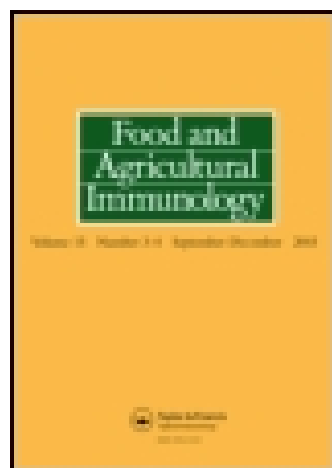




Food and Agricultural Immunology

Publication details, including instructions for authors and subscription information:

<http://www.tandfonline.com/loi/cfai20>

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Published online: 16 Sep 2008.

To cite this article: C. Castro, R. Martín, T. García, E. Rodríguez, I. González, B. Sanz & P. E. Hernández (1992) Indirect enzyme-linked Immunosorbent assay for detection of cow's milk in goat's milk, Food and Agricultural Immunology, 4:1, 11-18, DOI: [10.1080/09540109209354748](https://doi.org/10.1080/09540109209354748)

To link to this article: <http://dx.doi.org/10.1080/09540109209354748>

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Indirect Enzyme-linked Immunosorbent Assay for Detection of Cow's Milk in Goat's Milk

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(Received for publication 19 September 1991)

An indirect enzyme-linked immunosorbent assay (ELISA) has been developed for the specific detection of cow's milk (1-25%) in goat's milk. The test uses polyclonal antibodies raised in rabbits against bovine whey proteins (BWP). The anti-BWP antibodies were recovered from the crude antiserum by immunoabsorption and elution from a column containing immobilized BWP. The anti-BWP antibodies were biotinylated and rendered cow's milk specific by mixing them with lyophilized ovine and caprine whey proteins. Streptavidin-peroxidase was used to detect the biotinylated anti-BWP antibodies bound to bovine milk proteins immobilized on 96-well plates. The colour developed by the subsequent enzymic conversion of the substrate gave clear absorbance differences when assaying mixtures of goat's milk containing variable amounts of cow's milk.

Keywords: *Biotinylated antibodies, enzyme-linked immunosorbent assay, goat's milk adulteration, goat's milk specific antibodies.*

INTRODUCTION

Substitution of expensive milk with cheaper milk is unethical and may be objectionable for health and economic reasons. Also, there is increasing pressure from consumer organizations for quantitative ingredient declarations, and the current trend towards 'informative labelling' means that those monitoring composition have to ensure that food products are safe, unadulterated and accurately labelled. Accordingly, there is a need to develop good analytical procedures for use by dairy factories and law enforcement agencies in their attempts to combat the increasing problems of adulteration of expensive milk with cheaper milk intended for use in cheese manufacture.

Different methods are currently used for milk species identification. Non-immunological methods include gel electrophoresis (Aschaffenburg & Dance, 1968; Pierre &

Portmann, 1970; Ramos *et al.*, 1977; Furtado, 1983; Amigo *et al.*, 1986), isoelectrofocusing (Krause *et al.*, 1982; Addeo *et al.*, 1989; Rispoli & Saugues, 1989), gas-liquid chromatography (Iverson & Sheppard, 1989; Prager, 1989) and high performance liquid chromatography (Diosady *et al.*, 1980; Bican & Blanc, 1982; Bican, 1983; Pearce, 1983).

Immunological methods developed for milk species analysis include techniques such as agar-gel immunodiffusion (Durand *et al.*, 1974; Amigo *et al.*, 1989) and its 'dry disc' modification test known as the COMIT assay (García *et al.*, 1989), radial immunodiffusion (Levieux, 1977), rocket immunoelectrophoresis (Radford *et al.*, 1981), haemagglutination inhibition (Levieux, 1980), immunodotting (Aranda *et al.*, 1988) and several forms of enzyme-linked immunosorbent assay (ELISA) (García *et al.*, 1990, 1991; Rodríguez *et al.*, 1990).

Immunoassays are considered an excellent replacement for conventional analytical methods applied to milk species identification since they provide quantitative data faster than non-immunological techniques. ELISA is the most widely used form of immunoassay in food analysis on account of its simplicity and suitability as a screening method; it is also easy to use, cost-effective, reliable, rapid and readily automated. It can be designed either as an inexpensive qualitative method or as a reliable quantitative procedure.

