



Comparison of allergen quantification strategies for egg, milk, and peanut in food using targeted LC-MS/MS

Weili Xiong^{1,2} · Christine H. Parker¹ · Chelsea C. Boo^{1,3} · Katherine L. Fiedler¹

Received: 17 May 2021 / Revised: 12 July 2021 / Accepted: 13 July 2021

© This is a U.S. government work and not under copyright protection in the U.S.; foreign copyright protection may apply 2021

Abstract

Methods for the detection and quantification of food allergens in complex matrices are necessary to ensure compliance with labeling regulations and assess the effectiveness of food allergen preventive controls. Liquid chromatography–tandem mass spectrometry (LC-MS/MS) has emerged as an orthogonal technique in complement to immunochemical-based assays. However, the absence of established guidelines for MS-based quantification of allergens in food has limited harmonization among the method development community. In this study, different quantification strategies were evaluated using a previously developed multiplexed LC-MS/MS method for the detection of egg, milk, and peanut. Peptide performance criteria (retention time, signal-to-noise ratio, and ion ratio tolerance) were established and quantification approaches using varying calibrants, internal standards, background matrices, and calibration curve preparation schemes were systematically evaluated to refine the previous method for routine laboratory use. A matrix-matched calibration curve using allergen ingredients as calibrants and stable isotope-labeled peptides as internal standards provided the most accurate quantitative results. The strategy was further verified with commercially available reference materials and allowed for the confident detection and quantification of food allergens. This work highlights the need for transparency in calibration strategy and peptide performance requirements for effective evaluation of mass spectrometric methods for the quantification of food allergens.

Keywords Food allergens · Mass spectrometry · Targeted MRM proteomics · Quantification strategies · Reference materials

Introduction

Food allergies are a growing global health concern affecting up to 5% of the adult population and 8% of children in the USA [1, 2]. At present, there is no cure for food allergies and management relies on strict avoidance of allergenic foods and preparedness to treat allergic symptoms after accidental exposure to allergens. To enable food allergic consumers to avoid products that may pose a health risk, the Food Allergen Labeling and Consumer Protection Act (FALCPA) was

enacted in 2004 and requires all labels for packaged foods to list ingredients that contain any of the eight major food allergens (milk, eggs, fish, crustacean shellfish, tree nuts, peanuts, wheat, and soybeans) [3]. In 2011, the Food Safety and Modernization Act (FSMA) defined food allergens as hazards and requires that manufacturers incorporate food allergen management into safety control plans to prevent cross-contact [4]. To ensure compliance with current regulations, reliable analytical methods that can detect and quantify multiple food allergens in complex matrices are needed.

Enzyme-linked immunosorbent assay (ELISA) has been the conventional analytical platform used for the detection and quantification of allergens in food due to its sensitivity, robustness, and standardization for routine laboratory analysis [5]. However, the performance of commercial ELISA kits from different suppliers varies for the same food allergen due to different allergenic protein targets, calibration materials, reporting units, and antibody selectivity and cross-reactivity [6–9]. Additionally, the significant public health risk imposed by undeclared allergens and increasing population of consumers allergic to multiple food allergens has

✉ Katherine L. Fiedler
Katherine.L.Fiedler@fda.hhs.gov

¹ U.S. Food and Drug Administration, Center for Food Safety and Applied Nutrition, College Park, MD 20740-3835, USA

² Joint Institute for Food Safety and Applied Nutrition, University of Maryland, College Park, MD 20740, USA

³ Present address: Dynamic Omics, Antibody Discovery and Protein Engineering (ADPE), R&D, AstraZeneca, Gaithersburg, MD 20878, USA

established the need for multiplexed methods capable of the detection and quantification of multiple allergens in a single analysis [8, 10]. Therefore, single-analyte ELISA methods have limited usefulness as a screening tool for unknown allergens. A multiplexed immunochemical method has recently been developed, called the xMAP Food Allergen Detection Assay (xMAP FADA) [11–13], which is capable of simultaneously detecting and quantifying 14 food allergens plus gluten and sesame by employing complementary sets of antibody conjugated magnetic beads.

Targeted liquid chromatography–tandem mass spectrometry (LC-MS/MS) is a multiplexed technique complementary to antibody-based methods as it relies on the identification of allergen-specific peptides, offering high specificity [14]. In the past decade, a number of MS-based methods have been developed for the sensitive detection of multiple allergens in complex food matrices [15–24]. These methods describe the selection of reliable proteotypic peptides and detection of target allergens in a range of food matrix sources [25]. However, the extension of these methods to allergen quantification has remained challenging and different approaches have been proposed. Recently, it has become globally recognized that harmonization and transparency are needed in the development of mass spectrometric methods for allergen quantification in foods [26–28]. The proper assessment of a method requires explicit details on calibration strategy and data interpretation that are often not provided. Calibration can be achieved by standard addition, where calibrants are added directly to the experimental sample, or with an external calibration curve, which involves the generation of a separate calibration sample set. Standard addition has been used for the quantification of food allergens, but requires prior knowledge of the approximate allergen concentration so that calibrants can be added to the samples at the appropriate amount [24]. External calibration, on the other hand, has been more widely used for allergen quantification and published methods have used synthetic target peptides, allergenic proteins, or allergen ingredients as calibrants [21, 29–32].

The goal of this work was to optimize and evaluate a multi-allergen LC-MS/MS method which was previously developed for the detection and quantification of egg, milk, and peanut in commercially processed food products [21]. Different MS quantification approaches were compared to establish recommendations for calibration and standardized criteria for data processing. The first calibration strategy evaluated employs an external calibration curve generated by spiking synthetic target peptide calibrants into blank (allergen-free) food matrices [7, 21, 29]. The use of a peptide-based calibrant approach for allergen quantification requires conversion factors to convert the amount of target peptide measured to the concentration of allergen commodity [7, 21, 29]. Unfortunately, some of these values are not well characterized, especially for some of the lesser-studied allergens. The second calibration strategy

evaluated involves the generation of calibration curves by spiking and/or serially diluting known amounts of allergen ingredient standards into food matrices, followed by the same preparation procedure used for experimental samples [31, 32]. Theoretically, calibration curves should be prepared in a matrix that is matched or very similar to samples under investigation to address the effects of food matrix on sample processing and signal intensity. However, this requirement is not always practical when considering the broad range of possible food matrix sources; therefore, the impact of matrix on the performance of the different calibration strategies was also explored in this study.

Internal standards (IS) are another important aspect of a quantitative mass spectrometric method, allowing for data normalization and the correction of matrix effects and instrument variability. Although some methods do not employ internal standards [29], their use is highly recommended [30]. Internal standards are added at a constant amount to each calibration and experimental sample so the analyte concentration can be determined by comparing the analyte-to-IS ratio of the experimental sample to the calibrant-to-IS ratios from the external calibration curve. In the previously developed method, stable isotope–labeled (SIL) target peptides were used as internal standards in concurrence with guidelines set forth by the Clinical Proteomic Tumor Analysis Consortium (CPTAC) of the National Cancer Institute for peptide-based, targeted MS quantification of proteins for clinical applications [33]. Alternative labeled internal standards include extended SIL peptides [24], SIL concatemers [34], and SIL proteins [35]. SIL internal standards are ideal since they behave chemically identical to the target peptide during mass spectrometric analysis with only a slight mass difference; however, they are expensive. Therefore, the use of structurally analogous peptides as surrogate internal standards was explored as a cost-effective alternative [36]. Since peptide-based internal standards are usually added just prior to MS analysis, they do not undergo the same preparation procedure as the experimental samples and cannot correct for losses that occur during protein extraction and digestion. Surrogate standards, such as an exogenous protein, on the other hand, can be added earlier in the sample preparation protocol to account for variability during sample processing and MS analysis [36].

Overall, method performance was evaluated for different calibrants (synthetic target peptides and allergen ingredient standards), internal standards (SIL target peptides, exogenous peptides, and an exogenous protein), and matrix sources (matched matrix, similar matrix, independent matrix, and solvent-only) in allergen fortified cookies. Defined criteria used for objective data processing have also been provided in support of establishing guidelines for the standardized interpretation of mass spectrometric data for the quantification of food allergens. The optimized strategies were then tested against commercially available reference materials (MoniQA

milk incurred gluten-free cookies and LGC peanut incurred chocolate dessert) to benchmark the performance of the method. The workflow described herein provides a robust platform that may be used in support of allergen labeling regulations and the protection of consumers with food allergies.

