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ELISA for Raw and Heat-Treated Cow's and Buffalo's Milk in the Milk of Other Species and Sources

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Abstract

Due to the lower prices of cow's and buffalo's milk, fraudulent mixing with higher priced milk of other species and sources is economically attractive but illegal and dangerous for allergic consumers. For the rapid detection of cow's milk, several immunoassays are available but most of them lack the possibility to detect heat-treated cow's milk due to denaturation of the target protein(s). In the present indirect enzyme-linked immunosorbent assay (ELISA), a mouse monoclonal antibody (Mab) raised against the bovine milk protein κ-casein is labelled with the enzyme horseradish peroxidase and the binding to the bovine κ-casein-coated wells of a 96-wells microtiter plate is inhibited by free bovine κ-casein in the sample or standard. The Mab recognises a 5 amino acids-containing epitope on the glycomacropeptide (GMP) part of bovine κ-casein which is absent in κ-casein of goat, sheep, horse, donkey, camel, etc, but is present in κ-casein of buffalo. Due to the indirect assay format, the cow's and buffalo's milk ELISA also recognises heat-treated milk containing denatured κ-casein. The measurement range of the bovine κ-casein calibration standards is from 0.1 to 2.5 µg/ml, the limit of detection is 0.05 µg/ml and the only milk sample preparation needed is dilution (100 or 1000 times). This new fast and easy-to-apply ELISA is well suited for the detection of the lower priced raw and heated milk of cows and buffalos in the higher priced milk of other species and sources within the range of 0.25 till 50%.

Keywords: Cow's milk; Buffalo's milk; Fraud; ELISA; Heat-treated milk

Abbreviations:

ELISA: Enzyme-Linked Immunosorbent Assay; Mab: Monoclonal Antibody; HRP: Horseradish Peroxidase; GMP: Glycomacropeptide; FAO : Food and Agriculture Organization; LOD: Limit of Detection; DL: Decision Level; UHT: Ultra-High-Temperature

Introduction

The Food and Agriculture Organization (FAO) of the United Nations has estimated that 85% of all milk worldwide was produced by cows [1]. In addition to cattle, many other kinds of livestock provide milk used by humans for dairy products. These animals include buffalo, goat, sheep, camel, donkey, horse, reindeer and yak. The first four animals produce about 11, 2, 1.4 and 0.2 %, respectively of all milk worldwide [1]. As described by Barlowska et al. [2], cow's milk is the most universal raw material for processing, which is reflected in the broadest spectrum of manufactured products. The composition of goat's milk allows using it as the raw material for dairy processing also. Milk from sheep and buffalos, regarding their high content of protein and fat, make a very good material for processing, especially cheese making. Milk from donkeys, camels and horses have the most comparable protein composition to human milk and are consumed predominantly in a non-processed form and the first two sources are suggested as cheaper alternatives for human milk [3].

Due to these differences in production capacities, the consumer prices of milk from these different species differ greatly and can be up to 17 times higher than cow's milk (in the case of horse milk in the Netherlands) and this encourages fraudulent mixing for economic benefits. Similar illegal handlings can be expected with the more expensive grain milks (from barley, oats, rice and spelt), legume milks (from lupin, pea, peanut and soy), nut milks (from almond, cashew, hazelnut and walnut) and seed milks (from hemp, quinoa, sesame seed, sunflower seed and coconut).

As cow's and buffalo's milk are the cheapest of the described milk sources, this encourages fraudulent mixing for economic benefits and this is illegal and a risk for consumers with cow's milk allergy or intolerance [4]. The vast majority of children with persistent cow's milk allergy were positive on skin prick testing to water buffalo's milk [5], which indicates that water buffalo's milk is unlikely to be a fruitful alternative for children with cow's milk allergy and should be avoided as well. There are several analytical methods for species identification of milk and milk products [6] and, for instance with PCR, rapid and sensitive identification of buffalo's, cow's and sheep's milk is possible [7]. However, immunoassays are easier to apply in less equipped laboratories or closer to production facilities. At present, immunoassays for the detection of buffalo's milk are, to our knowledge, not available. For the immunochemical detection of cow's milk, the use of species specific immunoglobulins [8], whey proteins [6], or caseins [6] in sandwich and indirect formats are described and these are not or less suitable for the detection of heated cow's milk due to the denaturation of the target proteins. Song et al. [9] developed an ELISA suitable for the detection of heat-treated cow's milk in goat's milk, in the range from 2% to 50%, by the application of cow's milk-

