

SPECIAL GUEST EDITOR SECTION: FOOD ALLERGENS NEW METHODS

A Targeted LC-MS/MS Method for the Simultaneous Detection and Quantitation of Egg, Milk, and Peanut Allergens in Sugar Cookies

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Food allergy is a growing public health concern, with many individuals reporting allergies to multiple food sources. Compliance with food labeling regulations and prevention of inadvertent cross-contact in manufacturing requires the use of reliable methods for the detection and quantitation of allergens in processed foods. In this work, a novel liquid chromatography-tandem mass spectrometry multiple-reaction monitoring method for multiallergen detection and quantitation of egg, milk, and peanut was developed and evaluated in an allergen-incurred baked sugar cookie matrix. A systematic evaluation of method parameters, including sample extraction, concentration, and digestion, were optimized for candidate allergen peptide markers. The optimized method enabled the reliable detection and quantitation of egg, milk, and peanut allergens in sugar cookies, with allergen concentrations as low as 5 ppm allergen-incurred ingredient.

Food allergy prevalence, currently estimated to be between 3 and 4% of the general population (1), is increasing, posing a substantial concern to public health (2, 3). In order to better protect consumers with food allergies, the Food Allergen Labeling and Consumer Protection Act (4) was enacted in 2004 and mandates the identification of eight major allergens (crustacean shellfish, egg, fish, milk, peanut, soy, tree nuts, and wheat) on all labels for packaged foods sold in the United States. Presently, there is no cure for food allergies, which are otherwise managed by strict avoidance of the suspect food and the preparedness to address allergic reactions (5). To effectively safeguard the food-allergic population, the food industry and

regulators need reliable analytical methods for allergen detection and quantitation in a variety of food matrices.

Commercial immunochemical-based technologies (e.g., ELISA and lateral-flow devices) are the most commonly used analytical platforms for allergen quantitation in food. Despite the sensitivity and standardization of ELISA test kits for routine food analysis, quantitation may vary between manufacturers due to differences in protein extraction procedures, reference materials, selectivity of antibodies, and the format of analytical results (6–8). In recent years, a paradigm shift to targeted MS methods has been driven by the need for orthogonal confirmatory technologies for allergen quantitation. Liquid chromatography (LC)-tandem mass spectrometry (MS/MS) methods harness the capability of multiplex quantitation in a single analytical experiment. Proteolytic peptides are selected as a fingerprint for the allergen protein(s) of interest and selectively optimized under tunable extraction, digestion, and instrument conditions for allergen detection and quantitation. Although LC-MS/MS techniques are less time- and cost-efficient, there is no reliance on epitopes for peptide identification, making MS techniques more amenable to the analysis of thermally processed foods. Although the majority of current MS methods are based on single allergen detection (9–12), multiallergen LC-MS/MS methods have recently emerged as an efficient alternative for method development (6, 13–17).

This study aims to showcase the performance of a targeted LC-MS/MS method for the detection and quantitation of multiple food allergens (egg, milk, and peanut) in a thermally processed bakery product (sugar cookies). This matrix was chosen as a result of its complexity, multiallergen content, thermal-processing effects, and prior analysis by ELISA (18). Method development included optimization of the extraction, concentration, and digestion of samples for candidate peptide markers. General challenges affiliated with protein-based methods for allergen detection in food are also addressed as a means to characterize method abilities and assess limitations. Ultimately, this work serves as a foundation for implementing the quantitative analysis of allergens by LC-MS/MS to support food allergen management and the protection of consumers diagnosed with food allergies.

Experimental

Formulation of Allergen-Incurred Sugar Cookies

Sugar cookies were prepared at the U.S. Food and Drug Administration, Institute for Food Safety and Health (Bedford

Guest edited as a special report on “A Global Reflection on Food Allergen Regulations, Management, and Analysis” by Carmen Diaz-Amigo and Bert Popping.

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Supplemental information is available on the *J. AOAC Int.* Web site, <http://aoac.publisher.ingentaconnect.com/content/aoac/jaoac>

This research was funded by the U.S. Food and Drug Administration (FDA), Center for Food Safety and Applied Nutrition (College Park, MD). Chelsea C. Boo was supported, in part, by appointment to the Research Participation Program, administered by the Oak Ridge Institute for Science and Education (Oak Ridge, TN) through an interagency agreement between the U.S. Department of Energy and the FDA.

DOI: <https://doi.org/10.5740/jaoacint.17-0400>

Park, IL) as control (0 ppm) and allergen-incurred samples. Sugar cookies were formulated with spray-dried whole-egg powder, NIST 8445 reference material (National Institute of Standards and Technology, Gaithersburg, MD), nonfat dry milk, NIST 1549 standard reference material, and partially defatted (12%) light-roast peanut flour (Golden Peanut Co., Alpharetta, GA) at 5, 10, 25, 100, and 500 µg/g (ppm, w/w) for each of the three allergen ingredients prior to baking. The detailed preparation of incurred samples has previously been published (18). In brief, a premix containing 5000 ppm egg powder, nonfat dry milk, and peanut flour was prepared in all-purpose flour and mixed for 2 h with a KitchenAid mixer. For incurred samples, the appropriate concentration of flour premix was added to the remaining dry ingredients (all-purpose flour, baking powder, baking soda, and pure cane sugar) and mixed for 2 h with a Hobart mixer. Blending of dry and binder ingredients (vanilla extract, all-vegetable shortening, and water) was performed using the Hobart mixer for 20 min to create a dough mixture. The dough was baked for 25 min at 190°C (374°F). Baked cookies (approximately 600 g/batch) were ground in a food processor to achieve homogeneity prior to sampling. Control cookies (0 ppm allergen ingredient) were similarly prepared in the absence of the allergen ingredient.

