

Rocket immunoelectrophoresis and ELISA as complementary methods for the detection of casein in foods?

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Abstract

A rocket immunoelectrophoresis (RIE) method is described and compared with a commercial ELISA kit. Thirteen food products were selected from the categories sausage, rice pudding, fruit-ice, biscuit and baby-dinner. These had all tested negative for casein in a previous investigation using an older commercial ELISA kit. In the present study, using a more recent commercially developed ELISA kit and RIE, only three of the 13 products still tested negative for casein. However, the RIE detected levels of casein in ten of the remaining products, whereas the ELISA revealed the presence of casein only in the three products giving the highest levels of casein with RIE. Thus, the two methods both confirm the presence of casein when the levels in the samples are within the range of the detection limit for the ELISA method.

Keywords: Allergy, casein, consumer protection, food safety, milk, rocket immunoelectrophoresis, enzyme-linked immunoassays.

Introduction

Cows' milk protein allergy (CMA) is defined as an immunologically mediated reaction to cows' milk proteins. The occurrence of cows' milk allergy in infancy has been variously estimated at 2–5% in developed countries. In the first year of life, up to 5–15% of infants show symptoms suggestive of cows' milk allergy or intolerance (Høst 1994, Høst 2002). Patients with CMA must avoid the ingestion of food or beverages containing these allergens. Milk-free products may become contaminated due to inadequate cleaning routines between production batches, changes in the source of ingredients, substitutions in the recipes or deliberate adulteration of a food for economic reasons.

The principle proteins in cows' milk are α_{s1} -, α_{s2} -, β - and κ -caseins (80%) and the whey proteins (20%), mainly comprising β -lactoglobulin, α -lactalbumin, bovine serum albumin (BSA) and immunoglobulins (Wal 2002). A number of these proteins

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have been identified as allergens, i.e. recognized by protein-specific IgE (Wal et al. 1995).

The European Commission (2003) has recently amended food legislation and it is now mandatory to declare those ingredients listed in the European Food Labelling Directive 2003/89/EC, and all products thereof. In order to ensure compliance with the legislation and to protect CMA patients from ingesting milk-free products that may have been contaminated, reliable and sensitive detection methods are necessary.

Caseins are relatively heat-stable milk proteins, and immunological techniques allow detection of trace amounts both before and after food processing (Hischenhuber 2001). However, in immunoassays, antibodies can cross-react with other analytes present in a food matrix. Hence, to establish the credibility of results, two methods should be used to confirm positive findings.

To investigate the advantages of using a validated rocket immunoelectrophoresis (RIE) or a commercial ELISA kit as methods to confirm the presence of casein in foods, we present a small study of casein in selected food products intended for CMA patients. The results of both methods are qualitatively and quantitatively compared and discussed.

Material and methods

Food samples and food products

In a previous national study on casein in food products intended for CMA patients, 150 products, including sausage, rice pudding, fruit ice, biscuit and baby dinners had been purchased from local food stores in Oslo and analysed with an ELISA kit, commercially available on the market in 2000 (the manufacturer at that time declared a lower limit of detection (LOD) of 2000 mg kg^{-1}). For the present study, we selected 13 of the food products which in the previous study tested negative for casein. Products from all the previously studied matrix groups were included.

Reagents and equipment

Polyclonal rabbit anti-bovine casein antibody was supplied by Pharmacia Diagnostics, Uppsala, Sweden. Tris, glycine, agarose IEF, agarose M and GelBond films were all obtained from Amersham Biosciences, Uppsala, Sweden. PEG 8000 and Hammersten casein were products of USB, Cleveland, OH, USA. EZBlue colloidal Coomassie Blue staining solution was obtained from Sigma Aldrich, Steinheim, Germany. Bovine serum albumin (BSA, IgG- and LPS-free) was obtained from Sigma, St Louis, MO, USA. Goats' milk produced by TINE BA, Norway, was purchased from a local supermarket. Ewes' milk was a gift from André Mo-Gregersen, Norway. Total protein was determined using the DC Protein Assay (Bio-Rad, Hercules, CA, USA). The protein content of ewes' and goats' milk, used for testing cross-reactivity, was measured by using BSA as a standard. An indirect competitive casein ELISA kit, product no. 902062W was supplied by Tepnel, Flintshire, UK. Samples were sonicated in a Branson 3200 ultra-sonic bath and centrifuged at $39200 \times g$. Electrophoresis was carried out using a horizontal electrophoresis unit with a cooling plate (Multiphor II, Amersham Pharmacia Biotech, Uppsala, Sweden.) Absorbance

