Characterization of the heat treatment undergone by milk using two inhibition ELISA for quantification of native and heat denatured -lactalbumin

Sophie Jeanson, Didier Dupont, Nicolas Grattard, Odile Rolet-Répécaud

Characterization of the heat treatment undergone by milk using two inhibition ELISA for quantification of native and heat denatured α-lactalbumin

Running Title : ELISA for discrimination of heat-treated milks

Sophie Jeanson, Didier Dupont*, Nicolas Grattard, and Odile Rolet-Répécaud

INRA-Station de Recherche en Technologie et Analyses Laitières BP 89 F-39801 Poligny, France

* Author to whom correspondence should be addressed [fax +33 0 84 37 37 81 ; e-mail dupont@poligny.inra.fr]

ABSTRACT

Dairy industries are interested to know the heat treatment undergone by milk for controlling the quality of drinking milks or in order 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 had been submitted to, without disposing of the original raw milk. For this purpose, α-lactalbumin was chosen as a bio-indicator of heat treatment and monoclonal antibodies specific for its native (NAL) or heat-denatured (HDAL) form were raised and used in two inhibition ELISA.

ELISA allowed differentiation between raw, pasteurized, UHT 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 like 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 products 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 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 bio-indicator concentration that can fluctuate among milks, rending 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 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 thermal bio-indicator of heat treatments because its denaturation in milk occurs on a large scale of temperatures between 70°C to 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 appearance of new epitopes at the surface of the molecule or 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. But 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 of 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

Native α-lactalbumin purification. NAL was purified using a two-step procedure. First, NAL was isolated from a pH 4.6 lactoserum by ion-exchange chromatography on Q-Sepharose Fast Flow column (16 x 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. Then, fractions containing isolated NAL were electrophoresed by PAGE in 25 mM Tris, 192 mM glycine pH 8.3 buffer without Sodium Dodecyl Sulfate. After migration, the first lane was cut 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 6.5-45 kDa (Sigma).

Preparation of heat-denatured α-lactalbumin. 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 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 control that HDAL solution contains no detectable amount of NAL.

Monoclonal antibodies. 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, mice were bled and the 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% polyethylene glycol 1000 (Merck KGaA, Darmstadt, Germany), following the procedure described by Köhler and Milstein (1975). Supernatants of hybrid clones were assayed by 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 1 h 30 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 1 h 30 at 37°C. Bound antibodies were quantified with 100 µL donkey anti-mouse

immunoglobulin-alkaline phosphatase conjugate (Immunoresearch Laboratories Inc., West Grove, PA) diluted 1/5000 in PBS-T, after an incubation of 1 h 15 at 37°C. One hundred microliters paranitrophenyl 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-bottomed ELISA plates were coated with 1 µg/mL NAL in bicarbonate buffer 0.1 M pH 9.6 (100 µL per well) and incubated for 1 h 30 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, 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 h 30 at 37°C. One hundred microliters of the mixture were then added to each ELISA plate well and further incubated for 1 h 30 at 37°C. The reaction was revealed as described above. Same procedure was followed for HDAL quantification, except that NAL was replaced by HDAL and Mab 20 ascite 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, 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

120 temperature. Heat treatment was halted immediately by immersion in ice water.
121 Commercial milk samples were kindly furnished by SODIAAL (B. Le Révérend).
122
123 **Assessment of the ELISA**. Evaluation of the ELISA for quantification of NAL and
124 HDAL in milk was performed using repeatability and recovery studies. Repeatability
125 was assessed by determining the relative repeatability standard deviation (RSDr)
126 after quantification in triplicate of NAL and HDAL in a milk sample heated at the
127 different time x temperature combinations previously described (see above).
128 Accuracy of the method was assessed by adding different amounts of purified NAL
129 or HDAL (0.2, 0.5 and 1 mg/mL) to a raw milk sample for NAL and HDAL
130 quantification respectively. NAL or HDAL concentrations were determined before and
131 after this addition by inhibition ELISA as described above, and results were
132 expressed as percentage recovery of the amount of NAL or HDAL added.
133 Specificity of the method was estimated by testing by ACP-ELISA the reactivity of
134 Mabs 20 and 130 for possible cross reactivity against four major milk proteins :
135 purified caseins, immunoglobulin G, β-lactoglobulin and bovine serum albumin
136 (Sigma). These proteins were coated at 1 µg/mL on the plate and ACP-ELISA was
137 carried out following the procedure described above.
138
139 **Calculation of D and Z values**. D values (time required for 90% denaturation) were
140 calculated, by regression analysis, as the reciprocal of the slope of lines obtained for
141 each temperature by plotting the logarithm of residual native protein (%) as a
142 function of holding time. The effect of temperature on D value was also studied and
143 the Z value (degrees needed for 10 fold decrease in D) was calculated, by
144 regression analysis, as the reciprocal of the slope of the line obtained by plotting the

