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Short communication

Streamlining the analytical workflow for multiplex MS/MS allergen detection in processed foods

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ABSTRACT

Allergenic ingredients in pre-packaged foods are regulated by EU legislation mandating their inclusion on labels. In order to protect allergic consumers, sensitive analytical methods are required for detect allergen traces in different food products. As a follow-up to our previous investigations, an optimized, sensitive, label-free LC-MS/MS method for multiplex detection of five allergenic ingredients in a processed food matrix is proposed. A cookie base was chosen as a complex food matrix and home-made cookies incurred with whole egg, skimmed milk, soy flour, ground hazelnut and ground peanut were prepared at laboratory scale. In order to improve the analytical workflow both protein extraction and purification protocols were optimized and finally a sensitive streamlined SRM based analytical method for allergens detection in incurred cookies was devised. The effect of baking on the detection of selected markers was also investigated.

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1. Introduction

Contamination of foods by allergens is a matter of concern, especially for allergic consumers (Monaci, Pilolli, De Angelis, & Mamone, 2015), and specific legislation has been issued in different countries to regulate the proper labelling of foods.

In the European Union, the presence of a total of 14 allergenic ingredients must be indicated in the list of allergenic ingredients in food labels. However, only eight out of these 14 (milk, egg, fish, soy, crustacean shellfish, tree nuts, peanuts, wheat) have to be reported in the US, as for the Food Allergen Labelling and Consumer Protection Act (FALCPA, US FDA, 2004).

Adherence to labelling requirements is independent of the amount of allergens present in the food, with the only exception being gluten, for which limits have been established (EC Reg 41/2009/EC).

However, there is still the risk of accidental contamination due to “hidden” and unexpected allergens, which represents the main cause triggering allergic reactions in sensitive individuals.

To protect the health of allergic consumers effectively, both the food industry and regulatory bodies require reliable analytical methods, which target allergenic proteins or appropriate markers to monitor potential contamination throughout the food chain. In the last few years (Johnson et al., 2011; Monaci & Visconti, 2009; Monaci et al., 2015; Picariello, Mamone, Addeo, & Ferranti, 2011) mass spectrometric (MS) methods have received increasing attention in the identification of allergens in complex foods, due to a number of advantages over antibody-based methods such as enzyme linked immunosorbent assay, especially when applied to complex or processed food matrices (Török et al., 2015). In fact, the composition and processing of the matrix can mask or alter allergen epitopes, i.e., specific targets of the immunoassay, thereby impairing the detection and quantification of food allergens (Monaci & Visconti, 2010). By contrast, MS methods offer the advantages of multiplex allergen detection with high

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molecular-level specificity in one run, enabling unequivocal identification of the allergen or a marker in a variety of foods.

Several MS-based methods have been developed for multi-allergen screening in foods, by the detection of their representative peptide markers (Bignardi, Elviri, Penna, Careri, & Mangia, 2010; Cryar et al., 2013; Heick, Fischer, & Pöpping, 2011; Lutter, Parisod, & Weymuth, 2011; Monaci, Losito, De Angelis, Pilolli, & Visconti, 2013; Monaci, Losito, Palmisano, Godula, & Visconti, 2011; Monaci, Losito, Palmisano, & Visconti, 2011; Pilolli, De Angelis, Godula, Visconti, & Monaci, 2014; Pöpping & Godefroy, 2011; Weber, Raymond, Ben-Rejeb, & Lau, 2006); however, only a few of the several classes of food allergens in different food commodities have been studied.

Although good sensitivities have been achieved, a drawback of the MS methodology is the long analysis times, ranging between four and six hours, excluding the time for proteolytic digestion.

Recently, we contributed to this research field with the development of an analytical method based on micro HPLC–ESI–MS/MS for the simultaneous detection of soy, egg and milk allergens in artificially contaminated allergen-free cookie matrices (Monaci, Pilolli, De Angelis, Godula, & Visconti, 2014) but did not include incurred cookies. Food processing can affect allergen detection by inducing chemical modifications as well as changes in the protein structure and solubility, which impact the extraction efficiency (Downs & Taylor, 2010; Gomaa & Boye, 2013; Monaci, Brohé, Tregoe, & Van Hengel, 2011; Platteau et al., 2011; Poms & Anklam, 2004; Schmitt, Nesbit, Hurlburt, Cheng, & Maleki, 2010). Therefore, we aimed to ameliorate the analytical workflow by streamlining the sample pre-treatment protocol and then assess the sensitivity using home-made incurred cookies, the recipe for which was as close to real foods as possible. With this aim, we describe herein the optimization of a selective reaction monitoring method for the detection and label-free quantification of egg, milk, soy, hazelnut and peanut, all of which might typically contaminate a commodity. To speed up sample handling, a two-step protocol was proposed, based on ultrasound-assisted solvent extraction followed by rapid size-exclusion chromatography. After tryptic digestion, the peptide pool was subjected to an automated chromatographic separation, with a pre-enrichment/purification step realized using a 1 cm long C18 trap column mounted upstream the analytical column. The primary advantages of this on-line coupling of solid phase extraction (SPE) with RP-HPLC rely on the potential to automate the process, thereby shortening the analysis time (Barreiro, Luiz, Maciel, Maciel, & Lanças, 2015). Different instrumental features of a dual pressure linear ion trap MS instrument were exploited to create a rapid and sensitive multi-allergen SRM-based method for the identification of target peptide markers. Finally, this study focused on the evaluation of baking effects on the detection of peptide marker traces of each allergenic food under investigation.

