

Development and Validation of the Detection Method for Wheat and Barley Glutens Using Mass Spectrometry in Processed Foods

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Abstract Currently, the only effective treatment for coeliac disease is a gluten-free (GF) diet to avoid gluten-containing food intake. However, the current enzyme-linked immunosorbent assay (ELISA) method for detecting gluten lacks the ability to precisely detect and quantify gluten in fermented and hydrolyzed foods. The purpose of this study was to use liquid chromatography-tandem mass spectrometry (LC-MS/MS) to develop a robust and stable method for gliadin and hordein detection when assessing food safety. This study successfully extracted hordein from barley flour to use as a reference standard. Liquid chromatography/quadrupole time-of-flight mass spectrometer (LC/Q-TOF/MS) performed an

analysis to identify unique marker peptides from gliadin and hordein. For determining the limit of detection of gluten in GF products, gliadin and hordein were spiked into GF products at concentrations of 1–100 mg/kg with isotope-labeled peptides as internal standards (IS). The developed method enabled the detection of 2.5 mg/kg of wheat and barley gluten in GF products. Finally, compared with the immunochemical method, the ability for the MS-based method to detect and quantify gluten content from fermented and hydrolyzed foods was unaffected and demonstrated the potential to analyze suspected gluten-contaminated foods to assess product safety.

Keywords Celiac disease · Gluten-free · Liquid chromatography-mass spectrometry · Wheat · Gliadin · Barley · Hordein · Prolamins

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Introduction

Gluten is a storage protein present in the endosperm of cereals like wheat, barley, rye, and oat (Shewry et al. 2002; Commission Regulation 2009) and is a cross-linked protein comprising prolamins, monomeric, alcohol-soluble protein and glutelins, polymeric, and alcohol-insoluble protein (Wieser 2007). The cereal prolamins are namely gliadin in wheat, hordein in barley, secalin in rye, and avenin in oat. All prolamins contain high amounts of glutamine and proline but contain few basic amino acids, such as lysine (Shewry et al. 1992). Although starch is one of major compounds to supply calories in cereals, gluten proteins from these cereals can trigger three pathologies: (1) coeliac disease (CD), an autoimmune disorder of the small intestine affecting about 1% of the world's population, specifically for the Occident (Green et al. 2003; Abadie et al. 2011; Bernardo and Peña 2012); (2) food allergies, which affects a smaller group of

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people (about 0.2–0.5%) caused by IgE-mediated allergic responses that can be critical (Snégaroff et al. 2006; Zuidmeer et al. 2008); and (3) gluten sensitivity, a gluten intolerance that affects a larger group, but is largely unreported (Sapone et al. 2011; Biesiekierski et al. 2011). To avoid carelessly gluten-contained food intake, the GF food regulation is necessary (Ciclitira et al. 2005). Therefore, the improved sensitivity of analytical methods to detect gluten is imperative.

Several countries have established rules for GF food labeling that specifically inform tolerance standards for gluten compared to other requirements for food allergen labeling. According to the Codex Alimentarius Standards 118-1979 (revised 2015) adopted by the World Health Organization standard for GF foods, GF foods are defined as dietary foods consisting of ingredients that do not contain gluten at a level that exceeds 20 mg/kg (Codex Alimentarius Commission 2015). The US Food and Drug Administration (FDA) and European Commission also consider the Codex standard (Commission Regulation 2009; U.S FDA 2013). Although oats, like other gluten-containing cereals, belong to celiacogenic cereals, recent research has shown that gluten from oats (avenins) contains lower proline and glutamine than gliadin, hordein, secalin, or their crossbred varieties; therefore, oats do not trigger or induce an autoimmune response with CD (Comino et al. 2011). As a result, if not contaminated with other seeds, oats have been removed from gluten-containing grains in USA (U.S FDA 2013). However, in Europe country, oats still classed as gluten-containing grains (Commission Regulation 2009).

Many methods have been developed to detect gluten based on genomic and proteomic approaches, as well as immunological tests (Denery-Papini et al. 1999; Skylas et al. 2000; Sandberg et al. 2003). ELISA is currently the most common approach used to detect gluten in food. However, ELISA is unable to identify the cereal species, and recent research indicates that ELISA does not precisely detect and quantify gluten in fermented and hydrolyzed foods owing to a lack of proper reference material (Diaz-Amigo and Popping 2013). Efficiency of the ELISA antibodies is also affected by probable loss of immunoreactive epitopes of gluten after food processing, which could lead to inaccurate estimates of total gluten content (Kanerva et al. 2011; Diaz-Amigo and Popping 2012). Recently, mass spectrometry (MS) has been widely used to analyze biological samples for proteomic research. There are two ways for protein identification in proteomic: top-down and bottom-up method. Bottom-up method is based on detecting the specific peptides which are digested from targeted proteins (Aebersold and Mann 2003; Chait 2006); in fact, several studies have used MS-based methods to analyze gluten (Sealey-Voyksner et al. 2010; Simonato et al. 2011; Fiedler et al. 2014). In addition to discriminating species, the advantages of MS-based methods include their ability to overcome antibody cross-reactivity and loss of gluten

epitopes where the ELISA-based method is otherwise limited. Nevertheless, the sensitivity of MS-based methods is often affected by the complex matrix (Trufelli et al. 2011), and the ability to quantitate proteins could be restricted by a lack of standardized reference material.

The purpose of this study was to develop a simple and stable method for gliadin and hordein detection. The first approach of this study aimed to extract hordein from barley flour as a reference standard. Our second approach was to detect unique marker peptides from gliadin and hordein used to determine the detection limit of gluten in GF products. The last approach compared the results of the LC-MS/MS-based method against measurements from the ELISA method.

Material and Methods

Chemicals and Materials

Gliadin from wheat (powder), acetone, ethyl alcohol, urea, ammonium bicarbonate, Bradford reagent, dithiothreitol (DTT), iodoacetamide (IAA), trifluoroacetic acid (TFA), trypsin IX (trypsin), and formic acid (FA) were purchased from Sigma-Aldrich (St. Louis, USA). Acetonitrile (ACN) was obtained from Avantor Performance Materials (Center Valley, USA) and water from Scharlau (Barcelona, Spain). To confirm if the GF products were gluten-free, ELISA analysis was performed using RIDASCREEN® Gliadin (R-Biopharm, Darmstadt, Germany). The isotope-labeled IS peptides (N-LWQIPEQS(R)-C/R: 13C, 15N labeled; N-QQCCQLANINEQS(R)-C/Q: iodoacetamide modification/R: 13C, 15N labeled) were synthesized from Mission Biotech (Taipei, Taiwan) with a purity of approximately 95% according to the supplier. Unprocessed raw materials (seeds, flour), processed foods (beers, cookies, beverages), and GF products (GF flour, corn flour, apple wine, rice wine) were obtained from local markets.

Gliadin and Hordein Extraction/Purification

The gliadin extraction procedure was adopted from previous study (Tatham et al. 2000), and the final amount of gliadin in the Sigma gliadin powder was calculated with the correction factor of 0.4 following previous study (Manfredi et al. 2015). The gliadin stock solution was prepared by dissolving an aliquot of Sigma gliadin powder in 60% aqueous ethanol solution (EtOH), shaking vigorously at room temperature (RT) for 2 h, and centrifuging at 1900×g for 10 min, after which the supernatant was collected and stored frozen (−20 °C).

Hordeins were extracted from 1 g of whole grain barley flour. The extraction procedure was the same as for gliadin. The soluble crude protein was then dried in a centrifugal evaporator (Labconco, Kansas City, USA) at 65 °C, re-suspended with 1 mL of 6 M urea and 13 µL of neat glacial acetic acid

(final concentration 1.0%), and centrifuged at $18,000\times g$ for 10 min, after which the supernatant was filtered on a 0.2- μm polytetrafluoroethylene membrane (Thermo Fisher Scientific, Waltham, USA). Urea soluble proteins were analyzed on a HPLC using an Agilent chromatography system (1260 Infinity; Agilent Technologies, USA) with OpenLAB CDS ChemStation software (Agilent Technologies, Foster City, USA). For each fraction, 100 μL was injected onto a mRP Hi-Recovery Protein Column (2.1×75 mm; Agilent Technologies, Foster City, USA) at 25 °C using an isocratic gradient at 30% B for 2 min at 0.5 mL/min (solvent A, 0.1% TFA in water; solvent B, 0.1% TFA in ACN) and eluted with a linear gradient from 30 to 60% B over 8.4 min, followed by 5 min of column re-equilibration. Column effluent was monitored at 280 nm (Shahbazi et al. 2016). The fractions were collected, dried in a centrifugal evaporator, and dissolved in 1 mL of 60% EtOH.

Sample Preparation for the Spiking Experiments

The 100 mg/kg of gluten in GF products was prepared using 50 μg of gluten protein and 0.5 g of the GF product. This method was repeated dilution for the preparation of gluten in GF products at 80, 40, 20, 10, 5, 2.5, 1.25, and 1 mg/kg.

