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Optimization of a Competitive ELISA with Polyclonal Antibodies for Quantification of Prolamins in Foods

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The optimization of a sequential competitive ELISA for the quantification of prolamins in foods is described in this article. The assay was developed using polyclonal antibodies obtained by the hyper-immunization of rabbits with commercial gliadin. The ELISA developed in this way showed a very high degree of detectability (detection limit, 1 ng ml⁻¹), as well as the ability to discriminate between prolamins harmful to coeliac individuals from non-toxic prolamins. The influence of the solvent used for extraction of the samples on the detection capability of the test was also studied. The assay proved to be useful for the evaluation of gliadins in processed foods including meat products. The assay was applied to many types of foods and was compared with a commercial kit approved by the Association of Official Analytical Chemists.

Keywords: Gliadin, prolamins quantification, coeliac disease, ELISA

INTRODUCTION

Coeliac disease is a disorder of the small intestine. Its onset is triggered in susceptible individuals by ingestion of a group of proteins known as prolamins, found in wheat, barley, rye and triticale. Patients present with severe intestinal mucosal damage, which may result in severe malabsorption, diarrhoea and steatorrhoea (Branski *et al.*, 1992). The exact amount of toxic protein that produces the damage is unknown, but minimal doses induce a strong immune reaction in the intestinal mucosa, which is evidenced by lymphocyte infiltration. Strict adherence to a gluten-free diet usually results in complete reversion of the symptoms and mucosal histopathological changes (Marsh, 1992; Catassi *et al.*, 1993).

Gliadins (the prolamins fraction from wheat) are extracted from flour by alcoholic solvents, generally 70% ethanol, and the homologous proteins from barley and rye are

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extracted in a similar way. Prolamin fractions of these cereals consist of a complex mixture of proteins having a high sequence homology and large regions of repetitive sequences. This results in a high degree of cross-reactivity (Shewry *et al.*, 1986; Skerritt & Underwood, 1986; Festenstein *et al.*, 1987).

Cereal proteins, especially wheat prolamins, exhibit physico-chemical properties that are useful in food manufacturing. They are therefore used extensively in the food industry. The World Health Organization (WHO) recommends that a gluten-free product should contain less than 1 mg of gluten/100 g of dry product (Codex Alimentarius). As a consequence, analytical methods with a high detection capability are mandatory to certify the gliadin composition of the food for coeliac patients.

Several immunoassays have been developed using monoclonal and polyclonal antibodies. For instance, Friis (1988) developed a competitive ELISA with polyclonal antibodies which exhibits a good working range for concentrations covered by the standard curve ($10\text{--}250\text{ ng ml}^{-1}$ of gliadin), but can be used only with unprocessed foods. Troncone *et al.* (1986) developed a sandwich ELISA using gliadin-specific rabbit IgG antibodies. This assay has a high detection ability, but exhibits cross-reactivity with rice and maize prolamins. In turn, Freedman *et al.* (1987) developed a sandwich ELISA that used rabbit polyclonal anti-gliadin IgG as the capture antibody and an anti-gliadin monoclonal antibody as the developing agent. This ELISA has a detection level of 15 ng ml^{-1} , detecting all gliadin groups as well as prolamins from barley, rye and oats, and is non-reactive to non-toxic proteins. Skerritt and Hill (1990a) developed a sandwich ELISA using a mouse IgM monoclonal antibody to ω -gliadin, the gliadin having the highest stability to heat. This assay has a detection limit of 160 ng ml^{-1} of gliadin (16 mg/100 g of dry product). It detects prolamins of toxic cereals even in cooked or processed products. This assay has been endorsed by the Association of Official Analytical Chemists (AOAC) as an official method, and is commercially available (Skerritt & Hill, 1990b). Because of this assay's high cost, however, its use is quite limited in developing countries. Recently, a sandwich ELISA using a monoclonal antibody to a peptide of α -gliadin was reported. This assay showed a high detection level (15 ng ml^{-1} of gliadin) (Ellis *et al.*, 1994).

