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Didier Dupont, Odile Rolet-Repecaud, Stéphanie Muller-Renaud. Determination of the Heat Treatment Undergone by Milk by Following the Denaturation of α -Lactalbumin with a Biosensor. *Journal of Agricultural and Food Chemistry*, 2004, 52 (4), pp.677 - 681. 10.1021/jf034722w . hal-04304023

HAL Id: hal-04304023

<https://hal.inrae.fr/hal-04304023>

Submitted on 24 Nov 2023

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Determination of the Heat Treatment Undergone by Milk by Following the Denaturation of α -Lactalbumin with a Biosensor

DIDIER DUPONT,* ODILE ROLET-REPECAUD, AND STÉPHANIE MULLER-RENAUD

INRA, Unité de Recherches en Technologie et Analyses Laitières, Place du Champ de Foire,
 F-39800 POLIGNY, France

Dairy industries are interested in knowing the heat treatment undergone by milk so as to control the quality of drinking milks or to control their heating systems. Among the different techniques available to characterize the heat treatment of milk, estimation of the denaturation of proteins has been widely used. However, because the concentration of the proteins in raw milk can fluctuate significantly, determining only the concentration of a native protein without knowing its concentration in the raw milk before undergoing heat treatment can lead to significant imprecision. The objective of this study was to develop, on Biacore 3000, a biosensor assay for determining the denaturation index of α -lactalbumin by quantifying separately the native and "heat-denatured" forms of α -lactalbumin with specific monoclonal antibodies. α -Lactalbumin denaturation index is independent of the concentration of α -lactalbumin in the original raw milk. The technique developed is discriminating, fast, repeatable, fully automated, and requires no pretreatment of the milk sample.

KEYWORDS: Biosensor; milk; α -lactalbumin; heat treatment; denaturation; antibodies

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 the heat treatment milk has undergone.

Few techniques allow accurate determination of the rate of denaturation of milk proteins (1). Alkaline phosphatase and peroxidase determinations have been used for many years to assess the completeness of dairy products' pasteurization (2) and to evaluate the severity of milk thermization (3). In contrast, lactulose determination is a suitable technique used for strongly heated milks, such as UHT and sterilized milks (4). Unfortunately, none of these techniques allow the study of all types of heat treatment. Furthermore, they are based on the determination of a bio-indicator concentration that can fluctuate among milks, rendering the determination of the heat treatment undergone by a milk, without the reference of the original raw milk, difficult (3). 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 (5).

Among the major whey proteins, α -lactalbumin (14.2 kDa) is the most resistant to heat-denaturation in milk (6) and remains at measurable concentration, even after UHT-treatment (7). Because UHT milks represent around 90% of the drinking milks commercialized in France, α -lactalbumin was chosen as thermal bio-indicator for the present study. Additionally, it has been

shown that denaturation of α -lactalbumin causes unfolding of the molecule (8). 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 (7). 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 the concentration of NAL in individual raw milk fluctuates significantly with the stage of lactation and the casein and β -lactoglobulin phenotypes (9). Variation of NAL concentration is probably more limited in bulk than in individual milk, but may remain significant. Recently, we succeeded in producing two monoclonal antibodies (Mabs), one NAL specific (Mab 20) and the other specific to the "heat denatured" form (Mab 130) of α -lactalbumin (HDAL) (10). By use of these probes, two ELISAs for specific quantification of NAL and HDAL were developed. Results obtained with this method showed that classification of milks according to the heat treatment undergone was possible, without having to know the α -lactalbumin concentration of the original raw milk. However, this method, based on the use of two ELISAs, showed poor repeatability and was extremely cumbersome (4 to 8 samples analyzed per day). Therefore, its use as

* To whom correspondence should be addressed. Tel.: (+33)-3-84-73-63-00. Fax: (+33)-3-84-37-37-81. E-mail: dupont@poligny.inra.fr.

77 a control method for routine analysis of series of milk samples
78 was impossible.

79 Consequently, using the same immunological probes, the
80 present study shows the development of a technique using the
81 generic biosensor Biacore 3000 device. Biacore 3000 is an
82 optical biosensor that allows the progress of biomolecular
83 interactions to be monitored in real time. The technique
84 developed allows the denaturation index of α -lactalbumin
85 on milk to be determined within 4 min. It is fully automated, and
86 no pretreatment of the milk sample apart from dilution in the
87 course buffer is required.

