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Development of a biosensor immunoassay for the quantification of α_{S1}-casein in milk

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An immunoassay to quantify α_{S1}-casein (α_{S1}-CN) in milk using an optical biosensor, based on surface plasmon resonance (SPR) measurement, has been developed. The assay consists of a two-step sandwich strategy, with two anti-α_{S1}-CN antibodies directed against each extremity of the molecule. This strategy permits only intact α_{S1}-CN to be quantified and not its degradation products. The calibration curve was obtained using a reference milk powder with a known α_{S1}-CN concentration. Analysis time per sample was less than ten minutes. The antibody-coated surface could be used for more than 150 determinations. Detection limit was established at 0·87 µg/ml and the intra- and inter-assay variation coefficients were 2·86 and 5·31%, respectively. The method was applied to raw milk to quantify intact α_{S1}-CN, with no pre-treatment of the sample. An initial analysis of 48 milk samples permitted α_{S1}-CN concentrations ranging from 8·8 to 12·06 mg/ml to be obtained.

Keywords: α_{S1}-casein, biosensor, immunoassay, milk, quantification.

Cow milk contains around 33 g proteins/l. Milk proteins are divided into two principal families, whey proteins (WP; 20% of milk proteins) and caseins (CN; 80% of milk proteins). WP include β-lactoglobulin (β-lg), α-lactalbumin (α-la), serum albumin (BSA) and immunoglobulins (Igs). CN are represented by four major proteins named α_{S1}-, α_{S2}-, β- and κ-CN, respectively. The relative concentration of these four CN in milk can vary according to different factors, such as the breed (Storry et al. 1983), the lactation stage of the cow (Barry & Donnelly, 1980) and the feeding (Sutton, 1989). These variations can affect the coagulation properties of milk (Okigbo et al. 1985), the cheese yield (Montagne et al. 1995) and the general quality of cheese. Furthermore, indigenous milk enzymes can hydrolyse CN, leading to the formation of degradation products that, for the most part, are soluble. Among the different types of proteins present in milk, only intact CN will constitute the cheese matrix, WP and degradation products being mainly eliminated with the whey during draining. Therefore, the quality of milk will depend not only on the relative proportion of the four CN, but also on the state of hydrolysis of these CN. Thus, the determination of intact CN concentration seems to be important in obtaining an accurate estimation of the quality of milk used for cheesemaking.

Several biochemical methods have been developed to quantify the four major CN in milk, such as gel electrophoresis (Ng-Kwai-Hang & Kroeker, 1984; Collin et al. 1987; Deshmukh et al. 1989), Fast Protein (FPLC) (Andrews et al. 1985; Guillou et al. 1987; Collin et al. 1991) and High Performance Liquid Chromatography (HPLC) (Visser et al. 1991; Bobe et al. 1998; Mas et al. 1999; Bordin et al. 2001) and capillary electrophoresis (De Jong et al. 1993; Vallejo-Cordoba, 1997). But none of these methods gave an optimum separation of all the major milk proteins. In particular, separation between α_{S1}- and α_{S2}-CN by FPLC, and α_{S2}- and κ-CN by HPLC, were not always achieved (Leonil et al. 1995; Bobe et al. 1998). Moreover, these techniques required sample preparation before analysis, increasing the assay time. Finally, a microparticle-enhanced nephelometric immunoassay has been developed to quantify α_S-, β- and κ-CN (Collard-Bovy et al. 1991; El-Bari et al. 1991) and applied to milk and dairy products (Humbert et al. 1991). This technique used polyclonal antibodies directed against the whole CN, rending impossible the discrimination between intact CN and their degradation products.

