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Particle-packed column *versus* silica-based monolithic column for liquid chromatography–electrospray-linear ion trap-tandem mass spectrometry multiallergen trace analysis in foods

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ABSTRACT

A bicarbonate buffer-based extraction method for the simultaneous analysis of five nut allergens (Ana o 2, cashew-nut; Cor a 9, hazelnut; Pru 1, almond; Ara h3/4, peanut; Jug r 4, walnut) in cereals and biscuits using liquid chromatography–electrospray-linear ion trap-tandem mass spectrometry (LC–ESI-LIT-MS^2) was developed and validated. The method was based on our earlier published LC–MS^2-based method in a research frame aimed at the identification and determination of hidden allergens in foods by using selective biomarker peptides. A C18 particle-packed column and a silica-based C18 monolithic column were compared in terms of chromatographic performances, such as peak shape, resolution, analysis time and selectivity. The C18 particle-packed column exhibited better performances and was further used for method development and validation. By operating under MS^2 selected reaction monitoring (SRM) acquisition mode, linearity, limits of detection (LOD) and quantitation, trueness and precision were evaluated on breakfast samples enriched with a mix of the five nuts. Good linearity of the matrix matched-calibration curves was obtained and detection limit values generally varied from 14 to 55 mg nut/kg matrix. Recoveries were in the $76 \pm 4\%$ to $94 \pm 3\%$ range with RSD <15%. The capabilities of LIT to perform MS^n fragmentation was exploited to improve selectivity of the analysis, and the LC–(SRM) MS^2 method was compared in terms of LOD, linearity, precision and accuracy with a LC–(SRM) MS^3 method. Finally, both the LC–MS^2 and LC–MS^3 methods were successfully applied to the analysis of nut traces in commercially available breakfast cereals and biscuits.

1. Introduction

The presence in foods of undeclared allergenic ingredients or hidden allergens arising from accidental contamination can cause serious reactions to sensitised individuals and clearly represents a growing food-safety issue. In the European Union (EU) food-labeling regulations have recently been revised and the labeling of several allergenic ingredients is now mandatory (Directives 2003/89/EC and 2007/68/EC) [1–3]. However, little is known about the so called threshold doses, i.e. the minimum amount of an allergenic food which is able to cause an allergic reaction. For these reasons, the development of selective and sensitive analytical methods for allergen analysis at the trace level is recommended in order to improve food labeling directives and to increase consumer protection.

The most commonly methods used for the detection of allergens in foods have been reviewed [4,5] and include immunoblotting, rocket immunoelectrophoresis, radio-allergosorbent test, radioimmunoinhibition assay, enzyme-linked immunosorbent assay (ELISA), and liquid chromatography–mass spectrometry (LC–MS). In the last years, increasing emphasis has been put on the development of confirmatory methods based on the use of liquid chromatography–electrospray-tandem mass spectrometry (LC–ESI-MS/MS) techniques [6–9]. In such a context, our research group successfully developed and validated innovative confirmatory and screening mass spectrometry based-methods for the identification and determination of hidden peanut allergens in foods. By using a shotgun proteomic approach, peanut proteins were enzymatically degraded to peptides and two selected biomarker peptides for each allergen were then analyzed under selected reaction monitoring (SRM) mode in a triple quadrupole or a linear ion trap (LIT) mass analyzer [10–12].

In this work, our attention was paid to the evaluation of the LC–LIT-MS/MS capabilities to obtain useful and simultaneous detection and quantification of five different hidden allergens across different food samples in a single short run. Ana o 2 (cashewnut), Cor a 9 (hazelnut), Pru 1 (almond), Jug r 4 (walnut) and Ara h3/4 (peanut) proteins were investigated. These

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no-glycosylated seed storage proteins belong to the 11S-globulin family (Cupin superfamily) that accounts for a number of known major food allergens and are of interest to both the public and industry due to food safety concerns.

