Immunoreactivity of Goat's Milk Casein Fractionated by Ion-Exchange Chromatography

Ana I. Haza, Paloma Morales, Rosario Martín, Teresa García, Gonzalo Anguita, Isabel González, Bernabé Sanz, and Pablo E. Hernández*

Departamento de Nutrición y Bromatología III, Facultad de Veterinaria, Universidad Complutense, 28040 Madrid, Spain

Goat's milk casein was fractionated by anion-exchange fast protein liquid chromatography (FPLC) on a Mono Q HR 5/5 column and by cation-exchange chromatography on a column containing an S-Sepharose Fast Flow matrix. The identification of the resulting fractions was performed by SDS–PAGE using a PhastSystem electrophoresis unit. Three fractions (β-casein, κ-casein, and α_s-casein) were separated by anion-exchange FPLC, while cation-exchange chromatography allowed the identification of four fractions (β-casein, κ-casein, α_{s1}-casein, and α_{s2}-casein). The purified fractions were characterized immunologically by an indirect ELISA using polyclonal antibodies raised in rabbits against goat's casein and further purified by affinity chromatography in a column containing immobilized goat's caseins, biotinylated and blocked with lyophilized ovine and bovine caseins. The most immunoreactive caprine casein fraction was the α_{s2}-casein purified by cation-exchange chromatography, meaning this is the fraction with the largest number of epitopes specific of goat's milk casein.

Keywords: *Goat's milk casein; immunoreactivity of caprine caseins; anion-exchange FPLC; cation-exchange chromatography; enzyme-linked immunosorbent assay (ELISA)*

INTRODUCTION

The production of goat's milk has gained considerable economic importance in the past few years. European goat breeding has a strong dairy specialization; with 3% of the world goat population, Europe produces 17% of the world goat's milk. This production is mainly found in southern countries of Europe, with Greece, Spain, and France producing 86% of the milk in the European Union (Le Jaouen and Toussaint, 1993). Goat's milk is mainly transformed into cheeses. In Spain the total milk output has increased considerably over the past 10 years, rising from 290 million liters in 1980 to 473 million liters in 1990 (Ramos and Juárez, 1993).

The amino acid composition and sequence of the major caprine caseins, β, κ, α_{s1}, and α_{s2}, have been determined, and their similarity to bovine caseins has been established (Richardson and Creamer, 1974, 1975; Mercier et al., 1976; Brignon et al., 1989). The caprine caseins have been examined by electrophoresis, and genetic variants have been found (Boulanger et al., 1984; Di Luccia 1986; Addeo et al., 1988; Moio et al., 1989; Chianese et al., 1992). Furthermore, ion-exchange chromatography has been used successfully to separate and quantify individual casein fractions (Mikkelsen et al., 1987; Jaubert and Martín, 1992; Kaminarides and Anifantakis, 1993; Law and Tziboula, 1992).

Milk species identification represents a considerable problem for the food analyst and in many parts of the world also for law enforcement authorities. Accordingly, there is a need to develop good analytical procedures to combat the increasing problems of adulteration of expensive milk with cheaper milk intended for use in cheese manufacture.

* Author to whom correspondence should be addressed (fax +34-1-3943743; e-mail ehernan@eucmvx.sim.ucm.es).

Nonimmunological and immunological methods have been described for the identification of milk from closely related species (Ramos and Juárez, 1984). Nonimmunological methods are not convenient for routine analysis of milk samples since they are relatively costly and time-consuming and require technical expertise (Levieux and Venien, 1994). Immunoassays are considered an excellent replacement for the conventional analytical methods applied to milk species identification. The enzyme-linked immunosorbent assay (ELISA) is the most widely used immunoassay in food analysis, being low in cost and fast to operate; it is also easy to use, reliable, and readily automated (Allen, 1990).

Immunological methods require the production of specific antisera with high titers. The development of hybridoma technology (Köhler and Milstein, 1975) has provided the means for continuous production of monospecific antibodies of known biological activity and consistent specificity. Although hybridoma technology does not necessarily require highly purified antigens, selected antigens should produce a higher yield of monoclonal antibodies of interest. To obtain monoclonal antibodies specific for caprine caseins, it should be convenient to select as immunogen the casein fraction with the largest number of epitopes specific to goat's milk.

