

Marker peptide selection for the determination of hazelnut by LC–MS/MS and occurrence in other nuts

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Abstract The aim of this work was identifying and selecting hazelnut marker peptides and subsequently developing a complementary method of common immunoassay for the detection of hazelnut. For this purpose, at first, an in silico digestion of three major hazelnut allergens (Cor a 8, Cor a 9 and Cor a 11) was performed to get information about expected peptides. After extraction and trypsin digestion of hazelnut proteins, the samples were measured with tandem mass spectrometry (MS/MS) by direct infusion, which led to identification of 14 peptides. Eight of them with the highest MS signal were synthesized and used as standards for developing a liquid chromatography (LC)–MS/MS method in selected reaction monitoring (SRM) mode. Since almost all food allergens derived from nuts belong to the seed storage protein family and have homologue structure, a Basic Local Alignment Search Tool (BLAST) search was performed to identify the hazelnut specificity of the developed method. According to BLAST, only one peptide occurs in three other nuts, and the remaining seven selected peptides are hazelnut specific. Additionally to hazelnut, the eight other listed nuts in Directive 2003/89/EC as allergen were extracted, digested and measured with the developed method to prove the BLAST results. The analytical data confirmed that six peptides are hazelnut specific, on the contrary to anti-

hazelnut antibodies, which showed cross-reactivities to all other nut extracts. Comparing these results, it could be shown that with this LC–MS/MS method in SRM mode, the specific detection of hazelnut is possible.

Keywords Nut allergens · Cor a 8 · Cor a 9 · Cor a 11 · Hazelnut · LC–MS/MS

Introduction

Because of its special nutritional values, hazelnut (HN) is often used in human nutrition with a special emphasis in ice cream, chocolates and confectionary products such as nougat [1]. However, its proteins are in discussion as they can cause pollen- and food-related allergy. At least five protein types of hazelnut appear to be involved in these allergic reactions. The first major allergen, Cor a 1 (18 kDa, Bet v 1 family), is a typical pollen allergen. The second one, Cor a 2 (14 kDa), belongs to the profilin family and could be found in pollen as well as in seed. The other three belong to the seed storage protein family. Cor a 8 (9 kDa) is a nonspecific lipid transfer protein from the prolamin superfamily [2], which is associated with severe allergic reactions to hazelnuts. The other major food allergen present in hazelnut is Cor a 9 (Corylin), a 59 kDa protein which belongs to the legumin (11S globulin protein family). The last major food allergen from HN is Cor a 11 (48 kDa); as glycosylated vicilin, it belongs to the 7S seed storage globulin family [3].

It has to be stated that most of the listed major tree nut allergens are from the seed storage family such as legumines, 2S albumins and vicilins. For example, the major allergens Ara h 1 (peanut), Jug r 2 (walnut), Ana o 1 (cashew) and Cor a 11 (hazelnut) are vicilins (7S globulins) [3]. Recently, Bignardi et al. [4] investigated the detection

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of the 11S globulin family from five different nuts—Ana o 2 (cashew), Cora a 9 (hazelnut), Pru l 1 (almond), Jug r 4 (walnut) and Ara h3/4 (peanut)—in breakfast cereals and biscuits by liquid chromatography (LC)–linear ion trap–tandem mass spectrometry (MS/MS). Although there is homology between 11S globulin proteins from different legumes, this sequence homology is more definitive for 7S globulin proteins, where the variable domains are mainly found within the N- and C-terminal regions and not in the inside parts of the sequences, whereas in 11S proteins, only β -polypeptide is conservative and the α -polypeptide is variable [5]. This homologous structure can cause allergic cross-reactivity among these nuts; however, it is not a guaranty for cross-reactivity [6]. Goetz et al. [6] could show strong cross-reactivity of hazelnut to walnut and pecan, and moderate cross-reactivity to cashew, Brazil nut, pistachio and almond.

Since one third of all anaphylactic reactions were caused by tree nut ingestion [6], a major focus of allergenic food protein analysis is the detection and characterization of nuts listed in Annex IIIa of the EC-Directive 2007/86/EC, in which the indication of the ingredients present in foodstuffs was requested, especially those that cause allergies or intolerances in consumers. The list includes almonds (*Amygdalus communis* L.), hazelnuts (*Corylus avellana*), walnuts (*Juglans regia*), cashews (*Anacardium occidentale*), pecan nuts (*Carya illinoensis*), Brazil nuts (*Bertholletia excelsa*), pistachio nuts (*Pistacia vera*) and macadamia nuts (*Macadamia ternifolia*) [7]. A lot of research was done on allergen purification, characterisation [2, 3, 8] and expression of allergenic proteins [9], but for the determination of the hazelnut content in different food, efficient and reliable quantification and detection methods are needed. The immunochemical methods, which use specific antibodies for the detection of proteins, are mostly employed because antibodies can recognise very specific proteins in trace amounts in a complex food matrix [10]; however, the food analyst must be aware of the class of antibody being used in an immunoassay [11] and the used immunogen, as cross-reactivities can occur. Additional valuable information can be found in the botanical family tree of the eudicots. Walnut and pecan belong to Juglandaceae. Cashew and pistachio belong to Anacardiaceae. Hazelnut belongs to the family Betulaceae, which explains cross-reactivities to birch. Almond belongs to the family Rosacea, and all together, they appear within the rosids, which are beside the asterids the largest clades in the eudicots. As hazelnuts belong beside walnut and pecan to the same botanical order, cross-reactivities within them can occur. Therefore, antibodies have to be very well characterized according to their properties. This highlights the need for comparative methods, which are still scarce in the field of food allergen detection [12]. Besides rapid immunoanalytical methods,

advanced analytical methods based on LC–MS/MS are highly requested for structural investigation and quantification of allergenic proteins [13].