Taking into account that the chromatographic separation plays a fundamental role in the MS analysis of complex peptide mixtures, two different chromatographic columns (i.e. C18 particle-packed column and a silica-based C18 monolithic column) were compared in terms of resolution, peak shape and analysis time before MS method development. As for MS acquisition mode, the non-scanning nature of SRM analysis, usually performed on a triple quadrupole mass spectrometer allows to obtain excellent sensitivity and enables the detection of low-abundance proteins in highly complex mixtures. In the IT instruments, collision induced dissociation (CID) experiments allow to perform single-step fragmentation and to obtain product ions that are not subjected to further fragmentation. Thus, the analysis of large peptides by SRM in LIT mass spectrometers can be performed with improved detection limits owing to the formation of fewer but more intense product ions. In addition, the capability of LIT-MS of performing MS^n experiments allows to obtain increased selectivity on the target peptides in complex matrices. In this work, using one-dimensional chromatographic separation and linear ion trap MS detection, the quantitation of five allergens by SRM under MS^2 mode was evaluated in terms of sensitivity, selectivity, accuracy, recovery, limits of detection and quantitation. Performance of the LC–ESI-LIT-MS method under MS^3 acquisition mode was also assessed.

Finally, the LC–MS^2 and LC–MS^3 methods were applied to commercially available samples and the quantitative results were compared.

2. Experimental

2.1. Chemicals

Bicarbonate buffer (pH 8.0), acetonitrile (HPLC purity), trifluoroacetic acid (TFA), formic acid (analytical reagent grade) were purchased from Carlo Erba (Milan, Italy). Trypsin from bovine pancreas, Bradford reagent, leukine-enkefaline acetate hydrate were from Sigma-Aldrich (St. Louis, Missouri, USA).

Roasted peanuts (*Arachis hypogea*), walnuts (*Juglans regia*), hazelnuts (*Corylus avellana*), almonds (*Prunus dulcis*) and cashewnut (*Anacardium occidentale*), three different commercial biscuits and five breakfast cereals samples (cornflakes, breakfast cereals based on cereal flakes and dried fruit, cereal mix with fruit and chocolate) were obtained at a local food store. All the products analyzed reported the precautionary label “may contain trace of nuts, milk and soy”.

2.1.1. Sample treatment

Fortified samples were prepared by adding the five ground nuts at different amounts and by homogenizing the mixture before weighting and extraction.

Nuts-containing food extracts were prepared by adding 10 mL of 50 mM bicarbonate buffer (pH 8.0) to 1 g of ground sample. The efficiency of the protein extraction procedure was initially evaluated by varying the extraction time (2 h, 4 h and 6 h), extraction temperature (37 °C, 60 °C) and by quantifying the total protein content using a Bradford assay. The best results were obtained by performing a 6 h extraction at 60 °C.

Proteins were extracted by shaking for 6 h at 60 °C, then the extract was centrifuged (4000 g, 20 min) and filtered on 0.2 μm nylon filter before tryptic digestion. Enzymatic digestion was performed by adding a trypsin solution (12.5 μL, 200 μg/mL) to 100 μL of protein extract in order to obtain a protein:trypsin ratio 50:1 and carried out at 50 °C overnight. The digestion reaction was quenched with 2 μL TFA.

2.2. Liquid chromatography–ion trap mass spectrometry

LC separation was carried out on a C18 Kinetex (100 mm × 2.1 mm, 2.7 μm particles) (Phenomenex, CA, USA) column thermostated at 25 °C using a gradient solvent elution system [(A) aqueous formic acid 0.1% solution (v/v)/(B) 0.08% (v/v) formic acid in acetonitrile]. Gradient elution was as follows: solvent B was set at 5% for 3 min, then delivered by a linear gradient from 5% to 20% in 6 and to 60% in 1 min. Solvent B was maintained at 60% for 1 min before column reequilibration (5 min). The flow-rate was 250 μL/min. The same gradient was used to elute peptides on a C18 Chromolith Performance column (100 mm × 2 mm) (Merck KGaA, Darmstadt, Germany) at a flow-rate of 350 μL/min. The mobile phase was delivered by the Surveyor chromatographic system (ThermoElectron Corporation, San Josè, CA, USA) equipped with a 200-vial capacity sample tray. Injection volume was 10 μL.

A LTQ XL linear ion trap instrument (ThermoElectron Corporation) equipped with a pneumatically assisted ESI interface was used. The system was controlled by the Xcalibur software.

The sheath gas (nitrogen, 99.999% purity) and the auxiliary gas (nitrogen, 99.998% purity) were delivered at flow-rates of 45 and 5 arbitrary unit, respectively.

