



Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma



Multi-allergen detection in food by micro high-performance liquid chromatography coupled to a dual cell linear ion trap mass spectrometry

Linda Monaci^{a,*}, Rosa Pilolli^a, Elisabetta De Angelis^a, Michal Godula^b, Angelo Visconti^a

^a Institute of Sciences of Food Production, National Research Council of Italy (ISPA-CNR), Via Amendola 122/O, 70126 Bari, Italy

^b Thermo Fisher Scientific, Czech Republic, Slunecna 27, CZ-100 00 Prague, Czech Republic

ARTICLE INFO

Article history:

Received 2 May 2014

Received in revised form 25 June 2014

Accepted 26 June 2014

Available online xxx

Keywords:

Linear ion trap

Mass spectrometry

Food allergens

Multi-allergen analysis

Cookies

ABSTRACT

There is a raising demand for sensitive and high throughput MS based methods for screening purposes especially tailored to the detection of allergen contaminants in different food commodities. A challenging issue is represented by complex food matrices where the antibody-based kits commercially available might encounter objective limitations consequently to epitope masking phenomena due to a multitude of interfering compounds arising from the matrix. The performance of a method duly optimized for the extraction and simultaneous detection of soy, egg and milk allergens in a cookie food matrix by microHPLC–ESI–MS/MS, is herein reported. Thanks to the innovative configuration and the versatility shown by the dual cell linear ion trap MS used, the most intense and reliable peptide markers were first identified by untargeted survey experiment, and subsequently employed to design an ad hoc multi-target SRM method, based on the most intense transitions recorded for each selected precursor peptide. A sample extraction and purification protocol was optimized also including an additional step based on sonication, which resulted in a considerable improvement in the detection of milk allergen peptides. *Data Dependent*TM Acquisition scheme allowed to fill out a tentative list of potential peptide markers, which were further filtered upon fulfilling specific requirements. A total of eleven peptides were monitored simultaneously for confirmation purposes of each allergenic contaminant and the two most sensitive peptide markers/protein were selected in order to retrieve quantitative information. Relevant LODs were found to range from 0.1 µg/g for milk to 0.3 µg/g for egg and 2 µg/g for soy.

© 2014 Elsevier B.V. All rights reserved.

1. Introduction

Food allergy is a public health problem reported to be on the rise especially in industrialized countries and with a high prevalence in childhood [1]. This type of allergy referring to both Ig-E (type I) and non-Ig-E mediated reactions, is triggered by the ingestion of a food or food additive also known as “food allergen” [2]. The lethal risk associated with food allergies and the concern to protect public health have led the regulatory bodies to issue legislation concerning the labeling of the principal food allergens around the globe. Although a large number of foods are considered allergenic, legislation issued in several countries to protect allergic consumers is mainly targeted to a restricted list of food allergens. In the European Union the last Directive 2007/68/EC regulates the mandatory

labeling of a total of 14 allergenic ingredients that, whenever used, must be declared on the respective food label [3]. Nevertheless, some ingredients exempted from allergen declaration are also listed in Directive 2007/68/EC. Although threshold data could constitute the basis of regulatory thresholds, to date most regulatory agencies have focused on ingredients that must be reported in the label irrespective of the amounts in the food, the only exception being represented by gluten where limits have been set within EU.

Contamination of food by hidden allergens at the moment represents a major health problem for allergic patients. Allergens are defined hidden when they are not declared on the product label and unexpectedly might reach the end products through several routes. Apart from the intentional use of some allergenic ingredients in food manufacturing, a risk of an accidental contamination of foods by allergens is likely to exist.

Different analytical methods have been developed for monitoring food allergen contamination along the food chain. Methods address either the allergen itself or a marker contained in the allergenic food. To support allergen control within HACCP programs,

* Corresponding author. Tel.: +39 0805929343; fax: +39 0805929374.
E-mail address: linda.monaci@ispa.cnr.it (L. Monaci).

laboratory immunoassays have been proposed due to the ease in use, the relatively high throughputs and the low detection limits [4]. On the other hand, an analysis might be required for confirmation of the results obtained. Therefore, a more direct analytical technique with a high level of specificity is needed. In the last few years mass spectrometry (MS) methods [5,6] have been considered a promising analytical strategy for food allergens monitoring thanks to the advances made in this technology that enables the reduction of the risk of false positives compared to ELISA methods.

As well known, there are a number of limitations presented by the antibody-based methods especially when applied to complex or processed food matrices where allergen detection might be hampered as a consequence of epitope modification or masking effect. Among them also the impossibility to run a multi-allergen analysis. To overcome such limitations, several MS based methods have been developed for multiallergen detection in foods [7–12]. A review of the recent literature yielded only a few reports mainly directed to the multiallergen determination of several classes of food allergens in different food commodities. Among the last papers published on this regard, the method authored by Heick et al. is one of the first MS approach using a triple quadrupole instrument, enabling the simultaneous detection of seven allergens in bread chosen as food model [10]. In their paper the authors reported LODs achieved of 5, 24 and 42 µg/g for milk, soy and egg, respectively, whereas a lower limit was reached for almonds. In another work Bignardi et al. developed a MS-based method for tracing 5 different nuts in fortified cookies with LODs ranging between 10 and 50 mg nut/kg matrix [13]. In the mentioned work a comparison between two different acquisition schemes, either MS² or MS³ mode, was also carried out for detecting these classes of nuts, employing a linear ion trap based system. Other works reported methods mainly tailored to the detection of other allergenic categories including lupins, nuts and gliadin, milk, etc. in different food products with highly variable limits of detection achieved [14–17].

In general, sensitivity of a method strictly depends on different factors including the complexity of the food matrix, the extractability of the targeted proteins from the allergen-containing-food and the detection performance of the instrument used. On this regard, in order to assist the scientific community in properly designing MS-based methods for detection of allergens, some guidelines have been reported [18]. Among the recommendations, a preliminary proper selection of reliable and stable candidate peptide-markers tracing for the allergenic protein must be accomplished and the most reliable transitions effectively designed for each selected peptide. However, the effective validation of methods through the use of naturally incurred reference materials for each food matrix investigated should be accomplished for the harmonization of the final results.

In this work, we focus on three allergenic food categories including egg, milk and soy that might accidentally contaminate cookies chosen as food model. Beyond optimization of the best extraction conditions, different instrumental parameters were carefully investigated and evaluated in the work performed by using a linear ion trap MS instrument based on a differential pressure cell system and able to deliver a rapid and sensitive multi-allergen detection method.

Notably, while the first cell held at higher pressure than in a usual linear ion trap system produced an improvement in the efficiency of trapping, isolating and fragmenting ions of interest reducing the time required for precursor isolation, the second cell held at a lower pressure, instead, allowed for a faster mass analysis with increased resolution. This novel method for controlling the ion population in the trap produced a dramatic increase in the practical scan rate achievable in a typical data dependent tandem MS experiment. The features offered by this system have been in this work exploited to run a survey experiment followed by a

multiple selected reaction monitoring (SRM) experiment based on the best transitions selected to set up a sensitive multi-target method.