In this paper, the development of a sequential competitive ELISA of high specificity and detectability using polyclonal antibodies for the quantification of prolamins in foods is described. Interference by the solvent on immunodetection was also studied, and the performance of this assay was compared with that accepted by the AOAC.

MATERIALS AND METHODS

Production of Anti-gliadin Polyclonal Antibodies

New Zealand White rabbits were immunized intra-dermally with 1 ml (1 mg ml^{-1}) of commercial gliadin (Sigma Chemical Co., St. Louis, MO, USA) in phosphate-buffered saline (PBS) with Freund's complete adjuvant. They were later inoculated every 3 weeks with the same immunogen prepared in Freund's incomplete adjuvant. Serum samples were analyzed 7–10 days after each booster.

Preparation of Protein Extracts

Flours of wheat, barley, rye, triticale, oat, soy, rice and maize were extracted for 2 h at room temperature using 70% (v/v) aqueous ethanol (10 ml g^{-1}) after removal of the albumin-globulin fraction using 0.15 M-NaCl (10 ml g^{-1} , $4 \times 1\text{ h}$ at room temperature). Samples were centrifuged at $10\,000 \times g$ for 15 min at 8°C after each incubation step. Protein extracts were lyophilized, dissolved in and dialyzed against $0.01\text{ M-acetic acid}$, and were finally filtered through a $0.22\text{ }\mu\text{m}$ Nucleopore membrane (Millipore). The protein concentration was determined using Lowry's method (Lowry *et al.*, 1951) with the gliadin solution as a standard.

Preparation of Food Samples

Prolamins of foods were extracted with 70% aqueous ethanol at a 10 ml g⁻¹ ratio using an Omnimixer homogenizer (Sorvall), for 4 min at 50% maximal speed, in an ice bath. The extracts were centrifuged for 2 min at 15 000 × g.

Preparation of Gliadin Standard Solution

Gliadin (Sigma Chemical Co.) was dissolved in 70% aqueous ethanol at a concentration of approximately 2 mg ml⁻¹. The solution was filtered through filter paper (Whatman no. 1) and then through a 0.22 µm Nucleopore membrane (Millipore). A clear and stable gliadin solution was prepared by this procedure. Kjeldahl's method was employed to determine the protein concentration in the solution.

Sequential Competitive ELISA

Coating. Gliadin standard solution was dissolved in PBS at concentrations of 0.1, 1 and 10 µg ml⁻¹. Polystyrene strips (Maxisorp, Nunc, Denmark) were coated for 16 h at 4°C with 100 µl/well of gliadin in PBS. The wells were washed with PBS containing 0.5% (v/v) Tween 20 (PBS-T) and non-specific binding sites were blocked by incubating with 200 µl/well of 3% (w/v) dry skimmed milk in PBS for 2 h at 37°C. After each incubation step, the wells were washed with PBS-T.

Interaction between antigens and antibodies in liquid phase (pre-incubation). Gliadin standard solutions (of concentrations 0.64 ng ml⁻¹–10 µg ml⁻¹) and antibodies were dissolved in PBS-T containing 1% (w/v) of dry skimmed milk (diluent solution). Equal volumes of antigen and antibody solutions were mixed, and incubated under different temperature and time conditions (16 h at 4°C or 30 min, 1 h and 2 h at 37°C).

Competition with antigens in solid phase. A total of 100 µl/well of each sample from the previous step were incubated at 37°C, in wells coated with gliadin (10 µg ml⁻¹) as described, for different times (20, 30, 60, 120 or 240 min).

