

Quantification of Proteins in Dairy Products Using an Optical Biosensor

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Two recent techniques using optical immunosensor technology were developed for the quantification of milk proteins in dairy products. The first application is the simultaneous quantification of the 3 major caseins (α_{S1} , β , and κ). This assay consists of a 2-step sandwich strategy, with 2 monoclonal antibodies directed against the N- and C-terminal extremities of each of the caseins, respectively. This strategy permits only intact caseins to be quantified, and not their degradation products. The technique is fast (10 min), sensitive (detection limit about 0.87 $\mu\text{g/mL}$), and has been applied successfully to raw and drinking milks. In the second application, the severity of the heat treatment sustained by a milk of unknown origin is determined by quantifying separately the native and heat-denatured forms of α -lactalbumin with specific monoclonal antibodies. The technique allows discrimination of the different heat treatments studied (pasteurization, direct and indirect ultra-high temperature, sterilization), is fast (4 min), repeatable, fully automated, and requires no pretreatment of the milk sample.

The protein content of milk is probably the major constituent in milk that influences both quality payment schemes and breeding programs. Milk total proteins include whey proteins (WPs) such as β -lactoglobulin (β -lg), α -lactalbumin (α -la), bovine serum albumin (BSA), and immunoglobulins (Ig), as well as several caseins. Because the technological and nutritional behavior of a protein is unique, it is of paramount importance to have quantitative and qualitative information on at least all main protein species in food products. Such information can now be obtained on raw milk as well as on many simple dairy products [thermally treated milks, caseinates, whey and WP concentrates, and fermented dairy products (e.g., cheese, yogurt)] in which partial degradation of proteins has occurred.

The techniques that have been used to characterize the main proteins in milk and other dairy products include electrophoresis, liquid chromatography (LC), and immunochemical methods (1). Among those, immunochemical techniques are probably the most sensitive and specific. However, techniques such as enzyme-linked immunosorbent assay (ELISA) are too cumbersome to be used routinely because analysis of one sample can take several hours.

Biosensor systems using the optical detection principle of surface plasmon resonance (SPR) are a recent addition to research and analytical tools (2). The advantages of this technique include real-time measurement, freedom from labeling requirements, and rapidity and reproducibility of the analysis; there is also great interest in developing protein quantification techniques with this approach. Indeed, with specific antibodies showing significant affinities toward milk proteins, it seems possible, using this technology, to develop quantitative methods that are rapid, sensitive, automated, and cost-effective. Among the generic biosensors commercially available that exploit SPR, various Biacore instruments (Biacore International SA, Uppsala, Sweden) are the most widely used.

We present here a review of 2 recent applications on the use of optical SPR-biosensor technology for quantification of milk proteins in dairy products.

Application 1—Milk Caseins

The first application describes the characterization of raw milk through simultaneous quantification of the 3 major caseins (α_{S1} , β , and κ). Determination of the protein content consists of the quantification of both the casein and the WPs present. However, during cheese-making, only caseins will constitute the cheese matrix; the WPs and the major part of casein degradation products provided by natural primary proteolysis are mainly eliminated in the whey at draining (3). Therefore, in order to have an accurate estimation of the quality of milk used for cheese-making, it seems more important to determine the concentration of native caseins of milk rather than its total protein content.

The caseins include 4 principal proteins: α_{S1} -, α_{S2} -, β -, and κ -casein. Their concentrations range between 12–15 g/L for α_{S1} , 9–11 g/L for β , and 3–4 g/L for κ and α_{S2} , and can be influenced by different factors such as breed of animal (4), lactation stage (5), and feeding regimen (6). These variations