The objective of this study was in first instance extracting and digesting hazelnut proteins from processed hazelnuts in order to characterise and identify some marker peptides for further development of a complementary method to immuno-based assays for food analysis with a different technique. Since it has been proven that cross-reactivities especially can occur in tree nuts, where botanical family associations are given [6], in this work, in-house-produced anti-HN antibodies were not used for assay development but for immunoblotting experiments to characterise the cross-reactivities of the antibodies to other nut extracts, which can be found within the labelling list of the EC-Directive. The protein extracts of different nuts were detected by western blot (using two in-house-produced antibodies) as well as digested with trypsin. The sequence homology and specificity of target hazelnut peptides were searched with the Basic Local Alignment Search Tool (BLAST) [14] and compared with the protein sequences from numerous other organisms and within the rosid family. Digested hazelnut extract was measured by MS/MS, and subsequently, an LC–MS/MS method in selected reaction monitoring (SRM) mode was developed. Additionally, the other nut extracts were measured by this LC–MS/MS method. This leads to interesting differences in antibody detection and MS/MS measurements.

Materials and methods

Materials, chemicals and reagents

The nuts (hazelnut, walnut, pecan, pistachio, peanut, cashew, Brazil nut, macadamia and almond) were purchased from local markets.

NuPAGE® 12% Bis–Tris pre-cast gels (1.0 mm×15 well), See Blue® Plus2 prestained standard, MagicMark™ XP Western Standard, NuPAGE® LDS sample buffer and Simply Blue™ SafeStain were purchased from Invitrogen Corporation (Carlsbad, CA, USA).

The anti-HN antibodies (polyclonal from rabbit and monoclonal from mouse) were produced in-house. The labelled species-specific secondary antibody for western blot (anti-mouse-IgG-HRP and anti-rabbit-IgG-HRP) were obtained from Sigma-Aldrich (Steinheim, Germany) as well the chemicals ammonium acetate (MS grade), tris[hydroxymethyl]aminomethane (Trizma® base, 99.9%), albumin bovine serum (fraction V, $\geq 96\%$), ethylenediamine-tetraacetic acid (EDTA, 99%), Tween20, hydrogen peroxide (30 wt.%, semiconductor grade), 3,3',5,5'-tetramethylbenzidine (TMB, 89%), Bis(2-ethylhexyl) sulphosuccinate sodium salt (DONS)

and iodoacetamide. Ammonium hydrogen carbonate ($\geq 99\%$), acetonitrile (HPLC gradient grade), sodium dodecyl sulphate (SDS, $\geq 99\%$), 2-(*N*-Morpholino)ethanesulphonic acid (MES, PUFFERAN® 99%) and 1,4-dithiothreitol ($\geq 99\%$) were purchased from Carl Roth GmbH (Karlsruhe, Germany). Trypsin (Sequencing Grade, modified) was from Roche Applied Science (Mannheim, Germany). Urea, acetic acid (glacial) 100%, citric acid, sodium chloride (GR for analysis), sodium dihydrogen phosphate monohydrate (GR for analysis) and disodium hydrogen phosphate dihydrate were from Merck (Darmstadt, Germany). Ethanol 99%, denaturated, was from J.T. Baker chemicals B.V. (Deventer, Holland).

The eight selected peptides (one from Cor a 8, four from Cor a 9 and three from Cor a 11), which showed the highest MS signal, were ordered in lyophilized form from "Peptide 2.0" (Chantilly, VA, USA).

NuPAGE® MES SDS Running Buffer, 20 $\times$, was prepared by dissolving 60.6 g Trizma® base, 97.6 g MES, 10 g SDS and 3.0 g EDTA in 500 mL water. For electrophoresis, stock solution was diluted to 1 $\times$ with water just before use.

Phosphate-buffered saline (PBS), 0.2 M, pH 7.5, was prepared by dissolving 32.22 g Na₂HPO₄, 2.62 g NaH₂PO₄ and 21.18 g NaCl in 1,000 mL distilled water. For wash solution PBST, 0.1% Tween 20 was added to PBS buffer.

Substrate solution for western blot included 24 mg TMB, 80 mg DONS, 10 mL ethanol and 30 mL citrate buffer (0.15 M, pH 5.0). Per 10 mL of substrate solution, 5 μ L H₂O₂ was added short before beginning of the reaction.

The used water was either purified by reverse osmosis or drawn from a Milli-Q plus System from Millipore (Molsheim, France). If not stated otherwise, procedures were performed at room temperature.

