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Research Article

FOOD CHEMICAL CONTAMINANTS

Multi-Allergen Quantification in Food Using Concatemer-Based Isotope Dilution Mass Spectrometry: An Interlaboratory Study

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Abstract

Background: Food allergen analysis is essential for the development of a risk-based approach for allergen management and labeling. MS has become a method of choice for allergen analysis, even if quantification remains challenging. Moreover, harmonization is still lacking between laboratories, while interlaboratory validation of analytical methods is necessary for such harmonization.
Objective: This interlaboratory study aimed to evaluate the potential of MS for food allergen detection and quantification using a standard addition quantification strategy and a stable isotope-labeled (SIL) concatemer as an internal standard.
Methods: In-house-produced test material (cookies), blank and incurred with four allergens (egg, milk, peanut, and hazelnut), allergen standards, an internal standard, and the complete methodology (including sample preparation and ultra-HPLC–MS/MS method) were provided to nine laboratories involved in the study. Method sensitivity and selectivity were evaluated with incurred test material and accuracy with spiked test material. Quantification was based on the standard addition strategy using certified reference materials as allergen protein standards and a SIL concatemer as an internal standard.
Results: All laboratories were able to detect milk, hazelnut, and peanut in the incurred cookies with sufficient sensitivity to reach the AOAC INTERNATIONAL *Standard Method Performance Requirements* (SMPR® 2016.002). Egg detection was more complicated due to food processing effects, yet five laboratories reached the sensitivity requirements. Recovery results were laboratory-dependent. Some milk and hazelnut peptides were quantified in agreement with SMPR 2016.002 by all participants. Furthermore, over 90% of the received quantification results agreed with SMPR 2016.002 for method precision.
Conclusion: The encouraging results of this pioneering interlaboratory study represent an additional step towards harmonization among laboratories testing for allergens.

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Highlights: In this pioneering interlaboratory study, food allergens were analyzed by MS with characterized incurred and spiked test materials, calibrated with a certified reference material, and a single SIL concatemer used as an internal standard.

Food allergies are a growing health issue, especially in industrialized countries, where up to 10% of the population is affected (1, 2). In the absence of recognized and accepted treatments, dietary management based on the exclusion of allergenic food is the only solution for allergic consumers (3). In this regard, many jurisdictions have introduced labeling requirements for substances that cause allergies and intolerance reactions in sensitive individuals (4–6). These requirements refer to allergens used as ingredients, which could be foods or substances that are incorporated into the food products as part of the recipe. Consequently, this regulation does not address cross-contaminations, which are, together with mislabeling, major issues in food allergy management (7). Food allergen analysis is therefore an essential tool for the development of a risk-based approach to allergen management. Robust, specific, and sensitive detection methods are thus needed to protect allergic patients and guarantee correct food labeling.

During the last decade, MS has become a method of choice for allergen analysis (8–14). It predominantly focuses on the analysis of specific peptides obtained upon a proteolytic digestion of the proteins in a sample, including proteins from allergenic ingredients. A series of MS-based methods targeting single or multiple food allergen(s) have been recently developed (10). In some cases, validation was conducted by the laboratory that developed the method to evaluate its performance, including sensitivity, repeatability, and recovery. In other cases, the validation process was missing or only partially covered. For this purpose, AOAC INTERNATIONAL developed guidelines, that is, *Standard Method Performance Requirements* (SMPR®) 2016.002, for detecting and quantifying selected food allergens (egg, milk, peanut, and hazelnut) by MS-based methods to guide laboratories in method development and validation (15).

Harmonization efforts among laboratories of both the analytical pipelines and the obtained results are lacking yet strongly desired (11). Interlaboratory validation is a necessary step towards harmonization in food allergen analysis. The results of several interlaboratory studies using techniques based on antibodies such as ELISA or on DNA using PCR have been published (16–19). However, to the best of our knowledge, no such interlaboratory study focusing on an MS-based method is available. An interlaboratory comparison of targeted MS and ELISA test methods for the quantification of peanuts in the chocolate dessert matrix has been undertaken in the European Union-funded "Integrated Approaches to Food Allergen and Allergy Risk Management" (iFAAM) project (20). However, no results have so far been published. Stoyke and co-workers (12) detailed the objectives of the official German working group, which aims to standardize validated MS-based methods and develop general validation criteria. Here, a multi-laboratory validation study is also planned.

In 2016, the "Allersens" project (21, 22) was initiated with the objective of developing and validating an MS-based method for the detection and quantification of four allergens in processed food matrixes. These allergens (eggs, milk, peanuts, and hazelnuts) were selected based on their prevalence (23), severity of allergic reaction symptoms (24), and observed high frequency of accidental presence in food (25). The first step of the project was to identify potential peptide biomarkers for each allergen using high-resolution MS (HR-MS). The four allergens were separately subjected to representative food processing techniques and analyzed by HR-MS. Among the hundreds of identified peptides, potential peptide biomarkers were selected using criteria that ensured the specificity, sensitivity, and robustness of the quantitative method. The details of this selection and the identified peptide biomarkers were presented separately in research papers, one dedicated to each allergen (26–29). In parallel, a stable isotope-labeled (SIL) internal standard strategy was developed for allergen quantification (30). This strategy is based on the production and use of an SIL concatemer, an artificial protein recombinantly produced and composed of concatenated proteotypic peptides originating from the different proteins of interest. It must be noted that a concatemer is a compromise between the SIL synthetic peptides used by most laboratories and SIL proteins. SIL peptides are affordable, yet do not address and thus do not compensate for variations introduced during all sample preparation steps, such as proteolytic digestion. SIL proteins are, in this respect, more ideal, but unaffordable for routine testing by most laboratories. We developed, produced, and purified an ^{15}N SIL concatemer composed of 19 proteotypic peptides, which allows the analysis of four allergenic ingredients (egg, milk, peanut, and hazelnut). This concatemer was used to develop and validate an MS-based method for the detection and quantification of these allergens in processed food matrixes (cookie and chocolate). The method development for sample preparation and standard addition quantification strategy was based on the work of our colleagues (9). The AOAC performance requirements (SMPR® 2016.002) for repeatability, intermediate precision, reproducibility and recovery were reached for at least one peptide per allergen across both matrixes, and method quantification limits complied with the action levels of the Food Industry Guide to the Voluntary Incidental Trace Allergen Labelling (VITAL®) Program Version 3.0 (31).

To demonstrate the feasibility of method harmonization among analytical laboratories for the analysis of food allergens by MS, the developed method was submitted to nine laboratories across Europe involved in our study. The analytical procedure, blank and incurred cookie matrix, standards, and SIL concatemer internal standard were provided to participants. This paper describes the results obtained from this interlaboratory study, including method selectivity, sensitivity, trueness, and precision evaluations and discusses implications thereof.

Experimental

Participating Laboratories

Laboratories were recruited on a voluntary basis following communications at scientific congresses and through the European Network of Food Allergen Detection Laboratories (ENFADL) of the European Joint Research Centre (JRC). In all, nine laboratories from six European countries (Belgium, Czech Republic, Italy, the Netherlands, Spain, and Switzerland) were involved in the study. Invitations with clear instrumental requirements were first sent to the candidates. After acceptance, packages

containing samples and standards were sent, while documents (standard operating procedure for sample preparation and analysis and reporting form) were shared electronically. Laboratories were given 2 months to report the results. The nine laboratories involved in this interlaboratory study are named Laboratory 1 to Laboratory 9 in the manuscript.

Test Material Production

All test materials used in this work were produced at Food Pilot, a food processing test facility housing semi-industrial equipment at the Flanders Research Institute for Agriculture, Fisheries and Food (ILVO, Melle, Belgium). A complete description of cookies and protein standard preparation can be found in our previous study (31). Briefly, doughs for blank (containing no allergens) and highly contaminated (with a defined amount of the four allergens) cookies were separately produced, baked at 180°C, milled, and sieved. Different allergen concentration levels (expressed in parts per million, ppm, and corresponding to mg total protein from the allergenic food per kg of matrix) were obtained by mixing the blank cookie powder and the highly contaminated cookie powder in serial dilutions. Cookie powders incurred at 2.5, 5, and 25 ppm with each of the four considered allergens were taken for the interlaboratory trial.

Incurred materials at the different concentrations were tested for homogeneity of the four allergens, which was done by determining the allergen concentration in duplicate in 10 randomly selected subsamples at each concentration level using an in-house developed ELISA (CER Groupe, Marloie, Belgium). Homogeneity was evaluated using the Fearn and Thompson test (32). The homogeneity testing results can be found in Supplemental Material 1 and were extensively described in our previous study (31).

Protein Standards

Protein standards are here defined as extracts from reference material with certified protein content. Specifically, the following certified allergen reference materials were used: "hazelnut powder" (LGC7425 allergen reference material—hazelnut powder—partially defatted, LGC), "peanut flour" (LGCQC1020 light roasted, partially defatted peanut flour, LGC), "skimmed milk powder" (SMP-MQA 092014, MoniQA Association), and "spray-dried whole egg" (NIST SRMVR 8445, National Institute of Standards and Technology). Individual allergen stock solutions were generated by extracting the standards with the same protein extraction protocol applied for sample analysis in the detection method (liquid extraction, sonication, and centrifugation, see below). These stock solutions were used to prepare spiking and standard addition solutions containing all four allergens for method accuracy evaluation. Allergen content in the spike and standard addition solutions is summarized in Table 1. Allergen spiking levels were expressed in ppm (corresponding to mg total protein from the allergenic food per kg of matrix), considering the certified protein content of the standards and blank matrix test portion (2 g).

