

SPECIAL GUEST EDITOR SECTION

Investigation of Reduced ELISA Recovery of Almond and Hazelnut Traces from Roasted Nut Samples by SDS-PAGE and Mass Spectrometry

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Western society is facing an increase in the number of food-allergic individuals, with rising incidence in the past years. Therefore, allergen-free food and accurate and reliable analysis of allergen contamination are essential for the protection of consumers. Yet, there is limited understanding on the effect of food processing on allergenicity and on the ability of available methods to detect trace contamination in processed food. Available studies addressing this have relied on sample processing on a laboratory scale. In this study, industry-like processing under precisely defined conditions (ranging from 110 to 150°C roasting temperatures) was employed to better understand the limitations of state-of-the-art methods for detecting traces of hazelnut and almond in processed food. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis indicated an overall reduction in extracted proteins from roasted nut samples, and with matrix-assisted laser desorption ionization time-of-flight Cor a 9 and Prunin, were identified as majorly expressed proteins for hazelnut and almond, respectively. A commercial ELISA kit detected nut traces only up to a 130°C roasting temperature. Untargeted MS (Orbitrap) analysis was able to detect traces of nuts roasted up to 150°C while also confirming Cor a 9 and Prunin

as the major expressed proteins for hazelnut and almond, respectively. Preparing cookie dough spiked with roasted nut samples, a complex food matrix was simulated. Analysis by ELISA showed the same limitations encountered for pure nuts samples, hardly detecting traces of nuts roasted above 130°C. Targeted MS (linear ion trap) using multiple reaction monitoring methods for one proteotypic peptide for Cor a 9 and Prunin, respectively, enabled a detection of nut traces up to 150°C. The results indicated that a reduced extractability because of temperature-related effects (e.g., protein denaturation, cross-linking, poor solubility) caused the significant differences between the ELISA and MS analysis. Overall, the results of this study may form the basis to improve allergen detection after roasting through improved extraction methods and refined ELISA formats.

In recent years, the number of allergic individuals has increased, a process seemingly enhanced by the Western lifestyle (1). In the European Union (EU), roughly 1.5% of adults and 5–6% of children suffer from a food allergy (2). In the United States, on the other hand, 4% of the adult population and 5% of children suffer from food-induced allergies. In the United States, the prevalence in children has risen about 50% since 1997 (3). Among the most typical allergens, peanuts are a major cause for mediation of food-induced allergies, ranging from mild allergic symptoms (e.g., soreness, rashes, and sneezing/coughing) to severe reactions, including anaphylactic shock (4). Besides peanuts, hazelnuts and almonds are ingredients that have to be labeled prominently on products according to EU and U.S. regulations, as they alike represent common food allergens (5, 6). Labeling is an important tool to protect allergic consumers, as avoidance is currently the only preventive action against food allergies (7). Therefore, food producers need to check their products thoroughly using adequate test systems and risk assessment (8). Unintended contamination is one of the major

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causes of recalls of products by the food industry (9). Those contaminations can occur during production (e.g., when the same baking facility is used to produce hazelnut-containing and hazelnut-free cookies), packaging, and in the supply chain of the raw materials. To check for these contaminations, food producers require robust and reproducible methods to identify and quantify allergens in diverse food matrices (e.g., cookies). The most widely used method is ELISA, which relies on an antibody-antigen interaction to detect certain proteins (allergens or indicator proteins). ELISAs usually show a good sensitivity and selectivity, but are potentially susceptible to a common problem, as their robustness is closely tied to the structural integrity and accessibility of the specific antigen in question. Studies reported a reduced ELISA recovery for heat-treated samples; it was shown for peanuts that heating the samples to certain temperatures significantly reduced the recovery, ranging from 70-fold up to 2000-fold signal reduction (10). As heat treatment (e.g., roasting, baking, and heat sterilization) is a common working step during food production, this poses a significant risk for allergic consumers and producers. A reduced ELISA recovery because of heat treatment is not necessarily correlated with a decreased allergenicity of the proteins. It was shown for peanuts (11, 12) that heat treatment even increased the allergenic potential. This may lead to a higher risk for allergic consumers, as food products may not be labeled correctly because of insufficient test results.

Currently, little information about the allergenicity of roasted hazelnuts and almonds has been published (13–17). Furthermore, a limitation of most studies is the sample processing (e.g., roasting), which was mostly performed on a laboratory scale, leaving industrial processes underrepresented. However, using preparations close to real industrial processes might have a different influence on the allergenicity of proteins. This is especially problematic regarding the temperatures used. For example, the heat transfer in an industrial drum roaster is significantly different from the heat transfer in a conventional oven.

To investigate the reduced ELISA recovery under industry-like processing conditions, almonds and hazelnuts were roasted in an industrial drum roaster under precisely defined conditions at various roasting temperatures and times. After roasting, grinded nuts were used to spike cookie dough with known amounts of hazelnut and almond, baked under controlled conditions. Proteins from raw nuts, roasted nuts, and nut-spiked cookies were extracted and analyzed by ELISA, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), matrix-assisted laser desorption ionization–time-of-flight (MALDI-TOF) MS, untargeted (Orbitrap) and targeted [linear ion trap (LIT) LC–tandem MS (MS/MS)] MS, respectively. Our aim was to identify the factors influencing protein recovery. Data was used to assess potential factors that may influence protein integrity and extraction, both affecting allergen detection by ELISA.