Sample (Standard Proteins and Food Matrices) Digestion

An aliquot of each protein extraction solution was dried in a centrifugal evaporator and re-suspended with 1 mL of 8 M urea in 50 mM (mmol/L) ammonium bicarbonate (pH 8.0), and 10 μL of 1 mM (mol/L) DTT (final concentration 10 mmol/L) was added for the reduction reaction at RT for 60 min. For the alkylation reaction, 30 mM (mmol/L) IAA were then added at 37 °C for 30 min in the dark. After alkylation, 8 mL of 50 mM (mmol/L) ammonium bicarbonate (pH 8.0) was added to dilute the urea to less than 1 M (mol/L). The proteins were digested by trypsin IX (1:100 enzyme-to-protein ratio at 37 °C; Sigma-Aldrich, St. Louis, USA) overnight (approximately 18 h); then, tryptic digestion was stopped by acidifying with folic acid (FA) to a final concentration of 5%. The resulting peptides were desalted using Oasis HLB (500 mg; Waters, USA) according to the manufacturer's instructions, after which the desalted peptide solution was dried in a centrifugal evaporator and dissolved in 300 μL of aqueous solution containing 0.1% FA and 5% ACN for qualitative analysis. The peptide solutions were diluted 1:1 with the IS peptides in 0.1% FA and 5% ACN for reproducibility and quantitative analysis.

Protein and Peptide Identifications

The peptide mixtures were analyzed by an Agilent 1290 Infinity LC system equipped with an Agilent 6530 Accurate-

Mass Q-TOF MS system, which was electrosprayed using Agilent Jet Stream dual electrospray source (AJI ESI). The MassHunter Workstation Acquisition software was used to control the whole system (Agilent Technologies, Foster City, USA). Aliquots of 20 μL of each extract were injected, and peptides separated on a Polaris 3 C18 column (3- μm particle size, 2.0×150 mm; Agilent Technologies) using a ACN gradient of 5 to 60% B over 50 min at 40 °C; the flow rate was maintained at 0.3 mL/min. The ESI parameters were set as follows: capillary voltage at 4.0 kV, nozzle voltage of 0 V, nebulizer pressure at 35 lb per square inch (psi), drying gas 8 L/min of nitrogen, and gas temperature of 300 °C. For the TOF analyzer parameters, fragmentor voltage was set at 175 V, skimmer voltage at 65 V, and OCT 1 RF Vpp at 750 V. For the peptide mass analysis, the auto-MS/MS mode was used with a ramped collision energy (CE) slope of 3.1 and offset of 1 with charge 2^+ , a slope of 3.6, and offset of -4.8 with charge 3^+ or more. The mass range of the MS spectra was acquired in the 285–1700 m/z range, and the MS/MS spectra were acquired in the 100–1700 m/z range. The data were collected by auto-MS/MS acquisition with an MS acquisition rate of three spectra per second and MS/MS acquisition rate of two spectra per second. The instrument was calibrated using the Agilent tuning mix HP0321 (Agilent Technologies, Foster City, USA) in acetonitrile; continuous internal calibration used the following reference masses: 121.0509 and 922.0098 m/z (Agilent Technologies, Foster City, USA).

The information of peptides were extracted from raw data and identified using the Spectrum Mill MS proteomics workbench against the National Center for Biotechnology Information (NCBI) nonredundant (nr) protein sequence database. The parameters of search were as follows: mass tolerance window of 20 ppm for the precursor ions and 50 ppm for the produce ions (daughter ions), trypsin digestion no more than two miscleavages, carbamidomethylation of cysteine as a type of fixed modification, the scores greater than 10, and the percentage scored peak intensities (% SPI) greater than 80% of the database spectrum.

LC-MRM-Based Quantitative Detection of Marker Peptides

The peptide mixtures were resolved using 5% ACN contained 0.1% FA and analyzed by an Agilent 1290 Infinity LC system equipped with an Agilent 6490 Triple Quad MS system, which was electrosprayed using AJI ESI. The MassHunter Workstation Acquisition software was used to control the whole system (Agilent Technologies, Foster City, USA). The peptide separations were performed on a CSHTM 130 C18 column (1.7- μm particle size, 2.1×150 mm; Waters, Milford, USA) used with a column temperature of 40 °C. Measurements were performed in ESI+ dynamic multiple reaction monitoring (MRM). Twenty microliters of samples

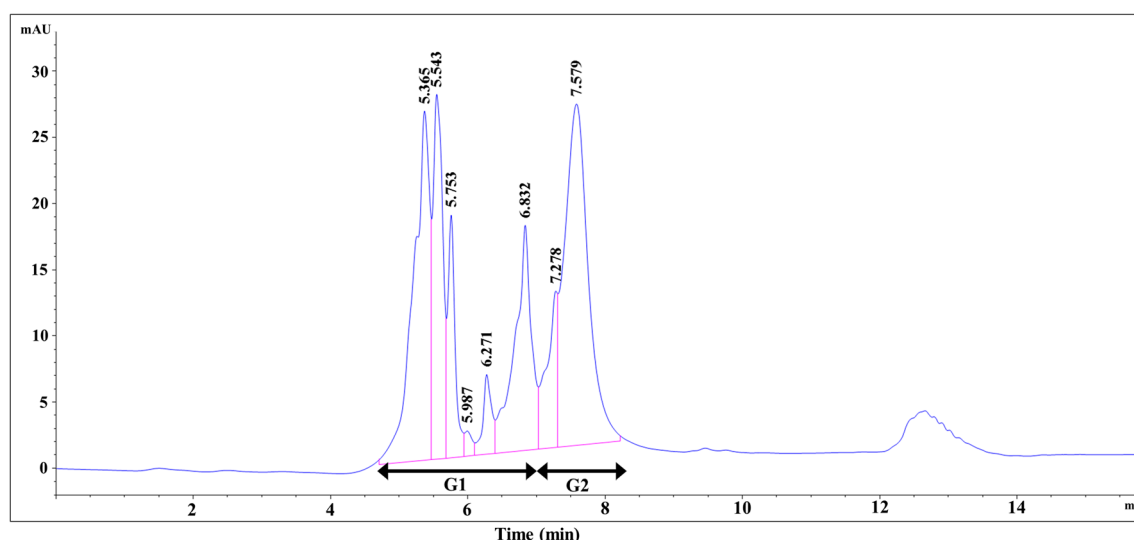


Fig. 1 HPLC chromatogram of barley proteins was divided into two main parts. One hundred microliter extraction solution was injected onto a mRP Hi-Recovery Protein Column (2.1×75 mm), and the proteins were eluted with a linear gradient. Column effluent was

monitored at 280 nm. The barley proteins were divided into two main parts: alpha-amylase/trypsin and chymotrypsin inhibitor (*G1*) and hordeins (*G2*)

were injected at a flow rate of 0.3 mL/min. The mobile phases A and B were the same as above. The LC of ACN gradient was performed of 5% B at 0 min, 15.5% B at 9.5 min, 20% B at 9.6 min, 47% B at 14 min, 100% B at 15 min, and post time for 8 min.

ESI parameters were as follows: gas temperature 250 °C, gas flow 15 L/min, nebulizer 30 psi, sheath gas heater 300 °C, sheath gas flow 11 L/min, and the capillary voltage was 4000 V. Dwell time was 20 ms/transition; the fragmentation voltage was set to 380 V, and optimized CE voltage of each peptide was measured by Optimizer software (Agilent Technologies, Foster City, USA). The MRM transitions were chosen for each peptide, as the double or triple-charged precursor ions paired with their resulting y fragment ions. From the product ion mass spectrum of each precursor ion, three of the most abundant product ions were selected.

MRM Data Analysis

MRM data were processed and visualized with MassHunter quantitative analysis software (Agilent Technologies, Foster

City, USA) and Skyline Software Systems (Skyline, Herndon, USA). The eight-point standard curves were prepared with a $1/x$ weighting. The calibration curve was established according to the measured analyte-to-IS peak area ratios of calibration standard samples. The R-squared values should be over 0.99. The qualification criteria followed FDA guidelines (U.S. Food and Drug Administration 2013); coefficient of variation (CV) for each quantitative peptide in GF matrices was evaluated at three concentration levels: the limit of quantification (LOQ) and two higher concentration levels (10 and 40 mg/kg) that should be below 15%, and accuracy (% (mean measured concentration/nominal concentration) $\times 100\%$) was 80 to 120%. The limit of detection (LOD) was based on a signal to noise (S/N) ratio of 3 to 1 and LOQ on 10 to 1.

