

# Simultaneous Detection of 13 Allergens in Thermally Processed Food Using Targeted LC–MS/MS Approach

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**Food allergy is a major concern for public health and food industries. Because of the large numbers of food ingredients to be tested, MS is considered an alternative to existing techniques in terms of high selectivity, sensitivity, and capability to analyze multiple allergens simultaneously. In this study, we developed the method for monitoring significant peptides derived from 13 food allergens (milk, eggs, cod, shrimp, lobster, almonds, brazil nuts, cashew nuts, hazelnuts, walnuts, peanuts, wheat, and soybeans) and evaluated it in thermally processed foods (bread, cookie, fried fish, and frozen pasta). To select significant peptides to monitor, we used a bioinformatics-based approach and experimental confirmatory analysis. It was demonstrated that the developed method could detect target food ingredients from thermally processed foods successfully.**

Food allergy refers to an abnormal response of the immune system to certain food proteins (1, 2). Food allergy prevalence is currently estimated to be up to 10% of population in the developed world (3, 4). Because there is currently no cure for food allergies, strict avoidance of allergic substances is the only effective strategy for the management of a food allergy. The Food Allergen Labeling and Consumer Protection Act (FALCPA) requires that food manufacturers label food products intentionally containing eight major allergenic food ingredients (5). Milk, eggs, fish, crustacean shellfish, tree nuts, peanuts, wheat, and soybeans are identified as the major food allergens. On the other hand, the unexpected contamination of food by these allergens because of cross-contact during manufacturing or undeclared ingredients may cause the mislabeling of food. In the United States, 53 recalls caused by undeclared allergens were reported in 2017, and the number of recalls increased at the rate of 15.9% per year on average from 2005 to 2017 (6).

Immunological assays (e.g., ELISA and lateral flow assays) and PCR assays are the most commonly used routine techniques to detect allergenic foods. These techniques allow researchers to detect protein or DNA of allergenic substances with quick and easy sample handling; however, issues with these detection methods have also been reported, such as false positives caused by cross-reactivity and false detection of certain proteins in processed foods (7–9). These days, LC–tandem MS (MS/MS) is becoming an alternative technique for the detection of allergenic proteins with high selectivity and sensitivity as well as the capability of simultaneously analyzing multiple allergens. LC–MS/MS detects peptides as a significant trace of the proteins after sample preparation including extraction, reduction, alkylation, and enzymatic digestion, including the denaturation process in general. Unlike immunological assays, which rely on monoclonal or polyclonal antibody recognition of the epitope of target antigens, LC–MS/MS identifies denatured peptides by their chemical properties based on chromatography and MS (10).

Data-independent acquisition on high-resolution mass spectrometers has been demonstrated for the quantitative analysis of food allergens (11–13); however, targeted data acquisition using triple quadrupole mass spectrometers has been the most extensively used technique. To establish robust targeted data acquisition methods, the significant peptides need to be carefully selected based on specificity to the background matrix proteome and vulnerability after food processing (14).

In this study, we demonstrate the simultaneous analysis of multiple allergens of milk, eggs, cod, shrimp, lobster, almonds, brazil nuts, cashew nuts, hazelnuts, walnuts, peanuts, wheat, and soybeans and the detection of allergens in thermally processed foods such as bread, cookies, frozen pasta, and fried fish. The target foods were chosen based on FALCPA regulations. The selection of peptides for monitoring wheat glutenin was also discussed as an example of target peptides selection.

## Experimental

### *Protein Extraction, Reduction, Alkylation, Enzymatic Digestion, and Solid-Phase Extraction (SPE) Purification*

All chemicals and reagents were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise specified. Water and organic solvents were CHROMASOLV™ LC/MS grade, obtained from Honeywell (Morris Plains, NJ). Commercially available foods were obtained at local grocery stores. The samples were ground

Guest edited as a special report on “Mass Spectrometry: Status Quo in Food Allergen and Food Authenticity Applications” by Bert Popping and Carmen Diaz-Amigo.

Color images are available online at <http://aoac.publisher.ingentaconnect.com/content/aoac/jaoac>

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DOI: <https://doi.org/10.5740/jaoacint.19-0060>

into fine powders with dry ice by a GM-200 knife mill (Retsch GmbH, Haan, Germany). Ground samples (0.5 g each) were weighed into 15 mL centrifuge tubes. Five milliliters hexane was added into the tubes for defatting, and after centrifugation for 5 min at  $5000 \times g$ , the hexane layer was discarded; the process was repeated a total of three times. Samples were dried using a nitrogen evaporator (MICROVAP; Organomation, Berlin, MA). The extraction buffer, containing 2 mol/L urea, 1 mmol/L EDTA, 0.1 mmol/L phenylmethylsulfonyl fluoride, and 1 mmol/L dithiothreitol in 50 mmol/L Tris-HCl (pH 8.0), was added for protein extraction. After incubation at room temperature for 3 h and centrifugation for 15 min at  $5000 \times g$ , the supernatant was collected into a new 2 mL tube. Protein concentration was calculated using the Bicinchoninic Acid Protein Assay Kit. Aliquots of extracts containing 200  $\mu$ g proteins were transferred into a new 2 mL tube and were mixed with a reduction solution containing 8 mol/L urea and 5 mmol/L dithiothreitol in 50 mmol/L Tris-HCl (pH 8.0) for 60 min. Proteins were mixed with 500 mmol/L iodoacetamide solution for 30 min in the dark for alkylation. For proteolysis with trypsin, the concentration of urea was diluted to 1 mol/L with a digestion solution containing 50 mmol/L Tris-HCl (pH 8.0), and MS grade Trypsin Gold (Promega, Madison, WI) was added into the tube at a ratio of 1:100 (total protein in sample:trypsin). Protein digestion was accomplished after incubation at 37°C for 12 h. Ten microliters 50% formic acid in water was added to quench the reaction. After quenching the reaction, samples were cleaned up with an ISOLUTE<sup>®</sup> C18 SPE cartridge (25 mg/1 mL; Biotage, Uppsala, Sweden) and lyophilized with a FreeZone freeze dryer system (Labconco, Kansas City, MO). Dried samples were stored below -20°C until analysis. Prior to analysis, dried samples were reconstituted with 50  $\mu$ L 1% formic acid and 2% acetonitrile in water and transferred into TORAST-H<sup>™</sup> Bio Vials (Shimadzu, Kyoto, Japan).