Materials and Methods

Chemicals and Materials

Hazelnuts (*Corylus avellana*) were purchased raw and unpeeled from Kessko (Cat. No. 07111; Bonn, Germany). Almonds (*Prunus dulcis*) were purchased raw and unpeeled from Kessko (Cat. No. 07001). Synthetic peptides were ordered from Thermo Fisher Scientific (Waltham, MA) in AQUA QuantPro quality. Acetonitrile (ACN), ammonium bicarbonate (AmBiC), formic acid (FA), NuPAGE™ 4–12% Bis-Tris

12-well mini gels, NuPAGE MES SDS Running Buffer (20X), NuPAGE LDS Sample Buffer (4X), NuPAGE Sample Reducing Agent (10X), PageRuler Prestained Protein Ladder (10–250 kDa), SimplyBlue™ SafeStain, sodium hydroxide pellets, trifluoroacetic acid (TFA), water, and 4 mm diameter (0.2 µm pore size) regenerated cellulose syringe filters were purchased from Thermo Fisher Scientific. Trypsin Gold Sequencing Grade was purchased from Promega (Madison, WI). Alpha-cyano-4-hydroxycinnamic acid (CHCA), dithiothreitol (DTT), iodoacetamide (IAA), tris(2-carboxyethyl) phosphine (TCEP), and tris(hydroxymethyl)aminoethane hydrochloride (tris-HCl) were purchased from Carl-Roth (Karlsruhe, Germany). All chemicals were purchased in highest available quality (minimum HPLC grade). ELISAs RIDASCREEN®FAST Hazelnut (Cat. No. R6802) and RIDASCREEN®FAST Almond (Cat. No. R6901) were purchased from R-Biopharm AG (Darmstadt, Germany).

Roasting Process

Nuts were roasted with a drum roaster PROBATino/1 (PROBAT-Werke, Gimborn Maschinenfabrik GmbH, Emmerich, Germany). Temperature, time, and gas supply were graphed for every roasting process using the Probat PILOT ROASTER Shop software. Each batch, containing 1000 g nuts, was roasted under defined conditions. To represent a practical range of process parameters, each batch was processed at a set time (10, 20, and 30 min) and temperature (110, 120, 130, 140, and 150°C) in repeat and randomized determination. After grinding, nuts were vacuum sealed and stored at –18°C.

Cookie Preparation

Cookies were baked based on an allergen-free recipe (MoniQA Association, Vienna; used for the recipe of milk and soy reference material). In short, the recipe includes sugar, salt, vanilla sugar, baking powder, flax seed flour, potato flour, carob gum, xanthan gum, and water. An amount of 4000 mg/kg nut sample was spiked into the cookie dough. Only batches with a roasting time of 30 min of all roasting temperatures (110–150°C) were used for spiking. The spiked cookie dough was portioned and baked at 150°C for 25 min in a conventional oven (accent line Series 8; Bosch, Gerlingen, Germany). After grinding, cookies were vacuum sealed and stored at –18°C.

Protein Extraction for SDS-PAGE and MS Analysis

The 30 mg samples were extracted using 1 mL extraction buffer (0.1 M tris-HCl, adjusted to pH 7.5 with 1 M sodium hydroxide solution) at 50°C for 1 h, shaking at 400 rpm on a thermo block. After the extraction, the samples were centrifuged at 13.000 × g for 5 min to sediment all residual sample material. The buffer was then exchanged to 0.1 M AmBiC (pH 8.0) using PD SpinTraps G-25 (GE Healthcare, Chicago, IL) according to the manufacturer protocol.

SDS-PAGE

The SDS-PAGE was performed according to the manufacturer protocol. In short, 1 µL extracted protein sample was mixed with 25 µL ultrapure water, 10 µL loading buffer, and 4 µL reducing

agent. The samples were heated to 70°C for 10 min, and 20 μ L were transferred to the gel pocket. The power supply was set to 200 V for 35 min. The gels were rinsed with ultrapure water three times for 5 min each and stained with SafeStain for 1 h. The gels were subsequently destained overnight in ultrapure water to increase contrast.

MALDI-TOF MS

Bands of interest were excised from the SDS-PAGE gel and transferred into reaction vessels. The gel pieces were destained with 100 μ L ultrapure water for 5 min, and the supernatant was discarded. One hundred microliters 0.1 M AmBiC-ACN (1+1, v/v) were added and incubated at room temperature for 10 min, and the supernatant was discarded. Then, 50 μ L ACN were added and incubated at room temperature for 5 min, discarding the supernatant. The gel pieces were dried for 10 min. Twenty microliters of reducing buffer (10 mM DTT in 0.1 M AmBiC) were added and incubated at 56°C for 30 min. Twenty microliters alkylating buffer (55 mM IAA in 0.1 M AmBiC) were added and incubated at room temperature for 30 min under exclusion of light. The supernatant was discarded, and the washing steps with ultrapure water, AmBiC-ACN, and ACN were repeated as described above. Fifteen microliters of trypsin buffer (20 ng/ μ L trypsin in 0.1 M AmBiC) were added and incubated at 37°C overnight. The next day, the supernatant was transferred into a reaction vessel. AmBiC-ACN (1+1, v/v) was added to the remaining gel piece until it was fully covered and incubated at room temperature for 5 min. The supernatant was again removed and combined with the previously retained supernatant. The combined supernatants were prepared on the MALDI-TOF target by applying the thin layer method; a saturated CHCA solution in acetone containing 0.1% TFA was prepared and a thin layer coated onto the target (AnchorChip, Bruker Daltonics). Then, 0.5 μ L of each sample were applied to the anchor spots and left to dry. All samples were analyzed in positive reflector mode with matrix suppression up to m/z 400 using a Bruker Daltonics Autoflex speed. The measurements were controlled by Bruker Daltonics ProteinScape 4.0 software, enabling an automated database search for the obtained results using our in-house Mascot server (version 2.2.0.4). The results were matched against a hazelnut and almond proteome database, respectively. The top five peptide hits for each protein were subjected to a LIFT-MS/MS measurement for additional confirmation.