Biosensor systems, using the detection principle of surface plasmon resonance (SPR) (Jonsson et al. 1991), are a

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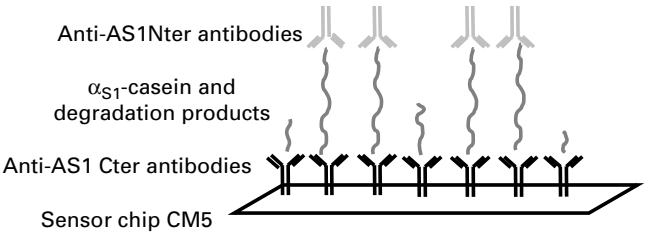

Fig. 1. Strategy developed for the quantification of α_{S1}-CN using a sandwich assay. α_{S1}-CN is taken between two antibodies specific of its N- and C-terminal extremities, allowing the detection of only the intact molecule.

recent addition to research and a new analytical tool. The biosensors enable study of all types of interactions between two molecules (protein–protein, enzyme–substrate, antigen–antibody, etc). Among the optical biosensors commercially available, the Biacore system (Biacore International SA, Uppsala, Sweden) is one of the most commonly used. Indeed, it has already been applied in the detection of enterotoxins (Rasooly & Rasooly, 1999; Rasooly, 2001; Homola et al. 2002), drug residues (Sternesjo et al. 1995; Baxter et al. 2001; Gaudin & Maris, 2001; Haasnoot & Verheijen, 2001; Ferguson et al. 2002) or vitamins (Caselunghe & Lindeberg, 2000; Indyk et al. 2000), in milk and dairy products. Several analysis kits are now commercialised to quantify contaminants or vitamins in food products.

α_{S1}-CN, the most abundant CN, accounts for 35–45% of the CN content in milk. In fresh raw milk, the α_{S1}-CN may be hydrolysed by indigenous milk enzymes, plasmin and cathepsin-D (Eigel, 1977; Kaminogawa et al. 1980). α_{S1}-CN plays a role in milk coagulation, in curd firmness (Grandison et al. 1985; Remeuf et al. 1989) and has an important structural role by contributing to the integrity of the cheese matrix (Lawrence et al. 1987).

The objective of the present study was to develop a method that specifically quantifies intact α_{S1}-CN only and not its degradation products. Therefore, the strategy used was a sandwich immunoassay, taking the protein to be quantified between two antibodies directed against the N- and C-terminals (Fig. 1). This method was developed and validated on milk.

Materials and Methods

Reagents

Unless otherwise stated, chemicals of analytical grade were purchased from VWR (VWR International, Fontenay-sous-Bois, France).

Sensor chip carboxymethyl 5 (CM5) (research grade), Rabbit anti-mouse Fc γ Igs (RAM), HEPES buffer saline (HBS-EP) pH 7·4 (10 mM-4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid, 150 mM-NaCl, 3·4 mM-EDTA, 0·005% Surfactant P20), Acetate pH 4·5 (10 mM-sodium acetate pH 4·5), 100 mM-N-hydroxysuccinimide (NHS), 400 mM-N-ethyl-N′-(3-di-ethylaminopropyl) carbodiimide (EDC) and ethanolamine hydrochloride pH 8·5 were all prepared with reagents from Biacore International SA (Biacore International SA, Uppsala, Sweden).

α_{S1}-, α_{S2}-, β-CN were purified as described previously with a minimum purity of 85% (Collin et al. 1991; Senocq et al. 2001b). κ-CN, α-la, β-lg, IgG and BSA were obtained from Sigma-Aldrich (Sigma-Aldrich, St Quentin Fallavier, France).

Milk samples

Milk powder: Skimmed low heat milk powder (SRTAL-INRA, Poligny, France) was used for biosensor calibration. Milk was reconstituted at 120 g/l, 10 mM-$CaCl_2$. The concentration of each CN in milk powder has been determined by reverse phase HPLC (RP-HPLC) (Nutrinov, Vezin-le-Coquet, France).

Milk samples: Six herds (three Montbeliarde and three Holstein) of ten cows each received different feeding and were milked 8 times over a three month period, providing a total of 48 milk samples. Samples were stored at −20 °C. Before analysis, the top fat layer was removed by centrifugation (1800 ***g***, 4 °C, 15 min). Thirty milk samples were selected to be analysed by infrared method (International Dairy Federation, 1996) and capillary electrophoresis.