2. Materials and methods

2.1. Reagents

Acetonitrile (LC–MS grade), formic acid, acetic acid, ammonium bicarbonate, trizma base, Tween 20, hydrochloric acid, iodoacetamide (IAA), dithiothreitol (DTT), egg powder (EP) and skimmed milk powder (MP) were obtained from Sigma–Aldrich (Milan, Italy). Trypsin (proteomic grade) was purchased by Promega (Milan, Italy); RapigestTM surfactant was purchased by Waters (Milford, MA, USA). Cellulose acetate syringe filters, 0.45 µm (size 30 mm), and polytetrafluoroethylene syringe filters, 0.45 µm (size 4 mm) were purchased by Sartorius (Stedim Biotech GmbH, Goettingen, Germany). Quick StartTM Bradford protein assay was purchased by Bio-Rad (Segrate, MI, Italy). Disposable cartridges PD-10 were purchased from GE Healthcare Life Sciences (Milan, Italy), while ultrafiltration (UF) tubes with 9 kDa cut-off membranes were from Thermo Fisher Scientific (Illkirch Cedex, France). Pre-cooked soy flour (SF) was purchased from a local retailer.

2.2. Stock solutions

Stock solutions of EP and MP were prepared dissolving 1 mg of commercial powder into 1 mL of 50 mM ammonium bicarbonate solution. SF extract was prepared by incubation for 2 h at 60 °C of 1 g of commercial flour with 20 mL of 20 mM Tris–HCl buffer added with 0.01% of Tween 20. The SF extract was collected and employed as stock solution together with EP and MP solutions for the preparation of artificially contaminated cookies. In order to align the concentration values among EP, MP and SF extract, the protein content of the aforementioned three stock solutions was evaluated by colorimetric Quick StartTM Bradford assay. Thus, all concentration reported in the following text were referred to µg of spiked proteins. A mixture of stock solutions EP, MP, and SF extract in 50 mM ammonium bicarbonate solution was employed for the identification of the best peptide markers.

2.3. Bradford assay for protein quantification

Protein quantification of stock solutions and cookie extracts were performed with the commercial Quick StartTM protein assay based on colorimetric Bradford assay. The Bradford assay involves the binding of Coomassie Brilliant Blue G-250 dye to proteins. First a calibration curve with BSA (bovine serum albumin), selected as reference protein, was obtained by serial dilution of BSA within the 0.125–1 µg/mL concentration range. 20 µL of each BSA solution were mixed to 1 mL of dye reagent, previously warmed up at room temperature. After 5 min incubation at room temperature, the absorbance at 595 nm was recorded. A standard curve was created by plotting the Abs₍₅₉₅₎ values versus their concentration in µg/mL. The unknown samples were analogously treated and the relevant concentration determined by extrapolation of the calibration curve. At least three replicates for each sample were analyzed.

2.4. Preparation of spiked samples

Commercial cookies declared egg, soy and milk-free were purchased from a local retailer. Cookies were milled coarsely for 30 s in a blender at 17000 rpm and passed through a sieve of 1 mm (Sterilmixer 12 model 6805-50; PBI International). 1 g of ground allergen free cookie powder was fortified with standard solutions of egg powder, milk powder and SF extract in order to obtain in the final

product concentrations (referred to the protein content) of 1, 2, 5, 20 $\mu\text{g/g}$ for EP, 0.5, 2, 5, 20 $\mu\text{g/g}$ for MP and 10, 20, 50, 100 $\mu\text{g/g}$ for SF. Samples were left drying for 12 h in the oven at 40 °C to facilitate evaporation and allow interaction with food components. The following day samples were extracted according to the protocol here following described.

2.5. Sample preparation

2.5.1. Extraction and purification

One gram of allergen-spiked ground cookie sample was combined with 20 mL of Tris–HCl buffer 20 mM at pH = 8.2 [19] and left shaking for 2 h at 60 °C. The extract was centrifuged for 10 min at 3000 rpm and the supernatant filtered through 0.45 μm acetate cellulose membranes. 2.5 mL of supernatant was loaded on a size exclusion column (G25 Sephadex column) as purification (pre-LC–MS purification) step. The eluted fraction (3.5 mL) was withdrawn and centrifugated for 60 min, at room temperature, in ultrafiltration tubes with 9 kDa cut-off membrane. The retained part of the sample was subsequently dialyzed against 4 mL of water to remove residual low molecular weight matrix components. A final volume of 100 μL was collected from the filter and submitted to further enzymatic digestion.

For the ultrasound treatment a sonicator Model Vibracell™ from Sonics (Sonics & Materials Inc., Newton, CT, USA) was introduced in the purification procedure after the 0.45 μm filtration step and applied in the following conditions: time 10 min, amplitude 60, pulse 4 s, and output ca 14 W. Ultrasound treated samples were subjected to the same purification procedure described before.

2.5.2. Enzymatic digestion

Protein enzymatic digestion was accomplished on the final cookie extract by employing trypsin as specific cleavage enzyme. The ultrafiltrate was denatured by heating for 15 min at 95 °C and subsequently diluted in the surfactant/denaturing agent Rapigest™ (dil 1/3) to reach a final volume of 100 μL . Before digestion protein reduction was performed by adding 5 μL of DTT solution 50 mM, followed by 30 min incubation in a thermoshaker at 60 °C. After cooling the sample at room temperature, 10 μL of IAA solution 100 mM were added and the mixture was placed in the dark for 30 min at room temperature. Finally 20 μL of a trypsin solution (1 $\mu\text{g}/\mu\text{L}$ in acetic acid 50 mM) were added to the sample so that a ratio of 1/50 (enzyme/protein) was attained to facilitate a complete enzymatic digestion. Reaction was halted after 12 h by acidifying the sample with HCl 1 M and the final extract was filtered through 0.45 μm PTFE filters before injection.