coated plates and a polyclonal antiserum raised against bovine β-casein with an enzyme-labelled secondary antibody. The cow's milk specificity was obtained by a pre-incubation of the anti-bovine-β-casein with goat β-casein. The production of more specific monoclonal antibodies (Mabs) against bovine κ-casein was described earlier [10]. Caseins are the major protein components of cow's and buffalo's milk [11], comprising 83% (23 g/L) and 90% (44 g/L), of the total amount of milk protein of respectively cow's and buffalo's milk, of which around 9% (2 g/L cow's milk) and 7% (3 g/L buffalo's milk) is κ-casein. In milk, the caseins, together with calcium phosphate, form aggregates of several thousand individual protein molecules with average diameters of 150 to 200 nm, known as casein micelles. Most, if not all, of the κ-casein is present on the surface of these micelles and, as such, easy to approach by antibodies.

The application of the anti-κ-casein Mabs in an optical biosensor for the detection of cow's milk in the milk of ewes and goats, with a measurement range from 0.1% to 1% cow's milk (in the inhibition assay format), has been described previously [10]. However, the applied optical biosensor (Biacore 3000) is very expensive and not suitable for routine control application. An alternative and cheaper sensor (Spreeta) has also been applied to this purpose [13] but is not commercially available. The Mabs were previously applied in an indirect ELISA format for the detection of bovine rennet whey powder in milk powder and buttermilk powder [14] using a κ-casein-coated plate. The dose-response curve for κ-casein in this ELISA ranged from 0.1 till 1 µg/ml.

In the present study, the same format is applied to develop a faster assay, with a stabilised (sealed) protein (κ-casein)-coated ready-to-use microtiter plate and ready-to-use calibration standards, for the detection of fraudulent additions (percentages) of cow's milk in milk of other species and sources. The applied Mab recognises a 5 amino acids-containing epitope on the glycomacropeptide (GMP) part of bovine κ-casein and, with the use of the indirect assay format, the ELISA should also recognise heat-treated cow's milk containing denatured κ-casein. This epitope is also present in κ-casein of buffalo's milk and, consequently, the ELISA should also detect buffalo's milk in the milk of other species and sources.

Materials and Methods

Chemicals and instrumentation

The cow's and buffalo's milk ELISA is a new, commercially available test kit (5171BKC) of EuroProxima (Arnhem, the Netherlands).

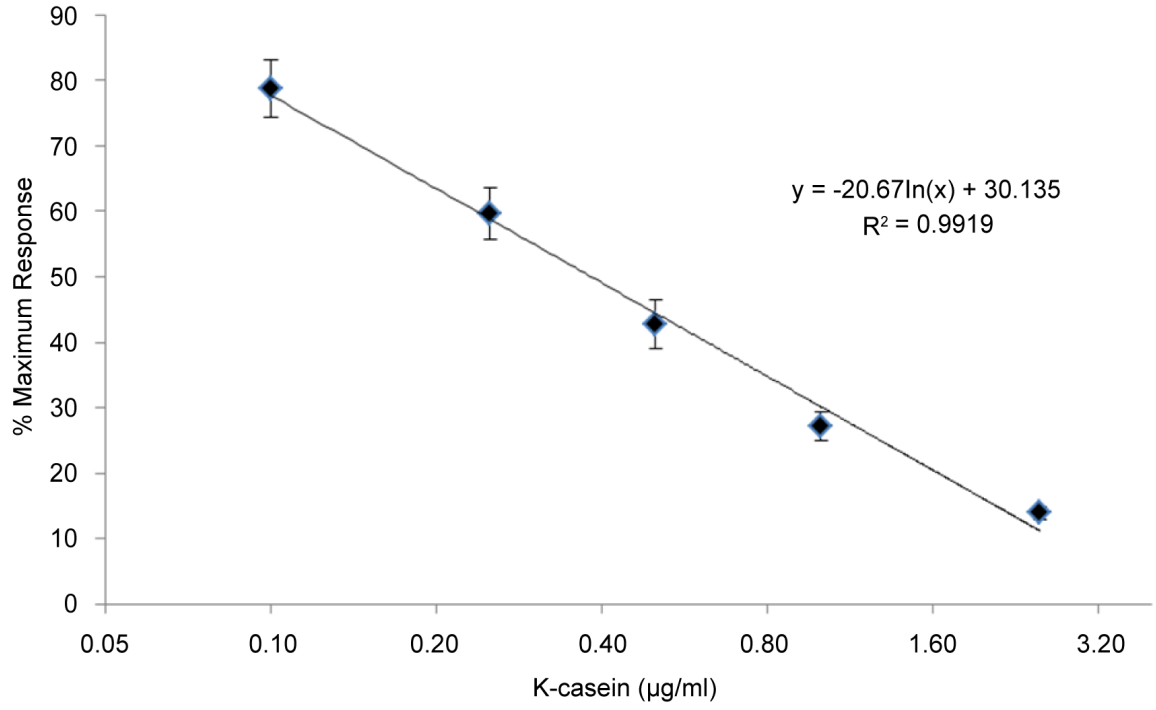

Figure 1: Average calibration curve (n=4) obtained over 4 different days with the ready-to-use standard solutions of bovine κ-casein supplied in the ELISA kit

