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vMethod™ Application for Food Testing

A Highly Selective and Sensitive LC-MS/MS Method for the Quantification of Gluten Proteins

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Measuring Multiple, Unique Signature Peptides to Determine Gluten Levels in Diverse Food Matrices

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Introduction

Gluten is a multi-protein complex located in the endosperm portion of wheat, rye, and barley grains that are commonly found in Western diets and are steadily becoming more prevalent in Eastern diets. Gluten ingestion has been linked to a number of gastrointestinal disorders, including celiac disease, wheat allergies, and non-celiac gluten sensitivity, with the epidemiologically relevant prevalence of these disorders estimated to be around 5% of the global poplulation.[1]

Gluten-related disorders result from diverse mechanisms: celiac's disease is caused by an autoimmune reaction to gliadin, a glycoprotein that, along with glutenin, helps to form the gluten complex; wheat allergies are mediated by IgE antibodies to allergenic gluten proteins; while the cause of non-celiac gluten sensitivity is not yet well understood. However, for all these gluten-related disorders, consuming gluten can have serious health consequences, making the detection and quantitation of dietary gluten in pre-packaged foods extremely important.

In 2013, the FDA established, among other criteria, a gluten limit of less than 20 parts-per-million (ppm) for foods that carry a gluten-free label (e.g., gluten-free, no gluten, free of gluten, without gluten). Before issuing this regulation, there were no U.S. standards or definitions for the food industry to reference when labeling a food's gluten levels, leaving many consumers unsure of the gluten content. Typically, gluten content has been determined using an ELISA assay, but these tests have a limited linear response and often generate false negative or false positive results.

Herein, we have developed and verified a selective and sensitive LC-MS/MS-based method for detecting and quantifying gluten signature peptides in a variety of food matrices. This method relies on the use of three MRM transitions for each unique gluten signature peptide released from the glutenin subunit (DY10). To increase assay precision, a stable isotope-labeled gluten standard was added to food homogenates with unknown gluten levels prior to enzymatic protein digestion. Assay performance was evaluated using raw cereal grains, as well as baked, dehydrated, and fermented products.

A major advantage of LC-MS/MS analysis over the ELISA assay is that multiple, unique peptide markers can be monitored simultaneously in a single injection, providing information on gluten content, as well as the identity of other grains (rye, barley, oats). Using this LC-MS/MS method, an accurate gluten concentration as low as 5 ppm can be obtained, with excellent repeatability (%CV) of less than 20%, along with information on a food product's grain composition.

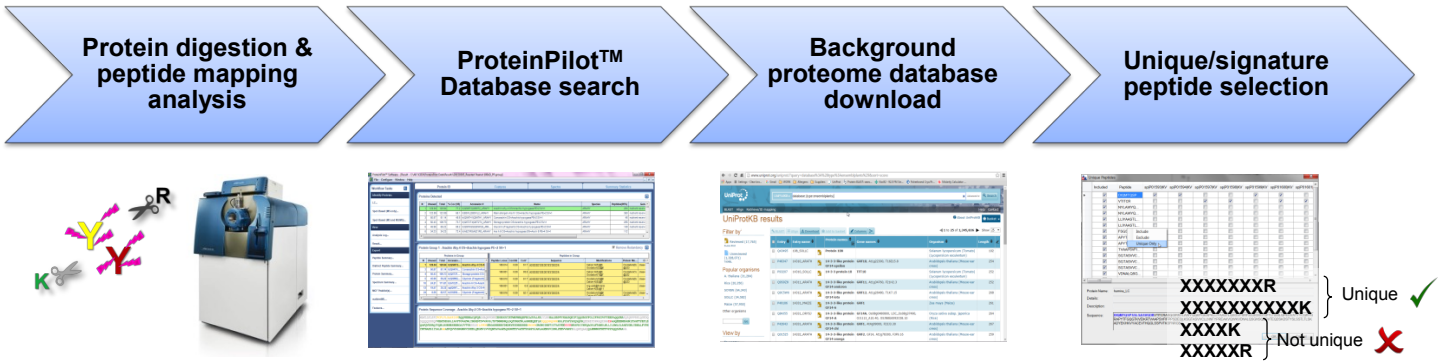

Figure 1. Signature peptide selection workflow using the SCIEX TripleTOF® 6600 system and ProteinPilot™ software

