

Effect of hot air and infrared roasting on hazelnut allergenicity

Cristina Lamberti^a, Stefano Nebbia^a, Sara Antoniazzi^a, Simona Cirrincione^{a,*}, Emilio Marengo^b, Marcello Manfredi^b, Denis Smorgon^c, Giovanna Monti^d, Antonella Faccio^e, Maria Gabriella Giuffrida^a, Raffaella Balestrini^e, Laura Cavallarin^a

^a Institute of the Science of Food Production – National Research Council, Largo Braccini 2, 10095 Grugliasco, TO, Italy

^b Center for Translational Research on Autoimmune and Allergic Disease – CAAD, University of Piemonte Orientale, Corso Trieste 15/A, 28100 Novara, Italy

^c Istituto Nazionale di Ricerca Metrologica, Strada delle Cacce 91, 10135 Torino, Italy

^d SC Pediatria, Regina Margherita Children's Hospital, Città della Scienza e della Salute, Piazza Polonia 94, 10126 Torino, Italy

^e Institute for Sustainable Plant Protection – National Research Council, Viale Mattioli 25, 10125 Torino, Italy

ARTICLE INFO

Keywords:

Corylus avellana
Hazelnut
Proteins
Allergens
Roasting

ABSTRACT

Roasting is known to affect the protein profile and allergenicity of hazelnuts (*Corylus avellana* cv TGL). The aim of the study was to investigate whether roasting techniques based on different heat transfer methods (hot air and infrared), differently affect the protein solubility and the IgE-binding capacities of both the soluble and insoluble hazelnut protein fractions. The immune-reactivity of the Cor a 9, Cor a 11 and Cor a 14 allergens resulted to be stable after roasting at 140 °C, for both types of treatment, while roasting at 170 °C caused a reduction in IgE-binding, which was particularly noticeable after infrared processing, that led to an almost complete disappearance of allergenicity. Microscopical analyses showed that roasting caused cytoplasmic network disruption, with a loss of lipid compartmentalization, as well as an alteration of the structure of the protein bodies and of the cell wall organization.

1. Introduction

The major hazelnut producing countries throughout the world are Turkey and Italy (Klockmann, Reiner, Bachmann, Hackl, & Fischer, 2016). Hazelnuts are marketed, both unshelled and shelled, as raw or roasted, and used as ingredients to obtain a wide variety of products, included baked goods, breakfast cereals, ice creams, various dessert formulations and chocolates (Alasalvar & Shahidi, 2008). Industrial thermal processing is applied to remove the kernel skin, to reduce the moisture and to develop a better aroma and taste (Marzocchi et al., 2017). Roasting also results in an extension of the shelf-life, thanks to the inactivation of enzymes and the eradication of undesirable micro-organisms (Özdemir et al., 2001). The control of the temperature and of the moisture distribution is crucial for the design of the thermal process, quality control, and for the choice of appropriate storage and handling practices. The most widely used nut roasting method is the convective heat transfer process, which is performed in a hot air (HA) oven,

working either in continuous mode or in batch systems (Perren & Escher, 2007). Another innovative possibility is infrared (IR) heating, which has been used successfully for the dry-roasting and pasteurization of almonds (Yang et al., 2010) and, more recently, for hazelnut roasting (Belviso et al., 2017). Unlike conventional heating mechanisms, in which heat is transferred from the surface to the interior, the main advantage of an IR treatment is that roasting proceeds from the inside of the hazelnut outward, without ventilation, which means that the loss of aroma is minimized (Rastogi, 2012). However, most of the effects of IR roasting on nut quality have only been partially studied. Belviso et al. (2017) established that a hot air system allowed roasted hazelnuts to be obtained with a lower rupture force and improved oxidative stability, compared to hazelnuts roasted with an IR system. Instead, Binello et al. (2018) found that an IR oven was better at preserving the antioxidant compound content of whole hazelnuts, than the other roasting systems. As far as pastes are concerned, hazelnuts treated in an IR oven at a high temperature showed higher viscosity and

Abbreviations: CBB, Coomassie Brilliant Blue; HA, hot air; HT, high temperature; I, water-insoluble protein; IR, infrared; LDS-PAGE, lithium dodecyl sulfate-PAGE; LT, low temperature; O, oil body enriched sample; OB, oil body; PAS, periodic acid-Schiff; PB, protein body; S, water-soluble protein; T, total protein sample

* Corresponding author.

