

Use of specific peptide biomarkers for quantitative confirmation of hidden allergenic peanut proteins Ara h 2 and Ara h 3/4 for food control by liquid chromatography–tandem mass spectrometry

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Abstract A liquid chromatography–electrospray–tandem mass spectrometry (LC–ESI–MS–MS) method based on the detection of biomarker peptides from allergenic proteins was devised for confirming and quantifying peanut allergens in foods. Peptides obtained from tryptic digestion of Ara h 2 and Ara h 3/4 proteins were identified and characterized by LC–MS and LC–MS–MS with a quadrupole–time of flight mass analyzer. Four peptides were chosen and investigated as biomarkers taking into account their selectivity, the absence of missed cleavages, the uniform distribution in the Ara h 2 and Ara h 3/4 protein isoforms together with their spectral features under ESI–MS–MS conditions, and good repeatability of LC retention time. Because of the different expression levels, the selection of two different allergenic proteins was proved to be useful in the identification and univocal confirmation of the presence of peanuts in foodstuffs. Using rice crispy and chocolate-based snacks as model food matrix, an LC–MS–MS method with triple quadrupole mass analyzer allowed good detection limits to be obtained for Ara h2

(5 $\mu\text{g protein g}^{-1}$ matrix) and Ara h3/4 (1 $\mu\text{g protein g}^{-1}$ matrix). Linearity of the method was established in the 10–200 $\mu\text{g g}^{-1}$ range of peanut proteins in the food matrix investigated. Method selectivity was demonstrated by analyzing tree nuts (almonds, pecan nuts, hazelnuts, walnuts) and food ingredients such as milk, soy beans, chocolate, cornflakes, and rice crispy.

Keywords Liquid-chromatography–tandem mass spectrometry · Hidden peanut allergens · Biomarker peptides

Introduction

In recent years peanut allergy seems to be on the rise in western countries, with a prevalence of 1% to 1.5% [1]. Together with tree nuts, peanuts account for the majority of food allergic reactions with fatal outcome both in children and in adults [2]. Since spontaneous resolution of peanut allergy is rare [3] and curative treatment is not yet available [4], strict elimination of peanuts is the rule, because traces of peanut can cause a severe allergic reaction in sensitive subjects [5, 6]. However, strict elimination is difficult, especially because food labeling practice can be still inadequate [7, 8] and contamination of nonallergenic foodstuffs with allergenic ingredients can easily occur during processing and transport. Recent developments intending to improve regulatory aspects of food labeling have led to increased advisory labeling [9–11].

Up to now several methods have been developed for the determination of peanut allergens in foods; these are mainly based on immunoassay (sandwich and competitive enzyme linked immunosorbent assay (ELISA) [12, 13], polymerase

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chain reaction (PCR), and real-time-PCR [14]. These approaches represent valid screening tools allowing simple analysis of a high number of samples. However, in such methods problems related to DNA–protein expression level, cross-reactivity, accuracy, and selectivity are still present. In a research program dealing with the development of new analytical strategies for the determination of hidden allergens in foods, very recently [15] we proposed a non-competitive sandwich immunoassay with inductively coupled plasma-mass spectrometric (ICP–MS) detection for the analysis of peanut proteins in different food matrices by using metal-tag antibodies. The method was demonstrated to be suitable for the detection of peanut allergens down to 1.5 mg peanuts kg⁻¹ matrix with good precision. The ICP–MS system presents several advantages, including high sensitivity and the capability of performing multi-tag detection. However, this technique does not overcome the limitations of immunoassay methods in terms of accuracy and selectivity.

In such a context, the high selectivity of mass spectrometry in protein analysis can overcome some of the difficulties associated with the correct identification of peanut proteins in complex food matrices. In this work we propose a proteomic-based approach for quantitative confirmation of peanut allergens in foods. In particular, we devised a liquid chromatography–electrospray-tandem mass spectrometry (LC–ESI–MS–MS) method with triple quadrupole (QqQ) analyzer for identification and determination of two major peanut allergens, i.e. Ara h 2 and Ara h 3/4 [16, 17], by using characteristic peptide biomarkers.

Recently, LC–ESI–MS–MS methods for the detection of Ara h 1 in ice-cream and dark chocolate have been published [18, 19]. In those papers, the approach proposed was based on the detection of biomarker peptides of one protein, i.e. Ara h 1, to directly identify peanut allergens at low parts per million level in food matrices.

