

A comparison of time-resolved fluoroimmunoassay and ELISA in the detection of casein in foodstuffs

G. B. SLETTEN, K. E. LØVBERG, L. H. MOEN, H.-J. SKARPEID, & E. EGAAS

National Veterinary Institute, Oslo, Norway

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Abstract

With the aim of lowering the detection limit for casein in foods, three competitive assays are described: direct time-resolved fluoroimmunoassay (TR-FIA), using europium-conjugated antibody, indirect TR-FIA, using biotinylated antibody with europium-conjugated streptavidin and ELISA, using a HRP-conjugated secondary antibody. Food samples (instant potato, flour mix, packet soup, spice-mix) were analysed. Standard curve sensitivities in direct and indirect TR-FIAs did not differ significantly ($p=0.097$), but both TR-FIAs were considerably less sensitive (both $p<0.0001$) than ELISA (LOQs 1.3, 1.5 and $<1.0 \text{ mg kg}^{-1}$, respectively). The precision and working analyte range was similar in all three methods. Casein content measured in food products was comparable, using the three assays and rocket immunoelectrophoresis. The TR-FIA approach provided no improvement over the ELISA. All three assays allowed quantification of casein in foodstuffs in the order of $1\text{--}1.5 \text{ mg kg}^{-1}$, providing a basis for more rigorous validation and collaborative testing.

Keywords: Casein, cow's milk allergy, food allergens, enzyme-linked immunosorbent assay, milk, time-resolved fluoroimmunoassay.

Introduction

Cow's milk allergy (CMA) occurs in approximately 2–5% of infants in developed countries; a further 5–15% may show symptoms suggestive of cow's milk allergy or intolerance (Høst 2002). The principle proteins in cow's milk are the caseins (80% total protein) and the whey proteins (20%), of which β -lactoglobulin (β -LG) is the most abundant (10%) (Wal 2002). All the common milk proteins have been implicated in CMA, as shown by the presence of specific IgE in the sera of CMA patients (Wal et al. 1995).

Exposure to very small amounts of the offending protein may be sufficient to initiate allergic reactions, but at the present time, insufficient evidence exists to establish threshold levels for milk allergens. Reactions to less than 8 mg casein (Gern et al. 1991) and to 120–180 μg of whey protein (Laoprasert et al. 1998) and a lethal case of anaphylaxis to around 60 mg casein (Malmheden Yman et al. 1994) have been documented, illustrating the risk of inadvertent exposure to CMA sufferers, but such

Correspondence: Gaynour B. Sletten, National Veterinary Institute, PO Box 8156 Dep., N-0033 Oslo, Norway. Tel: +47 23 21 62 63. Fax: +47 23 21 62 01. E-mail: gaynour-b.sletten@vetinst.no

anecdotal experience is unsuited to the determination of threshold values. Clinical evidence from double-blind, placebo-controlled food challenges provides better estimates of threshold dose; the lowest provoking dose for cow's milk was observed at 0.6 mg protein, corresponding to 0.02 ml milk (Poms *et al.* 2004, Taylor *et al.* 2002).

Patients with CMA must avoid the ingestion of foodstuffs or beverages containing cow's milk proteins, but there are several reasons why this may be problematic. Food products may be incorrectly or inadequately labelled, or initially allergen-free foods may be contaminated during processing due to inadequate cleaning routines, changes in the source of ingredients, substitutions in the recipes or deliberate adulteration for economic reasons (Poms *et al.* 2004). At present, insufficient information is available for establishing maximum acceptable levels of allergens in foodstuffs: general agreement places the detection limits for different foods between 1 and 100 mg kg⁻¹, depending on food type (Poms *et al.* 2004, Taylor *et al.* 2002).

Immunological assays have been used in the detection of cow's milk proteins in foodstuffs; highly sensitive assays for traces of β -LG in infant feeding formulas have been described (Makinen-Kiljunen and Palosuo 1992, Mariager *et al.* 1994) but assays for casein in foodstuffs are more appropriate, as caseins are more abundant in milk and have greater heat-stability, more easily retaining their immunogenicity after processing (Hischenhuber 2001). Previously described competitive enzyme-linked immunosorbent assays (ELISA) for bovine caseins, used for detecting the fraudulent addition of cow's milk to sheep or goat's milk, had limits of detection (LOD) for casein in excess of 30 mg kg⁻¹ (Anguita *et al.* 1997, Rolland *et al.* 1993). A rocket immunoelectrophoresis method has been described with a LOD for casein of 2.5 mg kg⁻¹ (Moen *et al.* 2005, but this method, while exceeding the performance of a commercially available casein ELISA kit (LOD 5 mg kg⁻¹), is relatively labour-intensive. Hefle and Lambrecht (2004) have recently described a sandwich ELISA for casein with a detection limit of 0.5 mg kg⁻¹. However, the competitive format has the advantage over sandwich assays of being a single-epitope assay, enabling smaller proteolytic allergen fragments to be detected.

