

Liquid Chromatography Mass Spectrometry

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Method Validation of Milk and Egg Allergens in Bakery Products by LC-MS/MS Using QSight Technology

labels whenever intentionally incorporated into foods³. Other's country regulations include the Food Allergen Labeling and Consumer Protection Act (FALCPA) of 2004⁴ in the United States, which sets a number of eight major allergenic foods (Big-8), Canada and Australia regulate 12, China 11 and Japan 7 allergenic foods⁵.

Beyond the voluntary addition of allergenic ingredients into foods to improve their characteristics, risk of cross-contamination is likely to exist, especially along the food chains where different foods are processed and strict risk assessment plans are not put in place by the food industry⁶. Current legislation does not address the accidental contamination of allergens due to cross-contamination. In order to protect consumers' health, different countries have set their own action levels for several allergens. On this regard, the Allergen Bureau of Australia proposed the VITAL (Voluntary Incidental Trace Allergen Labelling) program⁷ to help food industry in the allergen risk assessment process establishing reference doses for egg, milk, tree nuts, peanut, and soybean.

Introduction

Food allergies are an increasing health problem due to their growing prevalence worldwide¹. European Commission (EC) Regulation No. 1169/2011² established a list of 14 allergenic ingredients which need to be indicated in the respective food

To protect the health of allergic consumers from the inadvertent or intentional contamination by allergens, different analytical methods for the sensitive detection of allergens in food were developed over the years. These can be divided into two main classes, those that target characteristic proteins of the allergenic ingredient or those that use a marker DNA sequence that indicates the presence of the food that causes allergy. Among the first class of methods, the most commonly used are enzyme-linked immunosorbent assays (ELISA). These tests use specific antibodies for the detection of species-specific allergen proteins. Although these tests can be extremely sensitive and specific, they are highly dependent on the epitopes recognized by the antibodies, which can be influenced by cross-reactivity with other species. Moreover, some components of the food matrix can interfere with the absorption measurements done in the ELISA tests. The second class of methods are based on amplification and detection of specific DNA sequence encoding for the allergenic protein or species-specific DNA marker by PCR. Although DNA is a highly stable molecule, thermal treatment of in food such as baking cooking or roasting can reduce the DNA extraction yield and compromise DNA integrity. In addition, highly processed foods such as vegetable oils, gelatin, lecithin or starch contain little or no DNA and are a challenge for PCR testing. More recently, mass spectrometry (MS) methods have gained the primacy of being considered reliable and multi-target approaches for allergen identification. Indeed, MS enables identification of the contaminating allergens with the highest confidence through detection of specific and prototype signature peptides. Several methods have been developed over the last ten years, exploiting liquid chromatography (LC) coupled to different mass analyzers employing both high-resolution systems for marker peptide discovery and triple quadrupole mass spectrometers for quantification⁸. Harmonization and validation of a MS-based method capable of detecting several allergens in one run is among the primary objectives of recent European projects⁹.

Working towards this aim, in this work we present the performance of a targeted method using the QSight® 220 liquid chromatography triple quadrupole mass spectrometer (LC-MS/MS) for the simultaneous quantification of milk and egg allergens in thermally processed bakery products (cookie as representative

product). In-house validation results will be presented, following AOAC method performance requirements for the detection and quantification of food allergens, as well compliancy with currently proposed VITAL thresholds.

Experimental

Standard and Reagents

Method development was based on the use of native synthetic peptides. Since both milk and egg are widely investigated allergens and as such, a good consensus about the most reliable peptide markers has been reviewed and identified⁹. The selected custom marker peptide sequences (see Table 1) were synthesized by GenScript (Piscataway, NJ, USA) and distributed by Twin Helix (Milan, Italy). The provided lyophilized powder of each peptide was reconstituted with 100 mM Bicarbonate Ammonium:Acetonitrile 80:20 (v/v) to reach the concentration of 1 mg/mL.

