

Determination of Hidden Hazelnut Oil Proteins in Extra Virgin Olive Oil by Cold Acetone Precipitation Followed by In-Solution Tryptic Digestion and MALDI-TOF-MS Analysis

Cristina De Ceglie,[†] Cosima Damiana Calvano,^{*,†} and Carlo Giorgio Zambonin^{†,‡}

[†]Dipartimento di Chimica and [‡]Centro Interdipartimentale di Ricerca S.M.A.R.T., Università degli Studi di Bari, Aldo Moro Via Orabona, 4, 70126 Bari, Italy

ABSTRACT: Adulteration of extra-virgin olive oil (EVOO) with hazelnut oil (HO) is an illegal practice that could have severe health consequences for consumers due to the possible exposure to hidden hazelnut allergens. Here, matrix-assisted laser-desorption/ionization (MALDI) mass spectrometry (MS) was used as a rapid and sensitive technique for the detection of a low concentration of hazelnut proteins in oil samples. Different protocols were tested for protein extraction, and the most efficient (cold acetone) was applied to HO and EVOO adulterated with HO. The subsequent in-solution tryptic digestion of protein extracts and MALDI-MS analysis, using α -cyano-4-chlorocinnamic acid as matrix, allowed the detection of stable hazelnut peptide markers (i.e., the m/z ions 1002.52, 1356.71, 1394.70, 1440.81, 1453.85, 1555.76, 1629.83, 1363.73, and 1528.67) attributable to the main hazelnut proteins Cor a 9, Cor a 11, and Cor a 1. Thus, the approach might allow the direct detection of specific hazelnut allergens in EVOO at low concentration without time-consuming pretreatments.

KEYWORDS: olive oil, hazelnut, protein digestion, cold acetone, allergens, MALDI

■ INTRODUCTION

Extra-virgin olive oil (EVOO) is rich in important nutrients such as essential and monounsaturated fatty acids, phytosterols, vitamins, and natural antioxidants. It is very expensive and is often subjected to frauds involving replacement of high-cost ingredients with lower grade and cheaper substitutes. Adulteration of virgin olive oil with different seed oils, such as hazelnut and almond, in addition to economic implications, represents a serious problem for regulatory agencies, oil suppliers, and consumers.

Olive oil quality is governed by food safety legislation, with a particular attention on the exclusion of contaminants such as metals, pesticides, toxins, and allergens.¹ In recent years, the European Union has supported efforts to protect both producers and consumers from frauds involved in EVOO production, introducing regulations aimed at ensuring traceability, allowing the possibility of tracking batches throughout its production, processing, and distribution, to define its origin, and to rule its labeling.²

Hazelnut oil (HO) is obtained from *Corylus avellana L.* seeds. Hazelnuts have high lipid content (60%) but low amounts of proteins (16%) and carbohydrates (14%). More than 90% of the fat content is constituted by oleic (72.8–83.5%) and linoleic (7.6–16.6%) acids that are the most abundant fatty acids also in olive oil. Besides, HO contains low quantities of palmitic (4.1–6.8%), stearic (1.9–2.8%), and linolenic acids (0.1–0.6%).³

HO is used to adulterate EVOO because of its similar chemical composition with respect to both major and minor compounds;⁴ thus, it cannot be easily detected. However, this fraud may have health implications for some consumers, due to the hidden presence of hazelnut residual proteins in EVOO that can induce severe reactions in allergic subjects.

Hazelnut allergens are proteins with molecular masses ranging from 10 to 60 kDa; the main allergens known are Cor a 1 (17 kDa), Cor a 2 (14 kDa), Cor a 8 (12 kDa), a lipid transfer protein (LTP), Cor a 9 (globulin 11S), and Cor a 11 (globulin 7S).^{5,6} More recently, two oleosin isoforms (Cor a 12, Cor a 13), an isoflavone reductase homologue (Cor a 6), a luminal binding protein (Cor a 10), a 2S albumin (Cor a 14),⁷ and a Cor a 9 isoform⁸ have been identified as new potential allergens in hazelnut. As far as the protein Cor a 1 is concerned, it exists in different isoforms (i.e., Cor a 1.01, Cor a 1.02, Cor a 1.03, and Cor a 1.04). The first three isoforms are known to be pollen allergens, although Cor a 1.04, which has four similar isoforms (Cor a 1.0401–1.0404), has been found in hazelnut kernels.⁹

Many scientific papers argue about the allergenic power of refined seed oils and their real protein content.^{10–16} Hazelnut oil could be considered a protein-free food, although the presence of residual proteins depends on its processing method and on the kernels employed (raw or roasted). For instance, Bremer and co-workers¹⁷ reported, by using a biosensor immunoassay, a protein concentration from 0.35 to 1.670 $\mu\text{g/g}$ in different Greek virgin hazelnut oils, while Teuber et al.¹³ found a protein content of 62 $\mu\text{g/mL}$ in a refined/unrefined blend of hazelnut oil. In general, crude hazelnut oils have a certain protein amount (hundreds of $\mu\text{g/mL}$);^{11,17} partially refined oils may contain residual amounts of proteins (tens of $\mu\text{g/mL}$), thus representing a great risk for allergic subjects; refined hazelnut oils should be almost protein free.

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Several methods are available to detect EVOO adulteration with HO, targeting different chemical markers such as free fatty acids,^{4,18,19} triacylglycerols,^{20,21} sterols,²² volatile organic compounds (VOC)²³ and, in particular, filbertone,²⁴ a ketone absent in olive oils. Filbertone determination is generally performed by liquid or gas chromatography (L-GC) coupled to mass spectrometry.²⁵ However, this marker becomes useless in the case of adulteration of EVOO with refined HO, because it undergoes a partial or full degradation during the refining process.²⁶ Recently, a method based on hydrophilic interaction chromatography (HILIC) microsolid phase extraction (μ SPE) coupled to matrix assisted laser desorption ionization (MALDI) time-of-flight (ToF) mass spectrometry (MS) for the detection of the polar components in hazelnut and olive oil has been developed.²⁷ Consecutive analysis by tandem mass spectrometry allowed the identification of lysophosphatidylcholines as HO markers. More recently, phospholipids were confirmed²⁸ to be promising markers of EVOO adulteration with HO by a MALDI-MS approach involving an ionic liquid^{29,30} (tributylamine-CHCA) as matrix.

However, existing literature dealing with the direct determination of HO residual proteins in EVOO, in order to get an immediate evaluation of the allergenic potential, is poor, and the attention is mainly focused toward intact proteins rather than peptides. Furthermore, MALDI-MS has never been applied on this topic—regardless, this technique has widely demonstrated its great potential on similar analytical problems^{31,32} due to its rapidity and sensitivity.

A recent study¹⁷ developed a biosensor immunoassay for residues of hazelnut proteins in olive oil, whereas Arlorio et al.⁵ set up a simulation of olive oil adulterated with hazelnut oils to identify possible risks for allergic consumers, demonstrating the presence of hazelnut proteins in solvent-extracted hazelnut oil.

