

Detection and Quantitation of Selected Food Allergens by Liquid Chromatography with Tandem Mass Spectrometry: First Action 2017.17

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Background: In response to a need for accurate and reliable methods for food allergen regulatory compliance, a method for the detection and quantitation of whole egg, whole milk, peanut, and hazelnut in eight food matrices was developed and evaluated in a single-laboratory validation. The matrices include cookies, cookie dough, bread, breakfast cereal, salad dressing, ice cream, and red wine. **Objective:** The method was compared with *Standard Method Performance Requirements* (SMPR) 2016.002 established by the AOAC Stakeholder Panel on Strategic Food Analytical Methods. **Methods:** The method involves tryptic digestion of allergen proteins in food matrices incurred or spiked with allergen standards [reference materials (RMs), Standard RMs (SRMs), or in-house prepared standard] and uses labeled peptide internal standards. LC-tandem MS analysis of the signature tryptic peptides of the four allergens is performed using multiple reaction monitoring. **Results:** For 10 allergen/matrix combinations, the method demonstrated adequate sensitivity with a minimum quantitation limit of 3 mg/kg for whole egg and 10 mg/kg for milk, peanut, and hazelnut allergens. Repeatability precision across 3 days of analyses was <17% with analytical range of 10–1000 mg/kg. Recovery from incurred and spiked matrix-matched standards varied from 60 to 118%. **Conclusions:** The method met the minimum performance requirements of SMPR 2016.002 for whole egg in cookies, bread,

cookie dough, and salad dressing; whole milk in cookies and red wine; peanut in breakfast cereal; and hazelnut in cookies. **Highlights:** The ERP determined that the data presented met the SMPR and accordingly recommended the method to be granted First Action status. In September 2017, the Official Methods Board approved the method as First Action.

In response to a need for reference methods, the AOAC Stakeholder Panel on Strategic Food Analytical Methods (SPSFAM) developed *Standard Method Performance Requirement* (SMPR[®]) 2016.002 for the detection and quantitation of selected allergens in 10 food matrices (1–3). SMPR 2016.002 provides the performance criteria for whole egg, whole milk, peanut, and hazelnut in cookies as well as bread, cookie dough, salad dressing, wine, infant formula, dark chocolate, ice cream, breakfast cereal, and milk chocolate.

The key steps in selecting marker peptides for each allergen are summarized in Figure 1. The uniqueness of the marker peptides was evaluated by searching against the NCBI nr protein database using the protein-protein BLAST query. As shown in Table 1, YFGYTGLR was found in chicken, Japanese quail, and turkey eggs. However, food manufacturers are more inclined to use chicken eggs as a result of the lower cost compared with eggs from other avian species. Therefore, this homology is not considered as a disadvantage to the method. Cow, goat, sheep, and buffalo belong to the Bovidae family and share a high degree of homology among their milk proteins such as caseins (4). Ah-Leung et al. have also reported that most children who are allergic to cow's milk are also unable to tolerate goat and/or sheep's milk (5). Therefore, YLGYLEQLLR and EDVPSER were retained in the method. For GGLEPINFQTAADQAR (Ew1), peanut, and hazelnut, no homologies were found in common food ingredients such as tree nuts and grains.

Previously, two proteins with two marker peptides per protein and two multiple reaction monitoring (MRM) transitions per peptide were used for detecting whole egg, whole milk, peanut, and hazelnut. Despite the qualifier ion being able to meet most the SMPR criteria, some of the qualifier ions from other marker peptides were not sensitive enough to meet the method quantitation limit (MQL) and

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This method was approved by the AOAC Expert Review Panel for Food Allergens as First Action.

The Expert Review Panel invites method users to provide feedback on the First Action methods. Feedback from method users will help verify that the methods are fit-for-purpose and are critical for gaining global recognition and acceptance of the methods. Comments can be sent directly to the corresponding author or methodfeedback@aoac.org.

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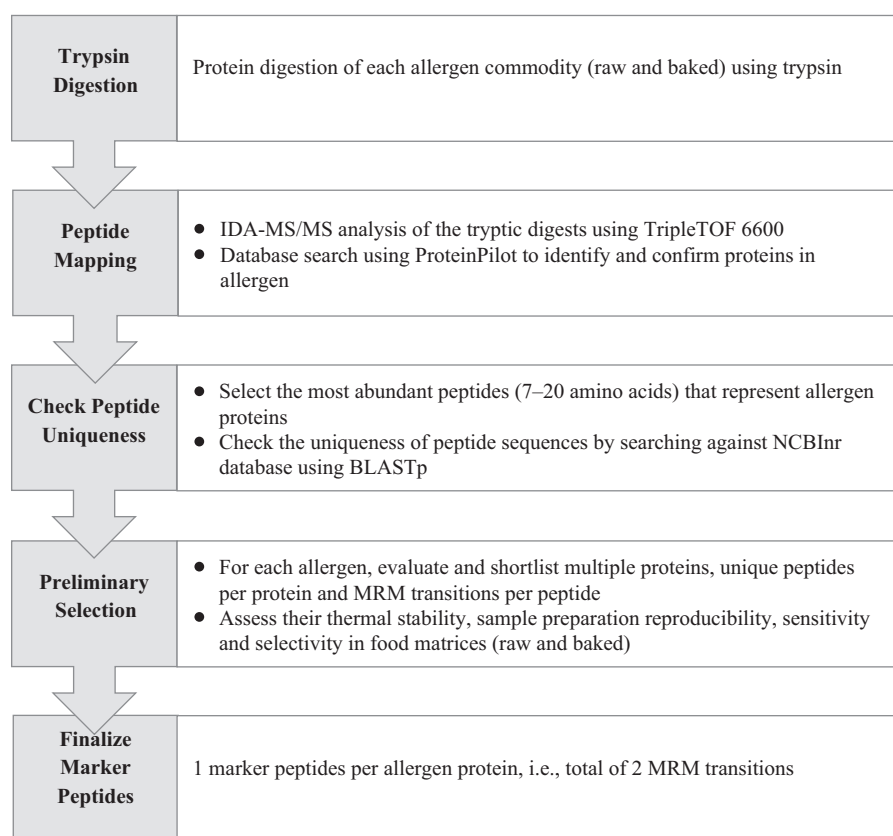


Figure 1. Workflow for the selection of marker peptides.

Table 1. Uniqueness of the marker peptides determined from database search using the NCBI Protein BLAST query

Allergen	Protein	Peptide sequence	BLAST search results	Peptide ID
Whole egg	Gal d 2	GGLEPINFQTAADQAR	Gallus gallus (chicken)	Ew1
	Gal d 3	YFGYTGALR	Gallus gallus (chicken), Coturnix japonica (Japanese quail), Meleagris gallopavo (Turkey)	Ew2
Whole milk	Bos d 9 (α -s1-casein)	YLGYLEQLLR	Bos taurus (cattle/cow), Capra hircus (domestic goat), Ovis aries (sheep), Bubalus bubalis (water buffalo)	M1
		EDVPSEK	Bos taurus (cattle/cow), Capra hircus (domestic goat), Ovis aries (sheep), Bubalus bubalis (water buffalo)	M2
Peanut	Ara h 3	WLGLSAEYGNLYR	Arachis hypogaea (peanut),	P1
Hazelnut	Cor a 9	ADIYTEQVGR	Corylus avellana (hazelnut)	H1

method detection limit (MDL) requirements [New, LS, AB SCIEX (Distribution); unpublished data]. The main challenge lies in being able to select multiple peptides that are able to fulfill the ideal characteristics of being unique to the allergen species, abundant, and selective. Therefore, in the current method, we chose to use one marker peptide with two MRM transitions for detecting each allergen. The main concern with using only one marker peptide is selectivity in different food matrices because they may contain diverse food ingredients and processing methods. To overcome this, ion ratios (relative ion ratio of the qualifier ion to quantifier ion) were used to facilitate allergen identification with an increased level of confidence.

Milk contains two protein fractions, i.e., caseins and whey, which may be used separately for food production. Because α -casein was used as the only marker protein for milk, this method may have limitation for foods that contain only whey protein as ingredient and foods such as infant formula that contain a different ratio of caseins and whey proteins than whole milk. For all analyses, β -lactoglobulin was initially included as the third marker protein for milk, as it is the major protein in the whey fraction. Although it was able to fulfill the minimum SMRP requirements in cookies (MQL=3 ppm), it had poor sensitivity in infant formula, dark chocolate and red wine (MQL=30 ppm; data not shown). Therefore, it was

removed from the final method. Likewise, only ovalbumin from egg white was used as the marker protein for egg. This method therefore has limitation for foods that solely or partly contain egg yolk as ingredient.

This manuscript reports the results of the single-laboratory validation (SLV) for the LC with tandem MS (LC-MS/MS) method for the four allergens and comparison to the SMPR acceptance criteria (Table 2). The SLV presented in the study has been approved by the respective AOAC Expert Review Panel (ERP) on September 25, 2017.

