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Research Article

Effects of the Varietal Diversity and the Thermal Treatment on the Protein Profile of Peanuts and Hazelnuts

Elisabetta De Angelis, Simona Lucia Bavaro, Linda Monaci, and Rosa Pilolli

Institute of Sciences of Food Production, National Research Council of Italy (CNR-ISPA), Via G. Amendola 122/O, 70126 Bari, Italy

Correspondence should be addressed to Rosa Pilolli; rosa.pilolli@ispa.cnr.it

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Several buffer compositions were compared for their efficiency in protein extraction from both raw and roasted peanut and hazelnut samples, the final goal being to understand the modification of protein solubility upon roasting and maximize the extraction yield. Denaturant conditions provided by urea-TBS buffer resulted in satisfactory extraction yields for both peanut and hazelnut samples, before and after the thermal treatment. In addition, different varieties of peanuts and hazelnuts were characterized to highlight the extent of variability in the protein profile accounted by the varietal factor and eventual differential resistance among cultivars to protein modification induced by the thermal processing. The protein profile was characterized by gel electrophoresis, and specific bands were analyzed by micro-HPLC-MS/MS coupled to software-based protein identification. No significant difference was observed for the investigated hazelnut cultivars, namely, *Campana*, *Romana*, and *Georgia*, whereas interesting features were presented for the peanut varieties *Virginia*, *Zambia*, and *China*. In particular, *Zambia* variety lacked two bands of approximately 36 and 24 kDa that were visible in *Virginia* and *China* varieties, which could suggest a lower allergenic potential of this particular variety which deserves to be further investigated before drawing final conclusions.

1. Introduction

Nuts represent a popular food in the common diet and are considered among the healthiest snack, thanks to the wide range of essential nutrients. Despite being high in fat, nuts have a number of impressive benefits on human health since they are rich in antioxidants, fibers, and unsaturated fatty acids and proteins [1]. On the other hand, due to the high content of allergenic proteins therein contained, tree nuts and peanuts are responsible for approximately 80% of anaphylactic reactions and account for over 50% of child fatalities related to food allergies in industrialized countries [2, 3]. Amongst food allergies, peanut and hazelnut allergies are considered the most dangerous and life-threatening allergies in the developed countries. The latter affects especially the US and Northern Europe, due to the common habit of youngsters to consume this food [4, 5]. Peanut allergy affects mostly the population of North America, United Kingdom, and Australia. It has been estimated that approximately 2% of children and 0.5–1% of US population is affected by peanut allergy, while in the United Kingdom, a birth cohort study from the Isle of Wight estimated the prevalence of peanut allergy at 1.3% [6, 7]. As for hazelnuts, an epidemiological study carried out by the EuroPrevall research consortium showed that the hazelnut was the most common sensitizing nut in the adult EuroPrevall cohort at 9.3% (lowest, Iceland 1.3%; highest, Switzerland 17.8%), followed by walnut at 3.0% (lowest, Iceland 0.1%; highest, Spain 7.7%), and peanut at 2.7% (lowest, Iceland 0.5%; highest, Spain 7.2%) [8]. Despite the increasing knowledge about the molecular and immunological properties of peanut and hazelnut allergens, the understanding of the processes and factors causing the severity of allergic reactions is still limited [9].

In order to improve the organoleptic properties and the shelf life of foods and to preserve their quality and safety, a number of new processing methods have been devised by the food industries. However, in many cases, it is not clear whether such treatments might alter the risk of eliciting an allergic reaction in sensitive consumers. These processing

methods may induce protein modification, altering its intrinsic allergenicity through protein aggregation via disulfides or other interchain covalent bonds; all structural changes at the protein level may induce a concomitant disappearance or appearance of new IgE-binding epitopes [10–12]. Several authors reported that the thermal treatment causes changes in the structure/conformation of proteins in different matrix [13–15], depending on the structural and chemical properties of the protein itself, the type of thermal processing and the operating conditions applied (temperature, pH, and time) [16–19].

In addition, the conformational changes of the protein allergen structure occurring during the thermal process result in the formation of protein aggregates or oligomers, with decreased protein solubility [20, 21]. In this regard, numerous methods to detect allergens contamination and/or to assess *in vitro* the allergenicity of specific ingredients rely on protein extraction systems. It was clearly demonstrated that the use of inappropriate extraction conditions can have a dramatic impact on protein extraction yield, decreasing the recovery of proteins which constrains the analysis sensitivity or, in the worst case, impairing the reliability of the immunoassay tests for allergenicity assessment [22]. Moreover, the proteins solubility is important not only in the determination of the allergen content or allergenicity assessment, but also in a variety of other applications such as clinical research studies and immunotherapy, as well as, evaluation of the quality and nutritional value of different foods.

In this work, we scouted several buffer compositions for their efficiency in protein extraction from both raw and roasted peanut and hazelnut samples, the final goal being to understand the modification of protein solubility upon roasting process and maximize the extraction yield. In addition, different varieties of peanuts and hazelnuts were characterized before and after the thermal treatment, in order to highlight the extent of variability in the protein profile accounted by the varietal factor, and eventual differential resistance to protein modification induced by thermal processing. The protein profile was characterized by gel electrophoresis, and specific bands were subjected to in-gel protein digestion and analyzed by micro-HPLC-MS/MS coupled to software-based identification.

