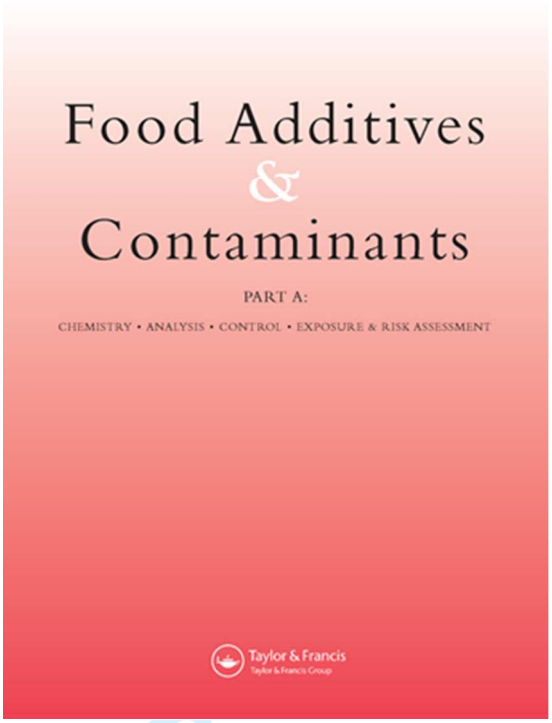

High Resolution – Orbitrap™ based Mass Spectrometry for rapid detection of peanuts in nuts

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Abstract:	Peanut represents one of the most harmful allergenic food capable of triggering severe and sometimes lethal reactions in allergic consumers upon ingestion of even little amounts. Several proteins capable of inducing allergic reactions have been identified from this nut source that have been recognized by patients' IgE antibodies. 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 been also devised for tracing peanuts contamination in different foods with low limits of detection reached. The applicability of a benchtop High Resolution Exactive™ Mass Spectrometer has never been investigated for the rapid screening of peanuts contamination in a complex food matrix like nuts mixtures. We report in this paper the design of suitable peanuts markers and the development of a HR-Orbitrap™ MS based method for peanuts detection in a mixture of nuts species. With this aim, different types of samples were prepared: i) "nuts-based powder" made up of a mixture of hazelnuts, pistachios, almonds and walnuts and ii) "nuts powder fortified with peanuts". Different levels of fortifications were produced and the applicability of the method was tested in this work. Finally, a subset of six peptides fulfilling specific analytical requirements were chosen for checking suitability of the method tailored the detection of peanuts in nuts-based products and two out 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 limit of detection as low as 4 µg of peanuts proteins or 26 µg of peanuts in 1 g of matrix.

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Table supplemental material.dotx

High Resolution – Orbitrap™ based Mass Spectrometry for rapid detection of peanuts in nuts

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Abstract

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 have been identified from this nut source that have been recognized by patients' IgE antibodies. 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 been also devised for tracing peanut contamination in different foods achieving low limits of detection. The applicability of a benchtop High Resolution Exactive™ Mass Spectrometer has never been investigated for the rapid screening of peanut contamination in a complex food matrices like mixtures of nuts. We report in this paper the design of suitable peanut markers and the development of a HR-Orbitrap™ MS based method for peanut detection in a mixture of nuts species. With this aim, different types of samples were prepared: *i)* "nuts-based powder" made up of a mixture of hazelnuts, pistachios, almonds and walnuts and *ii)* "nuts powder fortified with peanuts". Different levels of fortifications were produced and the applicability of the method was tested in this work. Finally, a subset of six peptides fulfilling

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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 Ig-E mediated food allergies (Pumphrey et al. 1999; Hourihane et al. 1998) with the highest rate of inducing anaphylactic shock especially in US and UK (Yunginger et al. 1988; Sampson HA 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 proved to persist into adulthood in the majority of sensitive individuals (Skolnick et al. 2001). As well known, peanut allergy can be triggered by the ingestion of even little amounts of peanuts from 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 better address peanut allergy diagnosis and therapies, 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 proteins were identified as capable of inducing allergic reactions that are recognized 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 hypogea)*. 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 recognized by 30-40% of patients's IgE antibodies (Lehmann et al. 2006; Suhr et al. 2004).

