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# **Characterization of the heat treatment undergone by milk using two inhibition ELISA for quantification of native and heat denatured $\alpha$ -lactalbumin**

Running Title : ELISA for discrimination of heat-treated milks

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## ABSTRACT

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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,  $\alpha$ -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  $\alpha$ -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  $\alpha$ -lactalbumin.

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**Keywords:**  *$\alpha$ -lactalbumin; denaturation; monoclonal antibodies; ELISA*

## 2 INTRODUCTION

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

6 Few techniques allow accurate determination of the rate of denaturation of  
7 milk proteins (Wilbey, 1996). Alkaline phosphatase and peroxidase determinations  
8 have been used for many years to assess the completeness of dairy products  
9 pasteurization (Monget and Laviolette, 1978) and to evaluate the severity of milk  
10 thermization (Pellegrino et al., 1996). In contrast, lactulose determination is a  
11 suitable technique used for high heated milks, such as UHT and sterilized milks (IDF,  
12 1991). Unfortunately, none of these techniques allow the study of all kinds of heat  
13 treatment. Furthermore, they are based on the determination of a bio-indicator  
14 concentration that can fluctuate among milks, rendering difficult the determination of  
15 the heat-treatment undergone by a milk without the reference of the original raw milk  
16 (Pellegrino et al., 1996). Lactulose concentration, for instance, has been shown to  
17 fluctuate in milk during storage due to evolution of phosphate, citrate and calcium  
18 concentrations that play a role in the formation of lactulose (Andrews, 1989).

19  $\alpha$ -lactalbumin (14.2 kDa) was chosen as thermal bio-indicator of heat  
20 treatments because its denaturation in milk occurs on a large scale of temperatures  
21 between 70°C to 96°C (Larson and Rolleri, 1955 ; Donovan and Mulvihill, 1987). In  
22 addition, it was shown that denaturation of  $\alpha$ -lactalbumin causes unfolding of the  
23 molecule (Chaplin and Lyster, 1986). These conformational modifications may  
24 induce appearance of new epitopes at the surface of the molecule or disappearance  
25 of some epitopes present in the native form of  $\alpha$ -lactalbumin. Thus, immunochemical  
26 techniques could be an attractive alternative for following heat denaturation of this  
27 protein because of their specificity and sensitivity. An Enzyme-Linked

28 ImmunoSorbent Assay (ELISA) was previously developed to quantify native  $\alpha$ -  
29 lactalbumin (NAL) in heat treated milks (Duranti et al., 1991). This technique which  
30 used a rabbit polyclonal serum specific to NAL showed a decrease of the NAL  
31 concentration proportional to the intensity of the heat treatment. But its application is  
32 limited because it is necessary to know the concentration of NAL prior to heat  
33 treatment. Moreover, it has been demonstrated that NAL concentration in individual  
34 raw milk fluctuates significantly with the stage of lactation and the casein and  $\beta$ -  
35 lactoglobulin phenotypes (Ng-Kwai-Hang et al., 1987). Variation of NAL  
36 concentration is probably more limited in bulk than in individual milk but may remain  
37 significant.

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

43

## 44 MATERIALS AND METHODS

45

### 46 **Development of ELISA**

47

48 **Native  $\alpha$ -lactalbumin purification.** NAL was purified using a two-step procedure.

49 First, NAL was isolated from a pH 4.6 lactoserum by ion-exchange chromatography

50 on Q-Sepharose Fast Flow column (16 x 100 mm ; Pharmacia LKB Biotechnology

51 AB, Uppsala, Sweden) equilibrated in 20 mM Tris-HCl pH 6.6 buffer. NAL was eluted

52 by a 1 M NaCl gradient. Then, fractions containing isolated NAL were

53 electrophoresed by PAGE in 25 mM Tris, 192 mM glycine pH 8.3 buffer without

54 Sodium Dodecyl Sulfate. After migration, the first lane was cut out and colored using

55 12% TCA to visualize the band corresponding to NAL. This band was removed from

56 the rest of the gel and purified NAL was taken off the gel by diffusion in 1 M

57 Phosphate buffer pH 7.2 for 10 h at 4°C.  $\alpha$ -lactalbumin purity was checked by SDS-

58 PAGE as previously described (Laemmli, 1970) using 13.3% acrylamide gels and

59 silver staining kit (Sigma, St Louis, MO) for coloration (Heukeshoven and Dernick,

60 1985). The molecular size of purified  $\alpha$ -lactalbumin was estimated by using SDS-

