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Original article

Determination of lactulose and furosine in milk using front-face fluorescence spectroscopy

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Abstract – A novel rapid and non-destructive front-face fluorescence method to quantify furosine and lactulose in heat-treated milk has been developed. The emission fluorescence spectra (305–450 nm) of tryptophans of proteins, and emission and excitation fluorescence spectra of fluorescent Maillard-reaction products were recorded directly on milk samples. Principal component analysis (PCA) and principal component regression (PCR) were applied to process the spectra obtained. The front-face fluorescence method can give valuable information on the quality of heat-treated milk because it allows the evaluation of furosine and lactulose content simultaneously. The results were well correlated with those of the reference methods. The front-face fluorescence technique is relatively low-cost, rapid and non-destructive and it could replace existing conventional analytical techniques practiced for lactulose and furosine evaluation in dairy products.

Milk / heat treatment / front-face fluorescence / tryptophan / Maillard-reaction products / furosine / lactulose

Résumé – **Détermination du lactulose et de la furosine dans le lait par spectroscopie de fluorescence frontale.** Une méthode nouvelle et non destructive basée sur la spectroscopie de fluorescence frontale a été mise au point pour quantifier la furosine et le lactulose dans les laits chauffés. Les spectres d'émission (305–450 nm) de fluorescence des tryptophanes des protéines et d'excitation et d'émission de fluorescence des produits de la réaction de Maillard du lait ont été enregistrés. L'analyse en composantes principales (ACP) et la régression en composantes principales (RCP) ont été appliquées à la collection de spectres. Les résultats montrent que la spectroscopie de fluorescence frontale permet de caractériser sur les plans des propriétés physico-chimiques et de la qualité les laits chauffés : en effet la furosine et le lactulose peuvent être déterminés simultanément. Les résultats ont été comparés à ceux obtenus aux moyens des méthodes de référence et de fortes corrélations ont été

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observées entre les différentes méthodes. La spectroscopie de fluorescence frontale, qui est une technique relativement peu coûteuse, rapide et non destructive pourrait remplacer à terme les méthodes conventionnelles de dosage de la furosine et du lactulose.

Lait / traitement thermique / fluorescence frontale / tryptophane / composés de la réaction de Maillard / furosine / lactulose

1. INTRODUCTION

Heat treatment results in many chemical and structural changes in milk, the extent of which depends on the temperature and duration of heating [14, 28]. One mechanism involved in the deleterious consequence of heat treatment is the Maillard reaction that leads to the decrease in nutritional value of proteins and formation of brown compounds in milk. The extent of the reaction can be evaluated by the determination of the content of furosine (ε-N-2-furoylmethyl-L-lysine), the main stable Amadori compound in the early Maillard reaction, and lactulose (4-0-β-galactopyranosyl-D-fructose), respectively. Furosine and lactulose can be used as indicators of heat damage of milk and for distinction of UHT milk, pasteurized milk and in-container sterilized milk [1, 2, 6, 7, 9, 15, 27]. The combination of lactulose and furosine values for UHT milk provides information on the quality and genuineness of commercial milks [23], particularly to detect the presence of reconstituted milk powder in consumption milk [11, 26]. It has been reported that there is a fairly good correlation between furosine and lactulose content in UHT milk, and the use of the ratio of both parameters provides better results in evaluation of the quality of milk [7, 22].

Many studies have been carried out to develop methods for the determination of furosine and lactulose in milk and a number of chromatographic, electrophoretic and enzymatic techniques have been proposed for practical use [1, 5, 8, 10, 18, 20, 24, 25, 29]. Ion-pair reversed-phase high-performance liquid chromatography (HPLC) appears to be the most developed method, and therefore, most popular technique to determine furosine in dairy products. HPLC has also been used as a reference method in almost all the studies intended to develop novel techniques for furosine determination.

Lactulose is usually determined by gas chromatography [7–9], HPLC [22] or by the enzymatic method based on the reactions involving lactulose and lactose hydrolysis [15]. The quantity of D-fructose is equal to the amount of lactulose present. Thus, by determining D-fructose one can quantitatively estimate lactulose in the milk being studied. But as free D-glucose – a product of the hydrolysis of lactose, interferes with D-fructose determination, it is eliminated using glucose oxydase.