Optimized conditions of the interface were as follows: ESI voltage 3.5 kV, capillary voltage 20 V, capillary temperature 270 °C. MS^2 and MS^3 experiments were performed under both product-ion and SRM conditions with a collision gas (He) pressure of 2.1×10^{-3} mbar in the collision cell. In the product-ion scan mode the 200–1600 m/z range was monitored. The SRM transitions monitored were reported in Table 1. For quantitative purposes, the most intense SRM transition was monitored for each allergen source, whereas the other transitions reported in Table 1 were monitored for confirmatory purposes.

2.3. Method validation

Validation of the whole analytical procedure was performed under MS^2 mode on fortified samples of breakfast cereals (mix of cereal flakes) according to Eurachem guidelines [13]. For this purpose, breakfast cereal samples were fortified with different nuts amounts and measurements were carried out by monitoring the most abundant MS/MS transition for each peptide (Table 1). The detection limits (LOD) and the quantitation limits (LOQ) were calculated from the calibration curve as 3 s/slope and 10 s/slope, respectively, where s is the standard deviation of the blank signal obtained by performing 10 independent blank measurements. Linearity was assessed over suitable mg nuts/kg matrix ranges, starting from LOQ values. Precision was evaluated as RSD for each compound in terms of intra-day repeatability and intermediate precision (inter-day repeatability). For this purpose, the within day repeatability was evaluated by performing three independent extractions of the matrix fortified with 50 and 100 mg nuts/kg matrix and three LC–MS^2 injections for each extract in the same day and the inter-day repeatability was calculated on five days by performing five independent extractions of the matrix fortified with 50 and 100 mg nuts/kg matrix and three LC–MS^2 injections for each extract. The matrix effect was assessed by using the recovery function. The matrix-matched calibration curve was obtained by analyzing the matrix extracts fortified with a mixture of the five nuts at six concentration levels and treated applying the whole analytical procedure. Each level was analyzed three times. The recovery of the protein extraction procedure was evaluated by performing a 6 h extraction at 60 °C on the blank matrix and on the matrix fortified at two different concentration levels (50 and 100 mg nuts/kg

Table 1
Allergen source, target peptides and SRM transitions monitored in MS^2 and MS^3 mode.

Allergen (*source*, UNIPROT ID)	Peptide	SRM transitions (*m*/*z*) (CE 30 eV)	
		MS^2	MS^3
Ana o 2 (*Anacardium occidentale*, Q8GZP6)	VFDGEVR	412/575[a] 412/460	575/460[a]
	ADIYTPEVGR	561/552 561/658	552/459
Cor a 9 (*Corylus avellana*, Q8W1C2)	ADIYTEQVGR	577/689 577/567	689/646[a]
	QEWER	374/365[a] 374/490	365/490
Pru 1 (*Prunus dulcis*, Q43607)	QQGQQEQQQER	694/685 694/677	685/677[a]
	QQEQLQQER	594/585[a] 594/576	585/576
Ara h3/4 (*Arachis hypogea*, Q8LKN1)	SPDIYNPQAGSLK	695/977[a] 695/815	977/700[a]
	YQQQSR	406/518 406/397	518/500
Jug r 4 (*Juglans regia*, Q2TPW5)	LDALEPTNR	516/487[a] 516/616	487/469[a]
	EFQQDR	412/403 412/290	403/387

[a] *m*/*z* transition monitored for the calculation of the validation parameters.

matrix). The total protein content was determined by using a Bradford assay.

3. Results and discussion

3.1. In silico selection of biomarker peptides

In this work the proteins that target the five allergen sources were initially selected on the basis of previous experiments and the scientific literature. The following major allergen proteins for the five nuts under investigation were selected: Ana o 2 (cashewnut), Cor a 9 (hazelnut), Pru 1 (Almond), Ara h3/4 (peanut), Jug r 4 (walnut). For all the allergens investigated no isomeric forms from the same gene are reported in the database sequences (http://www.uniprot.org/). As for biomarker peptide selection, different criteria, such as absence of missed cleavages, good ESI sensitivity, no post-translational modification sites and sequence specificity, were considered. Thereby, by analyzing a raw tryptic digest extract from each nut, a subset of peptides was directly identified by the mass spectrometer in a data-dependent acquisition mode to characterize each selected protein with a sequence coverage average of ~35%.

