

Fast biosensor immunoassays for the detection of cows' milk in the milk of ewes and goats

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Two monoclonal antibodies (MAb) raised against bovine κ -casein were developed and applied in an automated optical biosensor (Biacore 3000) to create easy and fast direct and inhibition biosensor immunoassays (BIA) for the detection of cows' milk in the milk of ewes and goats. With both assay formats, low limits of detection ($<0.1\%$) and fast run times (around 5 min) were obtained. For sample preparation, milk was diluted in buffer (direct assay) or in an antibody-containing buffer (inhibition assay) only. For quantitative analysis, calibrants of cows' milk in ewes' or goats' milk were used. Advantages of the direct BIA are: the single reagent format (biosensor chip immobilized antibodies only); the use of small amounts of antibodies (2 μg for >350 tests); and the wide measurement range (0.1 to 10% cows' milk). Despite these advantages, the inhibition BIA (using κ -casein immobilized on the chip) was preferred because of the possible application of non-purified Mab, the higher responses, the higher sensitivity at relevant low percentages of cows' milk and its robustness (>800 cycles per chip).

Keywords: Bovine κ -casein, monoclonal antibodies, goats' milk, ewes' milk, cows' milk, milk species detection, biosensor immunoassay.

Differences in price and seasonal availability might make it attractive for farmers to adulterate expensive ewes' and goats' milk with cheaper cows' milk. Council Regulation No. 1255/1999 of the commission of the European Communities requires producers to state the type of milk used for manufacturing cheese or other dairy products. Apart from the possible economic loss, correct species identification is important for consumers who may have specific food allergies.

To detect adulteration of ewes' and goats' milk with cows' milk before the manufacture of dairy products requires a fast method. According to Council Regulation No. 1255/1999, such a method should have a limit of detection (LOD) for cows' milk of $<1\%$. The most recently published methods for the detection of cows' milk in the milk or cheese of other species are based on detection of DNA (Bania et al. 2001; Maudet & Taberlet, 2001) in which LOD of 0.1% of cows' milk in goats' milk can be obtained. However, to detect species-specific DNA, the necessary extraction, PCR amplification and separation procedures can make these methods time-consuming. The most frequently published methods for identifying milk species focus on protein detection and are based mainly

on separation technologies and immunoassays. Using separation technologies, such as fast protein liquid chromatography (FPLC) on an anion-exchange column (Haasnoot et al. 1986), reversed-phase HPLC (De Frutos et al. 1992) and polyacrylamide gel electrophoresis (Furtado, 1983), LOD for cows' milk in the milk of other species is $>1\%$. Moreover, in general, the procedures are time-consuming. Several enzyme-linked immunosorbent assay (ELISA) techniques have been published, based on the detection of bovine caseins (Rodriguez et al. 1990, 1993; Bitri et al. 1993; Rolland et al. 1993; Anguita et al. 1997; Richter et al. 1997), native or heat-denatured bovine whey proteins (Castro et al. 1992; Levieux & Venien, 1994; Beer et al. 1996) or bovine immunoglobulin G (IgG) (Sauer et al. 1991). Although these assays can detect cows' milk in the milk of other species at levels below 0.1%, the analysis time is of the order of several hours.

In the present study, an automated optical biosensor (BIACORE 3000) was tested as a tool for the detection of cows' milk in the milk of ewes and goats. The detection principle of this biosensor is based on surface plasmon resonance (SPR), which has been described previously (Markey, 2000). A simplified explanation of the principle is that detection is based on changes in refractive index at the sensor chip surface, caused by concentration changes at the surface, e.g., when an analyte binds to an

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immobilized ligand. The major advantages of this system are that the detection can be performed without labelling of the reagents, results can be obtained within minutes and the procedure can be automated, which renders this system suitable for high-throughput screening. This system was applied previously to develop direct biosensor immunoassays (BIA) for the detection of non-milk proteins (soy, pea and soluble wheat proteins) in milk powders (Haasnoot et al. 2001). In this direct BIA format, antibodies were immobilized onto the biosensor chip surface and the binding of plant proteins was measured directly. The total run time of this assay was 5 min and LOD was below 0.1% of plant protein in milk protein.

In the present study, two BIA formats (direct and inhibition) for the detection of cows' milk in raw milk of ewes and goats were compared to investigate differences in performance (response differences, limits of detection, robustness, etc.). In both formats, monoclonal antibodies (Mab) developed against bovine κ -casein were used. This research was performed within an EU project (AFRAMILK, contract no. G6RD-CT2000-00420), dealing with the rapid detection of fraudulent additions to milk, which focuses on the development of a fast control system prior to the manufacturing of milk products. For that reason, this research focused on the fast detection of cows' milk in the raw milk of ewes and goats.

Materials and Methods

Milk samples

Raw milk samples from ewes ($n=14$) and goats ($n=5$), from 19 different farms, were obtained from MEVGAL S.A. (Thessaloniki, Greece). Sodium azide (tablets) was added to the samples for conservation. Milk samples were transported to The Netherlands under cooled conditions (ice packs) and after arrival they were stored in 50-ml portions at -20°C until used. Thawed samples were stored at $4-6^{\circ}\text{C}$ and used for a maximum of 5 d. Before analysis, the milk samples were brought to room temperature and mixed. Cows' milk was made by reconstituting a bovine skimmed milk powder (1 g+9 ml of water). A pasteurized full-fat bovine milk and a pasteurized full-fat goats' milk (both from Friesche Vlag, Ede, The Netherlands), a full-fat goats' milk powder (Mekkermeik from Henri Willig, Katwoude, The Netherlands) and a high temperature pasteurized soy milk (Alpro Soja, Vandemoortele, Roosendaal, The Netherlands) were purchased locally.