2. Materials and methods

2.1. Preparation of incurred cookie samples

Cookies incurred with egg, milk, soy, hazelnut and peanut were prepared according to the following recipe: 418 g of wheat flour, 180 g of sugar, 1 g of salt, 2 g of sodium bicarbonate, 90 g of extra virgin olive oil, 160 g of water and 6.4 g of each allergenic ingredient (skimmed milk powder, egg powder, pre-cooked soy flour, ground roasted peanut and ground roasted hazelnut). Allergenic ingredients were first homogenized and then added to the dry mixture (wheat flour, sugar, salt and sodium bicarbonate) at a final concentration of 10,000 µg/g. Subsequently, olive oil and water were added and the final total weight was 900 g. Virgin olive oil

was used to replace any milk-derived product in order to avoid contamination by milk traces in the blank. The incurred dough was left to mix for 40 min before further portioning into 10 g aliquots, which were spread in baking trays of approximately 7 cm in diameter and 1 cm in height and baked at 200 °C for 12 min. After cooling down, the cookies were weighed in order to re-scale the actual allergen concentration to the final baked matrix. The highest incurred cookies corresponded to 8756 µg/g (µg of allergenic ingredients per g of matrix). To make blank cookie samples, the amount of allergenic foods was replaced by wheat flour (a total of 32 g).

Both blank and incurred cookies were finely milled in a blender at 8000 g (Steril mixer 12 model 6805–50, PBI International) by iterations of four cycles of blending (30 s) and rest (10 s) in order to prevent overheating of the material. Ground blank and incurred cookies were passed through a 1 mm sieve, spread on a large tray (50 cm x 50 cm) and manually mixed for homogeneity. A total of 5 subsamples (10 g each) were taken from each stock powder produced (blank and incurred) and subsequently combined to form a single representative sample (approximately 50 g each). Two serial dry dilutions of the incurred sample were prepared by mixing the ground blank and incurred samples in the ratio 1:3, to reach the final concentration of 973 µg/g. These samples represented the reference concentration for all further experiments.

2.2. Preparation of spiked cookie samples

Blank ground cookies were dry-spiked with an appropriate amount of each allergenic ingredient (skimmed milk powder, egg powder, pre-cooked soy flour, ground roasted peanut and ground roasted hazelnut) in order to obtain a final concentration of 973 µg/g each. This was considered the “spiked” reference sample.

2.3. Calibration curves

Calibration curves of both incurred and spiked samples were prepared by serial dilutions of the reference cookie extracts of concentration 973 µg/g with appropriate volumes of the blank cookie extract. A total of five concentration levels were obtained in the range of 200–10 µg/g, with two analytical replicates for each level. The processing effect on the final detection was assessed by comparing the calibration slopes obtained for the incurred and spiked cookie curves and was defined as the loss of sensitivity expressed in percentage per individual allergen (Processing effect % = $(1 - \text{slope}_{\text{incurred}} / \text{slope}_{\text{spiked}}) \times 100$).

2.4. Sample purification protocol

2.5. g of ground cookies (blank or incurred at a concentration of 973 µg/g) were combined with 50 mL of Tris–HCl buffer, 20 mmol/Lat pH = 8.2, and then subjected to ultrasonic treatment with a probe sonicator (Vibracell™, Sonics & Materials Inc., Newton, CT, USA) under the following conditions: time of 30 min, amplitude of 70, pulse lengths of 10 s ON/2 s OFF, and output of ca 14 W (overheating was controlled with an ice bath). The extract was centrifuged for 15 min at 1800g and the supernatant filtered through 1.2 µm acetate cellulose membranes. Next, 2.5 mL of the sample was loaded on a disposable size exclusion column (G25 Sephadex column) and eluted with 3.5 mL of 50 mmol/L ammonium bicarbonate as the purification step. Protein quantification was accomplished on cookie extracts with the commercial Quick Start™ protein assay, which is based on the colorimetric Bradford assay. Instructions provided by the company were followed.

2.5. Enzymatic digestion

Protein enzymatic digestion was carried out on a 300 μ L aliquot of the eluted sample by using trypsin as the specific cleavage enzyme. Before digestion, protein denaturation (15 min at 95 °C), reduction (incubation for 30 min at 60 °C after addition of 15 μ L of 50 mmol/L DTT solution) and alkylation were performed (incubation for 30 min at room temperature after addition of 30 μ L of 100 mmol/L IAA solution). Finally, 4 μ L of a trypsin solution (1 μ g/ μ L in acetic acid 50 mmol/L) were added to the sample such that a ratio higher than 1/50 (enzyme/protein) was attained to facilitate complete enzymatic digestion. The reaction was stopped after approximately 14 h by acidifying the sample with 1 mol/L HCl and the final extract was filtered through a 0.2 μ m-filter before chromatographic injection.