We report in this paper the use of two chromatographic techniques for the fractionation of goat's milk casein and its partial immunological characterization by an indirect ELISA to identify those fractions displaying the highest specific immunogenicity.

MATERIALS AND METHODS

Preparation of Casein Samples. Fat was removed from goat's milk by centrifugation at 3000*g* for 10 min at 4 °C and filtration through glass wool. Skim milk was heated to 45 °C and acidified to pH 4.7 with 0.2 M

0021-8561/95/1443-2025$09.00/0

HCl. The acidified milk was kept at 40 °C for 30 min and centrifuged at 6000*g* for 10 min to recover the precipitated caseins. The pellet was washed three times with distilled water to eliminate remaining whey proteins and centrifuged at 6000*g* for 30 min (Rodríguez et al., 1991). The supernatant was discarded, and the pellet containing the precipitated caseins was lyophilized, placed in an airtight container, and stored at −20 °C until use.

Anion-Exchange Fast Protein Liquid Chromatography (FPLC). The lyophilized caprine caseins were fractionated by anion-exchange fast protein liquid chromatography (FPLC) on a Mono Q HR 5/5 column (Pharmacia AB, Uppsala, Sweden). Samples were prepared as previously described (Calvo et al., 1992). Briefly, 10 mg of freeze dried goat's casein was dissolved in 10 mL of 5 mM bistrispropane-HCl (pH 7.0) (BTP) containing 3.3 M urea, and after stirring for 5 min at 20 °C, the pH was adjusted to 7.0 and 10 μL of 14 mM 2-mercaptoethanol was added while stirring continued for 1 h at 20 °C in the dark. The casein samples were finally filtered through a 0.22 μm pore size GV filter (Millipore, Bedford, MA).

The caprine caseins were eluted from the Mono Q HR 5/5 anion-exchange column essentially following the method described by Davies and Law (1987), for the fractionation of bovine caseins. Briefly, the column was equilibrated with 2.5 mL of 5 mM BTP (pH 7.0) buffer followed by 3 mL of BTP buffer containing 1 M NaCl and then another 5 mL of BTP buffer. After this, 200 μL of the caprine caseins obtained as described above was applied to the column and eluted, at a flow rate of 1.0 mL/min, in a stepwise 0−0.4 M NaCl gradient. The concentration of NaCl being fed into the column reached 0.10 M after 2 mL of gradient, 0.11 M after 9 mL, 0.22 M after 14 mL, 0.28 M after 21.5 mL, 0.295 M after 29 mL, and 0.40 M after 35 mL.

Cation-Exchange Chromatography. The lyophilized caprine caseins were fractionated by cation-exchange chromatography essentially as described by Jaubert and Martín (1992). Briefly, 1 g of lyophilized caprine casein in 25 mL of 0.8 mM DTT, 25 mM sodium formate, and 7.5 M urea buffer (pH 4.0) was reduced for 2 h at 24 °C and then applied to a column (2.5 × 30 cm) containing 150 mL of S-Sepharose Fast Flow cation exchanger (Pharmacia). The column was previously equilibrated with 0.064 mM DTT, 75 mM sodium formate, and 7.5 M urea buffer (pH 4.0), while the elution was performed at room temperature at a flow rate of 1 mL/min using a linear NaCl gradient from 0 to 0.3 M for 18 h. Casein fractions were collected with a FRAC-100 collector (Pharmacia LKB), while absorbance of the samples was determined at 280 nm in a Hitachi Model U-2000 spectrophotometer.

Protein Determination. Protein concentration was determinated according to the method of Lowry et al. (1951) modified as described by Markwell (1978).