In order to protect allergic consumers from the risk to develop allergic reactions, legislation (EC 2007) has been issued in EU requiring the mandatory labelling of allergenic ingredients including peanuts whenever introduced into a food. In addition to the intentional addition 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 categories is likely to exist as a consequence of cross a contamination that can take place during nuts storage (same rooms 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 allergens 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 may differ significantly in terms of the antibodies emploted, target proteins, calibrators and extraction procedures, they can provide considerably different results for the same analyte (Torok et al. 2015). Methods so far devised for peanuts detection are mainly based on the antibody recognition of some target Ara h- allergens and generally employ an ELISA (enzyme linked immunosorbent assay) format. However, given to some 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 masking effect. in the last years mass spectrometry

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 mass spectrometers, have been reported in literature for the identification of peanuts markers in different types of food or in raw and roasted peanuts (Chassaigne et al. 2006). However the methods developed were mainly intended to detect traces of peanuts in food commodities like biscuits, chocolate and ice-creams with LODs ranging from 0.1 to 10 µg of peanut ingredient in 1 g of food matrix (Careri et al. 2007; Heick et al. 2011; Shefcheck et al. 2004; Shefcheck et al. 2006).

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

We 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; Monaci et al. 2013; Pilolli et al. 2014).

In the present paper we describe the development of a method based on the use of a benchtop Orbitrap™ based mass spectrometer 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 HCD chamber that is 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 was placed on the selection of specific and unique peptide markers that did not show any identity with other tree nuts coming from different sources. Afterwards, by monitoring the selected peptides at the highest mass accuracy, a rapid method was developed enabling the detection of peanuts in other nuts down to 4 µg/g of total

proteins by full scan high resolution MS acquisition. This proved the high versatility of HR-MS system for the rapid screening of peanuts traces in nuts and products thereof.

Material and methods

Nuts

Roasted peanuts (*Arachis hypogaea,* var. Zambia) and roasted nut sources composing the "nuts mix" were kindly provided by Besana S.p.A. (San Gennaro Vesuviano, NA, 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 trizma-base were obtained from Sigma–Aldrich (Milan, Italy). Trypsin (proteomic grade) was purchased by Promega (Milan, Italy) whilst 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 by Bio-Rad (Segrate, MI, Italy).

Preparation of spiked samples

Nuts 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 five volumes (w/v) of hexane and let it stirring for 1h. 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". Blank nuts sample, free from peanut contamination, was obtained by mixing together 1g of each nut defatted flour, namely

roasted hazelnuts, almonds, walnuts and pistachios. 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 µg/g. Starting from spiked mix extract a set of samples (n = 3) artificially contaminated with peanuts at four levels covering the concentration range 1251000 µg of peanut per gram of matrix were 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 different level was diluted 8 times with the mobile phase by keeping constant the matrix content and a calibration curve within the range of 8-62 µg/g was plotted.

Sample treatment

Extraction and purification

Protein extraction was accomplished by adding to 4g of samples 80ml of 20mM Tris HCl, pH 8.2 buffer (1:20 w/v) followed by shaking for 20min at room temperature 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 sec ON, 2 sec 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 on a size exclusion column (Bio-Spin® P-6, Biorad, cut off 6kDa) 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 specific cleavage enzyme. Samples were firstly 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 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 15 µL of a trypsin solution (1 µg/µL 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 a complete enzymatic digestion. Reaction was halted after 5 h by acidifying the sample with HCl 1 M. The final digests were diluted twice with mobile phase (H_2O/ CH_3CN 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 peanuts peptide markers was carried out by analyzing a nuts mixture contaminated at 1000µg/g level by HPLC-MS/MS on a Linear Ion Trap Mass Spectrometer Velos Pro™ (Thermo Fisher Scientific, San Josè, USA) equipped with a UHPLC pump and an ESI interface. Peptides separation was accomplished on the Acclaim™ PepMap100 analytical column (internal diameter 1 mm, length 150 mm, particle size 3 µm, porosity 100Å) provided by Thermo Fisher (San Josè, US), employing H_2O+0.1% formic acid and CH_3CN/H_2O, 80/20 v/v+0.1% formic acid as eluents (solvent A and solvent B, respectively). The chromatographic run started with 10% B, kept isocratic for 3min, followed by a linear gradient to 55% B in 48min and a second gradient to 90% in 2min. An isocratic step at 90% B continued for 20 min, followed by a return to10% B in 7 min. At the end of the run the column was allowed to equilibrate at 10% B for 25min. The flow rate was 60 µl/min and the injection volume was set to 20 µL. To investigate candidate peptide markers for peanuts 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*, 4 microscans; 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 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, normalized collision energy 35, activation q 0.25, activation time 10 ms. The ESI settings for interfaces were the following: 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, San Josè, US) interrogating a customized 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: *Arachis Hypogaea, Corylus Avellana Juglans Regia Pistacia Vera, Prunus Dulcis* Software results were filtered by peptide mass deviation (300 ppm) and peptide confidence (high = 1% confidence). Candidate peptides were then searched in 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 high resolution mass spectrometry analysis of nuts mixtures, an (U)HPLC pump equipped with an autosampler (Accela™, Thermo Fisher Scientific, San Jose, CA, USA) coupled with an Exactive™ non-hybrid single stage Orbitrap-mass spectrometer (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. The gradient used was: from 10% to 35% of solvent B (A = H_2O + 0.1% of formic