Biosensor assay

Apparatus: The optical biosensor used to perform the assay was a Biacore 3000 (Biacore International SA, Uppsala, Sweden).

Detection principle: The Biacore technology allows real-time measurement of molecule interactions using Surface Plasmon Resonance (SPR) detection (Jonsson et al. 1991). One of the reactants is immobilised onto an extended carboxymethyldextran matrix layered on a gold surface (Sensor chip CM5), and the other is introduced, in a continuous flow over the sensor surface. The SPR detects and measures changes in refractive index due to binding and dissociation of interacting molecules in proximity to the gold surface. The change in refractive index is proportional to the quantity of analyte interacting with the ligand and causes a shift in the angle of index at which the SPR phenomenon occurs. These shifts, monitored continuously over time, are shown as sensorgrams and expressed in resonance units (RU). The integration of SPR detection, a microfluidic system and operator designed sensor surfaces into one automated analytical system provides quantitative analysis of biospecific interactions.

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Peptide immunogens

The peptides were chosen in the α_{S1}-CN sequence and corresponded to the N- and C-terminal ends of the CN and were called AS1Nter and AS1Cter respectively. The sequence -rpkhpikhqglpqevlne-, corresponding to the fragment 1–18 of α_{S1}-CN, constituted the peptide AS1Nter. The sequence -pigsensekttmplw-, corresponding to fragment 185–199 of α_{S1}-CN, constituted the peptide AS1Cter.

The peptides were chemically synthesised by Synt:em (Nîmes, France), with a minimum purity of 85%. A cysteine residue was added at the C-terminal of the AS1Nter peptide and modified by C-terminal amidation. A cysteine residue was added at the N-terminal of the AS1Cter peptide.

The peptides were conjugated 2:1 w/w to Keyhole Limpet Hemocyanin (KLH) to increase their immunogenicity.

Polyclonal antibodies

Polyclonal antibodies (Pabs) directed against β-, α_{S2}- and κ-CN were produced as previously described (Senocq et al. 2001a).

Production of monoclonal antibodies (Mabs)

Female BALB/c mice (IFFA-CREDO, St-Germain sur l'Arbresle, France) were immunised with a mixture of the two conjugated peptides (5 µg of each) in Complete Freund Adjuvant (Difco laboratories, Detroit, MI, USA) distributed equally into the rear foot pads. After 14 d, mice were immunised using the same procedure (5 µg of each conjugated peptides) in Incomplete Freund Adjuvant (Difco laboratories). On day 17, draining lymph popliteal nodes were removed and pooled. The polyclonal serum of mice was collected to be tested. Fusion of lymphocytes and myeloma cells X63/Ag 8·653 (Kearney et al. 1979) was carried out with 1 ml 45% PEG 1000 following the procedure described by Köhler & Milstein (1975). Supernatants of hybrid clones were assayed by Antigen Coated Plate Enzyme-Linked Immunosorbent Assay (ACP-ELISA) (see below).

Ascites from interesting Mabs were obtained according to the procedure of Jones et al. (1990). Mabs were purified from ascitic fluid by affinity chromatography using a HiTrap NHS-activated HP column (Amersham Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer recommendations. Briefly, 10 mg of α_{S1}-CN in 1 ml coupling buffer (0·2 M-$NaHCO_3$, 0·5 M-NaCl, pH 8·3) were covalently immobilised on the column. Any excess active groups were deactivated by washing with 0·5 M-ethanolamine, 0·5 M-NaCl, pH 8·3 and the non-specifically bound ligands were washed out with 0·1 M-acetate, 0·5 M-NaCl, pH 4. Ascitic fluid (1 ml) diluted in 4 ml running buffer (75 mM-Tris-HCl, pH 8) was then injected. The column was washed with running buffer. Mabs were eluted with elution buffer (100 mM-glycine-HCl, 0·5 M-NaCl, pH 2·7) and the eluted fraction was neutralised by addition of $\frac{1}{4}$ volume of 1 M-Tris-HC,l pH 9. The purified antibodies were aliquoted and stored at −20 °C.

