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Gluten residues in gluten-free foods sold in Christchurch, New Zealand: comparison of LC-MS and ELISA methods

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RESEARCH PAPER

Abstract

Two distinct immunologically-mediated diseases, Coeliac disease and wheat allergy, are associated with ingestion of proteins from wheat and some related cereals. Both conditions are usually managed by adherence to a gluten-free (GF) diet. Two methods for detecting gluten protein were compared: enzyme-linked immunosorbent assay (ELISA) and liquid chromatography-mass spectrometry (LC-MS). The methods were compared for their specificity in analysis of a range of cereals and pseudocereals (buckwheat, quinoa) and the ability to detect gluten in foods claimed to be GF. The ELISA method detected gluten in 6/61 (9.8%) samples, with results in the range of 11-450 mg/kg. LC-MS detected gluten in five of these samples with results in the range of 6-130 mg/kg. These foods have the potential to cause adverse health effects in individuals with Coeliac disease or wheat allergy.

Keywords: allergens, contaminants, food safety, mycotoxins

1. Introduction

Two distinct immunologically-mediated diseases are associated with ingestion of proteins from wheat and some related cereals. Wheat allergy is an immunoglobulin E-mediated 'classical' food allergy, while Coeliac disease is an autoimmune inflammatory response in the small intestine leading to nutrient malabsorption (EFSA, 2004).

Wheat proteins are conventionally classified according to their solubility, molecular weight, function and location within the wheat grain. Albumins (water soluble) and globulins (salt soluble) are generally functional (enzymes, etc.) low-molecular weight proteins, concentrated in the bran and germ of the wheat grain and constituting approximately 20% of total grain protein (EFSA, 2004). The remainder of wheat protein is referred to as gluten protein and is involved in the formation of the rubbery gluten complex that enables the use of wheat for breadmaking. Gluten proteins are the major storage proteins of the wheat grain (Battais *et al.*, 2008). Gluten contains approximately equal amounts of alcohol soluble gliadin proteins and alcohol insoluble glutenin proteins (EFSA, 2004). Gliadin is monomeric, while glutenin is a highly viscous, heterogeneous polymer. These are high molecular weight storage proteins and are located predominantly in the starchy endosperm of the wheat grain. Consequently, gluten proteins are the main proteins present in white wheaten flour.

While a wide range of gliadin and glutenin proteins have been associated with wheat allergy, there is evidence to suggest two different profiles. Water/salt-soluble proteins and α-, β- and γ-gliadins appear to be more important allergens for children, while ω-gliadins are the major wheat allergens for adults (Battais *et al.*, 2008). The role of wheat in Coeliac disease has been shown to be due to the proline and glutamine-rich gliadins, particularly the α-, γ- and ω-gliadins (EFSA, 2004). A 33-amino acid peptide with high resistance to protease enzymes has been identified and is believed to be a primary initiator of the inflammatory response in Coeliac disease (Shan *et al.*, 2002). This peptide or homologues have only been identified in wheat gliadin, barley hordeins and rye secalins. Wheat, barley and rye may

all elicit adverse reactions in Coeliac disease sufferers. There is conjecture concerning the ability of oat protein to elicit adverse reactions in Coeliac disease sufferers. There is some evidence to suggest that the near-ubiquitous presence of wheat contamination in oats may be responsible for adverse reactions (Richman, 2012; Sey *et al.*, 2011).

Despite the huge quantity of cereals consumed worldwide, cereal allergies in adults are reported to be rare (EFSA, 2004). European estimates of the prevalence of wheat allergy in children (0-14 years) have ranged from 0.0 to 0.5% (Zuidmeer *et al.*, 2008). While no New Zealand estimates of the prevalence of wheat allergy are available, several estimates of the prevalence of Coeliac disease in New Zealand have been made (Carrington *et al.*, 1987; Cook *et al.*, 2000, 2004; Ussher *et al.*, 1994). Estimates have generally increased over time, but it is uncertain whether this reflects a true increase in prevalence or improvements in detection and diagnosis. A large (n=1,064) long-term study in Christchurch estimated the prevalence of Coeliac disease to be in the range 0.6-1.2% (Cook, 2000).