The kit contains a ready-to-use 96-wells microtiter plate (12 strips, 8 wells each) coated with bovine κ-casein, 6 ready-to-use bovine κ-casein standards (0, 0.10, 0.25, 0.5 1.0 and 2.5 µg/ml), sample dilution buffer, rinsing buffer, concentrated Mab-HRP conjugate solution, dilution buffer, substrate solution and stop solution. The ELx808 Absorbance microplate reader with Gen 5 Data Analysis Software from Bio-Tek Instrument, Inc. was obtained via Beun de Ronde (Abcoude, the Netherlands), the Well wash 4MK2 plate washer was from Thermo Scientific (Breda, the Netherlands), the Vortex Genie-2 was from Scientific Instruments (Bohemia, N.Y., USA) and the TiMix2 microtiter plate shaker from Edmund Bühler GmbH was obtained via Salm en Kipp (Breukelen, the Netherlands)

Samples

A sample of raw cow's tank milk was obtained from a local farm. Pasteurized and UHT-treated cow's milk were obtained from a local supermarket and the non-fat dry cow's milk (blotting grade blocker) was obtained from Bio-Rad (Veenendaal, the Netherlands). Raw milk samples of 5 individual buffalos were obtained from the farm De Stoerderij (Son en Breugel, the Netherlands). Raw milk samples from 5 camels were obtained from the farm Kamelenmelkerij Smits (Berlicum, the Netherlands). Raw milk samples from 5 individual sheep were obtained from Dr. S.E. Kakabakos INRASTES, Immunoassay-Immunosensors Lab (Athens, Greece).

Milk sample preparation

Depending on the required sensitivity, milk samples were analysed after a 1:100 or 1:1000 dilution. First, the milk sample was homogenised at room temperature after which 100 µl was pipetted into a tube to which 900 µl of sample dilution buffer was added (1:10 diluted sample). After homogenising on a vortex for 2 sec, 100 µl of the 1:10 diluted sample was pipetted into a tube and 900 µl sample dilution buffer was added and homogenised on a vortex for 2 sec (1:100 diluted sample). Another 10 times dilution was applied to obtain a 1:1000 diluted sample. Milk powders, after the preparation of milk (1 g of milk powder + 9 ml of water), were prepared in the same way.

ELISA procedure

Samples or standard solutions were pipetted (50 µl) in duplicate in the wells of the ready-to-use microtiter plate, followed by the addition of 50 µl of the diluted Mab-HRP conjugate. After sealing the plate and a short mixing step of 5 seconds on the microtiter plate shaker, the plate was incubated for 1 hour in the dark at room temperature. Then the plate was washed three times using the microtiter plate washer after which 100 µl of substrate solution was added to all wells. After an incubation of 30 minutes in the dark at room temperature, 100 µl of stop solution was added to all wells and the absorbance values were measured immediately (within 10 min) at 450 nm.

Results and Discussion

ELISA

Zachar et al. [6] described that caseins are more stable under high temperature conditions than whey proteins, but the immunogenicity of whey proteins is better. On the contrary, we experienced that after immunizing mice with a mixture of β-lactoglobulin and κ-casein only Mabs against the latter were obtained [10]. Their application in a biosensor [10], an ELISA [14] and a lateral-flow test strip [15] resulted in sensitive immunoassays and one of them (Mab 4G10) performed the best with a limit of detection (LOD) below 1 µg/ml of bovine κ-casein. Therefore, Mab 4G10 was selected for the development of the present indirect ELISA. Previously [14], the applied bovine κ-casein-coated plates were stored at -20°C with a shelf life of maximum 1 month and they had to be washed prior to use. The new ELISA kit contains a ready-to-use coated plate with a shelf life of 6 months and the combination with the ready-to-use bovine κ-casein calibration standards and the HRP-conjugated Mab, makes the assay much easier to perform with only four pipet handlings, one wash step and two incubation steps of 1 and 0.5 h. In comparison, the indirect competitive ELISA for bovine IgG in the milk of other species required seven pipet handlings, five wash steps and five incubation periods from overnight (ON) till 1 h [8] and the indirect ELISA for bovine β-casein [9] required six pipet handlings, five incubation periods (ON till 20 min) and three wash steps. The calibration curve in buffer is similar to the one in the previous assay [14], with a measurement range between 0.1 and 2.5 µg of bovine κ-casein per ml. This is more sensitive than the biosensor immunoassay using the same Mab [10] and the IgG ELISA with a limit of detection of 1 µg/ml [8].