Instruments and reagents

BIACORE 3000, sensor chips (CM5), HBS-EP buffer [pH 7.4, consisting of 10 mM-4-(2-hydroxyethyl)-piperazine-1-ethanesulphonic acid, 150 mM-sodium chloride, 3 mM-EDTA, 0.005% (v/v) surfactant polysorbate 20] and an amine coupling kit [containing

0.1 M-N-hydroxysuccinimide (NHS), 0.4 M-N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide (EDC), and 1 M-ethanolamine hydrochloride-NaOH (pH 8.5)] were supplied by Biacore AB (Uppsala, Sweden). The FPLC system and HiTrap protein G columns (1 ml) were supplied by Amersham Biosciences (Uppsala, Sweden). Bovine milk proteins, κ -casein (C0406), β -casein (C6905), α -casein (C6780), α -lactalbumin (α -la) (L6010), β -lactoglobulin (β -lg) (L2506), polyethylene glycol 1500 (PEG 1500, 50%), dimethyl sulphoxide (DMSO) and antifoam A [A5758] were obtained from Sigma-Aldrich Chemie (Zwijndrecht, The Netherlands). Another κ -casein was a gift from NIZO (Ede, The Netherlands). ICN Biochemicals (Cleveland, OH, USA) supplied γ -casein [100653]. Stock solutions of these milk proteins were prepared in PBS (0.5 mg/ml). The proteins were dissolved by gently mixing for 30–60 min. Further dilutions were prepared in HBS-EP buffer. COSTAR[®] ELISA microtitre plates, tissue culture plates (96 and 24 wells) and culture flasks (25, 75 and 162 cm²) were obtained from Corning Incorporated (Corning, NY 14831, USA). Dulbecco modified Eagle's medium (DMEM), L-glutamine and penicillin/streptomycin (P/S) were obtained from BioWhittaker Europe (Parc Industriel, De Petit-Rechain, B-4800 Verviers, Belgium) and Fetal Clone I (FCI) was from HyClone (925 West 1800 South Logan, Utah 84321, USA). Hybridocult and hybridokine were purchased from IQ Corporation (Rozenburglaan 13a, 9727 DL Groningen, The Netherlands). Hypoxanthine/thymidine (HT; 50 \times) and aminopterin (A; 100 \times) were obtained from Life Technologies (Breda, The Netherlands). Rabbit anti-mouse immunoglobulins-horseradish peroxidase (RAM-HRP) was purchased from DAKO A/S (Produktionsvej 42, DK-2600 Glostrup, Denmark) and solutions of tetramethylbenzidine (TMB) peroxidase substrate and peroxide from Kirkegaard and Perry Labs (Gaithersburg, MD 20879-9963, USA). Specol was obtained from the Animal Sciences Group (ASG, Edelhertweg 15, 8219 PH Lelystad, The Netherlands) and the BCA protein assay reagents from Pierce Biotechnology Inc. (Rockford, IL 61105, USA). Murine myeloma P3/NS-1/1-Ag4-1 (NS-1) was supplied by American Type Culture Collection (P.O. Box 1549, Manassas, VA 20108, USA) and iso-propanol by Merck (Darmstadt, Germany).

Production of monoclonal antibodies

Immunization of mice. Two female mice (Balb/c Ola Hsd), 10–12 weeks old, were immunized subcutaneously (sc) with a mixture of 75 μg of β -lg and 25 μg of κ -casein (obtained from NIZO) in 100 μl of PBS emulsified with 100 μl of Specol. Booster injections (sc) with a mixture of 37.5 μg of β -lg and 12.5 μg of κ -casein in 100 μl of PBS emulsified with 100 μl of Specol were given at 2-week intervals. Blood was taken from each mouse prior to the first immunization (pre-immune) and 1 week after each

booster injection. Sera were tested for antibodies against β -lg and κ -casein by indirect ELISA.

Fusion and cloning. The two mice were primed intraperitoneally with a mixture of 37.5 μ g of β -lg and 12.5 μ g of κ -casein in 200 μ l of PBS 4–5 d before spleen cell isolation. Hybridoma cells secreting Mab to κ -casein were prepared using murine myeloma (NS-1) as the fusion partner. Myeloma cells were fused with splenocytes from the mice at a ratio of 1:4 using PEG 1500 precipitation. Cells were suspended in culture medium (DMEM, supplemented with 8% FCI+1% hybridocult+0.5% P/S) and plated in 96-well tissue culture plates at 100 000 cells/100 μ l in each well. One hundred microlitres of two-times concentrated HAT selection medium (culture medium+2% HT+2% A) was added to each well after 4 h. After 1 week, the medium was changed to HT medium (culture medium+1% HT) and 5 d thereafter, by culture medium. From days 12 to 14, hybridoma supernatants were tested for antibodies to κ -casein and β -lg using indirect ELISA. Positive cultures were transferred from the culture in the 96-well plate to 1 ml of culture medium in a 24-well plate. After the 24-well culture became dense, the culture was transferred to 25 cm² culture flasks. Thereafter, the positive cultures were cloned by limiting dilution (average 0.2 cells/well) in a limiting dilution selection medium (DMEM supplemented with 8% FCI+1% hybridokine+0.5% P/S) until stable (2–3 clonings). Mab-producing clones of interest were grown in 162 cm² culture flasks. The cell culture supernatants obtained were ready for use in experiments. Mab-producing clones were propagated and frozen (overnight at –80 °C in a freezing container filled with isopropanol) in freezing medium (DMEM containing 12% dimethyl sulphoxide, 38% FCI) and stored in nitrogen. The immunoglobulin subclass of the Mab was determined by the Mouse Monoclonal Antibody Isotyping Kit (IsoStrip) of Roche (Mannheim, Germany).