AOAC Official Method 2017.17
Detection and Quantitation of Selected
Food Allergens LC-MS/MS
First Action 2017

[Applicable to the determination of whole egg in cookies, bread, dough, salad dressing, and wine; whole milk (excluding whey) in cookies, baked goods, and wine; Peanut in cookies, ice cream, and breakfast cereal; and hazelnut in cookie down to 1, 3, or 10 ppm, depending on the allergen/matrix combination.]

Caution: Refer to Safety Data Sheets (SDS) for safety precautions when using chemicals. Use personal protective equipment recommended in SDS. See Table 2017.17A for method performance data supporting acceptance of the method.

A. Principle

The test portion is defatted with hexane and dried. A denaturing buffer is added to the dried sample, and the mixture is shaken for 60 min to facilitate extraction of the allergen proteins. The proteins are reduced, alkylated, and digested into peptides using trypsin. The digestion reaction is quenched using formic acid and an internal standard mixture is added (a stable isotope-labeled peptide for each allergen). The sample is filtered prior to analysis. The peptides are separated from interfering compounds and each other using ultra HPLC (UHPLC) and quantitated using MS/MS.

B. Apparatus

(a) *HPLC column*.—Kinetex 2.6 μm C18 100Å, 100 $\times$ 2.1 mm i.d. column (Phenomenex Inc., Torrance, CA), or equivalent.

Table 2017.17A. LC conditions

LC parameter settings		
Mobile phase A	0.1% formic acid in water	
Mobile phase B	0.1% formic acid in acetonitrile	
Flow rate	300 µL/min	
Column	Phenomenex Kinetex 2.6 µm C18, 100 × 3.0 mm i.d.	
Guard column cartridge	Phenomenex SecurityGuard ULTRA guard column for C18 sub-2 µm core shell columns with 3 mm i.d.	
Injection vol., µL	10	
Autosampler parameter settings		
Needle rinse solvent (R0)	20+20+60 acetonitrile–methanol–isopropanol	
Rinsing volume	1000 µL	
Rinsing speed	35 µL/s	
Sampling speed	2.0 µL/s	
Rinse dip time	5 s	
Rinse mode	Before and after aspiration	
Cooler temperature	10°C	
Column oven		
Column temperature	30°C	
LC gradient		
Time, min	%A	%B
0.0	98	2
1.3	98	2
11.0	60	40
12.5	2	98
14.4	2	98
14.6	98	2
20.0	98	2

(b) *HPLC guard column*.—Phenomenex SecurityGuard ULTRA guard column for C18 sub-2 μm core shell columns with 2.1 mm i.d., or equivalent.

(c) *HPLC guard column holder*.—Phenomenex SecurityGuard ULTRA cartridge holder for UHPLC columns 2.1–4.6 mm i.d., or equivalent.

(d) *UHPLC system*.—Shimadzu Nexera UHPLC system consisting of CBM20Alite controller, two LC-30AD solvent

Table 2. Summary of method performance data and comparison with SMPR 2016.002 requirements

Parameter	Whole egg allergen					Whole milk allergen			Peanut allergen			Hazelnut allergen	
	SMPR 2016.002	Cookies	Bread	Cookie dough	Salad Dressing	SMPR 2016.002	Cookies	Red wine ^a	SMPR 2016.002	Cookies	Breakfast cereal	SMPR 2016.002	Cookies
Analytical range, mg/kg	10–1000	1–1000	1–1000	3–1000	1–1000	10–1000	10–1000	10–1000	10–1000	10–1000	3–1000	10–1000	10–1000
MQL, mg/kg	≤5	3.0	3.0	3.0	3.0	≤10	10.0	10.0	≤10	10.0	3.0	≤10	10.0
MDL, mg/kg	≤1.65	1.0	1.0	1.0	1.0	≤3	3.0	3.0	≤3	3.0	1.0	≤3	3.0
Rec., %	60–120	59.6–73.2	65.0–73.7	96.6–113.3	78.2–94.2	60–120	60.2–62.4	73.2–119.3	60–120	57.3–68.7	84.6–110.6	60–120	70.5–77.6
RSDr, %	≤20	0.9–7.3	1.3–16.2	1.3–8.5	1.6–8.9	≤20	1.6–6.0	0.2–6.2	≤20	1.2–5.5	0.7–7.5	≤20	1.2–8.4

^a Tested in red wine because white wine contained milk as fining agent.

pumps (equipped with 20 μ L mixer), SIL30AC autosampler, and CTO30A column oven (Shimadzu Corp., Kyoto, Japan), or equivalent.

(e) *MS/MS system*.—SCIEX QTRAP 6500 equipped with IonDrive Turbo V Ion Source, or equivalent.

(f) *Data acquisition software*.—SCIEX Analyst, version 1.6.2, or equivalent.

(g) *Data processing software*.—SCIEX MultiQuant, version 3.0.2, or equivalent.

(h) *Analytical balance*.—Readable to at least 0.1 mg (0.0001 g).

(i) *Food processor*.

(j) *Bead mill homogenizer*.—Bead Ruptor 24 (Omni International, Kennesaw, GA) with 7 mL polypropylene screw cap tubes prefilled with 2.8 mm ceramic beads, or equivalent.

(k) *Convection oven*.

(l) *Nitrogen evaporator or vacuum concentrator*.—TurboVap LV with 15 mL tube rack (Biotage AB, Uppsala, Sweden), or equivalent.

(m) *Multitube vortexer*.—For 15 mL tubes.

(n) *Centrifuge*.

(o) *Vortex mixer*.

(p) *Incubator shaker*.—Thermomixer comfort (Eppendorf AG, Hamburg, Germany), or equivalent.

(q) *Molecular weight cut-off (MWCO) filter unit*.—Amicon Ultra-0.5 centrifugal filter unit with Ultracel-10 membrane, 10 kDa NMWL (Merck Millipore, Billerica, MA), or equivalent.

(r) *Centrifuge tubes*.—Polypropylene, 15 mL with screw cap.

(s) *Microtubes*.—Polypropylene, 2.0 mL.

(t) *Volumetric flasks*.—Glass, 200 mL.

(u) *Pipets*.—20, 100, 200, 1000, and 5000 μ L, adjustable.

(v) *Pipet tips*.

(w) *HPLC autosampler vial*.—Polypropylene, 250 μ L.

C. Chemicals and Reagents

Where specific part number is listed, other brands may be used provided that they are of equivalent grade.

(a) *Hexane*.— $\geq 95\%$, anhydrous.

(b) *Urea*.— $\geq 98\%$.

(c) *Tris base*.— $\geq 99\%$.

(d) *Octyl β -D-glucopyranoside (OGS)*.— $\geq 98\%$ (Sigma-Aldrich Part No. O8001-5G, or equivalent).

(e) *Ammonium bicarbonate*.— $\geq 99\%$.

(f) *Tris(2-carboxyethyl)phosphine hydrochloride (TCEP)*.— $\geq 98\%$.

(g) *S-Methyl methanethiosulfonate*.— $\geq 97\%$, liquid at room temperature.

(h) *Calcium chloride solution*.—1 M (Sigma-Aldrich Part No. O8001-5G, or equivalent).

(i) *Trypsin*.—TPCK treated, lyophilized enzyme powder (SCIEX part No. 4445250, or equivalent).

(j) *Formic acid*.— $\geq 98\%$.

(k) *Water*.—HPLC grade.

(l) *Acetonitrile*.—HPLC grade.

(m) *Egg albumin*.— $\geq 98\%$ [for preparing System Suitability Test (SST) sample].

(n) *Milk α -casein*.— $\geq 70\%$ (for preparing SST sample).

(o) *Allergen-free food matrices*.—(1) *Cookie mix or premix*.—Egg-, milk-, peanut-, and hazelnut-free.

(2) *Bread mix or premix*.—Egg-free.

(3) *Salad dressing*.—Egg-free.

(4) *Wine*.—Milk-free.

(5) *Breakfast cereal*.—Peanut-free.

(6) *Ice cream*.—Peanut-free.

D. Standards

(a) *Allergen Standard Reference Materials (SRM; allergen commodities)*.—(1) *Whole egg RM*.—Spray-Dried Whole Egg Powder for Allergen Detection, NIST SRM 8445. Total protein = $48 \pm 1\%$ (mass fraction). Consult NIST website for material details.

(2) *Milk SRM*.—Whole Milk Powder, NIST SRM 1549a. Total protein = $25.64 \pm 0.31\%$ (mass fraction). (Consult NIST website for material details.)

(3) *Peanut SRM*.—Peanut Butter, NIST SRM 2387. Total protein = $22.2 \pm 0.5\%$ (mass fraction). (Consult NIST website for material details.)

(4) *Hazelnut*.—Prepare baked hazelnut in-house. Bake whole raw hazelnuts at 180°C in a convection oven for 15 min. Grind into a fine powder using a food processor and freeze at -80°C for 30 min. Mill into a smooth and fine paste using a bead mill homogenizer. Estimated total protein = 15.03% (mass fraction). (Total protein information obtained from U.S. Department of Agriculture Food Composition Database, accessed May 29, 2017).