### Quantitation of Wheat Allergens

Stable isotope-labeled synthetic peptides (heavy isotope-labeled peptides) were used for quantitation of native peptides (light peptides). Heavy-labeled peptides were labeled by incorporation of [13C615N2] lysine or [13C615N4] arginine located at each C terminus. Heavy-labeled peptides were purchased from Sigma-Aldrich. Lyophilized peptides were reconstituted to 50 pmol/L with 50% acetic acid in water as stock solutions. Lyophilized peptides and stock solutions were stored below -80°C until use. Heavy-labeled peptides were spiked into each sample vial at the final concentration of 100 fmol/ $\mu$ L. NIST wheat flour Standard Reference Material<sup>®</sup> (SRM) 1567B was used to prepare the calibration curve.

### LC-MS/MS Analysis

LC-MS/MS analysis was conducted using a Nexera X2 Ultra HPLC system coupled to a triple quadrupole mass spectrometer (LCMS-8050; Shimadzu). For mobile phases, 0.1% formic acid was used in water (A) and acetonitrile (B) at a flow rate of 0.5 mL/min. Ten microliters each sample was injected onto a Shim-pack XR-ODSH analytical column (2.0 mm id  $\times$  75 mm length, 1.6  $\mu$ m) maintained at 40°C. The gradient elution time program was set as follows: 2% B (0.0 min), 15% B (4.0 min), 40% B (7.0 min), 95% B (7.1–8.0 min), and 2% B

(9.1–10.0 min). For the rinse solvent, 70% methanol in water was used. Samples were stored in an autosampler at 4°C.

Peptides were monitored by single reaction monitoring, also known as multiple reaction monitoring, in positive electrospray ionization mode. The detection time window was set at 1 min. The target cycle time was set at 1000 msec with a 1 msec pause between each transition. The dwell time for each transition was automatically set at 6–32 msec. Other parameters for the mass spectrometer were set as follows: interface voltage of 1 kV, nebulizing gas flow of 1.5 L/min, heating gas flow of 20 L/min, drying gas flow of 5 L/min, interface temperature of 250°C, desolvation line temperature of 150°C, heat block temperature of 200°C, and collision-induced dissociation gas pressure of 270 kPa. All data were processed by LabSolutions (Shimadzu) and Skyline (15).

## Results and Discussion

### Selection of Significant Peptides from Wheat Glutenin

We monitored two subunits of glutenin as targets of wheat protein. Glutenin is the aggregate of high molecular weight glutenin subunit (HMW-GS) and low molecular weight glutenin subunit (LMW-GS). HMW-GS and LMW-GS account for 30 and 12% of storage protein in common wheat, respectively (16), and these are known as the major wheat allergens Tri a 26 and Tri a 36, respectively (17). First, four amino acid sequences for both glutenin subunits (P10385 and P10386 for Tri a 36, P10387 and P10388 for Tri a 26) were obtained from UniProt, and we prepared a method list for theoretically calculated tryptic peptides using Skyline. Among them, the only detectable peptides remaining in the list are potential target peptides. On the other hand, target peptides must be unique to background proteome matrices in order to avoid false positives. In the case of glutenin, the similar protein composites collectively referred to as gluten are found in other grains and grass families. We used a bioinformatics approach to select preferable peptides. The candidate peptides were gathered and used for a Basic Local Alignment Search Tool search using UniProt. The results were summarized in Tables 1 and 2. In Table 1, the results were merged into the genus. The “X” mark indicates that the peptide was found in the genus. The genus *Triticum* consists of many species of wheat, including common wheat (*T. aestivum*). All of the peptides were found in *Aegilops*, including *A. tauschii*, which is the ancestor of wheat. Peptides that were frequently found among other grasses and grains, such as AQLAQLPAMCR, ELQESSLEACR, and EGGDALSASQ, were removed from the candidate list to avoid false positives. However, candidate peptides derived from common wheat may not be found in the other species in the genus *Triticum*. There are no peptides preserved in all species. This means that it is important to monitor multiple peptides to reduce the risk of false positives. ELQELQER, SVAVSQVAR, AQQPATQLPTVCR, VFLQQQCIPVAMQR, and VFLQQQCSPVAMPQR were finally selected as the target peptides. In addition to the bioinformatics approach, we analyzed eight grains, including common wheat and durum wheat, to evaluate these peptides experimentally (Figure 1).