61 PAGE standards in the range 6.5-45 kDa (Sigma).

62

63 **Preparation of heat-denatured  $\alpha$ -lactalbumin.** HDAL was defined as the result of

64 the heating in a water bath of 1 mL of purified NAL at 1.42 mg/mL in 0.02 M Tris-HCl

65 buffer pH 6.9 in stoppered micro test tubes for 1 h at 95°C followed by cooling in an

66 ice bath for 5 min. Precipitated denatured proteins were separated by filtration

67 through 0.22  $\mu$ m cellulose acetate membrane (Minisart, Sartorius AG, Goettingen,

68 Germany). Differential Scanning Calorimetry (DSC) was used as described by De

69 Wit and Swinkels (1980) to control that HDAL solution contains no detectable  
70 amount of NAL.

71

72 **Monoclonal antibodies.** Female BALB/c mice were immunized with 30 µg of  
73 purified NAL or HDAL in complete Freund's adjuvant (IFFA-CREDO, St Germain-sur-  
74 L'Arbresle, France) distributed equally into the rear foot pads. After a rest period of  
75 14 days, mice were immunized using the same procedure with 30 µg of purified NAL  
76 or HDAL in incomplete Freund's adjuvant (IFFA-CREDO). On day 17, mice were  
77 bled and the draining lymph nodes (popliteal and inguinal) were removed and  
78 pooled.

79 Fusion of lymphocytes with myeloma cells Sp2/O-Ag14 (Shulman *et al.*, 1978) was  
80 carried out with 1 mL of 45% polyethylene glycol 1000 (Merck KGaA, Darmstadt,  
81 Germany), following the procedure described by Köhler and Milstein (1975).  
82 Supernatants of hybrid clones were assayed by Antigen Coated on Plate (ACP)  
83 ELISA (see below). Secreting hybridomas were subcloned by limiting dilution. Their  
84 monoclonal nature was checked at a confidence level of 95% according to Poisson's  
85 distribution (De Blas *et al.*, 1981).

86

87 **ELISA.** An ACP-ELISA for the detection of antibodies directed against NAL and  
88 HDAL in the culture supernatants was performed. Briefly, microtiter plates (Nunc F96  
89 Maxisorp, Nunc Kamstrup, Roskilde, Denmark) were coated with 100 µL of NAL or  
90 HDAL at 1 µg/mL in 0.1 M bicarbonate buffer pH 9.6 and incubated 1 h 30 at 37°C.  
91 Blocking of the remaining binding sites was performed with 250 µL of Phosphate  
92 Buffered Saline/0.05% Tween 20 (PBS-T). These plates were then filled with 100 µL  
93 per well of hybridoma supernatants diluted 1/2 in PBS-T and incubated 1 h 30 at  
94 37°C. Bound antibodies were quantified with 100 µL donkey anti-mouse

95 immunoglobulin-alkaline phosphatase conjugate (Immunoresearch Laboratories Inc.,  
96 West Grove, PA) diluted 1/5000 in PBS-T, after an incubation of 1 h 15 at 37°C. One  
97 hundred microliters paranitrophenyl phosphate at 1 mg/mL (Sigma) were used as a  
98 substrate. Absorbance was measured at 405 nm using an Anthos HT3 Autoreader  
99 (Anthos Labtec Instruments, Salzburg, Austria).

100 An inhibition ELISA was performed for NAL or HDAL quantification in milk. For NAL  
101 quantification, flat-bottomed ELISA plates were coated with 1 µg/mL NAL in  
102 bicarbonate buffer 0.1 M pH 9.6 (100 µL per well) and incubated for 1 h 30 at 37°C.  
103 Blocking of the remaining binding sites was performed with PBS-T. Serial dilutions of  
104 NAL (0-1000 ng/mL, 75 µL) in 0.4 M trisodium citrate, 75 mM EDTA, 0.05% Tween  
105 20 (TCST-EDTA) were used as standards. Milk samples diluted in TCST-EDTA (four  
106 dilutions from 1/1000 to 1/5000, 75 µL), or NAL standards, were incubated in test  
107 tubes with 75 µL of 1/1500 dilution of Mab 20 ascite for NAL quantification, for 1 h 30  
108 at 37°C. One hundred microliters of the mixture were then added to each ELISA  
109 plate well and further incubated for 1 h 30 at 37°C. The reaction was revealed as  
110 described above. Same procedure was followed for HDAL quantification, except that  
111 NAL was replaced by HDAL and Mab 20 ascite replaced by Mab 130 diluted  
112 1/500000. Each NAL or HDAL quantification was made in triplicate.