In-Solution Tryptic Digest

One hundred and sixty microliters of buffer-exchanged protein extract were reduced with 10 μ L reducing buffer (30 mg/mL TCEP in 0.1 M AmBiC) at 60°C for 15 min. Subsequently, the samples were alkylated with 10 μ L alkylating buffer (18 mg/mL IAA in 0.1 M AmBiC) at room temperature for 20 min under exclusion of light. Finally, the samples were digested using 10 μ L digestion buffer (20 ng/ μ L trypsin in 0.1 M AmBiC) at 37°C overnight.

LC-MS/MS (Orbitrap)

The samples obtained via in-solution digest were filtered using regenerated cellulose syringe filters to remove any particulate matter present and transferred to HPLC vials. Chromatographic

separation was performed on a Hypersil Gold C18 column (100 $\times$ 2.1 mm, 3.0 μ m; Cat. No. 25003-102130; Thermo Fisher Scientific) protected by matching guard cartridges (10 $\times$ 2.0 mm, 3.0 μ m; Cat. No. 25003-012101; Thermo Fisher Scientific) in a Surveyor HPLC system (Thermo Fisher Scientific). Using a quaternary HPLC system, eluent A was ultrapure water, eluent B was ACN, and eluent C was 10% FA in ultrapure water. The flowrate was 250 μ L/min, and channel C was kept constant at 1% (which was accounted to the part of aqueous eluent) to ensure an overall FA content of 0.1% in the eluent throughout the measurements. After injection, eluent B was held at 5% as initiation for 2 min. It was then ramped to 54% over the course of 25 min and held for 4 min, followed by a steep increase to 95% in 4 min. This plateau was held for 4 min and reverted to 5% B in 3 min. This plateau was held for 10 min prior to the next injection. The injection volume was 5 μ L. The HPLC system was coupled with an LTQ Orbitrap Velos Pro system (Thermo Fisher Scientific) via an electrospray ionization source. All measurements were performed in positive mode using a data-dependent full scan with top five fragmentation. The obtained results were processed with Bruker Daltonics ProteinScape 4.0 software linked to an in-house Mascot server (version 2.2.0.4). The results were matched against a hazelnut and almond proteome database, respectively.

LC-MS/MS (LIT)

The samples obtained via in-solution digest were filtered using regenerated cellulose syringe filters to remove any particulate matter present and transferred to HPLC vials after adding 5% ACN and 16.7 pmol/100 μ L stable-isotope-labeled standard. Chromatographic separation was performed on a Hypersil Gold C18 column (100 $\times$ 2.1 mm, 3.0 μ m; Cat. No. 25003-102130; Thermo Fisher Scientific) protected by matching guard cartridges (10 $\times$ 2.0 mm, 3.0 μ m; Cat. No. 25003-012101; Thermo Fisher Scientific) in a Series 200 HPLC system (Perkin Elmer). Eluent A was ultrapure water containing 0.1% FA, and eluent B was ACN containing 0.1% FA with a constant flow rate of 300 μ L/min. After injection, eluent B was held at 5% as initiation for 1 min. It was then ramped to 50% over the course of 8 min, followed by a steep increase to 95% in 1 min. This plateau was held for 2 min and reverted to 5% B in 1 min. This plateau was held for 4 min prior to the next injection. The injection volume was 20 μ L. The HPLC system was coupled with an API 3200 QTRAP system (Sciex) via an electrospray ionization source. All measurements were performed in positive mode using a scheduled multiple reaction monitoring (MRM) method with two transitions per selected peptide. All transitions and according settings for the MRM methods are summarized in Table 1. The obtained results were processed with Analyst software version 1.6.2 (Sciex).

ELISA

Fifty grams of sample (roasted hazelnut, almond, or spiked cookie) were grinded in a blender. One gram was extracted with commercially available extraction buffer as described in the instruction for use (R-Biopharm AG). Samples were then diluted to a level of 20 mg/kg with diluted Allergen Extraction buffer and analyzed using RIDASCREEN FAST Hazelnut and RIDASCREEN FAST Almond, respectively. Recovery was calculated using RIDASOFT Win.NET (version 1.103.0.0217).

Table 1. MS/MS transitions for quantifier and qualifier with parameters for the LIT-LC–MS/MS measurements

Peptide	Q1 ^a , <i>m/z</i>	Q3 ^b , <i>m/z</i>	Dwell, <i>ms</i>	DP ^c , V	EP ^d , V	CEP ^e , V	CE ^f , V	CXP ^g , V
Hn1_INTVNSNTLPVLR	720.8	711.8	20	41	9	88	29	30
Hn1_INTVNSNTLPVLR	720.8	484.2	20	41	9	88	32	58
Hn1_INTVNSNTL (¹³ C ₆ , ¹⁵ N)PVLR	724.3	715.3	20	41	9	88	29	30
Hn1_INTVNSNTL (¹³ C ₆ , ¹⁵ N)PVLR	724.3	484.2	20	41	9	88	32	58
Al1_TEENAFINTLAGR	718.4	631.3	20	36	10	84	33	8
Al1_TEENAFINTLAGR	718.4	545.2	20	36	10	84	29	24
Al1_TEENAFINTL (¹³ C ₆ , ¹⁵ N)AGR	721.9	637.7	20	36	10	84	33	8
Al1_TEENAFINTL (¹³ C ₆ , ¹⁵ N)AGR	721.9	544.9	20	36	10	84	29	24

^a Q1 = Quadrupole 1.^b Q3 = Quadrupole 3.^c DP = Declustering potential.^d EP = Entrance potential.^e CEP = Collision entrance potential.^f CE = Collision energy.^g CXP = Collision exit potential.

