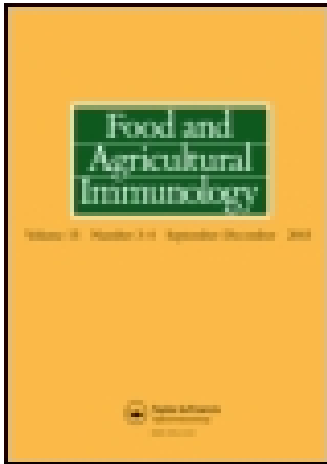




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Determination of Gluten in Foods Using a Monoclonal Antibody-based Competition Enzyme Immunoassay

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A sensitive competition enzyme-immunoassay for quantification of gluten in foods was developed, using horseradish peroxidase-labelled monoclonal antibodies. Selected antibodies specific for wheat omega-gliadin components were used, and these antibodies bound proteins from the related cereals, rye and barley, which are also toxic to individuals with gluten-intolerance (coeliac disease). Binding of these antibodies was not inhibited by heating of gluten during cooking or baking and the assay did not detect cereals not toxic in coeliac disease, such as maize or rice. Gluten could be quantified at higher levels in meat products or in cereal products such as flours or baked goods. Results were not affected by wheat variety. Quantitative results could be obtained using simple extraction techniques and solvents (40% or 70% ethanol). Detection of gluten was quantitative in a wide range of foods, except for certain products containing gluten proteins that had been subjected to severe heat, enzymic or chemical treatment. In these products overestimates rather than underestimates were usually obtained.

INTRODUCTION

Tests for the determination of gluten in foods have a wide variety of applications, ranging from the analysis of foods for individuals with dietary intolerances such as coeliac disease (Cole & Kagnoff, 1985) or forms of wheat allergy to the uses in food labelling monitoring and in quality control of cereal-based or cereal-containing foods. The levels of cereal (or specifically, of gluten) in processed foods are difficult to monitor because cereal proteins must be distinguished from other proteins (e.g. meat proteins). This problem is complicated when changes in protein solubilities and conformation have occurred during processing (Skerritt, 1990).

A number of sensitive radioimmunoassays (RIA) and ELISA assays for gluten have been developed, based on polyclonal antisera to gliadins (the monomeric protein

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portion of wheat gluten). However, these assays were only quantitative with uncooked foods, and did not detect (coeliac-toxic) rye or barley prolamins (Windemann *et al.*, 1982; Ciclitira & Lennox, 1983; Fritschy *et al.*, 1985). The polyclonal antibody sandwich assay of Troncone *et al.* (1986) weakly detected maize prolamins, while the sandwich assay of Freedman *et al.* (1987) which used one monoclonal and one polyclonal antibody, detected rye prolamins very weakly, and limited its reliable use to uncooked, wheat-based foods.

In our development of immunoassays for gluten in both cooked and uncooked foods, we (Skerritt *et al.*, 1984; Skerritt, 1985) developed monoclonal antibodies (MAb) specific for the unusually heat-stable ω -gliadin fraction of gluten (Schofield *et al.*, 1983). Earlier qualitative (Skerritt & Smith, 1985) and quantitative (Skerritt, 1985) ELISAs were developed to detect gluten proteins bound to discs prepared from a nitrocellulose solid phase, after they had been soaked in food extracts. While these assays provided reliable results, handling of nitrocellulose discs proved tedious, and the assay was lengthier than most microwell assays. The antibodies used in that work functioned poorly in microwell ELISA, owing to low affinities (Skerritt & Martinuzzi, 1986). In this paper, the use is of novel high-affinity ω -gliadin binding antibodies in a microwell-based antigen competition ELISA for quantification of gluten in foods is described. This assay format was chosen for initial detailed studies as it had earlier been used for monoclonal antibody-based quantification of specific gliadins (Skerritt *et al.*, 1987). Also, competition ELISA is finding increasing use in food analysis for the determination of contaminants such as pesticides, mycotoxins and antibiotic residues (Morris *et al.*, 1987). This paper describes the development of the competition ELISA and compares its properties and performance with a sandwich ELISA, also developed within our laboratory (Skerritt & Hill, 1990).

MATERIALS AND METHODS

Materials

Ammonium sulphate, sodium chloride, sodium dihydrogen phosphate, urea and disodium hydrogen phosphate, 30% hydrogen peroxide and all solvents and acids used were purchased from BDH Chemicals, Kilsyth, Australia. Horseradish peroxidase (HRP) and bovine serum albumin was purchased from Boehringer Mannheim, FRG. 2, 2'-azino (3-ethyl benzothiazolin)-6-sulphonic acid (ABTS) and Tween 20 were purchased from Sigma (St Louis, MO, USA). Immulon B plates were from Dynatech (Chantilly, VA, USA). Hydroxylapatite (Biogel HT) was purchased from Bio-Rad (Richmond, CA, USA).

Monoclonal Antibodies

Methods used for the preparation, purification, and characterisation and labelling of MAb to wheat gliadins and glutenins have been described in detail elsewhere (Skerritt & Underwood, 1986; Skerritt & Hill, 1990).

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Proteins in various flour extracts were analysed by SDS-PAGE (1200 Vh run) as described earlier (Skerritt & Underwood, 1986).

Antigen Competition EIA

The gliadin antigen used for microwell coating was prepared by 70% ethanol extrac-

tion of the residue following 10% sodium chloride treatment of Timgalen flour (Skerritt & Underwood, 1986). Immulon microwell plates were treated (18 h, 37°C) with gliadin (2 µg/100 µl well) in 50 mM-sodium carbonate buffer, containing 3% ethanol. Microwells were washed (three times) with 50 mM-sodium phosphate-0.9% sodium chloride, pH 7.2 (PBS) containing 0.05% (v/v) Tween 20, and non-specific antibody binding blocked with 150 µl 1% bovine serum albumin (BSA) in PBS (1 h, 20°C).