Time-resolved fluoroimmunoassay (TR-FIA) technology, based on the detection of fluorescence from lanthanide dissociation, has been suggested as more sensitive than enzyme-based or fluorescence immunoassays (Dickson *et al.* 1995, Jackson and Ekins 1986). Extended decay times, long Stokes' shifts and high fluorescence intensities, particularly of europium, increase assay signal: noise ratio. An additional advantage of TR-FIA is the use of multiplex assays, in which antibodies with differing specificities are labelled with different lanthanides, e.g., europium (Eu), samarium (Sm) and terbium (Tb). Lanthanides emit fluorescence at different wavelengths, thus allowing simultaneous determination of up to four analytes.

The aim of this study was to lower the detection limit for casein in foodstuffs, by means of TR-FIA, using an in-house polyclonal antiserum against total bovine casein. Polyclonal antisera have the advantage of recognizing multiple epitopes and thus may bind both to the native casein complex and novel casein fraction epitopes exposed during food processing; their superiority over monoclonal antibodies has been demonstrated in casein ELISA (Plebani *et al.* 1997). Direct and indirect competitive TR-FIAs and a competitive ELISA have been developed and compared in the detection of casein in foodstuffs.

Materials and methods

Reagents and buffers

Carbonate/bicarbonate buffer capsules, Tween-20, Tween-40, ethylenediaminetetraacetic acid (EDTA), Tris-HCl, diethylene-triaminepentaacetic acid (DTPA), α -, β - and κ -caseins, β -LG, ovalbumin, ovomucoid, fish gelatine, equine myoglobin and biotinamidocaproic acid 3-sulfo-NHS ester were all obtained from Sigma-Aldrich, Steinheim, Germany. Electrophoresis grade bovine serum albumin (BSA), IgG-free BSA and bovine γ -globulin were purchased from Sigma, St Louis, MO, USA. Hammersten casein was used as antigen for immunization and as the standard in all the assays (USB, Cleveland, OH, USA). Human serum albumin was obtained from Calbiochem (La Jolla, CA, USA) and extracts of beef and lamb from Tepnel Bio-Systems (Deeside, UK).

Phosphate-buffered saline (PBS), pH 7.4 was prepared from PBS tablets (Oxoid, Basingstoke, UK). Buffer capsules were used to prepare carbonate/bicarbonate coating and sample extraction buffer, pH 9.6. Washing buffer consisted of PBS+0.05% Tween-20. Blocking buffer contained 1% electrophoresis grade BSA in PBS or 1% gelatine in some cross-reactivity experiments. Twenty mM EDTA in blocking buffer was used as ELISA assay buffer. TR-FIA assay buffer consisted of 50 mM Tris-HCl with 0.5% IgG-free BSA, 0.05% bovine globulin, 0.01% Tween-40, 20 μ M DTPA and 20 μ g L⁻¹ amaranth (Fluka, Buchs, Switzerland). Horse-radish peroxidase-labelled goat anti-rabbit IgG (GAR-HRP) was used as secondary antibody in the ELISA (Zymed, San Francisco, CA, USA), detected using 3,3',5,5'-tetramethylbenzidine dihydrochloride substrate Enhanced K-Blue TMB (Neogen, Lexington, KY, USA). DELFIA/autoDELFIA enhancement solution and Eu-labelled streptavidin were used in the TR-FIAs (Wallac, Turku, Finland).