There are several analytical techniques currently being used for the determination of lactulose and furosine at the laboratory level. Nevertheless, their widespread application in the food industry is currently limited, because they are relatively expensive, time-consuming, require highly skilled operators and are not easily adapted to on-line monitoring. For this reason, there is a pressing need to develop new analytical techniques, which are rapid, non-destructive and relatively low-cost and can be applied in both fundamental research and in the factory as on-line sensors for monitoring the properties of milk during processing, storage and handling.

The past five decades have witnessed the emergence of fluorescence spectroscopy to become a useful tool in experimental biology and chemistry. Fluorescence spectroscopy is a sensitive, rapid and non-invasive analytical method which provides information on the presence of fluorescent

molecules, and on their environment, in biological samples. For example, the fluorescent properties of aromatic amino acids of proteins can be used to study protein structure or protein-hydrophobic molecule interactions [19].

A method for estimating the heat treatment of liquid milk based on right angle fluorescence was recently developed [4]. According to the method, protein denaturation is monitored by measuring the fluorescence intensity of tryptophan (exc.: 290 nm, em.: 340 nm) and detecting advanced Maillard products (exc.: 350 nm, em.: 440 nm). Since the method can only be applied to transparent samples, preliminary treatments of milk are required. To obtain a suitable transparent sample, milk is to be subjected to acidification, centrifugation, dilution and filtration.

Indeed, classical fluorescence spectroscopy cannot be applied directly to the study of turbid systems such as dairy products. When the absorbance of the sample is higher than 0.1, the screening and diffusion effects induce a decrease in fluorescence intensity and a distortion of excitation spectra [16]. To avoid these problems, an alternative method – front-face fluorescence spectroscopy – has been developed in order to allow the investigation of the fluorescence of powdered, turbid and concentrated samples. Despite its great potential, this technique has been seldom used in the characterization of food products. Nevertheless, front-face fluorescence has been applied to the study of proteins in wheat gluten [17], in milks [12] and in fats in cheeses [13]. Therefore, front-face fluorescence spectroscopy appears to be the most suitable analytical technique to study dairy products, which are also complex systems exhibiting intrinsic fluorescence compounds [19]. The main advantage of the method is the possibility of avoiding any sample preparation procedure.

Recently it has been demonstrated that front-face fluorescence spectroscopy can discriminate milk samples subjected to heat treatment from those subjected to homogenization [12]. In addition, examining the fluorescence spectra in different semi-hard cheeses, it was shown that front-face fluorescence spectroscopy allows monitoring of changes in cheese proteins during their ripening under controlled conditions [21]. It appears from the above mentioned studies that the fluorescence of protein tryptophan residues recorded by front-face fluorescence spectroscopy is a valuable tool to investigate the changes in the protein structure and interactions.

The objective of the present study is to demonstrate that front-face fluorescence spectroscopy can be a non-destructive and rapid method of measuring furosine and lactulose in heat-treated milk. The method does not involve any physical or chemical pre-treatment.

2. MATERIALS AND METHODS

2.1. Milk samples

Overheated half-cream UHT milk was provided by a dairy company. Normally-heated half-cream UHT and pasteurized milks were obtained from a dairy company and a local supermarket, respectively. Series of milk samples with different furosine and lactulose concentrations were prepared by diluting overheated UHT milk with two types of normally-heated UHT milks. A total of 13 samples were prepared and analyzed for furosine and lactulose. Milks were coded NUHT1, NUHT2, and OUHT for the first normal UHT milk, the second normal UHT milk and overheated UHT milk, respectively.

2.2. Reference method for the determination of furosine

For the determination of furosine a HPLC method [24] was used, with the exception that 0.1% acetic acid was buffer A and a common C8 250 × 4.6 mm column

from Waters (Wexford, Ireland) was used instead of the furosine-dedicated column mentioned in the conventional method. Powdered furosine dihydrochloride was supplied by Neosystem S.A. (Strasbourg, France). The manufacturer's analysis of the product was 77.2% furosine (dry weight basis), 16.1% Cl^-, and 3.2% H_2O. A series of standard solutions with different furosine contents varying from 0 to 0.121 mg·mL^{-1} were prepared by diluting furosine powder in 3 mol·L^{-1} HCl. 20 μL of each standard solution were injected to obtain the calibration curve.