While individuals with wheat allergy or Coeliac disease vary widely in their tolerance to gluten protein (Haraszi *et al.*, 2011), both conditions are usually managed by adherence to a gluten-free (GF) diet. In New Zealand and Australia for a food to carry a claim to be gluten free it must contain: (a) no detectable gluten; (b) no oats or oat products; and (c) no cereals containing gluten that have been malted, or products of such cereals (FSANZ, 2013).

There are now a wide range of GF foods available. These are generally analogues of gluten-containing foods, but based on flours from rice, maize, soya, amaranth or wheat starch, specially purified for use in gluten-free foods. Gluten may occasionally be present in such foods due to cross-contact with gluten-containing material, gluten contamination of GF ingredients, or inclusion of ingredients of unknown composition.

A proportion of GF products have been shown to contain gluten residues, as determined by a range of methods including enzyme-linked immunosorbent assay (ELISA) (Amaya-González *et al.*, 2011; Gélinas *et al.*, 2008; Laube *et al.*, 2011; Thompson *et al.*, 2010; West Australian Department of Health, 2004), polymerase chain reaction (PCR) (Mašková *et al.*, 2011; Mujico *et al.*, 2011; Olexová *et al.*, 2006) and matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI/TOF-MS) (Camafeita *et al.*, 1997; Méndez *et al.*, 2000).

A liquid chromatography-mass spectrometry (LC-MS) method has recently been published that detects six physiologically relevant peptides, resulting from enzymatic digestion of wheat gluten (Sealey-Voyksner *et al.*, 2010). The current study applied this method to the analysis of GF foods available in New Zealand and compared the results to a conventional ELISA assay.

2. Materials and methods

Chemicals

Calcium chloride dihydrate (BDH Analar Prod 10070; BDH Chemicals, Poole, UK), gluten from wheat (Sigma G5004; Sigma-Aldrich Co., St. Louis, MN, USA), hydrochloric acid 37% (Scharlau Reagent Grade AC07412500; Scharlab S.L., Sentmenat, Spain), tris(hydroxymethyl)methylamine (TRIS) (BDH Analar Prod 10315; BDH Chemicals), α-chymotrypsin from bovine pancreas (Sigma C4129; Sigma-Aldrich Co.), pepsin from porcine gastric mucosa (Sigma P6887; Sigma-Aldrich Co.) and trypsin from bovine pancreas (Sigma T8003; Sigma-Aldrich Co.).

Materials

Amicon ultra centrifugal filter units Ultracel-10,000 MWCO (Millipore MILUFC801008; Millipore Corporation, Billerica, MA, USA), 50 ml polypropylene conical tubes, 30 x 115 mm (Falcon 352070; BD, Franklin Lake, NJ, USA), safe-lock tubes, 1.5 ml (Eppendorf 0223632040; Eppendorf AG, Hamburg, Germany).

Food samples

Foods making a GF claim (n=61) were purchased from supermarkets (n=45), bakeries (n=15) or other retail outlets (n=1). Product types included biscuits (including crackers), bakery products (bread, cakes), bakery mixes for domestic use, breakfast cereals, sauces, pasta, snack bars, snack foods (crisps, pretzels) and confectionery. All foods were sampled in Christchurch, New Zealand during September - November 2011. All foods were homogenised in a food processor and stored at 4 °C prior to analysis.

Flour samples from a range of cereals and pseudocereals were obtained from supermarkets and specialist retail outlets in Christchurch.

ELISA assay

All samples were analysed using a Neogen Corporation BioKits gluten assay (Neogen Corporation, Lansing, MI, USA). The method is a direct sandwich ELISA, based on reaction of extracted proteins with monoclonal antibodies to ω-gliadins (Skerritt and Hill, 1990). Homogenised samples (2 g) were mixed with extraction solution (20 ml, 40% v/v ethanol in water, including Neogen extraction additive) and analysed according to manufacturer's instructions. Method performance was checked by analysis of the provided gluten control.

Prepared standards equivalent to 3, 5, 10, 20 and 50 mg/kg gluten protein, provided with the assay, were also analysed. Samples with absorbance values outside the calibrated range were diluted 50-fold and reanalysed. While the calibration curve is not linear over its whole range, it is substantially linear (R^2=0.9903) over the range 0-20 mg/kg and all final absorbance values were in this range. Levels of gluten in unknowns were determined by linear interpolation from the standards.