Materials and methods

Preparation of allergen fortified cookie test samples

Egg, milk, and peanut-free commercial cookies (wheat-based animal crackers) were purchased from a local grocery store and used as a blank matrix. Blank cookies (batches of approximately 10 g) were ground twice for 5 s at 25,000 rpm using an IKA Tube Mill (NC, USA) prior to fortification with the following allergen ingredients: spray-dried whole-egg powder (NIST 8445), nonfat dry milk (Nestle Carnation Inc., Solon, OH), and light-roast peanut flour (Golden Peanut and Tree Nut, LLC, Alpharetta, GA). For the dry mixing (DM) approach, ground blank cookies (9.7 g) were fortified with 100 mg of each dry allergen ingredient for a concentration of 10,000 mg egg, milk, and peanut/kg cookie (ppm, w/w). Test samples (10 g total) at 10, 25, and 100 mg egg, milk, and peanut/kg cookie were prepared by a parallel dilution of the 10,000 mg/kg allergen fortified sample with blank cookie. To ensure effective mixing, samples were homogenized in a 50 mL round bottom centrifuge tube five times at 1500 rpm for 1 min with a Spex SamplePrep 2010 Geno/Grinder (Thomas Scientific, Swedesboro, NJ). For the slurry mixing (SM) approach, allergen ingredient mixtures were prepared in tris-buffered saline-0.1% Tween® 20 (TBST) before fortifying the blank cookies. The slurry mixing approach has been used previously [13] and can accommodate for differences in particle size between allergen ingredients and sample matrix. Briefly, milk and egg stock solutions were prepared separately at 10% w/v (100,000 µg/mL; 0.5 g egg or milk allergen ingredient in 5 mL TBST) and the peanut stock solution was prepared at 2% w/v (20,000 µg/mL; 0.5 g peanut allergen ingredient in 25 mL TBST). The three allergen stock solutions were combined and diluted in parallel with TBST using wide bore pipette tips to generate allergen working solutions at 20× the desired final concentrations. Test samples at 10, 25, and 100 mg egg, milk, and peanut/kg cookie were achieved by fortifying 1 g blank cookies with 50 µL corresponding allergen working solutions. Test samples generated using both approaches were prepared in triplicate and analyzed.

Preparation of commercially available reference materials

Rice (gluten-free) cookie reference materials (RMs) for milk were purchased from MoniQA Association. The set contained

a negative control of blank rice cookie, a low-level milk incurred rice cookie (3.54 mg milk protein/kg cookie equivalent to 10 mg milk/kg cookie), and a high-level milk incurred rice cookie material (17.7 mg milk protein/kg cookie equivalent to 50 mg milk/kg cookie).

Chocolate mousse dessert RMs for peanut were purchased from LGC Group (Teddington, UK). The set contained a negative control of blank chocolate mousse dessert and a positive peanut incurred chocolate mousse dessert (10 mg peanut protein/kg mousse equivalent to 17.5 mg peanut/kg mousse). The peanut RMs were reconstituted before use according to the manufacturer's instructions. In brief, 2.333 g water was added to 1 g mousse paste (paste to water ratio of 3:7). The mixture was stirred until no lumps of paste were visible and left to stand at room temperature for 2 h before use.

Protein extraction and digestion

Samples were prepared according to a previously described protocol with modifications [21]. One gram of each sample was defatted three times with 3 mL hexane and vacuum-dried for 15 min. The defatted samples were spiked with 44.4 µL 10 pmol/µL yeast enolase and reconstituted in 10 mL protein extraction buffer containing 2 M urea, 50 mM Tris-buffered saline (TBS), and 25 mM dithiothreitol (DTT). Following 5 min of orbital shaking, 10 min of water bath sonication, and 15 min of end-over-end rotation, samples were centrifuged for 15 min and the supernatant (protein extract) was collected. The extraction process was repeated once more and both supernatants were combined. Total protein concentrations were estimated using a Qubit Protein Assay Kit (Invitrogen) according to the manufacturer's instructions.

Protein concentration and digestion were achieved using a modified filter-aided sample preparation (FASP) protocol as previously described [21]. In summary, 1 mg total protein (maximum 900 µL), theoretically containing 20 pmol yeast enolase, was transferred to a 1.5 mL Eppendorf centrifuge tube. Extracted proteins were reduced and alkylated in-solution and then concentrated on a 10 kDa molecular weight cutoff (MWCO) filter. The filtration membrane was washed and then the proteins were digested with trypsin (100:1 substrate to enzyme) at 37 °C for 4 h. Peptide digests were collected and prepared for MS analysis by dilution (1:10) in 94.9% water, 5% acetonitrile, 0.1% formic acid containing 2.5 fmol/µL rabbit phosphorylase B digest, 0.5 pmol/µL angiotensin I, and 2.5 fmol/µL stable isotope-labeled (¹³C¹⁵N) synthetic peptides.

Preparation of synthetic peptide calibrants and calibration curves

Lyophilized native and stable isotope-labeled (¹³C¹⁵N C-terminal amino acid) synthetic peptides were purchased from

New England Peptide, Inc. (Gardner, MA) and reconstituted as previously described at 100 pmol/ μ L in 94% water, 5% acetonitrile, 1% formic acid, 5 fmol/ μ L phosphorylase B digest, and 1 pmol/ μ L angiotensin I [16]. Synthetic peptide stock solutions were stored at -80°C . Native synthetic peptides were combined and diluted with 94.9% water, 5% acetonitrile, 0.1% formic acid, 5 fmol/ μ L phosphorylase B digest, and 1 pmol/ μ L angiotensin I to generate working solutions for the preparation of the calibration curves. SIL synthetic peptides were combined and diluted with 94.9% water, 5% acetonitrile, 0.1% formic acid, 5 fmol/ μ L phosphorylase B digest, and 1 pmol/ μ L angiotensin I to generate a working solution of 50 fmol/ μ L for the addition to each calibration and test sample.

Synthetic peptide calibration curve samples were prepared at 0, 0.1, 1, 10, and 100 fmol peptide on column in various background matrices to ensure peak intensities from test samples were within the range of the calibration curve. The matrix-matched and matrix-independent calibration samples were prepared with blank cookie or sausage digest, respectively, diluted 1:10. The solvent-only calibration curve was prepared in 97% water, 3% acetonitrile, and 0.1% formic acid. A separate calibration curve was prepared with a blank cookie digest, generated as described above, with the exception of 44.4 μ L 10 pmol/ μ L yeast enolase. These calibration curve samples were then spiked with yeast enolase digest prior to MS analysis at a final concentration of 15.4 fmol/ μ L, obtained by the in-solution digestion of 20 pmol intact yeast enolase (equivalent amount as the test samples). All calibration samples contained final concentrations of 2.5 fmol/ μ L phosphorylase B digest, 0.5 pmol/ μ L angiotensin I, and 2.5 fmol/ μ L SIL peptides.

Preparation of allergen ingredient calibrants and calibration curves

A separate batch of allergen ingredient stock solutions and working solutions were prepared as described above and used as calibrants. Blank cookie or sausage samples (1 g) were spiked with 50 μ L 20 $\times$ allergen working solutions to produce matrix-matched and matrix-independent calibration samples, respectively, at 0, 5, 12.5, 50, and 125 mg egg, milk, and peanut/kg food. Spiked calibration samples were subjected to protein extraction and digestion and prepared for MS analysis as described above. The preparation of individual calibration samples at each concentration of the calibration curve is labor-intensive; therefore, a “dilution preparation” strategy was used for the preparation of allergen ingredient calibration curves in blank cookie, blank rice cookie (MoniQA RM), and blank chocolate mousse dessert (LGC RM). In this strategy, a concentrated calibration sample (500 mg egg, milk, and peanut/kg food) was subjected to protein extraction and digestion as described above. The peptide digests were then diluted

in parallel with blank matrix peptide digests to generate calibration samples (20 μ L total) at 0, 5, 12.5, 50, and 125 mg egg, milk, and peanut/kg food. These calibration samples were then prepared for MS analysis as described above. Once again, all calibration samples contained final concentrations of 2.5 fmol/ μ L phosphorylase B digest, 0.5 pmol/ μ L angiotensin I, and 2.5 fmol/ μ L SIL peptides.

LC-MS/MS

Targeted mass spectrometric analyses were conducted as previously described [21] using a Sciex QTRAP 6500 (Framingham, MA) coupled with a Waters ACQUITY ultra-performance liquid chromatography (UPLC) M-Class System (Milford, MA, USA). Each sample was injected onto a nanoEase M/Z Symmetry C18 trap column (100 $\text{\AA}$, 5 μ m, 180 μ m $\times$ 20 mm) for 3 min at 5 μ L/min with 99.5% A (0.1% formic acid in water) and 0.5% B (0.1% formic acid in acetonitrile). The peptides were then separated on a nanoEase M/Z Peptide BEH C18 analytical column (130 $\text{\AA}$, 1.7 μ m, 100 μ m $\times$ 100 mm, 40 $^{\circ}\text{C}$) using a linear gradient of 3–40% B over 30 min at 300 nL/min, followed by an increase to 85% B (held constant for 4 min) and re-equilibration (55 min total run time). The Sciex QTRAP 6500 was operated in a scheduled multiple-reaction monitoring (MRM) mode with a 120 s detection window and a target scan time of 1.65 s. Source parameters were set as follows: curtain gas (CUR) at 30 psi, ion spray voltage at 2400 V, collision gas (CAD) at high, interface heater temperature (IHT) at 180 $^{\circ}\text{C}$, and ion source gases 1 and 2 (GS1 and GS2) at 20 and 0 psi, respectively. Resolutions at Q1 and Q3 were low and unit, respectively. Three MRM transitions were monitored for each native and stable isotope-labeled egg, milk, and peanut allergen peptide as well as each yeast enolase and rabbit phosphorylase B peptide (Supplementary Information (ESM) Table S1) with optimal values for declustering potential (DP), entrance potential (EP), collision energy (CE), and collision cell exit potential (CXP) as previously described [21]. In total, 102 peptide transitions were monitored simultaneously in one injection.