We report in this communication the use of an indirect ELISA and blocked, biotinylated antibodies against bovine whey proteins (BWP) to detect and quantify the presence of cow's milk in goat's milk.

MATERIALS AND METHODS

Preparation of the Antigenic Extracts

Fat was removed from bovine milk by centrifugation at $13\,000\times g$ for 10 min at 4°C and filtration through glass wool. The skimmed milk was further heated to 37°C and acidified to pH 4.6 with 0.2 M-HCl. It was then maintained at 40°C for 30 min and centrifuged at $5800\times g$ for 10 min to remove the precipitated caseins. The pH of the supernatant was adjusted to 7.0 with 1 M-NaOH and centrifuged at $5800\times g$ for 20 min to precipitate any remaining caseins. The supernatant containing the whey proteins was dialyzed overnight against phosphate-buffered saline (PBS, pH 7.2, containing 0.02% sodium azide) and lyophilized. The dried whey proteins were placed in an air-tight container and stored at -20°C until use.

Production of Polyclonal Antibodies

Polyclonal antibodies against BWP were raised in New Zealand male white rabbits, following a previously described procedure (García *et al.*, 1990). The anti-BWP antibodies were recovered from the crude antiserum by immunoadsorption and elution from a column containing immobilized BWP, also as previously described (García *et al.*, 1990).

Biotinylated Antibodies

The affinity recovered antibodies (anti-BWP) were conjugated to biotin according to the method described by Bonnard *et al.* (1984). Briefly, biotinamidocaproate *N*-hydroxysuccinimide ester (Sigma Chemical Co., St Louis, MO) dissolved at 1 mg ml^{-1} in dimethylsulphoxide (Merck, Darmstadt) was added (at a molar excess of 50) to the affinity-recovered antibodies (anti-BWP) dissolved in PBS pH 7.2 (1.25 mg ml^{-1}), and incubated for 2 h at room temperature. The unbound reagent was separated from the

antibodies by overnight dialysis against PBS. Aliquots of the biotinylated antibodies (0.1 ml) were stored at -20°C until use.

Blocking of the Biotinylated Antibodies

The biotinylated anti-BWP antibodies (1 mg ml^{-1}) diluted in PBST (PBS containing 5% of Tween 20) were rendered bovine milk-specific by mixing them with lyophilized ovine and caprine whey proteins (0.04 mg ml^{-1} of the diluted biotinylated antibodies) for 16 h at 4°C .

Reference Milk Samples

Reference milk mixtures were prepared mixing cow's and goat's milk at different percentages (1%–100%, v:v) and maintained frozen at -20°C until use. Samples of pasteurized and sterilized bovine milk were of commercial origin. Mixtures from two different batches of milk were prepared for testing by ELISA.

Indirect ELISA Procedure

Flat-bottomed micro-ELISA plates (Costar, Cambridge, MA) were filled with $100\text{ }\mu\text{l}$ of the milk samples diluted 1:100 (v:v) in PBS, pH 7.2 and maintained for 30 min at 37°C . The wells were washed five times with PBST and coated with $200\text{ }\mu\text{l}$ of gelatin (0.1% w/v) in PBS for 30 min at 37°C . After another five washes with PBST, $100\text{ }\mu\text{l}$ of the blocked biotinylated anti-BWP antibodies diluted 1/200 (v/v) in PBST were added to the wells and the plates were then incubated for 30 min at 20°C . After washing five more times with PBST to remove heterologous antigens and unattached antibodies, $100\text{ }\mu\text{l}$ of streptavidin-peroxidase (Dakopatts, Copenhagen) diluted 1:3000 (v:v) in PBST were added to the wells, and the plates incubated for 30 min at 20°C . The wells were washed five more times with distilled water before addition of $150\text{ }\mu\text{l}$ of the substrate solution consisting of 0.6 mg ml^{-1} of 2,2'-azinobis-3-ethyl-benzthiazoline sulphonic acid (ABTS, Sigma) in citric acid-phosphate buffer (pH 3.9) which contained H_2O_2 (110 vol, $0.2\text{ }\mu\text{l ml}^{-1}$). After incubation for 30 min, the reaction was stopped by addition to each well of $50\text{ }\mu\text{l}$ of sodium fluoride (2%, w/v, in distilled water). The green colour developed by conversion of the substrate was measured at 405 nm with a Titertek Multiskan Plus Spectrophotometer (Flow Laboratories, McLean, VA).

