Characterization of the Heat Treatment Undergone by Milk Using Two Inhibition ELISAs for Quantification of Native and Heat Denatured α-Lactalbumin

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Dairy industries are interested to know the heat treatment undergone by milk for controlling the quality of drinking milks or to control their heating systems. The purpose of this work was to develop a specific and sensitive technique for classification of the heat treatment a milk has been submitted to, without disposing of the original raw milk. For this purpose, α-lactalbumin was chosen as a bioindicator of heat treatment, and monoclonal antibodies specific for its native or heat-denatured form were raised and used in two inhibition ELISAs. ELISA allowed differentiation among raw, pasteurized, ultrahigh-temperature-treated, and sterilized milks without even having to know the α-lactalbumin concentration of the original raw milk. However, this technique was more suitable for intense heat treatments such as UHT treatment and sterilization because of the heat stability of α-lactalbumin.

Keywords: *α-Lactalbumin; denaturation; monoclonal antibodies; ELISA*

INTRODUCTION

Before or during processing, milk is usually submitted to heat treatment. For process control or regulation purposes, there is a need to know the severity of heat treatment milk has undergone.

Few techniques allow accurate determination of the rate of denaturation of milk proteins (Wilbey, 1996). Alkaline phosphatase and peroxidase determinations have been used for many years to assess the completeness of dairy product pasteurization (Monget and Laviolette, 1978) and to evaluate the severity of milk thermization (Pellegrino et al., 1996). In contrast, lactulose determination is a suitable technique used for high-heated milks, such as ultrahigh-temperature (UHT) and sterilized milks (IDF, 1991). Unfortunately, none of these techniques allow the study of all kinds of heat treatment. Furthermore, they are based on the determination of a bioindicator concentration that can fluctuate among milks, making difficult the determination of the heat treatment undergone by a milk without the reference of the original raw milk (Pellegrino et al., 1996). Lactulose concentration, for instance, has been shown to fluctuate in milk during storage due to the evolution of phosphate, citrate, and calcium concentrations that play a role in the formation of lactulose (Andrews, 1989).

α-Lactalbumin (14.2 kDa) was chosen as a thermal bioindicator of heat treatments because its denaturation in milk occurs on a large scale of temperatures between 70 and 96 °C (Larson and Rolleri, 1955; Donovan and Mulvihill, 1987). In addition, it was shown that denaturation of α-lactalbumin causes unfolding of the molecule (Chaplin and Lyster, 1986). These conformational modifications may induce the appearance of new epitopes at the surface of the molecule or the disappearance of some epitopes present in the native form of α-lactalbumin. Thus, immunochemical techniques could be an attractive alternative for following heat denaturation of this protein because of their specificity and sensitivity. An enzyme-linked immunosorbent assay (ELISA) was previously developed to quantify native α-lactalbumin (NAL) in heat-treated milks (Duranti et al., 1991). This technique, which used a rabbit polyclonal serum specific to NAL, showed a decrease of the NAL concentration proportional to the intensity of the heat treatment. However, its application is limited because it is necessary to know the concentration of NAL prior to heat treatment. Moreover, it has been demonstrated that NAL concentration in individual raw milk fluctuates significantly with the stage of lactation and the casein and β-lactoglobulin phenotypes (Ng-Kwai-Hang et al., 1987). Variation of NAL concentration is probably more limited in bulk than in individual milk but may remain significant.

In this study, we have succeeded in producing two monoclonal antibodies (Mabs), one NAL specific and the other specific for the heat-denatured form of α-lactalbumin (HDAL). Use of these probes in two ELISA tests allowed a classification of milks according to the heat treatment they had been submitted to, without having to know the α-lactalbumin concentration of the original raw milk.