was read at 450 nm on a VICTOR² 1420 multilabel plate counter (Wallac, Turku, Finland).

Rocket immunoelectrophoresis

Preparation of antibody-containing gel and buffer strips

The rocket immunoelectrophoresis was performed after Laurell (1966) with some modifications as described by Eriksson et al. (1992), and by Yman et al. (1994). Agarose buffer strips were prepared at least one day before use by dissolving Agarose IEF (2%, w/v) in 0.25 M Tris/0.5 M glycine buffer, pH 8.7, in a boiling water bath. After 30 min gelation, the buffer strips were stored at 4°C. Agarose M (1.2%, w/v) and PEG 8000 (polyethylene glycol, 3% w/v) were added to 0.1 M Tris/0.5 M glycine buffer, pH 8.7. After heating in a boiling water bath (7–8 min), the solution was allowed to cool to 60°C in a water bath before the antibody was added. A 1/300 dilution of rabbit anti-casein antiserum was used for the detection of casein in the range of 13–150 mg kg⁻¹. The gels were cast on GelBond films (84 mm × 94 mm) placed with the hydrophilic side up on a levelling table, to ensure a uniform gel thickness (1 mm). After gelation, the gels were kept in a humidified chamber overnight at 4°C, in order to allow the gel structure to develop. Gels and buffer strips could be stored at 4°C for up to one month.

Electrophoretic procedure

Buffer strips were 1 cm wide at the top and bottom edges of the gel. Sample extracts (4 µl) were pipetted into the wells of the antibody-containing gel immediately prior to starting electrophoresis, in order to prevent diffusion of the sample. Electrophoresis was performed for 2 hours at 150 V, 75 mA, 7 W, and with cooling. Immediately after the electrophoretic run, non-precipitated proteins were removed by washing, and the gel was pressed for 30 min to reduce its thickness and again washed for 30 min. The gel was then dried, stained overnight with colloidal Coomassie Blue staining solution and finally washed with water, 2 × 10 min, before drying again.

LOD was chosen as suggested by Standard Operation Procedure from the Swedish National Food Administration as the amount of casein producing a peak height of approximately 2 mm. The limit of quantification (LOQ) was determined by making buffer solutions with known amounts of casein. The lowest concentration which gave a confirmed rocket (4–6 mm) was chosen as the LOQ. The precision was performed by doing at least 10 independent analyses of some selected samples. The uncertainty of the trueness/bias was performed by doing recovery determinations of different spiked matrixes.

Protein extraction and food sample preparation

The samples (2 g) were homogenized in a mechanical blender and then extracted using 10 ml 0.1 M Tris/0.5 M glycine buffer, either overnight in a shaking water bath at 45°C or for 2 hr in an ultra-sonic bath (Standard Operation Procedure from the Swedish National Food Administration). The extracted mixtures were then centrifuged at 39 200 × g at 4°C for 30 min. Fat and larger particles from the matrix were removed by filtration through glass wool.

Standard curves and recovery studies

Hammersten casein was used as the standard, both for method validation (to determine recovery rates) and for quantification. Casein was either diluted with extraction buffer (0.1 M Tris/0.5 M glycine) or relevant food matrixes (which contained less casein than the LOD for the RIE method) to give concentrations between $2.5 \mu\text{g ml}^{-1}$ and $30 \mu\text{g ml}^{-1}$. Linear regression of peak height vs. μg casein standard was used to produce a standard curve to quantify the amount of casein in the sample.

Recovery studies were performed using relevant food samples which had been artificially spiked with casein standard at levels between 2.5 mg kg^{-1} and 50 mg kg^{-1} .