Quantitative Analysis of ELISA and LC-MRM-Based Methods

The gluten content of 12 food samples were tested using both the RIDASCREEN® Gliadin ELISA (AOAC Performance

Table 1 Q-TOF LC/MS characteristic of barley protein fractions for HPLC

Group	Fractions	No. of matched peptides (% sequence coverage)	Score	Accession no. ^a	Protein name	Mw
G1	F1–F6	5 (43)	93.81	225,172	Alpha-amylase/trypsin inhibitor	20,107.1
		4 (73.4)	73.40	124,125	Subtilisin-chymotrypsin inhibitor CI-1A	8882.1
G2	F7–F8	5 (26.6)	93.22	224,385	Hordein B	30,863.6
		2 (9.3)	37.93	1,708,280	Gamma-hordein-3	33,644.6

^a The proteomics workbench was against the National Center for Biotechnology Information (NCBI) nonredundant (nr) protein sequence database

Table 2 Number of gliadin and hordein peptides identified with trypsin

Prolamins	Protein class	Peptide count
Gliadin	Alpha-gliadin	8
	Gamma-gliadin	20
Hordeins	B-hordein	8
	Gamma-hordein	3

Tested Method 120601, R-Biopharm AG, Darmstadt, Germany) and LC-MRM-based methods. ELISA analysis was performed according to the manufacturer's instruction. For LC-MS/MS analysis, 0.5 g or 0.5 mL of food products was prepared from a sufficient amount of sample that was at least 5 g or 5 mL; then, the extraction procedure and MRM analysis were followed by the abovementioned procedures. The concentration were calibrated by the derived equation.

Results and Discussion

HPLC Fractionation and LC/Q-TOF/MS for Analysis of Barley Prolamin (Hordeins)

Unlike gliadin, the hordein proteins were prepared by self-extracting due to lack of a commercial standard. Hordeins were extracted with 60% EtOH from 1 g of whole grain barley flour, and the extracts were, respectively, separated into eight HPLC fractions shown in Fig. 1. Barley fractions were collected after their following retention times: 4.7–5.5, 5.5–5.7, 5.7–5.9, 5.9–6.1, 6.1–6.4, 6.4–7.0, 7.0–7.3, and 7.3–8.2 min. For protein identification, each HPLC fraction was then analyzed by LC/Q-TOF/MS. The identification of major proteins for each fraction and their molecular masses are listed in Table 1. The resulting barley fractions can be divided into two groups. Although numbers of peak were detected in the mass spectrum of each fraction, only the peptides in G2 group pertain to hordein proteins. Therefore, the G2 group was collected as the standard protein for hordeins. Due to the

Table 3 List of the MS condition of the selected peptides for each prolamin

Peptide code	Peptide sequence	Protein	Precursor ion, <i>m/z</i>	Charge state	Product ion, <i>m/z</i>	Collision energy, V	Retention time, min
W1	DVVLQQHNIAHGR	α-Gliadin	496.3	+3	636.9 (y11 ⁺²) 587.3 (y10 ⁺²) 424.9 (y11 ⁺³)	17 13 9	3.9
W2	LWQIPEQSR ^a	α-Gliadin	578.8	+2	857.4 (y7) 729.4 (y6) 616.3 (y5)	17 21 13	11.2
W3	APFASIVAGIGGQ (Srinivasan et al. 2015)	γ-Gliadin	594.3	+2	502.3 (y6) 431.2 (y5) 204.1 (y2)	9 9 21	12.6
W4	APFASIVADIGGQ (Altenbach et al. 2010)	γ-Gliadin	623.3	+2	560.3 (y6) 261.1 (y3) 204.1 (y2)	9 13 13	12.8
W5	SDCQVMQQCCQQLAQIPR (Simonato et al. 2011)	γ-Gliadin	793.4	+3	697.4 (y6) 385.3 (y3)	9 17	13.3
B1	ILPFGIDTR	B-hordein	516.3	+2	805.4 (y7) 561.3 (y5) 276.2 (y2)	10 10 10	11.4
B2	VFLQQQCSPVR	B-hordein	681.4	+2	1115.6 (y9) 1002.5 (y8) 874.4 (y7)	22 22 22	7.6
B3	VFLQQQCSPVPVQR (Guo et al. 2016)	B-hordein	892	+2	792.5 (y7) 596.4 (y5) 400.2 (y3)	29 29 29	9.4
B4	APFVGVTGVGGQ (Tanner et al. 2016)	γ-Hordein	594.3	+2	617.3 (y7) 518.3 (y6) 261.1 (y3)	10 10 10	11.7
B5	EFLQQTDLDEK	γ-Hordein	762.4	+2	1134.5 (y9) 1021.5 (y8) 893.4 (y7)	25 25 25	10.4
B6	QCCQQLANINEQSR ^a (Tanner et al. 2016)	γ-Hordein	626.3	+3	860.4 (y7) 746.4 (y6) 633.3 (y5)	10 10 10	6.3

^a Peptides were chosen for quantitative purposes

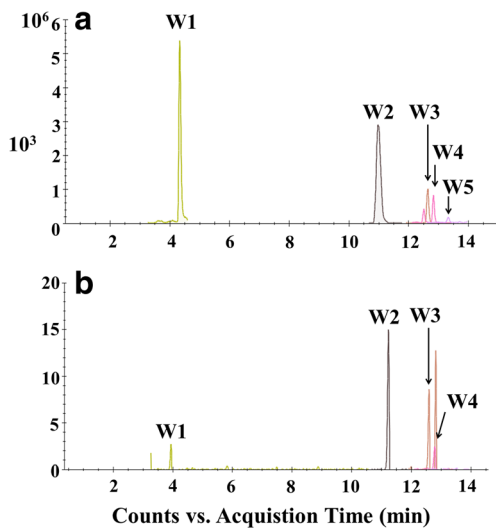


Fig. 2 LC-MS/MS extracted chromatograms of the gliadin marker peptides. The proteins in **a** wheat flour and **b** beer containing wheat and barley were analyzed by LC/MS/MS. The chromatograms of the gliadin marker peptides were extracted. Peptides W1 and W2 belonged to α -gliadin, and peptides W3 to W5 belonged to γ -gliadin

uncertain ability of the commercial ELISA kit to accurately quantify the hordein content (Kanerva et al. 2006; Tanner et al. 2013b; Colgrave et al. 2014), hordein protein concentrations were measured using a BCA Protein Assay Reagent Kit (Sigma-Aldrich, St. Louis, USA).

Optimized Selection of Specific Peptide for Gliadin and Hordein Proteins

The gliadin and hordein proteins were digested by trypsin and identified by LC/Q-TOF/MS. These data were searched using the NCBI nr protein sequence database on Spectra Mill software. All 39 peptides were identified from each prolamin family listed in Tables 2 and S1. Detected peptide counts were lower compared to other reports that used another enzyme (Fiedler et al. 2014), such as chymotrypsin, or two or three enzymes (Allred et al. 2014; Manfredi et al. 2015) that increased the cleavage site positions when detecting more peptides. Despite using just one common enzyme in this study, it still detected several peptides that are unique to gliadin or hordein. The details of identified prolamin peptides with sequence are presented as the Supporting Information (Table S1).

In these results, only B-hordein and γ -hordein peptides were identified, but not any peptide for C-hordein or D-hordein. Although C-hordein is one of the most components in barley, the trypsin cleavage sites of C-hordein are lower than B-hordein and γ -hordein. Most peptides of C-hordein cleaved by trypsin are too long to analyze. Furthermore, the contents of D-hordein in barley are far lower than B-hordein and C-hordein (Colgrave et al. 2012; Balakireva and Zamyatnin 2016). This could be the reason why specific

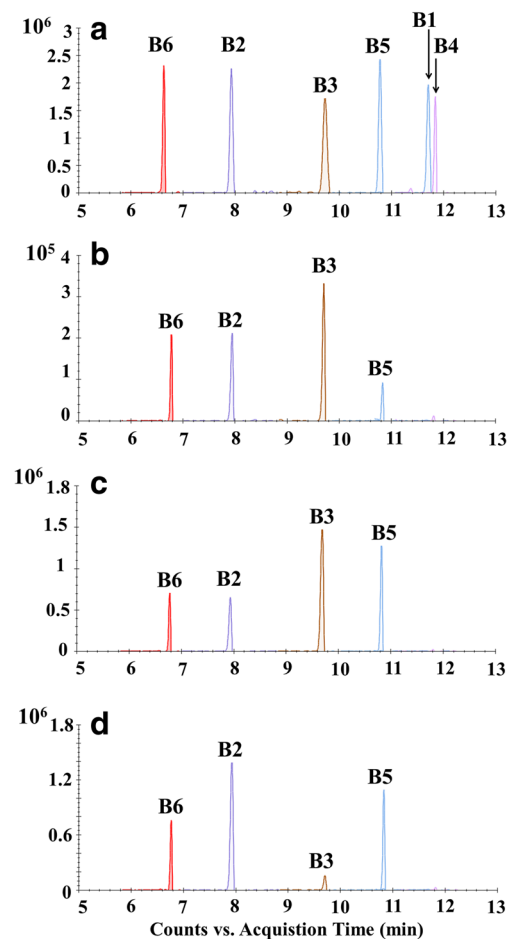


Fig. 3 LC-MS/MS extracted chromatograms of the hordein marker peptides. The proteins in **a** barley flour, **b** malt beverage, **c** beer, and **d** beer containing wheat and barley were analyzed by LC/MS/MS. The chromatograms of the hordein marker peptides were extracted. Peptides B1 to B3 belonged to B-hordein, and peptides B4 to B6 belonged to γ -hordein

peptides cannot be found in C-hordein and D-hordein by using LC/Q-TOF/MS. Furthermore, it is known that food processing could cause protein and DNA degradation. The peptides predicated by the theoretical search may not be detected in processed food. It could be better to choose the biomarker candidates without a priori (Marbaix et al. 2016). In this study, besides the identified peptides, the detection method for B-hordein and γ -hordein was established using MRM.

