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Novel aspects of quantitation of immunogenic wheat gluten peptides by liquid chromatography–mass spectrometry/mass spectrometry

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ABSTRACT

A novel, specific and sensitive non-immunological liquid chromatography–mass spectrometry (LC–MS) based assay has been developed to detect and quantify trace levels of wheat gluten in food and consumer products. Detection and quantification of dietary gluten is important, because gluten is a principle trigger of a variety of immune diseases including food allergies and intolerances. One such disease, celiac sprue, can cause intestinal inflammation and enteropathy in patients who are exposed to dietary gluten. At present, immunochemistry is the leading analytical method for gluten detection in food. Consequently, enzyme-linked immunosorbent assays (ELISAs), such as the sandwich or competitive type assays, are the only commercially available methods to ensure that food and consumer products are accurately labeled as gluten-free. The availability of a comprehensive, fast and economic alternative to the immunological ELISA may also facilitate research towards the development of new drugs, therapies and food processing technologies to aid patients with gluten intolerances and for gluten-free labeling and certification purposes. LC–MS is an effective and efficient analytical technique for the study of cereal grain proteins and to quantify trace levels of targeted dietary gluten peptides in complex matrices. Initial efforts in this area afforded the unambiguous identification and structural characterization of six unique physiologically relevant wheat gluten peptides. This paper describes the development and optimization of an LC–MS/MS method that attempts to provide the best possible accuracy and sensitivity for the quantitative detection of trace levels of these six peptides in various food and consumer products. The overall performance of this method was evaluated using native cereal grains. Experimental results demonstrated that this method is capable of detecting and quantifying select target peptides in food over a range from 10 pg/mg to 100 ng/mg (corresponding to approximately 0.01–100 ppm). Limits of detection (LOD) and quantification (LOQ) for the six target peptides were determined to range from 1 to 30 pg/mg and 10–100 pg/mg respectively. Reproducibility of the assay was demonstrated by evaluation of calibration data as well as data collected from the analysis of quality control standards over a period of four consecutive days. The average coefficient of determination (R^2) for each peptide was consistently found to be >0.995 with residuals ranging from approximately 80% to 110%. Spike recovery data for each peptide in various matrices was evaluated at a concentration level near the approximate LOQ for each, as well as at higher concentration levels (30 and 60 ng/mg). The average range of accuracy of detection for all peptides at the lower concentration level was determined to be 90% (± 11), while accuracy at the 30 and 60 ng/mg levels was 98% (± 5) and 98% (± 3), respectively. The usefulness and capabilities of this method are presented in a practical application to prospectively screen a variety of common commercially available (native and processed) gluten-containing and gluten-free foods and products.

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1. Introduction

Gluten proteins comprise a large family of storage proteins found in cereal grain seeds. This large protein family consists of

hundreds of proteins ranging in size from about 30 kDa into the millions of kDa [1]. Since the late 1700s, proteins in this family have been studied extensively and several theories have been applied to further subdivide or classify the family into sub-groups [2–4], according to their characteristics, amino acid sequences, locations within the grain, solubility and relationship within other grains. Today's nomenclature generally refers to “gluten” as the water-insoluble seed storage proteins found in the Triticeae tribe of the grass (Gramineae) family that includes wheat, barley and rye.

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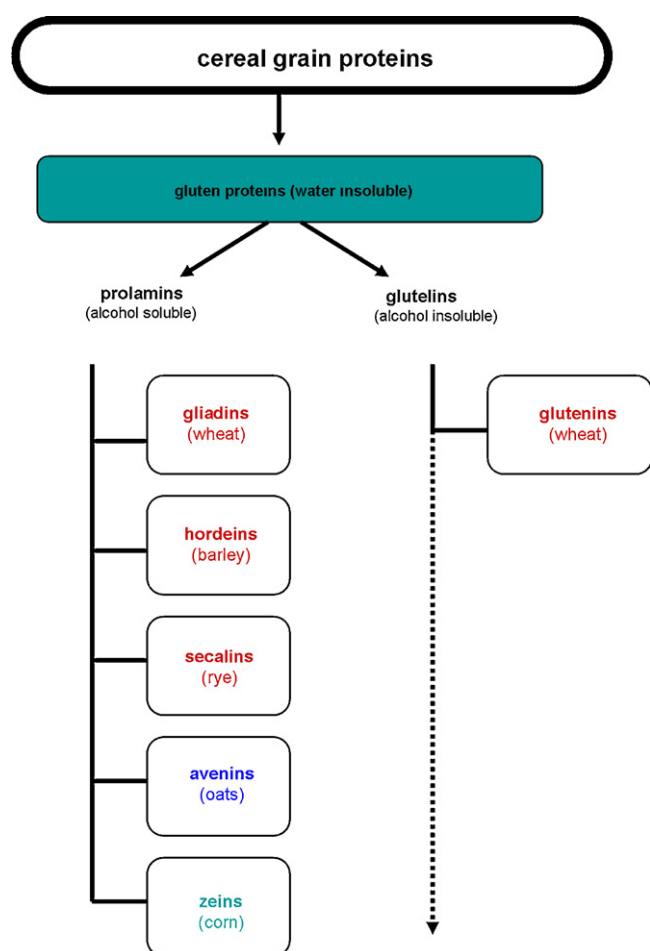


Fig. 1. Cereal grain proteins have been grouped according to their solubility characteristics. Prolamin and glutelin proteins from wheat, barley and rye are associated with gluten sensitivities; those from oats have a possible association and those from corn are considered non-toxic.

Some gluten proteins associated with wheat, rye and barley grains are considered a trigger of a variety of immune diseases, including food allergies and intolerances [5]. One such disease, celiac sprue, is a complex autoimmune disorder of the small intestine that affects close to 1% of the world's population [6,7]. Current knowledge about the pathogenesis of the disease involves environmental, genetic and immunological factors. In those who are genetically predisposed, exposure to gluten can trigger both innate and adaptive immune responses. Prolonged exposure to even modest quantities of dietary gluten can result in severe damage to the small intestine. In contrast, symptoms can vary from severe to limited or even absent [8,9].

Two interesting solubility-based subgroups of the large water-insoluble gluten protein family are: (1) the aqueous alcohol-soluble prolamins and (2) the alcohol-insoluble glutelins (i.e. “gliadins” and “glutenins”, respectively, in wheat). In the wheat kernel, the monomeric gliadins and polymeric glutenins are found in the relative ratio 65:35 and form most of the storage protein. Similar groups of proteins, illustrated in Fig. 1, are found in barley and rye grains [10]. The prolamins (originally so-named for their high proline and glutamine content) and more recently, the glutelins of wheat, barley and rye grains, have all been identified as the environmental triggers for the immune reactions in celiac patients [11–14]. Although all cereal grains contain prolamin and glutelin proteins, the amino acid sequences of the proteins in each grain are different. At present, only those of wheat, barley and rye have

been shown to trigger the abnormal immune response that affects the intestinal lining of those genetically susceptible to celiac disease.

Currently, the only effective therapy available for those who suffer with celiac disease is a strict lifelong gluten-free diet [15–17]. Maintaining a true gluten-free existence is difficult, because gluten is used (under a variety of names) extensively, throughout the world in many manufacturing arenas [18]. In food manufacturing, it is used as a flavor enhancer, a thickener, fortification ingredient, filler, whitener etc. Gluten is also used in the manufacturing of personal care products, nutritional supplements and drug products. Fortunately, the presence of gluten in a product is usually noted by its inclusion in the ingredient list on product packaging (albeit referenced by a variety of names). Unfortunately, there are also many hidden sources of gluten; from unlabeled ingredients and from cross-contamination of manufacturing and transportation equipment. Therefore, people with celiac disease are destined to a life of label-reading to ensure that products are safe for personal use and consumption. Several groups of researchers are working to establish if there actually exists a “safe” quantity of gluten, tolerable for celiac patients to ingest on a daily basis [19,20]. But in the meantime, a gluten content of “zero” is desired.

Therefore, from the perspectives of patient disease management, manufacturing quality control and worldwide consistency with respect to product labeling, there poses an obvious need to be able to accurately determine that consumer products are gluten-free and label them as such. However, the subject of product labeling is a controversial one and there is no concise definition of “gluten-free” that is accepted worldwide [21–23]. The United States Food and Drug Administration (FDA) has proposed a definition for the term “gluten-free”, which was posted in the Federal Register on January 3, 2007. The final form of the definition was expected to be published in August 2008; but it still remains in draft form [24].

Conversely, the Codex Alimentarius Commission (a joint committee with delegates from both the Food and Agriculture Organization of the United Nations [FAO-UN] and the World Health Organization [WHO]) revised its Standard for Foods for Special Dietary Use for Persons Intolerant to Gluten in July 2008 [25]. The Codex definition is now the standard for gluten content allowed in a food product to be considered gluten-free for international trade. In short, it states that for a product to be labeled “gluten-free”, it must not contain more than 20 ppm (parts per million) of wheat, barley or rye gluten. This correlates to about 1 mg of total gluten in 50 g of food.

There is also controversy surrounding the analytical method(s) considered acceptable to a variety of organizations to determine and quantify the amount of gluten in food and consumer products. Currently, both the FDA and the Codex endorse different methods, variations of an immunological antibody-based ELISA. Various ELISA methods are available. Each has proven to be efficient, yet each has limited application in trace gluten analysis [26,27]. It is imperative to know which method to use in each analytical application, thus accurately ensuring food safety.

The work described here details the development of a sensitive and specific non-immunological analytical method, which can be considered complementary to the ELISA assay. This liquid chromatography–mass spectrometry (LC–MS) based method has been developed for the quantitative detection of trace levels of immunogenic gluten marker peptides from complex mixtures, such as food samples. Data shown will emphasize how this direct enzymatic digestion–LC–MS/MS method releases the physiologically relevant marker peptides, then detects and quantifies them, in a wide variety of native and processed foods in order to form a correlation between the quantities of the gluten marker peptides in the digested food to a known gluten adverse reaction.

2. Experimental

2.1. Materials and reagents

2.1.1. Chemicals

Pepsin, trypsin, chymotrypsin, KH_2PO_4 and NaOH were obtained from Sigma–Aldrich (St. Louis, MO, USA). HPLC-grade acetonitrile dithiothreitol (DTT) ethanol, isopropanol, tris-(hydroxymethyl) aminomethane and HCl were obtained from Fischer Scientific. Water was obtained from an in-house Milli-Q water purification system (Millipore, Billerica, MA, USA).

2.1.2. Samples of food and consumer products

Quinoa flour, stone ground whole grain corn flour, stone ground whole grain soy flour, vital wheat gluten flour, stone ground whole wheat flour (brand 1), whole wheat flour (brand 2), rye flour, barley flour, rice flour, oat flour, powdered ice tea mix, pasta, orzo, chee-rios, hot sauce, bread, goldfish crackers, white vinegar, toothpaste, body lotion and body wash were all obtained at a local supermarket (Raleigh, NC, USA). Beer, gin, vodka, rum, red and white wine were obtained from a local specialty store (Durham, NC, USA). Gluten-free items (beer, rice + potato pasta, rice pasta, quinoa pasta, soy pasta, bread, pretzels, protein bar, quinoa cereal, rice seasoning mix, pad thai seasoning mix and crackers) were obtained from a local specialty store (Chapel Hill, NC, USA).