Isolation of monoclonal antibodies

Mab from the raw cell culture media (about 400 ml) were isolated by ammonium sulphate precipitation (Verheijen et al. 2000) followed by affinity chromatography using a HiTrap Protein G column in accordance with the manufacturer's instructions. With the Mab 6A10G12F8-containing medium, the total amount of IgG obtained was 7.4 mg in 2 ml and, with the Mab 4G10F10-containing medium, 4.2 mg in 3 ml.

Indirect ELISA

Microtitre plates were coated with κ -casein and β -lg solutions (100 ng/100 μ l in each well) in coating buffer (50 mM-sodium carbonate, pH 9.6) for 1 h at room temperature. Subsequently, wells were emptied and blocked

with 200 μ l ovalbumin solution (0.1% w/v in coating buffer) for 1 h at room temperature. Prior to use, the plate was washed three times with washing buffer (PBS containing 0.05% Tween-20 and 0.004% antifoam A). To each well, 100 μ l of a serial dilution of serum or culture supernatant was added. The plate was incubated at room temperature for 1 h and washed three times with washing buffer. Subsequently, 100 μ l of RAM-HRP in a 1:2500 dilution in PBST was added to each well and incubated for 1 h at room temperature. After washing the plate, the bound peroxidase was assessed by adding 100 μ l of a freshly prepared mixture of TMB peroxidase substrate and peroxidase (1:1; v/v). After incubation in the dark for 20–30 min at room temperature, the reaction was stopped by adding 100- μ l aliquots of 1 M-phosphoric acid and the coloured product was measured at 450 nm using an Argus 400 microplate reader (Canberra Packard, Downers Grove, IL 60515, USA).

Direct biosensor immunoassays

In the direct BIA format, affinity purified Mab were immobilized onto the biosensor chip (CM5) surface by the use of the amine coupling kit and the Surface Preparation Wizard (aiming for 10000 RU) as present in the BIACORE 3000 control software. The biosensor surface was activated by injecting (35 μ l at a flow rate of 5 μ l/min) a mixture of EDC and NHS (1:1; v/v) into one of the four flow channels (Fcs). Then the Mab, diluted (0.05 mg/ml) in coupling buffer (10 mM-sodium acetate; pH 4.5) was injected and attached to the carboxymethylated dextran surface via primary amine groups. After coupling, active groups were blocked with M-ethanolamine. Mab 6A10G12F8 was immobilized in Fc2 (final response of 9500 RU) and Mab 4G10F10 in Fc4 (final response of 9200 RU). Fc1 and Fc3 were used as blanks and were activated as described above and directly deactivated with ethanolamine. During all analysis, 20- μ l injections at a flow rate of 20 μ l/min were applied and for the regeneration, 10 μ l of a 10 mM-HCl solution was injected. The total time between two sample injections was 315 sec. Prior to the injection of ewes' and goats' milk, the samples were brought to room temperature, homogenized and diluted ten times in HBS-EP buffer (20 μ l milk plus 180 μ l buffer). For quantitative analysis, a calibration graph was prepared after analysing calibration standards (0.05; 0.15; 0.45; 1.35; 4.05 and 12.15% cows' milk in goats' milk).

Inhibition biosensor immunoassays

In the inhibition BIA format, κ -casein was immobilized onto the biosensor chip surface in Fc2 following the same amine coupling procedure as described for the immobilization of Mab (see Direct BIA). κ -Casein stock solution (0.5 mg/ml in PBS) was diluted (0.1 mg/ml) in 10 mM-acetate buffer (pH 4.0) and in the Surface Preparation Wizard we aimed for a level of 5000 RU and

obtained a final response of 4600 RU. Fc1 was used as blank and was activated and directly deactivated with ethanolamine as described above. Different mixtures of the standard solutions or samples with the two Mab (raw culture supernatants or affinity purified IgG) and different injection volumes and flow rates were tested. In the final protocol, 190 μ l of a mixture of the two Mab [raw cell culture supernatant of Mab 4G10F10 (0.4 ml) and Mab 6A10G12F8 (0.8 ml)+2.8 ml HBS-EP buffer] was mixed with 10 μ l of the homogenized milk samples in a microtitre plate and 40 μ l was injected at a flow rate of 40 μ l/min. For regeneration, 20 μ l of a 50 mM-sodium hydroxide solution was injected at 40 μ l/min. Total time between two sample injections was 260 sec.