For each targeted protein, those peptides providing good ESI-MS responses and unequivocally identifying the target protein were selected. For this purpose, BLAST (Basic Local Alignment Search Tool; www.ncbi.nml.nih.gov link NCBI BLAST) search was performed (algorithm: blastp; MATRIX PAM 30; GAP COASTS: existence 10, extension 1; DATABASE: non-redundant protein sequences) in order to evaluate the identity of each peptide with all the similar peptides present in the databases. To develop a reliable quantification method, two peptides were selected and monitored for each targeted protein (Table 1). In the case of the peptides containing a Q or E residue at the N-term, an in-source deamination or dehydratation process was found to occur before trap isolation. In a further step, product-ion LIT measurements were carried out on the biomarker peptides by varying collision energy (from 20 to 35 eV) to select specific SRM transitions for each biomarker peptide. The MS^2 and MS^3 spectra exhibited several fragments of the y- and b-series to cover and confirm their sequences. Attention was paid to the selection of those fragment ions that provide optimal signal intensity and that could discriminate the targeted peptides from other species present in the sample. The definitive assay was constituted of a series of transitions (precursor/fragment ion pairs) in combination with the retention time for each targeted peptide as reported in Table 1.

3.2. Packed versus monolithic column

For MS analysis of complex peptide mixture, suitable separation methods have to be developed to improve resolution, sensitivity and analysis time. With the aim of devising a separation method suitable for the MS detection of the ten biomarker peptides in complex samples, the separation performances of a C18 column packed with 2.6 μm particles, and a C18 monolithic column having the same length and internal diameter were compared. Generally, C18 monolithic columns allow to apply fast gradient at higher flow-rate with lower backpressure, obtaining fast analysis and excellent peak shape.

By analyzing a nut protein aqueous solution with the C18 monolithic column, the LC–IT-MS^2 elution profile (within 8 min) of the peptides resulted to match that observed using the C18 silica particle-packed column (within 9 min) (Figs. 1 and 2). The chromatographic profile of the ten peptides on the monolithic column showed both excellent peak shape (in-run-peak width (FWHM, average on the ten peptides) = 3.60 ± 0.07 s) and retention time stability (RSD < 2.3%). Also in the case of the C18 packed-particle column, satisfactory results in terms of in-run-peak width (5.10 ± 0.08 s) and retention time variation (RSD < 0.9%) were obtained. As for resolution, the two columns exhibited very similar values, ranging from 0.7 to 14 for the particle-packed column and from 0.8 to 12 for the monolithic column. The quantification of the tryptic digest on the monolithic column (flow-rate 350 μL/min) without flow-splitting evidenced approximately a 40% sensitivity reduction (average value for all the peptides) with respect to the particle-packed column (flow-rate 250 μL/min). As for quantitative results, selectivity of the two columns in terms of matrix effect (i.e. signal enhancement/suppression) was also investigated. For this purpose, an aqueous tryptic digest (n = 3, n = number of independent samples) and a sample tryptic digest (n = 3) were analyzed. The results evidenced a signal suppression degree ranging from −65% to