Characterisation of monoclonal antibodies

Characterisation by ACP ELISA. This method was used to test the hybrid clones against peptides AS1Nter, AS1Cter and α_{S1}-CN. Briefly, 100 µl each antigen at 0·5 µg/ml 0·1 M-bicarbonate buffer, pH 9·6 were coated onto a microtitre plate (NUNC F96 Maxisorp, Dominique Dutscher, Brumath, France) and incubated 1 h 30 min at 37 °C. The remaining binding sites were blocked by incubating 250 µl gelatin (VWR International) at 10 g/l phosphate-buffered saline, 0·05% Tween 20 (PBS-T) for 1 h at 37 °C. Culture supernatants (50 µl) were diluted 1:2 in PBS-T and incubated 1 h at 37 °C. Bound Ig were detected by incubating 100 µl of donkey anti-mouse immunoglobulin alkaline phosphatase conjugate (Jackson Immunoresearch, Interchim, Montluçon, France) diluted 1/3000 in PBS-T for 1 h at 37 °C. Wells were rinsed between each incubation step for 15 s with four changes of 250 µl PBS-T. Following the last rinsing, 100 µl *p*-nitrophenyl phosphate (Sigma-Aldrich) at 1 g/l 1 M-diethanolamine-HCl, 1 mM-$MgCl_2$, 0·1 mM-Zinc acetate were incubated in the wells. After 30 min, the absorbance at 405 nm was read against a blank, and corrected according to the background signal in the absence of antigen.

Characterisation by Biacore. Mabs recognising AS1Nter or AS1Cter peptides and α_{S1}-CN were tested in Biacore to detect if they were able to capture α_{S1}-CN.

Preparation of the sensor surface. RAM were immobilised covalently on a CM5 sensor chip by amine coupling, as described previously (Jonsson et al. 1991). Briefly, a continuous flow of HBS-EP passing over the sensor surface at 5 µl/min was maintained. The CM5 carboxymethylated dextran matrix was activated by the injection of 35 µl of a solution containing 0·2 M-EDC and 0·05 M-NHS. Next, 35 µl RAM (30 mg/l in Acetate, pH 4·5) were injected, followed by 35 µl 1 M-ethanolamine to block remaining NHS-ester groups. The immobilisation level was 10000 RU, corresponding to 10 ng/mm^2 RAM. RAM were immobilised on flow channel Fc2, with Fc1 being used as reference cell.

Assay principle: A continuous flow was maintained at 10 µl/min. 20 µl of supernatant of hybrid clones recognising AS1Nter or AS1Cter and α_{S1}-CN were injected onto the RAM sensor chip, followed by the injection of 20 µl α_{S1}-CN at 20 µg/ml HBS-EP. Regeneration was achieved with 10 µl 50 mM-NaOH.

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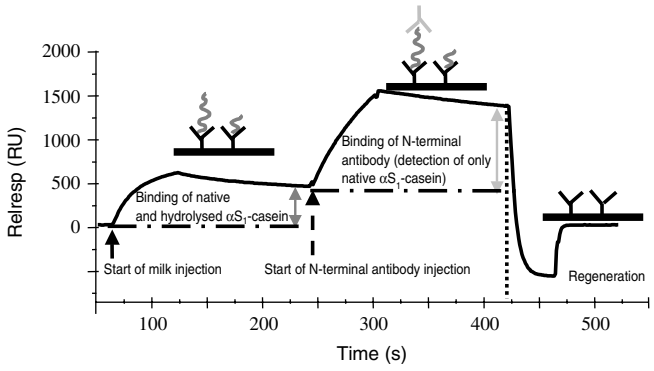

Fig. 2. Diagrammatic representation of a sensorgram of a typical milk sample showing the various steps involved in the sandwich method. The response taken into consideration for casein quantification corresponds to the one of the binding of anti-AS1Nter antibodies on the intact α_{S1}-CN.