For each calibration strategy, all calibration curves were run within the same batch with the respective test samples. Bovine serum albumin (BSA) digest was injected prior to each batch and a continuing calibration verification standard (mid-point concentration sample of the calibration curve) was injected after each batch as a quality control standard to monitor the instrument stability and performance. Each test sample was prepared in triplicate and analyzed with interleaved blanks and quality control standards mentioned above to ensure sample stability and minimize peptide carryover. All experimental MS/MS data were analyzed using Analyst 1.6.2 (Sciex) and Skyline 19.1 [37].

Results and discussion

Peptide performance criteria

In contrast to well-established MS-based methods for the analysis of small molecules in food, consensus guidelines on acceptable peptide performance for allergen analysis has yet to be determined. A clear set of criteria and consistent terminology is essential for the evaluation of MS-based food allergen detection methods. To increase confidence in the detection and quantification of food allergens, multiple proteins, peptides, and transitions are usually monitored per allergen. Given the possible diversity and complexity of food sample matrices, acceptance criteria such as retention time tolerance, signal-to-noise (S/N) ratio, and ion ratio tolerances are required to verify the identification of individual target peptides in an MRM method.

A total of 14 peptide markers from egg, milk, and peanut were monitored (two peptides per allergen protein and at least two allergen proteins per allergen; ESM Table S1). The selection of these peptide markers in past work was based upon previously established criteria (e.g., empirical MS identifications, physiochemical properties, and specificity), the assessment of peptide performance due to processing effects and cultivar variations, and MRM properties (e.g., chromatographic peak shape, relative abundance, matrix interference, and stability) [21]. Due to the difficulties in combining data from multiple peptides for quantification [28], one peptide is often used for the quantification of allergens [24, 32]. In this study, the following peptide markers were selected for quantification (quantitative peptides) for each allergen or allergen ingredient based on best overall performance: egg (GGLEPINFQTAADQAR), milk casein (YLGYLEQLLR), milk whey (LSFNPTQLEEQCHI), and peanut (NLPQQCGLR); while other peptide markers were utilized for confirmation (confirmatory peptides). Milk fractions were monitored separately to extend the scope of the quantitative method to products that may contain a non-standard casein to whey ratio, such as infant formula, or only one milk fraction. Three transitions were monitored per peptide and the first and second most abundant transitions were used to assess peptide performance.

For the confident detection of an allergen in a sample, at least two allergen-derived peptides were required to meet the following performance criteria, which were established based on previously published methods [21, 24, 38]:

1. Retention time (RT) within $\pm 5\%$ of the average RT for SIL peptide in the same matrix.
2. For quantitative peptide markers, quantitation ions (most abundant transition) with an S/N of $>10:1$ and confirmation ions (second most abundant transition) with an S/N of $>3:1$. For confirmatory peptide makers, two most

abundant transitions with an S/N of $>3:1$. Noise was defined as the background signal from blank matrix.

3. Ion ratio (peak area of second most abundant transition/peak area of most abundant transition) within $\pm 30\%$ (relative) of average ion ratios for SIL peptides in the same matrix.

Currently, no regulatory action limit has been established for the three allergens under investigation. Researchers commonly use the Voluntary Incidental Trace Allergen Labeling (VITAL) thresholds as a reference value to assess method sensitivity. Recently updated VITAL 3.0 guidelines recommend reference doses (ED_{01}) of 0.2 mg protein for egg, milk, and peanut, for the protection of 99% of the allergic population [39]. In the absence of appropriate legislation, we have relied on the precedence set forth in the gluten-free labeling of foods which designates mg allergen commodity per kg of food and converted the VITAL 3.0 reference doses to approximately 23 mg/kg for egg, 22 mg/kg for peanut, and 32 mg/kg for milk in a serving size of 50 g cookies [27, 30, 40]. Therefore, commercial cookies were fortified with egg, milk, and peanut at 10, 25, and 100 mg each allergen commodity/kg cookie and used as test samples for the comparison of the different quantification approaches. To ensure the preparation of homogeneous samples, two fortifying procedures were compared: dry mixing and slurry mixing [13]. Both fortifying strategies provided comparable linearities and R^2 values for all selected peptide markers over the concentration range of 10–100 mg/kg (ESM Fig. S1). However, higher CVs (%) were observed in replicate sample preparations using the dry mixing approach as compared to the slurry mixing approach (ESM Table S2), indicating that the dry mixing approach can produce inconsistent concentrations in fortified samples [41]. Additionally, the slurry mixing approach requires less blank matrix for preparation and was therefore chosen to prepare the allergen fortified test samples.

The detection of peptide markers in the allergen fortified cookies was evaluated according to the established data criteria (ESM Table S3). All four milk and six peanut peptide makers were detected in each sample at each of the three concentrations. In comparison, egg peptide markers exhibited lower signal intensities, among which both egg lysozyme C peptide markers, FESNFNTQATNR and GTDVQAWIR, failed to pass the required criteria at 10 mg/kg where the low abundance of lysozyme C peptides is mirrored by the low relative protein composition in egg (3.4%) [42]. Nevertheless, egg was confidently detected as both peptide markers from ovalbumin were detected at the lowest concentration. Therefore, reliable detection was achieved for egg, milk, and peanut in the cookies fortified at 10, 25, and 100 mg egg, milk, and peanut/kg cookie.

Optimization of quantitative strategy

To develop an optimal quantification strategy, careful consideration is needed in the selection of calibrant, internal standard, and background matrix for the external calibration curve. The extraction and digestion of allergen proteins in complex food matrix sources significantly impacts peptide recoveries and behavior; therefore, an appropriate approach is needed to correct for instrument variability, matrix effect, and sample loss during diverse preparation steps. Here, we investigated two main calibration approaches using either synthetic target peptides or allergen ingredients as calibrants (Fig. 1). For each approach, the quantitative performance was further evaluated for different internal standards and/or matrix compositions. Additionally, two different protocols were compared for the preparation of the calibration curve using allergen ingredients as calibrants. The objective was to formulate a quantification scheme that provides reliable quantitative results and is suitable for routine analysis.

Calibrant

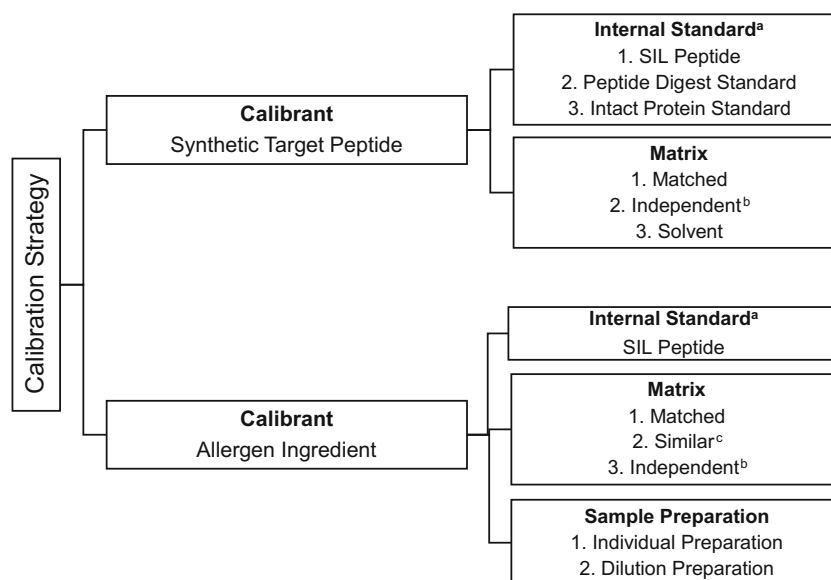
To assess the performance of different calibrants, separate calibration curves were prepared using either synthetic target peptides or allergen ingredients. Both calibration curves were prepared in a matched matrix and SIL target peptides (5 fmol) were added to each calibration sample prior to MS analysis as internal standards. The synthetic peptide-based calibration curve was prepared by spiking different amounts of synthetic

target peptides into diluted blank cookie digest prior to MS analysis. In contrast, the allergen ingredient-based calibration curve was prepared by spiking different amounts of allergen ingredient solutions into blank cookie followed by the same sample preparation process used for the test samples. The calibration curves were generated by plotting peak area ratios (peak area of target peptide normalized with peak area of SIL peptide) of quantitative peptides against the calibrant (synthetic target peptides or allergen ingredients) concentration, with linear regression and a $1/\times$ weighting factor. Overall, all quantitative peptides demonstrated a good R^2 of >0.99 and >0.95 for calibration curves using synthetic target peptides and allergen ingredients calibrants, respectively.

The method for determining the allergen concentration (mg/kg) of the test samples depends on which calibrant is employed (Fig. 2). When synthetic target peptides are used as calibrants, proper conversion factors are needed to convert the measured target peptide concentration, extrapolated from the external calibration curve, into the unit of mg allergen (egg, milk casein, milk whey, or peanut) per kg food; see ESM for more detail. Conversely, no conversion factors are required when allergen ingredients are used as calibrants; therefore, the allergen concentration of the test samples can be directly determined based on the external calibration curve and normalized peak area ratios.

