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Food Chemistry 98 (2006) 188–194
Food Chemistry
www.elsevier.com/locate/foodchem

Analytical, Nutritional and Clinical Methods

Evaluation of tracers for the authentication of thermal treatments of milks

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Received 31 January 2005; received in revised form 22 June 2005; accepted 30 July 2005

Abstract

For regulatory purposes, several methods have been proposed for defining biochemical tracers to identify heat treatments to which milks may be submitted. However, none of these methods is universal whereas they are usually restricted to one or two categories of treated milks and are not able to discriminate any unknown sample arriving in a control laboratory. Therefore, Arilait-Recherches and Syndilait (Syndicat National des Laits de Consommation) decided to undertake a study in order to define an efficient discrimination approach applicable to commercial milk samples. About 200 commercial samples were tested, produced in several plants where they received the standard heat treatments here-applied. Five types of heat treatments were studied: pasteurisation, high pasteurisation, direct UHT, indirect UHT, and sterilisation. In order to estimate the effect of storage on long-term storage milks, samples were submitted to two storage conditions: 90 days at 25 °C and 90 days at 35 °C. The selected tracers were: furosine; lactulose; native α-lactalbumin; denaturised α-lactalbumin; percent of denaturised α-lactalbumin; FAST index, tryptophan fluorescence, β-lactoglobulin, and lactoperoxidase: altogether 5000 measurements were collected. Analysis of variance clearly demonstrates that no tracer can be selected to universally discriminate milks from all these technologies. The only possible authentication technique must be based on a multivariate approach. The application of factorial discriminant analysis by combining at least five tracers, gives a possible solution to this question. Most discriminative tracers are those which globally measure the structural modifications of the milk protein rather than those which specifically quantify the metabolites of the Maillard reaction.
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Keywords: Milk heat treatments; Authentication; Discriminant analysis; Furosine; α-Lactalbumin

1. Introduction

Many thermal treatments are applied to bulk milk in order to improve its conservation. In Europe, a wide range of heating processes and time/temperature combinations can be found, leading to considerable diversity of commercial products. Attention was focused on the authentication of heat-treated milks and milk integrity whereas heat treatments always induce changes in the biochemical composition of milk.

The investigation for a universal and well adapted biochemical tracer for the heat treatment of milks began in 1992. Starting from the preliminary study and published data, a list of potential tracers was established (Council Regulation (EC) 2597/97, 1997; Pellegrino et al., 1995). Two major types of analytes were previously identified as potential tracers of heat treatments (Pellegrino, Tirelli, Masotti, & Resmini, 1996); they have been more recently

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doi:10.1016/j.foodchem.2005.07.052

described as potential intrinsic time temperature integrators (TTI) (Claeys, Van Loey, & Hendrickx, 2003). The first category includes compounds that appear in milk as a result of the Maillard reaction. Among these, analytical quantification techniques for tracers such as lactulose and Maillard products such as furosine have been standardised by international bodies. The second category of analytes measure milk proteins, structurally or functionally modified by the heat treatment: enzymes such as alkaline phosphatase and lactoperoxidase have been widely used as tracers to characterise low-intensity heat-treatments such as pasteurisation. Various techniques that measure the denaturation of whey proteins such as β-lactoglobulin or α-lactalbumin have also been shown to be suitable for evaluating the intensity of heat treatments. For instance, the quantification of sulfhydryl groups (Guingamp, Humbert, & Linden, 1993) and lactosylated proteins (Morgan, Leonil, Molle, & Bouhallab, 1997) were demonstrated as potential tracers. More recently, fluorimetric methods have also been proposed for overall characterisation of heat-treated milks (Birlouez-Aragon, Leclere, Quedraogo, Birlouez, & Grongnet, 2001; Birlouez-Aragon, Sabat, & Gouti, 2002).

A classic drawback in several of these studies is that the samples were prepared on the laboratory scale and did not represent the actual diversity of industrially processed milks. Therefore, Arilait-Recherches and Syndilait supported an experimental study on commercial milk samples, representative of French consumption and/or production, in order to review the possible application of proposed heat tracers in a practical context. The goals of this study can be simply summarised:

1. Assess whether heat treatments can be clearly authenticated on set of commercial milks representative of the classical heat treatment processes applied in France.
2. Assess whether one or several heat treatment tracers can be used to achieve this authentication.
3. Estimate the consequences of storage and shelf-life conditions on the discriminative performances of the selected tracers.