MATERIALS AND METHODS

Development of ELISA. *NAL Purification.* NAL was purified using a two-step procedure. First, NAL was isolated from a pH 4.6 lactoserum by ion-exchange chromatography on a Q-Sepharose Fast Flow column (16 × 100 mm; Pharmacia LKB Biotechnology AB, Uppsala, Sweden) equilibrated in 20 mM Tris-HCl, pH 6.6, buffer. NAL was eluted by a 1 M NaCl gradient. Fractions containing isolated NAL were then electrophoresed by polyacrylamide gel electrophoresis (PAGE) in 25 mM Tris/192 mM glycine, pH 8.3, buffer without sodium dodecyl sulfate (SDS). After migration, the first lane was cut

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out and colored using 12% TCA to visualize the band corresponding to NAL. This band was removed from the rest of the gel, and purified NAL was taken off the gel by diffusion in 1 M phosphate buffer, pH 7.2, for 10 h at 4 °C. α-Lactalbumin purity was checked by SDS−PAGE as previously described (Laemmli, 1970) using 13.3% acrylamide gels and silver staining kit (Sigma, St. Louis, MO) for coloration (Heukeshoven and Dernick, 1985). The molecular size of purified α-lactalbumin was estimated by using SDS−PAGE standards in the range of 6.5−45 kDa (Sigma).

Preparation of HDAL. HDAL was defined as the result of the heating in a water bath of 1 mL of purified NAL at 1.42 mg/mL in 0.02 M Tris-HCl buffer, pH 6.9, in stoppered micro test tubes for 1 h at 95 °C followed by cooling in an ice bath for 5 min. Precipitated denatured proteins were separated by filtration through a 0.22 μm cellulose acetate membrane (Minisart, Sartorius AG, Goettingen, Germany). Differential scanning calorimetry (DSC) was used as described by De Wit and Swinkels (1980) to ensure that the HDAL solution contains no detectable amount of NAL.

Mabs. Female BALB/c mice were immunized with 30 μg of purified NAL or HDAL in complete Freund's adjuvant (IFFA-CREDO, St. Germain-sur-L'Arbresle, France) distributed equally into the rear foot pads. After a rest period of 14 days, mice were immunized using the same procedure with 30 μg of purified NAL or HDAL in incomplete Freund's adjuvant (IFFA-CREDO). On day 17, draining lymph nodes (popliteal and inguinal) were removed and pooled.

Fusion of lymphocytes with myeloma cells Sp2/O−Ag14 (Shulman et al., 1978) was carried out with 1 mL of 45% poly(ethylene glycol) 1000 (Merck KGaA, Darmstadt, Germany), following the procedure described by Köhler and Milstein (1975). Supernatants of hybrid clones were assayed by using an antigen coated on plate (ACP) ELISA (see below). Secreting hybridomas were subcloned by limiting dilution. Their monoclonal nature was checked at a confidence level of 95% according to Poisson's distribution (De Blas et al., 1981).

ELISA. An ACP-ELISA for the detection of antibodies directed against NAL and HDAL in the culture supernatants was performed. Briefly, microtiter plates (Nunc F96 Maxisorp, Nunc Kamstrup, Roskilde, Danemark) were coated with 100 μL of NAL or HDAL at 1 μg/mL in 0.1 M bicarbonate buffer, pH 9.6, and incubated for 1.5 h at 37 °C. Blocking of the remaining binding sites was performed with 250 μL of phosphate-buffered saline/0.05% Tween 20 (PBS-T). These plates were then filled with 100 μL per well of hybridoma supernatants diluted 1:2 in PBS-T and incubated for 1.5 h at 37 °C. Bound antibodies were quantified with 100 μL of donkey anti-mouse immunoglobulin−alkaline phosphatase conjugate (Immunoresearch Laboratories Inc., West Grove, PA) diluted 1:5000 in PBS-T, after an incubation of 1.25 h at 37 °C. One hundred microliters of *p*-nitrophenyl phosphate at 1 mg/mL (Sigma) were used as a substrate. Absorbance was measured at 405 nm using an Anthos HT3 autoreader (Anthos Labtec Instruments, Salzburg, Austria).

An inhibition ELISA was performed for NAL or HDAL quantification in milk. For NAL quantification, flat-bottom ELISA plates were coated with 1 μg/mL NAL in 0.1 M bicarbonate buffer, pH 9.6 (100 μL per well), and incubated for 1.5 h at 37 °C. Blocking of the remaining binding sites was performed with PBS-T. Serial dilutions of NAL (0−1000 ng/mL, 75 μL) in 0.4 M trisodium citrate, 75 mM EDTA, and 0.05% Tween 20 (TCST-EDTA) were used as standards. Milk samples diluted in TCST-EDTA (four dilutions from 1:1000 to 1:5000, 75 μL), or NAL standards, were incubated in test tubes with 75 μL of 1:1500 dilution of Mab 20 ascite for NAL quantification, for 1.5 h at 37 °C. One hundred microliters of the mixture was then added to each ELISA plate well and further incubated for 1.5 h at 37 °C. The reaction was revealed as described above. The same procedure was followed for HDAL quantification, except that NAL was replaced by HDAL and Mab 20 ascite was replaced by Mab 130 diluted 1:500000. Each NAL or HDAL quantification was made in triplicate.

