

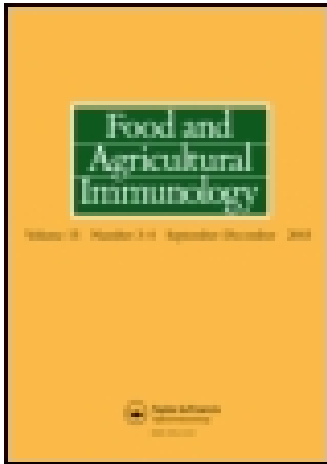
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Sandwich ELISA for Detection of Goats' Milk in Ewes' Milk and Cheese

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A sandwich ELISA has been developed for the detection of defined amounts of goats' milk (1–100%) in ewes' milk and cheese. Polyclonal antibodies were raised in rabbits against goat caseins (GC). The resultant antibodies were affinity purified by immunoabsorption of the crude antiserum on to columns containing immobilized cow, ewe and goat caseins, followed by elution of the goats' milk-specific antibodies (anti-GC) from the column containing the goat caseins. The anti-GC antibodies bound to the wells of a microtiter plate were used to capture the goats' caseins from milk or cheese mixtures. Further immunorecognition of the captured proteins was achieved with the same antibodies conjugated to biotin. ExtrAvidin–peroxidase was used to detect biotinylated antibodies bound to their specific antigens. Subsequent enzymic conversion of the substrate gave clear absorbance differences when assaying mixtures of ewes' milk and cheese containing variable amounts of goats' milk.

Keywords: Biotinylated antibodies, ELISA, ewes' milk adulteration, goats' milk identification

INTRODUCTION

Cheese manufacture in Mediterranean countries is often affected by fraudulent substitution of ewes' milk with goats' milk because of their difference in price. In the herds many goats and sheep often co-exist and farmers may send adulterated milk to the dairy factories as pure ewes' milk. This may become a serious problem in cheese manufacture as the composition of the milk influences the rennet clotting time, rate of curd formation and coagulum strength of the final product.

Several methods are available for the identification of goats' milk in ewes' milk.

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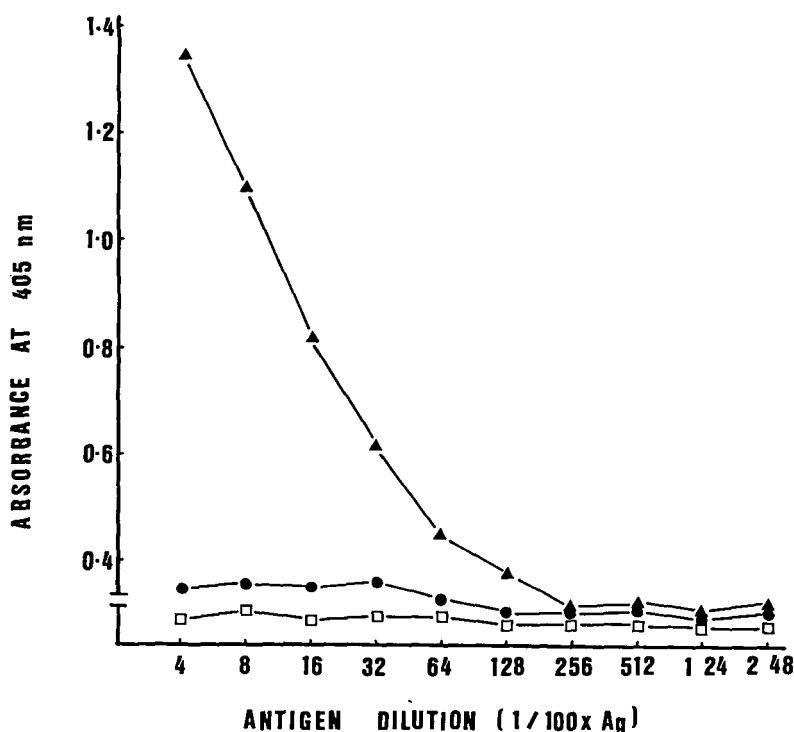


FIG. 1. Sandwich ELISA results for the identification of goats' (▲), ewes' (●) and cows' (□) milk using the capture anti-GC diluted 1:2000 and the biotinylated antibodies diluted 1:500.

Non-immunological methods, based on the identification of some specific components of milk fat or milk proteins, include gas-liquid chromatography (Smeyers-Verweke *et al.*, 1977), gel electrophoresis (Assenat, 1967; Amigo *et al.*, 1989) and isoelectrofocusing (Ruiz & Santillana, 1986). Although these methods have their own individual merits, they are not yet used routinely for analysis because they are either too cumbersome and time consuming or costly.