113

114

### 115 **Analytical performances of ELISA**

116

117 **Heat treatments.** Aliquots (1 mL) of milk were heated at 65, 70, 75, 80, 85, 95°C for  
118 0, 10, 20, 30, 40, 50 and 60 min in a thermostatically controlled water bath (Polystat  
119 33, Bioblock Scientific, Illkirch, France) maintained at  $\pm 0.05^\circ\text{C}$  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,  $\beta$ -lactoglobulin and bovine serum albumin  
136 (Sigma). These proteins were coated at 1  $\mu$ g/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,  $\alpha$ -  
150 lactalbumin (IDF, 1996),  $\beta$ -lactoglobulin (IDF, 1996), lactulose (IDF, 1991) and  
151 furosine (Resmini et al., 1990) concentrations were determined by HPLC.

152

153

154 RESULTS

155

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

160

161 **Monoclonal antibodies specificity.** One fusion experiment carried out using NAL  
162 as immunogen yielded 19 positive clones. Among those, 6 produced Mabs  
163 specifically directed against NAL. Another fusion experiment carried out using HDAL  
164 as immunogen yielded 749 positive clones. Among those, 159 produced Mabs  
165 specifically directed against HDAL, 15 were NAL specific and 575 produced NAL and  
166 HDAL cross-reacting Mabs. Mab 20 and Mab 130, respectively NAL and HDAL  
167 specific, were chosen for their affinity towards NAL and HDAL and further  
168 characterized.

169

170 **Inhibition ELISA.** Mabs 20 and 130 specificity was confirmed by inhibition ELISA  
171 (Figure 1a, b). With Mab 20, the standard curve obtained with purified solutions of  
172 NAL had a linear detection range between 10 and 500 ng/mL (Figure 1a). Only  
173 background inhibition values were found with HDAL, confirming that this antibody  
174 recognized only NAL. With Mab 130, the standard curve had a linear range between  
175 10 and 10000 ng/mL (Figure 1b). Only background inhibition values were found with  
176 NAL, confirming that this antibody recognized only HDAL. Each value given in Figure  
177 1a,b represents the average of 5 separate assays.

178

179 **Assessment of the ELISA for native and heat-denatured  $\alpha$ -lactalbumin**  
180 **quantification in milk.** RSDr for NAL and HDAL quantification in milk were  
181 respectively 11 and 12%.

182 For NAL quantification, the percentage of NAL measured after addition of 0.2, 0.5  
183 and 1 mg/mL NAL to a raw milk sample compared to the NAL theoretically present in  
184 this sample was 93, 96, 91% respectively with an average recovery of 93.3%. For  
185 HDAL quantification, the percentage of HDAL measured after addition of 0.2, 0.5 and  
186 1 mg/mL HDAL to a raw milk sample compared to the HDAL theoretically present in  
187 this sample was 80, 88, 109% respectively with an average recovery of 92%.

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

191

192 **Measurement of native and heat-denatured  $\alpha$ -lactalbumin in milk.** NAL (Figure  
193 2a) and HDAL (Figure 2b) concentrations in heated milk were determined by ELISA.  
194 Figure 2a shows that no significant decrease in NAL was observed for heating at  
195 65°C. NAL concentration started to decrease for a heat-treatment of 70°C/40 min.  
196 Conversely, appearance of HDAL occurred for heat-treatment of 70°C/40 min and  
197 treatments at 75°C or more. In order to compare these results to data published by  
198 other authors, we determined by extrapolation of Figure 2a and b the percentage of  
199 residual NAL of milk heated during 30 min at 70, 74, 77, 82 and 96°C (Table 1). The  
200 results obtained were in good agreement with those of the other studies. It was also  
201 interesting to note that, for each heat treatment, addition of NAL and HDAL gave the  
202 same concentration as the one measure for NAL in the raw milk. This demonstrates  
203 that the two ELISA were complementary and that all the  $\alpha$ -lactalbumin was  
204 quantified.

205

206 **D and Z values.** Determination of D and Z values by regression analysis was carried  
207 out for  $\alpha$ -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  $\alpha$ -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  $\alpha$ -lactalbumin  
218 ( $\text{HDAL}/\text{NAL}+\text{HDAL} \times 100$ ) 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  $\alpha$ -  
223 lactalbumin did probably not occur (milk n°8, Table 3).

