SPECIAL GUEST EDITOR SECTION: FOOD ALLERGENS NEW METHODS

Simultaneous Analysis of Multiple Allergens in Food Products by LC-MS/MS

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There is currently no cure for food allergies, and sufferers can only rely on the correct labeling of foods to avoid allergens. Hence, it is important that analytical methods are sensitive and accurate enough to screen for the presence of multiple allergens in food products. In this study, we developed an LC-tandem MS method that is able to simultaneously screen or quantify the signature tryptic peptides of multiple allergen commodities. This method is capable of screening and identifying egg white, skim milk, peanut, soy, and tree nuts (almond, Brazil nut, cashew, hazelnut, pecan, pine nut, pistachio, and walnut) at a detection limit of 10 ppm in incurred bread and cookies. It was further tested for the quantitative analysis of whole-egg, whole-milk, peanut butter, and hazelnut commodities, which are incurred or spiked into selected food matrixes as defined in AOAC INTERNATIONAL *Standard Method Performance Requirement* (SMPR®) 2016.002. The method demonstrated excellent sensitivity with a Method quantitative limit of 3 ppm for whole egg and 10 ppm for the remaining three allergen commodities. It also demonstrated good recovery (60–119%) and repeatability (RSD_r <20%), with an analytical range of 10–1000 ppm for each allergen commodity and was able to meet the minimum performance requirements of the SMPR.

Food allergy is an adverse immune response, mostly IgE-mediated, to an antigenic food protein (1). Even limited exposure to a food protein allergen can invoke significant reactions, such as hives, itching, nausea, vomiting, and asthma, in sensitive individuals. Additionally, food allergy is one of the common causes of anaphylaxis, an acute and potentially deadly allergic reaction (2, 3). The prevalence of food allergy is rising worldwide and is estimated that it will reach 10% in developed countries (4). It has been estimated that 5% of adults and 8% of children are affected by food allergies in the United States (5). In Europe, a meta-analysis of lifetime and point prevalence of self-reported food allergy in children and adults estimated these values to be 17.3 and 5.9%, respectively (6).

Presently, there is no cure for food allergies, and sufferers must rely on the correct labeling of foods to avoid consuming allergens. The Food Allergen Labeling and Consumer Protection Act (FALCPA) in the United States has identified eight major foods that account for 90% of food allergic reactions, and these must be declared on any processed food products. These are milk, eggs, fish, crustacean shellfish, tree nuts, peanuts, wheat, and soybeans (7). These allergens are also included in the list of foods and ingredients recommended to be declared on the labels of prepackaged foods by the Codex Alimentarius, a collection of food standards established by Food and Agriculture Organization of the United Nations and the World Health Organization (8). Authorities in other parts of the world, such as the European Union, Canada, Japan, and Australia–New Zealand, have subsequently developed their own set of mandatory food-labeling legislation based on the allergen list derived from Codex (9). So far, no regulatory thresholds have been established for most allergens, except in Japan, which requires food items containing ingredients such as egg, milk, wheat, and peanuts at an allergen protein concentration >10 mg/kg (10 ppm) be labeled (10).

Despite improvements in measures to protect allergic consumers, undeclared allergens remain one of the biggest reasons for food recalls in many countries, with milk being the main culprit, followed by gluten, soy, and eggs (11). Common reasons for these recalls include manufacturing operational errors, unintended cross-contamination, mislabeling, and mispackaging. Diverse food types, ingredients, and processing methods pose additional analytical challenges to the accurate detection of the presence of allergenic materials in finished food products. Therefore, a method that can unambiguously confirm and identify allergens would be invaluable for food screening.

At present, immunoassays and PCR are the main techniques routinely used for detecting allergens. Immunoassays detect allergenic proteins in foods using antibodies. In particular, ELISA is the most widely used technique for detecting and quantifying allergens. Although ELISA tests are simple to operate, able to provide quick results, and are sensitive, they tend to be susceptible to cross-reactivity with matrix components, which could lead to false-positive results. They also lack multiplexing capabilities and require multiple kits to simultaneously screen for more than one allergen in a food sample. The antibody-binding properties of ELISAs may also be severely affected when the target allergenic protein is denatured or modified during thermal or other food processing, leading to

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false-negative results or reduced quantifications (12–15). On the other hand, PCR detects and quantifies allergen markers by amplifying the DNA that encodes the allergenic protein. PCR is highly specific and sensitive and can be used to identify multiple allergens. However, because it only detects DNA and not protein, PCR is unable to prove the presence or absence of allergenic protein. It is also not suitable for allergens with minute amounts of DNA, such as egg white and milk, and may lead to false-negative results. Similar to ELISA, PCR analysis is negatively affected by thermal or other food processing, which could destroy DNA and generate matrix interference.

Over the last several years, LC-tandem MS (MS/MS) has been gaining traction as the newest analytical technology for allergen detection. LC-MS/MS directly analyzes digested peptide fragments of allergenic proteins using their distinct molecular masses (16, 17). MS was first applied for allergen detection in the late 1990s, but it is only in the last several years that there has been a gradual increase in the number of publications on LC-MS/MS-based qualitative and quantitative allergen analysis (15, 18–20). This is not surprising because of the rapid advancement in LC-MS technology, which has improved the detection and quantitation of large and complex molecules, such as proteins. LC-MS/MS also demonstrates the highest potential for future improvements due to its reliability, sensitivity, and specificity relative to conventional methodologies. In particular, its multiplexing capability is especially attractive for multiallergen detection given the increased complexity and diversity of food matrixes.

Making use of these advantages, we aimed to develop a specific, selective, and sensitive LC-MS/MS-based method to detect multiple allergens in a single injection. Peptide-mapping experiments were first performed to identify unique and selective peptides that could be used as signature markers of each allergen. Next, a targeted multiple-reaction monitoring (MRM) method was optimized to screen marker peptides of egg white, skim milk, peanut, soy, almond, Brazil nut, cashew, hazelnut, pecan, pine nut, pistachio, and walnut in incurred bread and cookies. It was also used to analyze 16 commercial bakery products to verify its applicability to screen the 12 above-mentioned allergen commodities. In the second phase of the study, the method was applied for the quantitative analysis of hazelnut and standard reference materials (SRMs) of whole egg (egg white and egg yolk), whole milk, and peanut butter in 10 different food matrixes. The method was assessed for linearity, sensitivity, recovery, and repeatability based on the performance parameters and acceptance criteria listed in AOAC INTERNATIONAL *Standard Method Performance Requirement* (SMPR®) 2016.002 (21).

Experimental

Chemicals and Reagents

Hexane (95%; anhydrous), urea, Trizma base (≥99.0%), octyl β-D-glucopyranoside (OGS; ≥98%), ammonium bicarbonate (≥99.0%), tris(2-carboxyethyl)phosphine hydrochloride (TCEP), *S*-methyl methanethiosulfonate (MMTS), calcium chloride solution (volumetric; 1.0 M), formic acid (approximately 98%), and acetonitrile (HPLC grade; ≥99.9%) were purchased from Sigma-Aldrich (St. Louis, MO). Water was purified using a Sartorius arium pro UV Ultrapure Water System (Sartorius, Göttingen, Germany). L-1-Tosylamide-2-phenylethyl chloromethyl ketone-treated trypsin was obtained from SCIEX (Framingham, MA). Stable isotope-labeled peptides (the first marker peptide of protein 1 in each allergen) for egg white, milk, peanut, and hazelnut were custom synthesized for a purity of more than 95% by New England Peptide, Inc. (Gardner, MA). They were labeled at the C-terminal arginine, ($^{13}C_6{}^{15}N_4$)R, and used as internal standards (ISs) for the quantitative analysis.

Preparation of Incurred and Spiked Food Matrixes

(a) *Incurred cookies and bread for the screening analysis.*—Egg-white powder and skim-milk powder were purchased from a local baking supplies store (Phoon Huat Pte Ltd, Singapore). Raw forms of peanuts (shelled), soy, and tree nuts (shelled) were purchased from a local wholesaler (Teck Sang Pte Ltd, Singapore). Gluten- and allergen-free cookie and bread premixes (labeled as gluten-free by the manufacturers on the packaging) were purchased from a local supermarket. Except for egg-white powder and skim-milk powder, each allergen commodity (in whole) was baked at 180°C for 10–15 min in a convection oven, cooled, and ground into a fine powder using a food processor. Next, the ground allergen commodity was frozen at –80°C for 30 min and milled into a fine paste using an Omni Bead Ruptor 24 (Omni, Inc., Kennesaw, GA). Prior to baking, 100 g cookie or bread premixes were supplemented with 10, 100, and 1000 ppm (10, 100, and 1000 μg/g) of each of the 12 allergen commodities. For each concentration, each of the 12 allergens was weighed, and 100 g preweighed premix added. Appropriate amounts of canola oil and water were added, and the ingredients were mixed for 20 min using a stand mixer (Model No. MK-GB1; Panasonic, Singapore) to form homogenous dough. Next, dough was baked at 180°C for 18 min (cookies) or 40 min (bread) in a convection oven. Blank cookies and bread (0 ppm) were also prepared using the same procedure without addition of allergen commodities. After cooling, the baked cookies and breads were homogenized into a fine powder using a food processor.