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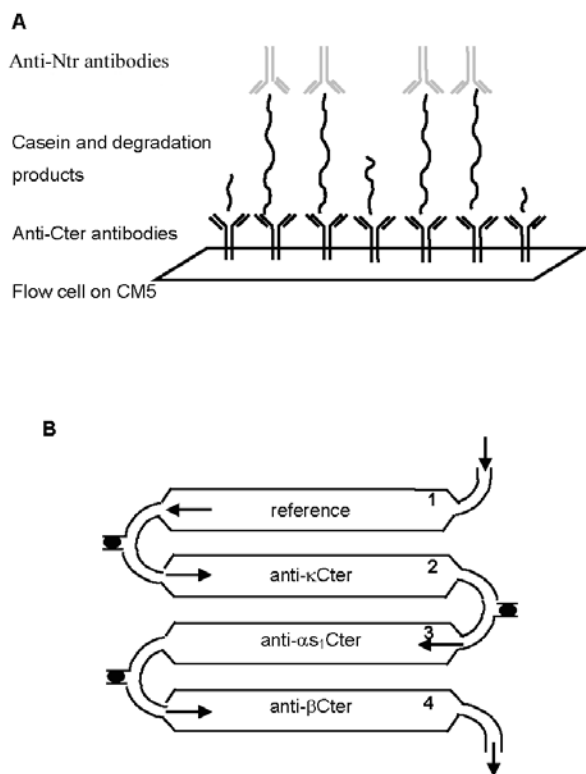


Figure 1. Strategy developed for the simultaneous quantification of α_{s1} -, β -, and κ -casein. (A) Principle of the immunoassay. (B) Schema of the sensor chip with the different flow cells.

in casein content can affect the coagulation properties of milk (7), the cheese yield (8), and the general quality of cheese. Therefore, the quality of milk will greatly depend on the relative proportion of the 4 caseins.

Several biochemical methods such as gel electrophoresis (9,10), fast protein LC (FPLC; 11), high-performance LC (HPLC; 12, 13), and capillary electrophoresis (14, 15) have been developed to quantify the 4 major caseins in milk. None of these techniques can be used to assess milk quality routinely. Further, these methods did not give an optimum separation of all major milk proteins and, in particular, separation between α_{s1} - and α_{s2} -casein by FPLC, and α_{s2} - and κ -casein by HPLC, was not always achieved (12, 16). A microparticle-enhanced nephelometric immunoassay was also developed to quantify α_{s1} -, β -, and κ -casein (17, 18) and was applied to milk and dairy products (19). This technique uses polyclonal antibodies directed against the whole casein, precluding discrimination between intact caseins and their degradation products.

We have developed an optical biosensor immunoassay allowing the simultaneous quantification of α_{s1} -, β -, and κ -casein in milk. Three flow cells (Fc) of a sensor chip were used to quantify separately each of the caseins, and the fourth was used as reference. Because the method required specific quantification of intact caseins, a sandwich immunoassay protocol was used with 2 monoclonal antibodies (Mabs)

specifically directed against the terminal extremities of the proteins. On each flow cell, the Mabs directed against the C-terminal extremity of each of the caseins was immobilized onto the sensor chip surface and used to capture their respective protein. Following injection of a milk sample, a mixture of the second Mabs (directed against the respective N-terminal extremity) was injected. The final binding response was therefore specific for native casein (Figure 1A). This method was developed and validated on raw milks and applied to commercial milks.

Application 2—Heat Denaturation of Milk

The second application describes the characterization of the heat treatment undergone by milk during conventional processing by following the thermal denaturation of α -la. 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.

Determination of the rate of denaturation of milk proteins has been used for many years to assess the heat treatment of milk. However, these techniques are based on the determination of a bioindicator concentration that can fluctuate among milks (20), making it difficult to determine the heat treatment undergone by milk, without the reference of the original raw milk.

The protein α -la (14.2 kDa) was chosen as thermal bioindicator of heat treatments because its denaturation in milk occurs over a large range of temperatures, from 70 to 96°C. Additionally, it has been shown that denaturation of α -la results in unfolding of the molecule (21). 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 -la. Thus, immunochemical techniques could be an attractive alternative for following heat denaturation of this protein because of their specificity and sensitivity. However, concentration of native α -la (NAL) can fluctuate significantly in milk with the stage of lactation and the casein and β -lg phenotypes.

Recently, we succeeded in producing 2 Mabs, one NAL-specific (Mab 20) and the other specific to the heat-denatured form (Mab 130) of α -la (HDAL). Using these probes, 2 ELISAs for specific quantification of NAL and HDAL were developed (22). However, this method, based on the combination of 2 ELISAs, showed poor repeatability and was highly manipulative, and its use as a control method for routine analysis of milk was therefore impractical.