2.6. HPLC–MS/MS analysis and database search

For HPLC–MS analyses a system composed of a UHPLC pump provided with a autosampler and ESI interface was Linear Ion Trap Dual pressure Mass Spectrometer Velos Pro™ (Thermo Fisher Scientific, San José, USA). For peptides separation an Acclaim™ PepMap analytical column provided by Thermo Fisher (San José, US), 1 mm $\times$ 15 cm $\times$ 3 μm , 100 Å porosity, was used by applying the following elution gradient program: 0–40 min from 85% to 45% A, 45–10% in 2 min and kept stable for 10 min. Then in the following 2 min solvent A was raised to 85% and left isocratic for 15 min to allow column equilibration (solvent A = H_2O + 0.1% formic acid; solvent B = $\text{CH}_3\text{CN}/\text{H}_2\text{O}$, 80/20 v/v + 0.1% formic acid). The flow rate was 60 $\mu\text{L}/\text{min}$. The system was run under two different acquisition schemes. First mode was a survey analysis also termed Nth order double play Data Dependent™ Acquisition (DDA) by activating the dynamic exclusion option. In particular, two events were set for this experiment: (1) full MS in the range 400–2000 m/z , 4 microscans, corresponding to a resolving power 5400 $M/\Delta M$ (with

a maximum injection time of 50 ms/1 microscan, for full MS, and 100 ms/4 microscan for MS/MS acquisitions); (2) full MS/MS DDA of the 20 most abundant ions in the MS spectrum using a normalized collision energy at 35%. Dependent settings for the DDA were the following. *Global*: dynamic exclusion enabled, repeated count 1, repeat duration 30 s, exclusion list size 500, exclusion duration 20 s, exclusion mass width by mass – low 0.5 and high 1.5. *Segment*: predict injection time enabled, charge state screening enabled, charge state rejection +1; +4 and up. *Scan event*: current scan event, minimum threshold 500 counts, Nth most intense $N=20$; activation: activation type CID, default charge state +3, isolation width 2.0, normalized collision energy 35, activation q 0.25, activation time 10 ms).

Raw data were processed via commercial software Proteome Discoverer™ version 1.3 (Thermo Fisher Scientific, San José, US) and identification was achieved by Sequest search by interrogating both Uniprot database (DB) and a customized food allergen DB built *in house* and containing approximately 150 entries (including milk, soy, egg proteins and typical protein contaminants usually detected in tryptic digest e.g. keratines). Software results reported in Table 1 were filtered by peptide mass deviation (400 ppm), and peptide confidence (high = 1% confidence). After selection of peptide markers and proper transitions, a five segments acquisition scheme was set up, screening a total of eleven peptides. Each segment counted up to three scan events, represented by selected reaction monitoring (SRM) MS/MS acquisition in which each selected peptide was isolated and activated by CID with a normalized collision energy of 35%, and ion current related to the two most intense transitions were recorded within a 3 m/z window.

3. Results and discussion

3.1. Optimization of extraction conditions

As a first step, the best conditions for the efficient extraction of total proteins from cookies were investigated, and the protein content estimated by using a Bradford assay, was chosen as discriminating factor. Protein extraction was accomplished on the same amount of milled sample kept under constant shaking at 60 °C with Tris–HCl buffer at pH = 8.2. Different conditions were tested and the following extraction parameters evaluated: (a) extraction volume, (b) extraction time and (c) addition of 0.1% (v/v) of detergent Tween 20. Our results show that the total protein content was not significantly affected by the investigated parameters, with the only exception represented by the reduction of the extraction yield evidenced when buffer volume was lowered from 20 to 10 mL (2.1 mg/mL versus 0.8 mg/mL). Also the addition of a surfactant such as Tween 20 did not show to significantly change the final protein content. Still, basing on the effectiveness of the total workflow for cookie sample purification and considering the final aim of detecting traces of allergenic proteins potentially contaminating foods, the use of 2 h long incubation with 20 mL of extraction buffer for 1 g of milled material, without surfactant addition, was preferred.

3.2. Selection of peptide markers

The first step in the development of a MS-based method for food allergen detection is the proper selection of peptide markers which should fulfil specific requirements to be considered reliable markers for the target allergen proteins. Thus, a preliminary untargeted MS analysis was carried out on an extract mixture (from milk powder, egg powder and soy flour) aimed at identifying the most abundant peptides attributed to each allergenic food under investigation, and potentially entering the list of candidate markers.

Table 1

List of proteins that correspond to the peptides identified by Proteome Discoverer™ software (version 1.3), through Sequest search of experimental Data Dependent™ MS/MS spectra against a customized food allergen database.

Allergenic ingredient	Uniprot code – protein	Score		Coverage		Peptides	
		Uniprot DB	Restricted DB	Uniprot DB	Restricted DB	Uniprot DB	Restricted DB
Soy	P19594 – 2S albumin	–	19.99	–	21.5%	–	3
	O64458 – 34 kDa maturing seed protein	–	22.42	–	13.7%	–	3
	P13916	45.83	85.17	21.0%	33.6%	6	14
	β-conglycinin-α-chain						
	P11827	17.71	51.52	7.51%	17.68%	3	8
	β-conglycinin-α'-chain						
	P25974	21.02	55.47	15.5%	27.8%	4	11
	β-conglycinin-β-chain						
	P04776 – glycinin G1	83.00	118.72	41.62%	59.2%	10	17
	P04405 – glycinin G2	62.97	125.35	32.4%	46.6%	8	14
	P11828 – glycinin G3		44.91		22.3%		8
	P02858 – glycinin G4	75.62	91.98	26.9%	33.1%	9	12
	P04347 – glycinin	41.53	67.77	18.8%	33.3%	6	11
Egg	Q9LD16		21.74		20.4%		4
	Kunitz trypsin inhibitor 3						
	P09186 – seed linoleate 9S-lipoxygenase-3	39.58		13.1%		6	
Milk	P01012 – ovalbumin	40.13	55.32	28.0%	34.5%	6	8
	P02789 – ovotransferrin		13.87		6.2%		3
Milk	P02662 – αS1-casein		38.13		32.7%		5
	P02754 – β-lactoglobulin	30.88	38.28	33.7%	41.6%	3	5

The experimental workflow implemented in this investigation is depicted in Fig. 1.

Velos Pro™ DDA mode enables untargeted or global proteomics analysis. In particular, DDA *Nth order double play* (top 20) option was used. In this frame the analyzer performs two dependent scan events. From the full MS acquired in the first DDA scan event the 20 most abundant ions were selected for subsequent MS/MS analysis sequentially of each of the 20 precursor ions in the second DDA event. In order to keep only useful information related to potential candidate peptide markers, ion charge screening was activated and only double and triple charged ions were selected for triggering CID activation.