Analytical Performances of ELISA. *Heat Treatments.* Aliquots (1 mL) of milk were heated at 65, 70, 75, 80, 85, and 95 °C for 0, 10, 20, 30, 40, 50, and 60 min in a thermostatically controlled water bath (Polystat 33, Bioblock Scientific, Illkirch, France) maintained at ±0.05 °C of the required temperature. Heat treatment was halted immediately by immersion in ice water. Commercial milk samples were kindly furnished by SODIAAL (B. Le Révérend).

Assessment of the ELISA. Evaluation of the ELISA for quantification of NAL and HDAL in milk was performed using repeatability and recovery studies. Repeatability was assessed by determining the relative repeatability standard deviation (RSDr) after quantification in triplicate of NAL and HDAL in a milk sample heated at the different time−temperature combinations previously described (see above).

Accuracy of the method was assessed by adding different amounts of purified NAL or HDAL (0.2, 0.5, and 1 mg/mL) to a raw milk sample for NAL and HDAL quantification, respectively. NAL or HDAL concentrations were determined before and after this addition by inhibition ELISA as described above, and results were expressed as percentage recovery of the amount of NAL or HDAL added.

Specificity of the method was estimated by testing by ACP-ELISA the reactivity of Mabs 20 and 130 for possible cross-reactivity against four major milk proteins: purified caseins, immunoglobulin G, β-lactoglobulin, and bovine serum albumin (Sigma). These proteins were coated at 1 μg/mL on the plate, and ACP-ELISA was carried out following the procedure described above.

Calculation of D and Z Values. D values (time required for 90% denaturation) were calculated, by regression analysis, as the reciprocal of the slope of lines obtained for each temperature by plotting the logarithm of residual native protein (percent) as a function of holding time. The effect of temperature on *D* value was also studied, and the *Z* value (degrees needed for 10-fold decrease in *D*) was calculated, by regression analysis, as the reciprocal of the slope of the line obtained by plotting the logarithm of *D* values as a function of temperature, in a range which showed a linear relationship.

Comparison with Other Methods for Milk Classification. To compare results obtained by ELISA on commercial heated milks with reference techniques, α-lactalbumin (IDF, 1996), β-lactoglobulin (IDF, 1996), lactulose (IDF, 1991), and furosine (Resmini et al., 1990) concentrations were determined by HPLC.

RESULTS

Antigens. SDS−PAGE analysis of purified α-lactalbumin showed only a single band at 14.2 kDa (results not shown). Analysis of the purified antigens by DSC confirmed that heating NAL for 1 h at 95 °C led to a total denaturation of α-lactalbumin (results not shown).

Mab Specificity. One fusion experiment carried out using NAL as immunogen yielded 19 positive clones. Among those, 6 produced Mabs specifically directed against NAL. Another fusion experiment carried out using HDAL as immunogen yielded 749 positive clones. Among those, 159 produced Mabs specifically directed against HDAL, 15 were NAL specific, and 575 produced NAL and HDAL cross-reacting Mabs. Mabs 20 and 130, respectively NAL and HDAL specific, were chosen for their affinity toward NAL and HDAL and further characterized.

Inhibition ELISA. The specificity of Mabs 20 and 130 was confirmed by inhibition ELISA (Figure 1). With Mab 20, the standard curve obtained with purified solutions of NAL had a linear detection range between 10 and 500 ng/mL (Figure 1a). Only background inhibition values were found with HDAL, confirming that this antibody recognized only NAL. With Mab 130, the standard curve had a linear range between 10 and 10000 ng/mL (Figure 1b). Only background inhibition values were found with NAL, confirming that this

Table 1. Comparison of Residual NAL of Heated Milk (Percent) Determined by Larson and Rolleri (1955), Lyster et al. (1974), Levieux (1980), and Jeanson et al. (This Paper)

		residual NAL of milk (%) during 30 min of heating				
reference	method	70 °C	74 °C	77 °C	82 °C	96 °C
Larson and Rolleri (1955)	electrophoresis	91	75	51	32	0
Lyster et al. (1974)	immunodiffusion		80[a]	64[a]	32[a]	1[a]
Levieux (1980)	immunodiffusion	82[b]	72[b]	62[b]	31[b]	
Jeanson et al. (this paper)	ELISA	95[b]	76[b]	56[b]	24[b]	2[b]

[a] Calculated value. [b] Extrapolated value.