(b) *Stable isotope-labeled peptide internal standards (ISs)*.—(1) *Whole Egg peptide (Ew1) internal standard*.—GG LEPINFQTAADQA($^{13}\text{C}_6^{15}\text{N}_4$)R, $\geq 95\%$ purity (New England Peptide Inc., Gardner, MA).

(2) *Whole Milk peptide (M1) IS*.—YLGYLEQLL($^{13}\text{C}_6^{15}\text{N}_4$)R, $\geq 95\%$ purity (New England Peptide Inc.).

(3) *Peanut peptide (P1) IS*.—WLGLSAEYGNLY($^{13}\text{C}_6^{15}\text{N}_4$)R, $\geq 95\%$ purity (New England Peptide Inc.).

(4) *Hazelnut peptide (H1) IS*.—ADIYTEQVG($^{13}\text{C}_6^{15}\text{N}_4$)R, $\geq 95\%$ purity (New England Peptide Inc.).

E. Preparation of Incurred and Spiked Matrix-Matched Standards

(a) *Cookie (incurred) standards*.—(1) *Cookie, blank*.—Weigh 100 ± 1 g of cookie mix. Add 18 mL canola oil and 9 mL water. Mix well for at least 10 min. Press gently to form dough with approximately 1 cm thickness. Bake at 180°C for 18 min.

(2) *Cookie, 10 mg/kg*.—Weigh whole egg RM, whole milk SRM, peanut SRM, and hazelnut (1 ± 0.1 mg each). Weigh accurately 100 ± 1 g cookie mix and add to the allergens. Add 18 mL canola oil and 9 mL water. Mix well for at least 10 min to ensure homogeneity of the allergens in dough. Press gently to form dough with approximately 1 cm thickness. Bake at 180°C for 18 min.

(3) *Cookie, 100 mg/kg*.—Weigh whole egg RM, milk SRM, peanut SRM and hazelnut (10 ± 1 mg each). Weigh accurately 100 ± 1 g of cookie mix and add to the allergens. Add 18 mL canola oil and 9 mL water. Mix well for at least 10 min to ensure homogeneity of the allergens in dough. Press gently to form dough with approximately 1 cm thickness. Bake at 180°C for 18 min.

(4) *Cookie, 1000 mg/kg*.—Weigh whole egg RM, milk SRM, peanut SRM and hazelnut (100 ± 10 mg each). Weigh accurately 100 ± 1 g of cookie mix and add to the allergens. Add 18 mL canola oil and 9 mL water. Mix well for at least 10 min to ensure homogeneity of the allergens in dough. Press gently to form dough with approximately 1 cm thickness. Bake at 180°C for 18 min.

(5) Separately homogenize the 10, 100, and 1000 mg/kg cookies into fine powder using the food processor. Make sure to

wash the food processor thoroughly with detergent in between grinding cookies with different concentrations of allergens.

Note: Keep in mind that the solid sample can be inhomogeneous. Therefore, grind the samples very well before weighing.

(6) *Cookie, 1 mg/kg*.—Weigh accurately 2 g 10 mg/kg cookie into 18 g blank cookie. Mix well for at least 5 min to combine.

(7) *Cookie, 3 mg/kg*.—Weigh accurately 6 g 10 mg/kg cookie into 14 g blank cookie. Mix well for at least 5 min to combine.

(8) *Cookie, 30 mg/kg*.—Weigh accurately 6 g 100 mg/kg cookie into 14 g blank cookie. Mix well for at least 5 min to combine.

(9) *Cookie, 300 mg/kg*.—Weigh accurately 6 g 1000 mg/kg cookie into 14 g blank cookie. Mix well for at least 5 min to combine.

(10) *Foreachconcentration(0,1,3,10,30,100,300,1000mg/kg)*, weigh 1 ± 0.1 g cookie into a 15 mL centrifuge tube. Subject standards to sample preparation as described in **H(b–h)**.

(b) *Bread (incurred) standards*.—(1) *Bread, blank*.—Weigh 100 ± 1 g bread mix. Add 6.7 mL canola oil and 95 mL water. Mix well for at least 15 min. Bake at 180°C for 40 min.

(2) *Bread, 10 mg/kg*.—Weigh 1 ± 0.1 mg whole egg RM. Weigh accurately 100 ± 1 g of bread mix and add to the whole egg RM. Add 6.7 mL canola oil and 95 mL water. Mix well for at least 10 min to ensure homogeneity of the allergens in dough. Bake at 180°C for 18 min.

(3) *Bread, 100 mg/kg*.—Weigh 10 ± 1 mg whole egg RM. Weigh accurately 100 ± 1 g bread mix and add to the whole egg RM. Add 6.7 mL canola oil and 95 mL water. Mix well for at least 10 min to ensure homogeneity of the allergen in dough. Bake at 180°C for 18 min.

(4) *Bread, 1000 mg/kg bread*.—Weigh 100 ± 10 mg whole egg RM. Weigh accurately 100 ± 1 g bread mix and add to the whole egg RM. Add 6.7 mL canola oil and 95 mL water. Mix well for at least 10 min to ensure homogeneity of the allergen in dough. Bake at 180°C for 18 min.

(4) Separately homogenize the 10, 100, and 1000 mg/kg breads into fine powder using the food processor. Make sure to wash the food processor thoroughly with detergent in between grinding breads with different concentrations of allergens.

Note: Keep in mind that the solid sample can be inhomogeneous. Therefore, grind the samples very well before weighing.

(5) *Bread, 1 mg/kg*.—Weigh accurately 2 g 10 mg/kg bread into 18 g blank bread. Mix well for at least 5 min to combine.

(6) *Bread, 3 mg/kg*.—Weigh accurately 6 g 10 mg/kg bread into 14 g blank bread. Mix well for at least 5 min to combine.

(7) *Bread, 30 mg/kg*.—Weigh accurately 6 g 100 mg/kg bread into 14 g blank bread. Mix well for at least 5 min to combine.

(8) *Bread, 300 mg/kg*.—Weigh accurately 6 g 1000 mg/kg bread into 14 g blank bread. Mix well for at least 5 min to combine.

(9) For each concentration, weigh 1 ± 0.1 g bread into a 15 mL centrifuge tube. Subject standards to sample preparation as described in **H(b–h)**.

(c) *Cookie dough (incurred) standards*.—(1) *Cookie dough, blank*.—Weigh accurately 100 g cookie mix. Add 18 mL canola oil and 9 mL water. Mix well for at least 10 min.

(2) *Cookie dough, 10 mg/kg*.—Weigh 1 ± 0.1 mg whole egg RM. Weigh accurately 100 ± 1 g cookie mix and add to whole egg RM. Add 18 mL canola oil and 9 mL water. Mix well for at least 10 min to ensure homogeneity of allergens in dough.

(3) *Cookie dough, 100 mg/kg*.—Weigh 10 ± 1 mg whole egg RM. Weigh accurately 100 ± 1 g cookie mix and add to whole egg RM. Add 18 mL canola oil and 9 mL water. Mix well for at least 10 min to ensure homogeneity of allergens in dough.

(4) *Cookie dough, 1000 mg/kg*.—Weigh 100 ± 10 mg whole egg RM. Weigh accurately 100 ± 1 g o cookie mix and add to whole egg RM. Add 18 mL canola oil and 9 mL water. Mix well for at least 10 min to ensure homogeneity of allergens in dough.

(5) *Cookie dough, 1 mg/kg*.—Weigh accurately 2 g 10 mg/kg cookie dough. Add to 18 g blank cookie dough. Mix well for at least 5 min to combine.

(6) *Cookie dough, 3 mg/kg*.—Weigh accurately 6 g 10 mg/kg cookie dough. Add to 14 g blank cookie dough. Mix well for at least 5 min to combine.

(7) *Cookie dough, 30 mg/kg*.—Weigh accurately 6 g 100 mg/kg cookie dough. Add to 14 g blank cookie dough. Mix well for at least 5 min to combine.

(8) *Cookie dough, 300 mg/kg*.—Weigh accurately 6 g 1000 mg/kg cookie dough. Add to 14 g blank cookie dough. Mix well for at least 5 min to combine.

(9) For each concentration, weigh 1 ± 0.1 g cookie dough into a 15 mL centrifuge tube. Subject standards to sample preparation as described in **H(b–h)**.

(d) *Salad dressing (spiked) standards*.—(1) *Spike solution, 2 mg/mL*.—Weigh 12 ± 0.4 mg whole egg RM. Add 6 mL water. Mix in a vortex mixer for 1 min.

(2) *Spike solution, 0.002–0.6 mg/mL*.—Serially dilute the 2 mg/mL spike solution using water to prepare at least 5 mL each remaining spike solutions at 0.6 mg/mL (add 1.8 mL 2 mg/mL spike into 4.2 mL water and mix in a vortex mixer), 0.2 mg/mL (add 2.0 mL 0.6 mg/mL spike into 4 mL water and mix in a vortex mixer), 0.06 mg/mL (add 1.8 mL 0.2 mg/mL spike into 4.2 mL water and vortex), 0.02 mg/mL (add 2.0 mL 0.06 mg/mL spike into 4 mL water and mix in a vortex mixer), 0.006 mg/mL (add 1.8 mL 0.02 mg/mL spike into 4.2 mL water and mix in a vortex mixer), and 0.002 mg/mL (add 2.0 mL 0.006 mg/mL spike into 4 mL water and mix in a vortex mixer).

