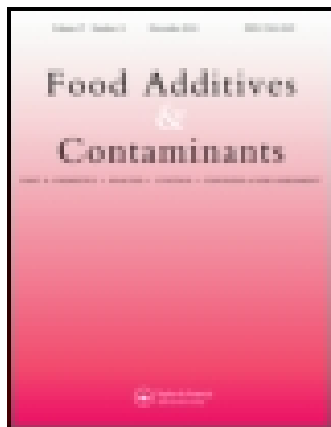




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High-resolution Orbitrap™-based mass spectrometry for rapid detection of peanuts in nuts

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High-resolution OrbitrapTM-based mass spectrometry for rapid detection of peanuts in nuts

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Peanut represents one of the most harmful allergenic foods capable of triggering severe and sometimes lethal reactions in allergic consumers upon ingestion of even small amounts. Several proteins capable of inducing allergic reactions that have been recognised by patients' IgE antibodies have been identified from this nut source. Methods mainly based on ELISA assays have been developed in order to detect peanuts in several food commodities. In addition LC-MS/MS methods based on different mass analysers have also been devised for tracing peanut contamination in different foods achieving low limits of detection. The applicability of a benchtop high-resolution ExactiveTM mass spectrometer has never been investigated for the rapid screening of peanut contamination in complex food matrices like mixtures of nuts. We report in this paper the design of suitable peanut markers and the development of an high-resolution OrbitrapTM mass spectrometer-based method for peanut detection in a mixture of nuts species. With this aim, different types of samples were prepared: (1) nuts-based powder made up of a mixture of hazelnuts, pistachios, almonds and walnuts; and (2) nuts powder fortified with peanuts. Different levels of fortifications were produced and the applicability of the method was tested. Finally, a subset of six peptides fulfilling specific analytical requirements was chosen to check the suitability of the method tailored to the detection of peanuts in nuts-based products, and two of them, peptides VYD and WLG, were selected as quantitative markers. The method proved to be a suitable screening tool to assess the presence of traces of peanuts in other tree nuts with a limit of detection as low as 4 µg of peanuts proteins or 26 µg of peanuts in 1 g of matrix.

Keywords: peanuts; tree-nuts; allergens; high resolution-mass spectrometry; Exactive; markers

Introduction

Peanut allergy represents a typical form of allergy to nuts, accounting for the majority of severe food-related allergic reactions (Bock et al. 2007). It is the major cause of immunoglobulin E (IgE)-mediated food allergies (Hourihane et al. 1998; Pumphrey et al. 1999), with the highest rate of induced anaphylactic shock, especially in the US and UK (Yunginger et al. 1988; Sampson et al. 1992; Bock et al. 2001). Peanut allergy affects both children in their early life and adults, but in contrast to other food allergies (like egg and milk allergy) this type of allergy has been proved to persist into adulthood in the majority of sensitive individuals (Skolnick et al. 2001). As is well known, peanut allergy can be triggered by the ingestion of even little amounts of peanuts by allergic consumers, irrespective of the processing the peanuts had undergone. Most patients suffering from this type of allergy display symptoms even after ingestion of one kernel of peanuts as low as 1 mg (Sicherer & Sampson 2007). To address peanut allergy diagnosis and therapies better, all allergenic proteins have been isolated from peanuts and tested for their allergic activity in order to identify the most harmful peanut allergens.

According to the Allergen Nomenclature Sub-Committee of the International Union of Immunological Societies, 11 Ara h proteins were identified as being

capable of inducing allergic reactions that are recognised by IgE antibodies from patients upon peanuts ingestion. Notably, most of the Ara h-type allergens are part of the storage proteins of peanuts (*Arachis hypogaea*). Among them it has been reported that Ara h 1 and Ara h 3 belonging to the cupin family and Ara h 2 and Ara h 6 belonging to the 2S albumin family are the relevant proteins responsible for serious allergic reactions to peanuts (Nicolaou et al. 2011). In particular, the heat and digestion-stable allergen Ara h 6 (conglutin) showed approximately 60% of sequence identity with Ara h 2 and was reported to be recognised by 30–40% of patients' IgE antibodies (Suhr et al. 2004; Lehmann et al. 2006).

In order to protect allergic consumers from the risk of developing allergic reactions, legislation (EC 2007) has been issued in European Union requiring the mandatory labelling of allergenic ingredients including peanuts whenever introduced into a food. In addition to the intentional introduction of allergenic ingredients in food commodities that is currently regulated by the enforced legislation, the risk of an inadvertent contamination of foodstuffs by allergenic ingredients is also likely to exist. This could be due to cross-contamination phenomena especially occurring in the food chains where there is not a dedicated line for the production of a particular food. In particular, the hazard of hidden peanuts contaminating different nut

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categories is likely to exist as a consequence of cross-contamination that can take place during nut storage (in the same areas where different nut species are stored) or due to the use of the same equipment for processing different nuts. Therefore, in order to safeguard the health of patients allergic only to peanuts, a method to detect eventual traces of peanuts in nuts as a consequence of cross-contamination is sought for.

Several methods have been developed in the past years for detection of allergen contamination in different food commodities. A number of ELISA tests are currently available for the analysis of allergens in foods. However, as these test kits can differ significantly in terms of the antibodies employed, target proteins, calibrators and extraction procedures, they can provide considerably different results for the same analyte (Török et al. 2015). Methods so far devised for peanut detection are mainly based on the antibody recognition of some target Ara h allergens and generally employ an ELISA format. However, given some of the limitations posed by the immunoassays (Khuda et al. 2012; Hebling et al. 2012) due to cross-reactivity phenomena existing between different classes of tree nuts and the risk of false-negatives as a consequence of epitope modification or a masking effect, in the last few years MS methods have been investigated as an alternative tool to monitor the presence of the offending allergens in complex food matrices.