For method performance evaluation, cookie reference materials (RM) for milk allergen detection was purchased from Monitoring and Quality Assurance in the Total Food Supply Chain (MoniQA)¹⁰ association. The kit contains the following four samples: (i) a negative control gluten-free cookie, (ii) a positive control consisting in characterized dried skim milk powder (SMP) with validated protein content, as well as two incurred materials (gluten free cookies) added with SMP at (iii) low (3.53 mg/kg) and (iv) high (17.7 mg/kg) concentration levels.

Trypsin Gold (MS grade) was purchased from Promega, disposable desalting cartridges PD-10 were purchased from GE Healthcare Life Sciences, while C18 cartridges (50 mg, 1 mL) were used for sample clean-up. Skim milk powder and whole egg powder were purchased by Sigma Aldrich.

Sample Preparation

Allergen-free (allergenic ingredients replaced) and incurred (with skimmed milk powder and whole egg powder) cookies were prepared according to an in-house protocol¹¹. RM were incorporated accordingly as ingredients in the baking process. Commercial cookies labeled by the manufacturer as "prepared without adding milk and eggs" were provided by Galbusera SpA and analyzed.

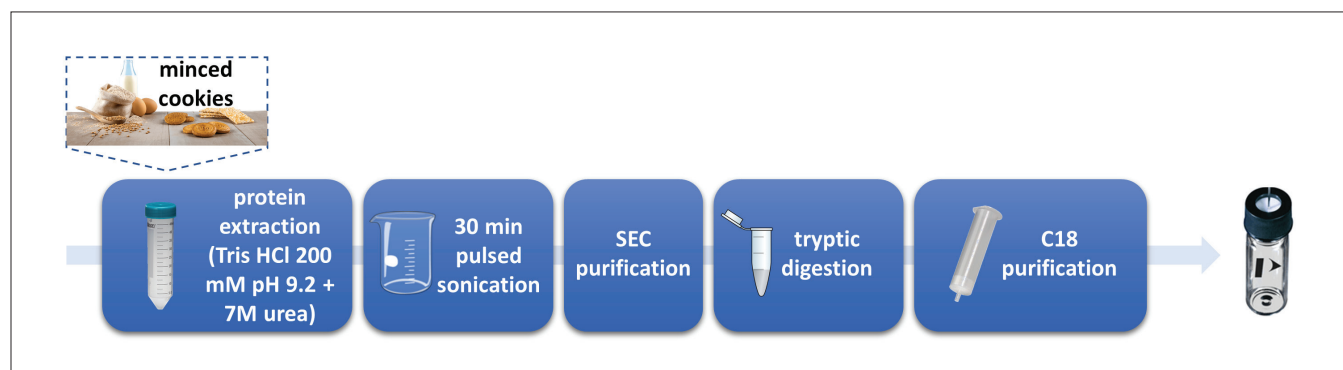


Figure 1. Experimental workflow for the simultaneous detection of milk and egg allergens in cookie samples (adapted from Monaci et al.)¹².

An overview of the sample preparation workflow is illustrated in Figure 1. In short, proteins from minced cookie samples (allergen free, incurred and commercial) were extracted with Tris-HCl buffer 200 mM with Urea 7 M at pH = 9.2 and the resulting extract was filtered through 5 µm acetate cellulose membrane. Extracted proteins were subjected to tryptic digestion for 14 hours, digestion was stopped by acidification (HCl 6 M) and the final digest was centrifuged at 1800 x g for 10 min before collecting the supernatant. Tryptic digest was then filtered through a 0.45 µm regenerated cellulose (RC) filter and 1 mL aliquot loaded on a C18 SPE column (previously conditioned with methanol and 50 mM ammonium bicarbonate) for a further purification step. C18-retained peptides were washed with 800 µl of 0.1% formic acid aqueous solution and eluted with 1.5 mL of methanol/water (90/10 v/v). The collected fraction was dried under gentle stream of nitrogen and suspended in 100 µl of 0.1% formic acid in acetonitrile/water (90/10, v/v) solution. Samples were finally filtered through a RC 0.45 µm syringe filter.