Results and Discussion

Investigation of Intact and Digested Proteins by SDS-PAGE and MALDI-TOF MS

Assessment of the recovery on protein level via SDS-PAGE indicated multiple prominent bands throughout different roasting conditions for hazelnut and almond. Figure 1 shows the stained SDS-PAGE gels for hazelnut; the most prominent bands were visible at approximately 38 and 24 kDa. From native hazelnut to hazelnuts roasted at 110°C for 30 min, the bands

became more prominent, and then, following the temperature and time gradient in rising order, the protein bands faded gradually until they completely disappeared at 140°C roasting temperature for 10 min. For almond (Figure 2), there were also two bands most prominently visible at approximately 48 and 24 kDa. The bands of the almond extract became more prominent from native to roasted at 120°C for 10 min, then again fading with increasing roasting temperature and time. In contrast to hazelnut, the bands deriving from the almond extract were hardly visible already after roasting at 130°C for 20 min. This observation indicates, that the problem might arise already

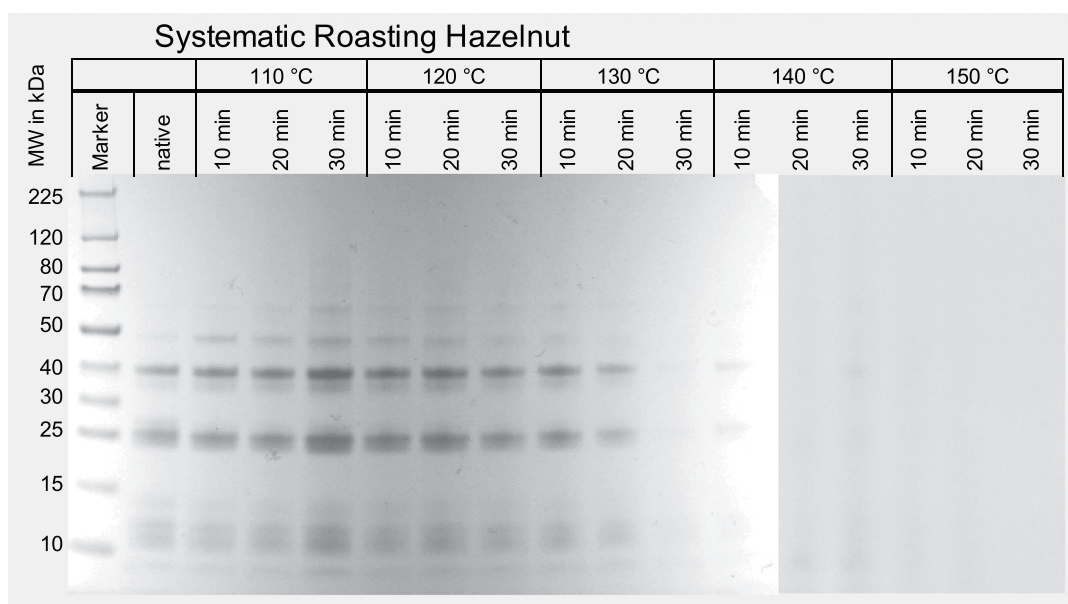


Figure 1. Coomassie-stained SDS-PAGE gels of extracted hazelnut proteins obtained under reducing conditions. Joint picture of two gels prepared in parallel. Marker weights are valid for both gels. MW = molecular weight, kDa = kilodalton.