LC-MS/MS methods based on the use of ion-trap, triple-quadrupole or QTOF MS have been reported in the literature for the identification of peanut markers in different types of food or in raw and roasted peanuts (Chassaigne et al. 2007). However, the methods developed were mainly intended to detect traces of peanuts in food commodities such as biscuits, chocolate and ice creams with LODs ranging from 0.1 to 10 µg of peanut ingredient in 1 g of food matrix (Shefcheck & Musser 2004; Shefcheck et al. 2006; Careri et al. 2007; Heick et al. 2011).

To the best of our knowledge, the applicability of such methods to nuts-based products has never been investigated, nor has a high-resolution (HR) Exactive™ system been exploited as a targeted screening tool to assess the presence of peanut contamination in other tree nuts.

We have already developed in our group a method using an Orbitrap™-based non-hybrid MS using HR full-scan MS acquisition to detect traces of egg and milk allergens in wines fined with allergenic fining agents. Challenging LODs were attained thanks to the application of post-acquisition filtering of the target peptides at the highest mass accuracy (Monaci et al. 2011, 2013; Pilolli et al. 2014).

The present paper describes the development of a method based on the use of a benchtop Orbitrap™-based MS without any quadrupole operating filtration of the precursor ion prior to ion fragmentation. In this

configuration all peptides eluted from the analytical column can straightaway undergo fragmentation in the high energy collision dissociation chamber (HCD) integrated within the system, thus enabling the system to perform a rapid and high-throughput screening of peanuts in nuts mixtures. In the first part of the paper attention is placed on the selection of specific and unique peptide markers that did not show any identity with other tree nuts coming from different sources. Following, a method using these specific peptides is described in the paper that enables the detection of peanuts in other nuts down to 4 µg g⁻¹ of total proteins by full-scan HRMS acquisition. This proves the high versatility of the HRMS system for the rapid screening of peanut traces in nuts and products thereof.

Material and methods

Nuts

Roasted peanuts (*Arachis hypogaea*, var. Zambia) and roasted nut sources composed of a 'nuts mix' were kindly provided by Besana S.p.A. (San Gennaro Vesuviano, Naples, Italy) within a national funded project (Safe&Smart, National CL.AN-Cluster Italy). In particular, roasted hazelnuts (*Corylus avellana*, var. Georgia), pistachios (*Pistacia vera*, var. Iran), almonds (*Prunus dulcis*, var. Italia) and walnuts (*Juglans regia*, var. Cile) were used to create the nuts-based mixture.

Chemicals

Acetonitrile (LC-MS grade), formic acid, ammonium bicarbonate, hydrochloric acid, iodoacetamide (IAA), dithiothreitol (DTT), hexane and trisma-base were obtained from Sigma-Aldrich (Milan, Italy). Trypsin (proteomic grade) was purchased by Promega (Milan, Italy). Polytetrafluoroethylene (PTFE) syringe filters, 0.2 µm (size 4 mm) were provided by Sartorius (Stedim Biotech GmbH, Goettingen, Germany). Bio-Spin® P-6 Gel Columns-Tris Buffer were purchased from Bio-Rad (Segrate, Milan, Italy).

Preparation of spiked samples

Nut-roasted kernels obtained from Besana S.p.A. were firstly ground in a blender (Sterilmixer 12 model 6805–50, PBI International, Milan, Italy) and then passed through a 2 mm sieve. The resulting flour was defatted by mixing the extract with 5 vols (w/v) of hexane and left stirring for 1 h. The hexane layer was then decanted and removed. (The procedure was repeated twice.) Finally, the defatted flour was dried under a gentle stream of air. Two different types of samples were produced, namely blank mix and spiked mix. A blank nuts sample, free from peanut contamination, was obtained by mixing together 1 g of

each nut-defatted flour, namely roasted hazelnuts, almonds, walnuts and pistachios. A spiked nuts mixture was produced by combining an appropriate amount of blank mix with a calculated amount of peanut-defatted powder to obtain the final contamination level of $1000 \mu\text{g g}^{-1}$. Starting from a spiked mix extract, a set of samples ($n = 3$) artificially contaminated with peanuts at four levels covering the concentration range 125–1000 μg of peanut per g of matrix was prepared. Each sample was prepared by combining appropriate volumes of spiked and blank mix nuts extracts just before Bio-Spin purification and then treated as described below.

Calibration curve in diluted samples

For the calculation of the LOD in the extract diluted with mobile phase, each sample spiked at a different level was diluted eight times with the mobile phase by keeping constant the matrix content, and a calibration curve within the range of 8–62 $\mu\text{g g}^{-1}$ was plotted.

Sample treatment

Extraction and purification

Protein extraction was accomplished by adding to 4 g of samples 80 ml of 20 mM Tris-HCl, pH 8.2 buffer (1:20 w/v) followed by shaking for 20 min at RT in an Orbital shaker (KS 4000 i-control shaker, IKA Works GmbH & Co. KG, Staufen, Germany). Subsequently, samples were submitted to ultrasound treatment (Q125, Qsonica, LLC Newton, CT, USA) at the following conditions: time 30 min, amplitude 70%, pulse 15 s on, 2 s off, and output around 18 W. After centrifugation (15 min, 1734g, 18°C), the supernatant was carefully collected and 100 μl of it were loaded onto a size-exclusion column (Bio-Spin® P-6, Bio-Rad, cut-off 6 kDa) for further purification. A final volume of 100 μl was eluted from the column and submitted to enzymatic digestion.