2. Materials and Methods

2.1. Peanut and Hazelnut Samples. Peanut (*Arachis hypogaea*) and hazelnut (*Corylus avellana*) samples were obtained from Besana S.p.A. (San Gennaro Vesuviano, NA, Italy) as raw and roasted kernels. In particular, three varieties of each nut were provided, namely, *Virginia, Zambia, and China* for peanuts and var. *Campana*, *Romana*, and *Georgia* for hazelnuts. Peanut kernels were roasted for 30 min at 165°C. Hazelnut kernels were subjected first to a drying procedure for 5 min at 90°C, followed by roasting at 135°C for 15 min.

2.2. Chemicals. Acetonitrile (Gold HPLC ultragradient) and trifluoroacetic acid were purchased from Carlo Erba Reagents (Cornaredo, MI, Italy). Ultrapure water was produced by a Millipore Milli-Q system (Millipore, Bedford, MA, USA) while formic acid (MS grade) was provided by Fluka (Milan, Italy). Acrylamide and bis-acrylamide solutions as well as Quick Start™ Bradford protein assay kit were purchased from Bio-Rad Laboratories (Segrate, MI, Italy). Phosphate buffer saline (PBS), Trizma-base, sodium chloride, urea, ammonium bicarbonate, and iodoacetamide (IAA) along with other chemicals for electrophoresis (dithiothreitol, sodium dodecyl sulfate-SDS, glycine, glycerol, Coomassie Brilliant Blue G-250, and methanol-HPLC grade) were obtained from Sigma-Aldrich (Milan, Italy). Bromophenol blue was provided by Carlo Erba Reagents (Cornaredo, Italy) whilst trypsin (proteomic grade) for in-gel protein digestion was purchased from Promega (Milan, Italy). Electrophoresis experiments were accomplished on Mini-Protean Tetra Cell equipment provided by Bio-Rad Laboratories (Segrate, MI, Italy). Cellulose acetate (CA) syringe filters of 1.2 μm were obtained from Labochem Science S.r.l. (Catania, Italy) whilst Polytetrafluoroethylene (PTFE) syringe filters of 0.45 μm were purchased from Sartorius (Gottingen, Germany).

2.3. Protein Extraction from Peanut and Hazelnut Samples. Raw and roasted kernels of peanuts and hazelnuts were ground in a blender (Sterilmixer 12 model 6805-50, PBI International, Milan, Italy) and then passed through a 2 mm sieve. The obtained mince was then extracted according to the following protocol: 1 g of sample was combined with 20 ml of extraction buffer and left for 1 h at 25°C in an orbital shaker (KS 4000 i-control shaker, IKA Works GmbH & Co. KG, Staufen, Germany). Four different buffer compositions were tested, namely, (i) 10 mM phosphate buffer saline pH 7.4 + NaCl 0.15 M, (ii) 0.1 M ammonium bicarbonate + NaCl 0.15 M, (iii) 20 mM Tris·Cl, pH 8.2, and (iv) 7 M Urea + TBS pH 8 (50 mM Tris·Cl, 150 mM NaCl) + 25 mM IAA. Afterwards, the samples were subjected to 10 min of ultrasound treatment in a water bath (Sonomatic water bath, Langford, Birmingham, England) and centrifuged for 15 min at 1734 g keeping the temperature at 18°C. Peanut and hazelnut extracts were separated into three distinct layers: a fatty upper layer, a central phase containing the soluble protein fraction, and a solid pellet (material debris). The protein fraction was carefully collected and then filtered on a 1.2 μm CA syringe filter. A further filtration of the samples on a 0.45 μm PTFE filter was accomplished just before electrophoretic runs and LC-MS/MS analysis.

2.4. Bradford Assay for Protein Quantification. Protein quantification of protein extracts were performed with the commercial Quick Start protein assay kit (Bio-Rad) based on the colorimetric Bradford assay. The assay was carried out according to the manufacturer instructions, and at least three extraction replicates for each sample were analyzed.

2.5. SDS-PAGE Analysis. Protein extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 15% homogeneous hand-cast gel (8.6 cm × 6.7 cm × 1 mm). The extracts were mixed with

Laemmli buffer (62.5 mM Tris·Cl, pH 6.8, 25% glycerol, 2% SDS, 0.01% bromophenol blue, and 100 mM DTT to a final 1 : 1 ratio) and then reduced by heat treatment at 100°C for 5 min. PAGE runs were carried out with Mini-Protean Tetra Cell (Bio-Rad Laboratories) using TGS (25 mM Tris, 192 mM glycine, and 0.1% SDS) as running buffer and by applying 50 V potential for 15 min and 80 V potential until the end of the run. Afterwards, the gels were stained by Coomassie Brilliant Blue G-250, and the images were recorded by a Gel Doc EZ Imager system (Bio-Rad Laboratories, Segrate, MI, Italy). On the first lane of each gel, Precision Plus Protein™ All Blue Standards mixture (10–250 kDa, Bio-Rad Laboratories) was loaded as molecular weight reference.

2.6. Protein In-Gel Digestion and LC-MS/MS Analysis. Selected protein bands of raw and roasted peanut were cut from polyacrylamide gel and subjected to in-gel protein digestion. Details about the digestion protocol were described elsewhere [23]. The obtained peptide pools were dried in a "Speed Vac" centrifuge and suspended in 80 μl of H_2O/ACN 90 : 10 + 0.1% formic acid (v/v).