SDS−PAGE PhastSystem Electrophoresis. Selected caprine casein fractions obtained from FPLC and cation-exchange chromatography were dialyzed exhaustively against distilled water and lyophilized. Fractions were later analyzed by SDS−PAGE using PhastSystem electrophoresis equipment (Pharmacia). Electrophoresis was performed on homogeneous 20% polyacrylamide gels and buffer strips containing 0.2 M tricine, 0.2 M tris, and 0.55% SDS (pH 8.1). The casein samples were dissolved in a buffer of 10 mM Tris-HCl, 1 mM EDTA (pH 8.0), 2.5% SDS, 5% 2-mercaptoethanol, and 0.003% bromophenol blue. The samples were boiled for 5 min; after cooling, approximately 5 μL of sample (10 μg of protein) was deposited in each lane of the gels. Electrophoretic conditions programmed into the PhastSystem were 250 V, 10 mA, and 3 W at 15 °C for 90 Vh (volthour). Gels were stained for 40 min at 50 °C in a 0.02% PhastGel Blue R (Pharmacia) solution and destained in a solution of 30:10:60 methanol/acetic acid/water until the background was clear.

Biotinylated Antibodies. Polyclonal antibodies against goat's casein (anti-GC) raised in rabbits were purified by immunoadsorption chromatography in a column containing immobilized goat's caseins, biotinylated and made species-specific by blocking with lyophilized ovine and bovine caseins, following a previously described procedure (Rodriguez et al., 1991).

Enzyme-Linked Inmunosorbent Assay (ELISA) Procedure. Flat bottom micro-ELISA plates (Nunc, Roskilde, Denmark) were filled with 100 μL of the purified casein fractions diluted in phosphate buffer saline (PBS) (pH 7.2) and incubated for 1 h at 37 °C. The wells were washed five times with PBST (PBS containing 5% Tween 20) and coated with 200 μL of 0.1% gelatin in PBS for 30 min at 37 °C. After another five washes with PBST, 100 μL of the biotinylated anti-GC antibodies (1.05 mg/mL) diluted 1/1000 in PBST was added to the wells, and the plates were incubated on a plate shaker (LKB, Pharmacia) for 1 h at room temperature. After washing five more times with PBST to remove free antibodies, 100 μL of streptavidin peroxidase conjugate (0.8 mg/mL) (Dakopatts, Denmark) diluted 1/3000 in PBST was added to the wells, and the plates were incubated with shaking for 1 h at room temperature. The wells were washed five more times with distilled water before the addition of 150 μL of the substrate enzyme solution consisting of 0.6 mg/mL of 2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid) (ABTS, Sigma) in citric acid−phosphate buffer (pH 3.9), with H_2O_2 (110 vol, 0.2 μL/mL of buffer). The green color developed by conversion of the substrate was measured at 405 nm with a Titertek Multiskan Plus spectrometer (Flow Laboratories, McLean, VA).

RESULTS

Fractionation of Caprine Casein by Ion-Exchange Chromatography. The fractionation of whole caprine casein by anion-exchange FPLC on a Mono Q HR 5/5 column using a discontinuous NaCl gradient is shown in Figure 1. Total goat's casein was separated into three main fractions, which after dialysis and lyophilization were analyzed by SDS−PAGE in a PhastSystem electrophoresis unit. Results of SDS−PAGE (Figure 2) indicated that fraction 1 contained κ-casein, fraction 2 contained β-casein, and fraction 3 contained the α_s-casein fraction (α_{s1}- and α_{s2}-caseins).

Whole caprine casein was also fractionated on an S-Sepharose Fast Flow cation exchanger using a linear NaCl gradient. Five component fractions were collected (Figure 3), which after dialysis and lyophilization were also analyzed by SDS−PAGE in a PhastSystem electrophoresis unit (Figure 4). The resultant electrophoretic patterns indicated that fraction 1 contained β_1- and β_2-caseins in a single band, since the two phosphorylated levels are only slightly different in molecular weight. Fraction 2 contained κ-casein which migrated ahead of the β-casein, in keeping with the smaller molecular weight of κ-casein. Fraction 3 contained

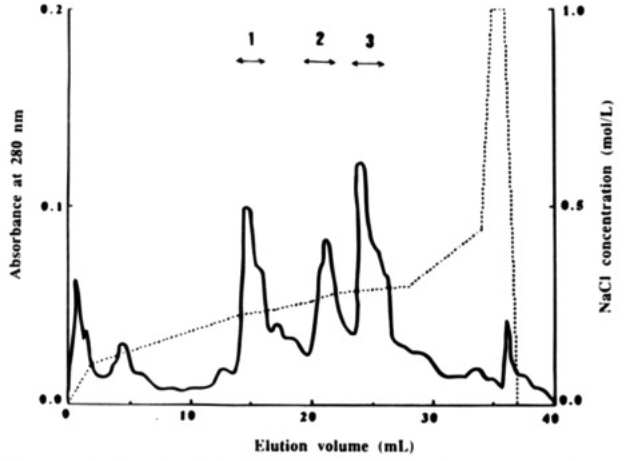

Figure 1. Goat's milk casein fractionation by anion-exchange FPLC on a Mono Q HR 5/5 column. Eluted caseins were pooled into three main fractions (1–3).