(3) *Spiked salad dressing (1, 3, 10, 30, 100, 300, and 1000 mg/kg)*.—Weigh 1 ± 0.1 g salad dressing into each of seven labeled 15 mL centrifuge tubes. Add 0.5 mL 2 mg/mL spike solution to the tube labeled 1000 mg/kg. Likewise, for each of the remaining spike solutions, add 0.5 mL to the appropriate tube. Mix in a vortex mixer tubes for 2 min.

(4) *Blank salad dressing*.—Weigh 1 ± 0.1 g salad dressing into a labeled 15 mL centrifuge tube and add 0.5 mL of water. Mix in a vortex mixer for 2 min.

(5) Subject the standards to sample preparation as described in **H(b–h)**.

(e) *Red wine (spiked) standards*.—(1) *Working solution, 5 mg/mL*.—Weigh 10 ± 0.4 mg of whole milk SRM. Add 2 mL of water. Mix in a vortex mixer for 1 min.

(2) *Working solution, 0.005–1.5 mg/mL*.—Serially dilute the 5 mg/mL spike solution using water to prepare at least 500 μL spike solutions at 1.5 mg/mL (add 0.6 mL 5 mg/mL spike solution to 1.4 mL water and mix in a vortex mixer), 0.5 mg/mL (add 1.0 mL 1.5 mg/mL spike solution to 2.0 mL water and mix in a vortex mixer), 0.15 mg/mL (add 0.6 mL 0.5 mg/mL spike solution to 1.4 mL water and mix in a vortex mixer), 0.05 mg/mL (add 1.0 mL 0.15 mg/mL spike solution to 2.0 mL water and mix in a vortex mixer), 0.015 mg/mL (add 0.6 mL 0.05 mg/mL spike solution to 1.4 mL water and mix in

a vortex mixer), and 0.005 mg/mL (add 1.0 mL 0.015 mg/mL spike solution to 2.0 mL water and mix in a vortex mixer).

(3) *Spiked red wine samples (1, 3, 10, 30, 100, 300 and 1000 mg/kg).*—For each concentration of spike solution, pipet 50 μ L into a 2 mL microtube. Bring to dryness using a vacuum concentrator without heating to avoid precipitation of the spikes. Add 250 μ L red wine to each tube. Mix in a vortex mixer tubes at high speed for 15 min. Add 675 μ L 100 mM ammonium bicarbonate solution to each tube.

(4) *Blank red wine.*—Pipet 250 μ L red wine into 2 mL microtube. Add 675 μ L 100 mM ammonium bicarbonate solution.

(5) Subject the blank and spiked red wine standards to reduction, alkylation and protein digestion as described in **H(d–h)**.

(f) *Breakfast cereal (spiked) standards.*—(1) *Blank breakfast cereal protein extract (Blank BCPE).*—Weigh approximately 100 g breakfast cereal. Homogenize into fine powder using the food processor. Weigh 1 ± 0.1 g into each of three 15 mL centrifuge tubes. Subject to predigestion sample preparation as described in **H(b)**.

(2) *BCPE, 1000 mg/kg.*—Weigh peanut SRM (1 ± 0.1 mg) into a 15 mL centrifuge tube. Weigh accurately 1 ± 0.1 g of homogenized breakfast cereal into the same tube. Mix it well for 2 min. Subject to predigestion sample preparation as described in **H(b)**.

(3) *BCPEs, 1–300 mg/kg.*—Serially dilute the 1000 mg/kg BCPE using blank BCPE to prepare at least 1 mL 300 mg/kg BCPE (add 0.3 mL 1000 mg/kg BCPE to 0.7 mL blank BCPE and vortex), 100 mg/kg BCPE (add 0.4 mL 300 mg/kg BCPE to 0.8 mL blank BCPE and vortex), 30 mg/kg BCPE (add 0.3 mL 100 mg/kg BCPE to 0.7 mL blank BCPE and vortex), 10 mg/kg BCPE (add 0.4 mL 30 mg/kg BCPE to 0.8 mL blank BCPE and vortex), 3 mg/kg BCPE (add 0.3 mL 10 mg/kg BCPE to 0.7 mL blank BCPE and mix in a vortex mixer), and 1 mg/kg BCPE (add 0.4 mL 3 mg/kg BCPE to 0.8 mL blank BCPE and mix in a vortex mixer).

(4) Transfer 500 μ L each blank and spiked BCPE standard to a fresh tube and subject to the rest of the sample preparation procedure as described in **H(d–h)**.

F. SST Sample

(a) *Protein stock solutions.*—(1) Weigh egg albumin and milk α -casein (5 ± 0.1 mg each) separately.

(2) To each protein, add 1 mL water and mix in a vortex mixer for 30 s to prepare the 5 mg/mL stock solution. Store at -20°C or below. Solutions can be used indefinitely until noticeable degradation of the solution concentration is observed (Shelf-life is up to 6 month in -20°C or lower without freeze-thaw circle.).

(b) *SST spike mixture (500 μ g/mL per protein).*—Add egg albumin, and α -casein stock solutions (50 μ L each) to 400 μ L of extraction buffer. Prepare fresh before use.

(c) *SST sample (12.5 μ g/mL per protein).*—(1) Add 100 μ L the SST spike mixture to 3.7 mL of extraction buffer, and 200 μ L of denaturant. Mix in a vortex mixer 1 min to mix.

(2) Transfer 500 μ L solution to a 2 mL microtube.

(3) Subject to protein digestion as described in **H(d–h)**.

G. Preparation of Reagents and Solutions

(a) *Protein extraction buffer and denaturant.*—(1) *Extraction buffer.*—50 mM Tris base and 2 M urea in water. Accurately weigh 1.2 g Tris base and 24 g urea into

a 200 mL volumetric flask. Dilute to volume with water. Prepare fresh before use.

(2) *Denaturant.*—20% (w/v) OGS in water. Add 20 mL water directly to the bottle containing OGS powder (5 g). Swirl to dissolve completely and prevent formation of bubbles. Store at $\leq -20^\circ\text{C}$. Expiration: 1 year.

(b) *Ammonium bicarbonate solution, 100 mM.*—Accurately weigh 395 mg ammonium bicarbonate and add 50 mL water. Mix well to dissolve. Prepare fresh before use.

(c) *Reducing reagent.*—50 mM TCEP. Accurately weigh 143 mg TCEP into a 10 mL volumetric flask. Dilute to volume with 100 mM of ammonium bicarbonate solution. Store at $\leq -20^\circ\text{C}$. Expiration: 6 months.

(d) *Alkylating reagent.*—100 mM S-Methyl methanethiosulfonate (MMTS). Dilute 13.2 μ L MMTS with 1385 μ L of 100 mM ammonium bicarbonate solution. Mix in vortex mixer for 30 s. Prepare fresh before use.

(e) *Digestion buffer.*—5 mM calcium chloride in 100 mM ammonium bicarbonate solution. Dilute 140 μ L 1 M calcium chloride solution with 27.86 mL 100 mM ammonium bicarbonate solution. Prepare fresh before use.

(f) *Trypsin solution (1 μ g/ μ L).*—Pipet appropriate volume of HPLC water to the lyophilized enzyme powder achieve a final concentration of 1 μ g/ μ L. Prepare fresh before use.

(g) *Peptide IS stock solutions (1 mg/mL each).*—(1) *Egg white peptide IS stock solution.*—Dissolve egg white peptide IS (1 mg) with 1 mL 0.1% formic acid in water. Mix in a vortex mixer for 1 min to dissolve completely.

(2) *M1 IS stock solution.*—Dissolve M1 IS (1 mg) with 1 mL of 20% acetonitrile in water with 0.1% formic acid. Mix in a vortex mixer for 1 min to dissolve completely.

(3) *P1 IS stock solution.*—Dissolve P1 IS (1 mg) with 1 mL of 20% acetonitrile in water with 0.1% formic acid. Mix in a vortex mixer for 1 min to dissolve completely.

(4) *H1 IS stock solution.*—Dissolve H1 IS (1 mg) with 1 mL of 20% acetonitrile in water with 0.1% formic acid. Mix in a vortex mixer for 1 min to dissolve completely.

Note: Store the stock solutions at $\leq -20^\circ\text{C}$. They can be used indefinitely until noticeable degradation of the solution concentration is observed.

(h) *Peptide IS mixture (ISM; 5 μ g/mL per peptide IS).*—Combine and serially dilute the egg white, milk, peanut, and hazelnut peptide IS stock solutions (1 mg/mL each) with 10% acetonitrile in water with 0.1% formic acid. Prepare fresh every week.

Note: Store the ISM at $\leq -20^\circ\text{C}$. They can be used indefinitely until noticeable degradation of the solution concentration is observed.

(i) *Mobile phases.*—(1) *Mobile phase A.*—0.1% formic acid in water (aqueous). Add 1 mL formic acid to 1 L HPLC grade water.