Food samples were extracted (see below) and gliadin standards prepared in 10 ml/g of the extractant indicated, diluted appropriately in 1% BSA in PBS—0.05% (v/v) Tween, and 50 µl of each dilution added to microwells. Gliadin was extracted from foods using an Ultra-Turrax homogenizer (Sorvall, Newtown, CT, USA) for 30 s at 50% maximal speed. HRP-labelled antibody (50 µl) diluted in BSA-PBS-Tween was immediately added, plate contents gently mixed and incubated (30 min, 20°C). After four washes, 100 µl ABTS in 100 mM-sodium citrate, pH 4.5, containing 0.003% (v/v) hydrogen peroxide was incubated (10 min, 20°C). Colour development was terminated by acidification, and product absorbances determined at 414 nm. Monoclonal antibody concentrations were selected to yield an absorbance of 1.0 in the absence of competing antigen.

Preparation of Meat/Gluten Blends and Flour/Starch Blends

Differing amounts of commercial vital gluten (NB Love Industries, Enfield, Australia) previously analysed for protein, were blended with pure beef mince (total mass = 500 g) for 3 min at 20°C, using a Morton (Morton Machinery Co. Ltd, Wishaw, Scotland) mixer. 100 g samples were then cooked in a domestic microwave (750 W) on the highest heat setting for 5 min. Gluten contents were calculated accounting for water loss and the protein content of the gluten used. The gluten content of a bread wheat flour (cv. Timgalen) was determined by machine washing in water using a Glutomatic (Falling Number AB, Stockholm, Sweden), freeze-drying and Kjeldahl nitrogen analysis. Protein was calculated as nitrogen $\times$ 5.7. Differing proportions of flour and prime commercial starch (0.3% protein) were blended by overnight rotation.

Flour, Cereal and Food Samples

Flours were milled from samples of nine Australian wheat varieties grown at two sites producing different characteristic protein contents. Other cereal samples used were: bread wheat (*Triticum aestivum*) cv. Timgalen (13.7% protein), durum wheat (*Triticum turgidum*) cv. Durati (13.3% protein), rye (*Secale cereale*) mixed varieties (8.9% protein), barley (*Hordeum vulgare*) cv. Clipper (6.3% protein), oats (*Avena sativa*) cv. Cooba (9.9% protein), maize (*Zea mays*) mixed varieties (6.5% protein) and rice (*Oryza sativa*) cv. Calrose (5.8% protein). Food samples and starches were obtained either directly from the manufacturers or from a retail store. The protein contents ($N \times 5.7$) of flours, starches and cereal samples used were determined by Kjeldahl analysis.

RESULTS

Choice of Extractant for Food Samples—Gel Electrophoresis Studies

Initially, a large number of gliadin solvents was used to extract wheat flour samples (10 ml/g) to develop a model sample for food extraction, and the extracts analysed by SDS-PAGE, and in some cases by polyacrylamide gradient gel electrophoresis under acidic buffer conditions (Skerritt & Underwood, 1986). Of a range of aqueous ethanol

concentrations tested (10–90%), 40% ethanol best extracted the full complement of gliadins (Figure 1). Higher concentrations (55–80%) extracted less ω -gliadin, while relatively little gliadin (by Coomassie blue staining of electrophoresis gels) was extracted by 10–25% or 90% ethanol (data not shown).