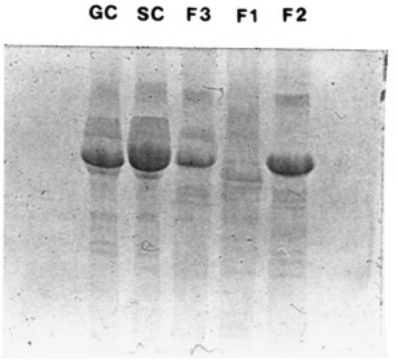

Figure 2. SDS–PAGE PhastSystem on 20% polyacrylamide gels of the caprine caseins fractionated by FPLC. Lane GC, whole goat's casein; lane SC, whole sheep's casein; lane F1, fraction 1 (κ-casein); lane F2, fraction 2 (β-casein); lane F3, fraction 3 (α_s-casein).

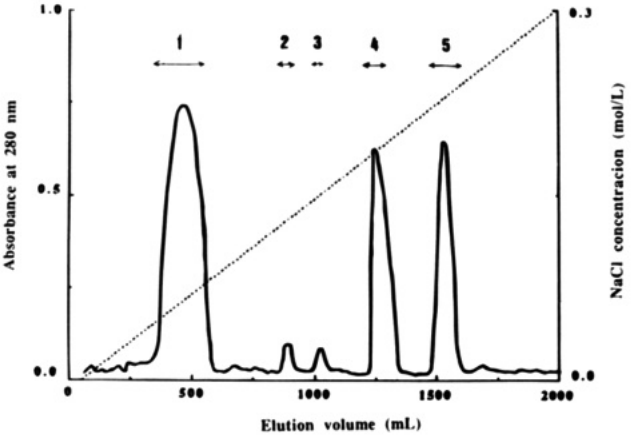

Figure 3. Goat's milk casein fractionation by cation-exchange chromatography on an S-Sepharose Fast Flow matrix. Eluted caseins were pooled into five main fractions (1–5).

diffuse protein bands in small amounts and was not used in further analyses. Fraction 4 probably consists of α_{s1}-casein with residual β-casein and κ-casein, while fraction 5 contained mostly α_{s2}-casein, the protein migrating behind the α_{s1}-casein, in keeping with its slightly higher molecular weight. These results confirm those previously described by Law and Tziboula (1992). Protein material, with slightly lower molecular weight than the main α_{s2}-band, was also detected in this fraction, being probably a minor α_s-casein fraction as previously identified by Grosclaude et al. (1987).

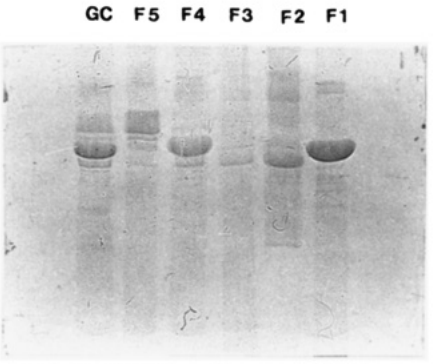

Figure 4. SDS–PAGE PhastSystem on 20% polyacrylamide gels of the caprine caseins fractionated by cation-exchange chromatography. Lane GC, whole goat's casein; lane F1, fraction 1 (β-casein); lane F2, fraction 2 (κ-casein); lane F3, fraction 3 (diffuse protein bands); lane F4, fraction 4 (α_{s1}-casein with residual β-casein and κ-casein); lane F5, fraction 5 (mostly α_{s2}-casein).