88 MATERIALS AND METHODS

89 **Chemicals.** NAL and HDAL were purified as previously described
90 (10). Mab 20 and Mab 130 monoclonal antibodies were raised on mice
91 as previously described (10). Ascites were obtained according to the
92 procedure of Jones et al. (11). They were purified by affinity
93 chromatography using NAL or HDAL covalently immobilized onto
94 Hi-Trap NHS-activated HP columns (Amersham pharmacia biotech,
95 Uppsala, Sweden) following the method proposed by the manufacturer.

96 **Milk Samples.** A total of 24 pasteurized, 10 highly pasteurized, 16
97 direct UHT, 29 indirect UHT, and 8 sterilized milks were collected,
98 by Arilait Recherches, in French factories during the winter and summer
99 periods. All the milk samples were analyzed directly after treatment.
100 Direct UHT, indirect UHT, and sterilized milks were stored for a further
101 90 days at either 25 or 35 °C and analyzed again after storage.

102 **ELISA.** Inhibition ELISAs were performed for NAL or HDAL
103 quantification in milk. For NAL quantification, flat-bottomed ELISA
104 plates were coated with 1 μ g/mL NAL in bicarbonate buffer 0.1 M pH
105 9.6 (100 μ L per well) and incubated for 1 h 30 at 37 °C. Blocking of
106 the remaining binding sites was performed with phosphate-buffered
107 saline/0.05% Tween 20 pH 7.2 (PBS-T). Serial dilutions of NAL (0–
108 1000 ng/mL, 75 μ L) in 0.4 M trisodium citrate, 75 mM EDTA, 0.05%
109 Tween 20 pH 6.3 (TSCT-EDTA) were used as standards. Milk samples
110 diluted in TSCT-EDTA (four dilutions from 1/1000 to 1/5000, 75 μ L),
111 or NAL standards, were incubated in test tubes with 75 μ L of 1/1500
112 dilution of Mab 20 ascites for NAL quantification for 1 h 30 at 37 °C.
113 A 100- μ L sample of the mixture was then added to each ELISA plate
114 well and further incubated for 1 h 30 at 37 °C. The reaction was revealed
115 with 100 μ L of donkey anti-mouse-immunoglobulin-alkaline phos-
116 phatase conjugate (Immunoresearch Laboratories Inc., West Grove, PA)
117 diluted 1/5000 in PBS-T and incubated 1 h at 37 °C. A 100- μ L sample
118 of *p*-nitrophenyl phosphate at 1 mg/mL (Sigma-Aldrich, St Quentin
119 Fallavier, France) was used as substrate. Absorbance was measured at
120 405 nm using a Ceres 900 microplate reader (Bio-Tek Instruments Inc.,
121 Winooski, VT). The same procedure was followed for HDAL quan-
122 tification, except that NAL was replaced by HDAL and Mab 20 ascites
123 replaced by Mab 130 diluted 1/500 000. Each NAL or HDAL
124 quantification was made in triplicate. Results were expressed by
125 calculating the mean α -lactalbumin denaturation index ((HDAL/(NAL
126 + HDAL)) \times 100).

127 **Biacore.** Biacore technology (Biacore International SA, Uppsala,
128 Sweden) uses Surface Plasmonic Resonance (SPR) to monitor the
129 reaction between antibody and antigen. SPR detects changes in the
130 refractive index close to a metal surface. One of the reactants is
131 immobilized, and the other is introduced in solution flowing over
132 the sensor surface. The sensor surface consists of a dextran matrix coupled
133 to a thin gold film. This matrix extends out from the sensor surface
134 and permits immobilization of biomolecules through amine groups. The
135 reaction between immobilized ligands and injected analyte takes place
136 in the hydrophilic environment defined by the dextran matrix. When
137 immobilized ligands bind the analyte, the angle at which SPR occurs
138 is changed. Changes in the angle are expressed in arbitrary units called
139 resonance units (RU).