Consequently, using the same immunological probes, we transferred this immunochemical technique to the SPR-based instrument, which allowed for the simultaneous quantification of both NAL and HDAL (23). The denaturation index of α -la in milk was determined within only 4 min; the technique is fully automated and no pretreatment of the milk sample is required apart from dilution in buffer.

Experimental

Instrumentation

The optical biosensor used to perform the assay was a Biacore 3000 (Biacore International SA). This technology allows real-time measurement of molecule interactions using SPR detection. Instrument operation and data processing were performed with BIAevaluation control software 3.1.

Reagents

Unless otherwise stated, chemicals were obtained from Sigma-Aldrich (Sigma-Aldrich, St. Quentin Fallavier, France). NAL and HDAL were purified as previously described (22).

Milk Samples

Milk powder.—For casein determination, serial dilutions (1:20 000 to 1:400) of a skimmed low-heat milk powder (URTAL-INRA, Poligny, France) were used for biosensor calibration. Milk was reconstituted at 12% (w/v) in CaCl_2 10 mM. The concentration of each casein in milk powder had been determined by reversed-phase HPLC (RP-HPLC; Nutrinov, Vezin-le-Coquet, France).

Milk samples.—For Application 1, 30 individual raw milk samples were aliquoted and stored at -20°C . Their total protein contents were determined by an infrared method (24). Before analysis, the top fat layer was removed by centrifugation ($1800 \times g$, 4°C , 15 min). Commercial pasteurized, highly pasteurized, direct ultra-high temperature (UHT), indirect UHT, and sterilized milk samples, kindly provided by Arilait Recherches (Paris, France) were aliquoted and stored at -20°C until analysis.

For Application 2, a total of 85 samples of pasteurized, highly pasteurized, direct UHT, indirect UHT, and sterilized milks were collected by Arilait Recherches in French factories during winter and summer periods. All the milk samples were analyzed directly after treatment. Direct UHT, indirect UHT, and sterilized milks were stored for a further 90 days at either 25 or 35°C and analyzed again after storage.

Monoclonal Antibodies

For casein quantification, 3 paired Mabs were used, each of them specific for a single casein. Each pair constituted a Mab directed against the N-terminal extremity of casein (called anti- κ Nter, anti- α_{S1} Nter, and anti- β Nter, respectively), and the other directed against the C-terminal extremity (anti- κ Cter, anti- α_{S1} Cter, and anti- β Cter, respectively). The Mabs directed against β -casein were produced by immunization with purified β -casein and characterized previously in our laboratory (25). The Mabs directed against α_{S1} - and κ -casein were produced after immunization by synthetic peptides corresponding to the N- and C-terminal sequences of the 2 proteins (26).

For the heat-treatment characterization, NAL-specific (Mab 20) and HDAL-specific (Mab 130) Mabs were raised in mice as previously described (22).

Biosensor Immunoassay

Preparation of the sensor surface.—For both applications, the 3 capturing anti-Cter Mabs were immobilized covalently on a carboxymethyl sensor chip (CM5; Biacore International SA) by amine coupling, as described previously (27).

Assay principle.—For casein quantification, the assay consisted of 3 simultaneous sandwich tests, each specific for 1 casein. A continuous flow of 0.01 M HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% Surfactant P20 (HBS-EP, Biacore International SA) was maintained at 30 $\mu\text{L}/\text{min}$. A 30 μL volume of sample was injected, followed by injection of 30 μL of a mixture of the 3 anti-Nter Mabs (anti- κ Nter and anti- α_{S1} Nter diluted 1:100, and anti- β Nter diluted 1:1000 in HBS-EP buffer). The sensor surface was regenerated by two 15 μL injections of 5 mM NaOH.