145 logarithm of D values as a function of temperature, in a range which showed a linear
146 relationship.
147
148 **Comparison with other methods for milk classification**. In order to compare
149 results obtained by ELISA on commercial heated milks with reference techniques, α-
150 lactalbumin (IDF, 1996), β-lactoglobulin (IDF, 1996), lactulose (IDF, 1991) and
151 furosine (Resmini et al., 1990) concentrations were determined by HPLC.
152
153

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).

Monoclonal antibodies 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. Mab 20 and Mab 130, respectively NAL and HDAL specific, were chosen for their affinity towards NAL and HDAL and further characterized.

Inhibition ELISA. Mabs 20 and 130 specificity was confirmed by inhibition ELISA (Figure 1a, b). 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 antibody recognized only HDAL. Each value given in Figure 1a,b represents the average of 5 separate assays.

Assessment of the ELISA for native and heat-denatured α-lactalbumin 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 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, 91% respectively with an average recovery of 93.3%. For HDAL quantification, the percentage of HDAL measured after 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, 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 native and heat-denatured α-lactalbumin 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 heat-treatment of 70°C/40 min and treatments at 75°C or more. In order to compare these results to data published by other authors, we determined by extrapolation of Figure 2a and b 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 measure for NAL in the raw milk. This demonstrates that the two ELISA were complementary and that all the α-lactalbumin was quantified.

205

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

209

210 **Quantification of native and heat denatured α-lactalbumin in commercial milks**
211 **by ELISA**. In 32 milk samples analyzed, NAL concentration varied respectively from
212 1.31 to 1.84 mg/mL for raw milks, from 1.10 to 1.57 mg/mL for pasteurized milks,
213 from 0.23 to 0.63 mg/mL for UHT milks and from 0.01 to 0.05 mg/mL for sterilized
214 milks (Table 3). HDAL concentration varied respectively from 0.02 to 0.04 mg/mL for
215 raw milks, from 0.04 to 0.23 mg/mL for pasteurized milks, from 0.72 to 1.21 mg/mL
216 for UHT milks and from 1.51 to 1.66 mg/mL for sterilized milks. Ranking the milk
217 samples according to their percentage of denatured α-lactalbumin
218 (HDAL/NAL+HDAL x 100) showed a percentage less or equal to 3% in raw milk,
219 between 2.9 and 13.9% in pasteurized milks, between 59.1 and 84.0% in UHT milks
220 and between 97.1 and 99.3% in sterilized milks (Figure 3). These results show that it
221 was possible to differentiate milks according to their heat treatment, except perhaps
222 for low temperature pasteurization (65°C/30 min), where denaturation of α-
223 lactalbumin did probably not occur (milk n°8, Table 3).

224

225 **Comparison of ELISA with reference techniques**. α-lactalbumin, β-lactoglobulin,
226 lactulose and furosine concentrations of milks n° 7, 8, 11, 16, 19, 29 and 31 were
227 determined (Table 4) and compared to the percentage of denatured α-lactalbumin
228 obtained by ELISA (Table 3). Except lactulose which did not allow discrimination
229 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 ELISA 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 concentration 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 can not 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 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) 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 ELISA, 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 between 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 most resistant protein to heat treatment (Resmini et al., 1989). 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. 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, 1978 a,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 constitute 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 really undergone by milk at the industrial level and could be applied to determination of the « thermal past » of milk powders that have usually received three to four consecutive heat treatments prior to commercialization. However, this technique can not be applied for characterization of mild heat treatments such as thermization (57-68°C/15-50s) because of the heat stability of α-lactalbumin under 70°C. This limitation could be overcame by developing another ELISA using a bio-indicator more sensitive to mild heat treatments such as furosine or alkaline phosphatase.

