Talanta 91 (2012) 33–40

Contents lists available at SciVerse ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Comprehensive analysis of gluten in processed foods using a new extraction method and a competitive ELISA based on the R5 antibody

María C. Mena*, Manuel Lombardía, Alberto Hernando, Enrique Méndez[1], Juan P. Albar

Proteomics Facility, Centro Nacional de Biotecnología (Consejo Superior de Investigaciones Científicas), Calle Darwin 3, Cantoblanco, 28049 Madrid, Spain

ARTICLE INFO

Article history:
Received 26 August 2011
Received in revised form
22 December 2011
Accepted 28 December 2011
Available online 18 January 2012

Keywords:
Enzyme-linked immunosorbent assay (ELISA)
Monoclonal antibody R5
Hydrolysed gluten
"Gluten-free" processed foods
Gluten extraction

ABSTRACT

The only treatment for coeliac disease is to follow a strict, life-long gluten-free diet. It is therefore essential to use a highly sensitive, specific technique for gluten analysis in foods. Nowadays, the usual method for determining gluten content in gluten-free foods, internationally accepted by the Codex Alimentarius Commission, is the R5 antibody-based sandwich ELISA, combined with the cocktail-extraction solution. This technique requires at least two epitopes in the protein, but in hydrolysed foods, proteins are fragmented during food processing and converted into peptides in which only one toxic epitope may appear. Consequently, it was necessary to develop a new competitive immunoassay that, together with a reliable, compatible extraction solution, would provide a complete gluten analysis in any kind of food. We analysed commercial foods and home-made maize breads spiked with a known amount of gliadins using the sandwich R5 ELISA and the new competitive R5 ELISA that has been developed. These foods had previously been extracted with 60% ethanol/water, the cocktail solution or the new extracting solution called UPEX (universal prolamin and glutelin extractant solution). The complementary SDS-PAGE and western blot techniques were also used to confirm the gluten content. The limits of detection and quantification of the competitive R5 ELISA were 0.36 and 1.22 ng/ml of gliadins, respectively. The intra- and inter-assay precisions based on two samples were, respectively, 7.3% and 5.4% for the first sample and 9.9% and 6.3% for the second. This new assay was a better technique than the sandwich R5 ELISA for detecting gliadins quantitatively in hydrolysed foods. Regarding the extraction procedure, we did not find any significant interference from components of the UPEX solution at the concentration used. In addition, the UPEX solution extraction was compatible with the R5 western blot and mass spectrometry techniques. The competitive R5 ELISA we developed, combined with the UPEX solution described here, is a very useful tool for detecting and quantifying gluten in any kind of food samples, including heat-treated and/or hydrolysed ones.

© 2012 Elsevier B.V. All rights reserved.

1. Introduction

Coeliac disease is a permanent intolerance to gluten proteins in wheat, barley, rye and possibly oats that, in genetically predisposed people, leads to villous atrophy of the small-bowel mucosal [1]. The only treatment for this disease is to follow a strict life-long gluten-free diet and it is therefore essential to use a highly sensitive, specific technique for gluten analysis in food. The prevalence of this disease is approximately 1% worldwide, but many coeliac disease cases are undiagnosed because they have atypical clinical presentations or are asymptomatic [2]. Diagnosis and treatment should be established early, given that the untreated disease causes several highly important clinical malignant complications [3].

Gluten contains hundreds of protein components that are present as either monomers or as oligomers and polymers linked by interchain disulphide bonds. The solubility of these proteins depends on the degree of aggregation; the monomeric proteins are soluble in an alcohol solution, while the polymeric ones are soluble in alcoholic solutions under reducing conditions [4]. Traditionally, gluten proteins have been classified as prolamins and glutelins; together, these are the main storage proteins in wheat, barley and rye kernels and other cereals such as oats, rice and maize [5].

In January 2009, the European Commission published a new European Regulation concerning the composition and labelling of foodstuffs suitable for people intolerant to gluten; this regulation indicates that foods may display the term "gluten-free" if the gluten content does not exceed 20 mg/kg in them as sold to the final consumer [6]. Consequently, methods for gluten analysis must be sensitive enough to quantify these levels of gluten in foods.

Nowadays, the method for determination of the gluten content in gluten-free foods accepted internationally by the Codex

* Corresponding author. Tel.: +34 915854670; fax: +34 915854506.
E-mail address: mcmena@cnb.csic.es (M.C. Mena).
[1] Deceased.

0039-9140/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
doi:10.1016/j.talanta.2011.12.073

Table 1
Analysis by competitive R5 ELISA of maize bread spiked with gliadins during bread processing. Bread A (54 ppm of intact gliadins) and Bread C (52 ppm of hydrolysed gliadins) were extracted using different concentrations of Sarkosyl (S) and TCEP (T) reagents. Results are expressed in ppm of gliadins (average ± standard deviation) and percentage of gliadin recovery (R).

Sample	60% ethanol		1% S—2 mM T		1% S—5 mM T		2% S—2 mM T		2% S—5 mM T		6% S—2 mM T		6% S—5 mM T	
	Gliadins	R	Gliadins	R	Gliadins	R	Gliadins	R	Gliadins	R	Gliadins	R	Gliadins	R
A	7.3 ± 1.3	13.5	37.8 ± 15.1	70.1	46.3 ± 7.4	85.6	42.8 ± 2.8	79.2	55.4 ± 8.8	102.5	46.8 ± 3.9	86.6	59.8 ± 6.3	110.8
C	7.6 ± 1.6	14.6	42.6 ± 17.6	82.0	44.8 ± 2.5	86.1	39.8 ± 12.4	76.4	51.0 ± 5.0	98.1	52.8 ± 1.8	101.4	51.8 ± 5.5	99.6

Alimentarius Commission is the sandwich ELISA based on the R5 antibody [7]. This is an immunological test based on the R5 monoclonal antibody recognising potential coeliac-toxic epitopes, which occur repeatedly in gliadins, hordeins and secalins (the prolamin proteins from wheat, barley and rye, respectively). This technique is used combined with the cocktail extraction solution, which is useful for extracting gluten even in heat-treated foods, in which the insoluble matrix makes complete extraction of gluten from foods more difficult [8]. This cocktail solution contains a reducing agent that, together with alcohol extraction, extracts both monomeric and polymeric gluten proteins [9].