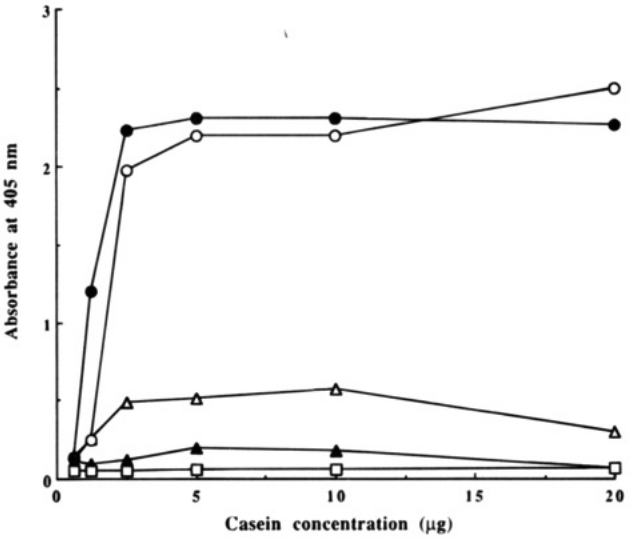

Figure 5. Indirect ELISA results of the purified caprine casein fractions by FPLC, against fraction 1 (▲, κ-casein), fraction 2 (△, β-casein), fraction 3 (○, α_s-casein), whole sheep's casein (□), and whole goat's casein (●), using biotinylated antibodies specific for caprine caseins (anti-GC).

Partial Immunological Characterization of Caprine Caseins. The purified caprine casein fractions fractionated by FPLC and cation-exchange chromatography were characterized immunologically by an indirect ELISA procedure. The assay used polyclonal antibodies raised in rabbits against goat's casein (GC). The anti-GC antibodies were further purified by affinity chromatography in a column containing immobilized goat's caseins, biotinylated and blocked with lyophilized ovine and bovine caseins (Rodriguez et al., 1991).

Extensive checkerboard titrations were performed to determine the adequate concentrations of components for the indirect ELISA procedure. Different concentrations of whole caprine and ovine caseins as well as purified caprine caseins were used (0–20 μg of protein in PBS), and the optimum conditions for the indirect ELISA developed in this work were obtained using the biotinylated anti-GC antibodies diluted 1:1000 and the streptavidin peroxidase conjugate diluted 1:3000.

Results from Figures 5 and 6, respectively, show the absorbance values from the three caprine casein fractions separated by anion-exchange FPLC and the four fractions separated by cation-exchange chromatography. In both figures, it is observed that the anti-GC antibod-

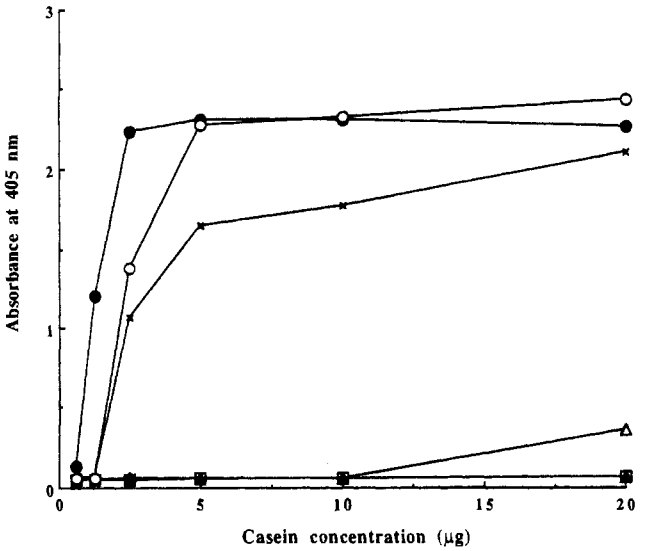

Figure 6. Indirect ELISA results of the purified caprine casein fractions by cation-exchange chromatography matrix, against fraction 1 (△, β-casein), fraction 2 (▲, κ-casein), fraction 4 (×, α_{s1}-casein with residual β-casein and κ-casein), fraction 5 (○, α_{s2}-casein), whole sheep's casein (□), and whole goat's casein (●), using biotinylated antibodies specific for caprine caseins (anti-GC).

ies effectively discriminate goat's casein from ewe's caseins. Similar results were obtained with cow's caseins (data not shown). On the other hand, the highest absorbance values were obtained with fraction 3 (α_s-casein) purified by anion-exchange FPLC (Figure 5), fraction 5 (α_{s2}-casein) purified by cation-exchange chromatography (Figure 6), and whole goat's caseins (Figures 5 and 6). The α_{s1}-casein-containing fraction showed a lower value of absorbance than the α_{s2}-casein (Figure 6). The β-casein and κ-casein fractions obtained by anion-exchange FPLC (Figure 5) and cation-exchange chromatography (Figure 6) showed much lower immunological reactivity.

