POLISH JOURNAL OF FOOD AND NUTRITION SCIENCES
Pol. J. Food Nutr. Sci. 2006, Vol. 15/56, SI 1, pp. 203–207

INVESTIGATION OF PROTEINS IN EWE, GOAT AND COW CHEESES BY GEL ISOELECTRIC FOCUSING

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Key words: IEF of cheese proteins and peptides, species identification, cheese adulteration

A method is described for the determination of bovine milk in cheeses by polyacrylamide gel isoelectric focusing according to EC reference method on ready-to-use gel plates. The advantages of this method are easy to use handling and good reproducibility by application of ampholytes with high concentration of urea. The method includes (i) recovery of cheese proteins fat and salt free; (ii) hydrolysis of cheese proteins with plasmin; (iii) separation by isoelectric focusing at pH 3–10 of bovine, ovine, caprine and buffalo γ-caseins according to their different isoelectric points and the densitometric evaluation of the electrophoretic bands. With the modification of the reference method the addition of cow milk to samples of goat and goat/ewe milk cheeses was proven.

INTRODUCTION

When ewe, goat or buffalo milks are blended with cow milk the resultant mixture is not obviously different from pure milk, especially if the level of addition is below 15%. This substitution can however become a serious problem in cheese manufacture as the composition of milk influences the organoleptic characteristics of the final product. Consumption of products containing undeclared milk may cause allergies in sensitised individuals [Miller, 1987]. Investigation of species origin of milk used in cheese manufacture is important in cheeses made from ewe, goat or buffalo milk, which are exported to or traded in European Union countries where product authenticity must be assured. Useful indices for identification of adulterations of ewe, goat and buffalo milk with cow milk are ratios of short chain fatty acids (especially C_{12}/C_{10}, C_{14}/C_{10} and C_{16}/C_{10}) or determination of ash constituents, investigation of β-carotene content or introduction of riboflavin-derived coenzyme of xanthine oxidase from cow milk into ewe milk and enzymatic conversion of formaldehyde to formic acid [Singhal *et al.*, 1997].

Nevertheless the investigations of possible adulteration are focused on the composition of proteins or peptides in the mixed milk and milk products.

A number of methods using, *e.g.* polyacrylamide gel electrophoresis [Furtado, 1983; Mayer & Hortner, 1992; Mayer, 2005; Molina *et al.*, 1995; Veloso *et al.*, 2002], capillary electrophoresis [Cartoni *et al.*, 1999; Herrero-Martinez *et al.*, 2000; Molina *et al.*, 2000], isoelectric focusing [Addeo *et al.*, 1990, 1995, Krause *et al.*, 1982, 1989], HPLC [Veloso *et al.*, 2002, Moatsou *et al.*, 2003], hydrophobic interaction chromatography [Bramanti *et al.*, 2003], immunological methods [Levieux, 1980; Elbertzhagen, 1987] especially a method of competitive enzyme immunoassay using monoclonal antibodies specific to bovine γ-caseins ELISA [Mayer & Mayr, 2005] and polymerase chain reaction (PCR) using species specific primers [Bottero *et al.*, 2003; Mayer & Mayr, 2005] were performed for species identification in dairy products.

There is an EEC reference method [Commission Regulation (EC) No 213/2001] and German standard [DIN 10469] to investigate and evaluate bovine caseins in products made from ewe, goat or buffalo milk.

In this paper a qualitative and quantitative procedure to detect cow milk caseins in commercial products from goat, ewe and buffalo milk by gel isoelectric focusing of peptides by using Blank Precotes® and Servalyte® 3–10 system is described.

MATERIAL AND METHODS

Cheese samples. The full ripened ewe, goat, mixes of both, buffalo and cow milk cheeses were taken from the supermarket in Berlin and stored in refrigerator for analysis.