Enzymatic digestion

Protein enzymatic digestion was carried out on the final extracts and trypsin was used as a specific cleavage enzyme. Samples were first denatured by heating for 15 min at 95°C and then treated with 5 μl of DTT solution 50 mM (30 min of incubation in a thermoshaker at 60°C) for protein reduction. After cooling the sample to RT, 10 μl of IAA solution 100 mM were added and the mixture was placed in the dark for 30 min at RT. Finally, 15 μl of a trypsin solution (1 $\mu\text{g } \mu\text{l}^{-1}$ in acetic acid 50 mM) were added to the sample so that a ratio of 1/50 (enzyme/protein based on the results of the Bradford protein assay) was attained to facilitate complete enzymatic digestion. The reaction was halted after 5 h by acidifying the sample with HCl 1 M. The final digests were diluted

twice with mobile phase ($\text{H}_2\text{O}/\text{CH}_3\text{CN}$ 90/10 v/v + 0.1% formic acid) and filtered through 0.2 μm PTFE filters before HPLC injection.

HPLC-MS/MS analysis

HPLC-Linear Ion Trap™ MS analysis

Identification of peanut peptide markers was carried out by analysing a nuts mixture contaminated at a $1000 \mu\text{g g}^{-1}$ level by HPLC-MS/MS on a Linear Ion Trap Mass Spectrometer Velos Pro™ (Thermo Fisher Scientific, San Jose, CA, USA) equipped with a UHPLC pump and an ESI interface. Peptide separation was accomplished on the Acclaim™ PepMap100 analytical column (internal diameter 1 mm, length 150 mm, particle size 3 μm , porosity 100 Å) provided by Thermo Fisher, employing H_2O + 0.1% formic acid and $\text{CH}_3\text{CN}/\text{H}_2\text{O}$, 80/20 v/v + 0.1% formic acid as eluents (solvents A and B, respectively). The chromatographic run started with 10% B, kept isocratic for 3 min, followed by a linear gradient to 55% B in 48 min and a second gradient to 90% in 2 min. An isocratic step at 90% B continued for 20 min, followed by a return to 10% B in 7 min. At the end of the run the column was allowed to equilibrate at 10% B for 25 min. The flow rate was 60 $\mu\text{l min}^{-1}$ and the injection volume was set to 20 μl . To investigate candidate peptide markers for peanut detection, the system was run in untargeted mode employing the *N*th order double-play Data Dependent™ acquisition (DDA) option. In particular, two events were set for this experiment: (1) full MS in the range 400–2000 *m/z*, four microscans; and (2) full MS/MS DDA of the 20 most abundant ions in the MS spectrum using a normalised 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 50 and high 1500.
- *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, *N*th most intense $N = 20$; activation: activation type CID, isolation width 2.0, normalised collision energy 35, activation *q* 0.25, activation time 10 ms.

ESI settings for interfaces were: sheath gas flow (a.u.) 25, auxiliary gas flow (a.u.) 5, spray voltage 4 kV, capillary temperature 320°C, heater temperature 30°C.

Raw data were processed via Proteome Discoverer™ software (version 1.4, Thermo Fisher Scientific) interrogating a customised food allergen database (DB) built in

house. The in-house DB contained approximately 2800 sequences imported from UniProt KB, including sequences derived from the following nuts species: *A. hypogaea*, *C. avellana*, *J. Regia*, *P. vera* and *P. dulcis*. Software results were filtered by peptide mass deviation (300 ppm) and peptide confidence (high = 1% confidence). Candidate peptides were then searched in the Protein Information Resource (PIR) online platform in order to check their uniqueness for peanut. Only peptide pool attributed exclusively to peanut were then screened to identify the most appropriate peptide markers.

MS-Orbitrap™ analysis

For HRMS analysis of nuts mixtures, an (U)HPLC pump equipped with an autosampler (Accela™, Thermo Fisher Scientific) coupled with an Exactive™ non-hybrid single-stage Orbitrap-MS (Thermo Fisher Scientific, Bremen, Germany), equipped with an HESI II interface, was employed. Chromatographic separation of peptides was accomplished by gradient elution on a Aeris peptide XB-C18 analytical column (internal diameter 2.1 mm; length 150 mm; particle size 3.6 µm; porosity 100 Å) at a flow rate of 200 µl min⁻¹. The gradient used was: from 10% to 35% of solvent B (A = H₂O + 0.1% of formic acid, reserve B = CH₃CN + 0.1% of formic acid) in 30 min, then to 70% B in 2 min and to 90% B in 3 min, kept stable for 9 min and back to 10% B in 1 min. This composition was maintained for 15 min to ensure column reconditioning. The sample volume injected into the HPLC column was 20 µl. MS acquisitions were based on a single-segment/two-events acquisition scheme where the two events were represented respectively by a MS full-scan and a HCD-MS all-ion fragmentation acquisition (collision energy = 35 eV), both accomplished in a 400–1300 *m/z* range. Other parameters defined for both acquisition events were: resolution high (50 000 FWHM), microscans 1, AGC balanced 1 × 10⁶, maximum IT 100 ms, sheath gas flow rate 30 (a.u.), auxiliary gas flow rate 15 (a.u.), spray voltage 4 kV, capillary temperature 320°C, capillary voltage 32.5 V, tube lens voltage 130 V, skimmer voltage 30 V, heater temperature 30°C. MS analysis was carried out by implementing the lock mass option for the corrections of any mass shift due to instrumental drift.