LC-MS assay

The LC-MS assay was based on the method of Sealey-Voyksner *et al.* (Sealey-Voyksner *et al.*, 2010). This method involves detection of specific peptides that remain intact after digestion with pepsin, trypsin and chymotrypsin by HPLC with triple quadrupole MS/MS. Analysis and protein database searches demonstrated the selected peptides were present in various wheat varieties and detectable at much lower concentrations in rye. Four of the peptides have been detected at very low concentrations in barley (Sealey-Voyksner *et al.*, 2010). The peptides were assessed to be potentially immunogenic, based on their interaction with human transglutaminase 2.

Sample preparation

A homogenised sample (0.6 g) was accurately weighed into a 50 ml centrifuge tube and made up to 20 ml with a freshly prepared pepsin solution (30 mg/50 ml in 0.01 M HCl). The mixture was incubated in a water bath for 2 hours at 37 °C and shaken every 10 minutes.

Gluten digest standards were prepared by accurately weighing 60 mg of gluten into a 50 ml centrifuge tube (equivalent to 10% or 10,000 mg/kg gluten in a 0.6 g sample approximately equivalent to the protein content of wheat bread; Ministry of Health/Plant and Food Research, 2009). The standard was digested with pepsin as above, and then serially diluted in 0.01 M HCl to give the following gluten concentrations (in a theoretical 0.6 g sample): 1000, 100, 50, 20, 10 and 1 mg/kg.

Gluten standard and sample pepsin digests were centrifuged for 5 minutes at 3,600 rpm. An aliquot (0.5 ml) of the clear supernatant was removed and pipetted into a 1.5 ml Eppendorf tube. Sixty three microliters of 0.2 M TRIS was pipetted into each tube followed by freshly prepared (immediately prior to use) solutions of trypsin (25 µl of 25 mg/5 ml in 1 mM HCl, 2 mM $CaCl_2.2H_2O$) and α-chymotrypsin (25 µl of 25 mg/5 ml in 1 mM HCl, 2 mM $CaCl_2.2H_2O$).

The tubes were sealed and incubated for 30 minutes at 37 °C and shaken every 10 minutes. The solutions were then transferred to an Amicon 10,000 MWCO filter and centrifuged for 25 minutes at 3,000 rpm prior to LC-MS analysis.

Instrumentation and analytical conditions

Six peptides indicative of gluten were assayed on an Agilent 1200 series HPLC (Agilent Technologies, Santa Clara, CA, USA) coupled to an Agilent 6410 triple quadrupole (QQQ) mass spectrometer (MS). The peptides were labelled P1 to P6, using the conventions adopted by the reference method (Sealey-Voyksner *et al.*, 2010). Yields of one of the six peptides (P2) were very low in the current study and it was excluded from the assay. The mass spectrometer was operated in positive ion multimode, a blend of electrospray ionisation (ESI) and atmospheric pressure chemical ionisation (APCI). Daily mass calibration was conducted using the Agilent tune compound according to the manufacturer's instructions. Sample analyses were carried out using multiple reaction monitoring, using time programming for optimum sensitivity and specificity for the selected peptides.

The APCI source was operated at a capillary voltage of 2,500 V, with a nitrogen drying gas flow rate of 5 l/minute and gas temperature of 325 °C and a vaporiser temperature of 200 °C. The nebuliser was operated at 60 psi. Optimum performance was obtained with a corona setting of 0 µA, indicating that ionisation was primarily through ESI. Charging voltage was 2,000 V. The cone voltage used for all peptides was 150 V, except P6 for which the cone voltage was 120 V. Parent to product ion transitions were monitored for 100 ms (dwell time) using wide resolution mode. Table 1 gives the details of the target peptide and transitions monitored.

Chromatographic separation was carried out on a Kinetex C18 reverse-phase column (150×2.1 mm, 2.6 µm with in-line filter; Phenomenex Inc., Torrance, CA, USA), operating at 40 °C. The gradient used was 10% B to 100% B over 15 minutes, then 10% B for 10 minutes, at a flow rate of 0.2 ml/minute. Mobile phase A was 95% H_2O/5% methanol (MeOH) + 0.02% v/v formic acid (FA). Mobile phase B was 100% MeOH + 0.02% v/v FA. Injection volumes were typically 40 µl.