Biosensor immunoassay

Sample preparation: To disrupt the casein micelles, several dissociating buffers were tested for milk samples dilution: HBS-EP, HBS-EP+EDTA (10, 30, 50 and 75 mM), 2 and 6 M-urea, 0·4 M-tri-sodium citrate, and SDS, Tween 80, Triton X100, CHAPS (3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate), sarcosine and Nonidet P40 (NP40) at 10 g/l.

Assay principle. Anti-AS1Cter antibodies (1/20 in Acetate pH 4·5) were immobilised on the sensor surface as described previously. A continuous flow was maintained at 30 µl/min. 30 µl of sample were injected, followed by the injection of 30 µl anti-AS1Nter monoclonal antibodies, diluted 1/100 in HBS-EP, as shown in Fig. 1. The regeneration of the sensor surface was accomplished with two 15 µl injections of 5 mM-NaOH.

Calibration curve. A Calibration curve was produced with serial dilutions (1/20 000 to 1/1250) of milk powder containing a known α_{S1}-CN concentration in HBS-EP. The calibration curve was established with the response corresponding to the binding of anti-AS1Nter antibodies on the intact α_{S1}-CN, as shown in Fig. 2.

Specificity of the assay. The specificity was assessed by replacing Mab anti-AS1Nter with Pab directed against β-, α_{S2}- and κ-CN.

Quantification in milk samples. Milk samples were diluted 1/5000 in HBS-EP buffer. To determine α_{S1}-CN concentration, each sample was analysed in duplicate.

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 of the mean (mean+3SD). Assay precision was defined by determining *intra-assay* (within run) and *inter-assay* (between runs) variation. Intra-assay variation was determined by ten successive analyses of the same sample at three different dilutions (1/6667, 1/5000 and 1/4000). Inter-assay variation was evaluated with the same samples in 10 different runs.

To determine the accuracy, the recovery of exogenous amounts of α_{S1}-CN added to a milk was calculated. Four different quantities of milk powder corresponding to 0·63, 1·26, 3·78 and 6·3 mg α_{S1}-CN/ml were added to a raw milk sample. Each sample was analysed, in duplicate, at a 1/6000 dilution.

Capillary electrophoresis

Capillary electrophoresis was carried out using an Agilent CE 1100 (Agilent GMBH, Karlsruhe, Germany) controlled by its software data system, Agilent chemstation for CE (version A09.01, Agilent). The separation was performed using a bare fused silica column Agilent 24·5 cm. The separation was conducted at 20 kV and sample solutions were injected at 500 mbarsec. The running buffer consisted in 50 mM-Phosphate buffer with 20 mM-DDT and 0·1% Hydroxyethylcellulose, 8 M-urea, pH 2·5. Samples were reduced in waterbath 2 h at 40 °C in reducing buffer (50 mM-Phosphate buffer with 10 mM-DDT and 8 M-urea, pH 8). The run time was 10 min per run; 30 min including conditioning (NaOH followed by H_2O and run buffer).

Results

Production and characterisation of monoclonal antibodies

One fusion experiment yielded 11 positive clones. Among those, 7 produced Mabs recognizing AS1Cter peptide and 4 Mabs recognizing AS1Nter peptides in ACP ELISA. Mabs produced by the 11 positive clones cross-reacted with α_{S1}-CN. These Mabs were tested in Biacore via a RAM sensor chip to see if they are able to capture α_{S1}-CN. Four Mabs directed against the C-terminal end of α_{S1}-CN and two directed against the N-terminal end recognized α_{S1}-CN after fixation on the RAM sensor chip. The Mab presenting the best fixation rate of α_{S1}-CN was chosen as the capture antibody for the immunoassay. It was a Mab directed against the C-terminal end of the protein that was called anti-AS1Cter (data not shown).