Specificity

Since recently we know that Mab 4G10 recognises a 5 amino acid-containing epitope on the GMP part of bovine κ-casein. This epitope is not present in the other caseins and the previously described cross-reactivities with β-, γ and α-casein (25, 7 and 1.5%, respectively [10]) must be due to impurities in the applied casein standards. This epitope is also absent in the κ-casein of goat, sheep, horse, donkey, camel, etc. but present in κ-casein of buffalo. The specificity of the test is shown in Figure 2A where the responses obtained with 1:100 diluted milk of different sources are presented. Raw cow's milk, heat-treated (pasteurized and UHT) cow's milk, cow's milk powder and raw buffalo's milk gave almost no responses (full inhibitions) which proves that the ELISA is specific for cow's and buffalo's milk.

Maximum responses were obtained with whole goat's milk, sheep's milk, camel's milk and milk of soy, almond, rice and oats which proves that the ELISA can be applied for the detection of cow's and buffalo's milk in milk of these sources. In Figure 2B, the responses obtained after analysing 1:100 000 diluted cow's and buffalo's milk samples show that small differences are observed between raw and pasteurized milk and cow's milkpowder but with higher inhibitions for the heat-treated milk samples. This proves that the ELISA suites for the detection of heat-treated cow's milk which is a big advantage because, at present, there is only a small number of ELISA tests with sufficient sensitivity for the detection of additives in heat-treated milk [6].The results obtained with the four buffalo's milk samples also show small differences which might be caused by differences in the indvidual κ-casein concentrations. Overall, the average response obtained with the cow's milk samples (41 ± 7%) was higher compared with the average response obtained with buffalo's milk (35 ± 5%) which means that higher concentrations of κ-casein are present in buffalo's milk which has been described previously (2 and 3 g/L for cow's and buffalo's milk, respectively [16,17]).

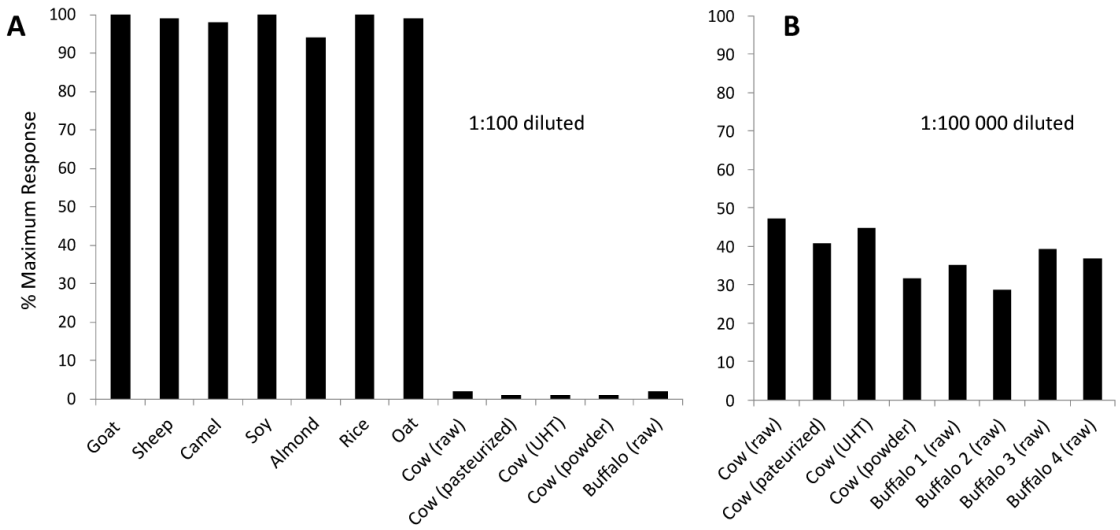

Figure 2: Percentages of the maximum response obtained with A) 1:100 diluted milk samples of a selection of different species and sources and B) 1:100 000 diluted milk of cows (raw and heat-treated) and raw buffalo's milk samples obtained from four different buffalos