Protocols for acceptance of LC-MS results

In the original work of Sealey-Voyksner *et al.* (2010) samples were considered to contain gluten residues if any of the six target peptides were detected at levels above the analytical limit of detection. In the current study, this approach was found to be unreliable. In particular, there was considerable interference observed with peptides P4 and P5. For both of these peptides, apparent detections were rarely supported by the presence of a qualifier transition. While peptides P1, P3 and P6 were generally well resolved, with acceptable

Table 1. Triple quadrupole MS/MS conditions for analysis of gluten peptides.

Analyte	Sequence	Precursor ion (charge state)[1]	Transition (charge state)[1]	Production type	Collision energy (V)
P1	LQPQNPSQQQPQEQVPL	*980.7 (+2)*	*1,150.7 (+1)*	b10	30
		980.7 (+2)	**866.6 (+2)**	b15	20
P3	VPVPQLQPQNPSQQQPQEQVPL	**1,240.9 (+2)**	**1,126.7 (+2)**[2]	b20	25
		1,240.9 (+2)	*762.4 (+1)*	b7	35
P4	RPQQPYPQPQPQY	*814.6 (+2)*	*1,221.8 (+1)*	b10	25
		814.6 (+2)	**407.3 (+1)**	y3	30
P5	QPQQPFPQTQQPQQPFPQ	**717.6 (+3)**	**2,44.1 (+1)**	y2	18
		1,075.9 (+2)	*1,308.8 (+1)*	b11	25
		1,075.9 (+2)	*726.3 (+1)*	b6	28
P6	PQQSPF	*703.4 (+1)*	*441.4 (+1)*	b4	25
		703.4 (+1)	**263.3 (+1)**[2,3]	y2	35

[1] Transitions in bold were used as primary indicators of the presence of the peptide, transitions in italics were used as qualifier; their presence and response ratio to the primary indicator was used to confirm the presence of the peptide.
[2] The presence of these transitions and their qualifiers was treated as an overall confirmation of the presence of gluten.
[3] This transition was used for overall quantitation of the amount of gluten present.

qualifiers in samples containing greater than 20 mg/kg of gluten, peptide P1 was often not detectable in samples containing less than 10 mg/kg and was considered to a relatively insensitive marker for the presence of gluten.

Based on these observations, a protocol was adopted where a sample was considered to contain gluten residues if both P3 and P6 were present above the analytical limits of detection (approximately 2 mg/kg). Limits of detection were calculated from the pooled standard deviation of low level (<10 mg/kg) samples (IANZ, 2004). Quantification was based on P6, the peptide with the strongest response under our analytical conditions. This is similar to the approach adopted by Heick *et al.* (2011b), which included monitoring of up to four peptides for each allergen source material, but based quantification on the most intense transition.

In addition, the Agilent system used employs a compound confirmation protocol based on so-called qualifiers (Agilent Technologies, 2012). This is used when two transition product ions are produced in measureable quantities. In such cases, Agilent suggest that the most abundant ion be used for quantification, while the less abundant ion is used as an identity confirmation check. The response ratio between the two ions should be approximately constant. Qualifier ratios for P3 and P6 were calculated from gluten standards and identity was considered to be confirmed if the qualifier ion was detected and if the qualifier ratio was within three standard deviations of the mean qualifier ratio.

3. Results and discussion

Specificity of gluten analytical methods

The specificity of both ELISA and LC-MS methods was examined by analysis of a range of cereals and pseudocereals, such as buckwheat and quinoa, purchased from retail outlets in Christchurch. Results are summarised in Table 2.

The positive response of the ELISA test to barley is consistent with validation data available for this test. Similarly, positive ELISA responses to rye, spelt and bread

Table 2. Specificity of enzyme-linked immunosorbent assay (ELISA) and liquid chromatography-mass spectrometry (LC-MS) gluten methods.

Sample	ELISA result	LC-MS result
Barley flour	positive	negative
Millet flour	negative	negative
Buckwheat flour	positive	negative
Corn flour	negative	negative
Quinoa flour	negative	negative
Oat flour	positive	positive (weak)
Rice flour	negative	negative
Rye flour	positive	positive
Purple wheat flour	positive	positive
Spelt flour	positive	positive
Standard white wheaten flour	positive	positive

wheat are consistent with the known specificity of this kit. It is contentious whether gluten ELISA kits exhibit true response to oat protein or whether responses seen are due to the known difficulty of obtaining oats without some level of wheat contamination (Gendel *et al.*, 2008; Thompson, 2004). A positive response of the gluten ELISA kit to buckwheat has been reported previously (Mašková *et al.*, 2011; Thompson *et al.*, 2010). However, again it is uncertain whether this is a true immunological cross-reaction or due to wheat contamination of buckwheat samples.