An untargeted MS analysis of a mixture of skim milk powder (MP), egg powder (EP) and soy flour (SF) extract solution was preliminary performed with the aim to identify the most intense peptides. Raw data were processed via commercial software Proteome Discoverer™ (version 1.3) and protein assignment was accomplished by Sequest scoring algorithm by searching MS/MS spectra against a large (Uniprot) and a small sized (restricted to food allergens and other contaminants) DB, respectively. Sequest scoring algorithm, allowing to search the MS/MS spectra against a certain protein DB, can yield possible peptide identifications from which source proteins can be inferred. The protein coverage (part of the protein covered by peptide matching), protein score (sum of all peptide X_{corr} values above the specified score threshold) and number of peptides identified for each allergenic protein were retrieved by interrogating both DBs (Uniprot versus restricted “food allergen”) and are reported in Table 1. As shown, the use of a larger DB resulted in a decreased protein coverage and score as well as a lower number of matching peptides attributed to that candidate protein. For this reason and in order to reduce the time needed for DB searching, the use of a restricted DB was preferred. DB searching was run at a high confidence level by setting FDR, decoy database search, indicating a measure of certainty in the identification, down to 0.01 meaning a strict confidence (FDR = is a statistical value that estimates the number of false positive identifications among all identifications found by a peptide identification search). The peptide spectra showing the highest matching with the predicted fragmentation patterns were further validated by visual

inspection of the analyst and only those MS/MS spectra-peptide sequence matches that met the following criteria were considered confident peptide identifications: (1) all fragments with an abundance higher than 10% relative to the most abundant fragment in each MS/MS spectrum should be correctly attributed, (2) at least three consecutive peptide fragments of either y- or b-ions should be clearly detected, (3) a significant total ion current (≥ 500) of the MS/MS spectra should be recorded. After the accurate validation of peptide-matches, preferring only the most abundant peptides from the list and showing a good reproducibility ($\leq 15\%$ variance) in the same charge state, and the evaluation of a satisfactory resolution obtained by chromatographic separation, a restricted list of candidate markers was compiled along with the two most intense transitions detected. Typical MS/MS spectra acquired in DDA mode and assigned via software to peptide markers belonging to the proteins of interest β-conglycinin, ovalbumin and α-casein, are depicted in Fig. 2. This information conveyed into a tailored SRM acquisition method devised to achieve a sensitive and selective detection of the target proteins in complex food matrices. Table 2 summarizes the list of peptides along with the selected transitions included in the acquisition method. The candidate peptide markers were further BLAST searched against the UniProt DB by setting an expectation value threshold (E) of 10 (E is a statistical measure of the number of expected matches in a random database) in order to avoid false positives. In addition, MS analysis was carried out in the allergen-free cookie extract in order to confirm the absence of any interfering peak at the expected retention times.

The triple quadrupole-like fragmentation spectra and the unique configuration of the dual pressure linear ion trap mass spectrometer offered several features. On one hand the presence of a high pressure cell enabled a maximum ion trapping capacity and isolation and fragmentation efficiency at the same time thus producing a general decrease in the time required for precursor isolation. On the other hand the second cell held at a lower pressure, allowed for a faster mass analysis with increased resolution. This novel method for controlling the ion population in the trap produced a dramatic increase in the practical scan rate achievable in a typical data dependent tandem MS experiment. The greater scan rate of the Velos Pro™ allows more frequent sampling of

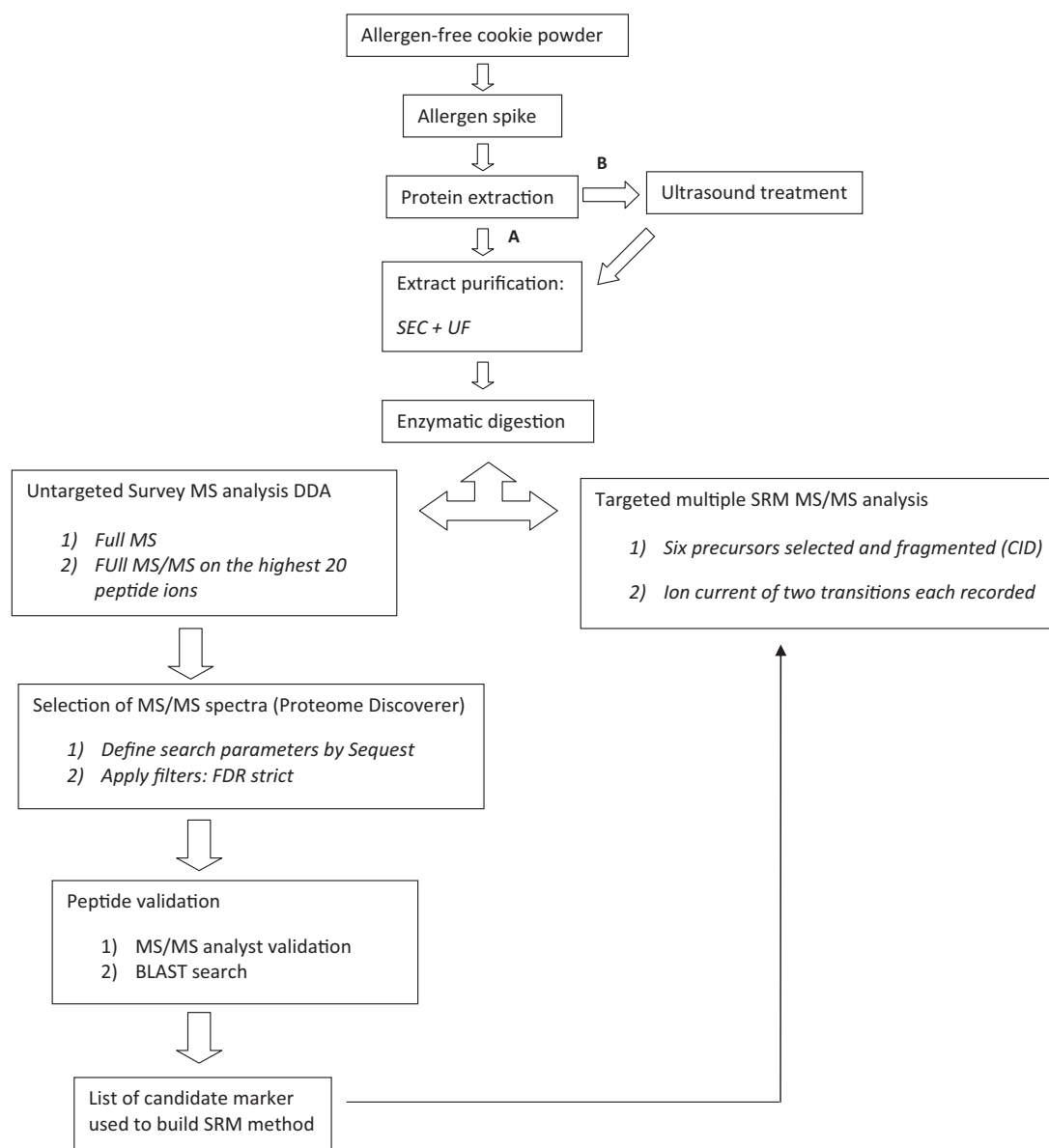


Fig. 1. Experimental workflow and double MS acquisition scheme designed for peptide markers discovery.

weak precursors resulting in a high number of peptides detected that might be used to interrogate the DB. A faster scan rate in general not only improves the duty cycle but also allows for the use of shorter chromatographic gradients favouring experimental

analysis throughput. The characteristics offered by this technology compared to other conventional linear ion trap mass spectrometers, turn beneficial for the method under development. Finally, aiming at designing a proper MS acquisition method, parameters

Table 2

Restricted list of candidate marker peptides selected for inclusion into the SRM-MS/MS acquisition method, with the relevant two-most intense transitions (P04405: glycine G2, P13916: β -conglycinin- α -chain, P02858: glycine G4, P01012: ovalbumin, P02754: β -lactoglobulin, P02662: α s1-casein).