Antibody production, purification and labelling

Rabbit serum was obtained from female Chinchilla rabbits K898, immunised subcutaneously with Hammersten casein in PBS, emulsified with Freund's complete adjuvant (1:1). Boosters of casein in incomplete Freund's adjuvant (1:1) were given at four-week intervals for three months. Antigen purity was confirmed with SDS-PAGE and Western blot, using chicken antibodies to bovine α -, β - and κ -casein, and β -LG (kind gift from AgResearch, Ruakura, NZ). K898 serum was desalted on a PD-10 column and purified by affinity chromatography on a casein-coupled NHS-activated column (both Amersham Biosciences, Uppsala, Sweden), eluted using 100 mM ethanolamine (BDH, Poole, UK). The antibody was concentrated in PBS, using Vivaspın 6 concentrators, 10 000 MWCO (Vivascience, Hannover, Germany). Total protein was determined using the DC Protein assay (Bio-Rad, CA, USA), based on the method of Lowry (Lowry et al. 1951) and the concentration adjusted to 1 mg/ml. Purified K898 antibody was covalently conjugated to a europium chelate (N1-ITC-Eu) (Wallac, Turku, Finland). Biotinylation was carried out in carbonate buffer, on a rotator at RT for 4 h, followed by neutralization with 1M NH₄Cl, and dialysis against PBS. The final concentration was adjusted to 1 mg ml⁻¹.

General assay procedure

Costar 9018 microtitre plates (Corning, NY, USA) were used in the ELISA and Maxisorp low fluorescence yellow plates (Nunc, Roskilde, Denmark) were used in the TR-FIAs. Plates were coated overnight at 4°C with an appropriate dilution of casein in carbonate buffer and blocked for 2 h at room temperature. Appropriate dilutions of K898 antibody/conjugate were incubated overnight at 4°C with the serially diluted standard or sample. In the secondary step, GAR-HRP was incubated for 1 h at room temperature in the ELISA and Eu-streptavidin in the indirect TR-FIA. TMB substrate or enhancement solution were added, as appropriate. Absorbance at 450 nm or time-resolved fluorescence at 615 nm (excitation 340 nm) were read on a VICTOR² 1420 multilabel plate counter (Wallac). Plates were washed after each step in a plate-washer (Skatron Instruments, Lier, Norway).

Specificity of antibody

To test for specificity of antibody binding, plates were coated with casein, β -LG or human serum albumin at 5 $\mu\text{g ml}^{-1}$. The specificity of purified K898/conjugate was confirmed using competitive ELISA, with K898 at 1/200 000 and GAR-HRP at 1/25 000. One percent gelatine in PBS was used initially as blocking/assay buffer, but as no cross-reactivity to BSA was observed, subsequently 1% BSA in PBS was used. All purified proteins and protein extracts were tested at concentrations from 1000–1.95 ng ml^{-1} : Hammerstein casein, α -, β - and κ -caseins, β -LG, ovalbumin, ovomucoid, BSA (A-7030), bovine γ -globulin, fish gelatine and equine myoglobin, protein extracts of beef and lamb (Tepnel), cod, tropomyosin, yellow pea, lupin, hazelnut, Brazil nut, almond and peanut (all prepared in-house). Hammersten casein was also tested at the same concentrations after heating at 100°C for 15, 30 and 60 min.

Indirect competitive ELISA

Checker-board titrations were carried out in order to establish optimal coating concentrations and antibody dilutions. Hammersten casein, in dilutions from 1000–7.8 ng ml^{-1} , was used for the standard curve. Optimal coat concentration was found to be 1 $\mu\text{g ml}^{-1}$, purified antibody was added at 1/300 000 and GAR-HRP was used at 1/35 000, giving OD values for maximum binding (B_0) not greater than 1.8–2.0.

Direct and indirect competitive TR-FIA

Checker-board titrations were carried out in order to establish optimal coating concentrations and antibody dilutions. Hammersten casein, in dilutions from 1000–7.8 ng ml^{-1} , was used for the standard curve. Optimal coat concentration for both direct and indirect TR-FIA was found to be 5 $\mu\text{g ml}^{-1}$. In the direct TR-FIA, Eu-labelled antibody was added at approximately 220 ng ml^{-1} , giving 500,000–700,000 counts at (B_0).

In the indirect TR-FIA, purified antibody was added at a dilution of 1/100 000 and Eu-streptavidin was used at 1/1000, giving approximately 100,000 counts at (B_0).

Sample analysis

Food samples containing low levels of casein (two brands of instant potato, flour mix), or none (packet soup, spice-mix), were selected from samples analysed previously by RIE (Egaas et al. 2002). The samples (2 g) were suspended in 18 ml carbonate buffer, vortexed for 1 min and centrifuged at $1250 \times g$ for 15 min, at room temperature. Each extract was then analysed using all three formats. Extracts were used directly or further diluted as necessary, in assay buffer.