When complex matrices are analyzed, the selection of adequate peptide markers derived from different proteins for detection of hidden allergens is of great importance. In this context, very recently a characterization of potential peptide biomarkers of Ara h 1, A h 2, and Ara 3/4 using capillary LC–nanoESI–quadrupole–time-of-flight (QTOF)–MS–MS has been proposed [20]. In that work, attention was paid to the establishment of a list of peptides that remain stable after peanut processing.

The key feature of the research study proposed here is the development of a LC–ESI–QqQ–MS–MS method for two different proteins of the same allergenic source with the goal of combining the selectivity of the approach based on the detection of peptide biomarkers for different allergens with the capability of the method of performing quantitative analysis. In addition, attention was paid to assessment of method performance for both qualitative and quantitative

purposes. The choice of two targets can offer the advantages of increasing the selectivity of allergen identification and better assessment of electrospray sensitivity for different peptides and thus the detection limits of the method.

Experimental

Chemicals

Ara h2 was purified from raw peanuts following a procedure reported in the literature [21], whereas Ara h3/4 purified from raw peanuts was kindly provided by Dr W. Becker from the Leibniz Center for Medicine and Biosciences at the Research Center Borstel (Borstel, Germany). Native protein solutions were prepared and diluted in deionized water (18 MΩ cm resistivity) which was obtained from a Milli-Q Element water-purification system (Millipore, Bedford, MA, USA). Acetonitrile (HPLC purity), trifluoroacetic acid, and formic acid (analytical reagent grade) were purchased from Carlo Erba (Milan, Italy).

Trypsin from bovine pancreas, iodoacetamide, and dithiothreitol were from Sigma–Aldrich (St Louis, Missouri, USA).

Liquid chromatography–quadrupole/time of flight-mass spectrometry

Characterization of Ara h 2 and Ara h 3/4 peptides was carried out using a microLC–ESI–Q–TOF Micro (Waters, Milford, MA, USA) mass spectrometer. LC elution was performed on a Symmetry C₁₈ capillary column (0.32 mm × 150 mm, 5 μm) (Waters) using a gradient solvent system [(A) 0.1% (v/v) formic acid in water; (B) 0.08% (v/v) formic acid in acetonitrile]. Gradient elution was as follows: solvent B was set at 5% for 6 min, then delivered by a linear gradient from 5% to 90% in 28 min. Solvent B was maintained at 90% for 2 min before column re-equilibration (10 min). Flow-rate was 6 μL min⁻¹. Mobile phase was delivered by a CapLC pump (Waters) equipped with a 48-vial capacity sample-management system. Standard or sample solution (10 μL) was loaded on a Symmetry 300 C₁₈ (Waters) precolumn (0.32 mm × 10 mm, 5 μm) and eluted with the mobile phase on to the chromatographic column for on-column analyte enrichment and to avoid column contamination.

The nebulizing gas (nitrogen, 99.999% purity) and the desolvation gas (nitrogen, 99.998% purity) were delivered at flow-rates of 10 and 600 L h⁻¹ respectively. Q-TOF external calibration was performed using a peptide mixture solution and a fifth-order nonlinear calibration curve was usually adopted. The interface parameters were: ESI

voltage 3.0 kV, cone voltage 30 V, rf lens 0.5 V, source temperature 50 °C, desolvation temperature 100 °C.

Identification with the Q-TOF instrument was performed using accurate MS and MS–MS measurements of the selected target peptides.

Continuum mode TOF mass spectra were acquired over the m/z range 400–2000 using an acquisition time of 1 s and an interscan delay of 0.1 s. When operating in product-ion mode, collision energy and the m/z range were optimized as a function of peptide charge and mass.

Liquid chromatography–triple quadrupole mass spectrometry

LC elution was carried out on a Poroshell C₁₈ 300 Å (75 mm×2.1 mm, 5 µm particles) (Agilent Technologies, Santa Clara, CA, USA) using a gradient solvent system [(A) aqueous formic acid (0.1% v/v)/(B) 0.08% (v/v) formic acid in acetonitrile]. Gradient elution was the same as reported above. The flow-rate was 200 µL min⁻¹.

The mobile phase was delivered by a Waters 2690 series Alliance quaternary pump equipped with a 120-vial capacity sample-management system. Injection volume was 30 µL.

