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Gluten Quantification of Foods via the Immunodominant Gliadin Epitope

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Abstract

Coeliac disease (CD) is an immune mediated condition involving dietary gluten, found in wheat, rye, and barley; avoiding them is the only treatment.

We generated a mAb to the immunodominant gliadin epitope alpha-2-gli-57-75 and developed assays to quantify gluten in foods, comparing the results with those from the R5 assay. Competition assays were developed using native gliadin as the competing and coating antigen. Results were compared with those from the Ingenesa Ingezim Gluten ® kit.

The mAb recognized prolamins from wheat, spelt, rye and barley. A competition assay that detected gliadin at 0.027ppm in a foodstuff revealed gliadin contamination of several foods labelled gluten-free.

Purified wheat starch, used as wheat flour substitute to generate gluten-free foods, contains quantifiable amounts of the immunodominant epitope. The assay is sufficiently sensitive to assess the suitability of foods for coeliac patients and could be used as an alternative to the R5.

Keywords: Coeliac disease, Immunodominant peptides, Gluten measurement, Gliadin measurement, ELISA, R5 assay.

Introduction

Gluten sensitivity, manifesting as coeliac disease (CD) and the associated skin lesion, dermatitis herpetiformis (DH) affects 1:100 people in Northern Europe and the USA. There is an acquired immune response directed against dietary gluten, mediated by activation of gluten specific T lymphocytes, although an earlier T-cell independent innate immune response to gluten may occur [1]. Ingestion of gluten, which comprises two groups of proteins, gliadins and glutenins, in susceptible individuals leads to inflammation of the small intestinal mucosa, with damage to the villous architecture. They suffer malabsorption, loss of nutrients and single or multiple deficiencies leading to, for example, anaemia or lack of bone calcification. In children, this may result in growth retardation, delayed development, behavioural problems, and poor educational achievement. In adults, there may be fatigue, low fertility, poor pregnancy outcome, or neurological defects. Long- term complications include osteoporosis [2], increased prevalence of malignancy and an increased mortality [3].

The only generally accepted treatment is a strict life-long gluten-free diet, with the complete avoidance of wheat, rye and barley. The related cereal oats appears to be disease triggering in a minority (5%) of CD patients [4]. Other cereals, including maize, rice, millet and sorghum do not trigger CD. Cereal derivatives are found in a vast array of processed foods, thus a gluten-free diet is very restrictive. Removal of the majority of gluten from wheat flour yields purified wheat starch that is widely used as a basis for production of nominally gluten-free foods for individuals with CD.

Assays for the measurement of gluten in foods intended for those who are intolerant to gluten proteins are required. The Codex Draft Revised Standard for Gluten-Free Foods [5], proposed limits. Rendered gluten-free products may contain 20-100 ppm gluten. A later amendment (previously in 2008 and more recently in 2015) stated that only foods with less than 20ppm gluten may be labelled gluten-free, whilst those containing amounts of gluten between 20 and 100ppm may be labelled reduced gluten [5]. This regulation was introduced within the European Union in 2012. An immunoassay is thus essential for the detection of gluten contamination at 20ppm.

Wheat gluten mainly comprises two proteins, gliadins and glutenins both of which exacerbate CD. Similar proteins exist within rye (secalins), barley (hordeins) and oats (avenins). Methods for measurement of gluten in foods have been based on the measurement of wheat gliadin, which is then multiplied by 2 to obtain total gluten. This is based on the assumption that gliadins and glutenins are found in roughly equal proportions in wheat flour. This has been shown to be incorrect, with the ratio varying considerably between wheat cultivars [6].

The R5 method of gluten analysis [7], based on an antibody

raised against rye secalins has been accepted as the standard method of gluten analysis to support the new legislation on gluten-free foods. This method, however, only weakly recognises the immunodominant gliadin T-cell stimulating epitope [8]. Not only wheat, but also rye, barley and oats contain peptides similar to the immunodominant gliadin peptide [9], and thus quantification of this important epitope in commercially available nominally gluten-free foods, including those based on purified wheat starch, is highly pertinent in the dietary management of CD.

We describe in this manuscript the development of a monoclonal antibody (mAb) against the immunodominant gliadin epitope, alpha-2-gliadin 57-75, which has permitted the development of a competition format assay based on the use of The Prolamin Working Group gliadin (PWG) standard, which was developed for use as a European Standard [10].We then compare the results for gluten quantification using our assay with those obtained employing the R5 assay.

