



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: T. Garcíá, R. Martín, E. Rodríguez, P. Morales, I. González, B. Sanz & P. E. Hernandez (1994) Detection of goats' milk in ewes' milk by an indirect elisa, Food and Agricultural Immunology, 6:1, 113-118, DOI: [10.1080/09540109409354819](https://doi.org/10.1080/09540109409354819)

To link to this article: <http://dx.doi.org/10.1080/09540109409354819>

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Detection of Goats' Milk in Ewes' Milk by an Indirect ELISA

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(Received for publication 12 September 1993)

An indirect ELISA has been developed successfully for the detection of defined amounts of goats' milk (1–100%) in ewes' milk. The assay uses polyclonal antibodies against goats' whey proteins (GWP) raised in rabbits. The anti-GWP antibodies were recovered from the crude antiserum by immunoadsorption and elution from a column containing immobilized GWP. The anti-GWP antibodies were biotinylated and rendered goats' milk-specific by mixing them with lyophilized cows' and ewes' whey proteins. ExtrAvidin–peroxidase was used to detect the specific anti-GWP antibodies bound to goats' milk proteins immobilized on 96-well plates. Subsequent enzymic conversion of substrate resulted in discernible differences in optical density between mixtures of ewes' milk containing variable amounts of goats' milk.

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

INTRODUCTION

The production of ewes' and goats' milk has considerable economic importance resulting from the widespread acceptance of traditional cheeses, many made exclusively of pure ewes' milk. Food inspectors are responsible for assuring consumers that the milk and dairy products they purchase are safe, unadulterated and accurately labelled. Unfortunately, fraudulent substitution of ewes' milk by the cheaper goats' milk is a common practice, and it is not always easy to detect and quantify.

A number of research groups have proposed different methods for detection of goats' milk in ewes' milk. Electrophoretic techniques include polyacrylamide gel electrophoresis (PAGE) (Amigo *et al.*, 1989) and isoelectrofocusing (IEF) (Ruiz & Santillana, 1986), while

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the most commonly used immunological technique is radial immunodiffusion (RID) proposed by Leveux (1977). The three methods have been compared (Amigo *et al.*, 1989, 1992) and they are reliable and comparable from a qualitative point of view. However, while RID has the disadvantage of being semiquantitative, PAGE and IEF allow quantification but require equipment and specially trained staff.

The ELISA is a widely used form of immunoassay for measuring components of foods that are capable of acting as antigens. This method can be applied to species identification to determine whether adulteration has taken place. (Morris & Clifford, 1985; Newsome, 1986; Rittenburg, 1990; Morgan *et al.*, 1992). An advantage of ELISA methods over conventional approaches is cost-effectiveness, which stems from the inherent simplicity of the procedure. Once the antiserum has been developed, the rest of the procedure may be readily automated. Moreover, the immunoassay formats can be designed to meet different needs—either qualitative assays for field use or accurate quantitative procedures.

In this paper the use of blocked biotinylated antibodies against goats' whey proteins (GWP) and the development of an indirect ELISA to detect and quantify the presence of goats' milk in ewes' milk are reported.

MATERIALS AND METHODS

Preparation of the Antigenic Extracts

Fat was removed from goats' milk by centrifugation at $13\,000 \times g$ for 10 min and filtration through glass wool. Skimmed milk was heated to 45°C and acidified to pH 4.47 with 0.2 M-HCl. The acidified milk was kept at 40°C for 30 min before centrifugation 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% (w/v) sodium azide) and lyophilized. The dried whey proteins were placed in an air-tight container and stored at -20°C until use.

Production of Caprine Milk-specific Antibodies

Polyclonal antibodies against GWP were raised in male New Zealand White rabbits. Immunization was initiated by subcutaneous injection of 1 mg lyophilized GWP emulsified in 1 ml of Freund's complete adjuvant (Difco Laboratories, Detroit, MI, USA) and 1 ml of deionized and distilled water at multiple sites along the rabbits' backs. Ten booster doses made in Freund's incomplete adjuvant were applied subcutaneously every 6 days. After 70 days the rabbits were bled and the blood was allowed to clot for 1 h at room temperature. Serum was collected by centrifugation at $2000 \times g$ for 10 min. Samples of crude antiserum (1 ml) were stored frozen at -20°C .

Antibodies against GWP were recovered from the crude antiserum by immunoabsorption chromatography. Their isolation was carried out by passing 15 ml samples of crude antiserum, diluted 1:2 with $2 \times$ concentrated PBS, pH 7.2 containing 0.5% Tween 80, through a CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden). This column contained 9 g of freeze-dried powder coupled, according to the manufacturer's instructions, to 158 mg of the lyophilized GWP. Antibodies against GWP (anti-GWP) were released from the 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.

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-GWP) 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.