2.1.3. Peptide standards

Synthetic standards of the six peptides (LQPQNPSQQQPQE-QVPL, TQQPQQPFPQPQQPFPQ, VVPVQLQPQNPSQQQPQE-QVPL, RPQQYPQPQPQY, QPQQPFPQTQQPQQPFPQ and PQQSPF) were prepared in order to confirm the identity of the six physiologically relevant peptides that have been identified by enzymatic digestion/fluorescent tagging experiments (manuscript in preparation). These peptides were prepared and purchased from (Thermo Electron, Ulm, Germany) and were analyzed using HPLC–MALDI-TOF mass spectrometry in order to determine their exact molecular weights and purity (>90% pure). The six synthetically prepared peptide standards were used in combination to serve as the calibration and quality control standards in this LC–MS assay.

2.1.4. Preparation of calibration and quality control standards, procedure and analytical blanks

Calibration standards were prepared as cocktails of the six synthetically prepared target peptides [(1) LQPQNPSQQQPQE-QVPL, (2) TQQPQQPFPQPQQPFPQ, (3) VVPVQLQPQNPSQQQPQE-QVPL, (4) RPQQYPQPQPQY, (5) QPQQPFPQTQQPQQPFPQ and (6) PQQSPF]. Standards were prepared using appropriate aliquots taken from individual stock peptide solutions of each peptide, prepared in 80%/20% water/acetonitrile. Calibration standard concentration levels were as follows: 0.1, 0.3, 1, 3, 10, 30, 100 and 300 pg/ μL and 1, 3, 10, 30 and 100 ng/ μL . This corresponded to a range of about 3 ppb to 100 ppm.

Two types of quality control standards were prepared. The first type was prepared by spiking stock peptide solutions at three concentration levels that matched the solvent calibration standards into aliquots of corn flour that had previously been enzymatically digested (as described in Section 2.2). The second type of quality control standard was prepared by spiking the stock peptide solutions at concentration levels that matched the solvent calibration standards into aliquots of water that had also been enzymatically treated.

Two types of procedure blanks were prepared. The first type used corn flour as a representative gluten-free matrix and was enzymatically digested (as described in Section 2.2) with no target peptides spiked in. The second type used water as the matrix and was also enzymatically treated with no target peptides spike in.

Analytical blanks were prepared as aliquots of the HPLC mobile phase A (95% water/5% acetonitrile (ACN) + 0.025% trifluoroacetic acid (TFA)).

2.2. Sample preparation

Prior to analysis by LC–MS, samples were treated with proteases using an *in vitro* procedure that utilized conditions and enzymes that model gastric and duodenal protein digestion in humans. This enzymatic digestion procedure (modeled after one such procedure reported by Marti et al. [28]) will release some or all of the six target peptides, should wheat gluten be present in the sample.

In brief, samples were proteolyzed with pepsin, trypsin and chymotrypsin (PTC) according to the following protocol: 30 mg of homogenized sample was dissolved into 1 mL pepsin solution (0.01 M HCl adjusted to pH 2), to establish a 1:100 pepsin to protein ratio. Samples were incubated (at 38 °C) with gentle shaking for 2 h. 50 μL of a 50 mM phosphate buffer and 35 μL of a 0.1 M NaOH solution were added to each sample, keeping the pH between 7 and 7.5. Thereafter, 25 μL of a 1:100 trypsin:chymotrypsin solution (in 50 mM phosphate) were added to each sample and the sample incubated (at 38 °C) and gently shaken for 30 min. Following brief centrifugation (~10 min), 200 μL of the supernatant was withdrawn from each sample and 200 μL of mobile phase A (95% water/5% acetonitrile (ACN) + 0.025% trifluoroacetic acid (TFA)) was added. Samples were again centrifuged for 10 min and a 200 μL aliquot was removed from each for LC–MS analysis.

2.3. Instrumentation and analysis conditions

HPLC–ESI-triple quadrupole (QQQ)–MS/MS was used in the development of this quantitative method for the analysis of the 6 target peptides. High performance liquid chromatography (HPLC) was performed using an Agilent 1200 Rapid Resolution LC system (Agilent Technologies, Santa Clara, CA, USA).

The HPLC was coupled to an Agilent 6410 Triple Quadrupole (QQQ) mass spectrometer (Agilent Technologies, Santa Clara, CA, USA), operated in positive atmospheric pressure electrospray ionization (AP-ESI) mode. Mass calibration of the mass spectrometer was conducted using the Agilent tune compound, according to the manufacturer's documented procedures. Daily mass calibration checks were performed in order to assure instrument response was accurate and consistent. Sample analyses were performed in multiple reaction monitoring (MRM) mode, employing time programming to obtain maximum possible sensitivity and specificity for each target peptide.

The atmospheric pressure ionization (API) source was operated at a capillary voltage of 3800 V (half moon electrode in the API chamber), using a nitrogen drying gas at a flow rate of 9.5 L/min and temperature heated to 350 °C. The nebulizer was operated at 45 PSI. The fragmentation voltage (capillary exit voltage) used for all target peptides was 150 V, except for peptide 6, whereby 120 V was used. Each parent to product ion transition was monitored for 100 ms, except for time segment 5, whereby each transition was monitored for 50 ms. Table 1 lists the six target peptide sequences, their respective monoisotopic molecular weights, time program segments, parent to product ion transitions and respective collision energies used for the analyses.

Typically, 20 μL of sample was injected into the LC–MS system. For the ultra-trace level analyses, a sample volume of 100 μL was used. Sample aliquots were injected onto a C18 reverse-phase Ascentis Express (Sigma–Aldrich/Supelco) column of dimensions 2.1 mm i.d. $\times$ 150 mm with 2.7 μm superficially porous silica particles for the separation of peptides. The gradient used for peptide separation was 0–22% B over 35 min, followed by 22–60% B over 10 min at 25 °C at a flow rate of 300 $\mu\text{L}/\text{min}$. Mobile phase A was

Table 1
MS/MS conditions.

Peptide #	Sequence	Monoisotopic MW (Da)	Time segment	MRM transition*	Product ion type	Collision energy (V)
1	LQPQNPSQQPQEQVPL	1958.0	4	980.7(+2)–866.6(+2) <i>980.7(+2)–1150.7</i>	b15 b10	20 30
2	TQQPQQPFPQQPQQPFPQ	2148.0	5	<i>1075.9(+2)–1195.6</i> 1075.9(+2)–956.6 <i>1075.9(+2)–244.4</i>	y10 b8 y2	30 25 17.5
3	VPVPQLQPQNPSQQPQEQVPL	2478.3	6	1240.9(+2)–1126.7(+2) <i>1240.9(+2)–762.4</i>	b20 b7	25 30
4	RPQQYPQPQPQY	1627.8	3	<i>814.6(+2)–1221.8</i> 814.6(+2)–407.3	b10 y3	25 30
5	QPQQPFPQTQQPQQPFPQ	2148.0	5	<i>1075.9(+2)–726.3</i> 717.6(+3)–244.1 <i>1075.9(+2)–1308.8</i>	b6 y2 b11	27.5 17.5 25
6	PQQSPF	702.3	2	703.4–441.4 <i>703.4–263.3</i>	b4 y2	25 35

Target peptide sequences, their respective monoisotopic molecular weights, time program segments, parent and product MRM ion transitions and respective collision energies used for all analyses (*charge state other than 1 is listed in parentheses: MRM transitions in bold were used for quantification; MRM transitions in italics were used for confirmation).

95% water/5% acetonitrile (ACN) + 0.025% trifluoroacetic acid (TFA). Mobile phase B was 5% water/95% acetonitrile (ACN) + 0.025% trifluoroacetic acid (TFA).

3. Results

3.1. Discovery of physiologically relevant immunogenic peptides

Previous work with recombinant gliadin proteins has led to the discovery of two highly inflammatory, physiologically relevant, multivalent 33- and 28-residue gluten oligopeptides [29,30]. In turn, those peptides have proven to be useful markers of gluten toxicity in several subsequent studies [31–34]. Motivated by those findings, we wished to identify additional physiologically relevant gluten peptides that could be detected and quantified in trace amounts with high sensitivity and specificity in complex foods. To this end, we studied native cereal grains, using an innovative approach involving fluorescence tagging of proteolytically digested wheat gluten peptides treated with recombinant human transglutaminase 2. This approach used LC-ESI-Ion Trap MSⁿ to identify and characterize potential immunogenic peptides. About 25 potential wheat gluten peptide sequences were thus identified that could potentially serve as markers for gluten content in food [manuscript in preparation].

From the list of 25 potential wheat gluten peptides originally identified, 6 of the most intensely MS-responding peptides (listed in Section 2.1.3) were chosen for further study. Confirmation of the identity of each of the peptides was determined by comparing each structure and molecular weight with synthetically prepared peptides of the corresponding sequences. HPLC–MS/MS analysis as well as NCBI protein database searches confirmed that these peptides were all present in various varieties of wheat and not present in non-immunogenic grains (i.e. corn and rice). These 6 wheat gluten peptides were then chosen as target peptides for the development of the quantitative LC–MS assay.

3.2. Quantitative LC–MS/MS assay development

The established analytical technique HPLC–MS/MS has been used successfully to study trace contamination of a variety of analytes in food [35–38]. The use of this technique in the determination of the presence/absence of trace amounts of immunogenic gluten peptides in food and consumer products would provide an overall indication of the toxicity of the product to those with

gluten sensitivities. Utilizing the excellent analytical capabilities of HPLC–MS/MS, a method was developed and optimized in order to provide the best possible accuracy, and sensitivity for the quantitative detection of trace levels of the 6 wheat gluten peptides in native and processed foods.

3.2.1. Direct enzymatic digestion

Prior to analysis by HPLC–MS/MS, samples were treated *in vitro* with various proteases. The *in vitro* enzymatic digestion procedure that was developed uses similar conditions and enzymes as those found in the human digestion system [28, manuscript in preparation]. The procedure was tested on a variety of gluten-containing and gluten-free food samples and was found to reproducibly yield the target peptides when gluten proteins were present. However, it was felt that in order to use this procedure in instances where trace analysis was required, it needed to be further optimized, in order to determine any conditions that would provide the best possible yield of the 6 immunogenic peptides, reduce sample preparation time and the amount of reagents needed. LC-ESI–MS/MS detection was used in the following series of experiments.

- (1) evaluation of alternative cocktails of digestive (gastric and pancreatic) enzymes including pepsin, trypsin, chymotrypsin, elastase and carboxypeptidase A;
- (2) evaluation of proteolytic digestion (reaction) times;
- (3) evaluation of alternative protein: enzyme ratios;
- (4) evaluation of alternative procedures to quench proteolytic activity;
- (5) evaluation of the need for sample pre-concentration;
- (6) evaluation of the need for an aqueous alcohol extraction procedure;
- (7) evaluation of the need to reduce disulphide bonds;
- (8) evaluation of sampling size with and without the alcohol extraction procedure.

The experiments evaluating alternative cocktails of digestive enzymes determined that essentially, pepsin, trypsin and chymotrypsin were all that were required in order to release a reasonable yield of the 6 target peptides. The absence of any of these 3 proteases from the digestion procedure did not release some of the 6 target peptides from the samples. Inclusion of elastase, and carboxypeptidase A resulted in a slight increase in target peptide yield, but an increase in sample preparation time of several hours.