Preparation of Cookie Extracts for LC-MS/MS

All chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless stated otherwise. All solvents were Optima LC/MS grade and purchased from Fisher Scientific (Pittsburgh, PA). Homogenized cookie samples were weighed (0.1000 ± 0.0002 g) in triplicate into 2.0 mL Eppendorf Protein LoBind centrifuge tubes (Hauppauge, NY) and defatted with 1400 µL hexane by end-over-end rotation for 15 min. Following 5 min of centrifugation at $12\,000 \times g$ in an Eppendorf centrifuge (Model No. 5424R), the hexane layer was discarded, and defatting was repeated two additional times. Samples were vacuum-dried for 15 min using a Labconco CentriVap (Kansas City, MO) and weighed to estimate total fat loss. The defatted samples were reconstituted in 1000 µL extraction buffer [2 M urea, 50 mM Tris-buffered saline (TBS), and 25 mM dithiothreitol (DTT)]. Protein extraction was achieved by incubation in an Eppendorf ThermoMixer for 5 min at 1000 rpm, water bath sonication at ambient temperature in a Branson sonicator (Danbury, CT) for 10 min, and end-over-end rotation for 15 min. Following 15 min of centrifugation at $14\,000 \times g$, the extraction supernatant was reserved and the extraction process repeated once more, after which both extraction supernatants were combined. Total protein concentrations were estimated using a Qubit Fluorometer (Life Technologies Corp., Carlsbad, CA).

Sample Concentration and Digestion

Sample concentration and digestion were achieved using a modified filter-aided sample preparation (FASP) method (19). For each extraction solution, volumes totaling 1.0 mg total protein were mixed with 2 M urea, 50 mM TBS, and 20 pmol yeast enolase (digestion quality control; QC) in 1.5 mL

Eppendorf Protein LoBind centrifuge tubes. Proteins were reduced with 10 mM DTT at 37°C at 1000 rpm for 1 h and alkylated with 25 mM iodoacetamide at ambient temperature and gentle shaking for 1 h. Protein solutions were concentrated at $14\,000 \times g$ (20°C) using pre-equilibrated Amicon Ultra 0.5 mL Centrifugal Filter Units with Ultracel-10 membranes (Millipore, Billerica, MA) and washed with 500 µL of 1 M urea and 50 mM ammonium bicarbonate. The concentrated sample (20 µL) was reconstituted with Waters RapiGest (RG) acid-labile surfactant (Waters Corp., Milford, MA) at a final concentration of 0.1% in 50 mM ammonium bicarbonate (v/v). On-membrane digestion was accomplished using Promega MS Grade Trypsin Gold (Madison, WI) at a 100:1 total protein-to-enzyme ratio. Solutions were diluted to volume (100 µL) with 50 mM ammonium bicarbonate and incubated in a ThermoMixer at 37°C at 650 rpm for 4 h. Digested peptides were collected by centrifugation for 30 min at $14\,000 \times g$ (20°C). The membrane was washed with 50 mM ammonium bicarbonate (50 µL) and the centrifugation repeated. Peptide digestion was quenched upon addition of 0.5% trifluoroacetic acid (TFA) and 2.0% acetonitrile. The RG surfactant was degraded during a 30 min incubation at 37°C (ThermoMixer). Digests were centrifuged for 5 min at $12\,000 \times g$ (20°C).

Directly prior to MS analysis, sample digests were diluted 1:2 in dilution buffer containing 94.9% water, 5% acetonitrile, 0.1% formic acid, 5 fmol/µL rabbit phosphorylase B digest (Waters Corp.), and 1 pmol/µL angiotensin I. In all samples, heavy-labeled ($^{13}\text{C}^{15}\text{N}$) synthetic peptide surrogates were spiked into diluted digests prior to injection at a final concentration of 2.5 fmol/µL. Peptide digests were transferred to Waters LC-MS-certified clear glass screw-neck total-recovery vials (12 × 32 mm) with cap and preslit PTFE/silicone septa.

Preparation of Light and Heavy-Labeled Synthetic Peptides

Light (native) and heavy-labeled ($^{13}\text{C}^{15}\text{N}$) synthetic peptides were synthesized and purchased from New England Peptide, Inc. (Gardner, MA). The following specifications were required for all synthesized peptides: $\geq 97\%$ purity confirmed by HPLC with UV detection, confirmation of mass by MS, peptide content determined by amino acid analysis, equimolar peptide distribution [light:heavy (L:H) peptide peak area ratio = 1.0 ± 0.2], and vial-to-vial reproducibility (L:H peptide peak area ratio RSD of $<20\%$). Lyophilized peptides were received as 10 nmol equimolar mixtures of each peptide marker separated by allergen (egg, milk, and peanut) and isotopic label (light and heavy). Synthetic peptides were prepared in accordance with recommendations by Hoofnagle et al. (20). Vendor tubes were centrifuged for 5 min and reconstituted to 100 pmol/µL with 94% water, 5% acetonitrile, 1% formic acid, 5 fmol/µL rabbit phosphorylase B digest, and 1 pmol/µL angiotensin I. Reconstituted peptides were mixed on a vortex mixer, sonicated, and centrifuged, each for 5 min, and aliquoted in 0.5 mL Eppendorf Protein LoBind centrifuge tubes. Lyophilized peptides and concentrated stock solutions were stored at -80°C for >1 year and up to 6 months, respectively (20).

Preparation of Standard Curves for Quantitation

A matrix-matched calibration curve was prepared over the concentration range of 0.05–100 fmol/μL using a neat stock of light synthetic peptides in a 1:2 diluted 0 ppm baked cookie lysate. In all samples, the heavy synthetic peptide surrogate concentration remained uniform at 2.5 fmol/μL. Interleaved blanks and QC standards (e.g. LC-MS/MS certified bovine serum albumin digest; Michrom, Auburn, CA) were incorporated throughout the sample sequence. The midpoint calibration curve solution (2.5 fmol/μL) was also injected every 12 h throughout the sample analysis to monitor sample stability and instrument performance.