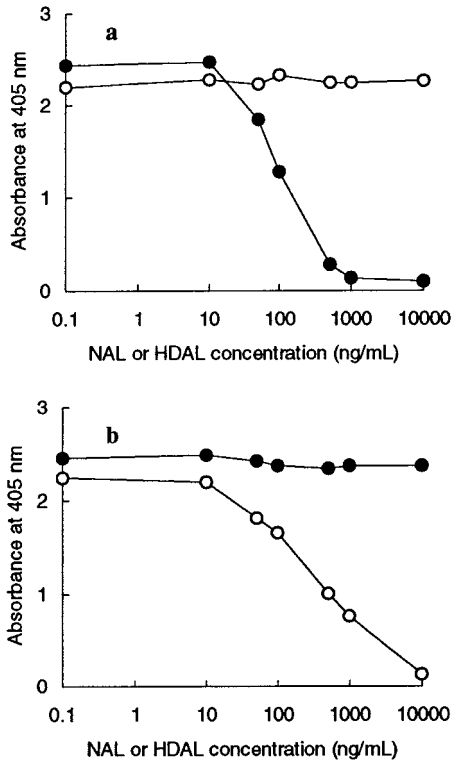

Figure 1. Specificity of Mabs 20 (a) and 130 (b) against NAL (●) and HDAL (○) as determined by inhibition ELISA.

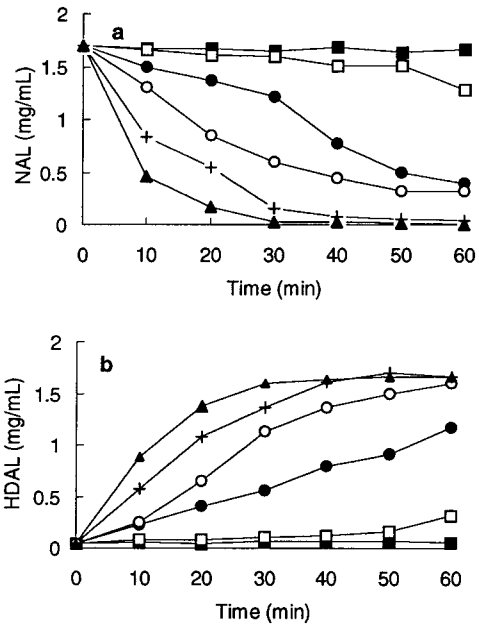

Figure 2. Concentrations (in milligrams per milliliter) of NAL (a) and HDAL (b) determined by ELISA in milk samples heated at 65 (■), 70 (□), 75 (●), 80 (○), 85 (+), and 95 °C (▲) during 0, 10, 20, 30, 40, 50, and 60 min.

antibody recognized only HDAL. Each value given in Figure 1 represents the average of five separate assays.

Assessment of the ELISA for NAL and HDAL Quantification in Milk. RSDr for NAL and HDAL quantification in milk were, respectively, 11 and 12%.

For NAL quantification, the percentage of NAL measured after the addition of 0.2, 0.5, and 1 mg/mL NAL to a raw milk sample compared to the NAL theoretically present in this sample was 93, 96, and 91%, respectively, with an average recovery of 93.3%. For HDAL quantification, the percentage of HDAL measured after the addition of 0.2, 0.5, and 1 mg/mL HDAL to a raw milk sample compared to the HDAL theoretically present in this sample was 80, 88, and 109%, respectively, with an average recovery of 92%.

ELISA was shown to be specific for NAL and HDAL quantification because no cross-reactions were observed between Mabs 20 and 130 and bovine caseins, β-lactoglobulin, immunoglobulins, and serum albumin.