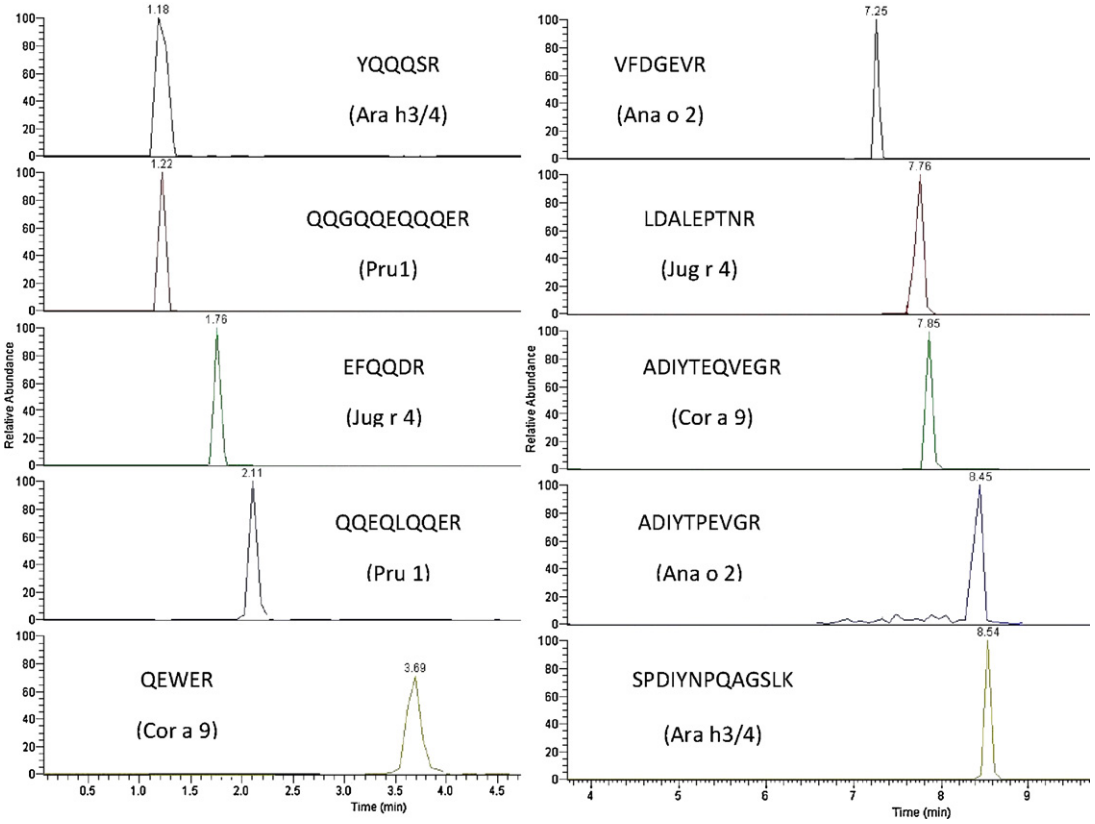

Fig. 1. LC–ESI-LIT-MS^2 separation of the ten targeted peptides from a matrix (cereals) tryptic digest on the C18 particle-packed column (100.0 mm × 2.1 mm, 2.6 μm). Blank matrix was fortified with a mixture of the five nuts 0.01% (w/w). Mobile phase: formic acid aqueous solution (0.1%, v/v) / acetonitrile (for gradient elution see Section 2), flow-rate 250 μl/min, injection volume: 10 μL.

−2% for the monolithic column for all the peptides, whereas in the case of the C18 particle-packed column the signal variation ranged from −58% to +12%. In particular, by using the particle-packed column three of 10 peptides exhibited a signal enhancement from +5% to +12%. On the basis of these findings, the particle-packed column that allowed to obtain a rapid LC separation of the analyzed peptides while reducing solvent consumption was selected for further validation studies.

3.3. LC–MS^2 method validation

The primary goal of this work was to validate a sensitive and robust LC–MS^2 method for the analysis of allergens at trace levels in foods. For this purpose, studies on linearity, trueness, precision, selectivity and recovery were performed.

Even if little is known about the minimum concentration level able to provide severe allergy reaction, detection limits between 1 and 100 mg of allergenic protein per kg food are accepted for all the analytical methods [4]. By operating under MS^2 SRM conditions, very good LOD values in the 10–55 mg allergen/kg food were determined from the matrix-matched calibration curves. LOQ values were found in the 37–180 mg allergen/kg food range (Table 2).

Generally, the LC–ESI-MS/MS linear range was explored over one order of magnitude of concentration for all the peptides (Table 2). After testing significance of the intercept (p value lower than 0.05 at 95% confidence level), linearity was mathematically verified by applying the Mandel fitting test. A p value higher than 0.05 demonstrated that the best data fit could be obtained using a first order regression model. Homogeneity of variance of replicates at different concentration levels was proved at 95% confidence level ($p > 0.05$). The method accuracy was then tested both in terms of precision and trueness. Excellent precision in terms of intra-day repeatability was calculated providing RSD% in the 3–10% ($n = 9$) range. The intermediate precision results were found not exceed 15% ($n = 15$), confirming good method precision. As for trueness, a calculation of the recovery function was performed to ascertain the influence of the matrix for the determination of all the peptides under investigation. The slope and the intercept of the recovery functions calculated for the analytes were compared respectively with 1 and 0 by means of a t-test. The t-test performed on the intercept provided a p value at the 95% confidence level higher than 0.05 ($p = 0.145$) demonstrating that the calibration equation is in the $y = b_1 x$ form and thus the absence of constant systematic errors. In the case of the slope, since the t-calculated resulted to be higher than the t-tabulated at the 95% confidence level (1.86), it can be inferred that the calibration curve obtained by spiking samples are significantly different from that obtained using standard solutions, except for the VFDGEVR (Ana o 2) peptide. In the case of the SPDIYNPQAGSLK (Ara h3/4) and QEWER (Cor a 9) peptides, a signal enhancement of 12% and 5%, respectively, was observed; in the case of all the other peptides a signal suppression ranging from 11 to 50% was calculated. To overcome matrix effect matrix-matched calibration curves were built up to perform a label-free quantification method.