Allergen concentrations measured in the 10, 25, and 100 mg/kg allergen fortified cookies using the two different calibration curves are illustrated in Fig. 3A. The calibration curve employing allergen ingredients as calibrants provided

Fig. 1 Overview of different calibration strategies



^a Stable isotope-labeled (SIL) peptides and peptide digest standard (phosphorylase B digest) were spiked prior to LC-MS/MS analysis; intact protein standard (yeast enolase) was spiked prior to protein extraction

^b Independent - sausage matrix

^c Similar - rice cookie matrix

Fig. 2 Calculation of allergen concentration using two different calibrants: synthetic target peptides or allergen ingredients

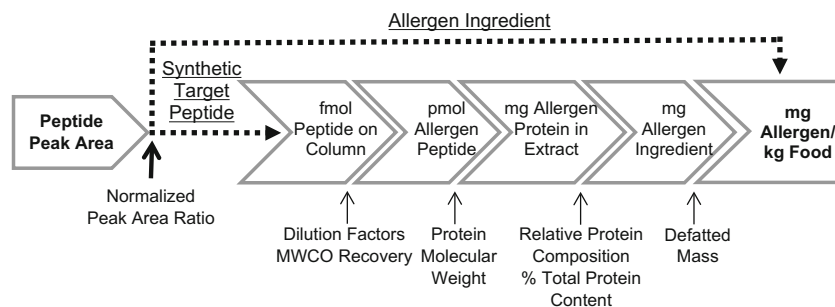
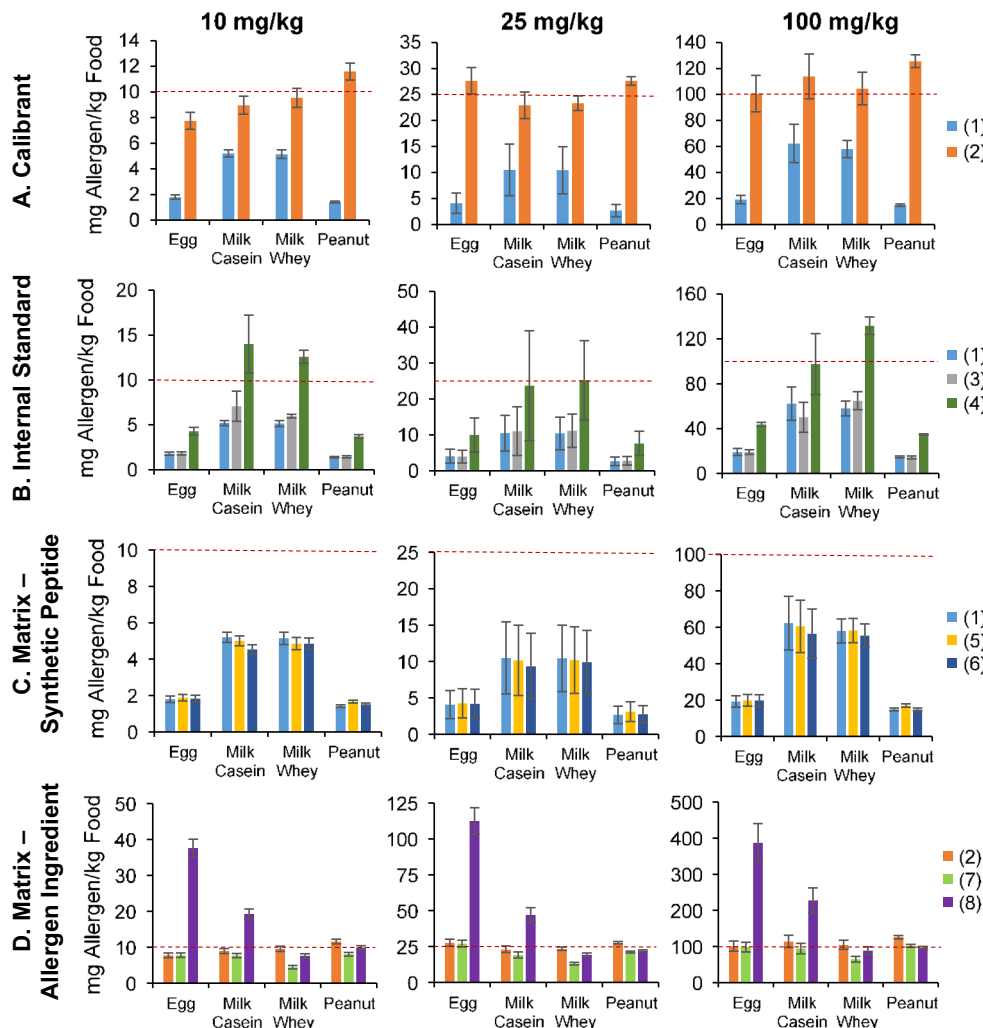


Fig. 3 Allergen concentration (mg each allergen commodity/kg cookie) inferred in allergen fortified cookies using different **A** calibrants: (1) synthetic target peptides and (2) allergen ingredients; **B** internal standards: (1) SIL peptide, (3) peptide digest standard, and (4) intact protein standard; **C** synthetic peptide calibration curves prepared in different matrices: (1) matched, (5) independent, and (6) solvent-only; **D** allergen ingredient calibration curves prepared in different matrices: (2) matched, (7) similar, and (8) independent, with quantification schemes shown in the table (error bars represent deviations between sample preparation triplicates and dashed lines indicate the actual allergen concentrations)



Curve	Calibrant	Internal Standard	Matrix	Test Sample
(1)	Synthetic Target Peptide	SIL Peptide	Matched	Fortified Cookie
(2)	Allergen Ingredient	SIL Peptide	Matched	Fortified Cookie
(3)	Synthetic Target Peptide	Peptide Digest Standard ^a	Matched	Fortified Cookie
(4)	Synthetic Target Peptide	Intact Protein Standard ^b	Matched	Fortified Cookie
(5)	Synthetic Target Peptide	SIL Peptide	Independent ^c	Fortified Cookie
(6)	Synthetic Target Peptide	SIL Peptide	Solvent	Fortified Cookie
(7)	Allergen Ingredient	SIL Peptide	Similar ^d	Fortified Cookie
(8)	Allergen Ingredient	SIL Peptide	Independent ^c	Fortified Cookie

^a Peptide Digest Standard – phosphorylase B peptide digest; ^b Intact Protein Standard – intact yeast enolase protein

^c Independent – sausage matrix; ^d Similar – rice cookie matrix

more accurate quantitative results for all allergens at all three concentrations. Method recoveries, defined as the percent ratio of the measured and fortified allergen concentration, over the concentration range of 10–100 mg/kg using the synthetic peptide calibration curve were 16–19%, 42–62%, 42–58%, and 11–15% for egg, milk casein, milk whey, and peanut, respectively; whereas method recoveries using the allergen ingredient calibration curve were 77–111%, 90–114%, 93–105%, and 110–126%, respectively. Despite the better recoveries achieved with the allergen ingredient calibration curve, this approach requires labor-intensive sample preparation procedures and is further optimized below (“[Preparation of allergen ingredient calibration curve](#)”).

Internal standard

Three different internal standards were compared using the synthetic peptide-based calibration strategy, due to the simpler sample preparation of this approach: SIL peptides, peptide digest standard, and intact protein standard (Fig. 3B). All calibration curves were prepared using synthetic target peptides as calibrants spiked into diluted blank cookie digest. Constant amounts of SIL target peptides (5 fmol) and digested rabbit phosphorylase B (5 fmol) were spiked into each calibration and test sample prior to MS analysis. Intact yeast enolase (20 pmol) was spiked into each test sample prior to extraction and an equivalent amount of digested yeast enolase (generated by in-solution digestion) was spiked into a separate set of calibration samples prior to MS analysis. The peak area of each target peptide was normalized with the peak area of either the SIL peptide or the average peak area of the three peptides for phosphorylase B or enolase. Phosphorylase B and enolase peptides were selected to cover the separation space of the chromatographic gradient.

Allergen concentrations measured in the 10, 25, and 100 mg/kg allergen fortified cookies using the three different internal standards are displayed in Fig. 3B. Comparable quantitative results were observed between SIL target peptides and peptide digest standard, indicating that phosphorylase B digest can be used as a cost-effective surrogate internal standard in the cookie matrix. However, due to matrix interference from complex samples, the use of SIL target peptides as internal standards is recommended to ensure peaks can be correctly assigned to synthetic and native target peptides using the criteria established earlier. Unlike SIL target peptides and peptide digest standard, which were spiked into samples prior to MS analysis, the use of an intact protein surrogate standard allows the internal standard to be incorporated earlier, providing correction for the entire sample preparation protocol. Even though method recoveries of allergens were improved when an intact protein was used as the internal standard, the measured concentration of egg and peanut were still underestimated in the test samples, possibly because a single

protein surrogate standard is not able to sufficiently represent the chemistry of all the different allergenic proteins.

Matrix–synthetic peptide calibration curve

The impact of matrix on the performance of the synthetic peptide-based calibration strategy was evaluated by comparing the quantification results for the 10, 25, and 100 mg/kg allergen fortified cookies achieved using calibration curves prepared in different background matrices (Fig. 3C). Largely distinct from matched cookie matrix, a previously studied sausage matrix was selected as an independent background matrix [13]. Synthetic target peptides were spiked into either diluted blank cookie digest, diluted blank sausage digest, or 94.9% water, 5% acetonitrile, and 0.1% formic acid to represent matrix-matched, matrix-independent, and solvent-only, respectively. Enhanced and more stable signals were observed for the calibrant peptides in cookie and sausage matrices compared to the neat solvent. However, peak area ratios were largely constant across all matrices as SIL target peptides normalized the raw peak area and angiotensin I was used as a carrier for the solvent-only calibration samples. As a consequence, comparable quantitative results were achieved in all matrices for synthetic peptide-based quantification when using SIL target peptides as internal standards.