2.6. On-line SPE-HPLC coupling with MS/MS analysis and software-based data processing

For HPLC–MS analyses, a system consisting of a dual UHPLC pump, provided with an autosampler and an ESI interface connected to a Dual Pressure Linear Ion Trap Mass Spectrometer Velos Pro™ (Thermo Fisher Scientific, San José, USA), was used. Peptide enrichment, purification and separation was accomplished by automated on-line coupling of solid phase extraction (SPE) and reversed phase (RP) HPLC, allowed by a low-dispersion 2-position switching valve (10-port). The same mobile phases were employed for both SPE and RP-HPLC: solvent A = H₂O + 0.1% formic acid and solvent B = CH₃CN + 0.1% formic acid. 100 μ L of digest (loading volume optimized by a specific data set, see Section 3.3 and Supporting information) were loaded on an Acclaim™ 300 C18 cartridge (2 $\times$ 10 mm, 3 μ m, 300 Å, Thermo Fisher –San José, US–) for SPE at 300 μ L/min of 100% solvent A for 2 min, followed by a brief washing of the cartridge by increasing the organic solvent B from 0 to 5% in 1 min, and then maintained isocratic at 5% of B for 1 more minute. After 4 min, the valve was switched to allow collection of the enriched peptide fraction from the cartridge and the chromatographic separation on an Aeris Peptide 3.6 μ XB-C18 analytical column (150 $\times$ 2.10 mm, 3.6 μ m, 100 Å, Phenomenex) at the flow rate of 200 μ L/min. The following elution gradient program was applied: 4–29 min solvent A reduced from 88% to 60%, 29–30 min of further reduction from 60% to 20%, constant for 15 min, 45–53 min at a constant 88% column conditioning before the next injection. The system was run under two different acquisition schemes: the first mode was a survey analysis in *Data Dependent™ Acquisition* (DDA), the second mode was a multiple SRM MS/MS acquisition. Raw data were processed by the commercial software Proteome Discoverer™ version 1.4 (Thermo Fisher Scientific, San José, US) and identification was accomplished by Sequest HT search, by referencing a customized food allergen database (FA-DB) built *in-house* and containing approximately 75600

entries (including milk, soy, egg, peanuts and hazelnut proteins and typical protein contaminants usually detected in tryptic digest, e.g., keratines). The software results reported in Table 1 were filtered by peptide confidence levels (high = 1% confidence), with the minimum number of peptides set at 3 per protein. After selection of peptide markers and proper transitions, a four-segment acquisition scheme was set up, screening for a total of ten peptides. Each segment included up to two scan events, represented by SRM acquisition in which each selected peptide was isolated and activated by CID with a normalized collision energy of 35%, and the ion currents related to the three most intense transitions were recorded within a 3 *m/z* window.

3. Results and discussion

As a first step, the mixture used to produce cookies was fortified with allergenic ingredients prior to heat treatment to mimic real cases of naturally contaminated food products. Such incurred materials allowed meaningful development of the sample pre-treatment protocol and a critical assessment of the performance of the analytical method, with particular attention being paid to the evaluation of the processing effects. Blank and incurred cookies were produced by the addition of equal amounts of five allergenic ingredients (egg, milk, soy, hazelnut and peanut) to wheat flour in the cookie dough, up to a final concentration of 10,000 μ g/g in the dry ingredient mixture. After dough-making and baking, the cookies were withdrawn and weighed in order to rescale the allergen concentration to the dry matrix, calculated to be approximately 8756 μ g/g for each allergenic ingredient. By serial dilutions based on the dry mixture of incurred and blank minces in a 1:3 ratio and proper homogenization of the sample, a “reference” minced sample at allergen concentration level of 973 μ g/g was produced and used for all further experiments.

3.1. Workflow optimization

To design the sample extraction workflow, the purification and pre-concentration methods used in our previous investigations on spiked allergen-free cookies (Monaci et al., 2014) were used. Route A of Fig. 1 summarizes the main steps of the developed procedure, which included 2 h of extraction with Tris–HCl buffer solution at 60 °C under shaking, ultrasound treatment of the collected and micro-filtrated protein extract and purification by size-exclusion chromatography (SEC) followed by pre-concentration using ultra-filtration (UF) cut-off membranes, for a total running time of approximately six hours (Monaci et al., 2014).

In order to reduce the overall time of sample handling, some modifications were introduced along the workflow and the relevant equivalence in terms of protein extraction yield and peptide detection efficiency were assessed. First, the optimization of step A.1 was evaluated and the protein content estimated by Bradford

Table 1

List of proteins identified through characteristic peptides by Proteome Discoverer™ software (version 1.4), based on Sequest HT search of experimental *Data Dependent™* MS/MS spectra against a customized food allergen database.

Allergenic Ingredient	Identified protein: Uniprot Accession Number-Description	Coverage	N° Peptides (Unique peptides)
Milk	P02662 – Alpha-S1-casein (Bos taurus)	19.2%	3 (3)
Egg	P01012 – Ovalbumin (Gallus Gallus)	10.6%	3 (3)
Soy	P04776 – Glycinin G1	10.5%	5 (3)
	P04405 – Glycinin G2	13.6%	7 (5)
Hazelnut	Q8W1C2 – 11S globulin-like protein (Corylus avellana)	15.5%	6 (6)
Peanut	Q9FZ11 – Gly1 (Arachis hypogaea)	25.3%	8 (2)
	Q647H3 – Arachin Ahy-2 (Arachis hypogaea)	19.4%	7 (1)
	Q5I6T2 – Arachin Ahy-4 (Arachis hypogaea)	19.6%	7 (1)
	A5Z1R0 – Arachin 6 (Arachis hypogaea)	21.7%	8 (3)
	Q6PSU4 – Conarachin (Arachis hypogaea)	12.4%	4 (4)