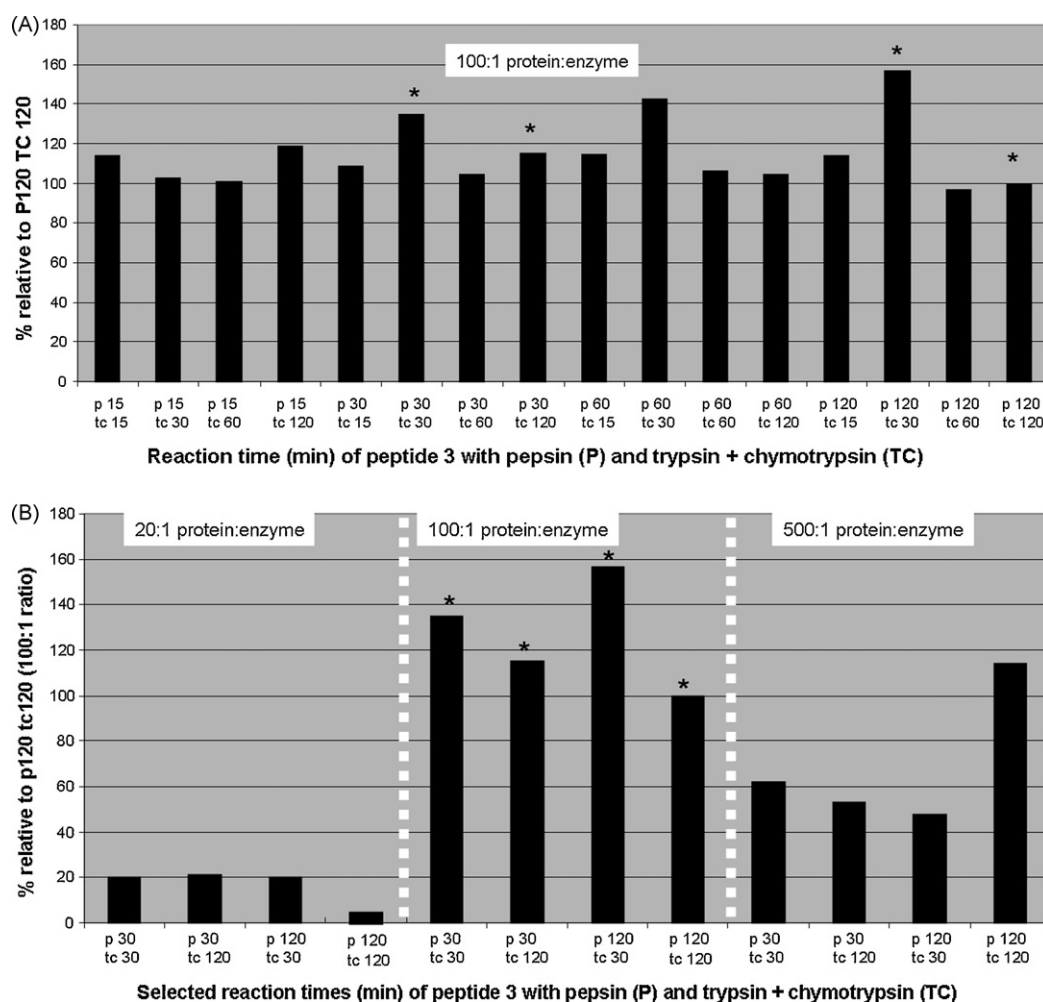


Fig. 2. Varying the protein:enzyme ratio, types of enzymes and enzymatic digestion times can affect the yield of the target peptides: (A) optimization of the yield of peptide 3 (VPVPQLQPQNPSQQPQEQVPL) by varying reactions times with pepsin, trypsin and chymotrypsin using a protein:enzyme ratio of 100:1; (B) optimization of the yield of peptide 3 by varying the protein:enzyme ratio at selected digestion times (*).

In general, the protein:enzyme ratio and the amount of time that enzymes are in contact with food during the digestion process corresponds directly to the yield of proteolyzed products. In an effort to investigate the time needed to produce the highest yield of the 6 target peptides, a series of experiments were performed that monitored peptide formation during a digest of wheat with pepsin, trypsin and chymotrypsin. Results showed that the highest yield of the target peptides was obtained with a pepsin reaction time of 2 h and a trypsin/chymotrypsin reaction time of 30 min. The results also showed that the protein:enzyme ratio that formed the highest yield of the peptides was 100:1. Data shown in Fig. 2 illustrates how varying the protein:enzyme ratio and digestion time affected the formation of target peptide 3 (VPVPQLQPQNPSQQPQEQVPL). Fig. 2(A) shows how the formation of peptide 3 varied with different digestion times of enzymes pepsin, trypsin and chymotrypsin, at a protein:enzyme ratio of 100:1. The graph is normalized to the longest digestion time of 120 min for pepsin and 120 min for trypsin and chymotrypsin. Four time points were chosen (labeled with a “*”) and these were used to study formation of peptide 3 while varying the protein:enzyme ratio. Fig. 2(B) shows results for protein:enzyme ratios of 20:1, 100:1 and 500:1.

One mechanism that is commonly used to terminate an enzyme's activity is to heat the reaction mixture in order to destroy the enzyme. It was postulated that this mechanism may also alter or hydrolyze some of the target peptides. Consequently, three alternative procedures to quench enzymatic reactivity were investigated:

(1) addition of a solution of 50% water/50% acetonitrile (ACN), (2) addition of HPLC mobile phase A (95% water/5% ACN + 0.025% trifluoroacetic acid (TFA)) and (3) chill on ice for 30 min. The highest yield of all peptides was achieved using the procedure whereby HPLC mobile phase A was added to quench the enzymatic reaction.

The inclusion of an aqueous alcohol extraction step is commonplace in ELISA analysis of wheat gluten proteins [17,39]. Its purpose is to pre-extract the alcohol-soluble monomeric gliadin proteins from the remaining alcohol-insoluble proteins in the sample, thus improving the detection capabilities of the assay. The efficiency of our direct proteolysis approach, with respect to the yield of the six target peptides, was compared with this alternate methodology (which employs the aqueous alcohol pre-extraction step). Using wheat gluten, experiments compared the direct proteolysis approach with procedures that employed a 50% and 60% ethanol, as well as a 50% and 60% isopropanol pre-extraction step, followed by proteolysis with pepsin, trypsin and chymotrypsin. The yield of peptides 3, 4, 5 and 6 was found to be higher without the alcohol pre-extraction step, while the yield of peptides 1 and 2 more than doubled using alcohol pre-extraction. Interestingly, experiments using wheat flour (versus wheat gluten) produced the opposite results for peptides 1 and 2.

Based on the similarities in structure of all six target peptides, an explanation for this was not forthcoming. A possible explanation for the higher yield of peptides 3, 4, 5 and 6 without any alcohol pre-extraction may involve a contribution of the target peptides that

had formed from the direct digestion of the alcohol-insoluble polymeric glutenin. It has only recently been determined that glutenin proteins are also immunostimulatory [40]. Glutenin proteins consist of multiple units of monomeric gliadins held together by an extensive network of cross-linked disulfide bridges [41]. The traditional understanding that gliadins and glutenins could be cleanly fractionated from each other, based on their solubility characteristics, using an aqueous alcohol extraction has given way to the more recent observations that some glutenin subunits actually are alcohol soluble and some gliadins are actually alcohol insoluble [40–42]. As such, the ELISA methodology for the detection of gluten could conceivably miss detecting some gluten proteins because their alcohol extraction step does not extract them from the sample. Further experiments involving alcohol pre-extraction with the addition of a reducing agent (DTT) to reduce glutenin disulfide bonds assessed the yield of the six target peptides as compared to the direct proteolysis approach. Even with the addition of a reducing agent, designed to catch the alcohol-soluble glutenin monomers, this method does not yield as high a yield of the target peptides as our direct proteolysis approach, described here.

Efforts to improve the overall sensitivity of this assay involved experiments that evaluated the pre-concentration of samples, prior to HPLC–MS analysis. Following the completed proteolysis procedure, the supernatant was removed, taken down to dryness and reconstituted to a smaller volume with an appropriate solvent. Various solvents and solutions were evaluated, but results showed very little increase in the final yield of peptides. It appeared that significant losses in peptide yield occurred during the various stages of this pre-concentration procedure. Further experiments involved increasing the sampling size of the initial wheat sample from 30 mg up to 300 mg. The enzyme concentrations were increased accordingly, in order to maintain the 100:1 (protein:enzyme) ratio. In theory, increasing the sample size ten times should have increased the yield of peptides by 10. However, losses in peptide yield from incomplete proteolysis, sample handling and an increased number of sample dry down/reconstitution steps needed in this procedure outweighed the benefits of using such a large sample size. An increase in the amount of sample injected (from 10 μ L to 100 μ L) into the HPLC–MS system did allow for a significant increase in sensitivity, which would be helpful for samples requiring ultra-trace component analysis.

The above experiments allowed the direct *in vitro* proteolytic procedure to be streamlined and the best possible conditions and proteases that yielded the highest levels of the six peptides to be determined. The optimized procedure that simulates actual digestion conditions in the alimentary tract is outlined in Section 2.2.

3.2.2. Liquid chromatography

Enzymatically digested food samples have hundreds of proteolyzed peptides present. Therefore, in order to achieve adequate separation and resolution of our target peptides from others present in such complex matrices, we worked with a variety of columns, stationary phases, particles and pore sizes.

The HPLC conditions and column chosen for a particular analysis should be done so as to provide adequate specificity and sensitivity for that analysis. For this type of HPLC–MS/MS analysis, chromatography conditions needed to be chosen to reduce the complexity of the sample entering the MS instrument. This was necessary to reduce matrix ion suppression and interferences from common product ions that could hinder the specificity or sensitivity achieved by MS/MS. To that extent, a short 1–10 min HPLC separation, typical of a quantitative analysis of one target analyte, would in no way be sufficient to reduce the number of co-eluting components that could interfere with the peptide analyses. Therefore, it was observed that HPLC separation times of approximately 60 min were needed, in order to minimize these matrix effects.

The choice of chromatographic column also plays a major role in the specificity and sensitivity for the analysis. Improving the separation of the components reduces the number of co-eluting components that can suppress ionization or interfere in the MS/MS analysis. Improved resolution also results in sharper, more concentrated peaks, which would result in a better response in a concentration detector, such as UV and electrospray ionization mass spectrometry.

The Ascentis Express C18 column packed with 2.7 μ m superficially porous fused core particles appeared to result in the best performance. The peaks were noticeably sharper than those seen with smaller (1.8 μ m) porous particles. This improved chromatographic performance using these particles could be a result of the nature of the particles in this column and/or the increased length of this column. By placing a solid core at the center of the particle, the potential diffusion path length for the peptides through the 0.5 μ m porous layer was shortened considerably, compared to that in a totally porous particle. This shorter diffusion path should reduce dispersion from resistance to mass transfer, thus reducing peak broadening. This type of particle, although larger in overall size, should theoretically, generate similar efficiency to a smaller totally porous particle without generating high backpressures. It was observed that the backpressures from this column were lower, as compared with those observed with the other columns evaluated.

For this application of gluten peptide analysis, the 2.7 μ m superficially porous silica particles in the Ascentis Express C18 column provided much better chromatographic peak shape and resolution for the separation of the six target peptides.