Matrix–allergen ingredient calibration curve

We also investigated the impact of matrix on the performance of the allergen ingredient-based calibration strategy using various food matrices (Fig. 3D). Matrix-matched and matrix-independent calibration curves were prepared by spiking allergen ingredient solutions into blank cookie and sausage matrix, respectively, followed by protein extraction and digestion procedures. The preparation of an allergen ingredient calibration curve in solvent is not feasible due to the vast differences in protein extraction and digestion for small amounts of pure allergen ingredient in solvent alone [28]. Lower peak areas were observed for selected quantitative peptides in the sausage matrix calibration curve compared to the cookie matrix calibration curve. The matrix effect on recovery observed for the sausage matrix calibration curve resulted in overestimation of egg and milk casein concentration in the cookie samples (Fig. 3D, curves 2 and 8). This indicates the use of allergen ingredients as calibrants requires the calibration curves are matrix-matched for accurate quantification. Considering the wide variety of possible food matrices, an exact matrix-matched calibration curve for each single food sample is impractical. It is generally assumed that related food commodities behave similarly [32]. Therefore, an allergen ingredient calibration curve was also prepared with blank rice cookie matrix as a matrix-similar background (Fig. 3D, curve 7). Interestingly, the matrix-similar calibration curve provided similar quantitative

results for all allergens compared to a matrix-matched calibration curve considering the differences in gluten content between the wheat (animal cracker) and rice (MoniQA) cookies. Future investigations are needed to determine if background matrices can be grouped into categories for comparable quantification, based on relative water, carbohydrate, fat, and protein content [43].

Preparation of allergen ingredient calibration curve

Two preparation strategies were evaluated to improve the practicality of allergen ingredient calibration curves for routine analysis, without reducing the number of calibration points. The first strategy relies on the individual preparation of calibration samples at each concentration along the calibration curve (4 points), while the second strategy involves the preparation of a single calibration sample at a high concentration of allergen ingredient (500 mg egg, milk, and peanut/kg cookie) followed by dilution with blank cookie digest. Both strategies provided comparable quantification results for the 10, 25, and 100 mg/kg allergen fortified cookies (Fig. 4) and the dilution preparation strategy is more suitable for routine analysis. We would recommend the preparation of a separate calibration QC sample at 20 mg/kg, in order to ensure the accuracy of the calibration curve generated by the “dilution preparation” strategy, and will employ this approach going forward.

Commercially available reference materials

The performance of the optimal synthetic peptide and allergen ingredient calibration-based strategies were further evaluated using commercially available reference materials: MoniQA milk incurred cookies at 10 and 50 mg milk/kg cookie and LGC peanut incurred chocolate mousse dessert at 17.5 mg

peanut/kg mousse (Fig. 5). Synthetic peptide calibration curves were prepared in solvent while allergen ingredient calibration curves were prepared with the above calibrants (whole-egg powder reference material, nonfat dry milk, and light-roast peanut flour) in a matched matrix (either blank MoniQA cookie or LGC chocolate mousse dessert) using the dilution preparation strategy; both calibration curves were prepared with SIL target peptides as internal standards. Both strategies were successful at detecting and quantifying milk in the MoniQA reference material at low (10 mg/kg) and high (50 mg/kg) concentration levels and peanut in the LGC reference material at 17.5 mg/kg (ESM Fig. S2). Further information on the detection of targeted peptides is provided in ESM Table S3. The two approaches provided similar quantification results for milk whey in the MoniQA reference material; whereas only slightly better method recoveries were observed for milk casein using the allergen ingredient calibration curve compared to the synthetic peptide calibration curve. Average method recoveries for the low- and high-concentration level reference materials were $40 \pm 3\%$ and $42 \pm 9\%$ for milk casein, respectively, and $58 \pm 5\%$ and $61 \pm 5\%$ for milk whey, respectively, using the synthetic peptide-based calibration strategy. Average method recoveries for the low- and high-concentration level reference materials were $59 \pm 3\%$ and $68 \pm 14\%$ for milk casein, respectively, and $52 \pm 5\%$ and $69 \pm 5\%$ for milk whey, respectively, using the allergen ingredient-based calibration strategy. It should be noted that different extraction efficiencies between the fortified calibration samples and incurred reference materials resulted in the underestimation of both milk whey and casein concentrations in the MoniQA rice cookies when using the allergen ingredient calibration strategy. On the other hand, the allergen ingredient calibration curve provided much more accurate quantification results for the LGC reference material compared to the synthetic peptide calibration curve. Specifically, an average

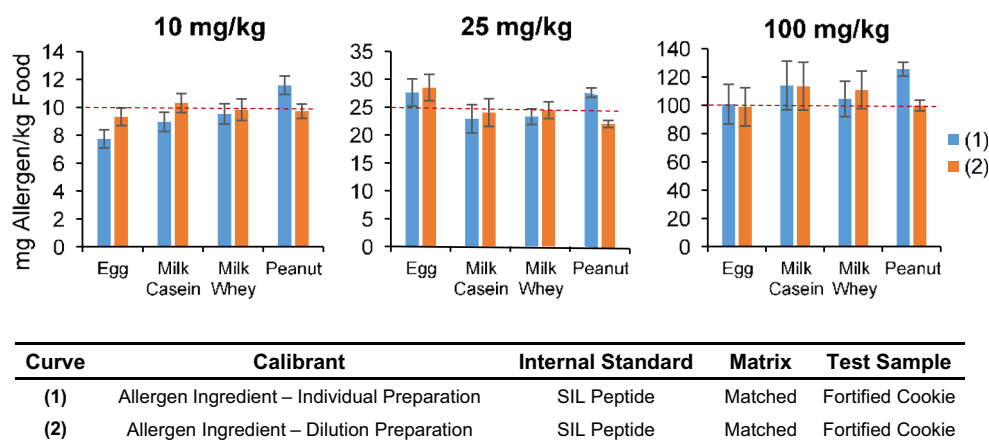
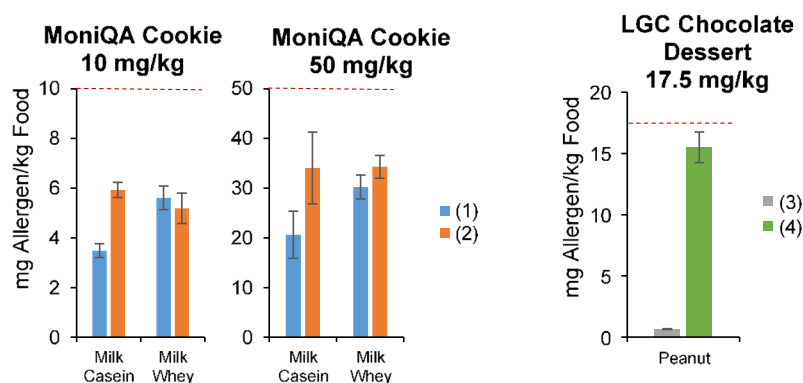


Fig. 4 Allergen concentration (mg each allergen commodity/kg cookie) inferred in allergen fortified cookies using different allergen ingredient calibration curve preparation strategies: (1) the individual preparation of each calibration sample at each concentration and (2) the dilution

preparation of a concentrated calibration sample followed by dilution prior to sample analysis, with quantification schemes shown in the table (error bars represent deviations between sample preparation triplicates and dashed lines indicate the actual allergen concentrations)

Fig. 5 Allergen concentration (mg allergen commodity/kg food) inferred in commercial reference materials using different calibration strategies: (1) and (3) synthetic target peptide calibrants, SIL peptide internal standards, solvent-only, and (2) and (4) allergen ingredient calibrants, SIL peptide internal standard, matched matrix, with quantification schemes shown in the table (error bars represent deviations between sample preparation triplicates and dashed lines indicate the actual allergen concentrations)



Curve	Calibrant	Internal Standard	Matrix	Test Sample
(1)	Synthetic Target Peptide	SIL Peptide	Solvent	MoniQA Milk Incurred Cookie
(2)	Allergen Ingredient	SIL Peptide	Matched	MoniQA Milk Incurred Cookie
(3)	Synthetic Target Peptide	SIL Peptide	Solvent	LGC Peanut Incurred Chocolate Dessert
(4)	Allergen Ingredient	SIL Peptide	Matched	LGC Peanut Incurred Chocolate Dessert

recovery of $89 \pm 7\%$ was achieved for peanut using the allergen ingredient-based calibration strategy. Chocolate is known to be a difficult matrix for allergen quantification [32] and these results demonstrate the benefit of alternate calibration strategies. In addition, if matrix interference prohibits the use of a certain peptide or transition, the peptide performance criteria can be used to select alternate quantification peptides or transitions from the data.