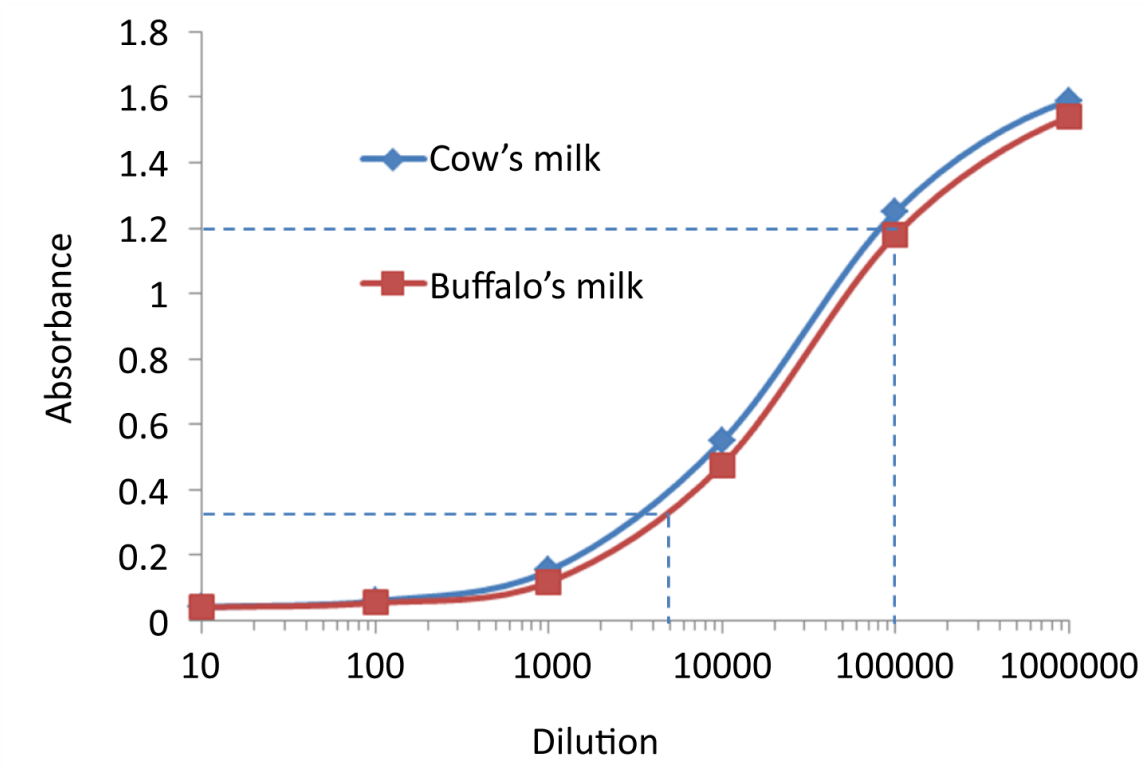

Figure 3: Absorbance values obtained in the ELISA with serially diluted raw cow's tank milk and raw buffalo's milk (mixture of milk from five buffalos)

Sensitivity

Measuring serial dilutions of raw cow's tank milk as well as of raw buffalo's milk (mixture of milk from 5 buffalos), the results for cow and buffalo are similar (Figure 3). Overall, some more inhibition is observed with buffalo's milk compared to cow's milk. Maximum responses were obtained when both milk samples were diluted 1:1 000 000 and higher. Significant response decreases (20%) were seen with

1:100 000 dilutions corresponding with approximately 0.1% of cow's and/or buffalo's milk in 1:100 and 1% in 1:1000 times diluted milk samples of other species. Almost full inhibition (80%) was observed with a dilution of 1:5000 corresponding to 2% and 20% of cows or buffalo's milk in 1:100 and 1:1000 diluted samples, respectively.

So, the two least expensive milk species [1] can be detected in the more expensive milk of other species and sources. For the detection of cow's milk in buffalo's milk, another Mab (6A10) is available [10]. The epitope of Mab 4G10 is located on the GMP part of κ-casein and during cheese making, this GMP part ends up into the whey. That is the reason why Mab 4G10 can be used for the detection of the GMP-containing whey powder in milk powder [14]. Consequently, this cow's and buffalo's milk ELISA is not suitable for the detection of cow's or buffalo's milk in cheese. For that, another Mab is available with its epitope on the para-κ-casein part of the κ-casein. For the detection of fraudulent additions, we have chosen to apply 1:100 or 1:1000 diluted milk samples in the test, which requires no extra sample preparation other than homogenization. In the previously developed biosensor with the same Mab [10], the samples were diluted 20 times only to obtain a calibration in the range of 0.1 to 1 % cow's milk in ewe's milk which confirms the better sensitivity of the new ELISA.