Methods

Study Design

The study involved generation of a murine mAb to the wheat immunodominant peptide, followed by use of the antibody to develop a competition assay to quantify the gluten content of foods, including those marketed as gluten-free. The results obtained were compared to those obtained using the commercially available R5 assay.

Antigens

Peptides: Alpha gliadin amino acids 56-75 (LQLQP-FPQPQLPYPQPQLPY) were prepared and donated by Dr Herbert Wieser, as described [11]. The peptide is highly stimulatory to coeliac small intestinal gluten sensitive T-cells [11], confirming the role of the motif in the disease pathogenesis.

Peptide conjugates: The peptide, to be used as an immunogen, was conjugated to Tubercullin Purified Protein Derivative (PPD) to act as a carrier. PPD was obtained in the form of Heaf Multiple Puncture test solution (Evans Vaccines, Liverpool, UK). The liquid form was precipitated by the addition of four volumes of acetone, and the PPD solid recovered by centrifugation. The PPD was then weighed and the molecular weight of the PPD-peptide complex calculated (MW 10,000). A molar ratio of PPD:peptide of 2:1 was dissolved in PBS at a concentration of 2mg PPD/ml. Glutaraldehyde was added to a final concentration of 0.05% v/v, and the mixture stirred in the dark for 24 hours. Ammonium chloride was added to give a 0.1 M solution and stirring continued for a further 30 minutes. Following this, the conjugate was precipitated using acetone at-70 °C for one hour. The suspension was then centrifuged and the supernatant discarded. The precipitate was allowed to dry and then diluted in PBS to a final concentration of 1mg conjugate/ml.

Other antigens utilized for characterisation: Prolamin Working Group gliadin (PWG)(batch IRMM480) standard was provided by Dr Herbert Wieser, German Research Centre for Food Chemistry, Garching, Germany[10]. Kolibri gliadin was prepared as described [12]. Pure milled flour from rye (variety Rheidol), barley (variety Porter) and oats (variety Peniarth) were supplied by Plant Breeding International, Trumpington, UK. Dr F Janssen of the Regional Food Inspection Service, Zuppen, Netherlands, kindly donated whole grains of rice, maize, millet and sorghum. Amidon wheat starches A, B and C were obtained from the Transia Gluten Plate Test Kit. These were designated as A when acceptable for a GFD (<100ppm); B when was in mid-range (300- 600ppm gluten) and C when containing high gluten content (1000-2500ppm gluten), all according to previous Codex Alimentarius guidelines.

Production of monoclonal antibodies

All animal experiments were performed under UK Home Office Project License number PPL 70/5688. BALB/c mice taking a gluten-free diet (Harlan UK, Oxford Code TD.94158) were used, since this leads to enhanced serum immune response to injected gliadin [13]. The animals were immunised as described and those with high serum titers of IgG antibodies to gliadin were used for mAb generation [13]. Cell culture was performed in the presence of a maintenance dose of Plasmocin (Invivogen, Autogen Bioclear, Calne, Wiltshire UK). Resultant hybridomas were tested for IgG antibody production against gliadin and the peptide. Limiting dilution was used to clone positive hybridomas. Clones were screened for IgG antibody production and positives expanded. Supernatants were concentrated using 50% ammonium sulphate precipitation.

Indirect Enzyme Linked-Immunosorbant Assay (ELISA) for characterisation of antibodies in mouse sera and hybridoma supernatants

To test for the presence of the murine antibody to the immunodominant peptide, U-shaped wells of plates were coated with immunodominant peptide at 1ug/ml in PBS. For testing of mAb supernatants, the wells were coated with whole Kolibri gliadin or PWG at 50µg/ml in 40% ethanol at 4°C for 18 hours. Antibody supernatants from fusions were tested undiluted, whilst supernatants from clones were diluted to 1:2 and titred to 1:2048. Following these experiments monoclonal antibody CDC-5 was selected for further experiments on the basis of its reactivity with peptide in PWG and Kolibri gliadin.

For testing reactivity of mAbs with wheat cultivars, pure grains, A B Semper (Oslo, Norway) kindly provided various European wheat cultivars, all of which have been used for bread making at various times.