RESULTS AND DISCUSSION

A problem currently concerning dairy factories is the detection of small amounts of bovine milk in caprine milk supplies intended for use in cheese manufacture, because the composition of the milk influences the organoleptic characteristics of the final product. The indirect ELISA described in this work uses polyclonal antibodies raised in rabbits against bovine whey proteins (BWP). To recover those antibodies specific for BWP, samples of the crude antiserum were circulated onto a column containing BWP coupled to CNBr-activated Sepharose 4B followed by elution of the adsorbed antibodies from the column (anti-BWP antibodies). Immunodiffusion experiments in agarose gels following the Ouchterlony technique (Ouchterlony, 1948) indicated that the anti-BWP antibodies gave reactions of identity not only against bovine milk but also against ovine and caprine milk (results not shown). This cross-reactivity was expected because the immunopurification step recovered antibodies that may recognize whey proteins in bovine milk and epitopes in the same proteins from closely related species.

To be used in the indirect ELISA developed in this work, the affinity recovered anti-BWP antibodies were conjugated to biotin. Since biotin is a small molecule, its binding to the probe antibody does not have the deleterious effects associated with linking an enzyme directly to the antibody (Wilchek & Bayer, 1988). The biotinylated anti-BWP antibodies were rendered bovine milk-specific by mixing them with lyophilized ovine and caprine whey proteins in a process termed as 'blocking'. The blocking treatment is an effective procedure to remove immunological cross-reactions (Durand *et al.*, 1974; Doberstein & Greuel, 1982; Jones & Patterson, 1986), without the need for affinity purification through columns containing heterologous antigens in a bound form.

Extensive checkerboard titrations were performed to determine the optimal level of antigen, antibody and conjugate dilution to be used in order to maximize the results. Optimum conditions for the indirect ELISA were obtained using the milk samples diluted 1:100 and the blocked biotinylated anti-BWP antibodies 1:200 (v:v), while the streptavidin-peroxidase conjugate was diluted 1:3000 (v:v).

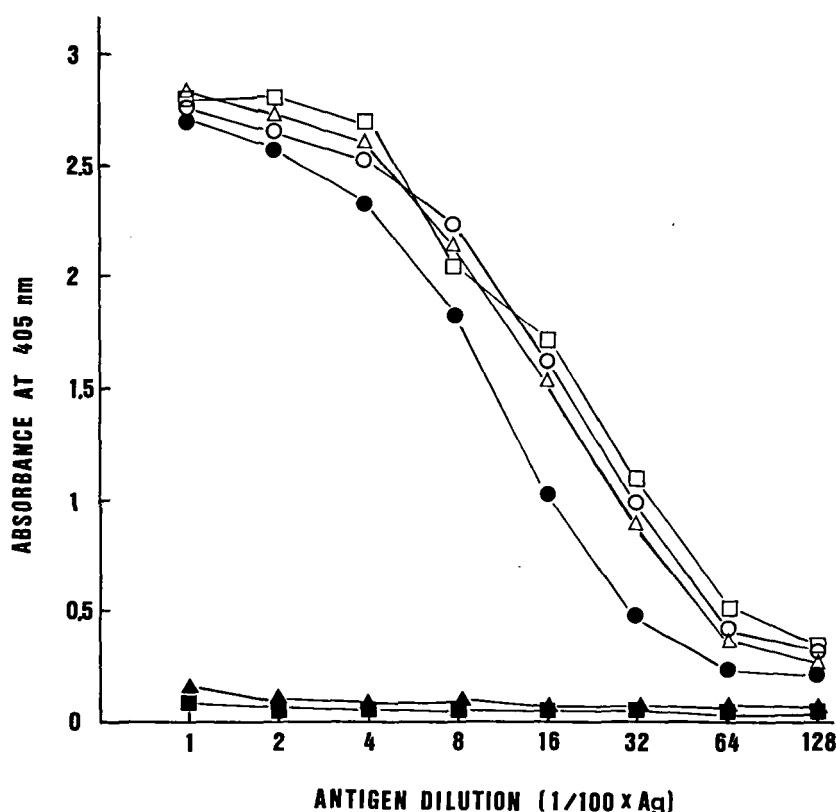


FIG. 1. Indirect ELISA results for the identification of cow's (O), goat's (□) and sheep's (Δ) milk using biotinylated anti-BWP antibodies. Effect of blocking of the same antibodies with lyophilized sheep's and goat's whey proteins to differentiate bovine (●), caprine (■) and ovine milk (▲).