Protein	Peptide sequence	X_{Corr}	m/z (charge)	Selected transitions	
Glycinin G2 β -Conglycinin- α -Chain Glycinin G4	VFDGELQEGGVLPQNFVAFAAK	5.53	1201.14 (+2)	y_9^+ 945.52	y_{10}^+ 1044.58
	ESYFVDAQPK	3.39	592.29 (+2)	y_5^+ 558.29	y_6^+ 657.36
	TISSEDKPFNLR	3.96	703.87 (+2)	y_5^+ 646.37	y_{10}^+ 1192.60
	AIPSEVLAHSYNLR	3.92	785.42 (+2)	y_7^+ 860.44	y_{12}^{+2} 693.36
Ovalbumin	EVVGSAAEAGVDAASVSEEF	7.03	1004.98 (+2)	y_{10}^+ 1110.51	y_{12}^+ 1266.60
	GGLEPINFQTAADQAR	5.82	844.42 (+2)	y_{12}^{+2} 666.34	y_{10}^+ 1121.53
	LTEWTSNNVMEER	4.44	799.36 (+2)	y_8^+ 867.41	y_9^+ 1068.46
	YPILPEYLQCVK	3.72	761.90 (+2)	y_9^+ 1149.60	y_8^+ 1036.51
β -Lactoglobulin	TPEVDDEALEK	3.95	623.30 (+2)	y_7^+ 819.37	y_{10}^+ 572.77
α s1-Casein	YLGYLEQLLR	3.61	634.36 (+2)	y_6^+ 771.47	y_8^+ 991.56
	FFVAPFPEVFCK	3.52	692.87 (+2)	y_8^+ 920.50	y_9^+ 991.52

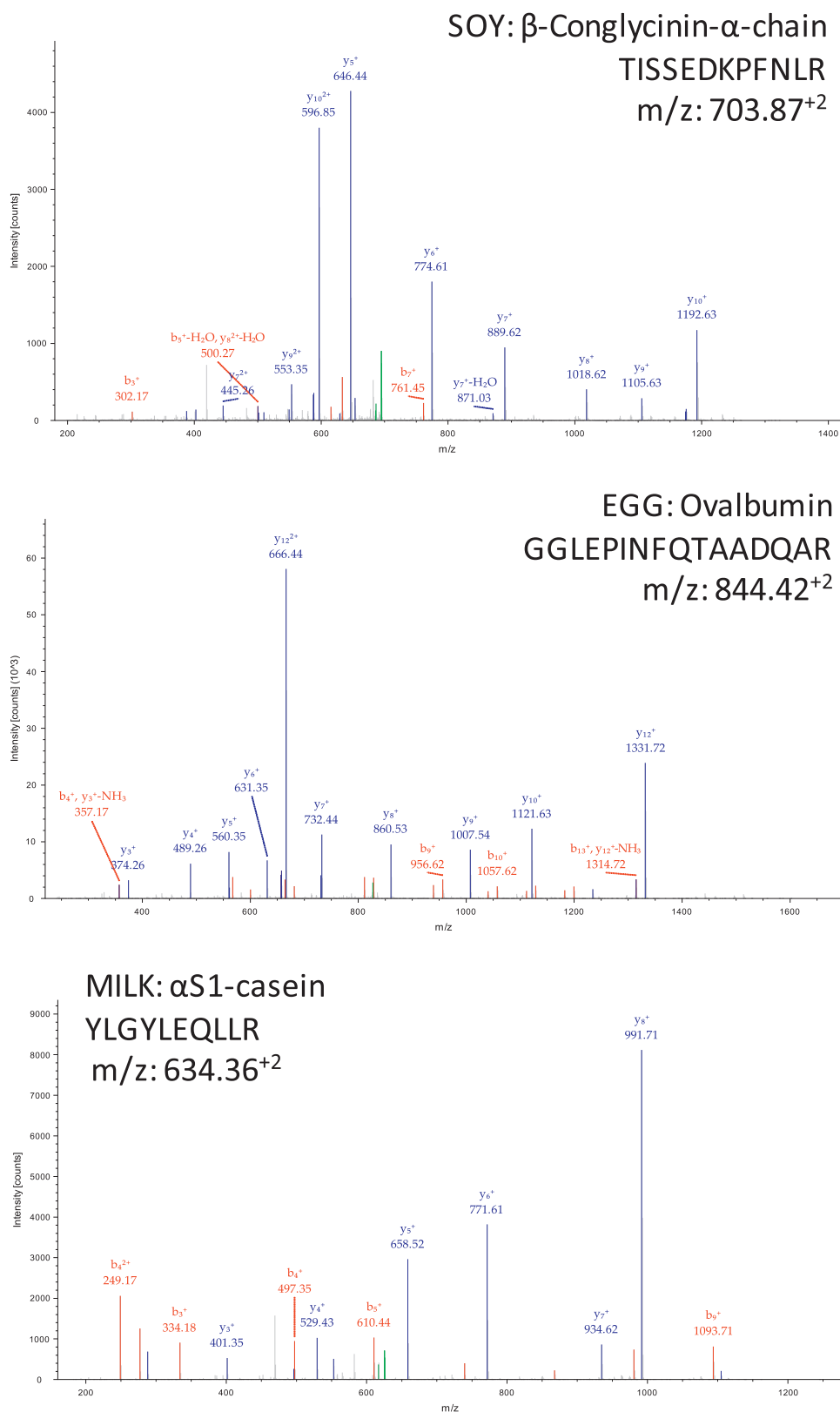


Fig. 2. Typical MS/MS spectra acquired in DDA mode and assigned via Sequest software to specific peptides belonging to soy, milk and egg proteins.

such as number of segments along the LC run, number of scan events per segment, acquisition scan rate (and consequently resolution $M/\Delta M$) and acquisition range, were optimized in order to find a good compromise among speed of execution, MS spectra resolution and chromatograms trace definition, i.e. number of

acquisition points per peak. Basing on the expected elution time for each peptide marker, the eleven peptides included into the SRM method were separated into five segments, with a maximum of three scan events per segment in which each selected peptide marker was isolated and activated by collision induced dissociation