Light (native) and heavy-labeled ($^{13}\text{C}^{15}\text{N}$) synthetic peptide peak area ratios were monitored and used for calculation of allergen ingredient concentration (Figure 1). It should be noted that the calculation scheme was applied to each individual allergen peptide marker. Thus, a final calculated concentration represents the parts-per-million (micrograms per gram of defatted food) amount of allergen ingredient (total egg, milk, or peanut) as derived from a single allergen peptide marker. Sample preparation recoveries were estimated by monitoring peak areas for heavy-labeled synthetic peptides in a digestion- and injection-fortified control (0 ppm) sample matrix. Digestion-fortified samples were prepared in triplicate and analyzed in duplicate. The average method recovery for all peptides was calculated to be $77 \pm 20\%$ for the baked matrix. The relative protein composition is expressed as the percent contribution of each individual allergen protein in the total protein content for egg, milk, and peanut (Supplemental Information Table S1). Although values in the literature for the relative allergen protein composition of egg (21) and milk (22) were used, the relative allergen protein composition for peanut was experimentally estimated from a light-roast peanut flour extract as the percentage of total identified MS/MS spectra (spectral counts) for all peptides within an allergen protein family (custom database curated with 357 664 protein sequences). Percent nitrogen content was measured using a micro-Kjeldahl method for each allergen source material (18) and is representative of the total protein content in egg, milk, and peanut allergen ingredients (Supplemental Information Table S2). Moisture loss from the baked cookies was minimal ($<2\%$) and, therefore, determined negligible in the calculated concentrations.

LC/MS/MS

Chromatographic separations were conducted using a Waters nanoACQUITY ultra-performance LC (UPLC) system operated in single-pump trapping mode. Sample injections were loaded

onto an ACQUITY UPLC M-Class symmetry trap column (180 μm id × 20 mm) packed with 5 μm C18 particles (100 Å) for 3 min at 5 μL/min. Solvent composition during trapping consisted of 99.5% A (0.1% formic acid in water) and 0.5% B (0.1% formic acid in acetonitrile). For separation, sample injections were loaded onto a reversed-phase ACQUITY UPLC analytical capillary column (100 μm id × 100 mm) packed with 1.7 μm C18 BEH particles (130 Å) and maintained at 40°C. Partial loop injection volumes (2 μL) were consistent for all samples (5 μL loop). Peptides were eluted with a linear gradient of 3–40% B in 30 min, followed by a ramp up to 85% B (4 min hold) prior to re-equilibration (55 min total run time). The column flow rate was maintained at 300 nL/min. The weak needle wash solvent (600 μL) was composed of 96.9% water, 3% acetonitrile, and 0.1% TFA (v/v). The strong needle wash solvent (200 μL) was composed of 0.1% formic acid in acetonitrile (v/v). The autosampler was maintained at 4°C.

Multiple-reaction monitoring (MRM) MS analysis was conducted using a Sciex QTRAP 6500 (Framingham, MA) operated in positive-ion mode and equipped with a Sciex NanoSpray III Ion Source and a precut uncoated silica tip emitter capillary (360 μm od × 20 μm id) with a 10 μm id tip (New Objective, Woburn, MA). MS/MS data were collected in a scheduled MRM mode with a 300 s detection window, a targeted scan time of 1.65 s, and a 5 ms pause between mass ranges. Q1 resolution and Q3 resolution were low and unit, respectively. Source parameters were as follows: curtain gas at 20 au, collision gas at high (12 au), ionspray voltage at 2400 V, ion source gas 1 at 20 au, ion source gas 2 at 0 au, and interface heater temperature at 150°C. The collision energy (CE) and collision cell exit potential (CXP) were optimized for each light and heavy-labeled peptide transition via syringe-assisted infusion. Values for declustering potential and entrance potential were 80 and 10 V, respectively, for each peptide transition. The final LC-MS/MS MRM method monitored 102 peptide transitions. Both light and heavy-labeled variations of egg, milk, and peanut allergen peptides were included (28 peptides, with 3 transitions per peptide) along with yeast enolase (digestion QC) and rabbit phosphorylase B (injection QC); 6 total peptides, with 3 transitions per peptide. LC-MS/MS data were processed using Analyst 1.6.2 (Sciex) and Skyline Version 3.6 (23).

Results and Discussion

Optimization of the Sample Preparation

For complex, processed food matrixes, sample preparation is generally regarded as the most significant element in proteomics method development (19). Despite challenges in

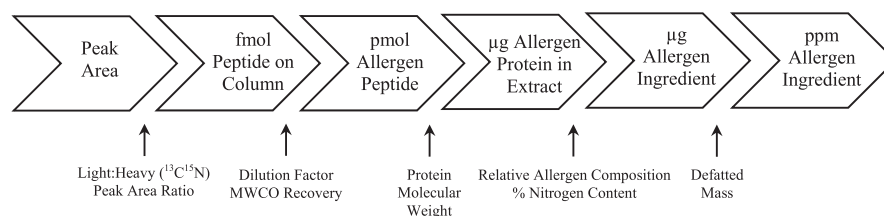


Figure 1. Flowchart calculation of allergen ingredient concentration (parts per million) by LC-MS/MS. MWCO = Molecular weight cut-off filter.

the development of a single, universal sample preparation methodology for all food allergens, matrix components, and processing conditions (24, 25), the current methodology provides an efficient and robust sample preparation procedure for the extraction and digestion of select egg, milk, and peanut protein allergens in allergen-incurred sugar cookies. Prior optimization of a protein extraction protocol incorporating the addition of a chaotropic denaturant (2 M urea) and a reducing agent (25 mM DTT) in a TBS buffer at pH 8.0 (14) was adopted in the current work to facilitate protein solubility across a diverse composition of protein chemistries in a heat-processed food matrix. Sugar cookies were incurred with egg, milk, and peanut allergen standards prior to baking to provide a representative test matrix similar to a commercially available food product (18). The 25 ppm allergen-incurred sugar cookie was selected for method development to enhance the recovery of candidate egg, milk, and peanut peptide markers.

A modified FASP protocol was incorporated into the method based on previous utilization and success in allergen-incurred bakery products (14). Protein concentration on-filter was evaluated to optimize peptide recovery and removal of denaturant/detergent prior to digestion (19). Four total protein concentrations on-filter were investigated: 0.5, 1.0, 1.5, and 2.0 mg. Due to reduced matrix interferences, a decreased protein concentration time, and reproducible digestion performance, a 1.0 mg total protein capacity was selected (Supplemental Information Figure S1).