Internal Standard

An in-house developed SIL concatemer was used as an internal standard. Complete information on the design, production, and characterization of this concatemer can be found in our previous study (30). Briefly, the concatemer is an artificial protein, recombinantly produced, labeled with ^{15}N isotopes, and composed of 19 concatenated tryptic peptides corresponding to the selected biomarkers for the four allergens targeted in the ultra-HPLC (UPLC)–MS/MS method.

Study Design

The study was designed to evaluate the performance of the quantitative method for multiple allergen detection in terms of selectivity, sensitivity, and accuracy.

Extracts of egg, milk, peanut, and hazelnut standards digested with trypsin were provided to the participants to develop and optimize the UHPLC–MS/MS method. Participating laboratories also received an extra cookie sample, incurred at 25 ppm with the four allergens, providing them with the opportunity to test sample preparation and the UHPLC–MS/MS method in real conditions.

To consider the effects of food processing, the participating laboratories were asked to evaluate the method selectivity and sensitivity on the incurred cookie samples, in which the four allergens were added to the food matrix before processing. The laboratories were asked to prepare and analyse a blank (allergen-free) cookie sample and cookie samples incurred at three distinct levels with the targeted allergens (2.5, 5, and 25 ppm). Note that these levels were not communicated to the laboratories. The participating laboratories were asked to provide chromatograms of the different targeted transitions in each sample and a peak-to-peak S/N estimation.

Blank cookie matrix and extracts of standards of the four allergens were provided for method accuracy evaluation and laboratories were asked to blindly spike blank cookie matrix with allergen extracts and to use a standard addition method at two levels for quantification. A total of six samples (standard addition at two levels and two replicates) were prepared and analyzed for method accuracy evaluation. The laboratories were asked to provide integrated chromatograms for each sample and the different targeted transitions. Using standard addition, the laboratories were also asked to estimate the concentration, in ppm, of the four allergens spiked in the blank matrices.

Materials and Apparatus

The following materials and apparatus were required at participating laboratories:

(a) General laboratory equipment: 15 and 50 mL polypropylene conical tubes, 1.5 mL microcentrifuge tubes, pipettes (20–

Table 1. Theoretical allergen concentration obtained with a 100 µl addition in a 2 g sample for method accuracy evaluation

Allergen	Spike solution, ppm	Standard Addition A, ppm	Standard Addition B, ppm
Milk	2.5	0.5	5
Egg	25	10	100
Hazelnut	5	1	10
Peanut	50	25	250

200, 200–1000, and 1000–10 000 μL), agitator for 50 mL conical tubes, centrifuge for 15 and 50 mL conical tubes, centrifuge for 1.5 mL microcentrifuge tubes, pH meter, and precision balance.
(b) Sonication bath.
(c) Water bath.
(d) Nitrogen evaporator sample concentrator.
(e) SPE vacuum manifold.
(f) Sep-Pak C18 6 cc Vac solid-phase extraction (SPE) cartridges, 500 mg sorbent per cartridge and 55–105 μm from Waters (WAT043395).
(g) Polypropylene injection vials.
(h) ACQUITY UPLC Peptide BEH C18 Column, 130Å, 1.7 μm, 2.1 mm × 150 mm from Waters (186003556) or equivalent.
(i) UPLC–MS/MS system with electrospray ionization (UHPLC–ESI-MS/MS)

Reagents

The following reagents and solutions were required at the participating laboratories to prepare samples and perform the UHPLC–MS/MS analysis:

(a) Urea, powder and BioReagent for molecular biology, suitable for cell culture (U5378 from Sigma-Aldrich or equivalent).
(b) Tris(hydroxymethyl)aminomethane (Tris-HCl) ACS reagent ≥99.8% (252859 from Sigma-Aldrich or equivalent).
(c) Ammonium bicarbonate (NH_4HCO_3), BioUltra, ≥99.5% (09830 from Sigma-Aldrich or equivalent).
(d) DL-Dithiothreitol (DTT) ≥98% (TLC; D0632 from Sigma-Aldrich or equivalent).
(e) Iodoacetamide (IAA), 98%, ACROS Organics™ (AC122270050 from Fisher Scientific or equivalent).
(f) Trypsin from bovine pancreas, TPCK-treated (T8802 from Sigma-Aldrich).
(g) Deionized water (18.2 $M\Omega.cm^{-1}$).
(h) Hydrochloric acid, fuming 37% (1003171000 from Sigma-Aldrich or equivalent).
(i) Acetic acid, 99.8%, for analysis, ACROS Organics (AC222142500 from Fisher Scientific or equivalent).
(j) Formic acid 99%, ULC/MS—CC/SFC (069141 from Biosolve or equivalent).
(k) Dimethyl sulfoxide (DMSO) for synthesis (8029121000 from Sigma-Aldrich or equivalent).
(l) Water ULC/MS—CC/SFC (232141 from Biosolve or equivalent).
(m) Acetonitrile ULC/MS—CC/SFC (012041 from Biosolve or equivalent).
(n) Extraction buffer: 200 mM Tris-HCl, pH 9.2 with 2 M urea, prepared the day of the extraction
(o) Digestion buffer: 200 mM NH_4HCO_3.
(p) 200 mM DTT prepared in digestion buffer: this solution was prepared extemporaneously.
(q) 400 mM IAA prepared in digestion buffer: this solution was prepared extemporaneously and protected from any source of light (with aluminum foil, for instance).
(r) 50 mM acetic acid.
(s) Trypsin solution (1 mg/mL) prepared in 50 mM acetic acid: this solution was prepared extemporaneously with gentle dissolution avoiding harsh use of a vortex mixer.
(t) Digestion stop solution (water–formic acid, 4 + 1, by volume).
(u) SPE conditioning/wash solution (water–formic acid, 999 + 1, by volume).
(v) SPE elution solution (acetonitrile–water–formic acid, 799 + 200 + 1, by volume).
(w) Sample solubilization solution (water–acetonitrile–formic acid, 949 + 50 + 1, by volume).
(x) LC solvent A (water ULC/MS–formic acid, 999 + 1, by volume).
(y) LC solvent B (acetonitrile ULC/MS–formic acid, 999 + 1, by volume).

Sample Weighing and Identification

Participating laboratories were asked to weigh 2 g ± 0.02 g of each one of the four cookie test matrixes dedicated to method selectivity and sensitivity evaluation, which were entitled Sensitivity Blank Cookie, Sensitivity Cookie A, Sensitivity Cookie B, and Sensitivity Cookie C in separate 50 mL conical tubes. These three levels—A, B, and C—corresponded to cookie test matrixes incurred at 2.5, 5, and 25 ppm with the four allergens, respectively. Laboratories were also asked to weigh, in separate 50 mL conical tubes, 6 × 2 g ± 0.02 g of the cookie dedicated to method trueness and precision evaluation, which was entitled Trueness/Precision Cookie.

Sample Fortification

The standards and internal standard solutions were added to the weighed matrix test portions according to the following procedure. The ^{15}N concatemer internal standard was added to each of the 10 portions. We asked laboratories to first dilute 200 μL of the provided internal standard with 2300 μL extraction buffer before spiking the matrix test portions with 100 μL of this diluted internal standard. No other standard was added to the four portions dedicated to method selectivity and sensitivity evaluation. The six portions for method trueness and precision evaluation were spiked with 100 μL of "Spike solution". Allergen levels in this "Spike solution" were not communicated to the participating laboratories. An additional 100 μL "Standard addition A" solution was spiked in the sample entitled "T/P Cookie 1 + SA-A" and "T/P Cookie 2 + SA-A". Finally, an additional 100 μL "Standard addition B" solution was added to the sample entitled "T/P Cookie 1 + SA—B" and "T/P Cookie 2 + SA—B". Allergen levels in these different solutions, in ppm, are shown in Table 1.

Sample Preparation and Purification

After fortification, all matrix test portions were extracted with 20 mL extraction buffer by shaking at room temperature for 30 min followed by 15 min of ultrasound treatment in a sonication bath filled with cold water. The samples were centrifuged at 4600 *g* for 15 min at 10°C, after which 10 mL of the middle liquid phase was transferred to a new 50 mL conical tube. A volume of 10 mL of digestion buffer was added to each sample. Next, the proteins were reduced for 45 min at room temperature with 1 mL 200 mM DTT solution and then alkylated for 45 min at room temperature and in the dark with 1 mL 400 mM IAA solution. For proteolytic digestion, 1 mL of the 1 mg/mL trypsin solution was added to the samples, which were incubated for 1 h at 37 ± 0.5°C. Digestion was then stopped by adding 300 μL digestion stop solution, and subsequently, samples were centrifuged at 4600 *g* for 10 min at 10°C. The obtained supernatant liquid was loaded on Sep-Pak C18 6 cc Vac SPE cartridges previously conditioned with 18 mL acetonitrile and then 18 mL SPE conditioning/wash solution. The loaded samples were washed with 18 mL SPE conditioning/wash solution and finally eluted in a new 15 mL conical tube with 6 mL SPE elution solution. A volume of 30 μL DMSO was added to each eluate before evaporation

under a nitrogen flow in a water bath at 40°C. Pellets were dissolved in 600 µL sample solubilization solution, centrifuged at 4660 *g* for 5 min at 10°C, transferred to 1.5 mL microcentrifuge tubes, and centrifuged again at 11 500 *g* for 10 min at 10°C. The obtained supernatant liquid was finally transferred to polypropylene injection vials.