Further approaches, based on DNA and immunoassays analysis, are also available in the literature, and they now include an array of platforms. The fundamental principle of this approach is that the amount of DNA remaining in processed foods is sufficient for analytical purposes to obtain traceability, to detect the presence of both genetically modified organisms and allergens.^{33–37}

Some of these methods are routinely used by industry and administration agencies even if they lack accuracy and robustness, especially in the case of processed foods.^{38,39}

In this work, we tested different approaches for the extraction of residual proteins from various hazelnut oils. The resulting extracts were in-solution digested and analyzed by MALDI-MS. The most efficient extraction protocol was based on cold-acetone protein precipitation. The whole procedure is rapid and simple and facilitated the detection of hazelnut marker peptides in both HO and EVOO adulterated with HO.

MATERIALS AND METHODS

Reagents. Acetone, water, chloroform (CHCl_3), acetonitrile (ACN), and methanol (MeOH) were LC-MS grade; hexane and *tert*-butyl methyl ether were HPLC grade. Trichloroacetic acid (TCA), trifluoroacetic acid (TFA), Trizma hydrochloride (Tris-HCl), sodium chloride (NaCl), all the reagents for the tryptic digestion (dithiothreitol (DTT), iodoacetamide, ammonium bicarbonate (NH_4HCO_3), urea, porcine trypsin), ACTH 18–39 fragment, angiotensin I, and renin were obtained from Sigma-Aldrich (St. Louis, MO). C₁₈Zip Tips were from Millipore (Billerica, MA). Tris-Glycine buffer, Laemmli buffer, 4–15% Mini-PROTEAN TGX precast

gels, molecular weight marker “Precision Plus Protein Standards Dual Color”, Mini-PROTEAN II electrophoresis cell, were acquired from Bio-Rad Laboratories (Hemel Hempstead, Hertfordshire, U.K.). α -Cyano-4-chlorocinnamic acid (CCICA), obtained as a synthetic product, was recrystallized four times before use.

Samples. Different hazelnut oils were analyzed: two Italian (Monferrato, Piedmont), cold-pressed, from roasted and raw hazelnuts, respectively; one German, cold-pressed, from roasted hazelnuts; one Turkish, refined, from raw hazelnuts; one Greek, refined, from raw hazelnuts (the last two were seized by Italian Customs agencies). Commercial (five brands) and raw Italian extra-virgin olive oils (homemade by various Apulia cultivars) were analyzed. EVOO was intentionally adulterated by cold-pressed HO at different percentages (50/50 and 80/20, w/w). Each experiment was performed in triplicate.

Protein Extraction Methods from Oils. Experimental protocols available in the literature were applied with slight modifications.

Cold Acetone Precipitation.¹⁰ To 5 g of pure hazelnut oil and pure olive oil, 12.5 mL of cold acetone (4 °C) was added, the mixture was shaken vigorously, kept for 1 h at 4 °C, and shaken every 10 min; then it was centrifuged at 4200g for 1 h, and the precipitate was washed twice with 1 mL of cold acetone. The pellet obtained from extraction was dried at 40 °C for 5 min in a vacuum concentrator.

Hexane/Acetone Precipitation.¹⁰ To 5 g of pure hazelnut oil or pure olive oil, 12.5 mL of cold hexane/acetone (1:1, v/v) was added, the mixture was shaken vigorously, kept for 1 h at 4 °C, and shaken every 10 min; then it was centrifuged at 4200g for 1 h, and the precipitate was washed twice with 1 mL of cold hexane/acetone. The pellet obtained from extraction was dried at 40 °C for 5 min in a vacuum concentrator.

Diethyl Ether/Trichloroacetic Acid Precipitation.⁵ Ten milliliters of *tert*-butyl methyl ether was added to 10 g of pure hazelnut oil and pure olive oil, and the mixtures were maintained at 18 °C for 30 min. Then, 10 mL of 20% w/v trichloroacetic acid (4 °C) was added, the mixture was transferred to a separating funnel, and the extraction was repeated three times. The three aqueous phases were collected and washed twice with 10 mL of *tert*-butyl methyl ether to remove lipids. The aqueous fractions were partially concentrated and centrifuged at 28 000g for 20 min at 4 °C. The supernatant was discarded; the pellet was washed twice with 1 mL of acetone and finally dried.

Ammonium Hydrogen Carbonate.⁴⁰ Hazelnut oil (7.5 mL) was extracted with 3.5 mL of ammonium hydrogen carbonate (0.2 M, pH 7.8) on a shaker for 2 h at room temperature. The mixture was centrifuged at 11 180g for 20 min at 4 °C, and the aqueous layer was dried in a vacuum concentrator.

TBS Extraction.⁴¹ Tris-buffered saline (TBS) was prepared with 40 mM Tris-HCl and 300 mM NaCl. In particular, to 2 g of hazelnut oil, 5 mL of TBS was added. Extraction was done at 60 °C for 20 min under shaking. The extract was centrifuged at 4200g for 20 min, defatted by extracting twice with *n*-hexane, and dried under a nitrogen stream.

Bligh–Dyer Extraction.⁴² CHCl_3 /MeOH (1:2, 1.87 mL) was added to 0.5 mL of hazelnut oil or olive oil followed by vigorous vortex-mixing. Then, 0.62 mL of CHCl_3 was added, and the solution was vortexed again. Afterward, 0.62 mL of H_2O was added, and the solution was vortexed again. Finally, the solution was centrifuged for 5 min at 4200g, and the

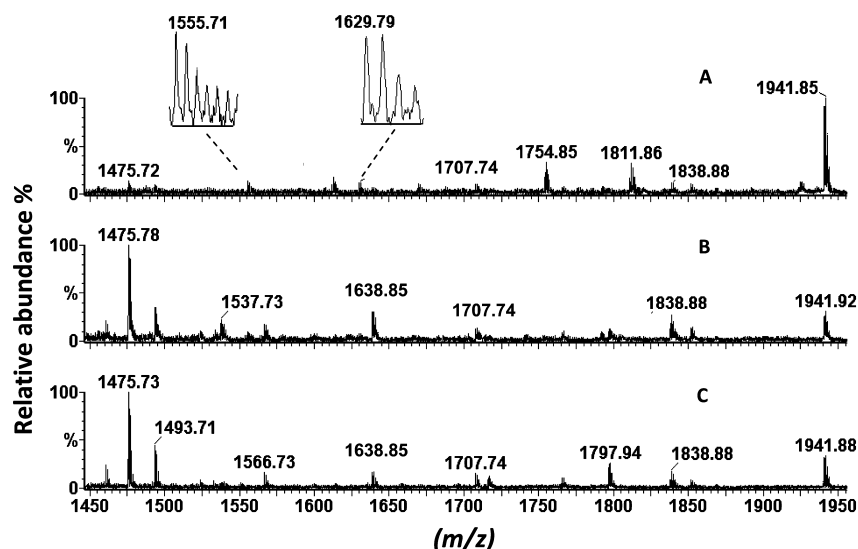


Figure 1. MALDI mass spectra relevant to the in-solution tryptic digests of hazelnut oil extracts obtained using Bligh–Dyer (A), diethyl ether/trichloroacetic acid (B), and hexane/acetone (C) protocols, respectively. The two peaks shown in figure (A), at m/z 1555.71 (RAESEGFEWVAFK) and 1629.79 (ALPDDVLANAFQISR), were attributed to Cor a 9.

aqueous upper layer, containing the proteins, was collected, divided in several aliquots, and dried under a stream of nitrogen. This aqueous extract was used for the following analysis steps.