Incubation with peroxidase conjugate and colour reaction. Goat anti-rabbit IgG horseradish peroxidase conjugate (Bio-Rad), diluted 1:1500 in diluent solution, was incubated (100 µl/well) for 1 h at 37°C. The colour reaction was developed by adding a solution containing *o*-phenylenediamine (1 mg ml⁻¹, Merck) and 30% H₂O₂ (1 µl ml⁻¹) in 0.1 M-citrate-phosphate buffer, pH 5.0. The enzymatic reaction was stopped after 20 min with 40 µl/well of 2 M-sulphuric acid. The optical density was determined at 490 nm. Absorbance values were transformed by a logit function (Tijssen, 1985):

$$\text{logit } p = \ln \frac{p}{(1-p)}$$

where

$$p = \frac{(A - A_0)}{(A_M - A_0)}$$

using *A*, *A_M* and *A₀* as absorbance values (OD_{490 nm}) from wells of samples and standard, well without antigen competitor and well without antibody solution respectively.

Gluten Assay Kit

The gluten assay kit (Cortecs Diagnostic, Deeside, UK) was used according to the

manufacturer's instructions. This assay is a sandwich ELISA which uses an anti- ω -gliadin monoclonal antibody for the coating and the same antibody as a peroxidase conjugate (Skerritt & Hill, 1990b).

RESULTS

Optimization of the Competitive ELISA

Anti-gliadin serum was obtained by rabbit hyper-immunization with commercial gliadin. When an extract of total wheat proteins was used, reactivity to α -, β -, γ - and ω -gliadins and high molecular weight glutenins was observed by immunoblotting. Furthermore, a good recognition towards the prolamin fraction of barley, rye and triticale was observed. Reactivity to oat protein was low, and no recognition of soy, maize and rice proteins was observed (data not shown).

In order to devise the sequential competitive ELISA, each step of the assay was analyzed as described below.

- (1) *Titration of antiserum and selection of the working dilution.* Coating was performed for 16 h at 4°C with three gliadin concentrations (0.1, 1 and 10 $\mu\text{g ml}^{-1}$). As can be seen in Figure 1, the optimal working conditions were found within the range 1/100 000–1/200 000. On the basis of the curves obtained for each coating concentration, a working dilution was chosen so that an OD_{490 nm} of 1.5–2 would be obtained after 20 min of colour development.
- (2) *Solid-phase competition with gliadin.* The competition between gliadin immobilized on a solid phase and the immunocomplexes formed by pre-incubation of gliadin standard solutions with immune serum at 4°C for 16 h were evaluated. Competition was carried out for 20, 30, 60, 120 or 240 min at 37°C. The results are shown in Figure 1(b) as the logit transformation of OD_{490 nm} versus the logarithms of the gliadin concentrations. As can be seen, the straight lines exhibit similar slopes (i.e. differences of competition times have no influence on the assay sensitivity). The highest detectability was obtained after competition times of 20 or 30 min, as, for example, a 50% shift (logit $p = 0$), observed in plots at 20 or 30 min, was attained with concentrations lower than those corresponding to the other times tested.
- (3) *Antigen–antibody interaction in a fluid phase (pre-incubation).* In order to carry out a competition step of 30 min at 37°C, the influence of different pre-incubation conditions (16 h at 4°C, 1 h and 2 h at 37°C) was then studied. The results (Figure 1(c)) show straight lines of similar slopes under different pre-incubation conditions tested. The highest detectability of the assay occurred at a pre-incubation time of 120 min at 37°C.
- (4) *Gliadin concentration in the coating.* For the coating, three antigen concentrations (1, 5 and 10 $\mu\text{g ml}^{-1}$) were tested under the conditions of pre-incubation and competition previously determined. The serum dilution that would produce an OD_{490 nm} = 1.5–2 after 20 min was chosen in each case, according to Figure 1(a). From the results plotted in Figure 1(d), it followed that the highest detectability was achieved by performing the coating with a 1 $\mu\text{g ml}^{-1}$ gliadin solution.

Quantification by Competitive ELISA

The mean standard curve corresponding to seven assays is shown in Figure 2. The logit transformation was chosen to obtain better accuracy of curve fit for the antigen dose–response curves. The assays exhibited a good linear response within a wide concentration range (0.6 ng ml⁻¹–10 $\mu\text{g ml}^{-1}$). Fifty per cent inhibition (logit $p = 0$) was obtained with a concentration of 5 ng ml⁻¹, and the limit of detection (as calculated with a t -test at 99% confidence) was 1 ng ml⁻¹. The detection level of gluten corresponding to a 1/50 sample dilution was 0.1 mg/100 g of dry product.