3.2.3. Mass spectrometry

In order to set up any quantitative analytical procedure for target analytes, the MS system first needed to be optimized for each analyte. Various mass spectrometric conditions and parameters were optimized in order to achieve the best possible sensitivity and specificity for the six target peptides. With the instrumentation used in our analyses, most instrument parameters were mass dependent and were optimized using a tuning mix (provided by the instrument manufacturer), in order to obtain the best possible ion transmission yet maintain mass resolution. This was initially done via an auto-tune function, which is available on the Agilent 6410 triple quadrupole system. Following the auto-tune, a fine tune was performed manually, in order to adjust any settings deemed necessary to improve the signal. Two key MS instrument parameters, however, were compound dependent, requiring individual optimization for each peptide. These parameters were fragmentor voltage and collision energy.

The “fragmentor” is the term Agilent Technologies uses to describe the variable voltage at the capillary exit in the electrospray interface. The potential drop between the capillary exit and first skimmer aids in the focusing and transmission of the ions through the skimmer region. As collisions occur between ions in this region, the internal energy of the ions can increase and this can result in fragmentation (sometimes termed “up front CID”). This type of fragmentation is not desired in this particular type of assay, because it could reduce the molecular ion precursors specifically chosen for each peptide in the MS/MS process. Therefore, the fragmentation voltage is compound dependent; the bond strengths of the atoms in the compound and the transfer of kinetic to internal energy in a compound are dependent on the structure and molecular weight of each peptide. In order to optimize the fragmentor voltage setting for each of peptides, flow injection analysis (FIA) was performed on each peptide individually. With each injection of a peptide standard, the fragmentor voltage was incremented by 30–40 V until the optimum voltage was determined.

Analogous to the fragmentation voltage, the collision energy (CE) voltage is also compound dependent. CE depends on the bond strength, molecular weight of the compound and the pathway in which the ion is formed (i.e. directly from the parent ion or through a series of sequential intermediates). The collision energy needs to be optimized in order to determine the optimal voltage to produce the most intense characteristic product ions that will be monitored for each peptide in the MS/MS analysis. The collision cell is a hexapole in the Agilent 6410 QQQ mass spectrometer. It uses nitrogen gas which serves as the collision gas used to transfer kinetic energy into internal energy. The voltage on the collision cell sets the kinetic energy for the ion. However, unlike the fragmentor voltage, which is chosen to maximize the precursor ion intensity, the CE voltage is chosen to produce the most abundant product ions from the chosen precursor ion.

The CE optimization for each peptide was also performed by FIA, whereby the CE voltage was incremented by 5 V with each subsequent injection of peptide standard until the optimum voltage was determined.

A time-programmed multiple reaction monitoring (MRM) MS acquisition profile was developed, using the instrumentation and conditions described in Section 2.3. Time programming, using multiple time segments based on the LC retention times of the peptides, was used to maximize the MS-response and achieve the highest sensitivity. Each segment was set to monitor only the chosen MRM transitions for the particular target peptides eluting during that particular time period. Each segment monitored one peptide, except segment 5 in which peptides 2 and 5 were monitored. Dividing the chromatogram into segments allowed for longer dwell times to monitor the target ions, compared to monitoring all ions (12 ions) over the course of entire analytical run. The longer dwell time improved the signal/noise ratio for the measurement by 2–3 times.

Complete characterization of each peptide sequence was accomplished using both HPLC and MS/MS. Many of the peptide sequences contained common motifs and consequently, the MS/MS spectra of some of the peptides consisted of common product ions. However, in order to assure specificity and the unambiguous identification of each target peptide, unique MRM parent to product ion transitions were used with each peptide in combination with the associated LC retention times for each. All peptides except peptide 6 used the doubly charged parent ion ($[M+2H]^{2+}$) as the precursor ion for the MRM experiment. Peptide 6, being of low molecular weight (702 Da), only formed the singly charged ion ($[M+H]^+$). Product ions from each target peptide were primarily chosen based on sensitivity of the respective “b” or “y” type fragments that formed by the MS/MS process. However, the choice of ion transition was not always the most intensely responding transition, but those that did not interfere with signals from other peptides or matrix components that were present in that particular time segment. Peptides 2 and 5 proved to be challenging, because they were isomers and were not able to be totally chromatographically resolved. Their sequences were also similar and they both fragmented to form similar product ions. However, they were able to be unambiguously resolved by MS/MS because both the primary and confirmatory sets of parent to product transitions chosen for each of these peptides were different in molecular weight and were specific for the respective sequences. It appeared the product ions for these two peptides favored cleavage between the amino acids F (phenylalanine) and P (proline), resulting in some similar and some different product ions that could be used to differentiate the compounds. A “b8” fragment ion at m/z 956.6 was used to identify peptide 2 and a “b6” ion at m/z 726.3 for peptide 5. Fig. 3(A) and (B) shows representative HPLC–MS/MS product ion mass spectra of peptides 2 and 5, illustrating several common product ions and how the two peptides could be discriminated using unique parent to product transition

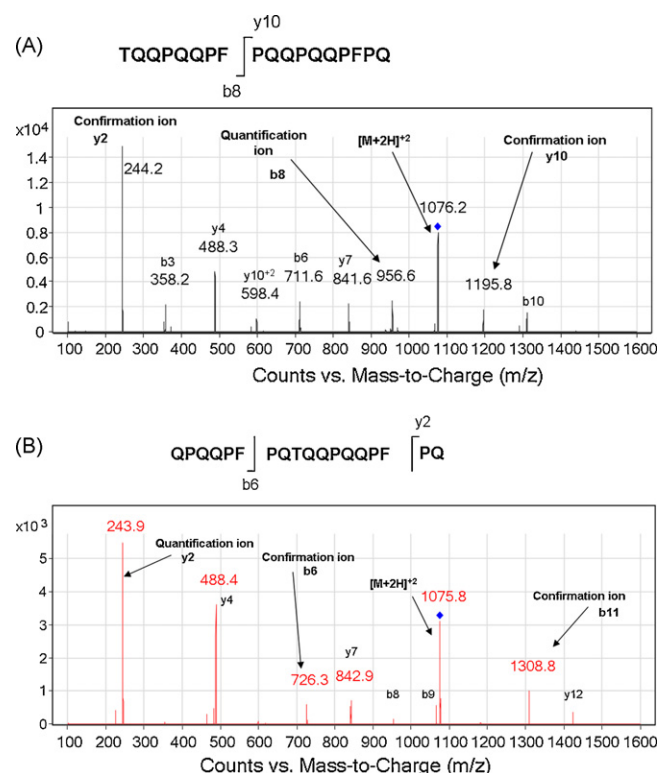


Fig. 3. HPLC–MS/MS product ion mass spectra of the two isomeric peptides: (A) peptide 2 and (B) peptide 5, generated from their respective $[M+2H]^{2+}$ precursor ions. The y2, y4 and y7 product ions are common to both peptides. The two peptides can be discriminated using other unique parent to product transition ions for quantification and confirmation purposes.

ions. Sequence information, types of product ions, quantification and confirmation parent to product transition ions are labeled for each peptide.

Table 1 lists the target peptide sequences for all six peptides, their respective monoisotopic molecular weights, time program segments, parent and product MRM ion transitions and respective collision energies used for the analyses. MRM transitions in bold are used for quantification; MRM transitions in italics are used for confirmation. A representative time-programmed MS/MS–extracted ion chromatogram (EIC) of a cocktail of all 6 synthetic peptide calibration standards [(1) LQPQNPSQQPQEQVPL, (2) TQQPQQPFPQQPQQPFPQ, (3) VPVQLQPQNPSQQPQEQVPL, (4) RPQQYPQPQPQY, (5) QPQQPFPQTQQPQQPFPQ and (6) PQQSPF] at concentration 3 ng/mg is shown in Fig. 4. Fig. 4(A) shows the primary MRM transitions used for each peptide. These are, in general, the most intensely MS-responding ions and were the transitions used for quantification purposes. Secondary MRM transitions are shown in Fig. 4(B). These are the confirmation ions used for positive identification of the presence of the peptides in samples. Peptides 2 and 5 have two sets of confirmation transitions. Only the primary confirmation transitions are shown here.

3.3. LC–MS/MS method performance

The overall performance of this HPLC–MS/MS assay was evaluated according to a variety of parameters, commonly used to assess the capabilities of such an assay and verify that it can meet the requirements deemed necessary to be used efficiently for the purposes of quantitative detection of target compounds in real samples.

Linearity, sensitivity and reproducibility of the LC–MS assay was demonstrated from the evaluation of calibration data, col-

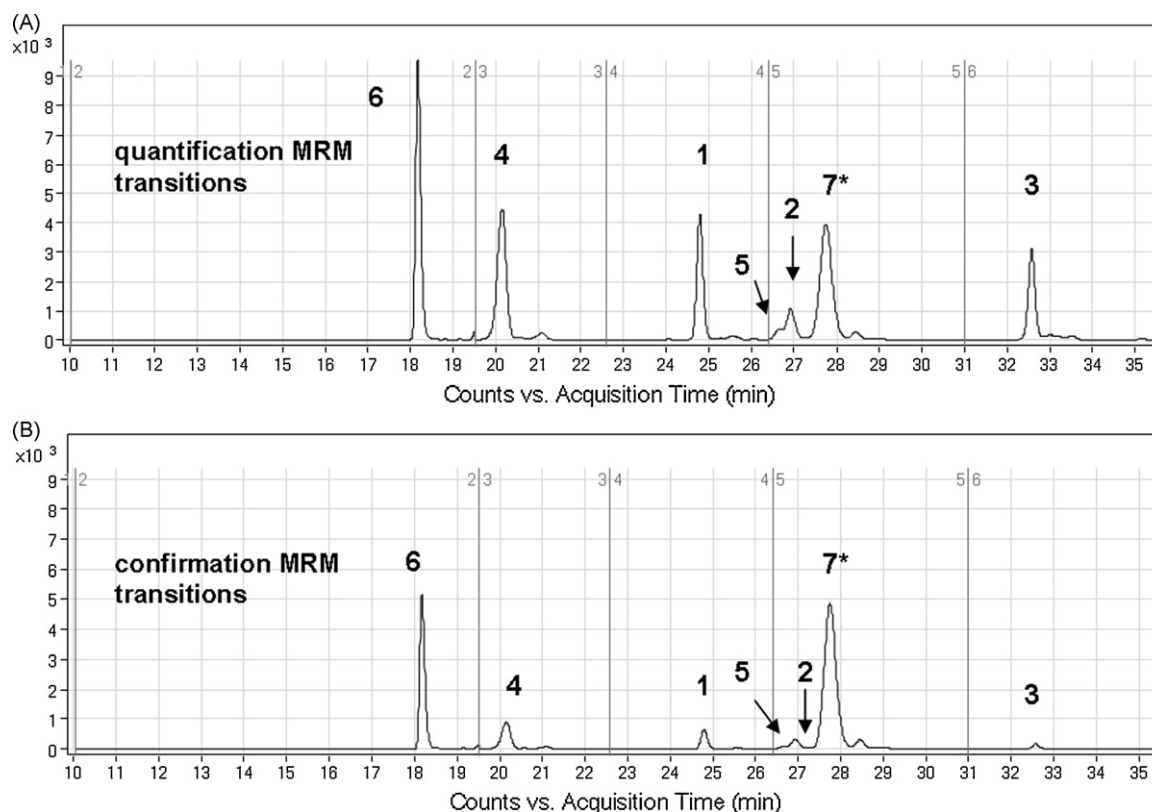


Fig. 4. HPLC–MS/MS–extracted ion chromatograms of a solvent standard cocktail of all six target peptides at a concentration of 3 ng/mg. The time programming scheme shows all 12 MRM parent to product transitions that are monitored: (A) parent to product transitions used for quantification and (B) parent to product transitions used for confirmation. Peptide 7* is discussed in Section 4.