The reported uncertainty of measurement was calculated with 95% level of confidence, according to International Standardization Organization (ISO 1993).

Casein ELISA

The samples (2 g) were extracted in a 0.05 M sodium carbonate/0.05 M sodium bicarbonate buffer (18 ml), pH 9.6 and diluted 1:50 according to the manufacturer's instructions. Biotinylated casein was detected by means of an avidin-peroxidase conjugate, using 3,3',5,5'-tetramethylbenzidine dihydrochloride (TMB) as substrate. Absorbance for unknown samples was read from a standard curve and casein content (mg kg^{-1}) calculated after correction for extraction/dilution. According to the manufacturer, the LOQ was determined by the lowest standard 50 ng ml^{-1} which is equivalent to 25 mg kg^{-1} at a standard dilution, i.e. 1/10 extraction and 1/50 dilution. LOD was declared to be 5 mg kg^{-1} , interpolated concentration at OD 450 nm of the zero standard ($n=3$) minus three standard deviations. In the present study, however, LOD was calculated based on eight instead of three parallels of the zero standard.

Results*Rocket immunoelectrophoresis*

The anti-bovine casein antibody cross-reacted with proteins from ewes' and goats' milk (see Figure 1) but not with other food constituents or potential allergens, such as BSA, β -lactoglobulin, myoglobin, ovalbumin, hazelnut extract or peanut extract.

A rocket peak height of approximately 2 mm corresponded to a casein concentration of $0.5 \mu\text{g ml}^{-1}$, giving an LOD of 2.5 mg kg^{-1} . The lowest casein concentration which gave a defined rocket ($2.5 \mu\text{g ml}^{-1}$), implied an LOQ of 13 mg kg^{-1} (see Figure 2, lane 5), and the uncertainty of measurement was calculated as 50%.

The standard curves prepared in food matrixes extracts were not superimposable on those prepared in buffer (see Figure 3). Recoveries in the range of 99–111% and a CV <10% were obtained from biscuits spiked with $13\text{--}50 \text{ mg kg}^{-1}$ casein. Spiked samples of sausage, fruit-ice, and margarine gave similar results.

Using single applications of five different standard concentrations, standard curves with Standard Error of Mean (SEM) values did not exceed $\pm 0.55 \mu\text{g ml}^{-1}$ (buffer), $\pm 0.63 \mu\text{g ml}^{-1}$ (sausage), $\pm 1.13 \mu\text{g ml}^{-1}$ (biscuit) and $\pm 1.5 \mu\text{g ml}^{-1}$ (fruit-ice) (not shown in Figure 3).