The transformation of proteins into peptides is considered the most critical step in a proteomics workflow. The applied

digestion protocol can drastically affect the yield of tryptic peptide formation, resulting in different (quantitative) results from the same peptide under various method conditions (26, 27). Common tryptic digestion parameters involve overnight incubation (12–16 h) at 37°C, but parameters are experiment-dependent and should be investigated accordingly (27). In this work, trypsin incubation time/temperature, protein:trypsin ratio, and RG surfactant incorporation were evaluated for selected egg, milk, and peanut peptide markers in the 25 ppm incurred cookie. Figure 2 provides a summary of data collected for all digestion optimization experiments. The light to heavy-labeled synthetic peptide peak area ratio (L:H) was used as a measure of digestion efficiency and compared for seven allergen peptide markers: egg peptides GGLEPINFQTAADQAR (ovalbumin) and FESNFNTQATNR (lysozyme C); milk peptides LSFNPTQLEEQCHI (β -lactoglobulin) and YLGYLEQLLR (α -S1 casein); and peanut peptides NNPFYFPSR (Ara h 1), NLPQQCGLR (Ara h 2), and SPDINYPQAGSLK (Ara h 3). Overall, modifications to the digestion protocol exhibited minimal change in the detection of egg, milk, and peanut allergen peptide targets, with the exception of the positive effects of RG incorporation on egg ovalbumin peptide GGLEPINFQTAADQAR. The final optimized digestion parameters were incorporated into all future experiments: incubation of 100:1 protein-to-trypsin ratio with RG at 37°C for 4 h. Despite efforts to globally improve recovery of individual peptide markers in heat-processed sugar cookie samples, results concluded that sample digestion parameters must be compromised to balance variations in protein chemistry for egg, milk, and peanut allergens.

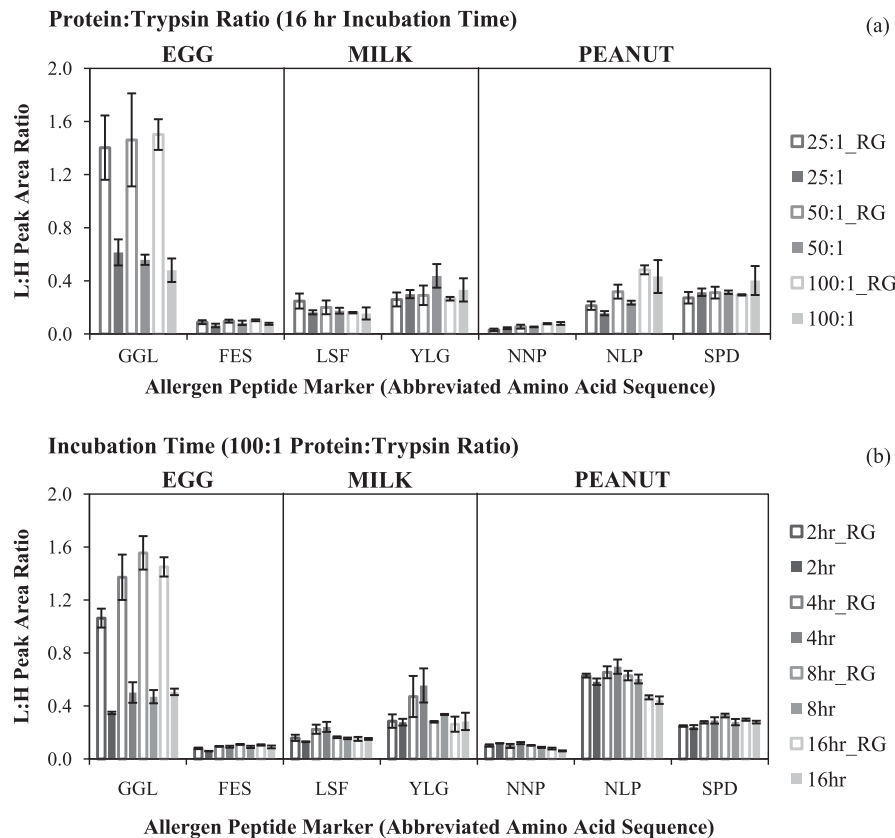


Figure 2. Optimization of trypsin digestion through investigation of (A) protein:trypsin ratio and (B) incubation time for 25 ppm-incurred sugar cookie. Both experiments incorporated 1 mg total protein with trypsin digestions performed at 37°C with and without RG surfactant. Error bars represent deviations between sample preparation ($n = 2$) and instrumental ($n = 2$) replicates. RG = RapiGest SF surfactant.

Optimization of Allergen Peptide Markers and Quantitative Method Development

Candidate peptide markers from egg, milk, and peanut protein allergens were selected using an MS1-based comparative proteomic survey of thermally processed food sample extracts and based on empirical MS identifications, physiochemical properties, specificity, processing effects, cultivar variations, and matrix influences (10, 14, 16, 18, 20, 28–31). Although all candidate peptide markers were selected from known protein allergens, the degree of protein allergenicity and correlation with immunoglobulin E epitope recognition were not considered. Optimization of reversed-phase LC separation, charge-state selection, and compound-dependent MS parameters was completed for each candidate peptide marker in neat solution and verified in sugar cookie digests. As a general rule, the three most intense y-ion transitions with masses greater than their corresponding precursor mass were monitored.