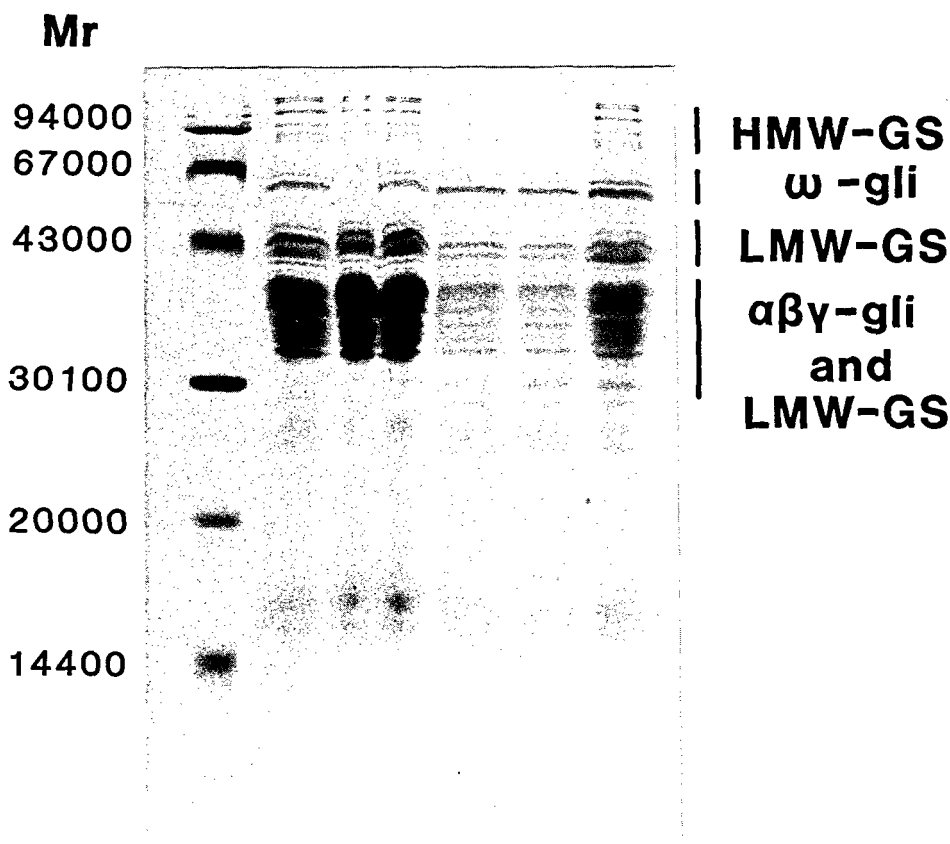


FIG. 1. Analysis of flour extracts by SDS-PAGE. From left, molecular weight markers: 40% ethanol; 70% ethanol; 55% isopropanol; 1 M-urea; 1 mM-HCl; 10 mM-acetic acid.

Trace amounts of high molecular weight glutenin subunits and M_r 15 000–18 000 'pseudogliadins' were extracted by 40% and 70% ethanol and 55% isopropanol, but 70% ethanol did not extract some of the ω -gliadins. Urea (1 M) and HCl (1 mM) appeared to be more gliadin-specific extractants, but gliadin extraction appeared to be less complete than that obtained with acetic acid (100 mM) (Figure 1). However, acetic acid (100 mM) extracted certain low molecular-weight glutenins. A variety of other extractants tested (not shown) either extracted a very high proportion of glutenin (e.g. 2-chloroethanol (25%), acetonitrile (50%), chloroform-methanol (1:1)), high amounts of albumin and globulin (dioxane, 60% ethylene glycol, 50%) or were poor gliadin extractants (HCl 0.1–0.5 mM; urea 0.2–0.5 M).

Choice of Antibody

Earlier work from our laboratory (Skerritt, 1985; Skerritt & Smith, 1985) demonstrated that ω -gliadin-specific antibodies enabled accurate determination of gluten in

flours, even after these flours had been heated or baked. Therefore, among high-affinity IgG antibodies, two ω -gliadin binding antibodies (401/21 and 405/7) were compared with 222/5 (an $\alpha\beta$ -gliadin binding MAb) for reaction with gliadins extracted from unheated and heat-treated flours, and with ω -gliadin purified by ion-exchange chromatography (Booth & Ewart, 1969). While binding of 222/5 to gliadin was decreased by heating (Figure 2A), binding of 401/21 (Figure 2b) and of 405/7 was not reduced (not shown). Similar immunoassay results were obtained with gliadins extracted from heated (100°C, 60 min) and unheated flour/water (1:2) slurries, using 1 M-urea as well as 40% and 70% ethanol (not shown). 222/5 bound relatively poorly to purified ω -gliadin, while the binding of 401/21 and 405/7 was similar to ω -gliadin and total gliadin (Figure 2).

Antibodies 222/5 and 401/21 were also compared in the competition assay format for reaction with extracts of several different foods and cereals (Tables 1 and 2). In these pilot experiments, 70% ethanol was used as the extractant. A range of foodstuffs based on egg, milk, soya and meat products known not to contain gluten, did not react with the antibodies. Detection of gluten in starches was possible with each antibody, and a starch quite unsuitable for use in 'gluten-free' foods (0.61% protein) was discriminated readily from a suitable starch (0.28% protein) (Table 1). While gluten was readily detected in standard baked goods such as bread or cookies (4–15% gluten) by both antibodies, moderate levels in processed foods such as soups and meats were poorly detected by 222/5, but readily by 401/21 (Table 1).

Cereal Protein Specificity of Antibodies

The cross-reactions of the three antibodies described above plus two others, which from preliminary experiments reacted with wheat, rye, barley and/or oat prolamins (403/8) and 404/6) were studied using 70% ethanol extracts of the different cereals (Table 2). The extracts dilution producing 50% inhibition binding was determined for each cereal, and results expressed relative to those obtained for bread wheat. 401/21 and another ω -gliadin binding MAb, 304/13, were also studied with 40% ethanol extracts of each cereal (see below). Cross reaction of 401/21 was similar with both extracts (Table 2). Detection of 'gluten protein' from bread and durum wheats, rye and barley is necessary for coeliac-toxic cereals to be identified (Anand *et al.*, 1978). For this purpose, antibodies 222/5 and 405/7 had too narrow a cross-reaction, while 304/13, 401/21 and 404/6 had appropriate cereal cross-reaction; 401/21 bound better to barley protein than the other antibodies (Table 3). 403/8 bound oat proteins well, but only very weakly to barley prolamins. 404/6 was excluded for further study as it bound mainly to high-mobility gliadins (Skerritt & Lew, 1990). 304/13 did have suitable cereal cross-reaction characteristics and was studied in more detail in the sandwich assay format (Skerritt & Hill, 1990).