For the analysis of commercial bakery products, 12 cookies and 4 bread products were purchased from a local supermarket and bakeries and homogenized into a fine powder using the food processor. They were allergen-free, contained ingredients derived from allergens, or had precautionary allergen labeling.

(b) *Incurred and spiked food matrixes for the quantitative analysis.*—SRMs for whole-egg powder (SRM 8445), whole-milk powder (SRM 1549a), and peanut butter (SRM 2387) were purchased from the National Institute of Standards and Technology (Gaithersburg, MD). For hazelnut, the SRM recommended in AOAC SMPR 2016.002 is formulated in liquid chocolate and, therefore, not appropriate for preparing other incurred or spiked food matrixes. Hence, in-house prepared baked hazelnut was used instead. Briefly, it was prepared by baking raw whole hazelnuts at 180°C in a convection oven for 15 min and milled into a fine paste using the Omni Bead Ruptor 24. The allergen commodities were incorporated into food matrixes in two ways: before baking at 180°C (incurred) and after processing (spiked). For our study, processed foods (purchased from local supermarkets) stated or tested to be free of the allergens of interest and were used to prepare the spiked matrixes. The exception was red wine, which was used as the matrix for whole-milk powder because milk was listed as the fining agent in white wine. The food matrixes that were incurred and spiked with allergen commodities are summarized in Table 1.

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For cookies and bread, incurred matrixes were fortified with 10, 100, and 1000 ppm of each allergen commodity prior to baking and prepared using the same recipe as described for the screening analysis. Blank matrixes (0 ppm) not fortified with any allergen commodity were also prepared. After baking and homogenization into a fine powder, the 10, 100, and 1000 ppm incurred matrixes were serially diluted (based on weight) using blank matrixes to prepare 1, 3, 30, and 300 ppm incurred samples. Briefly, 1 and 3 ppm incurred samples were prepared by diluting the 10 ppm incurred matrix using appropriate amounts of blank matrixes. Similarly, 30 and 300 ppm samples were prepared by diluting 100 and 1000 ppm incurred matrixes, respectively, with blank matrixes. Blank and incurred cookie dough were prepared using the same recipe, but without baking. For the rest of the food matrixes (Table 1), allergen-free foods were used as blank matrixes (0 ppm). These were spiked at 1, 3, 10, 30, 100, and 300 ppm by either serially diluting the 1000 ppm spiked sample with blank food matrix or adding allergen spike solutions to the blank food matrix (for whole-egg powder and whole-milk powder).

Sample Preparation

(a) *Lipid removal and protein extraction.*—1 g of each homogenized food sample was defatted by extraction twice with hexane and dried by evaporation in the fume hood overnight. 4 mL of protein extraction buffer containing urea, Trizma base, and OGS were added to each dried defatted sample and shaken at high speed using a vortex mixer for 1 h to extract allergen proteins. Next, samples were centrifuged for 15 min, and 500 µL supernatant (protein extract) were transferred into a clean 2 mL microcentrifuge tube.

For ice cream and salad dressing, the same volume of buffer was first added to dilute and extract the proteins from 1 g food sample. Samples were subsequently defatted by extraction twice with hexane (liquid–liquid extraction). They were centrifuged for 15 min, and 500 µL of the bottom aqueous phase (protein extract) were transferred into a clean 2 mL microcentrifuge tube.

(b) *Reduction, alkylation, and protein digestion.*—50 mM of TCEP were added to each 500 µL protein extract and the extracts reduced for 1 h. Next, the reduced extracts were alkylated by adding 100 mM MMTS and incubated for 20 min. Samples were diluted with 425 µL digestion buffer (5 mM calcium chloride in 100 mM ammonium bicarbonate solution) and enzymatically digested with 20 µL of 1 µg/µL trypsin at 37°C overnight (12–14 h). The reaction was quenched using 30 µL formic acid (approximately 98%) and mixed well on a vortex mixer. 450 µL of the digested sample were centrifuge-filtered using a 10 kDa MWCO filter (Merck Millipore, Billerica, MA). 200 µL of the filtrate were transferred into a 250 µL vial for LC-MS/MS analysis. For the quantitative analysis, 10 µL IS mix solution (containing 5 µg/mL of each labeled peptide IS) were added prior to filtration using the MWCO filter.

Recovery (for Quantitative Analysis)

For all food matrixes, recovery of the whole assay was assessed at 10, 100, and 1000 ppm (seven independent analyses per concentration) by comparing the area ratios of the spike-before sample with the spike-after sample and expressed as a percentage. In the context of this study, the spike-before samples refer to the incurred or spiked food samples. The spike-after samples refer to samples prepared by combining blank food matrix protein digests with allergen commodity protein digests.

Peptide-Mapping Analysis

The tryptic digests of the allergen commodities (raw and baked) were analyzed using an ExionLC AD HPLC system (20 µL mixer) interfaced with a TripleTOF 6600 system equipped with a DuoSpray Ion Source (SCIEX, Singapore). The autosampler and column oven temperatures were set to 10 and 30°C, respectively. The mobile phases consisted of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). The flow rate was set to 0.3 mL/min. Peptides were separated using a Phenomenex Kinetex C18 column (100 × 3 mm id, 2.6 µm; Torrance, CA), with a linear gradient program of 2–40% solvent B for 20 min. The column was flushed

Table 1. Priority allergen–matrix combinations listed in AOAC SMPR 2016.002 (21)[a]

		Egg	Milk	Peanut	Hazelnut
Food matrix	Incurred or spiked food matrix tested	Whole-egg powder, SRM 8445	Whole-milk powder, SRM 1549a	Peanut butter, SRM 2387	Baked hazelnut, prepared in-house
Cookies	Incurred	✓	✓	✓	✓
Bread	Incurred	✓	NR[b]	NR	NR
Dough	Incurred	✓	NR	NR	NR
Salad dressing	Spiked	✓	NR	NR	NR
Wine	Spiked	✓ (white wine)	✓ (red wine)	NR	NR
Infant formula	Spiked	NR	✓	NR	NR
Dark chocolate	Spiked	NR	✓	NR	NR
Ice cream	Spiked	NR	NR	✓	✓
Breakfast cereal	Spiked	NR	NR	✓	✓
Milk chocolate	Spiked	NR	NR	✓	✓

[a] Incurred or spiked food matrixes were prepared for quantitative analysis.

[b] NR = Not required by AOAC SMPR 2016.002.

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Table 2. MRM transitions of egg white and egg yolk (for whole eggs), milk, peanut, and hazelnut for quantitative detection on the QTRAP 6500 system