Thus, results from this work indicate that the α_{s2}-casein fraction from caprine milk was the most immunologically reactive fraction against species-specific anti-GC antibodies, meaning this is the fraction with the largest number of epitopes specific to goat's milk casein.

DISCUSSION

The fractionation of caprine milk caseins has been performed by anion-exchange FPLC on a Mono Q HR 5/5 column. The order of elution was κ-casein, β-casein, and α_s-casein (Figure1), which is consistent with previous results obtained with cow's milk (Davies and Law, 1987) and from SDS−PAGE (Figure 2). This method permits a good resolution of the β- and κ-caseins, while the α_{s1}- and α_{s2}-caseins are eluted close together. Our results confirm those previously obtained by others (Jaubert and Martín 1992), suggesting that anion-exchange chromatography under conditions usually used to fractionate bovine caseins does not allow separation of the four caprine caseins. From previous studies using anion-exchange FPLC (Law and Tziboula, 1992) it appeared to be difficult to obtain consistent separation of the α_{s1}- and α_{s2}-caseins at alkaline pH, because of differences in the net negative charges of the variants.

The caprine milk caseins also have been fractionated by cation-exchange chromatography on an S-Sepharose Fast Flow matrix as described by Jaubert and Martin (1992). The order of elution of the four major casein fractions was β-casein, κ-casein, α_{s1}-casein, and α_{s2}-casein (Figure 3), as shown by the SDS−PAGE mobility patterns (Figure 4).

The goat's milk casein fractionated by anion-exchange FPLC and cation-exchange chromatography were characterized immunologically by an indirect ELISA procedure using polyclonal antibodies raised in rabbits against goat's casein, purified by affinity chromatography in a column containing immobilized goat's caseins, biotinylated and rendered goat-specific by blocking. Similarly obtained antibodies were previously shown to be useful in an indirect ELISA for the detection and quantification of goat's milk in ewe's milk and cheese differentiating 1−25% of goat's milk in prepared milk and cheese mixtures. With the anti-goat specific antibodies used in this work, the results obtained (Figures 5 and 6) indicate that the α_{s2}-casein purified by cation-exchange chromatography is the most immunoreactive fraction, probably because this is the fraction with the largest number of epitopes specific to goat's milk casein.

The immunological methods have potential value for the analysis of food products, particularly for detecting the presence of foreign milk in milk or dairy products. An enzyme-linked immunosorbent assay (ELISA) has been developed in several versions for the identification of milk species, thus demonstrating the effectiveness of this approach for the analysis of milk mixtures. The antiserum is the most important ingredient in any immunoassay. Its specificity and affinity determine the specificity and sensitivity of the assay. Polyclonal antisera against goat's caseins or unspecified whey proteins have been used in sensitive enzyme immunoassays to detect the presence of goat's milk in ewe's milk and cheese (García et al., 1993, 1994; Rodriguez et al., 1991, 1994). However, all of these antisera required affinity purification and blocking by heterologous proteins or purification by immunoabsorption chromatography against milk whey proteins or caseins. Hence, the main thrust of new developments of such enzyme immunoassays is highly dependent on the improvement of antibody quality.

To reduce problems with cross-reactions and immunoaffinity purification steps, Rolland et al. (1993) have developed a strategy based on research into the specific amino acid sequence of milk proteins. Comparison of the primary sequences of bovine and ovine milk proteins shows that certain short α_{s1}-casein peptide fragments are cow's milk specific. A peptide was chemically synthesized and directly used as an immunogen to produce polyclonal monospecific antibodies in rabbits. Thus, a competitive ELISA technique, based on the detection and measurement of bovine α_{s1}-casein, was successfully developed to detect and quantify the presence of bovine milk in ovine milk and cheese.