Hardware/Software

The chromatographic separation was conducted by a PerkinElmer LX50 ultra high performance liquid chromatography (UHPLC) system and detection was achieved using a PerkinElmer QSight 220 LC/MS/MS, equipped with ESI and APCI ionization sources. All instrument control, data acquisition and data processing were performed using the Simplicity™ 3Q software.

Methods Performance Evaluation

A matrix matched calibration curve was prepared over the concentration range of 0.0125 - 0.25 µg/ml (four concentration levels, measured in duplicate) by spiking a defined amount of synthetic peptide stock solutions to tryptic digest of allergen-free cookie extract. Limit of detection (LOD) and limit of quantifications (LOQ) were calculated according to the interpolation parameters as 3-times and 10-times, respectively, the standard deviation of the line intercept of the calibration curve divided by its slope.

One of the most controversial subjects in food allergens detection has been the appropriate scale to measure contamination level, ranging from allergen labelling as whole ingredient, to clinical

studies and potential threshold levels referring to total protein content of the allergenic ingredient. In this study the following conversion factors were employed, based on prior research.

Starting from synthetic peptide concentrations in $\mu\text{g}_{\text{peptide}}/\text{mL}_{\text{extract}}$ and using appropriate conversion factors (see Figure 2) stepwise were ultimately converted into $\mu\text{g}_{\text{total protein}}/\text{g}_{\text{matrix}}$. All method analytical features i.e. sensitivity, repeatability/reproducibility, recovery and processing effects, were evaluated according to the final reporting unit.

Method precision was calculated based on five replicates of a $100 \mu\text{g}_{\text{allergenic ingredient}}/\text{g}_{\text{matrix}}$ contamination level sample for intra-day repeatability and repeated over three days to evaluate inter-day reproducibility. Method recovery was evaluated only for milk by means of the validated RMs. The blank sample provided in the kit was used to create a new matrix-matched calibration curve with synthetic peptides. The low and high incurred samples were analyzed and the percent ratio between the measured and the validated concentration values defined as the method recovery.

Final method performance was evaluated according to the AOAC Standard Method Performance Requirements (SMPR) for Detection and Quantitation of Selected Food Allergens¹³.

Processing Effects

In order to estimate the food processing effects of the selected marker peptides, both spiked and incurred materials were prepared. Spiked material is produced from the addition of allergenic ingredients to processed blank food matrix, whereas incurred materials derives from their inclusion in blank raw materials before food processing¹⁴. In both cases a final concentration of $300 \mu\text{g}_{\text{allergenic ingredient}}/\text{g}_{\text{matrix}}$ was aimed at. The incurred cookie was obtained by cookie matrix enriched with $3000 \mu\text{g}/\text{g}_{\text{allergenic ingredient}}$ which was diluted 1:10 with an appropriate amount of allergen-free cookie matrix before dough-making and baking. The spiked cookies, on the other hand, were produced by adding the skim milk powder and the whole egg powder directly on the blank cookie mince after the baking and grounding step.