224

225 **Comparison of ELISA with reference techniques.**  $\alpha$ -lactalbumin,  $\beta$ -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  $\alpha$ -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

230 techniques.  $\beta$ -lactoglobulin determination appeared to be more appropriate for  
231 characterization of mild heat treatments such as pasteurization.  
232

233 DISCUSSION

234

235 We have developed two inhibition ELISA for quantification of NAL and HDAL  
236 that allow classification of milk samples according to the severity of the heat  
237 treatment from pasteurization to sterilization, even if the  $\alpha$ -lactalbumin concentration  
238 of the original raw milk is unknown.

239

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

253

254 Values obtained for denaturation of  $\alpha$ -lactalbumin in milk were comparable  
255 with those found by Larson and Rolleri (1955) using moving-boundary  
256 electrophoresis and those of Lyster et al. (1974) and Levieux (1980) using  
257 immunodiffusion. Addition of NAL and HDAL concentrations found by ELISA for  
258 each heat-treatment studied gave approximately the NAL concentration of the

259 original raw milk. This result suggested that by using jointly these two ELISA,  $\alpha$ -  
260 lactalbumin was quantified independently of its denaturation state. Application of this  
261 method to the characterization of  $\alpha$ -lactalbumin heat denaturation in fluid milk  
262 allowed discrimination between raw, pasteurized, UHT and sterilized milks and  
263 provided a range of variation for each category.

264

265 Heat denaturation of  $\alpha$ -lactalbumin has been extensively studied  
266 (Elfagm and Wheelock, 1977; Levieux 1980; Dalgleish et al., 1997).  $\alpha$ -lactalbumin  
267 has been shown to be in milk one of the most resistant protein to heat treatment  
268 (Resmini et al., 1989). Z value of 20.54 obtained here for  $\alpha$ -lactalbumin was higher  
269 than the value of 18.06 obtained by Lucisano et al. (1994) probably because of the  
270 different techniques used. However it confirmed the heat resistance of  $\alpha$ -lactalbumin.  
271 Z value is higher for  $\alpha$ -lactalbumin than for IgG (6.79),  $\beta$ -lactoglobulin B (8.33),  $\beta$ -  
272 lactoglobulin A (10.64) and bovine serum albumin (12.35) (Lucisano et al., 1994).  
273 Elfagm and Wheelock (1977, 1978 a,b) found that irreversible interactions between  
274  $\alpha$ -lactalbumin and  $\beta$ -lactoglobulin occurred after heat treatment through disulfide  
275 bonds and hydrophobic interactions (Dalgleish et al., 1997) and that this complex  
276 can form a new aggregate with  $\kappa$ -casein bound to the micelle. Thus, renaturation of  
277  $\alpha$ -lactalbumin in milk becomes impossible because of the formation of this  $\alpha$ -  
278 lactalbumin- $\beta$ -lactoglobulin-casein complex.

279

280 Measurement of  $\alpha$ -lactalbumin denaturation by ELISA was shown to be of  
281 great interest for discriminating raw, pasteurized, UHT and sterilized milks. It seemed  
282 to be more efficient for characterization of pasteurization than lactulose  
283 determination. In contrast, determination of  $\beta$ -lactoglobulin by HPLC appeared to be



284 more suitable for characterization of mild heat treatments such as pasteurization and  
285 constitute an excellent marker for low heat treated milks (Negroni et al. 1998).  
286 Determination of the percentage of denatured  $\alpha$ -lactalbumin by ELISA could also be  
287 extremely useful for controlling the heat treatment really undergone by milk at the  
288 industrial level and could be applied to determination of the « thermal past » of milk  
289 powders that have usually received three to four consecutive heat treatments prior to  
290 commercialization. However, this technique can not be applied for characterization of  
291 mild heat treatments such as thermization (57-68°C/15-50s) because of the heat  
292 stability of  $\alpha$ -lactalbumin under 70°C. This limitation could be overcome by  
293 developing another ELISA using a bio-indicator more sensitive to mild heat  
294 treatments such as furosine or alkaline phosphatase.

295

296

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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  $\alpha$ -lactalbumin. *J. Dairy Res.* **1986**, 53, 249-258.

Dalgleish, D.G.; Senaratne, V.; Francois, S. Interactions between  $\alpha$ -lactalbumin and  $\beta$ -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  $\beta$ -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.  $\alpha$ -lactalbumin detection in heat treated milks by competitive ELISA. *Milchwissenschaft*, **1991**, 46, 230-232.