Results

Production of monoclonal antibodies

After two fusions and sub-cloning, we found no stable hybrids that produced antibodies against β -lg; however, five hybrids produced antibodies against κ -casein (two IgM and three IgG1 with kappa light chain). In the indirect ELISA, the three IgG1 Mab-containing cell cultures were tested for optimum dilution and for competition with free κ -casein. With two of these Mab [Mab 4G10F10 (further named 4G10) and 6A10G12F8 (further named 6A10)] inhibition was obtained with κ -casein concentrations in the 0.1 to 1 μ g/ml range. These two Mab were applied in the BIA.

Direct biosensor immunoassays

In the direct BIA format, affinity purified Mab were immobilized onto the biosensor chip surface. Following the immobilization procedure in the Surface Preparation Wizard, 40 μ l of a Mab-containing solution (0.05 mg/ml) was injected into the Biacore (2 μ g of each Mab). After immobilization, final responses of 9500 RU (Mab 6A10 in Fc2) and 9100 RU (Mab 4G10 in Fc4) were obtained. These responses corresponded to absolute amounts of about 11 ng of immobilized Mab in each Fc (Stenberg et al. 1991), which means that only small portions (about 0.5%) of the injected Mabs were immobilized onto the sensor surface.

Standard solutions with different concentrations of the milk proteins in HBS-EP buffer were injected (20 μ l) over the immobilized Mab (20 μ l/min) and the responses obtained were plotted against the protein concentrations. Results obtained with Mab 4G10 are shown in Fig. 1. The highest responses were obtained after the injection of κ -casein solutions (up to 1200 RU). Although with lower responses, binding of the other caseins was also observed with both Mab. Concentrations of the casein solutions corresponding to a response of 200 RU were 1, 4, 15 and 70 μ g/ml for κ -casein, β -casein, γ -casein and α -casein, respectively. From these concentrations, cross-reactivities

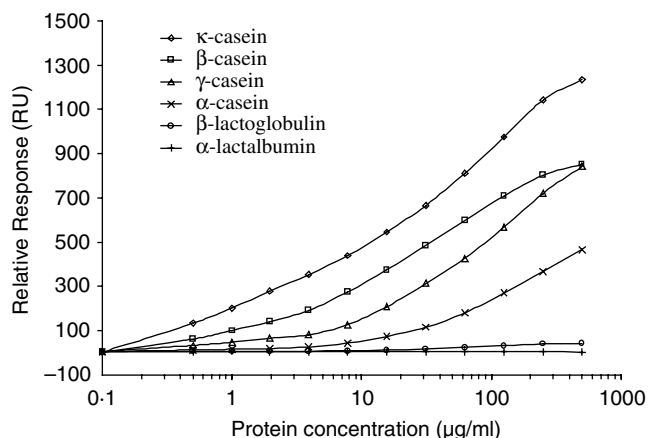


Fig. 1. Relative responses (RU) obtained in the direct BIA due to binding of different concentrations of bovine milk proteins (diluted in HBS-EP buffer) to immobilized Mab 4G10. Injections (20 μ l) were performed at 20 μ l/min.

(CR) were calculated as 100% (κ -casein), 25% (β -casein), 7% (γ -casein) and 1.5% (α -casein). Whey proteins α -la and β -lg did not react with the Mab. Comparable CR was found with Mab 6A10, but with about 40% lower responses.

After injection of κ -casein over the immobilized Mab, second injections of the two Mab showed that the Mab did not bind to κ -casein captured by the same Mab (see Fig. 2). However, as shown in Fig. 2, Mab 6A10 bound to κ -casein captured by Mab 4G10 and vice versa. Comparable results were obtained with the other three bovine caseins. This suggests that the Mab recognized one epitope on the caseins only and that the two Mab reacted with different epitopes on the caseins.

Different dilutions of bovine milk (reconstituted from the skimmed milk powder) were injected (20 μ l) over the immobilized Mab (20 μ l/min). High responses (970 RU with Mab 4G10 and 780 RU with Mab 6A10) were observed after injection of 10 and 100-times diluted bovine milk. These responses fell to about 50% after injection of 1000-times diluted bovine milk. Injection of 10-times diluted milk from goats and sheep resulted in low responses (30 RU) only. This means that, from the milk species tested, the two Mab were specific for cows' milk caseins only.

Cows' milk was added to goats' milk (0.05 to 12.15%) and the samples were analysed after a 10-times dilution in HBS-EP buffer. Responses obtained were plotted against the percentages of cows' milk and the calibration graphs obtained with the two Mab are shown in Fig. 3. The better performance (higher responses) of Mab 4G10 is obvious. Such calibration graphs for both Mab were used to calculate the limits of detection (LOD=average percentage of cows' milk plus three-times SD) using the responses obtained after analysing the 19 blank raw milk samples from sheep and goats (see Table 1). LOD obtained with Mab