Allergen (species)	ID No.	Protein No.	Peptide No.	Frag No.	Q1, *m/z*	Q3, *m/z*
Egg white (*Gallus gallus*)	Ew1-1[a]	1	1	1	844.4	666.3
	Ew1-2			2		1121.5
	Ew1-1-IS	1	1	1	849.4	671.3
	Ew1-2-IS			2		1131.5
	Ew2-1	1	2	1	930.0	1017.5
	Ew2-2			2		888.5
	Ew3-1[b]	2	1	1	524.3	737.4
	Ew3-2			2		283.1
	Ew4-1	2	2	1	434.7	584.3
	Ew4-2			2		453.2
Egg yolk (*Gallus gallus*)	Ey1-1[c]	1	1	1	524.8	468.3
	Ey1-2			2		725.4
	Ey2-1	1	2	1	479.8	731.4
	Ey2-2			2		228.1
	Ey3-1	2	1	1	480.6	709.4
	Ey3-2			2		355.2
	Ey4-1	2	2	1	457.8	617.4
	Ey4-2			2		730.5
Milk (*Bos taurus*)	M1-1[a]	1	1	1	634.4	991.6
	M1-2			2		249.2
	M1-1-IS	1	1	1	639.4	1001.6
	M1-2-IS			2		249.2
	M2-1[d]	1	2	1	416.2	488.3
	M2-2			2		244.6
	M3-1	2	1	1	623.3	572.8
	M3-2			2		819.4
	M4-1	2	2	1	415.7	330.7
	M4-2			2		400.2
Peanut (*Arachis hypogea*)	Pn1-1[c]	1	1	1	771.4	1242.6
	Pn1-2			2		272.2
	Pn1-1-IS	1	1	1	776.4	1252.6
	Pn1-2-IS			2		272.2
	Pn2-1	1	2	1	684.4	748.4
	Pn2-2			2		836.4
	Pn3-1	2	1	1	688.8	300.2
	Pn3-2			2		930.5
	Pn4-1	2	2	1	786.9	804.4
	Pn4-1			2		1118.5
Hazelnut (*Corylus avellana*)	^{1}H-1[c]	1	1	1	576.3	689.4
	^{1}H-2			2		852.4
	^{1}H-1-IS	1	1	1	581.3	699.4
	^{1}H-2-IS			2		862.4
	H2-1	1	2	1	720.9	484.3
	H2-2			2		1013.6
	H3-1	2	1	1	682.4	872.5
	H3-3			2		743.5
	H4-1	2	2	1	524.8	822.4
	H4-2			2		227.2

[a] Quantifier ion for all allergens except in white and red wines.

[b] Quantifier ion for egg white in white wine.

[c] Quantifier ion.

[d] Quantifier ion for whole milk in red wine.

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with 90% solvent B for 5 min and equilibrated with 2% solvent B for 5 min. The injection volume was 10 µL. Information-dependent acquisition (IDA) was performed in positive electrospray ionization mode and consisted of a time-of-flight-MS survey scan from *m/z* 100 to 1250, followed by automated MS/MS product ion scans from *m/z* 100 to 1500 for the top 20 ions with the highest intensity. The MS/MS trigger criteria, including precursor intensity, charge state, and dynamic exclusion for the precursor ions, were applied. IDA data were acquired using Analyst TF 1.7 and processed using ProteinPilot Software 5.0 (SCIEX). The selection of marker peptides using the IDA data is discussed in more detail in *Results and Discussion*.

LC-MS/MS Analysis

(a) *Screening analysis.*—Digested samples were analyzed using a Prominence $UFLC_{XR}$ system with a 50 µL mixer (Shimadzu Corp., Kyoto, Japan) and interfaced with a QTRAP 4500 equipped with a Turbo V Ion Source (SCIEX). The HPLC column, autosampler temperature, column oven temperature, mobile phases, and flow rate were the same as for the peptide-mapping analysis. 30 µL of each sample were injected and separated with a linear gradient of 2–40% solvent B for 11 min. Following this, the column was flushed with 98% solvent B for 3.4 min and equilibrated with 2% solvent B for 5.6 min. MRM scans were performed in positive electrospray ionization mode for the 12 allergens (egg white, milk, peanut, soy, almond, Brazil nut, cashew, hazelnut, pecan, pine nut, pistachio, and walnut). The collision energy (CE) and declustering potential (DP) for each MRM transition were optimized using Skyline v3.5 to ensure maximum sensitivity. The data were acquired using Analyst 1.6.3 with the Scheduled MRM algorithm activated with a target scan time of 0.5 s for the multiplex detection of multiple MRM transitions for the marker peptides in a single injection of the food sample.

(b) *Quantitative analysis.*—For the quantitative method, analyses were performed using the ExionLC AD HPLC system (20 µL mixer) interfaced with a QTRAP 6500 equipped with an IonDrive Turbo V Ion Source. The HPLC column and LC-MS/MS parameters were the same as for the screening method. The MRM transitions of the marker peptides for egg (white and yolk), milk, peanut, and hazelnut and their optimized DP and CE for the QTRAP are summarized in Table 2. The same scan time for the screening analysis was used in the Scheduled MRM algorithm.

Data Processing

Peak integration and data processing were performed using the MQ4 algorithm in MultiQuant v3.0.2 software (22). A minimum Gaussian smooth width of 1 point was used for peak integration. When necessary, integration parameters, such as minimum peak height, noise percentage, or peak splitting factor, were adjusted. Due to the complexity of different food matrixes, peak integration was also checked to ensure that the analyte peak was selected and integrated correctly. The calibration curves were generated by plotting either the integrated peak areas or the area ratios (normalized with the labeled peptide IS) against incurred or spiked allergen commodity concentrations, with 1/x fitting.

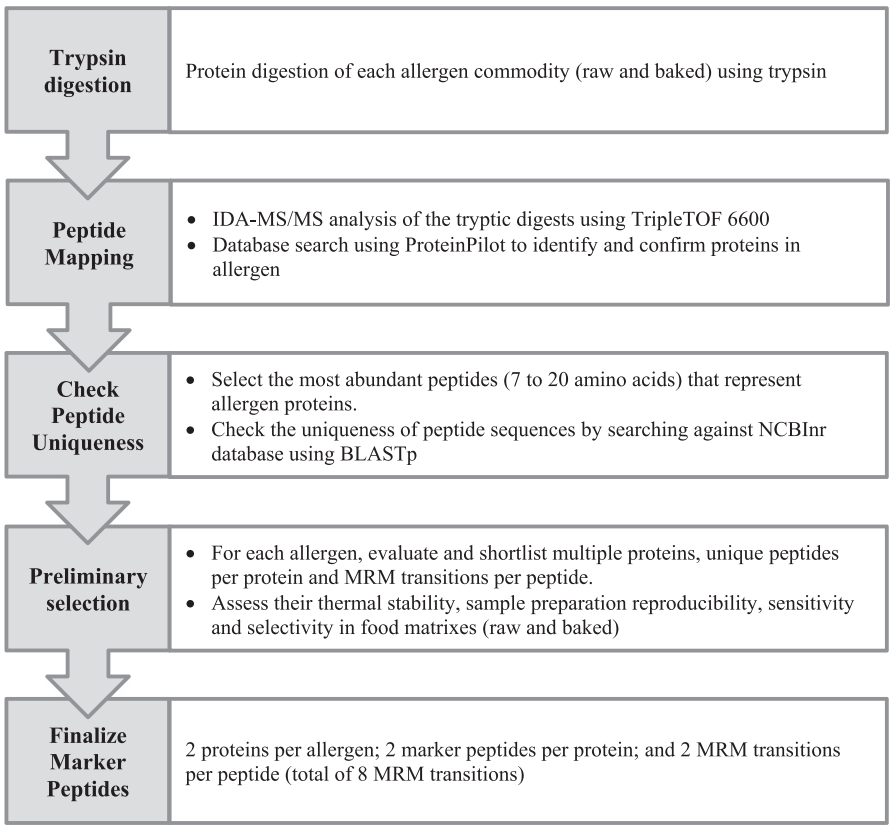

Figure 1. Workflow for the selection of unique marker peptides.