As for recovery, one batch of breakfast cereals was enriched with a mixture of the five nuts (see Section 2) and values ranging from 76 ± 4% to 94 ± 3% were obtained.

3.4. Comparison of the LC–MS^2 and the LC–MS^3 methods

By operating under MS^3 SRM conditions, LOD and LOQ of all the peptides except for the allergen Jug r 4 resulted to be higher than those obtained under MS^2 mode (Table 3). The LC–ESI-MS^3

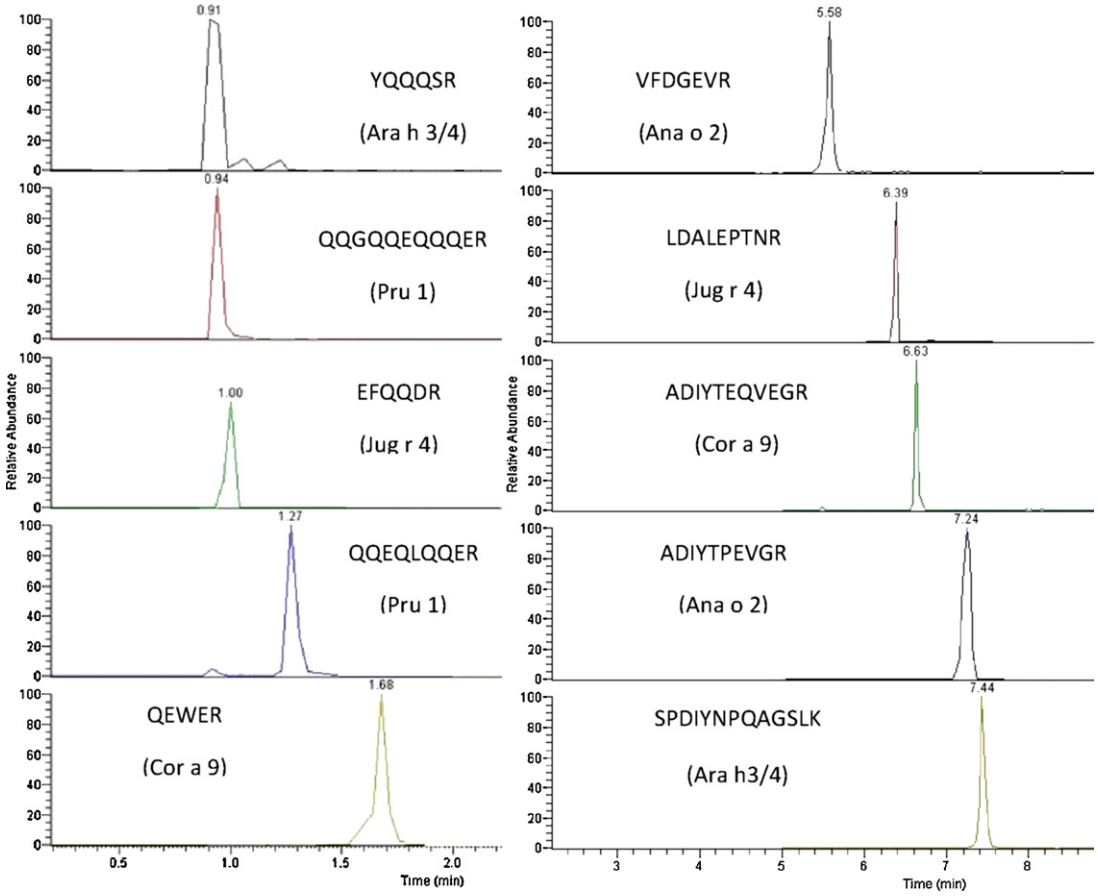

Fig. 2. LC–ESI-LIT-MS^2 separation of the ten targeted peptides from a matrix (cereals) tryptic digest on the C18 monolithic column (100.0 mm × 2.1 mm). Blank matrix was fortified with a mixture of the five nuts 0.01% (w/w). Mobile phase: formic acid aqueous solution (0.1%, v/v)/acetonitrile (for gradient elution see Section 2), flow-rate 350 μl/min, injection volume: 10 μL.