Soft spring wheats: Sverno, Ring, Rang, Draband, Pompe

Strong spring wheats: Dragon, Kadet

Soft autumn wheats: Folke, Hilder, Portal, Solid, Kosack

Strong autumn wheats: Holme, Stark

Spelt wheat: Dinkle

The milled flours from these grains were extracted (1mg/ml) with 40% v/v ethanol to solubilise gliadins and the extracts were used to coat ELISA plates at 50µg/ml. Anti-gliadin antibody CDC-5 was titred to 1:2048 in flat-bottomed shaped wells of plates coated with these extracts.

For the sera or supernatants, following 45 minutes incubation at 37°C, the bound antibody was detected using rabbit anti-mouse IgG, conjugated to alkaline phosphatase (Sigma A4132) at a 1:2000 dilution. The plates were then again incubated for 45 minutes at 37 °C, followed by detection with p-nitrophenyl

phosphate solution (Sigma 7998) and the optical density read at 405 nm.

Competition assay for measurement of gluten in foods

To establish the optimal reagent concentrations for the competition assay, "checker board" assays were carried out, with whole gliadin (PWG) serving as the coating antigen. Competition occurs between immobilised gliadin and the gliadin sample or standard in solution to be detected. Three assay formats were tested.

Indirect one-step assay: mAb CDC-5, horseradish peroxidise (HRP) labelled-anti-mouse IgG (Sigma A9044) and gliadin standard were pre-incubated together, prior to addition to the plate.

Indirect two-step assay: CDC-5 and gliadin were incubated together, followed by addition of HRP labelled anti-mouse and incubated in the U-shaped wells of theplate.

Direct one-stepassay: CDC-5 directly labelled with HRP was incubated with gliadin standard prior to on-plate incubation.

Pre-incubation and incubation times were studied, over ranges of 10, 30 and 60 minutes for on plate incubation, and at 10, 20, 30, 40 and 60 minutes for pre-incubation. Pre- incubation and on plate incubation temperatures were studied at 4°C, 22°C and 37°C, as explained in the results and discussion. The final optimised conditions were shown to involve an indirect one step assay where the PWG gliadin was coated on to the microtitre plate at 500ng/ml and a standard curve prepared over a range between 1.22 and 5000ng/ml PWG. CDC-5 antibody at 0.48µg/ml together with anti-mouse-HRP at 1 in 10,000 were pre-incubated for 60 minutes at 37°C with the gliadin standards, followed by an on-plate incubation time of 15 minutes at 22°C (ambient temperature). Cross-reactivity assays were carried out with ethanol extracts of rice, maize, rye, barley, and oats. Compatibility of the developed assay with the optimised in-house extraction buffer used for extraction of gluten from foodstuffs was studied at various dilutions (1:3, 1:20, 1:40, 1:60, 1:80, 1:100 and 1:200). Food samples were homogenised and extracted at 100mg/ml. The extraction solution (Patente Nacional 200802393 por "Procedimientos Para La Extracción De Prolaminas Tóxicas Del Gluten En Celiaquía") is prepared as follows: 0.01g of TCEP (Tris (2- carboxyethyl) phosphine hydrochloride) (Fluka Biochemika Ref.93284) is added to 4ml of Tris-HCl 50mM pH 7.4. The latter is prepared as follows: 2.422g of TRIZMA base (Tris [hydroxymethyl] aminomethane) (Sigma Ref. T-6066), is added to 2.76ml of 6 N HCl (Scharlau Ref. AC0752) in water up to 400ml and the pH checked. Once the TCEP is dissolved, 6ml of glycerol (Scharlau GL0026) is added and the solutions mixed.

Results and Discussion

Table 1 shows the serum titres for IgG antibodies to immunodominant peptide for mice immunised twice with peptide-PPD conjugate, which ranged from 1:3,200 to 1:50,000. A number of mAbs were produced from fusions using these immunised animals; the antibody CDC-5 was selected for further experiments. In addition to the reaction of the antibody with PWG, CDC-5 also had titres in excess of 1:2048 to all 15 varieties of bread wheats tested, including spelt wheat.

Table 1: Serum IgGtitres to immunodominant peptide of immunised mouse

Mouse 1	1:50000
Mouse 2	1:25000
Mouse 3	1:3200
Unimmunised mouse	1:400

Competition format assay for detection of gluten in Foods

The results obtained with one and two-step indirect and direct competition assays were compared in order to determine the best experimental configuration (Table 2).