The specific peptides were further optimized by LC/MS/MS and were selected for MRM analysis according to the abundant peaks. The m/z values of product ions, collision energy, and retention time are shown in Table 3. For each marker peptide, two or three product ions were monitored. Peptides W1 and W2 belonged to α -gliadin, peptides W3 to W5 to γ -gliadin, peptides B1 to B3 to B-hordein, and peptide B4 to B6 to γ -hordein. The peptides W3, W4, W5, B3, B4, and B6 were among the marker peptides reported in the literature (Altenbach et al. 2010; Simonato et al. 2011; Srinivasan et al. 2015; Tanner et al. 2016; Guo et al. 2016).

Table 4 Validation of the method for quantitative peptides in GF sample

Peptide code	Peptide sequence	For 2.5 ppm		For 10 ppm, %		For 40 ppm, %		Coefficient of determination (R^2)	LOQ (ppm), $n = 5$	LOD (ppm), $n = 5$
		CVs %	Mean accuracy (%)	CVs %	Mean accuracy (%)	CVs %	Mean accuracy (%)			
W2	LWQIPEQSR	6.6	97.3	6.9	94.0	4.5	105.9	0.9980	2.5	2.5
B6	QCCQQLANINEQSR	8.0	91.8	10.4	95.9	7.3	103.3	0.9936	2.5	2.5

Unprocessed raw materials and processed food products were performed to characterize the specificity of selected peptides. In particular, the processed food products were processed by hydrolysis, cooking, and fermentation. The detected results are shown in Figs. 2, 3, and S1–S3. For raw materials, all gliadin and hordein peptides were detected except W4 peptide that was observed a peak splitting phenomenon (Figs. 2a and 3a). For processed food products, only a few peptides, W5, B1, and B4, were undetectable from the hydrolyzed or fermented products (Figs. 2b and 3b) and other specific peptides were successfully detected. The intensity variable of peptides B2, B3, and B5 in several processed foods was observed (Fig. 3a–d). Previous studies show that W3 peptide (APFASIVAGIGGQ) is one of the sequence responses for immunoglobulin A (IgA) reactivity in serum of CD patients (Srinivasan et al. 2013, 2015). This representative peptide was also considered as a biomarker for gliadin in this study. However, W3 peptide observed both matched the

sequence of γ -gliadins and a hypothetical protein (EMT15786.1) and a peak splitting phenomenon in processed food (Fig. 2b). To make sure the accuracy of our method, we did not establish the detection method of gliadin using W3 peptide. Based on the abundance, reproducibility, and stability, peptides W2 and B6 were chosen as potential peptides for quantitative analysis.

Quantitative LC/MS/MS Analysis

The marker peptides analyzed by LC/MS/MS used the conditions shown in Table 3. For quantitative analysis of gluten, gliadin or hordein proteins were spiked into GF products at concentrations of 1–100 mg/kg with IS peptides (Figs. S4–S5). The isotope-labeled IS peptides were synthesized based on quantitative peptides diluted 1:1 (v/v) with digested calibration samples (IS peptides of W2 and B6 were C-terminal [13C]/[15N]-arginine labeled, final concentration of 1 pmol/

Table 5 Comparison of ELISA and LC-MS/MS-based methods for gliadin and hordein detection

Product type	Food product	Gluten-free claims (Y/N)	Measured amount of gluten content (ppm $\pm$ SE)		
			Gluten by ELISA ^a	Gliadin (α -gliadin) by MS ^b Peptide W2	Hordein by MS ^b Peptide B6
Liquid	Gluten-free beer 1	Y	<Value for LOD	<Value for LOD	12.6 $\pm$ 0.05
	Gluten-free beer 2	Y	<Value for LOD	3.25 $\pm$ 0.14	>100
	Rice wine	N	<Value for LOD	<Value for LOD	<Value for LOD
	Malt beverage	N	18.5 $\pm$ 5.7	2.8 $\pm$ 0.6	>100
	Low-gluten beer	N	<Value for LOD	<Value for LOD	68.0 $\pm$ 4.2
	Beer 1	N	<Value for LOD	<Value for LOD	>100
	Beer 2	N	<Value for LOD	<Value for LOD	>100
	Wheat beer ^c	N	47.7 $\pm$ 25.3	>100	>100
Solid	Tortilla chips	Y	<Value for LOD	<Value for LOD	<Value for LOD
	Wheat flour	N	>80	>100	4.3 $\pm$ 0.7
	Cookies ^d	N	50.7 $\pm$ 0.67	>100	>100
	Wheat cookies	N	>80	>100	10.3 $\pm$ 1.4

^a The Gluten ELISA kit is designed with a range of detection from 5 to 80 ppm. The LOQ is 5 ppm gluten. The LOD is 3 ppm gluten

^b The LC-MS/MS methods are designed with a range of detection from 2.5 to 100 ppm. Both LOQ and LOD are 2.5 ppm prolamin

^c The ingredients of beer were containing wheat and barley

^d The ingredients of cookies were containing wheat and barley

μL) before analysis. According to different types of ingredients commonly used in food manufacturing, the matrices of GF flour were used for gliadin and the matrices of apple wine for hordein. The LOQ and LOD, CV%, coefficient of determination (R^2), and mean accuracy (%) for each peptide are summarized in Table 4. For gliadin, the LOD was 2.5 mg/kg, and residual range (accuracy, %) was in the 83.6–113.8 range. For hordein, the LOD was 2.5 mg/kg, and residual range (accuracy, %) was in the 82.9–112.6 range. These data demonstrate that the correlation coefficient for each quantitative peptide was greater than 0.995 with approximately 80–100% accuracy. The values of R^2 and accuracy showed that W2 and B6 are the suitable peptides for gluten quantitation. Furthermore, the CV values ($n = 5$) of all quantitative peptides were approximately below 10% for each spike level, indicating good precision of the analytical method.

Comparison of ELISA and LC-MS/MS-Based Methods

Previous studies have indicated that the results of ELISA could be inaccuracies in gluten content of processed foods. Several unprocessed and processed foods were used to compare the ability for the ELISA and MS-based methods to detect gluten. To eliminate the matrix effect, IS peptides were used.

The analysis results of all 12 samples are summarized in Table 5. The results showed that gliadins can be detected in wheat flour, cookies, and beer for the ELISA method. However, as expected, the ELISA method clearly lacked the ability to accurately measure hordein in fermented and hydrolyzed foods. Changes in immunoreactive epitopes of gluten during food processing may lead to a significant impact on the antigen-antibody interaction in ELISA. Therefore, it could not estimate gluten content by adopting ELISA. Conversely, an improved detection capability for the LC-MS/MS method to detect gliadin and hordein in both unprocessed and processed foods was observed. Specifically, the LC-MS/MS method also detected hordein from gluten-free beer 1 produced from barley that claimed to have undergone a process to remove the gluten content. Our results showed that the MS strategy presents a high degree of identification reliability.

Conclusion

It is the first study that developed a purification method for hordeins and established a quantitative method for gliadins and hordeins using LC-MS/MS. Furthermore, this study demonstrated that the LC-MS/MS method is more capable of detecting the prolamins, gliadins, and hordeins compared with the ELISA method. Two quantitative peptides were selected from a number of potential prolamins marker peptides based on

their abundance, reproducibility of MS-based signals, and the intensity stability in different processed foods. Compared with the immunochemical method, LC-MS/MS was able to detect and quantify the gluten content from fermented and hydrolyzed foods. This method provides a reliable reproducible approach for detecting gliadin and hordein with the potential to analyze suspected gluten-contaminated foods when assessing product safety.

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Compliance with Ethical Standards

Conflict of Interest Yi-Shun Liao declares that he has no conflict of interest. Je-Hung Kuo declares that he has no conflict of interest. Bo-Lin Chen declares that he has no conflict of interest. Hsiu-Wei Tsuei declares that he has no conflict of interest. Che-Yang Lin declares that he has no conflict of interest. Hsu-Yang Lin declares that he has no conflict of interest. Hwei-Fang Cheng declares that she has no conflict of interest.

Ethical Approval Human and animal studies were not performed in this study by any authors.

Informed Consent None.