acid, reserve B = CH_3CN + 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 assure column reconditioning. The sample volume injected in 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 1×10^6, 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 were 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 nuts mixture

With the aim 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 4 different nut species (namely hazelnuts, almonds, walnuts and pistachios) spiked with peanuts at 1000 µg/g. Selected cultivars representative for each species (as detailed in the materials and methods) were used in the present study. Due to the high level of homology shared between proteins belonging to different peanuts varieties, one among them namely the *Arachis hypogaea* var. Zambia was chosen as the representative variety. An extraction procedure tailored to allergens extraction from cookies and using a ultrasound step (Monaci et al., 2014), was utilized and applied in this work in order to shorten the time needed for the extraction of peanuts proteins. As first step, a scouting experiment in DDA acquisition mode was performed on the linear ion trap mass spectrometer 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: i) full scan MS and ii) sequential MS/MS event triggered by the twenty 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 database compiled in-house (for details see materials and methods section). The number and sequence of the identified peptides were retrieved for each allergenic protein family by interrogating the database at a high confidence, FDR set at 0.01 (False Discovery Rate: 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, is schematized 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 from 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-transitional modification sites, sequence specificity, stability and good intensity of the spectra produced. As a result, a total of six peptides met the mentioned requirements and 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 hours of incubation with trypsin, the efficiency of the enzymatic reaction reached the plateau and no further increase in the intensity of peanuts markers was recorded compared to 12 hours incubation time. Nuts fortified with peanuts at the following levels 125-250-500-1000 µg/g, were analysed by HPLC-HR-MS and a subset of six potential markers shown in table 1 (VYD, WLG, FNL, SPD, DLA, GTG) was employed for

checking suitability of the method for the detection of peanuts in nuts-based products. The criteria followed for peptide markers detection were: a) reproducibility of the retention time ≤ 0.1 min b) mass accuracy better ≤ 5 ppm c) at least two fragment ions detected in the HR-MS/MS spectrum. Data resulted from a preliminary investigation gave rise to exclude two peptides since they did not display a sufficient sensitivity being instead only detectable at the highest levels tested. In total, four out of six peptides proved to be suitable for HR-MS analysis at the fixed analytical conditions and 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 and 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 resumed in the same table. Basing on the results obtained, the mentioned peptides were deemed good marker 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 pictured in Figure 1.