140 The reaction is monitored continuously, and the binding curve is
141 directly visualized on a computer screen. The integration of SPR
142 detection, a microfluidic system and operator designed sensor surfaces
143 in one automated analytical system provides easy, flexible, and
144 quantitative analysis of biospecific interactions.

Immobilization Procedure. Monoclonal antibodies 20 and 130 were 145
immobilized covalently on a CM5 sensorchip by amine coupling as 146
described previously (12). All the chemicals used for antibody 147
immobilization were from Biacore International SA. Briefly, a contin- 148
uous flow of HBS-EP (10 mM Hepes, 0.15 M NaCl, 3 mM EDTA, 149
0.005% surfactant P20) pH 7.4, over the sensor surface at 5 μ L/min, 150
was maintained. The CM5 carboxymethylated dextran matrix was 151
activated by the injection of 35 μ L of a solution containing 0.2 M 152
N-ethyl-*N'*-(3-diethyl-aminopropyl)-carbodiimide (EDC) and 0.05 M 153
N-hydroxysuccinimide (NHS). Next, 35 μ L of Mab 20 or 130 1/10 in 154
sodium acetate pH 4.5 were injected, followed by 35 μ L of 1 M 155
ethanolamine to block remaining NHS-ester groups. The immobilization 156
level was 10 000 RU corresponding to 10 ng/mm² of Mab. Mab 20 157
was immobilized on flow channel (Fc) 2, with Fc1 being used as a 158
reference cell. Mab 130 was immobilized on Fc4, with Fc3 being used 159
as a reference cell. 160

Biacore Assay. Whole milk samples (10 μ L) diluted 1/1000 in HBS- 161
EP were injected on Fc1 and Fc2 and on Fc3 and Fc4 for NAL and 162
HDAL determinations, respectively. Fc1–Fc2 and Fc3–Fc4 were 163
regenerated with 5 μ L of 10 mM Glycine–HCl pH 2.7 and 5 μ L of 15 mM 164
NaOH, respectively. Each NAL or HDAL quantification was made 165
in triplicate. Results were expressed by calculating the mean α -lactal- 166
bumin denaturation index ((HDAL/(NAL + HDAL)) \times 100). 167

168 Repeatability of the Biacore assay was assessed by determining the
169 relative repeatability standard deviation (RSDr) after quantification in
170 triplicate of NAL and HDAL in the 87 commercial milk samples
171 supplied by Arilait Recherches. Accuracy of the method was assessed
172 by comparison of the denaturation index obtained using Biacore on
173 these 87 commercial milk samples with those obtained by inhibition
174 ELISA.

175 Finally, the possible influence of EDTA on the structure of native
176 α -lactalbumin was checked by comparing the results obtained on milk
177 samples diluted in HBS-EP or in HBS-P (same buffer without EDTA)
178 from Biacore.

179 **Statistical Analysis.** Data obtained with the Biacore assay on 193
180 milk samples were processed by factorial discriminant analysis (FDA)
181 using the DISCRIM procedure on SAS (13). Five classes of milk
182 samples (pasteurized, highly pasteurized, direct UHT, indirect UHT,
183 and sterilized) were proposed and validated by FDA. Results were
184 expressed as percent correctly classified into each group.

185 RESULTS AND DISCUSSION

186 **Analytical Characteristics of the Biacore Assay.** No
187 particular influence of the EDTA contained in the HBS-EP
188 buffer used to dilute milk samples was observed when the same
189 samples were injected with an HBS buffer containing no EDTA
190 (data not shown). Sensorgrams obtained by injecting raw, 190
pasteurized, highly pasteurized, direct UHT, indirect UHT, and 191
sterilized milk samples on sensorchips coated with Mab 20 and 192
Mab 130 as capturing antibodies are represented **Figure 1**, parts 193
A and **B**, respectively. For NAL determination, the intensity of 194
the response increases as the intensity of the heat treatment 195
decreases (raw > pasteurization > high pasteurization \geq direct 196
UHT > indirect UHT > sterilization). In contrast, for HDAL 197
determination, the intensity of the response increases as the 198
intensity of the heat treatment increases. 199

200 For NAL determination, regeneration occurred by using one
201 5 μ L injection of 10 mM Glycine–HCl, pH 2.7 onto the
202 sensorchip. In contrast, for HDAL determination, regeneration
203 occurred by using one 5 μ L injection of 15 mM sodium
204 hydroxide onto the sensorchip.