The micro-HPLC-MS/MS analyses were performed on a UHPLC system, with an ESI interface and a linear ion trap dual pressure mass spectrometer (Velos Pro™, Thermo Fisher Scientific, San José, USA). 2 μl of tryptic digests were injected on an Acclaim PepMap100 column (15 × 1 mm, 3 μm, 100 Å, Thermo Fisher) at a flow rate of 60 μl/ml. A multistep gradient was applied for separation: solvent A from 85% to 45% in 40 min, then down to 10% in 2 min, kept stable for 10 min, and back to 85% in 2 min with a final 15 min long column conditioning (solvent A = H_2O + 0.1% formic acid; solvent B = CH_3CN : H_2O 80 : 20 (v/v) + 0.1% formic acid). MS system was run in *Nth order double play* (Data Dependent™ Acquisition, DDA) mode by activating the dynamic exclusion option. Full description of such analysis mode was detailed elsewhere [23, 24]. MS/MS spectra were processed via the commercial software Proteome Discoverer™ version 1.4 (Thermo Fisher Scientific, San José, US) and protein identification was achieved by Sequest searching algorithm against a customized database compiled by downloading from Swiss Prot DB the taxonomy code of *Arachis hypogaea* (ID: 3818, containing about 1200 sequences). The results were filtered according to specific constrains detailed elsewhere [23, 24], and only proteins identified by at least three unique peptides were taken into consideration for any further comment.

3. Results and Discussion

3.1. Buffer Extraction Efficiency for Raw and Roasted Peanuts and Hazelnuts. In the optimization of a reliable method for protein extraction, the main limitations for protein solubility are the structural and/or chemical modifications induced by thermal processing. In the present study, we investigated the suitability of four different buffers commonly employed for peanut and hazelnut samples for the effective extraction of proteins from both unprocessed and roasted nuts. The best extraction yield and the influence on the quality of protein electrophoretic profile were taken into account in the final evaluation.

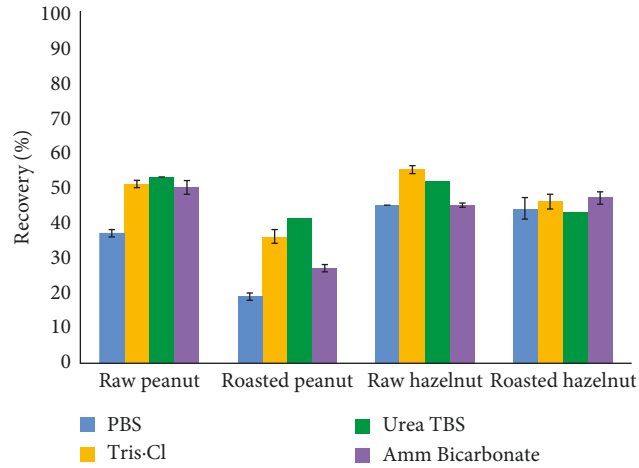

Figure 1: Comparison of extraction yields for raw and roasted peanut and hazelnut samples provided by different buffer compositions: PBS (10 mM phosphate buffer saline pH 7.4 + NaCl 0.15 M), Tris·Cl (20 mM Tris·Cl, pH 8.2), urea-TBS (7 M urea + 50 mM Tris·Cl, 150 mM NaCl pH 8 + 25 mM IAA); ammonium bicarbonate (0.1 M ammonium bicarbonate + NaCl 0.15 M).

The extraction yield of the different buffer was assessed by a commercial protein assay kit. An averaged value over three replicates for each sample was provided. Protein recovery was calculated by comparing the experimental protein concentration (mg/mL) assayed in nut extracts with the expected values estimated to be around 25% and 14%, for peanuts and hazelnuts, respectively, (USDA Branded Food Products Database) both for raw and roasted samples. Figure 1 reported the extraction yield obtained for each buffer investigated in all samples analyzed. Concerning peanuts, the extractive capacity of the different buffers strictly depended to the starting material, either raw or roasted. In raw peanuts, a similar extraction yield between Tris·Cl, urea-TBS, and ammonium bicarbonate buffers was observed (51%, 53%, and 50%, respectively). On the contrary, PBS showed the lowest recovery (37%). Interestingly, the extraction efficiency decreased when the same procedure was applied to the thermal-processed product. In this case, the values of protein recovery were 41%, 36%, 27%, and 19% for urea-TBS, Tris·Cl, ammonium bicarbonate, and PBS, respectively. This trend was in agreement with that reported by Chassagne et al., which optimized the extraction procedure of raw and processed peanut proteins from different varieties, using two sequential steps based on 50 mM Tris·Cl, 150 mM NaCl, pH 7.4 (first step), and ethanol/water 20 : 80 (v/v) (second step) buffers [25]. They showed that the total protein content of the different peanut varieties was comparable, but their extraction efficiency was variable. In addition, they reported that the mild sequential extraction method withdrew two distinct sets of proteins, increasing not only the amount of individual peanut proteins that can be extracted compared to the one-step method but also extending the total number of proteins extracted. Here, we observed that increasing the pH of Tris·Cl buffer to 8, higher protein recoveries were obtained, specifically 51% and 36% for raw and roasted peanuts, respectively. This evidence was