Selectivity of the chosen peptide marker

The nuts mixture was first screened for assessing 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 triply charged ion by ESI MS analysis and due to its length it produced a peculiar isotopic pattern with the M+1 isotope showing a higher intensity than the isotope M. Therefore the accurate mass of the isotope M+1 was chosen as 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 ionization features and non presenting co-eluting interferences along the run, was also employed as quantifier ion to increase the confidence in the analytical measurement. As a sake of example Figure 2 reports an overlay of XIC traces referred to a peanut-free nuts-mixture (Figure 2(a)) and 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 years already reported the identification of peanut peptides in different food matrices like biscuits, chocolate, cereal based products with challenging limits of detection achieved. In table 3 is pictured an overview of the peptides designed by other authors as markers for peanuts 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 food matrix was expected to pose not negligible issues to the detection of peanut allergens. To the best of our knowledge no papers have appeared in the literature reporting validation of peptide markers for tracing peanuts in nuts products. In light of this, we report in this paper the selection of specific peptides unique for peanuts that can be therefore employed as reliable markers for detecting contamination by peanuts in nuts based products. It is worthy to be underlined that compared to other food matrices nuts-mixture represents a very complex food matrix highly rich in fats and proteins and most of proteins present a high degree of homology among a subclass of allergenic proteins like the storage proteins. By taking this into account our efforts were aimed at the selection of unique and sensitive peanut peptides and at the development of an easy to use screening HR MS-based method for the fast monitoring of peanuts 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* s.d. intercept/slope of the calibration curve) were calculated as appearing in Table 4. According to the data shown, LODs comprised between 26 and 27 µg/g 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 performances of the method and to calculate the instrumental LOD, four spiked 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-Orbitrap™ based Exactive MS analysis and the minimum amount of peanut detectable was estimated according to the calibration equations. Limits ranged from 13 ng injected for nut extracts to 4 ng injected referred to the diluted extracts. As expected, the reduction of the matrix content in this extracts also produced a decrease in the matrix effect displayed by MS and consequently a lower value was obtained in this case. At last, to show the quality of the HPLC-MS traces recorded in proximity of the estimated LODs, reconstructed ion chromatograms for peptide ion 848.116 both in nuts-based extracts and in the diluted extracts are depicted in Figure 3 and Figure 4, showing a retention time of 17.3 min.

Finally the total protein content of peanuts extracts was estimated by 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 µg/g ($\mu g_{peanut\ protein}/g_{matrix}$) referred to the protein content (or 26 and 88 µg/g referred to the peanut powder, $\mu g_{peanut}/g_{matrix}$). This turns to be a very useful information since it refers to the minimum detectable amount of peanuts proteins likely able to trigger allergic reactions.

Finally LODs for peanuts were calculated in spiked nuts extracts diluted 8 times with mobile phase and a value ranging from 1 to 2 $\mu g_{peanut}/g_{matrix}$ (using as markers peptides 848 and 771) were calculated. 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 a considerable matrix effect displayed in case of analysis of nuts mixture.

Compliance of the method with the VITAL reference dose for peanuts

In general, with the aim to help manufacturers, a standardized allergen risk assessment tool for food producers named VITAL (Voluntary Incidental Trace Allergen Labelling) system has been developed. This system originally created by the Allergen Bureau of Australia and New Zealand, is currently adopted by numerous countries EU included. The VITAL grid helps food producers to assess the impact of allergen cross-contact and provide 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. On 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 action levels, depending if the allergen level falls in the green or yellow zone, no precautionary statement or only the precautionary labelling is recommended (action levels change in dependence of the portion size). In contrast to that, the red zone refers instead to total peanuts proteins exceeding the reference dose of 0.2 mg consumed by the consumption of a certain amount of food. In that case the mandatory allergen labelling is required. By looking at the sensitivity attained by the developed method, with this approach a level approximately 50 folds lower than the reference dose foreseen for peanuts proteins was attained.

Conclusions

A rapid method based on HPLC-HR-MS detection and the identification of suitable and unique peanut markers have been reported in this paper. LOD down to 4 µg/g referred to the total peanut

protein per gram 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 peanuts traces in nuts samples and can serve as a screening tool for assessing any eventual cross-contamination occurring along the food chain. According to the reference dose established for peanut proteins the method herein proposed enables to reach a value that is approximately 50 folds lower than the threshold value .

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Captions for figures

Figure 1.Overlay of the Orbitrap™ eXtracted Ion Current (XIC) chromatograms filtered on the accurate mass of the four selected peptides, in a nuts mixture sample contaminated with peanuts at 1000 µg/g level (mass tolerance 5ppm).

Figure 2.Overlay of the Orbitrap™ XIC chromatograms for peptide VYD (*m/z* 848.116) in nuts mixture spiked at different concentration levels comprised in the range 125-1000 µg/g (mass tolerance 5ppm).