Ultra-HPLC Coupled to Tandem Mass Spectrometry

Peptides were separated by UPLC using a C18 reversed-phase column heated at 50°C. Laboratory 7 did not follow the recommendations and used a C4 reversed-phase column. A gradient of 26 min was applied to 20 µL injected sample volume with the following specifics: 0–3 min: 8% LC solvent B; 3–18 min: linear gradient from 8 to 42% LC solvent B; 18.1–22.5 min: 85% LC solvent B; 22.6–26 min: 8% LC solvent B at a flow rate of 0.2 mL/min. Eluted peptides were ionized by positive electrospray (ESI+) and analyzed in multiple reaction monitoring (MRM) mode. Most laboratories used mass spectrometers equipped with triple quadrupole mass analyzers. Laboratories 2 and 4 used Q-Orbitrap instruments. The following instrument parameters were recommended: capillary voltage at 2.5 kV; cone voltage at 30 V; source temperature at 150°C; desolvation temperature at 500°C; source gas flow at 50 L/h; and desolvation gas flow at 1200 L/h. Targeted transitions for the four considered allergens and the 19 peptides are summarized in Table 2. Three transitions for each peptide were analyzed, together with the corresponding quantification transition of the ^{15}N-labeled internal standard. Retention time and collision energy optimized on a Waters Xevo TQ-S instrument were given as indications to participating laboratories. Extracts of egg, milk, peanut, and hazelnut standards prepared as described above and ready to inject were provided to the participants to develop and optimize the UHPLC–MS/MS method.

Table 2. Overview of the precursor and product ions and their corresponding charge state (+) used in the multiple reaction monitoring method for the UHPLC-MS/MS-based detection of milk, egg, hazelnut and peanut

Allergen	Protein	Peptide	Precursor[a]	Product ions (fragments)[a]	RT[b], min	CE[b], V
Milk	α_{s1}-casein	FFVAPFPEVFGK	693.3++ 699.8++[c]	[y_9] 992.2+, [y_8] 921.1+, [y_6] 676.8+ [y_8] 930.0+ [c]	15.7	16
		HQGLPQEVLNENLLR	587.7+++ 595.6+++[c]	[y_7] 872.0+, [b_7] 790.8+, [y_6] 758.8+ [y_6] 769.8+[c]	11.6	12
		YLGYLEQLLR	634.7++ 641.7++[c]	[y_8] 992.2+, [y_6] 771.9+, [y_5] 658.8+ [y_8] 1004.1+[c]	14.7	16
	β-lactoglobulin	IDALNENK	459.0++ 464.5++[c]	[y_7] 803.8+, [y_6] 688.8+, [y_4] 504.5+ [y_6] 697.7+ [c]	4.9	13
		TPEVDDEALEK	623.7++ 629.6++[c]	[y_8] 919.0+, [y_7] 819.8+, [y_{10}] 573.1++ [y_{10}] 578.6++[c]	7.8	19
		VYVEELKPTPEGDLEILLQK	772.2+++ 779.8+++[c]	[y_{18}] 1026.7++, [y_{17}] 977.1++, [y_{11}] 628.2++ [y_{18}] 1037.1++[c]	13.4	17
Egg	Ovalbumin	GGLEPINFQTAADQAR	844.9++ 855.8++[c]	[y_{10}] 1122.2+, [y_7] 732.8+, [y_{12}] 666.7++ [y_{12}] 675.7++[c]	11.2	22
		HIATNAVLFFGR	673.8++ 682.7++[c]	[y_{10}] 1096.3+, [y_9] 1025.2+, [y_8] 924.1+ [y_{10}] 1110.2+[c]	12.0	22
	Ovotransferrin	SAGWNIPIGTLIHR	512.6+++ 519.6+++[c]	[y_8] 907.1+, [y_6] 696.8+, [y_4] 538.7+ [y_8] 920.0+[c]	13.3	15
		FYTVISSLK	529.6++ 534.6++[c]	[y_7] 747.9+, [y_6] 646.8+, [y_5] 547.7+ [y_7] 755.9+[c]	11.8	12
	Vitellogenin-1	NVNFDGEILK	575.1++ 581.6++[c]	[y_8] 936.0+, [y_6] 674.8+, [y_5] 559.7+ [y_8] 946.0+[c]	10.9	13
		TVIVEAPIHGLK	639.3++ 646.7++[c]	[y_8] 865.0+, [y_7] 735.9+, [y_6] 664.8+ [y_6] 673.8+[c]	10.3	19
Hazelnut	Cor a 9	ADIYTEQVGR	576.6++ 583.6++[c]	[y_7] 852.9+, [y_6] 689.7+, [y_5] 588.6+ [y_6] 699.7+[c]	7.7	16
		ALPDDVLANAFQISR	815.9++ 825.8++[c]	[y_8] 907.0+, [y_7] 835.9+, [y_{13}] 723.8++ [y_{13}] 732.7++[c]	14.2	19
		LNALEPTNR	514.6++ 521.5++[c]	[y_6] 729.8+, [y_5] 616.6+, [y_4] 487.5+ [y_4] 495.5+[c]	7.7	14
		TNDNAQISPLAGR	679.2++ 688.7++[c]	[y_7] 713.8+, [y_6] 600.7+, [y_5] 513.6+ [y_6] 609.6+[c]	8.5	19
Peanut	Ara h 1	GSEEEDITNPINLR	794.3++ 803.8++[c]	[y_7] 828.0+, [y_6] 726.8+, [y_5] 612.7+ [y_5] 621.7+[c]	10.7	19
		GSEEEGDITNPINLR	822.9++ 832.8++[c]	[y_7] 828.0+, [y_6] 726.8+, [y_5] 612.7+ [y_5] 621.7+[c]	10.5	22
		GTGNLELVAVR	565.2++ 572.6++[c]	[y_7] 800.0+, [y_6] 686.8+, [y_5] 557.7+ [y_5] 565.7+[c]	10.6	18

[a] The transitions correspond to the isotopic average mass

[b] Retention time (RT) and collision energy (CE), optimized on a Waters Xevo TQ-S instrument, were given as indications to participating labs.

[c] Indicates the ^{15}N stable isotope-labelled internal standard corresponding transition for each peptide.

Data Treatment and Concentration Calculation

A reporting form was electronically provided to the participating laboratories to estimate method sensitivity and accuracy. The content of the form was as follows:

(a) *Selectivity and sensitivity.*—Method sensitivity was evaluated based on S/N measurements on the different chromatograms with peak-to-peak analysis. For each of the four analyzed samples (Sensitivity Blank Cookie, Sensitivity Cookie A, Sensitivity Cookie B, and Sensitivity Cookie C) and for the different targeted transitions of the 19 peptides (three from the analyte and one from the ^{15}N internal standard), laboratories were asked to provide chromatograms and S/N estimations. The LOQ was defined as the lowest allergen concentration (expressed in ppm) with S/N >10 for one analyte transition, on the condition that the blank matrix sample showed no peak at the specific retention time, thus ensuring specificity.

(a) *Trueness and precision.*—Laboratories were asked to evaluate the allergen concentration in the matrix test portions fortified with the "Spike solution". This evaluation was done using a standard addition quantification method. A standard addition curve was generated using three data points (matrix test portions fortified with the "Spike solution", matrix test portions fortified with the "Spike solution" + "Standard addition A", and matrix test portions fortified with the "Spike solution" + "Standard addition B"). The standard addition curve was constructed with the internal standard concatemer-based signal response (area ratio between analyte and internal standard chromatographic peaks) in each sample. The allergen concentration (expressed in ppm) was calculated using the concentration of the different allergens in the standard addition solutions (Table 1) and a linear regression model and corresponded to the ratio of the intercept to the slope of the equation of the curve (Figure 1).

The allergen concentration measurement in the matrix test portions fortified with the "Spike solution" was repeated with biological duplicates to evaluate method precision based on repeatability and reproducibility. Within-laboratory precision was evaluated using the relative standard deviation (RSD_r). The results obtained for this parameter had to be considered with caution, since only two biological replicates were prepared by the participating laboratories (to limit the workload). Reproducibility was calculated from among-laboratory data expressed as reproducibility relative standard deviation (RSD_R). Method trueness evaluation was based on recovery, calculated as the ratio of the average determined concentration to the spiked concentration.

For each of the six analyzed samples and for the 19 targeted peptides, laboratories were asked to report the signal response, considering the corresponding transitions between analyte and ^{15}N internal standard. Allergen concentration was automatically calculated, in ppm, by the reporting form.