Assay characterization

Each assay was performed six times, independently. The working range for each format was taken to be I_{20} – I_{80} (the analyte concentrations at 20 and 80% reduction in signal strength, respectively). In this study, I_{20} was considered to be the lower limit of quantification (LOQ).

Statistical analysis

CurveCalc ELISA processing software was used to analyse the standard curves and calculate the sample data (with kind permission of AgResearch, Ruakura, NZ). Standard curves were compared, using analysis of variance (ANOVA) with repeated measurements and the significance of differences between the three formats was calculated using Student's *t*-test.

Results

Total casein, α -, β - and κ -caseins competed with Hammersten casein coat protein for binding to antibody K898. Heating casein at 100°C for 15, 30 and 60 min had no effect on its ability to compete for antibody. No competitive binding was observed from bovine serum albumin, β -LG or γ -globulin, or from any of the other food proteins tested. The K898 antibody was demonstrated to be specific for all the caseins and thus suitable for use in the analysis of casein content in foodstuffs.

A comparison of the I_{20} and LOQ for the three methods showed the performance of the ELISA to be superior (see Table I), with an LOQ of $<1.0 \text{ mg kg}^{-1}$ (ppm).

Comparing each pair of standard curves at optimized conditions (see Figure 1), by Student's *t*-test, showed that:

- Direct and indirect TR-FIA were not significantly different ($p=0.097$);
- Direct TR-FIA and ELISA were significantly different ($p<0.0001$); and
- Indirect TR-FIA and ELISA were significantly different ($p<0.0001$).

Table I. Characteristics of direct and indirect TR-FIA and indirect ELISA ($n=6$ for all formats). Standard error of the mean (SEM) was calculated from six standard curves. I_{20} was taken to be the limit of quantification. Sample LOQ was corrected for dilution on extraction and expressed as mg casein per kilo foodstuff.

Methods	$I_{20} \pm \text{SEM}$ (ng ml ⁻¹)	Sample LOQ (mg kg ⁻¹)
Indirect ELISA detected with HRP	34 ± 4	<1
Direct TR-FIA detected with Eu	60 ± 6	1.3
Indirect TR-FIA detected with Eu	68 ± 6	1.5

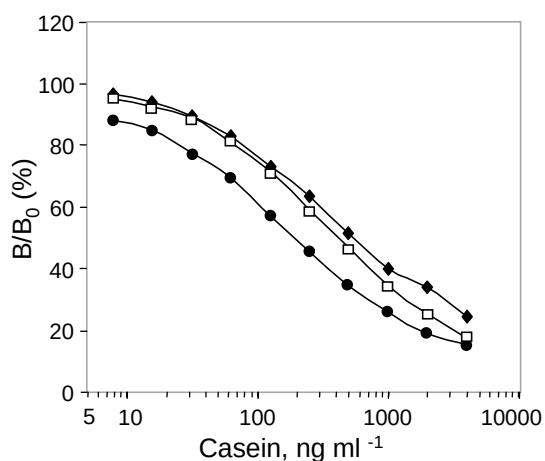


Figure 1. Standard curves for competitive immunoassays: a) direct TR-FIA (◆), b) indirect TR-FIA (□), c) ELISA (●). Each symbol represents the mean of six duplicate determinations. SEM for all values in all three assays was < 2.5 %. B₀ denotes max. antibody binding for each technique (fluorescence counts for TR-FIA, OD for ELISA). B/B₀ expresses actual bound antibody relative to B₀.

Calculated values for the assays confirmed that all three methods were applicable in approximately equal ranges of analyte concentrations and with comparable precision (see Table I).

Casein was measured in five food products, using the three immunoassays and the results were compared with those achieved using rocket immunoelectrophoresis (Egaas et al. 2002). The results for all four methods were comparable (see Table II); nevertheless, the determined values for flour and one instant potato product, in particular, differed somewhat between the direct TR-FIA and the two indirect methods. Two products were below the LOD for the RIE method, one of which (spice-mix) was also below the LODs for both TR-FIAs. The ELISA was able to quantify casein in both of these products.

Discussion

The direct and indirect TR-FIAs did not differ significantly ($p=0.097$) in their performance, having LOQs of 60 and 68 ng ml⁻¹, respectively. The high value for LOD in the direct TR-FIA reflects the much greater variation in counts observed with

Table II. Casein content in food products, measured using indirect ELISA, direct TR-FIA and indirect TR-FIA, compared with rocket immunoelectrophoresis (LOD 2.5 mg kg⁻¹). Each food extract was analysed in all three formats.