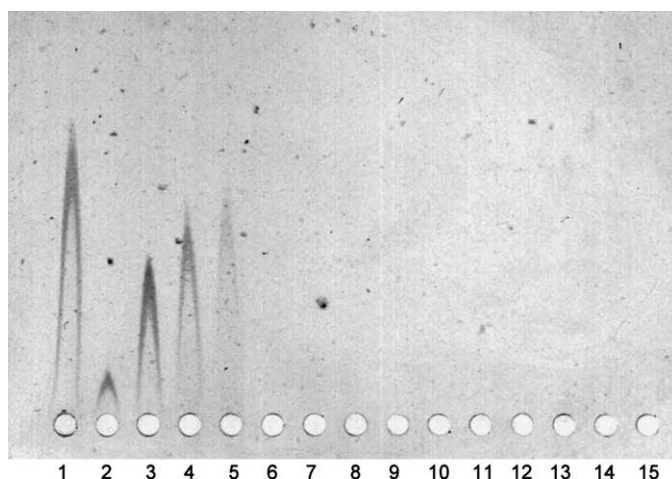


Figure 1. Cross-reactivity studies of food components with Coomassie Blue-stained rocket immunoelectrophoresis precipitates. Samples and standards were applied at 4 μl /well. In lane 1–2: standard protein 30 and 2.5 $\mu\text{g/ml}$. Number in brackets represent total protein; lane 3: cows' milk (13 $\mu\text{g ml}^{-1}$); 4: ewes' milk (13 $\mu\text{g ml}^{-1}$); 5: goats' milk (13 $\mu\text{g ml}^{-1}$); 6: BSA (13 $\mu\text{g ml}^{-1}$); 7: β -lactoglobulin (50 $\mu\text{g ml}^{-1}$); 8: β -lactoglobulin (10 $\mu\text{g ml}^{-1}$); 9: myoglobin (50 $\mu\text{g ml}^{-1}$); 10: myoglobin (10 $\mu\text{g ml}^{-1}$); 11: ovalbumin (13 $\mu\text{g ml}^{-1}$); 12: hazelnut (25 $\mu\text{g ml}^{-1}$); 13: peanut (30 $\mu\text{g ml}^{-1}$); 14/15: blank.

Eight out of the 13 different food products analysed contained levels of casein from 16–320 mg kg^{-1} and two contained traces between the LOD and the LOQ (2.5–<13 mg kg^{-1} (see Table I)).

ELISA

Using our own criteria for the determination of LOD using eight replicates instead of three for the zero standard, the LOD was 25 mg kg^{-1} , which was similar to the LOQ

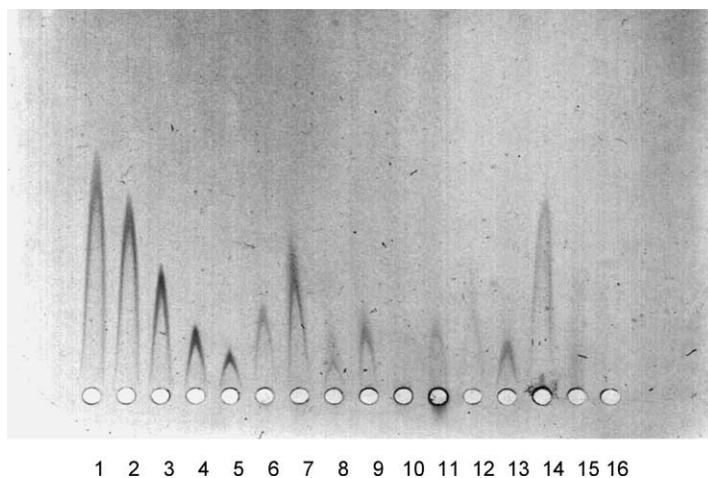


Figure 2. Rocket immunoelectrophoresis precipitates, stained with Coomassie Blue. In lanes 1–5: standard protein 30, 20, 10, 5 and 2.5 $\mu\text{g ml}^{-1}$, respectively; lane 6: sausage; 7 & 8: rice pudding; 9 & 10: biscuit; 11: sausage; 12–14: fruit ice; 15: baby food; 16: blank.

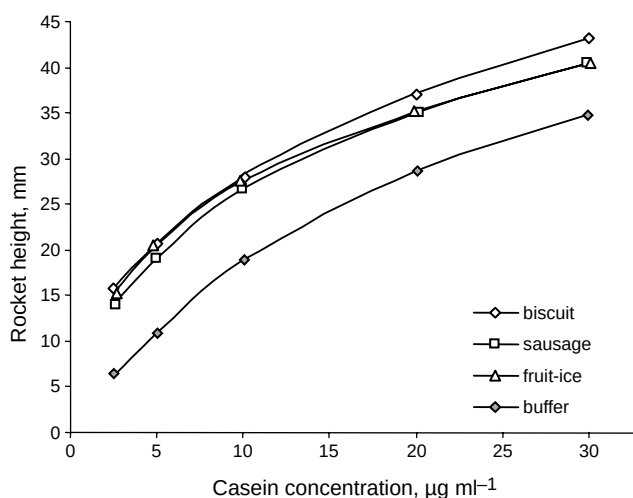


Figure 3. Casein standard curves for various matrices: Extraction buffer, sausage, biscuit and fruit-ice (ice-lolly). SEM values did not exceed $\pm 0.55 \mu\text{g ml}^{-1}$ (buffer), $\pm 0.63 \mu\text{g ml}^{-1}$ (sausage), $\pm 1.13 \mu\text{g ml}^{-1}$ (biscuit) and $\pm 1.5 \mu\text{g ml}^{-1}$ (fruit-ice) (not shown on the figure).