Considerations for the final peptide markers were based on chromatographic peak shape, relative abundance, matrix interference, and stability. The following peptides (quantitation and confirmation, respectively listed) were selected as optimized allergen peptide markers: egg peptides GGLEPINFQTAADQAR and YPILPEYLQCVK (ovalbumin) and FESNFNTQATNR and GTDVQAWIR (lysozyme C); milk peptides LSFNPTQLEEQCHI and TPEVDDEALEK (β -lactoglobulin) and YLGYLEQLLR and FFVAPFPEVFGK (α -S1 casein); and peanut peptides NNPFFYPSR and GTGNLELVAVR (Ara h 1), NLPQQCGLR and CCNELNEFENNQR (Ara h 2), and SPDIYNPQAGSLK and FNLAGNHEQEFLR (Ara h 3). A complete peptide transition list for allergen peptide markers, accompanied by CE, CXP, and approximate retention time (RT) values, is presented in Table 1. A representative extracted ion chromatogram of a 500 ppm incurred cookie illustrates the elution profile of the 14 equimolar heavy-labeled ($^{13}\text{C}^{15}\text{N}$) allergen peptide markers across a 30 min gradient (Figure 3). Yeast enolase (ENO1_YEAST) and rabbit phosphorylase B (PYGM_RABIT) peptides, incorporated as digestion and injection QC standards, respectively, were selected to provide wide coverage across the same elution gradient (Supplemental Information Figure S2). Instrument conditions for QC standards (ENO1_YEAST and PYGM_RABIT) are summarized in Table 2.

The targeted LC-MS/MS method provided the simultaneous detection of 14 peptide targets from egg, milk, and peanut protein allergens. Matrix-matched calibration solutions were prepared in a control cookie matrix (0 ppm) over the concentration range of 0.1–200 fmol on the column. Quantitation was performed by isotope-dilution MS and the matrix-matched calibration curves generated based on the ion signal ratio for the most abundant light (native) and heavy ($^{13}\text{C}^{15}\text{N}$) transition of each peptide. Linear calibration regressions had an R^2 value of >0.99 for all seven quantitation peptide markers. The RT variations for all light and heavy-labeled peptides in each sample were within $\pm 3\%$ of the average RT for the calibration standards. RT alignments and confirmed ion ratios between quantitation and confirmation transitions ($\pm 20\%$ tolerance limit of relative abundance) authenticate the detection of an individual peptide. For each peptide, quantitation ions were reported with an S/N of $>10:1$ and confirmation ions with an S/N of $>3:1$. Consistency in instrument performance was observed, with technical replicates having RSDs of $<5\%$.

Application of the Multiallergen LC-MS/MS Method to Allergen-Incurred Sugar Cookies

Method performance for the multiallergen MRM method was evaluated in egg, milk, and peanut allergen-incurred baked cookies. Reliable detection was achieved in the 5 ppm incurred cookie for three of four egg peptides, all milk peptides, and two peanut peptides. All allergen peptide markers were detectable in the 25 ppm allergen-incurred cookie (Figure 4). Variances in relative abundances of allergen peptide markers are indicative of ionization efficiency (Figure 3), respective relative allergen protein compositions (Supplemental Information Table S1), and food matrix/processing effects. For most proteins, thermal heating results in the formation of insoluble protein aggregates and oligomers, reduced protein solubility, and a propensity for chemical modification (28). As previously demonstrated, the extent of heat processing has been shown to alter the peptide relative abundance profile for α -S1 casein peptides YLGYLEQLLR and FFVAPFPEVFGK as compared with heavy synthetic peptide surrogates (9). A similar phenomenon is observed between peanut Ara h 3 peptides SPDIYNPQAGSLK and FNLAGNHEQEFLR. Considering extraction efficiency in matrix, peptide ionization, and chromatographic features, a condensed list of allergen peptide markers was selected for quantitation from each allergen protein: GGLEPINFQTAADQAR (ovalbumin), FESNFNTQATNR (lysozyme C), LSFNPTQLEEQCHI (β -lactoglobulin), YLGYLEQLLR (α -S1 casein), NNPFFYPSR (Ara h 1), NLPQQCGLR (Ara h 2), and SPDIYNPQAGSLK (Ara h 3). In most cases, quantitation peptide markers were selected as exhibiting equivalent (or near-equivalent) solubilization in a pre- and post-processed matrix (14).

In this work, allergen concentration is represented as parts-per-million (micrograms per gram) allergen ingredient and is defined in Figure 1. All values were experimentally determined for each allergen ingredient, with the exception of the protein molecular weight (MW) and relative allergen protein composition for egg (21) and milk (22), which were adopted from the literature. The relative allergen protein composition for peanut was estimated from a light-roast peanut flour extract as the percentage of total identified spectral counts for all peptides within an allergen protein family and incorporates artifacts of the current extraction method. The allergen protein composition of peanut is dependent on thermal processing (raw versus roasted), cultivar, and geographic varieties (32) and should be taken into consideration as a variable when evaluating sample matrixes with incurred peanut allergen. Percent nitrogen content was determined for each allergen source material and incorporated into the calculation of the total available protein in each allergen ingredient. Inconsistencies between total available protein content and extractable protein content; however, will result in underestimations of total allergen ingredient. Such discrepancies are less pronounced for the milk and egg protein allergens as compared with roasted peanut (14). As MS-based methods transition toward use as confirmation or quantitative applications for allergen detection, the need for harmonization between reporting units, diversification of food commodities, and method validation will ultimately help refine current methods for use in routine analysis.

Figure 5 portrays a comparison of allergen ingredient (parts per million) calculated for the seven quantitation peptides in five

Table 1. Sciex QTRAP 6500 instrument conditions for target light and heavy-labeled ($^{13}\text{C}^{15}\text{N}$) peptide markers for egg (ovalbumin and lysozyme C), milk (beta-lactoglobulin and alpha-S1-casein), and peanut (Ara h 1, Ara h 2, and Ara h 3) allergen proteins.