Milk samples 1:100 diluted

Samples of a serial dilution of pasteurized cow's milk in the milk prepared from goat's milk powder (1 g + 9 ml water) from 50% to 0.001% cow's milk, were analysed (after 1:100 dilution) and the absorbance values were used to calculate the corresponding concentrations (µg/ml) of bovine κ-casein by means of the calibration curve. As shown in Figure 4, the relationship is linear from 0.1 up to 1.2 µg/ml of bovine κ-casein and from 0.25% to 2.5% of cow's milk, with a slope of 2.5 (1 µg/ml of κ-casein compares with 2.5% of cow's milk). The limit of detection (LOD) with 1:100 diluted milk samples lies below the lowest calibration point (at 85% of the maximum response (15% inhibition)) and is about 0.05% of cow's milk. The decision level (DL) is set at the lowest calibration standard of 0.1 µg/ml which compares with 0.25% of cow's and/or buffalo's milk in the milk of other species and sources.

Therefore, for qualitative interpretations, milk samples (1:100 diluted) giving absorbance values higher than the average absorbance value obtained with the lowest standard (0.1 µg/ml) are considered as negative (no cow's and/or buffalo's milk present (<0.25%)), whereas milk samples (1:100 diluted) giving absorbance values lower than the absorbance value obtained with the lowest standard (0.1 µg/ml) are considered as positive (cow's and/or buffalo's milk present (>0.25%)). The lower the absorbance value of the sample, the higher the concentration of cow's and/or buffalo's milk in the sample. Above the 1 µg/ml of κ-casein, the relationship with the percentage of cow's milk is non-linear due to the saturation of the Mabs with the high amounts of κ-casein.

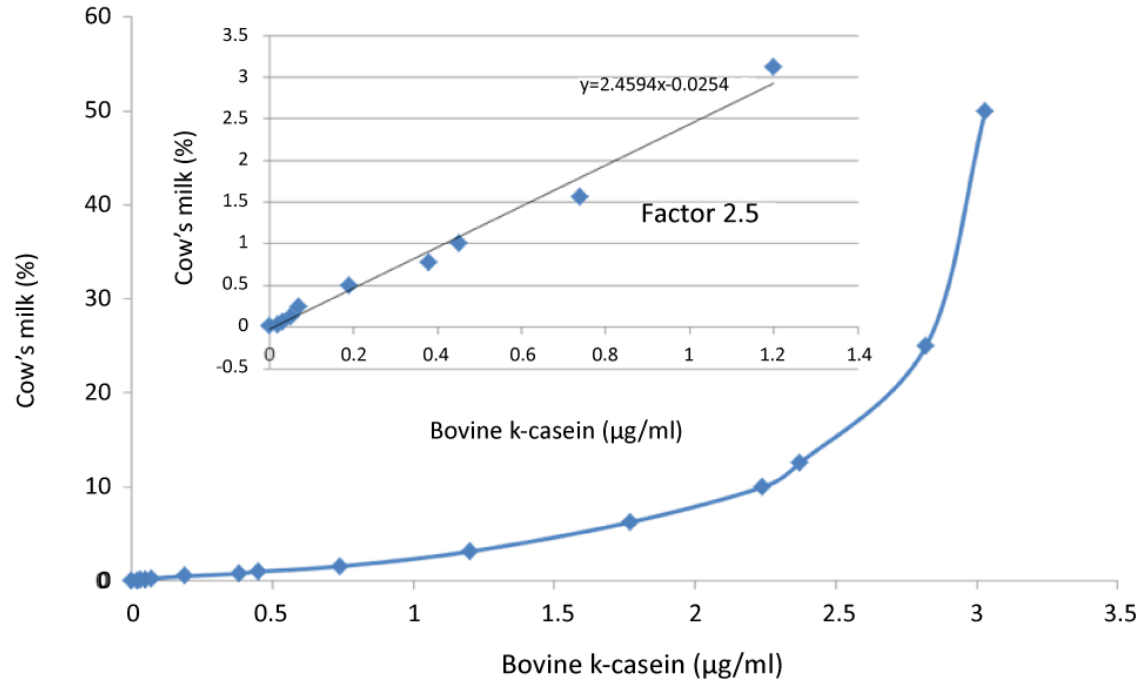

Figure 4: Non-linear relationship between the high percentages of cow's milk (>2.5%) and the calculated concentrations of bovine κ-casein and linear relationship (insert) at low concentrations of cow's milk (<2.5%) in 1:100 diluted goat's milk

The differences between the absorbance values obtained with 1:100 diluted blank raw milk samples of 5 different sheep (1.44 ± 0.11) and 5 different camels (1.40 ± 0.05) and those from the same samples spiked with 0.25% of raw cow's milk (1.01 ± 0.03 for sheep and 1.06 ± 0.04 for camel) clearly show that there is no data overlap between the blanks and spikes at the proposed DL of 0.25% cow's milk with 1:100 diluted samples.