Milk samples were transferred into 10 mL screw-cap Pyrex vials and then subjected to hydrolysis in the presence of 7 mol·L^{-1} HCl for 23 h at 110 °C [7]. Hydrolysis was performed in duplicate for each milk sample.

Before injection, the hydrolysate was filtered through a 0.2 μm Millipore filter, diluted two times in distilled water and then injected into the volume of 50 μL.

A modular HPLC analytical system 400 from Kontron Instruments and a UV-VIS HPLC detector 430 were used.

Chromatographic conditions were as follows. The elution gradient expressed as solvent B proportion was: 0–13.5 min, 0%; 13.5–20.5 min, 50%; 20.5–21.5 min, 50%; 21.5–23.0 min, 0%. The flow rate and column temperature were set at 1.2 mL·min^{-1} and 35 °C, respectively. UV detection was carried out at 280 nm.

The results are reported as the average of four separate injections per sample.

2.3. Reference method for the determination of lactulose

Lactulose was dertermined using an enzymatic method [4]. It was measured using a Boehringer Mannheim D-Glucose/D-Fructose test kit combined with β-galactosidase (1500 U), triethanolamine hydrochloride, glucose oxidase (5000 U) and catalase. The content of lactulose in the samples was determined by measuring light absorbance at 340 nm using a Cary 100 Bio UV-Visible spectrophotometer (Varian, Mulgrave, Australia). The analysis was performed in duplicate for each milk sample.

2.4. Fluorescence spectroscopy

Fluorescence spectra of the milk samples were obtained using a FluoroMax-2 spectrofluorometer (Instruments S.A., Longjumeau, France) equipped with a model 1962A single-position (56°) cell holder for surface fluorescence. Milk samples were placed in a 5 mL quartz cuvette and emission spectra of tryptophan residues (305–450 nm, resolution: 0.5 nm) were recorded with excitation wavelength set at 290 nm. The spectra of each sample were recorded in triplicate using different aliquots. Emission (380–600 nm) and excitation (250–420 nm) spectra of fluorescent Maillard-reaction products in the milk samples were also recorded at excitation and emission wavelengths set at 360 nm and 440 nm, respectively.

2.5. Mathematical evaluations

The fluorescence spectra were normalized by reducing the area under each spectrum to a value of 1 according to Bertrand and Scotter [3].

Principal component analysis (PCA) was applied to the normalized spectra in order 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. This statistical multivariate treatment makes it possible to draw similarity maps of the samples and to get spectral patterns. While the similarity maps allow the comparison of the spectra in such a way

that two neighbouring points represent two similar spectra, the spectral patterns exhibit the absorption bands that explain the similarities observed on the maps. The PCA software was written in the C language [3].

Lactulose and furosine variables were predicted from the fluorescence spectra using the principal component regression (PCR) technique. The method is a multiple regression applied from the principal components of the samples rather than from the raw data. PCR is a popular technique for forming regression models. It works by doing a PCA decomposition of the predictor variables, then regressing the PCA scores against the predicted variable. Prediction equations for the furosine and lactulose contents were obtained using PCR.

3. RESULTS AND DISCUSSION

3.1. Evaluation of furosine and lactulose content as determined using conventional methods

The concentrations of furosine in the 13 milk samples are presented in Figure 1. In general, the quantity of furosine in both NUHT1 and NUHT2 milks containing an equal quantity of OUHT milk is approximately the same and the data obtained can be described by a linear function ($R^2 = 0.90$). NUHT1 and NUHT2 milks exhibited about 56 mg·L^{-1} furosine content. When the OUHT milk concentration increased from 0 to 100% the furosine content also increased, reaching 87 mg·L^{-1} for pure OUHT milk.