Results and Discussion

Data Handling and Statistics

The reporting form was correctly filled out by all participating laboratories. Based on the feedback, there were no major difficulties encountered in the method application, even for the laboratories not trained for allergen analysis. The only exception was Laboratory 4, which experienced some difficulties in providing data for method accuracy evaluation. These difficulties were identified and were due to the analytical system used to acquire the data. Laboratory 4 used an Orbitrap Q-Exactive instrument in SRM (selected reaction monitoring) mode with a relatively slow acquisition rate. Some chromatographic peaks were defined only by three acquisition points. The resulting data were therefore invalid and not provided for more than half of the considered peptides. It was therefore decided to exclude the data from Laboratory 4 for method accuracy evaluation.

Chromatograms related to method selectivity and sensitivity evaluation were inspected, and no statistical treatment was applied to the received data. As detailed in the following section, this evaluation was based on S/N measurement of the different chromatograms with peak-to-peak analysis.

The data related to method accuracy evaluation were checked for outliers according to AOAC Appendix D (33). A 1-tail Cochran test at a *P* value of 2.5% was first used to assess the presence of significant variability among the duplicate within-laboratory analysis. Values obtained by Laboratory 7 were found to be outliers according to the Cochran test for 11 out of the 19 considered peptides. Most of these peptides (10 out of 11) correspond to egg and milk proteins. Peptide GSEEEDITNPINLR from peanuts was also found to be an outlier in the data obtained by Laboratory 1. The Grubbs test was applied as a single-value 2-tail test with a *P* value of 2.5% to identify participant data with extreme results. The results of this test indicated that the data obtained by Laboratory 7 were outliers for 17 of the 19 considered peptides. Based on these statistical analyses, it was decided to exclude the data provided by Laboratory 7 for method accuracy evaluation.

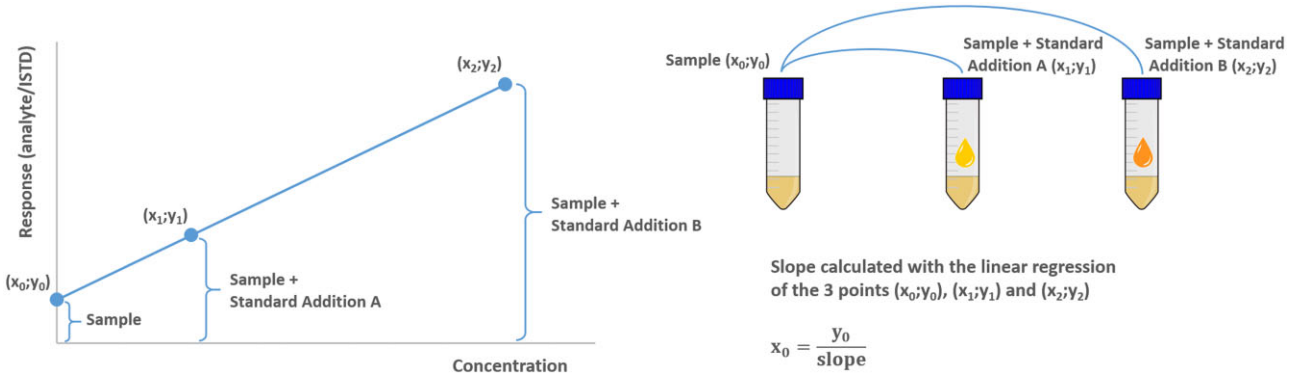

Figure 1. Layout of quantification strategy. For quantification, a standard addition calibration curve is constructed with the ^{15}N SIL internal standard concatemer-based response (defined as the ratio of the quantification peak area to internal standard ion peak area in each sample). The allergen concentration (x_0) of the sample is determined from the ratio of the intercept (y_0) to the slope of the equation of the curve.

Method Selectivity and Sensitivity Evaluation

Blank cookies and cookies incurred at several levels with the four allergens considered in the study (egg, milk, peanut, and hazelnut) were prepared by participating laboratories. Method selectivity and sensitivity evaluation were based on S/N measurements on the different chromatograms with peak-to-peak analysis. Complete results with measured S/N values for each transition of the 19 peptides are described in Table 3.

The ^{15}N-labeled peptides obtained from the proteolysis of the concatemer internal standard were detected with a S/N >10 in 98% of the cases. No general trend was observed for the remaining 2%. Laboratory 4 was, for example, unable to detect the ^{15}N-labeled peptide TPEVDDEALEK in any of the four samples, whereas Laboratory 2 detected the ^{15}N-labeled peptides HQGLPQEVLNENLLR and SAGWNIPIGTLIHR with S/N values between 7 and 10. The 19 ^{15}N-labeled peptides were all detected with S/N values above 10 in all samples by six laboratories.

A relatively high method selectivity was observed with more than 95% of the analyte targeted transitions with S/N <10 in the blank samples. In some cases, as with peptides FFVAPFPEVFGK and YLGYLEQLLR from milk with Laboratory 2, contaminations during the sample preparation and/or in the LC–MS instrument were suspected. The three analyte transitions of both peptides were detected with S/N >10 in the blank sample. Both peptides were characterized by high detection sensitivity and were highly hydrophobic. This combination of factors led to a high risk of contamination between samples. The same transitions were detected by Laboratory 2, with an intensity more than 20 times higher in the cookie incurred with 2.5 ppm of milk. In these cases, the signal obtained was more likely due to contamination than to the method's lack of selectivity. This contamination example highlights the challenges of routine food allergen analysis. In the laboratory, samples need to be handled, prepared, and analyzed with great care to avoid contaminations leading to false-positive results. For the analysis of small molecules such as pesticides, veterinary drugs, or natural toxins, the targeted compounds are usually minority components. For food allergen analysis, it can be imagined that a laboratory searches for traces of milk in a dark chocolate sample after having prepared and analyzed ice cream in which milk proteins were present in high abundance. Material and instrument cleaning between the different samples and great care during the sample preparation (from the crushing to the transfer of the sample ready for analysis to the injection vial) are of high importance to avoid undesired contamination.

A single transition per peptide was detected in the blank sample for the egg peptide FYTVISSLK by Laboratory 8 and the milk peptide TPEVDDEALEK by Laboratory 9. In these cases, the interfering signal could be due to sample preparation or matrix effects. For these particular transitions and this matrix, the method was not selective enough and the related transitions had to be rejected. During the development and validation of a food allergen analysis method, multiple blank matrixes have to be considered and analyzed to strengthen method specificity (34). An additional and theoretical verification of peptide specificity can be performed. This step is generally accomplished by querying the selected peptide sequence against publicly available protein sequence databases (8). However, a high diversity of food matrixes, with their associated matrix effects and without possible blank versions, can be encountered in food allergen routine analysis. To ensure method specificity, it is recommended to consider multiple peptides per protein and multiple transitions per peptide, as we did, or even to monitor the ion ratios between these transitions (11, 35).

All participating laboratories were able to detect the presence of milk with S/N >10 in the cookie incurred at the lowest level (2.5 ppm). For hazelnut, only Laboratory 9 failed to detect the allergen at this lowest level. All laboratories were also able to detect peanut and hazelnut at the 5 ppm level. For these three food allergens, all participants were able to reach, in terms of sensitivity, SMPR 2016.002 (method quantification limit ≤10 ppm) established by AOAC (15). The detection of egg was more difficult with four and five participating laboratories succeeding with the cookies incurred at 2.5 and 5 ppm levels, respectively. For this particular allergen, the AOAC method quantification limit is ≤5 ppm. All participants succeeded in the detection of egg in the cookie incurred at 25 ppm. Moreover, a lower sensitivity for egg detection has already been observed during the validation of the method (31) and was clearly attributed to the effects of food processing. Modifying the sample preparation protocol could be an option to increase the egg detection sensitivity. However, the impact on the detectability of the other allergens has to be monitored. It was, for instance, demonstrated that the introduction of an additional protein purification step, between the extraction and the enzymatic digestion, could improve allergen detection with MS-based method (36).

This first part of our interlaboratory study dedicated to method selectivity and sensitivity evaluation demonstrated the challenges associated with food allergen analysis and the potential of our MS-based method.

Method Trueness and Precision Evaluation

A duplicate preparation and analysis of cookies fortified with standard extracts of the four allergens and a standard addition quantification strategy were considered to evaluate the accuracy of the method. Although the raw ingredients used for test material production of cookies were characterized in terms of protein content, the allergen concentration in the finished product was not. Despite being theoretically calculated, the true amount of protein from each allergen remains uncertain due to the lack of a reference detection method for allergens in these materials. For these reasons, method accuracy was evaluated using blank formulation of the cookies, fortified with the reference allergen protein standards described above. The materials used for the production of these allergen protein extracts are characterized with a higher confidence than our in-house incurred test material and are used in other laboratories and studies (37) as well, thus gaining harmonization. However, certified reference materials, incurred with one or multiple allergen(s) as the cookie incurred with milk protein produced by the MoniQA Association (38), are strongly required by testing laboratories. These materials would allow the evaluation of the accuracy of quantitative methods, including the impact of food processing.

The method trueness evaluation was based on recovery, calculated as the ratio of the average determined concentration to the spiked concentration (Table 4) and precision based on repeatability with RSD_r and reproducibility with RSD_R (Table 5). The results obtained by the participating laboratories for method trueness and precision evaluation were compared after removing outliers, with SMPR 2016.002 established by AOAC (15): a recovery in the range of 60–120%, RSD_r <20% and RSD_R < 0%.