Experimental

Sample Preparation and Digestion of Bakery Products and Baby Formula

Baked goods (1 g) were homogenized by grinding and then defatted with hexane (5 mL). Excess hexane was removed by oven-drying samples at 60ºC. Eight-point calibration lines were constructed over a broad range of concentrations (5 to 1000 ppm gliadin in 1 g gluten- free homogenate). Food samples were extracted (3 mL of gluten extraction solvent) and centrifuged. Supernatants (1 mL) were then collected and dried under vacuum to 0.25 mL.

After adding diluent (100 µL), samples were denatured and reduced with denaturant (5 µL) and reductant (5 µL) at 60ºC for 1 hr. After cooling to room temperature, samples were alkylated with a cysteine blocking reagent (15 µL) at 20ºC for 1 hr. Samples were incubated with a reductant (2.5 µL) at 20 ºC for 1 hr to stop alkylation,. The reduced and alkylated protein samples were digested with trypsin (50 µg) for 3 hr at 37ºC to release signature peptides.

Sample Preparation and Digestion of Fermented Beverages

An eight-point calibration line was constructed over a broad range (5 ppm to 1000 ppm of gliadin in 1 mL gluten- free beverage). Fermented beverage samples were dried to 0.25 mL, prior to dilution, denaturation and reduction of proteins as described for bakery products. Beverage samples were digested following the above protocol for bakery products.

Sample Cleanup

A stable isotope-labeled internal standard (IS) was added to the sample after digestion, followed by solid phase extraction to remove excess polar and non-polar contaminants. Strong cation-exchange cartridges (Phenomenex) were used for sample cleanup following the manufacturer's protocol.

LC Separation

Peptides were chromatographically separated using a C18 column (2.6 µm, 100 x 2.1 mm) and a 9-min gradient at a flow rate of 300 µL/min. The mobile phases were 0.1% formic acid in water and 0.1% formic acid in acetonitrile. The injection volume was set to 5 µL.

MS/MS Detection

A SCIEX TripleTOF® 6600 LC-MS/MS system was used for identifying allergen proteins and selecting signature peptides. The peptide mapping experiments were performed by analyzing protein digests of rye, barley, oats and wheat. The detailed selection strategy is discussed in the results section.

For quantitative analyses of signature peptides of glutenin, food samples were evaluated using a SCIEX QTRAP® 4500 LC-MS/MS system with Turbo V™ source in positive electrospray ionization mode.

Results and Discussion

During method development, signature peptides were chosen based on: 1) the uniqueness of their sequence; and 2) their sensitivity of detection. Information on peptide sequences, relative abundance, and post-translational modifications was generated using a ProteinPilot™ software database search and LC-MS/MS analysis of peptides using a TripleTOF® 6600 System (Figure 1). Results were compared to information on the tryptic peptides generated from the food homogenate's background proteins.

After peptide selection, the effects of acute conditions during baking (such as high acidity and heat) on the stability of signature peptides were evaluated using the QTRAP® 4500 System. Similarly, the efficiency of the protein extraction protocol was optimized by testing various solvents and enhancing SPE procedures and monitoring peptide levels.

To gauge the linearity of the response, signals generated from the MRM transitions of signature peptides from various grains (wheat, rye, barley, and oats) were compared to peak areas generated from known concentrations of gluten standards added to food homogenates. MRM transitions that produced linear signals over the broadest dynamic range were selected for the final method. Three signature peptides and three MRM transitions for each peptide were selected for the final quantitation and screening of proteins from various food homogenates.

To quantify gluten peptides, calibration curves were constructed over a broad range of gluten concentrations (5 to 1000 ppm). The signature peptide MRM transitions were tested for selectivity and linearity in three different food matrices (Figures 2a-c). The most selective transition with the best regression value was employed for the remaining quantitation experiments. The other two MRM transitions were used in qualitative screens for gluten.