The immunological methods most commonly applied to goats' milk identification include techniques such as the precipitin test (Pinto, 1966), radial immunodiffusion (Levieux, 1977, Amigo, *et al.*, 1989) and an indirect ELISA (Rodríguez *et al.*, 1991). Immunoassay techniques provide complementary and/or alternative approaches to reduce the use of costly, sophisticated equipment and analysis time while still maintaining reliability and improved sensitivity. ELISA is the most widely used form of immunoassay in food analysis and has advantages of being low in cost and fast to carry out. It is also easy to use, reliable, rapid and readily automated (Rittenburg, 1990). It can be designed either as a cheap qualitative method or as a reliable quantitative procedure. The development of immunoassay techniques useful to the food industry may contribute to the quality control and safety of our food supply.

In this paper the use of affinity-purified antibodies against goats' caseins (GC) and the development of a sandwich ELISA to detect and quantify the presence of goats' milk in ewes' milk and cheese are reported.

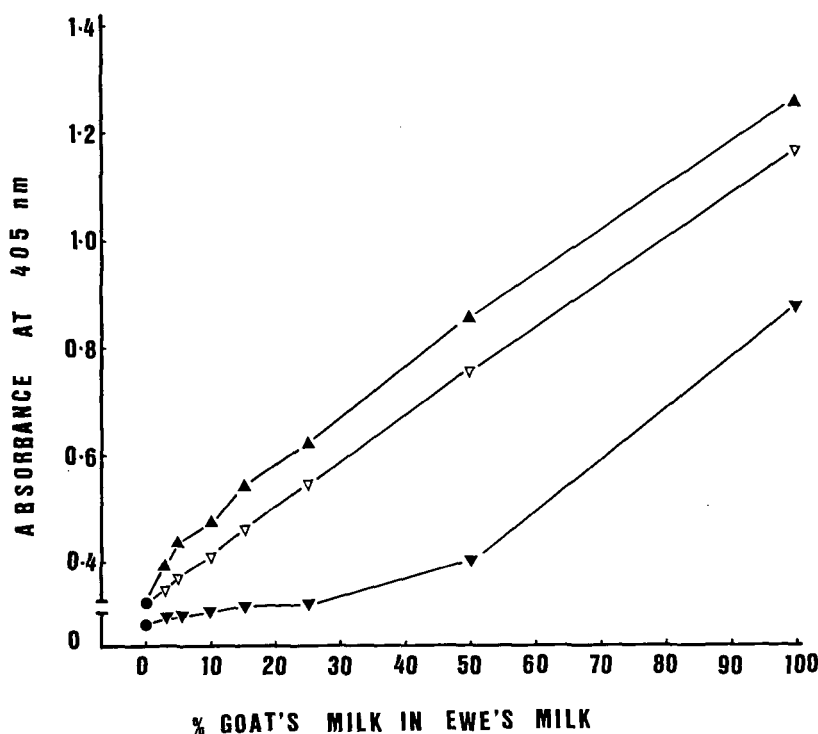


FIG. 2. Sandwich ELISA responses to ewes' milk substitution (●) with goats' milk (▲). Symbols represent the mean value of triplicate assays from two independent milk mixtures.

MATERIALS AND METHODS

Preparation for the Antigenic Extracts

Fat was removed from goats' milk by centrifugation at $13\,000 \times g$ for 10 min and filtration through glass wool. The skim milk was further heated to 37°C and acidified to pH 4.47 with 0.2 M-HCl, held at 40°C for 30 min and centrifuged at $6000 \times g$ for 30 min to recover the precipitated caseins. The pellet was washed three times with 20 ml of distilled water to eliminate remaining whey proteins and centrifuged at $6000 \times g$ for 10 min. The supernatant was discarded and the pellet containing the precipitated caseins was lyophilized, placed in an air-tight container and stored at -20°C until use.

Production of Goats' Milk Specific Antibodies

Polyclonal antibodies against GC were raised in male New Zealand White rabbits. Immunization was initiated by subcutaneous injection of 8 mg lyophilized GC emulsified in 0.5 ml of Freund's complete adjuvant (Difco Laboratories, Detroit, Michigan, MI, USA) and 2 ml of deionized and distilled water at multiple sites along the rabbits' backs. Booster doses ($n = 20$) made in Freund's incomplete adjuvant were applied subcutaneously every 6 days. After 120 days the rabbits were bled and the blood was allowed to clot for 1 h at room temperature. Serum was collected by centrifugation at $800 \times g$ for 30 min. Samples of crude antiserum (1 ml) were stored frozen at -20°C .