The results received for method trueness evaluation seemed to be laboratory-dependent. Indeed, quantification results sent by Laboratory 2 led to recoveries in agreement with AOAC requirements for all 19 targeted peptides. In contrast, only five peptides

Table 3. Overview of method selectivity and sensitivity evaluation based on the analysis of blank cookie and cookie incurred at several levels with the four allergens considered in the study (egg, milk, peanut and hazelnut)[a]

					Blank Cookie									Cookie A (2.5 ppm)									Cookie B (5 ppm)									Cookie C (25 ppm)								
Allergen	Protein	Peptide	Precursor	Product ions (fragments)	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8	Lab 9	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8	Lab 9	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8	Lab 9	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8	Lab 9
Milk	αs1-casein	FFVAPFPEVFGK	693.3++	[y_8] 921.1+	6.0	>10	1.0	2.0	1.0	8.5	7.5	1.0	1.6	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10
				[y_6] 676.8+	1.0	>10	1.0	2.0	1.0	3.2	9.0	1.0	3.5	3.0	>10	>10	>10	>10	9.7	>10	>10	3.4	7.0	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10
				[y_9] 992.2+	7.0	>10	1.0	1.0	1.0	>10	7.8	2.3	1.0	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10
			699.8++[b]	[y_8] 930.0+[b]	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10
		HQGLPQEVLNENLLR	587.7+++	[y_6] 758.8+	1.0	1.0	1.0	1.0	1.0	1.0	4.0	1.0	3.0	>10	5.0	>10	>10	>10	6.9	>10	>10	9.0	>10	5.0	>10	>10	>10	>10	>10	>10	>10	>10	6.0	>10	>10	>10	>10	>10	>10	>10
				[b_7] 790.8+	1.0	1.0	1.0	1.0	1.0	1.5	3.4	1.0	2.3	9.0	5.0	2.5	>10	>10	>10	>10	>10	8.7	>10	5.0	>10	>10	>10	>10	>10	>10	>10	>10	5.0	>10	>10	>10	>10	>10	>10	>10
				[y_7] 872.0+	1.0	1.0	1.0	1.0	1.0	1.8	5.0	1.0	2.0	>10	5.0	>10	>10	>10	>10	>10	>10	>10	>10	5.0	>10	1.0	9.0	>10	>10	>10	>10	>10	6.0	>10	>10	>10	>10	>10	>10	>10
			595.6+++[b]	[y_6] 769.8+[b]	>10	9.0	>10	>10	>10	>10	>10	>10	>10	>10	8.0	>10	>10	>10	>10	>10	>10	>10	>10	9.0	>10	>10	>10	>10	>10	>10	>10	>10	10.0	>10	>10	>10	>10	>10	>10	>10
		YLGYLEQLLR	634.7++	[y_8] 992.2+	>10	>10	1.0	1.0	1.0	>10	9.5	9.6	8.2	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10
				[y_5] 658.8+	1.0	>10	1.0	2.0	1.0	6.2	1.2	1.0	7.7	>10	>10	8.3	>10	9.0	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10
				[y_6] 771.9+	1.0	>10	1.0	1.0	1.0	5.5	2.4	4.3	2.0	4.0	>10	8.0	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10
			641.7++[b]	[y_8] 1004.1+[b]	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10
	β-lactoglobulin	IDALNENK	459.0++	[y_6] 688.8+	1.0	1.0	1.0	1.0	1.0	4.5	1.0	8.5	6.2	3.0	4.0	1.0	1.0	>10	>10	2.1	6.8	>10	5.0	3.0	1.0	1.0	>10	>10	3.1	>10	>10	>10	>10	5.7	1.0	>10	>10	8.7	>10	>10
				[y_7] 803.8+	2.0	1.0	1.0	1.0	1.0	3.0	1.2	>10	6.6	2.0	>10	2.1	1.0	>10	>10	3.3	>10	>10	4.0	>10	1.0	1.0	>10	>10	4.0	>10	>10	>10	>10	7.6	1.0	>10	>10	>10	>10	>10
				[y_4] 504.5+	2.0	1.0	1.0	1.0	1.0	1.0	3.5	1.0	2.4	2.0	>10	3.4	1.0	2.0	4.7	3.2	1.0	3.4	2.0	>10	3.1	1.0	1.0	5.4	6.5	7.3	5.1	>10	>10	>10	1.0	7.0	>10	>10	>10	>10
			464.5++[b]	[y_6] 697.7+[b]	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10
		TPEVDDEALEK	623.7++	[y_{10}] 573.1++	5.0	1.0	1.0	1.0	1.0	2.3	2.5	5.4	2.9	>10	1.0	1.0	1.0	2.0	3.5	2.7	1.5	5.2	>10	1.0	3.7	1.0	5.0	1.0	4.4	1.8	>10	>10	3.0	>10	1.0	10.0	9.4	>10	>10	>10
				[y_7] 819.8+	2.0	1.0	1.0	1.0	1.0	2.3	1.0	3.2	3.2	4.0	1.0	1.0	1.0	1.0	1.4	1.0	1.0	3.6	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.1	8.8	2.0	1.0	4.8	1.0	1.0	3.8	3.8	4.5	8.5
				[y_8] 919.0+	4.0	1.0	1.0	1.0	1.0	3.8	1.4	1.0	>10	1.0	1.0	1.0	1.0	1.0	2.0	1.0	1.0	9.1	1.0	1.0	1.0	1.0	1.0	2.3	1.0	1.0	>10	6.0	1.0	6.7	5.0	9.0	4.1	4.9	4.4	>10
			629.6++[b]	[y_{10}] 578.6++[b]	>10	>10	>10	2.0	>10	>10	>10	>10	>10	>10	>10	>10	>10	2.0	>10	>10	>10	>10	>10	>10	>10	>10	2.0	>10	>10	>10	>10	>10	>10	>10	>10	2.0	>10	>10	>10	>10
		VYVEELKPTPEGDLEILLQK	772.2+++	[y_{18}] 1026.7++	1.0	1.0	1.0	1.0	1.0	1.6	2.0	1.0	1.0	>10	>10	4.3	1.0	2.0	>10	>10	1.0	7.5	8.0	>10	7.0	1.0	7.0	>10	>10	2.5	8.8	>10	>10	>10	1.0	>10	>10	>10	>10	>10
				[y_{17}] 977.1++	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	8.0	3.0	1.0	>10	1.0	1.0	3.4	1.0	1.1	10.0	3.0	2.9	>10	1.0	1.0	4.6	1.0	3.1	>10	8.0	>10	>10	8.0	>10	>10	6.8	7.6
				[y_{11}] 628.2++	1.0	1.0	1.0	1.0	1.0	1.3	3.2	1.0	2.5	6.0	1.0	1.0	1.0	1.0	1.0	2.3	1.0	2.5	8.0	1.0	1.0	1.0	1.0	1.0	6.5	1.0	3.4	>10	6.0	6.0	1.0	2.0	>10	>10	1.5	6.7
			779.8+++[b]	[y_{18}] 1037.1++[b]	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10
Egg	Ovalbumin	GGLEPINFQTAADQAR	844.9++	[y_{12}] 666.7++	1.0	1.0	1.0	1.0	1.0	1.4	1.0	1.0	1.0	>10	>10	>10	1.0	2.0	1.0	>10	2.2	3.8	>10	>10	>10	5.0	3.0	9.6	>10	3.9	8.2	>10	>10	>10	>10	>10	>10	>10	>10	>10
				[y_7] 732.8+	1.0	1.0	1.0	1.0	1.0	2.6	1.0	1.0	1.1	1.0	7.0	7.9	1.0	1.0	1.0	1.0	1.0	3.2	2.0	8.0	3.9	1.0	1.0	6.4	2.0	1.0	2.4	2.0	>10	>10	>10	2.0	>10	1.9	3.4	4.9
				[y_{10}] 1122.2+	1.0	1.0	1.0	1.0	1.0	1.1	1.0	1.0	2.0	1.0	>10	4.2	1.0	1.0	1.0	4.5	2.4	5.7	7.0	10.0	2.8	2.0	2.0	7.3	1.0	4.1	4.8	>10	>10	>10	>10	1.0	>10	>10	>10	>10
			855.8++[b]	[y_{12}] 675.7++[b]	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10
		HIATNAVLFFGR	673.8++	[y_{10}] 1096.3+	1.0	1.0	1.0	1.0	1.0	1.0	1.9	1.2	>10	3.0	1.0	1.8	1.0	2.0	2.9	5.6	5.2	2.9	10.0	7.0	2.7	5.0	1.0	1.0	8.4	5.2	4.7	>10	>10	8.7	10.0	6.0	>10	>10	>10	6.6
				[y_9] 1025.2+	2.0	1.0	1.0	1.0	1.0	1.0	1.0	1.1	3.7	1.0	1.0	1.0	1.0	1.0	1.1	1.0	1.5	4.0	1.0	4.0	1.0	1.0	1.0	1.9	1.0	2.4	4.1	3.0	>10	1.7	1.0	1.0	5.5	1.0	3.5	1.7
				[y_8] 924.1+	3.0	1.0	1.0	1.0	1.0	1.0	1.0	3.0	1.0	1.0	>10	1.0	1.0	1.0	1.0	1.0	4.1	1.9	1.0	>10	1.0	1.0	1.0	1.2	1.0	5.6	1.5	2.0	>10	1.4	1.0	1.0	1.0	1.0	4.5	1.4
			682.7++[b]	[y_{10}] 1110.2+[b]	>10	>10	7.0	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10
	Ovotransferrin	SAGWNIPIGTLIHR	512.6+++	[y_8] 907.1+	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.0	1.0	1.0	2.7	1.0	1.0	1.0	1.0	1.0	9.0	2.4	2.9	>10	6.8	8.0	4.0	1.0	1.0	>10	>10	9.2	>10	>10
				[y_6] 696.8+	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.6	1.2	1.0	1.0	1.0	1.0	2.0	1.0	2.1	3.6	1.0	1.0	1.0	1.0	>10	1.0	1.6	1.1	3.5	1.1	1.0	5.0	1.0	>10	7.0	8.1	2.0	7.9	1.2
				[y_4] 538.7+	1.0	1.0	1.0	1.0	1.0	1.0	1.8	1.0	1.0	2.0	4.0	1.0	1.0	1.0	1.0	1.3	1.0	2.8	1.0	4.0	1.0	1.0	1.0	1.0	1.0	1.2	1.0	1.0	5.0	1.0	1.0	>10	2.0	3.2	3.1	1.7
			519.6+++[b]	[y_8] 920.0+[b]	>10	9.0	>10	>10	>10	>10	>10	>10	>10	>10	7.0	>10	>10	>10	>10	>10	>10	>10	>10	8.0	>10	>10	>10	>10	>10	>10	>10	>10	10.0	>10	>10	>10	>10	>10	>10	>10
		FYTVISSLK	529.6++	[y_7] 747.9+	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.4	1.0	4.0	1.0	1.0	1.0	2.0	1.0	1.0	2.4	1.0	3.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	7.0	1.0	1.0	1.0	>10	6.4	3.8	2.9
				[y_6] 646.8+	>10	1.0	1.0	1.0	1.0	1.0	1.0	>10	3.6	4.0	1.0	1.0	2.0	1.0	1.0	1.0	>10	1.3	4.0	1.0	1.0	2.0	1.0	1.0	1.0	>10	6.5	5.0	1.0	1.0	2.0	2.0	3.6	1.0	>10	3.5
				[y_5] 547.7+	>10	1.0	1.0	1.0	1.0	1.3	1.0	4.1	3.2	>10	7.0	1.0	1.0	>10	1.0	1.0	3.7	1.8	>10	8.0	1.0	1.0	1.0	1.0	1.0	6.3	2.5	>10	8.0	1.0	1.0	1.0	4.3	1.0	4.2	4.2
			534.6++[b]	[y_7] 755.9+[b]	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10
	Vitellogenin-1	NVNFDGEILK	575.1++	[y_8] 936.0+	1.0	1.0	1.0	1.0	1.0	1.0	1.1	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.1	1.0	1.4	1.0	1.0	1.0	1.0	1.0	1.0	1.6	1.0	1.9	1.0	1.0	1.0	5.0	6.0	3.7	>10	1.8	1.7
				[y_5] 559.7+	1.0	1.0	1.0	1.0	1.0	1.0	2.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.8	2.3	1.0	1.0	2.0	1.0	1.0	1.0	1.0	3.7	1.3	1.0	2.5	1.0	1.0	1.0	1.0	1.0	1.5	1.0	1.0	2.9