Sample preparation (extraction and digestion)

The nuts were ground and mixed with 0.1 M NH₄HCO₃, pH 7.6 (1:10). The extraction was performed with a rotary shaker at 50 rpm and at 4 °C for 2 h. The mixture was centrifuged for 30 min at 6,500 $\times$ g and 4 °C using a Conical Tube Fixed-angle Rotor (C1015) and an ALLEGRA® X-22 centrifuge (Beckman Coulter, Inc., Fullerton, CA, USA). The supernatant was filtered through glass fibre MN85/70 BF from Macherey-Nagel (Macherey-Nagel GmbH & Co., Düren, Germany) and cellulose acetate membrane filter, 0.2 μ m (Sartorius Stedim Biotech GmbH, Goettingen, Germany). The filtrate was dried completely overnight using a vacuum centrifuge, Model VR-1/120/240 (Heraeus Instruments, Denmark).

Proteolytic digestions with trypsin were carried out using a protocol for in-solution digestion [15] with some modifications; in short, the extracted proteins were resuspended in 6 M urea, 0.1 M NH₄HCO₃ buffer. One hundred-

microlitre aliquots were reduced and alkylated using 0.2 M DTT and 0.2 M iodoacetamide in 0.1 M NH₄HCO₃. Twenty microgrammes of trypsin was added to the samples and incubated at 37 °C overnight. The reaction was stopped by addition of acetic acid.

SDS-PAGE and western blot

Extracted proteins were separated via gel electrophoresis. Discontinuous SDS-polyacrylamide gel electrophoresis (PAGE) was performed using Invitrogen pre-cast NuPAGE® 12% Bis-Tris Gels and MES SDS running buffer, following the manufacturer's instructions. The proteins were either visualized with Coomassie staining (with SimplyBlue™ SafeStain according to manufacturer's recommendations) or blotted (western blot).

For blotting, the XCell II™ Blot Module (Invitrogen Corporation, USA) was used. The proteins were transferred to a Protran® 0.45 μ m nitrocellulose membrane (Schleicher & Schuell BioScience, Dassel, Germany) and blocked with 2% bovine serum albumin. After washing with PBST (2 $\times$ 10 min), the membrane was incubated with primary antibodies, which were in-house-produced anti-hazelnut antibodies. Another wash step (3 $\times$ 10 min with PBST) was followed before incubation with the secondary anti-species antibodies, which were linked to horseradish peroxidase (HRP) and used for the colorimetric detection of the proteins. The reaction of hydrogen peroxide (included in substrate solution) with peroxidase converts the soluble dye into an insoluble form. It precipitates next to the enzyme and thereby stains the proteins on the membrane.

Liquid chromatography–tandem mass spectrometry (LC–MS/MS)

The chromatographic separation of tryptic-digested samples was carried out on an Aquasil C18 reversed phase column (50 $\times$ 2.1 mm, 3 μ m particle size from Thermo Electron Corporation, Marietta, GA, USA), equipped with a C18 4 $\times$ 3 mm i.d. security guard cartridge (Phenomenex, Torrance, CA, USA), using an 1100 series HPLC (Agilent, Waldbronn, Germany).

Injection volume was 5 μ L, and the flow rate was 0.5 mL/min. Eluent A consisted of 10% acetonitrile, 5 mM ammonium acetate, and eluent B was 95% acetonitrile, 5 mM ammonium acetate. The HPLC conditions were initial hold of 0.5 min, linear gradient from 3% to 40% B within 11.5 min, rapidly up to 100% B and 4 min hold, switch back to 3% B within 2 min and equilibration for 5 min. MS measurement was done between 4 and 14 min.

A QTrap 4000 LC–MS/MS system (Applied Biosystems, Foster City, CA, USA), equipped with a Turbo V Ion Spray (ESI) source was used. Screening and identification

of tryptic peptides, as well as optimization of SRM parameters for profiling of selected tryptic peptides, was carried out by directly infusing tryptic-digested hazelnut proteins or peptide standard solutions, respectively, into the mass spectrometer at a flow rate of 10 $\mu\text{L}/\text{min}$. Chromatographic and (tandem) mass spectrometric data were evaluated using the AnalystTM software version 1.5.

The used ESI source parameters were positive ionization mode; curtain gas, 20 psi; both ion source gas 1 and 2, 50 psi; source temperature, 150 $^{\circ}\text{C}$ for directly infusion and 535 $^{\circ}\text{C}$ for LC-MS/MS; and ion spray voltage, +4,000 V. Collision gas (nitrogen) was set to high. For each SRM transition, a dwell time of 50 ms was chosen, and the pause between mass ranges was 5 ms. Optimized SRM parameters (Table 1) were used for profiling the tryptic peptides of hazelnut allergens by LC-MS/MS.

Quantification of peptides in SRM mode

For the determination of the peptide concentrations in the tryptic-digested samples by the LC-MS/MS method in

SRM mode, linear working ranges were evaluated. Calibration curves were created by analyzing eight dilution levels of a mixture containing eight synthetic standard peptides (concentration of each peptide, 125 $\mu\text{g}/\text{mL}$). This mixture was diluted with 10% acetonitrile and 5 mM ammonium acetate in nine steps (concentration range from 3.1 to 4,166.7 ng/mL per peptide).

Results and discussion

Gel electrophoresis and western blot

The gel electrophoresis is an important part of proteomics analysis because in one single step, a complex mixture of proteins can be separated. Staining reveals information about characteristic band distribution of the applied extracts and protein mixtures throughout the used separation gel. An additional blotting step offers the advantage using an antibody-antigen reaction for further characterisation of the blotted proteins with the developed antibodies.