Results and discussion

Selection of unique peptides tracing peanuts in a nuts mixture

Aiming to identify the most sensitive and reliable peanuts markers, a preliminary investigation was carried out in a complex food matrix composed by a mixture of four different nut species (namely, hazelnuts, almonds, walnuts and pistachios) spiked with peanuts at 1000 µg g⁻¹.

Selected cultivars representative for each species (as detailed in the materials and methods section) were used. Due to the high level of homology shared between proteins belonging to different peanuts varieties, one among them, namely the *A. hypogaea* var. Zambia, was chosen as the representative variety. An extraction procedure tailored to allergen extraction from cookies and using an ultrasound step (Monaci et al. 2014) was utilised and applied in this work in order to shorten the time needed for the extraction of peanuts proteins. As a first step, a scouting experiment in DDA acquisition mode was performed on the linear ion-trap MS to draw a potential list of candidate peptides markers for peanuts. This type of acquisition is mainly based on two consecutive and dependent scan events: (1) full-scan MS and (2) a sequential MS/MS event triggered by the 20 highest precursor ions exceeding a defined threshold. Raw data were then processed through the commercial software Proteome Discoverer™ and protein assignment was accomplished via Sequest scoring algorithm by searching MS/MS spectra against a small-sized DB compiled in-house (for details, see the materials and methods section). The number and sequence of the identified peptides were retrieved for each allergenic protein family by interrogating the DB at a high confidence and a false discovery rate (FDR) set at 0.01 (indicating a measure of certainty in the identification). A summary of all peptides detected with the relevant Xcorr and the allergenic protein which belong to it is schematised in Table 1. In turn, peptides showing MS/MS spectra highly matching with the predicted fragmentation patterns and displaying the highest Xcorr were further analysed by the analyst by visual inspection of the generated spectra in order to select those showing the highest intensity and reproducibility in the same state charge (variance ≤ 15%) and the best chromatographic resolution from matrix interferences. This subset of peptides was further submitted to PIR search in order to filter only unique and specific peptides tracing for peanut proteins. In order to narrow down the list of potential markers, some criteria were adopted based on the following: absence of missed cleavages, no post-translational modification sites, sequence specificity, stability and good intensity of the spectra produced. As a result, a total of six peptides met the mentioned requirements, which are highlighted in bold in Table 1.

Performances of the Exactive-Orbitrap™ MS-based method

In order to shorten the analysis time, different incubation times with trypsin were investigated in terms of efficiency of the enzymatic reaction. After 5 h of incubation with trypsin, the efficiency of the enzymatic reaction reached a plateau and no further increase in the intensity of peanuts markers was recorded compared with a 12-h incubation

Table 1. Peptides identified by Proteome Discoverer™ software (version 1.4) through a Sequest search of experimental data-dependent MS/MS spectra against a customised nuts allergen database.

Peptide sequence	Allergenic group	Xcorr ^a	PEP ^b	Charge	MH+ (Da)	ΔM (ppm)	m/z (Da)
VYDEELQEGHVLVVPQNFAVAGK	Ara h 3	5.56	5.60*10⁻⁶	3	2541.8	212.24	847.9
WLGLSAEYGNLYR		4.76	3.16*10⁻⁶	2	1542.0	153.31	771.5
FNLAGNHEQEFLR		4.66	4.66*10⁻⁶	2	1574.6	-139.21	787.8
SPDIYNPQAGSLK		3.92	5.23*10⁻⁵	2	1389.4	-237.36	695.2
AGQEEENEGGNIFSGFTPEFLAQAFQVDDR		3.74	1.03*10 ⁻⁵	2	3303.0	160.36	1652.0
TANDLNLILR		3.44	5.32*10 ⁻³	2	1255.7	-34	628.4
SQSDNFEYVAFK		3.23	1.21*10 ⁻³	2	1434.3	-227.42	717.7
GYFGLIFPGcPSTYEPAQQR		3.00	1.80*10 ⁻³	2	2474.8	259.67	1237.9
GENESDEQGAIVTVR		2.96	2.30*10 ⁻²	2	1603.6	-125.85	802.3
FFVPPSQSLR		1.97	2.52*10 ⁻⁴	2	1305.5	-125.38	653.3
IFLAGDKDNVIDQIEK	Ara h 1	4.35	1.14*10⁻⁵	3	1817.8	-99.97	606.6
GSEEDITNPINLR		3.57	1.17*10⁻³	2	1586.6	-134.21	793.8
DLAFPGSGEQVEK		3.02	3.62*10⁻³	2	1376.5	-96.28	688.8
GTGNLELVAVR		4.12	9.71*10⁻²	2	1128.7	74.44	564.9
EGEPDLSNNFGK	Ara h 2	1.69	3.32*10 ⁻³	2	1306.5	-45.62	653.8
QQWELQGDR		2.68	1.99*10 ⁻¹	2	1159.4	-168.68	580.2

Notes: Sequences shown in bold refer to peptides selected as candidate marker for HRMS method development.

^aXcorr is the peptide score, which refers to the number of fragment ions common to two different peptides with the same precursor mass. It calculates the cross-correlation score for all candidate peptides queried from the database.

^bPEP is the posterior error probability, i.e. the probability that the observed peptide-spectrum match is incorrect.

time. Nuts fortified with peanuts at the following levels: 125–250 to 500–1000 $\mu\text{g g}^{-1}$, were analysed by HPLC-HRMS, and a subset of six potential markers shown in Table 1 (VYD, WLGL, FNL, SPD, DLA, GTG) was employed to check for the suitability of the method for the detection of peanuts in nuts-based products. The criteria followed for peptide markers detection were: (1) reproducibility of the retention time ≤ 0.1 min, (2) mass accuracy better than ≤ 5 ppm; and (3) at least two fragment ions detected in the HRMS/MS spectrum. Data resulting from a preliminary investigation gave rise to the exclusion of two peptides since they did not display sufficient sensitivity, being instead only detectable at the highest levels tested. In total, four of six peptides proved suitable for HRMS analysis at the fixed analytical conditions, and they were thereby used for the following investigations. Calibration curves were constructed by plotting the signal of each candidate peptide marker against the spike level in nuts-based samples; the relevant calibration parameters calculated are reported in Table 2. Both the theoretical and the experimental peptide ions were recorded and the relevant mass deviation was estimated.