A Quattro LC triple-quadrupole instrument (Micromass, Manchester, UK) equipped with a pneumatically assisted ESI interface was used. The system was controlled by Masslynx software version 4.0 (Micromass).

The nebulizing gas (nitrogen, 99.999% purity) and the desolvation gas (nitrogen, 99.998% purity) were delivered at flow-rates of 55 and 500 L h⁻¹, respectively.

Full-scan, selected ion monitoring (SIM), product-ion, and selected-reaction monitoring (SRM) experiments were performed operating the mass spectrometer in PI mode.

Optimized conditions of the interface were as follows: ESI voltage 3.5 kV, cone voltage 35 V, rf lens 0.3 V, source temperature 130 °C, desolvation temperature 250 °C. Full-scan mass spectra were acquired over the scan range m/z 200–2000 using a step size of 0.1 Da. SIM experiments were carried out on the doubly charged ions of selected peptides. For MS–MS experiments collision-induced dissociation (CID) was performed with a collision gas pressure of 2.3×10^{-3} mbar in the collision cell. The collision energies (CE) were optimized for the mass and charge state of each peptide in order to obtain selective and intense fragmentation. The SRM transitions monitored were as follows: m/z 807/1050 CE 35 eV (CCNELNEFENNQR, Ara h 2), m/z 950/1120 CE 35 eV (CMCEALQQIMENQSDR, Ara h 2), m/z 649/1089 CE 30 eV (AHYQVVDSNGDR, Ara h 3/4), m/z 695/700 CE 35 eV (SPDIYNPQAGSLK, Ara h3/4). Leucine–enkephalin was used as internal standard (m/z 557/397; CE 20 eV). A ± 0.5 mass resolution was set in Q1 and Q3.

Samples

Raw peanut samples (*Arachis hypogaea*) of the variety red skin from five different producers were purchased in commercial stores.

Sample treatment

Method 1

Peanut extracts and peanut-containing food extracts were prepared by adding 10 mL Tris HCl 0.2 mol L⁻¹ (pH 8.2) to 1 g ground sample. Proteins were extracted by shaking for 2 h at 60 °C, then the extract was centrifuged (3000 g, 5 min) and filtered through a 0.2-µm Nylon filter. Sample concentration was performed on a 5 kDa MW cut-off cellulose filter (Millipore Billerica, MA, USA) by adding 5 mL protein extract and reducing the volume to 0.5 mL. The sample was then digested with trypsin (5 µL, 0.6 mmol L⁻¹ in tris HCl, pH 8.2). Different digestion conditions in terms of time (12 and 24 h) and temperature (37 °C, 50 °C) were tested. The final digestion conditions were: 50 °C for 24 h and reaction blocking with 1 µL trifluoroacetic acid.

Milk, cacao powder, soy beans, tree nuts, rice crispy, cornflakes (all purchased from a major store chain) were extracted by applying the same procedure. Peptide extracts were stored at -20 °C until analysis.

Method 2

The same procedure as for method 1 except for a further liquid–liquid extraction step with hexane before sample concentration on the cut-off filter.

Method 3

Sample (1 g) was incubated overnight in 10 mL 0.2 mol L⁻¹ Tris HCl (pH 8.2) and 100 µL trypsin 0.6 mmol L⁻¹ at 50 °C with vigorous shaking. The resulting mixture was centrifuged at 14000 rpm for 5 min. The supernatant was filtered through a 0.45-µm centrifugal filter (Millipore).

The extract was further purified using Strata-X polymeric multimode solid-phase cartridges (Phenomenex, Torrance, CA, USA). The cartridge was washed with 2 mL 95:5 (v/v) water–acetonitrile and peptides were eluted with 1 mL acetonitrile. The eluate was dried under a nitrogen stream and re-suspended in 200 µL 60:40 (v/v) water–acetonitrile containing 0.1% formic acid.

LC–QQ–MS–MS method performance

Method performance was assessed in terms of limit of detection (LOD), limit of quantitation (LOQ), linearity,

accuracy, and selectivity. For this purpose a rice crispy/cacao-based snack was chosen as the matrix.