The authors wish to thank R. Vaivre for technical assistance and B. Le Révérend (SODIAAL) for furnishing commercial milk samples. They are grateful to R. Grappin for comments on the manuscript and to D. Lorient (ENSBANA) for his scientific advice. The authors are also grateful to M. Nicolas (CNEVA) for conducting reference analysis on milk samples. This study received the financial support of SODIAAL.

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. Meth.* **1981**, *45*, 109-115.

De Witt, J.N.; Swinkels, G. A differential scanning calorimetry study of the thermal denaturation of bovine β-lactoglobulin. Thermal behaviour 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.* **1978**a, *61*, 28-32.

Elfagm, A.A.; Wheelock, J.V. Heat interaction between α-lactalbumin, β-lactoglobulin and casein in bovine milk. *J. Dairy Sci.* **1978**b, *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. Determination of acid-soluble β-lactoglobulin content. **1996**, Provisional International Standard 178.

IDF. Heat treated milk-determination of lactulose content. **1991**, Provisional International Standard 147.

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. Vét.* **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 ultra-high 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*, Ed.; International Dairy Federation: Brussels : 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. *It. 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. *It. 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.

Figure 1. Specificity of monoclonal antibodies 20 (a) and 130 (b) against native (NAL, 🕯) and heat-denatured (HDAL, ☎) α-lactalbumin as determined by inhibition ELISA.

Figure 2. Concentrations in mg/mL of native (NAL, a) and heat-denatured (HDAL, b) α-lactalbumin 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.

Figure 3. Scale representing the different percentages of heat-denatured α-lactalbumin determined by inhibition ELISA in industrial raw, pasteurized, UHT and sterilized milks.

Table 1. Comparison of residual native α-lactalbumin of heated milk (%) determined by Larson and Rolleri (1955), Lyster *et al*. (1974), Levieux (1980) and the authors.

		Residual native α-lactalbumin of milk (%) Temperature of heating during 30 min (°C)				
References	Methods	70	74	77	82	96
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*.	ELISA	95[b]	76[b]	56[b]	24[b]	2[b]

[a] : calculated value ; [b] : extrapolated value

Table 2. D and Z values for bovine α-lactalbumin

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

Correlation coefficients in parentheses

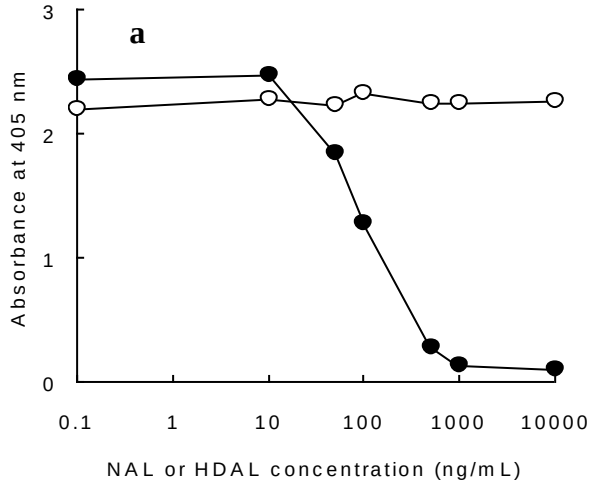
Table 3. Native (NAL), heat-denatured (HDAL) α-lactalbumin concentrations (in mg/mL) and resulting percentage of denatured α-lactalbumin of industrial milk samples determined by ELISA

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**

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/100g protein	α-lactalbumin mg/L	β-lactoglobulin mg/L	Lactulose mg/100g protein
7	Raw	2.9	4	1057	3598	Abs*
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

* Absence



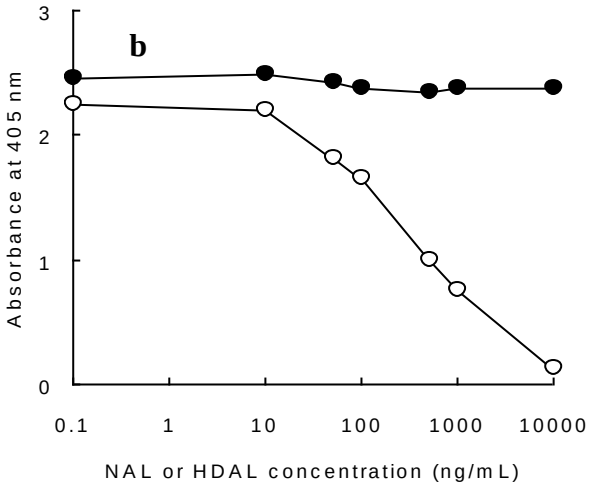


Figure 1

Jeanson *et al*.