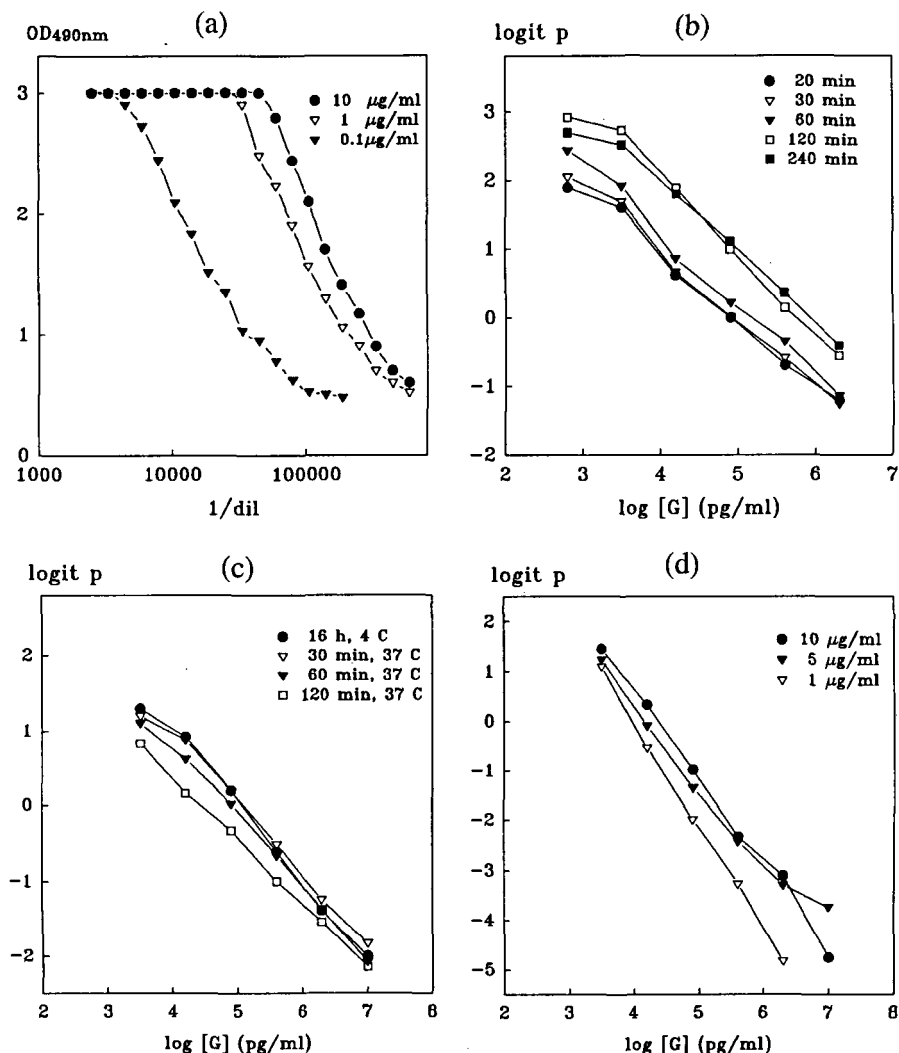


FIG. 1. Optimization of ELISA steps. (a) Titration of anti-gliadin sera using 0.1, 1 and 10 μg gliadin ml^{-1} for coating. Logit p versus logarithm of gliadin concentration ($\log [G]$) of: (b) competition at different times using a coating solution of 10 μg gliadin ml^{-1} and a pre-incubation of 2 h at 37°C; (c) analysis of pre-incubation conditions (16 h at 4°C or 30 min, 1 h and 2 h at 37°C, coating 10 μg gliadin ml^{-1} and competition during 30 min at 37°C); (d) analysis of coating concentration with coating solutions of 1, 5 and 10 μg gliadin ml^{-1} , pre-incubation for 2 h at 37°C and competition for 30 min at 37°C.