(2) *Mobile phase B.*—0.1% formic acid in acetonitrile (organic). Add 1 mL formic acid to 1 L HPLC grade acetonitrile.

H. Sample Preparation

(a) Weigh 1 ± 0.1 g homogenized solid or liquid sample into a 15 mL centrifuge tube.

(b) *Predigestion sample preparation for solid foods, including cookies, bread, cookie dough, and breakfast cereal.*—

(1) *Lipid removal.*—Add 5 mL hexane and shake at high speed for 5 min.—(a) Centrifuge at $4000 \times g$ for 20 min.

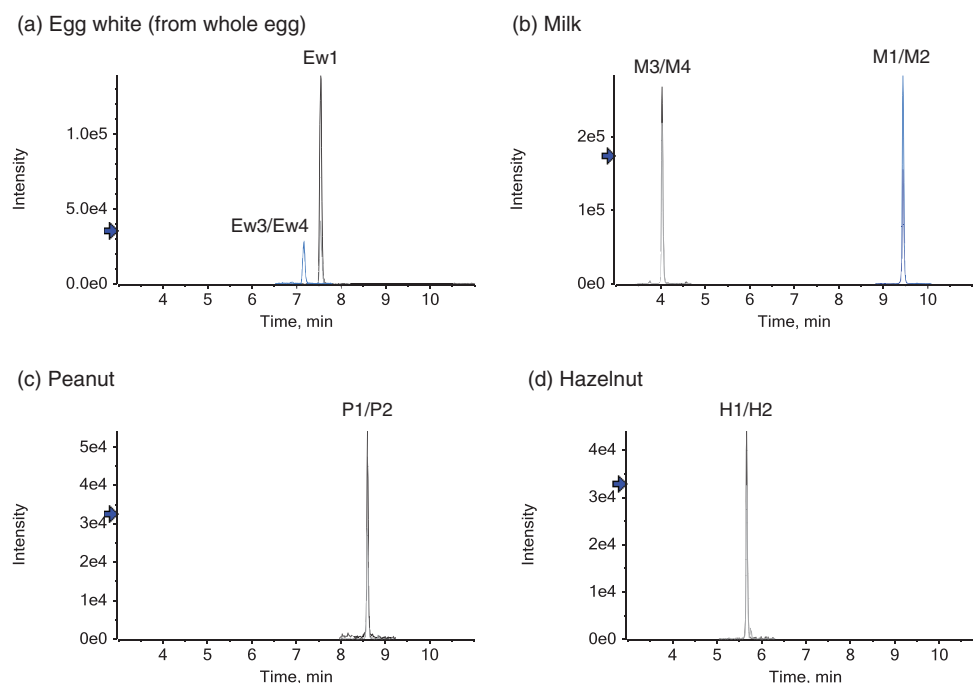


Figure 2017.17A. Extracted ion chromatograms of the marker peptides for (a) egg white (from whole egg), (b) whole milk, (c) peanut, and (d) hazelnut in 100 ppm incurred cookies (arrows indicate the peaks with lowest intensity).

(b) Discard 3 mL the supernatant (solvent).
 (c) Repeat steps (a) and (b) and discard 5 mL supernatant.
 (d) Remove the excess residual hexane for 30 min under a gentle flow of nitrogen without heating. Tap the tube to loosen the dried sample before the next step.
 (2) *Protein extraction and denaturation.*—(a) Add 7.8 mL extraction buffer for breakfast cereal or 3.8 mL extraction buffer for all other matrices. Add 200 μ L denaturant.
 (b) Cap the tube and shake until the dried sample is sufficiently wetted.
 (c) Shake vigorously using the multitube vortexer for 50 min at high speed.
 (d) Using a pipet tip with wide bore (e.g., 5 mL), transfer 1.2 ± 0.2 mL the sample into a 2 mL microtube.
 (e) Centrifuge at $16000 \times g$ for at least 15 min (or longer to ensure the supernatant is clear).
 (f) Transfer 500 μ L of supernatant to a clean 2 mL microtube for reduction reaction. See **H(d)**.
 (c) *Predigestion sample preparation for salad dressing and ice cream.*
Note: Wine samples are subjected to reduction directly, **H(d)**.
 (1) *Dilution and protein extraction and denaturation.*—(a) Add 3.8 mL extraction buffer for ice cream or 3.3 mL extraction buffer for salad dressing. Add 200 μ L denaturant.
 (b) Shake vigorously using the multitube vortexer for 10 min at high speed.
 (2) *Lipid removal.*—(a) Add 5 mL hexane and inverting the sample tube gently 30 times (to prevent formation of emulsion).
 (b) Centrifuge at $4000 \times g$ for 20 min.
 (c) Discard 3 mL top hexane layer.
 (d) Repeat steps (a) and (b) and discard 5 mL top hexane layer.
 (e) Carefully transfer 1.2 ± 0.2 mL bottom aqueous layer to a 2 mL microtube.
 (f) Centrifuge at $16000 \times g$ for 15 min (or longer to make sure that the supernatant is clear).

(g) Transfer 500 μ L the bottom aqueous layer to a clean 2 mL microtube for reduction reaction. See **H(d)**.
 (d) *Reduction.*—(1) Add 50 μ L reducing reagent and mix in a vortex mixer for 10 s to.
 (2) Incubate at 60°C for 60 min with shaking at 1000 rpm.
 (e) *Alkylation.*—(1) Add 25 μ L alkylating reagent and mix in a vortex mixer for 10 s.
 (2) Incubate at room temperature ($25 \pm 1^\circ\text{C}$) for 20 min.
 (f) *Protein digestion.*—(1) Add 425 μ L digestion buffer.
 (2) Add 20 μ L trypsin solution (1 $\mu\text{g}/\mu\text{L}$) and mix in a vortex mixer gently for 10 s.
 (3) Incubate at 37°C overnight (12 ± 4 h).
 (4) Add 30 μ L formic acid to quench the digestion activity and mix in a vortex mixer well.
 (5) Centrifuge the samples for 1 min at $16000 \times g$.
 (g) *Addition of combined stable isotope-labeled peptide IS solution.*—(1) Add 10 μ L ISM and mix in a vortex mixer for 10 s.
 (2) Centrifuge for at least 15 min at $16000 \times g$ (or longer to make sure that the supernatant is clear).
 (h) *Postdigestion sample cleanup.*—(1) Pipet 500 μ L supernatant to a 10 kDa MWCO filter unit.
 (2) Centrifuge at $12000 \times g$ for 15 min.
 (3) Pipet 150 μ L filtrate to a HPLC autosampler vial for LC-MS/MS analysis. Store the remaining filtrate at -20°C and below for further analysis (if necessary).

I. Instrumental Conditions

(a) *HPLC conditions.*—See Table 2017.17A (settings can be varied across different auto-samplers to achieve optimal performance). An example chromatogram is provided in Figure 2017.17A for reference. Where a specific instrument is cited, an alternative may be used provided it has the same or

Table 2017.17B. MS/MS parameters and settings for operation

Instrument parameter	Value
MS parameters	
Scan type	MRM
Experiment type	Scheduled MRM
Duration	20 min
Polarity	Positive
MRM detection window	75 s
Target scan time	0.5 s
Source parameters	
IonSpray voltage (IS)	5500 V
Curtain gas	35 psi
Source temperature	550°C
Ion source gas 1	50 psi
Ion source gas 2	55 psi
Collision gas	Medium
Compound parameters	
Entrance potential	10 V
Collision cell exit potential	18 V
Diverter valve: 0–3 min to waste; after 12 min to waste. Users can adjust the divert times based on the retention times of the peaks that elute first and last.	

better characteristics. Likewise, an alternative HPLC column may be used provided it allows a good separation between peptide peaks and matrix interferences.

(b) MS/MS conditions.—See Tables 2017.17B and 2017.17C for MS/MS operating conditions and MRM parameters, respectively (settings can be varied across different instruments to achieve optimal performance). To obtain the maximum sensitivity, source parameters such as electrode tip protrusion, CUR, TEM, GS1, and GS2 may need to be optimized prior to analysis. Where a specific instrument is cited, an alternative may be used provided it has the same or better characteristics.

(d) Retention time check (e.g., for new HPLC column).—Inject the 100 mg/kg sample incurred or spiked incurred food (10 µL) and analyze it in MRM mode with dwell time 50 ms to check the retention times of the peptide markers (Table 2017.17C).

(e) System suitability check.—Before batch analysis, ensure that the LC-MS/MS system is working in conditions such as the method remains fit for purpose. Inject 10 µL SST sample using the LC-MS/MS conditions as shown in Tables 2017.17A–C. Compare the integrated peak areas of the marker peptides with the acceptance limits provided in Table 2017.17D. It is recommended that the SST be injected during the following interval:

(1) *Prior to batch analysis, and*

(2) *During batch analysis.*—Inject SST sample approximately every 4 h of sample extraction analysis (e.g., 12 samples with analysis cycle time of 20 min) and after the analysis of the last sample.