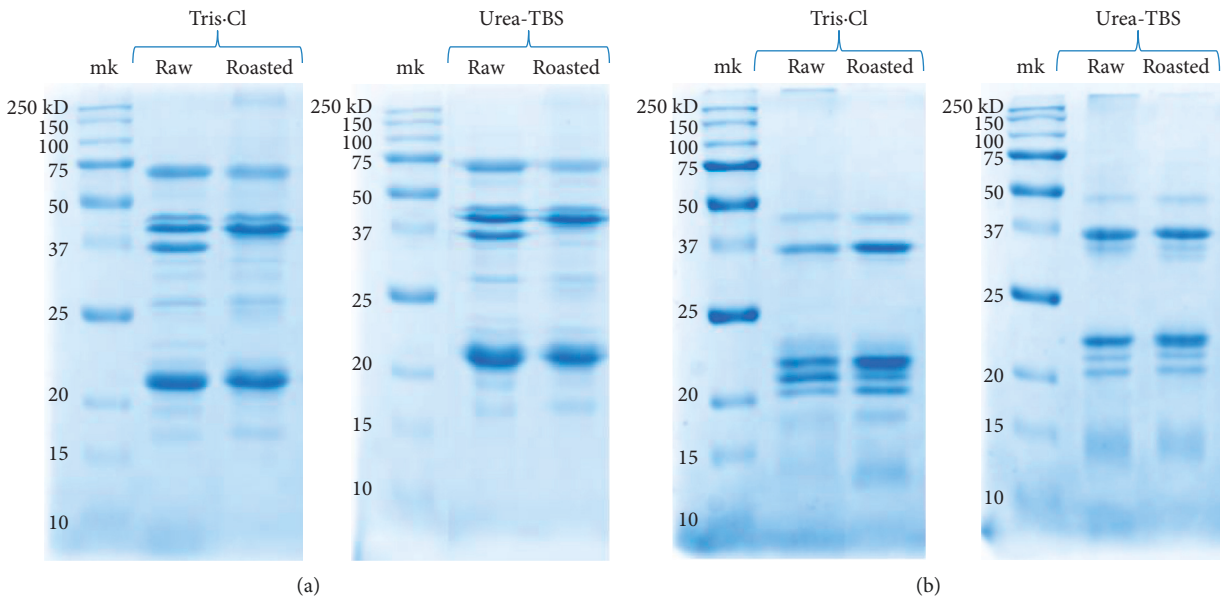

Figure 2: Typical electrophoretic profile of raw and roasted samples extracted with Tris·Cl and urea-TBS buffers (SDS-PAGE, 15% acrylamide gel). (a) Comparison of peanut protein profiles extracted with Tris·Cl and urea-TBS. (b) Comparison of hazelnut protein profiles extracted with Tris·Cl and urea-TBS. The first lane refers to protein markers (mk) for molecular weight reference.

also suggested by previous studies on processed peanuts, which confirmed that extraction solutions with pH ≥ 8.5 improved the protein recovery [18, 26, 27]. Besides the good observed results by Tris·Cl buffer for protein extraction of raw and treated peanuts, best recovery values were obtained with urea-TBS buffer. Hebling and co-workers previously demonstrated the efficacy of this buffer [28]. They compared PBS and urea-TBS buffer for the extraction of proteins from raw and roasted peanuts and reported that in roasted samples, urea-TBS buffer resulted in an increase of total protein extraction yield of approximately 250% compared to PBS. Likewise, we found that urea-TBS extraction yield was proportionally 200% higher than PBS, the latter producing the worst results for processed nuts.

Thermal processing of peanut samples resulted in the generation of insoluble protein aggregates, oligomers, and reduced protein solubility. The use of denaturant conditions and/or acid conditions in protein extraction could cause a displacement of protein complex, promoting an improved extraction also of thermal-treated proteins. It was proved that Ara h 1 detection was extremely high in the presence of urea and SDS, under acidic conditions. Moreover, a partial or total unraveling of the tertiary structure was detected, as showed by the presence of lower molecular weight bands in the 1D-gels and 1D-western blot analysis [29].

As for ammonium bicarbonate, this buffer composition was scarcely investigated for peanut protein extraction. However, the high recovery percentage found in raw material suggested that it could be effectively employed for this kind of material, whereas processed foods should be better handled in denaturant conditions for best protein extraction yield.

Figure 1 also shows the results obtained for raw and roasted hazelnuts. As general statement, smaller extent of variability in the total protein recovery was observed upon modification of buffer composition and extraction of raw or roasted hazelnut. The best results were obtained for Tris·Cl buffer which showed a recovery percentage of 55% in raw and 46% in roasted hazelnut material, whereas extraction yields of 52% and 43% for raw and processed hazelnuts, respectively, were calculated for urea-TBS solvents. Both the last two buffer compositions confirmed the decreased protein solubility caused by the thermal processing; however, the similarity of results obtained by working in either denaturant or nondenaturant conditions may suggest that hazelnut proteins could be less subjected to interproteins aggregation which would require harsher conditions for solubilization. Differently, the ammonium bicarbonate buffer provided lower recovery values with a high similarity of the extraction yields for raw and processed materials.

Besides total protein recovery, differential extraction of specific protein classes can occur as a function of the buffer composition and the processing of the food. Therefore, a qualitative evaluation of a protein electrophoretic profile in terms of band resolution and type of proteins extracted was carried out on both peanut and hazelnut samples. Specifically, extracts obtained from raw and roasted peanuts and hazelnuts by using Tris·Cl and urea-TBS buffers were analyzed.