Isolation of cheese proteins. The amount equivalent to 5 g dry mass of cheese was weighed into a 100-mL centrifuge tube. After adding 60 mL of distilled water the mixture was homogenised with a rod homogeniser (IKA Labortechnik) at 8 000 to 10 000 rpm. The pH was adjusted to 4.6 with

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diluted acetic acid and the suspension was centrifuged (5 min, 3 000 g). The fat and whey were decanted and the residue was homogenised at 20 000 rpm in 40 mL distilled water adjusted to pH 4.5 with diluted acetic acid. After adding 20 mL of dichloromethane the homogenisation was repeated again and centrifugation was carried out with Sigma 3 K-1 equipment (5 min, 3 000 g). The aqueous and organic phases were decanted and the casein layer that lies between both phases was removed, rehomogenised in 40 mL of distilled water and 20 mL of dichloromethane and centrifuged. This procedure was repeated until both extraction phases were colourless (two to three times). At last the protein residue was homogenised with 50 mL of acetone, filtered through a medium-speed folded filter paper and washed with two separate 25 mL portions of acetone each time and allowed to dry in a stream of nitrogen, then finely pulverised in a mortar and stored at -20°C.

Plasmin cleavage of β-caseins to intensify γ-caseins. 25 mg of isolated caseins were dispersed in 0.5 mL of 0.2 mol/L ammonium carbonate buffer with 0.05 mol/L EDTA, pH 8 and homogenised in a 1.5 mL Eppendorf-Vial for 20 min by using *e.g.* ultrasonic treatment. The sample was heated to 40°C for 5 min and 50 μL of plasmin (EC 3.4.21.7) were added. After mixing the sample was incubated for one hour at 40°C with continuous shaking. To inhibit the enzyme 50 μL of α-aminocaproic acid solution were added, and the solution was placed in an ice bath, for a few minutes, to cool at 4°C. 50 μL of 1 g/mL (100%) trichloroacetic acid was carefully added to this solution (500 μL), the release of CO_2-bubbles was awaited, then it was closed and – after mixing gently – the vial was carefully reopened again to reduce pressure built up by further release of CO_2. This step was repeated once and finally the vial was left on the ice bath for 5 min to allow complete precipitation of protein. Afterwards, the protein was recovered by centrifugation (14000 rpm, 5 min). The supernatant was removed thoroughly and the precipitated protein was shortly washed in the vial with 200 μL of bidest. H_2O. After additional centrifugation (14000 rpm, 1 min), the washing liquid was removed and the residue was dissolved in 0.5 mL of protein dissolving buffer (dissolve 3.45 g of glycerol (87% w/w), 14.42 g urea ultrapure (ICN or Merck) and 150 mg dithiothreitol (ICN) in bidest. H_2O and fill up to 30 mL).

Isoelectric focusing. The procedures of polyacrylamide gel isoelectric focusing (PAGIF) was carried out as described in the EU-Reference Method [Commission Regulation (EC) No 213/2001, Annex XV] with the exception of using blank Precotes® (SERVA Electrophoresis GmbH) 245×125 mm, 0.3 mm, 5% T, 3% C ready-to-use, onto Gel-Fix support film precasted gels after equilibration at room temperature with carrier ampholytes blends solution. The equilibration solution for denaturing runs was obtained by mixing of 36 g of urea, 6 mL of Servalyt® 3–10, 0.75 mL of Servalyt® 5–7, 7.5 mL of glycerol (99%) and supplemented with bidest water up to the volume of 75 mL. The equilibration time for the plate 245×125 mm at room temperature was 50 min. Isoelectric focusing was carried out using MULTIPHOR II flat-bed equipment with an ECPS 3000/150 power supply (Amersham Biosciences). Few drops of Bayol (SERVA Electrophoresis GmbH) were dripped onto the centre of the cooling block and spread over the surface. The reverse of the gel carrier sheet was wiped with Bayol too. The cooling thermostat was adjusted to 12°C–15°C. After rehydration, the ready-to-use gel was placed with the carrier sheet side onto the centre of the cooling block by avoiding trapping of air bubbles. The electrode strips were cut to gel length, soaked with the electrode solutions (anode: 1 mol/L of phosphoric acid and cathode: 1 mol/L sodium hydroxide) and placed in the position provided (9.5 cm distance of electrodes).