Table 2
Sensitivity and linearity.

Peptide #	Sequence	Coefficient of determinatic (R^2)	Residual range (% accuracy)	LOD (pg/mg)	LOQ (pg/mg)
1	LQPQNPSQQPQEQVPL	0.9936	78–104	3.5	20
2	TQQPQQPFPQQPQQPFPQ	0.9960	91–102	25	100
3	VPVQLQPQNPSQQPQEQVPL	0.9992	93–103	14	50
4	RPQQPYQPQPQY	0.9916	85–103	3	20
5	QPQQPFPQTQQPQQPFPQ	0.9963	77–109	30	100
6	PQQSPF	0.9977	83–112	1	10
Average		0.9967			

Regression analysis showing representative data to support sensitivity and linearity of the assay. The LOD was calculated at a signal:noise (S/N) level 3.1. Values used for the LOQ were calculated to be approximately 5 times the LOD and as close as possible to lowest point on tie calibration curve.

lected over the four consecutive days. Table 2 summarizes data from a representative regression analysis, done using a corn flour matrix (proven free of gluten) spiked with a cocktail of standards of the six target peptides. The respective limits of detection (LOD) and quantification (LOQ), coefficients of determination (R^2), as well as residuals for each peptide is shown. These data demonstrate that this assay can detect and quantify select immunogenic wheat gluten peptides over a range of 10–1000 pg/mg in food. This corresponds to being able to measure the peptides over a range of approximately 10–1000 ppb (parts per billion). Detection and quantification at higher levels was also achieved, using a second calibration curve, covering a range from 1 ng/mg to 100 ng/mg, which corresponds to a measurement range of approximately 1–100 ppm (data not shown). The average coefficient of determination (R^2) for each peptide was consistently found to be >0.995 with residuals ranging from approximately 80% to 110%.

Specificity of the assay was demonstrated by monitoring two or three different MRM MS/MS transitions for each of the target peptides (refer to Table 1). In general, the most intense MS-responding

ions constituted the primary MRM transition, which was used for quantification purposes. The less intense ions constituted the secondary MRM transitions, which were used for the confirmation purposes. Specificity of the cocktail of proteases with respect to the six target peptides was demonstrated by analyzing a sample which consisted of all reagents required in the analysis, but without matrix or peptide standards present. No ions were found to be present that would interfere with the MS/MS signals for any of the target peptides. Experiments also determined that none of the six target peptide standards showed any further degradation upon direct treatment with the cocktail of proteases. Specificity of the protease cocktail with respect to the six target peptides was also demonstrated via spike recovery experiments in matrix.

Spike recovery experiments were conducted to test the assay's absolute ability to detect the six peptides, free of interferences, when spiked in both an analyte-free and analyte-containing matrix (i.e. corn versus wheat). Two spike recovery experiments were performed, whereby a standard cocktail of the six peptides was spiked: (1) into corn flour matrix at two levels: (a) representing 0.06 ng/mg

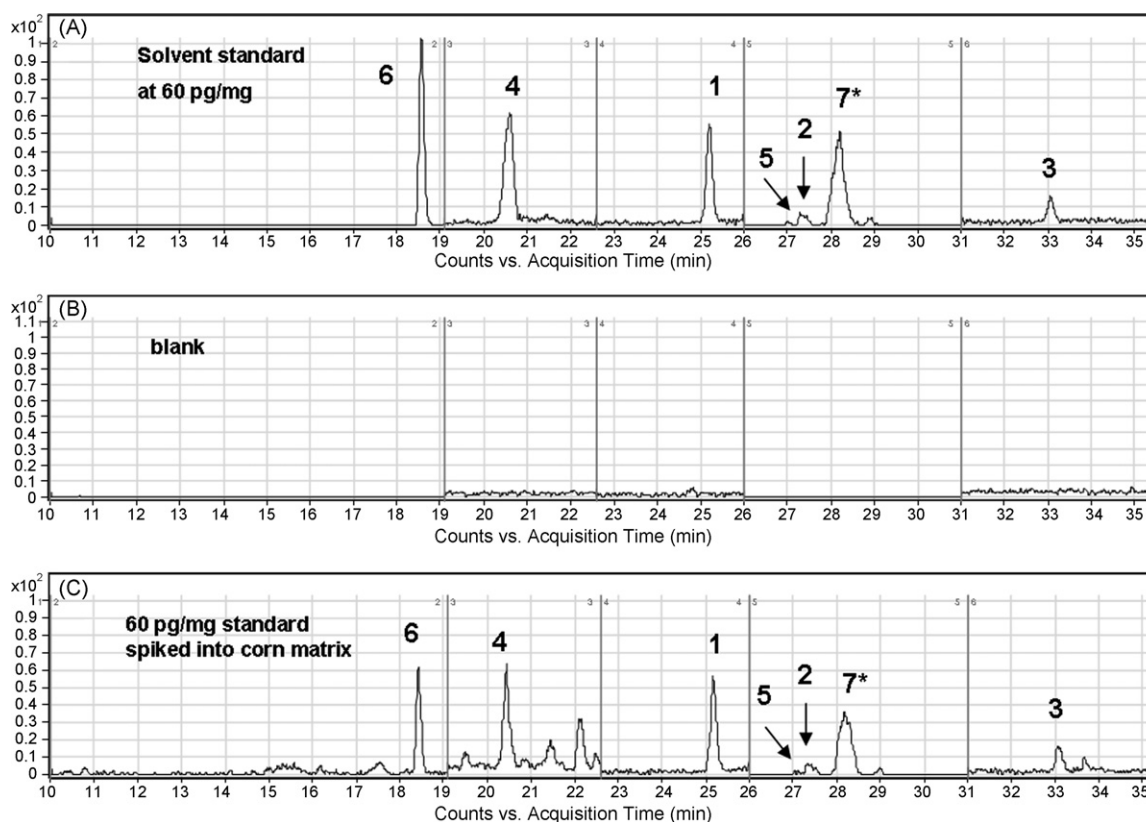


Fig. 5. HPLC–MS/MS-extracted ion chromatograms from the spike recovery experiments: (A) a 60 ppb solvent standard cocktail of peptides 1–6; (B) analytical blank and (C) a 60 ppb standard cocktail of peptides 1–6 spiked into native proteolyzed corn flour. These are representative views of peptide responses near their respective LOQ. Peptide 7* is discussed in Section 4.

Table 3
Spike recovery.

Peptide #	Sequence	%Accuracy in corn (0.06 pg/mg)	%Accuracy in corn (30 pg/mg)	%Accuracy in wheat (60 pg/mg)
1	LQPQNPSQQPQEQVPL	96.2	92.8	99.4
2	TQQPQQPFQPPQ	85.6	95.8	94.1
3	VPVPQLQPQNPSQQPQEQVPL	103.5	97.1	97.4
4	RPQQPYPPQPPQ	90.4	92.3	97.5
5	QPQQPFQPPQPPQ	90.1	99.1	96.8
6	PQQSPF	68.7	108.3	102.2
Average		89.1	97.6	97.9
Relative standard deviation (%)		11.7	5.9	2.7

Accuracy of the recovery of the six target peptides spiked at 0.06 ng/mg and 30 ng/mg into corn and 60 ng/mg spiked into wheat.

peptide in food and (b) representing 30 ng/mg peptide in food; and (2) into wheat flour matrix representing 60 ng/mg peptide in food. Fig. 5 shows representative HPLC–MS/MS-extracted ion chromatograms of spike recovery experiments: (A) a 60 ppb solvent standard cocktail of peptides 1–6, (B) an analytical blank and (C) a 60 ppb standard cocktail of peptides 1–6 spiked into corn flour. A calibration curve was prepared for each target peptide which bracketed the concentration of peptide spiked into each matrix (proteolyzed corn and wheat). The areas of each of the peptide peaks in the spiked samples were measured and the corresponding quantity of that peptide in the samples was determined using the respective calibration curve. The calculated results of these recovery experiments are tabulated in Table 3. Percent (%) accuracy was calculated as [(measured value/theoretical value) × 100]. The “measured value” refers to the peak area of each peptide measured in matrix and the “theoretical value” refers to the corresponding peak area measured in the solvent standard.

Results (Table 3) show that the range of accuracy of detection of the 0.06 ng/mg and 30 ng/mg standards spiked into corn

were 69–104% and 92–108% respectively. The lower level spike (0.06 ng/mg) represented a level near the LOQ of the assay and is equivalent to approximately 60 ppb. The 30 ng/mg level represented a higher level, perhaps more representative of wheat contamination found in food. An even higher concentration (60 ng/mg) was required to determine spike recovery in wheat, due to the presence of native peptides in the matrix. Recovery from wheat was based on subtracting endogenous peptide concentrations from a wheat sample that had not been spiked (in other words, background subtraction). The peak area for each peptide in the spiked wheat sample was determined by subtracting the corresponding background concentration from the un-spiked wheat sample.

Evaluation of matrix effects and robustness of the assay involved the examination of procedure blanks and analytical blanks (Section 2.1.4). Neither showed matrix effects or carryover. Solvent standards, quality controls and procedure blanks were all found to be stable for well over 8 months, kept at 0 °C.

The use of isotopically labeled internal standards was considered, but not implemented, because of cost and also because