(continued)

Table 3. (continued)

Allergen	Protein	Peptide	Precursor	Product ions (fragments)	Blank Cookie									Cookie A (2.5 ppm)								
					Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8	Lab 9	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8	Lab 9
				[y_6] 674.8+	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.3	1.4
			581.6++[b]	[y_8] 946.0+[b]	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**
		TVIVEAPIHGLK	639.3++	[y_8] 865.0+	1.0	1.0	1.0	1.0	1.0	1.0	2.2	1.0	1.5	1.0	1.0	1.0	1.0	4.0	1.4	1.2	1.1	1.9
				[y_6] 664.8+	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.1	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.2
				[y_7] 735.9+	1.0	1.0	1.0	1.0	1.0	1.0	3.4	1.0	2.1	1.0	1.0	1.0	1.0	4.0	1.0	9.3	1.0	2.4
			646.7++[b]	[y_6] 673.8+[b]	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**
Hazelnut	Cor a 9	ADIYTEQVGR	576.6++	[y_6] 689.7+	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.7	5.0	9.0	**>10**	**>10**	**>10**	9.4	9.9	**>10**	6.3
				[y_7] 852.9+	1.0	1.0	1.0	1.0	1.0	6.1	1.0	1.0	1.0	9.0	**>10**	**>10**	**>10**	5.0	**>10**	**>10**	**>10**	7.5
				[y_5] 588.6+	3.0	1.0	1.0	1.0	1.0	1.4	1.0	1.0	5.3	7.0	10.0	**>10**	**>10**	1.0	3.1	5.5	**>10**	7.6
			583.6++[b]	[y_6] 699.7+[b]	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**
		ALPDDVLANAFQISR	815.9++	[y_{13}] 723.8++	2.0	1.0	1.0	1.0	1.0	**>10**	1.0	1.0	1.2	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	6.8
				[y_8] 907.0+	1.0	1.0	1.0	1.0	1.0	1.3	1.0	1.0	2.4	3.0	**>10**	5.9	**>10**	4.0	5.3	**>10**	**>10**	3.6
				[y_7] 835.9+	2.0	1.0	1.0	1.0	1.0	1.0	1.0	1.4	1.0	2.0	10.0	1.9	**>10**	3.0	2.1	4.4	**>10**	2.9
			825.8++[b]	[y_{13}] 732.7++[b]	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**
		LNALEPTNR	514.6++	[y_4] 487.5+	**>10**	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.4	**>10**	8.0	**>10**	**>10**	6.0	2.7	3.8	8.8	3.3
				[y_5] 616.6+	1.0	1.0	1.0	1.0	1.0	1.6	1.0	1.0	2.1	10.0	7.0	**>10**	2.0	**>10**	4.8	4.6	9.9	4.3
				[y_6] 729.8+	1.0	1.0	1.0	1.0	1.0	2.5	1.0	1.0	1.6	5.0	6.0	**>10**	1.0	**>10**	**>10**	4.5	**>10**	3.3
			521.5++[b]	[y_4] 495.5+[b]	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**
		TNDNAQISPLAGR	679.2++	[y_6] 600.7+	1.0	1.0	1.0	1.0	1.0	2.2	1.0	2.1	2.9	**>10**	**>10**	**>10**	1.0	4.0	6.5	**>10**	**>10**	8.4
				[y_5] 513.6+	**>10**	1.0	1.0	1.0	1.0	1.8	1.0	1.6	3.3	**>10**	**>10**	**>10**	5.0	4.0	8.3	**>10**	4.0	3.9
				[y_7] 713.8+	1.0	1.0	1.0	1.0	1.0	2.3	1.0	1.6	2.3	3.0	**>10**	**>10**	5.0	7.0	7.7	**>10**	6.8	2.1
			688.7++[b]	[y_6] 609.6+[b]	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**
Peanut	Ara h 1	GSEEEDITNPINLR	794.3++	[y_5] 612.7+	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.4	2.3	4.0	1.0	1.0	2.0	2.0	1.0	1.4	2.5	4.1
				[y_7] 828.0+	1.0	1.0	1.0	1.0	1.0	1.0	1.0	**>10**	2.5	7.0	1.0	1.0	1.0	1.0	1.0	1.8	2.1	**>10**
				[y_6] 726.8+	4.0	1.0	1.0	1.0	1.0	1.0	1.0	1.5	3.0	2.0	1.0	1.0	1.0	1.0	1.0	1.0	8.7	6.0
			803.8++[b]	[y_5] 621.7+[b]	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	1.0	**>10**	**>10**	**>10**	**>10**	**>10**
		GSEEEGDITNPINLR	822.9++	[y_5] 612.7+	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	8.0	1.0	5.0	3.0	1.0	1.0	1.0	1.0
				[y_7] 828.0+	9.0	1.0	1.0	1.0	1.0	1.0	1.0	**>10**	1.0	4.0	1.0	1.0	10.0	1.0	2.0	2.5	**>10**	1.0
				[y_6] 726.8+	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	5.0	1.0	1.0	1.0	1.0	1.0
			832.8++[b]	[y_5] 621.7+[b]	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**
		GTGNLELVAVR	565.2++	[y_6] 686.8+	**>10**	1.0	1.0	1.0	1.0	2.4	1.0	1.3	2.4	**>10**	**>10**	**>10**	1.0	7.0	5.7	2.6	9.4	6.1
				[y_5] 557.7+	10.0	1.0	1.0	1.0	1.0	4.0	1.0	1.1	2.0	**>10**	8.0	**>10**	1.0	8.0	4.7	**>10**	3.8	2.5
				[y_7] 800.0+	**>10**	1.0	1.0	**>10**	1.0	6.4	1.0	4.7	7.9	**>10**	**>10**	1.0	**>10**	3.0	6.4	3.8	**>10**	8.2
			572.6++[b]	[y_5] 565.7+[b]	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**