After 30 min of pre-focusing, 18 μL of sample or standard solution was pipetted onto the paper applicator piece (5 x 5 mm), 17 sample applicator pieces were placed onto the gel at 5 mm intervals from each other and from the end of the gel too and 5 mm in longitudinal direction from the anode, and lightly pressed. The sample focusing was carried out for 60 min, then the sample pieces were carefully removed (without damaging the gel surface) and focusing was then continued with step 3 (final focusing) of Table 1.

TABLE 1. Denaturing run limited by current and power for the gel size 245 mm×125 mm and the separation distance of 9.5 cm.

Step	Time (min)	Voltage (V)	Current (mA)	Power (W)
Pre-focusing	30	2 000 max.	14 max.	4 constant
Sample focusing	60	2 000 max.	9.5 max.	13 max.
Final focusing	120	2 000 max.	6.5 max.	15 max.

Protein fixation. Immediately after turning the power off the electrode strips were removed and the gel was transferred into staining/destaining container filled with 200 mL of 15% TCA and held for 15 min by continuously shaking. After this time the fixative was drained off and the gel plate was short washed twice each time with 100 mL of destaining solution (500 mL of ethanol and 200 mL of glacial acetic acid was diluted to 2000 mL with distilled water).

Staining and destaining. After removing the wash solution the container was filled with 250 mL of staining solution and the gel plate was stained for 45 min with gentle shaking. The staining solution contains Coomassie Brilliant Blue G-250 (3.0 g of Coomassie Brilliant Blue G-250 (C.I. 42655) in 1000 mL of 90% (v/v) ethanol) and copper sulphate (5.0 g of copper sulphate pentahydrate in 1000 mL of 20 % (v/v) acetic acid) mixed together (1:1) prior to staining. The stained gels were washed twice using a 100 mL destaining solution each time, then shaken with 200 mL destaining solution for 15 min and the destaining step was repeated at least two or three times until the background was clear and uncoloured. The gel plate was rinsed with distilled water (2 x 2 min) and dried in the air (for 2 to 3 h) or with a hair dryer (for 10 to 15 min).

Densitometric quantitation. Densitometric evaluation of dried gels was performed with LAS 3000 image analyser (Fujifilm) with 3.2 mega pixels CCD camera with white light source trans illuminator for gel documentation by exposure

time of 250 msec. The system functions of the LAS-3000 were controlled remotely through Windows® software application Aida Dev. Ver. 4.10.016 *via* a standard USB interface. The relative intensity integrals were calculated as the percentage of the sum of integrated peaks.

RESULTS AND DISCUSSION

Compared with gels of "cast-in" ampholytes and urea [Commission Regulation of EC No 213/2001] the use of precasted gels is advantageous for the reproducibility and easy handling. The important benefit of the using almost empty precasted gels is the option of adding a high concentration of urea and ampholytes to the equilibration mixture without the danger of disturbing the gel polymerisation process. Gel after equilibration can be stored with incorporated urea up to 2 weeks. The used IEF method with blank Precotes in the pH range between 3 and 10 pH is especially suitable for investigation of complex samples, the most important pH range for separation of γ-caseins between 5 and 7 pH can be supported through addition of secondary ampholytes but it is not obligatory for good results (results not shown).

Within a broad pH range it is possible to distinguish para-κ-caseins of ewes', goats' and cows' milk as an additional parameter of species identification too [Mayer, 2005]. The use of restricted pH range from 5 to 8 is advantageous in extending the distance between the γ-caseins [Addeo *et al.*, 1990]. In not proteolysed milk products, bovine α_{s1}-casein can be used as a marker for added cow milk to ewes' and goats' milk. In the alkaline urea-polyacrylamide gel electrophoresis it is the fastest moved protein band [Sienkiewicz *et al.*, 1994]. However, in ripened cheeses α_{s1}-casein is partially degraded and the casein hydrolysis products show an electrophoretic mobility similar to that of bovine α_{s1}-casein [Mayer, 2005].

Figure 1 shows the results of the PAGIF separation of ovine cheese with the addition of increased quantity of cow milk caseins. The measurements of pH on the urea-gel surface show for bovine-γ_3-casein pI 6.5, for bovine-γ_2-casein pI 7.0, for ovine-γ_3-casein pI 6.7, for ovine-γ_2-casein pI 7.2 value respectively.