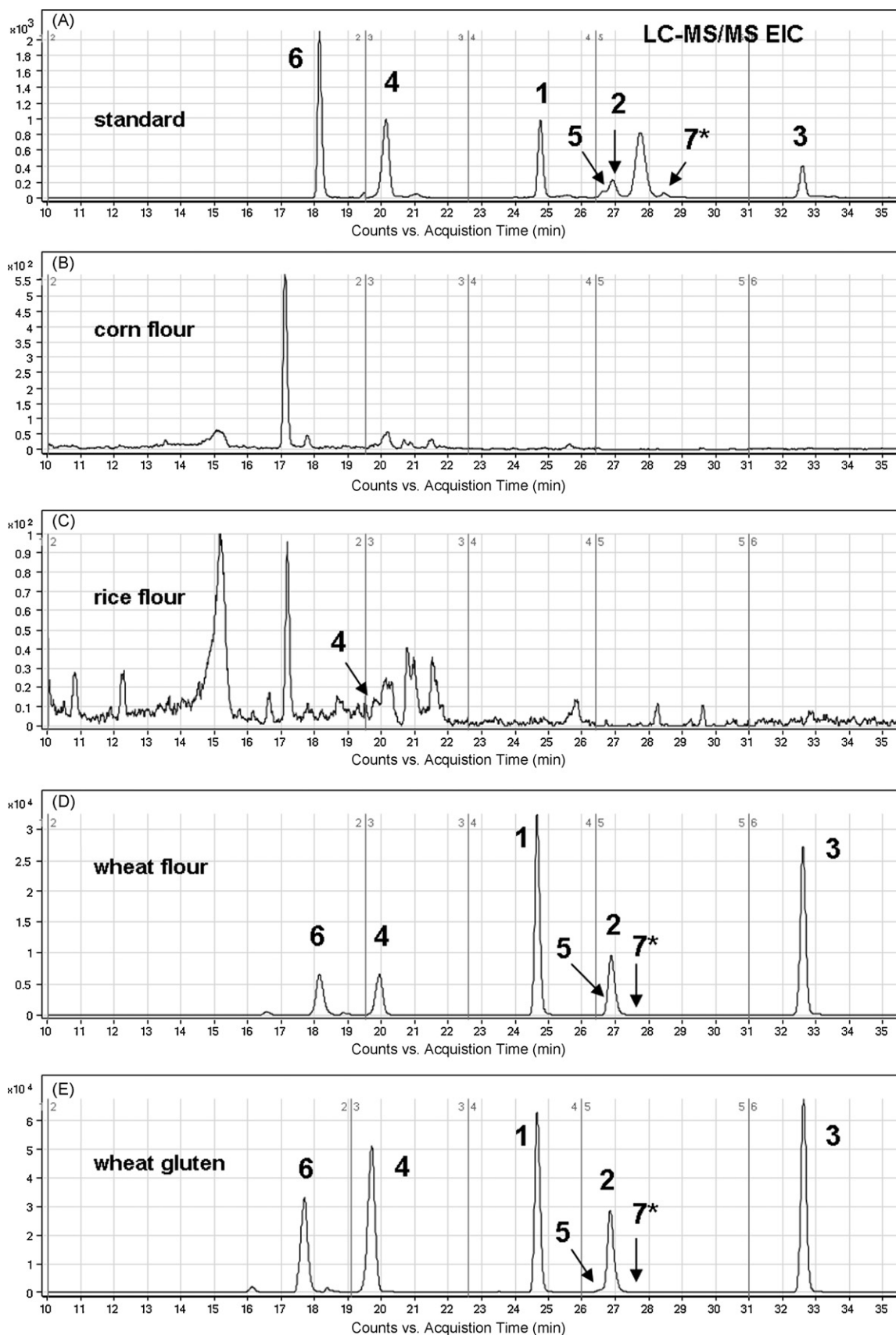


Fig. 6. Presence/absence of immunogenic wheat peptides profiles in various proteolyzed native grains as shown by these representative HPLC-MS/MS-extracted ion chromatograms: (A) a solvent standard of the six target peptides, (B) corn flour, (C) rice flour, (D) wheat flour and (E) wheat gluten. Peptide 7* is discussed in Section 4.

the results from the other qualification experiments demonstrated the method to be acceptable. Furthermore, because a target analyte extraction step was not required and because isotopically labeled standards could not be used to gauge the formation of the target peptides from the direct enzymatic digestion, their incorporation would only serve as an injection standard. An injection standard was deemed unnecessary, considering that the injection-to-injection precision, demonstrated from the analysis of various quality control samples over the course of the entire assay evaluation period, was found to be more than adequate (<12% overall).

3.4. LC–MS/MS detection and quantification of immunogenic peptides in food and consumer products

An overall demonstration of the capabilities of the above optimized HPLC–MS/MS assay involved the analyses of samples of a variety of common commercially available food and consumer products. The products were manufactured in various countries around the world. Some products contained gluten, others were naturally gluten-free or labeled as “gluten-free”. The detection of

any of the six peptides was intended to represent a positive indication of the presence of wheat gluten in the product.

Following the completion of the *in vivo* digestion procedure, HPLC–MS/MS analysis was performed on all sample extracts. Confirmation of the presence of the target peptides in the samples was based upon the following criteria, adapted from FDA guidelines on qualitative target compound identification: (1) target compound retention time (within 2% of that of its representative calibration standard); (2) detection of all MRM product ions (all MRM parent to product ion transitions must be seen); (3) MRM parent to product ion ratios (within 30% of those in the representative calibration standard) and (4) absence of target peptides in all procedure blanks and analytical blanks.

These experiments were carried out over the course of many months. Over this timeframe, normal variations in laboratory conditions, such as temperature, mobile phase preparation, age and status of the chromatography columns used caused retention times of the peptides and segment times in the MS-time-programmed acquisition to shift around slightly. However, this was not seen as a problem because the peptides were always analyzed in

Table 4

Quantitative results from the analysis of native and processed products by LC–MS/MS.

Product description	Product	Measured amount of peptide (ng/mg)					
		Peptide 1	Peptide 2	Peptide 3	Peptide 4	Peptide 5	Peptide 6
Native flours	Corn flour*	HD	ND	ND	ND	ND	ND
	Soy flour*	ND	ND	ND	ND	ND	ND
	Wheat flour	240	240	250	43	250	14
	Wheat gluten	510	730	700	220	600	65
	Rye flour	0.22	0.36	0.16	0.12	D	D
	Barley flour	0.02	ND	1.1	D	ND	0.015
	Quinoa flour*	ND	ND	ND	ND	ND	ND
	Oat flour*	D	ND	D	ND	ND	ND
	Rice flour*	ND	ND	ND	D	ND	ND
Processed foods	Bread	91	140	130	67	150	14
	Sun chips	13	28	9.7	11	28	26
	Goldfish crackers	190	180	140	14	140	11
	Cheerios	3.3	2.3	3.1	0.63	3.7	0.06
	Wheat pasta	4.4	52	29	26	D	4.4
	Orzo	12	90	160	150	2.9	12
	Rice crackers*	ND	ND	ND	ND	ND	ND
	Powdered ice tea mix*	0.046	ND	0.25	0.16	ND	0.025
Gluten-free products	Gluten-free pasta (1)*	ND	ND	ND	ND	ND	ND
	Gluten-free pasta (2)*	ND	ND	D	0.11	ND	ND
	Gluten-free bread*	ND	ND	ND	ND	ND	ND
	Gluten-free pretzels*	ND	ND	ND	ND	ND	ND
	Gluten-free rice seasoning mix*	ND	ND	ND	ND	ND	ND
	Gluten-free cracker*	ND	ND	ND	ND	ND	ND
	Gluten-free beer*	ND	ND	ND	ND	ND	ND
	Gluten-free pad thai seasoning*	ND	ND	ND	ND	ND	ND
	Quina pasta*	ND	ND	ND	ND	ND	ND
	Soy pasta*	ND	ND	ND	ND	ND	ND
	Protein bar*	ND	ND	ND	ND	ND	ND
	Quinoa cereal*	ND	ND	ND	ND	ND	ND
Beverages and sauces	Beer (light)	D	D	1.6	0.088	D	0.091
	Gin	ND	ND	0.06	ND	ND	ND
	Potato vodka*	ND	NO	ND	ND	ND	ND
	Hot sauce	0.15	ND	ND	ND	ND	ND
	Vinegar	D	D	2.6	0.12	1.5	D
	Rum*	ND	ND	ND	ND	ND	NO
	Red wine*	ND	ND	ND	ND	ND	ND
	White wine*	ND	ND	ND	ND	ND	ND
Consumer products	Body wash	0.49	0.23	0.7	0.83	0.32	0.12
	Toothpaste*	ND	ND	ND	ND	ND	ND
	Body lotion*	ND	ND	ND	ND	ND	ND
	LOD (ng/mg)	0.0035	0.025	0.014	0.003	0.03	0.001
	LOQ (ng/mg)	0.02	0.1	0.05	0.02	0.1	0.01

Summary of target peptides 1–6 detected and quantified in a variety of common commercially available food and consumer products.

*: expected to be gluten-free; LOD: limit of detection; LOQ: limit of quantification; ND: not detected (below LOD); D: value between the LOD and LOQ.

combination with standards and quality controls, thus allowing the proper retention times to be verified on a run to run basis.

Fig. 6 shows representative HPLC-MS/MS-extracted ion chromatograms of various gluten-containing and gluten-free native grain samples, evaluated by the LC-ESI-MS/MS assay in order to determine the presence/absence of the six target peptides. Traces shown in Fig. 6 include: (A) a solvent standard cocktail of the six target peptides; (B) corn flour; (C) rice flour; (D) wheat flour; and (E) wheat gluten. Database searches were also conducted using the National Center for Biotechnology Information (NCBI) protein database. The database searches revealed that each of the six target peptide sequences was found to be present in various varieties of wheat, but absent from non-immunogenic grains (corn and rice).

Table 4 presents the quantitative results from all the native and processed food and products analyzed with this assay. The detection of any of the six peptides represents a positive indication of the presence of wheat gluten in the sample. No particular peptide ratio profile was noted in any of the samples. All six peptides were detected in native wheat and rye flour samples. Peptides 1, 3, 4 and 6 were also detected in native barley flour. Oat and rice flour was shown to contain very low levels of two of the peptides. This could be a result of gluten contamination that occurred during the milling of the grains because most manufacturers handle many different types of grains. Oats has not been completely ruled out as a celiac-active grain. As expected, all of the wheat containing processed foods contained various concentrations of all six peptides. Interestingly, the powdered ice tea mix contained four of the seven peptides. None of the six target peptides were detected in most of the gluten-free products, with the exception of one brand of gluten free pasta and a pad thai seasoning mix. Potato vodka, rum and wine did not contain any of the peptides. As expected, beer was found to contain five of the seven peptides. Distilled products, such as gin are thought to be free of gluten, owing to the nature of the distillation process. However, this study found gin to contain a very low level of peptide 3. Vinegar and products that contain vinegar are expected to contain gluten, unless the vinegar has been distilled. The vinegar analyzed in this study was not distilled and did show the presence of four of the peptides. Hot sauce was found to contain a very low level of peptide 1. The label listed vinegar as an ingredient, but did not list the vinegar as “distilled”. The only consumer product that was found to contain any of the peptides was the body wash. Several peptides were found to have concentrations that were above the upper limit of the calibration range (100 ng/mg). These samples were diluted and re-analyzed to ensure that their results were within the calibration range.

An unexpected observation was the presence of some of the peptides in native barley and rye flour samples. Future research will aim to study these grains in depth.

4. Discussion

Increased interest, awareness and research surrounding gluten-related conditions, including celiac disease, over the last two decades has resulted in a wealth of new information, thus allowing proper diagnoses in both symptomatic and asymptomatic patients [43]. There is a growing understanding of the pathogenesis of celiac disease, new methods for screening patients and novel methodologies for analyzing gluten proteins and gluten-containing foods and products.

Nonetheless, the analysis, characterization and quantification of cereal grain gluten peptides and proteins is complicated. Protein composition, profile and natural sequence fluctuations of grains are in a constant state of flux, due to variances in cultivars, areas grown, climate and the emergence of genetic engineering. Gluten protein composition also changes during processing stages of manufacturing. Several analytical techniques, such as SDS-PAGE, capillary electrophoresis, PCR [44], RP-HPLC and mass spectrometry, have been used to study cereal grain proteins [45,46]. Mass spectrometry has only recently become involved in both genomic and proteomic areas of gluten analysis [47], MS-based methods have focused mainly on applications involving the qualitative detection and characterization of gluten proteins through the use of MALDI-TOF-MS [48,49] and LC-MS-based methods [50,51]. Research in the area of assessing the total gluten content and detecting gluten contamination in food has been traditionally done using immunochemical methods [5,20,26,27,44]. In fact, the only currently commercially available methods for determining the presence of gluten in foods are antibody-based ELISA methods. Various ELISA methods are endorsed by both the FDA and Codex Alimentarius. The practical application of each of these ELISA methods is limited [21,27], therefore, in order to accurately ensure food safety, it is imperative for manufacturers of gluten-free products, as well as governing agencies around the world, to know which method to use in each analytical application. Considering this existing controversy, a complementary analytical method to the ELISA should be well received.