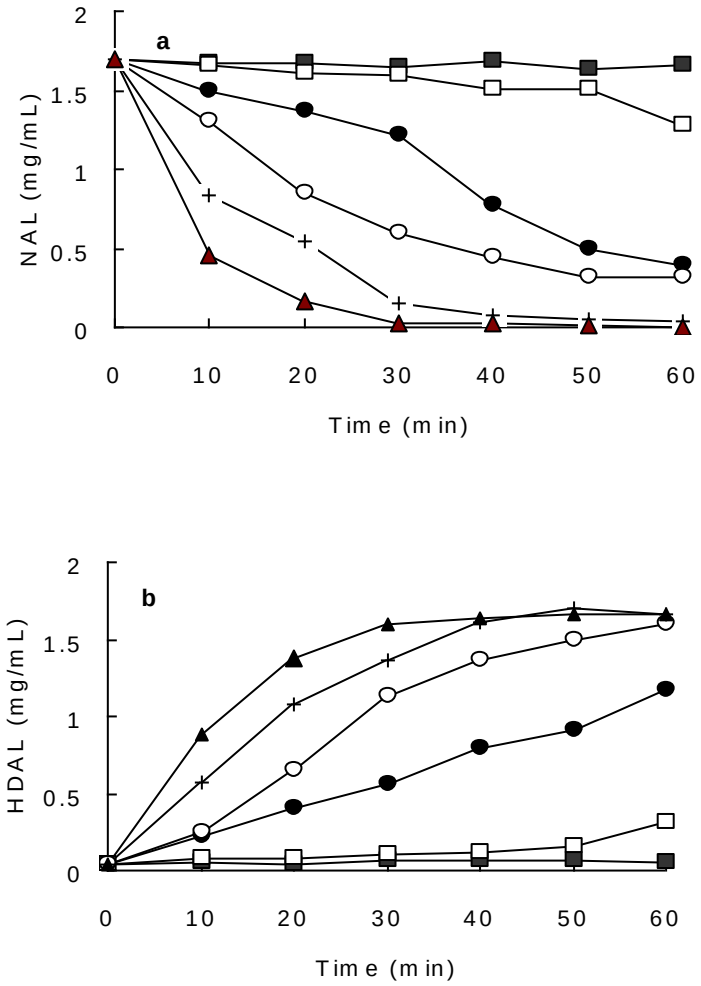


Figure 2

Jeanson *et al*.

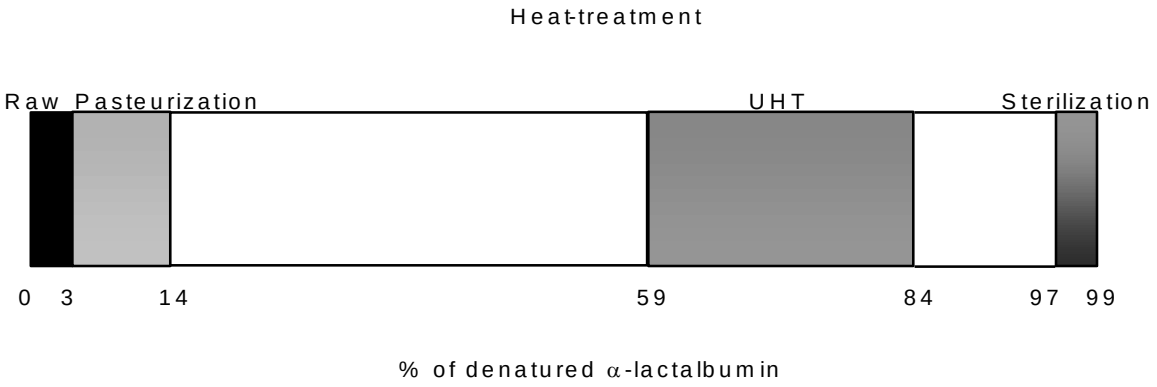


Figure 3

Jeanson *et al*.