Milk samples 1:1000 diluted

For quantitative interpretations above 1 µg/ml of κ-casein or 2.5% of cow's or buffalo's milk, an extra 10 times' dilution (1:1000 diluted milk) needs to be applied. The relationship is then linear (Figure 5) over a broad range from 2% up to 50% cow's milk with a slope of 17 (1 µg/ml is 17% cow's milk).

The differences between the absorbance values obtained with 1:1000 diluted blank raw milk samples of 5 different sheep (1.34 ± 0.04) and 5 different camels (1.34 ± 0.03) and those from the same samples spiked with 2% of raw cow's milk (0.86 ± 0.09 for sheep and 0.92 ± 0.02 for camel) clearly show that there is no data overlap between the blanks and spikes at the proposed DL of 2% cow's milk with 1:1000 diluted samples.

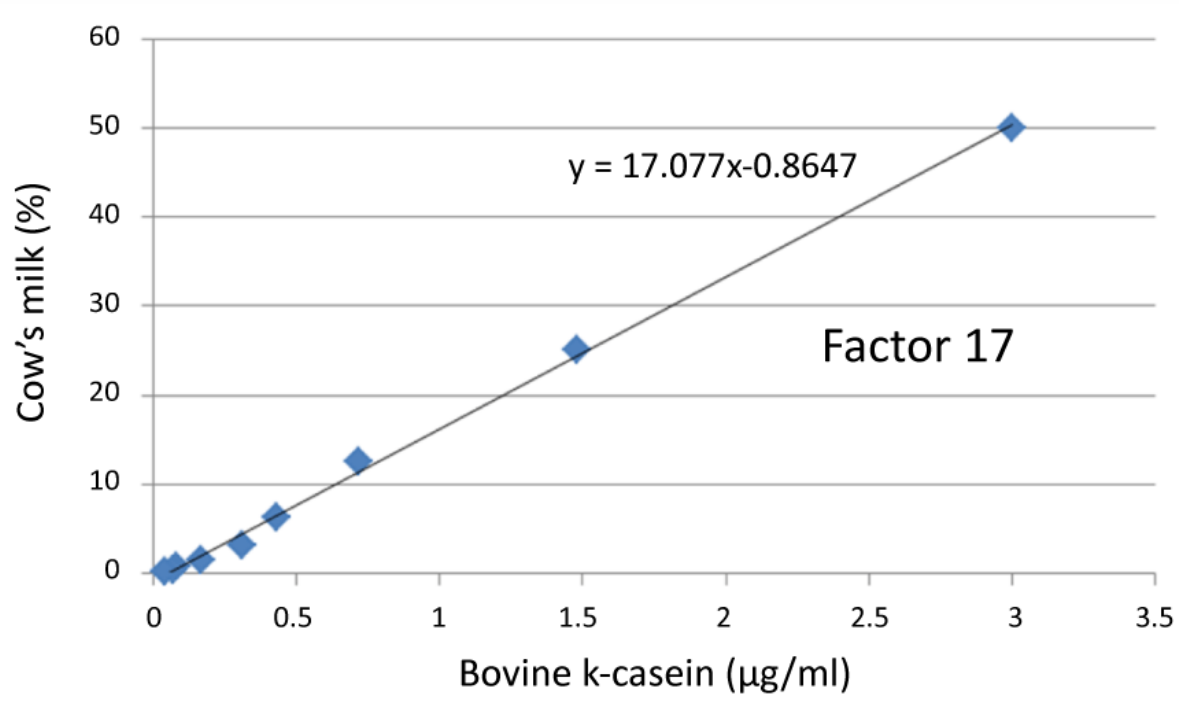

Figure 5: Linear relationship between the amount of calculated bovine κ-casein and the percentage of cow's milk in 1:1000 diluted milk samples of other species or sources