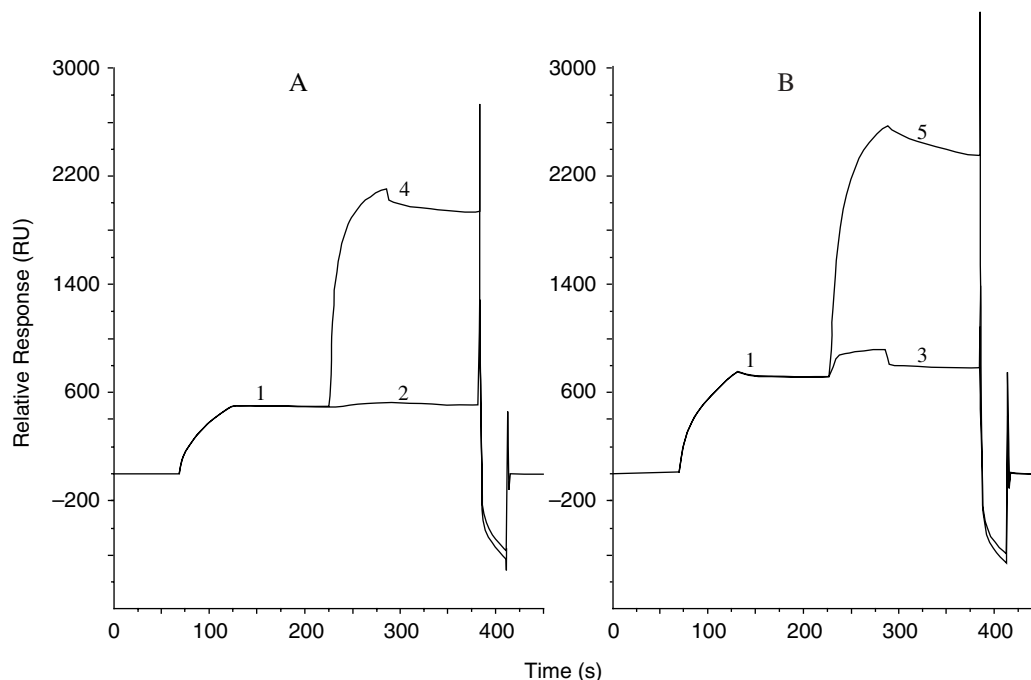


Fig. 2. Sensorgrams obtained after the injection of κ -casein (20 μ l of 50 μ g/ml) over immobilized Mab 6A10 in Fc2 (A) and immobilized Mab 4G10 in Fc4 (B), followed by second injections (20 μ l) of the Mab (350 μ g/ml) at a flow rate of 20 μ l/min. The numbers indicated in the four sensorgrams show: 1. the binding of κ -casein to immobilized Mab 6A10 [A] and Mab 4G10 [B], 2. no binding of Mab 6A10 to κ -casein captured by the same Mab [A], 3. no binding of Mab 4G10 to κ -casein captured by the same Mab [B], 4. binding of Mab 4G10 to κ -casein captured by Mab 6A10 [A], 5. binding of Mab 6A10 to κ -casein captured by Mab 4G10 [B].

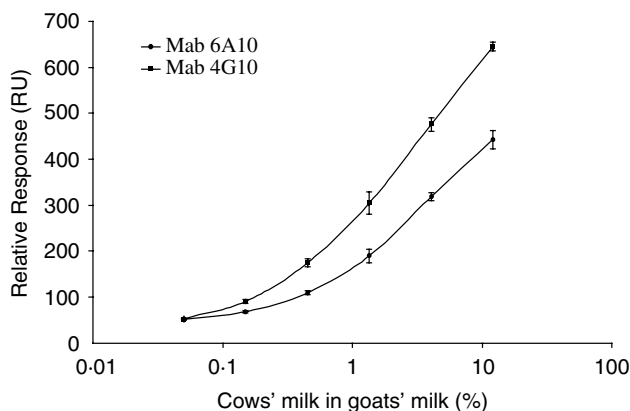


Fig. 3. Calibration graphs (average of 8 calibration graphs for each with SD) of cows' milk in goats' milk obtained in the direct BIA with the two Mab using 20 μ l injections (20 μ l/min) of 10-times diluted milk samples.

6A10 and Mab 4G10 were calculated as 0.07% and 0.06% cows' milk, respectively. Blank milk samples were spiked with different percentages of cows' milk and the results after analysing with the direct BIA are summarized in Table 1. With both Mab, calculated recoveries varied between 80 and 110%. The rather high SD was similar within the two groups of raw milk samples (ewes' and goats' milk). Responses obtained with calibration standards were followed during about 200 cycles and a 20%

decrease in response was observed. Owing to these reductions, calibration graphs have to be included within every series for quantitative analysis. In practice, acceptable calibration graphs were still obtained with both Mab after 350 cycles but, owing to the higher responses, Mab 4G10 performed better in the direct BIA.