Quantitation of food ingredients involves the normalization of multiple factors, such as extraction efficiency and the recovery of peptides during proteolysis. We used reference

Table 1. Search results of tryptic peptides calculated from target proteins (P10385, P10386, P10387, and P10388) among grasses and grains

	<i>Triticum</i> (wheat)	<i>Aegilops</i> (grass)	<i>Agropyron</i> (grass)	<i>Australopyrum</i> (grass)	<i>Brachypodium</i> (grass)	<i>Citropsis</i> (grass)	<i>Dasyphyrum</i> (grass)	<i>Elymus</i> (grass)	<i>Erenopyrum</i> (grass)	<i>Henrardia</i> (grass)	<i>Heteranthellum</i> (grass)	<i>Leymus</i> (grass)	<i>Psathyrostachys</i> (grass)	<i>Pseudoroegneria</i> (grass)	<i>Taeniatherum</i> (grass)	<i>Thinopyrum</i> (grass)	<i>Hordeum</i> (barley)	<i>Secale</i> (rye)
ACQVMDDQLR	X <sup>a</sup>	X														X		
AQQLAAQLPAMCR	X	X				X	X	X	X	X	X	X		X	X	X	X	X
AQQPATQLPTVCR	X	X					X									X		
ELQELQER	X	X														X		
ELQESSLEACR	X	X	X				X	X	X	X	X	X	X	X	X	X	X	X
GGSFYPGETTPPQLQQR	X	X					X	X	X							X		
IFWGIPALK	X	X														X		
ILPTMCSNVNPLYR	X	X												X				
LEGGDALSASQ	X	X			X		X	X	X	X	X	X		X	X	X	X	X
LPWSTGLQMR	X	X					X	X	X			X	X	X	X	X	X	X
MEGGDALSASQ	X	X					X	X	X				X	X	X	X	X	X
QGSYYPGQASPPQPGGQPGK	X	X						X	X				X			X		X
QDPGQGHPEQK	X	X																
QQQIPVHPSVLOQLNPCK	X	X				X								X				
QVVDQQLAGR	X	X							X						X			X
QYEQTVPK	X	X							X									
SQMLQQSICHVMQQCCQLR	X	X				X								X				
SVAVSQVAR	X	X							X									
TTTSVPFGVGTGVGAY	X	X					X	X	X					X		X		
VFLQQCCIPVAMQR	X	X				X								X				
VFLQQCCSPVAMPQR	X	X				X				X	X	X	X	X			X	

<sup>a</sup> X = The peptide was found in the genus.

Table 2. Search result of tryptic peptides calculated from target proteins (P10385, P10386, P10387, and P10388) in genus *Triticum*

	<i>Triticum aestivum</i> subsp. <i>tibeticum</i>	<i>Triticum aestivum</i> subsp. <i>yunnanense</i>	<i>Triticum compactum</i>	<i>Triticum dicoccoides</i>	<i>Triticum dicocon</i>	<i>Triticum macha</i>	<i>Triticum monococcum aegilopoides</i>	<i>Triticum monococcum subsp. monococcum</i>	<i>Triticum polonicum</i>	<i>Triticum spelta</i>	<i>Triticum timopheevii araraticum</i>	<i>Triticum timopheevii subsp. araraticum</i>	<i>Triticum turgidum subsp. durum</i>	<i>Triticum urartu</i>
ACQVMDQQLR	X <sup>a</sup>									X				
AQLAALPAMCR	X			X				X	X	X	X	X	X	X
AQPATQLPTVCR	X	X	X							X				
ELQELQER	X									X				
ELQESSLEACR	X	X	X	X			X	X		X	X	X	X	X
GGSFYGETTPPQQLQQR	X									X				
IFWGIPALLK	X									X				
ILPTMCSNVPLYR	X					X								
LEGGDALSASQ	X							X		X			X	X
LPWSTGLQMR	X	X	X	X	X		X	X		X	X	X	X	X
MEGGDALSASQ	X	X	X	X	X		X	X		X			X	X
QGSYYPGQASPQQPGGQQPGK	X	X	X							X				
QQPGQGQHPEQGGK	X													
QQQIPVHPVSLQQLNPCK	X			X		X	X	X		X	X	X	X	X
QVVDQQLAGR	X	X	X	X	X		X	X		X	X	X	X	X
QYEQTVWPPK	X	X	X	X						X				
SQMLQQSICHVMQQCCQQLR	X	X		X		X	X	X		X				X
SVAVSQVAR	X	X	X							X				
TTTSVPFGVGTGVGAY	X			X		X								
VFLQQQCIPVAMQR	X	X		X		X	X			X		X	X	X
VFLQQQCSPVAMPQR	X			X			X	X						X

<sup>a</sup> X = The peptide was found in the genus.

materials to assess the method's sensitivity. A calibration curve was prepared by plotting a peak ratio of light- and heavy-labeled peptides against the concentration of food ingredients. A 1/concentration-weighted linear regression was used (Figure 2). The linear range was set at 3.9–250 ppm (mg/kg), and its  $R^2$  value was >0.995.

### Detection of Multiple Allergens from Thermally Processed Foods

We expanded the target of the method to detect tryptic peptides derived from 13 allergenic food ingredients: milk, eggs, cod,

shrimp, lobster, almonds, brazil nuts, cashew nuts, hazelnuts, walnuts, peanuts, wheat, and soybeans. The final method contained 245 transitions of light peptides for simultaneous monitoring (Table 3). To evaluate the performance of this method, we analyzed a mixture of peptides prepared from raw food materials of milk, eggs, cod, shrimp, lobster, almonds, brazil nuts, cashew nuts, hazelnuts, walnuts, peanuts, wheat, and soybeans, and we confirmed that this method can detect all of the signals from the mixture of peptides prepared from raw materials (Figure 3). We also analyzed thermally processed foods such as bread, cookie, fried fish, and frozen pasta (Figure 4). These samples have labels declaring that they contain