All parameters referred to the four candidate markers such as retention time reproducibility and mass accuracy are also reported in Table 2. Based on the results obtained, the mentioned peptides were deemed good markers for qualitative purposes. An overlay of typical reconstructed ion chromatograms filtered on the accurate masses of the four selected peptides in a peanut spiked sample is shown in Figure 1.

Selectivity of the chosen peptide marker

The nuts mixture was first screened to assess the absence of any interfering isobaric compound falling at the same retention time of any of the four peptides. According to the calibration parameters calculated by the interpolation of the respective concentration peak areas for each peptide and reported in Table 2, peptides VYDEELQEGHVLVVPQNFAVAGK ($m/z = 848.116$) and WLGLSAEYGNLYR ($m/z = 771.391$) displayed the highest sensitivity and were therefore chosen as quantitative markers. Peptide VYDEELQEGHVLVVPQNFAVAGK was preferentially detected as a triply charged ion by ESI MS analysis, and due

Table 2. Summary of the features of candidate peptide markers and the relevant calibration curves parameters.

Allergen source	Sequence	Calculated m/z	Experimental m/z	Charge	Δ ppm	RT (±SD)	Slope ± SD	R ²
Ara h 1	GTGNLELVAVR	564.822	564.830	2	0.71	11.52 ± 0.02	73 ± 7	0.990
	DLAFPGSGEQVEK	688.838	688.848	2	0.14	11.17 ± 0.02	80 ± 5	0.996
Ara h 3	WLGLSAEYGNLYR	771.391	771.403	2	0.65	19.74 ± 0.03	176 ± 3	0.999
	VYDEELQEGHVLVVPQNFAVAGK	848.116	848.117	3	-0.94	17.25 ± 0.04	213 ± 3	0.999

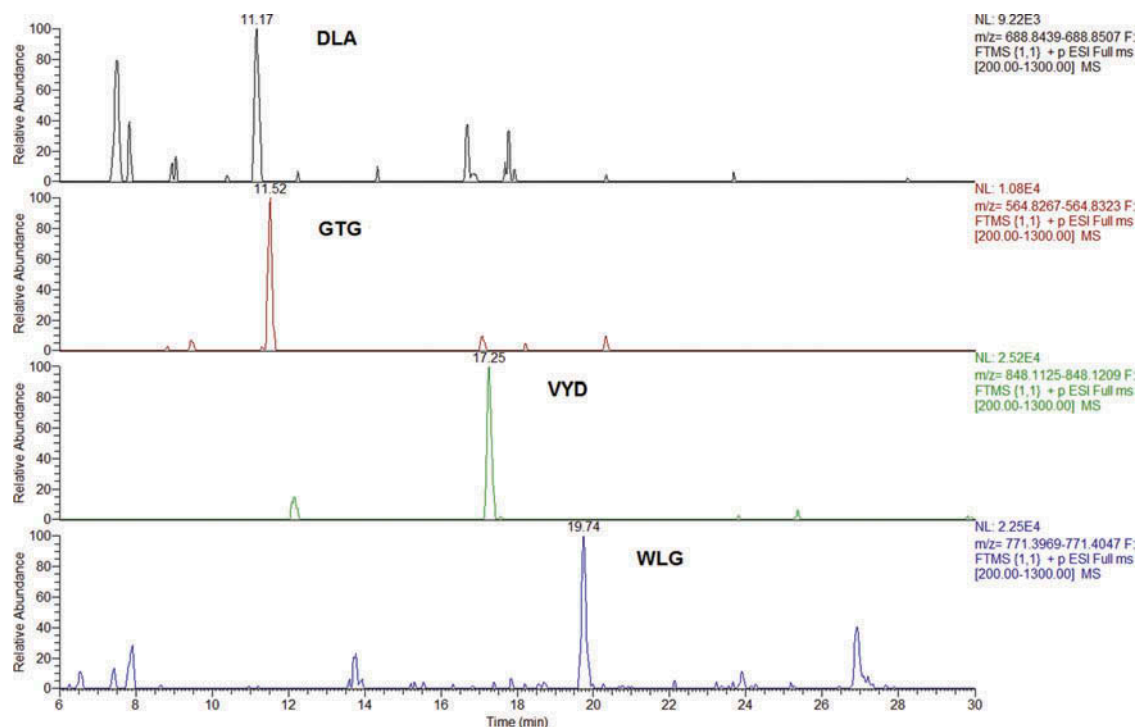


Figure 1. Overlay of the Orbitrap™ extracted ion current (XIC) chromatograms obtained for selected peptide markers of Ara h 1 and Ara h 3 in a nuts mixture sample contaminated with peanuts at $1000 \mu\text{g g}^{-1}$ (mass tolerance = 5 ppm).

to its length it produced a peculiar isotopic pattern with the $M + 1$ isotope showing a higher intensity than isotope M . Therefore, the accurate mass of isotope $M + 1$ was chosen as the leading ion for post-acquisition ion current filtration to plot the reconstructed ion chromatograms. For confirmation purposes, a second peptide WLGLSAEYGNLYR (m/z 771.391), showing good ionisation features and non-presenting co-eluting interferences along the run, was also employed as a quantifier ion to increase the confidence in the analytical measurement. As an example, Figure 2 reports an overlay of XIC traces referring to a peanut-free nuts mixture (Figure 2(a)) and a nuts-based product spiked with peanuts at different levels (Figures 2(b–e)). Chromatograms were obtained by filtering the current on the accurate mass of the 848.116 ion and by applying a mass tolerance as low as 5 ppm.