Allergen	Protein name (UniProt)	Peptide sequence ^a	Precursor (m/z)	Product transition	Product (m/z)	CE (V) ^b	CXP (V) ^c	Approximate RT (min)
Egg	OVAL_CHICK	GGLEPINFQTAADQAR ^d	844.4235++	y10 ^e	1121.5334+	42	32	22.5
				y9	1007.4905+	43	29	22.5
				y8 ^f	860.4221+	43	27	22.5
		GGLEPINFQTAADQA[13C-15N-R] ^d	849.4277++	y10 ^e	1131.5417+	42	32	22.5
				y9	1017.4988+	43	29	22.5
				y8 ^f	870.4303+	43	27	22.5
		YPILPEYLQCVK ^g	761.9023++	y9 ^f	1149.5973+	37	43	25.8
				y8 ^e	1036.5132+	37	32	25.8
				y6	810.4178+	44	26	25.8
		YPILPEYLQCV[13C-15N-K] ^g	765.9094++	y9 ^f	1157.6115+	37	43	25.8
				y8 ^e	1044.5274+	37	32	25.8
				y6	818.4320+	44	26	25.8
Egg	LYSC_CHICK	FESNFNTQATNR ^d	714.8288++	y10 ^f	1152.5392+	34	36	16.3
				y8	951.4643+	34	35	16.3
				y7 ^e	804.3959+	34	30	16.3
		FESNFNTQATN[13C-15N-R] ^d	719.8329++	y10 ^f	1162.5475+	34	36	16.3
				y8	961.4725+	34	35	16.3
				y7 ^e	814.4041+	34	30	16.3
		GTDVQAWIR ^g	523.2749++	y7 ^f	887.4734+	24	27	21.0
				y6	772.4464+	29	22	21.0
				y5 ^e	673.3780+	24	23	21.0
		GTDVQAWI[13C-15N-R] ^g	528.2790++	y7 ^f	897.4816+	24	27	21.0
				y6	782.4547+	29	22	21.0
				y5 ^e	683.3863+	24	23	21.0
Milk	LACB_BOVIN	LSFNPTQLEEQCHI ^d	858.4065++	y7 ^f	928.4193+	41	31	24.7
				y6	815.3352+	39	23	24.7
				y10 ^e	627.7928++	41	22	24.7
		LSFNPTQLEEQCH[13C-15N-I] ^d	861.9151++	y7 ^f	935.4365+	41	31	24.7
				y6	822.3524+	39	23	24.7
				y10 ^e	631.3014++	41	22	24.7
		TPEVDDEALEK ^g	623.2959++	y8	918.4415+	31	29	16.7
				y7 ^f	819.3731+	30	24	16.7
				y10 ^e	572.7721++	31	18	16.7
		TPEVDDEALE[13C-15N-K] ^g	627.3030++	y8	926.4557+	31	29	16.7
				y7 ^f	827.3873+	30	24	16.7
				y10 ^e	576.7791++	31	18	16.7
Milk	CASA1_BOVIN	YLGYLEQLLR ^d	634.3559++	y8 ^e	991.5571+	31	33	28.9
				y6 ^f	771.4723+	28	22	28.9
				y5	658.3883+	29	26	28.9
		YLGYLEQLL[13C-15N-R] ^d	639.3600++	y8 ^e	1001.5654+	31	33	28.9
				y6 ^f	781.4806+	28	22	28.9
				y5	668.3965+	29	26	28.9
		FFVAPFPEVFGK ^g	692.8686++	y10	1090.5932+	27	37	30.2
				y9 ^f	991.5247+	27	30	30.2
				y8 ^e	920.4876+	26	28	30.2
		FFVAPFPEVFG[13C-15N-K] ^g	696.8757++	y10	1098.6074+	27	37	30.2
				y9 ^f	999.5389+	27	30	30.2
				y8 ^e	928.5018+	26	28	30.2

Table 1. (continued)

Allergen	Protein name (UniProt)	Peptide sequence ^a	Precursor (m/z)	Product transition	Product (m/z)	CE (V) ^b	CXP (V) ^c	Approximate RT (min)
Peanut	ALL12_ARAHY	NNPFYFPSR ^d	571.2749++	y7 ^e	913.4567+	29	27	22.3
				y6	816.4039+	31	26	22.3
				y5 ^f	669.3355+	31	22	22.3
		NNPFYFPS[13C-15N-R] ^d	576.2790++	y7 ^e	923.4649+	29	27	22.3
				y6	826.4122+	31	26	22.3
				y5 ^f	679.3438+	31	22	22.3
		GTGNLELVAVR ^g	564.8222++	y7	799.5036+	31	24	21.0
				y6 ^e	686.4196+	27	23	21.0
				y5 ^f	557.3770+	30	22	21.0
	GTGNLELVAV[13C-15N-R] ^g	569.8263++	569.8263++	y7	809.5119+	31	24	21.0
				y6 ^e	696.4278+	27	23	21.0
				y5 ^f	567.3852+	30	22	21.0
Peanut	CONG7_ARAHY	NLPQQCGLR ^d	543.2797++	y7 ^e	858.4250+	29	29	15.3
				y6	761.3723+	31	27	15.3
				y5 ^f	633.3137+	32	24	15.3
		NLPQQCGL[13C-15N-R] ^d	548.2838++	y7 ^e	868.4333+	29	29	15.3
				y6	771.3806+	31	27	15.3
				y5 ^f	643.3220+	32	24	15.3
		CCNELNEFENNQR ^g	863.8490++	y9 ^f	1163.5440+	41	33	18.3
				y8 ^e	1050.4599+	42	30	18.3
				y7	936.4170+	41	29	18.3
	CCNELNEFENNQ[13C-15N-R] ^g	868.8532++	868.8532++	y9 ^f	1173.5522+	41	33	18.3
				y8 ^e	1060.4682+	42	30	18.3
				y7	946.4253+	41	29	18.3
Peanut	O82580_ARAHY	SPDIYNPQAGSLK ^d	695.3541++	y9 ^f	977.5051+	34	28	18.5
				y8	814.4417+	34	26	18.5
				y7 ^e	700.3988+	35	24	18.5
		SPDIYNPQAGSL[13C-15N-K] ^d	699.3612++	y9 ^f	985.5193+	34	28	18.5
				y8	822.4559+	34	26	18.5
				y7 ^e	708.4130+	35	24	18.5
	Q9SQH7_ARAHY	FNLAGNHEQEFLR ^g	525.5952+++	y11 ^e	657.3335++	23	19	20.8
				y10 ^f	600.7914++	23	17	20.8
				y9	565.2729++	26	16	20.8
		FNLAGNHEQEFL[13C-15N-R] ^g	528.9313+++	y11 ^e	662.3376++	23	19	20.8
				y10 ^f	605.7956++	23	17	20.8
				y9	570.2770++	26	16	20.8

^a C = Carbamidomethyl cysteine.^b Collision Energy (V).^c Collision Cell Exit Potential (V).^d Quantitation peptide.^e Quantitation ion.^f Confirmation ion.^g Confirmation peptide.

allergen-incurred baked cookie matrixes. Quantitation in the 5 ppm allergen-incurred cookie was achieved for at least one peptide per allergen, whereas all seven allergen peptide markers were quantitated in the 25 ppm allergen-incurred cookie. For all allergens, the multiallergen LC-MS/MS method underestimated the incurred allergen amounts at all concentrations. Despite low allergen recoveries, these data

correlate with recoveries from ELISA analyses previously acquired for the same allergen-incurred sugar cookie sample set (18). This indicates that allergen recoveries for the current sample test matrix were more dependent on matrix composition than analytical technique.