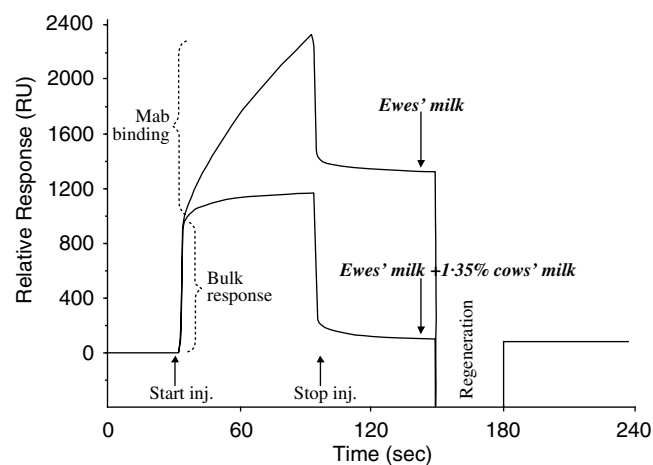
Inhibition biosensor immunoassays

In the inhibition BIA format, κ -casein was immobilized onto the chip surface with a final response of 4500 RU (about 5 ng absolute). Protein concentrations of the affinity purified fractions of the cell culture supernatants were determined as 1.4 mg/ml (Mab 4G10) and 3.7 mg/ml (Mab 6A10). Injections of 20 μ l at 20 μ l/min of the 100-times diluted Mab-containing fractions (0.3 to 0.7 μ g of Mab per injection) resulted in maximum responses of 1730 RU (Mab 4G10) and 2620 RU (Mab 6A10). Compared with the direct BIA, using 2 μ g of a Mab for more than 350 cycles (about 6 ng per cycle), the inhibition assay consumes a lot more Mab (300 to 700 ng per cycle) if high responses are desired.

After injection of undiluted Mab-containing raw cell culture supernatants (20 μ l at 20 μ l/min), high responses due to the binding of the Mabs were observed also (3800 RU for Mab 4G10 and 2600 RU for Mab 6A10). To save the energy necessary for the preparation of the affinity purified Mabs, further experiments were performed with

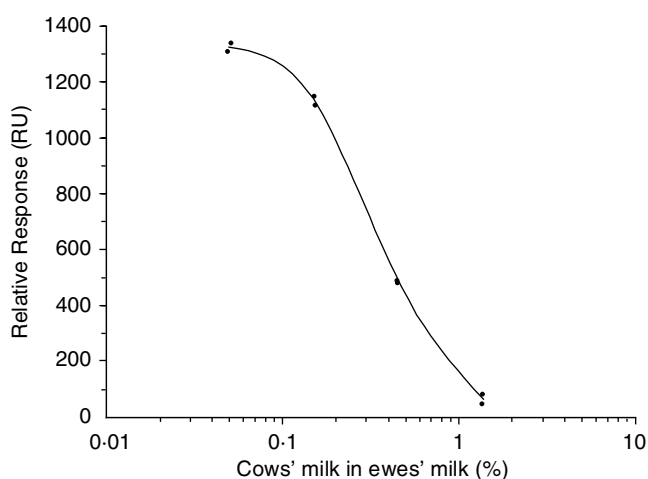
Table 1. Summary of the results obtained with the 19 raw milk samples (mean±SD) from ewes and goats analysed in the direct BIA format

Cows' milk addition (%)	Relative response (RU)		Cows' milk (%)		Recovery (%)	
	Mab 6A10	Mab 4G10	Mab 6A10	Mab 4G10	Mab 6A10	Mab 4G10
0	37±3	30±3	0.037±0.01	0.027±0.01	—	—
0.25	77±4	101±8	0.20±0.04	0.21±0.03	80±16	84±12
0.5	104±8	156±18	0.54±0.09	0.55±0.12	108±18	110±24
1	147±10	234±25	0.93±0.14	1.01±0.26	93±14	101±26
2	218±11	339±23	1.98±0.22	1.99±0.33	99±11	99±16

**Fig. 4.** Sensorgrams obtained in the inhibition BIA after injection (40 µl at 40 µl/min) of a blank ewes' milk and the same milk spiked with 1.35% cows' milk. The milk samples were diluted 20 times in HBS-EP buffer containing a mixture of the two Mab.

diluted raw cell culture supernatants. Ten-times diluted Mab containing cell culture supernatants (20 µl) were mixed with κ-casein solutions with different concentrations (80 µl) and injected (50 µl at 20 µl/min) over the sensor surface. Maximum responses (600 RU for Mab 4G10 and 350 RU for Mab 6A10) fell to 50% at κ-casein concentrations of 0.4 µg/ml (Mab 4G10) and 0.8 µg/ml (Mab 6A10). Comparable injections with the other milk proteins resulted in calculated CR of 22–28% for β-casein, 6–10% for γ-casein and <1% for α-casein, α-la and β-lg.

From the experiments with the direct BIA it was shown that the two Mab recognized different epitopes on the bovine caseins. In the final inhibition BIA, a mixture of the two Mab (0.4 ml of supernatant with Mab 4G10 and 0.8 ml of supernatant with Mab 6A10 plus 2.8 ml of HBS-EP buffer), in which each Mab contributed about half of the total response, was applied. Maximum responses obtainable in the inhibition BIA can be influenced by various parameters (amount of Mab, injection volume, flow rate and amount of milk sample injected). Different combinations were tested, aiming for a fast assay with a 50% inhibition at around 0.5% cows' milk in the milk of ewes and goats.

**Fig. 5.** Calibration graph of cows' milk in ewes' milk obtained in the inhibition BIA using 40 µl injections at 40 µl/min. The milk samples were diluted 20 times in HBS-EP buffer containing a mixture of the two Mab.

In the final protocol, 10 µl of homogenized raw milk, pipetted in a microtitre plate, was mixed with 190 µl of the antibody mixture and 40-µl injections were performed at 40 µl/min. Total time between two sample injections (including the regeneration step with a 20 µl injection of 50 mM sodium hydroxide) was 260 sec. Sensorgrams obtained with a blank ewes' milk sample and the same sample spiked with 1.35% cows' milk are presented in Fig. 4 and the difference in response obtained after the sample injections is obvious.