Measurement of NAL and HDAL in Milk. NAL (Figure 2a) and HDAL (Figure 2b) concentrations in heated milk were determined by ELISA. Figure 2a shows that no significant decrease in NAL was observed for heating at 65 °C. NAL concentration started to decrease for a heat treatment of 70 °C/40 min. Conversely, appearance of HDAL occurred for a heat treatment of 70 °C/40 min and treatments at ≥75 °C. To compare these results to data published by other authors, we determined by extrapolation of Figure 2 the percentage of residual NAL of milk heated during 30 min at 70, 74, 77, 82, and 96 °C (Table 1). The results obtained were in good agreement with those of the other studies. It was also interesting to note that, for each heat treatment, addition of NAL and HDAL gave the same concentration as the one measured for NAL in the raw milk. This demonstrates that the two ELISAs were complementary and that all of the α-lactalbumin was quantified.

***D* and *Z* Values.** Determination of *D* and *Z* values by regression analysis was carried out for α-lactalbumin (Table 2). The *Z* value of 20.54 obtained was higher than the value of 18.06 found by Lucisano et al. (1994).

Quantification of NAL and HDAL in Commercial Milks by ELISA. In 32 milk samples analyzed, the NAL concentration varied, respectively, from 1.31 to 1.84 mg/mL for raw milks, from 1.10 to 1.57 mg/mL for pasteurized milks, from 0.23 to 0.63 mg/mL for UHT milks, and from 0.01 to 0.05 mg/mL for sterilized milks (Table 3). HDAL concentration varied, respectively, from 0.02 to 0.04 mg/mL for raw milks, from 0.04 to 0.23 mg/mL for pasteurized milks, from 0.72 to 1.21 mg/mL for UHT milks, and from 1.51 to 1.66 mg/mL for sterilized milks. Ranking the milk samples according to their percentage of denatured α-lactalbumin (HDAL/NAL + HDAL × 100) showed a percentage less than or equal to 3% in raw milk, between 2.9 and 13.9% in pasteurized milks, between 59.1 and 84.0% in UHT milks, and

Table 2. *D* and *Z* Values for Bovine α-Lactalbumin

	D values (s $\times 10^{-3}$)				*Z* value
	75 °C	80 °C	85 °C	95 °C	
α-lactalbumin	5.389 (0.961)[a]	4.598 (0.983)	2.134 (0.986)	0.680 (0.971)	20.54 (0.985)

[a] Correlation coefficients in parentheses

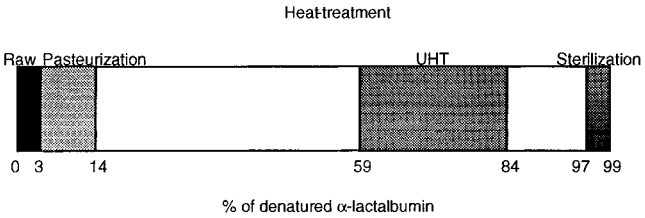

Figure 3. Scale representing the different percentages of HDAL determined by inhibition ELISA in industrial raw, pasteurized, UHT, and sterilized milks.

Table 3. NAL and HDAL Concentrations (in Milligrams per Milliliter) and Resulting Percentage of Denatured α-Lactalbumin of Industrial Milk Samples Determined by ELISA