Table 1 The parameters of the LC-MS/MS method in SRM mode for profiling of tryptic peptides of hazelnut allergens

Protein	Tryptic peptide	MW (Da)	Q1 (m/z)	DP (V)	Q3 (m/z)	Q3 ion	CE (V)	
Cor a 8	GIAGLNPNLAAGLPK	1,461.8	732.2	61	171.3	b_2	47	q_2
					301.3	y_3	33	Q
					937.7	y_{10}	50	q_1
Cor a 9	ALPDDVLANAFQISR	1,628.9	815.6	76	175.2	y_1	63	q_2
					185.2	b_2	87	q_1
					906.6	y_8	47	Q
	QGQVLTPQNFAVAK	1,612.9	807.8	71	186.2	b_2	69	q_2
					314.2	b_3	51	q_1
					874.6	y_8	37	Q
	INTVNSNTLPVLR	1,439.8	721.1	66	228.2	b_2	51	q_1
					484.4	y_4	35	Q
					1,013.7	y_9	35	q_2
Cor a 11	WLQLSAER	1,001.5	501.9	56	159.2	a_1	45	q_1
					272.3	a_2	29	Q
					575.5	y_5	27	q_2
	AFSWEVLEAALK	1,362.7	682.7	61	191.3	a_2	43	Q
					402.3	y_4	41	q_1
					644.4	y_6	31	q_2
	LLSGIENFR	1,047.6	524.9	56	199.3	a_2	29	Q
					565.4	y_4	25	q_2
					822.5	y_7	27	q_1
	ELAFNLPSR	1,045.6	524.0	56	215.2	a_2	35	q_1
					359.3	y_3	25	Q
					586.4	y_5	27	q_2

These peptides were used as standards for calibration curve. All of the precursor ions were doubly charged ($[M+2H]^{2+}$)

MW neutral experimental mass of peptide, Q1 precursor ion, DP declustering potential, Q3 product ion, CE collision energy, Q quantifier, q_1 first qualifier, q_2 second qualifier

In this work, at first, the profiles of proteins and differences between nut extracts were visualised by Coomassie staining (Fig. 1a), and then western blot provides analytical approaches for protein characterisation of prepared extracts. Nut extraction was done with NH_4HCO_3 at a neutral pH (7.6). Figure 1a shows the obtained profiles of the different nuts. In the molecular weight range from 70 to 30 kDa, where 11S and 7S seed storage proteins can be found, different bands were visualized, but also in the low molecular weight range below 17 kDa, proteins can be detected. As expected, anti-hazelnut antibodies showed cross-reactivity to almost all of different nut extracts. In case of the polyclonal antibody (Fig. 1b), which was raised against a whole hazelnut protein extract, several proteins within all nut extracts could be detected across the whole molecular weight range. Even the monoclonal anti-hazelnut antibody (Fig. 1c) showed cross-reactivity to different proteins of nut extracts. However, it seems as the reaction of the monoclonal antibody is more specific to the proteins in the molecular weight range between 50 and 40 kDa. This would be the region of the 7S storage proteins, which have their variable domains mainly located in the outer part of the proteins. However in contrast to Goetz et al., where a strong cross-reactivity between walnut, pecan and hazelnut was observed, our tested monoclonal anti-hazelnut antibody showed strong cross-reactivity to almond, Brazil nut and pistachio and moderate cross-reactivity to the other nuts. The reason of the high recognition pattern of the anti-hazelnut antibodies could be the fact that most of these nut allergens are seed storage proteins and some of these nuts are from the same botanical family and order, and additionally, the antibodies were raised against a complex protein mixture.

Identification of hazelnut allergen marker peptides by MS

In silico digestion

For facilitating the search of possible hazelnut marker peptides, *in silico* tryptic digestions of hazelnut protein sequences were performed as described earlier for milk [16]. The software tool “PeptideMass” [17] was used for *in silico* trypsin digestion of three hazelnut allergen sequences available in the UniProt database (Universal Protein Resource) [18] with accession numbers Q9ATH2 (Cor a 8), Q8W1C2 (Cor a 9) and Q8S4P9 (Cor a 11). The obtained peptides (data not shown) were further used for marker search by MS.

Identification of hazelnut marker peptides by MS/MS

After tryptic digestion of hazelnut extracts, a MS full scan was performed, and the obtained peptides from *in silico* digestion were sought. The next steps were enhanced resolution mode (ER), where the accurate masses of peptides found in full scan were determined, followed by enhanced product ion mode (EPI), where their compounds were fragmented and the amino acid sequences of the peptides were deduced. The MS/MS data were confirmed by *in silico* fragmentation of peptides using the software “Peptide Fragment Ion Analyser” (PFIA-II) [19].