Analysis of Cross-reactivity among Proteins of Different Origin

Extracts of wheat, barley, rye, triticale, oats, soy, maize and rice in 70% ethanol were analyzed using the competitive ELISA described above. To this end, the protein extracts were incubated with anti-gliadin serum, and the immune complexes formed were made to compete with gliadin immobilized in the solid phase. Figure 3(a) shows the results obtained by plotting logit p versus the logarithm of the protein concentration. There was a distinct difference in the competition ability of the different protein extracts, evidenced by the shifting of the straight lines along the x -axis. The results are shown as inhibition curves in

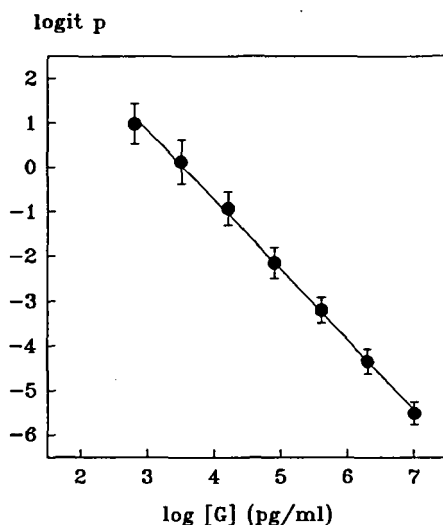


FIG. 2. Mean standard curve for gliadin determination. Vertical bars represents standard deviations for seven assays.

Figure 3(b). In Figures 3(a) and (b), shifting toward the right indicates a reduced competition ability, since higher concentrations of proteins in the extract were required by a given inhibition value. Figure 3(c) shows the competition index (CI) of the different extracts, calculated from the concentration producing a 50% inhibition ($\logit p = 0$), when the wheat extract was taken as reference.

Analysis of the Effect of the Solvent on Immunodetection

Most of the immunochemical tests employed for gliadin quantification use either 70 or 40% ethanol as the extraction solvent. By and large, since the objective is to determine low gliadin contents, analyses are carried out at low dilutions of the sample extracts in the assay diluent. Therefore, the influence of the ethanol present in the reaction medium on the antigen–antibody interaction was investigated. Solutions of 100 ng ml^{-1} gliadin in different ethanol concentrations were prepared, and analyzed by means of the ELISA described above. Figure 4 shows the inhibition obtained with each ethanol concentration, expressed as the percentage of the control with no ethanol. The increase in ethanol concentration results in a substantial decrease in the competition ability, and, consequently, in an under-valuation of the gliadin concentration in the sample.

This result should be taken into account, since foods with low gluten content are analyzed at dilutions ranging from 1/50 to 1/10. Under those conditions, the resulting ethanol concentration would range from 0.8 to 4% when 40% ethanol had been used, or from 1.4 to 7% in the case of 70% ethanol. For ethanol concentrations of 0.8–7%, the results indicate a decrease of reactivity, varying from 98 to 60% respectively. This leads to an under-valuation of the actual gliadin content from 2 to 40%.

Comparison between Results Obtained with the Competitive ELISA and a Commercial Kit

The comparison with a commercial kit was performed by processing standards, samples and controls simultaneously. The protocols of the kit and the assay developed in this study were followed. The gluten assay kit (Cortecs Diagnostics, UK) used was the one accepted as the