Antibody Cross-reaction with Wheat Grain Protein Fractions

Antibody 401/21 was assessed in the competition assay using purified wheat albumin, globulin, gliadin (prepared by either 40% ethanol, 70% ethanol or 1 M-urea treatment) or glutenin (Skerritt & Underwood, 1986). The albumin and globulin fractions did not produce significant inhibition, even at the highest concentration tested (Figure 3). Gliadin produced by 40% ethanol extraction was a more potent inhibitor of antibody binding (IC_{50} ~500 ng) than gliadin prepared by either 70% ethanol or 1 M-urea (Table 3, IC_{50} ~1100–1200 ng). The glutenin fraction was considerably more potent (IC_{50} ~12 ng), although the solvent used (100 mM-KOH) contributed largely to this increased potency (Skerritt & Martinuzzi, 1986). Lyophilized gliadin, or alcoholic

TABLE 1. Approximate gluten content of selected foods, determined using an $\alpha\beta\gamma$ -gliadin binding (222/5) and a ω -gliadin binding antibody (401/21)

Food type	Gluten ^a (%)	
	$\alpha\beta\gamma$ -gliadin-binding antibody	ω -gliadin binding antibody
Cooked meat products		
Sausage 1	0.2	1.0
Sausage 2	0.2	1.0
Veal	— ^b	—
Ham	—	—
Beef	—	—
Starch		
1 (0.28% protein)	0.02	0.02
2 (0.61% protein)	0.2	0.2
Soups		
Tomato	—	—
Mushroom (+flour)	0.002	1.0
Baked goods		
Breadcrumb	>1	>1
Cookies	>1	>1

^aGluten determined (to the nearest order of magnitude) to the following approximations: 0.002%, 0.02%, 0.2% and 1%. ^bNot detected (<0.001% gluten for 222/5; <0.01% gluten for 401/21).

TABLE 2. Cross-reaction of monoclonal antibodies with extracts of different cereal grains relative to bread wheat^a

Antibody	Cereal							
	Bread wheat 1/IC ₅₀ ^b	Bread wheat	Durum wheat	Rye	Barley	Oats	Maize	Rice
1. 70% ethanol extracts ^c								
222/5	20000	100	120	0.9	— ^d	—	—	—
401/21	2600	100	110	110	41	—	—	—
403/8	540	100	23	22	—	78	—	—
404/6	2700	100	81	64	7	—	—	—
405/7	70	100	27	14	—	—	—	—
2. 40% ethanol extracts ^c								
304/13	700	100	100	230	26	—	—	—
401/21	700	100	83	120	43	—	—	—

^aDetermined by comparison of cereal extract dilutions yielding 50% inhibition of binding with those from bread wheat=100.

^bReciprocal of the bread wheat extract dilution yielding 50% inhibition of antibody binding.

^cDetermined in a two-step competition assay, using an HRP-rabbit anti-mouse antibody, as not all of these antibodies were labelled.

^dNot detected (<10% inhibition of binding to gliadin).

^eAbsolute potencies relatively low, as a high concentration of labelled antibody was used.

TABLE 3. Effect of food extractant on determination of 'gluten' in different cereals

Extractant	Bread Wheat 1/IC ₅₀ ^b	Relative detectability ^a						
		Bread	Durum	Rye	Barley	Oats	Maize	Rice
Ethanol (40%)	1/1200	100	110	190	60	—	—	—
Ethanol (40%)	1/600	100	160	150	40	—	0.15	—
Urea (1 M)	1/1300	100	83	202	68	0.6	1.1	—
HCl (1 mM)	1/900	100	100	40	24	—	—	—
Acetic acid (10 mM)	1/900	100	100	110	25	—	0.3	—

^aDefined as the extract dilution at which 10% competition occurred, relative to that for bread wheat=100. Antibody 401/21 used.

^bIC₅₀=grain extract dilution at which 50% binding occurred. Higher numbers indicate more sensitive detection.

gliadin solutions treated with KOH, increased in inhibition potency by a factor of 3–4 (data not shown).

Choice of Solid-phase Coating Antigen

A variety of solvents has been used to prepare 'pure' gliadin from wheat flour, including aqueous ethanols, 1–2 M-urea and dioxane (Lee, 1968; Payne & Rhodes, 1982). These antigens were first assessed in a direct ELISA (Hill & Skerritt, 1989) using 401/21, and 2 µg/well 'gliadin'. There were not significant differences in assay performance obtaining with gliadin extracted with 40% ethanol ($A=1.0$ at 1/13000 dilution of HRP-labelled antibody) or with 70% ethanol ($A=1.0$ at 1/12000–1/13000 dilution); the preparations made with dioxane (1/12000) gave similar results. Some antigens which gave strong inhibition in the competition assay, such as gliadin from heated flour or purified ω -gliadin, performed poorly in indirect ELISA ($A=1.0$ not achieved at 1/100 antibody dilution). Therefore, gliadin extracted by 70% ethanol was chosen as the coating antigen for the competition ELISA.

Choice of Extractant—ELISA Studies

On the basis of the PAGE results, five extractants were chosen for further study in pilot competition ELISAs using 401/21: (1) 40% ethanol; (2) 70% ethanol; (3) 1 M-urea; (4) 1 mM-HCl; and (5) 10 mM-acetic acid. Antibody cross-reaction was studied with extracts of the various cereals (Table 3) as well as results obtained using a set of 'key' food samples. Essential to the use of an antibody test for identifying foods unsuitable for the coeliac diet, is: (i) minimal cross-reaction with maize protein, (ii) good discrimination of suitable and of unsuitable wheat starches, and (iii) sufficient and equally-sensitive detection of gluten in flours, before and after baking.