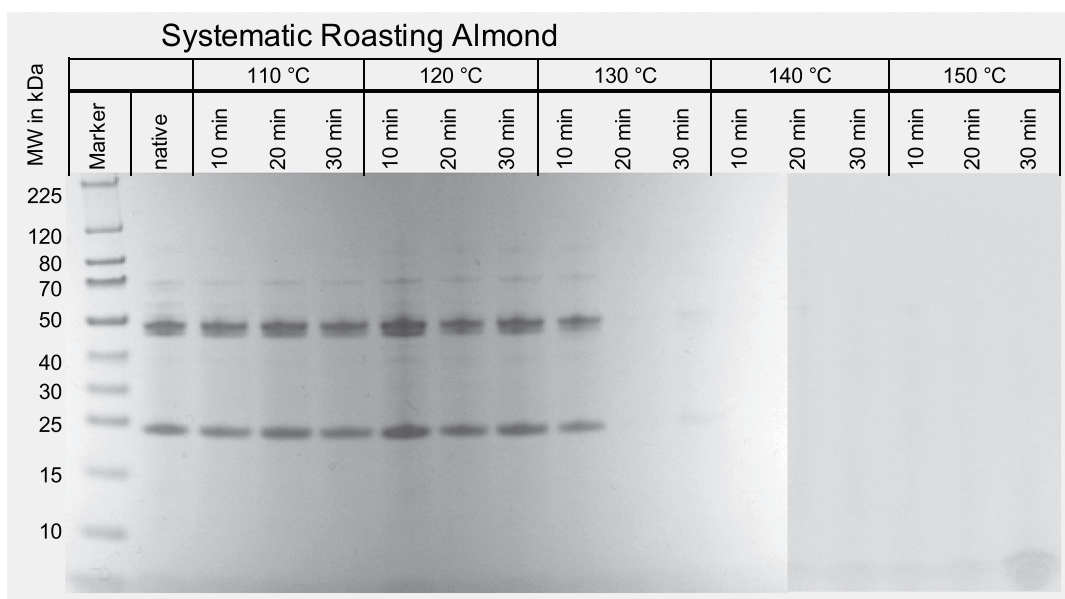


Figure 2. Coomassie-stained SDS-PAGE gels of extracted almond proteins obtained under reducing conditions. Joint picture of two gels prepared in parallel. Marker weights are valid for both gels. MW = molecular weight, kDa = kilodalton.

on the protein level. A reduction in overall protein content, without visible lower molecular weight degradation products, indicates a reduced extractability of the proteins because of cross-linking or other structural changes, leading to aggregation and poor solubility as a consequence. A similar behavior was previously reported for peanuts (10, 18).

In addition to the visual evaluation, in-gel digestion followed by MALDI-TOF MS peptide mapping of the aforementioned

bands was performed to identify the underlying proteins. For hazelnut, both the 38 and 24 kDa band resulted in a positive match for Cor a 9. The appearance of Cor a 9 in two separate bands was likely due to different subunits or fragments of this protein, visible on the gel as distinct bands. This hypothesis was supported by the sequence coverage maps for both bands, which are shown in Figure 3A and 3B. Each of the two bands solely contained peptides from separate parts of the

A	1	MAKLILVSFS	LCLLVLFNGC	LGIDVGLRRQ	QQRHFGEENL	DRLNALEPTN
	51	RIEAEAGQIE	SWDHNDQQFQ	CAGVAVIRRT	IEPNGLLLPQ	YSNAPELIYI
	101	ERGRGITGVL	FPGCPETFED	PQQQSQQGQG	QQQSQRSEQD	RHQKIRYFQE
	151	GDIIALPAGV	AHWCYNDGDS	PVVAVSLLHT	NNYANQLDEN	PRHFYLAGNP
	201	DDEHQRRQQQ	QFGQRRRQQQ	HSRGKEGEQE	QQGEGNNVFS	GFDAEFLADA
	251	FNVDVDTARR	LQSNQDKRRN	IVKVEGRLQM	VRPERSRQEW	ERQERQERES
	301	EQERERQRRQ	GGRGRDVNGF	EETICSLRLM	ENIGSRSRAD	IYTEQVGRIN
	351	TVNSNTLPVL	RWLQLSAERG	DLQREGLYVP	HWNLNAHSV	YAIRGRARVQ
	401	VDDNGNTVF	DDELRRQQVL	TIPQNFVAVK	RAESEGFWEV	AFKTNDAQI
	451	SPLAGRTSAI	RALPDDVLAN	AFQISREEAR	RLKYNRQETT	LARSSRSSE
	501	RMRRRSESEG	RAEA			
B	1	MAKLILVSFS	LCLLVLFNGC	LGIDVGLRRQ	QQRHFGEENL	DRLNALEPTN
	51	RIEAEAGQIE	SWDHNDQQFQ	CAGVAVIRRT	IEPNGLLLPQ	YSNAPELIYI
	101	ERGRGITGVL	FPGCPETFED	PQQQSQQGQG	QQQSQRSEQD	RHQKIRYFQE
	151	GDIIALPAGV	AHWCYNDGDS	PVVAVSLLHT	NNYANQLDEN	PRHFYLAGNP
	201	DDEHQRRQQQ	QFGQRRRQQQ	HSRGKEGEQE	QQGEGNNVFS	GFDAEFLADA
	251	FNVDVDTARR	LQSNQDKRRN	IVKVEGRLQM	VRPERSRQEW	ERQERQERES
	301	EQERERQRRQ	GGRGRDVNGF	EETICSLRLM	ENIGSRSRAD	IYTEQVGRIN
	351	TVNSNTLPVL	RWLQLSAERG	DLQREGLYVP	HWNLNAHSV	YAIRGRARVQ
	401	VDDNGNTVF	DDELRRQQVL	TIPQNFVAVK	RAESEGFWEV	AFKTNDAQI
	451	SPLAGRTSAI	RALPDDVLAN	AFQISREEAR	RLKYNRQETT	LARSSRSSE
	501	RMRRRSESEG	RAEA			

Figure 3. Sequence coverage maps for the identified peptide sequences (marked red) found in the hazelnut bands at (A) 41 kDa and (B) 26 kDa detected during the MALDI-TOF MS analysis. Both maps show the complete amino acid sequence of Cor a 9 in one-letter code. Color images are available online at <http://aoac.publisher.ingentaconnect.com/content/aoac/jaoac>.

complete sequence. This might be explained by the separation of the protein's C- and N-terminal subunit (predicted to be held together by a disulfide bond) of the hexameric Cor a 9 as a member of the 11S seed storage (globulin) protein family (19). The N-terminal subunit is reported to show two close bands around 35 kDa on the SDS gel, while the C-terminal subunit shows two close bands around 24 kDa (20, 21). The band identified here fit with these reported bands quite closely. The small deviation might be caused by sequence alterations, deriving from different origins of the hazelnuts used, which was previously reported (20). For almond, all positive matches resulted in Prunin being detected in both the 48 and 24 kDa bands. This might again be an indication for different subunits separated by the gel electrophoresis. In contrast to Cor a 9, the sequences obtained in the different bands for almond were distributed among the complete sequence, not allowing for an assignment of each band to separate parts of the protein sequence (data not shown). As another member of the 11S seed storage protein family, a subunit of the hexameric Prunin is also predicted to be composed of a C- and N-terminal subunit, linked by a disulfide bond. The occurrence of two bands and the lack of sequence correlation with predicted domains may indicate a more complex processing of the proform of the protein. Reported protein identifications were consistent throughout all

samples with visible bands on the gel; however, the number of identified peptides per protein was decreasing with increasing roasting temperature and time (data not shown).