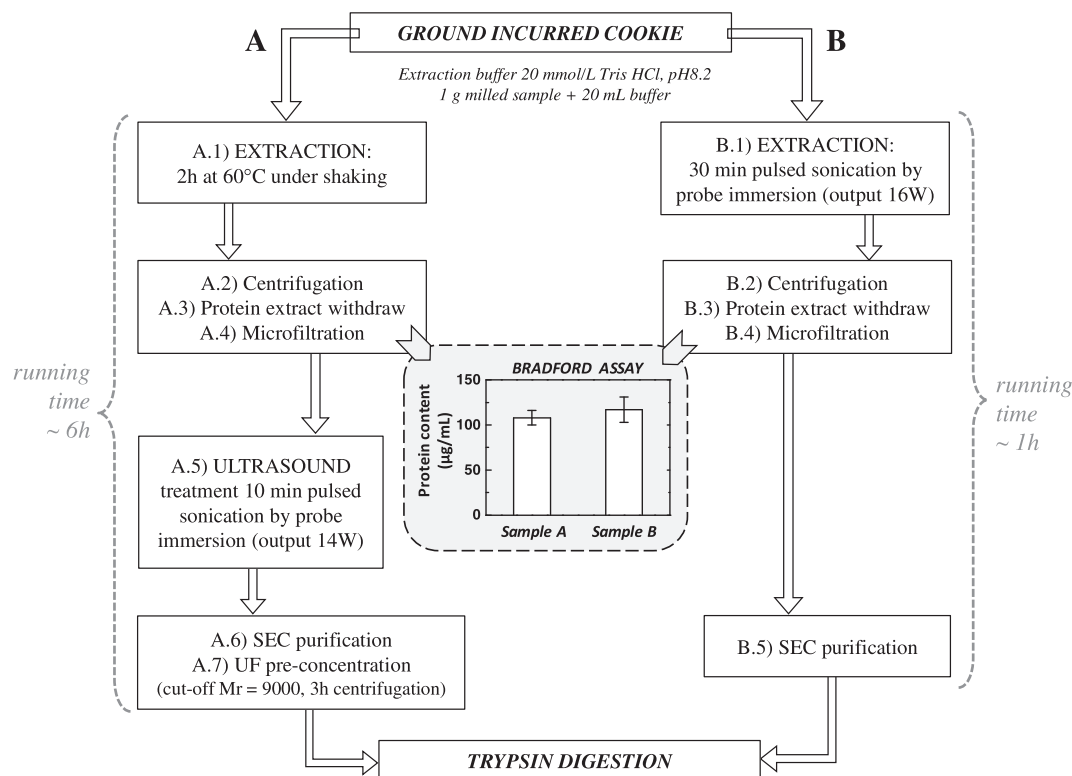


Fig. 1. Experimental workflow adopted for multiplex detection of egg, milk, soy, hazelnut and peanut in cookie samples.

assay was chosen as discriminating factor, as shown in Fig. 1. In particular, two sets of samples were prepared: one followed the procedure depicted in Fig. 1 from A.1 to A.4, while the other underwent an alternative extraction based on 30 min ultrasound treatment by probe immersion (see steps B.1 to B.4 of route B). Both samples were subjected to a colorimetric assay. According to the results obtained, the total protein content did not significantly when the 2 h-extraction step under shaking was replaced with a shorter extraction that was assisted and expedited by exposure to probe-based ultrasound treatment (see histogram included in Fig. 1). Even so, based on the effectiveness of the total workflow for cookie sample purification, the use of a faster extraction equivalent in terms of extraction yield was preferred. In addition, given the introduction of the ultrasound aid, which facilitated protein extraction, step A.5 was deemed negligible because it did not provide any increase in the detection sensitivity of milk markers. Therefore, it was removed from the protocol.

The next step in the workflow was the purification of the micro-filtered protein extracts by a disposable size exclusion column (step A.6/B.5). Due to the rapidity of this passage (only 15 min) and the need to selectively deplete the extract, this purification step was maintained in order to remove low molecular weight matrix interferences (cut-off Mr = 5000).

The advantage of using ultrafiltration cut-off devices for protein pre-enrichment was evaluated as the last step. Pre-concentration by an ultrafiltration cut-off filter was deemed unworthy. As a result, to streamline the analytical workflow, the whole running time of the method was reduced to one hour compared to the six-hour approach foreseen by our previous protocol (Monaci et al., 2014).

3.2. Selection of peptide markers

In our previous paper, a list of peptide markers was compiled for egg, milk, and soy allergens that was demonstrated to be

reliable for confirmative and quantitative purposes in the detection of multiple allergens. These markers were reconsidered in this study in order to confirm that they could also be used as markers in a processed food matrix. In addition, ex-novo information related to peanut and hazelnut peptide markers was required to compile a proper SRM instrumental method enabling sensitive multiplex detection of the five allergenic ingredients.

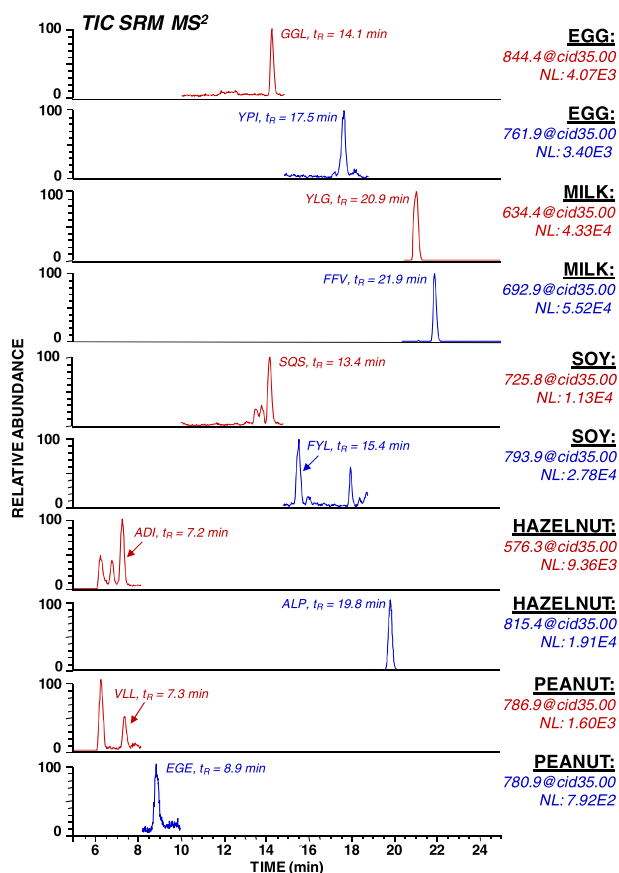
A preliminary untargeted MS analysis in the data dependent acquisition (DDA) mode was carried out on the protein extract of cookies, incurred at 973 µg/g (diluted 1:3 before injection). Raw data were processed using the commercial software Proteome Discoverer™ (version 1.4) and protein assignment was accomplished using the Sequest scoring algorithm by searching MS/MS spectra against a customized database (DB), restricted to food allergens and other contaminants. The protein coverage (part of the protein covered by peptide matching) and number of peptides identified for allergen proteins are reported in Table 1. Browsing the list of peptide sequences assigned by the software, the MS/MS spectra showing the highest match with the predicted fragmentation patterns were further validated by inspection, and only those spectra matching specific criteria were taken into consideration (Monaci et al., 2014).