Antibodies from crude antisera were made species-specific by immunoadsorption chromatography. Isolation of goats' milk-specific antibodies was carried out by passing 10 ml samples of crude antiserum, diluted 1:2 with $2 \times$ concentrated phosphate-buffered saline (PBS), pH 7.2, containing 0.5% Tween 80, through three affinity chromatography columns.

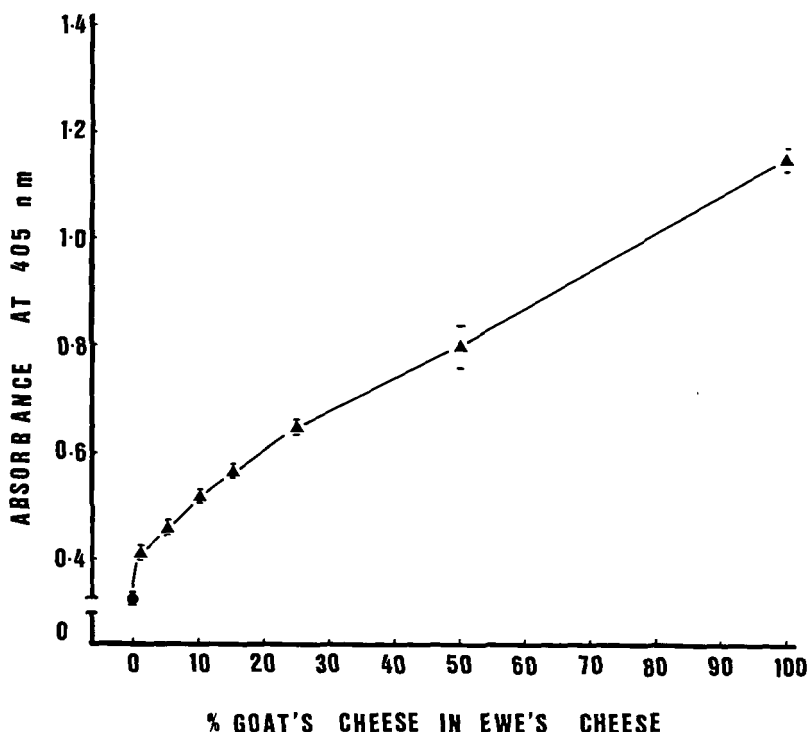


FIG. 3. Sandwich ELISA results to ewes' cheese substitution (●) with goats' cheese (▲). Symbols represent the mean value of triplicate assays from two independent cheese mixtures.

Each column contained 10 g of CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) coupled, according to the manufacturer's instructions, to 350 mg of the lyophilized cow, ewe and goat caseins. After passage of the diluted crude antisera through the three columns, specific antibodies against GC (anti-GC) were released from the goat column by elution with 0.1 M-glycine-HCl buffer, pH 2.5. The eluted fractions with an absorbance at 280 nm of over 0.1 were pooled, adjusted to pH 7.2 with solid Tris (Merck, Darmstadt, Germany), dialyzed overnight against PBS and lyophilized. Affinity chromatography was performed at 4°C. The immunopurification step was performed several times in order to concentrate sufficient anti-GC antibodies for use in subsequent ELISA experiments.

Biotinylated Antibodies

Biotinamidocaproate *N*-hydroxysuccinimide ester (Sigma Chemical Co., St Louis, MO, USA), dissolved at 1 mg ml⁻¹ in dimethyl sulphoxide (DMSO, Merck, Darmstadt, Germany), was added at a molar excess of 50 to the affinity-recovered antibodies (anti-GC) dissolved in PBS (1.25 mg ml⁻¹), and incubated for 2 h at room temperature (Bonnard *et al.*, 1984). Unbound reagent was separated from the conjugated antibodies by dialysis overnight at 4°C against PBS. Samples of biotinylated antibodies (0.1 ml) were stored at -20°C until use.