Table 2
Validation results obtained for the determination of allergens in biscuits under MS^2 acquisition mode. Concentrations are referred to the matrix fortified with a mix of the five nuts (mg nut/kg matrix).

Allergen	Peptide	LOD (mg/kg)	LOQ (mg/kg)	Concentration range (mg/kg)	Linearity $Y = b_1(\pm s_{b1})X$	r^2 ($n = 21$)
Ana o 2	VFDGEVR	14	46	50–500	30,783 (±407)	0.997
Cor a 9	QEWER	30	90	90–1000	8259 (±523)	0.998
Pru 1	QQEQLQQER	17	58	60–500	10,373 (±213)	0.986
Ara h3/4	SPDIYNPQAGSLK	10	37	40–400	32,979 (±1565)	0.998
Jug r 4	LDALEPTNR	55	180	200–1000	3627 (±358)	0.991

Table 3
Validation results obtained for the determination of allergens in biscuits under MS^3 acquisition mode. Concentrations are referred to the matrix fortified with a mix of the five nuts (mg nut/kg matrix).

Allergen	Peptide	LOD (mg/kg)	LOQ (mg/kg)	Concentration range (mg/kg)	Linearity $Y = b_1(\pm s_{b1})X$	r^2 ($n = 21$)
Ana o 2	VFDGEVR	30	98	100–1000	10,020 (±77)	0.953
Cor a 9	QEWER	35	110	110–1000	7350 (±29)	0.991
Pru 1	QQEQLQQER	25	80	80–1000	5109 (±106)	0.995
Ara h3/4	SPDIYNPQAGSLK	27	90	90–1000	10,178 (±21)	0.988
Jug r 4	LDALEPTNR	50	160	160–1000	2924 (±44)	0.992

Table 4
LC–ESI-MS^2 and MS^3 determination of the hidden allergens detected in the different food samples investigated ($n = 3$).

Sample	Almond (mg/kg)		Hazelnut (mg/kg)	
	MS^2	MS^3	MS^2	MS^3
Cereal mix with dried fruits	–	–	–	–
Exotic muesli	–	–	–	–
Cornfalkes	–	–	–	–
Muesli with dried fruits	98 ± 12	–	–	–
Natural cereal mix	100 ± 8	117 ± 2	50 ± 5	66 ± 3
Biscuits	–	–	–	–

–, not detected.