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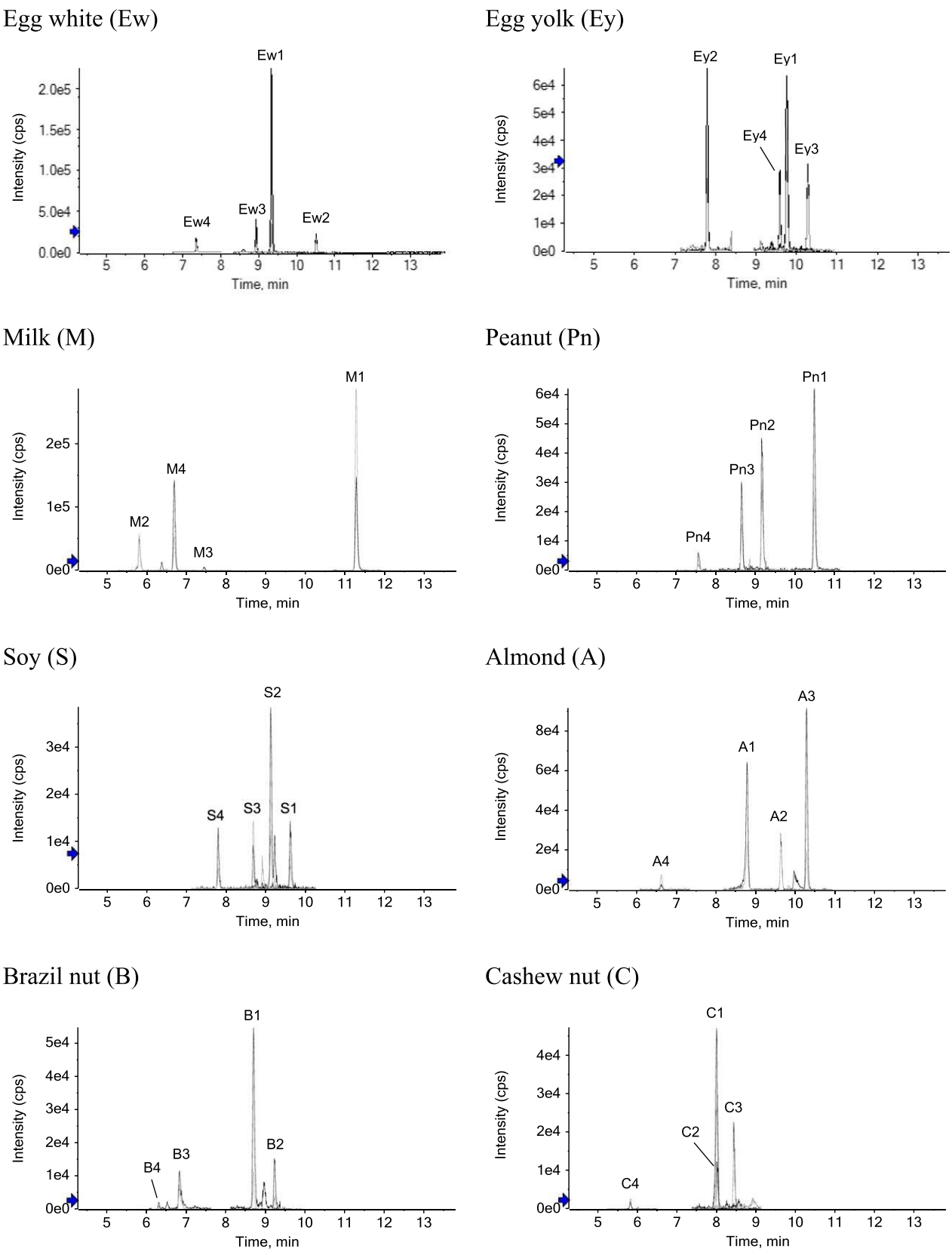

Figure 2. Extracted ion chromatograms (XICs) of the marker peptides of egg (egg white and egg yolk), milk, peanut, soy, and tree nuts in the cookie matrix incurred with 100 ppm of each allergen commodity. Thirty microliters of the sample were injected and analyzed using a Shimadzu Prominence UFLC$_{XR}$ system (50 µL mixer) interfaced with a QTRAP 4500 system. The first and second fragments for each marker peptide are shown as black and gray traces, respectively.

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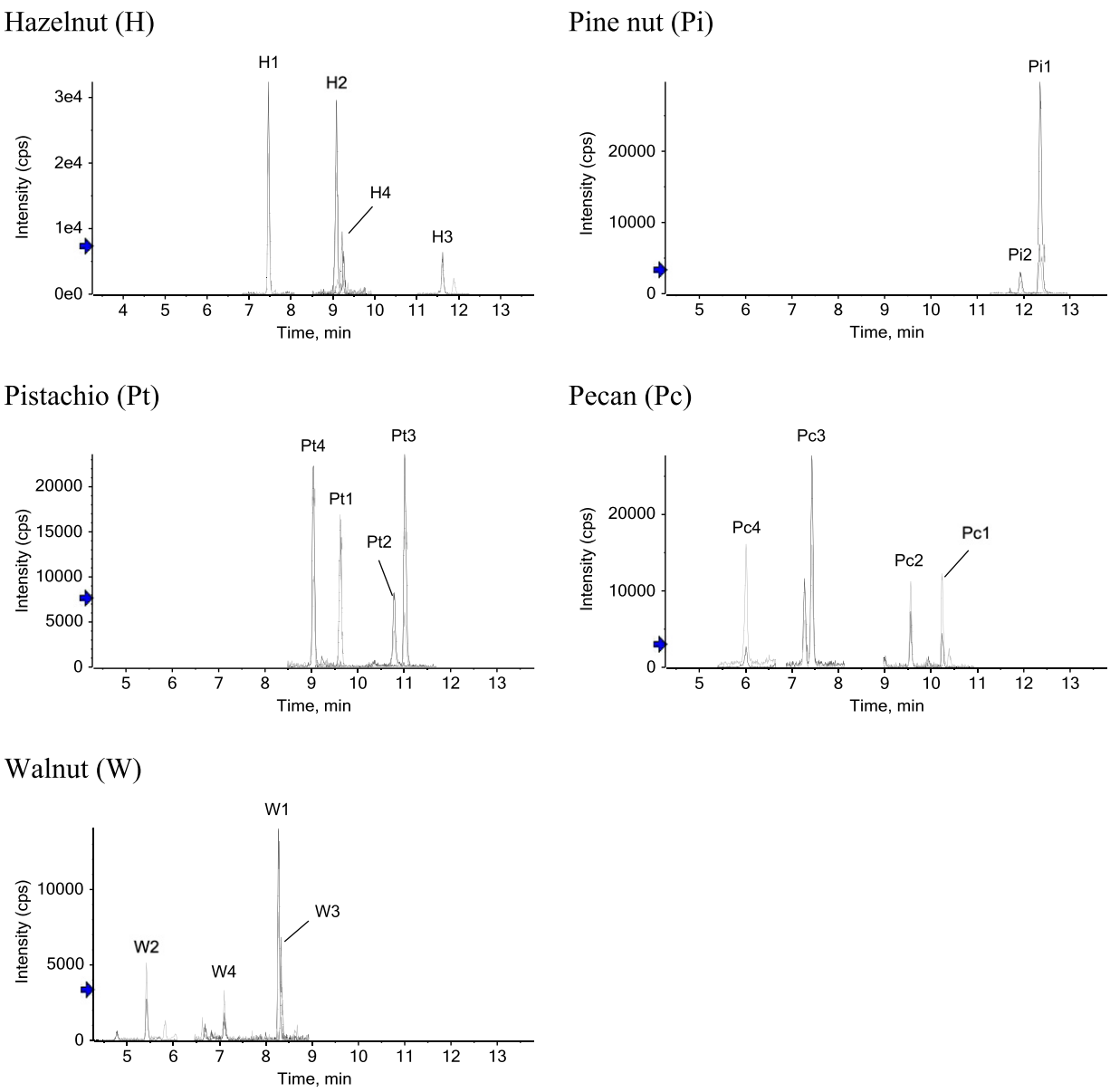

Figure 2. Continued.

Data Analysis and Interpretation

For the screening method, data were accessed qualitatively to determine whether the allergen commodity concentration, as represented by each marker peptide, could be detected at a threshold of more than 10 ppm (S/N >10). Hence, a MultiQuant query was developed for this purpose to facilitate data processing and interpretation. In the query, the peptide ratio for each peptide is calculated using the relative ion ratio of the second product ion to the first (Equation 1 and Table 2).

$$\text{Peptide ratio} = \frac{\text{Integrated area of second fragment ion}}{\text{Integrated area of first fragment ion}} \quad (1)$$

If an unknown sample contains an allergen, the following criteria must be fulfilled: (*1*) The difference for the peptide ratio between an unknown sample and a standard sample is less or equal to 30%; and (*2*) three out of the four peptides, i.e., 75%, for each allergen must fulfill the first criteria. These thresholds can also be adjusted based on user needs.