The secondary antibody was chosen among the Mabs recognising the N-terminal end of α_{S1}-CN. The choice was made by a sandwich test via the RAM sensor chip. The antibody was chosen for its capacity to detect the α_{S1}-CN after capture of the protein by the anti-AS1Cter antibody and was called anti-AS1Nter.

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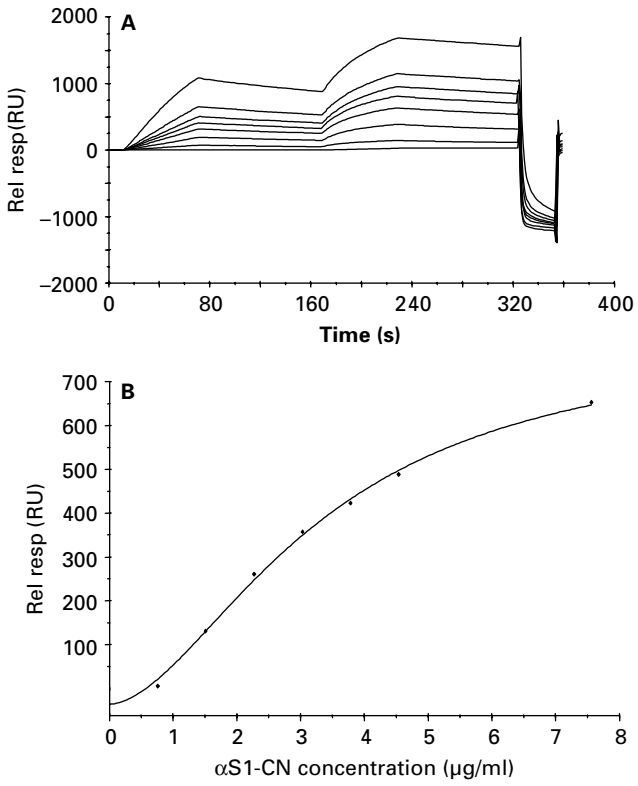

Fig. 3. Determination of standard curve A: sensorgram obtained for α_{S1}-CN concentrations ranging from 0 to 12·1 µg/ml. B: Resulting standard curve calculated with N-terminal antibody response.

Characteristics of the assay

Specificity of the assay. The specificity of the assay was assessed in two different ways. Firstly, the specificity of the anti-AS1Cter antibodies immobilised on the chip was tested against the principal milk proteins (α_{S2}-, β-, κ-CN, α-la, β-lg, IgG and BSA). The different proteins were injected onto the anti-AS1Cter coated sensor chip at 10 µg/ml. No cross-reaction was observed.

In milk, 95% of CN are organised in a micellar structure in association with calcium and phosphate. Therefore, the micelles have to be disrupted for the quantification of single CN. The HBS-EP buffer contains EDTA known to chelate calcium and destabilise the CN association. Several dissociating buffers containing detergents and chelating agents at different concentrations have been tested for their effects on the release of individual casein. Results obtained with these solutions did not differ from those obtained with HBS-EP (data not shown). So, the HBS-EP buffer was used for sample dilution. To verify if the response observed with milk or milk powder was due to the binding of a CN complex and not only α_{S1}-CN, the secondary antibodies anti-AS1Nter was replaced by Pabs, specific for β-, α_{S2}- or κ-CN. No binding of these probes on the immobilised antigen was observed, demonstrating that only α_{S1}-CN had been captured by the anti-AS1Cter.