Elfagm, A.A.; Wheelock, J.V. Effect of heat on  $\alpha$ -lactalbumin and  $\beta$ -lactoglobulin in bovine milk. *J. Dairy Res.* **1977**, *44*, 367-371.

Elfagm, A.A.; Wheelock, J.V. Interaction of bovine  $\alpha$ -lactalbumin and  $\beta$ -lactoglobulin during heating. *J. Dairy Sci.* **1978a**, *61*, 28-32.

Elfagm, A.A.; Wheelock, J.V. Heat interaction between  $\alpha$ -lactalbumin,  $\beta$ -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. Determination of acid-soluble  $\beta$ -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.; Roller, 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.

Negrini, L.; Bernard, H.; Clement, G.; Chatel, J.M.; Brune, P.; Frobort, Y.; Wal, J.M.; Grassi, J. Two-site enzyme immunometric assays for determination of native and denatured  $\beta$ -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.

Regeester, G.O.; Smithers, G.W. Seasonal changes in the  $\beta$ -lactoglobulin,  $\alpha$ -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, ☎)  $\alpha$ -lactalbumin as determined by inhibition ELISA.

Figure 2. Concentrations in mg/mL of native (NAL, a) and heat-denatured (HDAL, b)  $\alpha$ -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  $\alpha$ -lactalbumin determined by inhibition ELISA in industrial raw, pasteurized, UHT and sterilized milks.

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

References	Methods	Residual native $\alpha$ -lactalbumin of milk (%)				
		Temperature of heating during 30 min (°C)				
		70	74	77	82	96
Larson and Rolleri (1955)	Electrophoresis	91	75	51	32	0
Lyster <i>et al.</i> (1974)	Immunodiffusion	-	80 <sup>a</sup>	64 <sup>a</sup>	32 <sup>a</sup>	1 <sup>a</sup>
Levieux (1980)	Immunodiffusion	82 <sup>b</sup>	72 <sup>b</sup>	62 <sup>b</sup>	31 <sup>b</sup>	-
Jeanson <i>et al.</i>	ELISA	95 <sup>b</sup>	76 <sup>b</sup>	56 <sup>b</sup>	24 <sup>b</sup>	2 <sup>b</sup>

<sup>a</sup> : calculated value ; <sup>b</sup> : extrapolated value

Table 2. D and Z values for bovine  $\alpha$ -lactalbumin

Temperature (°C)	D values (s.10 <sup>-3</sup> )				Z value
	75	80	85	95	
$\alpha$ -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)  $\alpha$ -lactalbumin concentrations (in mg/mL) and resulting percentage of denatured  $\alpha$ -lactalbumin of industrial milk samples determined by ELISA

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

Table 4. Comparison of percentage of denatured  $\alpha$ -lactalbumin obtained using ELISA on commercial milks 7, 8, 11, 16, 19, 29 and 31 with  $\alpha$ -lactalbumin,  $\beta$ -lactoglobulin, lactulose and furosine concentrations.

Milk	Type	% HDAL (ELISA)	Furosine mg/100g protein	$\alpha$ - lactalbumin mg/L	$\beta$ - 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

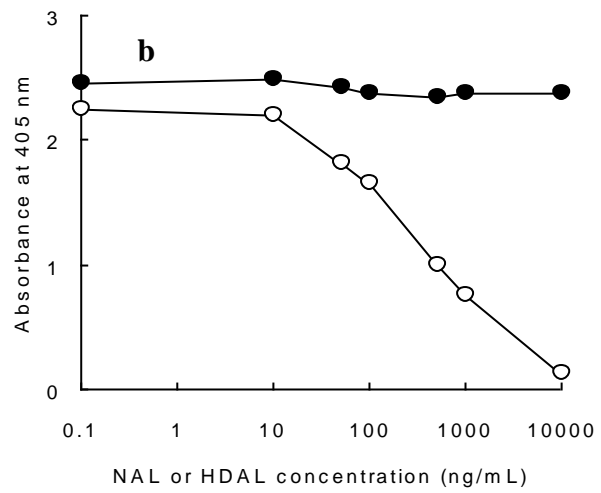
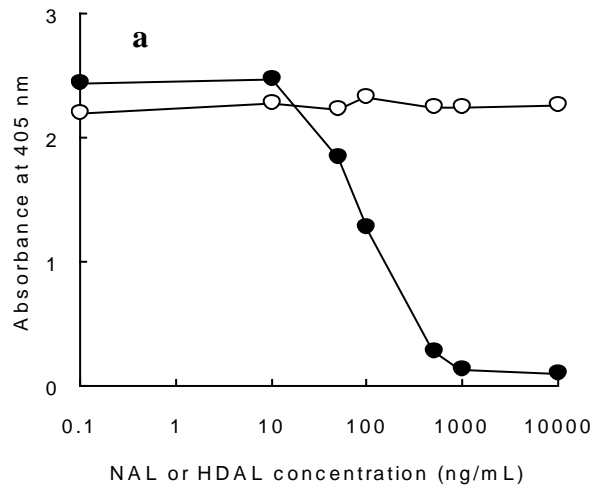