Results and Discussion

Selection of Marker Peptides

For a multiplexed method to work reliably, it is critical to select marker peptides that are not only sensitive and selective, but also unique to the allergen species. For this purpose, the key steps in the workflow for selecting unique marker peptides for each allergen are summarized in Figure 1. Peptide mapping was performed by running tryptic digestion of each allergen commodity using IDA analysis for protein identification. The resulting MS/MS spectra were searched with a ProteinPilot Paragon algorithm against protein databases [downloaded either from National Center for Biotechnology Information

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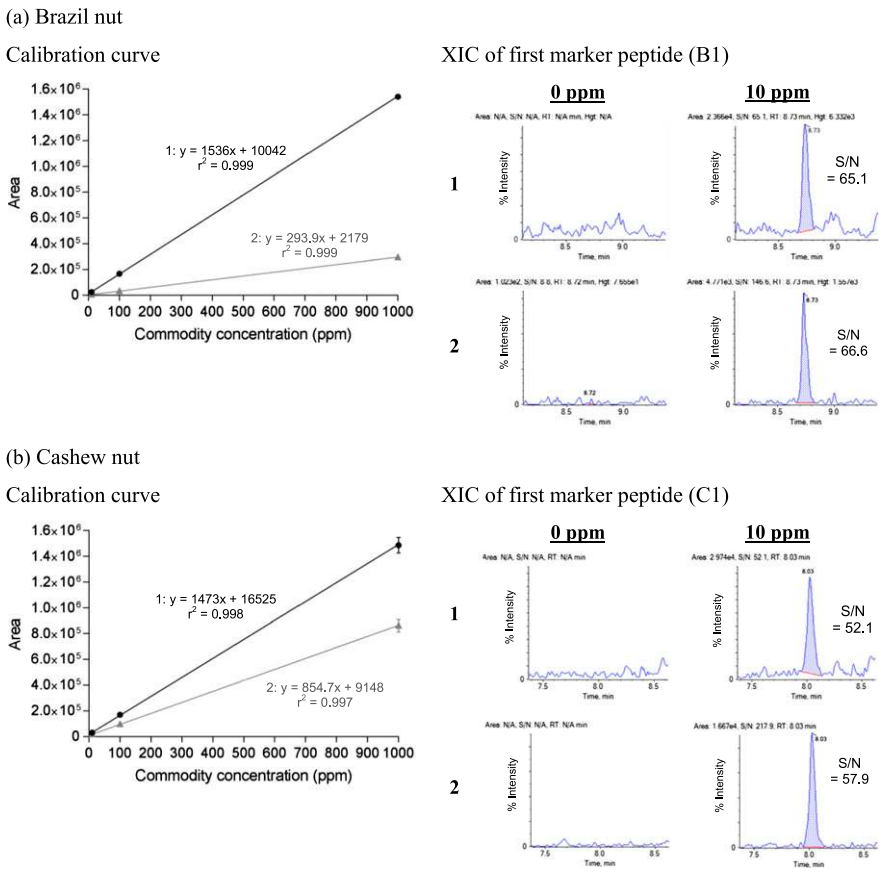

Figure 3. Calibration curves of the first marker peptide for (a) brazil nut and (b) cashew nut from 0 to 1000 ppm in incurred cookies (n = 5). For each peptide, XICs in blank (0 ppm) and 10 ppm (LOD) are also shown. Two MRM transitions, fragments 1 and 2 (labeled 1 and 2, respectively), were monitored for each peptide.

(NCBI) Protein or UniProtKB] to generate the list of proteins, peptide sequences, and abundance and modification information.

During the method development phase, the peptides that were most abundant (based on their MS intensities) with sequences between 7 and 20 amino acids were selected. This is because short peptides are less likely to be unique, whereas peptides with long sequences have poorer MS ionization efficiency. Peptides that contain cysteines (prone to oxidation),

Table 3. Intra- and interday precision and accuracy data of the quantifier ion for Brazil nut, cashew, pine nut, and pecan in the incurred cookie matrix

		Measured concn in cookie, ppm				
		Intraday, n = 3		Interday, n = 5		
Allergen	Concn of allergen commodity, ppm	Mean ± SD	RSD, %	Mean ± SD	RSD, %	Mean accuracy, %; n = 5
Brazil nut (LAENIPSR)[a]	10	10.1 ± 0.3	3.3	9.8 ± 0.6	5.8	97.5
	100	98.7 ± 3.6	3.7	102.8 ± 6.2	6.0	102.8
	1000	1001.2 ± 3.3	0.3	997.5 ± 5.6	0.6	99.7
Cashew (ADIYTPEVGR)	10	9.8 ± 0.4	3.7	9.7 ± 0.5	4.9	96.8
	100	101.9 ± 3.9	3.9	103.5 ± 5.2	5.0	103.5
	1000	998.3 ± 3.6	0.4	996.9 ± 4.7	0.5	99.7
Pine nut (ALPNFGEVSELLEGISR)	10	10.9 ± 0.0	0.4	10.5 ± 0.6	5.7	105.4
	100	90.2 ± 0.4	0.5	94.1 ± 6.6	7.0	94.1
	1000	1008.9 ± 0.4	0.0	1005.4 ± 6.0	0.6	100.5
Pecan (NFLAGQNNIINQLER)	10	10.3 ± 0.2	2.1	10 ± 0.4	4.2	100.1
	100	97.2 ± 2.4	2.5	99.9 ± 4.6	4.6	99.9
	1000	1002.6 ± 2.2	0.2	1000.1 ± 4.2	0.4	100.0

[a] The peptide sequence for the quantifier ion is shown in parentheses for each allergen commodity.

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post-translational modification sites, and missed cleavage sites were also removed from consideration. The uniqueness of the selected peptides was evaluated by searching against the NCBI non-redundant (NCBInr) database using the protein–protein BLAST query, BLASTp, to verify that these peptides are specific to their respective allergens and do not exist in other species, especially plants and mammals that are commonly used as ingredients in food products. At this point, peptides that were unique and reproducibly found in both the raw and baked allergen commodities with minimal variation in abundance were shortlisted. This is important because food processing, especially thermal processing, is known to affect the physiochemical properties of allergen proteins (23). Likewise, these peptides were also assessed for their sensitivity in raw and baked food matrixes (e.g., cookie dough and baked cookies) to make sure that they were minimally affected by heat exposure. The MRM transitions of the shortlisted peptides were also checked for specificity to ensure they were absent in other food allergens because food products may contain more than one allergen, and different allergenic ingredients may contain taxonomically related allergens. To minimize false-positive or -negative results, blank and incurred/spiked food matrixes were also checked to confirm the selectivity of the peptides and their MRM transitions in order to ensure minimal interference from food matrixes.

To ensure selectivity and specificity of the method, multiple proteins, peptides, and MRM transitions for each allergen were evaluated during the method development phase. After analyzing incurred bread and cookie matrixes, two proteins, three marker peptides per protein, and three MRM transitions for each peptide were initially shortlisted and optimized for each allergen. This resulted in at least 216 MRM transitions for the 12-allergen screening method. When the rest of the food matrixes were analyzed using this method, we observed that sensitivity and data quality were compromised for several allergen commodities, especially for the samples incurred or spiked at 10 ppm and below. This observation was especially prominent for marker peptides with lower sensitivity and could decrease the level of confidence in assay specificity. Hence, for the screening method, two proteins, two marker peptides per protein, and two MRM transitions per peptide, i.e., a total of eight MRM transitions, were selected for each allergen, except for egg white and pine nut (one protein was selected for these two allergens). With a reduced number of MRM transitions, a good compromise between sensitivity and specificity would be achieved while ensuring that the assay is accurate for multiple food matrix analysis. Furthermore, by using the peptide ratio calculation during data analysis, the identity of the allergen could be confirmed with an increased level of confidence. For the quantitative method, we further improved the selectivity of the method by using two proteins, two marker peptides per protein, and two MRM transitions per peptide to detect whole egg (egg white and egg yolk), milk, peanut, and hazelnut (Table 2).

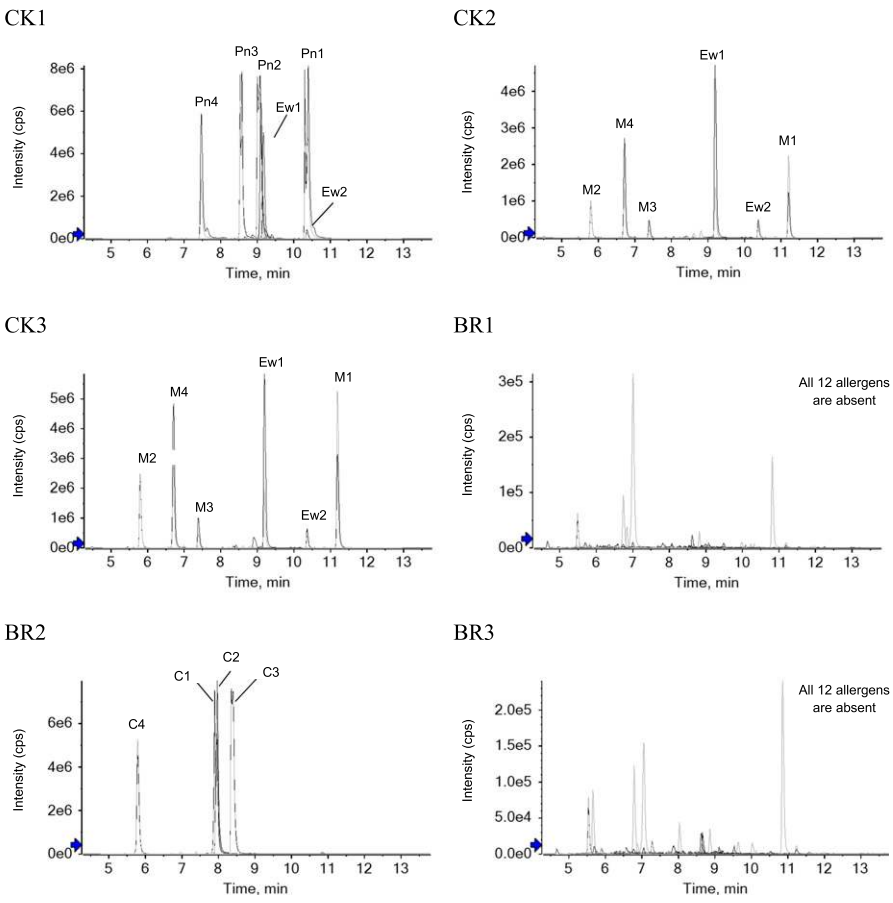

Figure 4. XICs of commercial cookie (CK1–CK3) and bread (BR1–BR3) products.