Figure 2 shows the separation plate of plasmin hydrolysed ovine cheese protein with added 25% cow casein (lane A), different commercial cheese samples from milk of ewe, goat and the mix of both, two of them (lane G and I) contain bovine caseins (bands no. 2 and 4) respectively. Standard β-casein sample after plasmolysis was separated as lane L.

The bovine β-casein standard (Figure 2 lane L) shows, after 60-min hydrolysis at pH 8.0, no more native β-casein, the predominant stable peptide fractions were γ_2-casein (β-CN f106-209) and γ_3-casein (β-CN (f108-209) with 26.8% and 26.1% of the total content of detected peptides, respectively. Plasmin is a serine proteinase which is optimally active at about pH 7.5 and 37°C, and is highly specific for peptide bonds on the C-terminal side of lysyl, and to a lesser extent, arginyl residues [Weinstein & Doolittle, 1972]. It is particularly active on α_{s2}- and β-caseins. Hydrolysis of the β-caseins at the Lys_{105}-His_{106} or Lys_{105}-Gln_{106} bond leads to the formation of γ_2-casein (C-terminal fragment) and proteose-peptone PP5 (N-terminal fragment). After hydrolysis of the Lys_{107}-Glu_{108} bond in the β-caseins are formed γ_3-casein and proteose-peptone PP8 fast (β-CN f1-28) and PP8 slow (β-CN f29-107). The third of the γ-caseins is γ_1-casein (β-CN (f29-209). It is formed after hydrolysis of Lys_{28}-Lys_{29} bond of β-casein but is no very stable and in the course of prolonged action of plasmin, it is degraded to γ_3-casein and PP8 slow. The γ_2-casein contains two positively charged amino acids more as γ_3-casein and a higher isoelectric point (Figure 1).

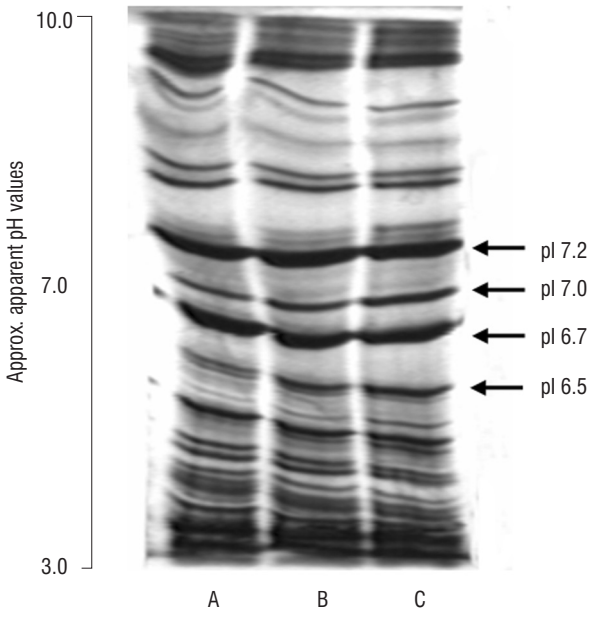

FIGURE 1. Thin layer gel isoelectric focusing of the plasmin hydrolysed ovine cheese proteins with A=10%, B=15% and C=25% of cow casein.
pI 7.2 = ovine-γ_2-casein; pI 7.0 = bovine-γ_2-casein; pI 6.7 = ovine-γ_3--casein; pI 6.5 = bovine-γ_3-casein