2. Materials and methods

2.1. Samples and experimental design

In order to have samples representative of French consumption, milk samples were directly collected in several plants, using variable technologies, at different seasons and different times during the process. Table 1 summarizes the experimental factors which were assumed to influence the concentrations of the tracers studied. First factor "Technology" consists in five different thermal treatments: temperature and time programs as applied by participating milk producers are indicated in fifth column. Sample codes used in the rest of the text are given in third column and number of sampling points (plants) in fourth. Second factor "Storage conditions" illustrates the fact that three technologies give long-term storage products which may be consumed after a variable storage period. Thus, sample aliquots were collected and submitted to two kinds of storage before analysis: S1, 90 days in a 25 °C incubator; S2, 90 days at 35 °C. S0 identifies non-stored samples.

Third factor "Season" was introduced to assess possible seasonal effects, and samples were collected during two different seasons: summer 2001 and winter 2001–2002. Moreover, the possible influence of the "Process" factor was controlled by collecting samples at the beginning and at the end of the batch. This fourth factor was only studied for long-term storage milks from 15 plants.

Twenty three plants participated in the study and prepared 92 samples. It must be underlined that these samples were collected during the normal production process, without any special intervention. Moreover, 15 plants provided triplicate samples that were stored under three storage conditions as described in Table 1. Finally, this gives a theoret-

Table 1
Factors of variation and level codes used in this study

Factors	Levels	Group codes	Number of sampling points	Heating treatment conditions
Technology	Pasteurised	1_Past	6	74 °C/30 s
	High pasteurised	2_Hpast	2	85 °C/30 s
	Direct UHT[a]	3_Dire	6	87 °C/20 s, and 140°/2 s
	Indirect UHT[a]	4_Indi	7	78 °C/15 s, and 137 °C/4 s
	Sterilised[a]	5_Ster	2	115 °C/12 min
Storage conditions	No storage	S0		
	90 days at 25 °C[b]	S1		
	90 days at 35 °C[b]	S2		
Season	Summer	S		
	Winter	W		
Time in the process	Beginning	B		
	End	E		

[a] Long-term storage milks.
[b] These storage conditions can only be applied to long-term storage milks.

ical total of supplementary 120 samples. As some samples were not correctly conserved or were damaged during transport, a final total of 194 samples, instead of $92 + 120 = 212$, was analysed and used for the statistical analysis.

2.2. Methods of analysis

Table 2 presents the list and the references of the standards of analytical methods used for the determination of studied heat tracers. Furosine (FURO) was determined according to ISO:DIS 18329 standard (2001) and the basic principle of the technique consists in an inverse phase HPLC, an elution with ion-paired buffer and a UV detection at 280 nm. ISO 11868 (1998) standard was applied to the determination of lactulose (LACT). After removing of fat and protein, lactulose is measured on the filtrate by HPLC using ion-exchange column and thermostatic refractive index detector. Quantification is achieved by reference to standard samples. β-Lactoglobulin (BLGN) was determined according to, ISO 13875/IDF 178 (1996). This standard specifies a method for the quantitative determination of the β-lactoglobulin content, soluble at pH 4.6 in liquid milk, by HPLC on a Chrompack 300 RP column and UV detection at 280 nm. Lactoperoxidase (LAPE) assay was the method of Shindler and Bardsley (1975). This method consists in removing precipitated milk proteins by filtration, adding ABTS [2,2′azino-di-(3-ethyl-benzthiazoline-6-sulphonic acid)] to supernatant and incubating at 37 °C for 1 h. LAPE is quantified by reading absorbance at 420 nm and converting this measurement by using a molar extinction coefficient of 43.2.

New candidate tracers were also tested: α-lactalbumin determined by the Biacore™ method and the FAST index determined by the FAST method®.

Native and denatured α-lactalbumin are determined by two surface plasmonic resonance (SPR) measurements. More details on this technique and the specific application to α-lactalbumin using the Biacore AB system are available in the recent publication by Dupont, Rolet-Répécaud, and Muller-Renaud (2004). For this study, monoclonal antibodies (Mab) 20 and 130, immobilised on a CM5 sensorchip as described by Löfas and Johnsson (1990), were used. They were demonstrated to be, respectively, specific of native and heat-denatured α-lactalbumin (Jeanson, Dupont, Grattard, & Rolet-Répécaud, 1999). For each assay, 10 µl of milk samples were diluted 1/1000 in adequate buffer (HBS) and injected in Biacore AB system: two separate signals were recorded and transformed by external calibration, one for native α-lactalbumin (N_ALB), the other for denatured (D_ALB) determinations, respectively. Each N_ALB and D_ALB measurement was made in triplicate. Results were expressed by calculating the average α-lactalbumin denaturising rate %ALB and expressed as

$$\%\text{ALB} = 100 \times \frac{\text{D_ALB}}{\text{D_ALB} + \text{N_ALB}}. \quad (1)$$