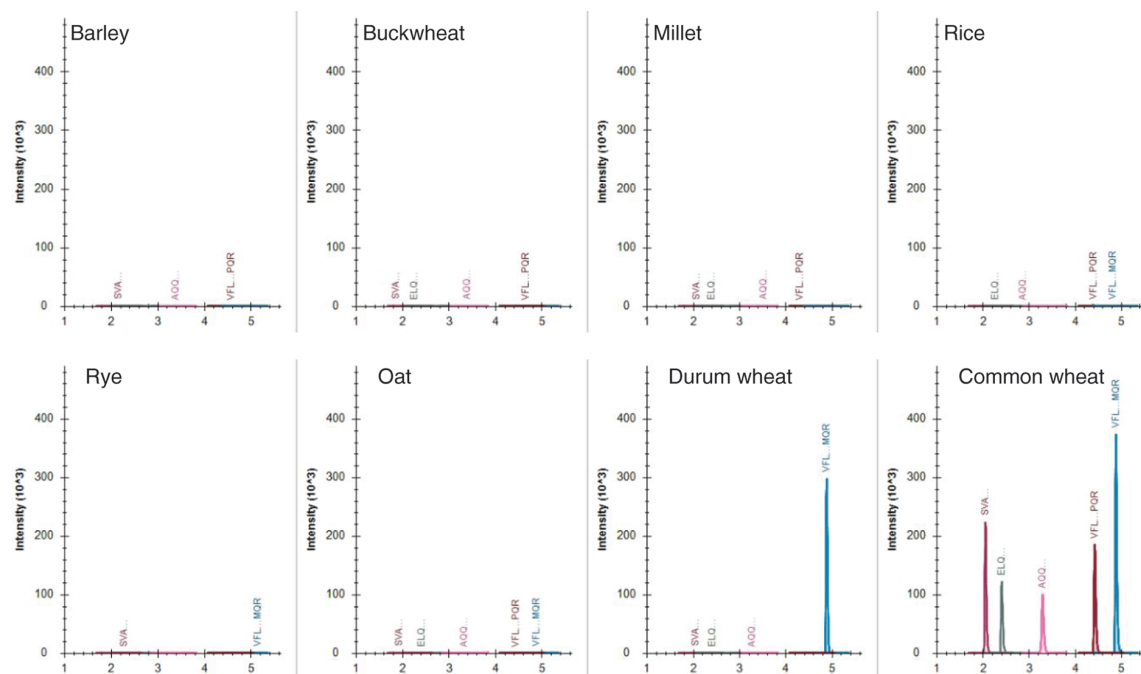


Figure 1. Chromatograms of five peptides derived from common wheat in barley, buckwheat, millet, rice, rye, oat, durum wheat, and common wheat.

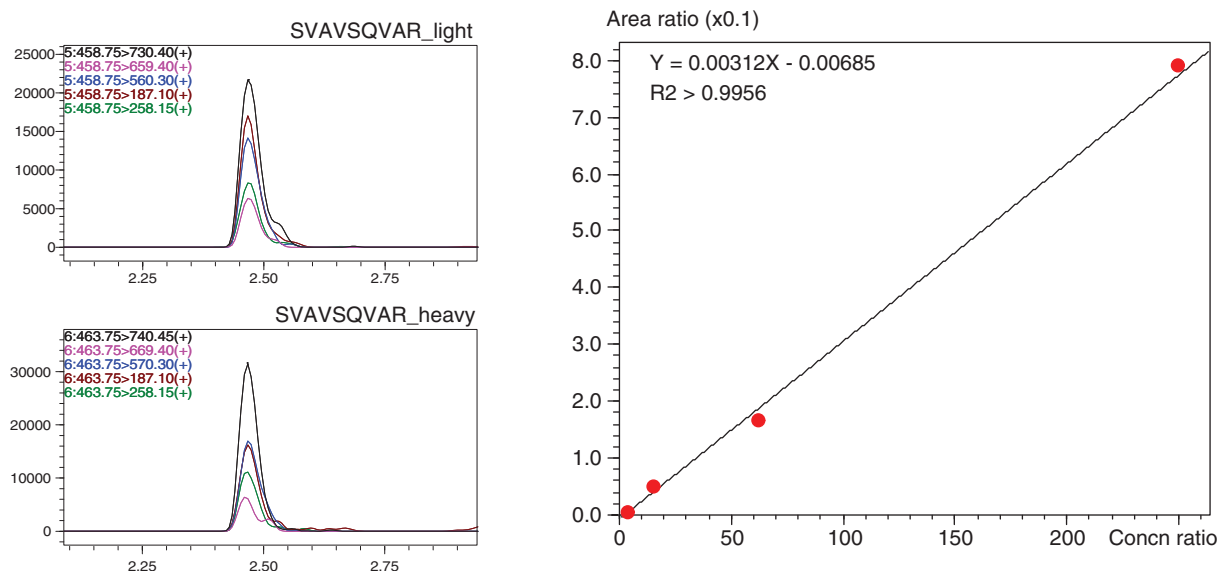


Figure 2. Representative chromatograms at 62.5 ppm and calibration curve using NIST wheat flour SRM 1567B.

**Table 3. Transitions for target light peptides for milk, eggs, cod, shrimp, lobster, almonds, brazil nuts, cashew nuts, hazel nuts, walnuts, peanuts, wheat, and soybeans**