With this strategy, the structures of one tryptic peptide from Cor a 8, eight peptides of Cor a 9 and five peptides from Cor a 11 (each consisting of 8–17 amino acids) were determined by MS/MS (data not shown). From these peptides, a total of eight peptides with the highest signal intensities in EPI mode were synthesized (one peptide from Cor a 8, four from Cor a 9 and three from Cor a 11, listed in

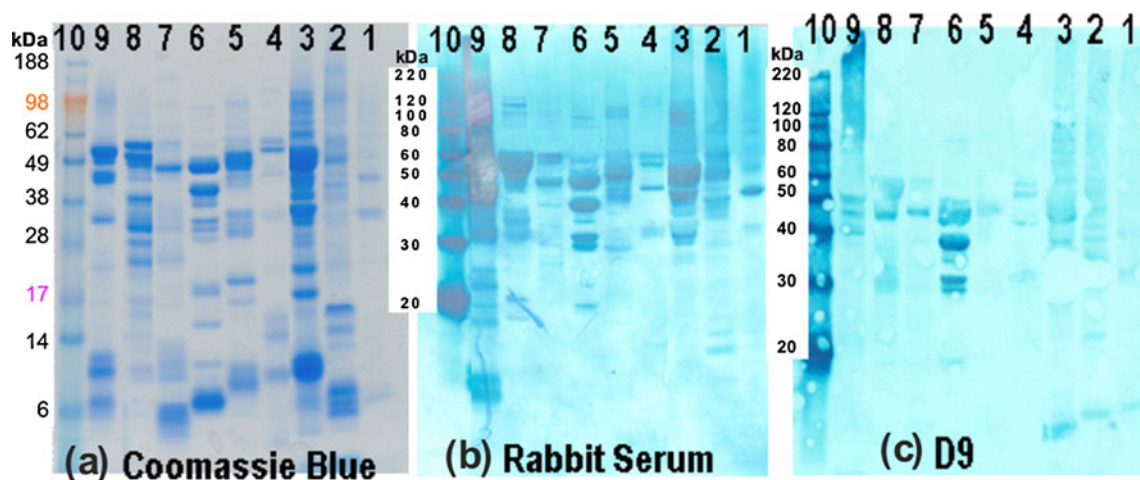


Fig. 1 Coomassie staining (a) and western blot of nut extracts with anti-hazelnut polyclonal rabbit serum (b) and monoclonal antibody D9 (c). Lane 1: walnut extract, lane 2: pecan extract, lane 3: pistachio

extract, lane 4: peanut extract, lane 5: cashew extract, lane 6: Brazil nut extract, lane 7: macadamia extract, lane 8: almond extract, lane 9: HN extract and lane 10: MW marker

Table 1) and used as standard peptides for the development of an LC–MS/MS profiling method. They were delivered in lyophilized form with high purity (from 95.0% for QGQVLTIPQNFAVAK to 99.4% for ELAFNLPSR) and then dissolved in methanol with the concentration of 1 mg/mL as stock solutions. The purity of these stock solutions was also checked via capillary electrophoresis (Hewlett Packard G1600AX 3D CE, Hewlett-Packard Company, USA); in each stock solution, only one peak was identified. The analyses were performed at optimized CE separation conditions.

BLAST search

For the determination of hazelnut specificity of these selected tryptic peptides, a BLAST search was applied [14]. According to BLAST (BLAST 2.2.22, first access 1 March 2010), all of them are hazelnut specific except WLQLSAER from Cor a 9, which could occur, besides in hazelnut, also in *P. vera* (pistachio), *C. illinoensis* (pecan), *Ficus pumila* var. *awkeotsang* and *J. regia* (walnut). It has to be mentioned that these results are data bank dependent; it could be possible that they occur in more plants, but probably the proteins of these plants are not investigated, and at the moment, no database entry can be found.

LC–MS/MS method development

For the establishment of a profiling method, the MS/MS SRM mode was employed, which has a very high specificity and sensitivity for the respective compound. Its chromatogram represents only product ions of a particular *m/z* ratio and is generated by fragmentation of a selected precursor ion. In this method, the direct infusion of synthetic peptides into the mass spectrometer was used to optimize the parameters. With the combination of them

with an LC gradient program, a multi-analyte LC–MS/MS method for determination of up to eight peptides from hazelnut was developed. For each of them, the most intense SRM transition served as quantifier (*Q*), whereas the second and third SRM transitions were chosen as qualifiers (*q*₁ and *q*₂) for the identification of the target compound (Table 1).

Using the synthetic standard peptides, appropriate calibration curves could be created with this method. Depending on the peptide, the lowest determined concentrations were either 3.1 or 4.2 ng/mL, and the linear range extends up to 3,125 or 4,167 ng/mL, respectively. The coefficients of determination of calibration curves were between 0.9914 and 1.000.

Occurrence of selected hazelnut marker in other nut extracts

In general, the protein databases are often incomplete; therefore, all of the nuts listed as allergen in the EC-Directive 2007/86/EC (including almond, hazelnut, walnut, cashew, pecan, Brazil nut, pistachio, macadamia and peanut) were used in this study. Nuts were extracted, digested and measured with the MS method mentioned above to observe if the selected hazelnut peptides could also be found in the other nuts (Table 2). The first criteria for detection of a peptide were the mass, the retention time and three SRM transitions, which should be matched to the standards (three examples shown in Fig. 2). If there were mismatches with the theoretical results, the ratios of the SRM intensities were compared with the synthetic standards. If necessary, nut extracts were also measured in EPI mode.