The FAST® method is a rapid method based on the determination of maximal fluorescence emission in total milk sample at 350/440 nm. This signal can be related to the modifications of molecular structures formed between reducing sugars or oxidizing lipids and lysine residues of proteins. This raw fluorescence signal is not directly used but corrected for the protein concentration. Protein concentration is also estimated by measuring tryptophan fluorescence (FTRP) at 290/340 nm in the pH 4.6 soluble fraction of the milk sample. Finally, the FAST index (FAST) is defined as the ratio of both these signals and is interpreted as a global indicator of milk protein changes (Birlouez-Aragon et al., 2001). When applied to heat-treated milk samples, FAST was demonstrated to be well correlated with lysine modification, briefly known as "lysine loss" and estimated by the specific determination of acid-released lysine, fluorescamine-reactive lysine, or more globally by infrared spectroscopy (Birlouez-Aragon et al., 2002). When calibrated with pertinent biomarkers, the FAST index is an efficient means of controlling heat treatment for a given food product.

Depending on the cost of the technique, measurements were replicated several times, so that a total of about 5000 measurements were collected.

2.3. Statistical analysis

Two types of statistical techniques were used: univariate and multivariate. Univariate techniques, mainly analysis of variance (ANOVA), were used for overall detection of any statistically significant effects of the factors studied. The experimental design applied for this study is a 4-factor nested experimental design – technology (5 levels), season (2 levels), time of the process (2 levels) and storage (3 levels)

Table 2
Tracers, codes and analytical method references (AU represents an arbitrary unit)

Tracer	Code	Unit	Method
Lactulose	LACT	mg/L	FIL 147B:1998
Furosine	FURO	mg/100 g protein	ISO:DIS 18329 (2001)
Lactoperoxidase	LAPE	IU/mL	Spectrophotometry
β-lactoglobulin	BLGN	mg/L	FIL 178:1996
Tryptophan fluorescence	FTRP	AU	FAST method
FAST index	FAST	AU	
Native α-lactalbumin	N_ALB	AU	BIACORE method
Denatured α-lactalbumin	D_ALB	AU	
% Denatured α-lactalbumin	%ALB	–	

– with an unequal number of replicates. The Technology factor was considered as a random effect factor.

Discriminant analysis (DA) was used as a multivariate technique. This well known technique has been extensively applied and described in many textbooks and publications (Cooley & Lohnes, 1971). It is often presented as an extension of the multivariate analysis of variance (MANOVA). The mathematical rationale consists in combining the original variables into new ‘‘canonical variables’’, computed in order to optimize the dispersion of groups; i.e. by looking for the combination of variables which gives the highest inter-group variance. Discriminative functions represent planes or hyperplanes which attempt to correctly separate the individuals of different groups of samples. These planes are obtained by combining the initial variables into a polynomial whose coefficients are the eigenvectors of the inter-group correlation matrix. In order to avoid a scale effect due to the different ranges of variation of the variables, raw data are standardised before computing this matrix. The great advantage of a multivariate technique, compared to univariate or bivariate methods, is that the correlation between variables can be used to better identify and select the most pertinent variable combinations. All calculations were performed using JMP® software from SAS Institute.

3. Results

In the framework of an unpublished preliminary study, 15 heating technologies or processes were studied leading to 35 types of laboratory-prepared samples on which 23 methods of analysis were applied including heat-treatment tracers and proximate. Data were used to prepare the present study. For instance, it was demonstrated that total fat had no significant influence on tracers' levels and could be discarded in future studies. Finally, nine methods of analysis for tracers were shown to be relevant, but, none could be claimed as universal and unambiguously discriminate one technology.

For this study, ordinary commercial samples were collected during the normal production process of participating plants. The goal was to better reflect the variations of milk that is actually consumed and give more robust discrimination models than those calculated with artificially prepared samples.