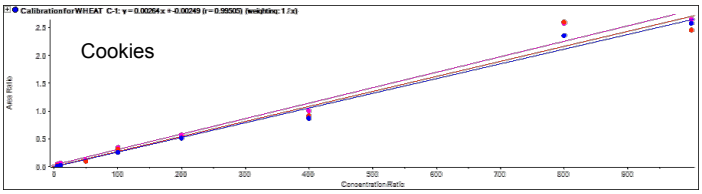

Figure 2a. Calibration lines for gluten quantification. A gluten signature peptide, selected for its stability under acute conditions, was detected over a broad range of concentrations (5 to 1000 ppm) in cookies. Three different MRM transitions were evaluated for linearity for each food product: Blue) wheat peptide C-1, Pink) wheat peptide C-2, and Orange) wheat peptide C-3.

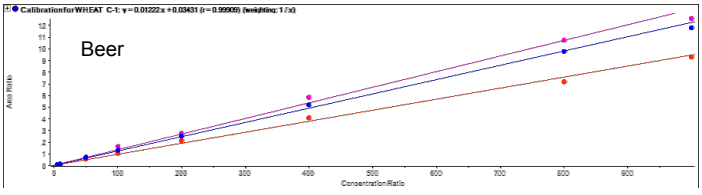

Figure 2b. Calibration lines for gluten quantification. A gluten signature peptide, selected for its stability under acute conditions, was detected over a broad range of concentrations (5 to 1000 ppm) in beer. Three different MRM transitions were evaluated for linearity for each food product: Blue) wheat peptide C-1, Pink) wheat peptide C-2, and Orange) wheat peptide C-3.

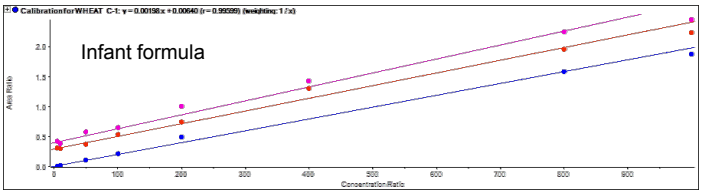

Figure 2c. Calibration lines for gluten quantification. A gluten signature peptide, selected for its stability under acute conditions, was detected over a broad range of concentrations (5 to 1000 ppm) in infant formula. Three different MRM transitions were evaluated for linearity for each food product: Blue) wheat peptide C-1, Pink) wheat peptide C-2, and Orange) wheat peptide C-3.

Quality control samples were tested at three different levels, LQC (10 ppm), MQC (400 ppm), and HQC (800 ppm), for each food homogenate (bakery products, fermented beverages, baby formula). Table 1 shows that intra-batch precision and accuracy results were within the specified limits for each matrix type and QC sample, with %CVs < 20% for all gluten levels tested. To ensure stability, solutions of gluten standards and IS were also tested over time and found to be stable for seven days at 4 ºC.

To identify other major grains (aside from wheat) in food homogenates, signature peptides for rye, barley and oats were identified. Three MRM transitions were chosen for each unique peptide from each grain type, and the highest performing transition for the most stable peptide was used to quantity levels of each grain type, while the remaining two MRM transitions were used for screening samples qualitatively.

The most selective gluten MRM transition was used to evaluate the effects of various food matrices (cookies, beer, and baby formula) on gluten detection. Increasing amounts of gluten standard (0, 5, and 1000 ppm) were added to food homogenates, which were then analyzed by LC-MS/MS for the gluten signature peptide content. Representative chromatograms for the selected gluten MRM transition showed a corresponding increase in instrument response when gluten levels were increased with no interference from the matrix regardless of food homogenate type (Figure 3).

Table 1. Within-batch precision and accuracy for the repeat measurement of gluten signature peptide levels in various food matrices (n=6)

Food Matrix	QC Sample	Concentration (ppm)	Mean (ppm)	Accuracy (%)	%CV
Cookies	LQC	10.0	11.2	111.7	8.1
	MQC	400	391.4	97.8	4.0
	HQC	800	735.9	92.0	3.2
Beer	LQC	10.0	9.4	93.7	17.4
	MQC	400	355.6	88.9	2.6
	HQC	800	924.0	115.5	2.5
Baby formula	LQC	10.0	9.9	98.8	18.8
	MQC	400	447.3	111.8	7.2
	HQC	800	767.4	95.9	3.5