Approximately 20 μg and 15 μg of protein were loaded in gel lanes for peanut and hazelnut extracts, respectively, and Figures 2(a) and 2(b) reported the protein patterns obtained through SDS-PAGE run. As illustrated in Figure 2(a), the peanut protein pattern did not change significantly between the two selected buffers Tris·Cl and urea-TBS for raw extracts, meaning that both buffers promoted the extraction of the same protein pool. Moreover, protein bands appeared resolved and defined, highlighting that the extraction buffer compositions did not affect the quality of SDS-PAGE run. Similar results were observed comparing protein profiles of Tris·Cl

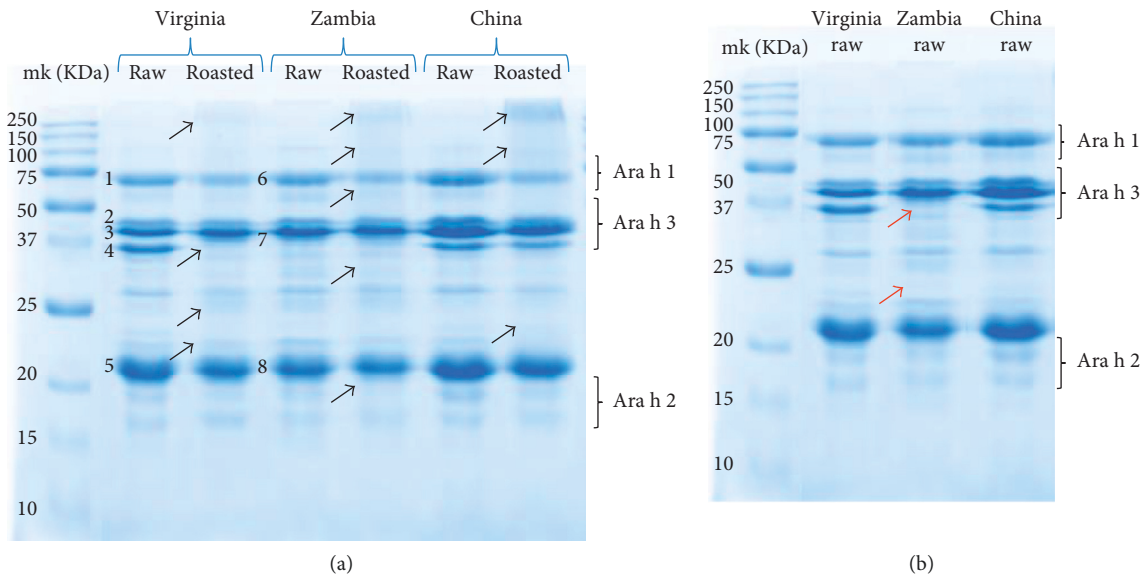

Figure 3: Typical electrophoretic profile of different peanut varieties (SDS-PAGE, 15% polyacrylamide gel). (a) Comparison of protein extracts from raw and roasted samples of three different varieties *Virginia, Zambia,* and *China*; the black arrows highlight the differences in protein profile induced by the roasting process for each variety; the bands marked with numbers (1–8) were subjected to LC-MS/MS analysis for protein identification. (b) Excerpt of the previous profile comparing profiles of raw peanut samples as a function of the variety (the red arrows show the main differences).

and urea-TBS extracts from roasted peanuts. Figure 2(b) reported SDS-PAGE profiles of hazelnut extracts; in this case, slight differences induced by the buffer composition were retrieved for both raw and roasted samples. The protein profile of Tris·Cl raw hazelnut extract presented two additional bands around 18 and 24 kDa molecular weight, which were missing in the corresponding lane of urea-TBS extract. Noteworthy, in the urea-TBS raw hazelnut extract, two additional bands of approximately 33–35 kDa molecular weight were highlighted that were weakly present in the Tris·Cl profile. Similar findings were observed for roasted hazelnut where the two bands of 18 and 24 kDa were observed only in the Tris·Cl profile and the two bands of approximately 33–35 kDa were present only in the urea-TBS extract. As for resolution, satisfactory band separation was achieved with both extraction buffers for unprocessed and processed materials, thus confirming what was already discussed for peanuts.

The results provided by the colorimetric assay on protein extracts and the electrophoretic profiling by SDS-PAGE confirmed that Tris·Cl and urea-TBS buffers could be similarly used for protein extraction from processed and unprocessed peanuts. On the contrary, hazelnut protein profiles obtained by gel electrophoresis demonstrated that the extracted protein composition was slightly different for the two buffers, although they showed similar total protein extraction yield. Therefore, since the aim of this study was to identify a single buffer capable of effectively extracting proteins simultaneously from both peanuts and hazelnuts, urea-TBS was the buffer selected for the following analysis on different varieties of peanuts and hazelnuts.

3.2. Varietal Diversity and Thermal Treatment Effects on Protein Profile. Food processing may deeply affect the protein structure depending on the intrinsic characteristics of the protein, the severity of the treatment applied, and the environment where it takes place. In particular, thermal processing has the potential to modify the structure, solubility, and immunochemical reactivity of allergen proteins, affecting the conformation, epitope antigenicity, and hydrophobicity [21, 30, 31].