Reference Milk Samples

Goats' and ewes' milk samples were mixed at different percentages (1–100% v/v) and maintained frozen at -20°C until use. Mixtures from two independent batches of milk were prepared to be tested by ELISA. Samples of milk mixtures (1 ml) were also

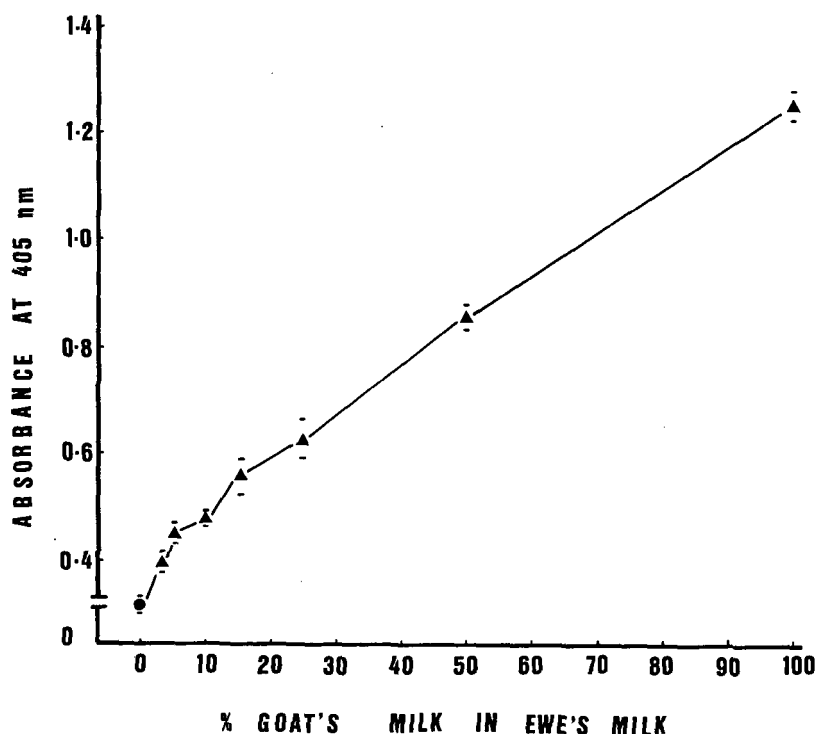


FIG. 4. Sandwich ELISA response to ewes' milk substitution (●) with unheated goats' milk (▲), pasteurized goats' milk (△) and sterilized goats' milk (▼).

pasteurized (62°C, 30 min) or sterilized (121°C, 20 min). Mixtures of goats' and ewes' cheese of known composition for use as controls in the assay were prepared by mixing ewes' cheese with different quantities (w/w) of goats' cheese. Standard extracts were prepared by homogenizing samples (25 g) of these cheese mixtures in 500 ml of PBS, pH 7.2. Aliquots of the extracts (1 ml) were diluted 1:5 in PBS and stored at -20°C until use.

Sandwich ELISA

Flat-bottomed micro-ELISA plates (Costar, Cambridge, MA, USA) were filled with 0.1 ml of the capture anti-GC antibodies (1.05 mg ml^{-1}) diluted 1:2000 in carbonate coating buffer (sodium carbonate-bicarbonate buffer, pH 9.6) and incubated at 37°C for 1 h. The wells were washed five times with PBS containing 5% Tween 20 (PBST) and coated with 0.2 ml of 0.1% gelatin in PBS for 1 h at 37°C . After five washes with PBST, 0.1 ml of milk samples diluted in PBST was added to the wells and the plates were incubated on a plate shaker (LKB, Pharmacia, Uppsala, Sweden) for 1 h at 20°C . Following another washing step with PBST, the biotinylated anti-GC antibodies diluted 1:500 in PBST were added to the wells, and the plates incubated with shaking for 1 h at 20°C . After washing five more times with PBST, 0.1 ml of ExtrAvidin-peroxidase (Sigma Chemical Co., St Louis, MO, USA), diluted 1:3000 in PBST was added to the wells, and the plates were incubated with shaking for 1 h at 20°C . Wells were washed five more times with distilled water before addition of 0.15 ml of the substrate (a solution consisting of 0.6 mg ml^{-1} 2,2'-azinobis-3-ethyl-benzthiazoline sulphonic acid, ABTS, Sigma) in citric acid-phosphate buffer (pH 3.9) with H_2O_2 (110 volume, $0.2 \text{ } \mu\text{l ml}^{-1}$ buffer). After a 10 min incubation the reaction was stopped by addition of 0.05 ml sodium fluoride (2% in distilled water) to

each well. The green colour from conversion of the substrate to product was measured at 405 nm with a Titertek Multiskan Plus spectrophotometer (Flow Laboratories, McLean, VA, USA).

RESULTS AND DISCUSSION

To detect and quantify the presence of goats' milk in ewes' milk and cheese, the sandwich ELISA described uses polyclonal antibodies raised in rabbits against GC. To remove those antibodies which could cross-react with cows' or ewes' milk proteins from the crude antiserum, portions of this antiserum were circulated once through three columns each containing immobilized cow, ewe or goat caseins, followed by elution of the goats' milk-specific antibodies (anti-GC) from the goat column. Although immunoadsorption chromatography is time consuming and requires technical expertise to obtain monospecific antibodies, it is considered to be an excellent procedure to remove cross-reaction antibodies.