For heat-treatment characterization, Mabs 20 and 130 were immobilized covalently as described previously (27). Then, 10 μL aliquots of whole milk samples diluted 1:1000 in HBS-EP were injected on Fc1 and Fc2, and on Fc3 and Fc4 for NAL and HDAL determinations, respectively. Fc1–Fc2 and Fc3–Fc4 were regenerated with an injection of 5 μL 10 mM glycine–HCl, pH 2.7, and 5 μL 15 mM NaOH, respectively. Each NAL or HDAL quantification was made in triplicate. Results were expressed by calculating the mean α -la denaturation index $[(\text{HDAL}/(\text{NAL} + \text{HDAL})) \times 100]$.

Validation of the Assays

Assay specificity.—For both assays, the binding specificity of the immobilized antibody was confirmed by injection of 10 μL purified solution of the major milk proteins at 10 $\mu\text{g}/\text{mL}$ (α_{S1} -, α_{S2} -, β -, and κ -casein; α -la, β -lg, IgG, and BSA). The extent of potential nonspecific binding from both target analyte and sample matrix components to the dextran support was assessed over a reference ligand-free surface.

Assay validation.—The detection limit was calculated from the mean of the measured content of a representative blank sample ($n = 20$) plus 3 times the standard deviation (SD) of the mean (mean + 3 SD).

Assay precision.—The repeatability was defined by determining intra-assay (within run) and interassay (between runs) variation. Intra-assay variation was determined by 10 successive analyses of the same sample at 3 different dilutions. Interassay variation was evaluated using the same sample in 10 different runs.

Results and Discussion

Application 1—Simultaneous Quantification of the Three Major Caseins (α_{S1} , β , and κ) in Raw and Drinking Milks

Anti- κ Cter, anti- α_{S1} Cter, and anti- β Cter Mabs were immobilized by amine coupling on flow cells 2, 3, and 4 of a CM5 sensor chip, respectively (Figure 1B). For each casein species, calibration curves were established with casein concentrations in reconstituted milk ranging from 0 to 15 $\mu\text{g}/\text{mL}$ for κ -casein, 0 to 12.6 $\mu\text{g}/\text{mL}$ for α_{S1} -casein, and 0 to 9.73 $\mu\text{g}/\text{mL}$ for β -casein, corresponding to the binding

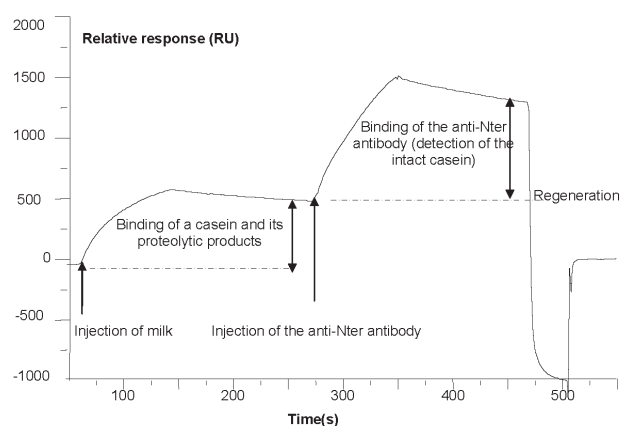


Figure 2. Diagrammatic representation of a sensorgram of a typical milk sample showing the various steps involved in the sandwich method. The response taken into consideration for casein quantification corresponds to that of binding the anti-Nter antibodies on the intact casein.

response of anti-Nter antibodies on the native casein (Figure 2).

(a) Characteristics of the assay.—Each assay was shown to be specific for 1 of the 3 caseins tested. The analysis for 1 sample, including regeneration time, was around 10 min. The regeneration conditions used on all 4 flow cells were identical. Each sensor surface could be used for more than 150 cycles without significant decrease in measured baseline, although a new calibration may be necessary after 60 cycles due to a slight decrease in the ability of the first Mab to capture casein.

The detection limit was estimated at 85 ng/mL for β -casein, 870 ng/mL for α_{S1} -casein, and 470 ng/mL for κ -casein. Therefore, the global detection limit of the assay was determined by the α_{S1} -casein assay. Repeatability determined with different concentrations of a raw milk sample showed intra-assay coefficient of variation (CV) of 2.6% for κ -casein, 4.3% for α_{S1} -casein, and 2.3% for β -casein. The interassay

CVs were 5.6% for κ -casein, 6.9% for α_{S1} -casein, and 6.2% for β -casein. The accuracy of the assay was estimated by recovery of exogenous additions of different concentrations of milk powder in a raw milk sample. Recovery ranged from 93 to 106.6% for κ -casein, 94.2 to 102.2% for α_{S1} -casein, and 101.2 to 105.7% for β -casein.