3.1. Exploratory data analysis

In order to identify variables presenting strong differences between sample groups, hierarchical 4-way ANOVA was applied to each tracer listed in Table 2. For all variables, 'Season' and 'Time in the process' have no significant effects and were not taken into account for the rest of the study. Moreover, the random factor 'Technology' often explained more than 90% of the total variance. ANOVA was completed by comparing all possible pairs of group means. Whereas many group mean differences were not significant, no tracer can be elected as an acceptable candidate to unambiguously separate all milk groups. Table 3 presents group means and standard deviations for each tracer as a function of the technology and storage conditions: according to the group, the numbers of replicates varied from 10 to 58.

Lactoperoxidase (LAPE) and β-lactoglobulin (BLGN) demonstrated a special behaviour while these tracers can be used to only identify one specific heating technology: this is not surprising because they only measure specific chemical modifications. Consequently, these tracers are not well suited to the goals of this study, which requires tracers with a continuous range of variations over all types of milk. Therefore, LAPE and BLGN were not taken into consideration in the rest of the study.

In order to better understand data structure, bivariate projections were used. Fig. 1 illustrates the scattering of samples for FTRP and FAST, for samples that were not submitted to storage. For the commodity of representation data were transformed into their decimal logarithms. According to Fig. 1, it can be concluded that the combination of FTRP and FAST is: (i) well adapted to discriminate sterilised milks; (ii) probably suitable for pasteurised milks; (iii) inefficient for other types of milks, although it may indicate trends. In this figure, the role of the 95% confi-

Table 3
Group mean and standard deviation (between parenthesis) for each tracer according to type of milk and storage conditions

Technology	%ALB	D_ALB	N_ALB	BLGN	FAST	FTRP	FURO	LACT	LAPE
No storage									
1. Pasteurized	11.0% (3.18%)	25 (7.7)	203 (12.3)	3550 (540.7)	18.1 (3.3)	5.0 (0.4)	4 (2.3)	15 (12.1)	0.52 (0.224)
2. High. Pasteur.	32.0% (6.15%)	81 (18.4)	171 (11.2)	1105 (623.2)	39.4 (10.8)	2.1 (0.7)	29 (33.6)	31 (13.6)	0.05 (0.000)
3. UHT direct	34.8% (6.33%)	69 (12.8)	130 (14.3)	660 (372.4)	54.7 (13.6)	1.9 (0.5)	59 (34.8)	144 (48.6)	
4. UHT indirect	73.6% (8.25%)	166 (38.0)	57 (14.7)	44 (123.8)	115.0 (19.1)	1.0 (0.1)	188 (51.8)	400 (134.4)	
5. Sterilized	98.6% (0.71%)	244 (24.2)	3 (1.5)	0 (0.0)	257.1 (20.4)	0.5 (0.1)	371 (88.7)	834 (218.1)	
Storage 90 days at 25 °C									
3. UHT direct	24.1% (3.83%)	43 (6.5)	136 (15.9)	0 (0.0)	46.3 (10.7)	2.2 (0.5)	169 (30.9)	207 (83.6)	0.05 (0.000)
4. UHT indirect	60.0% (9.65%)	100 (24.4)	65 (15.0)		90.0 (16.6)	1.2 (0.2)	264 (51.9)	462 (104.1)	
5. Sterilized	97.5% (1.31%)	176 (17.2)	4 (2.2)		183.1 (25.5)	0.6 (0.0)	417 (81.3)	967 (110.5)	
Storage 90 days at 35 °C									
3. UHT direct	31.9% (4.45%)	56 (7.5)	121 (16.0)	0 (0.0)	47.4 (11.3)	2.5 (0.5)	464 (58.4)	415 (83.6)	0.05 (0.000)
4. UHT indirect	60.5% (5.91%)	88 (12.0)	58 (14.0)		80.7 (10.8)	1.4 (0.1)	529 (83.3)	609 (101.7)	
5. Sterilized	97.6% (1.31%)	163 (14.0)	4 (2.3)		127.3 (20.2)	0.9 (0.1)	570 (81.8)	1063 (241.1)	