In comparison with milk and peanut, egg allergen markers exhibited lower recoveries, on average, for the allergen-incurred

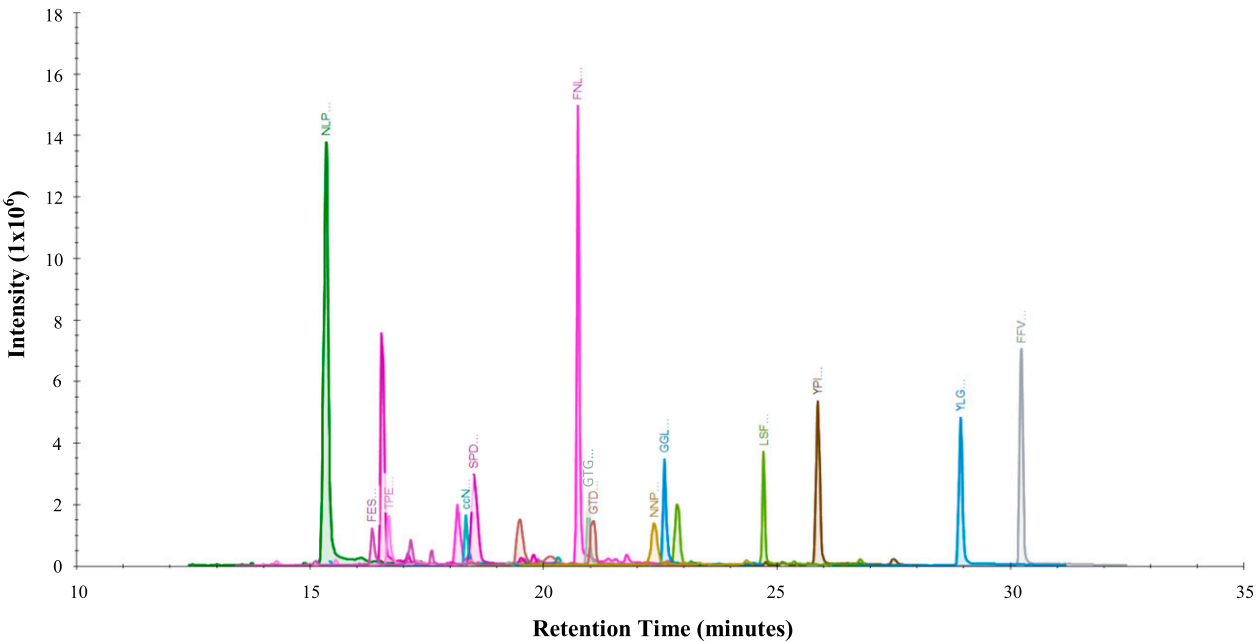


Figure 3. Extracted ion chromatogram of heavy-labeled ($^{13}\text{C}^{15}\text{N}$) synthetic peptide surrogates of targeted allergen markers in a 500 ppm incurred baked sugar cookie by LC-MS/MS.

sugar cookies. Within a food matrix, allergenic egg proteins ovalbumin and lysozyme C can be denatured or chemically altered at elevated heat treatments, resulting in reduced protein solubilization and determination of egg concentration (33, 34). In raw peanut extracts, Ara h 3 is identified as the most abundant allergen, followed by Ara h 1 (35). These seed globulin proteins;

Table 2. Sciex QTRAP 6500 instrument conditions for selected peptide transitions from yeast enolase (ENO1_YEAST) and rabbit phosphorylase B (PYGM_RABIT) quality control (QC) standards.

Protein name (UniProt)	Peptide sequence ^a	Precursor (m/z)	Product transition	Product (m/z)	CE (V) ^b	CXP (V) ^c	Approximate RT (min)
ENO1_YEAST	IGSEVYHNLK	580.3089++	y9 ^e	1046.5265+	29	30	15.4
			y6	773.4304+	30	28	15.4
			y5 ^f	674.3620+	31	23	15.4
	VNQIGTLSESIK	644.8590++	y10	1075.5994+	27	34	20.0
			y9 ^e	947.5408+	30	29	20.0
			y8 ^f	834.4567+	29	26	20.0
	AVDDFLISLDGTANK	789.9041++	y10	1031.5732+	36	36	26.9
			y9 ^e	918.4891+	37	31	26.9
			y8 ^f	805.4050+	33	27	26.9
PYGM_RABIT	APNDFNLK	459.7376++	y6 ^f	750.3781+	23	24	17.4
			y5 ^e	636.3352+	25	25	17.4
			y4	521.3082+	28	22	17.4
	LITAIGDVVNHDPPVVGDR	630.6742+++	y6 ^e	642.3570+	41	21	23.0
			y16 ^f	832.4235++	23	25	23.0
			y13	689.8391++	25	20	23.0
	LLSYVDDEAFIR	720.8721++	y10 ^f	1214.5688+	31	43	24.8
			y8	964.4734+	33	30	24.8
			y7 ^e	865.4050+	35	26	24.8

^a C = Carbamidomethyl cysteine.
^b Collision Energy (V).
^c Collision Cell Exit Potential (V).
^d Quantitation peptide.
^e Quantitation ion.
^f Confirmation ion.
^g Confirmation peptide.