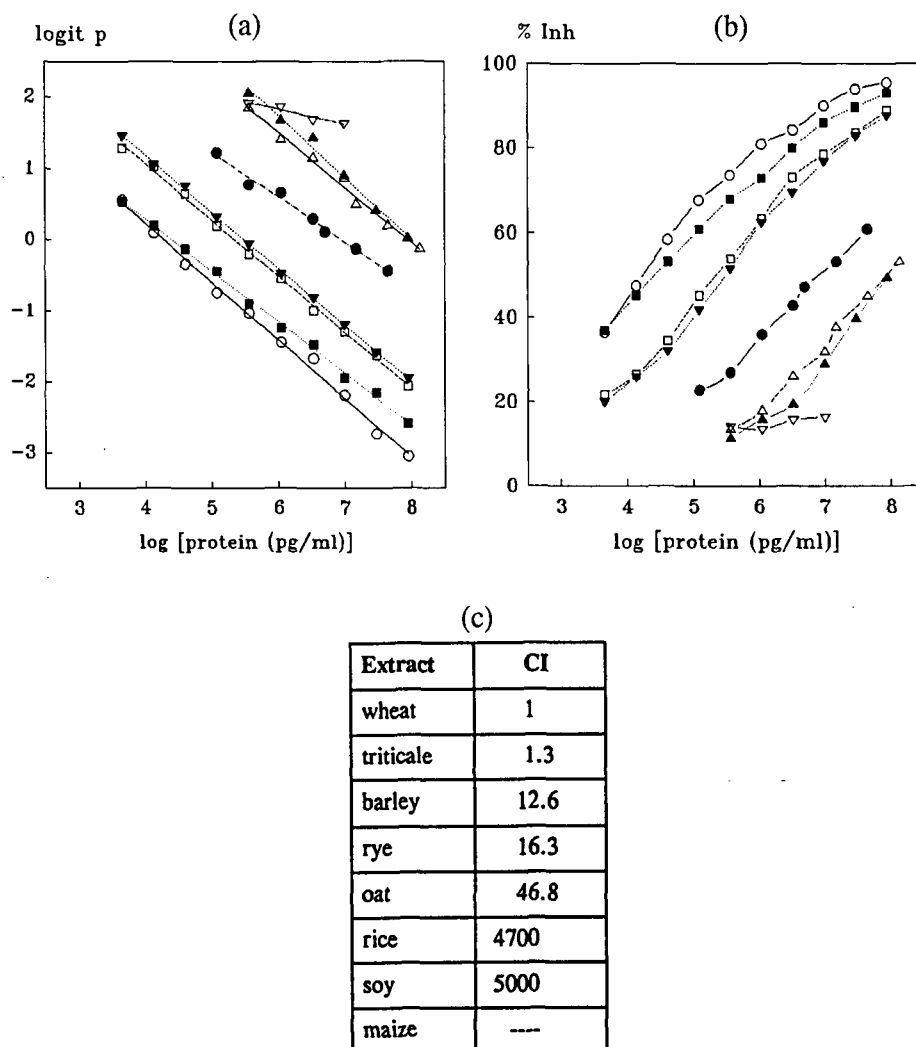


FIG. 3. Analysis of cross-reactivity among 70% ethanol extracts from flours of wheat (○), barley (□), rye (▽), triticale (■), oat (●), soy (▲), rice (△), and maize (▽). (a) Logit p versus logarithm of protein concentration; (b) percentage of inhibition; (c) competition index calculated from A at logit $p = 0$, using wheat as the reference.

official method by the AOAC. The kit includes a lyophilized gliadin standard and three controls of high, medium and low gluten content.

A good agreement was found between the results obtained by the authors' assay and the AOAC method for medium and high controls. Results of triplicate analyses performed with both the commercial kit and the new assay were respectively as follows: medium control, 23 ± 2 mg% and 27 ± 3 mg%; and high control, 100 ± 12 mg% and 115 ± 9 %. For low gluten content controls, the newly developed method detected 0.5 ± 0.1 mg% (four-fold determinations). The commercial kit was unable to detect values below 16 mg%.