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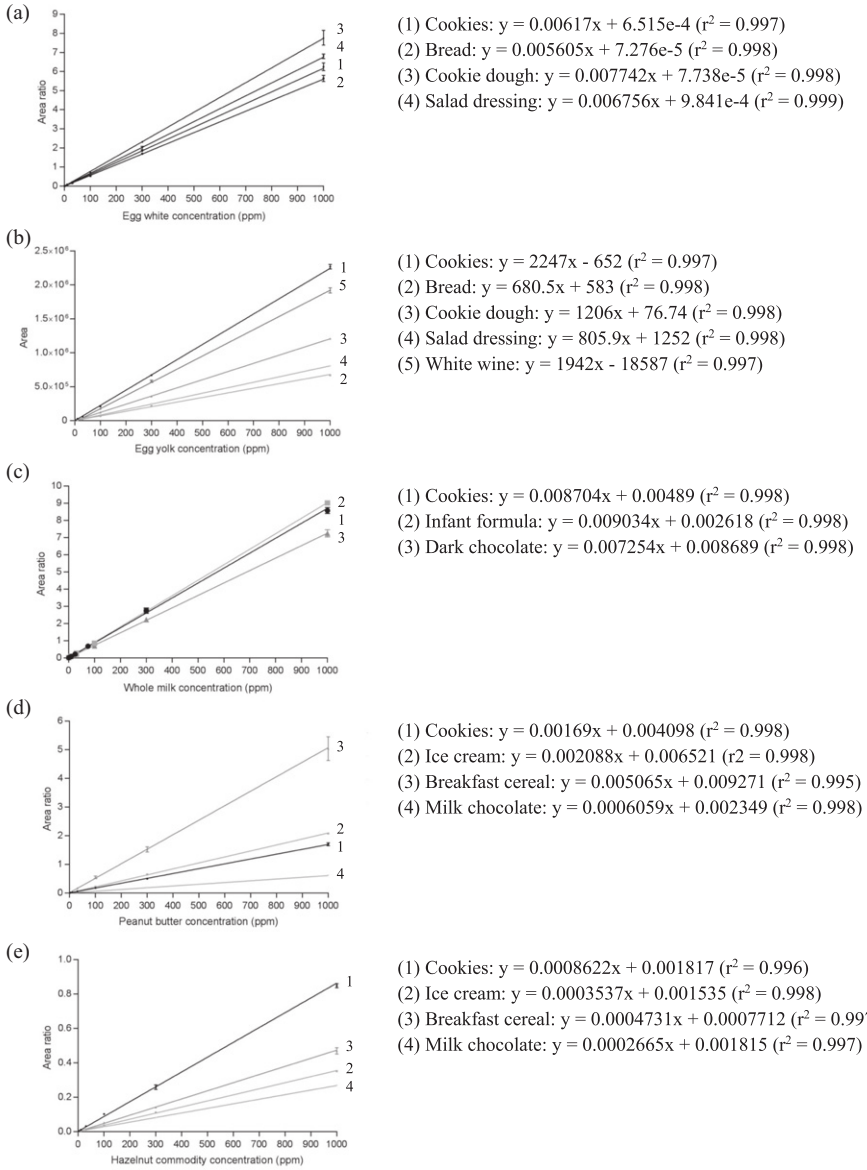

Figure 5. Calibration curves (n = 7) of the quantifier ion for (a) egg white, (b) egg yolk, (c) whole milk, (d) peanut butter, and (e) hazelnut in the various food matrixes defined in the AOAC SMPR (except for egg white and whole milk in white and red wines, respectively).

Twelve-Allergen Screening Analysis

Although most allergic individuals have reactions to a specific food, multiple food allergies are not uncommon and have been reported in adults and children (5). Eggs, milk, soy, and tree nuts are among the most commonly used ingredients in a large number of food products all over the world. However, immunoassays and PCR have some limitations when it comes

Table 4. Egg white: Ew1-1 (GGLEPINFQTAADQAR)[a]

Parameter	Acceptance criteria	Cookies	Bread	Cookie dough	Salad dressing	White wine[b]
Analytical range, ppm	10–1000	1–1000	1–1000	1–1000	1–1000	3–1000
MQL, ppm	≤5	≤1	≤1	≤1	≤1	≤3
MDL, ppm	≤1.65	≤0.1	≤0.1	≤0.1	≤0.1	≤0.25
Recovery, %	60–120	59.6–73.2	65.0–73.7	96.6–113.3	78.2–94.2	60.3–64.2
RSD_r, %	≤20	0.9–7.3	1.3–16.2	1.3–8.5	1.6–8.9	0.7–10.8

[a] Comparison of the SMPR acceptance criteria and quantitative method performance with respect to the quantifier ions of egg white and egg yolk (in whole egg), whole milk, peanut butter, and hazelnut in the different food matrixes. The peptide sequence for the quantifier ion is shown in parentheses in the table title for each allergen commodity. The recovery is the mean of the spiked or incurred recovery tested at 10, 100, and 1000 ppm. The MDL is estimated based on an S/N of 3:1 with respect to the MQL.

[b] For white wine, Ew3-1 (YFGYTGALR) was used as the quantifier ion instead of Ew1-1.

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Table 5. Egg yolk: Ey1-1 (LPLSLPVGPR)[a]

Parameter	Acceptance criteria	Cookies	Bread	Cookie dough	Salad dressing	White wine
Analytical range, ppm	10–1000	3–1000	3–100	3–1000	3–1000	10–1000
MQL, ppm	≤5	≤3	≤3	≤3	≤3	≤10
MDL, ppm	≤1.65	≤0.15	≤0.5	≤0.6	≤0.4	≤0.5
Recovery, %	60–120	59.8–67.0	61.0–67.5	86.6–103.3	39.9–40.7	98.8–117.5
RSD_r, %	≤20	0.4–6.9	2.3–10.6	1.9–12.2	0.9–8.2	0.8–10.4

[a] Comparison of the SMPR acceptance criteria and quantitative method performance with respect to the quantifier ions of egg white and egg yolk (in whole egg), whole milk, peanut butter, and hazelnut in the different food matrixes. The peptide sequence for the quantifier ion is shown in parentheses in the table title for each allergen commodity. The recovery is the mean of the spiked or incurred recovery tested at 10, 100, and 1000 ppm. The MDL is estimated based on an S/N of 3:1 with respect to the MQL.

to detecting multiple allergens in processed foods. Therefore, we aimed to bridge this gap by developing a method that is able to simultaneously screen multiple allergens in a single analysis. As shown in Figure 2, the LC-MS/MS-based screening method deployed in the present study is able to simultaneously detect 12 allergens, including egg white, milk, peanut, soy, and tree nuts (almond, Brazil nut, cashew, hazelnut, pecan, pine nut, pistachio, and walnut), which covers the five major classes of food allergens listed by the FALCPA and Codex Alimentarius. To facilitate the monitoring of many MRM transitions, the Scheduled MRM algorithm was used for monitoring each peptide transition only around its expected elution time. This decreases the total number of concurrent MRM experiments during a cycle and allows the cycle time and dwell time to be maintained. This approach not only maximizes the sensitivity of marker peptide detection, but also allows the method to be expanded as new allergen peptide markers are identified.