(f) Batch analysis.—Injection volume is 10 µL. For each analyte/matrix combination, make a single injection of blank matrix and a single injection of each of the seven matrix-matched standards. Next, make single injections of the SST sample and

unknown samples such that the SST sample is analyzed at the beginning and at the end of the batch and intermittently after no more than 12 unknown samples.

J. Calculations and Confirmation

(a) Calibration curve for quantitation.—Perform peak integration using the SignalFinder algorithm in SCIEX MultiQuant v 3.0.2 software. Generate the calibration curve by plotting the peak area ratio of the quantifier ion against the incurred or spiked allergen commodity concentration (microgram per kilogram). Do not include the matrix blank. The peak area ratio is calculated by normalizing the peak area of the quantifier ion with the peak area of its corresponding peptide IS transition (Table 2017.17C)

$$\text{Peak area ratio} = \frac{\text{Peak area of quantifier ion}}{\text{Peak area of IS transition}}$$

Plot the curve using least square regression analysis with a linear model and 1/x weighting. Do not force the regression through zero.

Note: For milk in red wine, generate calibration curve by plotting the peak area of the quantifier ion (M2-1) against the allergen commodity concentration.

(b) Calculation of whole egg, whole milk, peanut, or hazelnut allergen concentration.—Use the equation to calculate the concentration of allergen in an unknown sample

$$\text{Allergen Conc. (mg / kg)} = \frac{(\text{Area ratio of quantifier ion}) - (\text{y-intercept of linear calibration curve})}{\text{slope of linear calibration curve}} \times D$$

where y = the value of peak area read from the instrument output; and D = the dilution factor. If the result from an unknown sample is above the calibration curve, the user may dilute and reanalyze the sample or report the sample concentration to be above the highest calibration concentration, e.g., allergen concentration of unknown > 1000 mg/kg.

(c) Allergen confirmation.—Confirm the presence of the allergen in the food matrix by analyzing the ion ratios

$$\text{Ion ratio} = \frac{Q1 > Q3 \text{ peak area for qualifier ion}}{Q1 > Q3 \text{ peak area for quantifier ion}}$$

The allergen is confirmed if the ion ratio of the unknown is within ±20% of the mean value of ion ratio of the matrix-matched standards, in Table 2017.17C.

Validation Study

The SLV was conducted in accordance with SMPR 2016.002 (1) and AOAC guidelines (2, 3) and included determinations of analytical range, MQL, MDL, recovery, and repeatability of matrix-matched standards in a subset of the priority analyte/matrix combinations identified by SPSFAM. This SLV was performed by one analyst using a SCIEX QTRAP 6500 coupled to Shimadzu Nexera UHPLC system.

Method performance was determined using matrix-matched calibration standards prepared according to the method. For each calibration standard, three independent sample preparations were made. The first and second sample preparations were injected twice each, and the third sample preparation was

Table 2017.17C. MRM parameters

Allergen	Peptide sequence	RT ^a , min	ID	Q1 ^b , m/z	Q3 ^c , m/z	DP ^d , V	CE ^e , V	Use	Ion ratio
Whole egg	GGLEPINFQTAADQAR	7.50	Ew1-1	844.4	666.3	92.7	38.4	Quantifier ion	0.197
			Ew1-2	844.4	1121.5	92.7	40.4	Qualifier ion	
	GGLEPINFQTAADQA (¹³ C ₆ ¹⁵ N ₄)R	7.50	Ew1-IS1	849.4	671.3	92.7	38.4	IS for Ew1-1	
			Ew1-IS2	849.4	1131.5	92.7	40.4		
Whole milk	YLGYLEQLLR ^f	9.45	M1-1	634.4	991.6	75.5	33.4	Quantifier ion	1.955
			M1-2	634.4	249.2	75.5	29.4	Qualifier ion	
	YLGYLEQLL (¹³ C ₆ ¹⁵ N ₄)R	9.45	M1-IS1	639.4	1001.6	75.5	33.4	IS for M1-1	
			M1-IS2	639.4	249.2	75.5	29.4		
	EDVPSER ^g	3.95	M2-1	416.2	488.3	61.5	19.3	Quantifier ion	0.848
			M2-2	416.2	244.6	61.5	19.3	Qualifier ion	
Peanut	WLGLSAEYGNLYR	8.60	P1-1	771.4	1242.6	87.4	35.9	Quantifier ion	1.807
			P1-2	771.4	272.2	75.5	33.4	Qualifier ion	
	WLGLSAEYGNLY (¹³ C ₆ ¹⁵ N ₄)R	8.60	P1-IS1	776.4	1252.6	87.4	35.9	IS for P1-1	
			P1-IS2	776.4	272.2	87.4	38.9		
Hazelnut	ADIYTEQVGR	5.70	H1-1	576.3	689.4	73.1	27.4	Quantifier ion	0.753
			H1-2	576.3	852.4	73.1	29.4	Qualifier ion	
	ADIYTEQVG (¹³ C ₆ ¹⁵ N ₄)R	5.70	H1-IS1	581.3	699.4	73.1	27.4	IS for H1-1	
			H1-IS2	581.3	862.4	73.1	29.4		

^a RT=Approximate retention time.^b Q1=Precursor mass.^c Q3=Fragment mass.^d DP=Decustering potential.^e CE Collision energy.^f For all food matrices except red wine.^g For red wine only.**Table 2017.17D. Acceptance limits for SST sample**

ID (see Table 2017.17C)	Approximate integrated area (±30%)
Ew1-1	3.2 × 10 ⁶
Ew1-2	5.5 × 10 ⁵
Ew1-IS1	1.6 × 10 ⁶
Ew1-IS2	2.8 × 10 ⁵
M1-1	1.6 × 10 ⁷
M1-2	2.0 × 10 ⁷
M1-IS1	2.8 × 10 ⁶
M1-IS2	3.5 × 10 ⁶
P1-IS1	1.0 × 10 ⁶
P1-IS2	8.0 × 10 ⁵
H1-IS1	1.0 × 10 ⁶
H1-IS2	8.5 × 10 ⁵

injected thrice for LC-MS/MS analysis across 3 days ($n=7$). For milk allergen in wine, red wine was used as the matrix standard because milk is often used as a fining agent in white wine.

Results and Discussion

Calibration Curves and Analytical Range

Calibration curves for each analyte/matrix combination are presented in Figure 2. The curves were generated from the data collected over 3 days (seven data points per concentration).

All allergen commodities were able to meet or exceed the SMPR defined analytical range of 10–1000 mg/kg. All calibration curves also had good correlation coefficients (r^2) of >0.99 (Figures 2 and 3).

In red wine, nonspecific binding was observed for M1-1 and its peptide IS resulting in a calibration curve with quadratic fitting (Figure 3, inset). It is likely that the nonspecific binding is a result of the presence of tannins in wine. This is not unexpected because milk (caseins) is commonly used as a fining agent to remove tannins for reducing astringency in wines. Therefore, M2-1 was used as the quantifier ion (Figure 3). Because an IS was not available for M2, the peak area of the quantifier ion was plotted against the matrix-matched standard concentration, producing a linear fit. In the future, peptide internal standard should be synthesized and used for quantitating milk (M2) in wine.

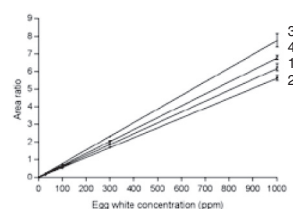
Notably, the slopes of the calibration curves for whole egg in the cookies, bread, cookie dough, and salad dressing differed by <14% (Figure 2a). Similarly, for milk, the calibration curves in cookies demonstrated less than 12% difference in their slopes (Figure 2b). This indicates the possibility of using a single calibration curve for different food matrices or preparing allergen commodities directly in LC-MS solvent (to reduce sample preparation time). However, more studies are required to evaluate this possibility.

MDL and MQL

MDL and MQL were estimated from the seven independent analyses of food matrices without allergens (matrix blanks).

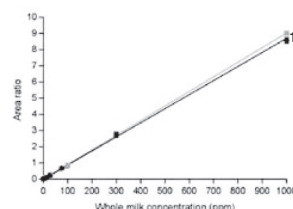
(a) Egg white (from whole egg)

- (1) Cookies: $y = 0.00617x + 6.515e-4$ ($r^2 = 0.997$)
 (2) Bread: $y = 0.005605x + 7.276e-5$ ($r^2 = 0.998$)
 (3) Cookie dough: $y = 0.007742x + 7.738e-5$ ($r^2 = 0.998$)
 (4) Salad dressing: $y = 0.006756x + 9.841e-4$ ($r^2 = 0.999$)



(b) Milk

- (1) Cookies: $y = 0.008704x + 0.00489$ ($r^2 = 0.998$)



(c) Peanut

- (1) Cookies: $y = 0.00169x + 0.004098$ ($r^2 = 0.998$)
 (3) Breakfast cereal: $y = 0.005065x + 0.009271$ ($r^2 = 0.995$)

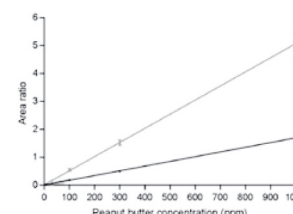


Figure 2. Calibration curves ($n=7$) of the quantifier ion for each allergen [(a) egg white, (b) milk, (c) peanut] in incurred or spiked food matrices (except for milk in red wine).