Comparative Investigation of Nut Samples by ELISA and Orbitrap LC-MS/MS

ELISA analysis of samples roasted under controlled industry-like conditions demonstrated reduced recoveries with increasing roasting temperature and time for both hazelnuts and almonds, respectively (Figure 4A and 4B). The decline markedly set on at 130°C roasting for 10 min and quickly intensified with rising time and temperature. At 140°C, almost no signals were detectable, leading to an immense underestimation of the allergens present. Comparing the influence of roasting temperatures and times on encountered recoveries, it is obvious that temperature has a bigger influence compared with time. Regarding the illustrated error bars, hazelnut showed higher average deviations overall compared with all almond measurements. For both nuts, the native, nonprocessed samples consistently showed an ELISA recovery of 100% (data not shown).

A reduced ELISA recovery was already reported for peanuts, which were thermally processed; the authors accounted the reduced recovery to either structural changes of the antigen's

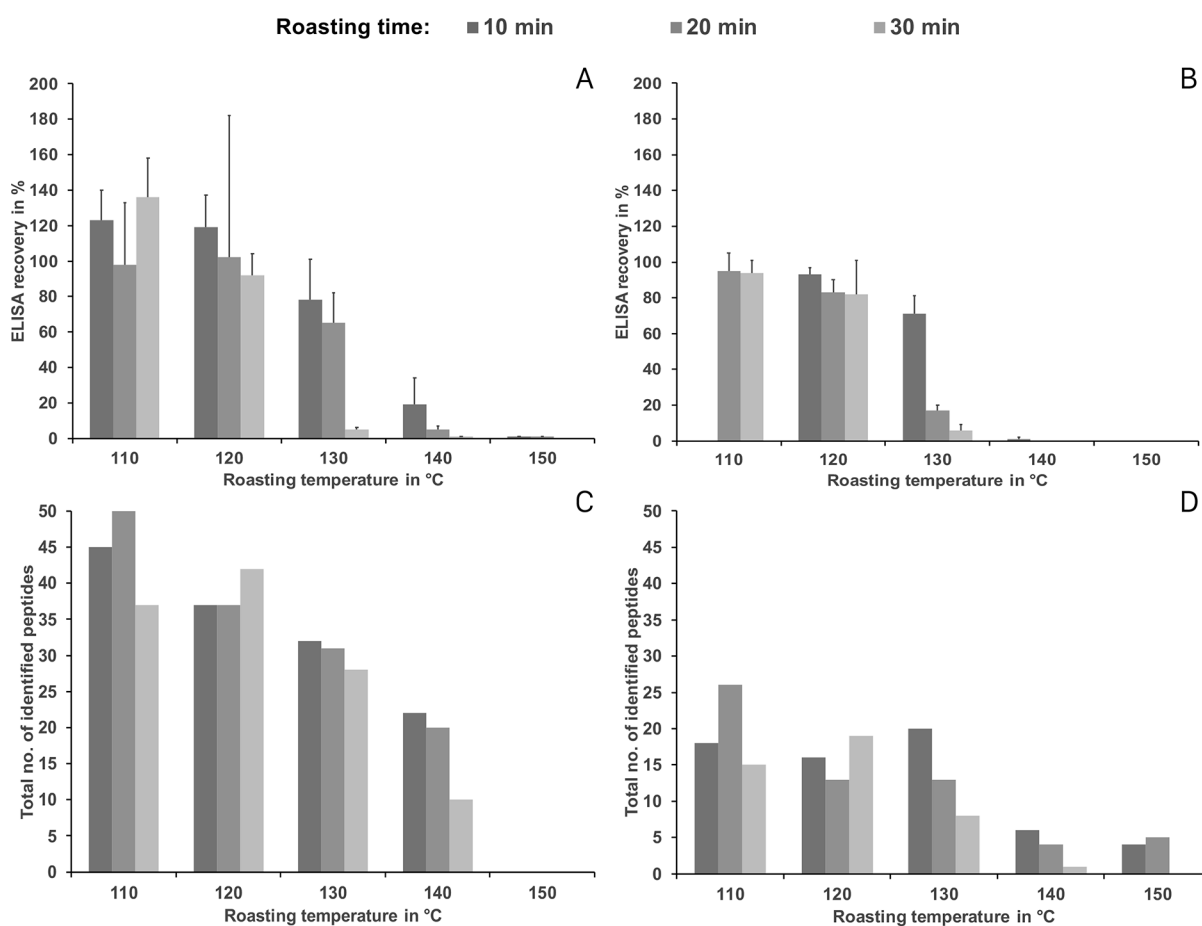


Figure 4. Graphical illustration of the obtained ELISA recoveries analyzing the samples roasted under varying conditions for (A) hazelnut and (B) almond. Error bars are calculated with standard variation based on $n = 3$ and graphical illustration of the total number of assigned peptides identified during the Orbitrap LC-MS/MS measurements of the samples roasted under varying conditions for (C) hazelnut and (D) almond.

epitope or a reduced extractability (10). Comparing SDS-PAGE and ELISA data presented here, a reduced extractability is the most probable explanation in our experimental setup, as a mere structural change of the epitope would not result in the observed fading of the protein bands with rising temperature and time gradient. Nevertheless, reduced recognition of proteins in roasted samples by the used antibodies may additionally contribute to the lower recoveries.