LOD and LOQ values were calculated for standard solution and for matrix as amounts for which the signal-to-noise ratios were 3:1 and 10:1, respectively. Instrumental linearity was calculated by spiking aqueous solutions with different amounts (0.1–20% w/v) peanut extract. A matrix-matched calibration curve was built-up by spiking matrix extract with different amounts of peanut extract (1–20% w/v). Method precision was assessed by performing two extractions of matrix fortified at two peanut concentration levels (1.25 and 5% w/w peanuts/matrix) and performing five LC injection replicates for each extract. Method selectivity was evaluated by analyzing tryptic digests of tree nuts, soybeans, milk, cacao powder, and rice crispy samples.

Results and discussion

LC–ESI-QTOF MS and MS–MS identification of Ara h 2 and Ara h 3/4 peptide biomarkers

Ara h 2 and Ara h 3/4 whole proteins were initially characterized by ESI-QTOF MS analysis. No protein interferences were found in the mass spectra, suggesting an excellent degree of purification of both proteins. In particular, Ara h 2, which was purified from peanut seeds in our laboratory, exhibited the typical doublet in the 16–18 kDa range, as reported in the literature [21].

In a further step, the tryptic digests of purified Ara h 2 and Ara h 3/4 were individually analyzed in LC–QTOF-MS in order to identify putative peptide biomarkers. Proteins were digested both in the native and reduced/alkylated (using dithiothreitol and iodoacetamide) forms. The results did not show significant differences among the most abundant peptides observed for both proteins digested under the two different conditions. Since no analytical advantages were obtained by performing digestion of reduced/alkylated proteins, the work was performed on the native forms.

Several doubly charged ions of the tryptic digest peptides were identified for both proteins allowing a good degree of sequence coverage to be obtained (74% for Ara h 2 and 60% for Ara h 3/4). Peptides were selected on the basis of the following criteria: signal intensity, retention time repeatability, lack of missed cleavages, specificity, and presence in the different Ara h 2 and Ara h 3/4 isoforms.

In the case of Ara h 2 the peptides at m/z 807.2451 (CCNELNEFENNQR; doubly charged ion) and m/z 949.8894 (CMCEALQQIMENQSDR; doubly charged ion of the cysteine oxidized form) were selected. Accurate mass measurements and product-ion MS–MS experiments were

carried out to identify the peptide sequences. No peptides covering the IgE-binding epitope giving the allergic reaction (two epitopes in the region aa57–74 contain the amino acid sequence DPYSP necessary for IgE binding, [22]) were found in the ESI mass spectrum of the tryptic digest of Ara h 2.

Concerning Ara h 3/4, the peptides at m/z 648.8109 (AHYQVVDSNGDR, doubly charged ion), m/z 695.3468 (SPDIYNPQAGSLK, doubly charged ion), and at m/z 732.8384 (AQSENYEYLAFK) were found to be the most abundant.

However, considering the protein sequences reported in databases it was possible to observe that the sequence at m/z 732 is not present in all isoforms thus suggesting that this peptide can not be a useful biomarker. To demonstrate this, peanut extracts from different providers were analyzed and the presence of the peptide at m/z 732 was observed only in two of the five samples analyzed. In addition, taking into account recovery of the extraction procedure and that the concentration of the protein extract is determined and made uniform before digestion, the peptide was detected with different abundances in the two varieties in which it was present. These findings suggest that the peptides useful as biomarkers must be present in all the different isoforms of the investigated proteins in order to detect them when different peanut cultivars are used in the food industry. Further, the analysis of more than one allergenic protein for confirmation purposes allows reduction of the problem of a different level of expression of a protein in the vegetable system, which can significantly affect the detection limit of the peptides. In the case of Ara h 2, the peptides at m/z 807 and 950 were found to be present with comparable signal intensity in all the varieties selected. In fact, the sequence coverage we obtained for Ara h 2 was predominantly on the conserved sequences and not on the sequences that differ in the various isoforms.