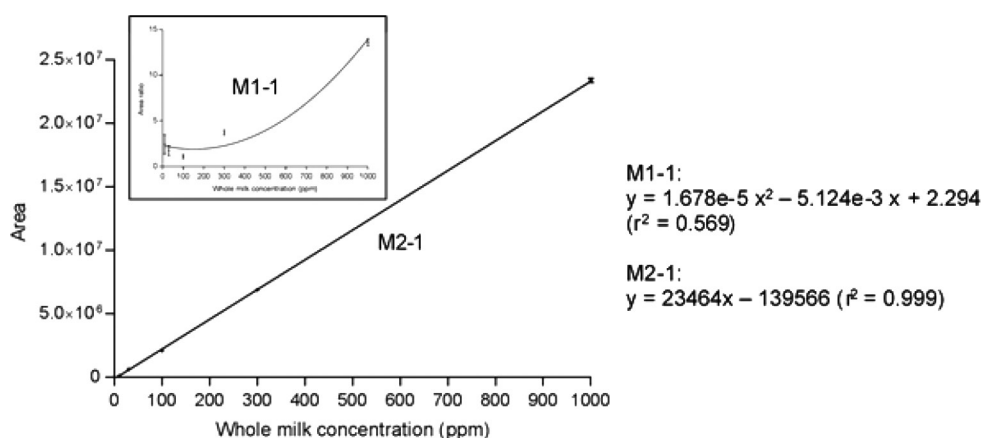


Figure 3. Calibration curve ($n=7$) for M2-1 (milk) in spiked red wine. The inset shows the calibration curve if M1-1 was used as quantifier ion.

If there was no detectable blank signal, then matrix blanks were spiked at a low concentration near the suspected MDL (spiked blanks). MQL and MDL (microgram per kilogram) were estimated using

$$\text{Est. MQL} = \text{Mean} + (10 \times \text{SD}) \quad (1)$$

$$\text{Est. MDL} = \text{Mean} + (3 \times \text{SD}) \quad (2)$$

where Mean=mean concentration of matrix blanks or spiked blanks; and SD=standard deviation of the replicate matrix blank or spiked blank analyses. The estimated MDL and MQL values are shown in Table 3.

The MQL was then validated at or near the estimated MQL concentration by analyzing blank matrix spiked with allergen commodities and determining the mean and standard deviation

of the results. In cases where the estimated MQL was below the lowest matrix-matched calibration standard, the MQL was validated at the concentration of the lowest matrix-matched calibration standard. The MQL was considered validated at the tested concentration if the repeatability relative standard deviation (RSD_r) was $\leq 20\%$. Table 4 shows the validated MQL concentrations with RSD_r values ranging from 3.9 to 11.1%. Figure 4 shows the chromatograms of the allergens in each matrix at the MDL and MQL. Overall, the method demonstrated adequate sensitivity as required by the SMPR for the claimed analyte/matrix combinations.

Extraction Recovery

Extraction recovery was assessed at 10, 100, and 1000 mg/kg by comparing the area ratios of matrix spiked before sample

Table 3. Estimation of MDL and MQL

Analysis No.	Whole egg, mg/kg				Whole milk, mg/kg		Peanut, mg/kg		Hazelnut, mg/kg
	Cookies	Bread	Cookie dough	Salad dressing	Cookies	Red wine	Cookies	Breakfast cereal	Cookies
1	0.047	0.100	0.150	0.022	0.571	1.83	-0.883	0.834	2.40
2	0.181	0.039	0.334	-0.044	0.689	1.82	0.397	1.015	2.54
3	0.064	0.243	0.290	0.024	0.501	1.83	0.347	0.763	1.65
4	0.028	0.024	0.712	0.038	1.599	2.07	-0.188	0.816	2.03
5	0.043	0.018	0.599	0.023	0.403	1.41	0.352	0.673	2.33
6	0.030	0.022	0.372	0.041	0.223	1.14	0.146	0.636	1.89
7	0.130	0.170	0.680	0.043	0.489	2.20	1.264	0.728	2.17
Mean	0.075	0.088	0.448	0.021	0.639	1.76	0.205	0.781	2.14
SD	0.058	0.088	0.216	0.030	0.447	0.37	0.651	0.126	0.31
Est. MQL	0.7	1.0	2.6	0.3	5.1	5.4	6.7	2.0	5.3
Est. MDL	0.2	0.4	1.1	0.1	2.0	2.9	2.2	1.2	3.1

Table 4. Validation of MQL determined from seven independent analyses of blank food matrices incurred or spiked with allergen commodities at or near the estimated MQL concentrations

		Whole egg, mg/kg				Whole milk, mg/kg		Peanut, mg/kg		Hazelnut, mg/kg
Parameter		Cookies	Bread	Cookie dough	Salad dressing	Cookies	Red wine ^a	Cookies	Breakfast cereal	Cookies
Est. MQL		0.7	1.0	2.6	0.3	5.1	5.4	6.7	2.0	5.3
Tested MQL		3.0	3.0	3.0	3.0	10.0	10.0	10.0	3.0	10.0
Analysis No.	1	3.2	3.5	3.1	3.5	11.6	9.7	9.3	3.0	9.6
	2	3.4	3.2	2.6	3.4	11.0	9.8	9.9	2.8	8.3
	3	3.4	2.9	3.1	3.0	10.8	9.9	10.6	2.5	10.2
	4	2.9	2.8	2.6	3.1	10.3	11.2	9.5	2.5	9.5
	5	3.5	2.5	3.0	2.9	10.1	11.1	9.8	2.5	8.0
	6	3.5	3.0	3.2	3.0	9.9	10.8	10.7	2.6	8.6
	7	3.3	3.3	2.9	3.4	10.1	10.8	10.5	2.6	9.1
Mean		3.3	3.0	2.9	3.2	10.5	10.5	10.0	2.6	9.0
SD		0.2	0.3	0.2	0.2	0.6	0.6	0.6	0.2	0.8
RSD _n %		6.4	11.1	8.3	7.6	5.8	6.2	5.6	7.2	8.7

^a Tested in red wine since white wine contained milk as fining agent.

preparation with matrix spiked after sample preparation (Figure 5). For the claimed analyte/matrix combinations, the method demonstrated adequate recovery between 57 and 119% (Table 5). Whole egg and peanut in cookies at 1000 mg/kg were the only instances where the recovery was just below 60% (59.6% and 57.3%, respectively). At the 10 and 100 mg/kg levels, however, the recoveries in these cases were above 60%.

Accuracy and Repeatability Precision (RSD_r)

Accuracy is defined as the closeness of the calculated concentration obtained by the method to the actual incurred or spiked concentration of the allergen commodity. It was calculated as the mean method result of the seven replicates divided by the known allergen commodity concentration and expressed as a percentage. Accuracy was calculated for each

of the seven matrix-matched calibration standards for each allergen/matrix combination and the data are presented in Table 6. Calibration residual errors must be within $\pm 20\%$ nominal concentration for the calibration curve to be acceptable. Repeatability precision, expressed as RSD_r, is also presented in Table 6 for each of the calibration standards and must be $\leq 20\%$ to meet the SMPR criterion. The method demonstrated good accuracy (87.9–118.7%) and good repeatability precision (0.2–16.2%) across 3 days of analyses and fulfilled the SMPR requirements ($\leq 20\%$).

Comparison with SMPR Requirements

The overall method performance results are summarized in Table 2 and compared with the SMPR 2016.002 requirements. For the 10 allergen/matrix combinations claimed by the