The three most intense transitions were chosen for each internally validated peptide and this information was used to build up a tailored SRM acquisition scheme to obtain the highest method sensitivity and selectivity for the detection of target proteins in a complex matrix. Table 2 summarizes the restricted list of peptides that met the requested criteria along with the specific transitions.

In order to design a proper MS acquisition method, different parameters were optimized to find the best conditions for LC-MS analyses. Based on the elution time of each candidate peptide marker, the acquisition run was split into six segments as detailed in Table 2. MS analyses were carried out both on blank and incurred cookie extracts in order to confirm the absence of any interfering peak at the expected retention times and to evaluate the resolution

Table 2Instrumental set-up for the devised SRM acquisition: list of candidate marker peptides with the relevant amino-acid sequence, *m/z* value, retention time and selected transitions.

MS segment	Allergen/protein	Peptide sequence (<i>m/z</i>)	<i>t_R</i> (±0.1 min)	Transitions
(I) 0–8.2 min	HAZELNUT/11S globulin-like protein	ADIYEQVGR (576.3)	7.2	[<i>M</i> +2 <i>H</i>] ²⁺ -H ₂ O; <i>y</i> ₆ ⁺ ; <i>y</i> ₇ ⁺
	PEANUT/Conarachin	VLEENAGGEQEER (786.9)	7.3	<i>y</i> ₁₂ ⁺ ; <i>y</i> ₇ ⁺ ; <i>y</i> ₈ ⁺
(II) 8.2–10.0 min	PEANUT/Conarachin	EGEQEWGTPGSEVR (780.9)	8.9	<i>y</i> ₆ ⁺ ; <i>y</i> ₈ ⁺ ; <i>y</i> ₉ ⁺
(III) 10.0–14.8 min	SOY/Glycinin G1-G2	SQSDNFEYVSFK (725.8)	13.4	[<i>M</i> +2 <i>H</i>] ²⁺ -H ₂ O; <i>y</i> ₃ ⁺ ; <i>y</i> ₁₀ ⁺
	EGG/Ovalbumin	GGLEPINFQTAADQAR (844.4)	14.1	<i>y</i> ₁₂ ⁺ ; <i>y</i> ₇ ⁺ ; <i>y</i> ₁₀ ⁺
(IV) 14.8–18.8 min	SOY/Glycinin G1-G2	FYLAGNQEQEFLK (793.9)	15.4	<i>y</i> ₁₁ ⁺ ; <i>y</i> ₉ ⁺ ; <i>y</i> ₁₀ ⁺
	EGG/Ovalbumin	YPILPEYLQCVK (761.9)	17.5	<i>b</i> ₄ ⁺ ; <i>y</i> ₈ ⁺ ; <i>y</i> ₉ ⁺
(V) 18.8–20.3 min	HAZELNUT/11S globulin-like protein	ALPDDVLANAFQISR (815.4)	19.8	<i>y</i> ₇ ⁺ ; <i>y</i> ₈ ⁺ ; <i>y</i> ₁₃ ⁺
(VI) 20.3–28 min	MILK/αS1-casein	YLGYLEQLLR (634.4)	20.9	<i>y</i> ₅ ⁺ ; <i>y</i> ₆ ⁺ ; <i>y</i> ₈ ⁺
	MILK/αS1-casein	FFVAPFPEVFGK (692.9)	21.8	<i>y</i> ₈ ⁺ ; <i>y</i> ₉ ⁺ ; <i>y</i> ₁₀ ⁺

**Fig. 2.** Overlay of typical ion chromatograms recorded in the SRM acquisition mode for the selected peptide markers shown in Table 2. The entire chromatographic run was divided into six acquisition time segments.

obtained by chromatographic separation. A typical representation of the multi-target analysis achieved in the SRM mode is shown in Fig. 2, in which an overlay of ten chromatographic traces is reported.

3.3. Automated pre-enrichment by on line coupling of SPE and RP-HPLC

To streamline the analytical workflow, the ultrafiltration step was avoided. However, in order to compensate for this pre-concentration in the sample handling, special instrumental features of the available chromatographic tools were implemented: an automated on-line selective enrichment procedure for peptides on was devised on a C18 cartridge, by the use of a dual pump configuration and a 10-port switching valve. On-line SPE is an

innovative sample preparation approach that is particularly useful for the analysis of complex matrices such as food commodities (Barreiro et al., 2015). The main expectations from such a configuration are the reduction of analysis time, automation, and potential to cleanup and simultaneously pre-concentrate target analytes with increased selectivity. These advantages become particularly relevant for a MS-based method tailored to the detection of multiple allergens in complex food matrices.