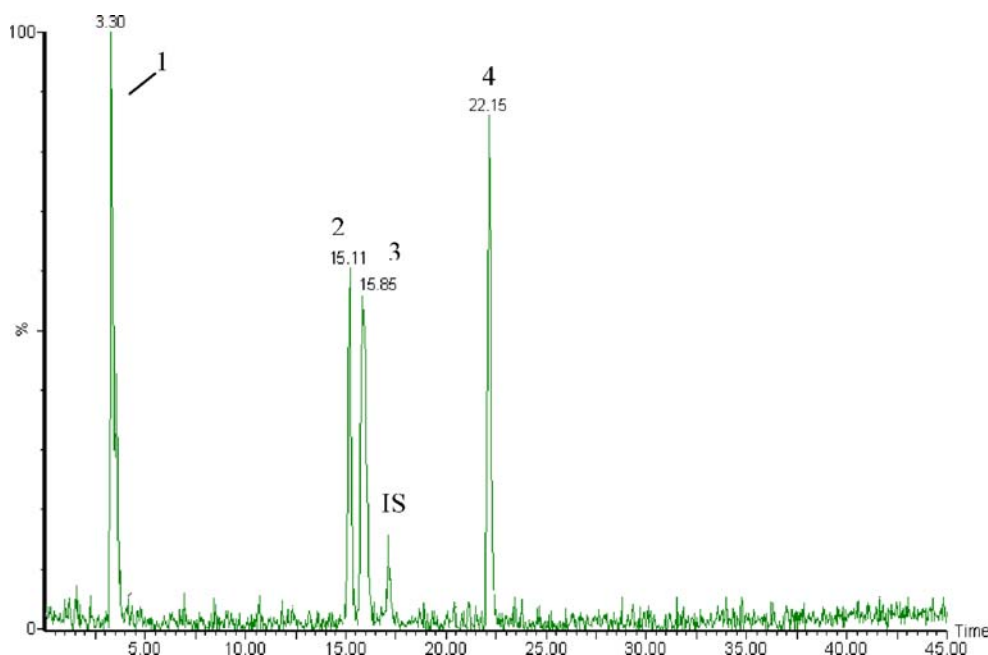
On the basis of these results, the LC tandem mass spectrometry method using a triple-quadrupole was developed.

LC–ESI-QqQ-MS–MS method

The chromatographic behavior of the four selected peptides (Fig. 1) exhibited excellent stability of the retention times (RSD < 1.5%, $n = 10$).

In a preliminary step ESI ionization efficiency of the selected peptides was evaluated to study analytical sensitivity for quantitative purposes. In fact, since different ESI sensitivity is expected, it should be assessed to verify which peptides can be useful for obtaining the lowest detection limits. Calibration curves were calculated in SIM mode by performing several dilutions of the tryptic digests of Ara h 2 and Ara h 3/4 standard solutions. From the slope values obtained it was possible to observe comparable sensitivity

Fig. 1 LC–ESI–QqQ–MS–MS SRM chromatograms of a tryptic digest of Ara h2 and Ara h3/4 standard solutions (20 µg mL⁻¹). Peaks: 1, *m/z* 649/1089 (Ara h3/4); 2, *m/z* 695/700 (Ara h3/4); 3, *m/z* 807/1050 (Ara h2); 4, *m/z* 950/1120 (Ara h2)



for the peptides at *m/z* 807 [$y = 3593(\pm 260)x$] and *m/z* 950 [$y = 2517(\pm 320)x$] (Ara h 2). Instead, in the case of the Ara h 3/4 peptide *m/z* 695 [$y = 22110(\pm 1140)x$], remarkably, the highest sensitivity was observed. The peptide at *m/z* 649 provided a sensitivity comparable to that observed for the Ara h 2 peptides [$y = 3720(\pm 167)x$]. These findings suggest that the peptide at *m/z* 649 can be a good marker to improve method detectability.

Using a low-resolution mass analyzer it is necessary to perform SRM experiments to obtain good selectivity in peptide identification in a complex matrix. For this reason, the product-ion mass spectra of the target peptides were acquired by optimizing the collision energy in order to obtain selective fragment ions with high intensity. The MS–MS spectra of peptides at *m/z* 807 (Ara h2), 950 (Ara h2), 695 (Ara h3/4), and 649 (Ara h3/4) showed fragment ions distributed under and above each selected precursor ion mass yielding useful analytical data for the quantitative method.

Sample treatment procedure

In the development of a sensitive method for the detection of hidden allergens in food products, sample preparation should be optimized to obtain the best sensitivity improving peptide detection limits. For this purpose, three different extraction methods were applied to peanuts.