No differences were found between the authors' standard and that of the commercial kit, when both standards were used in simultaneous assays using the two methods. Similar

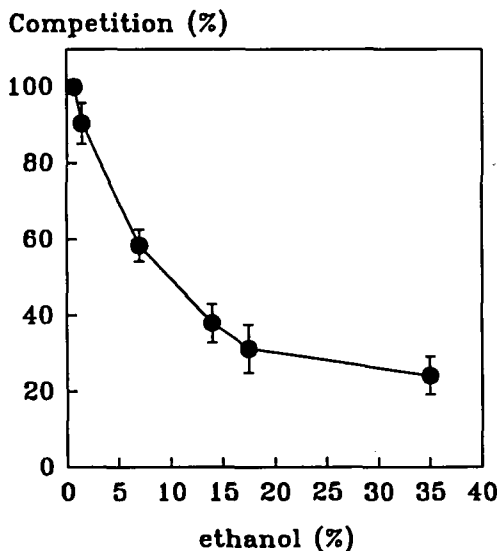


FIG. 4. The effect of different ethanol concentration on the competition of a 100 ng ml^{-1} gliadin solution. Points are means ($n = 4$) and vertical bars represents the standard deviation.

results were obtained in analyses of different foods performed using both methods. As these assays were performed at high extract dilution, the influence of the solvent was negligible.

Analysis of Food Samples by Competitive ELISA

The ELISA described above was used with commercial samples of different types of foods. The results are listed in Table 1.

It can be seen that the assay can be applied to a wide variety of foods, and that it maintains the ability to recognize prolamins in thermally processed foods, such as crackers and sausages. All products tested that here formulated for coeliac patients showed gliadin levels lower than that recommended by the WHO. Furthermore, the assay showed a good discrimination between foods containing no gluten and those containing a low amount of these proteins.

DISCUSSION

Coeliac disease is a chronic gastrointestinal condition and the only treatment available is adherence to a strict gluten-free diet (Branski *et al.*, 1992). The WHO has established that, the content of prolamins in any given type of food should not exceed $1 \text{ mg}/100 \text{ g}$ of the dry product (Codex Alimentarius Commission) in order to be considered gluten-free.

The need to certify the composition of foods for use by coeliac patients has resulted in the development of quantitative immunochemical methods (Troncone *et al.*, 1986; Freedman *et al.*, 1987; Friis, 1988).

Several problems such as the complexity of prolamins occurring in harmful cereals, their insolubility in aqueous solvents, their high cross-reactivity, the efficiency of the solvent used in the extraction procedure, the effects on the immunodetection caused by the presence of the solvent in the extraction medium and the changes produced by the thermal processes used in food manufacture, produce serious difficulties for optimization of the quantitative immunoassays.

The sequential competitive ELISA is a non-equilibrium assay, in which the pre-incu-

TABLE 1. Determination of gluten in foods by competitive ELISA^a

Food	Gluten (mg/100 g)	Products (labelled gluten-free)	Gluten (mg/100 g)
Cake flours	4000	Cookies	
Breakfast baked cereals	360	1	ND ^b
Cookies		2	0.2
1	2900	Spaghetti	0.6
2	770	Chocolate	
3	1800	1	ND
4	320	2	ND
Processed meats		Baking mixes	
Hamburgers 1	ND ^c	Cake 1	ND
Hamburgers 2	10 ^c	Cake 2	ND
Sausage 1	180 ^c	Bread	0.1
Sausage 2	35 ^c	Pizza	0.1
Soup, vegetable-based	900	Chocolate	ND
Chocolate		Maize starch	ND
1	ND		
2	ND		
3	0.3		
Mustard	100		

^aData shown are mg of gluten by 100 g of dry product.

^bND = not detectable (<0.1 mg/100 g).

^cData shown are mg of gluten by 100 g of net weight.

bation and competition steps with the immobilized antigen have a marked effect on the protein detection level. Long pre-incubation times favour the formation of immunocomplexes with the soluble antigen. Shorter competition times decrease the likelihood of interaction of the free antibody with the antigen in the solid phase, and of dissociation of those complexes formed during pre-incubation. The results shown in Figure 1(b) and (c) confirm both considerations. The concentration of gliadin in the coating also has an effect on detectability (Figure 1(d)), which increases as the gliadin concentration decreases.

Therefore, to attain the maximum possible detection level, the following working conditions were chosen: a coating of 1 µg ml⁻¹, pre-incubation for 2 h at 37°C and a competition time of 30 min at 37°C. Measurable competitions are achieved in this way, even when low gliadin amounts are present in the sample.