Several papers published over the last few years have already reported the identification of peanut peptides in different food matrices like biscuits, chocolate and cereal-based products with challenging LODs achieved. Table 3 overviews the peptides designed by other authors as markers for peanut detection in different food commodities. According to the data collected, although a number of peptides were identified for peanuts, only those proving to be the most sensitive were further employed for quantification purposes. However, due to the high degree of homology among storage proteins of the different nuts, such a food matrix was expected not to pose negligible issues in the detection of peanut allergens. To the best of

our knowledge no papers have appeared in the literature reporting the validation of peptide markers for tracing peanuts in nut products. In light of this, we report in this paper the selection of specific peptides unique for peanuts that can therefore be employed as reliable markers for detecting contamination by peanuts in nuts-based products. Note that compared with other food matrices, a nuts mixture represents a very complex food matrix highly rich in fats and proteins, and most of the proteins present a high degree of homology among a subclass of allergenic proteins such as the storage proteins. By taking this into account our efforts aimed at the selection of unique and sensitive peanut peptides and at the development of an easy-to-use screening HRMS-based method for the fast monitoring of peanut contamination in nuts products.

Calculation of LODs

Once the quantitative peptide markers had been selected, calibration curves were constructed in nuts-based products by monitoring both peptides and the respective LODs and LOQs (3^* and 10^* SD intercept/slope of the calibration curve) were calculated (Table 4). According to the data shown, LODs between 26 and $27 \mu\text{g g}^{-1}$ were attained in the nut matrix, proving that both peptides can be considered reliable peanuts markers for quantitative purposes. In addition, in order to evaluate the performances of the method and to calculate the instrumental LOD, four spiked

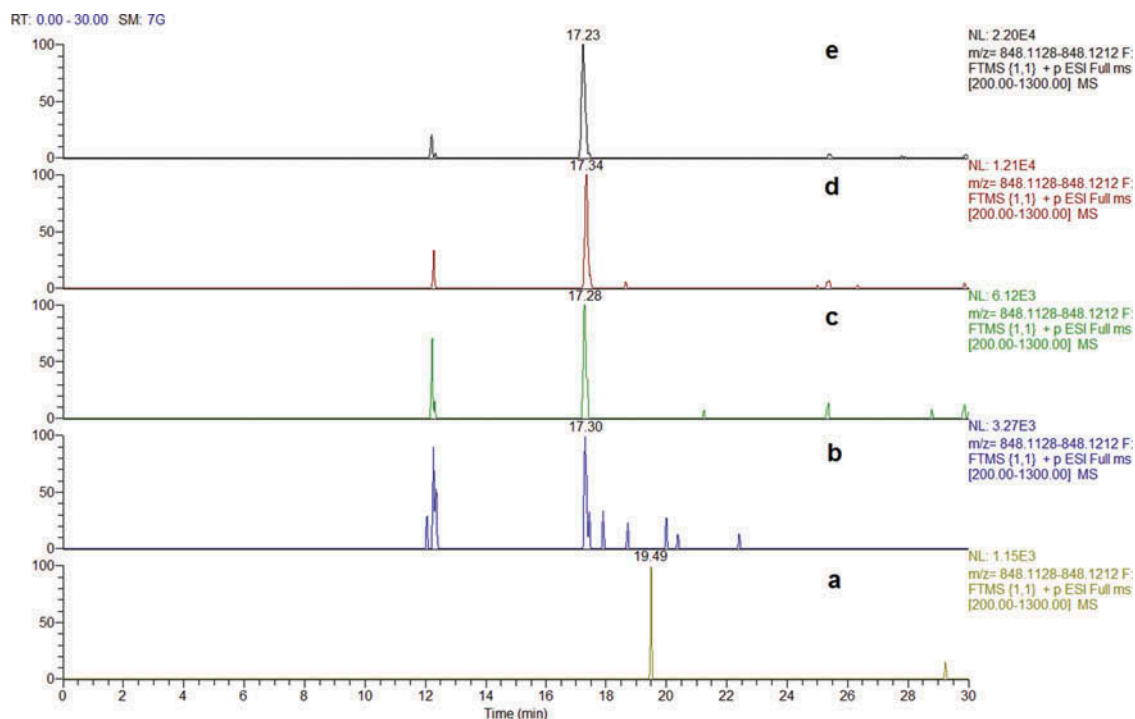


Figure 2. Overlay of the OrbitrapTM XIC chromatograms of peptide VYD (m/z 848.116) in a nuts mixture spiked at different concentration levels (mass tolerance = 5 ppm).

Table 3. Overview of validated peptides selected as quantitative markers for tracing peanuts in food products by using LC-MS/MS quantitative methods.