Extracts of maize meal, diluted 1/10 and 1/40, did not significantly react in the assay, except for those produced using 1 M-urea (18% inhibition at 1/40). A low-protein (0.25%) wheat starch, which would contain virtually no gluten (as this protein is starch-granule associated (Greenwell & Schofield, 1986)) produced little or no reaction (Table 4). Differences between sensitivities obtained with different extractants were seen for a high protein (0.46%) wheat starch; only 33% and 35% inhibition was seen for 70% ethanol and 1 mM-HCl extractants while 51% and 53% inhibition occurred with 1 M-urea and 40% ethanol, respectively. Equivalent dilutions of a wheat

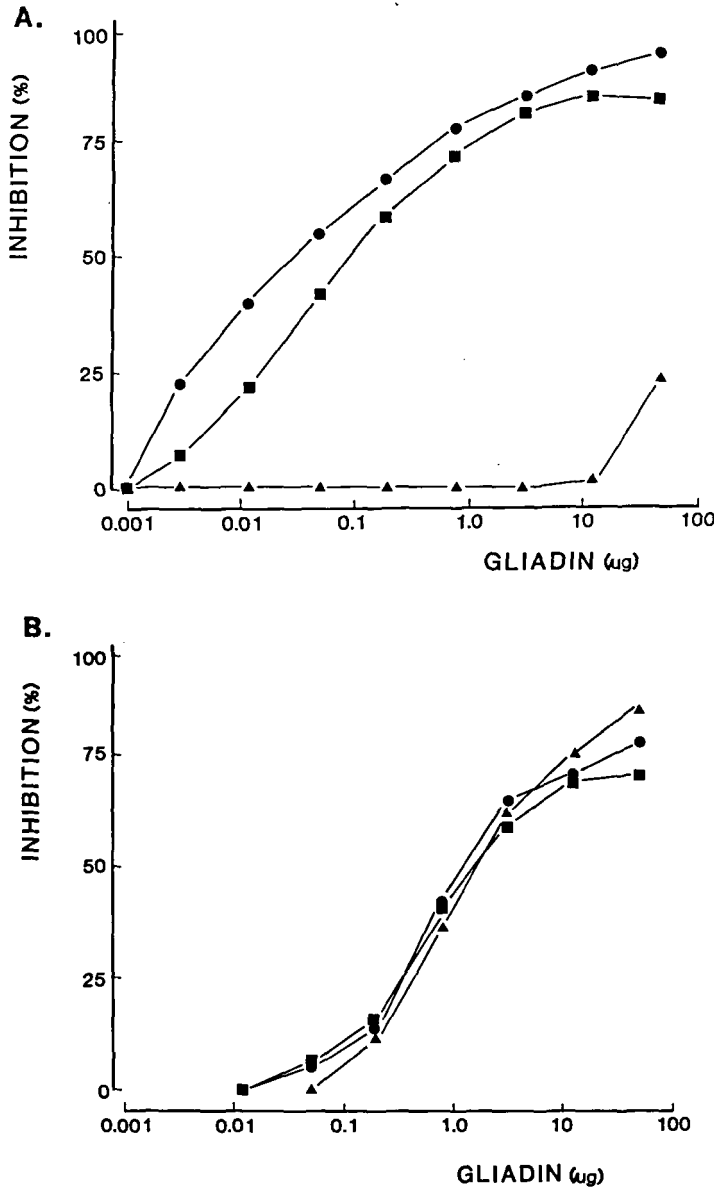


FIG. 2. Cross-reaction of (a) an $\alpha\beta$ -gliadin-binding monoclonal antibody (222/5), and (b) an ω -gliadin-binding antibody (401/21) with total gliadin (●) and ω -gliadin (▲) and gliadin (■) from heated flour.

flour extract had a breadcrumb extract produced similar inhibition of antibody binding with each extractant.

Assay Sensitivity

The limit of detection ('sensitivity') was defined as the gliadin concentration yielding 10% inhibition of antibody binding or 0.1 absorbance unit lower than antigen-free

TABLE 4. Determination of gluten in foods by competition ELISA

Wheat starches		Infant foods	
(1) 0.28% protein	n.d. ^a	(1) Vegetable	n.d. ^a
(2) 0.28% protein	n.d. ^a	(2) Beef	n.d. ^a
(3) 0.32% protein	0.04 ^a	(3) Beef and Vegetable	n.d. ^a
(4) 0.46% protein	0.18 ^a	(4) Chicken	0.86
'Gluten-free' bread mixes		Breakfast cereals	
(1)	n.d.	Rice-based	n.d. ^a
(2)	0.31 ^c	Granola	0.16
(3)	0.02		
Baked goods		Soups	
Crispbread	2.7 ^a	Tomato	n.d. ^a
Crumbing mix	9.0 ^a	Pea and ham	0.86
Cracker	5.0 ^b	Pumpkin	1.2 ^a
Cookie (1)	3.4 ^a		
(2)	12.4 ^a	Processed meats	
(3)	20.0 ^b	Beef-based (1)	n.d. ^a
Commercial (1)	9.6 ^a	Salami	n.d. ^a
flours (2)	12.0 ^a	Ham and chicken	0.80
(3)	9.2 ^a	Beef-based (2)	0.80 ^a
(4)	9.1 ^a		

^aActual or expected value known; value determined by ELISA was correct $\pm$ 50%.
^bValue determined > 50% greater than actual or expected value.
^cReported to have caused adverse reactions in several coeliacs.