In order to have more information about the influence of thermal treatment on peanut and hazelnut proteins, raw and roasted extracts were separated by using SDS-PAGE and the corresponding protein profiles compared. In addition, the investigation was widened to several peanut and hazelnut varieties in order to highlight the effects of natural genetic variability on the protein profile and its resistance to the thermal treatment applied. In particular, *Virginia*, *Zambia*, and *China* varieties were characterized as peanut samples and *Campana*, *Romana*, and *Georgia* varieties were investigated as hazelnut samples. Proteins were extracted by means of the previously selected urea-TBS buffer and separated by using SDS-PAGE. The same volumes of raw and roasted materials for each variety were loaded in the gel lanes, and a typical electrophoretic profile was reported in Figures 3 and 4 for peanut and hazelnut samples, respectively. Figure 3(a) showed the differences between banding patterns of peanut samples, and black arrows marked the modifications in the protein profile induced by thermal processing. Noteworthy, the profile of raw peanuts deeply changed after roasting, and this could be observed with different extent in all the three varieties analyzed. In particular, looking at the *Virginia* roasted peanut profile, the absence of the band of approximately 36 kDa, likely belonging to the Ara h 3 allergen group, and the band of about 21–23 kDa (Ara h 2 region) was noted together with the appearance of a weak band with molecular weight of

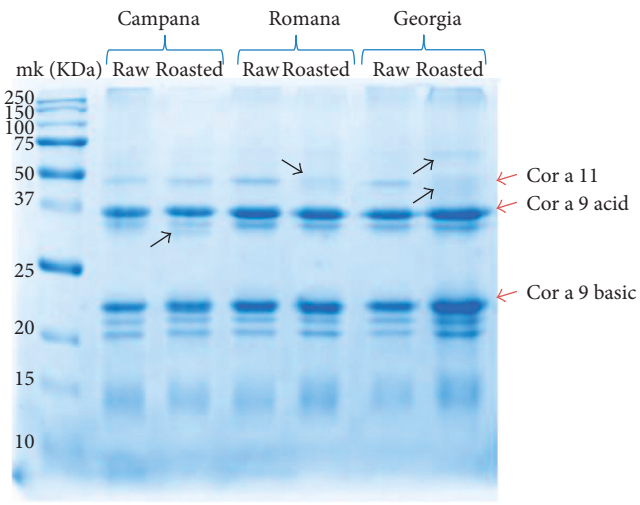

Figure 4: Typical electrophoretic profiles of raw and roasted samples of different hazelnut varieties obtained by SDS-PAGE electrophoresis on 15% polyacrylamide gel. The black arrows show the differences in protein profile induced by the roasting process for each variety.

approximately 38 kDa and two additional bands at 250 kDa and 25 kDa, respectively. As for *Zambia* variety, a new protein band in the 250 kDa region was detected for the roasted extract, whereas the loss of four bands was observed approximately at 100, 50, 30, and 20 kDa. Similarly, roasted peanut extracts from *China* variety showed an additional band at 250 kDa and the loss of three bands, one at 100 kDa and two in the range of 22–24 kDa. On the contrary, the protein banding at 36 kDa seemed to survive the roasting treatment, suggesting that in *China* variety this protein could be not susceptible to the heat treatment effect or that some protecting mechanism occurred during processing, differently from what was highlighted in *Virginia* variety. Noteworthy, for all varieties analyzed, roasting treatment induced the generation of the additional high molecular weight band (approximately 250 kDa), which was likely explained by the formation of protein complexes, confirming what was already extensively documented in the literature. Indeed, thermal processing caused the formation of new oligomeric conformations of peanut proteins, deeply influencing the SDS-PAGE migration [16, 17, 32–34]. Moreover, a substantial decrease in Ara h 1 and 2 detection by using ELISAs was also previously observed [35].

Focusing on the varietal diversity of the protein profile, an excerpt of Figure 3(a) was provided on panel B in order to appreciate the main differences due to the genetic polymorphism. *Virginia* and *China* varieties displayed a very similar protein profile, whereas *Zambia* variety showed three main differences, lacking the 36 kDa and the 23 kDa bands, whilst showing the additional band at approximately 38 kDa in molecular weight (see the red arrows in Figure 3(b)). No specific difference was noted for the three varieties in the regions of Ara h 1 and Ara h 2 proteins. These results suggested that the protein composition among peanut varieties could disclose significant differences by genetic polymorphism, and this could account for a natural variability in the final allergenicity of the specific variety. Similar findings were already demonstrated for tomato cultivars which promoted a differential clinical reactivity in allergic subjects, with less positive skin reactions and fewer symptoms after oral challenge [36].

In order to deepen the knowledge about the peanut protein composition, detailed LC-MS/MS experiments were carried out in order to identify the specific proteins of interest. To this aim, some selected protein bands from the *Virginia* electrophoretic pattern were in-gel digested by trypsin enzyme, and the resulting peptide pool was separated by using micro-HPLC and subjected to MS/MS analysis according to the experimental conditions detailed in Materials and Methods. Protein identification was achieved via commercial software by using Sequest HT searching algorithm against a customized database containing all the *Arachis hypogaea* accessions recorded in the Swiss Prot DB. Table 1 summarizes all the identified proteins and the relevant features provided by the Proteome Discoverer software for the validation of the identification provided such as protein score, coverage, and number of identified peptides. The digested bands labeled with progressive numbers in Figure 3 were mainly attributed to the major peanut allergens Ara h 1 and Ara h 3, and similar results were obtained for both raw and roasted peanuts. Specifically, proteins banding approximately at 75 kDa (band 1 and 6 of raw and roasted materials, resp.) were mainly attributed to Ara h 1 and Ara h 3, while protein bands ranging between 20 and 50 kDa (bands 2, 3, 4, and 5 from raw peanuts and 6, 7, and 8 of processed peanuts) were mainly assigned to Ara h 3 allergens. Some peptides belonging to Ara h 2 allergens were also found in band 5 from raw profile and the corresponding band 8 of roasted materials. It should be highlighted that due to the low resolution of SDS-PAGE technique, it was not possible to have a unique identification for each protein bands. Interestingly, protein band 1 from raw materials was almost exclusively assigned to Ara h 1 (as confirmed by the high number of specific peptides generated by tryptic digestion), whereas the corresponding band in processed peanuts, band 6, was a mixture of Ara h 1 and Ara h 3 (three proteins attributed to Ara h 1 and five proteins to Ara h 3); likely, the thermal processing induced the aggregation of Ara h 3 proteins to form a new high molecular weight complex of approximately 75 kDa. The 36 kDa band from raw materials that was found susceptible to thermal process (band 4) was attributed to Ara h 1 and Ara h 3. Similar composition was observed for the new protein band of approximately 38 kDa that appeared in roasted peanuts (band 7), highlighting that roasting could promote the aggregation of the aforementioned proteins generating new high molecular weight complexes. On the contrary, the bands 5 and 8 of raw and processed peanuts, respectively, resulted in similar composition (mainly Ara h 3 and Ara h 2), suggesting that these proteins were scarcely affected by the roasting process. Such results were in accordance with what was reported in previous studies [37, 38].