The improvement of antibody quality can also be obtained with hybridoma technology, which permits by adequate selection and cloning the production of monoclonal antibodies of defined specificity and affinity, without the requirements of extensive purification and testing (Johnston et al., 1985). Recently, Levieux and Venien (1994) have developed a two-site ELISA that uses two monoclonal antibodies, one of them species-specific for bovine β-lactoglobulin, to detect and quantify the presence of cow's milk in goat's or sheep's milk.

On the other hand, immunological methods for detecting milk adulteration based on the detection of heat-

sensitive proteins such as whey proteins often become ineffective if the heat-treated milk is used for cheese processing (Calvo et al., 1989; Amigo et al., 1991), while the caseins are more heat resistant (Rolland et al., 1993). On the basis of the results obtained in this work, further studies are in progress to produce monoclonal antibodies for caprine α_{s2}-casein to be used through the development of adequate ELISA procedures to detect and quantify the presence of goat's milk in cow's or ewe's milk and cheese.

ACKNOWLEDGMENT

We are grateful to a number of industries for their generous gifts of raw milk samples.

LITERATURE CITED

Addeo, F.; Mauriello, R.; Di Luccia, A. A gel electrophoretic study of caprine casein. *J. Dairy Res.* **1988**, *55*, 413–422.

Allen, J. C. The value of immunoassay to food analysis. In *Development and Application of Immunoassay for Food Analysis*; Rittenburg, J. H., Ed.; Elsevier Applied Science: London, 1990; pp 59–80.

Amigó, L.; Ramos, M.; Martín-Alvarez, P. J.; Barbosa, M. Effect of technological parameters on electrophoretic detection of cow's milk in ewe's milk cheeses. *J. Dairy Sci.* **1991**, *74*, 1482–1490.

Boulanger, A.; Grosclaude, F.; Mahé, M. F. Polymorphism of caprine (*Capra hircus*) α_{s1}- and α_{s2}-caseins. *Genet. Sel. Evol.* **1984**, *16*, 157–175.

Brignon, G.; Mahé, M. F.; Grosclaude, F.; Ribadeu-Dumas, B. Sequence of caprine α_{s1}-casein and characterization of those of its genetics variants which are synthesized at a high level. α_{s1}-CnA, B and C. *Protein Sequences Data Anal.* **1989**, *2*, 181–188.

Calvo, M.; Amigó, L.; Olano, A.; Martín, P. J.; Ramos, M. Effect of thermal treatments on the determination of bovine milk added to ovine or caprine milk. *Food Chem.* **1989**, *32*, 99–108.

Calvo, M.; Leaver, J.; Law, A. J. R.; Banks, J. M. Analysis of caseins in cheese using ion-exchange chromatography. *Milchwissenchaft* **1992**, *47*, 417–419.

Chianese, L.; Mauriello, R.; Intorcia, N.; Moio, L.; Addeo, F. New α_{s2}-casein variant from caprine milk. *J. Dairy Res.* **1992**, *59*, 299–305.

Davies, D. T.; Law, A. J. R. Quantitative fractionation of casein mixtures by fast protein liquid chromatography. *J. Dairy Res.* **1987**, *54*, 369–376.

Di Luccia, A.; Mauriello, R.; Addeo, F. Identification of caprine casein variants by electrophoretic techniques. *Protides Biol. Fluids* **1986**, *34*, 903–905.

García, T.; Martín, R.; Morales, P.; González, I.; Sanz, B.; Hernández, P. E. Sandwich ELISA for detection of caprine milk in ovine milk. *Milchwissenchaft* **1993**, *48*, 563–566.

García, T.; Martín, R.; Morales, P.; González, I.; Sanz, B.; Hernández, P. E. Detection of goat's mik in ewe's milk by an indirect ELISA. *Food Agric. Immunol.* **1994**, *6*, 113–118.

Grosclaude, F.; Mahé, M. F.; Brignon, G.; Di Stasio, L.; Jeunet, R. A mendelian polymorphism underlying quantitative variations of goat α_{s1}-casein. *Genet. Sel. Evol.* **1987**, *19*, 399–412.

Jaubert, A.; Martín, P. Reverse phase HPLC analysis of goat caseins. Identification of α_{s1} and α_{s2} genetic variants. *Lait* **1992**, *72*, 235–247.