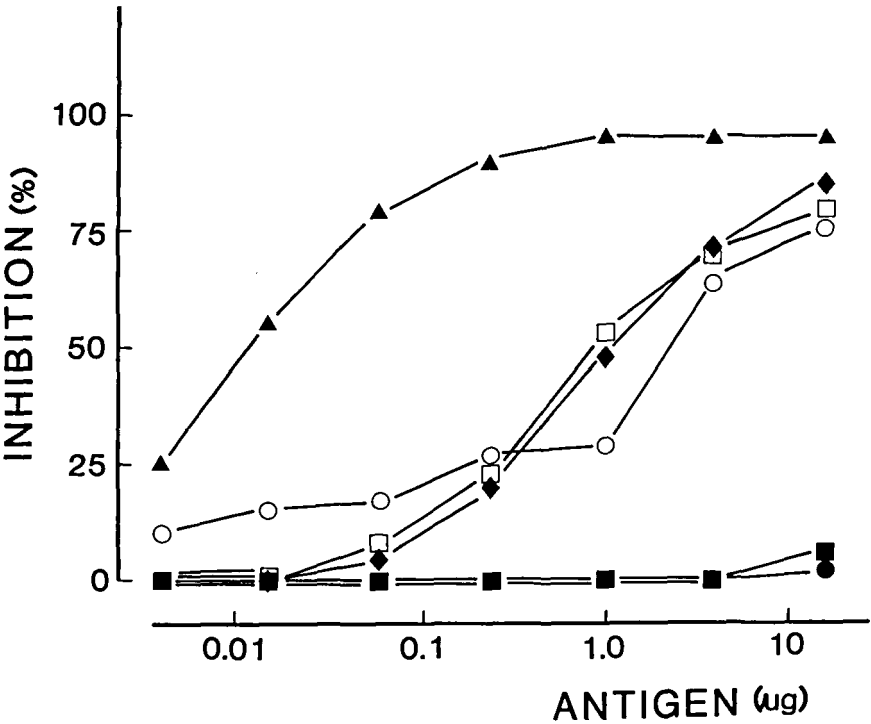


FIG. 3. Cross-reaction of 401/21 in competition ELISA with wheat protein fractions: (■) albumin; (●) globulin; (▲) glutenin; (□) gliadin extracted with 40% ethanol; (○) gliadin extracted with 70% ethanol; (◆) gliadin extracted with 1 M-urea.

controls. As the labelled antibody concentration was chosen to yield a control absorbance of 1.0 ± 0.1 , these definitions were equivalent. For 10 assays using 401/21 and 40% ethanol solvent, sensitivities in the range of $0.05\text{--}0.10\ \mu\text{g}$ gliadin was obtained (e.g. Figures 2(b), 3). Using a one-in-five dilution of a food extract and a 10 ml/g extraction ratio, this is equivalent to $0.01\text{--}0.02\%$ (w/w) gliadin or $0.02\text{--}0.04\%$ (w/w) gluten.

Quantitative Analysis of Gluten in Model Starch/Flour and Meat/Gluten Blends

The extractants (other than 10 mM-acetic acid) were further used to investigate their performance in quantification of gluten in model starch/flour and meat/gluten blends. A gluten standard curve was constructed for each of these studies, using a flour of known gluten content, treated with the particular extractant under study. For each of the four extractants tested, the relationships between gluten present in the starch/flour blends and gluten measured were reasonably close, although 70% ethanol provided values that were slightly high at gluten levels above 1% (Figure 4). In contrast, 40% and 70% ethanol produced more accurate results than the other extractants in determination of gluten in meat blends (Figure 5). While slight overestimates were seen with each extractant at low gluten levels, 1 mM-HCl produced excessively high values at all gluten additions. Cooking increased apparent levels of gluten determinant with each extractant, especially at low ($<0.5\%$) additions. Results for the 40% ethanol extractant were closest to actual, and 70% ethanol reasonably close; values with 1 mM-HCl and 1 M-urea were somewhat elevated.

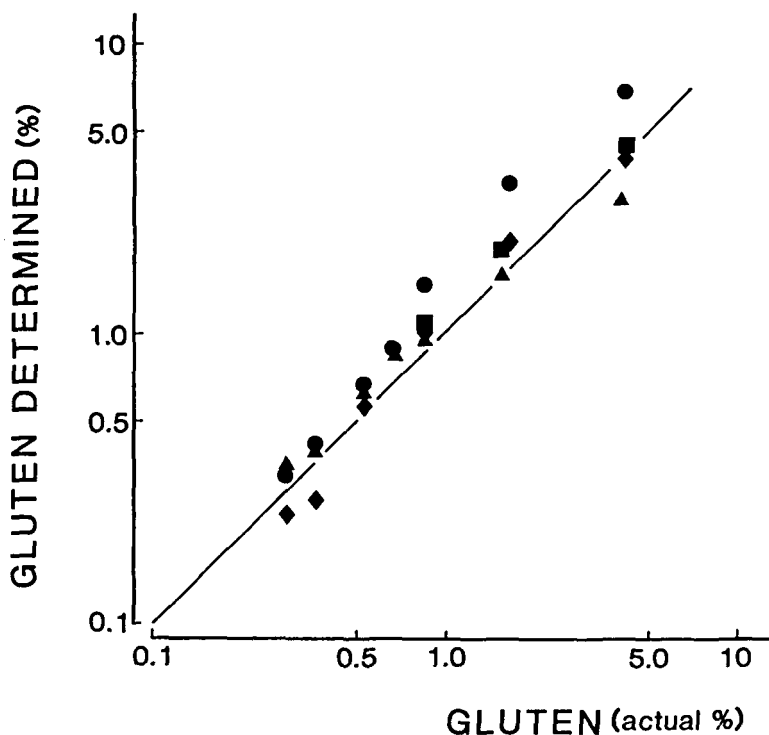


FIG. 4. Assay of gluten in flour/starch blends, extracted using: (■) 40% ethanol; (●) 70% ethanol; (◆) 1 M-urea; and (▲) 1 mM-HCl. The line indicating 100% recovery is indicated. Antibody: 401/21.