Finally, the investigation was focused on the effect of roasting on the protein profile of three different hazelnut varieties, namely, var. *Campana*, *Romana*, and *Georgia*. As

Table 1: Identification of protein bands from SDS-PAGE of raw and roasted peanut samples by LC-MS/MS analysis of tryptic peptides produced by in-gel protein digestion, coupled to commercial software-based protein identification.

	Band	Accession	Protein	Allergens	AAs	MW (kDa)	Score	Coverage	Number of peptides
Raw peanut	1	Q6PSU3	Conarachin (fragment)	Ara h 1	580	66.5	136.09	42.24	21
		P43238	Allergen Ara h 1, clone P41B	Ara h 1	626	71.3	124.07	36.42	19
		Q6PSU4	Conarachin (fragment)	Ara h 1	428	48.1	107.74	49.77	17
		Q9FZ11	Gly1	Ara h 3	529	60.4	9.53	7.56	3
		Q647H3	Arachin Ahy-2	Ara h 3	537	61.5	79.11	24.39	10
	2	Q9FZ11	Gly1	Ara h 3	529	60.4	42.03	22.12	8
		Q5I6T2	Arachin Ahy-4	Ara h 3	531	60.7	41.56	22.03	8
		B5TYU1	Arachin Arah3 isoform	Ara h 3	530	60.6	40.48	22.08	8
		A1DZF0	Arachin 6	Ara h 3	529	60.3	35.02	17.96	6
		Q6PSU6	Conarachin (fragment)	Ara h 1	303	34.1	13.49	14.19	3
		Q6PSU4	Conarachin (fragment)	Ara h 1	428	48.1	13.06	9.81	3
	3	Q9FZ11	Gly1	Ara h 3	529	60.4	125.79	38.19	13
		Q5I6T2	Arachin Ahy-4	Ara h 3	531	60.7	109.41	31.07	11
		A1DZF0	Arachin 6	Ara h 3	529	60.3	81.86	30.62	10
		Q647H3	Arachin Ahy-2	Ara h 3	537	61.5	80.69	26.63	8
		O82580	Glycinin (fragment)	Ara h 3	507	58.3	55.51	19.72	6
		Q6PSU4	Conarachin (fragment)	Ara h 1	428	48.1	14.62	14.02	4
		P43238	Allergen Ara h 1, clone P41B	Ara h 1	626	71.3	38.09	19.17	9
		Q6PSU3	Conarachin (fragment)	Ara h 1	580	66.5	37.94	20.52	9
	4	Q9FZ11	Gly1	Ara h 3	529	60.4	29.99	14.56	4
		Q647H4	Arachin Ahy-1	Ara h 3	536	61.5	29.42	14.37	4
		Q5I6T2	Arachin Ahy-4	Ara h 3	531	60.7	29.13	14.50	4
		A1DZF0	Arachin 6	Ara h 3	529	60.3	28.99	15.12	4
		A7LB59	Steroleosin-B		353	39.6	14.07	13.60	4
	5	A1DZF0	Arachin 6	Ara h 3	529	60.3	192.15	39.70	13
		Q647H4	Arachin Ahy-1	Ara h 3	536	61.5	175.31	41.42	15
		B5TYU1	Arachin Arah3 isoform	Ara h 3	530	60.6	170.96	42.45	13
		Q647H3	Arachin Ahy-2	Ara h 3	537	61.5	162.72	37.06	13
		Q9FZ11	Gly1	Ara h 3	529	60.4	152.40	39.13	14
		O82580	Glycinin (fragment)	Ara h 3	507	58.3	100.85	29.78	11
		Q6IWG5	Glycinin (fragment)	Ara h 3	510	58.0	81.33	24.90	7
		Q647H1	Conarachin		662	75.9	39.54	18.58	8
		Q6PSU2-4	Isoform 4 of Conglutin-7	Ara h 2	158	18.4	12.26	26.58	4
		Q647H2	Arachin Ahy-3	Ara h 3	484	54.5	11.27	8.06	3
Roasted peanut	6	P43238	Allergen Ara h 1, clone P41B	Ara h 1	626	71.3	204.92	43.77	25
		Q6PSU3	Conarachin (fragment)	Ara h 1	580	66.5	198.71	50.00	28
		Q6PSU4	Conarachin (fragment)	Ara h 1	428	48.1	171.83	58.41	22
		Q6PSU6	Conarachin (fragment)	Ara h 1	303	34.1	121.33	54.13	17
		Q9FZ11	Gly1	Ara h 3	529	60.4	63.33	45.18	14
		Q647H4	Arachin Ahy-1	Ara h 3	536	61.5	58.21	45.90	14
		A1DZF0	Arachin 6	Ara h 3	529	60.3	53.55	33.65	11
		Q5I6T2	Arachin Ahy-4	Ara h 3	531	60.7	52.19	37.29	12
		Q6IWG5	Glycinin (fragment)	Ara h 3	510	58.0	11.63	12.16	3
	7	Q9FZ11	Gly1	Ara h 3	529	60.4	127.85	42.34	18
		Q5I6T2	Arachin Ahy-4	Ara h 3	531	60.7	124.39	42.18	18
		Q647H4	Arachin Ahy-1	Ara h 3	536	61.5	112.15	37.69	15
		A1DZF0	Arachin 6	Ara h 3	529	60.3	100.26	38.94	15
		Q6PSU3	Conarachin (fragment)	Ara h 1	580	66.5	44.45	28.62	12
		P43238	Allergen Ara h 1, clone P41B	Ara h 1	626	71.3	39.35	24.44	10
		B5TYU1	Arachin Arah3 isoform	Ara h 3	530	60.6	189.34	40.00	13
		A1DZF0	Arachin 6	Ara h 3	529	60.3	177.93	42.91	14
		Q647H3	Arachin Ahy-2	Ara h 3	537	61.5	164.93	40.22	13
		Q5I6T2	Arachin Ahy-4	Ara h 3	531	60.7	159.65	36.53	13
	8	Q9FZ11	Gly1	Ara h 3	529	60.4	158.21	36.67	13
		Q647H4	Arachin Ahy-1	Ara h 3	536	61.5	136.54	42.35	14
		Q647H1	Conarachin	Ara h 3	662	75.9	60.25	15.26	7
		Q6IWG5	Glycinin (fragment)	Ara h 3	510	58.0	34.85	23.33	6
		Q6PSU2-4	Isoform 4 of Conglutin-7	Ara h 2	158	18.4	18.98	36.71	5