The principal limitation of the sandwich R5 ELISA is that it is essential that at least two epitopes of the sequences recognised by the monoclonal antibody R5 be present simultaneously in a protein or peptide. However, in hydrolysed foods (such as baby foods, syrups and beers), gluten proteins are fragmented during food processing and converted into peptides in which only one toxic peptide may appear. In this case, the quantification of gluten by sandwich R5 ELISA would be incorrect, yielding less than the real gluten content. The Codex Alimentarius Commission states that "for the detection of hydrolysed gluten a modification of the R5 assay (competitive ELISA) has to be applied" [7].

Some authors have used other antibodies different from the R5 antibody to develop competitive ELISA systems for analysis of gluten in foods but these antibodies are not the accepted by the Codex Alimentarius [10,11]. In addition, Laube et al., have developed both an electrochemical magneto immunosensor and a magneto ELISA based in a competitive assay to quantify gliadins in foods. These techniques are promising approaches, but are not compatible with solutions containing disaggregating agents required to achieve complete gluten extraction [12].

Based on the type of assay, the competitive technique is not compatible with the cocktail extraction solution and it is only possible to use an alcoholic solution for gluten extraction. As mentioned previously, the ethanol extraction itself does not lead to complete gluten extraction in heat-treated foods and the quantification is therefore not complete.

The aim of this study was to develop a competitive ELISA assay based on the R5 antibody, as the Codex Alimentarius recommends, together with a reliable and compatible extraction solution, that would lead to accurate gluten analysis in any kind of food, including heat-treated and/or hydrolysed foodstuffs.

2. Materials and methods

2.1. Reagents

All chemicals were of analytical grade or higher. All aqueous solvents and solutions were prepared with ultrapure water purified by a Milli-Q purification system (Millipore, Bedford, USA).

2.2. Samples

2.2.1. Food samples

We analysed a wide variety of commercial foods, both labelled and not labelled as gluten-free, that were bought in grocery stores in different regions of Spain during different seasons to maximise the chances of getting food products with a different production code. Solid samples were thoroughly ground to a fine, homogenised powder using an IKA A11 analytical mill (IKA®, Staufen, Germany). Liquid samples were homogenised by vigorous vortexing for 30 s.

2.2.2. Reference material and controls

We used the gliadin standard provided by the Working Group on Prolamin Analysis and Toxicity (2 mg/ml in 60% ethanol/water [v/v]). This gliadin standard is a mixture of 28 wheat cultivars representative of the European wheat-producing countries [13].

The positive control used in all the ELISA analyses was a commercial wheat starch containing 55 ppm of gliadins (110 ppm of gluten) as determined by the sandwich R5 ELISA. The negative control used in the ELISA analyses was a commercial maize flour sample containing less than 1.5 ppm of gliadins, as determined by the sandwich R5 ELISA.

2.2.2.1. Digestion of the gliadin standard. Three different gliadin solutions (1 mg/ml) were digested for 4 h at 37 °C and 600 rpm in a thermomixer (Eppendorf Thermomixer comfort) with different enzymes: trypsin (from bovine pancreas type I, Sigma, T8003) at 0.1 and 1 U/ml in an ammonium bicarbonate buffer, pH 8; subtilisin (from *Bacillus licheniformis* type VIII, Sigma, P5380) at 0.05 and 0.1 mU/ml in Tris–HCl, pH 7; and pepsin (from porcine gastric mucosa, Sigma, P7012) at 2 and 10 U/ml in 1% formic acid. Reactions were stopped by heating the reaction mixture at 100 °C for 10 min to inactivate the enzymes.

2.2.2.2. Preparation of self-made maize breads spiked with gliadins. Firstly, we tested all the ingredients using the sandwich R5 ELISA to ensure that they were gluten free. For preparation of the self-made breads A–E (Tables 1 and 2), maize flour (10 g), water (2000 g), baker's yeast (0.7 g), NaCl (0.2 g), glucose (0.4 g) and egg whites (1000 g) were kneaded into a dough, fermented for 1 h at 37 °C and baked at 230 °C in an oven (Heraeus) for 10 min. We allowed water evaporation until the bread weight was stable; the breads were then weighed using a precision balance (Boeco, Germany) and ground into a powder using an A11 analytical mill (IKA®, Staufen, Germany).

Bread E was used as negative control, without adding gliadins. For the preparation of the gliadin-spiked breads, a gliadin extract (either intact or hydrolysed) was added to the water used in the recipe before kneading the dough. Likewise, 0.75 mg of intact gliadins (375 μl of 2 mg/ml intact gliadins) was added to Bread A, 1.5 mg of intact gliadins (750 μl of 2 mg/ml intact gliadins) to Bread B, 0.75 mg of hydrolysed gliadins (375 μl of 2 mg/ml gliadins hydrolysed with trypsin) to Bread C and 1.5 mg of hydrolysed gliadins (750 μl of 2 mg/ml gliadins hydrolysed with trypsin) to Bread D.

The final amount of gliadins in the breads was calculated by the formula: gliadins (ppm) = [gliadins added (mg)/weight of bread (kg)] × 0.864 (correction factor for purity). The degree of purity of gliadins was calculated by protein determination according to the Dumas method and gliadins were determined by size-exclusion high-performance liquid chromatography [13].

Table 2
Analysis by competitive and sandwich R5 ELISA, after extraction with cocktail solution, 60% ethanol or UPEX solution, of maize breads spiked with gliadins during the bread manufacturing process. Results are expressed in ppm of gliadins (average ± standard deviation) and percentage of gliadin recovery (R). *N* = number of analysis.

			Sandwich R5 ELISA									Competitive R5 ELISA					
	Gliadins		Cocktail solution			60% ethanol			UPEX			60% ethanol			UPEX		
Sample	Type	Amount	*N*	Gliadins	*R*	*N*	Gliadins	*R*	*N*	Gliadins	*R*	*N*	Gliadins	*R*	*N*	Gliadins	*R*
A	Intact	54	9	53.4 ± 6.4	99.0	7	8.7 ± 1.6	16.1	15	54.6 ± 7.1	101.2	14	7.4 ± 1.7	13.6	28	55.4 ± 7.0	102.6
B	Intact	109	5	102.0 ± 9.5	93.6	5	12.4 ± 1.7	11.4	4	112.4 ± 10.7	103.1	5	10.4 ± 2.3	9.5	13	107.6 ± 11.2	98.7
C	Hydrolysed	52	5	35.7 ± 6.0	68.7	10	8.1 ± 2.0	15.5	13	22.2 ± 2.0	42.8	17	7.9 ± 2.4	15.1	31	52.7 ± 7.8	101.4
D	Hydrolysed	110	5	62.3 ± 6.2	56.6	5	11.2 ± 0.8	10.2	4	72.4 ± 8.6	65.8	5	13.8 ± 1.5	12.5	15	109.2 ± 8.4	99.2
E	No addition	0	6	<1.5	–	5	<1.5	–	3	<1.5	–	5	<1.2	–	21	<1.2	–

2.2.2.3. Food samples spiked with gliadins. We spiked 21 selected food samples with 55 ppm of gliadins. To obtain a homogenous powder and to check for a possible matrix effect that could interfere with the analysis, the gliadins were weighed and added to the food sample and the mixture was ground with an IKA A11 analytical mill (IKA®, Staufen, Germany). The foods were selected to get a widespread range of materials and different matrices.