Peanuts markers	Protein	Matrix	MS apparatus	Reference
m/z 606.6	Ara h 1	Vanilla ice cream	HPLC/QTOF-MS/MS	Shefcheck and Musser (2004)
m/z 688.9		Dark chocolate	HPLC-QqQ-MS/MS	Shefcheck et al. (2006)
m/z 688.9		Bread		Heick et al. (2011)
m/z 807.0	Ara h 2	Rice crispy/chocolate-based snacks	HPLC-QqQ-MS/MS	Careri et al. (2007)
m/z 695.7	Ara h 3/4	Biscuit/cereal breakfast	HPLC-LIT-MS/MS HPLC-QqQ-MS/MS	Careri et al. (2008)
m/z 695.7		Biscuit/cereal breakfast		Bignardi et al. (2010)
m/z 695.0		Rice crispy/chocolate-based snacks		Bignardi et al. (2013)
m/z 695.7		Biscuits		Careri et al. (2007)
m/z 526.6		Dark chocolate		Careri et al. (2008)

Table 4. Comparison of slopes, LOD and LOQ values calculated for the selected quantifier peptides.

Peptide	Slope ($\pm$ SD)	R^2	LOD _{matrix} ($\mu\text{g g}^{-1}$)	LOQ _{matrix} ($\mu\text{g g}^{-1}$)	LOD _{protein} ($\mu\text{g g}^{-1}$)	LOQ _{protein} ($\mu\text{g g}^{-1}$)
848.116	213 ($\pm$ 3)	0.999	26	88	4	14
771.403	175 ($\pm$ 3)	0.996	27	90	4	15

Notes: LOD_{matrix}/LOQ_{matrix} = μg of peanut g^{-1} of matrix.
LOD_{protein}/LOQ_{protein} = μg of peanut proteins g^{-1} of matrix.

samples were subdivided in two aliquots and submitted as such or after (1/4 v/v) dilution with the mobile phase keeping constant the matrix content in all diluted extracts.

Finally, both types of extracts were submitted to HPLC-OrbitrapTM-based Exactive MS analysis and the minimum amount of peanut detectable was estimated according to

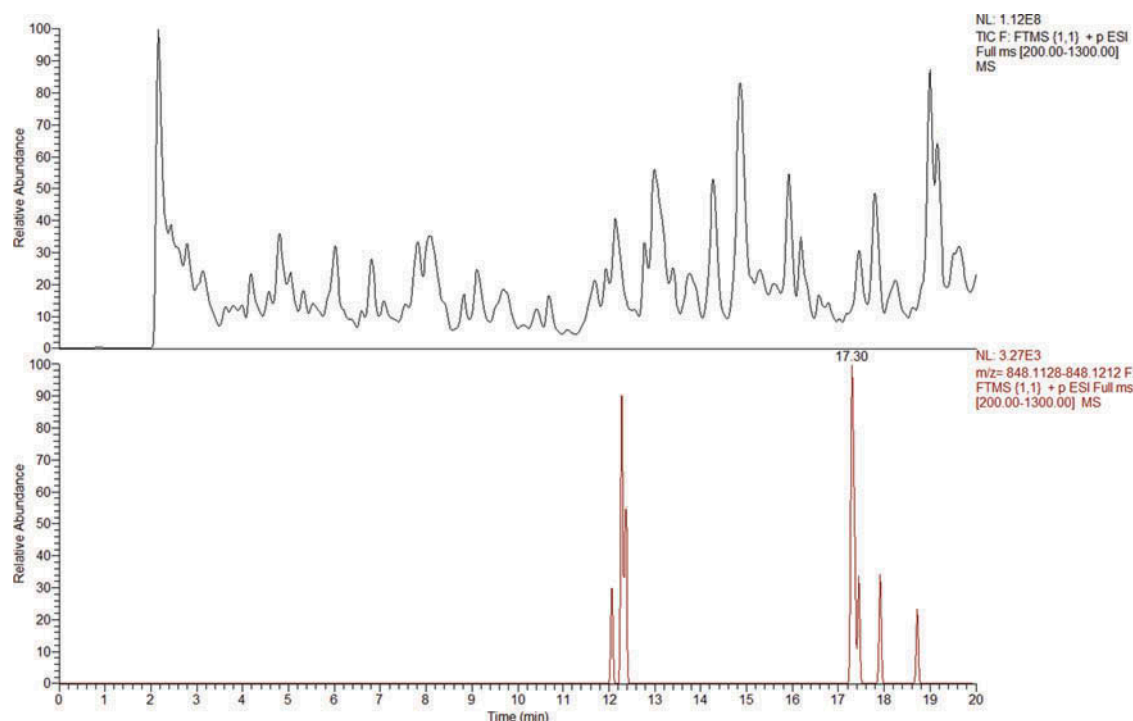


Figure 3. Overlay of the Orbitrap™ total ion current (TIC) and XIC chromatograms of peptide VYD (m/z 848.116) in a nuts mixture spiked at the minimum detectable concentration (mass tolerance = 5 ppm).

the calibration equations. Limits ranged from 13 ng injected for nut extracts to 4 ng injected referring to the diluted extracts. As expected, the reduction of the matrix content in this extract also produced a decrease in the matrix effect displayed by MS and, consequently, a lower value was obtained in this case. Finally, to show the quality of the HPLC-MS traces recorded in proximity of the estimated LODs, reconstructed ion chromatograms for peptide ion 848.116 in both nuts-based extracts and diluted extracts are depicted in Figures 3 and 4, showing a retention time of 17.3 min.

Finally, the total protein content of peanuts extracts was estimated by the Bradford assay, allowing the conversion of the previous matrix LOD (referred to whole peanut content) to protein LOD, referred to the total peanut proteins in 1 g of nuts mixture.

As reported in Table 4, LODs and LOQs were found to be respectively of 4 and 14 $\mu\text{g g}^{-1}$ ($\mu\text{g}_{\text{peanut protein}} \text{g}_{\text{matrix}}^{-1}$), referring to the protein content (or 26 and 88 $\mu\text{g g}^{-1}$ referring to the peanut powder, $\mu\text{g}_{\text{peanut}} \text{g}_{\text{matrix}}^{-1}$). This turns to be very useful information since it refers to the minimum detectable amount of peanut proteins likely able to trigger allergic reactions.