For quantitative analysis, calibration standards (0.05; 0.15; 0.45 and 1.35% cows' milk in ewes' milk) were analysed within every series of samples. Percentages of cows' milk were calculated with the help of a calibration graph as shown in Fig. 5, which shows a 50% inhibition at 0.3%.

The 19 blank raw milk samples from ewes and goats were analysed and the average background, expressed as concentration of cows' milk, was calculated as 0.039±0.013% which resulted in an LOD of 0.08%. Raw milk samples ($n=19$) were spiked with different percentages of cows' milk (0.1; 0.5 and 1%) and the averages

recovered were 0.13 ± 0.04 ; 0.44 ± 0.04 and $1.12 \pm 0.11\%$, respectively.

So far we had used bovine milk reconstituted from skimmed milk powder for the preparation of the calibration graphs. A comparable calibration graph was obtained using a pasteurized full-fat bovine milk obtained from the local market, which means that the binding of the Mab to bovine caseins is not influenced by the pasteurization of the bovine milk. A pasteurized full-fat goats' milk and a pasteurized full-fat goats' milk powder were also tested and no inhibition of the maximum response was observed (no cows' milk detected ($< \text{LOD}$)), which means that the temperature treatment of the milk had no measurable effect on this BIA. A high-temperature pasteurized soy milk was analysed also and no inhibition was detected, which indicates that this BIA is suitable for the detection of cows' milk in soy milk too.

The biosensor chip with immobilized κ -casein was used for about 800 cycles. During these cycles, the maximum response reduced to about 50% (from 2000 to 1000 RU). However, at the end of the experiments, acceptable calibration graphs were still obtained, which makes this BIA very robust. For quantitative analysis, a calibration graph has to be included within every series of samples.

Discussion

By immunizing mice with a mixture of β -Ig and κ -casein, we hoped to obtain Mab against both milk proteins. However, κ -casein seemed to be a better immunogen than β -Ig, which does not agree with Levieux & Venien (1994) who reported that caseins are poor immunogens and whey proteins are generally good immunogens. Reported production of antibodies against bovine κ -casein (Feng & Cunningham-Rundles, 1989; Kuzmanoff et al. 1990), α_{s1} -casein (Rollad et al. 1993) and β -casein (Senocq et al. 2001) confirms that caseins are good immunogens.

The two selected Mab developed against bovine κ -casein recognized two different epitopes of κ -casein and other bovine caseins and no reaction was observed with the whey proteins of bovine milk and milk from ewes and goats. Comparable results were found by Feng & Cunningham (1989) who produced a Mab against bovine κ -casein which did not bind to bovine serum albumin, bovine gammaglobulin, β -Ig and α -1a and with human milk. Kuzmanoff et al. (1990) isolated two Mab against bovine κ -casein of which one showed CR with bovine α -casein (13%) and β -casein (7%) and the other was more specific (about 1% CR with these bovine caseins). Senocq et al. (2001) developed several Mab against bovine β -casein and from the 21 Mab tested with milk samples from different species (cow, ewe, goat, human and equine), no Mab was bovine-specific and only a single Mab discriminated bovine milk from ewes' and goats' milk.

In our study, the two tested Mab raised against κ -casein were specific for cows' milk caseins and for the detection

of this milk species in the milk of ewes and goats, a further identification of the epitopes involved was not necessary. For possible application in cheese, a further identification should be applied to find out if the epitopes are located on the para- κ -casein part or on the glycomacropeptide part of κ -casein.

Mab were used to develop and evaluate two different BIA formats (direct and inhibition) with comparable LOD ($< 0.1\%$ cows' milk) and time of analysis (around 5 min). Advantages of the direct BIA are: the single reagent format (biosensor chip immobilized antibodies only); the use of low amounts of antibodies (2 μg for more than 350 analyses); and the wide measurement range (0.1 to 10% cows' milk). Despite these advantages, the inhibition BIA (using κ -casein immobilized on the chip) was preferred because high responses could be obtained; non-purified Mab-containing cell culture media could be used; a higher sensitivity (larger response difference) was obtained at relevant percentages of cows' milk (0.1 to 1%); and it was robust (> 800 analyses per chip). For quantification of adulterations at higher levels ($> 1\%$), milk samples should be re-analysed after a 5-times or 10-times dilution.

Rozenfeld et al. (2002) developed a polyclonal antibody (Pab) against cows' milk proteins and specific Mab against bovine α -casein, β -casein and κ -casein. With the Pab, a low CR (about 0.02%) was observed with a soy protein extract and a 30-kD component was responsible for this reaction. In immunoblots, their specific Mab also reacted with this component. Mab developed within our study did not show any activity against soy proteins from the undiluted soy milk (containing 7.2% soy beans) tested (CR $< 0.01\%$) and the detection of cows' milk in expensive soy milk seems to be a possible future application.