Food (binomial name)	Protein (IUIS <sup>a</sup> name)	Compound name	Precursor ion	Product ion	Collision energy, V	Ion name
Common wheat ( <i>Triticum aestivum</i> )	High molecular weight glutenin (Tri a 26)	SVAVSQVAR	458.75	730.40	15.2	y7
				659.40		y6
				560.30		y5
				187.10		b2
				258.15		b3
		AQQPATQLPTVCR	735.40	1142.60	23.8	y10
				632.30		y5
				200.10		b2
				328.15		b3
				490.60		y5
	Low molecular weight glutenin GluB3-23 (Tri a 36)	ELQELQER	522.75	802.40	17.2	y6
				674.35		y5
				545.30		y4
				432.20		y3
				304.15		y2
		VFLQQQCIPVAMQR	859.45	701.40	27.6	y6
				505.25		y4
				247.15		b2
			573.30	701.40	15.8	y6
				904.45		b7
		VFLQQQCSPVAMPQR	894.95	400.25	28.7	y3
			596.95	798.45	16.7	y7
				602.30		y5
				400.25		y3
				247.15		b2
Milk ( <i>Bos taurus</i> )	Caseins (Bos d 8)	FFVAPFPEVFGK	692.85	1090.60	22.5	y10
				991.50		y9
				920.50		y8
				676.35		y6
				351.20		y3
		YLGYLEQLLR	634.35	991.55	20.7	y8
				771.45		y6
				658.40		y5
				277.15		b2
				334.20		b3
		NAVPIPTLNR	598.35	911.55	19.5	y8
				701.40		y6
				600.35		y5
				186.10		b2
				285.15		b3
		FALPQYLK	490.30	832.50	16.2	y7
				761.45		y6
				648.35		y5
				423.25		y3
				147.10		y1

Table 3. (continued)

Food (binomial name)	Protein (IUIS <sup>a</sup> name)	Compound name	Precursor ion	Product ion	Collision energy, V	Ion name
Egg ( <i>Gallus gallus</i> )	Beta-lactoglobulin (Bos d 5)	IDALNENK	458.75	803.40	15.2	y7
				688.35		y6
				504.25		y4
				229.10		b2
				300.15		b3
	Ovalbumin (Gal d 2)	NVLQPSSVDSQTAMVLVNAIVFK	820.80	214.10	24.7	b2
				327.20		b3
				455.25		b4
				903.55		y8
				790.50		y7
	Ovotransferrin (Gal d 3)	ATYLDCK	492.25	811.40	16.3	y6
				648.35		y5
				535.25		y4
				420.25		y3
				173.10		b2
		TDERPASYFAVAVAR	551.60	586.35	15.1	y6
				515.35		y5
				416.25		y4
				345.20		y3
				920.40		b8
Atlantic cod ( <i>Gadus morhua</i> )	Beta-parvalbumin (Gad m 1)	ALTDKET	424.70	664.30	14.2	y6
				563.25		y5
				448.25		y4
				248.15		y2
				185.15		b2
		AFFVIDQDK	541.80	864.45	17.8	y7
				717.40		y6
				618.30		y5
				219.10		b2
				366.20		b3
		SGFIEDELK	583.80	875.45	19.1	y7
				762.35		y6
				633.30		y5
				504.25		y4
				260.20		y2
Whiteleg shrimp ( <i>Litopenaeus vannamei</i> )	Tropomyosin (Lit v 1)	IQLLEEDLER	629.35	242.15	20.5	b2
				355.25		b3
				1016.55		y8
				903.45		y7
				790.35		y6
		IVELEELR	565.30	213.15	18.5	b2
				342.20		b3
				917.45		y7
				788.40		y6
				675.35		y5

Table 3. (continued)

Food (binomial name)	Protein (IUIS <sup>a</sup> name)	Compound name	Precursor ion	Product ion	Collision energy, V	Ion name
	Sarcoplasmic calcium-binding protein (Lit v 4)	VFIANQFK	483.75	247.15	16	b2
				867.45		y7
				720.40		y6
				607.30		y5
				536.30		y4
		AGGLTLER	408.75	129.05	13.7	b2
				745.40		y7
				688.40		y6
				518.30		y4
				417.25		y3
	Myosin, light chain 2 (Lit v 3)	EGFQLMDR	498.25	662.35	16.4	y5
				534.25		y4
				421.20		y3
				290.15		y2
				175.10		y1
		GTFDEIGR	447.70	736.35	14.9	y6
				589.30		y5
				474.25		y4
				345.20		y3
				232.15		y2
American lobster ( <i>Homarus americanus</i> )	Tropomyosin (Hom a 1)	ALQNAEGEVAALNR	728.40	1029.55	23.6	y10
				958.50		y9
				829.45		y8
				544.30		y5
				636.30		y12
		FLAEEADR	475.75	690.30	15.7	y6
				619.25		y5
				490.25		y4
				175.10		y1
				261.15		b2
		SITDELDQTFSELSGY	902.90	439.20	29	y4
				326.15		y3
				239.10		y2
				182.10		y1
				120.05		y2
Soybean ( <i>Glycine max</i> )	Trypsin inhibitor (Gly m TI)	NKPLVVEFQK	601.35	243.15	19.6	b2
				959.55		y8
			401.25	453.30	9.6	b4
				552.35		b5
		NKPLVVQFQK	600.85	422.25	19.6	y3
				958.55		y8
			400.90	453.30	9.6	b4
				552.35		b5
		CPLTVVQSR	530.30	649.35	17.4	y5
				550.30		y4
				371.15		b3
				802.50		y7



Table 3. (continued)