Figure 3. Overlay of the Orbitrap™ Total Ion Current (TIC) and XIC chromatograms for peptide VYD (*m/z* 848.116) in nuts mixture spiked at the minimum detectable concentration (mass tolerance 5ppm).

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

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Figure 1

Overlay of the Orbitrap™ eXtracted Ion Current (XIC) chromatograms obtained for selected peptide markers of Ara h1 and Ara h3, in a nuts mixture sample contaminated with peanuts, at 1000 µg/g level (mass tolerance 5ppm).

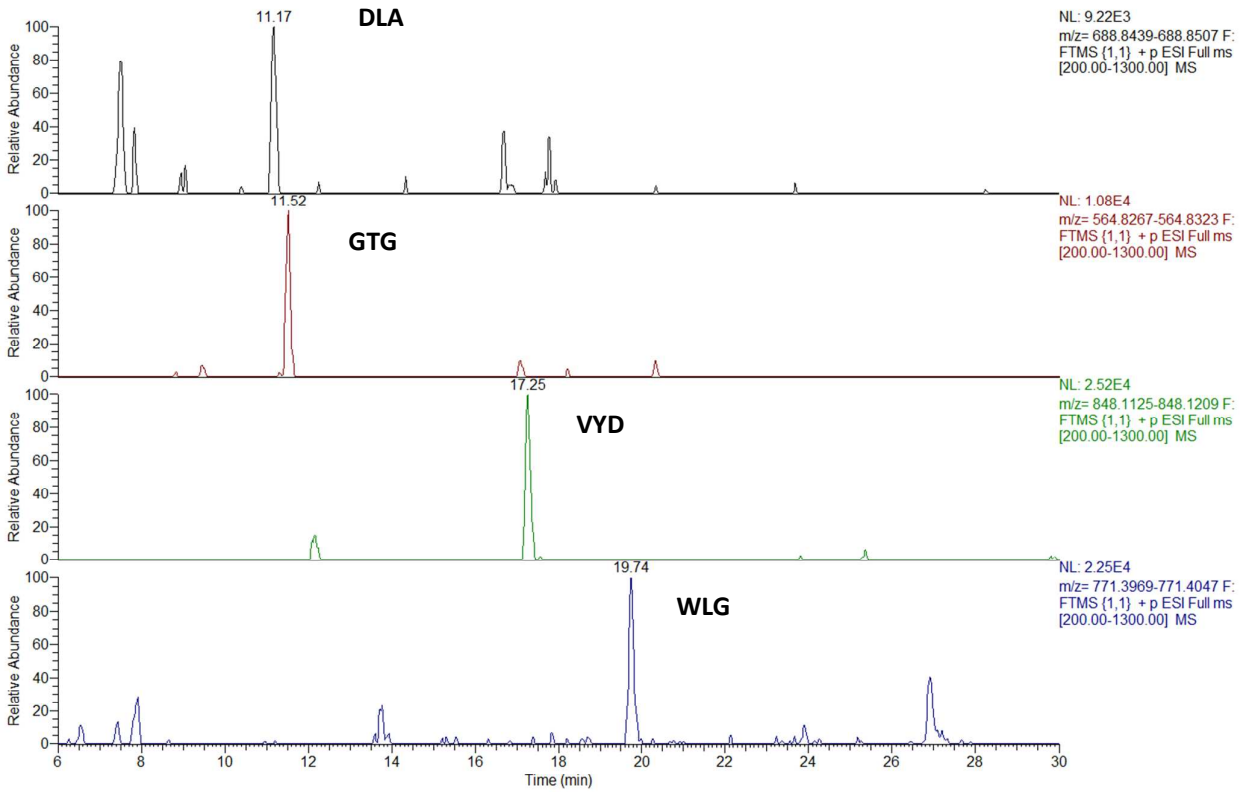

Figure 2

Overlay of the Orbitrap™ XIC chromatograms of peptide VYD (*m/z* 848.116) in nuts mixture spiked at different concentration levels (mass tolerance 5ppm).