According to BLAST results, theoretically, only WLQLSAER should be detected in walnut, pecan and pistachio. In the first instance, it seemed that the MS data showed different results. WLQLSAER was found in walnut

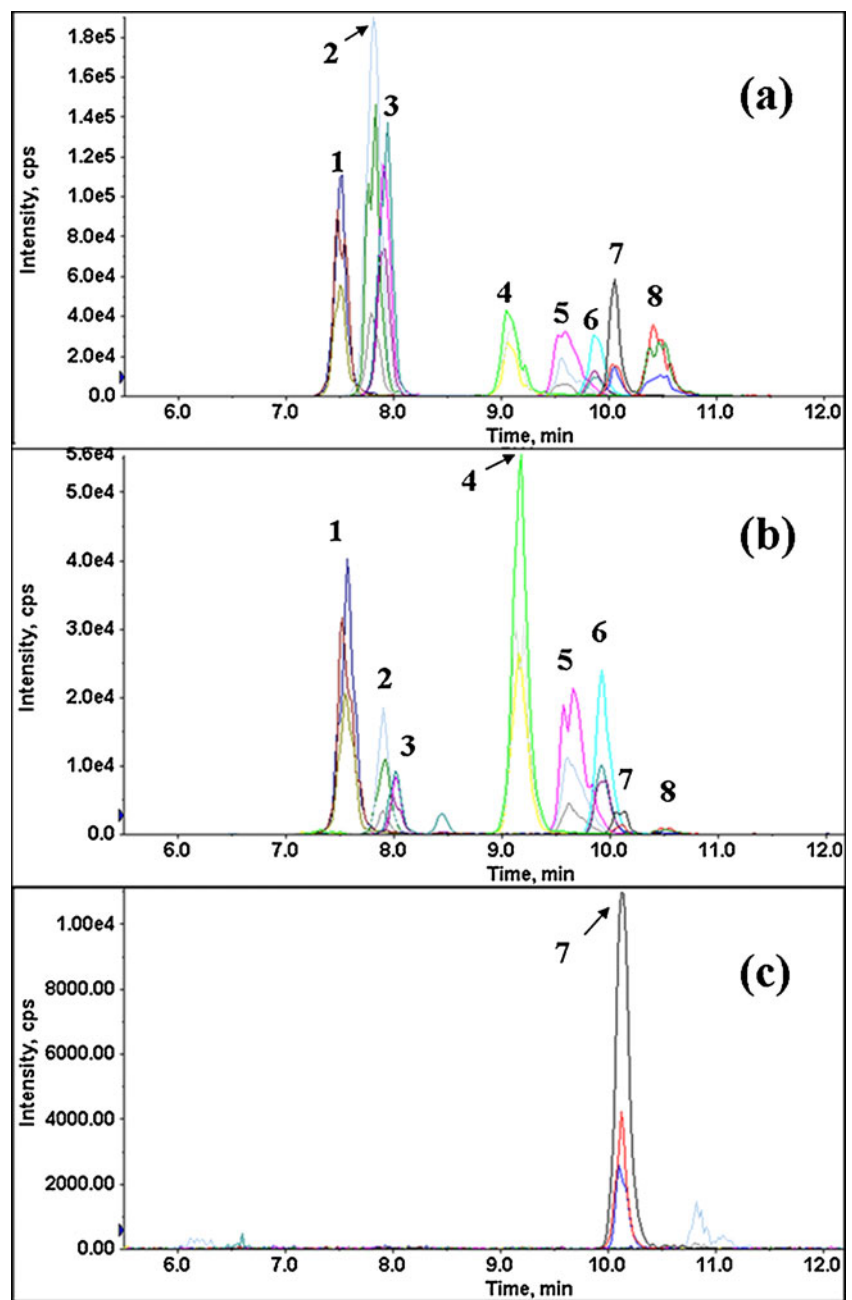
Table 2 Concentrations (microgrammes per gramme) of selected hazelnut peptides detected in digested hazelnut, walnut, pecan, pistachio and cashew samples

	Hazelnut	Walnut	Pecan	Pistachio	Cashew
GIAGLNPPLAAGLPGK	34.1	–	–	–	–
ALPDDVLANAFQISR	200	–	–	–	–
QGQVLTIPQNFAVAK	250	–	–	–	–
INTVNSNTLPVLR	397	–	–	–	–
WLQLSAER	114	6.4	–	13.7	64.2 ^a
AFSWEVLEAALK	7.1	3.0 ^a	21.6 ^a	–	–
LLSGIENFR	5.4	–	–	–	–
ELAFNLPSR	4.4	–	–	–	–

None of these peptides could be found in digested peanut, Brazil nut, macadamia and almond samples

^a Due to sequence homology, hazelnut peptides are falsely detected at the given concentrations

Fig. 2 Total ion chromatogram of (a) synthesized standard mixture, (b) digested HN extract and (c) digested pecan extract. Peak 1: WLQSAER, peak 2: LLSGIENFR, peak 3: ELAFNLPSR, peak 4: INTVNSNTLPVLR, peak 5: ALPDDVLANAFQISR, peak 6: QGQVLTIPQNFAVAK, peak 7: AFSWEVLEAALK and peak 8: GIAGLNPNAAGLPGK. Different transitions were shown with different colours



and pistachio (not in pecan) but additionally also in cashew. In contrast to BLAST information, AFSWEVLEAALK was likely detected in walnut and pecan in very low concentrations (Table 2). In this case, all of the three transitions were detected in the extracts at retention times very near to that from synthesized standard. Since these results did not fit the BLAST search results, the intensity ratios of three SRM transitions were also checked, which actually did not match with the synthetic standard.