Conclusions

An indirect ELISA, applying a Mab recognizing a 5 amino acids-containing epitope on the GMP part of bovine κ-casein, is developed in a 96-wells microtiter plate format for the detection of raw and heat-treated cows and buffalo's milk in milk of other species and sources. The measurement range of the calibration standard (bovine κ-casein) is from 0.1 to 2.5 µg/ml and the limit of detection is 0.05 µg/ml. Using 1:100 diluted milk of other species and sources, the decision level is 0.1 µg/ml of κ-casein which compares with 0.25% of cow's and/or buffalo's milk and the measurements range is linear till 2.5% of cow's and buffalo's milk. Using 1:1000 diluted milk, this range is from 2 till 50% cow's and buffalo's milk. Because the Mabs epitope is on the GMP part of κ-casein, which ends up into the whey, the test is not suitable for the detection of cow's and/or buffalo's milk in cheese of other species. For this application another Mab is available as well as for the detection of cows milk in the milk of buffalos. Due to differences in the concentrations of κ-casein in the milk of different cows and buffalos, the calculated percentages of cow's and/or buffalo's milk are indicative but that counts for all immunoassays where marker milk proteins are used.

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References

1. Gerosa S, Skoet J (2012) Milk availability – Trends in production and demand and medium-term outlook. ESA Working paper no. 12-01.
2. Barlowska J, Szwaikowska M, Litwińczuk Z, Krol J (2011) Nutritional value and technological suitability of milk from various animal species used for dairy production. Comprehensive Reviews in Food Science and Food Safety, 10, 291-302.
3. Rathore M, Sharma M, Mahich M (2011) Camel and donkey milk based nutritive powder: a cheaper alternative of human milk. Internation Journal of Pharmaceutical Studies and Research. II (IV), 20-22.
4. Bahna SL (2002) Cow's milk allergy versus cow milk intolerance. Ann Allergy Asthma Immunol., 89(6 Suppl 1):56-60.
5. Sheehan, WJ, Gardynski A, Phipatanakul W. (2009) Skin testing with water buffalo's milk in children with cow's milk allergy. Pediatr. Asthma Allergy Immunol. 22(3), 121-125.

6. Zachar P, Šoltés M, Kasarda R, Novotny J, Novikmecová M, et al. (2011) Analytical methods for the species identification of milk and milk products. Mljekarstvo 61 (3) 199-207.
7. Abdel-Rahman SM, Ahmed MMM (2007) Rapid and sensitive identification of buffalo's, cattle's and sheep's milk using species-specific PCR and PCR-RFLP techniques. Food Control 18, 1246-1249.
8. Hurley IP, Coleman RC, Ireland HE, Williams JHH (2004) Measurement of bovine IgG b indirect competitive ELISA as a means of detecting milk adulteration. J. Dairy Sci. 87, 543-549.
9. Song H, Xue H, Han Y (2011) Detection of cow's milk in Shaanxi goat's milk with an ELISA assay. Food Control 22, 883-887.
10. Haasnoot W, Smits NG, Kemmers-Voncken AE, Bremer MG (2004) Fast biosensor immunoassays for the detection of cows' milk in the milk of ewes and goats. J Dairy Res. 2004 Aug;71(3):322-9.
11. Borkova M, Snaselova J (2005) Possibilities of different animal milk detection in milk and dairy products – a review. Czech J. Food Sci. 23 (2), 41-50.
12. Dalgleish DG, Corredig M (2012) The structure of the casein micelle of milk and its changes during processing. Annu. Rev. Food Sci. Technol. 3: 449-467.
13. Haasnoot, W., Marchesini, G. and Koopal, K. (2006) Spreeta-based biosensor immunoassays to detect fraudulent adulteration in milk and milk powder. J. AOAC International, Vol. 89, No. 3, 849-855.
14. Bremer MGEG, Kemmers-Voncken AEM, Boers EAM, Frankhuizen R, Haasnoot W (2008) Enzyme-linked immunosorbent assay for the detection of bovine rennet whey powder in milk powder and buttermilk powder. International Dairy Journal 18, 294-302.
15. Martín-Hernández C, Muñoz M, Daury C, Weymuth H, Kemmers-Voncken AEM, et al. (2009) Immunochromatographic lateral-flow test strip for the rapid detection of added bovine rennet whey in milk and milk powder. International Dairy Journal 19, 205-208.
16. Ahmad S, Anjum FM, Huma N, Sameen A, Zahoor T (2013) Composition and physic-chemical characteristics of buffalo milk with particular emphasis on lipids, proteins, minerals, enzymes and vitamins. The Journal of Animal and Plant Sciences, 23 (1 Suppl.), 62-74.
17. Fox PF (2008) Chapter 1 Milk: an overview in Milk Proteins. From Expression to Food. Elsevier Inc., Eds. Thompson A, Boland M, Singh H. Food Science and Technology, 1-54.