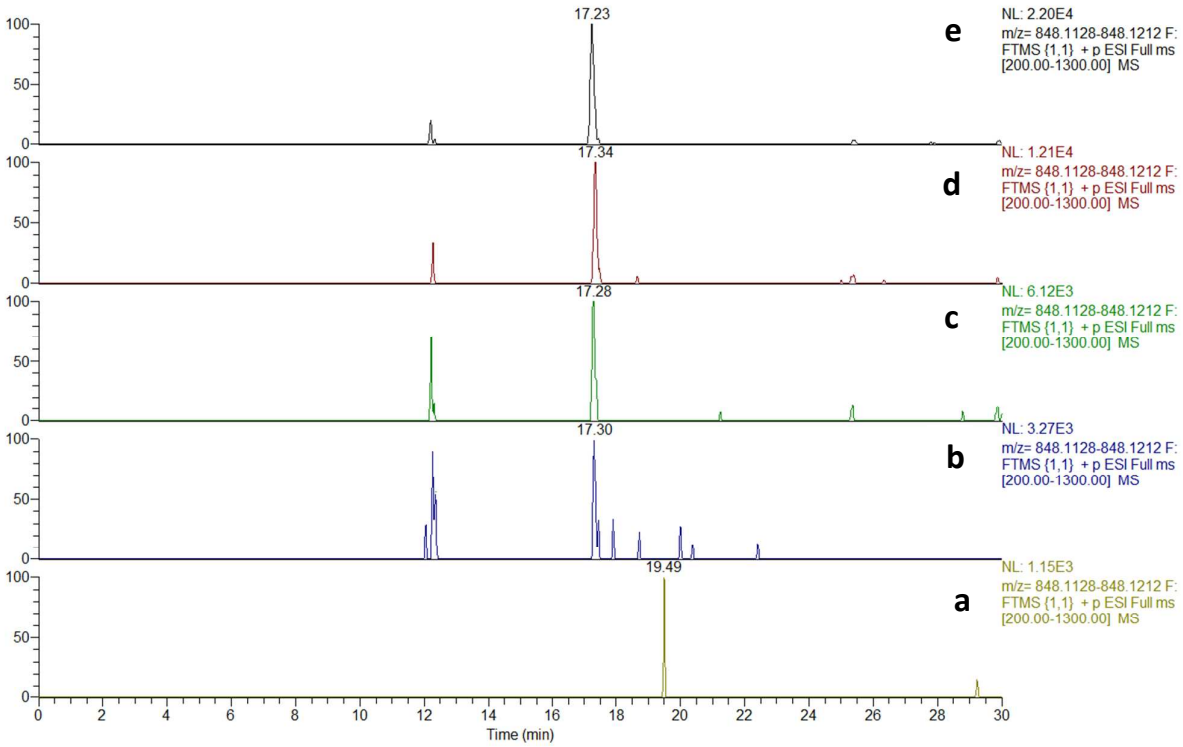

Figure 3.

Overlay of the Orbitrap™ Total Ion Current (TIC) and XIC chromatograms of peptide VYD (*m/z* 848.116) in nuts mixture spiked at the minimum detectable concentration (mass tolerance 5ppm).