According to previous studies, the furosine content in different normal UHT milks depends on the heat intensity and heating system used (direct or indirect) and varies in the range of 17.4 to 68.2 mg·L^{-1} [23], 17.6 to 85.4 mg·L^{-1} [7], and 33.5 to 74.4 mg·L^{-1} [25]. Our results (Fig. 1) are in good agreement with the data above, as is the furosine content of 1.3 mg·L^{-1} found

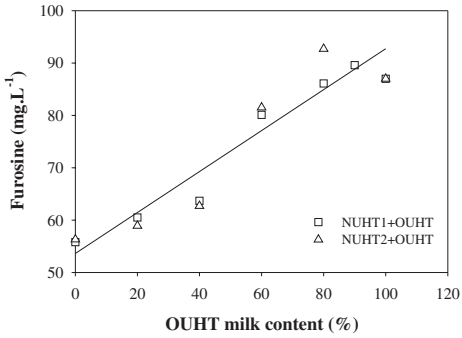

Figure 1. Furosine content in NUHT1, NUHT2 and OUHT milks and their mixtures, as determined using HPLC.

in our pasteurized milk sample with those reported as 1.1–1.5 mg·L^{-1} [4] and 1.2–2.2 mg·L^{-1} [26].

The lactulose contents of the 13 samples examined are given in Figure 2. The lactulose concentrations were slightly different in NUHT1 (42 mg·mL^{-1}) and NUHT2 (34 mg·mL^{-1}) milks and varied from these low values to the maximal value measured in OUHT milk (110 mg·mL^{-1}).

Both sets of data are well correlated by the linear functions with regression coefficients of 0.999 and 0.997 for NUHT1 and NUHT2 milk samples, respectively. The amount of lactulose in the studied milk

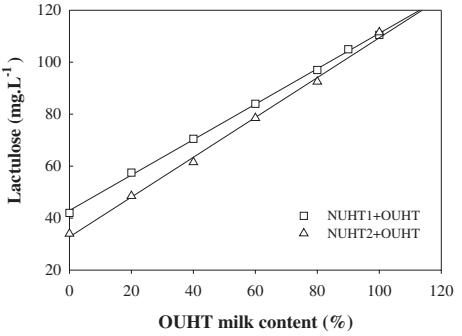

Figure 2. Lactulose content in NUHT1, NUHT2 and OUHT milks and their mixtures, as determined using the enzymatic method.

samples is lower than that reported before [7, 22]. Corzo et al. [7] reported that lactulose contents in commercial UHT milks varied between 154 and 1065 mg·L^{-1}. This difference could be explained by the fact that lactulose, as well as furosine formation, depends on many factors such as the heating rate and time, milk composition and storage conditions.

3.2. Front-face fluorescence investigation of milk

An intrinsic fluorophore of proteins, tryptophan, was excited at 290 nm and the emission spectra were recorded between 305 and 450 nm. In fact, the emission fluorescence region (305–450 nm) considered in this study allowed the study of the fluorescence of tryptophans (maximum emission at about 335 nm), as well as the fluorescent Maillard-reaction products (maximum emission at 440 nm). As fluorescent Maillard-reaction products are bound to the proteins, fluorescence transfer may occur between tryptophanyl residues in proteins and those molecules exhibiting a maximum excitation wavelength at about 340 nm [4]. Normalized emission fluorescence spectra of NUHT1, NUHT2, and OUHT milks and their mixtures were similar to those presented in Figure 3. All the maxima of tryptophan emission peaks were located at 343 nm. A shoulder was also observed at about 440 nm. Heat treatment significantly alters fluorescence properties of milk, that is clearly illustrated by the emission spectra obtained. As OUHT milk was added to NUHT milks, the fluorescence emission at 340 nm of the mixtures obtained decreased while the fluorescence at 440 nm increased (Fig. 3). Heat treatment causes partial denaturation of milk proteins and formation of fluorescent Maillard-reaction products. As a consequence of the denaturation, part of the tryptophan environments in proteins is modified.

Emission and excitation spectra of milks were recorded between 380–600 nm and 250–420 nm, respectively. The emission spectra obtained exhibited two peaks. As shown in Figure 4, the emission maxima of the first peaks were found at 416 nm, 429 nm and 434 for NUHT1, OUHT and pasteurized milks, respectively; and for the second peak at 518 nm, 515 nm and 517.5 nm for NUHT1, OUHT and pasteurized milks, respectively.