(b) Analysis of raw milk samples.—The concentrations of caseins in the 30 raw milk samples determined using the optical biosensor ranged from 7.25 to 14.52 g/L for α_{S1} -casein, 6.35 to 14.77 g/L for β -casein, and 2.5 to 4.68 g/L for κ -casein. A comparison of the addition of the 3 casein concentrations ($\alpha_{S1} + \beta + \kappa$) with total nitrogen content determined by infrared spectroscopy analysis showed a significant correlation between the 2 different techniques ($p < 0.001$).

(c) Analysis of heated milk samples.—Quantification of the individual caseins in heated milk is difficult using either chromatography or electrophoresis. Indeed, for a given protein, protein denaturation leads to products that may differ according to the treatment, often with an ultimate transformation into insoluble aggregates. Therefore, resolution of the bands or the peaks corresponding to the different caseins can often be poor. The biosensor technique was applied to the quantification of α_{S1} -, β -, and κ -casein in pasteurized, direct, indirect UHT, and sterilized milks, and concentrations obtained were in accordance with those found in raw milks (Table 1).

Application 2—Determination of the Heat Treatment Undergone by Milk by Following the Thermal Denaturation of α -Lactalbumin

(a) Analytical characteristics of the Biacore assay.—The technique was shown to be highly repeatable (RSD = 1.5 and 2.4% for NAL and HDAL determination, respectively), fast and fully automated, requiring only 2 μ L milk sample and no pretreatment of the sample apart from dilution in the assay buffer. Approximately 100 samples can be analyzed on the sensor chip before a slight decrease is observed in the ability of the Mab to capture the analyte. Analysis in parallel of 85 commercial milk samples by the described SPR

Table 1. Determination of α_{S1} -, β -, and κ -casein concentrations in different heated milk samples

Sample	Heat treatment	β -casein, mg/mL	α_{S1} -casein, mg/mL	κ -casein, mg/mL
PA-1	Pasteurized	8.39	11.63	4.26
PA-2		8.06	11.19	3.54
UI-1	Indirect UHT	8.06	11.36	4.07
UI-2		6.51	8.12	4.18
UD-1	Direct UHT	9.82	12.66	4.96
UD-2		8.93	12.94	4.56
S-1	Sterilized	9.20	10.79	4.23
S-2		10.84	11.32	5.26

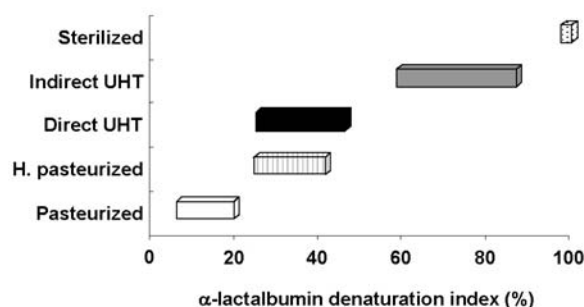


Figure 3. Minimal and maximal α -la denaturation index determined using Biacore on 22 pasteurized, 10 highly pasteurized, 16 direct UHT, 29 indirect UHT, and 8 sterilized commercial milks directly after manufacture.

immunoassay and by ELISA showed a satisfactory correlation between the 2 techniques ($r^2 = 0.92$).

(b) *Discrimination of the heat treatment of milk after manufacture.*—Figure 3 represents, for each type of heat treatment, the minimal and maximal α -la denaturation index observed using Biacore data. It shows that different heat treatments studied were easily discriminated, with the exception of the high pasteurization and direct UHT treatments. Traditionally, the range of the time/temperature factors used for these 2 heat treatments in France is 95–110°C/3 s–1 min and 140–148°C/2–10 s for high pasteurization and direct UHT, respectively. It is thus probable that these 2 treatments have similar denaturing effects on milk proteins.