Blocking of the Biotinylated Antibodies

The biotinylated anti-GWP antibodies diluted in PBS containing 5% Tween 20 (PBST) were rendered goats' milk-specific by mixing them with lyophilized cows' and ewes' whey proteins (1.3 mg of each per ml of biotinylated antibodies) and maintained overnight at 4°C before use in the ELISA experiments.

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 goats' and ewes' milk (1 ml) were also pasteurized (72°C , 15 s) or sterilized (121°C , 20 min).

Indirect ELISA

Flat-bottomed micro-ELISA plates (Costar, Cambridge, MA, USA) were filled with 0.1 ml of the milk samples diluted 1:50 in PBS, pH 7.2, and incubated for 1 h at 37°C . The wells were washed five times with PBST and coated with 0.2 ml of 0.1% gelatin in PBS for 30 min at 37°C . After five washes with PBST, 0.1 ml of the blocked biotinylated anti-GWP antibodies (0.55 mg ml^{-1}) diluted 1:25 in PBST was added to the wells and the plates incubated on a plate shaker (LKB, Pharmacia, Uppsala, Sweden) for 1 h at 20°C . After washing five more times with PBST to remove heterologous antigens and unattached antibodies, 0.1 ml of ExtrAvidin-peroxidase (Sigma), 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, $0.2 \mu\text{l ml}^{-1}$ 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

Polyclonal antibodies raised in rabbits against GWP have been used to detect and quantify the presence of goats' milk in ewes' milk by an indirect ELISA. To recover those antibodies specific for goats' whey proteins, samples of the crude antiserum were circulated through a column containing GWP coupled to CNBr-activated Sepharose 4B, followed by elution of the adsorbed antibodies off the column. Immunodiffusion experiments in agarose gels demonstrated that the anti-GWP antibodies released from the column gave reactions of identity not only against goats' milk, but also against ewes' and cows' milk (results not shown). This cross-reactivity was expected because the immunopurification step recovered antibodies that recognize whey proteins of goats' milk but also epitopes in the same proteins from closely related species.

The anti-GWP antibodies released from the affinity column were conjugated to biotin to amplify the antigen-antibody reaction in the indirect ELISA. Although the biotinylation

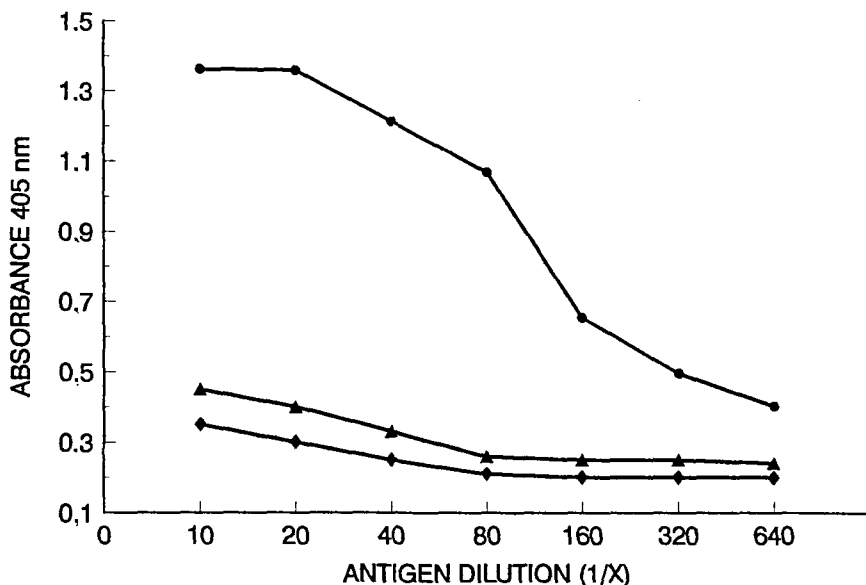


FIG. 1. Indirect ELISA results for the identification of goats' (●), ewes' (▲) and cows' (◆) milk using the blocked biotinylated antibodies diluted 1:25.

step could be performed directly with the crude antiserum, the use of affinity purified antibodies eliminated the binding of biotin to other proteins present in the antiserum.

Once biotinylated, the anti-GWP antibodies were rendered goats' milk-specific by mixing them with lyophilized ovine and bovine whey proteins in a process known as 'blocking'. The blocking treatment is an effective procedure to remove cross-reactions (Durand *et al.*, 1974; Jones & Patterson, 1986; García *et al.*, 1990; Rodríguez *et al.*, 1991) without the need for affinity purification through columns containing the heterologous antigens in a bound form. In the indirect ELISA, GWP bound to the wells of a microtiter plate were detected with the biotinylated, blocked anti-GWP antibodies. After the desired antigen-antibody reactions took place in the wells, the washing steps remove the remaining heterologous antigens and the products of the blocking interaction. Further recognition of the biotinylated anti-GWP antibodies bound to the wells was carried out with ExtAvidin-Peroxidase, a modified avidin reagent that combines the high specific activity of egg avidin with the low background of streptavidin (Hiller *et al.*, 1987).