2.3. Gluten extraction procedures

2.3.1. Aqueous ethanol and cocktail extraction

Samples (0.25 g) were weighed and transferred to a propylene tube. They were then extracted with 10 ml of 60% aqueous ethanol (v/v) or the cocktail solution, based on reducing 2-mercaptoethanol (Sigma M-6250, St. Louis, MO, USA) and chaotropic guanidine hydrochloride (Fluka 50940, Buchs, Switzerland) reagents in phosphate buffered saline (PBS) as previously described (patent WO 02/092633 A1) [9].

2.3.2. UPEX extraction

The gluten extraction procedure described here is based on reducing Tris (2-carboxyethyl)-phosphine (TCEP) (Sigma 32497LJ, St. Louis, MO, USA) and anionic surfactant N-lauroylsarcosine (Sarkosyl) (Aldrich 61745, St. Louis, MO, USA) reagents in PBS (patent WO 2011/07039 A2).

(1) A 0.25-g sample of a ground food was weighed and transferred to a 10-ml polypropylene tube.
(2) A 2.5-ml aliquot of the UPEX solution (5 mM TCEP, 2% N-lauroylsarcosine in PBS, pH 7) was added to the tube containing the sample. For preventing inactivation of the reducing agent, we prepared UPEX solution immediately before to use it.
(3) The tubes were closed tightly and the caps were covered with film to avoid evaporation.
(4) The contents of the tubes were mixed thoroughly by vortexing (5–10 s) and the tubes were placed in a rack.
(5) The tubes were incubated in an oven (Heraeus) at 50 °C for 40 min.
(6) The tubes were allowed to cool for 5 min at room temperature.
(7) A 7.5-ml aliquot of 80% ethanol/water (v/v) was added and the samples were thoroughly dispersed by vortexing for 10–60 s (until total dispersion of the sample was achieved) and then incubated for 1 h at room temperature in a rotary (head-over-head) shaker (Labinco B.V. model L26) at 45 turns/min.
(8) The tubes were centrifuged in a bench-top centrifuge (Eppendorf model 5810) for 10 min at 2500 g at room temperature.
(9) Using fresh Pasteur pipettes, the supernatant from each tube was transferred to a clean 10-ml polypropylene tube. The solution was then ready for the analysis by the different techniques. The analyses were performed within 24 h of extraction.

For the extraction of gluten in food samples containing chocolate or other ingredients that might affect the system, we added 0.25 g of gelatine (from cold water fish skin [Sigma No. G-7041]) and 0.1 g of polyvinylpyrrolidone (PVP) (Sigma PVP-360) to the sample weighed, before extracting with the UPEX solution.

2.4. Techniques employed

2.4.1. Sandwich R5 ELISA

We used a homemade sandwich R5 ELISA based on the unique monoclonal antibody R5 [8,14]. This antibody reacts with the epitope QQPFP and other amino acid motifs such as QLPFP, LQPFP and QQQFP present in coeliac-toxic sequences [15,16] from gliadins, hordeins and secalins; the antibody was found to be highly sensitive towards these prolamins [8].

2.4.2. Competitive R5 ELISA

A competitive system based on the R5 antibody was developed. A 96-well microplate (EIA/RIA flat bottom polystyrene microwell plate without a lid [Product 3590, Corning, NY, USA]) was coated with 100 μl of gliadins at 50 ng/ml in 50 mM sodium carbonate/bicarbonate buffer (pH 9.6), for 2 h at room temperature. The wells were then washed 3 times with 370 μl of PBS containing 0.05% Tween 20 (v/v) (Sigma, St Louis, MO, USA) (PBS-T), pH 7.4, using an automated Bio-Rad Model 1575 Immunowash microplate washer. To block unoccupied binding sites and to prevent possible non-specific adsorption, we performed a subsequent incubation with 300 μl of the blocking buffer (1% bovine serum albumin fraction V [BSA] [Sigma Chemical Co., USA] in PBS-T) for 1 h at room temperature. Afterwards, the microplate was washed three times as described above and then the standard antigen solutions or samples were added to each well. The samples consisted of 50 μl of sample extracts diluted in PBS-T plus 1% BSA (dilution buffer) at an appropriate dilution, which were dispensed into the plate wells in duplicate. In addition, both duplicates were also diluted 1/2 with dilution buffer to get 4 quantification points for every sample. The standard solution was prepared from a stock solution of 0.5 mg/ml of gliadins; it was diluted 1:6400 in dilution buffer to 78.13 ng/ml, then serially diluted to 0.61 ng/ml and dispensed into the plate wells in duplicate. The horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG was obtained from monoclonal antibody R5 (R5-HRP). Afterwards, a 50-μl aliquot of the purified antibody R5-HRP (1:50,000 in PBS-T) was added to each sample well, mixed thoroughly and incubated for 1 h at room temperature.

After washing 6 times with PBS-T, the wells were filled with 100 μl of K-Blue™ substrate 3,3′,5,5′-tetramethylbenzidine (TMB) (Neogen, Kentucky, USA). After incubation for 10 min at room temperature in darkness, 50 μl of H_2SO_4 2.5 M were added to each well to stop the enzyme reaction. Next, the immunoassay optical density absorbance of each well was measured with a Bio-Rad Model 680 Microplate Reader at 450 nm. A computer with a spreadsheet program to convert optical densities to concentrations (Biorad program Microplate Manager® 5.2.1) was used for analysing the results.