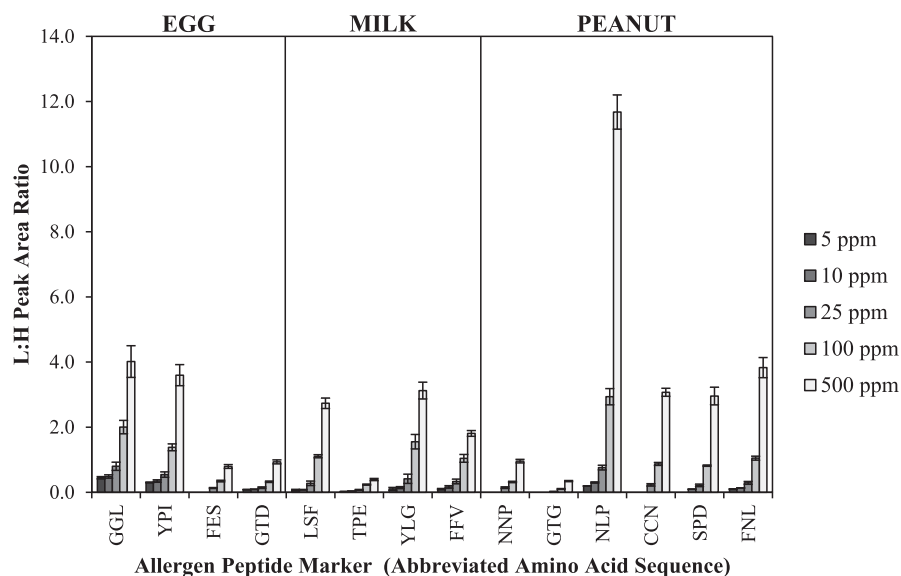


Figure 4. LC-MS/MS extracted ion peak areas for detected allergen peptide targets of egg (ovalbumin and lysozyme C), milk (β -lactoglobulin and α -S1 casein), and peanut (Ara h 1, Ara h 2, and Ara h 3) in allergen-incurred sugar cookies. Error bars represent deviations between sample preparation ($n = 3$) and instrumental ($n = 2$) replicates.

however, have a propensity for heat-induced advanced glycation end-product modification, which is demonstrated by reduced relative allergen protein composition values for roasted peanut extracts (Supplemental Information Table S1). Consequently, the reliable quantitation for peanut is preferential to the less chemically reactive 2S albumin peanut protein, Ara h 2 (10). Despite variations in chemical reactivity among allergen proteins, the multiallergen LC-MS/MS method demonstrates near-equivalent (unbiased) detection of selected egg, milk, and peanut peptides in the baked sugar cookie matrix.

The potential for food processing-induced changes in allergen protein structure and protein interactions with other food ingredients complicates the detection of allergen proteins and necessitates an allergen analysis strategy that can monitor multiple peptides from specific allergen proteins for reliable food allergen detection and quantitation. Although improved allergen detection and quantitation were achieved, the current method highlights the persistent challenges faced in food- and allergen-

based method development. Optimizations in multiallergen methods are challenging due, in part, to the extraction of proteins with varying physical and chemical properties. Often times, method and quantitation parameters are compromised in order to better accommodate all target allergen proteins. Yet, matrix interferences are largely unavoidable and can affect allergen recoveries, as demonstrated in the current work. Lastly, as a result of limited availability of allergen reference materials, the allergen community must resort to matrix-matched calibration curves, which are laboratory-intensive and must be prepared for each sample matrix.

Conclusions

The LC-MS/MS method described herein provides an optimized sample preparation protocol and MRM method for the simultaneous quantitation of egg, milk, and peanut in a model bakery product matrix (sugar cookie). The method provides good recovery of selected egg, milk, and peanut peptide markers in

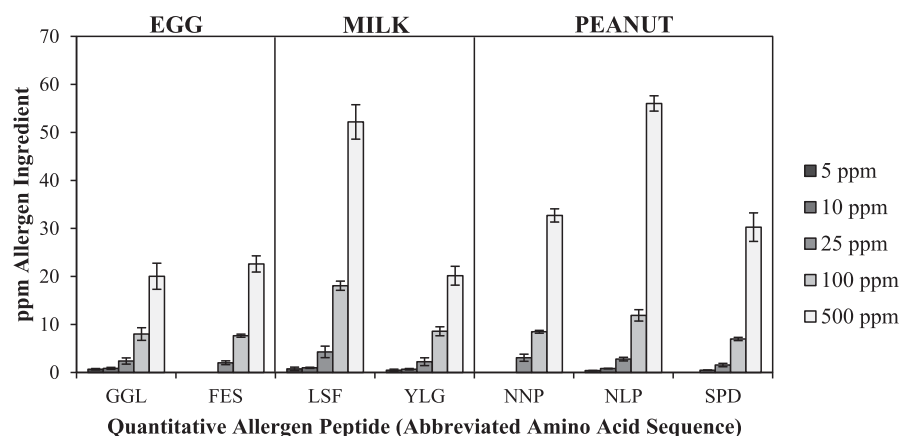


Figure 5. Calculated allergen ingredient (parts per million) of egg (ovalbumin and lysozyme C), milk (β -lactoglobulin and α -S1 casein), and peanut (Ara h 1, Ara h 2, and Ara h 3) in allergen-incurred sugar cookies analyzed by LC-MS/MS. Error bars represent deviations between sample preparation ($n = 3$) and instrumental ($n = 2$) replicates.

allergen-incurred sugar cookies (5–500 ppm), while improving method efficacy (efficiency, robustness, and cost) for use in routine analysis. We anticipate that the method can be applied to various types of bakery products, as well as other allergen-containing commercial food products. As a means to formulate a more universal quantitation scheme, various calibration curves and modes of quantitation are being investigated. A critical evaluation of proteomic quantitative platforms will yield a foundation for which the multiallergen method can be expanded to include other target allergen proteins. Understanding the limitations of analytical methods for food allergen quantitation with respect to sample preparation, peptide marker selection, allergen quantitation, and matrix constraints is crucial to enforce regulatory compliance, support allergen management in the food industry, and mitigate risk to consumers who have food allergies.

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