Our initial work in this area utilized the analytical power and capabilities of liquid chromatography in combination with mass spectrometry, to identify a subset of the small peptides (5–50 amino acids in length, rich in proline and glutamine) that remain in the body as a result of incomplete metabolism of wheat gluten proteins (manuscript in preparation). These types of peptides have been

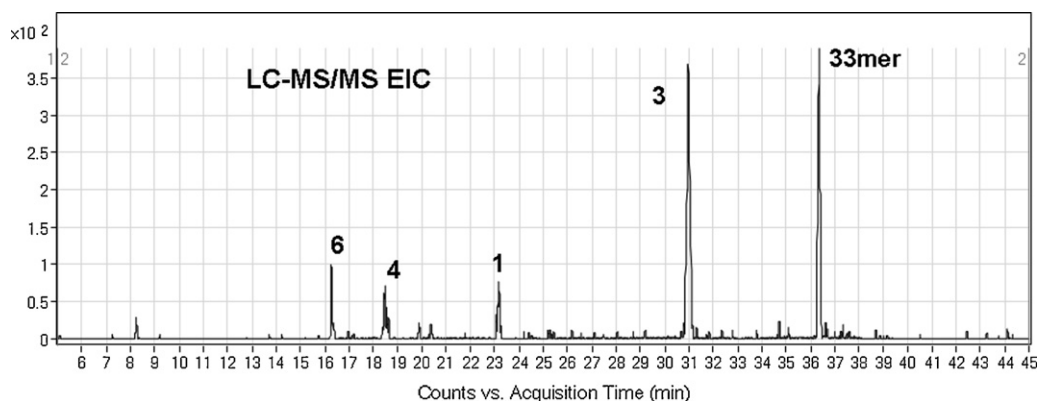


Fig. 7. A representative HPLC-MS/MS-extracted ion chromatogram of a popular powdered ice tea mix (from the USA) shows trace levels of target peptides 1, 3, 4 and 6. Another immunogenic peptide, a 33mer (LQLQPFQPQLPYQPQLPYQPQLPYQPQPF), is also identified in this sample. Gluten is not listed in as an ingredient on the product label of this product.

shown to trigger both innate and adaptive immune responses in those predisposed to celiac disease [8,30,52].

The focus of the work described here was to develop an LC–MS/MS assay, with the ability to successfully detect and quantify trace amounts of these uniquely identified immunogenic peptides. It should be noted that the emphasis of this work did not focus on determining the total gluten content in samples, nor the fingerprinting samples for a gliadin/glutenin profile, nor the identification of which exact proteins were present in samples. This novel analytical methodology is a combination enzymatic digestion–LC–MS procedure that was developed specifically to determine if wheat gluten is present, by forming, detecting and quantifying any of six immunogenic wheat gluten peptides, representative of

various varieties of wheat commonly found in a wide variety of consumer products and food (native or processed). Therefore, the presence of any of these peptides would render the product unsafe for use by people with gluten sensitivities. The data shown demonstrate that this assay can detect and quantify select immunogenic wheat gluten peptides, in a variety of food and consumer products, down to the low ppb (part per billion) level. In comparison, ELISA methods (currently endorsed by the FDA and Codex for the quantification of trace levels of wheat gluten) can at best and with limited applications, quantify down to low ppm range [26,27,39]. Therefore, this LC–MS assay is currently the most comprehensive, sensitive and versatile assay available. Given that the Codex standard for gluten content in a food product is 20 ppm (or 1 mg/50 g

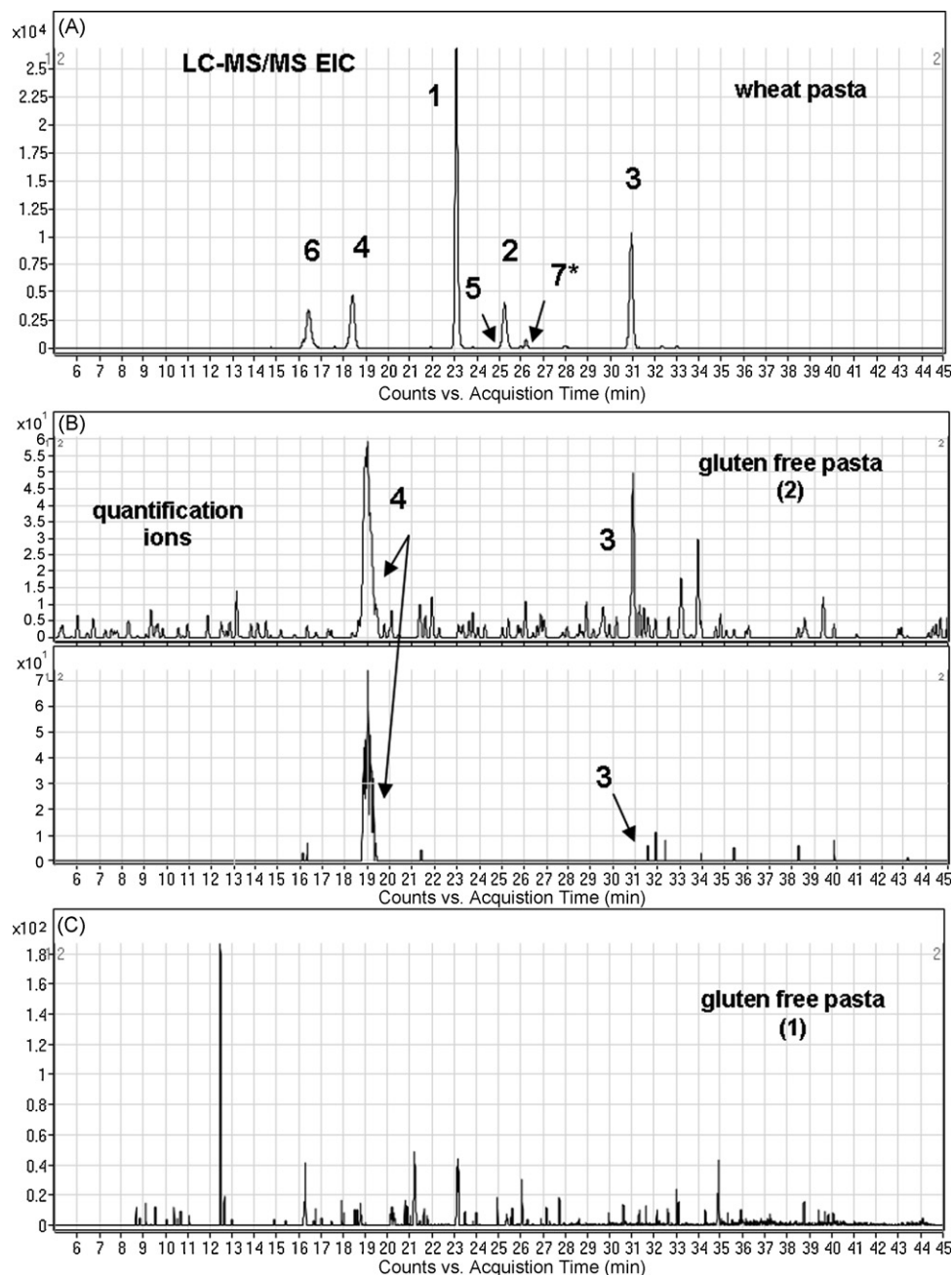


Fig. 8. HPLC–MS/MS-extracted ion chromatograms of three pasta products: (A) the target peptide profile from a regular pasta product (from Italy); (B) MRM chromatograms of peptide 4 for the quantifying ion (m/z 407.3 Da) on the top and the confirming ion (m/z 1221.8 Da) on the bottom in a gluten-free pasta product (from Italy) that was manufactured in a facility that also handles wheat and (C) no wheat target peptides are present in this gluten-free pasta (from the USA) manufactured in a gluten-free dedicated facility.

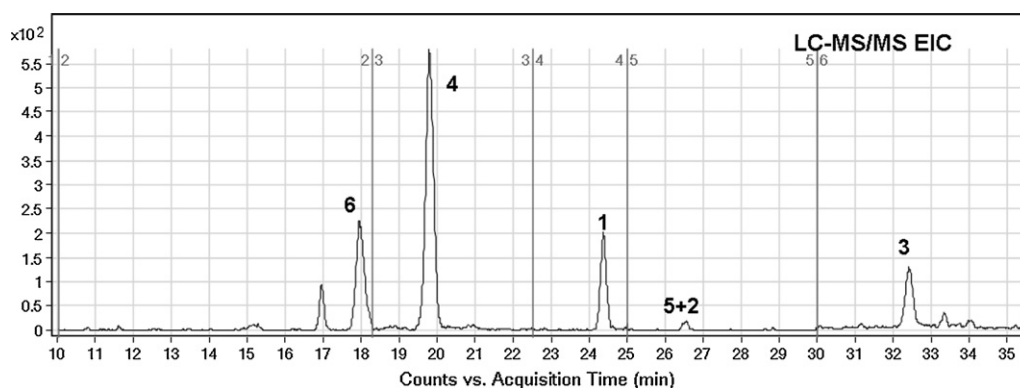


Fig. 9. HPLC-MS/MS-extracted ion chromatogram of a body wash product (from the USA) showing that target peptides 1, 2, 3, 4, 5 and 6 are present.

food), this assay can confirm the presence of wheat gluten well below this, down to 0.5 µg/50 g (corresponding to approximately 10 ppb) of food.

A unique ability of this novel methodology allows the detection of peptides that originate from both monomeric gliadins and the polymeric glutenins, because the sample is digested directly without alcohol extraction and reduction of disulfide bonds. This is a major advantage over the ELISA methodology. ELISA methods use an alcohol pre-extraction step in order to separate the alcohol-soluble monomeric prolamins [17,39], then detect them using immunochemistry. Intact glutenins are polymeric proteins and do not dissolve in aqueous alcohol. Neither do hydrolyzed nor denatured gluten proteins that come about from heat processing. This is one of the limitations encountered with some ELISA methods, which can lead to inaccurate quantitative results and calculated total gluten content in food [21,26,27]. The direct proteolysis approach provides additional advantages over ELISA methodologies in that it is a more streamlined procedure, reducing the total analysis time as well as the amount of reagents required.

The ability to detect and ultimately quantify any of the six immunogenic wheat peptides, provides an excellent indication of trace levels of gluten (from gliadins and/or glutenins) that are present, due to possible cross-contamination in ingredient handling, transporting or equipment maintenance procedures, hidden or unlabeled gluten in a product, or possibly the incomplete

removal of gluten during processing. Three of these conditions can be illustrated from data obtained in this study. [Fig. 7](#) shows a representative HPLC–MS/MS-extracted ion chromatogram of a popular powdered ice tea mix (manufactured in the USA), revealing the presence of trace levels of wheat gluten peptides 1, 2, 3 and 6. “Wheat gluten” (or gluten referenced by any number of alternate names) is absent from the ingredient list for this product. It is noteworthy to report that a trace level of another immunogenic peptide was identified in this particular sample. The last peak in [Fig. 7](#) was identified as a 33mer wheat gluten peptide, a known exceptionally immunoreactive peptide (LQLQPF-PQPQLPYQPQLPYQPQLPYQPQPF) from α -2 gliadin [\[29\]](#). Future work will optimize the LC–MS/MS assay to include this important immunogenic gluten peptide in its list of target peptides.