In-Solution Tryptic Digestion. The pellets obtained from the different extraction methods were digested in solution following a reported protocol.³² Dried pellets were resuspended in 40 μ L of 400 mM NH_4HCO_3 in 8 M urea. Five microliters of DTT (50 mM) was added, and the mixture was incubated for 40 min at 60 °C and chilled at room temperature. Next, 5 μ L of iodoacetamide (150 mM) was added, followed by an incubation in the dark at room temperature for 45 min. water (120 μ L) and 20 pmol of porcine trypsin were added, and the solution was incubated overnight at 37 °C. The digestion was stopped with 10% TFA. The tryptic digests were mixed 1:1 (v/v) with the matrix α -cyano-4-chloro-cinnamic acid (5 mg/mL), and then 1 μ L of the sample/matrix solution was spotted, dried, washed at least once on target, and analyzed by MALDI-TOF-MS. In some cases, due to the low concentration of hazelnut derived peptides in the samples, a procedure of desalting and preconcentration using C_{18} Zip Tips was employed.

SDS-PAGE. As a reference, a roasted hazelnut protein extract was separated on SDS-PAGE gel. The extract for SDS-PAGE was prepared according to Sheoran et al.⁴³ with slight modifications. To 0.03 g of hazelnut powder, 1 mL of Tris-HCl buffer (50 mM) was added, and the mixture was kept at 4 °C for 30 min and then centrifuged at 16 000g for 20 min at 4 °C. The supernatant was collected, the pellet re-extracted, and the supernatants pooled. The proteins were precipitated with 5 vols (v/v) of 100% acetone and incubated at –20 °C for 2 h. After centrifugation at 4000g for 40 min, the pellet was washed twice with acetone, dried for 5 min at 40 °C, and suspended in Laemmli buffer (1:1, v/v). Then, 1 μ L of DTT 50 mM was added and the samples were reduced at 90 °C for 5 min. Twenty microliters were loaded onto the gel, and electrophoresis was carried out at two different voltages—50 V to compact the proteins in the stacking gel and 80 V until the end of the run.

In-Gel Digestion of Hazelnut Proteins. The protein bands were cut and digested by porcine trypsin using a specific

protocol. At first, the bands were cut in small pieces that were covered with 200 μ L of 200 mM NH_4HCO_3 in 40% of ACN, and the samples were incubated at 37 °C for 30 min in a thermo shaker. The step was repeated until the gel pieces were decolorized. The gel fragments were dried in a speed vacuum for 15 min. Porcine trypsin (0.05 μ g/ μ L) and 50 μ L of 40 mM NH_4HCO_3 in 9% ACN were added, and the samples were incubated at 37 °C overnight. After the incubation, the liquid fraction, containing peptides, was collected and acidified with TFA before MALDI analysis.

MALDI-ToF-MS. MALDI-ToF-MS analyses were performed on a Micromass M@LDI - LR time-of-flight mass spectrometer (Waters MS Technologies, Manchester, U.K.) equipped with a nitrogen UV laser (337 nm wavelength), reflectron optics, and a fast dual microchannel plate (MCP) detector.

Positive ion spectra were acquired in reflectron mode. The following voltages were applied: pulse, 2610 V; source, 15 000 V; reflectron, 2000 V; MCP 1900 V. The laser firing rate was 5 Hz, and, unless otherwise specified, 50 laser shots were used for each well. The 50 resulting spectra were averaged, background subtracted, and smoothed by a Savitzky–Golay algorithm.

For peptide analysis, the explored mass range was 800–4000 m/z , and mass calibration was performed using a peptide mixture composed of angiotensin I, ACTH 18–39, and renin substrate tetradecapeptide porcine.

Database Search. For the attribution of hazelnut tryptic peptides, FindPept was employed as a main tool (www.expasy.org). The set parameters were the following: cysteines treated with iodoacetamide as a fixed modification and oxidation of methionines as a variable modification for in-solution digestion and in-gel digestion. A mass tolerance of 0.1 Da was set. Another useful tool was PeptideMass (http://web.expasy.org/peptide_mass/), accepting up to three missed cleavages.

ClustalO software was used for alignment study and the sequences of hazelnut, and avena 11S globulins, in FASTA format, were provided as an input.

RESULTS AND DISCUSSION

Methodology Development on Pure Hazelnut Oil. At first, different protein extraction approaches were tested on

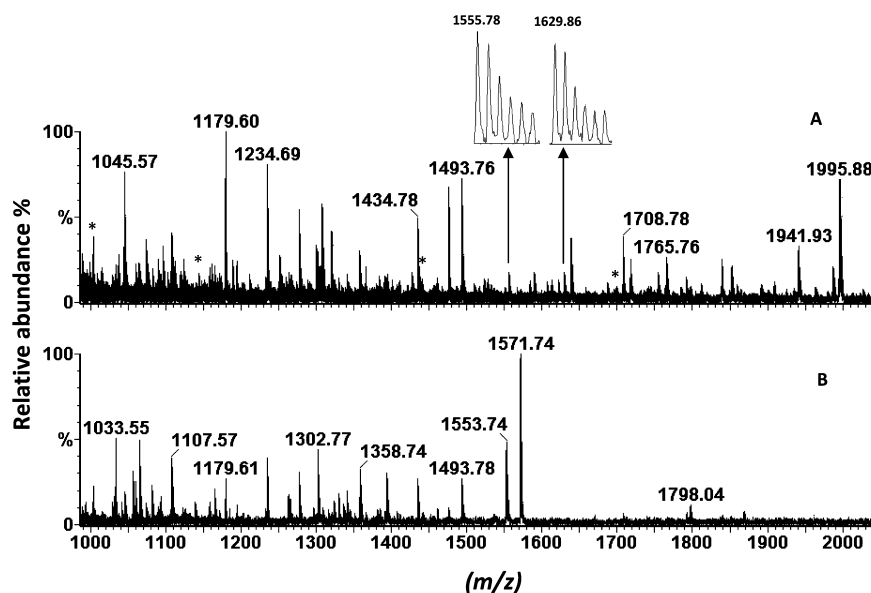


Figure 2. MALDI mass spectra (range 1000 to 2000 m/z) relevant to the in-solution tryptic digests of hazelnut oil extracts obtained using TBS (A) and ammonium hydrogen carbonate (B) protocols, respectively. In figure (A), the isotopic profiles of the peaks at m/z 1555.78 and 1629.86 from Cor a 9 were magnified, and peptides from the same protein at m/z 1002.53, 1144.54, 1440.76, and 1698.82 were marked with an asterisk.