For on-line SPE, a 2D set-up system was employed: in the first step, the sample (100 µg/g) was loaded by the autosampler onto the C18 cartridge for peptide pre-enrichment and removal of interfering species. In the second step, the retained peptides were eluted with a solvent of proper composition from the trap to the analytical column; the switching valve regulated this coupling. In order to obtain the maximum trapping efficiency under our experimental conditions, two main parameters were investigated, namely, the composition of the mobile phase and the injection/loading volume. The variation in peptide peak areas of SRM chromatograms was monitored as distinctive factor for their appropriate tuning.

First, the tryptic digests were injected with a 2-min isocratic flow of the water-based mobile phase (100% solvent A: H₂O + 0.1% formic acid); a rapid rinsing step of the cartridge (increase of the organic phase up to 5%) was also included before switching the valve, to allow the removal of major interferences with low affinity to the trap column, without significant loss of analytical signal. The devised gradient extended the whole chromatographic run by only four minutes.

After evaluating the eluent composition, a targeted experiment was performed to evaluate the maximum capacity of the cartridge, within the upper limit of 100 µL defined by the loop size in our equipment. Several loading volumes were tested in the range of 5–100 µL (matching an injected amount of matrix ranging between 0.18 and 3.6 mg), resulting in the proportional increase of the analytical response with good linear correlation, as shown in Fig. S1. Such evidence also avoided cartridge overloading with full-loop injections and was thus preferred for all further experiments.

3.4. Assessment of quantitative performances

Matrix-matched calibration curves of incurred cookies were carefully inspected in order to identify the best quantitative peptide markers for each allergenic ingredient, based on the list of candidate peptides included in the SRM method. Progressive dilutions in the range of 10–200 µg/g were considered and the relevant chromatographic runs recorded. The linearity of the response was assessed in the whole concentration range under investigation, with a linear correlation and coefficients better than 0.995 for the most sensitive peptide markers per allergenic ingredient. Limits of detection (LOD) and quantification (LOQ) were estimated as the minimum concentration of an added

Table 3
Overview of method performance.

PEPTIDE m/z	Sequence (Allergen)	LOD ($\mu\text{g/g}$)	LOQ ($\mu\text{g/g}$)	Slope	R ²
844.4	GGLEPINFQTAADQAR (EGG)	9	30	152 $\pm$ 4	0.998
692.9	FFVAPFPEVFGK (MILK)	7	20	5240 $\pm$ 120	0.998
793.9	FYLAGNQEQFLK (SOY)	6	19	149 $\pm$ 3	0.999
815.4	ALPDDVLANAFQJSR (HAZELNUT)	7	20	1000 $\pm$ 20	0.998
786.9	VLEENAGGEQEER (PEANUT)	13	40	68 $\pm$ 3	0.995

allergenic ingredient that can be detected, at signal-to-noise (S/N) ratios of 3 and 10, respectively (the standard deviation of the calibration line intercept was used in this case as an estimate of noise). Subsequently, the most sensitive peptides for each allergenic food category were selected as quantifier peptides among the list of ten peptide markers included into the SRM instrumental method. Table 3 reports an overview of the performance of the method for the five selected peptides. As shown, the limit values for each allergenic ingredient in the incurred cookies were found to be quite similar, ranging from 6 $\mu\text{g/g}$ ($\mu\text{g}_{\text{allergenic ingredient}}/\text{g}_{\text{food matrix}}$) for soy, which had the highest sensitivity, down to 7 $\mu\text{g/g}$ for milk and hazelnut, 9 $\mu\text{g/g}$ for egg and 13 $\mu\text{g/g}$ for peanut.

3.5. Evaluation of processing effects

Thermal processing can affect allergen detection by inducing chemical modifications as well as changes in the protein structure and solubility. In order to evaluate such effects on the detection of each candidate peptide marker, two sets of samples were considered in this work: i) cookies incurred with allergenic ingredients before dough-making and baking and ii) blank cookies spiked after baking; both samples were made with a concentration of 973 $\mu\text{g/g}$. At the same time, a blank allergen-free cookie sample was considered, and the three samples (incurred, spiked and blank) were subjected to ultrasound-assisted extraction, as depicted in Fig. 1. Protein extracts were withdrawn and used to prepare matrix-matched calibration curves by progressive dilutions (see Materials and methods for details) with the blank cookie extract. All the samples underwent SEC purification, and enzymatic digestion, as schematized in Fig. 1 (route B). The peak areas were plotted versus the incurred/spiked allergen concentrations, and matrix-matched calibration curves were finally obtained for milk, egg, soy, hazelnut and peanut. By comparing the calibration slopes obtained in the differently contaminated samples (incurred versus spiked), the effect of a typical food processing procedure, such as baking, on the detection of the chosen peptide markers could be assessed.