The results of the LC-MS analyses confirm that this assay is specific for gluten-containing cereals (Sealey-Voyksner *et al.*, 2010). The weak positive response to oat flour is almost certainly due to wheat contamination of this material. The lack of a positive response to buckwheat suggests that the positive result to this product for the ELISA method is probably due to immunological cross-reaction.

ELISA analysis of gluten-free foods

Gluten was detected in 6 of 61 (9.8%) of GF samples analysed, with measured gluten concentrations in the range 11-450 mg/kg. Five of the samples containing gluten came from a single bakery outlet. All samples taken from this outlet were found to contain gluten residues.

It is not possible to say what the mechanism of gluten contamination was in these samples. Concentrations are too low for the products to have been made from wheaten flour, which would result in gluten concentrations of several thousand mg/kg.

LC-MS analysis of gluten-free foods

Results of the LC-MS analyses confirmed those obtained by ELISA. A comparison of the quantitative results for gluten-positive samples by the two methods is given in Table 3.

Table 3. Gluten content of claimed gluten-free foods.

Sample description	Gluten content (mg/kg)	
	ELISA	LC-MS
Chocolate chip biscuit	11	<2
Afghan biscuit	22	6
Ginger bread man	51	20
Buns	12	11
Novelty biscuit	450	130
Yoghurt cookie	119	73

ELISA = enzyme-linked immunosorbent assay; LC-MS = liquid chromatography-mass spectrometry.

While the absolute values obtained for the gluten content of these samples differs considerably between the two methods, the relative ordering of the concentrations are similar. Given that the two methods used quite different standard materials for calibration and are measuring different characteristics of the gluten protein, it is not surprising that differences in measured concentration were observed. A similar lack of quantitative agreement between ELISA and LC-MS methods has been reported by studies of other allergenic source materials (milk, egg, soy, peanut, hazelnut, walnut, almond) (Heick *et al.*, 2011a).

No true 'standard' exists for gluten, although attempts have been made to develop well characterised materials (Van Eckert *et al.*, 2006). This is in line with standardised material development for other allergenic source materials (Dumont *et al.*, 2010). Both of the methods of gluten quantification employed in the current study rely on significant assumptions. The ELISA method assumes that the target gliadin proteins are present as a stable proportion of the total gliadin protein. It is further assumed that gliadin protein is half of the total gluten protein present. The LC-MS method also assumes a consistent ratio between the concentrations of target peptides and the total amount of gluten protein present. These assumptions are unlikely to be strictly correct. For example, a recent study demonstrated that the ratio of gliadin to glutenin in bread wheats can vary by a factor of two (Žilić *et al.*, 2011).

For this set of samples, LC-MS analysis offered no immediate advantages in specificity and sensitivity over the traditional ELISA method, but did provide an independent method for confirming the results obtained from ELISA.

There are no internationally-accepted guidelines for 'safe' levels of gluten exposure for wheat allergy and Coeliac disease sufferers and individuals will have variable tolerance to gluten. A double-blind placebo-controlled trial of patients with Coeliac disease found changes in small intestine mucosa at a dose level of 50 mg gluten/day, but not at 10 mg/day (Catassi *et al.*, 2007). Objective symptoms were observed in wheat allergic individuals receiving as little as 0.1 g of wheat or 10 mg of gluten for wheat containing 10% protein (Scibilia *et al.*, 2006). For the most contaminated product in the current survey, a biscuit containing 450 mg gluten/kg, consumption of 25 g of biscuit would have the potential to cause objective symptoms in the most sensitive wheat allergy sufferers, while 125 g/day of this product could produce symptoms in individuals with Coeliac disease. A large biscuit may weigh 60-70 g (Ministry of Health/Plant and Food Research, 2009).

While some of the gluten residues detected in the current study might be tolerated by most wheat allergy or Coeliac disease sufferers, their presence in GF foods is inconsistent with consumer expectations.

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