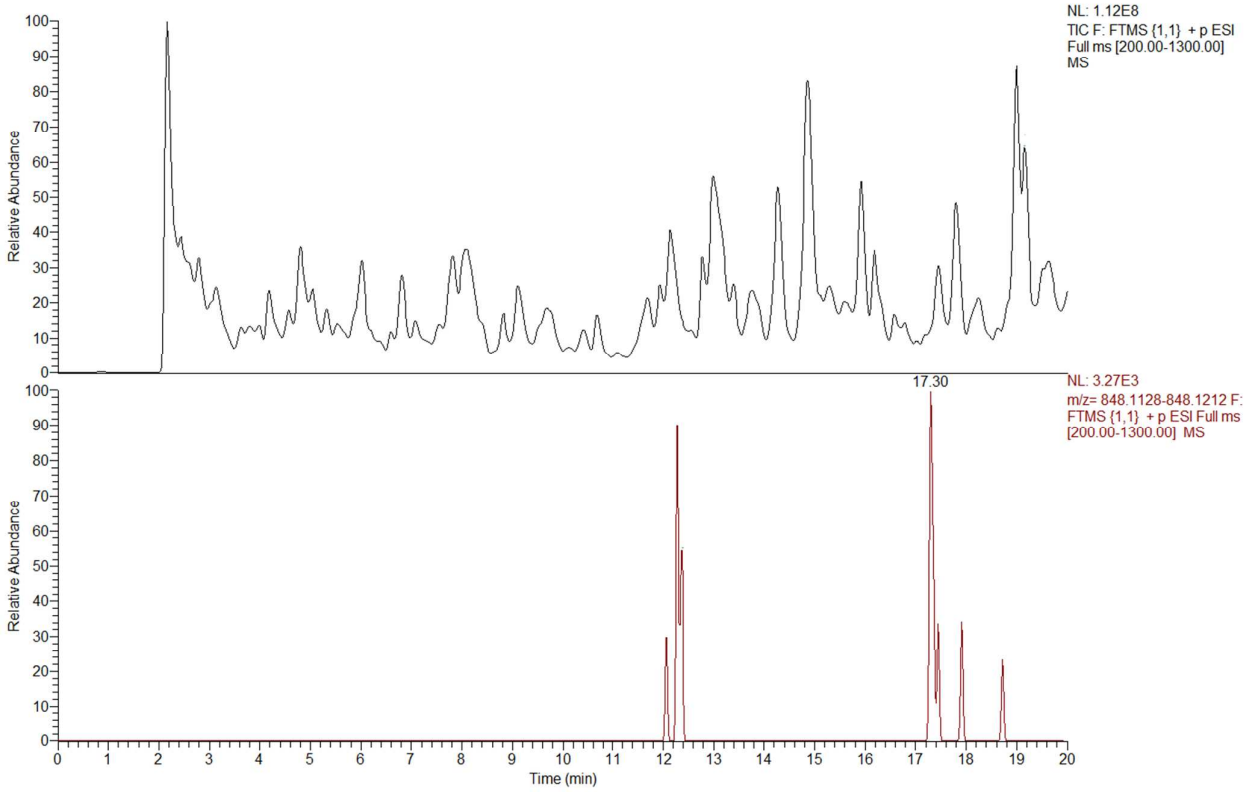

Figure 4.