Thus, two databases (NCBI [20] and UniProt [18]) were searched for proteins of walnut, cashew and pecan. Each found protein was *in silico* digested with trypsin using

PeptidMass [17] and the obtained peptides were compared with our standard peptides. In cashew allergen Ana o 2, which belongs to the 11S seed storage globulin family, a similar peptide could be found: WLQLSVEK with $[M+H]^+$ 1,002.2 Da. Therefore, digested cashew extract was measured in EPI mode (Q1, 501.9; CE, 35 V), and its spectrum was compared with the spectrum of the synthesized standard measured at the same EPI conditions, and also with the product ions obtained by *in silico* fragmentation using PFIA II (Fig. 3). As expected, the spectrums are very similar; however, two characteristic fragments (175.1 and 304.2) could not be found in cashew. Since all

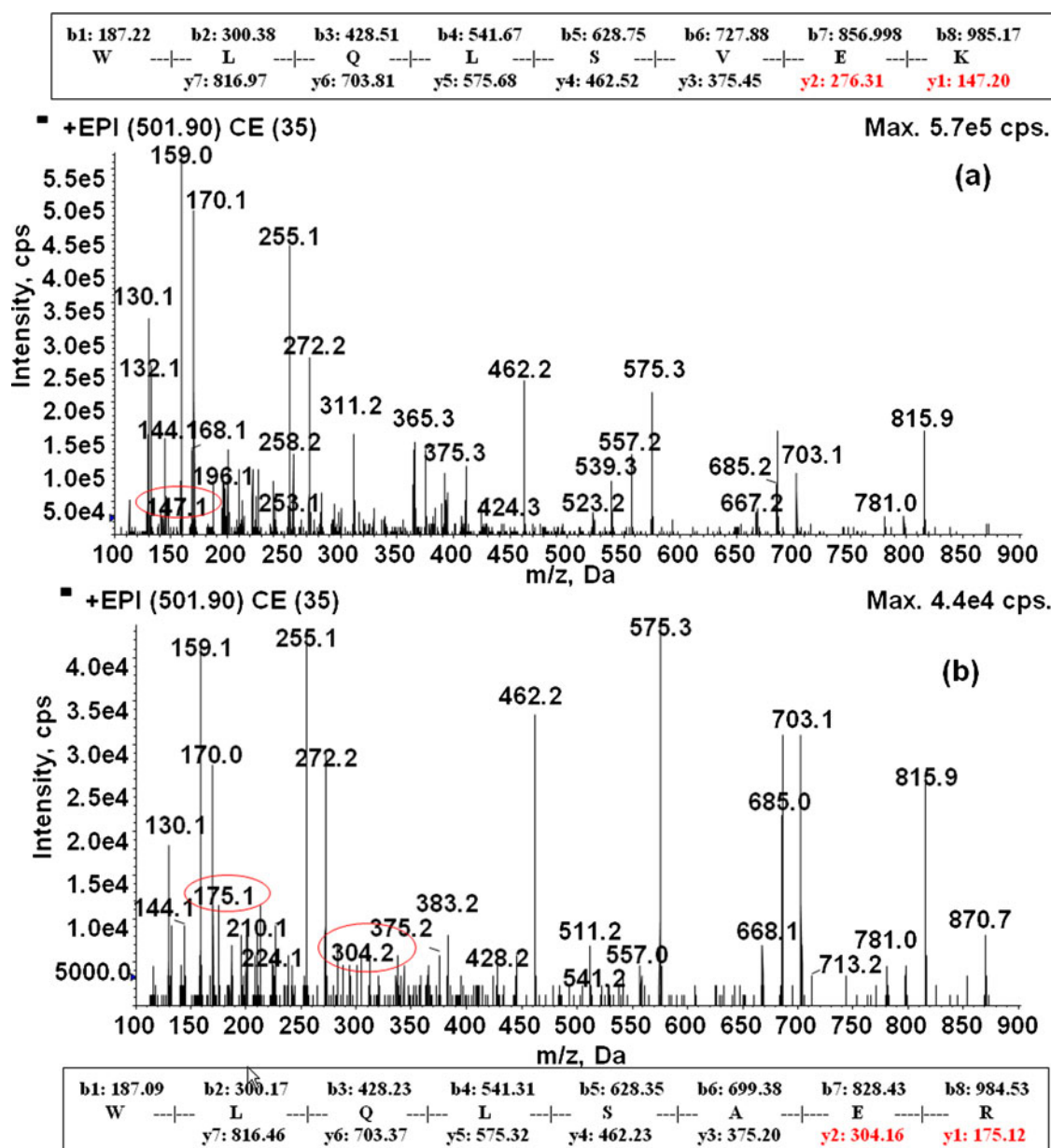


Fig. 3 Product ion spectrum ($[M+2H]^{2+}$, 501.90) of (a) digested cashew extract and (b) synthesized standard mixture. The product ions obtained from PFIA-II were shown in boxes above or below related spectra. y1 and y2 are characteristic for each peptide

of the chosen transitions for WLQLSAER with MW 1,001.5 (Q1, 501.9; Q3, 159.2, 272.3 and 575.5) were from the homologue part of the WLQLSVEK sequence with 1,002.2 Da (a1, a2 and y5 according to PFIA-II), it could also be detected with the developed LC-MS/MS method.

The product ion spectra of digested walnut and pecan extracts ($[M+2H]^{2+}$ m/z, 682.7; CE, 35 V) were also similar to the spectrum of the standard; however, the search in the database for a similar peptide to AFSWEVLEAALK in walnut and pecan proteins was not successful. It only can be speculated that in these two nuts, there is again a sequence akin to AFSWEVLEAALK, which is under investigation at the moment.