205 The technique was shown to be highly repeatable (intraCV
206 = 1.5 and 2.4% for NAL and HDAL determination, respec-
207 tively), fast, fully automated, and required only 2 μ L of milk
208 sample and no particular pretreatment of the sample apart from
209 dilution in the course buffer. Approximately 100 samples can
210 be analyzed on the sensorchip before the binding capacity of
211 the two monoclonal antibodies is altered. Analysis in parallel

Heat Treatment Undergone by Milk

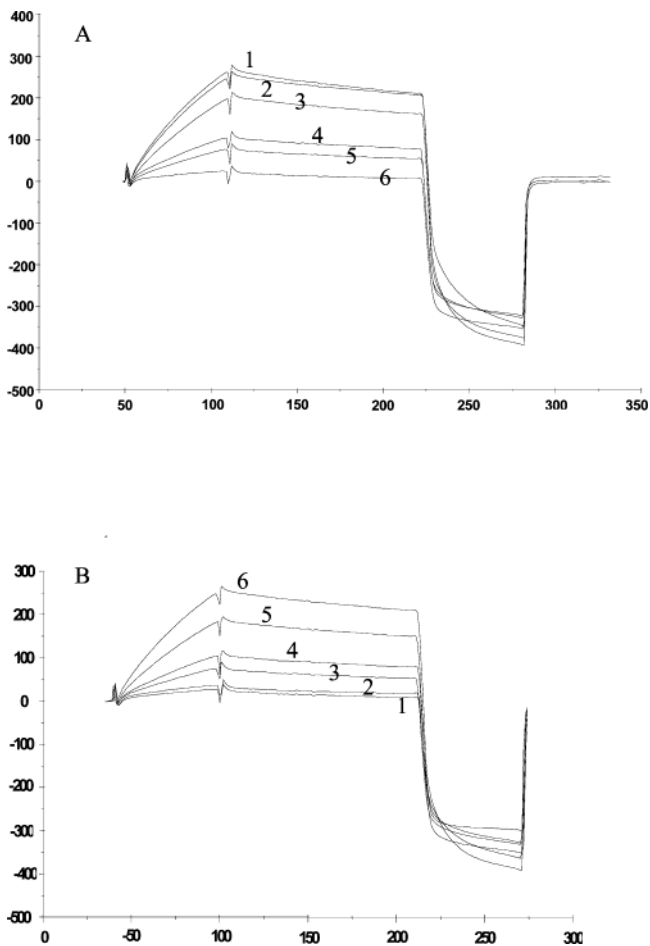


Figure 1. Overlay plot of binding to the antibody 20 (A) and 130 (B) of commercial raw (1), pasteurized (2), highly pasteurized (3), direct UHT (4), indirect UHT (5) and sterilized milks (6).

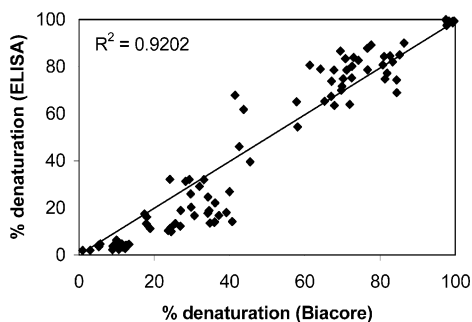


Figure 2. Correlation between the percentage of denaturation of α -lactalbumin as determined by ELISA and Biacore.

of 87 commercial milk samples on Biacore and by ELISA showed a satisfactory correlation between the two techniques ($r^2 = 0.92$) (Figure 2)

Discrimination on Biacore of the Heat Treatment of Milk after Manufacture. Figure 3 represents, for each type of heat treatment, the minimal and maximal α -lactalbumin denaturation index observed using Biacore. Denaturation index ranged from 5.2 to 18.9% for pasteurized milks, from 23.7 to 40.7% for highly pasteurized milks, from 24.2 to 45.4% for direct UHT milks, from 57.8 to 86.4% for indirect UHT milks, and from 97.2 to 99.7% for sterilized milks. Figure 3 shows that the different heat treatments studied were easily discriminated, except for the high pasteurization and direct UHT treatments that were found to be extremely close. Usually, the range of the time/temperature couples used for these two heat treatments