The analysis of the shape of the first peak (Fig. 4) shows that it depends on the quantity of fluorescent Maillard-reaction products present, which result from the

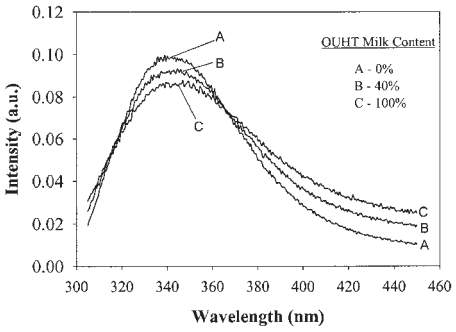

Figure 3. Normalized tryptophan emission spectra (exc. 290 nm) of NUHT2 milk containing different quantities of OUHT milk.

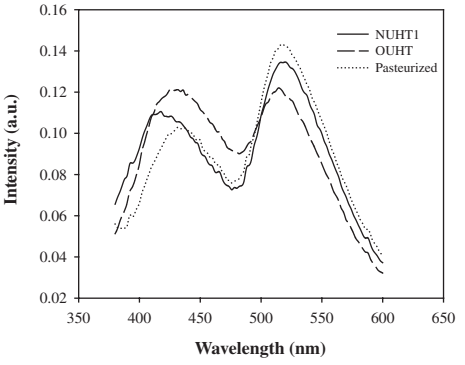

Figure 4. Normalized emission spectra (exc. 360 nm) of advanced Maillard-reaction products in NUHT1, OUHT and pasteurized milks.

heating rate. According to the method of Birlouez-Aragon et al. [4], a global quantification of the advanced Maillard-reaction products is given by measuring the fluorescence intensity at 440 nm. Our data (Figs. 3 and 4) clearly indicate that front-face fluorescence spectroscopy may be able to monitor protein structure changes and furosine content in milks caused by heat treatment of different rates. The fluorescence spectra of the milks showed an evolution of the fluorescence at about 430 nm related to the intensity of the heat treatment. The changes in the fluorescence in this region suggested that a fluorescent probe, different from tryptophanyl residues, is formed during severe heat treatment. Following excitation at 290 nm, the spectra for UHT milks showed a typical peak at about 340 nm, corresponding to the fluorescence emission of tryptophan residues, and an additional fluorescence band exhibiting a maximum near 430 nm (Figs. 3 and 4). This last fluorescence band was not observed for pasteurized milk (data not shown).

As milk contains a large number of molecules able to fluoresce, there is no certain explanation for the nature of the fluorophore emitting at about 515 nm (Fig. 4). The observed fluorescence is probably due to the contribution of FADH, which is a co-factor of enzymes found in milk. Whatever the nature of the observed fluorophore, our results show that the intrinsic fluorescence of milk has a huge potential to develop methods allowing the determination of the heating processes applied to milk.

The excitation spectra of fluorescent Maillard-reaction products (emission at 440 nm) are shown in Figure 5. Each excitation spectra is also characterized by the presence of two peaks, the second of which is located at 354 nm, 363 nm and 365 nm for NUHT1, OUHT and pasteurized milks, respectively. Considering that furosine is determined by measuring the fluorescence at 440 nm following excitation at 360 nm [4],

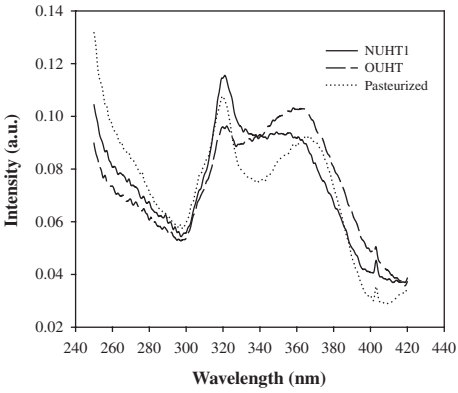

Figure 5. Normalized excitation spectra (em. 440 nm) of advanced Maillard-reaction products in NUHT1, OUHT and pasteurized milks.

this peak may be assigned to fluorescent Maillard-reaction products. However, NADH presents a maximum excitation wavelength at about 350 nm and may also contribute to the fluorescence observed in this region (Fig. 5).