Gluten Determination in Flours

Wheat varietal effects on the binding of antibody 401/21 were minimal, that is, extracts of different wheat varieties of the same total protein content and gluten content (by glutomatic analysis) performed very similarly in the ELISA. Analysis of the relationship between gluten content and protein content for 70% ethanol extracts of flour from 11 wheat samples (seven varieties grown at one or two sites) demonstrated that it was linear ($r=0.89$, $P<0.001$). The zero gluten intercept occurred at 2.7% protein. This value is equivalent to the amount of non-gluten protein found in most flours. Similar results were obtained with 1 M-urea extracts.

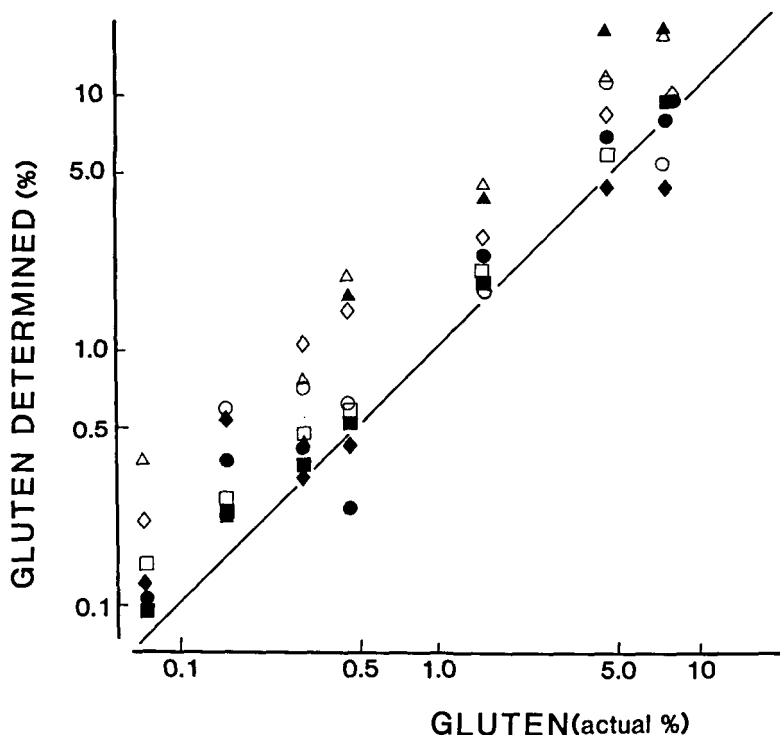


FIG. 5. Assay of gluten in gluten/meat blends, extracts using; 40% ethanol (■) raw (□) cooked; 70% ethanol (●) raw (○) cooked; 1 M-urea (◆) raw, (◇) cooked; and 1 mM-HCl (▲) raw (△) cooked. The line indicating 100% recovery is indicated. Antibody: 401/21.

Analysis of Foods

On the basis of these results, a large number of foods of known composition were analysed using both 40% and 70% ethanol extractants; some results with 40% ethanol are shown in Table 4. The limit of detection of this particular assay, using food extracts diluted 1/10 (final) was 0.025% gluten. Well-washed wheat starches (protein $\leq 0.28\%$) did not contain detectable gluten, although some commercially available starches with slightly higher protein contents (0.32–0.46%) produced positive results. Gluten was also detectable in one pre-gelatinized starch. Trace amounts of gluten were found in a number of 'gluten-free' dietary foods; batches of one baking mix, submitted to our laboratory after being suspected of coeliac-toxicity, contained 0.31–0.42%

gluten. Other non-wheat flours based on maize starch, rice, buckwheat, soya and pea did not react. Gluten was detectable in a range of foods, including processed meats and soups. While gluten was not detectable in most lager beers, one lager yielded 0.04%, an ale 0.2%, and a stout 0.8%. The latter beers would contain residual barley storage protein. While a large number of wheat flours tested gave correct results, even after chlorination for cake-baking, some biscuits and crackers gave artefactually high results, e.g. for some baked goods containing 2.0% to 4.0% gluten, results of 5–20% were obtained.

Two commercial caramels prepared from the acid and enzymic treatment of a prime wheat starch (which contained no detectable gluten) gave high apparent gluten contents (0.39% and 3.5% respectively, when extracted into 40% ethanol). In contrast, several candies, desserts and beverages contained caramel or caramel flavouring did show detectable gluten. Some samples containing cereal protein fragments also gave elevated determinations. A series of 17 enzymically and chemically modified glutens gave analyses of 0.6–1200% gluten (mean 470%).

DISCUSSION

Due to the low extractability of the glutenin fraction of gluten, each group attempting to determine gluten in foods has actually aimed to extract and analyse the gliadin fraction. Results are then expressed as gliadin or converted to gluten by multiplication by two, as the proportion of gliadin in gluten is close to 50% in different wheat varieties (Huebner, 1970). The most common extractant used for microwell ELISA of 'gluten' (gliadin) in foods has been 70% ethanol (Windemann *et al.*, 1982; Meier *et al.*, 1984; Fritschy *et al.*, 1985; McKillop *et al.*, 1985; Ayob *et al.*, 1988); in our hands, 40% ethanol and 70% ethanol performed equally well for gluten determination in the competition ELISA. Nevertheless, the greater potency of 40% ethanol flour extracts would suggest that this extractant removes more 'immunoreactive gluten'. PAGE analysis of flour extracts prepared with a range of gliadin solvents showed that many other 'gliadin solvents' either did not extract gliadin proteins well, or else removed substantial amounts of protein additional to gliadin, predominantly glutenin. The ω -gliadin binding antibodies used in the present study also bound glutenins. However, the antibodies could be made functionally gliadin-specific by use of 40% or 70% ethanol as a flour or food extractant. In addition to the potential problems with precipitation of glutenin-containing extracts upon dilution prior to immunoassay, this enables the potential loss of immunoreactivity upon cooking due to glutenin denaturation (Schofield *et al.*, 1983) to be avoided. Freedman *et al.* (1987) used a similar approach (55% ethanol extractant) to overcome cross-reaction with glutenins in the determination of gluten with broad-specificity antibodies.