Table 1. Determination of recovery, after exogenous addition of α_{S1}-CN, in a raw milk sample. Four different quantities of reconstituted milk powder corresponding to 0·63, 1·26, 3·78 and 6·3 mg α_{S1}-CN/ml were added to a raw milk sample. Each sample analysed was diluted 1/6000. Responses were the mean of two determinations

	Theoretical α_{S1}-CN concentration (mg/ml)	α_{S1}-CN concentration obtained (mg/ml)	Recovery (%)
Milk		6·32	
Milk+0·63 mg/ml	6·95	6·96	100·1
Milk+1·26 mg/ml	7·58	7·75	102·2
Milk+3·78 mg/ml	10·10	9·52	94·24
Milk+6·3 mg/ml	12·62	12·35	97·9

Time of analysis. The analysis of one sample, including regeneration, takes around 10 minutes.

Stability. The regeneration conditions were optimal by addition of 5 mM-NaOH. A sensor surface could be used for more than 150 cycles without any significant baseline decrease. However, a new calibration may be necessary after 60 cycles.

Standard curve. The curve was established with the anti-AS1Nter antibodies response to α_{S1}-CN concentrations in reconstituted milk ranging from 0 to 12·1 µg/ml (Fig. 3).

Detection limit. The detection limit, determined from the mean of the measured content of a representative blank sample (n=20; mean+3SD), was established at 0·87 µg/ml.

Reproducibility. The reproducibility determined with different concentrations of a raw milk sample, established for intra- and inter-assays was 2·86% and 5·31% respectively.

Accuracy. Exogenous additions of four known α_{S1}-CN concentrations in a raw milk sample were recovered between 94·2 and 102·2% (Table 1).

Analysis of milk samples

The 48 milk samples from 6 cow herds [3 Holstein (herds 1, 2, 3) and 3 Montbeliarde (herds 4, 5, 6)] collected at 8 different milking times were analysed. The concentrations of intact α_{S1}-CN obtained for milk samples ranged from 8·58 to 12·06 mg/ml (Fig. 4).

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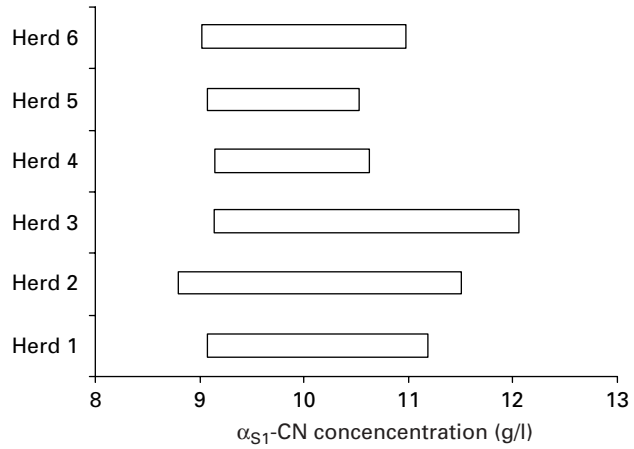

Fig. 4. Minimal and maximal values obtained for the α_{S1}-CN concentration (in g/l) calculated in 48 samples of raw milk. Six herds, three Holstein (Herds 1, 2 and 3) and three Montbeliarde (Herds 4, 5, 6) of ten cows each, were milked 8 times over a three month period.

Thirty milk samples selected to have a large range of concentration were analysed by infrared method and capillary electrophoresis. The correlation coefficients between the different methods were presented in Table 2.

Table 2. Correlation coefficients observed between the α_{S1}-CN concentration determined with the Biacore assay, the α_{S1}-CN concentration determined by capillary electrophoresis and the total protein concentration determined by infrared spectroscopy

	TP/Biacore	TP/CE	CE/Biacore
Correlation coefficient	0·614***	0·059	0·005

*** $P<0·001$
TP: total protein content determined by infrared method includes all milk proteins
CE: capillary electrophoresis