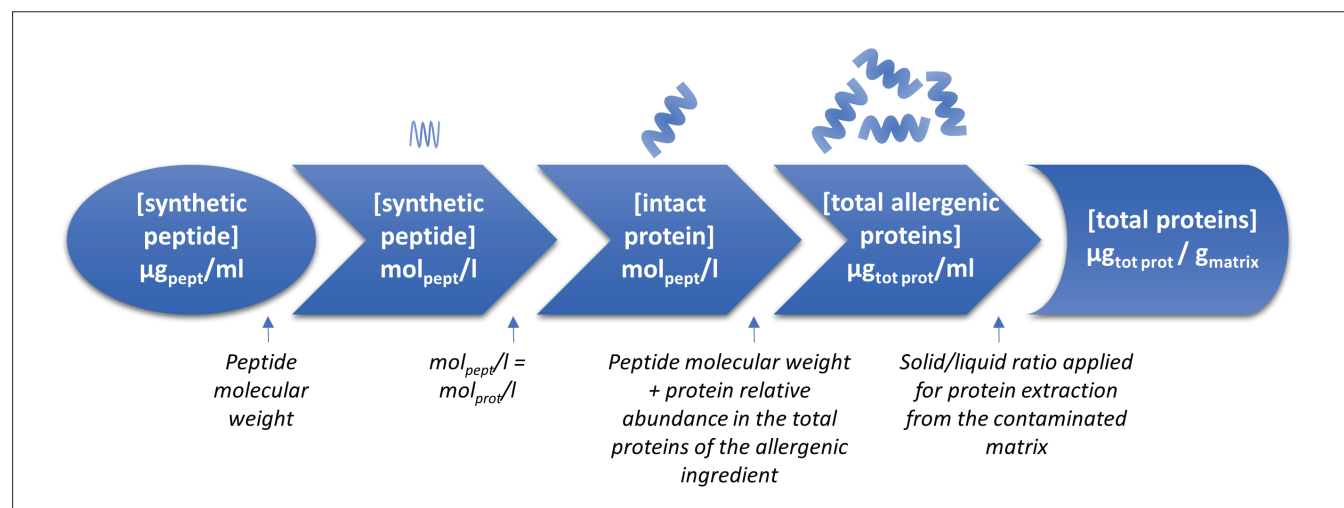


Figure 2. Flowchart calculation for the conversion of egg and milk synthetic peptides concentration ($\mu\text{g}_{\text{peptide}}/\text{ml}_{\text{extract}}$) into total protein concentration ($\mu\text{g}_{\text{tot prot}}/\text{g}_{\text{matrix}}$) (adapted from Monaci et al.)¹².

VITAL Reference Dose

The VITAL system, developed in 2007, has the aim to assess the impact of allergen cross-contamination in a quantitative risk-based methodological approach and to make decisions regarding proper precautionary allergen management and labeling. This is based on input from industry, consumers and global scientific risk assessment, all with the aim to protect allergic consumers. The likelihood to develop an adverse reaction in allergic people depends on the total amount of allergenic proteins consumed during a meal, and on the level of sensitization of each individual person. VITAL system relies on three key values which calculations are based on: (i) "reference amount" that represents the portion size, namely maximum amount of a food eaten in a typical eating occasion, (ii) the "reference dose" which refers to the total protein in milligrams from an allergenic food below which only the most sensitive individuals (between 1 and 5) in the allergic population are likely to experience an adverse reaction and (iii) the "action

levels" that are threshold levels of protein concentrations in food guiding the labelling (action level 1: no precautionary labelling required, action level 2: "may contain" labelling required and action level 3: "contain" labelling required).

LC Conditions and MS Parameter Settings

10 μ L of the final extract solution was injected directly onto a PerkinElmer Brownlee™ Validated Aqueous C18 reverse phase analytical column. The employed LC conditions are shown in Table 2, while the MS source settings are listed in Table 3. Source parameters including gas flows, temperatures and position settings, were optimized for maximum sensitivity using the prepared synthetic peptide solutions and flow injection analysis (FIA). In addition, compound-dependent parameters (CE – collision energy, EV – entrance voltage and CCL2 – collision cell lens 2 voltage) for multiple reaction monitoring (MRM) transitions were optimized in FIA for all peptides and are specified in Table 1.

Table 1. Optimized MRM and compound-dependent parameters for all peptides, ¹milk and ²egg marker peptides, underlined part is used as common abbreviation, quantifier transitions marked in bold.

