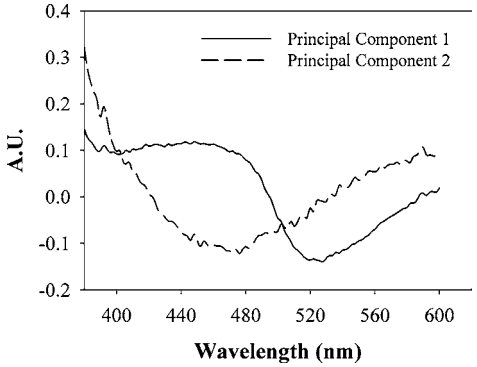

Figure 6. Spectral patterns corresponding to the principal components 1 and 2. Emission spectra of NADH/FADH (excitation 360 nm).

ing to the principal components 1 (87.8% of the variance) and 2 (7.5% of the variance) for NADH/FADH emission spectral data are given **Figure 6**. The spectral pattern 1 showed an opposition between a negative peak at 460 nm and a positive one at 525 nm: a decrease of the peak at 520 nm and an increase of the shoulder at 460 nm in the NADH/FADH emission spectra (**Figure 2B**) were induced by heating. The spectral pattern 2 was more difficult to analyze. It, however, exhibited a minimum at 470 nm and positive values at 420 and 600 nm suggesting a shift in the band located at 520 nm.

Prediction of Thermal Denaturation of Milk Proteins. Fluorescence measurements conducted on the milk samples and principal component analysis showed that the recorded emission and excitation spectra of milk are sensitive to the rate of thermal treatment. Therefore, we assumed that the investigated fluorophores could act as sensors to evaluate thermal denaturation of the individual milk proteins both qualitatively and quantitatively.

The combination of the results obtained by the front-face fluorescence spectroscopy and the immunological method was processed using principal component regression (PCR). A series of matrixes were built grouping data resulting from the PCA of the NADH/FADH emission and excitation spectra, which gave a better discrimination, and from the concentrations of the native residues of ALP, lactoferrin, IgG, BSA, β-lactoglobulin, and α-lactalbumin in the milk samples determined by enzymatic and immunological methods.

The PCR analysis showed that there is a correlation between the content of the native protein residues in milk measured by using enzymatic and immunological methods and results predicted by the front-face fluorescence spectroscopy. The best correlation between measured and predicted quantities of the native proteins studied was found for ALP (**Figure 7**) and β-lactoglobulin (**Figure 8**). For the other proteins studied, regression coefficients were below 0.55.

The concentration of the native ALP in the milk samples upon heating was better predicted when merged results of the principal component analysis of both emission and excitation spectra of NADH/FADH were used to build a data matrix for PCR. The regression coefficient in this case was 0.80 taking into account nine principal components as independent variables recommended by the PCR program (**Figure 7B**). PCR conducted on the excitation spectra of NADH/FADH only gave a linear correlation between measured and predicted ALP with a regression coefficient of 0.75 when seven principal components were considered (**Figure 7A**).

Results of PCR to predict the content of the native β-lactoglobulin in the heated milk samples are presented in **Figure 8**. In contrast with ALP, the content of the native β-lactoglobulin was predicted better using the NADH/FADH emission spectra

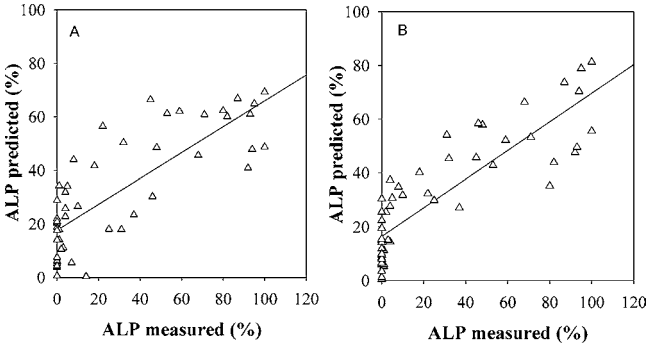

Figure 7. Correlation between the determination by enzymatic technique of native ALP content in heated milk samples and front-face fluorescent spectra. PCR was applied to (**A**) NADH/FADH excitation spectral data and (**B**) merged NADH/FADH emission and excitation spectral data.

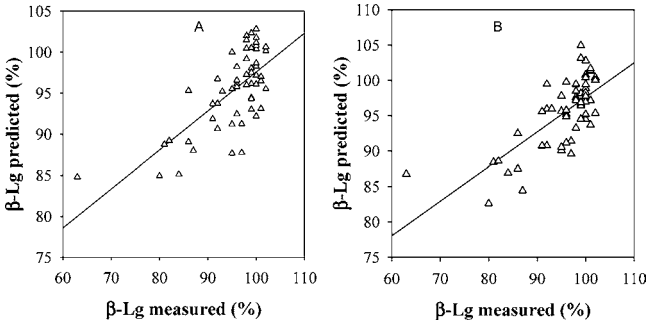

Figure 8. Correlation between the determination by immunological technique of native β-lactoglobulin content in heated milk samples and front-face fluorescent spectra. PCR was applied to (**A**) NADH/FADH emission spectral data and (**B**) merged NADH/FADH emission and excitation spectral data.

(**Figure 8A**). Considering five principal components, the regression coefficient was 0.67. Analysis of the merged NADH/FADH emission and excitation spectral data resulted in a linear correlation between measured and predicted β-lactoglobulin with a regression coefficient of 0.68 considering seven principal components as variables (**Figure 8B**). Ultimately, the results of the statistical analysis showed that the temperature-dependent concentration of the native milk proteins determined by fluorescence spectroscopy have a strong correlation with the results obtained by enzymatic and immunological techniques.

Front-face fluorescence spectroscopy has the potential to be a rapid and cheap method for the determination of residual native proteins in milk samples subjected to the mild thermal treatments. Particularly, predicted contents of alkaline phosphatase and β-lactoglobulin were the most accurate and well correlated with those measured by use of enzymatic and immunological techniques. We have shown that fluorescence properties of intrinsic fluorophores present in milk are modified following thermal treatment. Therefore, these fluorophores appeared to be sensitive and useful sensors to evaluate the intensity of heat treatment given to milk in the pasteurization range.

The most attractive advantages of the front-face fluorescence technique are that no preliminary sample preparation is needed prior to actual measurement and results are obtained in a relatively short period of time compared with conventional techniques. These advantages are based on the ability of front-face fluorescence to operate with optically opaque systems.

Infrared spectroscopy was also an adequate method for determination of milk composition. However, several factors may affect the accuracy of this technique (*24*, *25*). For instance, sample thickness is an essential factor influencing the accuracy

of total protein determination by near-infrared (NIR) spectroscopy. Moreover, sample thickness is to be chosen depending on the spectral region to provide high accuracy of determination (*24*). These disadvantages may hold back the implementation of NIR spectroscopy as an online sensor for quality management in milk processing. Front-face fluorescent spectroscopy is independent of sample thickness and other factors that may greatly affect NIR spectroscopy.

Thus, front-face fluorescence technique in conjunction with chemometric tools has a great potential to become a useful quality control method in rapid online, as well as offline, analysis of milk.

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