Conclusion

Eight tryptic peptides of three hazelnut allergens Cor a 8, Cor a 9 and Cor a 11, which gave high MS signals, were selected and synthesized. Using them as standards, an LC-MS/MS method in SRM mode was developed for the detection of hazelnut in food products. According to a BLAST search, only one of the four Cor a 9 peptides occurs in different nuts (walnut, pecan and pistachio). However, the MS measurements showed that this peptide occurs also in cashew. Additionally, one peptide from Cor a 11, which according to BLAST is hazelnut specific, showed a significant SRM signal for walnut and pecan as well. Using

product ion spectra, we demonstrated that the detected cashew, walnut and pecan peptides are very similar to our synthetic peptides, but not the same.

In this work, we could show that anti-hazelnut antibodies are cross-reactive also to other nuts. A reason for it is the sequence homology of proteins from different nuts, which can also be a problem for LC–MS/MS measurements for the detection of hazelnut in different food samples. However, the specificity of MS/MS full scans allows the unambiguous determination of peptides, strongly minimising the cross-reactivity encountered with immunoassays. Thus, we could develop a sensitive and selective method for the detection of hazelnut proteins in food samples, alternative to immunoanalytical methods. In further studies, we intend to use this method for the determination of trace amounts of allergic nut proteins in processed food.

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References

- Köksal AI, Artik N, Simsek A, Günes N (2006) Nutrient composition of hazelnut (*Corylus avellana* L.) varieties cultivated in Turkey. *Food Chem* 99:509–515
- Rigby NM, Marsh J, Sancho AI, Wellner K, Akkerdaas J, van Ree R, Knulst A, Fernandez-Rivas M, Brettlova V, Schilte PP, Summer C, Pumphrey R, Shewry PR, Mills ENC (2008) The purification and characterisation of allergenic hazelnut seed proteins. *Mol Nutr Food Res* 52:S251–S261
- Lauer I, Foetisch K, Kolarich D, Ballmer-Weber BK, Conti A, Altmann F, Vieths S, Scheurer S (2004) Hazelnut (*Corylus avellana*) vicilin Cor a 11: molecular characterization of a glycoprotein and its allergenic activity. *Biochem J* 383:327–334
- Bignardi C, Elviri L, Penna A, Careri M, Mangia A (2010) Particle-packed column versus silica-based monolithic column for liquid chromatography-electrospray-linear ion trap-tandem mass spectrometry multiallergen trace analysis in foods. *J Chromatogr A* 1217(48):7579–7585
- Belitz HD, Grosch W, Schieberle P (2001) *Lehrbuch der Lebensmittelchemie*. Springer Verlag, Berlin
- Goetz DW, Whisman BA, Goetz AD (2005) Cross-reactivity among edible nuts: double immunodiffusion, crossed immunoelectrophoresis, and human specific IgE serologic surveys. *Ann Allergy Asthma Immunol* 95:45–52
- EC-Directive 2007/86/EC of 27 November 2007
- Sathe SK, Venkatachalam M, Sharma GM, Kshirsagar HH, Teuber SS, Roux KH (2009) Solubilization and electrophoretic characterization of select edible nut seed proteins. *J Agric Food Chem* 57:7846–7856
- Lauer I, Alessandri S, Pokoj S, Reuter A, Conti A, Vieths S, Scheurer S (2008) Expression and characterization of three important panallergens from hazelnut. *Mol Nutr Food Res* 52: S262–S271
- Schubert-Ullrich P, Rudolf J, Ansari P, Galler B, Führer M, Molinelli A, Baumgartner S (2009) Commercialized rapid immunoanalytical tests for determination of allergenic food proteins: an overview. *Anal Bioanal Chem* 395:69–81
- Bonwick GA, Smith CJ (2004) Immunoassays: their history, development and current place in food science and technology. *Int J Food Sci Technol* 39:817–827
- Van Hengel AJ (2007) Food allergen detection methods and the challenge to protect food-allergic consumers. *Anal Bioanal Chem* 389:111–118
- Poms RE, Klein CL, Anklam E (2004) Methods for allergen analysis in food: a review. *Food Addit Contam* 21(1):1–31
- BLAST. Available at <http://blast.ncbi.nlm.nih.gov/Blast.cgi>. Accessed 1 Mar 2010
- In-solution digestion. Available at <http://www.osa.sunysb.edu/Proteomics/ProteinDigestPrep.pdf>. Accessed 14 Jan 2008
- Ansari P, Stoppacher N, Rudolf J, Schuhmacher R, Baumgartner S (2011) Selection of possible marker peptides for the detection of major ruminant milk proteins in food by liquid chromatography-tandem mass spectrometry. *Anal Bioanal Chem* 399:1105–1115
- PeptideMass. Available at <http://www.expasy.org/tools/peptide-mass.html>. Accessed 15 Feb 2010
- UniProt database. Available at <http://www.uniprot.org/uniprot>. Accessed 15 Feb 2010
- Peptide Fragment Ion Analyser (PFIA-II). Available at <http://hodgkin.mbu.iisc.ernet.in/~pfia/>
- NCBI (National Center for Biotechnology Information). Available at <http://www.ncbi.nlm.nih.gov/>. Accessed 28 Mar 2011