Protein	Marker Peptide Sequence	Precursor [m/z]	Retention Time [Min]	Product [m/z] (Fragment)	CE	EV	CCL2
α S1-Casein ¹	<u>FFV</u> APFPEVFGK	692.9 (+2)	13.1 \pm 0.02	920.3 (<u>y</u> ₈₊)	-26	15	-168
				991.4 (<u>y</u> ₉₊)	-26	17	-168
				1090.4 (<u>y</u> ₁₀₊)	-26	25	-172
	<u>YLG</u> YLEQLLR	634.6 (+2)	11.98 \pm 0.01	658.2 (<u>y</u> ₅₊)	-28	31	-124
				771.3 (<u>y</u> ₆₊)	-28	23	-152
				991.4 (<u>y</u> ₈₊)	-27	24	-140
β -Lactoglobulin ¹	<u>TPE</u> VDDEALEK	623.3 (+2)	5.8 \pm 0.01	572.5 (<u>y</u> ₁₀ ²⁺)	-30	34	-164
				819.1 (<u>y</u> ₇₊)	-29	41	-196
				1047.0 (<u>y</u> ₉₊)	-30	45	-170
	<u>VLV</u> LDTDYK	533.2 (+2)	8.3 \pm 0.01	640.8 (<u>y</u> ₅₊)	-24	24	-108
				753.9 (<u>y</u> ₉₊)	-22	18	-100
				853.0 (<u>y</u> ₇₊)	-22	22	-100
Ovalbumin ²	<u>GG</u> LEPINFQTAADQAR	844.7 (+2)	8.9 \pm 0.01	1007.4 (<u>y</u> ₉₊)	-51	50	-272
				1121.2 (<u>y</u> ₁₂₊)	-40	38	-380
				1331.6 (<u>y</u> ₁₀₊)	-35	29	-336
	<u>ISQ</u> AVHAAHAEINEAGR	592.1 (+3)	4.3 \pm 0.01	545.9 (<u>y</u> ₅₊)	-40	20	-200
				778.5 (<u>y</u> ₇₊)	-31	42	-128
				858.9 (<u>y</u> ₈₊)	-29	30	-204
Vitellogenin-2 ²	<u>NIP</u> FAEYPTYK	671.6 (+2)	9.1 \pm 0.01	507.9 (<u>y</u> ₄₊)	-39	33	-180
				557.9 (<u>y</u> ₉ ²⁺)	-25	25	-140
				1114.9 (<u>y</u> ₉₊)	-23	25	-132
	<u>NIG</u> ELGVEK	479.7 (+2)	6.5 \pm 0.01	228.0 (<u>b</u> ₂₊)	-18	24	-88
				544.8 (<u>y</u> ₉₊)	-24	27	-124
				673.9 (<u>y</u> ₉₊)	-21	27	-120

Table 2. LX50 UHPLC Parameters.

Column	Brownlee Validated Aqueous C18, 150 X 2.1 mm, 3 μ m (Part Number: N9303539)				
Mobile Phase	Solvent A: 0.1% formic acid in H ₂ O Solvent B: 0.1% formic acid in methanol				
Gradient	Step	Time [min]	Flow Rate [ml/min]	%A	%B
	1	0	0.2	90	10
	2	17	0.2	50	50
	3	17.2	0.2	10	90
	4	27	0.2	10	90
	5	27.2	0.2	90	10
	6	44	0.2	90	10
Injection Volume	10 μ L				
Temperature	Oven 30 $^{\circ}$ C Autosampler 10 $^{\circ}$ C				

Table 3. QSight ESI Source Parameters.

Ionization Mode	ESI in positive ionization mode
Drying Gas	120
HSID Temperature	250 $^{\circ}$ C
Nebulizer Gas	300
Spray Voltage	+4000 V
Source Temperature	400 $^{\circ}$ C

Results

A sensitive method based on LC separation and MS/MS analysis for the simultaneous detection of milk and egg allergens in cookie matrix was developed. The chromatographic conditions for peptide separation were optimized and the best compromise between total running time and peak resolution was investigated. Synthetic peptides were used for method optimization and for each selected marker peptide, the three most intense MRM transitions selected during tuning and FIA.