The specific anti-GC antibodies for use as an immunorecognition reagent in the sandwich ELISA were conjugated to biotin. Since biotin is a small molecule, when it binds to the probe antibody it does not have the damaging effect that an enzyme has when this is directly conjugated to the antibody (Wilcheck & Bayer, 1988). In the sandwich ELISA described here, the biotinylated anti-GC antibodies were detected with ExtrAvidin-peroxidase.

Extensive checkerboard titrations were performed to determine the dilutions of antigens and antibodies required to optimize the assay. Optimum conditions were obtained using the capture anti-GC antibodies (1.05 mg ml^{-1}) diluted 1:2000, the milk samples diluted 1:400, the biotinylated anti-GC antibodies diluted 1:500 and the ExtrAvidin-peroxidase conjugate diluted 1:3000. Using the optimized sandwich ELISA, it was observed (Figure 1) that the anti-GC antibodies effectively distinguished goats' milk from ewes' and cows' milk. From these results, it is clear that the immunoadsorption step removed most cross-reacting antibodies and enabled positive identification of goats' milk.

The sandwich ELISA was further used to detect the substitution of ewes' milk by goats' milk in prepared milk mixtures. Figure 2 shows the absorbance values from two independent batches of ewes' milk containing 0, 1, 5, 10, 20, 30, 50 and 100% goats' milk. For these milk samples, absorbance was related to goats' milk content (G) in the range 1–100% by the equation:

$$\text{Absorbance } (A_{450}) = 0.3799 + 0.0089 (\%G)$$

with a correlation coefficient of $r = 0.995$. This relationship could be used as a reference curve for measuring the addition of goats' milk to ewes' milk. However, since the reference curve is likely to differ slightly under the conditions given on different days of work and in different laboratories, it is advisable to run a reference curve with known percentages of goats' milk in ewes' milk along with the unknown samples. The coefficient of variation (CV) of optical densities for a standard sample of goats' milk allocated over two entire 96-well plates was 10% for intra- and inter-assay results.

To measure the response of the sandwich ELISA to detect and quantify the presence of goats' milk in ewes' cheese, two independent batches of ewes' cheese were prepared to include 0, 1, 5, 10, 15, 25, 50 and 100% of goats' cheese. Optimum conditions for the assay were obtained using the cheese samples diluted 1:50 while maintaining the capture antibodies diluted 1:2000 and the biotinylated antibodies diluted 1:500. Figure 3 shows that in these cheese batches absorbance was related to goats' cheese content (G) in the range 1–100% by the equation:

$$\text{Absorbance } (A_{450}) = 0.424 + 0.007 (\%G)$$

with a correlation coefficient of $r = 0.991$.

A control experiment was also performed to measure the response to different heat treatments of the specific anti-GC antibodies against goats' milk samples. Figure 4 shows

that batches of ewes' milk containing 0, 1, 5, 10, 15, 25, 50 and 100% of pasteurized or sterilized goats' milk gave lower optical density responses compared with the absorbance values shown by the goats' raw milk. Using an indirect ELISA (Rodríguez *et al.*, 1991), it was observed that mixtures of ewes' milk with pasteurized and sterilized goats' milk gave essentially similar absorbance values to those prepared with raw goats' milk. The differences in the two ELISA procedures in their sensitivity to detect heated goats' milk in the milk mixtures may be due to the different way in which the GC are presented to the antibodies. In the indirect ELISA, the heated caseins directly absorbed in the wells may still present a large number of conserved epitopes to the anti-GC antibodies. However, in the sandwich ELISA, the capture anti-GC may mask a number of unmodified epitopes that are no longer presented to the detector biotinylated antibodies. According to these results, the sandwich ELISA developed would indicate lower percentages of substitution if defined amounts of ewes' raw milk were substituted by either pasteurized or sterilized goats' milk.

The sandwich ELISA developed for the identification and quantification of goats' milk in ewes' milk is more sensitive than some reported electrophoretic, immunological and chromatographic methods (Assenat, 1967; Ruiz & Santillana, 1986; Amigo *et al.*, 1989; Iverson & Sheppard, 1989), being in the range of sensitivity reported by others (Levieux, 1987; Rodríguez *et al.*, 1991). This ELISA, using specific anti-GC antibodies, was accurate, easy to perform, sensitive and specific. It could also be suitable for the commercial production of stable kits, readily available for species verification of milk in large- or small-scale inspection programmes.

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