Food (binomial name)	Protein (IUIS <sup>a</sup> name)	Compound name	Precursor ion	Product ion	Collision energy, V	Ion name
Peanut ( <i>Arachis hypogaea</i> )	Cupin vicillin-type, 7S globulin (Ara h 1)	GIGTIISSPYR	582.30	689.40	19.1	y6
				588.35		y5
				489.30		y4
				993.55		y9
				835.45		y7
				722.40		y6
				609.30		y5
				522.25		y4
		NNPFYFPSR	571.25	229.10	18.7	b2
				669.35		y5
				506.25		y4
				686.40	18.5	y6
		GTGNLELVAVR	564.80	557.40		y5
				444.30		y4
Brazil nut ( <i>Bertholletia excelsa</i> )	11S globulin (Ber e 2)	NTIRPQGLLLPVYTNAPK	665.70	889.50	19.2	y8
				890.55		y16
				445.25		y8
				880.50		b8
				440.75		b8
		LYYVTQGR	500.25	723.40	16.5	y6
				560.30		y5
				461.25		y4
				277.15		b2
				440.20		b3
		LNANSVVYAVR	603.35	978.55	19.7	y9
				907.50		y8
				607.35		y5
				228.15		b2
				299.15		b3
		GIPVGVLANAYR	615.35	962.55	20.1	y9
				863.45		y8
				707.40		y6
				530.30		y10
				707.45		b8
Cashew ( <i>Anacardium occidentale</i> )	11S seed storage protein (Ana o 2)	NLFSGFDELLAEAFQVDER	767.70	993.45	22.8	y8
				793.40		y6
				646.30		y5
				304.15		y2
		ADIYTPEVGR	560.80	821.40	18.4	y7
				658.35		y6
				557.30		y5
				300.15		b3
				564.25		b5
		AMTSPLAGR	452.25	701.40	15	y7
				600.35		y6
				513.30		y5
				175.10		y1
				203.10		b2

Table 3. (continued)

Food (binomial name)	Protein (IUIS <sup>a</sup> name)	Compound name	Precursor ion	Product ion	Collision energy, V	Ion name
		TSVLGGMPEEVLANAFQISR	707.05	906.50	20.7	y8
				835.45		y7
				650.35		y5
				503.30		y4
				262.15		y2
English walnut ( <i>Juglans regia</i> )	Seed storage protein (Jug r 4)	TIEPNGLLLPQYSNAPQLVYIAR	857.45	1144.65	26.1	y10
				959.55		y8
				522.30		y4
				810.45		y14
				480.30		y8
		NEGFVWSFK	621.80	999.50	20.3	y8
				942.45		y7
				795.40		y6
				381.20		y3
				244.10		b2
Black walnut ( <i>Juglans nigra</i> )	Legumin (Jug n 4)	LVALEPSNR	499.80	786.40	16.5	y7
				715.35		y6
				602.30		y5
				473.25		y4
				213.15		b2
		GITGVLFPGCPETFEESQQGQSR	842.05	919.40	25.5	y8
				790.40		y7
				447.25		y4
				992.45		y17
				918.90		y16
European hazel ( <i>Corylus avellana</i> )	48 kDa glycoprotein (Cor a 11)	GNIVNEFER	539.25	793.40	17.7	y6
				580.25		y4
				226.10		y3
				172.05		b2
				285.15		b3
		ELAFNLPSR	523.80	804.45	17.2	y7
				733.40		y6
				586.35		y5
				472.30		y4
				359.20		y3
	11S globulin-like protein (Cor a 9)	ADIYTEQVGR	576.30	852.40	18.9	y7
				689.35		y6
				588.30		y5
				459.25		y4
				300.15		b3
		INTVNSNTLPVLR	720.90	1013.55	23.3	y9
				484.30		y4
				228.15		b2
				329.20		b3
			480.95	484.30	12.5	y4

Table 3. (continued)

Food (binomial name)	Protein (IUIS <sup>a</sup> name)	Compound name	Precursor ion	Product ion	Collision energy, V	Ion name
Almond ( <i>Prunus dulcis</i> )	Prunin 1 (Pru du 6)	ALPDDVLANAFQISR	815.45	1019.55	26.3	y9
				906.50		y8
				835.45		y7
				723.35		y13
			543.95	723.35	14.8	y13
	Prunin 2 (Pru du 6)	ALPDEVLANAYQISR	830.45	738.40	26.7	b7
				1035.55		y9
				922.45		y8
				738.40	15.1	b7
				503.30		y4
		ALPDEVLQNAFR	686.85	185.15	22.3	b2
				1188.60		y10
				748.40		y6
				635.35		y5
				507.25		y4

<sup>a</sup> IUIS = International Union of Immunological Societies.

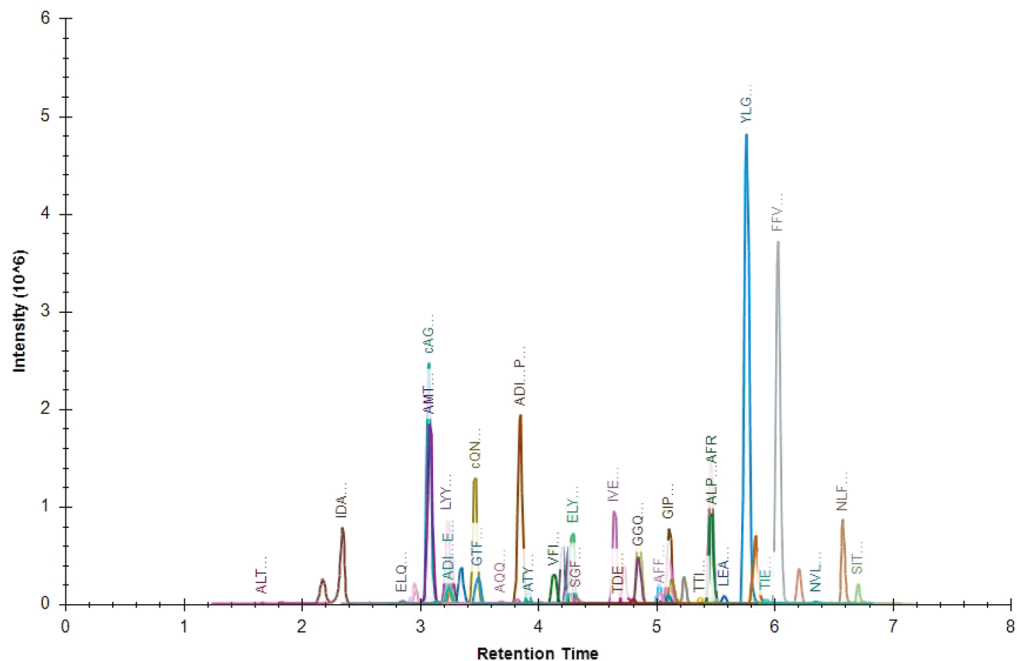


Figure 3. Chromatogram of a mixture of milk, eggs, cod, shrimp, lobster, almonds, brazil nuts, cashew nuts, hazelnuts, walnuts, peanuts, wheat, and soybeans.