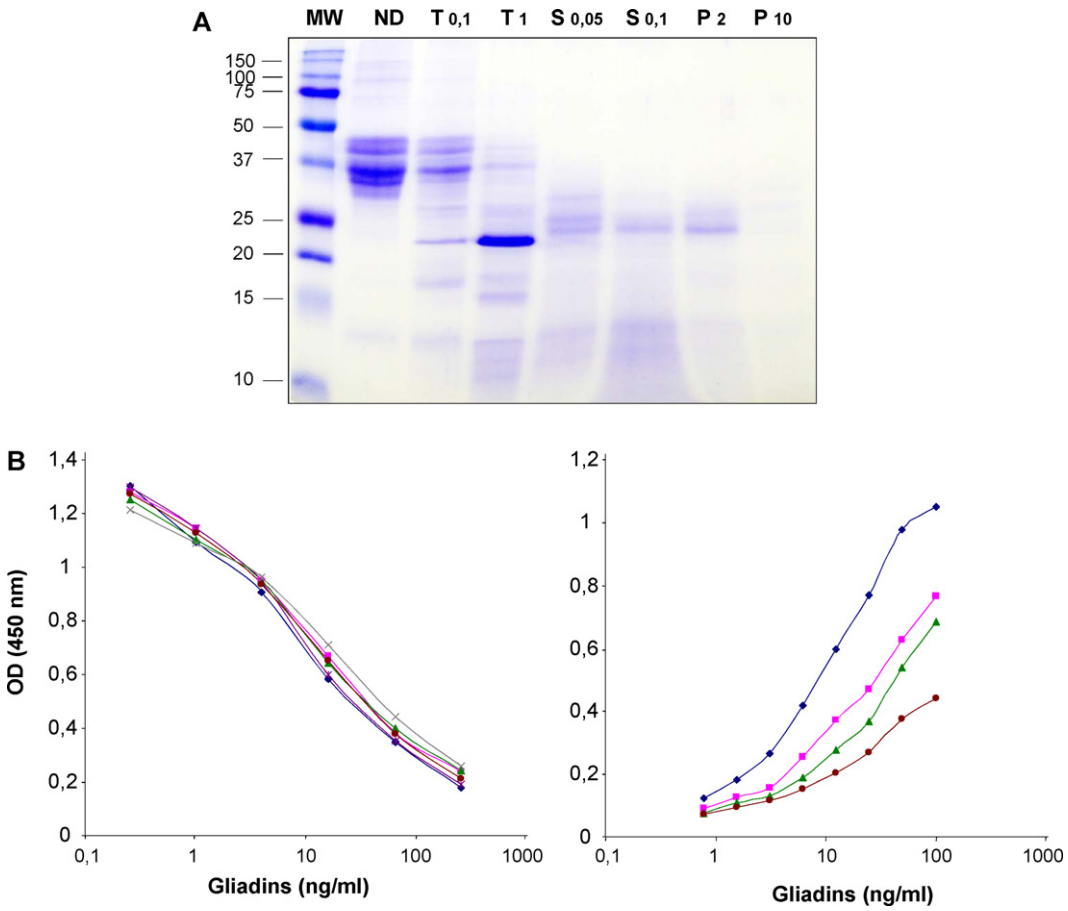

Fig. 1. (A) SDS-PAGE analysis of gliadins using Coomassie Blue staining. MW: molecular weight marker (shown on the left in kDa); ND: not digested; T 0.1 and T 1: digested with trypsin 0.1 and 1 U/ml, respectively; S 0.05 and S 0.1: digested with subtilisin 0.05 and 0.1 U/ml, respectively; P 2 and P 10: digested with pepsin 2 and 10 U/ml, respectively. (B) Competitive (left) and sandwich (right) R5 ELISA curves obtained with undigested gliadins (rhombus) and gliadins digested with trypsin 1 U/ml (square), subtilisin 0.05 U/ml (triangle), subtilisin 0.1 U/ml (cross), pepsin 2 U/ml (asterisk) and pepsin 10 U/ml (circle).

The microplate was placed in a humid box (moist chamber) during all of the incubation procedures to avoid evaporation.

2.4.3. SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis)

Discontinuous gel electrophoresis was performed using a Mini-PROTEAN III (Bio-Rad, Hercules, CA, USA). In this study, 0.5 μl of the intact and hydrolysed standards of gliadins (2 mg/ml solution in 60% ethanol) or the appropriate volume of each sample was dried in a Speed-Vac centrifuge (Eppendorf Concentrator plus) for 45–55 min and dissolved in electrophoresis loading buffer (60 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.1% bromophenol blue). These samples were vortexed with a Vortex mixer (IKA model Ms1) and boiled for 5 min in a thermomixer (Eppendorf Thermomixer comfort). Afterwards, 20 μl of each sample was loaded onto the polyacrylamide gel (a stacking gel containing 5% acrylamide and a running gel containing 18% acrylamide, at a gel size of 0.75 mm). Electrophoresis was run at a constant 25 mA/gel. Protein and peptide bands were visualised with Coomassie Blue staining.

2.4.4. Western blot

After one-dimensional SDS-PAGE, proteins were electrotransferred onto polyvinylidene difluoride membranes, incubated with R5-HRP and immunodetected using enhanced chemiluminescence (Amersham Pharmacia, Buckinghamshire, UK), as previously described [8].

3. Results and discussion

3.1. Characterisation of the competitive R5 ELISA

The sandwich immunoassay is a method using two antibodies that bind to different sites on the antigen. The primary antibody, which is highly specific for the antigen, is attached to a solid surface, while the detection antibody binds the antigen to a different epitope than the primary antibody, requiring at least two epitopes that do not overlap on the antigen. Consequently, if a sandwich ELISA is used to assess the gluten content of a product containing hydrolysed gluten, the reported gluten content is likely to be underestimated.

During the manufacture of many foods, some processes including thermal and enzymatic reactions may lead to protein hydrolysis [17], finding peptides and proteins that contain only one epitope but are still potentially toxic for coeliacs. This is very important because gluten is found in up to 70% of manufactured food products and manufacturing regulations are not standard in all countries [18]. As it is not always possible to know for certain, prior to the analysis, which food may contain gluten in a hydrolysed form, it is very important to use a technique that guarantees correct analysis, whether in the case of intact or hydrolysed proteins. Consequently, a competitive ELISA might properly quantify both intact and fragmented gluten because this technique uses only one antibody and requires only one epitope. In addition, the competitive system is cheaper and faster than the sandwich system, given that only one

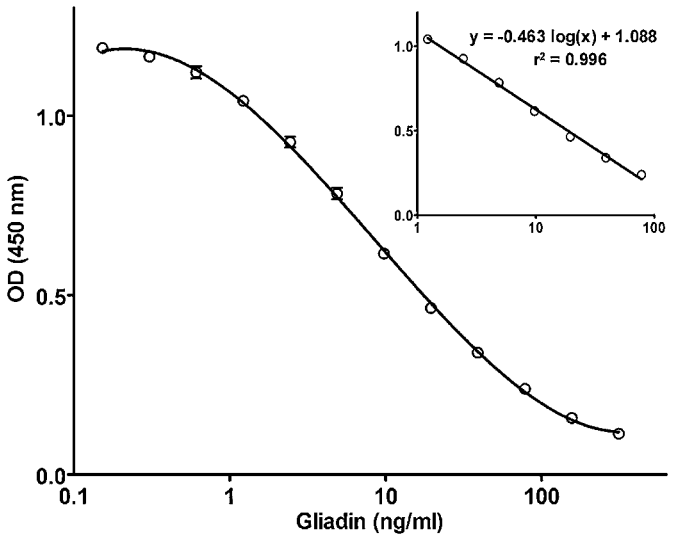

Fig. 2. Standard curve for gliadin determination by competitive R5 ELISA. Vertical bars represent standard errors of 8 independent assays. The inset shows the linear range (1.22–78.12 ng/ml) of gliadins.

antibody is used and the incubation of the sample and the conjugated antibody is performed together in the competitive ELISA.