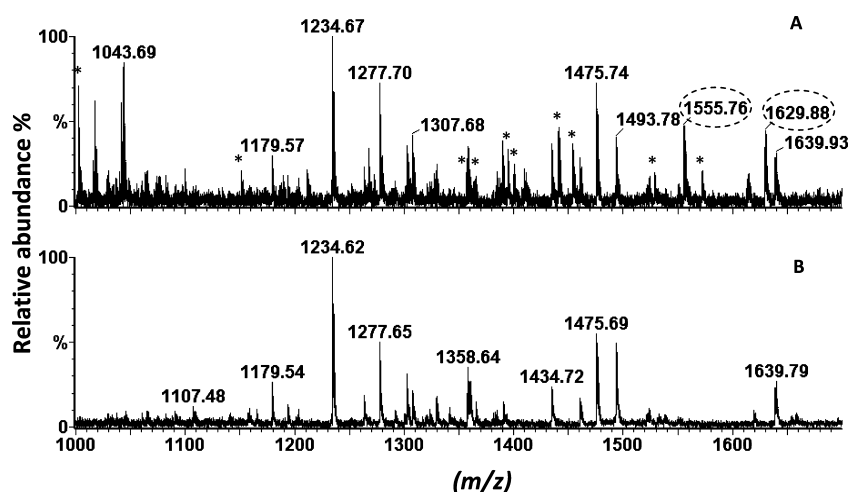


Figure 3. MALDI mass spectra (range shown 1000 to 1700 m/z) relevant to the in-solution tryptic digests of (A) an Italian cold-pressed hazelnut oil extract and (B) an extra-virgin olive oil extract, obtained using the cold acetone protocol. In 3(A), the two main peptides of Cor a 9 (at m/z 1555.76 and 1629.88) were encircled. The peptides at m/z 990.52, 1151.57, 1356.71, 1394.70, 1399.71, 1440.81, and 1571.84 from Cor a 9, 1528.67 from Cor a 1, 1363.73 from Cor a 11 and 1453.89 most likely from Cor a 14, were marked with an asterisk.

various raw hazelnut oil samples; it is worth noting that a very low quantity of oil (5–10 g) was used if compared to the usually employed amount (100 g).^{5,10,40,41} After the extraction, all samples were in-solution digested and analyzed by MALDI-MS. Eventually, the best method was selected on the basis of the simplicity, rapidity, and reproducibility of the procedure and according to the number of detected hazelnut peptides. Because of the sample complexity and the high number of existing allergen isoforms, their identification was achieved by using a combination of literature data^{5,8,44–46} and two different informatics tools, that is, the classic peptide mass fingerprint and FindPept (in the latter, proteins are individually compared with a reference list of hazelnut proteins).

Figure 1 reports the MALDI mass spectra relevant to the in-solution tryptic digests of hazelnut oil extracts obtained using Bligh–Dyer (A), diethyl ether/trichloroacetic acid (B), and hexane/acetone (C) protocols, respectively. Only two peptides

at m/z 1555.71 and 1629.79, originating from the hazelnut protein *Cor a 9*, were observed in the MALDI spectrum relevant to the Bligh–Dyer extract (Figure 1A), although no peptides arising from hazelnut proteins were detected by using the other two extraction procedures (diethyl ether/TCA and hexane/acetone). Here, due to the very low protein content in the oil extracts, the level of background proteins introduced during sample preparation (in the form of dust or contaminated chemicals) might mask the presence of the target proteins; indeed, the spectra of Figure 1B,C were dominated by intense signals attributable to human keratins (for instance, the m/z ions 1475.78, 1707.74, and 1638.85).

Then, protein extraction was carried out by using different buffers; the first method was based on the use of ammonium hydrogen carbonate, successfully applied by Rigby et al.⁴⁰ in the extraction of proteins from soybean oil, whereas the second protocol was based on the use of TBS as reported by Poms et

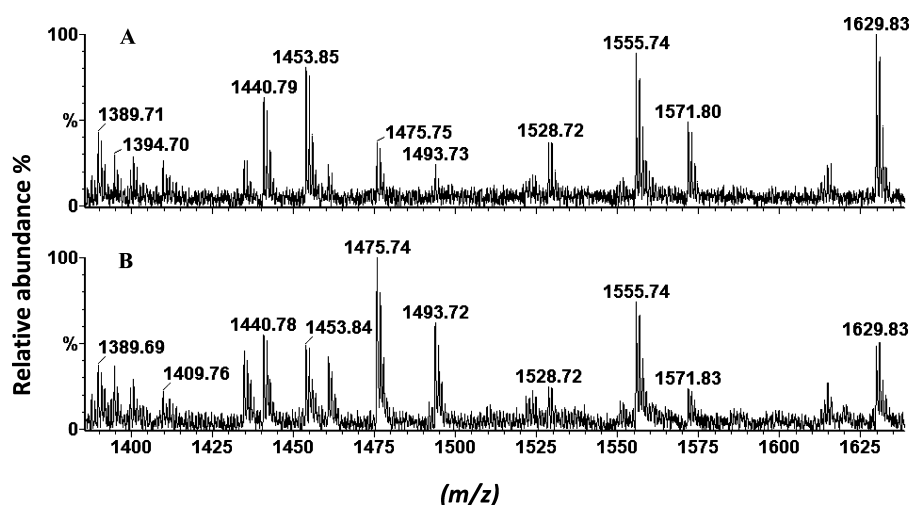


Figure 4. Representative m/z range of the MALDI spectra relevant to the in-solution tryptic digests of EVOO extracts adulterated with 50% (A) and 20% (B) of HO obtained from roasted hazelnuts.

Table 1. List of Hazelnut Peptides Identified in HO by Using Different Protein Extraction Methods

protein extraction method	number and related m/z ratios of identified hazelnut peptides
Bligh–Dyer	2:1555.71, 1629.77
diethyl ether/TCA	-
hexane/acetone	-
TBS	8:990.51, 1002.53, 1144.54, 1440.76, 1555.75, 1629.83, 1695.84, 1698.82
ammonium hydrogen carbonate	-
cold acetone (100% hazelnut oil)	13:990.52, 1002.52, 1151.57, 1356.71, 1363.73, 1394.70, 1399.71, 1440.81, 1453.89, 1528.67, 1555.76, 1571.84, 1629.88, 1934.87
cold acetone (50% hazelnut oil)	11:1002.52, 1356.71, 1363.73, 1394.70, 1399.71, 1440.79, 1453.85, 1528.72, 1555.74, 1571.80, 1629.83, 2643.44
cold acetone (20% hazelnut oil)	11:1002.52, 1151.57, 1356.71, 1363.73, 1394.70, 1399.71, 1440.78, 1453.84, 1528.72, 1555.74, 1571.83, 1698.76

al.⁴¹ in the case of peanut proteins. Figure 2 shows the MALDI spectra (range 1000 to 2000 m/z) relevant to the in-solution tryptic digests of hazelnut oil extracts obtained using TBS (A) and ammonium hydrogen carbonate (B) protocols, respectively. The inset in Figure 2A reports the close-up image of two *Cor a 9* peptides (m/z 1555.75 and 1629.83), whereas the other peptides of the same protein at m/z 1002.53, 1144.54, 1440.76, 1698.82 have been marked with an asterisk. On the contrary, by using ammonium hydrogen carbonate as extraction buffer (Figure 2B), no hazelnut peptides were detected.