Food product	Indirect ELISA with HRP (mg kg ⁻¹)	Direct TR-FIA with Eu (mg kg ⁻¹)	Indirect TR-FIA with Eu (mg kg ⁻¹)	Rocketimmuno- electrophoresis (mg kg ⁻¹)
Pea soup	5	10	8	<2.5
Instant potato 1	33	34	50	76
Instant potato 2	39	22	39	2.5–12.5
Spice-mix	2	<1.3	<1.5	<2.5
Flour	74	10	52	65

the relatively high concentration of Eu-labelled antibody. The ELISA standard curve differed significantly from direct and indirect TR-FIAs (both $p < 0.0001$) and performed considerably better than either TR-FIA, having a lower LOQ (I_{20}) of $34 \pm 4 \text{ ng ml}^{-1}$, comparable to $< 1.0 \text{ mg kg}^{-1}$ in food samples.

It remains to be clarified in further experiments whether the variable food sample results are due to differing matrix effects, uneven distribution of casein in the food samples or reflect a low precision of the assays close to their detection limits, particularly in the direct TR-FIA. There are no standard reference materials of known casein composition and homogeneity available at present; such standards would be of advantage in the development and evaluation of new methods.

In the present study, an indirect TR-FIA using biotinylated antibody and Eu-labelled streptavidin offers no significant improvement over the direct TR-FIA with Eu-conjugated antibody. Direct and indirect competitive TR-FIAs were compared in the measurement of methamphetamine in urine (Kimura et al. 1999), showing that the indirect competitive format, using biotinylated antigen, was 100–1000 times more sensitive than the direct, using Eu-labelled antigen. Hale et al. (2001) compared direct with indirect TR-FIA in the measurement of serum immunoglobulins: Biotinylated antibody with Eu-streptavidin was found to be 100 times more sensitive than the same antibody conjugated to samarium and in sandwich TR-FIAs to determine human FSH and TSH (Suonpaa et al. 1992), indirect TR-FIA using Eu-streptavidin was shown to be 2–5 times more sensitive than direct TR-FIA.

Hale et al. (2001) also compared direct TR-FIA with ELISA, in the measurement of serum IgG and IgA, using the same antibodies conjugated to Eu, Sm or alkaline phosphatase (AP). The TR-FIAs were found to be respectively 10 and 37 times more sensitive than the corresponding ELISAs, but no comparison was made with HRP-conjugated antibody.

However, when Paugam et al. (1998) compared direct sandwich TR-FIA and ELISA (HRP) in the detection of fungal galactomannan, no major improvement in sensitivity was achieved by using TR-FIA. Roberts et al. (1991) compared a direct TR-FIA with a direct ELISA, using both HRP- and AP-conjugated antibody, in the detection of serum immunoglobulins. Additionally, they compared the performance of the two enzymes each with chromogenic and fluorogenic substrates. Eu-based TR-FIA was found to have only intermediate sensitivity; ELISAs using HRP (fluorogenic or TMB substrates) and AP (fluorogenic substrate) were, respectively, 1.6, 1.7 and 1.9 times more sensitive than Eu, whereas ELISAs with AP (chromogenic substrates) and HRP (less-than-optimal chromogenic substrates) were less sensitive than Eu. These findings are confirmed by the results of the present study, with respect to HRP.

TR-FIA has been claimed to have greater sensitivity than enzyme-based or fluorescence immunoassays (Dickson et al. 1995). The results of this study indicate, however, that in the analysis of casein in food matrices, conventional ELISA is somewhat more sensitive than either direct or indirect TR-FIA.

The assays described in this work are of a competitive format and have the advantage of being single-epitope assays, applicable in the detection of degraded or denatured allergens.

The recent inclusion of 11 major groups of food allergens, including milk and milk products, in Annex IIIa of the European Food Labelling Directive 2003/89/EC (European Commission 2003), heightens the need for sensitive and dependable

methods for the detection of these allergens in foodstuffs. The results presented here give an indication that the more economical ELISA systems may confidently be used in favour of the more expensive TR-FIA systems in the detection of casein in foodstuffs. Experimenting with alternative detection systems, such as the use of fluorogenic substrates, may yield a further improvement in the sensitivity of the ELISA.

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