The fluorescence emissions of the first peaks located at about 320 nm also vary depending on the quantity of fluorescent Maillard-reaction products in the milk samples, i.e., the heat treatment applied. This peak may be mainly assigned to vitamin A since this fluorophore presents emission and excitation maxima at 410 nm and 322 nm [12], respectively. Therefore, useful information can be extracted from the milk fluorescence spectra evaluated by chemometric tools despite the large number of fluorophores found in milk.

Considering the excitation maxima in the vicinity of 360 nm (Fig. 5), the highest fluorescence intensity is exhibited by OUHT milk, which is mainly determined by the Maillard-reaction products. The pasteurized milk sample containing a relatively low content of Maillard-reaction products should have minimal fluorescence emission compared with NUHT and OUHT milks.

Whatever the nature of all the fluorescent compounds contributing to the excitation spectrum recorded in the 250–420 nm wavelength range, it appears that the main two peaks are located in the region where tryptophan residues emit photons. Considering the short distance between tryptophan residues and fluorescent Maillard-reaction products in a protein, fluorescence transfer between a donor (tryptophan) and an acceptor may be observed. After excitation at 290 nm, photons emitted by tryptophan residues are absorbed by the second probe (fluorescent Maillard-reaction product) fluorescing with maximum emission at about 440 nm (Fig. 1). It is concluded that a spectrum recorded on a milk sample between 305 and 450 nm following excitation at 290 nm contains information on protein structure (denaturation), as well as on Maillard-reaction product content.

Thus, measurements of fluorescence properties recorded directly on different milks and their mixtures modeling variable heat treatment rates have demonstrated the potential of front-face fluorescence technique to be a powerful instrument to evaluate the quality of milk, regarding Maillard-reaction products.

3.3. Multivariate analysis of milk fluorescence spectra

Principal component analysis (PCA) was applied to the spectral data to extract information on the content of advanced Maillard products in the milk samples investigated. Using this technique, the emission spectra can easily be compared with each other in the way that two similar spectra are represented by two neighbouring points on the plot called a similarity map. PCA is well suited for optimizing the evaluation of data with a minimum loss of information.

The spectra were normalized by reducing the area under each spectrum to a value of 1. In this way, only the shapes (shift of the peaks and broadening of the bands) of the spectra were considered in the analyses [12]. The results of PCA applied to the normalized tryptophan emission spectra of NUHT1, NUHT2 and OUHT milks and their mixtures recorded in triplicate between 305 nm and 450 nm (resolution: 0.5 nm) are presented in Figure 6. To build the similarity map, two principal components, A1 and A2, were used. The variance explained by A1 and A2 is 95.7% and 2.8%, respectively. Both principal components

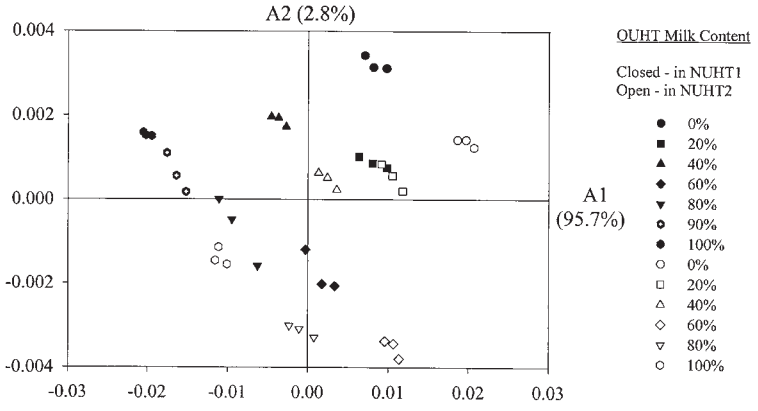

Figure 6. Principal component analysis similarity map defined by the principal components A1 and A2 for NUHT1 and NUHT2 milks containing different quantities of OUHT milk.

clearly discriminate different samples, grouping together replicates. This confirms that the technique is quite sensible and allows one even to distinguish between the two normal UHT milks examined (NUHT1 and NUHT2), which are supposed to be of the same quality, but were not manufactured in the same plant. The principal component 1 discriminated the milk samples according to the content of fluorescent Maillard-reaction products. The samples with the lowest furosine content were observed on the far right, while the samples with the highest furosine content were observed on the far left.