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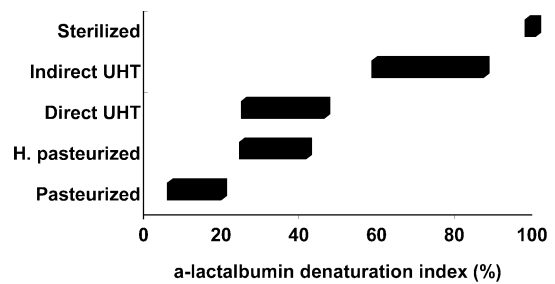


Figure 3. Minimal and maximal α -lactalbumin denaturation index determined using Biacore on 24 pasteurized, 10 highly pasteurized, 16 direct UHT, 29 indirect UHT, and 8 sterilized commercial milks directly after manufacture.

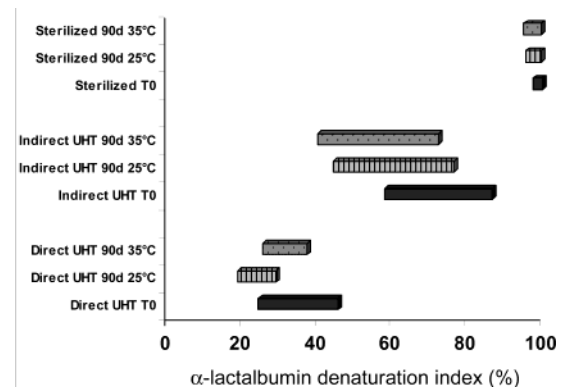


Figure 4. Effect of storage on the evolution of α -lactalbumin denaturation index in commercial direct UHT, indirect UHT, and sterilized milks analyzed directly after manufacture (solid bar) and after 90 days at 25 °C (bar with lines) or 35 °C (bar with dots).

in France is 95–110 °C/3s-1min and 140–148 °C/2s-10s for high pasteurization and direct UHT, respectively. It is thus probable that these two treatments have similar denaturing effects on milk proteins.

Effect of Milk Storage on α -Lactalbumin Denaturation Index Determined by Biacore. The evolution of α -lactalbumin denaturation index, as determined by Biacore during milk storage, was further studied. For this purpose, direct UHT, indirect UHT, and sterilized milks that were analyzed directly after manufacture were stored for a further 90 days at 25 or 35 °C. Results obtained showed that the denaturation index of α -lactalbumin decreased significantly after storage, at both temperatures (Figure 4). This decrease was shown to be the result of a decrease in the response obtained for HDAL, the response corresponding to NAL being quite stable (data not shown). This decrease in the response obtained with HDAL after storage does not seem to be the result of further proteolytic degradation of HDAL by residual heat-resistant proteases. Indeed, no significant proteolysis of α -lactalbumin was detected, after 90 days storage at 25 or 35 °C, using Western-blotting and revelation of the reaction with α -lactalbumin specific antibodies (data not shown). However, a possible explanation is that Maillard reaction may occur during storage and modify some epitopes on α -lactalbumin rendering their recognition by Mab 130 difficult.

Although we observed that the denaturation index of α -lactalbumin was not stable during milk storage (Figure 4), the different types of milk samples were still discriminated when results were plotted on a two-dimensional graph with NAL response on the X-axis and HDAL response on the Y-axis (Figure 5). FDA statistical analysis determined that only 6 milk samples out of the 193 analyzed on Biacore were not correctly

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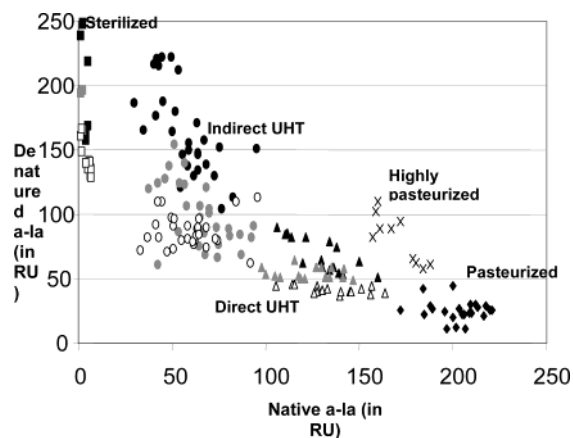