Figure 1  
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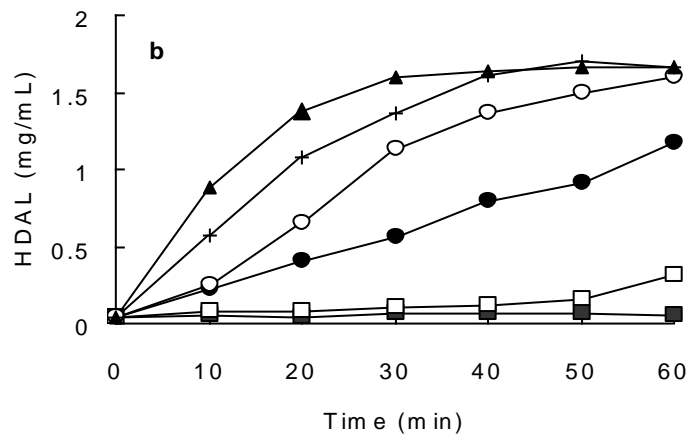
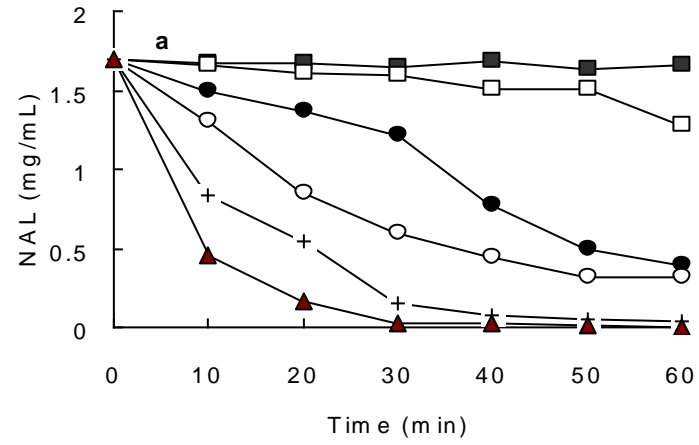


Figure 2  
Jeanson *et al.*

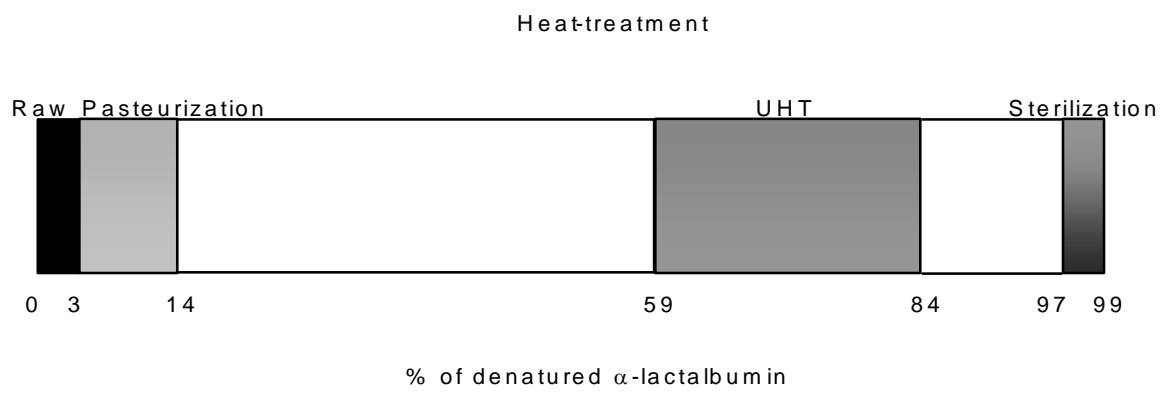


Figure 3  
Jeanson *et al.*