Allergen	Protein	Peptide	Precursor	Product ions (fragments)	Cookie B (5 ppm)									Cookie C (25 ppm)								
					Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8	Lab 9	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8	Lab 9
				[y_6] 674.8+	2.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.4	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.3	2.2
			581.6++[b]	[y_8] 946.0+[b]	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**
		TVIVEAPIHGLK	639.3++	[y_8] 865.0+	2.0	1.0	1.0	1.0	1.0	1.2	3.0	1.0	1.1	1.0	1.0	1.0	1.0	2.0	1.0	1.8	1.0	2.4
				[y_6] 664.8+	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.1	1.0	5.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
				[y_7] 735.9+	1.0	1.0	1.0	1.0	**>10**	1.0	3.4	1.0	1.7	1.0	1.0	1.0	1.0	4.0	1.5	1.8	1.0	2.0
			646.7++[b]	[y_6] 673.8+[b]	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**
Hazelnut	Cor a 9	ADIYTEQVGR	576.6++	[y_6] 689.7+	**>10**	7.0	**>10**	**>10**	5.0	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**
				[y_7] 852.9+	**>10**	**>10**	**>10**	**>10**	4.0	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**
				[y_5] 588.6+	1.0	**>10**	7.2	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	8.0	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**
			583.6++[b]	[y_6] 699.7+[b]	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**
		ALPDDVLANAFQISR	815.9++	[y_{13}] 723.8++	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**
				[y_8] 907.0+	6.0	**>10**	4.9	**>10**	4.0	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**
				[y_7] 835.9+	5.0	10.0	2.7	**>10**	3.0	6.4	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**
			825.8++[b]	[y_{13}] 732.7++[b]	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**
		LNALEPTNR	514.6++	[y_4] 487.5+	**>10**	8.0	**>10**	**>10**	**>10**	6.2	3.4	**>10**	**>10**	10.0	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**
				[y_5] 616.6+	**>10**	8.0	**>10**	2.0	**>10**	**>10**	4.4	**>10**	7.9	**>10**	10.0	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**
				[y_6] 729.8+	2.0	7.0	**>10**	1.0	**>10**	**>10**	5.4	**>10**	**>10**	**>10**	10.0	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**
			521.5++[b]	[y_4] 495.5+[b]	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**
		TNDNAQISPLAGR	679.2++	[y_6] 600.7+	10.0	**>10**	**>10**	5.0	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**
				[y_5] 513.6+	**>10**	9.0	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**
				[y_7] 713.8+	3.0	**>10**	**>10**	10.0	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**
			688.7++[b]	[y_6] 609.6+[b]	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**
Peanut	Ara h 1	GSEEEDITNPINLR	794.3++	[y_5] 612.7+	3.0	1.0	1.0	1.0	**>10**	1.9	1.2	3.0	6.5	**>10**	6.0	**>10**	5.0	5.0	**>10**	**>10**	8.9	8.9
				[y_7] 828.0+	10.0	1.0	1.0	1.0	1.0	2.2	2.1	2.7	**>10**	8.0	**>10**	7.2	1.0	1.0	4.0	**>10**	**>10**	**>10**
				[y_6] 726.8+	7.0	1.0	1.0	1.0	5.0	5.7	1.0	9.9	7.2	3.0	1.0	5.3	1.0	4.0	6.5	4.0	3.7	9.2
			803.8++[b]	[y_5] 621.7+[b]	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	5.0	**>10**	**>10**	**>10**	**>10**
		GSEEEGDITNPINLR	822.9++	[y_5] 612.7+	4.0	3.0	1.0	1.0	1.0	2.0	1.0	1.0	1.1	**>10**	**>10**	**>10**	**>10**	6.0	6.4	6.8	2.7	2.3
				[y_7] 828.0+	6.0	8.0	1.0	2.0	2.0	1.0	2.6	**>10**	2.4	**>10**	9.0	**>10**	**>10**	1.0	**>10**	**>10**	**>10**	2.4
				[y_6] 726.8+	1.0	1.0	1.0	5.0	1.0	1.0	1.0	1.0	1.4	1.0	1.0	**>10**	2.0	2.0	1.3	8.8	1.3	1.4
			832.8++[b]	[y_5] 621.7+[b]	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**
		GTGNLELVAVR	565.2++	[y_6] 686.8+	1.0	1.0	1.0	3.0	**>10**	**>10**	5.2	**>10**	8.2	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**
				[y_5] 557.7+	**>10**	**>10**	**>10**	**>10**	**>10**	6.4	**>10**	7.4	7.1	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	8.8	**>10**	**>10**
				[y_7] 800.0+	1.0	**>10**	6.7	**>10**	1.0	**>10**	4.1	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**
			572.6++[b]	[y_5] 565.7+[b]	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**

[a] The evaluation was based on signal-to-noise ratio (S/N) measurements on the different chromatograms with peak-to-peak analysis. Values with S/N > 10, criteria to define method LOQ, are highlighted in bold.
[b] Indicates the ^{15}N stable isotope-labelled internal standard corresponding transition for each peptide.

Table 4. Overview of method trueness evaluation, after outliers removal, based on the duplicate analysis of cookie fortified with the four allergens and using a standard addition quantification method[a]

Allergen	Protein	Peptide	Precursor[b]	Product ions (fragments)[b]	Recovery, %							
					Lab 1	Lab 2	Lab 3	Lab 5	Lab 6	Lab 8	Lab 9	Inter Lab
Milk 2.5 ppm	αs1-casein	FFVAPFPEVFGK	693.3++	[y_8] 921.1+	**111.4**	**97.8**	**108.7**	**111.2**	**104.3**	**108.5**	**117.6**	**108.5**
		HQGLPQEVLNENLLR	587.7+++	[y_6] 758.8+	120.5	**76.0**	**105.0**	**105.0**	**94.4**	**120.0**	121.2	**106.0**
		YLGYLEQLLR	634.7++	[y_8] 992.2+	**113.7**	**98.8**	**108.4**	**108.6**	**112.6**	121.1	126.2	**112.8**
	β-lactoglobulin	IDALNENK	459.0++	[y_6] 688.8+	**99.2**	**105.8**	141.7	133.8	**81.2**	**108.6**	**114.6**	**112.1**
		TPEVDDEALEK	623.7++	[y_{10}] 573.1+	**97.0**	**86.4**	141.3	**92.5**	**119.7**	**103.2**	142.8	**111.8**
		VYVEELKPTPEGDLEILLQK	772.2+++	[y_{18}] 1026.7+	**117.1**	**95.2**	127.0	**114.6**	**97.0**	**103.5**	**112.1**	**109.5**
Egg 25 ppm	Ovalbumin	GGLEPINFQTAADQAR	844.9++	[y_{12}] 666.7+	**110.5**	**90.8**	265.5	**119.7**	**62.2**	57.3	**76.5**	**111.8**
		HIATNAVLFFGR	673.8++	[y_{10}] 1096.3+	126.2	**88.7**	275.7	138.0	**63.7**	55.0	**79.2**	**118.1**
	Ovotransferrin	SAGWNIPIGTLIHR	512.6+++	[y_8] 907.1+	**106.9**	**90.1**	124.1	**105.5**	**85.1**	56.6	57.8	**89.4**
		FYTVISSLK	529.6++	[y_7] 747.9+	269.5	**92.3**	**115.6**	**118.6**	**96.0**	144.1	**93.8**	132.8
	Vitellogenin-1	NVNFDGEILK	575.1++	[y_8] 936.0+	**118.3**	**89.5**	121.4	**76.8**	**101.8**	120.9	**103.9**	**104.7**
		TVIVEAPIHGLK	639.3++	[y_6] 664.8+	46.6	**93.0**	122.2	320.8	138.2	**111.9**	134.0	138.1
Hazelnut 5 ppm	Cor a 9	ADIYTEQVGR	576.6++	[y_6] 689.7+	**111.4**	**102.0**	**119.9**	**112.2**	**101.2**	**107.3**	**104.5**	**108.4**
		ALPDDVLANAFQISR	815.9++	[y_{13}] 723.8+	**120.0**	**102.2**	125.3	**117.3**	**100.4**	**105.8**	**93.8**	**109.3**
		LNALEPTNR	514.6++	[y_4] 487.5+	**108.1**	**102.7**	124.4	**114.2**	**109.7**	**110.2**	**108.1**	**111.1**
		TNDNAQISPLAGR	679.2++	[y_6] 600.7+	137.2	**111.4**	125.4	121.0	128.9	**110.2**	**115.6**	121.4
Peanut 50 ppm	Ara h 1	GSEEEDITNPINLR	794.3++	[y_5] 612.7+	179.2	**79.9**	213.8	128.7	**91.0**	164.1	175.4	147.4
		GSEEEGDITNPINLR	822.9++	[y_5] 612.7+	185.9	**83.7**	157.6	172.5	**66.9**	169.8	132.3	138.4
		GTGNLELVAVR	565.2++	[y_5] 557.7+	134.4	**81.1**	158.1	**112.7**	**100.0**	**102.7**	**112.8**	**114.5**

[a]The evaluation was based on recovery, calculated as the ratio of average determined concentration to spiked concentration. The inter-laboratory recovery was also calculated. Values meeting the AOAC requirement criteria (recovery in the 60–120% range) are highlighted in bold.

[b]The transitions correspond to the isotopic average mass.