milk	type	NAL	HDAL	% HDAL
1	raw	1.47 ± 0.07	0.02 ± 0.003	1.3
2		1.50 ± 0.05	0.02 ± 0.005	1.3
3		1.67 ± 0.04	0.03 ± 0.01	1.8
4		1.47 ± 0.03	0.03 ± 0.004	2.0
5		1.50 ± 0.14	0.03 ± 0.001	2.0
6		1.84 ± 0.06	0.04 ± 0.01	2.1
7		1.31 ± 0.11	0.04 ± 0.007	3.0
8	pasteurized	1.35 ± 0.05	0.04 ± 0.008	2.9
9		1.52 ± 0.06	0.08 ± 0.01	5.0
10		1.10 ± 0.13	0.08 ± 0.01	6.8
11		1.33 ± 0.20	0.11 ± 0.01	7.6
12		1.57 ± 0.02	0.16 ± 0.01	9.3
13		1.42 ± 0.08	0.23 ± 0.05	13.9
14	UHT	0.63 ± 0.07	0.91 ± 0.13	59.1
15		0.54 ± 0.06	0.80 ± 0.05	59.7
16		0.53 ± 0.07	0.79 ± 0.06	59.8
17		0.42 ± 0.08	0.72 ± 0.04	63.1
18		0.49 ± 0.04	0.85 ± 0.04	63.4
19		0.48 ± 0.11	0.86 ± 0.04	64.3
20		0.49 ± 0.005	1.06 ± 0.09	68.4
21		0.45 ± 0.11	1.12 ± 0.05	71.3
22		0.39 ± 0.01	1.05 ± 0.05	72.9
23		0.36 ± 0.02	1.18 ± 0.08	76.6
24		0.38 ± 0.02	1.10 ± 0.17	74.3
25		0.32 ± 0.02	1.08 ± 0.01	77.1
26		0.33 ± 0.05	1.13 ± 0.07	77.4
27		0.32 ± 0.01	1.10 ± 0.17	77.5
28		0.30 ± 0.04	1.07 ± 0.06	78.1
29		0.23 ± 0.11	1.21 ± 0.09	84.0
30	sterilized	0.05 ± 0.009	1.66 ± 0.14	97.1
31		0.03 ± 0.001	1.66 ± 0.03	98.2
32		0.01 ± 0.008	1.51 ± 0.09	99.3

between 97.1 and 99.3% in sterilized milks (Figure 3). These results show that it was possible to differentiate milks according to their heat treatment, except perhaps for low-temperature pasteurization (65 °C/30 min), when denaturation of α-lactalbumin probably did not occur (milk 8, Table 3).

Comparison of ELISA with Reference Techniques. α-Lactalbumin, *β*-lactoglobulin, lactulose, and furosine concentrations of milks 7, 8, 11, 16, 19, 29, and 31 were determined (Table 4) and compared to the percentage of denatured α-lactalbumin obtained by ELISA (Table 3). Except lactulose, which did not allow discrimination between raw and pasteurized milks, the same ranking was obtained for the various techniques. *β*-Lactoglobulin determination appeared to be more appropriate for characterization of mild heat treatments such as pasteurization.

DISCUSSION

We have developed two inhibition ELISAs for quantification of NAL and HDAL that allow classification of milk samples according to the severity of the heat treatment from pasteurization to sterilization, even if the α-lactalbumin concentration of the original raw milk is unknown.

Quantification of NAL in different raw milks showed that the concentration of this protein in milk could greatly fluctuate (from 1.31 to 1.84 mg/mL). Several factors have been shown to cause variation of NAL concentrations in raw milk such as herd, stage of lactation, food intake, casein phenotype, milk yield, and health status of the mammary gland (Gray and Mackenzie, 1987; Ng Kwai-Hang et al., 1987; Regester and Smithers, 1991). Until then, most of the authors expressed α-lactalbumin denaturation in milk as the loss in percentage of NAL, taking the original raw milk as a reference (Resmini et al., 1989; Duranti et al., 1991). Because NAL concentration varies significantly in raw milk, these techniques cannot be applied to milk of unknown origin. On the contrary, the quantification of both NAL and HDAL in milk by ELISA, together with expression of the results as the percentage of denatured α-lactalbumin, allowed determination of the heat treatment the sample was submitted to, without knowing the α-lactalbumin concentration of the original raw milk.

Values obtained for the denaturation of α-lactalbumin in milk were comparable with those found by Larson and Rolleri (1955) using moving-boundary electrophoresis and those of Lyster et al. (1974) and Levieux (1980)

Table 4. Comparison of Percentage of Denatured α-Lactalbumin Obtained Using ELISA on Commercial Milks 7, 8, 11, 16, 19, 29, and 31 with α-Lactalbumin, *β*-Lactoglobulin, Lactulose, and Furosine Concentrations

milk	type	% HDAL (ELISA)	furosine (mg/100 g of protein)	α-lactalbumin (mg/L)	*β*-lactoglobulin (mg/L)	lactulose (mg/100 g of protein)
7	raw	2.9	4	1057	3598	abs[a]
8	pasteurized	2.6	3	1046	3542	abs
11	pasteurized	7.5	10	853	1606	abs
16	UHT	59.8	75	293	174	291
19	UHT	64.3	95	215	128	318
29	UHT	83.9	164	56	50	733
31	sterilized	98.5	207	9	10	917

[a] Absence.

using immunodiffusion. Addition of NAL and HDAL concentrations found by ELISA for each heat treatment studied gave approximately the NAL concentration of the original raw milk. This result suggested that by using jointly these two ELISAs, α-lactalbumin was quantified independently of its denaturation state. Application of this method to the characterization of α-lactalbumin heat denaturation in fluid milk allowed discrimination among raw, pasteurized, UHT, and sterilized milks and provided a range of variation for each category.