The R5 antibody recognises small and linear epitopes highly repeated and widely distributed among the different prolamin types from wheat, barley [8,16] and rye and are present is several sequences described as immunogenic or toxic for coeliacs [19]. These facts make the R5 antibody suitable for detecting hydrolysed prolamins, despite of some of the harmful small peptides that may remain undetected.

3.1.1. Gliadin standard

In our study, we digested gliadins with trypsin and partially digested them with subtilisin or pepsin. We assessed the extent of digestion using SDS-PAGE (Fig. 1A). These digested gliadins were used to perform the corresponding standards curves for both the competitive and sandwich R5 ELISA as compared with the standard curve. The resulting curves were similar in each case for the competitive assay. However, the curves were quite different in the sandwich ELISA (Fig. 1B) demonstrating that sandwich ELISA is not suitable for hydrolysed samples as expected.

We noticed that exhaustive digestion, with pepsin, subtilisin and other enzymes breaking the epitopes recognised by the R5 antibody, affected the ELISA system (data not shown). That is the reason why further research on this matter should be done in order to develop a more suitable standard because difference in the degree of hydrolysis can affect the antibody detection. This is a great challenge taking into account that it is very difficult to know beforehand the degree of hydrolysis of every product. In this aim, some authors have made efforts to achieve a better hydrolysed prolamin standard from wheat, barley and rye [20], although testing and validation is needed before using them as standards.

Nevertheless, commercial foods usually have only partial hydrolysis and, when the proteins are exhaustively hydrolysed, the toxicity for coeliac patients of the peptides generated usually disappears [21]. Taking our results in consideration and the fact that intact gliadin are so far the internationally accepted standard, we used intact gliadins in standard curves in subsequent assays.

3.1.2. Reference curve

The calibration curve for the competitive ELISA ($n = 8$) was generated with 12 different gliadin concentrations, ranging from 0.15 to 312.5 ng/ml in serial dilutions of 1:2 (Fig. 2) and was established based on absorbance (OD) versus log concentration of gliadins. The linear range was found to be 1.22–78.12 ng/ml with the regression equation of $y = -0.463 \log(x) + 1.088$, $r^2 = 0.996$ (Fig. 2, inset). The standard curve of a competitive binding assay has a negative slope, in which higher antigen values in the samples or standards yield lower absorbance.

3.1.3. Limits of detection and quantification of the system

The limit of detection (LOD) was defined as the lowest prolamin concentration detectable in the assays and calculated as 3 times the standard deviation (SD) ± the mean of 46 replicates of the maximum binding well (PBS-T in place of the standard). In our study, the LOD was 0.36 ng/ml of gliadins. The linear range of the standard curve was 1.22–78.12 ng/ml of gliadins (Fig. 2, inset). Although this is a narrow linear range, it is wide enough to quantify gluten contamination in foods for coeliacs and similar to other existing ELISA-based systems [10,11].

We established the limit of quantification (LOQ) as 1.22 ng/ml of gliadins. This allowed the analysis of food samples with an amount of gluten as low as 2.44 ppm, when working with a minimum sample dilution of 1:25 of the food extracted at a concentration of 250 mg/10 ml of 60% aqueous ethanol. However, the extraction is easier in liquid foods (such as beer) and we can use a lower dilution factor, lowering the LOQ to 0.61 ppm of gluten. The calculation of the gluten content is based on the assumption of a 1:1 ratio between gliadin and glutenin, although this is controversial [22].

3.1.4. Assessment of the assay precision of competitive R5 ELISA

To determine the reproducibility and repeatability of the competitive R5-ELISA, inter- and intra-assay coefficients of variation were calculated by performing 16 measurements of 2 samples containing 10 and 22 ppm of gluten. For intra-assay variability, we obtained coefficients of variation of 7.3 and 5.4%, respectively; for inter-assay variability, the coefficients of variation were 9.9 and 6.3%, respectively.

3.2. Optimisation of the new extraction system

An important focus in gluten analysis is to use an extraction system leading to a recovery near 100%, to guarantee that products suitable for coeliacs are really gluten free. During the processing of some foods, proteins are treated at high temperatures in a dry state at a neutral pH, forming isopeptide bonds between the e-amino groups of lysine and the β- or γ-carboxamide groups of asparagines and glutamine residues [17]. In addition, both prolamins and glutelins have to be extracted completely. This is because it was traditionally said that only prolamins were toxic for coeliacs, but a more recent classification (according to primary amino acid structure) reveals not only a great heterogeneity but also similarities between different gliadin and glutenin proteins. Peptides derived from both groups are immunostimulatory in coeliac disease and it is highly probable that wheat glutelins (glutenins) [23–25] and barley and rye glutelins would be also toxic for coeliacs [26].

Taking these facts into account, it is necessary to combine reducing and disaggregating agents to extract gluten proteins completely. However, the cocktail solution (patent WO 02/09263 A1), which contains these types of reagents, is not compatible with the competitive assay because β-mercaptoethanol interferes with the specific binding of the antibody, obtaining false results. Some authors have reported the interfering effect of reducing agents in the immunoassays [27,28]. In the same way our R5 competitive was affected by 2-mercaptoethanol (data not shown) and that is the reason why we tried to solve this problem and to test other reducing agents. To avoid this problem, we first tried to block the sulfhydryl groups of 2-mercaptoethanol with 4-vinylpyridine or iodoacetamide, but the results were inconsistent (data not shown).

After testing several reagents (data not shown), we found that the best combination for gluten analysis was the reducing agent TCEP and the surfactant N-lauroylsarcosine. We called this extraction solution UPEX (universal prolamin and glutelin extractant solution). We also proved that each reagent used separately or together, does not interfere with the analysis using the ELISA and western blot techniques. In addition, this extraction solution is compatible with analysis by mass spectrometry (data not shown).