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References

- Anguita G, Martin R, Garcia T, Morales P, Haza AI, Gonzalez I, Sanz B & Hernandez PE 1997 A competitive enzyme-linked immunosorbent assay for detection of bovine milk in ovine and caprine milk and cheese using a monoclonal antibody against bovine β -casein. *Journal of Food Protection* **60** 64–66
- Bania J, Ugorski M, Polanowski A & Adamczyk E 2001 Application of polymerase chain reaction for detection of goats' milk adulteration by milk of cow. *Journal of Dairy Research* **68** 333–336
- Beer M, Krause I, Stapf M, Schwarzer C & Klostermeyer H 1996 Indirect competitive enzyme-linked immunosorbent assay for the detection of native and heat-denatured bovine β -lactoglobulin in ewes' and goats' milk cheese. *Zeitschrift für Lebensmittel Untersuchung und Forschung* **203** 21–26

- Bitri L, Rolland MP & Besançon P** 1993 Immunological detection of bovine caseinomacropeptide in ovine and caprine dairy products. *Milchwissenschaft* **48** 367–371
- Castro C, Martin R, Garcia T, Rodriguez E, Gonzales I, Sanz B & Hernandez PE** 1992 Indirect enzyme-linked immunosorbent assay for detection of cows' milk in goats' milk. *Food and Agricultural Immunology* **4** 11–18
- Commission Regulation (EC)** No. 213/2001 *Official Journal of the European Communities* **L37** 51–59
- De Frutos M, Cifuentes A, Amigo L, Ramos M & Diez-Masa JC** 1992 Rapid analysis of whey proteins from different animal species by reversed-phase high performance liquid chromatography. *Zeitschrift für Lebensmittel Untersuchung und Forschung* **195** 326–323
- Feng Z-K & Cunningham-Rundles C** 1989 Production of a monoclonal antibody to bovine κ -casein. *Hybridoma* **8** 223–230
- Furtado MM** 1983 Detection of cow milk in goat milk by polyacrylamide gel electrophoresis. *Journal of Dairy Science* **66** 1822–1824
- Haasnoot W, Venema DP & Elenbaas HL** 1986 Determination of cow milk in the milk and cheese of ewes and goats by fast protein liquid chromatography. *Milchwissenschaft* **41** 642–645
- Haasnoot W, Olieman K, Cazemier G & Verheijen R** 2001 Direct biosensor immunoassays for the detection of nonmilk proteins in milk powder. *Journal of Agricultural and Food Chemistry* **49** 5201–5206
- Kuzmanoff KM, Andresen JW & Beattie CW** 1990 Isolation of monoclonal antibodies monospecific for bovine κ -casein. *Journal of Dairy Science* **73** 2741–2748
- Levieux D & Venien A** 1994 Rapid, sensitive two-site ELISA for detection of cows' milk in goats' or ewes' milk using monoclonal antibodies. *Journal of Dairy Research* **61** 91–99
- Markey F** 2000 Principles of surface plasmon resonance. In *Real-time Analysis of Biomolecular Interactions. Applications of BIACORE*, pp. 13–22 (Eds K Nagata & H Handa). Tokyo, Japan: Springer-Verlag
- Maudet C & Taberlet P** 2001 Detection of cows' milk in goats' cheeses inferred from mitochondrial DNA polymorphism. *Journal of Dairy Research* **68** 229–235
- Richter W, Krause I, Graf C, Sperrer I, Schwarzer C & Klostermeyer H** 1997 An indirect competitive ELISA for detection of cows' milk and caseinate in goats' and ewes' milk and cheese using polyclonal antibodies against γ -caseins. *Zeitschrift für Lebensmittel Untersuchung und Forschung* **204** 21–26
- Rodriguez E, Martin R, Garcia T, Gonzalez I, Morales P, Sanz B & Hernandez PE** 1993 Detection of cows' milk in ewes' milk and cheese by a sandwich enzyme-linked immunosorbent assay (ELISA). *Journal of the Science of Food and Agriculture* **61** 175–180
- Rodriguez E, Martin R, Garcia T, Hernandez PE & Sanz B** 1990 Detection of cows' milk in ewes' milk and cheese by an indirect enzyme-linked immunosorbent assay (ELISA). *Journal of Dairy Research* **57** 197–205
- Rolland MP, Bitri L & Besançon P** 1993 Polyclonal antibodies with pre-determined specificity against bovine α_{s1} -casein: application to the detection of bovine milk in ovine milk and cheese. *Journal of Dairy Research* **60** 413–420
- Rozenfeld P, Docena GH, Añón MC & Fossati CA** 2002 Detection and identification of a soy protein component that cross-reacts with caseins from cow's milk. *Clinical and Experimental Immunology* **130** 49–58
- Sauer S, Dietrich R, Schneider E & Terplan G** 1991 A rapid enzyme-immunoassay for the detection of cows' milk in ewes' and goats' milk. *Archiv für Lebensmittel Hygiene* **42** 151–154
- Senocq D, Dupont D, Rolet-Répécaud O & Levieux D** 2001 Monoclonal antibodies against bovine β -casein: production and epitope characterization. *Food and Agricultural Immunology* **13** 213–224
- Stenberg E, Persson B, Roos H, & Urbaniczky C** 1991 Quantitative determination of surface concentration of protein with surface plasmon resonance using radiolabeled proteins. *Journal of Colloid and Interface Science* **143** 513–526
- Verheijen R, Osswald IK, Dietrich R & Haasnoot W** 2000 Development of a one step strip test for the detection of (dihydro)streptomycin residues in raw milk. *Food and Agricultural Immunology* **12** 31–40