By using the method involving extraction of whole proteins followed by enzymatic digestion (method 1), the signals of all selected peanut peptides were observed with excellent signal-to-noise ratio in the LC–MS–MS SRM chromatogram. However, it was possible to observe an

increase of column back-pressure and a signal intensity decrease as a consequence of ESI source dirt when a number of subsequent analyses were run (i.e. more than 20 injections). In order to reduce sample impurities, an additional extraction step with hexane was introduced to eliminate the lipid fraction of the sample (method 2). However, even though the problems related to unclear extracts were solved, a significant decrease (between 20 and 50%) in the peak area of the investigated peptides was observed with respect to method 1.

To improve sample treatment the simultaneous extraction and digestion of peanut proteins was performed followed by an SPE clean-up step (method 3). This resulted in a decrease of peptide signal intensity (between 50 and 90%) with respect to method 1. This behavior could be the consequence of incomplete recovery of the peptides during digestion and purification or of a different matrix effect that can affect ESI ionization efficiency.

On the basis of these findings, method 1 was chosen. Different digestion conditions were then assessed, as reported in the experimental section. The best result in terms of recovery of the peptides from peanut-containing foods was observed when operating at 50 °C for 24 h. In particular, these conditions led to an increase of the peak area of the four peptides monitored of between 20 and 120% compared with the other milder digestion conditions.

Assessment of LC–ESI–QqQ–MS–MS method performance with foods

To assess the applicability of the LC–ESI–MS–MS SRM method for detection of peanut allergens in food, rice

crispy/cacao-based snacks were fortified with different amounts of raw peanuts.

Instrumental linearity calculated by using aqueous solutions spiked with percentages from 0.1 to 20% of a peanut peptide extract in general demonstrated better sensitivity for the peptides selected for Ara h3/4 ($y = 10.48(\pm 0.23)x$ and $y = 3.78(\pm 0.12)x$ for the peptides at m/z 695 and m/z 649 respectively) with respect to those selected for Ara h2 ($y = 4.23(\pm 0.22)x$ and $y = 2.68(\pm 0.18)x$ for the peptides at m/z 807 and m/z 950 respectively). The peptide m/z 695 provided the lowest detection limit ($1.5 \mu\text{g mL}^{-1}$) and quantitation limit ($4.8 \mu\text{g mL}^{-1}$). The LOD and LOQ values for matrix were calculated for all the peptides investigated. The best result was obtained for the peptide at m/z 695 (Ara h3/4); the LOD and LOQ were 1 and $3.7 \mu\text{g protein g}^{-1}$ matrix, respectively (Fig. 2a). In the case of Ara h2, the peptide exhibiting the lowest LOD and LOQ values was that at m/z 807 (LOD and LOQ values were 5 and $14 \mu\text{g g}^{-1}$, respectively; Fig. 2b). Method precision, expressed as RSD, was lower than 10% when calculated for 1.25 and 5% w/w peanuts/matrix concentration levels for all the peptides investigated. Trueness was evaluated as matrix effect and the results exhibited the presence of a significant suppression effect (from 30 to 50%) on the signals of the peptides of Ara h2, whereas in the case of Ara h3/4 peptides the suppression effect was lower than 10%. In general, the use of the internal standard selected did not completely overcome the matrix suppression effect but

reduced it to within 5 and 30% for Ara h3/4 and Ara h2 peptides, respectively.

Further, specificity of the peptide biomarkers was assessed for the matrix. In fact, Ara h 2 is a glycoprotein having sequence homologies with conglutin, the 2S albumin seed protein families, and the α -amylase from wheat. Ara h 3/4 is a protein belonging to the glycinin protein family and some of the IgE-binding epitopes (the C-terminal fragment appeared to bind more IgE) are conserved regions present in legumes (e.g. soy beans). Such IgE-binding sites provide a molecular explanation for the IgE cross-reactivity observed between soybean and peanut proteins [23].

For this purpose, research on protein databases was performed and the sequences of the selected peptides were found to be unique for peanuts. In fact, by analyzing peptide extracts from different food sources (tree nuts, soybeans, milk, cacao powder, rice crispy) no interfering signals were observed at the SRM transitions monitored for the peanut peptides. It should be noted that such selectivity was obtained by keeping a very narrow mass window (± 0.5 Th) on the first quadrupole, thus reducing the number of ions transmitted to the collision cell in spite of method sensitivity. In fact, using a larger mass window (± 1 Th), several signals were observed in the matrices tested. Such findings suggest that when a low-resolution mass analyzer is used, optimum selectivity can be obtained by selecting specific transitions and keeping a narrow m/z window on the first quadrupole for the MS–MS experiments.