An odourless reducing agent, TCEP is more specific for disulfide bridges and less toxic than the other reducing agents commonly used [29,30]. N-lauroylsarcosine is a detergent, widely used in plant cell lysis, which contributes to opening polypeptide chains and is even more efficient than guanidine hydrochloride [31]. Other authors have also used the reducing agent TCEP for gluten analysis but combined with guanidine hydrochloride [32]. For our purpose, we tested several concentrations of both reagents and found that the lowest concentration that achieves complete gluten extraction and is the most compatible with all the techniques was 5 mM of TCEP and 2% of N-lauroylsarcosine (Table 1).

TCEP is not particularly stable in phosphate buffers especially at neutral pH. For this reason, we prepared the solution immediately before use. However, stability of TCEP in solution must be study to increase the time of use of the ready-prepared solution. On the other hand, other buffer such as Tris–HCl could be used instead of phosphate.

To prove the efficiency of the competitive immunoassay combined with the extraction system developed here, we analysed the self-made maize breads spiked with gliadins. Each sample was extracted and analysed several times (the total number of analyses was 255) to obtain reliable results (Table 2).

Table 2 presents the comparison between the two ELISA techniques (sandwich and competitive) and the different procedures of extraction compatible with each system. The standards used in this study (self-made maize breads) are very homogeneous samples and present both the heating and hydrolysing treatments, with the results being representative of what occurs in commercial foods affected by these processes. This table shows that quantification with the competitive R5 ELISA after gluten extraction with UPEX is the only method that leads to an almost full recovery of the gluten present in these types of samples.

3.3. Determination of gluten in food samples

3.3.1. Recovery of gliadins in spiked food samples

To determine the possible effect of food matrices on the competitive assay, we spiked food samples not containing gluten (gluten below 3 ppm) with 55 ppm of gliadins. We selected samples from a wide variety of ingredients to ensure that the matrix composition did not affect the system of analysis. The data were expressed as the mean of four replicates (Table 3). The recovery results given by the competitive R5 ELISA were between 90% and 110% in most of the foods analysed. Nevertheless, in the case of chocolate and others foods containing tannins, we observed a lower recovery than expected. Tannins are plant polyphenols that bind and precipitate proteins (such as gliadins) and yield large tannic acid-gliadin complexes, therefore interfering in the determination of the gluten content in food. As well as gliadins, other proteins such as gelatine are susceptible to binding to polyphenols. Taking our previous results [33] into account, we optimised a protocol to measure gluten in foods by combining the UPEX solution with fish gelatine and PVP. We proved that, after extracting gluten by means of the UPEX solution together with gelatine and PVP, the recovery of gluten was also complete in these spiked samples. This modified protocol should be applied routinely or at least when analysing foods containing unknown ingredients.

Table 3
Analysis by competitive R5 ELISA of food samples spiked with gliadins extracted with UPEX solution or the modified protocol UPEX-gelatine-PVP. Results are expressed in percentage of gliadin recovery.

Food sample	UPEX	UPEX-gelatine-PVP
Baked snack	92	ND
Baby food	101	ND
Biscuits	105	ND
Bread flour	109	ND
Breakfast cereals	102	ND
Chips	103	ND
Chocolate biscuits	41	99
Chocolate cake	25	98
Chocolate cookies	8	101
Cold meat	94	ND
Cooked ham	103	ND
Curry powder	21	102
Custard	95	ND
Maize pancakes	94	ND
Paprika	99	ND
Pepper	44	122
Pizza dough	107	ND
Pudding	108	ND
Sausages	106	ND
Sliced bread	104	ND
Strawberry jam	32	102

ND: not determined.

Consequently, based on these data, it was confirmed that no significant matrix influence was produced when using this competitive system combined with the UPEX extraction for gluten analysis in food samples. In addition, these recovery rates proved that UPEX did not produce any interference with the analysis. However, when we used the cocktail solution, we found erroneous results due to interferences (value not quantifiable).

3.3.2. Analysis of food samples by competitive R5 ELISA and sandwich R5 ELISA

Once it had been demonstrated that the combined UPEX-competitive ELISA system worked properly in the standards and in the spiked foods, we assessed the performance of the new system by analysing commercial foods containing different gluten levels and with different treatments during the manufacturing process. The results of gluten quantification in representative foods are shown in Table 4. In the table, these foods were classified based on the theoretical processing during product manufacture.

We found that, in foods that were heated and hydrolysed, the values of gluten obtained using the UPEX solution extraction together with the competitive R5 ELISA were higher than those obtained by either extracting the gluten with 60% ethanol and analysing with the competitive R5 ELISA, or by extracting the gluten with the cocktail solution and analysing with the sandwich R5 ELISA. The use of this new extraction solution together with the competitive R5 ELISA is the only system capable of extracting and quantifying the totality of the hydrolysed gluten in heated processed foods.

In foods that were heated but not hydrolysed, the results obtained with both the cocktail extraction and the UPEX extraction combined with the sandwich R5 ELISA were similar to those obtained after extraction with UPEX solution combined with the quantification by the competitive R5 ELISA. Nevertheless, after extraction with 60% ethanol, the data obtained were lower in both assays, as expected.

In foods that were hydrolysed but not heated, the results obtained with the UPEX extraction were similar to those obtained with 60% ethanol extraction and quantification by competitive R5

Table 4
Gluten quantification in commercial foods by competitive and sandwich R5 ELISA using different extraction solutions. Results expressed in ppm of gluten.