The targeted LC-MS/MS method described in this study provided the simultaneous detection and quantification of egg, milk, and peanut allergens in fortified cookies over the concentration range of 10–100 mg each allergen commodity/kg cookie. Acceptance criteria for peptide performance, including retention time deviation, signal-to-noise ratio, and ion ratio threshold, were established to ensure confident detection of allergens at the lowest concentration. With the aim of developing a quantification approach suitable for routine use, we investigated various calibration strategies employing different calibrants, internal standards, background matrices, and preparation schemes. Method performance for two optimized calibration strategies was assessed with MoniQA milk reference materials and LGC peanut reference materials. The use of a solvent-only, synthetic peptide calibration curve with stable isotope-labeled peptides as internal standards provided comparable recoveries for milk casein and milk whey from the milk reference materials and is a viable option for some allergens and matrices. However, an allergen ingredient calibration curve prepared in a matched matrix using the dilution preparation from a concentrated calibration sample with stable isotope-labeled peptides as internal standards is recommended overall. This approach provided better recoveries for milk casein, milk whey, and peanut from the appropriate allergen reference material. An independent preparation of a calibration QC sample is highly recommended in the future to verify the accuracy of calibration curve preparation. We anticipate that the

optimized method can be applied to various types of food commodities and expanded to include peptide targets from other allergen ingredients. Moving forward, single lab validation of the multi-allergen MS-based method will determine the analytical metrics in multiple representative matrices, in order to provide a robust workflow that can be used in support of regulatory enforcement and the protection of consumers with food allergies.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s00216-021-03550-x>.

Availability of data and material The datasets generated and/or analyzed during the current study are available in the Panorama repository, <https://panoramaweb.org/TMPKrz.url>.

Funding This research was funded by the US Food and Drug Administration (FDA), Center for Food Safety and Applied Nutrition (College Park, MD). Weili Xiong 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 inter-agency agreement between the US Department of Energy and the FDA.

Declarations This article is not an official US Food and Drug Administration (FDA) guidance or policy statement. No official support or endorsement by the US FDA is intended or should be inferred.

Conflict of interest The authors declare no competing interests.

References

1. Sicherer SH, Sampson HA. Food allergy: a review and update on epidemiology, pathogenesis, diagnosis, prevention, and

- management. *J Allergy Clin Immunol.* 2018;141(1):41–58. <https://doi.org/10.1016/j.jaci.2017.11.003>.
2. Sicherer SH, Sampson HA. Food allergy: epidemiology, pathogenesis, diagnosis, and treatment. *J Allergy Clin Immunol.* 2014;133(2):291–307; quiz 308. <https://doi.org/10.1016/j.jaci.2013.11.020>.
3. Public Law 108–282, Title II. Food Allergen Labeling and Consumer Protection Act of 2004. Silver Spring: US Food and Drug Administration; 2004.
4. Public Law 111–353. Food Safety Modernization Act (FSMA) of 2011. Silver Spring: US Food and Drug Administration; 2011.
5. Schubert-Ullrich P, Rudolf J, Ansari P, Galler B, Fuhrer M, Molinelli A, et al. Commercialized rapid immunoanalytical tests for determination of allergenic food proteins: an overview. *Anal Bioanal Chem.* 2009;395(1):69–81. <https://doi.org/10.1007/s00216-009-2715-y>.
6. Khuda S, Slate A, Pereira M, Al-Taher F, Jackson L, Diaz-Amigo C, et al. Effect of processing on recovery and variability associated with immunochemical analytical methods for multiple allergens in a single matrix: sugar cookies. *J Agric Food Chem.* 2012;60(17):4195–203. <https://doi.org/10.1021/jf3001839>.
7. Parker CH, Khuda SE, Pereira M, Ross MM, Fu TJ, Fan X, et al. Multi-allergen quantitation and the impact of thermal treatment in industry-processed baked goods by ELISA and liquid chromatography-tandem mass spectrometry. *J Agric Food Chem.* 2015;63(49):10669–80. <https://doi.org/10.1021/acs.jafc.5b04287>.
8. Cho CY, Oles C, Nowatzke W, Oliver K, Garber EAE. Cross-reactivity profiles of legumes and tree nuts using the xMAP(R) multiplex food allergen detection assay. *Anal Bioanal Chem.* 2017;409(25):5999–6014. <https://doi.org/10.1007/s00216-017-0528-y>.
9. Holzhauser T, Johnson P, Hindley JP, O'Connor G, Chan CH, Costa J, et al. Are current analytical methods suitable to verify VITAL(R) 2.0/3.0 allergen reference doses for EU allergens in foods? *Food Chem Toxicol.* 2020;145:111709. <https://doi.org/10.1016/j.fct.2020.111709>.
10. Garber EA, Parker CH, Handy SM, Cho CY, Panda R, Samadpour M, et al. Presence of undeclared food allergens in cumin: the need for multiplex methods. *J Agric Food Chem.* 2016;64(5):1202–11. <https://doi.org/10.1021/acs.jafc.5b05497>.
11. Cho CY, Nowatzke W, Oliver K, Garber EA. Multiplex detection of food allergens and gluten. *Anal Bioanal Chem.* 2015;407(14):4195–206. <https://doi.org/10.1007/s00216-015-8645-y>.
12. Cho CY, Ivens KO, Nowatzke WL, Robotham J, Samadpour M, Grace T, et al. Extension of xMAP food allergen detection assay to include sesame. *J Food Prot.* 2020;83(1):129–35. <https://doi.org/10.4315/0362-028X.JFP-19-304>.
13. Garber EAE, Cho CY, Rallabhandi P, Nowatzke WL, Oliver KG, Venkateswaran KV, et al. Multi-laboratory validation of the xMAP-food allergen detection assay: a multiplex, antibody-based assay for the simultaneous detection of food allergens. *PLoS One.* 2020;15(7):e0234899. <https://doi.org/10.1371/journal.pone.0234899>.
14. Picariello G, Mamone G, Addeo F, Ferranti P. The frontiers of mass spectrometry-based techniques in food allergenomics. *J Chromatogr A.* 2011;1218(42):7386–98. <https://doi.org/10.1016/j.chroma.2011.06.033>.
15. Bignardi C, Elvirio L, Penna A, Careri M, Mangia A. Particle-packed column versus silica-based monolithic column for liquid chromatography-electrospray-linear ion trap-tandem mass spectrometry multiallergen trace analysis in foods. *J Chromatogr A.* 2010;1217(48):7579–85. <https://doi.org/10.1016/j.chroma.2010.10.037>.
16. Monaci L, Pilolli R, De Angelis E, Godula M, Visconti A. Multi-allergen detection in food by micro high-performance liquid chromatography coupled to a dual cell linear ion trap mass spectrometry. *J Chromatogr A.* 2014;1358:136–44. <https://doi.org/10.1016/j.chroma.2014.06.092>.
17. Korte R, Brockmeyer J. MRM(3)-based LC-MS multi-method for the detection and quantification of nut allergens. *Anal Bioanal Chem.* 2016;408(27):7845–55. <https://doi.org/10.1007/s00216-016-9888-y>.
18. Planque M, Arnould T, Dieu M, Delahaut P, Renard P, Gillard N. Advances in ultra-high performance liquid chromatography coupled to tandem mass spectrometry for sensitive detection of several food allergens in complex and processed foodstuffs. *J Chromatogr A.* 2016;1464:115–23. <https://doi.org/10.1016/j.chroma.2016.08.033>.
19. Sealey-Voyksner J, Zweigenbaum J, Voyksner R. Discovery of highly conserved unique peanut and tree nut peptides by LC-MS/MS for multi-allergen detection. *Food Chem.* 2016;194:201–11. <https://doi.org/10.1016/j.foodchem.2015.07.043>.
20. Planque M, Arnould T, Dieu M, Delahaut P, Renard P, Gillard N. Liquid chromatography coupled to tandem mass spectrometry for detecting ten allergens in complex and incurred foodstuffs. *J Chromatogr A.* 2017;1530:138–51. <https://doi.org/10.1016/j.chroma.2017.11.039>.
21. Boo CC, Parker CH, Jackson LS. A targeted LC-MS/MS method for the simultaneous detection and quantitation of egg, milk, and peanut allergens in sugar cookies. *J AOAC Int.* 2018;101(1):108–17. <https://doi.org/10.5740/jaoacint.17-0400>.
22. Pilolli R, De Angelis E, Monaci L. In house validation of a high resolution mass spectrometry Orbitrap-based method for multiple allergen detection in a processed model food. *Anal Bioanal Chem.* 2018;410(22):5653–62. <https://doi.org/10.1007/s00216-018-0927-8>.
23. Jira W, Munch S. A sensitive HPLC-MS/MS screening method for the simultaneous detection of barley, maize, oats, rice, rye and wheat proteins in meat products. *Food Chem.* 2019;275:214–23. <https://doi.org/10.1016/j.foodchem.2018.09.041>.
24. Planque M, Arnould T, Delahaut P, Renard P, Dieu M, Gillard N. Development of a strategy for the quantification of food allergens in several food products by mass spectrometry in a routine laboratory. *Food Chem.* 2019;274:35–45. <https://doi.org/10.1016/j.foodchem.2018.08.095>.
25. Pilolli R, Nitride C, Gillard N, Huet AC, van Poucke C, de Loose M, et al. Critical review on proteotypic peptide marker tracing for six allergenic ingredients in incurred foods by mass spectrometry. *Food Res Int.* 2020;128:108747. <https://doi.org/10.1016/j.foodres.2019.108747>.
26. Stoyke M, Becker R, Brockmeyer J, Jira W, Popping B, Uhlig S, et al. German government official methods board points the way forward: launch of a new working group for mass spectrometry for protein analysis to detect food fraud and food allergens. *J AOAC Int.* 2019;102(5):1280–5. <https://doi.org/10.5740/jaoacint.19-0056>.
27. Mills ENC, Adel-Patient K, Bernard H, De Loose M, Gillard N, Huet AC, et al. Detection and quantification of allergens in foods and minimum eliciting doses in food-allergic individuals (ThRAIL). *J AOAC Int.* 2019;102(5):1346–53. <https://doi.org/10.5740/jaoacint.19-0063>.
28. Johnson PE, Downs M. From signal to analytical reporting for allergen detection by mass spectrometry. *J AOAC Int.* 2019;102(5):1255–62. <https://doi.org/10.5740/jaoacint.19-0053>.
29. Monaci L, De Angelis E, Guagnano R, Ganci AP, Garaguso I, Fiocchi A, et al. Validation of a MS based proteomics method for milk and egg quantification in cookies at the lowest VITAL levels: an alternative to the use of precautionary labeling. *Foods.* 2020;9(10):1489. <https://doi.org/10.3390/foods9101489>.
30. Planque M, Arnould T, Renard P, Delahaut P, Dieu M, Gillard N. Highlight on bottlenecks in food allergen analysis: detection and quantification by mass spectrometry. *J AOAC Int.* 2017;100(4):1126–30. <https://doi.org/10.5740/jaoacint.17-0005>.