Due to the lack of commercially available SRMs, a majority of the allergen commodities were prepared in-house for this study. Instead of spiked food matrixes, incurred cookies and bread were prepared with the allergen commodities had been incorporated before processing (or baking, in this case) to mimic typical manufacturing conditions as closely as possible. The other key challenge was that there was a lack of regulatory guidance or industry agreement on whether the concentration of allergen commodity or protein should be quantified. Hence, for our study, we chose to quantify the concentration of each allergen at the commodity level, i.e., the actual food allergen commodity or physical substance, because this was more reflective of the scenarios in which contamination could possibly occur.

Despite being used for qualitative purposes, the screening method was still assessed for analytical range, linearity, sensitivity, and inter- and intraday precision, which were performed by preparing incurred cookie and bread matrixes fortified with 10, 100, and 1000 ppm of each allergen commodity. Because the first fragment of the first peptide (Table 2) typically demonstrated the highest S/N, it was chosen as the quantifier ion and used to plot the calibration curve for each allergen. The rest of the MRM transitions were used as qualifier ions to provide secondary confirmation for the identification of the presence of the allergen. Because this is a screening method, the S/N acceptance criteria were set as ≥ 10 and 3 for quantifier and qualifier ions, respectively, at 10 ppm allergen commodity concentration. As shown in Figure 3 and Supplemental Information Figure 1, the marker peptide signals for each allergen demonstrated a wide linearity range, from 10 to 1000 ppm, with good correlation coefficients ($r^2 \geq 0.995$) for both incurred matrixes (five independent analyses for each concentration per matrix). At 10 ppm, the allergens in both food matrixes had S/N of more than 10. For incurred cookies, the accuracy of the assay was found to be within 88.2–120.0%, whereas the intra- and interday precision of the assay were 0.0–9.7 and 0.4–15.3%, respectively (Table 3). For incurred bread, the accuracy of the assay was found to be within 86.6–113.0%, whereas the intra- and interday precision of the assay were 0.2–9.0 and 0.4–15.7%, respectively (Supplemental Information Table 1). These results confirmed that the method was suitable for the screening of the 12 allergens in food samples because both the accuracy and precision parameters were <20%, and demonstrated good sensitivity at 10 ppm.

To assess the applicability of the method to screen allergens in commercial food products, 12 cookies and 4 breads purchased from local bakeries or supermarkets were analyzed. As shown in Supplemental Information Table 2, a query was developed

Table 6. Whole milk: M1-1 (YLGYLEQLLR)[a]

Parameter	Acceptance criteria	Cookies	Infant formula	Red wine[b]	Dark chocolate
Analytical range, ppm	10–1000	3–1000	1–1000	10–1000	1–1000
MQL, ppm	≤5	≤3	≤1	≤10	≤1
MDL, ppm	≤1.65	≤0.75	≤0.5	≤0.5	≤0.5
Recovery, %	60–120	60.2–62.4	70.9–82.6	73.2–119.3	31.8–50.6
RSD_r, %	≤20	1.6–6.0	0.3–4.4	0.2–6.2	1.8–10.3

[a] Comparison of the SMPR acceptance criteria and quantitative method performance with respect to the quantifier ions of egg white and egg yolk (in whole egg), whole milk, peanut butter, and hazelnut in the different food matrixes. The peptide sequence for the quantifier ion is shown in parentheses in the table title for each allergen commodity. The recovery is the mean of the spiked or incurred recovery tested at 10, 100, and 1000 ppm. The MDL is estimated based on an S/N of 3:1 with respect to the MQL.

[b] For red wine, M2-1 (EDVPSER) was used as the quantifier ion instead of Ew1-1.

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Table 7. Peanut butter: Pn1-1 (WLGLSAEYGNLYR)[a]

Parameter	Acceptance criteria	Cookies	Ice cream	Breakfast cereal	Milk chocolate
Analytical range, ppm	10–1000	3–1000	10–1000	1–1000	10–1000
MQL, ppm	≤5	≤3	≤10	≤1	≤10
MDL, ppm	≤1.65	≤0.3	≤2.0	≤0.1	≤1.5
Recovery, %	60–120	57.3–68.7	86.6–96.5	84.6–110.6	16.7–22.7
RSD_r, %	≤20	1.2–5.5	1.5–9.3	0.7–7.5	1.2–10.3

[a] Comparison of the SMPR acceptance criteria and quantitative method performance with respect to the quantifier ions of egg white and egg yolk (in whole egg), whole milk, peanut butter, and hazelnut in the different food matrixes. The peptide sequence for the quantifier ion is shown in parentheses in the table title for each allergen commodity. The recovery is the mean of the spiked or incurred recovery tested at 10, 100, and 1000 ppm. The MDL is estimated based on an S/N of 3:1 with respect to the MQL.

and used to calculate the peptide ratio of each marker peptide for each allergen (Equation 1) and the peptide ratio of 10 ppm standard sample compared with the unknown sample to identify the presence of any allergen in the bakery products. As shown in Figure 4 and Supplemental Information Table 3, allergen-related signals were not detected in bread samples that were listed as egg-, milk-, and nut-free (BR1 and BR3). However, cookies and bread products that included allergens as ingredients, such as egg, milk, cashew, and peanut (CK1–CK3 and BR2), tested positive using the LC-MS/MS method. Notably, the method was able to detect soy in one of the cookie products (CK10) that was manufactured on equipment that also processed products that contain soy (indicated on the product packaging by the manufacturer). It should be noted that for the query to work properly, samples containing high concentrations of allergens should be diluted appropriately to ensure that the peptide ratios would not be skewed due to saturated signals.

Quantitative Analysis of Whole Egg, Whole Milk, Peanut Butter, and Hazelnut

To increase the sensitivity of the assay, the QTRAP 6500 was used for the quantitative method. The quantitative method was evaluated following the requirements stated in AOAC SMPR 2016.002 with respect to the commodity analytical range, method quantitation limit (MQL), method detection limit (MDL), linearity, recovery, and repeatability (RSD_r; 21). For all these parameters, the results were determined from seven independent analyses per concentration for each food matrix.

The calibration curves were generated by plotting the area ratio (of the quantifier ion to the labeled peptide IS) against the incurred or spiked allergen commodity concentration. One of the exceptions was egg yolk, for which the calibration curves were plotted using the area of the quantifier ion against the incurred allergen commodity concentration because the labeled peptide IS was not synthesized due to time constraints. Instead of using the first fragment, Ew3-1 and M2-1 were used as the quantifier ion for egg white and whole milk in white and red wines, respectively (Table 2). This is because, for white wine, protein 1 of the egg white (Ew 1 and Ew 2) demonstrated nonspecific interaction with certain matrix components and resulted in quadratic calibration curves (Supplemental Information Figure 2A and B). On the other hand, the calibration curves for protein 2 were linear (Supplemental Information Figure 2C and D). Likewise, such interaction was also observed for M1 and its labeled peptide IS in red wine (Supplemental Information Figure 3A); hence, M2-1 was used as the quantifier ion instead. Tannins most likely caused the nonspecific interaction with selected egg-white protein and milk peptides. This is not unexpected because egg (particularly egg white) and milk are commonly used as fining agents to remove tannins to reduce astringency in white and red wines. Overall, all allergen commodities demonstrated a wide analytical range of at least 3 orders with good r^2 (≥0.995) in the food matrixes, as required by the SMPR (Figure 5 and Tables 4–8). Notably, the gradients of the calibration curves for egg white in the four food matrixes differed by <14%, and a similar observation was seen for milk (<12% difference; Figure 5A and C). This seemed to indicate the possibility of using a single calibration curve for different food matrixes. However, this was not reproducible for peanut butter and hazelnut (Figure 5D and E) and highlights the importance of generating calibration curves in the food matrix of interest for quantitative analyses.

In general, the method demonstrated adequate sensitivity at either an MQL of ≤3 or 10 ppm in most food matrixes, as required by the SMPR (Tables 4–8, Figure 6, and Supplemental Information Figures 4–7). However, the sensitivity of the quantifier ions for hazelnut in milk chocolate was low and unable to reach the stipulated MQL of 10 ppm (Supplemental

Table 8. Hazelnut: ^{1}H-1 (ADIYTEQVGR)[a]

Parameter	Acceptance criteria	Cookies	Ice cream	Breakfast cereal	Milk chocolate
Analytical range, ppm	10–1000	3–1000	10–1000	3–1000	30–1000
MQL, ppm	≤5	≤3	≤10	≤3	≤30
MDL, ppm	≤1.65	≤0.25	≤2.0	≤0.45	≤10
Recovery, %	60–120	70.5–77.6	23.9–31.7	39.8–43.9	5.8–8.8
RSD_r, %	≤20	1.2–8.4	2.1–12.9	0.4–8.0	0.3–6.9

[a] Comparison of the SMPR acceptance criteria and quantitative method performance with respect to the quantifier ions of egg white and egg yolk (in whole egg), whole milk, peanut butter, and hazelnut in the different food matrixes. The peptide sequence for the quantifier ion is shown in parentheses in the table title for each allergen commodity. The recovery is the mean of the spiked or incurred recovery tested at 10, 100, and 1000 ppm. The MDL is estimated based on an S/N of 3:1 with respect to the MQL.