The aqueous alcohol extractants also enabled antibody cross-reaction with durum wheat and rye (thought to be as toxic as wheat (Anand *et al.*, 1978) and barley (less toxic (Baker & Read, 1976)) to mirror relative toxicity of these cereals in coeliac condition; other extractants and antibodies produced different cross-reaction patterns (Table 2 and 3). While some coeliac patients avoid oats, the toxicity of oats in coeliac disease is not firmly established (Baker & Read, 1976; Anand *et al.*, 1978). As the presence of oats in snack foods and cereals is usually obvious, and as oat flour is not used as meat extenders, failure to detect oats is not a shortcoming of this gluten assay. If detection of oats were required, antibody 403/8 could be used, perhaps in conjunction with 401/21.

Furthermore, the ω -gliadin binding antibody chosen did not show inherent varietal differences in binding to extracts of different wheats, a problem with some antisera used for gluten detection (Fritschy *et al.*, 1985). The linear relationship between gluten content and flour protein did not go through the origin, as a constant amount of flour

protein (2–2.5% by flour mass) is metabolic protein rather than storage (gluten) protein. The ability to specifically determine gluten in flours or grain meals by immunoassay suggests an alternative to labour-intensive gluten washing methods (American Association of Cereal Chemists, 1983). The alcohol solvents also provided accurate results with cooked meats and also starch blends containing known amounts of gluten. While others have identified that certain food types may yield anomalous results (Windemann, 1987), detailed studies on gluten recovery determined by EIA have not been performed by other laboratories.

Antibody specificity was important for detection of gliadin in cooked foods, as the $\alpha\beta$ -gliadin binding antibody, 222/5, detected gluten very weakly in processed meats or soups. Others using polyclonal antisera to total gliadin, have noted that gliadin detection is decreased by up to 50-fold by heating, as most of the antibody activity is directed towards α - and β -gliadins (Ciclitira & Lennox, 1983; Meier *et al.*, 1984). Attempts to develop polyclonal antibodies to ω -gliadin with sufficiently high affinity for use in a gluten detection ELISA have not been successful (Windemann, 1987). Thus, several groups have proposed the use of SDS-PAGE and immunoblotting for gluten detection in cooked foods (Janssen *et al.*, 1987; Windemann, 1987), though this approach would be cumbersome and only semi-quantitative at best.

The assay sensitivity (0.02–0.04% gluten) was suitable for analyses of foods labelled as 'gluten-free', but based on wheat starch. Such foods must contain no more than 0.3% wheat protein (Codex Alimentarius Commission, 1981); as wheat starch contains 0.24–0.28% non-gluten protein, a starch of 0.3% protein contains 0.02%–0.06% gluten. Slight overestimates for gluten content were obtained with cooked meats and greater overestimates obtained for cookies and crackers. This may be due to gliadin extracts of these foods being enriched in ω -gliadin, and not being affected by (low-affinity) competition by other gliadin fractions for the enzyme-labelled antibody, as would be the (usual) case with total gliadin extracts. In addition, overestimates produced with enzymically modified glutes in the competition assay, may arise from the repeating epitopes for this antibody thought to be present in ω -gliadin (Charbonnier & De Wolf, 1983; Kasarda *et al.*, 1983). This is because digestion would produce a larger number of independent molecular-species bearing epitopes, which would compete more effectively than when the epitopes were contiguous on large proteins. The ability of this assay to detect extensively modified or digested gluten proteins would be of practical use, as many such digests retain their toxicity to coeliacs (Cole & Kagnoff, 1985).

The competition microwell ELISA format enabled gluten to be rapidly and accurately determined in a wide variety of foods including, for the first time, cooked foods. With the exception of the high results discussed above, only two samples (certain caramel concentrates) gave 'false positive' results. These probably arose from (non-immunological) inhibition of HRP activity rather than from binding to the antibody; unlike sequential sandwich ELISA, the most common technique for detection of protein antigens (Tijssen, 1985), diluted crude food extracts and enzyme contact one another during the competition ELISA incubation. Technically, competition assays are slightly more difficult to interpret than sandwich assays, as results from test samples must be read with respect to (antigen-free) control, although with increasing penetration of hapten-competition immunoassays in food analysis, such analysis is becoming increasingly commonplace. Incubation times and coating antigen and labelled antibody concentrations are also more critical for competition assays (Tijssen, 1985). For these reasons, a sandwich ELISA for gluten in foods using ω -gliadin-binding monoclonal antibodies was developed (Skerritt & Hill, 1990).

This sandwich assay provided quantitative results with a wide range of foods, including caramel-based confectionery, biscuits and crackers. However, it was less sensitive to barley-based foods or barley-derived gluten (e.g. in beers). The assay was

also four- to eight-fold more sensitive than the competition assay, enabling any wheat-starch based product to be identified. This may be of advantage to very gluten-sensitive coeliacs (Cooke & Holmes, 1984) but means that the sandwich assay requires greater dilution of food extracts in routine use. The sandwich assay also required an extra incubation and washing step, increasing labour requirements and analysis time. To conclude, it is therefore likely that both sandwich and competition ELISA assay formats will have application in food analysis.

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