stated in the manufacturer's instructions. Eight of the 13 food products tested by RIE were found to contain casein above the LOD (see Table I). In three of these, casein was also quantified by ELISA, giving, respectively 320, 135 and 33 mg kg^{-1} (RIE), and 65, 119 and 61 mg kg^{-1} (ELISA). However, in the remaining five products found to contain 16, 21, 18, 26 and 33 mg kg^{-1} using RIE, casein was not detected by ELISA. Thus, the estimated LOD of 25 mg kg^{-1} determined in our laboratory by using eight replicates of the zero standard appears too low.

Discussion

The specificity test of the antibody used in the RIE showed cross-reactivity against ewes' and goats' milk. According to the manufacturer, the antibodies used in the

Table I. Analysis of various food products for casein with RIE (detection limit 2.5 mg kg^{-1}) compared to ELISA (claimed detection limit 5 mg kg^{-1}).

Food product	Milk protein declared	Results RIE mg kg^{-1}	Results ELISA mg kg^{-1}
sausage 1	No	320	65
sausage 2	No	nd	nd
sausage 3	No	21	nd
rice pudding 1	No	d*	nd
rice pudding 2	No	135	119
fruit-ice 1 (ice-lolly)	No	18	nd
fruit-ice 2 (ice-lolly)	No	33	nd
fruit-ice 3 (ice-lolly)	No	16	nd
fruit-ice (carton)	No	93	61
biscuit 1	No	nd	nd
biscuit 2	No	26	nd
baby food 1 (jar)	No	d*	nd
baby food 2 (jar)	No	nd	nd

nd =not detected, d* =detected below quantification limit.

ELISA kit also react to a small extent to casein from sheep, goats and buffalo. The α -caseins from cows', goats' and ewes' milk share more than 85% identical amino acids. According to Spuergin et al. (1997) and Muñoz et al. (2004) these species should not be recommended as substitutes for cows' milk for CMA patients. No cross-reactivity was found between the casein antibody used in the RIE and BSA, β -lactoglobulin, myoglobulin, ovalbumin, hazelnut extract and peanut extract. The ELISA kit reacts predominantly with α -casein and has been standardized using casein, giving a very similar response.

The Standard Operation Procedure from the Swedish National Food Administration included a centrifugation rate of $11000 \times g$. By increasing this to $39200 \times g$ we obtained the removal of particles which had previously necessitated a greater extract dilution prior to the RIE run. This procedure may explain the lower LOD achieved.

Standard curves prepared in blank matrix extracts were not superimposable on those prepared in buffer which demonstrates the importance of using matrix-dependent standard curves. The height of the rocket increases using matrix-dependent standard curves. This effect makes it possible to further increase the sensitivity of the method (Holzhauser et al. 1998). However, this has not been attempted in the present study. The importance of using standard curves in the representative food matrix has earlier been demonstrated for ELISA (Yeung & Collins 1996) and RIE (Holzhauser et al. 1998).

The LOD/LOQ of the RIE method was lower than the presently used ELISA kit. Commercial ELISA kits are continuously being improved by the manufacturers, and the currently available kit claims a LOQ of 1.6 mg kg^{-1} . However, this is still in the same range as the presently presented RIE method, and the two methods can still be used to confirm each other. Similarly, the RIE method can be used to confirm the casein analyses from an indirect ELISA recently developed in our laboratory; with a LOD of $<1 \text{ mg kg}^{-1}$ (Sletten et al. unpublished data). A recently developed sandwich ELISA has also been described, with a claimed detection limit of 0.5 mg kg^{-1} (Hefle & Lambrecht 2004).

Thus, even though the RIE method has the disadvantage of being somewhat time-consuming, the performance is still comparable to the ELISA kit currently commercially available, and the stability of the reagents was comparable to that of ELISA reagents.

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