To compare the results obtained by the newly developed front-face fluorescence method and the reference methods (HPLC and enzymatic), principal component regression (PCR) was applied. For this purpose, two data matrices were built: the first one contained the spectral data and the second one grouped results on lactulose and furosine determined with the reference methods. The analysis showed that there is a high correlation between the results. Observed and predicted values for both furosine and lactulose were described well by linear functions (Figs. 7 and 8). Correlation equations taking into account the principal components as independent variables were obtained as follows:

furosine ($r^2 = 0.956$)

$$F = 73.993 - 886.944 \times A1 - 4448.71 \times A2 + 4841.938 \times A3 - 3085.314 \times A13;$$

lactulose ($r^2 = 0.987$)

$$L = 76.385 - 1985.003 \times A1 - 5816.084 \times A2 + 3443.946 \times A3 - 4126.431 \times A4.$$

Thus, front-face fluorescence technique can provide distinct qualitative as well as quantitative information on the compounds that define physicochemical properties of milk submitted to heat treatment. A high correlation existing between the data measured by the reference and new methods indicates that front-face fluorescence spectroscopy is a very sensible technique for determining furosine and lactulose in milk. Whereas the determination of furosine content from the fluorescence spectra can be easily explained by the fluorescence of advanced Maillard products emitting in the 440 nm region following excitation at 290 nm, the correlations between the fluorescence spectra recorded in the 305–450 nm wavelength range and the lactulose content is more difficult to explain since lactulose is not a fluorescent molecule. Nevertheless, we can hypothesize that

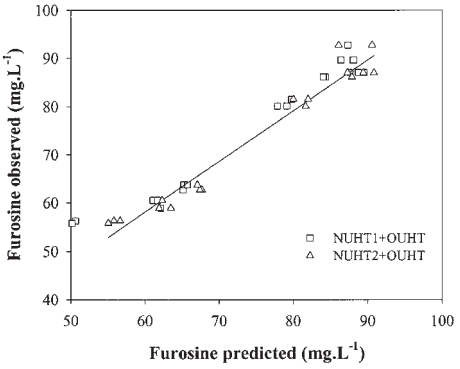

Figure 7. Prediction of furosine content in NUHT1 and NUHT2 milks containing different quantities of OUHT milk and comparison with observed values determined by HPLC.

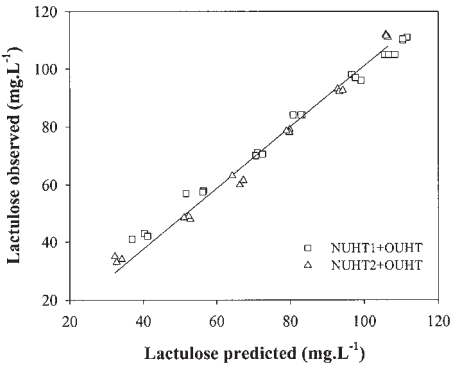

Figure 8. Prediction of lactulose content in NUHT1 and NUHT2 milks containing different quantities of OUHT milk and comparison with observed values determined by the enzymatic method.

the fluorescence properties of proteins in a heat-treated milk sample are correlated to the lactulose content.

4. CONCLUSION

The potential of front-face fluorescence spectroscopy in combination with chemometric methods to evaluate lactulose and furosine products in heat-treated milk has been demonstrated.

The most important advantage of the front-face fluorescence technique is that no physical or chemical treatment is needed to provide samples with specific properties that allow them to be examined using one of the practiced techniques. This is possible because of the ability of front-face fluorescence to deal with optically opaque systems [12, 16, 17]. Therefore, the front-face fluorescence technique provides unique information, which is a real reflection of the natural state of milk compounds. The FAST method, proposed previously as a rapid technique to determine advanced Maillard products in milk [4], is based on fluorescence too. However, prior to fluorescence measurements, a milk sample should be converted into a transparent liquid by applying several specific procedures, which requires some time and increases the cost of experimentation. Considering the overall time needed to obtain an analytical result, none of the techniques can compete with the front-face fluorescence technique developed here. Therefore, front-face fluorescence as a progressive technique may successfully be applied in evaluation of the quality of heat-treated milk and attract the interest of food scientists as well as food processors, since on-line measurements may be performed using an optic fiber.

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