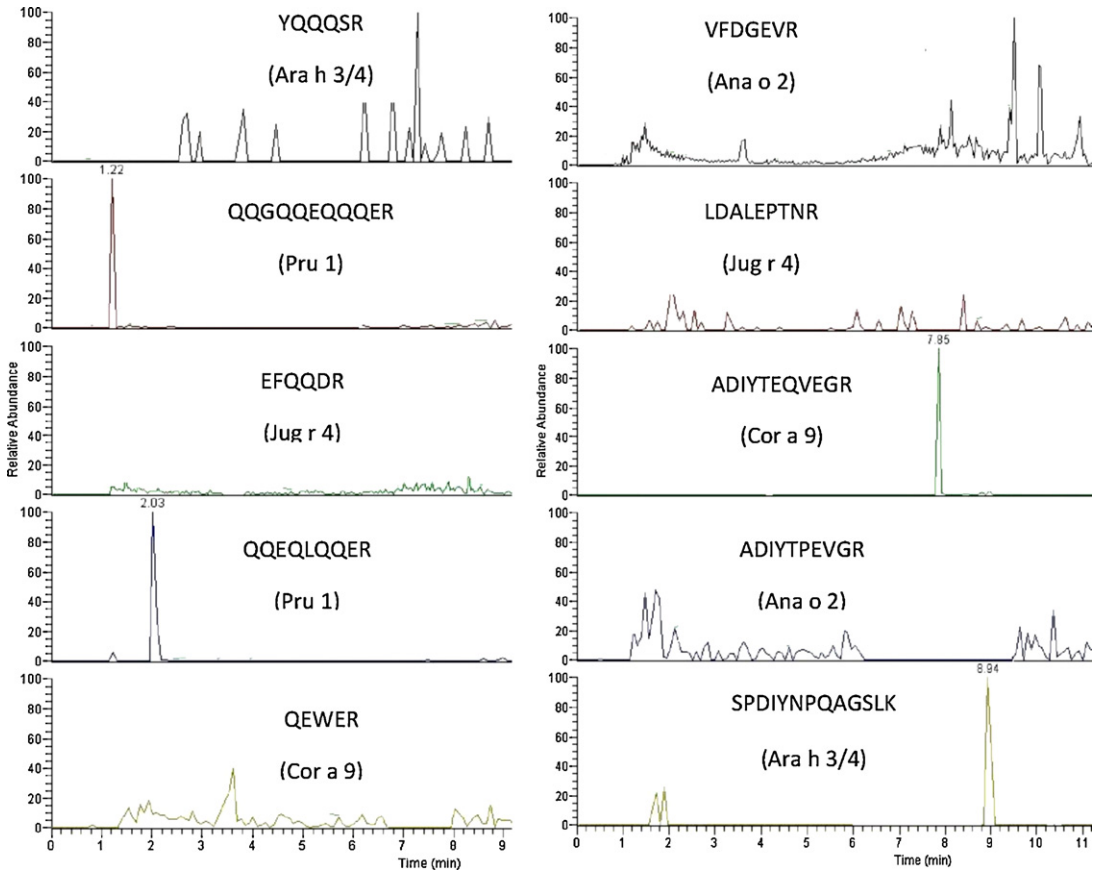

Fig. 3. LC–ESI-LIT-MS^2 separation of the breakfast cereal sample extract on the C18 particle-packed column (100.0 mm × 2.1 mm, 2.6 μm). Mobile phase: formic acid aqueous solution (0.1%, v/v)/acetonitrile (for gradient elution see Section 2), flow-rate 250 μl/min, injection volume: 10 μL.

linear range was established in general over one order of magnitude of concentration for all the peptides (Table 3). For this purpose, homogeneity of variance, the significance of the intercept and the best fitting at 95% confidence level were assessed. Within day repeatability and inter-day repeatability were excellent and ranged from 2 to 13%, and from 5 to 15%, respectively. As for matrix effect, results similar to those obtained under MS^2 mode were obtained in terms of peptides affected by matrix effect. In this case, the signal suppression percentage resulted in the range between the 6% and the 28%, suggesting a slightly reduction in the matrix effects.

3.5. Sample analysis

In the last step of the work the LC–MS^2 and LC–MS^3 methods were applied to a variety of commercial biscuits and breakfast cereal products to evaluate the presence of the hidden allergens under investigation. Among all samples analyzed, under MS^2 mode two of height resulted contaminated by traces of almond, hazelnut and peanut (Fig. 3, Table 4). However, by analyzing the same sample under MS3 mode the peanut peptide signal was not observed. As for confirmatory purposes, these samples were run under MS^2 and MS^3 product ion scan mode in order to acquire the whole fragmentation pattern of the targeted peptides, and their identity was univocally verified. In particular, in the case of the peanut peptide a isobaric interferent was detected demonstrating that MS^3 acquisition mode allows to improve selectivity of the analysis and unequivocally confirm the presence of the peptides found in the samples. The quantitative results are reported in Table 3.

The results of the analysis of these samples showed that the sample procedure in combination with LC–ESI-LIT-MS^2 and MS^3 analysis of sample extracts is capable of pin-pointing and quantifying the presence of hidden allergens in foods at the levels of interest.

4. Conclusions

A new rapid method for simultaneous detection of five allergens in foods was developed and successfully validated. Two LC columns were evaluated to improve chromatographic performances and the best results in terms of selectivity and sensitivity were obtained using a C18 particle-packed column. By operating under MS^2 conditions, five of the biomarker peptides can be detected quantitatively with LODs from 10 to 50 mg nut/kg matrix and precision from 3 to 15%. Applying the method to 8 commercial samples, the Cor a 9 and Pru 1 hidden allergens were detected at trace level. This study proved the possibility to increase in the future the number of allergens to investigate simultaneously in one single LC–MS^2 run, in order to provide a rapid and selective detection method useful for food control analysis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2010.10.037.

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