References

- Abadie V, Sollid LM, Barreiro LB, Jabri B (2011) Integration of genetic and immunological insights into a model of celiac disease pathogenesis. *Annu Rev Immunol* 29:493–525. doi:10.1146/annurev-immunol-040210-092915
- Aebersold R, Mann M (2003) Mass spectrometry-based proteomics. *Nature* 422:198–207. doi:10.1038/nature01511
- Allred LK, Sealey Voyksner JA, Voyksner RD (2014) Evaluation of qualitative and quantitative immunoassays to detect barley contamination in gluten-free beer with confirmation using LC/MS/MS. *J AOAC Int* 97:1615–1625. doi:10.5740/jaoacint.14-058
- Altenbach SB, Vensel WH, DuPont FM (2010) Analysis of expressed sequence tags from a single wheat cultivar facilitates interpretation of tandem mass spectrometry data and discrimination of gamma gliadin proteins that may play different functional roles in flour. *BMC Plant Biol* 10:7. doi:10.1186/1471-2229-10-7
- Balakireva AV, Zamyatnin AA (2016) Properties of gluten intolerance: gluten structure, evolution, pathogenicity and detoxification capabilities. *Nutrients*. doi:10.3390/nu8100644
- Bernardo D, Peña AS (2012) Developing strategies to improve the quality of life of patients with gluten intolerance in patients with and without coeliac disease. *Eur J Intern Med* 23:6–8. doi:10.1016/j.ejim.2011.09.016
- Biesiekierski JR, Newnham ED, Irving PM et al (2011) Gluten causes gastrointestinal symptoms in subjects without celiac disease: a double-blind randomized placebo-controlled trial. *Am J Gastroenterol* 106:508–514. doi:10.1038/ajg.2010.487quiz 515
- Chait BT (2006) Mass spectrometry: bottom-up or top-down? *Science* 314:65–66. doi:10.1126/science.1133987

- Ciclitira PJ, Ellis HJ, Lundin KEA (2005) Gluten-free diet—what is toxic? *Best Pract Res Clin Gastroenterol* 19:359–371. doi:[10.1016/j.bpg.2005.01.003](https://doi.org/10.1016/j.bpg.2005.01.003)
- Codex Alimentarius Commission (2015) STAN 118–1979, Revised 2015
- Colgrave ML, Goswami H, Howitt CA, Tanner GJ (2012) What is in a beer? Proteomic characterization and relative quantification of hordein (gluten) in beer. *J Proteome Res* 11:386–396. doi:[10.1021/pr2008434](https://doi.org/10.1021/pr2008434)
- Colgrave ML, Goswami H, Blundell M et al (2014) Using mass spectrometry to detect hydrolysed gluten in beer that is responsible for false negatives by ELISA. *J Chromatogr A* 1370:105–114. doi:[10.1016/j.chroma.2014.10.033](https://doi.org/10.1016/j.chroma.2014.10.033)
- Comino I, Real A, de Lorenzo L et al (2011) Diversity in oat potential immunogenicity: basis for the selection of oat varieties with no toxicity in coeliac disease. *Gut* 60:915–922. doi:[10.1136/gut.2010.225268](https://doi.org/10.1136/gut.2010.225268)
- Commission Regulation HAT (2009) COMMISSION REGULATION (EC) No 1201/2009. *Off J Eur Union L* 329:29
- Denery-Papini S, Nicolas Y, Popineau Y (1999) Efficiency and limitations of immunochemical assays for the testing of gluten-free foods. *J Cereal Sci* 30:121–131. doi:[10.1006/jcrs.1999.0268](https://doi.org/10.1006/jcrs.1999.0268)
- Diaz-Amigo C, Popping B (2012) Gluten and gluten-free: issues and considerations of labeling regulations, detection methods, and assay validation. *J AOAC Int* 95:337–348
- Diaz-Amigo C, Popping B (2013) Accuracy of ELISA detection methods for gluten and reference materials: a realistic assessment. *J Agric Food Chem* 61:5681–5688. doi:[10.1021/jf3046736](https://doi.org/10.1021/jf3046736)
- Fiedler KL, McGrath SC, Callahan JH, Ross MM (2014) Characterization of grain-specific peptide markers for the detection of gluten by mass spectrometry. *J Agric Food Chem* 62:5835–5844. doi:[10.1021/jf500997j](https://doi.org/10.1021/jf500997j)
- Green PHR, Fleischauer AT, Bhagat G et al (2003) Risk of malignancy in patients with celiac disease. *Am J Med* 115:191–195. doi:[10.1016/S0002-9343\(03\)00302-4](https://doi.org/10.1016/S0002-9343(03)00302-4)
- Guo B, Luan H, Lin S et al (2016) Comparative proteomic analysis of two barley cultivars (*Hordeum vulgare* L.) with contrasting grain protein content. *Front Plant Sci*. doi:[10.3389/fpls.2016.00542](https://doi.org/10.3389/fpls.2016.00542)
- Kanerva PM, Sontag-Strohm TS, Ryöppy PH et al (2006) Analysis of barley contamination in oats using R5 and ω -gliadin antibodies. *J Cereal Sci* 44:347–352. doi:[10.1016/j.jcs.2006.08.005](https://doi.org/10.1016/j.jcs.2006.08.005)
- Kanerva P, Brinck O, Sontag-Strohm T et al (2011) Deamidation of gluten proteins and peptides decreases the antibody affinity in gluten analysis assays. *J Cereal Sci* 53:335–339. doi:[10.1016/j.jcs.2011.02.003](https://doi.org/10.1016/j.jcs.2011.02.003)
- Manfredi A, Mattarozzi M, Giannetto M, Careri M (2015) Multiplex liquid chromatography-tandem mass spectrometry for the detection of wheat, oat, barley and rye prolamins towards the assessment of gluten-free product safety. *Anal Chim Acta* 895:62–70. doi:[10.1016/j.aca.2015.09.008](https://doi.org/10.1016/j.aca.2015.09.008)
- Marbaix H, Budinger D, Dieu M et al (2016) Identification of proteins and peptide biomarkers for detecting banned processed animal proteins (PAPs) in meat and bone meal by mass spectrometry. *J Agric Food Chem* 64:2405–2414. doi:[10.1021/acs.jafc.6b00064](https://doi.org/10.1021/acs.jafc.6b00064)
- Sandberg R, Bränden C-I, Emberg I, Cöster J (2003) Quantifying the species-specificity in genomic signatures, synonymous codon choice, amino acid usage and G+C content. *Gene* 311:35–42
- Sapone A, Lammers KM, Casolaro V et al (2011) Divergence of gut permeability and mucosal immune gene expression in two gluten-associated conditions: celiac disease and gluten sensitivity. *BMC Med* 9:23. doi:[10.1186/1741-7015-9-23](https://doi.org/10.1186/1741-7015-9-23)
- Sealey-Voyksner JA, Khosla C, Voyksner RD, Jorgenson JW (2010) Novel aspects of quantitation of immunogenic wheat gluten peptides by liquid chromatography–mass spectrometry/mass spectrometry. *J Chromatogr A* 1217:4167–4183. doi:[10.1016/j.chroma.2010.01.067](https://doi.org/10.1016/j.chroma.2010.01.067)
- Shahbazi Y, Malekinejad H, Tajik H (2016) Determination of naturally occurring estrogenic hormones in cow's and river buffalo's meat by HPLC-FLD method. *J Food Drug Anal* 24:457–463. doi:[10.1016/j.jfda.2016.02.014](https://doi.org/10.1016/j.jfda.2016.02.014)
- Shewry PR, Tatham AS, Kasarda DD (1992) Cereal proteins and coeliac disease. *Coeliac Dis Oxf Blackwell Sci Publ* 305:348
- Shewry PR, Halford NG, Belton PS, Tatham AS (2002) The structure and properties of gluten: an elastic protein from wheat grain. *Philos Trans R Soc B Biol Sci* 357:133–142. doi:[10.1098/rstb.2001.1024](https://doi.org/10.1098/rstb.2001.1024)
- Simonato B, Mainente F, Tolini S, Pasini G (2011) Immunochemical and mass spectrometry detection of residual proteins in gluten fined red wine. *J Agric Food Chem* 59:3101–3110. doi:[10.1021/jf104490z](https://doi.org/10.1021/jf104490z)
- Skyllas DJ, Mackintosh JA, Cordwell SJ et al (2000) Proteome approach to the characterisation of protein composition in the developing and mature wheat-grain endosperm. *J Cereal Sci* 32:169–188. doi:[10.1006/jcrs.2000.0321](https://doi.org/10.1006/jcrs.2000.0321)
- Snégárov J, Bouchez-Mahiot I, Pecquet C et al (2006) Study of IgE antigenic relationships in hypersensitivity to hydrolyzed wheat proteins and wheat-dependent exercise-induced anaphylaxis. *Int Arch Allergy Immunol* 139:201–208. doi:[10.1159/000091165](https://doi.org/10.1159/000091165)
- Srinivasan B, Focke-Tejkl M, Swoboda I et al (2013) A combined biochemical, biophysical and immunological approach towards the identification of celiac disease-specific wheat antigens. *Amino Acids* 45:889–900. doi:[10.1007/s00726-013-1537-6](https://doi.org/10.1007/s00726-013-1537-6)
- Srinivasan B, Focke-Tejkl M, Weber M et al (2015) Usefulness of recombinant γ -gliadin 1 for identifying patients with celiac disease and monitoring adherence to a gluten-free diet. *J Allergy Clin Immunol* 136:1607–1618.e3. doi:[10.1016/j.jaci.2015.04.040](https://doi.org/10.1016/j.jaci.2015.04.040)
- Tanner GJ, Colgrave ML, Blundell MJ et al (2013a) Measuring hordein (gluten) in beer—a comparison of ELISA and mass spectrometry. *PLoS One* 8:e56452. doi:[10.1371/journal.pone.0056452](https://doi.org/10.1371/journal.pone.0056452)
- Tanner GJ, Colgrave ML, Blundell MJ et al (2013b) Measuring hordein (gluten) in beer—a comparison of ELISA and mass spectrometry. *PLoS One* 8:e56452. doi:[10.1371/journal.pone.0056452](https://doi.org/10.1371/journal.pone.0056452)
- Tanner GJ, Blundell MJ, Colgrave ML, Howitt CA (2016) Creation of the first ultra-low gluten barley (*Hordeum vulgare* L.) for coeliac and gluten-intolerant populations. *Plant Biotechnol J* 14:1139–1150. doi:[10.1111/pbi.12482](https://doi.org/10.1111/pbi.12482)
- Tatham A, Gilbert S, Fido R, Shewry P (2000) Extraction, separation, and purification of wheat gluten proteins and related proteins of barley, rye, and oats. In: Marsh M, FRCP MNMM DSc (eds) *Celiac disease*. Humana Press, pp 55–73
- Trufelli H, Palma P, Famiglini G, Cappiello A (2011) An overview of matrix effects in liquid chromatography–mass spectrometry. *Mass Spectrom Rev* 30:491–509. doi:[10.1002/mas.20298](https://doi.org/10.1002/mas.20298)
- U.S. FDA (2013) Food labeling: gluten-free labeling of foods. *Fed Regist* 78:47154–47179
- U.S. Food and Drug Administration (2013) Guidance for industry—bioanalytical method validation. <http://www.fda.gov/downloads/Drugs/Guidances/ucm070107.pdf>
- Wieser H (2007) Chemistry of gluten proteins. *Food Microbiol* 24:115–119. doi:[10.1016/j.fm.2006.07.004](https://doi.org/10.1016/j.fm.2006.07.004)
- Zuidmeer L, Goldhahn K, Rona RJ et al (2008) The prevalence of plant food allergies: a systematic review. *J Allergy Clin Immunol* 121:1210–1218.e4. doi:[10.1016/j.jaci.2008.02.019](https://doi.org/10.1016/j.jaci.2008.02.019)