Johnston, L. A. Y.; Tracey-Patte, P. D.; Pearson, R. D.; Hurrell, J. G. R.; Aitken, D. P. Identification of the species of origin of meat in Australia by radioimmunoassay and enzyme immunoassay. In *Immunoassays in Food Analysis*; Morris, B. A., Clifford, M. N., Eds.; Elsevier Applied Science: London, 1985; pp 95–109.

Kaminarides, S. E.; Anifantakis, E. M. Comparative study of the separation of casein from bovine, ovine and caprine milks using FPLC. *J. Dairy Res.* **1993**, *60*, 495–504.

Köhler, G.; Milstein, C. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* **1975**, *256*, 495–497.

Law, A. J. R.; Tziboula, A. Quantitative fractionation of caprine casein by cation-exchange FPLC. *Milchwissenschaft* **1992**, *47*, 558–561.

Le Jaouen, J. C.; Toussaint, J. Goat's milk in Europe. *Lait* **1993**, *73*, 405–415.

Levieux, D.; Venien, A. Rapid, sensitive two-site ELISA for detection of cow's milk in goat's milk or ewe's milk using monoclonal antibodies. *J. Dairy Res.* **1994**, *61*, 91–99.

Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. Protein measurement with the pholin phenol reagent. *J. Biol. Chem.* **1951**, *193*, 265–275.

Markwell, M. A. K.; Hass, S. M.; Bieber, L. L.; Tolbert, N. E. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Anal. Biochem.* **1978**, *81*, 206–210.

Mercier, J. C.; Addeo, F.; Pelissier, J. P. Primary structure of the caseino macropeptide from goat κ-casein. *Biochemie* **1976**, *58*, 1303–1310.

Mikkelsen, J.; Hojrup, P.; Knudsen, J. Purification of goat's milk casein by reversed-phase high performance liquid chromatography and identification of α_{s1}-casein. *J. Dairy Res.* **1987**, *54*, 361–367.

Moio, L.; Chianese, L.; Di Luccia, A.; Mauriello, R.; Stingo, C.; Addeo, F. Identification of casein variants by immunobloting. In *Electrophoresis Forum;* Radola, B. J., Ed.; München, 1989; pp 475–478.

Ramos, M.; Juárez, M. Update on existing analytical methods for detecting mixtures of cow's, ewe's and goat's milk. *Int. Dairy Fed. Bull.* **1984**, *Document 181*, 3–9.

Ramos, M.; Juárez, M. Current research on goat's milk in Spain. *Lait* **1993**, *73*, 417–424.

Richardson, B. C.; Creamer, L. K. The isolation and chemical characterization of caprine β_1-casein and β_2-casein. *Biochim. Biophys. Acta* **1974**, *365*, 133–137.

Richardson, B. C.; Creamer, L. K. Comparative micelle estructure. The similarity between caprine α_s-casein and bovine α_{s3} casein. *Biochim. Biophys. Acta* **1975**, *393*, 37–47.

Rodríguez, E.; Martín, R.; García, T.; Azcona, J. I.; Sanz, B.; Hernández, P. E. Indirect ELISA for detection of goat's milk in ewe's milk and cheese. *Int. J. Food Sci. Technol.* **1991**, *26*, 457–465.

Rodríguez, E.; Martín, R.; García, T.; Azcona, J. I.; Sanz, B.; Hernández, P. E. Sandwich ELISA for detection of goat's milk in ewe's milk and cheese. *Food Agric. Immunol.* **1994**, *6*, 105–111.

Rolland, M. P.; Bitri, L.; Besançon, P. Polyclonal antibodies with predetermined specificity against bovine α_{s1}-casein: application to the detection of bovine milk in ovine milk and cheese *J. Dairy Res.* **1993**, *60*, 413–420.

Received for review November 29, 1994. Revised manuscript received May 18, 1995. Accepted May 30, 1995.[⊗] This work is partially supported by Grant ALI91-0229 from the Comisión Interministerial de Ciencia y Tecnología (CICYT). A.I.H. and G.A. are recipients of Fellowships from the Ministerio de Educación y Ciencia, Spain.

JF940674R

[⊗] Abstract published in *Advance ACS Abstracts,* July 15, 1995.