Table 2: CDC-5 competition assays comparison: Indirect one-step, indirect two-step and direct competition

	Indirect One-Step	Indirect Two-Step	Direct Competition
A_{min}	0.1±0.01	0.25±0.02	0.12±0.02
A_{max}	1.95±0.02	2.29±0.02	1.95±0.04
EC50 (ng/ml)	43.12±0.12	260.1±0.15	166.1±0.14
LOD (ng/ml)	0.62±0.02	11.23±0.04	9.00±0.02
Assay time	3h 10 min	4h 10 min	3h 10 min

Amin- minimum absorbance;

Amax- maximum absorbance;

LOD- limit of detection;

EC50- concentration of gliadin that provided a response halfway between baseline and maximum

The indirect one-step assay, that is pre-incubation of PWG, CDC-5 and anti-mouse-HRP together before incubation on the plate, gave the best results in terms of assay time and limit of detection (LOD) and was adopted for further studies. Pre-incubation times and on plate incubation times for the selected assay were studied and the results are shown in Table 3. As can be seen for the one-step indirect assay, the best LOD was obtained using 60 minutes pre-incubation time and 10 minutes incubation time. Tables 4 and 5 show the results obtained for optimisation of incubation temperatures. For the pre-incubation, the best results were obtained at 37°C, while for incubation the best results were obtained at 22°C.

The optimised assay was used to test the cross-reactivity between various cereal prolamins. As can be seen in Figure 1, there was cross-reactivity between PWG gliadin but none with oats, rice or maize, whilst detection of rye and barley prolamin extracts was obtained. The greater reactivity to barley hordeins compared to PWG gliadin can be explained by the fact that gel electrophoresis of PWG gliadin reveals that it is 30% contaminated with wheat glutenins.

The assay was performed with the gliadin standard made up in various concentrations of the extraction buffer.

As can be seen in Figure 2, the assay format is highly compatible with the extraction buffer of glycerol and TCEP, with no attenuation of the curve, except at the highest concentration of the buffer (that is a 1:3 dilution), demonstrating the applicability

Table 3: CDC-5 pre-incubation and incubation time study (one-step indirect assay)

Pre-Incubation/ Time	Incubation Time	10 min	30 min	60 min
10 min	LOD ng/ml	5.51±0.07	15.93±0.42	22.16±0.91
	Amax	2.52±0.03	2.88±0.00	3.03±0.00
	Amin	0.36±0.06	0.63±0.00	1.10±0.00
20 min	LOD ng/ml	10.61±0.09	10.06±0.12	45.18±0.43
	Amax	2.25±0.02	2.81 ± 0.00	2.96±0.00
	Amin	0.33±0.06	0.62±0.00	1.15±0.00
30 min	LOD ng/ml	5.43±0.12	11.39±0.03	16.36±0.30
	Amax	2.25±0.01	2.83±0.01	3.01±0.00
	Amin	0.39±0.01	0.66±0.04	1.00±0.00
40 min	LOD ng/ml	5.2±0.06	14.02±0.59	20.37±0.88
	Amax	2.39±0.05	2.91±0.04	3.07±0.00
	Amin	0.39±0.04	0.71±0.03	1.06±0.00
60 min	LOD ng/ml	3.06±0.32	5.55±0.59	17.44±3.63
	Amax	2.11±0.023	2.99±0.00	3.10±0.03
	Amin	0.24±0.01	0.74±0.00	1.16±0.03

Amin- minimum absorbance,
Amax- maximum absorbance,
LOD- limit of detection

Table 4: CDC-5 pre-incubation temperature study (one-step indirect assay)

	LOD ng/ml	R2	Amax	Amin
22°C	4.05±0.01	0.10±0.00	2.79±0.01	0.28±0.00
37°C	1.03±0.19	1.00±0.00	2.58±0.01	0.14±0.00
4°C	9.81±3.20	0.99±0.00	2.29±0.09	0.23±0.06

Amin-minimum absorbance,
Amax-maximum absorbance,
LOD- limit of detection

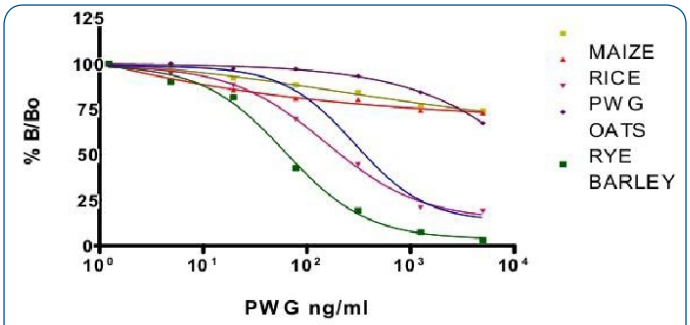

Figure 1: Cross reactivity of cereals prolamin working group gliadin (%B/BO- percentage of antibody bound to immobilised PWG gliadin on plate, at various concentrations of pre-incubated gliadin)

of the assay to the detection of real samples.