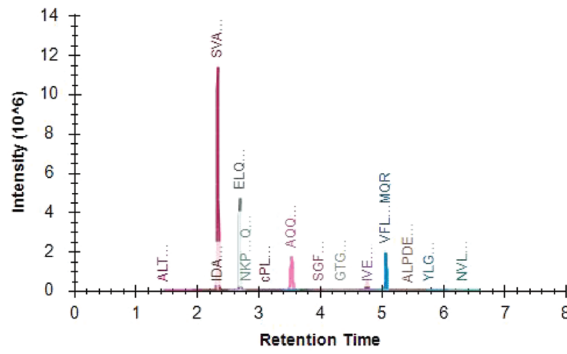
or can reasonably be expected to contain wheat, milk, eggs, peanuts, soybeans, cod, or shrimp as allergenic food ingredients (Table 4). A comparison of chromatograms of frozen pasta and standard samples is shown in Figure 5. The ratio of wheat-related peptides in frozen pasta suggested the existence of both common wheat and durum wheat, and a majority was durum wheat, which is commonly used to produce dry pasta (Figure 5c and 5d). In contrast, soybean peptides were not detected in frozen pasta despite their declaration (Figure 5e and 5f). The manufacturer listed soybean oil as an ingredient on the product label of the

frozen pasta, although it is known that highly purified oil does not contain protein. It suggested that the food samples did not contain soybean proteins.

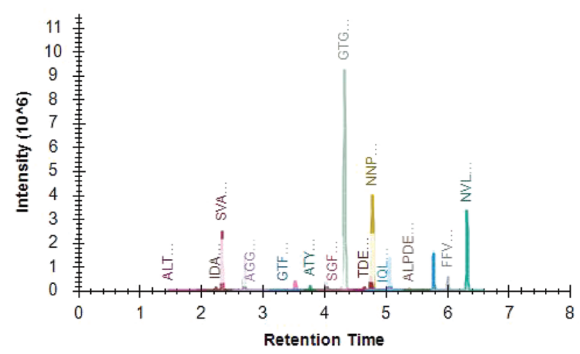
Conclusions

We developed the LC–MS/MS method for simultaneous monitoring of 13 allergenic food ingredients, including at least one food ingredient from the eight foods and food groups required by FALCPA. A total of 245 transitions were

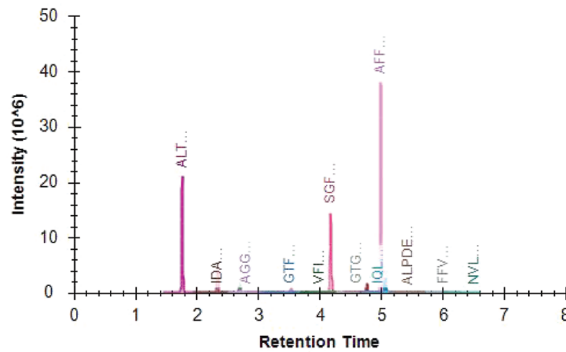
a) Bread



b) Cookies



c) Fried fish "beer batter cod"



d) Frozen pasta "garlic shrimp"

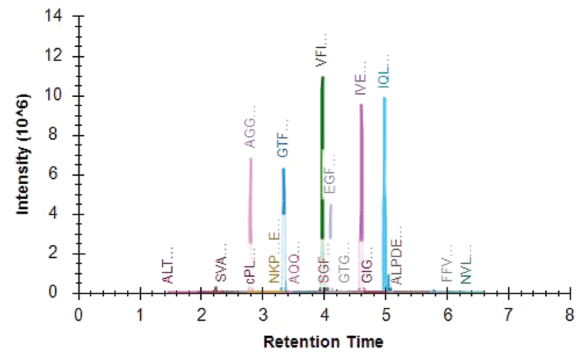


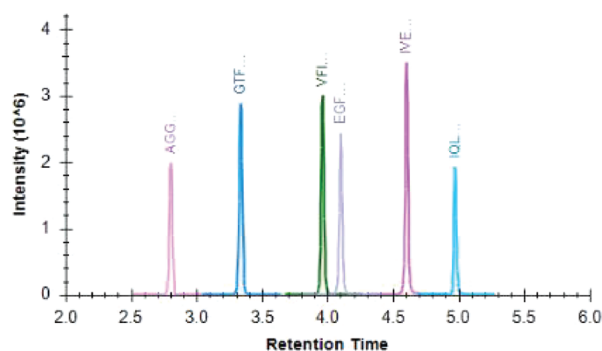
Figure 4. Chromatograms of four thermally processed foods: (a) bread, (b) cookies, (c) fried fish, and (d) frozen pasta.