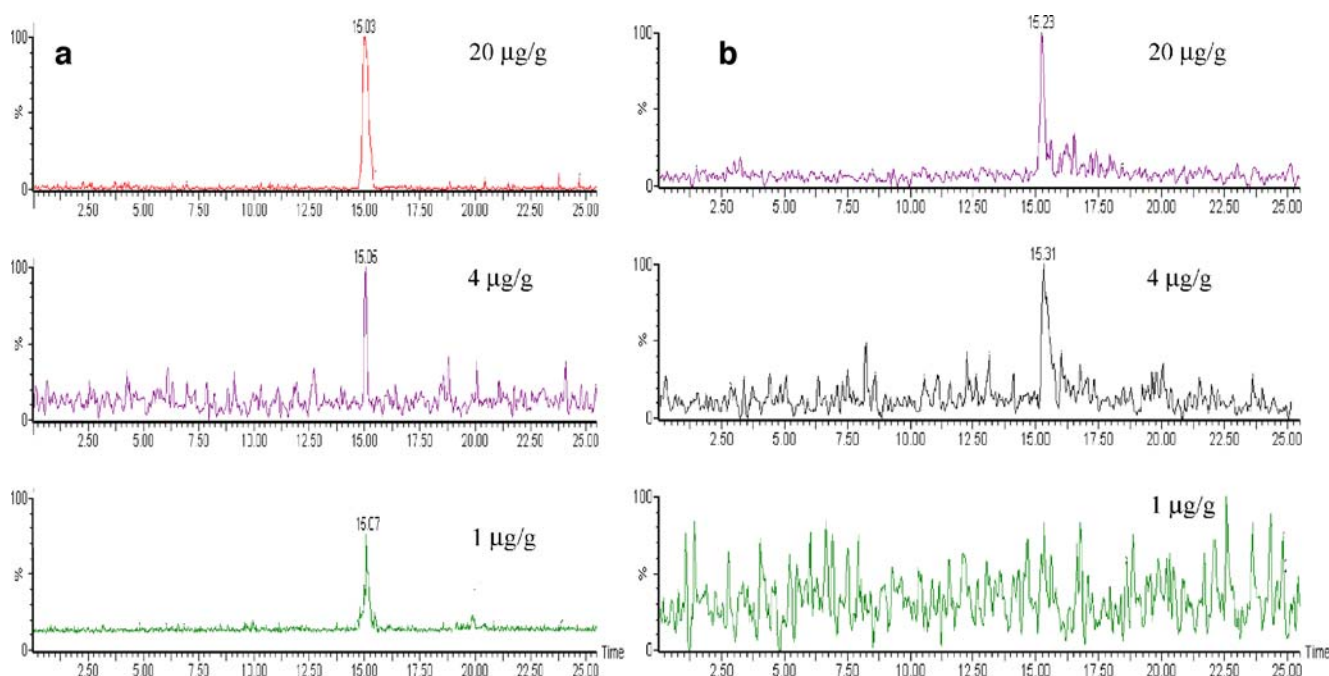


Fig. 2 LC-ESI-QqQ-MS-MS SRM chromatograms of peptides (a) m/z 695/700 [SPDIYNPQAGSLK, Ara h3/4] and (b) m/z 807/1050 [CCNELNEFENNQR, Ara h2] in chocolate/rice crispy-based matrix

fortified with Ara h2 and Ara h3/4 protein solutions at different concentrations

Conclusions

The approach based on the simultaneous identification of biomarker peptides derived from two different proteins makes the proposed method highly selective towards peanut residues in foods. The capability of identification of both Ara h 2 and Ara h 3/4 at concentrations as low as $1 \mu\text{g g}^{-1}$ was demonstrated in a complex matrix such as rice crispy/chocolate-based snacks.

Since the method combines highly selective identification with the capability of performing quantitative analysis at low concentration levels, it can be proposed as an effective quantitative confirmatory method for the detection and determination of hidden allergens in food.

Further developments will concern the strategy of sample treatment with the aim of obtaining simultaneous extraction, purification, and enrichment of the investigated peanut proteins. The final goal is to reduce the time required for sample preparation and to make detection and quantification limits less prone to the effects of the complexity of the matrices.

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