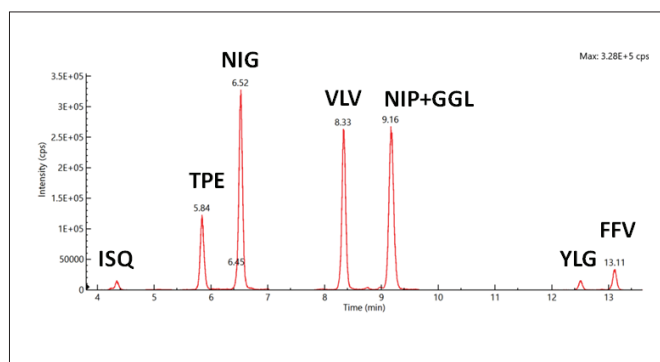


Figure 3. Representative total ion chromatogram (TIC) recorded for synthetic peptides in cookie matrix with a peptide concentration level of 0.166 μ g/mL.

Method Optimization

In order to confirm the absence of interfering peaks from the matrix background, a blank cookie sample was prepared according to the sample preparation protocol and spiked with synthetic peptides at fixed concentration. In Figure 3 a representative chromatogram acquired under the best separation conditions is presented and averaged peak retention times are reported.

Method Performance Parameters

Linearity, LOD and LOQ

Matrix-matched calibration curves in the range of 0.125-0.25 μ g/mL showed excellent linearity with $R_2 > 0.999$. From these calibration curves, LOD and LOQ were calculated according to the interpolation parameters as 3-times and 10-times, respectively, the standard deviation of the line intercept divided by the slope. The careful evaluation of LOD/LOQ values for the detected transitions allowed to identify the best quantifier marker and its most sensitive transition as reported in Table 4. Based on the conversion factors, values from the matrix-matched calibration curve values in μ g_{peptide}/mL_{extract} were recalculated to μ g_{total protein}/g_{matrix} and all further performance parameters referred to them.

Very challenging LODs were achieved for milk allergens, as low as 0.1 and 3 μ g/g, respectively referred to FFV (α S1-casein) and TPE (β -lactoglobulin) peptides. As for egg, LODs of 0.3 and 3 μ g/g were found for ISQ (ovalbumin) and NIP (vitellogenin - 2) peptides, respectively. Representative chromatograms of quantifier / qualifier of the four identified marker peptides are illustrated in Figure 4 at the lowest calibration point of 0.125 μ g/mL.

Method Validation According to VITAL Thresholds

Good sensitivity is demanded to new analytical methods complying or better overcoming the action levels prescribed by the VITAL program. Such levels are periodically updated according to new allergological data available from clinical studies, and last values from the version 3.0 of VITAL program were revised and released in October 2019. As for milk and egg, equal reference doses of 0.2 mg total protein was set, with an estimated portion size of 50 g for cookies, resulting in an action level one of 4 mg_{total protein}/kg. Noteworthy, the sensitivity achieved by this method not only complies with the prescribed values but is able to detect even tiny amounts of allergens, being approximately 40- and 13-fold lower than the action levels one foreseen for milk and egg proteins, respectively.

Precision

Intra-day and inter-day precision of the analytical method were evaluated to test the method repeatability and reproducibility within the same laboratory, with detailed values found in Table 4. The intra-day repeatability (RSD_r) was calculated within five independent replicates and values always lower than 10% were obtained in all cases. On the contrary, inter-day repeatability (RSD_R) was calculated over three days by analyzing the same fortified samples. Obtained values were always lower than 9% for both for milk and egg quantifier peptides. The mean values obtained on different days were compared by a one-way ANOVA test at 95% confidence level, resulting in no significant difference for all peptide markers.