Figure 5. NAL/HDAL response (in resonance units) determined by Biacore on commercially pasteurized (◆), highly pasteurized (×), and direct UHT analyzed after manufacture (▲), 90 days at 25 °C (▲) or 90 days at 35 °C (△); indirect UHT analyzed after manufacture (●), 90 days at 25 °C (●), or 90 days at 35 °C (○); and sterilized milks analyzed after manufacture (■), 90 days at 25 °C (■), or 90 days at 35 °C (□).

classified. Indeed, 100% of the highly pasteurized and the sterilized milks were correctly classified when analyzed using the Biacore assay. In contrast, among the 24 pasteurized milks analyzed on Biacore, 2 were wrongly classified as highly pasteurized milks. Three out of the 48 direct UHT milks were wrongly classified as highly pasteurized milks. Finally, only 1 out of the 87 indirect UHT milk samples analyzed on Biacore was incorrectly clustered as a sterilized milk.

In this study, the development of an optical immunosensor for the determination of the heat treatment a milk has been subjected to is presented. The assay is based on the quantification of the native and the heat-denatured form of a protein marker, α -lactalbumin, using two specific monoclonal antibodies. Until now, 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 (7, 14). The NAL concentration varies significantly in raw milk, therefore these techniques cannot be applied to milk of unknown origin. On the contrary, the quantification of both NAL and HDAL in milk, 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.

The assay is fast, repeatable, sensitive, automated, and requires no pretreatment of the sample apart from a dilution in the course buffer. It could therefore be of great interest for routine analysis. However, the Biacore device used to detect the antigen–antibody interactions is a tool for research applications that remains expensive.

Using this technique, we demonstrated that discrimination of pasteurized, direct UHT, indirect UHT, and sterilized milks was easy when the samples were analyzed directly after manufacture. Furthermore, statistical analysis of the data obtained on commercial milks analyzed on Biacore before and after a 90d storage at 25 or 35 °C showed that only 6 out of the 193 milks analyzed were incorrectly classified. These results reinforce the idea that the assay could be of great interest for manufacturer in controlling their heating process.

However, a 3-month storage at either 25 or 35 °C resulted in a significant decrease in the HDAL concentration in milk. Only a few studies have been carried out on the evolution of proteins during the storage of milk. Proteolysis is susceptible to occur during storage of UHT-milks and is caused mainly by either

indigenous or bacterial proteases. In particular, plasmin and its zymogen plasminogen have been found to be resistant to heat inactivation (15), and psychrotrophic bacteria are known to produce extracellular proteinases partly resistant to UHT treatment (16). Therefore, could the proteolysis phenomenon be responsible for the decrease observed in HDAL in milk upon storage? Probably not. In fact, no significant proteolysis of α -lactalbumin was detected, after 90 days storage at 25 or 35 °C, using Western-blotting and revelation of the reaction with α -lactalbumin specific antibodies (data not shown).

It is therefore more probable that the decrease in HDAL observed during storage of UHT and sterilized milk is the result of increased Maillard reactions causing a decrease in the accessibility of the epitope recognized on HDAL by monoclonal antibody 130. This hypothesis is supported by the fact that during storage at 25 or 35 °C of UHT or sterilized milk, the furosine concentration of the milk samples significantly increased (17).

Finally, characterization of HDAL and the epitope recognized by the monoclonal antibody according to the conformation of the molecule could help in understanding the biochemical processes that occur during storage of UHT and sterilized milks. Such work is currently in progress.

ACKNOWLEDGMENT

We wish to thank Arilait Recherches for collecting the milk samples and for its collaboration throughout the study. We thank Fabienne Genet, H el ene Carrez, Nicolas Grattard, and Sophie Jeanson, for their technical help, and Helen Lamprell, for revising the English. Finally, the authors wish to thank Christine Achilleos for carrying out statistical analyses.

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Received for review July 4, 2003. Revised manuscript received November 18, 2003. Accepted December 8, 2003. Financial support provided by Arilait Recherches and Biacore AB.

JF034722W