Table 4. Summary of food labeling and analytical results of thermally processed foods (bread, peanuts cookie, fried fish, and frozen pasta)

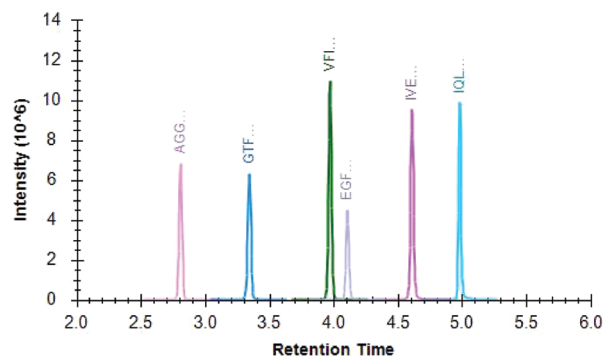
Food	Allergens	Bread		Cookie <sup>a</sup>		Fried fish, "beer butter cod"		Frozen pasta, "garlic shrimp"	
		Label	Detect	Label	Detect	Label	Detect	Label	Detect
Wheat	High molecular weight glutenin (Tri a 26)	x <sup>b</sup>	x	— <sup>c</sup>	x	x	x	x	x
	Low molecular weight glutenin (Tri a 36)		x		x		x		x
Milk	Caseins (Bos d 8)			—	x	x	x	x	x
	Beta-lactoglobulin (Bos d 5)				x		x		x
Eggs	Ovalbumin (Gal d 2)	x	x	—	x				
	Ovotransferrin (Gal d 3)		x		x				
Peanuts	Cupin, vicillin-type, 7S globulin (Ara h 1)			—	x				
Soybeans	Trypsin inhibitor (Gly m TI)			—				x	
Atlantic cod	Beta-parvalbumin (Gad m 1)			—		x	x		
Whiteleg shrimp	Tropomyosin (Lit v 1)			—				x <sup>d</sup>	x
	Myosin, light chain 2 (Lit v 3)								x
	Sarcoplasmic CBP (Lit v 4)								x

<sup>a</sup> No allergen information was provided.<sup>b</sup> x = Declared on label and/or detected from samples.<sup>c</sup> — = Not applicable.<sup>d</sup> It was declared as shrimp.

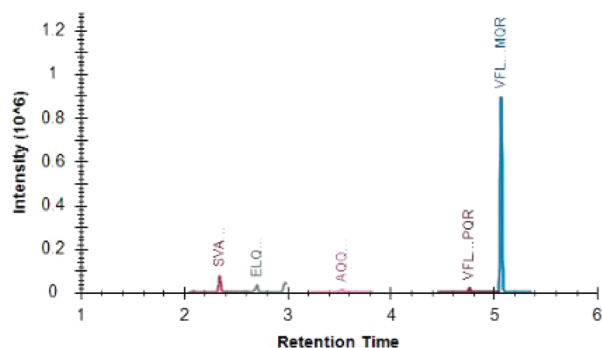
a) Shrimp peptides in frozen pasta



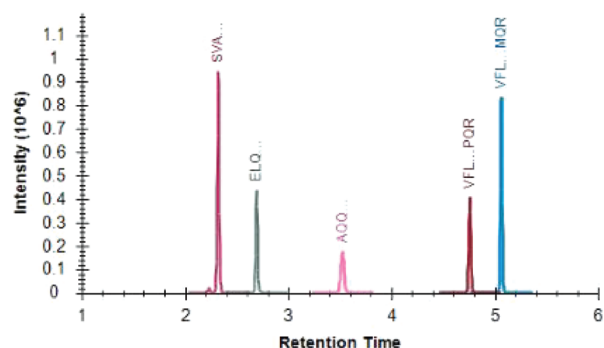
b) Shrimp peptides in whiteleg shrimp



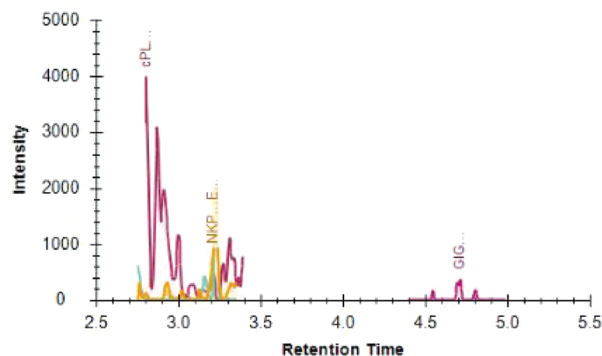
c) Wheat peptides in frozen pasta



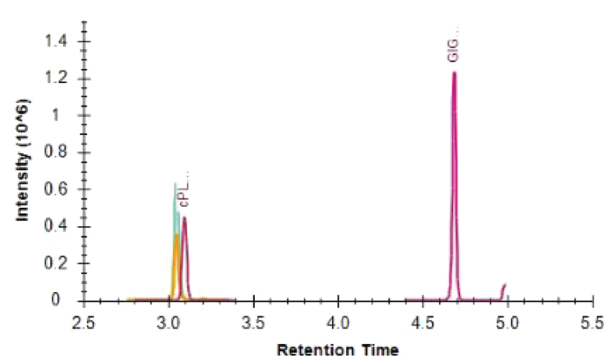
d) Wheat peptides in common wheat



e) Soybeans peptides in frozen pasta



f) Soybeans peptides in soybeans



**Figure 5.** Comparison of chromatograms of each ingredients in pasta and row materials: (a) shrimp peptides in frozen pasta, (b) shrimp peptides in whiteleg shrimp, (c) wheat peptides in frozen pasta, (d) wheat peptides in common wheat, (e) soybeans peptides in frozen pasta, and (f) soybeans peptides in soybeans.

set to monitor 50 peptides selected from 21 proteins. We successfully detected multiple allergens from raw materials and thermally processed foods. We also demonstrated the method's ability to quantitate wheat peptides ranging from 3.9 to 250 ppm (mg/kg) using NIST SRM 1567b wheat flour. These results confirm that this developed method could be used for monitoring of the 13 allergenic food ingredients from both raw material and thermally processed food.

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