In parallel to the ELISA measurements, the whole protein extracts of the roasted nut samples were digested using trypsin and analyzed via high-resolution LC-MS/MS (Orbitrap). The resulting data was subjected to a database search to identify proteins present. For all hazelnut extracts, the allergenic protein Cor a 9 was the major protein identified with the overall highest score. For almond, Prunin was the major protein identified. Both identifications are in agreement with the fact that Cor a 9 and Prunin are commonly expressed storage proteins for hazelnut and almond, respectively (20–22). These results further confirmed the previous findings of the MALDI-TOF MS analysis independently.

To evaluate the influence of the roasting process on the protein level present after extraction, the total number of all peptides matching to extracted proteins was calculated for each sample and plotted in Figure 4C and 4D. It is evident that the overall peptide number decreased with increasing roasting temperature and time. For hazelnut, no peptides were identified in all samples roasted at 150°C, whereas almond showed signals for samples roasted up to 20 min at 150°C. The peptides matched at higher roasting times and temperatures were exclusively derived from Cor a 9 (hazelnut) and Prunin (almond), respectively. Overall, LC-MS/MS appeared slightly advantageous for the detection, as the total drop in signal occurred at a higher roasting temperature compared with the ELISA results. This might be caused by the ELISA antigen detected being more susceptible to roasting compared with the proteins detected by LC-MS/MS or may simply be expressed at lower levels compared with the main storage proteins Cor a 9 and Prunin. Another possible explanation for the heat susceptibility might be the recognition of a conformational epitope by the antibody used in the commercial ELISA kit, which denatured after heat treatment. For this latter explanation, MS analysis would not be affected, as it is focusing on linear parts of the sequence, which are less likely to be altered by heat treatment.

From all peptides observed from samples after roasting at high temperatures, one proteotypic sequence present in all samples was selected for each nut and used to develop a quantitative LC-MS/MS method. The selected sequences were INTVNSNTLPVLR (Cor a 9) for hazelnut and TEENAFINTLAGR (Prunin) for almond. Both sequences were previously reported in literature and linked to the identification of nuts roasted in a conventional oven (23).

Comparative Investigation of Spiked Cookie Samples with ELISA and LC-MS/MS

Using stable-isotope-labeled peptide standards of the aforementioned proteotypic peptides (mass shift +7 Da achieved by six ¹³C and one ¹⁵N isotope on Leucine), LC-MS/MS MRM methods were developed for each of the peptides, observing two mass transitions (qualifier and quantifier) each. Both the

peptides of interest and the internal standard eluted at the same retention time consistently while still being well distinguishable by mass, fulfilling the fundamental requirements for a proper MRM method. Using these developed methods, relative quantitation of the selected peptides was enabled. Extracts from self-made cookie dough spiked with 4000 mg/kg roasted nut samples, simulating a more realistic and complex matrix, were analyzed. Baking of the cookies in a conventional oven represents a second processing step for the roasted nuts added to the dough, whose influence needs to be assessed adequately. Figure 5 comparatively displays the data of the spiked cookies from analysis by ELISA (Figure 5A and 5B) and by LC-MS/MS (Figure 5C and 5D). Nut samples were roasted at listed temperatures for 30 min. The ELISA results were comparable to those obtained in the analysis of the pure nut samples, showing a decreasing recovery with rising roasting temperature. For hazelnut, the last detectable response was obtained for cookies containing 4000 mg/kg hazelnut sample roasted at 140°C for 30 min. For almond-spiked cookies, the last signal occurred for the sample roasted at 120°C for 30 min. These results indicated a similar recovery in the more complex matrix compared with the analysis of the pure nut samples while not showing any evidence that the additional heat treatment derived from the baking process of the cookies resulted in a further decrease of the ELISA recovery.

The LC-MS/MS results showed a consistent analyte-to-standard ratio for all roasting temperatures of the hazelnut samples for the quantifier mass transition m/z 720.8 to 711.8. This indicated a good and stable choice of peptide and mass transition, enabling a comparative detection of the peptide over all temperatures during roasting for 30 min. For almond, the results show a significantly lower analyte-to-standard ratio throughout all temperatures compared with hazelnut while still showing a stable response for the mass transition m/z 718.4 to 545.2 for all investigated temperatures. In summary, the established MRM method provided a significant improvement compared with both ELISA and Orbitrap LC-MS/MS, enabling the detection of nut samples spiked at a concentration of 4000 mg/kg in cookies up to roasting at 150°C for 30 min.

Conclusions

Lack of suitable reference standards and the difficulty to reproduce industry-processing conditions of allergen-containing food in controlled small-scale experiments hamper the analysis of allergens in complex food matrices. In particular, there is limited knowledge on the effect of processing procedures (e.g., in roasting processes of almonds and hazelnuts) on allergenicity and how different processing parameters may influence recovery in analytical methods for allergen detection. Hence, experiments were undertaken to process almonds and hazelnuts under defined industry-like conditions and to prepare well-defined food standards through preparation of cookies that were spiked with the aforementioned roasted hazelnuts and almonds. These samples were used to investigate the decrease in ELISA recovery from almond and hazelnut samples roasted at various temperatures and times and in cookies spiked with these processed samples.