(c) *Effect of milk storage on α -la denaturation index.*—The change in α -la denaturation index during milk storage, as determined by the optical biosensor technique, was further investigated by the analysis of direct UHT, indirect UHT, and sterilized milks directly after manufacture and following storage for 90 days at 25 or 35°C. Results showed that the denaturation index of α -la decreased significantly after storage at both temperatures (Figure 4). Although we observed that the denaturation index of α -la was not stable during milk storage, the different types of milk samples were still discriminated when results were plotted on a 2-dimensional graph with NAL response on the X-axis and HDAL response on the Y-axis (Figure 5).

Conclusions

We have developed SPR-based immunosensors for the simultaneous quantification of intact κ -, β -, and α_{S1} -casein in milk and for the determination of the heat treatment a milk has been subjected to by the quantification of the native and the heat-denatured form of a protein marker, α -la. Both methods were shown to be fast (a few minutes per analysis), repeatable, sensitive, and automated, requiring no

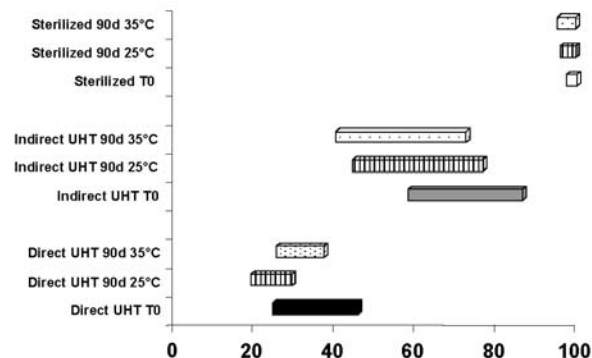


Figure 4. Effect of storage on the evolution of α -la denaturation index in commercial direct UHT, indirect UHT, and sterilized milks analyzed directly after manufacture and after 90 days at 25 or 35°C.

pretreatment of the sample apart from dilution in buffer. The applications for milk quality testing referred to in this review demonstrate the power that biosensor technology could have in providing reliable and fast results for the milk industry.

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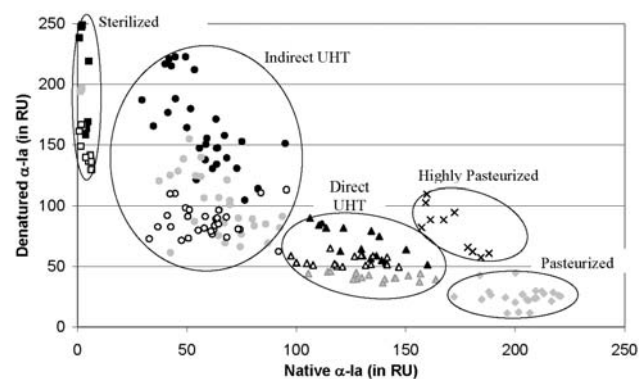


Figure 5. NAL/HDAL response (in resonance units) determined by Biacore on commercial pasteurized ($\diamond$), highly pasteurized ($\times$), direct UHT analyzed after manufacture ($\blacktriangle$) or 90 days at 25°C ($\blacktriangle$) or 90 days at 35°C ($\triangle$), indirect UHT analyzed after manufacture ($\bullet$) or 90 days at 25°C ($\bullet$) or 90 days at 35°C ($\circ$), and sterilized milks analyzed after manufacture ($\blacksquare$) or 90 days at 25°C ($\blacksquare$) or 90 days at 35°C ($\square$).