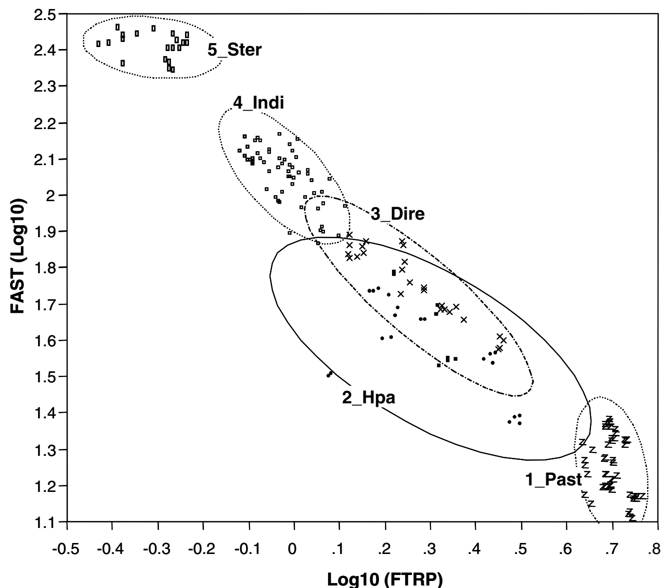

Fig. 1. Graphic display of individual values of the FAST index and tryptophan fluorescence (expressed as decimal logarithms). The 95% confidence ellipses are drawn for each type of milk and for all samples not submitted to storage. Legend: 1. Pasteurised milks (1_Past); 2. High pasteurised milks (2_Hpast); 3. Direct UHT milks (3_Dir); 4. Indirect UHT milks (4_Indir); 5. Sterilised milks (5_Steril).

dence ellipses is to indicate that, although a statistical test may lead to the conclusion that the arithmetic means are statistically different, many false positive samples may be considered as correctly classified.

This situation is degraded when long-term storage milk samples submitted to storage are included in display. Fig. 2 illustrates, for the same tracers, the increased dispersion of samples and the overlapping of groups: S0 represents no-storage samples; S1 90 days at 25 °C and S2 90 days at 35 °C. Moreover, it is possible to see a gradient within groups: for sterilised milks, the tougher the storage conditions, the lower the values of FAST and FTRP. This remark is also applicable to UHT milks but it is less visible.

In conclusion, univariate and bivariate data analysis allow selecting most promising tracers and illustrating that commercial milk samples present an almost continuous spectrum of variation in regard of measured tracers. Then, an alternative consists in exploring if a combination of tracers cannot help in discriminating samples.

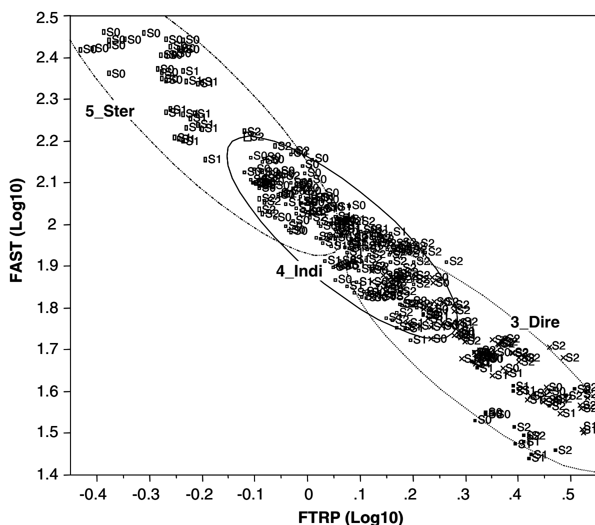

Fig. 2. Influence of storage on the dispersion of concentration with the same tracers as in Fig. 1 for long-term storage milks (see the text for codes). Legend: 3. Direct UHT milks (3_Dir); 4. Indirect UHT milks (4_Indir); 5. Sterilised milks (5_Steril); S0. No storage; S1. 90 days at 25 °C; S2. 90 days at 35 °C.

3.2. Discriminant analysis

Therefore, it was decided to apply discriminant analysis (DA) in order to prevent this overlapping by combining several tracers. It was performed with all samples, whatever the storage conditions, using 'Technology' as a grouping factor with the seven selected variables. Fig. 3 illustrates the projections of all samples, whatever stored or not, on canonical variables 1 and 2. This figure shows that some groups of samples can be easily separated from the others, while others still overlap. Variables are displayed on the same figure as segments linked to the centre of the diagram. The lengths of these segments indicate their discriminative power and their directions their relations to sample groups. However, this 2D representation is still rather confusing. For instance, the two groups 'high pasteurised' and 'direct