Heat denaturation of α-lactalbumin has been extensively studied (Elfagm and Wheelock, 1977; Levieux, 1980; Dalgleish et al., 1997). α-Lactalbumin has been shown to be in milk one of the proteins most resistant to heat treatment (Resmini et al., 1989). The *Z* value of 20.54 obtained here for α-lactalbumin was higher than the value of 18.06 obtained by Lucisano et al. (1994) probably because of the different techniques used. However, it confirmed the heat resistance of α-lactalbumin. The *Z* value is higher for α-lactalbumin than for IgG (6.79), *β*-lactoglobulin B (8.33), *β*-lactoglobulin A (10.64), and bovine serum albumin (12.35) (Lucisano et al., 1994). Elfagm and Wheelock (1977, 1978a,b) found that irreversible interactions between α-lactalbumin and *β*-lactoglobulin occurred after heat treatment through disulfide bonds and hydrophobic interactions (Dalgleish et al., 1997) and that this complex can form a new aggregate with *κ*-casein bound to the micelle. Thus, renaturation of α-lactalbumin in milk becomes impossible because of the formation of this α-lactalbumin−*β*-lactoglobulin−casein complex.

Measurement of α-lactalbumin denaturation by ELISA was shown to be of great interest for discriminating raw, pasteurized, UHT, and sterilized milks. It seemed to be more efficient for characterization of pasteurization than lactulose determination. In contrast, determination of *β*-lactoglobulin by HPLC appeared to be more suitable for characterization of mild heat treatments such as pasteurization and constituted an excellent marker for low-heat-treated milks (Negroni et al., 1998). Determination of the percentage of denatured α-lactalbumin by ELISA could also be extremely useful for controlling the heat treatment undergone by milk at the industrial level and could be applied to the determination of the "thermal past" of milk powders that have usually received three or four consecutive heat treatments prior to commercialization. However, this technique cannot be applied for the characterization of mild heat treatments such as thermization (57−68 °C/15−50 s) because of the heat stability of α-lactalbumin at <70 °C. This limitation could be overcome by developing another ELISA using a bioindicator more sensitive to mild heat treatments such as furosine or alkaline phosphatase.

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LITERATURE CITED

Andrews, G. Lactulose in heated milk. *IDF Bull.* **1989**, *238*, 45−52.

Chaplin, L. C.; Lyster, R. L. J. Irreversible heat denaturation of bovine α-lactalbumin. *J. Dairy Res.* **1986**, *53*, 249−258.

Dalgleish, D. G.; Senaratne, V.; Francois, S. Interactions between α-lactalbumin and *β*-lactoglobulin in the early stages of heat denaturation. *J. Agric. Food Chem.* **1997**, *45*, 3459−3464.

De Blas, A. L.; Ratnaparkhi, M. V.; Mosimann, J. E. Estimation of the number of monoclonal hybridomas in cell fusion experiment. Effect of post-fusion cell dilution on hybridoma survival. *J. Immunol. Methods* **1981**, *45*, 109−115.

De Witt, J. N.; Swinkels, G. A differential scanning calorimetry study of the thermal denaturation of bovine *β*-lactoglobulin. Thermal behavior at temperatures up to 100 °C. *Biochim. Biophys. Acta* **1980**, *624*, 40−50.

Donovan, M.; Mulvihill, D. M. Thermal denaturation and aggregation of whey proteins. *Ir. J. Food Sci. Technol.* **1987**, *11*, 87−100.

Duranti, M.; Carpen, A.; Iametti, S.; Pagani, S. α-lactalbumin detection in heat treated milks by competitive ELISA. *Milchwissenschaft* **1991**, *46*, 230−232.