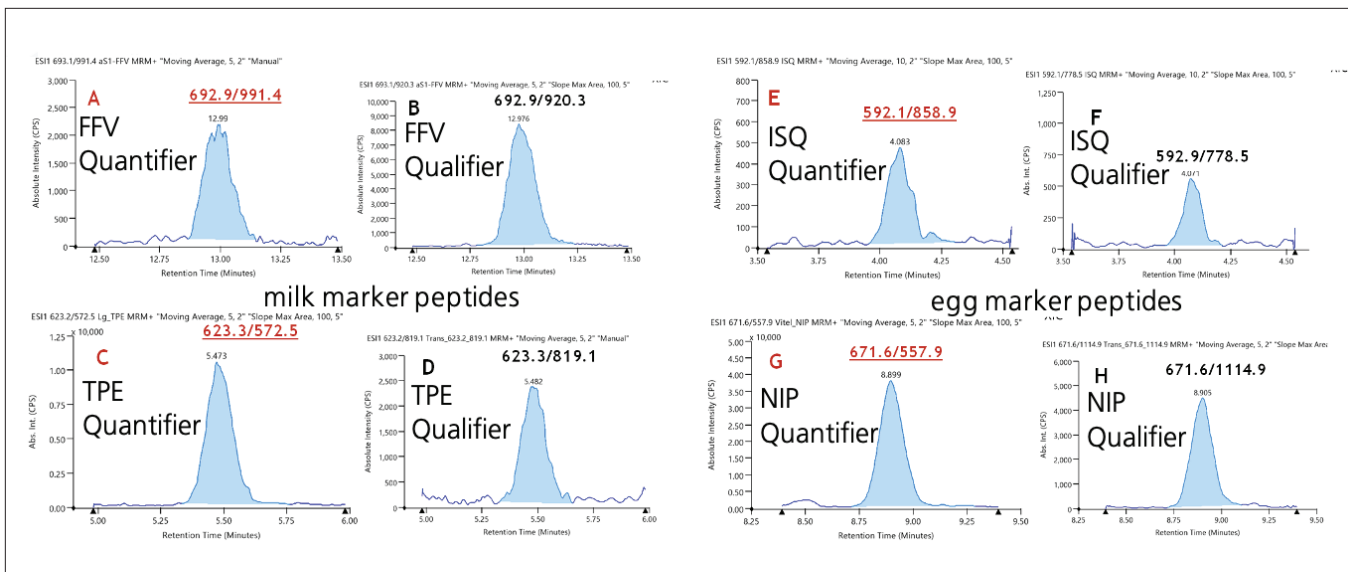


Figure 4. Typical chromatograms acquired for synthetic peptides in cookie matrix: XIC of quantifier transitions for most sensitive marker peptides of milk (A - FFV ; C - TPE) along with their relevant qualifier transition (B - FFV; D - TPE) and egg, quantifier transitions (E - ISQ; G - NIP); qualifier transitions (F - ISQ; H - NIP), all at a level of 0.0125 µg/mL.

Table 4. Method performance parameters.

Allergenic Ingredient: Protein	Marker	Transitions	LOD/LOQ (µg _{TOT PROT} /g _{MATRIX})	R ₂	RSDr% n=5 Day 1	RSDr% n=5 Day 2	RSDr% n=5 Day 3	RSDr%
Milk: αS1-Casein	FFV	692.9/991.4	0.1 / 0.3	1.0000	2	1	6	4
Milk: β-Lactoglobulin	TPE	623.3/572.5	3 / 8	0.9992	8	9	10	9
Egg: Ovalbumin	ISQ	592.1/858.9	0.3 / 1	1.0000	2	5	2	4
Egg: Vitellogenin-2	NIP	671.8/557.9	3 / 9	1.0000	3	3	4	6

Evaluation of Processing Effect

In order to evaluate possible food processing effects on the detection of each milk and egg peptide marker, two type of samples were compared, (i) incurred, where allergenic ingredients are added before dough-making and baking and (ii) spiked, where powdered allergenic ingredients are added to blank cookies after baking and grinding. Results shown in Figure 5 clearly indicate that food processing affects the detection of the selected markers for all the allergenic ingredients under consideration. A calculation of the % processing effect shows that milk peptides appear to be the most susceptible to baking, showing a reduction in peak area higher than 50%. On the contrary, egg peptides proved to be more resistant with reduction in peak area ranging from 17 to 26%.

Trueness

Trueness evaluation was limited to milk allergen since no reference materials for baked food is available yet for egg allergen. The low (3.54 µg/g) and high (17.7 µg/g) incurred samples were both analyzed in triplicate subjected to the whole sample preparation protocol along with reference allergen-free cookie sample. The percentage ratio between the experimental and theoretical values provided an estimate of the method recovery for milk allergen. Method recovery calculated with YLG peptide was 57 ± 6%, and 56 ± 7% at low and high concentration levels respectively, whereas the recovery calculated with the peptide FFV was 57 ± 4% and 50 ± 3% at low and high concentration levels, respectively.