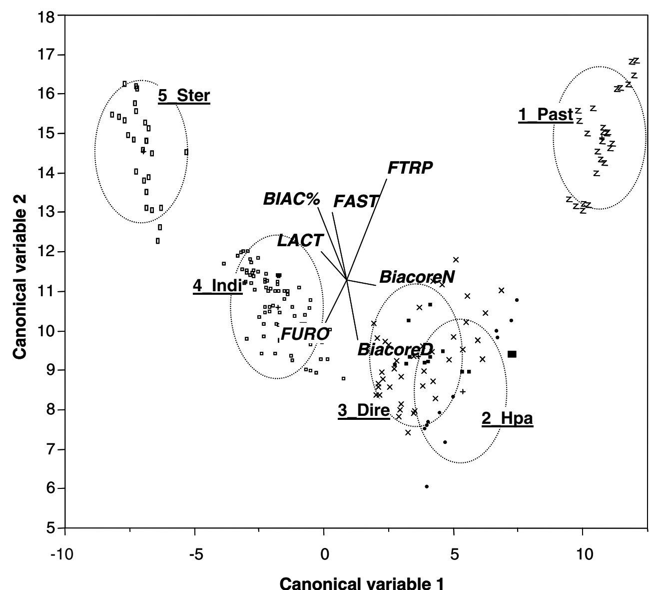

Fig. 3. Projections on canonical variables 1 and 2 computed by discriminant analysis for all milk samples, whatever the storage conditions. Density ellipses, around each centroid, represent 50% dispersion probability. Groups: 1. Pasteurised milks (1_Past); 2. High pasteurised milks (2_Hpast); 3. Direct UHT milks (3_Dir); 4. Indirect UHT milks (4_Indir); 5. Sterilised milks (5_Steril). Variables: LACT: lactulose; FTRP: tryptophan fluorescence; FAST: measurement of the FAST index; N_ALB: native α-lactalbumin; D_ALB: denatured α-lactalbumin; %ALB: % denatured α-lactalbumin.

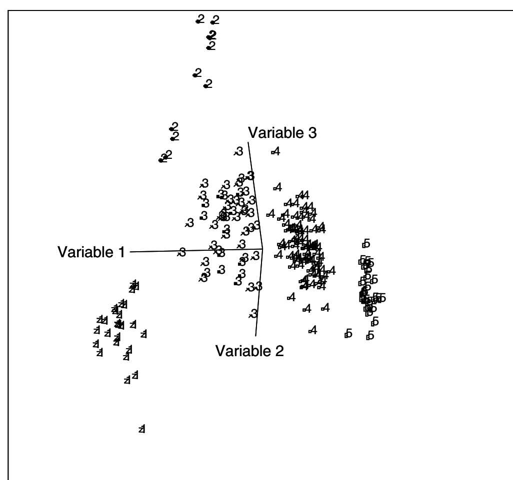

Fig. 4. Projections of samples on three canonical variables giving satisfactory separation of the five milk sample groups. Only group numbers are indicated, whatever the storage conditions. Legend: 1. Pasteurised milks; 2. High pasteurised milks; 3. Direct UHT milks; 4. Indirect UHT milks; 5. Sterilised milks.

UHT' appear to completely overlap and cannot be separated. With 3D representation and selecting an adequate angle of projection, the five groups are seen to be perfectly separated, as shown in Fig. 4. This visual interpretation can be confirmed by computing the posterior probability for a sample to belong to a given group. These probabilities were computed for all samples and all groups, and each sample was classified in the group for which its probability was the highest. All samples were correctly classified and a 100% score was reached for all groups. It can be concluded that it is possible to determine the heat treatment of a new sample from its biochemical analysis. But several tracers have to be combined to reach this goal.

On the other hand, it is possible to find out which tracer is the most pertinent, even if it cannot be used alone. This can be achieved by examining the eigenvectors that were used to build the canonical variables. These values are reported in Table 4 and indicate the important roles played by FTRP and %ALB: the higher the value, the more influential (in terms of discrimination) the variable. However, these two tracers, when used in bivariate diagrams, were not able to unambiguously separate the five groups and correctly sort out the composition modifications which occur during the storage. This means that a correct discrimination model needs more than these two tracers. By applying LDA to a reduced number of variables, we were able to show that four tracers (%ALB, FTRP, FAST and N_ALB) were sufficient to correctly classify nearly all samples. However, some groups were not 100% correctly classified, while this goal was achieved when using all variables.

Table 4
The first three eigenvectors measuring the discriminative power of each tracer

Tracers	Vector 1	Vector 2	Vector 3
FTRP	<u>0.0866</u>	<u>0.2072</u>	<u>−0.1206</u>
FAST	−0.0005	0.0020	0.0015
N_ALB	0.0018	−0.0002	0.0103
D_ALB	0.0002	−0.0013	−0.0021
%ALB	<u>−0.3188</u>	<u>0.7477</u>	<u>1.8175</u>
FURO	−0.0001	−0.0002	0.0003
LACT	−0.0002	0.0002	−0.0000

The highest coefficients correspond to the most discriminative variables and are underlined.