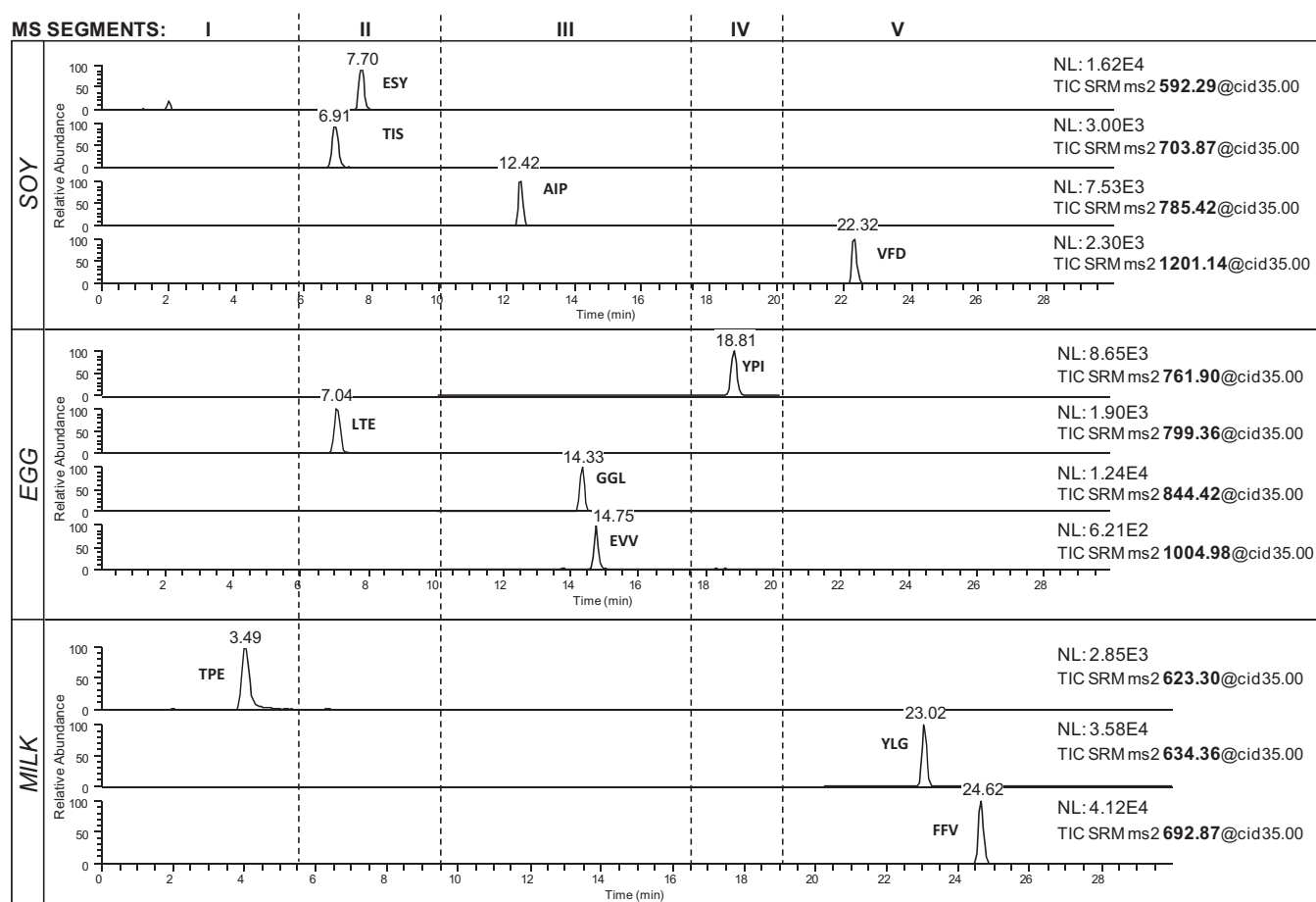


Fig. 3. Typical reconstructed ion chromatograms recorded in SRM-MS/MS acquisition mode for the selected list of peptide markers shown in Table 2. The dashed lines denote the five acquisition time segments into which the entire chromatographic run was divided.

and ion current related to the two most intense transitions recorded within a 3 m/z window. A typical representation of a multi-target analysis achieved in the SRM mode is shown in Fig. 3 where an overlay of eleven chromatographic traces is reported, and was measured by SRM monitoring the most abundant transitions. As shown, the chromatographic conditions yielded excellent peptide separation.

3.3. Workflow design and effect of sonication

After identification of suitable peptide markers, the following step aiming at developing an efficient and sensitive analytical method for the simultaneous screening of allergens in cookies, consisted in designing a proper purification protocol for protein extracts aimed at reducing the contribution of matrix components on the sensitivity and selectivity of the final detection method. With this aim, artificially contaminated cookies were prepared by addition of EP, MP and SF stock solutions to milled cookies. After overnight solvent evaporation, samples were extracted under the optimized conditions (see previous section) and subjected to purification. The scheme reported in Fig. 1 (route A) reports the workflow of the extraction/purification protocol referred to contaminated cookies. Protein extracts were purified by a combination of size exclusion chromatography (SEC) on disposable cartridges, with ultrafiltration of SEC eluates. This additional step aimed at removing the majority of interferences with molecular weight lower than 5 kDa and followed by pre-concentration of the final depleted protein extract. By implementing this procedure, followed by tryptic digestion of dialyzed ultrafiltrates, it was possible to confirm in all spiked samples, the presence of a subset of peptides corresponding

to the target proteins already identified into each stock solution previously analyzed. Besides, in order to improve the sensitivity of the whole analytical method, an additional step based on the ultrasound treatment with a 6 mm probe of the cookie extract was introduced along the workflow (see route B in Fig. 1). The goal of this sample manipulation step was disrupting protein aggregates eventually formed, with a consequent increase of the final protein yield and the expected peptides generated through the enzymatic digestion. Milled cookies fortified with the three allergenic ingredients at a fixed concentration level were extracted and two sub-samples were treated differently. In one route (A), protein extract was subjected to the purification procedure while in the other (route B) the extract underwent 10 min pulsed sonication by probe immersion with an output of 14 W before purification. Both sub-samples were afterwards processed (SEC and ultrafiltration) and digested in the same conditions.

By comparing integrated peak areas of SRM-MS/MS chromatographic traces recorded for both kind of sub-samples, it appeared that the introduction of the ultrasound step along the sample-preparation workflow, resulted in a considerable increase of milk allergen protein peptide markers (referred to α -S₁-CN). This is clearly shown in Fig. 4 that depicts peak areas referred to each representative allergen peptide marker monitored. The observed signal enhancement, especially highlighted for milk allergens, reflects the disruption action promoted by the ultrasound treatment taking place on the whole extract with a clear disaggregation of eventual casein micelles that might affect the enzymatic digestion yield and consequently impair method sensitivity.