Discussion

The development of an immunoassay for the quantification of α_{S1}-CN in milk using Biacore is presented in this study. The strategy used is a sandwich assay with two monoclonal antibodies directed against each extremity of the protein. To develop this assay, the immunological tools have been produced using synthetic peptides as immunogens to target the specificity of the antibodies against the selected parts of the CN. Two peptides have been chosen, corresponding to each extremity of the α_{S1}-CN. The strategy relied on the hypothesis that the peptide would mimic the antigenic determinant exposed by the CN. A primary selection of antibodies produced was realised by ACP-ELISA. All of the positive antibodies obtained recognised the peptide and the entire α_{S1}-CN but few of them, especially antibodies directed against the N-terminal end of the protein, showed a weaker signal in ELISA for the α_{S1}-CN. Among these antibodies, a second selection was accomplished with Biacore to choose the antibodies which were used immobilised on the sensor chip to capture the α_{S1}-CN. The choice was made according to two criteria. The first was the ratio between the binding level of the antibody on the RAM-sensor chip and the binding of α_{S1}-CN on this antibody. The second was the stability of the antibody-antigen complex. Actually, among the eleven antibodies recognising α_{S1}-CN in ELISA, five were not able to capture the α_{S1}-CN after immobilization on the RAM sensor chip and one captured the α_{S1}-CN but the complex formed was not stable.

To quantify α_{S1}-CN in milk, a calibration curve was established with a reference milk powder with a known α_{S1}-CN concentration in order to reduce the matrix effect.

This technique was applied to quantify α_{S1}-CN in 48 milk samples. The values obtained in this study varied significantly, with a large range of concentrations. Nevertheless, 87·5% ranged from 9 to 11 mg/ml, which corresponds to the values generally observed in milk. These important variations could be explained by the size of the herd. The limited size of the herds was deliberately chosen in order to increase the variability in the concentrations. The results did not permit a relation to be established between the variation and the different characters (breeds, feeding and lactation stage). The variations seem to be less important for the Montbéliarde herds. The α_{S1}-CN concentration calculated with the primary response after milk injection, was compared with the concentration determined with the secondary response after the binding of the AS1Nter antibodies (data not shown). For the majority of samples, α_{S1}-CN concentrations determined with the primary response were higher than those determined with the secondary response. The primary response corresponds to the fixation of α_{S1}-CN and C-terminal fragments of α_{S1}-CN onto the chip, whereas the secondary response corresponds to the detection of the entire protein by the specific antibodies from the N-extremity of α_{S1}-CN. These results confirmed that the strategy developed here quantified only intact α_{S1}-CN and not its proteolysis products. The difference in concentration between the two responses was not always the same, which tended to demonstrate the fact that the level of proteolysis was different in the milk samples. In whole fresh milk, α_{S1}-CN may be hydrolyzed by indigenous milk enzymes, plasmin and cathepsin-D (Eigel, 1977; Kaminogawa et al. 1980) and this enzymatic activity can vary from one milk to another (Andrews, 1983; Reimerdes, 1983).

The method was compared with total protein content determination and capillary electrophoresis. A significant correlation was observed between the total protein content and α_{S1}-CN but not between capillary electrophoresis and the two other methods. α_{S1}-CN is the most abundant protein in milk, so it seems to be normal to have a correlation between its concentration and the total protein content. So, the results obtained with capillary electrophoresis

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seem surprising. Furthermore, no correlation was observed between the total protein content and the additional concentration of the six major milk proteins determined by capillary electrophoresis (data not shown). This absence of correlation is probably due to the fact that CE, as it was used in the present study, is a semi-quantitative technique. Indeed, CN concentrations were determined by integration of the peak areas. A better accuracy could be reached by evaluating the concentrations with a calibration curve obtained with standards ran in parallel.

The immunoassay developed here is able to quantify intact α_{S1}-CN in milk. It is fast ($\approx$10 min per analysis), repeatable, sensitive, automated and requires no pre-treatment of the sample, apart from dilution in the course buffer. It could therefore be of great interest in routine analysis.

The same strategy will be applied to quantify the other CN (β-, α_{S2}- and κ-CN) using specific antibodies on the same sensor surface. This technique could constitute a progress in the characterization of the quality of milk and could be used for diverse applications in cheese.

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