	Food sample	Sandwich R5 ELISA			Competitive R5 ELISA	
		Cocktail solution	UPEX	60% ethanol	UPEX	60% ethanol
Heated and hydrolysed	Baby food A	25	26	10	35	8
	Baby food B	<3	<3	<3	<2.4	<2.4
	Baby food C	8	7	5	8	4
	Biscuits A	19	15	8	24	5
	Biscuits B	<3	<3	<3	4	3
	Biscuits C	3	4	3	9	5
	Breadcrumbs A	20	23	6	41	8
	Breadcrumbs B	<3	<3	<3	<2.4	<2.4
	Breakfast cereals A	3	3	<3	3	<2.4
	Breakfast cereals B	<3	3	<3	4	3
	Cake A	7	8	6	9	6
	Cake B	4	5	2	9	2
	Cake C	13	16	9	20	8
	Chocolate biscuits	<3	3	<3	5	3
	Cooking dough	25	32	15	93	68
	Infant cereals A	ND	25	15	27	14
	Infant cereals B	<3	<3	<3	4	<2.4
	Maize syrup	ND	93	ND	189	ND
	Malt drink A	ND	79	78	376	321
	Malt drink B	ND	188	167	437	359
	Maize pancakes	<3	<3	<3	3	3
	Muffins	<3	<3	<3	6	3
	Wheat starch A	102	118	75	155	93
Heated and not-hydrolysed	Bread	<3	<3	<3	<2.4	<2.4
	Gofio (Toasted cornmeal)	ND	69	47	71	45
	Pasta A	9	13	7	11	3
	Pasta B	25	35	16	27	14
	Pasta C	30	40	9	48	8
	Pasta D	13	26	8	36	10
	Pastries	5	3	<3	6	3
	Wheat starch B	20	22	9	21	6
Not-heated and hydrolysed	Beer A	ND	11	12	218	220
	Beer B	ND	3	<3	68	61
	Beer C	ND	4	ND	79	ND
	Beer D	ND	9	ND	141	ND
	Beer E	ND	5	ND	70	ND
	Beer F	ND	11	ND	175	ND
Not-heated and not-hydrolysed	Custard	<3	<3	<3	<2.4	<2.4
	Food colouring	4	5	3	3	3
	Pudding	<3	<3	<3	<2.4	<2.4
	Rice noodles	11	14	8	11	9
	Soluble cocoa	<3	<3	<3	<2.4	<2.4
	Soybeans	<3	<3	<3	<2.4	<2.4
	Soy drink A	<3	<3	165	<2.4	367
	Soy drink B	ND	ND	ND	<2.4	308
	Soy drink C	ND	ND	ND	<2.4	526

ND: not determined.

ELISA. However, they were higher than the results from the sandwich R5 ELISA.

In foods that were neither heated nor hydrolysed, the results obtained after extraction with the UPEX solution and quantification with sandwich or competitive R5 ELISA were similar to those obtained after extraction with 60% ethanol or cocktail extraction analysed by sandwich R5 ELISA.

We found surprising results when we analysed soy drinks as we reported previously [34]. When soy drink proteins were extracted with 60% ethanol, the values of gluten determined by both sandwich and competitive R5 ELISA were very high, whereas extractions by the UPEX solution or the cocktail solution showed values below the limit of quantification (Table 4). In addition, the analysis of the main ingredient in soy drinks (soybeans) using any of the extraction systems also resulted in values below the limit of quantification (Table 4).

To explain and to confirm these results, we performed the R5 western blot technique on these samples (Fig. 3). After extraction of proteins with 60% ethanol, soy drinks (but not soybeans)

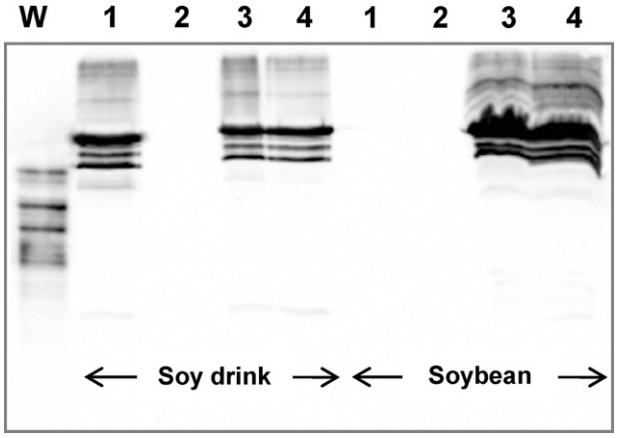

Fig. 3. Analysis by R5 western blot of wheat proteins extracted with 60% ethanol (W), soy drink and yellow soybeans extracted with 60% ethanol (1), UPEX solution supernatant (2), UPEX solution pellet (3) and electrophoresis loading buffer (4).

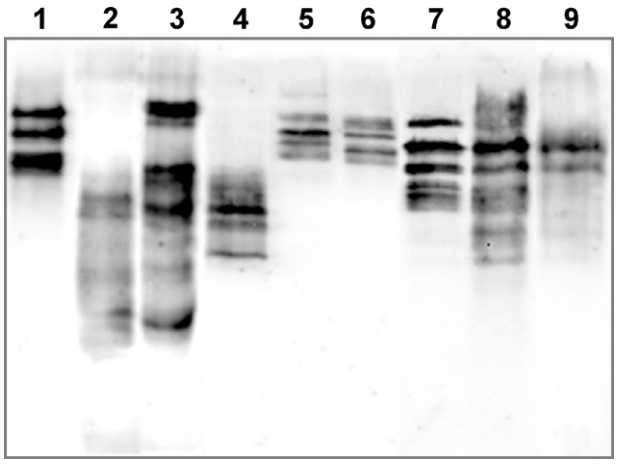

Fig. 4. Analysis by R5 western blot of food samples extracted with the UPEX solution. 1: wheat; 2: beer A; 3: malt drink A; 4: beer B; 5: rice noodles; 6: pasta A; 7: cooking dough; 8: wheat starch A; 9: maize syrup. Gluten values are presented in Table 4.

showed the presence of immunoreactive bands on the gel, mainly in a molecular weight region higher than that of gliadins. In contrast, we did not observe these bands in the supernatant after extraction with the UPEX solution, but we found them both in the pellet and after dissolution of the sample in the electrophoresis-loading buffer.

These data suggest that the R5 antibody cross-reacts with certain soy proteins that remain in suspension in ethanol extracts but precipitate when sample is preincubated with UPEX solution. Consequently, we assumed that processing soybeans to produce soy drinks might cause changes in the solubility of these proteins leading them to remain in suspension in 60% ethanol but nor in UPEX/60% ethanol. These results demonstrate that R5 ELISA combined with extraction with the UPEX solution is a reliable way to analyse gluten in soy foods.

We used the R5 western blot confirmatory technique to analyse selected commercial foods having different compositions and textures and manufactured with different treatments, which could affect gluten extraction and analysis (Fig. 4). For optimum band visualisation, the amount of gluten proteins loaded onto the gel was adapted for each sample. In some foods, we found bands only in the typical gel region corresponding to intact prolamins, while in others we found bands also corresponding to hydrolysed prolamins in the lower molecular weight region according to the results obtained using the ELISA systems. On the other hand, in contrast to when we used the cocktail solution, we did not observe structural deformations in the gel due to the UPEX solution reagents. Consequently, these data prove that combining the UPEX extraction with the R5 western blot technique is an optimum method for confirming gluten content in foods.