References

- (1) Tremblay, L., Laporte, M.F., Léonil, J., Dupont, D., & Paquin, P. (2002) in *Advanced Dairy Chemistry*, Vol. 1, P.F. Fox & P.L.H. McSweeney (Eds), Kluwer Academic/Plenum Publishers, New York, NY, pp 49–138
- (2) Rich, R.L., & Myszka, D.G. (2004) in *Drug Discovery Today: Technologies*, Vol. 1, K. Lam & H. Timmerman (Eds), Elsevier Ltd, London, UK, pp 301–308
- (3) Emmons, D.S., Dube, C., & Modler, H.W. (2003) *J. Dairy Sci.* **86**, 469–485
- (4) Storry, J.E., Grandison, A.S., Millard, D., Owen, A.J., & Ford, G.D. (1983) *J. Dairy Res.* **50**, 215–299
- (5) Barry, J.G., & Donnelly, W.J. (1980) *J. Dairy Res.* **47**, 71–82
- (6) Sutton, J.D. (1989) *J. Dairy Sci.* **72**, 2801–2814
- (7) Okigbo, L.M., Richardson, G.H., Brown, R.J., & Ernstrom, C.A. (1985) *J. Dairy Res.* **68**, 1887–1892
- (8) Montagne, P., Cuilliere, M.L., Marchal, E., El-Bari, N., Montagne, M., Benali, M., Faure, G., Duheille, J., Humbert, G., Linden, G., Heurtaux, N., Blesche, J.L., Gosselin, D., Desmares, A., & Delahaye, D. (1995) *Lait* **75**, 211–237
- (9) Ng-Kwai-Hang, K.F., & Kroeker, E.M. (1984) *J. Dairy Sci.* **67**, 3052–3056
- (10) Deshmukh, A.R., Donker, J.D., Addis, P.B., & Jenness, R. (1989) *J. Dairy Sci.* **72**, 12–17
- (11) Andrews, A.T., Taylor, M.D., & Owen, A.J. (1985) *J. Chromatogr.* **348**, 177–185
- (12) Bobe, G., Beitz, D.C., Freeman, A.E., & Lindberg, G.L. (1998) *J. Agric. Food Chem.* **46**, 458–463
- (13) Bordin, G., Cordeiro Raposo, F., De la Calle, B., & Rodriguez, A.R. (2001) *J. Chromatogr. A* **928**, 63–76
- (14) De Jong, N., Visser, S., & Olieman, C. (1993) *J. Chromatogr. A* **652**, 207–213
- (15) Vallejo-Cordoba, B. (1997) *J. Cap. Electrophor.* **4**, 219–224
- (16) Leonil, J., Molle, D., Gaucheron, F., Arpino, P., Guenot, P., & Maubois, J.L. (1995) *Lait* **75**, 193–210
- (17) El-Bari, N., Montagne, P.M., Humbert, G., Cuilliere, M.L., Varcin, P., Linden, G., & Duheille, J. (1991) *Food Agric. Immunol.* **3**, 63–71
- (18) Collard-Bovy, C., Marchal, E., Humbert, G., Linden, G., Montagne, P.M., El-Bari, N., Duheille, J., & Varcin, P. (1991) *J. Dairy Sci.* **74**, 3695–3701
- (19) Humbert, G., Collard-Bovy, C., Marchal, E., Linden, G., Montagne, P.M., Duheille, J., & Varcin, P. (1991) *J. Dairy Sci.* **74**, 3709–3715
- (20) Ng-Kwai-Hang, K.F., Hayes, J.F., Moxley, J.E., & Monardes, H.G. (1987) *J. Dairy Sci.* **70**, 563–570
- (21) Chaplin, L.C., & Lyster, R.L.J. (1986) *J. Dairy Res.* **53**, 249–258
- (22) Jeanson, S., Dupont, D., Grattard, N., & Rolet-Répécaud, O. (1999) *J. Agric. Food Chem.* **47**, 2249–2254
- (23) Dupont, D., Rolet-Répécaud, O., & Muller-Renaud, S. (2004) *J. Agric. Food Chem.* **52**, 677–681
- (24) IDF (1996) *Guide for the Operation of Mid Infrared Instruments Standard 141 B*, International Dairy Federation, Brussels, Belgium
- (25) Senocq, D., Dupont, D., Rolet-Répécaud, O., & Levieux, D. (2001) *Food Agric. Immunol.* **13**, 213–224
- (26) Muller-Renaud, S., Dupont, D., & Dulieu, P. (2004) *J. Agric. Food Chem.* **52**, 659–664
- (27) Löfås, S., & Johnsson, B. (1990) *J. Chem. Soc.* **21**, 152–158