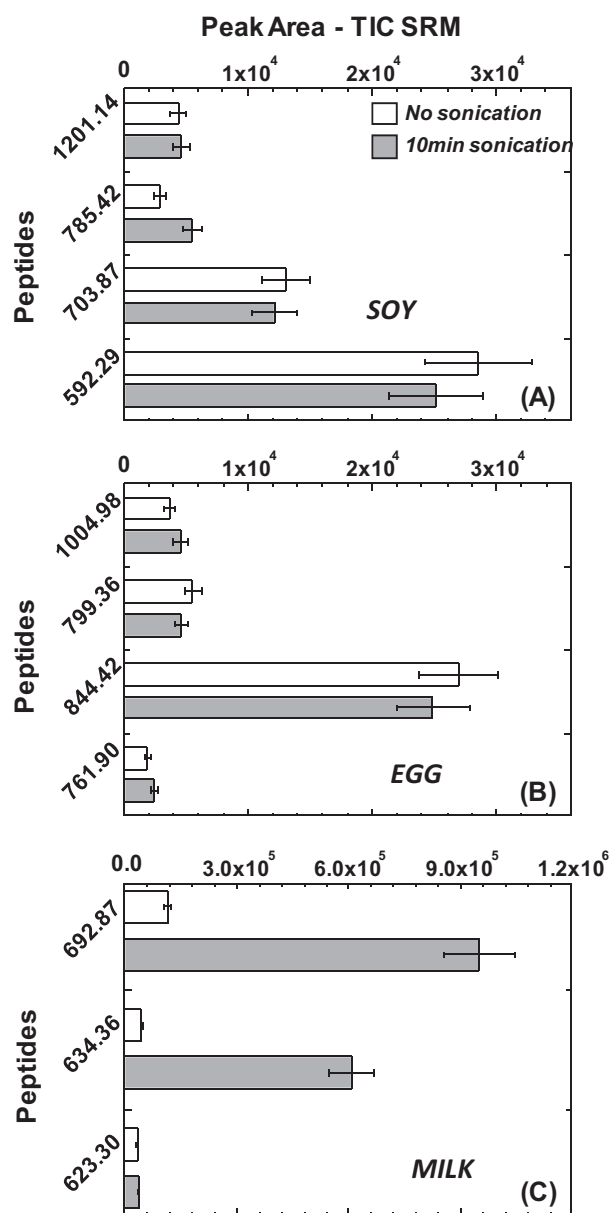


Fig. 4. Evaluation of the effects of sonication on the detection sensitivity for soy (A), egg (B) and milk (C) peptide markers.

3.4. Evaluation of the analytical method performances: linearity, sensitivity and recovery

Finally, the performance of the developed analytical method was assessed. A set of artificially contaminated samples with increasing amount of each allergenic ingredient was prepared in the concentration range of 1–20 µg/g for EP, 0.5–20 µg/g for MP and 10–100 µg/g for SF (µg of allergenic proteins/g of cookie matrix). The whole set of samples was processed simultaneously according to route B of Fig. 1 and the relevant chromatographic runs recorded. Reporting integrated peak areas versus the spike concentrations, calibration curves with R^2 better than 0.98 were obtained for milk and egg detection while values comprised between 0.92 and 0.96 were obtained for soy allergens depending on the specific peptide. The response linearity was assessed on the whole concentration range under investigation and limits of detection (LOD) and quantification (LOQ) were estimated as the minimum concentration of an added allergenic ingredient that can be detected, at S/N equals

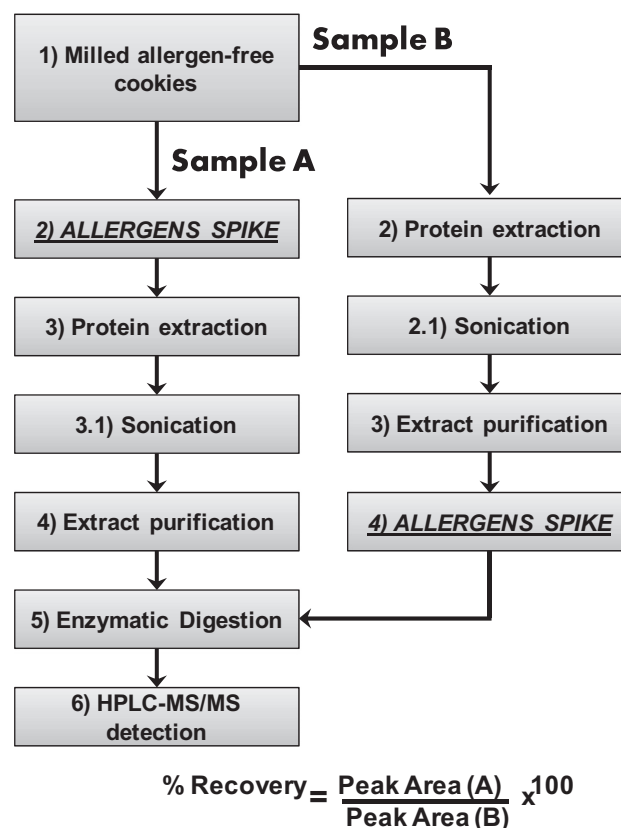


Fig. 5. Scheme of the workflow followed for extraction, purification and analysis of artificially contaminated cookie samples and for recovery estimation.

3 and 10, respectively (the standard deviation calculated on ten replicates of the lowest detected concentration was here used as an estimate of noise). Among the list of eleven peptide markers included into the SRM-MS/MS instrument method, the two most sensitive peptides for each allergenic food category were highlighted and assumed to be most reliable markers for quantitative purposes.

Table 3 reports an overview of method performances for these six peptides. As shown, couples of limit values for each allergenic ingredient resulted very similar irrespective of the specific marker monitored, thus confirming the reliability of both peptides selected for quantitative analysis. Besides, LODs were found to be quite different for each allergenic ingredient under consideration, ranging from 2 µg/g for soy to 0.3 µg/g for egg until the highest sensitivity obtained for milk down to 0.12 µg/g (allergenic protein content/food matrix).

Finally, recovery of the whole analytical method was assessed. Ad hoc experiments were performed and two types of samples were produced as pictured in Fig. 5: (A) allergen-free cookie preliminary fortified with the three allergenic ingredients (at 5 µg/g for milk and egg proteins and 50 µg/g for soy proteins) and then submitted to the whole pre-treatment procedure and (B) allergen-free cookie processed in the same way and fortified only after purification with an amount of each allergenic ingredient corresponding to the theoretical 100% recovery. Both samples (A and B) were then subjected to digestion and from the ratio of peak areas for each peptide marker, the actual recovery percentage was calculated. Again, values varied considerably according to the allergenic food ingredient considered. In general, they were calculated to be ranging from 23% for soy markers to 40 and 45% for egg and milk markers, respectively. This finding is in agreement with the higher limit of detection and quantification calculated for soy peptides

Table 3
Overview of method performances.