After replicating the procedure on other oils, it was possible to confirm that the most recurrent hazelnut peptides were at m/z 1555.75, 1629.83, 1002.53, 1440.76, 1698.82.

Finally, protein precipitation was carried out by using the cold acetone protocol described by Martin-Hernandez et al.¹⁰ The MALDI spectrum (range shown 1000 to 1700 m/z) relevant to the in-solution tryptic digest of an Italian cold-pressed hazelnut oil extract is reported in Figure 3A. Despite the intense signals arising from human keratins (m/z ions 1234.67, 1277.70, and 1475.74), typical hazelnut peptides at m/z values 1555.76, 1629.88, 990.52, 1151.57, 1356.71, 1394.70, 1399.71, 1440.81, 1571.84 (*Cor a 9*), 1528.67 (*Cor a 1*), 1363.73 (*Cor a 11*), and 1453.89 (most likely *Cor a 14*), were detected (marked with an asterisk). Of course, the peptide at m/z 1528.67 attributed to *Cor a 1* arises⁷ from the isoform *Cor a 1.0401–1.0404*.

When replicating the whole strategy on other oils, the number of recognized peptides was between 10 and 15, even if the stable markers found in all the replicates were at m/z 1002.53, 1151.57, 1356.71, 1394.70, 1440.76, 1555.75, 1629.83, and 1698.82.

The same procedure was then applied to extra virgin olive oil samples; the relevant MALDI spectrum is shown in Figure 3B. As expected, no peptides arising from hazelnut proteins were detected. Moreover, even peptides attributable to EVOO proteins were not observed probably due to their very low concentration (10–50 $\mu\text{g}/100\text{ g}$).⁴⁷ It was noted that the pellets obtained after cold-acetone protein precipitation of the EVOO samples investigated were not observable. On the contrary, in the case of hazelnut oil samples, visible pellets were obtained; their estimated protein concentration, determined by the Bradford method,⁴⁸ was in the range 20–100 $\mu\text{g}/\text{mL}$.

The cold acetone protocol proved to be the most efficient method for protein extraction and detection and was used for further experiments.

Methodology Application on Adulterated Samples.

The cold acetone protocol was applied to the analysis of EVOO samples adulterated by cold-pressed hazelnut oil (50/50 and 80/20, w/w). Figure 4 shows the MALDI spectra relevant to the in-solution tryptic digests of EVOO extracts adulterated with 50% (A) and 20% (B) of HO obtained from roasted hazelnuts; only the most informative m/z range was reported. The detected m/z ions belong to *Cor a 9* (m/z 1394.70, 1440.79, 1555.74, 1571.80, 1629.83), *Cor a 1* (1528.72 m/z), and *Cor a 14* (1453.85 m/z); Table 1 depicts the most significant hazelnut peptides showed in the spectra of Figure 4 (three replicates of the whole procedure were performed). It is worth noting that, among the various peptides detected in this work, the one at 1440.81 m/z (INTVNSNTLPVLR) was recently included⁴⁶ in a list of the most stable peptide markers for hazelnut detection in processed food, because it does not contain amino acids susceptible to oxidation.

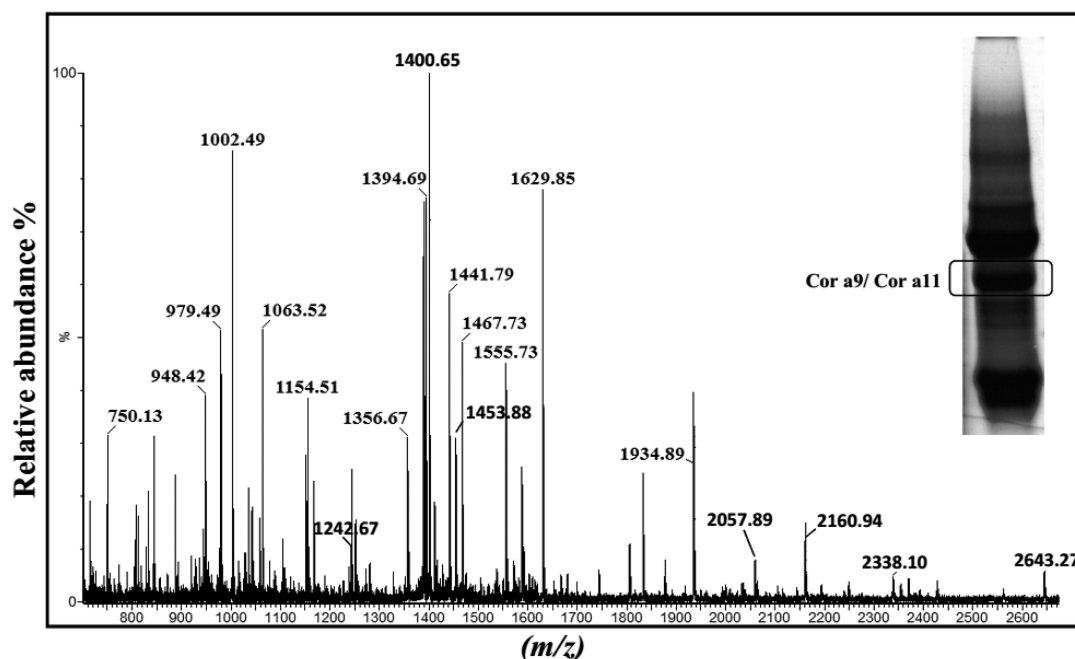


Figure 5. SDS-PAGE of a hazelnut kernels protein extract (inset) and MALDI mass spectrum relevant to the in-gel digestion of the band at 55 kDa.

The procedure was subsequently applied to different kinds of hazelnut oils of different origin (see experimental section), obtained from raw and roasted hazelnuts. Similar results were obtained for all samples, even if, as already reported,¹⁷ the amount of extracted protein was always higher in the case of raw hazelnut oils.

As clearly stated in the introduction, the objective of the present work was the detection of hidden hazelnut allergens in EVOO samples. In general, the lowest dose of allergens that provokes a reaction is called LOAEL or lowest observed adverse effect level. Recent clinical studies^{49,50} reported that the most sensitive patients had a LOAEL eliciting objective signs after the ingestion of only 1 mg of hazelnut protein, whereas the dosage eliciting reaction in a 10% of the food-allergic population is 133.8 mg. The present approach permitted the assessment of a total protein concentration in the range 20–100 μg per mL of hazelnut oil; then, 1 mL of EVOO adulterated with 20% of HO would contain a total protein concentration between 4 and 20 μg . It means that this method was able to reach detection limits well below the LOAEL value.