Real sample analysis using the optimised CDC5 one step indirect assay

The assay detected gliadin and the results multiplied by 2 in order to give the derived gluten content.

Wheat Starches: Table 6 shows the derived gluten content (gliadin x2) of five wheat starches. All wheat starches tested contained less than the 100ppm gluten proposed for foods to be labeled as reduced gluten, and one had less than 20ppm, so could

Table 5: CDC-5 incubation temperature study (one-step indirect assay)

	LOD ng/ml	Amax	Amin
22°C	2.71 ±0.25	2.51±0.00	0.12±0.01
37°C	14.09±5.95	1.49±0.00	0.28±0.05
4°C	3.35±0.3	1.75±0.02	0.14±0.02

Amin- minimum absorbance,
Amax- maximum absorbance,
LOD- limit of detection

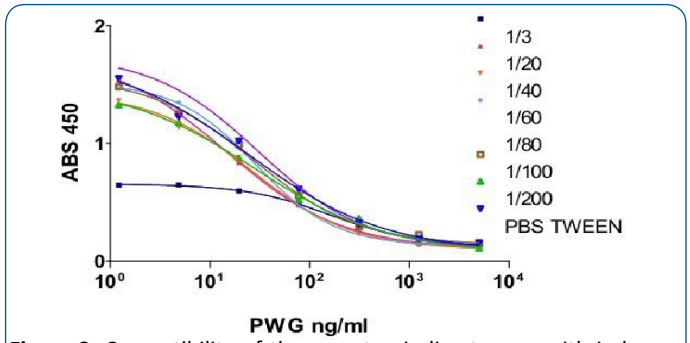

Figure 2: Compatibility of the one-step indirect assay with in-house extraction buffer(ABS 450- absorption at 450 NM when the PWG gliadin standard curve is measured in various concentrations of the extraction buffer, compared to PBS/Tween)

Table 6: Gliadin and derived gluten content (gliadin x2) of wheat starch samples using the CDC-5 indirect one-step

Name	Gliadin ppm	Gluten ppm
Wheat Starch 1	2.4	4.8
Wheat Starch 2	38.47	76.94
Wheat Starch 3	38.50	77.0
Wheat Starch 4	36.00	72.0
Wheat Starch 5	37.10	74.2

be labelled as gluten- free.

Samples from gluten-free food manufacturers: The majority of these were made from naturally gluten-free ingredients. Table 7 shows a corn flour with 14.2ppm gluten, being <20ppm, thus this product could be labelled as gluten-free. However, Table 8 shows that many products intended for CD sufferers contained quantities of gluten up to 100ppm, so under the new regulations would need to be labelled as reduced gluten. Table 9 reveals that certain foods intended for CD sufferers contained >100ppm gluten and thus would not be suitable for CD sufferers.

The reference laboratory at Universidad del País Vasco also provided coded samples. These were analysed by the described CDC-5 one-step indirect assay and by the commercially available R5 assay. Both assays measure gliadin, which is then multiplied by 2 to give the gluten content. Table 10 shows that there are differences between the results obtained using our developed

Table 7: Gliadin content of food samples with under 20ppm total gluten (gliadin x2) as measured using the CDC-5 one-step indirect assay

Manufacturer	Product	Naturally GF ingredients?	Gliadin ppm	Gluten ppm
Donarepa	Corn flour	Yes	7.1	14.2

Table 8: Gliadin content (in ppm) of food samples with under 100ppm total gluten (gliadin x2) as measured using the CDC-5 one-step indirect assay

Manufacturer	Product	Naturally GF ingredients?	Gliadinppm	Gluten ppm
Maizena	Corn flour sauce thickener	Yes	42.5	85.0
Glutafin	GF Biscuits	Yes	20.2	40.4
Dr Schar	Vanilla Wafers	Yes	39.7	79.4
Oleander	Onion snack	Yes	17.6	35.2
Sanavi	Rosceli Cream	Yes	11.8	23.6
Odlums	Titamyl white flour	No	15.4	30.8