In the indirect ELISA described, the biotinylated anti-BWP antibodies bound to the BWP immobilised on 96-well plates were detected with streptavidin-peroxidase. The effectiveness of the blocking procedure may be observed in Figure 1, where the blocked biotinylated anti-BWP antibodies effectively discriminate cow's milk from goat's and sheep's milk in a wide range of milk dilutions from 1:100 to 1:12 800 (v:v).

From these results, it is clear that the blocking of the biotinylated anti-BWP antibodies with the lyophilized ovine and caprine whey proteins eliminated most of the cross-reactions found in caprine and ovine milk.

Figure 2 shows the absorbance values from two different batches of caprine milk prepared to include 0%, 1%, 2.5%, 5%, 10%, 15%, 25% and 100% of bovine milk. In these milk batches, absorbance was related to cow's milk content (%C) in the range 1%–25% by the equation

$$\text{Absorbance} = 0.2274 + 0.0736 (\%C)$$

with a correlation coefficient of $r=0.999$. This relationship may be used as a standard curve when trying to evaluate the addition of bovine milk to caprine milk. However, since the reference curve is likely to differ slightly under given conditions on different days in different laboratories, it is advisable to run a reference curve with known percentages of bovine milk in caprine milk at the same time as the unknown samples.

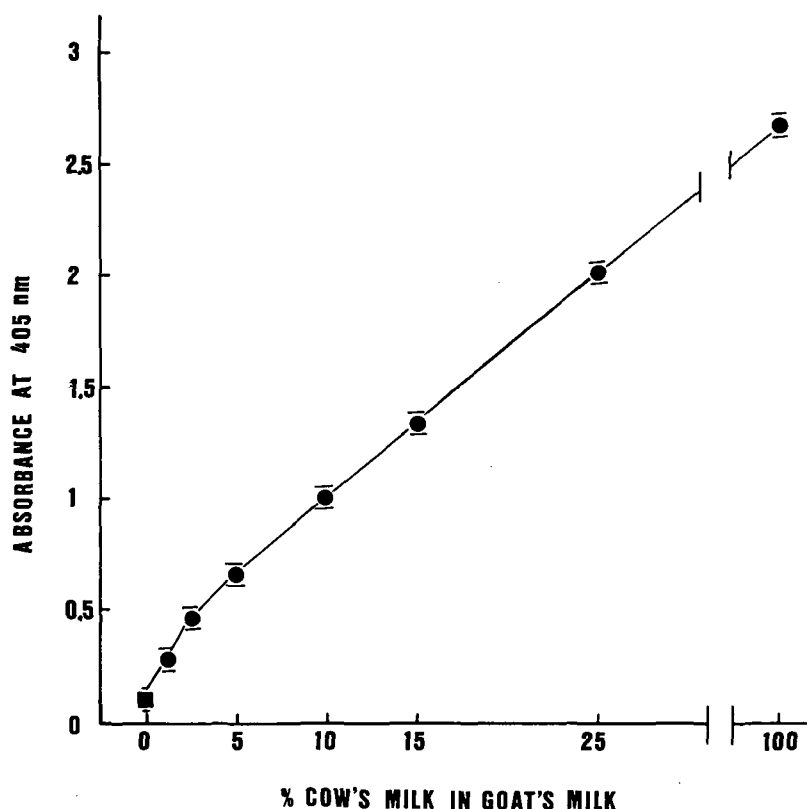


FIG. 2. Indirect ELISA response to goat's milk substitution (■) by cow's milk (●). Bars represent standard deviation of six assays from two independent milk mixtures.

A control experiment was also performed to measure the response of the blocked biotinylated anti-BWP antibodies and the developed indirect ELISA to bovine milk samples subjected to different heat treatments. Figure 3 shows that batches of pasteurized and sterilized bovine milk when mixed with goat's milk gave lower optical density responses as compared with the absorbance values shown by the bovine raw milk samples. These responses were about 25% lower for the pasteurized milk and

about 50% lower for the sterilized milk. Whey proteins are not as heat resistant as caseins (Calvo *et al.*, 1989), and their heat denaturation alters the immunoreactivity of the antisera raised against native whey proteins, thus decreasing the ability of the indirect ELISA to detect and quantify milk adulterations.