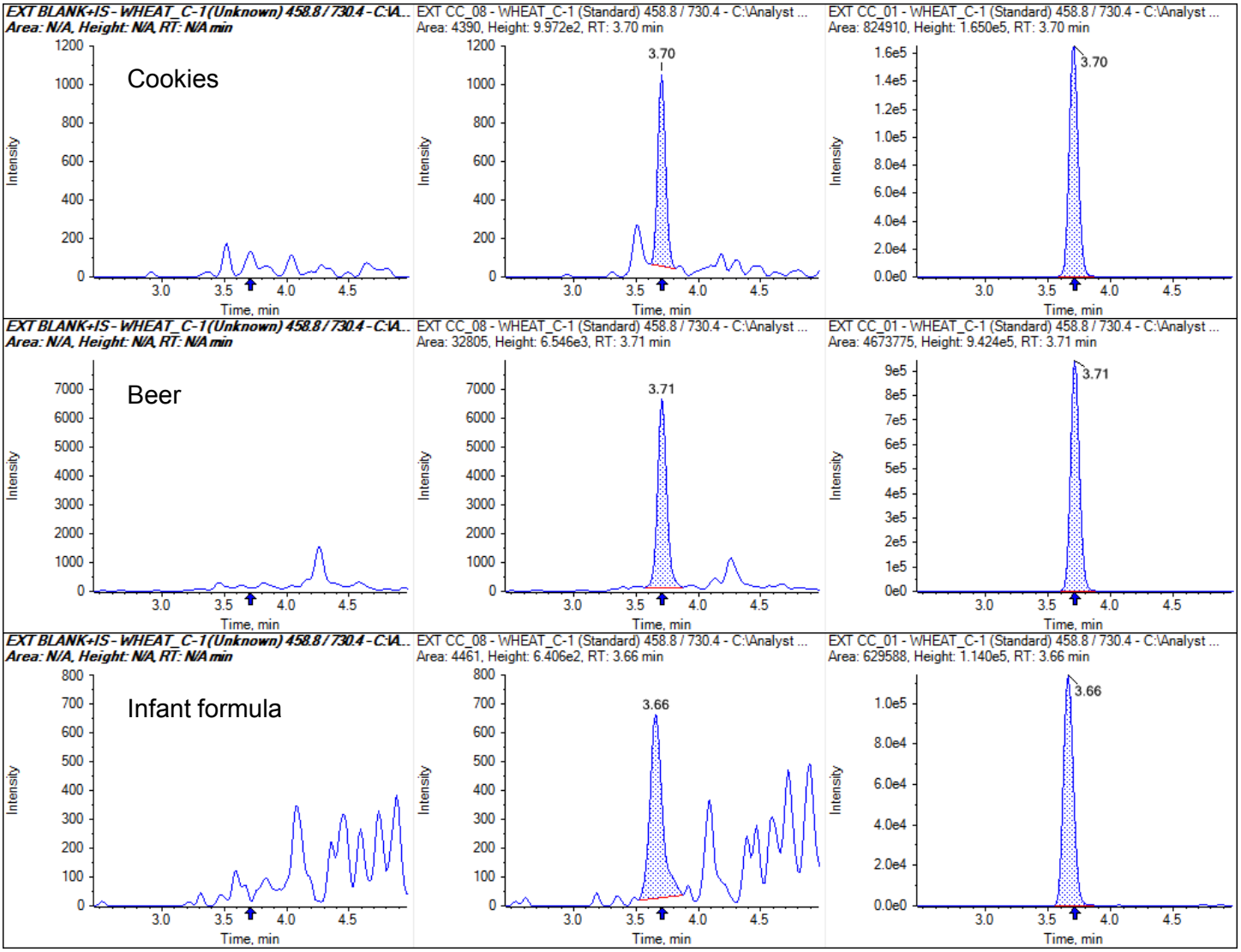

Figure 3. Representative chromatograms from MRM detection of gluten in A) cookies, B) beer, and C) infant formula. To detect a range of gluten levels, increasing amounts of gluten standard were fortified to the food homogenate: 0 ppm (left), 5 ppm (middle), and 1000 ppm (right).