31. New LS, Schreiber A, Stahl-Zeng J, Liu HF. Simultaneous analysis of multiple allergens in food products by LC-MS/MS. *J AOAC Int.* 2018;101(1):132–45. <https://doi.org/10.5740/jaoacint.17-0403>.
32. New LS, Stahl-Zeng J, Schreiber A, Cafazzo M, Liu A, Brunelle S, et al. Detection and quantitation of selected food allergens by liquid chromatography with tandem mass spectrometry: first action 2017.17. *J AOAC Int.* 2019;103(2):570–83. <https://doi.org/10.5740/jaoacint.19-0112>.
33. Hoofnagle AN, Whiteaker JR, Carr SA, Kuhn E, Liu T, Massoni SA, et al. Recommendations for the generation, quantification, storage, and handling of peptides used for mass spectrometry-based assays. *Clin Chem.* 2016;62(1):48–69. <https://doi.org/10.1373/clinchem.2015.250563>.
34. Gavage M, Van Vlierberghe K, Van Poucke C, De Loose M, Gevaert K, Dieu M, et al. Comparative study of concatamer efficiency as an isotope-labelled internal standard for allergen quantification. *Food Chem.* 2020;332:127413. <https://doi.org/10.1016/j.foodchem.2020.127413>.
35. Newsome GA, Scholl PF. Quantification of allergenic bovine milk alpha(S1)-casein in baked goods using an intact (1)(5)N-labeled protein internal standard. *J Agric Food Chem.* 2013;61(24):5659–68. <https://doi.org/10.1021/jf3015238>.
36. Domon B, Aebersold R. Options and considerations when selecting a quantitative proteomics strategy. *Nat Biotechnol.* 2010;28(7):710–21. <https://doi.org/10.1038/nbt.1661>.
37. MacLean B, Tomazela DM, Shulman N, Chambers M, Finney GL, Frewen B, et al. Skyline: an open source document editor for creating and analyzing targeted proteomics experiments. *Bioinformatics.* 2010;26(7):966–8. <https://doi.org/10.1093/bioinformatics/btq054>.
38. Mol HG, Zomer P, Garcia Lopez M, Fussell RJ, Scholten J, de Kok A, et al. Identification in residue analysis based on liquid chromatography with tandem mass spectrometry: experimental evidence to update performance criteria. *Anal Chim Acta.* 2015;873:1–13. <https://doi.org/10.1016/j.aca.2015.03.007>.
39. Remington BC, Westerhout J, Meima MY, Blom WM, Kruizinga AG, Wheeler MW, et al. Updated population minimal eliciting dose distributions for use in risk assessment of 14 priority food allergens. *Food Chem Toxicol.* 2020;139:111259. <https://doi.org/10.1016/j.fct.2020.111259>.
40. Food, Drug Administration HHS. Food labeling: gluten-free labeling of foods. Final rule. *Fed Regist.* 2013;78(150):47154–79.
41. Abbott M, Hayward S, Ross W, Godefroy SB, Ulberth F, Van Hengel AJ, et al. Validation procedures for quantitative food allergen ELISA methods: community guidance and best practices. *J AOAC Int.* 2010;93(2):442–50.
42. Caubet JC, Wang J. Current understanding of egg allergy. *Pediatr Clin N Am.* 2011;58(2):427–43, xi. <https://doi.org/10.1016/j.pcl.2011.02.014>.
43. Guidelines for the Validation of Chemical Methods in Food, Feed, Cosmetics, and Veterinary Products, 3 rd ed., Appendix 4 (2019). US Food and Drug Administration, Foods Program. <https://www.fda.gov/media/81810/download>. Accessed 7/1/2021.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Supplementary Information

Comparison of allergen quantification strategies for egg, milk, and peanut in food using targeted LC-MS/MS

Weili Xiong^{1,2}, Christine H. Parker¹, Chelsea C. Boo^{1†}, and Katherine L. Fiedler^{1*}

¹U.S. Food and Drug Administration, Center for Food Safety and Applied Nutrition,
College Park, MD, USA

²Joint Institute for Food Safety and Applied Nutrition, University of Maryland, College Park,
MD, USA

*Corresponding author: Katherine.L.Fiedler@fda.hhs.gov

[†]Present address: Dynamic Omics, Antibody Discovery and Protein Engineering (ADPE), R&D,
AstraZeneca, Gaithersburg, MD, USA

ORCID iD

Weili Xiong: 0000-0001-7208-917X

Katherine L. Fiedler: 0000-0002-8347-9373

Supplementary Materials and Methods

Conversion of Peptide Concentration into Allergen Ingredient Concentration

Applying proper conversion factors as previously described [1], target peptide concentrations were converted into allergen ingredient concentrations. Briefly, an estimated average recovery of 66% was applied for peptides on the MWCO filters by comparison of peak areas from synthetic peptides spiked prior to versus after filtration in blank cookie matrix. The total protein content was determined using a micro-Kjeldahl method for spray-dried whole egg powder (NIST 8445, 48%), nonfat dry milk (Nestle Carnation, 34.78%), and partially defatted (12%) light-roast peanut flour (Golden Peanut and Tree Nut, 50.39%) [2]. The relative protein composition for each allergen ingredient was previously determined from literature values or experimental estimations as follows [3,4]: egg ovalbumin (54%), egg lysozyme C (3.4%), milk beta-lactoglobulin (10%), milk alpha-S1-casein (32%), peanut Ara h 1 (13%), peanut Ara h 2 (21%), and peanut Ara h 3 (40%).

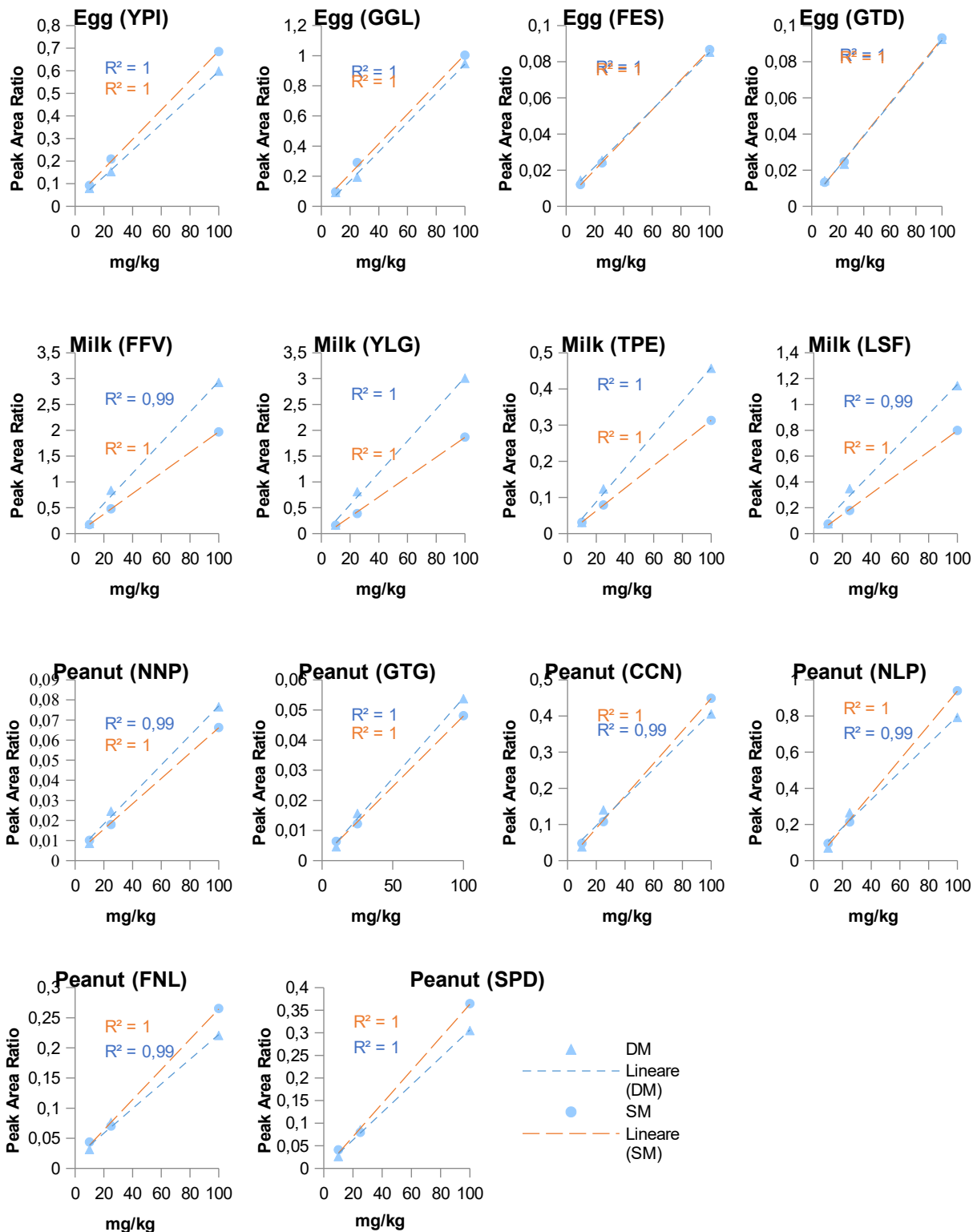
Fig. S1 Linear regression of peptide peak area ratio as a function of allergen concentration in allergen (egg, milk, and peanut) fortified cookies