4. Discussion

From this study, it can be concluded that nearly all milk samples can be correctly classified by combining two tracers only, if samples were not subjected to a storage step. In fact, storage conditions seem to highly modify the concentrations of the different markers and change reference values so that the various technological treatments can no longer be differentiated. In order to reach an acceptable level of discrimination, it is necessary to combine at least four tracers into a multivariate model. However, the performances of DA are strongly dependent on the number of data used to build the model; better performances should be achieved with a larger number of samples in each group. This technique is very sensitive to the structure of the data set used for building the discriminant functions and the number of samples in each group can be critical. However, other discriminant techniques can be applied and model validation techniques, such as cross-validation of jacknifing, can also be useful.

It is interesting to note that the most discriminative tracers are those which measure overall evolution of the protein fraction, such as determination of α-lactalbumin or the FAST index. However, the ability of protein fraction determination to discriminate heat treatments also depends on the analytical technique used. Indeed, when α-lactalbumin and β-lactoglobulin were determined by HPLC, storage led to significant modifications of milk chromatograms which became impossible to interpret (data not shown). In contrast, when the analytical signal is based on immunochemical mechanisms, such as in the Biacore technique, the performance is not affected by storage. These observations confirm the findings of Pellegrino, Resmini, and Luf (1995) who demonstrated that the acid-soluble β-lactoglobulin contents remained unchanged during milk storage, although the shape of the HPLC peaks progressively changed (Corzo, Lopez-Fandino, Delgado, Ramos, & Olano, 1994).

Tracers which are assumed to measuring the emergence of degradation metabolites, such as furosine or lactulose, are less efficient. In the present study, we observed a significant increase during storage of lactulose and furosine concentrations in UHT and sterilised milks. The influence of storage on the monosaccharide composition of UHT milks was already demonstrated by (Belloque, Villamiel, Lopez-Fandin, & Olano, 2001). These results are in accordance with earlier observations. In this respect, Corzo et al. (1994) demonstrated that storage for 90 days at 20 °C in-

creased the furosine concentration in UHT-treated milk samples by a range of 8.3–48.6 mg/100 g protein. At 30 and 40 °C, the increase ranged from 77 to 157 mg/100 g and 166 to 312 mg/100 g protein. For lactulose, an increase of 20 ± 4 and 95 ± 10 mg/l during 10 weeks was observed at 22 and 30 °C, respectively (Van Renterghem & De Block, 1996). Moreover, Pellegrino, De Noni, and Resmini (1995) showed that furosine concentration increased in a wide range of storage temperatures (between 4 and 35 °C), but lactose isomerisation occurred only on storage at 35 °C leading to an increase in lactulose concentration. The formation of furosine during storage of sterilised milk was shown to be independent of the initial concentration of this compound (Pellegrino et al., 1995).

Moreover, this study gave us the opportunity to compare new analytical methods which may be interesting candidates for forthcoming works. But, at this stage of development of these methods, it would be premature to try to define reference values for these tracers in relation to authentication of heat-treated milks: better standardisation and interlaboratory validation are necessary for improved reproducibility. It is now necessary to check how far these new tracers are discriminant in respect of storage conditions. Generally, this stage is not well defined in terms of duration and temperature, i.e. the "history" of a stored milk sample cannot be easily assessed. A recent work demonstrated that changes in the concentration of several tracers can be easily modelled as a function of temperature and time (Claeys et al., 2003). But this was achieved under laboratory conditions which are much better controlled than those of commercial samples. Therefore, the levels of both storage factors – temperature and time – applied for this study were defined on a rather subjective basis and would require closer attention. The variability of the usual storage conditions is also very difficult to assess. On the other hand, as the evolution of the protein fraction seems to be the key issue, more attention must to be paid to the qualitative composition of the protein of heat-treated milks. Many studies have already been done in this field but a better understanding of the biochemical modifications could be an interesting issue. If it becomes possible to model the thermal history of a milk sample as a function of its tracer composition, this history would need to be accurately standardised. This point rejoins our earlier remarks on storage conditions.

Acknowledgements

The authors thank the associate companies of Syndilait (Syndicat National des Laits de Consommation) for financial support and supply of milk samples.