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

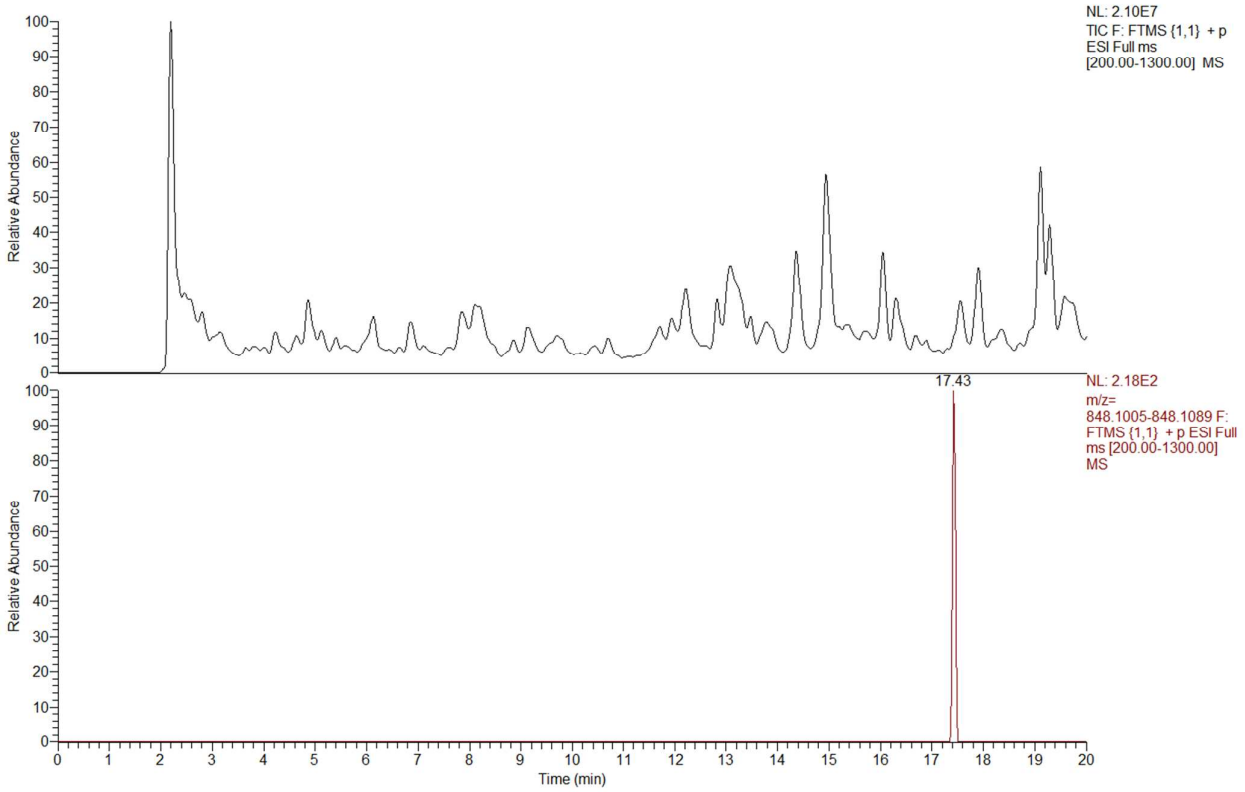

Table 1. List of peptides identified by Proteome Discoverer ™ software (version 1.4), through Sequest search of experimental Data Dependent MS/MS spectra against a customized nuts allergen database. Bold sequences refers to peptides selected as candidate marker for HR-MS method development.

Peptide sequence	Allergenic group	XCorr*	PEP**	Charge	MH+ [Da]	ΔM [ppm]	*m/z* [Da]
VYDEELQEGHVLVVPQNFAVAGK		**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	Ara h3	3.74	1.03 * 10^{-5}	2	3303.0	160.36	1652.0
TANDLNLLILR		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
GYFGLIFPGcPSTYEEPAQQGR		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
FFVPPSQQSLR		1.97	2.52 * 10^{-4}	2	1305.5	-125.38	653.3
IFLAGDKDNVIDQIEK		4.35	1.14 * 10^{-5}	3	1817.8	-99.97	606.6
GSEEEDITNPINLR		3.57	1.17 * 10^{-3}	2	1586.6	-134.21	793.8
DLAFPGSGEQVEK	Ara h1	**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		1.69	3.32 * 10^{-3}	2	1306.5	-45.62	653.8
QQWELQGDR	Ara h2	2,68	1.99 * 10^{-1}	2	1159.4	-168.68	580.2

*Xcorr: peptide score which refers to the number of fragment ions common to two different peptides with the same precursor mass and calculates the cross-correlation score for all candidate peptides queried from the database.

**PEP: posterior error probability is the probability that the observed peptide-spectrum match is incorrect.

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

Allergen source	Sequence	calculated *m/z*	experimental *m/z*	charge	Δ ppm	RT (±s.d.)	Slope ± s.d.	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

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 icecream	HPLC/QTOF-MS/MS	Shefcheck et al. 2004
m/z 688.9		Dark chocolate	HPLC-QqQ-MS/MS	Shefcheck et al. 2006
m/z 688.9		Bread		Heick J et al. 2011
m/z 807.0	Ara h 2	Rice crispy/Chocolate-basedsnacks	HPLC-QqQ-MS/MS	Careri et al. 2007 Careri et al. 2008
m/z 695.7	Ara h 3/4	Biscuit/Cereal Breakfast	HPLC-LIT-MS/MS	Bignardi et al. 2010
m/z 695.0		Rice crispy/Chocolate-based snacks		Bignardi et al. 2013
m/z 695.7		Biscuits	HPLC-QqQ-MS/MS	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 (±s.d.)	R^2	LOD_{matrix} (µg/g)	LOQ_{matrix} (µg/g)	$LOD_{protein}$ (µg/g)	$LOQ_{protein}$ (µg/g)
848.116	213 (±3)	0,999	26	88	4	14
771.403	175 (±3)	0,996	27	90	4	15

LOD_{matrix}/ LOQ_{matrix} : µg of peanut per g of matrix.

$LOD_{protein}$/ $LOQ_{protein}$: µg of peanut proteins per g of matrix.