Table 2. Gluten concentration in different food samples

	Labeled as Gluten-free		Gluten containing	
Food Matrix	**Sample ID**	**Gluten (ppm)**	**Sample ID**	**Gluten (ppm)**
Cookies	Choco Chip	1.3	Biscuit 1	7990
	Lemon	1.1	Biscuit 2	557
	Nutty Fibre Choco Chip	1.9	Biscuit 3	7970
Baby formula	Baby rice	n.d.	Infant Cereal multi-grain	493
	Baby rice	n.d.	Infant Cereal wheat apple	833
			Infant Cereal wheat honey	538
			Infant Cereal wheat mix fruit	504

The protein extraction efficiency for each matrix was calculated by comparing the gluten peak areas for food homogenate samples prior to the addition of gluten standards with gluten peak areas obtained post-extraction. The peak areas of six samples were averaged, and extraction efficiency was found to be around 50%, 80%, and 90% for bakery products, baby formula, and beer, respectively.

The cleanup efficiency of the SPE procedure was calculated by comparing peak areas from extracted samples to peak areas obtained from food homogenates prior to extraction. The SPE cleanup efficiency (from an average of six samples) was approximately 90%, 85% and 75 % for bakery products, fermented beverages, and baby formula, respectively.

A diverse range of food products, including gluten-free and gluten-containing foods with unconfirmed gluten levels, were analyzed for each food matrix. To simulate blinding, samples with unknown gluten levels were interspersed amongst QC samples for each batch of replicates.

Table 3. Gluten concentration in different beer samples

Food Matrix	Sample ID	Gluten (ppm)
Beer	Brand 1	2.4
	Brand 2	5.7
	Brand 3	n.d.
	Brand 4	2.3
	Brand 5	4.9
	Brand 6	1.4

Table 2 shows that food products that were advertised as gluten-free met those standards, and consistently revealed a gluten concentration under 20 ppm, while gluten-containing foods displayed much higher levels of gluten (86- to 1400-fold greater than the highest gluten level found in the food labeled gluten-free).

Table 3 shows the gluten concentration measured in different beer samples. The gluten concentration was calculated based on the signature peptides of glutenin was below 20 ppm, however, much higher concentrations of hordein were detected.

Figures 4 shows the peak area of a signature peptide of hordein for different beers.

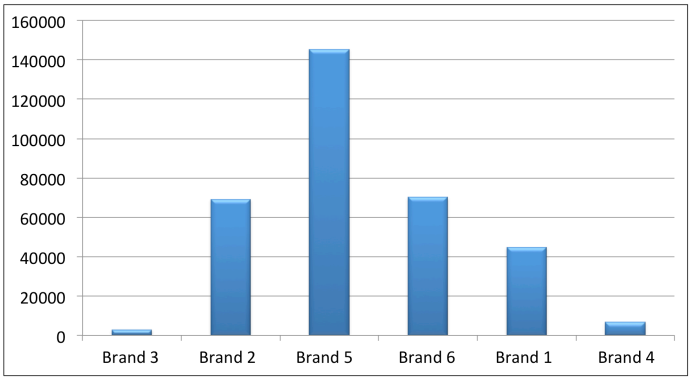

Figure 4. Peak area for a signature peptide of hordein in different beers

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Summary

A highly selective and sensitive LC-MS/MS method was developed to quantitate and screen for wheat gluten proteins in various food matrices, including bakery products, fermented beverages, and baby formula. This method measures unique, stable signature peptides using pre-determined MRM transitions for accurate quantitation and detection. The method was able to detect accurately gluten protein concentration as low as 5 ppm with a CV less than 20%.

Signature peptides for rye, barley, and oats were also established so that other major grains could be identified in various food matrices. LC-MS/MS methods permit the analysis of several MRM transitions simultaneously, offering a significant advantage over ELISA-based assays by supporting the detection of multiple grain species from the same food homogenate in a single injection.

References

1 L. Elli, et al.: 'Diagnosis of gluten related disorders: Celiac disease, wheat allergy and non-celiac gluten sensitivity' World J Gastroenterol. 6 (2015) 7110-7119

2 S. Lock: 'Gluten Detection and Speciation by Liquid Chromatography Mass Spectrometry (LC-MS/MS)' Foods 3 (2014) 13-29

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