References

Belloque, J., Villamiel, M., Lopez-Fandin, R., & Olano, A. (2001). Release of galactose and *N*-acetylglucosamine during the storage of UHT milk. *Food Chemistry, 72*, 407–412.

Birlouez-Aragon, I., Leclere, J., Quedraogo, C. L., Birlouez, E., & Grongnet, J. F. (2001). The FAST method, a rapid approach of the nutritional quality of heat-treated foods. *Nahrung, 45*(3), 201–205.

Birlouez-Aragon, I., Sabat, P., & Gouti, N. (2002). A new method for discriminating milk heat treatment. *International Dairy Journal, 12*, 59–67.

Claeys, W. L., Van Loey, A. M., & Hendrickx, M. E. (2003). Influence of seasonal variation on kinetics of time temperature integrators for thermally processed milk. *Journal of Dairy Research, 70*, 217–225.

Cooley, W. W., & Lohnes, P. R. (1971). *Multivariate data analysis*. New York: Wiley.

Corzo, N., Lopez-Fandino, R., Delgado, T., Ramos, M., & Olano, A. (1994). Changes in furosine and proteins of UHT-treated milks stored at high ambient temperatures. *Zeitschrift für Lebensmittel-Untersuchung und-Forschung, 198*, 302–306.

Council Regulation (EC) 2597/97. (1997). Laying down additional rules on the common organization of the market in milk and milk products for drinking milk. *Official Journal L 351* (pp. 0013–0015), 23/12/1997.

Dupont, D., Rolet-Répécaud, O., & Muller-Renaud, S. (2004). Determination of the heat treatment undergone by milk by following the denaturation of α-lactalbumin with a biosensor. *Journal of Agricultural and Food Chemistry, 52*, 677–681.

Guingamp, M. F., Humbert, G., & Linden, G. (1993). Determination of sulfhydryl groups in milk using Ellman's procedure and clarifying reagent. *Journal of Dairy Science, 76*, 2152–2156.

ISO 11868/IDF 147B standard. (1998). Heat-treated milk: determination of lactulose content. Method using high-performance liquid chromatography, Afnor, Paris la Défense.

ISO 13875/IDF 178 standard. (1996). Liquid milk – determination of acid-soluble beta-lactoglobulin content – reverse-phase HPLC method. International Standardisation Organisation, Geneva.

ISO:DIS 18329 draft standard. (2001). Détermination de la teneur en furosine – Méthode par chromatographie liquide à haute performance en phase inverse par paire d'ions. Afnor, Paris la Défense.

Jeanson, S., Dupont, D., Grattard, N., & Rolet-Répécaud, O. (1999). Characterization of the heat treatment undergone by milk using two inhibition ELISAs for quantification of native and heat-denatured α-lactalbumin. *Journal of Agricultural and Food Chemistry, 47*, 2249–2254.

Löfas, S., & Johnsson, B. (1990). A novel hydrogel matrix on gold surfaces in surface plasmon resonance sensors for fast and efficient covalent immobilisation of ligands. *Journal of the Chemistry Society, 21*, 152–158.

Morgan, F., Leonil, J., Molle, D., & Bouhallab, S. (1997). Non-enzymatic lactosylation of bovine β-lactoglobulin under mild heat treatment leads to structural heterogeneity of the glycoforms. *Biochemical and Biophysical Research Communications, 236*, 413–417.

Pellegrino, L., De Noni, I., & Resmini, P. (1995). Coupling of lactulose and furosine indices for quality evaluation of sterilized milk. *International Dairy Journal, 5*, 647–659.

Pellegrino, L., Resmini, P., & Luf, W. (1995). Assessment (indices) of heat treatment. In P. F. Fox (Ed.). *Heat induced changes in milk* (pp. 409–457). Brussels: International Dairy Federation.

Pellegrino, L., Tirelli, A., Masotti, F., & Resmini, P. (1996). Significance of the main chemical indicators of heat load for characterizing raw, thermized, pasteurized and high-temperature pasteurized milk. In *Heat treatments and alternative methods* (pp. 373–388). Brussels, Belgium: International Dairy Federation.

Shindler, J. S., & Bardsley, W. G. (1975). Steady-state kinetics of lactoperoxidase with ABTS as chromogen. *Biochemical and Biophysical Research Communications, 67*, 1307–1312.

Van Renterghem, R., & De Block, J. (1996). Furosine in consumption milk and milk powders. *International Dairy Journal, 6*, 371–382.