Elfagm, A. A.; Wheelock, J. V. Effect of heat on α-lactalbumin and *β*-lactoglobulin in bovine milk. *J. Dairy Res.* **1977**, *44*, 367−371.

Elfagm, A. A.; Wheelock, J. V. Interaction of bovine α-lactalbumin and *β*-lactoglobulin during heating. *J. Dairy Sci.* **1978a**, *61*, 28−32.

Elfagm, A. A.; Wheelock, J. V. Heat interaction between α-lactalbumin, *β*-lactoglobulin and casein in bovine milk. *J. Dairy Sci.* **1978b**, *61*, 159−163.

Gray, R. M.; Mackenzie, D. D. S. Effect of plane of nutrition on the concentration and yield of whey proteins in bovine milk. *N. Z. J. Dairy Sci. Technol.* **1987**, *22*, 157−165.

Heukeshoven, J.; Dernick, R. Simplified method for silver staining of proteins in polyacrylamide gels and the mechanism of silver staining. *Electrophoresis* **1985**, *6*, 103−112.

IDF. *Heat Treated Milk-Determination of Lactulose Content*; Provisional International Standard 147; IDF: Brussels, Belgium, 1991.

IDF. *Determination of Acid-Soluble β-Lactoglobulin Content*; Provisional International Standard 178; IDF: Brussels, Belgium, 1996.

Köhler, G.; Milstein, C. Continuous cultures of fused cells secreting antibodies of predefined specificity. *Nature* **1975**, *256*, 495−497.

Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 680−685.

Larson, B. L.; Rolleri, G. D. Heat denaturation of the specific serum proteins in milk. *J. Dairy Sci.* **1955**, *38*, 351−360.

Levieux, D. Heat denaturation of whey proteins: comparative studies with physical and immunological methods. *Ann. Rech. Vet.* **1980**, *11*, 89−97.

Lucisano, M.; Pompei, C.; Casiraghi, E.; Rizzo, A. M. Milk pasteurization: evaluation of thermal damage. *Ital. J. Food Sci.* **1994**, *2*, 185−196.

Lyster, R. L. J.; Wyeth, T. C.; Perkin, A. G.; Burton, M. Comparison of milks processed by the direct and indirect methods of ultrahigh temperature sterilization. V. Denaturation of the whey proteins. *J. Dairy Res.* **1974**, *38*, 403−408.

Monget, D.; Laviolette, P. Alkaline phosphatase and peroxidase microtests for the control of pasteurization of cow milk. *Lait* **1978**, *58*, 595−605.

Negroni, L.; Bernard, H.; Clement, G.; Chatel, J. M.; Brune, P.; Frobert, Y.; Wal, J. M.; Grassi, J. Two-site enzyme immunometric assays for determination of native and denatured *β*-lactoglobulin. *J. Immunol. Methods* **1998**, 220, 25−37.

Ng-Kwai-Hang, K. F.; Hayes, J. F.; Moxley, J. E.; Monardes, H. G. Variation in milk protein concentrations associated with genetic polymorphism and environmental factors. *J. Dairy Sci.* **1987**, *70*, 563−570.

Pellegrino, L.; Tirelli, A.; Masotti, F.; Resmini, P. 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*; International Dairy Federation: Brussels, Belgium, 1996; pp 373−388.

Regester, G. O.; Smithers, G. W. Seasonal changes in the β-lactoglobulin, α-lactalbumin, glycomacropeptide and casein content of whey protein concentrate. *J. Dairy Sci.* **1991**, *74*, 796−802.

Resmini, P.; Pellegrino, L.; Hogenboom, J. A.; Andreini, R. Thermal denaturation of whey protein in pasteurized milk. Fast evaluation by HPLC. *Ital. J. Food Sci.* **1989**, *3*, 51−62.

Resmini, P.; Pellegrino, L.; Battelli, G. Accurate quantification of furosine in milk and dairy products by a direct HPLC method. *Ital. J. Food Sci*. **1990**, *3*, 173−183.

Shulman, M.; Wilde, C. D.; Köhler, G. A better cell line for making hybridomas secreting specific antibodies. *Nature* **1978**, *276*, 269−270.

Wilbey, R. A. Estimating the degree of heat treatment given to milk. *J. Soc. Dairy Technol*. **1996**, *49*, 109−112.

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