Fig. S2 Extracted ion chromatograms (XICs) of MRM transitions monitored for quantitative peptides YLGYLEQLLR (milk casein) and LSFNPTQLEEQCHI (milk whey) from 10 mg milk/kg MoniQA cookies, and NLPQQCGLR (peanut) from 17.5 mg peanut/kg LGC chocolate mousse dessert

Table S1 Targeted peptide markers and transitions for egg (ovalbumin and lysozyme C), milk (alpha-S1-casein and beta-lactoglobulin), peanut (Ara h 1, Ara h 2, and Ara h 3) allergen proteins, and yeast enolase and rabbit phosphorylase B standard proteins. The table was modified from a previous study [1]

Table S2 CVs for peptide peak area ratio in test cookie samples fortified by dry mixing (DM) versus slurry mixing (SM) (n=3)

Table S3 Detection of peptide markers in allergen (egg, milk, and peanut) fortified cookies and commercially available allergen incurred reference materials (MoniQA milk incurred rice cookie and LGC peanut incurred chocolate mousse dessert)



DM – Dry mixing
SM – Slurry mixing

Fig. S1

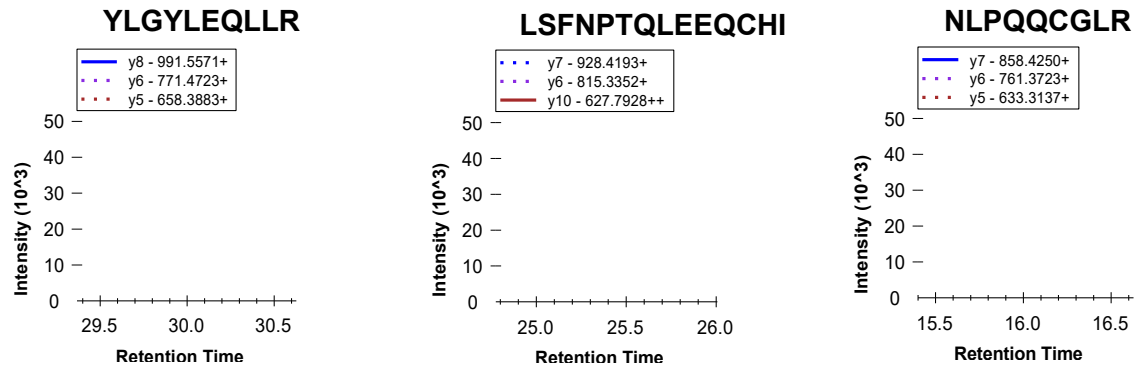


Fig. S2

Allergen	Target Protein	RT in Cookie (min)	Peptide Sequence ^{a,b}	Most Abundant Transition	2 nd Transition	3 rd Transition
Egg	Ovalbumin	23.3	<u>GGLEPINFQTAADQAR</u>	y10	y8	y9
		26.7	YPILPEYLQcVK	y8	y9	y6
	Lysozyme C	16.8	FESNFNTQATNR	y7	y10	y8
		22.0	GTDVQAWIR	y5	y7	y6
Milk	alpha-S1-casein	29.9	<u>YLGYLEQLLR</u>	y8	y6	y5

		31.3	FFVAPFPEVFGK	y8	y9	y10
	beta-lactoglobulin	25.4	<u>LSFNPTQLEEQcHI</u>	<u>y10</u>	y7	y6
		17.0	TPEVDDEALEK	y10	y7	y8
	Ara h 1	23.2	NNPFYFPSR	y7	y5	y6
		21.9	GTGNLELVAVR	y6	y5	y7
Peanut	Ara h 2	15.9	<u>NLPOQcGLR</u>	<u>y7</u>	y5	y6
		18.9	ccNELNEFENNQR	y8	y9	y7
	Ara h 3	19.1	SPDIYNPQAGSLK	y7	y9	y8
		21.7	FNLAGNHEQEFLR ⁺	y11	y10	y9
	Yeast enolase	16.0	IGSEVYHNLK	y5	y9	y6
		20.8	VNQIGTLSESIK	y8	y9	y10
		27.7	AVDDFLISLDGTANK	y8	y9	y10
	Rabbit phosphorylase B	18.0	APNDFNLK	y6	y5	y4
		23.9	LITAIGDVVNHDPPVVGDR ⁺	y16	y6	y13
		25.6	LLSYVDDEAFIR	y10	y7	y8

^a c = Carbamidomethyl (Cysteine)

^b Values for declustering potential (DP) and entrance potential (EP) were 80 and 10 V, respectively; the collision energy (CE) and collision cell exit potential (CXP) were previously optimized and described [1]

Quantitation Peptide and Quantitation Ion

⁺ triply charged precursor (all others doubly charged precursor)

Table S1

Sample	Mixing	Egg (%CV)				Milk (%CV)				Peanut (%CV)					
		YPI	GGL	FES	GTD	FFV	YLG	TPE	LSF	NNP	GTG	CCN	NLP	FNL	SPD
10 mg/kg	DM	15	11	6	17	14	10	20	15	13	14	10	12	18	7
	SM	4	7	15	6	4	7	7	8	12	6	15	5	15	22
25 mg/kg	DM	9	9	7	5	38	42	43	43	33	36	53	49	32	41
	SM	6	8	10	11	3	11	11	6	10	8	5	3	5	11
100 mg/kg	DM	7	4	7	3	14	13	12	13	15	15	5	6	11	9
	SM	9	14	9	10	7	15	14	12	1	8	10	4	12	5

DM – Dry Mixing

SM – Slurry Mixing

Table S2

Sample	Egg				Milk				Peanut					
	YPI	GGL	FES	GTD	FFV	YLG	TPE	LSF	NNP	GTG	CCN	NLP	FNL	SPD
AC 10 mg/kg	D	✓	x	x	✓	✓	✓	✓	D	✓	✓	✓	D	✓
AC 25 mg/kg	✓	✓	x	D	✓	✓	✓	✓	D	✓	✓	✓	D	✓
AC 100 mg/kg	✓	✓	D	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
MQC 10 mg/kg	NA	NA	NA	NA	✓	✓	✓	✓	NA	NA	NA	NA	NA	NA
MQC 50 mg/kg	NA	NA	NA	NA	✓	✓	✓	✓	NA	NA	NA	NA	NA	NA
LGC CP 17.5 mg/kg	NA	NA	NA	NA	NA	NA	NA	NA	D	✓	✓	✓	D	D

AC – milled commercial egg, milk, and peanut-free animal cracker fortified with allergen ingredient solutions (egg, milk, and peanut) at 10, 25, and 100 mg/kg

MQC – MoniQA milk incurred rice cookie at 10 and 50 mg/kg

LGC CP – LGC peanut incurred chocolate pudding dessert at 17.5 mg/kg

✓ – peptide marker met the performance criteria with S/N >10:1

D – peptide marker met the performance criteria with S/N > 3:1

x – peptide marker failed to meet the performance criteria

NA – not applicable

Table S3

References

1. Boo CC, Parker CH, Jackson LS (2018) A Targeted LC-MS/MS Method for the Simultaneous Detection and Quantitation of Egg, Milk, and Peanut Allergens in Sugar Cookies. *J AOAC Int* 101 (1):108-117. doi:10.5740/jaoacint.17-0400
2. Khuda S, Slate A, Pereira M, Al-Taher F, Jackson L, Diaz-Amigo C, Bigley EC, 3rd, Whitaker T, Williams KM (2012) Effect of processing on recovery and variability associated with immunochemical analytical methods for multiple allergens in a single matrix: sugar cookies. *J Agric Food Chem* 60 (17):4195-4203. doi:10.1021/jf3001839
3. Caubet JC, Wang J (2011) Current understanding of egg allergy. *Pediatr Clin North Am* 58 (2):427-443, xi. doi:10.1016/j.pcl.2011.02.014
4. Wal JM (2002) Cow's milk proteins/allergens. *Ann Allergy Asthma Immunol* 89 (6 Suppl 1):3-10. doi:10.1016/s1081-1206(10)62115-1