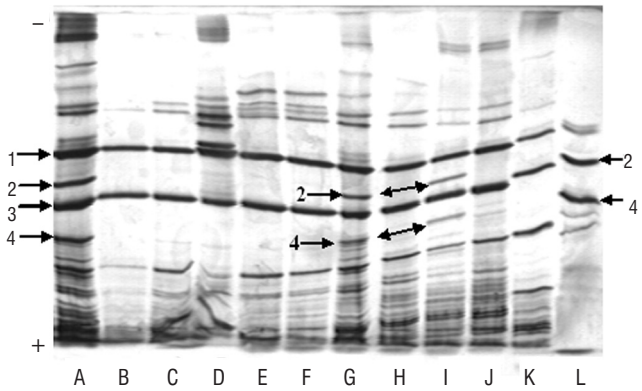

FIGURE 2. Thin layer gel isoelectric focusing of the plasmin hydrolysed cheeses proteins of commercial samples. A: ewe cheese with 25% bovine caseins; B–D: commercial ewe cheese samples not falsificated; E, F, H: commercial goat cheese samples not adulterated; G: commercial sample declared as goat milk cheese indicated cow milk; I: commercial sample declared as goat and ewe mix cheese indicated cow milk; J: commercial cheese sample declared as goat and ewe, not adulterated; K: commercial pure buffalo milk cheese; L: plasmin degradation products of bovine β-casein.
1 ovine-, caprine- und buffalo-γ_2-casein, 2 bovine-γ_2-casein, 3 ovine-, caprine- und buffalo-γ_3-casein, 4 bovine-γ_3-casein

Defatted and plasmolysed Tilsit cheese sample from bovine milk after longer ripening time contains 16.8 % and 17.8 % of γ_2-caseins and γ_3-caseins respectively (Figure 3).

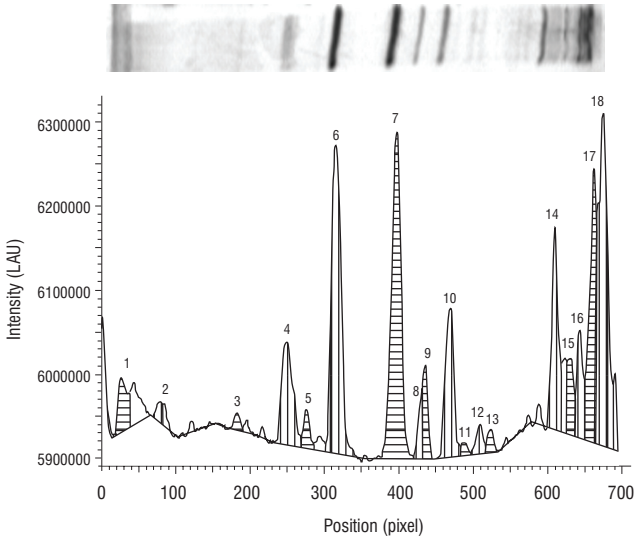

FIGURE 3. Densitometric analysis of plasmolysed Tilsit cheese caseins. Peak 6 is bovine-γ_2-casein and peak 7 is bovine-γ_3-casein.

CONCLUSIONS

On IEF plates with Servalyte® 3–10 with 8 mol incorporated urea the cheese samples made from cow milk showed clear γ_3- and γ_2-bands on pH points 6.5 and 7.0. The use of blank Precotes® with the described equilibration procedure is easy to do and advantageous for exact repeatability of separation results. The γ_3- and γ_2-bands of ewe, goat and buffalo milk cheeses (nearly at pH 6.7 and 7.2 respectively) are in clear different positions as γ_3- and γ_2-bands in cow milk products. The clear visualization of γ-casein bands in fermented milk products (*e.g.* cheeses) is well possible only after enhancing the content of these bands with plasmin. Any other overlapped peptide bands nearly on the same position disappeared as plasmin degraded that. From the position of cow milk γ_3- and γ_2-bands in ewe, goat and buffalo milk products it is possible to quantify the addition of cow milk to these products. It is important to control the content of labeled milk products. In the set of investigated samples there are 2 products with the addition of cow milk proteins from the producer, who labeled his product as pure goat milk product or as pure mix from ewe and goat milk. The positions of buffalo γ-caseins bands are slightly different from ewe and goat γ-caseins and distinctly different in position on IEF plates from cow milk γ-casein bands.

ACKNOWLEDGEMENTS

This study was supported by the Erciyes University Scientific Research Project Unit and Berlin Technical University Department of Food Chemistry. Thanks are due to chemist Mrs. Marina Konter, Research Lab. SERVA Electrophoresis GmbH, Heidelberg, Germany for the test of pH development on blank Precotes® with urea and for supplying Servalytes®.

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