Supplementary Material

Development and Validation of the Detection Method for Wheat and Barley Glutens using Mass Spectrometry in Processed Foods

Food Analytical Methods

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Figure S1.

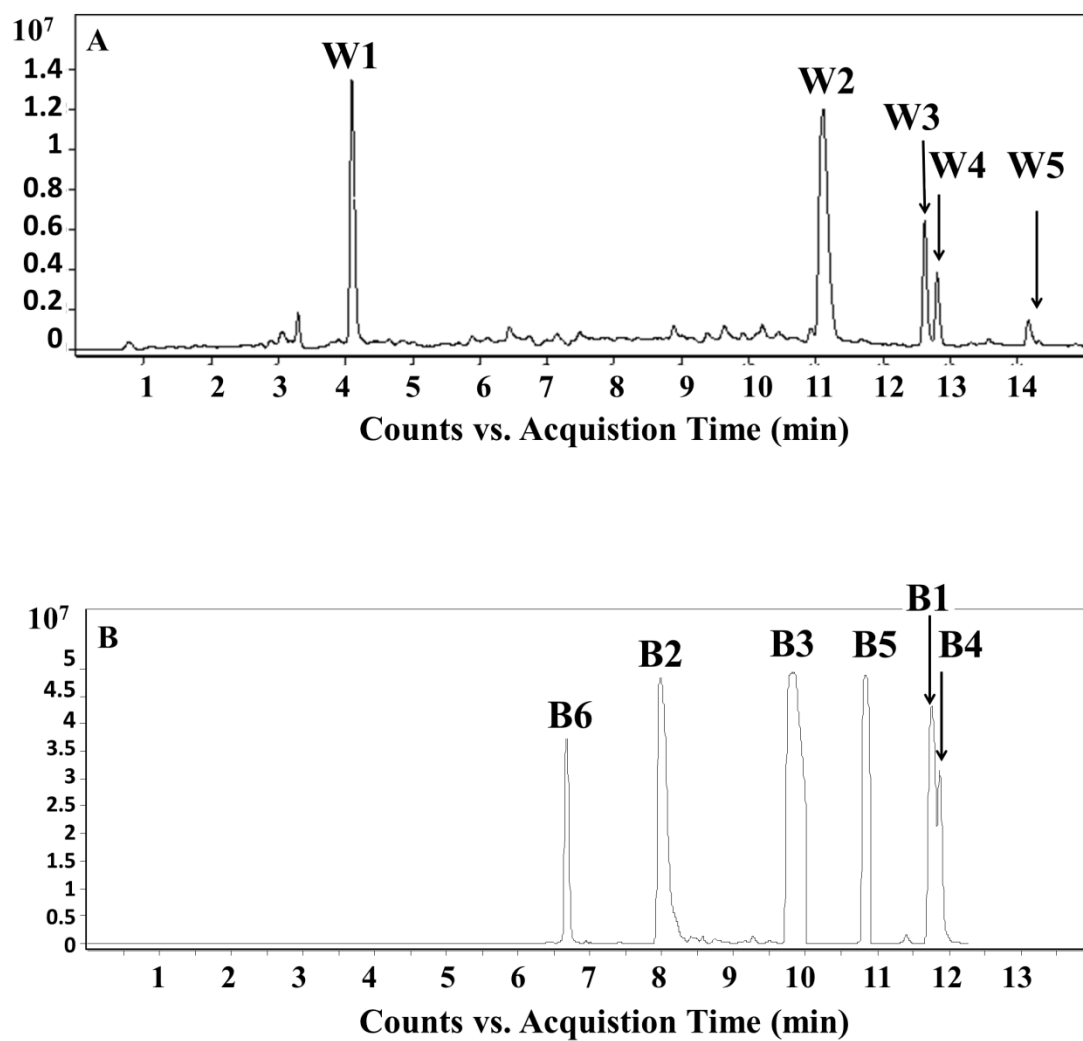


Figure S1. The total ion current (TIC) chromatograms of marker peptides. (A) wheat flour for the gliadins method; (B) barley flour for the hordeins method.

Figure S2.