Another example of the unexpected determination of trace levels of gluten is shown in Fig. 8. Fig. 8(A)–(C) is HPLC–MS/MS-extracted ion chromatograms of various pasta products: (A) wheat pasta; (B) and (C) are two different brands of “gluten-free” labeled pasta. The wheat pasta, shown in Fig. 8(A) contains all six target peptides. Fig. 8(B) shows the results for one “gluten-free” labeled pasta product. The upper trace in Fig. 8(B) shows the primary MRM parent to product transition ions, those used for quantification purposes. The bottom trace in Fig. 8(B) shows the parent to product MRM transition ions that are used for confirmation purposes. It can be seen that a trace level of target peptide 4, along with a small

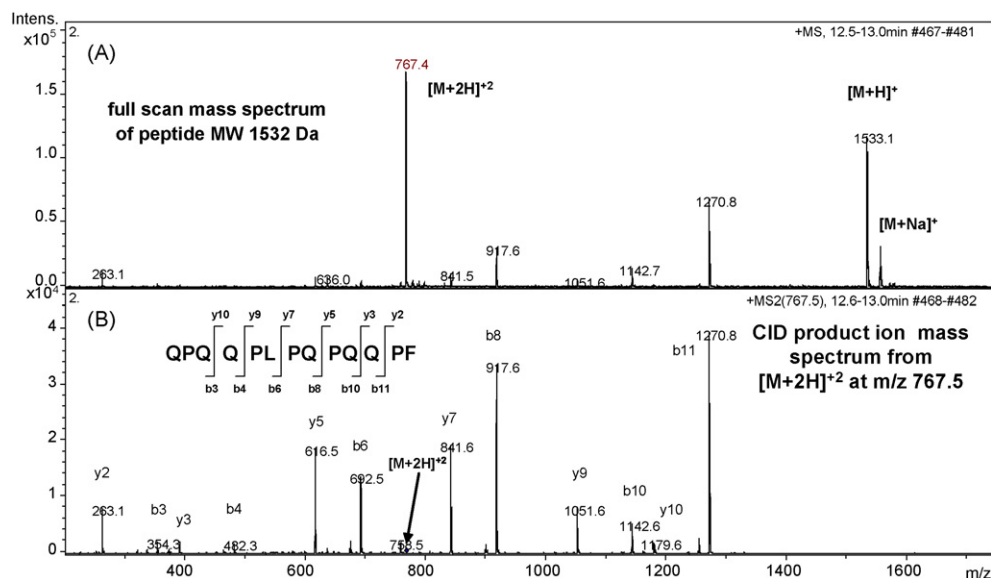


Fig. 10. Ion trap-MS/MS data showing sequence and structural information about newly identified peptide 7 (QPQQPLPQQPF): (A) full scan mass spectrum of the peptide and (B) CID product ion mass spectrum. This target peptide has been detected in other samples and is presented in some of the other figures, labeled as peptide 7*.

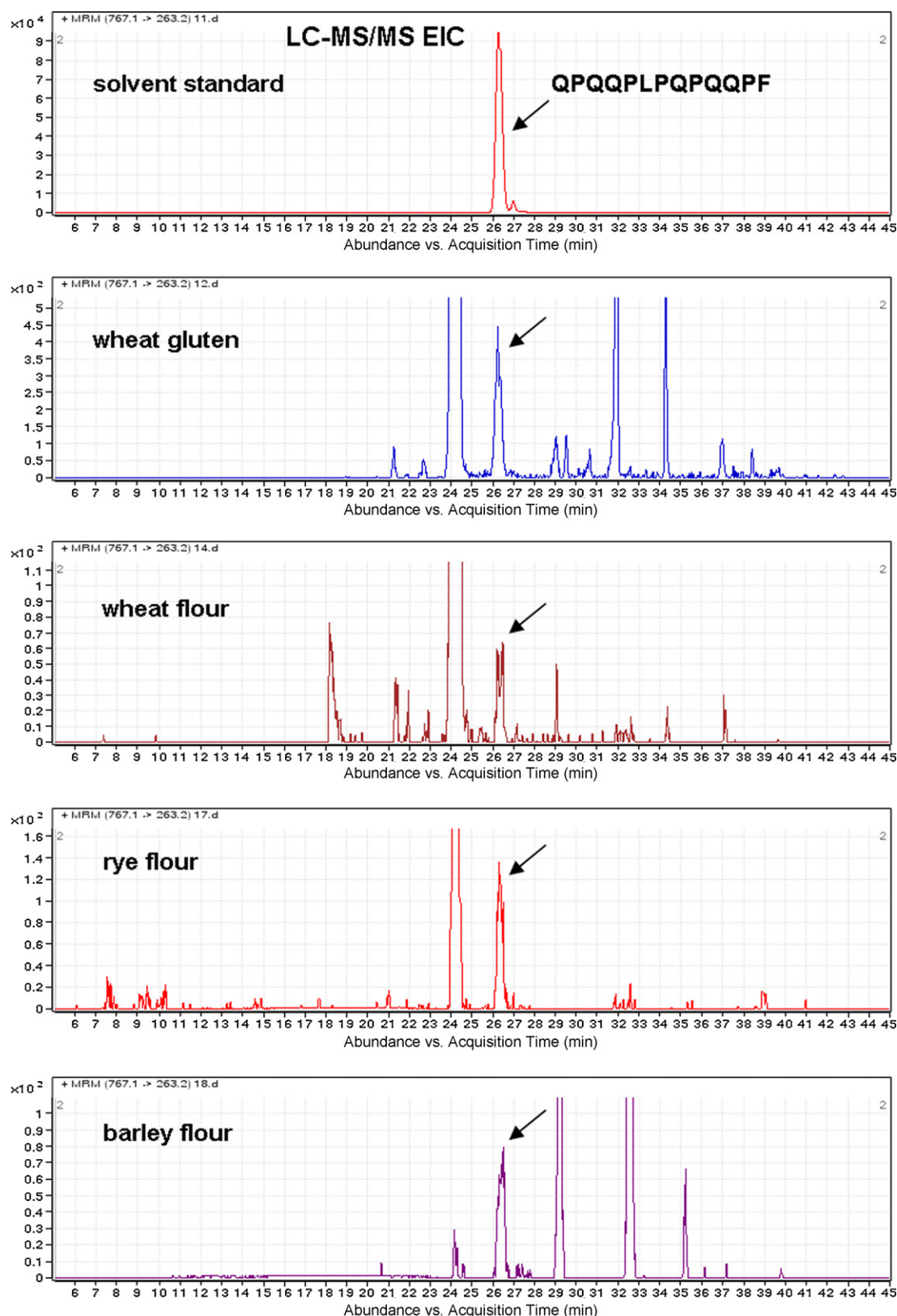


Fig. 11. HPLC–QQQ–MS/MS–extracted ion chromatogram of peptide 7 (QPQQPLPQPQQPF) monitoring the MRM transition m/z 767.1 (+2)–263.2 Da, which has been identified in native proteolyzed wheat, barley and rye flours. Preliminary data about peptide 7 has detected it in other samples and as such it has been included in other figures, labeled as peptide 7*.

detected peak corresponding to peptide 3, can be seen in the product (manufactured in Italy). However, only by looking at the lower part of Fig. 8(B), it can be seen that the confirming transition ion for peptide 4 is present, but is absent for peptide 3. Therefore, a positive

identification of peptide 3 in this product cannot be made. Interestingly, this product is labeled “gluten-free”, however, its package also displays a disclaimer that the product was manufactured in a facility that also manufactures products containing wheat. The

other “gluten-free” labeled pasta, shown in Fig. 8(C) shows no trace of wheat gluten contamination. As noted from this product’s package labeling, this product was manufactured in a facility dedicated only to gluten-free processing.

It was especially interesting to note, that for MRM transitions where the product is at a higher m/z value than the precursor ion, noise was significantly reduced. This has important connotations when performing trace component analysis, such as contamination in food. This condition happened to be applicable for the MRM transitions for three of our peptides. The m/z of the singly charged confirming product ion for peptides 1, 2 and 4 was above that of the doubly charged precursor ion. While the MS-intensity of these product ions (above the precursor m/z) was lower than that of other product ions (below the precursor m/z), the level of noise for the transitions was 5–7 times lower. Therefore, the signal to noise (S/N) ratio was ultimately higher, allowing much better sensitivity. This indicates a real advantage in choosing product ions above the multiply charged precursor ion, whenever possible. An example of this situation is seen in the bottom part of Fig. 8(B), where the EIC of the confirming ion for peptide 4 (m/z 1221.8) shows a very low level of noise.

Fig. 9 shows the profile of target peptides present in a dermatologist recommended daily moisturizing body wash. Peptides 1, 3, 4 and 6 can be seen clearly, along with a small contribution from peptides 2 and 5. Although hydrolyzed wheat protein is listed as an ingredient, this example is one where exposure to wheat may not be obvious. These examples provide much needed evidence as to how important it is to ascertain the presence of trace levels of wheat gluten in commercially available food and consumer products. From the consumer’s perspective, it is perhaps not evident that wheat gluten is present in these products, but our LC–MS/MS assay provides evidence that it could be unhealthy for a person with celiac disease, or any other form of gluten sensitivity, to be exposed to these products. From a manufacturing perspective, the abilities of this LC–MS assay provide an effective tool to ensure food safety and permit compliance with worldwide food labeling guidelines.

Future work will continue to attempt to identify more immunogenic wheat peptides (from both gliadins and glutelins) and to study rye and barley grains in similar detail. Our interest lies in the identification of peptides, some common to all grains and some unique to individual grains, in order to form an even more comprehensive list of marker peptides for use with this LC–MS/MS assay. Initial data, shown here, with native rye and barley grains has indicated that some of the six marker peptides identified in wheat have been observed in barley and rye flour samples. Future work will involve further sample analyses of food containing these grains and utilize relevant protein database searching to determine if these sequences are present in the barley and rye proteomes. Recent work has also identified one additional peptide sequence that has been observed in samples of all three celiac-active grain flours (wheat, barley and rye) and appears to be more sensitive in rye and barley flour samples than some of the other six target peptides identified to date. Fig. 10 shows ion trap-MS/MS spectra showing structural and sequence information about this peptide (PQQPLPQQPFF). Fig. 11 shows HPLC–QQQ-MS/MS-extracted ion chromatograms of this peptide (termed “peptide 7”) indicating its presence in native wheat, rye and barley flour samples.

5. Conclusion

High performance liquid chromatography–mass spectrometry is a powerful analytical technique for use in the analysis of food proteins. A rapid, sensitive and specific analytical LC–MS/MS method has been developed for the quantitative detection of immunogenic gluten marker peptides from complex mixtures. This versatile

methodology allows both cooked and native food to be analyzed for the presence of wheat gluten.

LC–MS/MS was used to both detect and quantify the marker peptides in enzymatically digested food samples using conditions and enzymes that model the human digestion system. This is a refreshing new approach, complimentary to the traditional immunochemical methods for the detection of gluten in food. Continued efforts in this area will hopefully pave the way for eventual commercial application, as a service to both the celiac community and the food industry, by providing an accurate and economic means to generate much needed data for researchers developing treatments for patients with gluten sensitivities and manufacturers producing and labeling products that are safe.

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