Thus, the present strategy allowed fast detection of a very low hazelnut protein content in complex matrices as edible oils by recurring to a simple acetone precipitation and in solution tryptic digestion.

SDS PAGE for Confirming Hazelnut Proteins Attribution. A more conventional approach, based on SDS-PAGE followed by in gel tryptic digestion, was then applied to a hazelnut kernels protein extract, with the aim to create a small reference database. All the bands obtained (see gel in the inset of Figure 5) were eventually analyzed by MALDI-MS. It should be noted that a univocal identification of these proteins is difficult to achieve, in particular by using a monodimensional gel, due to the presence of different isoforms, multiple glycoforms, and post-translational protease-cleaved subunits.⁵¹ In fact, some peptides attributable to Cor a 9 and Cor a 11 were detected in all the processed bands at different molecular weights. Considering that hazelnut proteins are present as high molecular weight complexes (300–450 kDa) of minor subunits

linked by noncovalent disulfide bridges, these weak bonds can be broken during the extraction processes, thus originating lower molecular weight units.

For instance, in the MALDI spectrum shown in Figure 5, relevant to the in gel digestion of the band at 55 kDa, the most intense peak was attributed to Cor a 9 even if some Cor a 11 peptides were also present. The complete attribution of the main peaks observed in the spectrum of Figure 5 is reported in Table 2. Other significant m/z ions (750.13, 1154.51, 1242.67, 1400.65, 1453.88, 1467.73, 2057.89, and 2160.94) were not attributable with any known identification tool. Only the m/z ions 1400.65 and 1453.88 were identified as the alkaline and immunogenic subunit of a new hazelnut allergen, an isoform of Cor a 9, recently identified by Nitride et al.⁸

Furthermore, by using FindPept and ClustalO (UniProt), it was possible to propose an attribution for the m/z ion 1242.67 by homology with similar proteins. Indeed, from the in-silico digestion of the Avena globulin 11S (*Avena sativa*), a peptide at 1242.66 m/z is generated. Then, aligning the sequences of *Avena sativa* globulin 11S (Q38780) and *Corylus avellana* 11S globulin-like protein (Q8W1C2), a similarity of 70% between the peptide QEARNLKNNR from *Avena s.* and the peptide EEARRLKYNR from *Corylus a.* was found suggesting an attribution as possible protein variants (Figure 6). Of course, de novo sequencing is needed to confirm this hypothesis and suggest possible protein variants.

In the present study, a cold-acetone protein precipitation followed by in-solution tryptic digestion and MALDI-MS analysis was applied for the first time for the direct determination of hidden hazelnut oil proteins in extra virgin olive oil.

The method, applied on oils of different geographical origin, showed to be reproducible and independent from oil processing and cultivars. In fact, contrary to usual markers (fatty acids, triacylglycerols, volatile compounds, and tocopherols) that are affected by the environmental conditions of plant growth, proteins, being more stable and species-specific, could

Table 2. Probable Attributions of the Peptides Observed in the MALDI Mass Spectrum Relevant to the Tryptic Digest of a SDS-PAGE Band (55 kDa) from a Hazelnut Protein Extract

<i>m/z</i>	position	sequence	protein
935.41	507–515	SESEGRAEA	Cor a 9
976.49	505–512	RRSESEGR	Cor a 9
979.49	333–340	<u>E</u> NICTRSR ^a	Cor a 9
1002.49	364–371	WLQLSAER	Cor a 9
1027.50	139–146	SEQDRHQK	Cor a 9
1027.50	43–51	LNALPTNR	Cor a 9
1063.52	301–308	ESE <u>Q</u> ERER ^a	Cor a 9
1077.49	395–403	GNIVNEFER	Cor a 11
1089.53	5–13	EDPELKKCK	Cor a 11
1151.53	341–350	ADIYTEQVGR	Cor a 9
1220.55	258–268	GSM _{ox} AGPYNSR	Cor a 11
1226.54	270–279	RRNIVKVEGR	Cor a 9
1279.65	486–495	YNRQETTLVR	Cor a 9
1327.62	1–11	MLPKEDPELKK	Cor a 11
1356.67	446–458	TNDNAQISPLAGR	Cor a 9
1394.69	339–350	SRADIYTEQVGR	Cor a 9
1400.65		HDGQNLFDWLR	Cor a 9 isoallergen
1416.75	94–105	ESFNVEHGDIIR	Cor a 11
1440.81	351–363	INTVNSNTLPVLR	Cor a 9
1453.88		ITSLNSLNPILR	Cor a 9 isoallergen
1555.73	433–445	RAESEGFVWVAFK	Cor a 9
1577.75	216–228	REQLEKVFGEQSK	Cor a 11
1587.72	364–376	WLQLSAERGDQQR	Cor a 9
1609.84	486–498	YNRQETTLVRSSR	Cor a 9
1629.85	464–478	ALPDDVLANAFQISR	Cor a 9
1698.75	195–208	HFYLAGNPDEHQR	Cor a 9
1805.93	75–89	TEEGRVQVLENFTKR	Cor a 11
1832.83	288–300	SRQEWERQERQER	Cor a 9
1876.90	73–88	VKTEEGRVVLENFTK	Cor a 11
1934.89	401–417	VQVDDNGNTVFDELRL	Cor a 9
2338.10	377–396	EGLYVPHWNLNHNSVYAIR	Cor a 9
2643.27	80–102	TIENGLLLPQYSNAPELIYIER	Cor a 9
2943.3	128–154	ILQPVSAPGHFEAFYAGGDEPESFYR	Cor a 11

^aUnderlined amino acids in bold are probably deamidated.

	* * *
Q8W1C2 (<i>Corylus avellana</i>)	479 (R)/EEARRLKYNR/(Q) 488
Q38780 (<i>Avena sativa</i>)	484 (R)/QEARNLKNNR/(G) 493

Figure 6. Alignment of the peptide at 479–488 from Cor a 9 with the peptide at *m/z* 1242.66 of 11S globulin from *Avena sativa*. The differences in the amino acid sequences are shown in bold with an asterisk and suggest possible protein variants.

be used for identifying each vegetable oil used for EVOO adulteration.

Stable hazelnut peptide markers in HO were detected at *m/z* 1002.52, 1356.71, 1394.70, 1399.71, 1440.81, 1555.76, 1571.84, 1629.83 from Cor a 9, at *m/z* 1453.85 from Cor a 9 isoform, at *m/z* 1363.73 from Cor a 11, and at *m/z* 1528.67 from Cor a 1. The peptides found in hazelnut oils can potentially be analytical targets for the determination of hazelnut traces in other processed foods.

AUTHOR INFORMATION

Corresponding Author

*E-mail: cosimadamiana.calvano@uniba.it. Fax: +39-080-5442026. Tel: +39-080-5443589

Notes

The authors declare no competing financial interest.