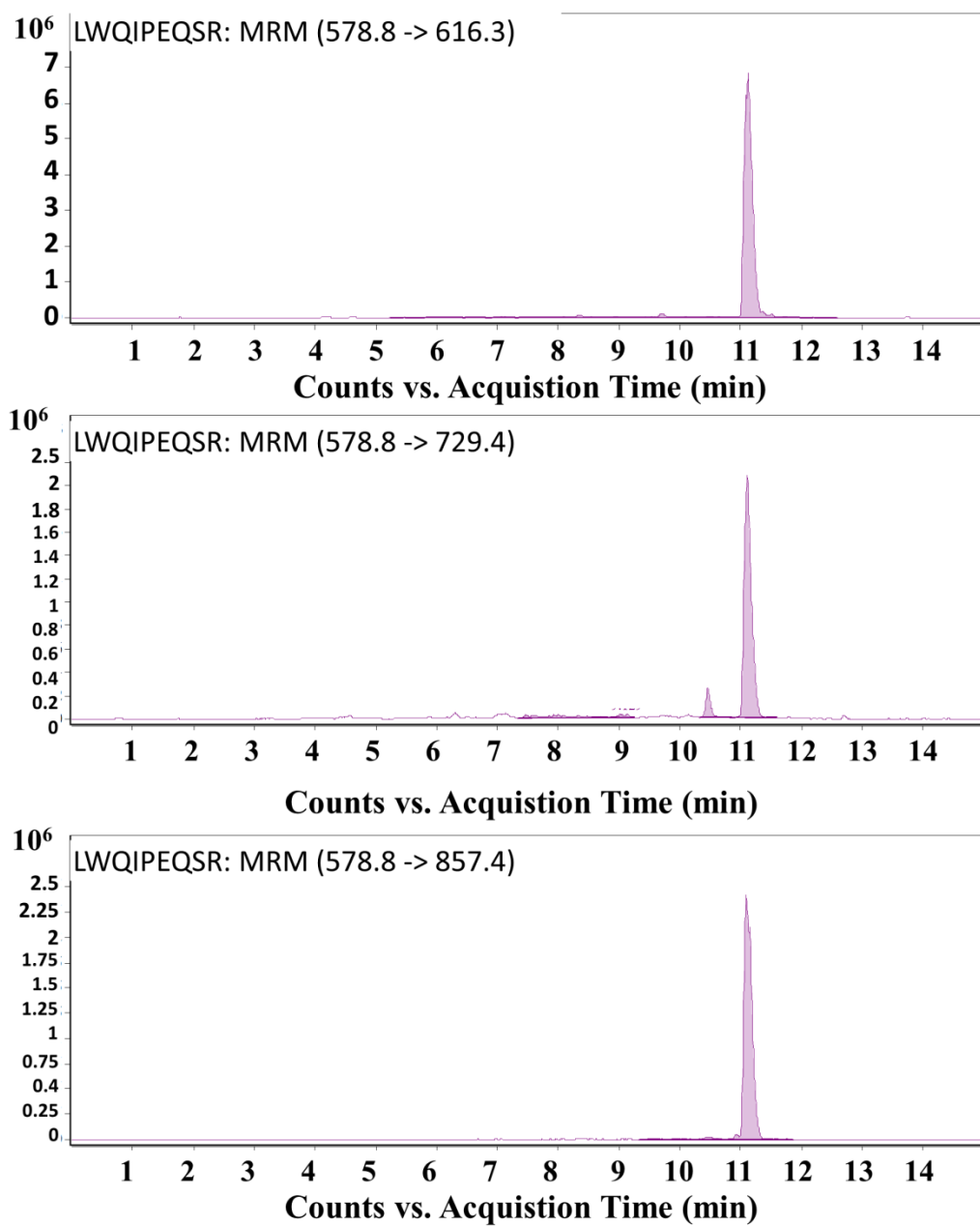


Figure S2. The MS/MS spectra of quantitative gliadin peptide W2 (LWQIPEQSR).

The MRM transition (m/z): 578.8 -> 616.3; 578.8 -> 729.4; 578.8 -> 857.4.

Figure S3.

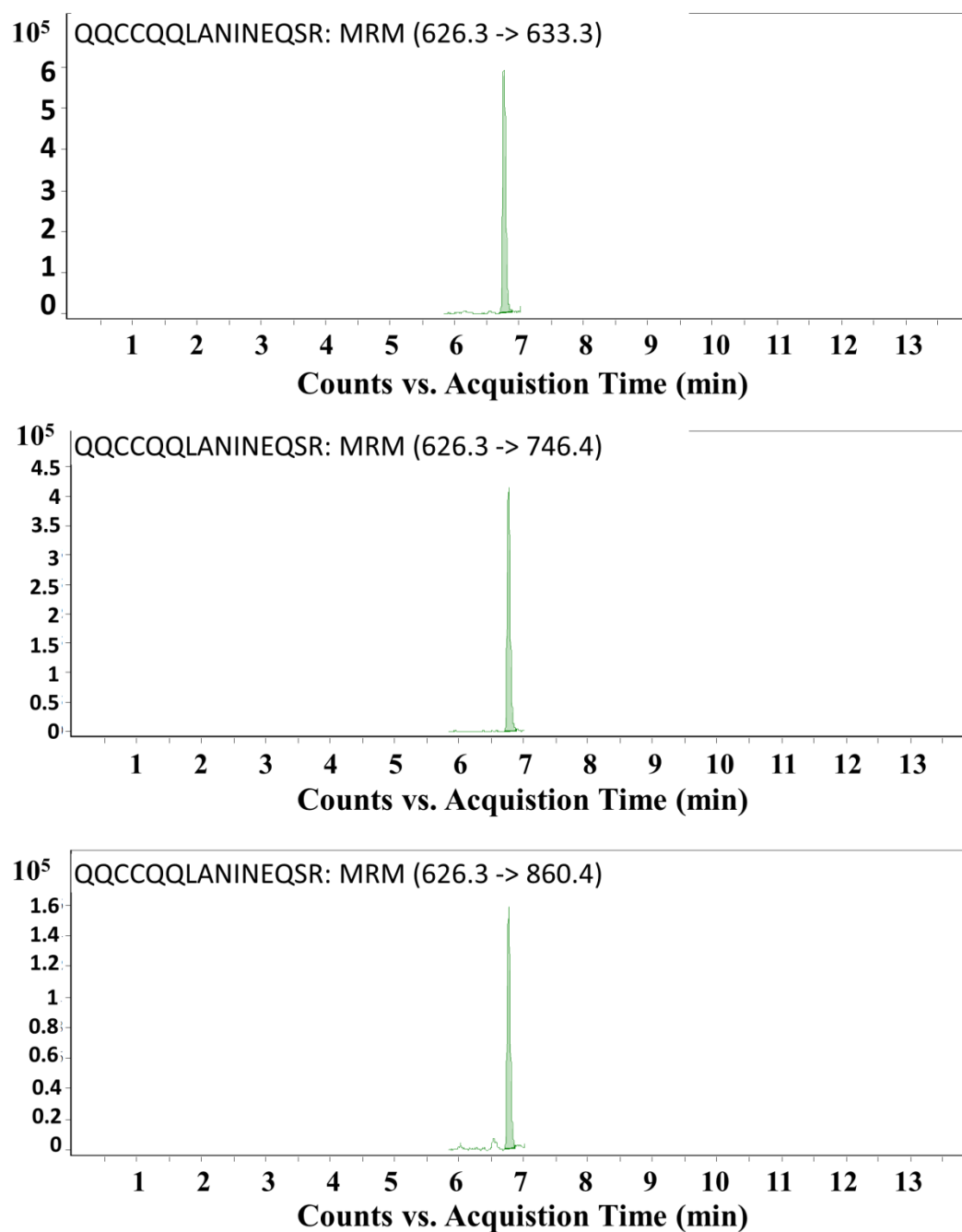
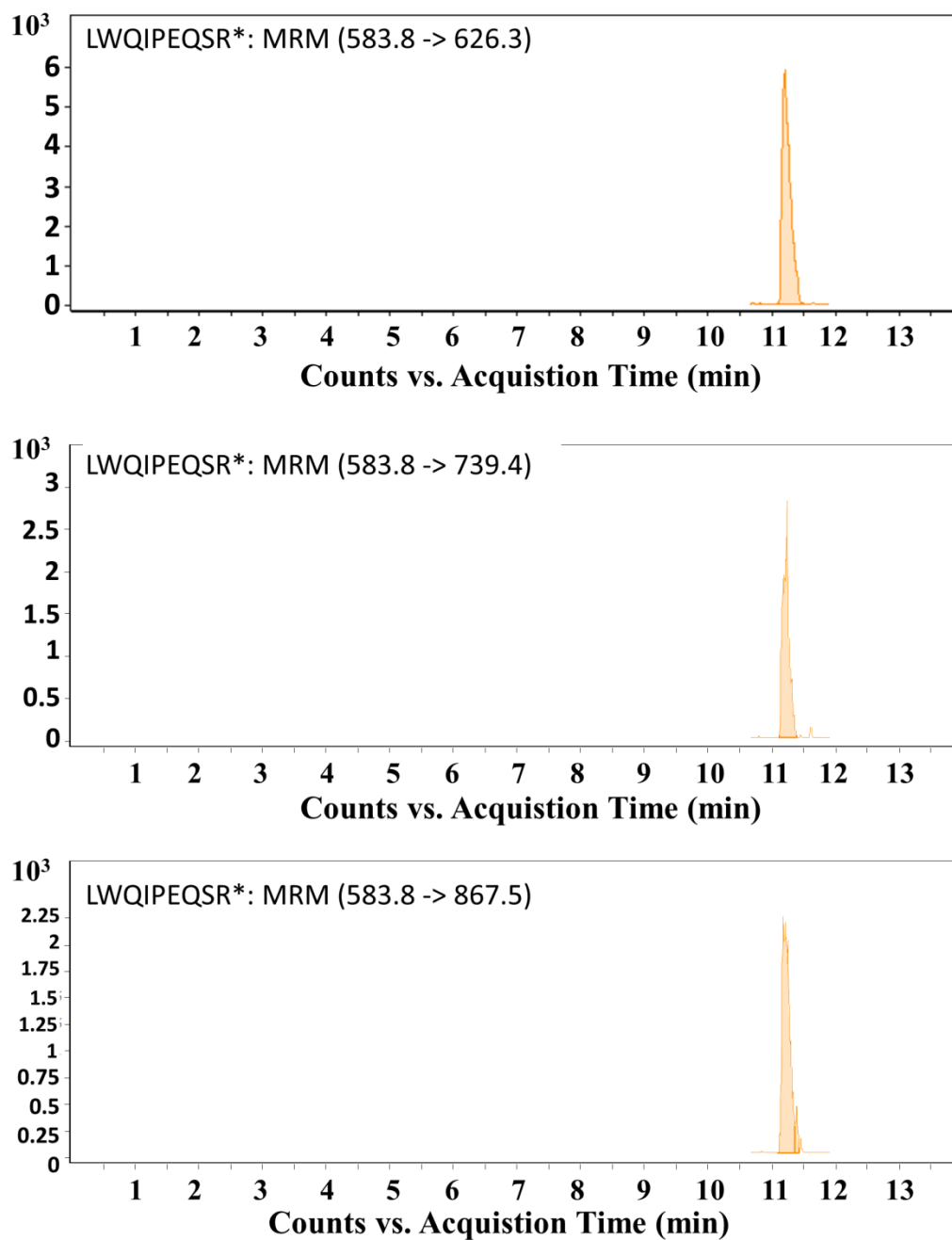


Figure S3. The MS/MS spectra of quantitative hordein peptide B6 (QQCCQQLANINEQSR). The MRM transition (m/z): 626.3 -> 633.3; 626.3 -> 746.4; 626.3 -> 860.4.