Peptide	Sequence (allergen)	LOD	LOQ	Slope	R ²
592.29	ESYFVDAQPK (soy)	2 µg/g	8 µg/g	(3.0 ± 0.2)*10 ³	0.927
703.87	TISSDKPFNLR (soy)	2 µg/g	7 µg/g	(9.5 ± 0.9)*10 ²	0.964
799.36	LTEWTSSNVMEER (egg)	0.3 µg/g	1 µg/g	(7.1 ± 0.4)*10 ³	0.984
844.42	GGLEPINFQTAAQAR (egg)	0.3 µg/g	1 µg/g	(2.82 ± 0.10)*10 ⁴	0.994
634.36	YLGYLEQLLR (milk)	0.14 µg/g	0.5 µg/g	(7.5 ± 0.2)*10 ⁴	0.994
692.87	FFVAPFPEVFGK (milk)	0.12 µg/g	0.4 µg/g	(7.6 ± 0.3)*10 ⁴	0.989

compared to the other two allergenic food proteins. In lights of these results an improvement of the extraction and/or purification protocol, especially tailored to soy proteins analysis, should be envisaged to increase the overall recovery of these food proteins.

4. Conclusions

Performance of a MS analytical method based on a linear ion trap dual cell system was assessed, for the simultaneous detection of soy, egg and milk allergens at the trace levels of few µg/g in cookie, chosen as complex reference matrix. After identification of the most reliable markers and the relative transitions, the method was duly optimized in order to maximize the ion signals for milk, egg and soy allergen detection in cookies spiked with increasing amounts of allergenic food. Very challenging LODs were obtained by following this approach, comprised between 0.12 and 0.3 µg/g referred respectively to the protein content of milk and egg ingredients in cookie, while a LOD of 2 µg/g was calculated for soy. However, in order to confirm the reliability of such markers and sensitivity of this method also in processed foods, an in thorough investigation should be undertaken also extended to allergen-incurred cookies where the processing might affect the overall sensitivity of the method. In conclusion, flexibility and speed scanning capabilities provided by such MS configuration allowed to perform scouting untargeted global experiments for identification of the suitable peptide markers and the relevant peptide transitions to be further implemented in a highly selected multi-target SRM screening method for achieving the desired sensitivity. All these features enabled to considerably streamline the time requested for these two essential steps demonstrating very good potentials for its applicability in food monitoring as a valuable tool for multi-allergen analysis.

Acknowledgements

Dr. Rossella Carone is kindly acknowledged for her technical assistance during sample preparation. The authors thank Dr. Dipankar Ghosh (Thermo Fisher Scientific, San José, USA) for providing the analytical column implemented in this work and Prof. Francesco Palmisano for the fruitful discussions and for the interest shown in this work. The work was funded by the project Safe&Smart – Nuove tecnologie abilitanti per la food safety e l'integrità delle filiere agro-alimentari in uno scenario globale – National CLAN-Cluster agroalimentare nazionale programma area 2.

References

- [1] R.S. Gupta, E.E. Springston, M.R. Warrier, B. Smith, R. Kumar, J. Pongracic, J.L. Holl, The prevalence, severity, and distribution of childhood food allergy in the United States, *Pediatrics* 128 (2011) e9.
- [2] L. Monaci, A. Visconti, Allergens, in: Y. Picó (Ed.), *Chemical Analysis of Food: Techniques and Applications*, Academic Press, MA, USA, 2012, p. 695.
- [3] European Commission, Commission Directive 2007/68/EC, Off. J. Eur. Union 310 (2007) 11.
- [4] L. Monaci, A. Visconti, Immunochemical and DNA-based methods in food allergen analysis and quality assurance perspectives, *Trends Food Sci. Technol.* 21 (2010) 272.
- [5] G. Picariello, G. Mamone, F. Addeo, P. Ferranti, The frontiers of mass spectrometry-based techniques in food allergenomics, *J. Chromatogr. A* 1218 (2011) 7386.
- [6] L. Monaci, A. Visconti, Mass spectrometry-based proteomics methods for analysis of food allergens, *TrAC* 28 (2009) 581.
- [7] A. Cryar, C. Pritchard, W. Burkitt, M. Walker, G. O'Connor, D.T. Burns, M. Quaglia, 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, *J. AOAC Int.* 96 (2013) 1350.
- [8] B. Pöpping, S.B. Godefroy, Allergen detection by mass spectrometry—the new way forward, *J. AOAC Int.* 94 (2011) 1005.
- [9] L. Monaci, I. Losito, F. Palmisano, A. Visconti, Reliable detection of milk allergens in food using a high-resolution, stand-alone mass spectrometer, *J. AOAC Int.* 94 (2011) 1034.
- [10] J. Heick, M. Fischer, B. Pöpping, First screening method for the simultaneous detection of seven allergens by liquid chromatography mass spectrometry, *J. Chromatogr. A* 1218 (2011) 938.
- [11] P. Lutter, V. Parisod, H. Weymuth, Development and validation of a method for the quantification of milk proteins in food products based on liquid chromatography with mass spectrometric detection, *J. AOAC Int.* 94 (2011) 1043.
- [12] L. Monaci, I. Losito, F. Palmisano, M. Godula, A. Visconti, Towards the quantification of residual milk allergens in caseinate-fined white wines using HPLC coupled with single-stage Orbitrap mass spectrometry, *Food Addit. Contam.* 28 (2011) 1304.
- [13] C. Bignardi, L. Elviri, A. Penna, M. Careri, A. Mangia, Particle-packed column versus silica-based monolithic column for liquid chromatography–electrospray–linear ion trap–tandem mass spectrometry multiallergen trace analysis in food, *J. Chromatogr. A* 1217 (2010) 7579.
- [14] C. Bignardi, M. Mattarozzi, A. Penna, S. Sidoli, L. Elviri, M. Careri, A. Mangia, A rapid size-exclusion solid-phase extraction step for enhanced sensitivity in multi-allergen determination in dark chocolate and biscuits by liquid chromatography–tandem mass spectrometry, *Food Anal. Methods* 6 (2013) 1144.
- [15] L. Monaci, J. Nørgaard, A.J. van Hengel, Feasibility of a capillary LC/ESI-Q-TOF MS method for the detection of milk allergens in an incurred model food matrix, *Anal. Methods* 2 (2010) 967.
- [16] L. Monaci, I. Losito, E. De Angelis, R. Pilolli, A. Visconti, Multi-allergen quantification of fining-related egg and milk proteins in white wines by high-resolution mass spectrometry, *Rapid Commun. Mass Spectrom.* 27 (2013) 2009.
- [17] D. Weber, P. Raymond, S. Ben-Rejeb, B. Lau, 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, *J. Agric. Food Chem.* 54 (2006) 1604.
- [18] P.E. Johnson, S. Baumgartner, T. Aldick, C. Bessant, V. Giosafatto, J. Heick, G. Mamone, G. O'Connor, R. Poms, B. Pöpping, A. Reuter, F. Ulberth, A. Watson, L. Monaci, E.N. Mills, Current perspectives and recommendations for the development of mass spectrometry methods for the determination of allergens in foods, *J. AOAC Int.* 94 (2011) 1026.
- [19] A. Goma, S. Ribereau, J. Boye, Detection of allergens in a multiple allergen matrix and study of the impact of thermal processing, *J. Nutr. Food Sci.* (2012) S9.