Compliance with AOAC SMPR 2016.002

Finally, the method performance was assessed following the minimum performance characteristics required by with the AOAC guideline (SMPR 2016.002) for the detection and quantification of allergens by mass spectrometry. Table 5 gives an overview of the obtained results for each of the parameters. For comparability purpose, LOD and LOQ referred to the quantitative milk and egg marker peptides were re-converted into µg_{allergenic ingredient}/g_{matrix}. The developed method met all the prescribed requirements and very challenging LOD and LOQ were achieved for both milk and egg allergen in cookie matrix.

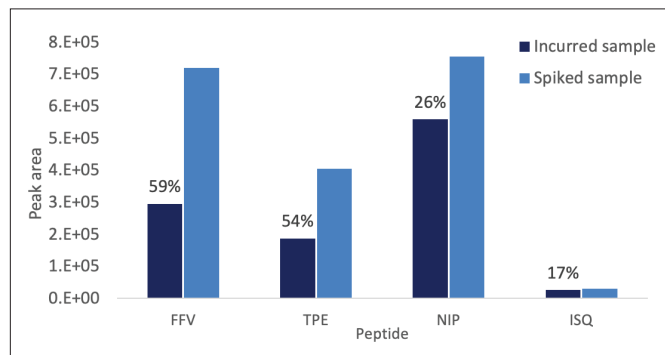


Figure 5. Impact of baking on the detectability of proteotypic peptides of milk and egg allergens calculated by comparing the peak area detected for marker peptides in incurred and spiked cookie contaminated at the level of 300 µg_{allergenic ingredient}/g_{matrix}. For each peptide/transition the percentage of signal suppression due to baking is reported.

Table 5. Comparison between method performance and the minimum requirements set by AOAC SMP 2016.002 for allergens detection in food. *Reported as ppm of the target allergen in food commodity, i.e. 25 ppm of “whole egg” in cookies.

Parameter	AOAC Method Performance Requirements		This Method	
	Whole Egg	Milk	Whole Egg	Skim Milk
Analytical Range (ppm)*	10-1000	10-1000	4-90	4-80
MQL (ppm)*	≤5	≤10	2	0.9
MDL (ppm)*	≤1.65	≤3	0.7	0.3
Recovery, %	60-120	60-120	N.A.	57±4
RSD _r , %	≤20	≤20	≤ 5	≤ 6
RSD _R , %	≤30	≤30	≤ 4	≤ 4

Sample Measurement

The validated method was applied to samples taken from different lots of commercial cookies and labelled as “prepared without adding of milk and eggs” in order to assess the actual absence of any trace of milk and egg allergens, according to the sensitivity of the method. No quantifiable peaks areas were detected for milk and egg quantifier peptides, which indicates that no accidental contamination occurred on these samples, within the sensitivity limits reported by the developed method.

Conclusion

In this work, we report the optimization of a LC-MS/MS method that exploits the features of the PerkinElmer QSight 220 for milk and egg allergens detection in cookie, chosen as a representative complex food matrix. Very challenging LOD and LOQ values were obtained for both egg and milk allergens that allowed detecting contamination levels lower than the reference threshold (action level 1) set for egg and milk by the VITAL program v 3.0. The in-house validation performed provided analytical features that complied with the minimum requirements set in the AOAC SMPR 2016.002 for allergen detection in food.

Method and Instrument Benefits:

- Very challenging LOD and LOQ values are obtained for both egg and milk allergens compared to current reference thresholds
- The method performance meet requirements as set by AOAC SMPR 2016.002
- QSight 220 LC/MS/MS meets requirements of sensitivity and selectivity for allergens detection and quantification

Further details of this study can be found in the published peer-reviewed paper¹².

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