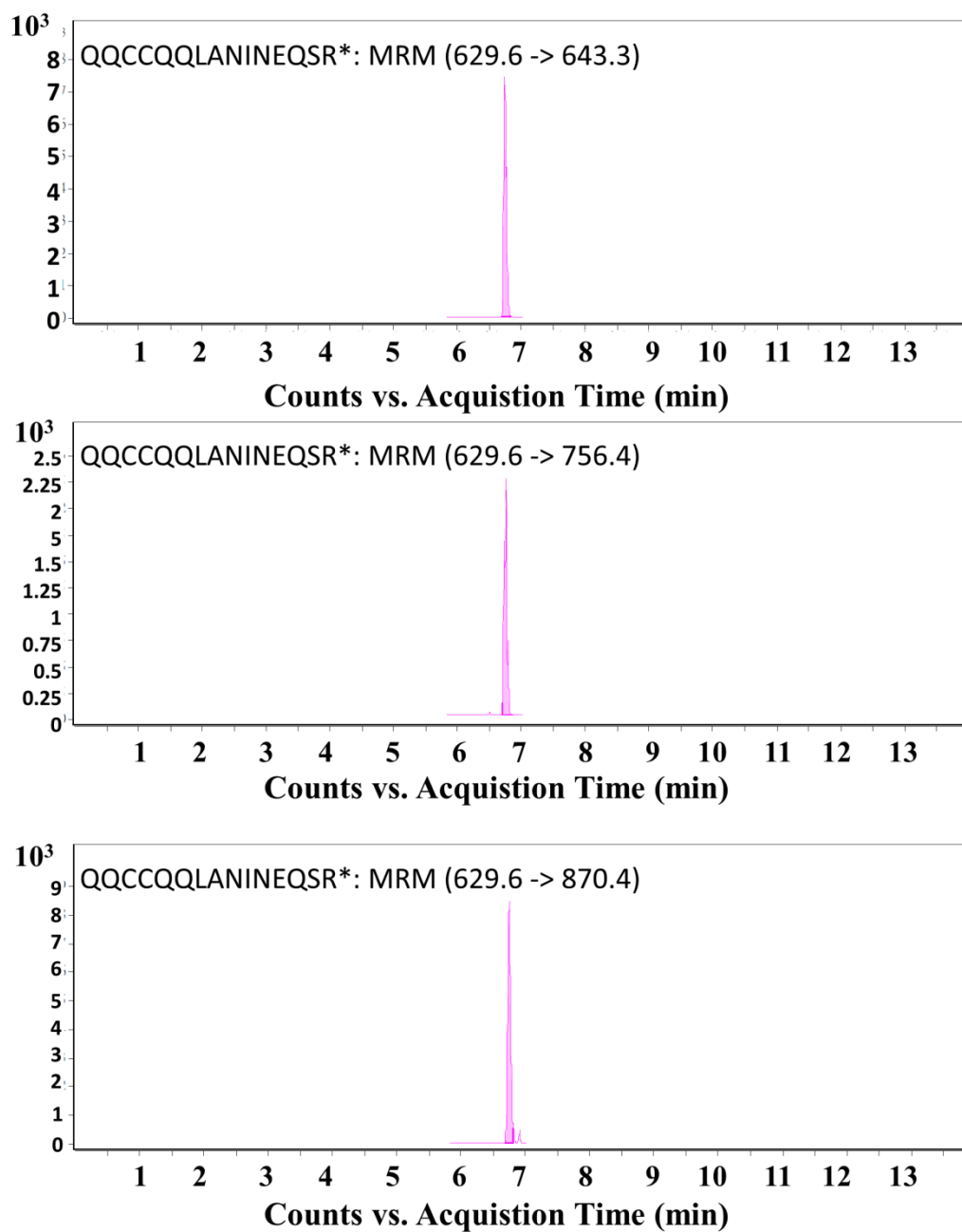
Figure S4.



*C- terminal [13C]/[15N]-arginine labeled

Figure S4. The MS/MS spectra of isotope-labeled IS peptides (LWQIPEQS(R)-R: ¹³C, ¹⁵N labelled) for gliadin quantitation. The MRM transition (m/z): 583.8 → 626.3; 583.8 → 739.4; 583.8 → 867.5

Figure S5.



*C- terminal [^{13}C]/[^{15}N]-arginine labeled

Figure S5. The MS/MS spectra of isotope-labeled IS peptides (QQCCQQLANINEQS(R)-Q: iodoacetamide modification /R: ^{13}C , ^{15}N labelled) for hordein quantitation. The MRM transition (m/z): 629.6 -> 643.3; 629.6 -> 756.4; 629.6 -> 870.4

Table S1.

Number of gliadin and hordein peptides identified with trypsin.

Prolamins	Protein class	Peptide Count	Score	Local	Global	False	Global	False	Discovery	Sequence	Specificity for Gliadin or Hordeins* (Y/N)
				Discovery Rate (FDR, %)		Rate (FDR, %)					
Gliadin	Alpha-gliadin	8	15.33	40			10			LWQIPEQSR	Y
			23.43	<0.1			<0.1			DVVLQQHIAHGR	Y
			20.14	<0.1			<0.1			GSVQPQLPQFEEIR	Y
			22.94	<0.1			<0.1			VPVPQLQPQNPSQQQPQK	Y
			13.7	40			17			QQQQQQQQILQQILQQQLIPCR	Y
			20.91	<0.1			<0.1			NLALETLPAMCNVYIPPYCTIAPVGIFGTN	Y
			12.98	66			22			NLALQTLPAMCNVYIPPYCTIAPFGIFGTN	Y
			22.72	<0.1			<0.1			NLALQTLPAMCNVYIPPHCSTTIAPFGIFGTN	Y
	Gamma-gliadin	20	11.1	77			35			LNPKK	N
			15.45	40			9			SDCQVMR	N
			18.22	<0.1			<0.1			APFASIVAGIGGQ	Y
			17.67	25			1			APFSSVVAGIGGQ	N
			19.58	<0.1			<0.1			APFASIVADIGGQ	Y
			19.8	<0.1			<0.1			LSLLQSVIPFLRPK	N
			19.47	<0.1			<0.1			SFIQPSLQQQLNPKK	Y
			24.38	<0.1			<0.1			SDCQVMQQCCQQLAQIPR	Y
			19.95	<0.1			<0.1			SLVLGTLPTMCNVFVPPECSTTK	Y
			22.91	<0.1			<0.1			NFLQQCNPVSLVSSLVSIILPR	Y

			22.94	<0.1	<0.1	NFLQQCNPVSLVSSLISMILPR	Y
			26.33	<0.1	<0.1	NYLLQQCNPVSLVSSLVSMILPR	Y
			22.51	<0.1	<0.1	NFLQQCNHVSLVSSLVSIILPR	Y
			20.86	<0.1	<0.1	SLVLQTLPTMCNVYVPPECSIIK	N
			18.69	<0.1	<0.1	SLVLQTLPSMCNVYVPPECSIMR	N
			16.18	25	5	SLVLQTLPTMCNVYVPPYCSTIR	Y
			21	<0.1	<0.1	RPLFQLVQGQGIIQPQPAQLEVIR	Y
			24.5	<0.1	<0.1	TLPNMCNVYVRPDCSTINAPFASIVAGISGQ	Y
			19.84	<0.1	<0.1	NFLQQCCKPVSLVSSLWSIILPPSDCQVMR	Y
			16.18	25	5	NILLQQCCKPASLVSSLWSIHWPPQSDCQVMR	N
Hordeins	B-hordein	8	10.2	84	40	HEAVR	N
			19.52	<0.1	<0.1	ILPFGIDTR	Y
			18.16	<0.1	<0.1	IVPLAIDTR	Y
			21.58	<0.1	<0.1	VFLQQQCSPVR	Y
			21.62	<0.1	<0.1	TLPTMCNVNPLYR	N
			23.89	<0.1	<0.1	VFLQQQCSPVAMSQR	Y
			24.17	<0.1	<0.1	VFLQQQCSPVPVPQR	Y
			19.35	<0.1	<0.1	SQMLQQSSCHVLQQCCQQLPQIPEQLR	N
	Gamma-hordein	3	20.62	<0.1	<0.1	EFLQQCTLDEK	Y
			17.75	<0.1	<0.1	APFVGVTGVGGQ	Y
			22.06	<0.1	<0.1	QQCCQQLANINEQSR	Y

* The specificities of the peptides were performed BLAST searches using the NCBI nonredundant protein sequences (nr) database.