Table 9: Gliadin content ppm of food samples obtained from manufacturers with over 100ppm total gluten (gliadin x2) as measured by the CDC-5 one-step indirect assay

Manufacturer	Product	Naturally GF ingredients?	Gliadin ppm	Gluten ppm
Hacendado	Cheese Chips	Yes	182.5	365
Hacendado	Potato Sticks	Yes	73.6	147.2
Hacendado	Milled black pepper	Yes	54.3	108.6
Hacendado	Yeast powder	Yes	123.8	247.6
Sanavi	GF pasta	Yes	75.2	150.4
Nomen	Rice flour	Yes	78.2	156.4
Maizena	Corn flour	Yes	50.6	101.2
Glutafin	White bread mix	No	123.8	246.6

Table 10: Comparison of results for coded samples gained using the R5 method (IngenesaIngezim gluten ® kit) and the CDC-5 one-step indirect assay

Sample Type	Code #	Gluten ppm (gliadin x2) R5 method	Gluten ppm (gliadin x2) CDC-5method
Corn Flakes	05061005 L1318	42	62
Hot paprika	13020206	30.6	10.3
Chocokrispies	01230206	38	28.8
Strawberry cookies	04080206	9	34.4
Nachos	14270706	58.4	16.8
Cream cake	13270406	31.6	51.4
Rice flour	01120406	11	0.5
Ketchup flavored crisps	04140606	11	13.5
Sweetened Corn Flakes	09060206	8.8	33.4
Maize flour	06120406	9.8	15.7
Rice flour	01270406	97	59.2
Choco rice	10160306	61	33.2

CDC-5 assay and those using the Ingenesa Ingezim Gluten ® kit, one of the forms of the R5 assay. Additionally, the anomalies followed no particular pattern. Thus whilst rice flour, measured by our method, had a derived gluten content of 0.46ppm, the result measured by the R5 method was 24 times greater at 11ppm. On the other hand strawberry cookies measured 34.4ppm gluten by our method, but 4 times less by the R5 method. These differences could affect the labelling category into which the products fall. For example ground chilli and nachos, measured by our method have under 20ppm gluten and are thus eligible to be labelled as "gluten-free", whereas measured by the R5 method they would only qualified as reduced gluten. On the other hand, the converse is true for strawberry cookies and sweetened cornflakes.

Conclusions

A mAb to the coeliac toxic immunodominant gliadin epitope can detect this peptide sequence in a wide variety of bread wheat cultivars. There is a clear reaction with spelt wheat, even though some producers of this wheat sub-species sometimes claim that it is gluten-free. The reaction of the antibody with rye and barley coincides with the observation of Vader [9] of similar epitopes in these cereals. While it has been well established that rye and barley are coeliac toxic, the toxicity of oats has been much disputed. Recent evidence suggests the presence of some gliadin-like epitopes within oats (avenins), with which gluten-sensitive T-cells may react [14]. However, our developed competition assay did not detect oats.

Analysis of real-food samples by our developed method demonstrates that many products marketed as gluten-free contain considerable quantities of gluten, even those based on naturally gluten-free ingredients. The accepted method for measurement of gluten in foods to accompany the new regulations is the R5 method. It is clear from our data that considerable differences exist in the values measured by the two methods. However, it is also clear that, whichever set of results one considers, a large number of products would contain levels of gluten greater than would be permitted to be labelled gluten-free (<20ppm). The reason for the differences obtained between the two methods is most likely due to differences in the frequency of the epitopes measured by the two antibodies between cereal cultivars. We would argue that measurement of the immunodominant T-cell epitope is the method more likely to give a true indication of the potential coeliac toxicity of a foodstuff.

The currently accepted and universally used practice of multiplying the gliadin contents of a foodstuff by 2 to obtain total gluten may help to amplify differences between the two methods. In the future, measurement of the glutenin fraction of wheat as well as the gliadin fraction could give a better indication of the true gluten content of foods intended for consumption by individuals who are intolerant of this group of proteins.

Authors Contribution

HJE made the mAb, participated in assay development and sample analysis, and co-wrote the paper. BC-R co-wrote the manuscript. MCB-R participated in assay development and sample analysis, TS performed initial characterisation of the antibody and co-wrote the paper, and PJC supervised the project and co-wrote the paper.

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