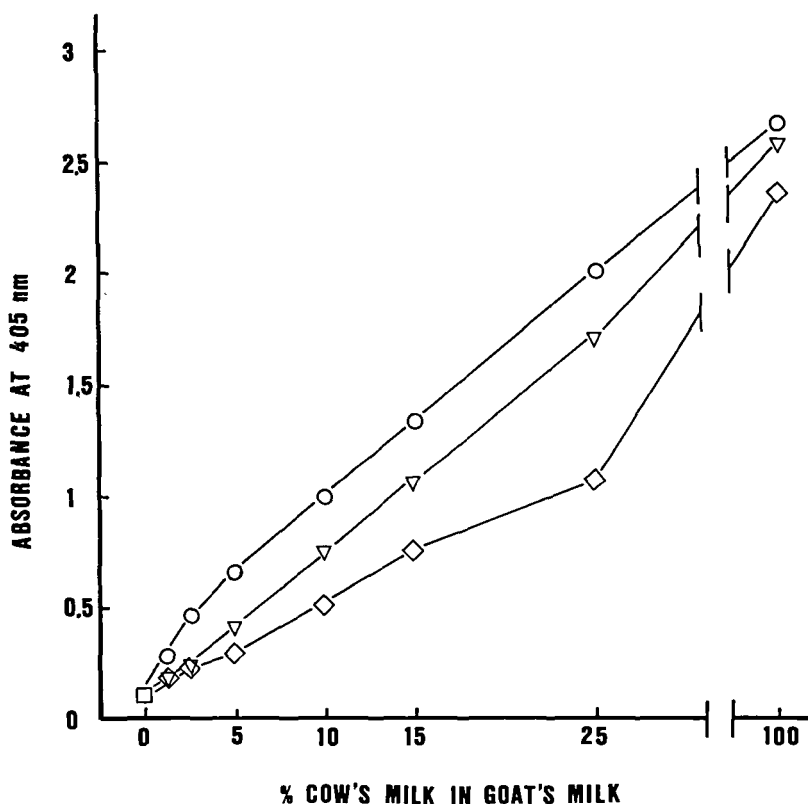


FIG. 3. Indirect ELISA response to goat's milk substitution (□) by unheated cow's milk (○), pasteurized cow's milk (△) and sterilized cow's milk (◇).

The indirect ELISA procedure described facilitates the detection and quantification of cow's milk in goat's milk differentiating 1%–25% of cow's milk in prepared milk mixtures. It should be noted that a large difference in the absorbance values was achieved within 1%–15% substitution of caprine milk by bovine milk. This resolution is very important for the detection and quantification of small amounts of bovine milk in caprine milk, since a problem currently concerning dairy factories is the detection of small amounts of milk from different animal species in batches which had been declared to be derived from one single species.

The indirect ELISA developed in this work is more sensitive than some reported chromatographic (Iverson & Sheppard, 1989), electrophoretic (Pierre & Portman, 1970; Ramos *et al.*, 1977; Amigo *et al.*, 1989) and immunological methods (Durand *et al.*, 1974; Leveux, 1977; García *et al.*, 1989), being in the range of the results reported by others (Leveux, 1980; Aranda *et al.*, 1988; Kaiser & Krause, 1985; Addeo *et al.*, 1989; Rispoli & Saugues, 1989; García *et al.*, 1990, 1991; Rodríguez *et al.*, 1990).

The indirect ELISA test as described here using blocked biotinylated anti-BWP antibodies offers a fast, accurate and possibly inexpensive method for milk species

identification. Nevertheless, a limiting factor for application of this technique in the dairy industry is the time-consuming isolation of the anti-BWP antibodies, since careful adsorption procedures need to be carried out in order to render the antiserum species-specific. Instead, adoption of enzyme immunoassays with the use of milk species-specific monoclonal antibodies would cut the cost of obtaining the immunological reagents and would permit its commercialization into kits containing all necessary reagents for species verification of milk in large or small scale inspection programmes.

ACKNOWLEDGEMENTS

This work is supported by Grant AL188-0111 from the Comisión Interministerial de Ciencia y Tecnología (CICYT). Cristina Castro, Teresa García, Elena Rodríguez and Isabel González are recipients of Fellowships from the Ministerio de Educación y Ciencia, Spain. We are grateful to the Cooperative Castellana de Ganaderos, Campo Real, Madrid, for their generous gift of the raw milk samples.

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