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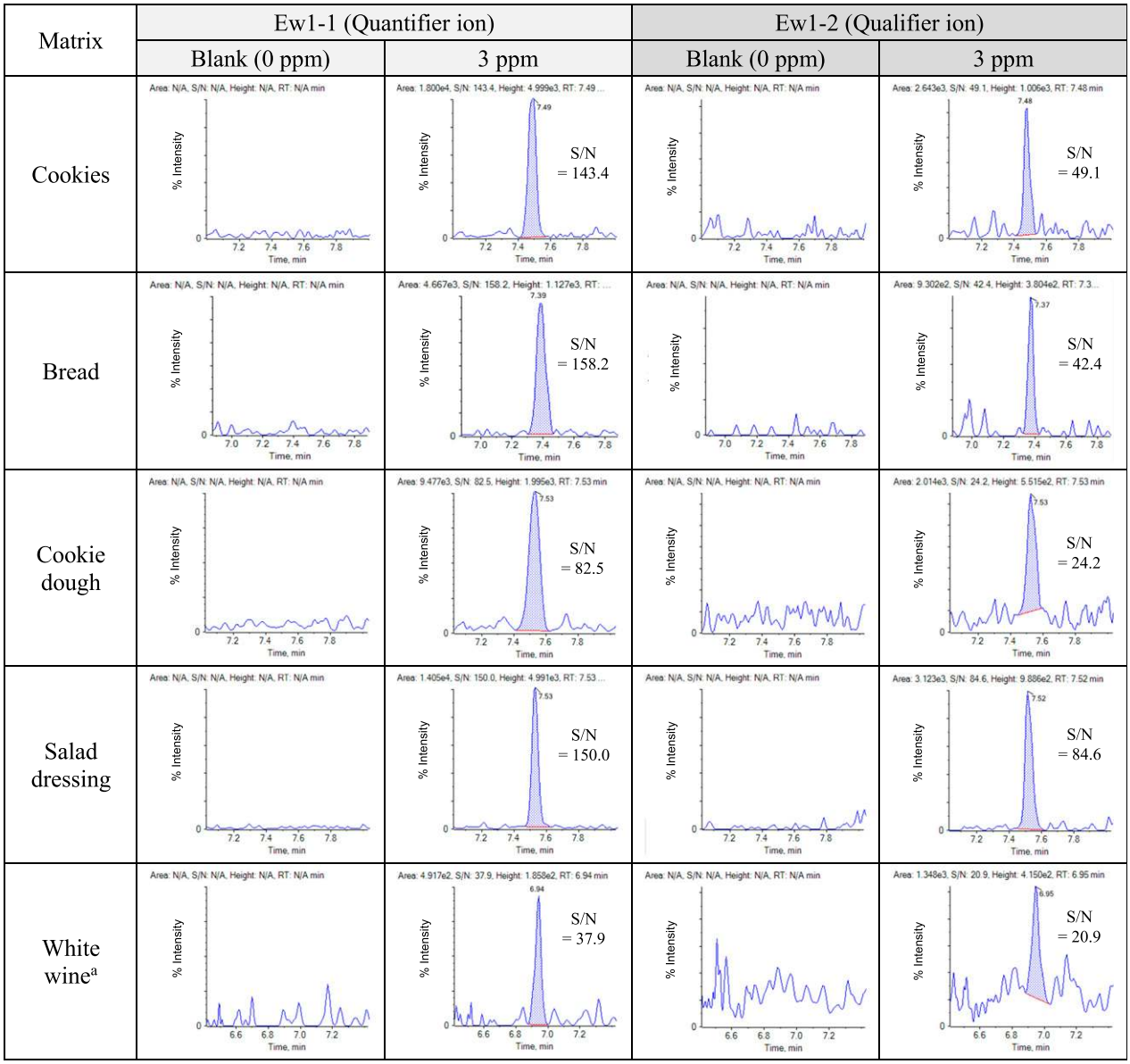
Information Figure 7). The MQLs for the rest of the qualifier ions were also at 30 ppm (except for H3, with an MQL of 10 ppm). Although the quantifier ion for peanut was able to attain an MQL of ≤10 ppm, the rest of the qualifier ions were unable to do so. The poor sensitivity of these two allergen commodities is likely due to matrix effect, which was exacerbated by poor recoveries (discussed in detail below). It was also observed that the overall sensitivity of egg yolk was approximately five times lower than egg white (based on MQLs), and a similar observation had been reported by Planque et al. (24). Although egg yolk peptides demonstrated a much lower sensitivity that egg white, there are food products that contain egg white and egg yolk exclusively. Hence, it is still necessary to include egg yolk in the method.

For most food matrixes, the method demonstrated adequate recovery: from 60 to 119% (Tables 4–8). Although the recovery of egg yolk in salad dressing is approximately 40%, it was still able to meet the SMPR requirement for sensitivity (Supplemental Information Figure 4). The recoveries of whole milk in milk chocolate, peanut butter in milk chocolate, and hazelnut in ice cream and milk chocolate matrixes are significantly lower (6–51%). The poor recovery could be the key reason why hazelnut was unable to reach the minimum SMPR requirement for the MQL and MDL in milk chocolate. This is not surprising, as chocolate has been reported to be a challenging matrix because it contains high concentrations of tannins/polyphenols, which have a high propensity to bind to proteins (25). To improve sensitivity and recovery, solid-phase extraction is recommended as a postdigestion sample cleanup step to improve sensitivity and recovery for food matrixes such as chocolate and ice cream.

Lastly, the method demonstrated good precision, was repeatable (with an overall RSD_r of 0.4–16.2% across 3 days of analyses), and fulfilled the SMPR requirements (≤20%).



[a] For white wine, Ew3-1 and Ew3-2 were used as quantifier and qualifier ions, respectively

Figure 6. XICs of the quantifier ion and one of the qualifier ions for egg white (Ew1-1 and Ew1-2, respectively) in blank and 3 ppm incurred or spiked food matrixes.

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Conclusions

An LC-MS/MS-based method for detecting and identifying 12 allergens, including egg white, skim milk, peanut, soy, and tree nuts (almond, Brazil nut, cashew, hazelnut, pecan, pine nut, pistachio, and walnut), was developed for rapid screening in bread, cookies, and bakery products. Unlike immunoassays, this screening method detects multiple peptides from each allergen protein, thus improving method specificity and minimizing the potential for false-positive and -negative results. Using only a single sample preparation method and multiplexed data acquisition, more allergens than previously reported were screened and differentiated from other food ingredients contained in the baked food matrixes.

The method was further tested for the quantitation of whole egg (egg white and egg yolk), whole milk, peanut butter, and hazelnut in food matrixes listed in AOAC SMPR 2016.002. Overall, the quantitative method was able to meet the minimum method performance requirements stated in AOAC SMPR 2016.002. For all food matrixes except chocolate (peanut butter and hazelnut), the target commodity analytical range of 10–1000 ppm was achievable, and the method demonstrated good repeatability, with an RSD_r of <20%. For most food matrixes tested, the recoveries of the allergen commodities ranged from 60–119%. The method demonstrated good sensitivity and was able to detect whole egg and all other studied allergen commodities at an MQL of 3 or 10 ppm, respectively (or lower for some food matrixes). Similar results were also achieved for the rest of the qualifier ions. These results confirmed that the method was suitable for screening the four allergen commodities in the selected food matrixes. Great care was taken, as much as possible, to ensure peptide uniqueness, especially by testing the common raw materials used in the food matrixes listed in the AOAC SMPR. Thus, the scope of the method is limited to these matrixes and allergen commodities. Because there is neither database nor empirical evidence that these marker peptides exist in the common ingredient(s) for these matrixes, we could only assume that they are unique for the products within the scope of this method. For other food matrixes beyond the scope, it is highly recommended that the user analyze blank food matrixes to verify the presence of interferences and re-evaluate method performance.

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