REFERENCES

- (1) Foods—Food and Drug Administration. <http://www.fda.gov/Food/default.htm> (Released on August 2014).
- (2) European Commission. http://ec.europa.eu/energy/oil/index_en.htm (Released on August 2014).
- (3) Savage, G. P.; McNeil, D. L.; Dutta, P. C. Lipid composition and oxidative stability of oils in hazelnuts (*Corylus avellana* L.) grown in New Zealand. *J. Am. Oil Chem. Soc.* **1997**, *74*, 755–759.
- (4) Vichi, S.; Pizzale, L.; Toffano, E.; Bortolomeazzi, R.; Conte, L. Detection of hazelnut oil in virgin olive oil by assessment of free sterols and triacylglycerols. *J. AOAC Int.* **2001**, *84*, 1534–1541.
- (5) Arlorio, M.; Coisson, J. D.; Bordiga, M.; Travaglia, F.; Garino, C.; Zuidmeer, L.; Van Ree, R.; Giuffrida, M. G.; Conti, A.; Martelli, A. Olive oil adulterated with hazelnut oils: simulation to identify possible risks to allergic consumers. *Food Addit. Contam., Part A* **2010**, *27*, 11–18.
- (6) Rigby, N. M.; Marsh, J.; Sancho, A. I.; Wellner, K.; Akkerdaas, J.; van Ree, R.; Knulst, A.; Montserrat, F. R.; Brettlova, V.; Schilte, P. P.; Summer, C.; Pumphrey, R.; Shewry, P. R.; Mills, E. N. The purification and characterisation of allergenic hazelnut seed proteins. *Mol. Nutr. Food Res.* **2008**, *S2*, S251–S261.
- (7) Zuidmeer, L.; Garino, C.; Marsh, J.; Lovegrove, A.; Morati, M.; Versteeg, S.; Schilte, P.; Shewry, P.; Arlorio, M.; van Ree, R. Isolation,

cloning and confirmation as an allergen of the 2S albumin from hazelnut. *Allergy* **2009**, *64*, 235–242.

(8) Nitride, C.; Mamone, G.; Picariello, G.; Mills, C.; Nocerino, R.; Berni Canani, R.; Ferranti, P. Proteomic and immunological characterization of a new food allergen from hazelnut (*Corylus avellana*). *J. Proteomics* **2013**, *86*, 16–26.

(9) Luttkopf, D.; Muller, U.; Skov, P. S.; Ballmer-Weber, B. K.; Wuthrich, B.; Skamstrup-Hansen, K.; Poulsen, L. K.; Kastner, M.; Hausteiner, D.; Vieths, S. Comparison of four variants of a major allergen in hazelnut (*Corylus avellana*) Cor a 1.04 with the major hazel pollen allergen Cor a 1.01. *Mol. Immunol.* **2002**, *38*, 515–525.

(10) Martin-Hernandez, C.; Benet, S.; Obert, L. Determination of Proteins in Refined and Nonrefined Oils. *J. Agric. Food Chem.* **2008**, *56*, 4348–4351.

(11) Crevel, R. W. R.; Kerkhoff, M. A. T.; Koning, M. M. G. Allergenicity of refined vegetable oils. *Food Chem. Toxicol.* **2000**, *38*, 385–393.

(12) Olszewski, A.; Pons, L.; Moutete, F.; Aimone-Gastin, I.; Kanny, G.; Moneret-Vautrin, D. A.; Gueant, J. L. Isolation and characterization of proteic allergens in refined peanut oil. *Clin. Exp. Allergy* **1998**, *28*, 850–859.

(13) Teuber, S. S.; Brown, R. L.; Haapanen, L. A. Allergenicity of gourmet nut oils processed by different methods. *J. Allergy Clin. Immunol.* **1997**, *99*, 502–507.

(14) Moneret-Vautrin, D. A.; Rance, F.; Kanny, G.; Olszewski, A.; Guéant, J. L.; Dutau, G.; Guérin, L. Food allergy to peanuts in France—evaluation of 142 observations. *Clin. Exp. Allergy* **1998**, *28*, 1113–1119.

(15) Moneret-Vautrin, D. A. Food allergy: present problems and perspectives. In *Advances in Allergology and Clinical Immunology: The Proceedings of the Plenary Sessions and Afternoon Symposia of the Xvth European Congress of Allergy*; Godard, P., Bousquet, J., Michel, F.B., Eds.; Parthenon Publishing Group Ltd.: U.K., 1992; pp 473–483.

(16) Moneret-Vautrin, D. A.; Hatahet, R.; Kanny, G.; Ait-Djafer, Z. Allergenic peanut oil in milk formulas. *Lancet* **1991**, *338*, 1149–1152.

(17) Bremer, M. G. E. G.; Smits, N. G. E.; Haasnoot, W. Biosensor immunoassay for traces of hazelnut protein in olive oil. *Anal. Bioanal. Chem.* **2009**, *395*, 119–126.

(18) Ayorinde, F. O.; Garvin, K.; Saeed, K. Determination of the fatty acid composition of saponified vegetable oils using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Rapid Commun. Mass Spectrom.* **2000**, *14*, 608–615.

(19) Park, K. H.; Kim, H. J. Analysis of fatty acids by graphite plate laser desorption/ionization time-of-flight mass spectrometry. *Rapid Commun. Mass Spectrom.* **2001**, *15*, 1494–1499.

(20) Jakab, A.; Nagy, K.; Heberger, K.; Vekey, K.; Forgacs, E. Differentiation of vegetable oils by mass spectrometry combined with statistical analysis. *Rapid Commun. Mass Spectrom.* **2002**, *16*, 2291–2297.

(21) Cozzolino, R.; De Giulio, B. Application of ESI and MALDI-TOF MS for triacylglycerols analysis in edible oils. *Eur. J. Lipid Sci. Technol.* **2011**, *113*, 160–167.

(22) Cercaci, L.; Rodriguez-Estrada, M. T.; Lercker, G. Analysis of the oxidation products of cis- and trans-octadecenoate methyl esters by capillary gas chromatography-ion-trap mass spectrometry. I. Epoxide and dimeric compounds. *J. Chromatogr. A* **2003**, *985*, 211–220.

(23) Bail, S.; Stuebiger, G.; Unterwieser, H.; Buchbauer, G.; Krist, S. Characterization of volatile compounds and triacylglycerol profiles of nut oils using SPME-GC-MS and MALDI-TOF-MS. *Eur. J. Lipid Sci. Technol.* **2009**, *111*, 170–182.

(24) Pavon, J. L. P.; del Nogal Sanchez, M.; Fernandez Laespada, E. M.; Cordero, B. M. Determination of filbertone in spiked olive oil samples using headspace-programmed temperature vaporization–gas chromatography–mass spectrometry. *Anal. Bioanal. Chem.* **2009**, *394*, 1463–1470.