Preliminary experiments to optimize the indirect ELISA suggested that the use of milk samples diluted 1:50, the blocked, biotinylated anti-GWP antibodies (0.55 mg ml^{-1}) diluted 1:25 and the ExtrAvidin-peroxidase conjugate diluted 1:3000, was the best combination of reagents required to achieve a reliable quantification of goats' milk in milk mixtures. The effectiveness of the blocking procedure may be seen in Figure 1, where the blocked, biotinylated anti-GWP antibodies effectively distinguish goats' milk from the ewes' and cows' milk.

The indirect 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 goat milk content (G) in the range 1–100% by the equation:

$$\text{Absorbance } (A_{405}) = 0.413 + 0.157 \text{ Ln } (\%G)$$

with a correlation coefficient of $r = 0.98$. This relationship could be used as a reference

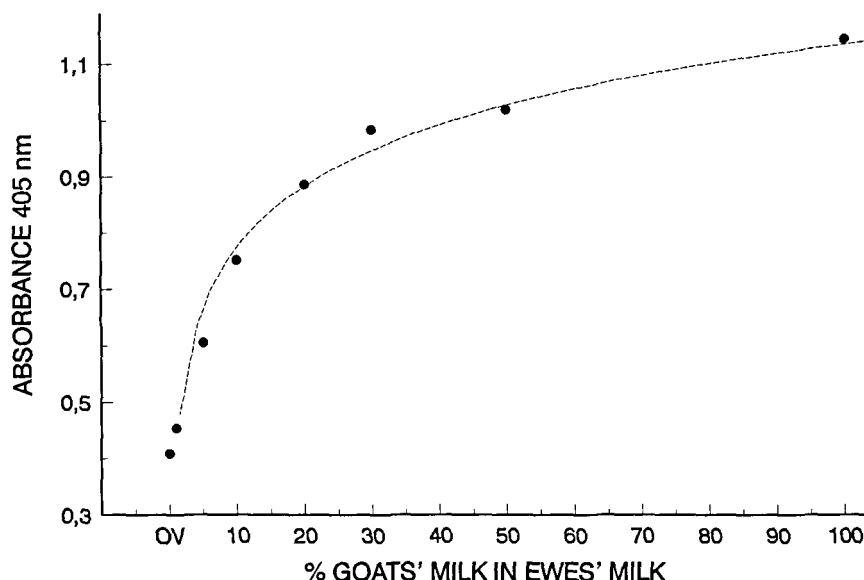


FIG. 2. Indirect ELISA response to ewes' milk substitution (▲) by goats' milk (●). Symbols represent the mean value of triplicate assays from two independent milk mixtures.

curve for evaluating the addition of goats' milk to ewes' milk. However, since the reference curve is likely to differ slightly under conditions on different days 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. It should be noticed (Figure 2) that the largest differences in the absorbance values were achieved when 1–20% of ewes' milk was substituted by goats' milk prepared in mixtures. In routine analysis this substitution is very important because a problem currently concerning the dairy industry is the detection of small amounts of goats' milk in ewes' milk.

A control experiment was performed to measure the response of the specific anti-GWP against goats' milk samples subjected to different heat treatments. Even though whey proteins are not as heat resistant as caseins (Calvo *et al.*, 1989; Calvo & Olano, 1992) and their denaturation might decrease the sensitivity of the immunological procedures, there were no meaningful differences in the absorbance values of the unheated, pasteurized and sterilized samples when assayed by the indirect ELISA described above (Table 1).

The indirect ELISA developed for the identification and quantification of goats' milk in ewes' milk is more sensitive than some electrophoretic, immunological and chromatographic methods reported (Assenat, 1967; Ruiz & Santillana, 1986; Amigo *et al.*, 1989;

TABLE 1. Results of the indirect ELISA for the identification of goats' and ewes' milk subjected to different heat treatments

Antigen dilution	Goats' milk			Ewes' milk		
	Unheated	Pasteurized	Sterilized	Unheated	Pasteurized	Sterilized
1:25	1.430	1.389	1.376	0.463	0.468	0.456
1:50	1.299	1.268	1.291	0.422	0.386	0.412
1:100	1.156	1.040	0.999	0.373	0.306	0.319

Iverson & Sheppard, 1989), being in the sensitivity range reported by others (Levieux, 1978; Rodriguez *et al.*, 1991). The ELISA test described here using blocked, biotinylated anti-GWP antibodies is easy to perform, sensitive and specific. There are no obvious barriers for its commercial production in the form of stable kits, containing all the necessary components and reagents for milk species identification in dairy factories or inspective controls.

ACKNOWLEDGEMENTS

This work was supported by a grant No. ALI88-0111 from the Comisión Interministerial de Ciencia y Tecnología (CICYT). T. G. was the recipient of a Fellowship from the Ministerio de Educación y Ciencia, Spain. We are grateful to the Cooperativa Castellana de Ganaderos, Campo Real, Madrid, for its generous gift of the raw milk samples.

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