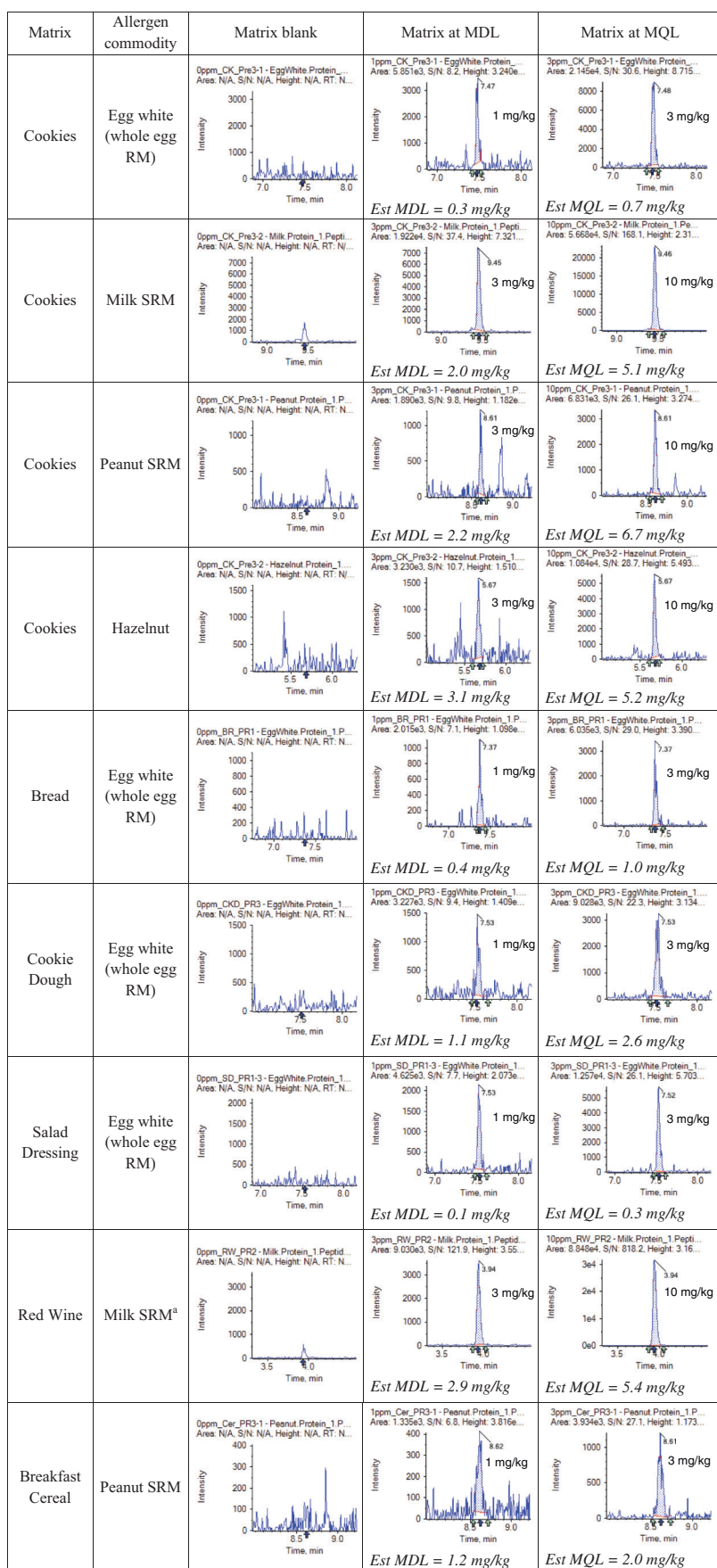


Figure 4. Extracted ion chromatograms of the quantifier ion in matrix blanks and matrices spiked with allergen commodities at or near the estimated MDL and MQL.

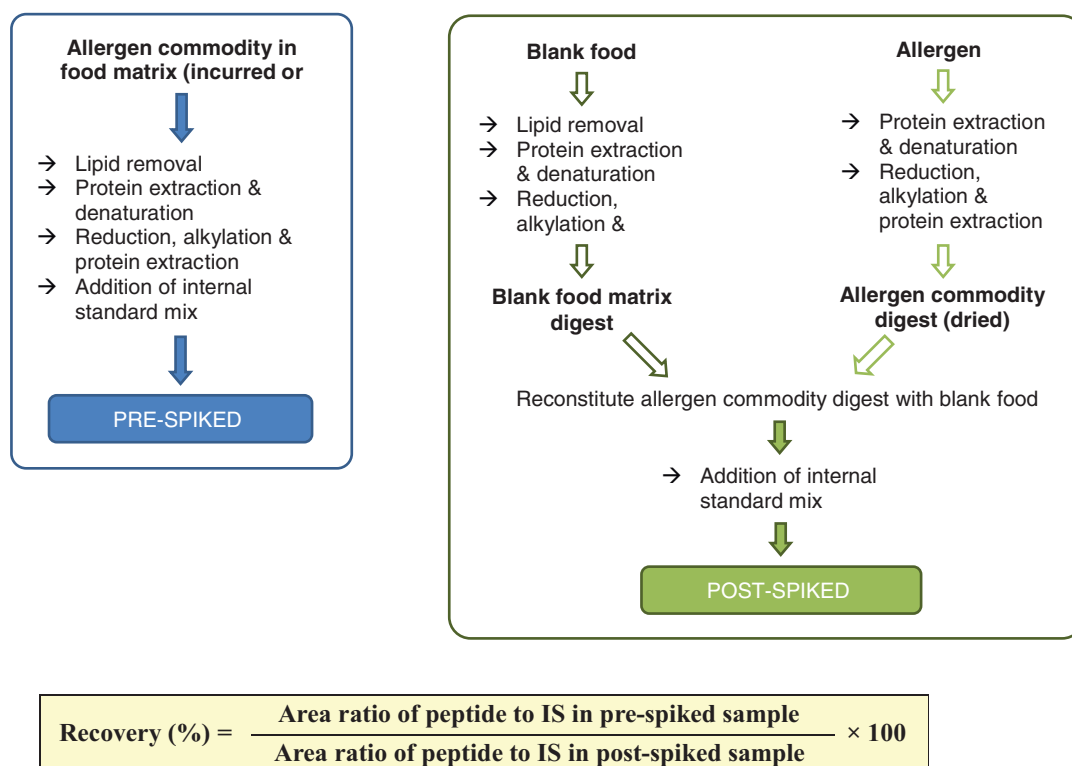


Figure 5. Workflow for preparing recovery samples and calculating recovery (percentage).

Table 5. Recovery of the allergen commodities from various food matrices^a

Calibration standard	N	Rec., %								
		Whole egg				Whole milk		Peanut		Hazelnut
		Cookies	Bread	Cookie dough	Salad dressing	Cookies	Red wine	Cookies	Breakfast cereal	Cookies
10	7	73.2	73.7	113.3	94.2	62.4	73.2	68.7	94.9	77.6
100	7	71.1	69.7	96.6	88.2	61.0	100.6	61.9	110.6	77.1
1000	7	59.6	65.0	100.6	78.2	60.2	119.3	57.3	84.6	70.5

^a Similar recoveries were obtained for the qualifier ion.

method, all met the SMPR requirements using matrix-matched calibration standards to define method performance (the closest higher calibration points were selected as MQL and MDL results). A study using blinded spiked and incurred samples as unknowns calculated against a calibration curve generated from a single set of matrix-matched standards should be undertaken to further evaluate the performance of the method.

Future Directions

Poor recovery is the main reason why most of the remaining SPSFAM priority allergen/matrix combinations were unable to reach the SMPR required MQL and MDL and therefore are not part of the method claim. In particular, chocolate has been reported to be a challenging matrix because it contains high concentrations of tannins and polyphenols, which have a high propensity to bind to proteins (7). To improve sensitivity and

recovery in cases such as chocolate and ice cream, optimization of postdigestion sample cleanup using solid-phase extraction should be explored. Additional work, including synthesizing labeled peptide ISs for M2 in wine, egg yolk peptide, and an alternative egg white peptide for use in wine, is needed to make the method more robust and to expand the method scope to include the remaining priority allergen/matrix combinations.

Conclusions

The method meets the SMPR criteria for the subset of allergen/matrix combinations claimed including egg white in cookies, bread, cookie dough, and salad dressing; for whole milk in cookies and wine; peanut in cookies and breakfast cereal; and hazelnut in cookies. All the allergens were not tested in all the matrices. The SLV presented in the study has been approved by the respective AOAC ERP on September 25, 2017.

Table 6. Accuracy and repeatability of the method determined from the mean of values from seven independent analyses over 3 days

Calibration standard	N	Parameter	Whole egg				Whole milk		Peanut		Hazelnut
			Cookies	Bread	Cookie dough	Salad dressing	Cookies	Red wine	Cookies	Breakfast cereal	Cookies
1	7	Accuracy, %	99.4	99.5	BQL ^a	95.4	BQL	BQL	BQL	BQL	BQL
		RSD _n , %	7.3	16.2	BQL	8.9	BQL	BQL	BQL	BQL	BQL
3	7	Accuracy, %	110.5	101.1	97.9	105.8	BQL	BQL	BQL	87.6	BQL
		RSD _n , %	6.0	10.9	8.5	8.1	BQL	BQL	BQL	7.5	BQL
10	7	Accuracy, %	111.2	96.4	105.5	101.2	105.4	104.9	100.3	100.7	90.4
		RSD _n , %	5.3	8.2	4.9	5.8	6.0	6.2	5.5	4.1	8.4
30	7	Accuracy, %	87.9	93.8	91.2	96.5	94.3	106.3	100.2	106.1	105.7
		RSD _n , %	6.7	7.1	6.7	5.0	4.4	5.4	4.2	3.9	3.6
100	7	Accuracy, %	90.1	95.3	96.1	99.8	101.3	95.5	102.5	105.8	118.7
		RSD _n , %	2.8	4.0	1.6	4.4	3.2	4.4	2.9	2.1	4.6
300	7	Accuracy, %	99.6	100.6	100.9	97.8	98.5	99.7	96.1	100.7	97.9
		RSD _n , %	2.5	2.6	3.4	4.2	5.2	4.4	3.6	2.5	4.5
1000	7	Accuracy, %	101.3	100.5	100.3	100.8	100.4	100.4	100.9	99.0	99.9
		RSD _n , %	0.9	1.3	1.3	1.6	1.6	0.2	1.2	0.7	1.2

^a BQL = Below quantitation limit.

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