Finally, LODs for peanuts were calculated in spiked nuts extracts diluted eight times with mobile phase and a value ranging from 1 to 2 $\mu\text{g}_{\text{peanut}} \text{g}_{\text{matrix}}^{-1}$ (using markers peptides 848 and 771) were calculated. The slopes of the

calibration curves obtained in spiked nuts samples and in samples diluted with mobile phase were $y = 400\,221$ and $y = 212\,641$ respectively, accounting for the considerable matrix effect displayed in the case of the analysis of a nuts mixture.

Compliance of the method with the VITAL reference dose for peanuts

In general, with the aim to help manufacturers, a standardised allergen risk-assessment tool for food producers named the VITAL (Voluntary Incidental Trace Allergen Labelling) system was developed. This system, originally created by the Allergen Bureau of Australia and New Zealand, is currently adopted by numerous countries, the European Union included. The VITAL grid helps food producers to assess the impact of allergen cross-contact and provides appropriate precautionary allergen labelling on their products. The system also includes a 'traffic light' labelling system based on the content of the allergenic ingredient present that refers to the VITAL grid. In general, the total amount of consumed proteins is an important factor linked to the likelihood of triggering an adverse reaction in sensitive individuals. In this regard, VITAL operates with this reference dose for a certain allergen (representing a part of the total proteins contained into the allergenic food), which is transferred into action levels according to the portion size. According to the defined

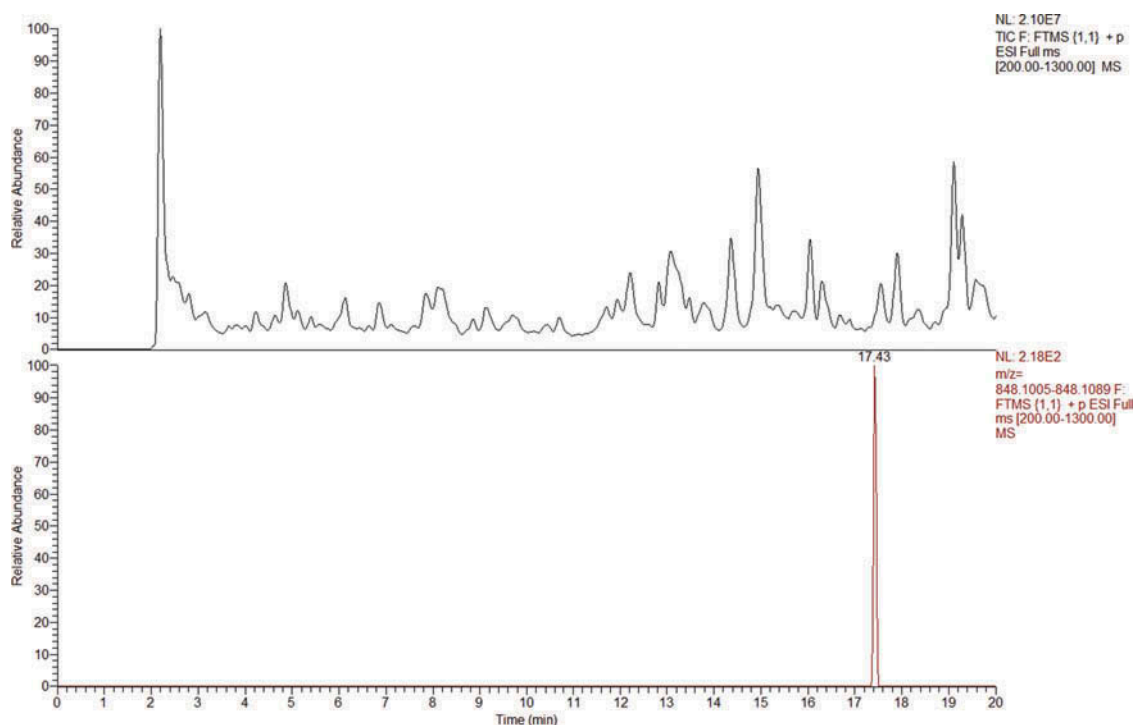


Figure 4. Orbitrap™ TIC and XIC chromatograms of peptide VYD (m/z 848.116) in a spiked nuts mix sample diluted in mobile phase at the minimum detectable concentration (mass tolerance = 5 ppm).

action levels, depending on whether the allergen level falls into the green or the yellow zone, no precautionary statement or only the precautionary labelling is recommended (action levels change depending on portion size). In contrast, the red zone refers instead to total peanut proteins exceeding the reference dose of 0.2 mg consumed by the consumption of a certain amount of food. In that case mandatory allergen labelling is required. By looking at the sensitivity attained by the developed method, with this approach a level of approximately 50-fold lower than the reference dose foreseen for peanut proteins was attained.

Conclusions

A rapid method based on HPLC-HRMS detection and the identification of suitable and unique peanut markers has been reported in this paper. An LOD down to $4 \mu\text{g g}^{-1}$ referring to the total peanut protein per g of nuts was achieved, and two different markers, peptides VYDEELQEGHVLVVPQNFAVAGK and WLGLSAEYGNLYR, were identified and selected as the best quantitative markers. The method enables the detection of peanut traces in nut samples and can serve as a screening tool for assessing any eventual cross-contamination that occurs along the food chain. According to the reference dose established for peanut proteins, the method

herein proposed enables a value that is approximately 50-fold lower than the threshold value to be reached.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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