It was shown that increasing roasting temperature and time led to a reduction in ELISA recovery. SDS-PAGE

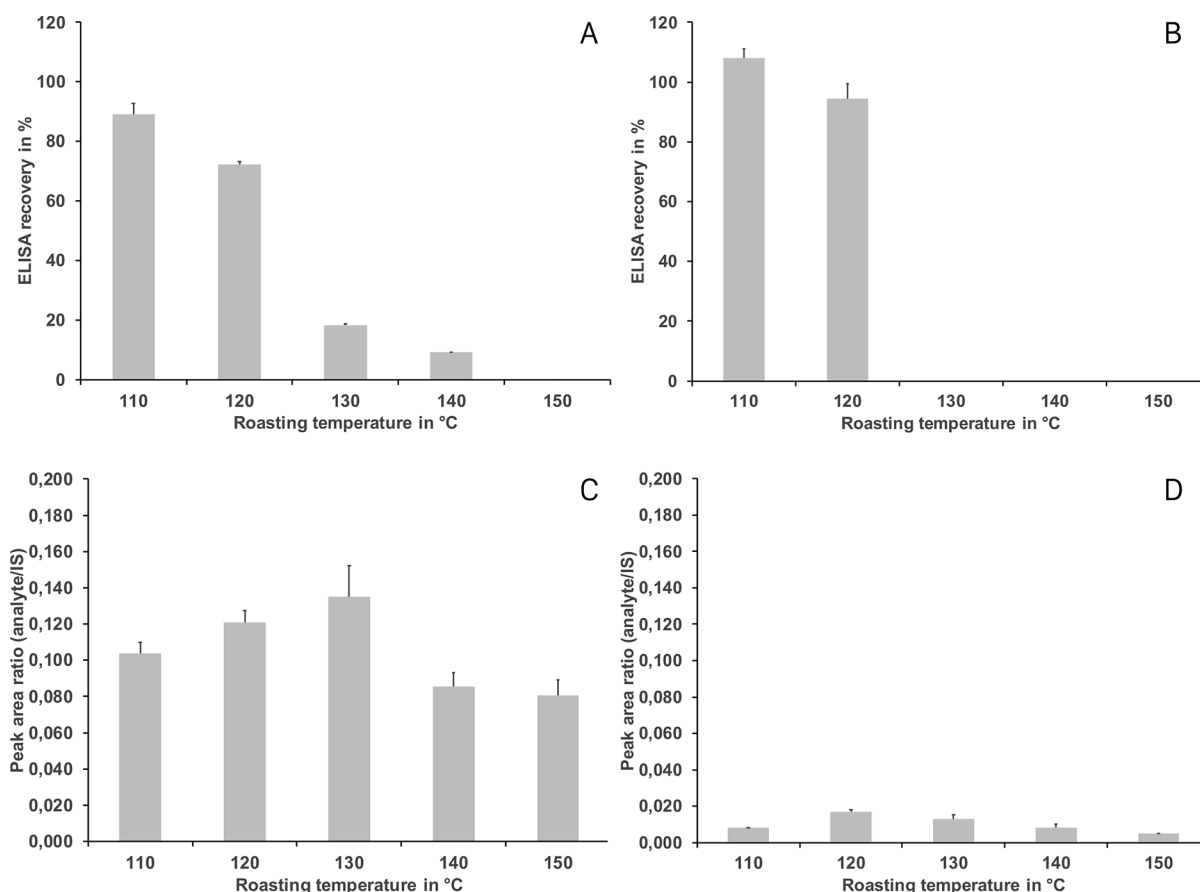


Figure 5. Graphical illustration of the obtained ELISA recoveries analyzing the cookie samples spiked with 4000 mg/kg (A) hazelnuts and (B) almonds roasted at various temperatures for 30 min; graphical illustration of the area ratios obtained via LIT-LC-MS/MS measurements of the cookie samples spiked with 4000 mg/kg (C) hazelnut and (D) almond roasted at various temperatures for 30 min showing the quantifier mass transitions per selected peptide (hazelnut: m/z 720.8 to 711.8; almond: m/z 718.4 to 545.2). Error bars are calculated with standard variation based on $n = 3$.

data indicated that this might be caused by reduced protein extraction, which may be due to heat denaturation and possible aggregation or cross-linking of heated proteins. A loss of a conformational epitope because of heat denaturation may be another explanation, although this appears in parallel with the reduced extractability, as the decrease in ELISA recovery mirrors the diminishing intensity of protein bands in SDS-PAGE. Detection sensitivity of LC-MS/MS was higher than for the particular ELISA used in this study. As the exact antigens of the primary ELISA antibodies are not known, they might target a weakly expressed protein or a conformational epitope, which is lost during heat treatment. The combined data from SDS-PAGE, MALDI-TOF MS, ELISA, high-resolution LC-MS/MS, and LIT-LC-MS/MS clearly indicated that the reduction in recovery is closely tied to a reduced extractability of the proteins in question.

It was demonstrated that the roasting of almonds and hazelnuts under industry-like conditions can affect ELISA recovery, while allergenic proteins are still present at higher roasting temperatures. Further investigations are required to assess the relevance of these findings for routine monitoring of allergen contamination. Furthermore, the results of this study may support efforts to improve allergen detection after roasting by improved extraction methods and refined ELISA formats.

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