previously described for peanuts, proteins of raw and roasted hazelnut samples were extracted in urea-TBS buffer and then separated by using SDS-PAGE. Figure 4 showed typical electrophoretic profiles. As first overview, the banding profiles of raw and roasted extracts for each variety (Figure 4) were not dramatically affected by the thermal treatment, different from what was observed for peanuts. Specifically, *Campana* variety showed only an additional band with mass of approximately 33–35 kDa in the processed sample compared to the raw extract. On the contrary, in *Romana* and *Georgia* roasted extracts, the band at 50 kDa belonging to Cor a 11 allergen became weak compared to the relative raw sample [39]. Moreover, in *Georgia* roasted sample, a new band of approximately 73 kDa was highlighted, although with a weak signal. So far, only a few papers were reported on the evaluation of food processing effects on hazelnut proteins, and most of them focused on the fate of allergenic proteins upon thermal treatment. In particular, Cor a 1, Cor a 2, and Cor a 8 were proved to be thermolabile proteins [40–42], whereas Cor a 9 and Cor a 11 allergens were considered more thermostable [43–45]. In addition, Cor a 9 and Cor a 11, under particular processing condition, could generate Maillard reactions and advanced glycation end products [44, 45]. Our findings were in accordance with what was previously reported. After roasting, acid and basic subunits of Cor a 9 were conserved in all hazelnut varieties investigated. Indeed, intense bands that corresponded to allergen subunits were observed in each banding profile of roasted samples. On the contrary, like reported in the literature, Cor a 11 banding at 50 kDa showed a signal decrease upon roasting in both *Romana* and *Georgia* profiles, suggesting that it could have been subjected to chemical modification altering its banding profile.

Concerning the varietal diversity in protein composition, the three cultivars selected in the present investigation presented no significant differences in the electrophoretic profile. Finally, since only slight differences were recorded upon by thermal treatment, no in-depth investigation by micro-HPLC-MS/MS analysis was required for this set of samples.

4. Conclusions

In this study, we investigated the suitability of four different buffer compositions for the effective and simultaneous extraction of total protein content from raw and roasted peanuts and hazelnuts. For peanut samples, the best extraction efficiency was obtained with urea-TBS buffer, with percent recoveries of 53% and 41% for raw and roasted samples, respectively. Our findings confirmed that by working in denaturant conditions, the total protein content extracted increased, likely due to an improved solubilization of more hydrophobic proteins or oligomers/aggregates induced by the heat treatment. The same buffer provided satisfactory extraction yields also for raw and roasted hazelnut kernels with percent recoveries resulted to be 52% and 43%, respectively. Peanut and hazelnut protein extracts were characterized by using SDS-PAGE in order to investigate the effects of thermal treatment on protein structure and solubility. Electrophoretic profiles of raw and roasted peanuts were proved sensitive to the roasting process with marked differences highlighted in the number and intensity of specific bands. LC-MS/MS analysis confirmed the assignment of these protein bands to the major peanut allergens, Ara h 1 and Ara h 3. As for hazelnuts, only few differences were observed by comparing banding profiles of raw and roasted extracts, suggesting that probably very few chemical modifications triggered by thermal processing occurred. Finally, difference between three different varieties of peanuts and hazelnuts were evaluated by comparing the electrophoretic patterns of raw extracts. No difference was observed for hazelnuts, whereas peanut varieties resulted slightly different. In particular, *Zambia* variety lacked two bands of approximately 36 and 24 kDa, which were detected in both *Virginia* and *China* varieties. This finding is worthy to be further investigated since *Zambia* could represent a variety with lower allergenic potential.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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