A comparison of the curve slopes obtained for each monitored peptide in both contaminated food samples is reported in Fig. 3: the white columns refer to incurred cookie calibration curves and the grey columns refer to the relevant spiked cookie calibration curves. The histogram clearly highlights how food processing affects, to different extents, the detection of the selected markers for all the allergenic ingredients under consideration. A calculation of the % processing effect (see Fig. 3 for details) shows that egg peptides appear to be the most susceptible to baking, followed by milk, soy and peanut, which showed comparable recoveries, whereas hazelnut peptides proved to be more resistant to the effect of baking. In general, the incorporation of allergens into the matrix before baking accounts for the reduction of the MS signal recorded in baked goods. As a consequence of such thermal treatment, a higher number of reactions between protein allergens and matrix components can take place along with the structural and/or chemical modification of allergens, causing a reduction in protein solubility/extractability and detection. In general, a reduction in allergen detection sensitivity in thermally processed foods has been previously documented in literature (Faeste, Løvberg, Lindvik, & Egaas, 2007; Westphal, Pereira, Raybourne, & Williams, 2004). On the other hand, it has been reported that detection of peanut, egg and milk allergens by immunoassay is compromised when analysing processed food matrices. This can be due to the effect of food processing on the antigenic determinants spread along the allergenic proteins, which can impair antibody recognition (Diaz-Amigo, 2010; Khuda, Jackson, Fu, & Williams, 2015; Monaci, Brohée et al., 2011; Pele, Brohée, Anklam, & Van Hengel, 2007).

In addition, other physiochemical interactions, occurring between allergens and other food components during baking can affect allergen detection: for example, it has been reported that interactions between soy and flour proteins during dough-mixing, and subsequent formation of a high-molecular-weight complex between soy 11S globulin and prolamin of wheat, contributes to underestimated soy allergen recovery.

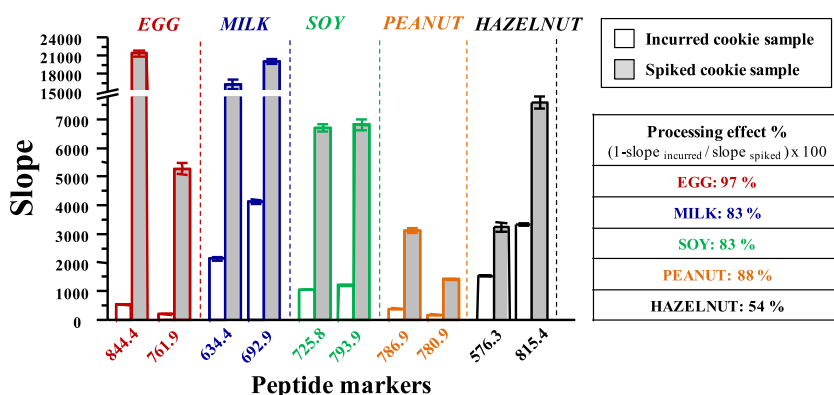


Fig. 3. Comparison of the calibration curve slopes obtained by interpolation of peak areas in the SRM mode for incurred and spiked samples. The table on the right reports the % processing effect expressed as the percent decrease of the sensitivity calculated in incurred samples compared to spiked samples.

4. Conclusions

In the present investigation, a LC-MS method for the sensitive multiplex detection of egg, milk, soy, hazelnut and peanut allergens in home-made cookies was developed. The design of a relatively fast sample handling procedure based on ultrasound-assisted extraction and purification by SEC, as well as a multidimensional chromatographic set-up, yielded an effective and streamlined analytical workflow. The automated multidimensional chromatographic separation allowed pre-enrichment and partial purification of peptides of interest on a C18 one-cm-long trap-column before classical reverse-phase separation. The use of an on-line SPE provided an advantage in the final SRM detection, resulting in higher sensitivity while still maintaining a reasonable run time. Final LODs obtained from calibration curves for all analyzed allergens were in the low-mg/kg range.

Regarding the effects of thermal processing on allergen peptide detection, egg peptides appeared to be the most susceptible to baking, followed by milk, soy and peanut with a comparable recovery, whereas hazelnut peptides proved to be more resistant to thermal processing.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.foodchem.2016.10.110>.