The competitive assay described exhibits a wide linear response range (0.6 ng ml⁻¹–10 µg ml⁻¹), the limit of detection being 1 ng ml⁻¹. The detection limit makes it possible to analyze samples with a gluten content as low as 0.1 mg/100 g of dry product (sample dilution, 1/50). This means that when working with the sample at a 1/10 dilution, 0.02 mg of gliadin/100 g can be detected.

Because of the high homology of cereal prolamins, and because food products for coeliac individuals often employ (among others) soy, maize and rice products to replace sources of harmful proteins, the study of cross-reactivity among these proteins is imperative for the development of quantification methods (Shewry *et al.*, 1986; Skerritt & Underwood, 1986; Festenstein *et al.*, 1987). This prompted analysis of protein extracts of the different cereals using the competitive ELISA. This system was chosen because of its closer similarity to the actual analytical situation that occurs in the prolamins quantification in foods.

A good degree of competition was seen between protein extracts of wheat, triticale, barley and rye (Figure 4(a) and (b)). It was somewhat lower in oats, whereas protein extracts of rice, soy and maize did not compete significantly when analyzed by this assay. These results show that the ELISA described above exhibits a high specificity for proteins harmful to coeliac patients, and, moreover, shows no cross-reactivity with unrelated proteins.

Different extraction solvents have been employed for gliadin quantification (Skerritt & Hill, 1990a). Most researchers prefer the use of alcoholic solvents, mainly ethanol. The extracting solvent becomes important in quantification. On one hand, it determines the extent of recovery of the sample prolamins during the extraction step. On the other hand, it can affect the antigen-antibody interaction as it is present in the reaction medium in which the immunoassay is performed (Skerritt & Martinuzzi, 1986; Skerritt & Hill, 1990c). The effect of the presence of different concentrations of ethanol on the ELISA was then studied. Results indicated that, for concentrations of ethanol in the reaction medium from 0.8 to 7%, an important decrease of the competition ability existed. This leads to reporting a result as 98% or 60% of the actual value respectively. This is a particularly relevant problem, since those ethanol concentrations in the reaction medium are present when samples with low gluten content are analyzed, where those ethanol concentrations arise from the 1/50 to 1/10 dilution of the sample extract. Thus, the ELISA would undervalue the actual gluten content of the sample. Despite this, gliadins can still be detected using the author's ELISA system, even in samples containing the minimal amount of gluten recommended by the WHO (1 mg/100 g). Even though the solvent effect is lower when 40% ethanol is used for the extraction, 70% ethanol has a higher capacity of extracting prolamins, and is thus frequently chosen for gliadin quantification.

The gluten assay kit, the official method accepted by the AOAC, was compared with the competitive ELISA. Similar values were obtained for the samples analyzed, as well as in the controls included in the kit corresponding to the medium and high gluten content. As far as the control of low gluten content is concerned, the assay developed in this study detected 0.5 ± 0.1 mg%, whereas the commercial reagent was unable to report values lower than 16 mg%.

The sandwich ELISA described by Ellis *et al.* (1994) which shows a very high sensitivity, has a detection limit of 15 ng ml^{-1} , while the ELISA described here has a limit of detection of 1 ng ml^{-1} .

It should be borne in mind that, as a result of the lack of adequate food control in many developing countries, a large number of products need to be analyzed to identify those suitable for coeliac patients. Therefore, the high cost of the commercial kit with monoclonal antibodies is a drawback limiting its use. A suitable ELISA using polyclonal antibodies may be afforded by many laboratories, and its widespread use is possible because of its low cost.

CONCLUSION

The assay developed in the authors' laboratory has a high level of detection together with an adequate accuracy, even at very low gliadin concentrations. It may be used with a wide variety of food samples. This method is suitable for use with both raw products and thermally processed foods, including meat products. Furthermore, it is in accordance with the recommendations of the WHO regarding the analysis of foods for consumption by coeliac patients, and it shows no cross-reactivity with unrelated plant proteins.

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