Table 5. Overview of method precision evaluation, after outliers removal, based on the duplicate analysis of cookie fortified with the four allergens and using a standard addition quantification method[a]

Allergen	Protein	Peptide	Precursor[b]	Product ions (fragments)[b]	RSDr, %							RSDR, %
					Lab 1	Lab 2	Lab 3	Lab 5	Lab 6	Lab 8	Lab 9	
Milk 2.5 ppm	αs1-casein	FFVAPFPEVFGK	693.3++	[y_8] 921.1+	**8.7**	**3.1**	**2.0**	**9.1**	**1.2**	**8.2**	**8.6**	**8.7**
		HQGLPQEVLNENLLR	587.7+++	[y_6] 758.8+	**12.3**	**15.8**	**3.8**	**8.5**	**0.3**	**3.7**	**11.7**	**17.3**
		YLGYLEQLLR	634.7++	[y_8] 992.2+	**9.1**	**5.3**	**7.2**	**3.0**	**6.7**	**6.5**	**6.7**	**9.9**
	β-lactoglobulin	IDALNENK	459.0++	[y_6] 688.8+	**6.7**	**2.8**	**6.9**	**6.4**	**2.5**	**3.7**	**5.3**	**17.9**
		TPEVDDEALEK	623.7++	[y_{10}] 573.1+	**4.1**	**2.8**	**2.2**	**0.7**	**6.4**	**11.4**	**12.6**	**20.7**
		VYVEELKPTPEGDLEILLQK	772.2+++	[y_{18}] 1026.7+	**4.1**	**2.9**	**3.2**	**12.0**	**2.5**	**0.6**	**8.1**	**11.6**
Egg 25 ppm	Ovalbumin	GGLEPINFQTAADQAR	844.9++	[y_{12}] 666.7+	**8.8**	**0.4**	**2.4**	31.9	**9.2**	**11.9**	**2.5**	60.9
		HIATNAVLFFGR	673.8++	[y_{10}] 1096.3+	**5.0**	**0.6**	**0.8**	32.1	**9.4**	**4.5**	**5.2**	61.3
	Ovotransferrin	SAGWNIPIGTLIHR	512.6+++	[y_8] 907.1+	**5.0**	**1.1**	**0.0**	**0.9**	**0.8**	**11.4**	**4.2**	**26.6**
		FYTVISSLK	529.6++	[y_7] 747.9+	21.3	**0.2**	**10.1**	**7.8**	**1.2**	**5.2**	**1.0**	47.1
	Vitellogenin-1	NVNFDGEILK	575.1++	[y_8] 936.0+	**4.8**	**14.0**	**0.7**	20.4	**3.2**	**4.0**	**0.4**	**17.0**
		TVIVEAPIHGLK	639.3++	[y_6] 664.8+	**14.5**	**4.1**	**5.0**	**15.3**	**1.4**	**9.3**	**4.9**	59.6
Hazelnut 5 ppm	Cor a 9	ADIYTEQVGR	576.6++	[y_6] 689.7+	**0.0**	**10.0**	**6.2**	**3.0**	**0.3**	**1.8**	**1.6**	**7.3**
		ALPDDVLANAFQISR	815.9++	[y_{13}] 723.8+	**10.4**	**4.5**	**2.9**	**6.5**	**3.3**	**3.3**	**8.4**	**11.7**
		LNALEPTNR	514.6++	[y_4] 487.5+	**17.8**	**0.5**	**6.7**	**2.3**	**2.3**	**3.9**	**4.5**	**9.5**
		TNDNAQISPLAGR	679.2++	[y_6] 600.7+	22.3	**6.1**	**8.8**	**8.9**	**2.0**	**2.3**	**1.9**	**13.3**
Peanut 50 ppm	Ara h 1	GSEEEDITNPINLR	794.3++	[y_5] 612.7+	49.1	**7.9**	**0.1**	**9.0**	**2.3**	**3.4**	**11.1**	38.8
		GSEEEGDITNPINLR	822.9++	[y_5] 612.7+	35.7	**2.1**	**4.3**	37.5	**0.6**	29.1	**15.7**	42.7
		GTGNLELVAVR	565.2++	[y_5] 557.7+	**17.4**	**7.9**	**0.3**	21.2	**2.1**	**2.2**	**0.9**	**23.0**

[a] The evaluation was based on repeatability defined as within laboratory relative standard deviation (RSDr) and on reproducibility with the relative standard deviation calculated from among-laboratory data (RSDR). Values meeting the AOAC requirement criteria (RSDr < 20% and RSDR < 30%) are highlighted in bold.
[b] The transitions correspond to the isotopic average mass.

met the criteria for Laboratory 5. Recoveries for FFVAPFPEVFGK (milk peptide) and ADIYTEQVGR (hazelnut peptide) were in the 60–120% range for all participating laboratories. Multiple peptides from the allergens were also quantified in this range by five or six laboratories (such as VYVEELKPTPEGDLEILLQK (milk), GGL EPINFQTAADQAR or SAGWNIPIGTLIHR (egg), ALPDDVLANAFQISR or LNALEPTNR (hazelnut), and GTGNLELVAVR (peanut)). The general method performance was evaluated with the interlaboratory average recovery evaluation. Of the 19 targeted peptides, 15 were in the 60–120% range, and egg and peanut stood out as difficult allergens to quantify with high trueness.

More than 90% of the received quantification results complied with the AOAC requirements for method precision ($RSD_r < 20\%$). As for trueness, differences between the participating laboratories were observed. Indeed, out of the 10 RSD_r values that were outside of the AOAC requirements, nine were obtained by Laboratories 1 and 5. A higher variability was observed for egg and peanut, with nine out of the 10 RSD_r values out of the AOAC requirements obtained for these two allergens. As mentioned earlier, these RSD_r values have to be considered with caution, as they were based on only two biological replicates (to limit the workload) prepared by the participating laboratories. Regarding reproducibility, 13 peptides were quantified in agreement with AOAC requirements ($RSD_R < 30\%$). Again, egg and peanut were the two allergens quantified with most variability among the laboratories.

Some aspects related to the application of such a quantitative method in a routine laboratory, out of the scope of this study but mandatory, were covered in a previous paper from our team (9). Before proceeding to the allergen quantification, some acceptance criteria have to be fulfilled by a given unknown sample to be declared as positive. The main question, much debated among laboratories, is whether a sample should be considered positive when a single allergen peptide is detected or whether at least two peptides should be detected. To answer this question, different parameters have to be considered, such as the S/N, the tolerated retention time deviations, and the relative ion intensity.

Despite the difficulties encountered by some participating laboratories, especially for the quantification of egg and peanut, at least one peptide per allergen was quantified with an average recovery and reproducibility in agreement with the AOAC requirement. These results again demonstrate the potential of this MS-based method and the proposed quantification strategy based on standard addition.

Conclusions

Allergen analysis is essential for the control and development of a risk-based approach for allergen management. As described in a recent review on food allergen detection methods (39), most of the current analytical issues are related to a lack of standardization, including appropriate reference materials, expression units for results, and calculation of method performance characteristics. With this interlaboratory study, we aimed to contribute to overcome these current limitations. This study is the final step of a multi-year project aimed at developing and validating a MS-based method for the detection and quantification of four allergens in processed food matrixes. It also represents an additional step towards the harmonization of food allergen analysis.

A complete methodology, including the sample preparation protocol, a quantification strategy based on standard addition and a SIL concatemer internal standard, and UHPLC–MS/MS method parameters, was provided to the study participants. Encouraging results were obtained in terms of method sensitivity and accuracy, even when some participants were totally inexperienced with food allergen analysis. As already observed during method validation, the results of our interlaboratory study highlight and confirm the difficulties encountered in food

Table 6. Summary of the results of the interlaboratory study and evaluation of the compliance rate of the participating laboratories (after outlier removal for accuracy results) with AOAC requirements

	Method LOQ		Recovery		Repeatability (RSD_r)	
	AOAC SPMR 2016.002, ppm	Compliance rate	AOAC SPMR 2016.002	Compliance rate	AOAC SPMR 2016.002	Compliance rate
Milk	≤10	9/9 Labs	60–120% range	7/7 Labs	RSD_r <20%	7/7 Labs
Egg	≤5	5/9 Labs	60–120% range	7/7 Labs	RSD_r <20%	7/7 Labs
Hazelnut	≤10	9/9 Labs	60–120% range	7/7 Labs	RSD_r <20%	7/7 Labs
Peanut	≤10	9/9 Labs	60–120% range	5/7 Labs	RSD_r <20%	7/7 Labs

allergen analysis, such as the required low LOD and the effects of food processing on analytical performance. Practical challenges associated with food allergen analysis in a routine analytical lab were also highlighted. Great care needs to be taken at any time to avoid contamination between samples, where traces of a given allergen could be detected after having analyzed a sample where the same allergen was the main ingredient. Notwithstanding these challenges, some laboratories were able to detect and quantify the different allergens with sensitivity and accuracy in agreement with AOAC requirements. Moreover, on average, at least one peptide per allergen was quantified with an accuracy that fulfilled these requirements. All the results of our interlaboratory study are summarized in Table 6, along with the compliance rate of the participating laboratories with AOAC SMPR.

The results obtained in this interlaboratory study demonstrate the potential of MS and of our method and quantification strategy for food allergen analysis. Effective methods are essential for allergen management and guaranteeing food safety for allergic consumers.

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Conflict of Interest

All authors declare no conflict of interest.

Supplemental Information

Supplemental information is available on the *J. AOAC Int.* website.

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