(25) Flores, G.; del Castillo, M. L. R.; Herraiz, M.; Blanch, G. P. Study of the adulteration of olive oil with hazelnut oil by on-line coupled high-performance liquid chromatographic and gas chromatographic analysis of filbertone. *Food Chem.* **2006**, *97*, 742–749.

(26) Cert, A.; Moreda, W.; Pérez-Camino, M. C. Chromatographic analysis of minor constituents in vegetable oils. *J. Chromatogr. A* **2000**, *881*, 131–148.

(27) Calvano, C. D.; Aresta, A.; Zambonin, C. G. Detection of hazelnut oil in extra-virgin olive oil by analysis of polar components by micro-solid phase extraction based on hydrophilic liquid chromatography and MALDI-ToF mass spectrometry. *J. Mass Spectrom.* **2010**, *45*, 981–988.

(28) Koidis, A.; Boskou, D. The contents of proteins and phospholipids in cloudy (veiled) virgin olive oils. *Eur. J. Lipid Sci. Technol.* **2006**, *108*, 323–328.

(29) Calò, V.; Nacci, A.; Monopoli, A. Pd-benzothiazol-2-ylidene complex in ionic liquids: Efficient catalyst for carbon-carbon coupling reactions. *J. Organomet. Chem.* **2005**, *690*, 5458–5466.

(30) Calvano, C. D.; De Ceglie, C.; D'Accolti, L.; Zambonin, C. G. MALDI-TOF mass spectrometry detection of extra-virgin olive oil adulteration with hazelnut oil by analysis of phospholipids using an ionic liquid as matrix and extraction solvent. *Food Chem.* **2012**, *134*, 1192–1198.

(31) Cozzolino, R.; Passalacqua, S.; Salemi, S.; Garozzo, D. Identification of adulteration in water buffalo mozzarella and in ewe cheese by using whey proteins as biomarkers and matrix-assisted laser desorption/ionization mass spectrometry. *J. Mass Spectrom.* **2002**, *37*, 985–991.

(32) Calvano, C. D.; Monopoli, A.; Loizzo, P.; Faccia, M.; Zambonin, C. G. Proteomic Approach Based on MALDI-TOF MS To Detect Powdered Milk in Fresh Cow's Milk. *J. Agric. Food Chem.* **2013**, *61*, 1609–1617.

(33) Cucu, T.; Platteau, C.; Taverniers, I.; Devreese, B.; De Loose, M.; De Meulenaer, B. ELISA detection of hazelnut proteins: effect of protein glycation in the presence or absence of wheat proteins. *Food Addit. Contam., Part A* **2011**, *28*, 1–10.

(34) Breton, C.; Claux, D.; Metton, I.; Skorski, G.; Bervillé, A. Comparative study of methods for DNA preparation from olive oil samples to identify cultivar SSR alleles in commercial oil samples: possible forensic applications. *J. Agric. Food Chem.* **2004**, *52*, 531–537.

(35) Busconi, M.; Foroni, C.; Corradi, M.; Bongiorno, C.; Cattapan, F.; Fogher, C. DNA extraction from olive oil and its use in the identification of the production cultivar. *Food Chem.* **2003**, *83*, 127–134.

(36) Pafundo, S.; Gulli, M.; Marmiroli, N. Multiplex real-time PCR using SYBR® GreenER for detection of DNA allergens in food. *Anal. Bioanal. Chem.* **2010**, *396*, 1831–1839.

(37) Agrimonti, C.; Vietina, M.; Pafundo, S.; Marmiroli, N. The use of food genomics to ensure the traceability of olive oil. *Trends Food Sci. Technol.* **2011**, *22*, 237–244.

(38) Downs, M. L.; Taylor, S. L. Effects of thermal processing on the enzyme-linked immunosorbent assay (ELISA) detection of milk residues in a model food matrix. *J. Agric. Food Chem.* **2010**, *58*, 10085–10091.

(39) Scaravelli, E.; Brohee, M.; Marchelli, R.; van Hengel, A. J. The effect of heat treatment on the detection of peanut allergens as determined by ELISA and real-time PCR. *Anal. Bioanal. Chem.* **2009**, *395*, 127–137.

(40) Rigby, N. M.; Sancho, A. I.; Salt, L. J.; Foxall, R.; Taylor, S.; Raczynski, A.; Cochrane, S. A.; Crevel, R. W.; Mills, E. N. Quantification and partial characterization of the residual protein in fully and partially refined commercial soybean oils. *J. Agric. Food Chem.* **2011**, *59*, 1752–1759.

(41) Poms, R. E.; Capelletti, C.; Anklam, E. Effect of roasting history and buffer composition on peanut protein extraction efficiency. *Mol. Nutr. Food Res.* **2004**, *48*, 459–464.

(42) Bligh, E. G.; Dyer, W. J. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **1959**, *37*, 911–917.

(43) Sheoran, I. S.; Ross, A. R. S.; Olson, D. J. H.; Sawhney, V. K. Compatibility of plant protein extraction methods with mass spectrometry for proteome analysis. *Plant Sci.* **2009**, *176*, 99–104.

(44) Lauer, I.; Foetisch, K.; Kolarich, D.; Ballmer-Weber, B. K.; Conti, A.; Altmann, F.; Vieths, S.; Scheurer, S. Hazelnut (*Corylus*

avellana) vicilin Cor a 11: molecular characterization of a glycoprotein and its allergenic activity. *Biochem. J.* **2004**, 383, 327–334.

(45) Ansari, P.; Stoppacher, N.; Baumgartner, S. Marker peptide selection for the determination of hazelnut by LC-MS/MS and occurrence in other nuts. *Anal. Bioanal. Chem.* **2012**, 402 (8), 2607–2615.

(46) Cucu, T.; De Meulenaer, B.; Devreese, B. MALDI-based identification of stable hazelnut protein derived tryptic marker peptides. *Food Addit. Contam., Part A* **2012**, 29, 1821–1831.

(47) Hidalgo, F. J.; Alaiz, M.; Zamora, R. Determination of peptides and proteins in fats and oils. *Anal. Chem.* **2001**, 73, 698–702.

(48) Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, 72, 248–54.

(49) Eller, E.; Hansen, T. K.; Bindslev-Jensen, C. Clinical thresholds to egg, hazelnut, milk and peanut: results from a single-center study using standardized challenges. *Ann. Allergy Asthma Immunol.* **2012**, 108, 332–336.

(50) Allen, K. J.; Remington, B. C.; Baumert, J. L.; Crevel, R. W. R.; Houben, G. F.; Brooke-Taylor, S.; Kruizinga, A. G.; Taylor, S. L. Allergen reference doses for precautionary labeling (VITAL 2.0): Clinical implications. *J. Allergy Clin. Immunol.* **2014**, 133, 156–164.

(51) Guo, F.; Kothary, M. H.; Wang, Y.; Yu, X.; Howard, A. J.; Fu, T. J.; Zhang, Y. Z. Purification and crystallization of Cor a 9, a major hazelnut allergen. *Acta Crystallogr., Sect. F: Struct. Biol. Cryst. Commun.* **2009**, 65, 42–46.