References

- Barreiro, J. C., Luiz, A. L., Maciel, S. C., Maciel, E. V., & Lanças, F. M. (2015). Recent approaches for on-line analysis of residues and contaminants in food matrices: A review. *Journal of Separation Sciences*, 38, 1721–1732.
- Bignardi, C., Elviri, L., Penna, A., Careri, M., & Mangia, A. (2010). Particle-packed column versus silica-based monolithic column for liquid chromatography-electrospray-linear ion trap-tandem mass spectrometry multiallergen trace analysis in food. *Journal of Chromatography A*, 1217, 7579–7585.
- Cryar, A., Pritchard, C., Burkitt, W., Walker, M., O'Connor, G., Burns, D. T., et al. (2013). Towards absolute quantification of allergenic proteins in food-lysozyme in wine as a model system for metrologically traceable mass spectrometric methods and certified reference materials. *Journal of AOAC International*, 96, 1350–1361.
- Diaz-Amigo, C. (2010). Towards a comprehensive validation of ELISA kits for food allergens. Case 2 – Milk. *Food Analytical Method*, 3, 351–356.
- Downs, M. L., & Taylor, S. L. (2010). Effects of thermal processing on the enzyme-linked immunosorbent assay (ELISA) detection of milk residues in a model food matrix. *Journal of Agricultural and Food Chemistry*, 58, 10085–10091.
- Faeste, C. K., Løvberg, K. E., Lindvik, H., & Egaas, E. (2007). Extractability, stability, and allergenicity of egg white proteins in differently heat-processed foods. *Journal of AOAC International*, 90, 427–436.
- Gomaa, A., & Boye, J. I. (2013). Impact of thermal processing time and cookie size on the detection of casein, egg, gluten and soy allergens in food. *Food Research International*, 52, 483–489.
- Heick, J., Fischer, M., & Pöpping, B. (2011). First screening method for the simultaneous detection of seven allergens by liquid chromatography mass spectrometry. *Journal of Chromatography A*, 1218, 938–943.
- Johnson, P. E., Baumgartner, S., Aldick, T., Bessant, C., Giosafatto, V., Heick, J., et al. (2011). Current perspectives and recommendations for the development of mass spectrometry methods for the determination of allergens in foods. *Journal of AOAC International*, 94, 1026–1033.
- Khuda, S. E., Jackson, L. S., Fu, T.-J., & Williams, K. M. (2015). Effects of processing on the recovery of food allergens from a model dark chocolate matrix. *Food Chemistry*, 168, 580–587.
- Lutter, P., Parisod, V., & Weymuth, H. (2011). Development and validation of a method for the quantification of milk proteins in food products based on liquid chromatography with mass spectrometric detection. *Journal of AOAC International*, 94, 1043–1059.
- Monaci, L., & Visconti, A. (2009). Mass spectrometry-based proteomics methods for analysis of food allergens. *Trends in Analytical Chemistry*, 28, 581–591.
- Monaci, L., & Visconti, A. (2010). Immunochemical and DNA-based methods in food allergen analysis and quality assurance perspectives. *Trends in Food Science & Technology*, 21, 272–283.
- Monaci, L., Brohée, M., Tregoat, V., & Van Hengel, A. (2011). Influence of baking time and matrix effects on the detection of milk allergens in cookie model food system by ELISA. *Food Chemistry*, 127, 669–675.
- Monaci, L., Losito, I., Palmisano, F., Godula, M., & Visconti, A. (2011). Towards the quantification of residual milk allergens in caseinate-fined white wines using HPLC-coupled with single-stage Orbitrap mass spectrometry. *Food Additives and Contaminants*, 28, 1304–1314.
- Monaci, L., Losito, I., Palmisano, F., & Visconti, A. (2011). Reliable detection of milk allergens in food using a high-resolution, stand-alone mass spectrometer. *Journal of AOAC International*, 94, 1034–1042.
- Monaci, L., Losito, I., De Angelis, E., Pilolli, R., & Visconti, A. (2013). Multi-allergen quantification of fining-related egg and milk proteins in white wines by high-resolution mass spectrometry. *Rapid Communication Mass Spectrometry*, 27, 2009–2018.
- Monaci, L., Pilolli, R., De Angelis, E., Godula, M., & Visconti, A. (2014). Multiallergen detection in food by micro high-performance liquid chromatography coupled to a dual cell linear ion trap mass spectrometry. *Journal of Chromatography A*, 1358, 136–144.
- Monaci, L., Pilolli, R., De Angelis, E., & Mamone, G. (2015). Mass Spectrometry in food allergen research. In Y. Picó (Ed.), *Comprehensive analytical chemistry* (pp. 359–393). Elsevier Science and Technology.
- Pele, M., Brohée, M., Anklam, E., & Van Hengel, A. J. (2007). Peanut and hazelnut traces in cookies and chocolates: Relationship between analytical results and declaration of food allergens on product labels. *Food Additives and Contaminants*, 24, 1334–1344.
- Platteau, C., Cucu, T., De Meulenaer, B., Devreese, B., De Loose, M., & Taverniers, I. (2011). Effect of protein glycation in the presence or absence of wheat proteins on detection of soybean proteins by commercial ELISA. *Food Additives and Contaminants-Part A*, 28, 127–135.
- Picariello, G., Mamone, G., Addeo, F., & Ferranti, P. (2011). The frontiers of mass spectrometry-based techniques in food allergenomics. *Journal of Chromatography A*, 1218, 7386–7398.
- Pilolli, R., De Angelis, E., Godula, M., Visconti, A., & Monaci, L. (2014). Orbitrap™ monostage MS versus hybrid Linear ion trap MS: Application to multi-allergen screening in wine. *Journal of Mass Spectrometry*, 49, 1254–1263.
- Poms, R. E., & Anklam, E. (2004). Effects of chemical, physical, and technological processes on the nature of food allergens. *Journal of AOAC International*, 87, 1466–1474.
- Pöpping, B., & Godefroy, S. B. (2011). Allergen detection by mass spectrometry the new way forward. *Journal of AOAC International*, 94, 1005–1005.
- Schmitt, D. A., Nesbit, J. B., Hurlburt, B. K., Cheng, H., & Maleki, S. J. (2010). Processing can alter the properties of peanut extract preparations. *Journal of Agricultural and Food Chemistry*, 58, 1138–1143.
- Török, K., Hajas, L., Horváth, V., Schall, E., Bugyi, Z., Kemény, S., et al. (2015). Identification of the factors affecting the analytical results of food allergen ELISA methods. *European Food Research and Technology*, 241, 127–136.
- U.S. Food and Drug Administration. Food Allergen Labeling and Consumer Protection Act of 2004 (Public Law 108–282, Title II), (2004); www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/Allergens/ucm106187.htm.
- Weber, D., Raymond, P., Ben-Rejeb, S., & Lau, B. (2006). Development of a liquid chromatography tandem mass spectrometry method using capillary liquid chromatography and nanoelectrospray ionization-quadrupole time-of-flight hybrid mass spectrometer for the detection of milk allergens. *Journal of Agricultural and Food Chemistry*, 54, 1604–1610.
- Westphal, C. D., Pereira, M. R., Raybourne, R. B., & Williams, K. M. (2004). Evaluation of extraction buffers using the current approach of detecting multiple allergenic and nonallergenic proteins in food. *Journal of AOAC International*, 87, 1458–1465.

SUPPLEMENTARY MATERIAL

Figure S.1 Optimization of loading volume for on-line SPE