4. Conclusions

The main advantages of the competitive R5 ELISA combined with the new extraction system developed (UPEX solution) are: (1) gluten is accurately determined in all kind of foods, including heat-treated and hydrolysed foods; (2) the UPEX solution is suitable for using together with both the sandwich and competitive R5 ELISA and also for using with confirmatory techniques for gluten analysis (such as R5 western blot and mass spectrometry); and (3) the competitive system is cheaper and faster than the currently available sandwich system.

Acknowledgements

The PWG standard of gliadins was kindly provided by Professor Martin Stern, chairman of the Working Group on Prolamin Analysis and Toxicity. This research was supported by the Spanish Ministerio de Educación y Ciencia (AGL 2004-02721) and Consejo Superior de Investigaciones Científicas (PIE 200420E442).

References

[1] K. Kaukinen, K. Lindfors, P. Collin, O. Koskinen, M. Maki, Clin. Chem. Lab. Med. 48 (2010) 1205–1216.
[2] C. Catassi, A. Fasano, Curr. Opin. Gastroenterol. 24 (2008) 687–691.
[3] H.J. Freeman, Gut Liver 3 (2009) 237–246.
[4] H. Wieser, Food Microbiol. 24 (2007) 115–119.
[5] P.R. Shewry, N.G. Halford, J. Exp. Bot. 53 (2002) 947–958.
[6] Commission of the European Communities, Official Journal of the European Union, 2009, pp. 16/13–16/15.
[7] Codex Alimentarius Commission, 2006, ALINORM 06/29/23.
[8] I. Valdes, E. Garcia, M. Llorente, E. Mendez, Eur. J. Gastroenterol. Hepatol. 15 (2003) 465–474.
[9] E. Garcia, M. Llorente, A. Hernando, R. Kieffer, H. Wieser, E. Mendez, Eur. J. Gastroenterol. Hepatol. 17 (2005) 529–539.
[10] B. Moron, A. Cebolla, H. Manyani, M. Alvarez-Maqueda, M. Megias, C. Thomas Mdel, M.C. Lopez, C. Sousa, Am. J. Clin. Nutr. 87 (2008) 405–414.
[11] E.H. Spaenij-Dekking, E.M. Kooy-Winkelaar, W.F. Nieuwenhuizen, J.W. Drijfhout, F. Koning, Gut 53 (2004) 1267–1273.
[12] T. Laube, S.V. Kergaravat, S.N. Fabiano, S.R. Hernandez, S. Alegret, M.I. Pividori, Biosens. Bioelectron. 27 (2011) 46–52.
[13] R. van Eckert, E. Berghofer, P.J. Ciclitira, F. Chirdo, S. Denery-Papini, H.J. Ellis, P. Ferranti, P. Goodwin, U. Immer, G. Mamone, E. Méndez, T. Mothes, S. Novalin, A. Osman, M. Rumbo, M. Stern, L. Thorell, A. Whim, H. Wieser, J. Cereal Sci. 43 (2006) 331–341.
[14] E. Mendez, C. Vela, U. Immer, F.W. Janssen, Eur. J. Gastroenterol. Hepatol. 17 (2005) 1053–1063.
[15] F. Kahlenberg, D. Sanchez, I. Lachmann, L. Tuckova, H. Tlaskalova, E. Méndez, T. Mothes, Eur. Food Res. Technol. 222 (2006) 78–82.
[16] A.A. Osman, H.H. Uhlig, I. Valdes, M. Amin, E. Mendez, T. Mothes, Eur. J. Gastroenterol. Hepatol. 13 (2001) 1189–1193.
[17] H.D. Belitz, W. Grosch, P. Schieberle, in: D.H. Belitz, W. Grosch, P. Schieberle (Eds.), Food Chemistry, 4th ed., Springer-Verlag, Berlin, Germany, 2009, pp. 8–92.
[18] A. Garcia-Manzanares, A.J. Lucendo, Nutr. Clin. Pract. 26 (2011) 163–173.
[19] R. Ciccocioppo, A. Di Sabatino, G.R. Corazza, Clin. Exp. Immunol. 140 (2005) 408–416.
[20] B. Gessendorfer, P. Koehler, H. Wieser, Anal. Bioanal. Chem. 395 (2009) 1721–1728.
[21] D. Stepniak, L. Spaenij-Dekking, C. Mitea, M. Moester, A. de Ru, R. Baak-Pablo, P. van Veelen, L. Edens, F. Koning, Am. J. Physiol. Gastrointest. Liver Physiol. 291 (2006) G621–G629.
[22] A. Hernando, J.R. Mujico, M.C. Mena, M. Lombardia, E. Mendez, Eur. J. Gastroenterol. Hepatol. 20 (2008) 545–554.
[23] D.H. Dewar, M. Amato, H.J. Ellis, E.L. Pollock, N. Gonzalez-Cinca, H. Wieser, P.J. Ciclitira, Eur. J. Gastroenterol. Hepatol. 18 (2006) 483–491.
[24] P.D. Howdle, Eur. J. Gastroenterol. Hepatol. 18 (2006) 703–706.
[25] M.V. Barone, M. Nanayakkara, G. Paolella, M. Maglio, V. Vitale, R. Troiano, M.T. Ribecco, G. Lania, D. Zanzi, S. Santagata, R. Auricchio, R. Troncone, S. Auricchio, PLoS One 5 (2010) e12246.
[26] G.J. Tanner, C.A. Howitt, R.I. Forrester, P.M. Campbell, J.A. Tye-Din, R.P. Anderson, Aliment. Pharmacol. Ther. 32 (2010) 1184–1191.
[27] V.V. Doña, C.A. Fossati, F.G. Chirdo, Eur. Food Red. Technol. 226 (2008) 591–602.
[28] M.C. Bermudo Redondo, P.B. Griffin, M. Garzon Ransanz, H.J. Ellis, P.J. Ciclitira, C.K. O'Sullivan, Anal. Chim. Acta 551 (2005) 105–114.
[29] J.A. Burns, J. Org. Chem. 56 (1991) 2648–2650.
[30] J. Han, Previews 2 (1999) 16–21.
[31] K. Schlink, R. Reski, Plant Mol. Biol. Report. 20 (2002), 423a–423f.
[32] B. Gessendorfer, H. Wieser, P. Koehler, J. Cereal Sci. 52 (2010) 331–332.
[33] M.C. Mena, A. Hernando, E. Méndez, Proceedings of the 21st Meeting Working Group on Prolamin Analysis and Toxicity, 2007, pp. 47–51.
[34] M.C. Mena, A. Hernando, M. Pité, M. Lombardía, E. Méndez., Proceeding of the 21st Meeting Working Group on Prolamin Analysis and Toxicity, 2007, pp. 41–47.