E-mail addresses: cristina.lamberti@ispa.cnr.it (C. Lamberti), stefano.nebbia@ispa.cnr.it (S. Nebbia), sara.antoniazzi@ispa.cnr.it (S. Antoniazzi), simona.cirrincione@ispa.cnr.it (S. Cirrincione), emilio.marengo@uniupo.it (E. Marengo), marcello.manfredi@uniupo.it (M. Manfredi), d.smorgon@inrim.it (D. Smorgon), giovanna.monti@email.it (G. Monti), antonella.faccio@ipsp.cnr.it (A. Faccio), gabriella.giuffrida@ispa.cnr.it (M.G. Giuffrida), raffaella.balestrini@ipsp.cnr.it (R. Balestrini), laura.cavallarin@ispa.cnr.it (L. Cavallarin).

<https://doi.org/10.1016/j.foodchem.2020.128174>

Received 25 March 2020; Received in revised form 26 August 2020; Accepted 21 September 2020

0308-8146/© 2020 Published by Elsevier Ltd.

density, in addition to a stronger aroma.

At a molecular level, thermal treatments lead to changes in the carbohydrates, proteins, fats, vitamins and amino acids, according to the temperature and time settings. Fats can be oxidized, vitamins can be inactivated, oligosaccharides can be decomposed hydrolytically or caramelized, and proteins and amino acids can cross-link with other ingredients (Masthoff et al., 2013). Amino acids and carbohydrates can react, during the Maillard reaction, and give rise to Amadori compounds, (Göncüoğlu Taş & Gökmen, 2017). Moreover, proteins can be affected by denaturation and/or aggregation, thereby causing a reduction in solubility and altering immunochemical recognition by disrupting conformational IgE-binding epitopes or exposing new epitopes that had previously been hidden in the native protein. Downs et al. (2016), when studying the retained IgE reactivity of insoluble and soluble roasted walnut proteins, demonstrated that the more intense the thermal treatment is, the more relevant the decrease in protein solubility and the higher the loss in immunoreactivity. Many authors have tried to clarify the direct effect of thermal processing on food allergenicity, but a general consensus is still lacking (Vanga & Raghavan, 2017).

Tree nuts belong to the “big eight” food group (milk, soybean, crustacean shellfish, eggs, fish, tree nuts, wheat, peanuts) that include the foods responsible for almost 90% of human food allergies (CODEX STAN1, 1985). Moreover, hazelnuts are the third food to cause anaphylactic shock in children, after cow's milk and eggs and (Costa, Mafra, Carrapatoso, & Oliveira, 2016). The effect of roasting on hazelnut allergenicity is controversial. Hansen et al. (2003) demonstrated a reduction in immune-recognition after processing at 140 °C for 40 min, albeit without any clinical significance, as 29% of the subjects showed allergic symptoms upon consumption of roasted hazelnuts. Worm et al. (2009) reported that roasting hazelnuts at 144 °C (the time was not specified) resulted in a reduction in immunoreactivity, thus suggesting that roasting might lower the risk of an adverse reaction. However, we have recently demonstrated that oleosins maintain their immunoreactivity after roasting (Nebbia et al., unpublished results).

The objective of this study was to determine whether: (i) the use of different roasting techniques could result in different roasted hazelnuts protein profiles and solubility, and (ii) how these alterations could affect hazelnut allergenicity. To this aim, the capacity of children IgEs to bind both the soluble and insoluble hazelnut protein fractions was investigated before and after roasting with two different techniques: hot air (HA) and infrared (IR).

2. Material and methods

2.1. The thermal processing of hazelnuts

Raw hazelnut (*Corylus avellana* cv. Tonda Gentile delle Langhe, TGL), were obtained from experimental fields in Cortemilia (Cuneo, Italy) and a pilot-scale roasting experiment was set up at Brovind (Cortemilia, Italy). The moisture content of the raw hazelnuts was $3.56 \pm 0.107\%$. Two pilot-scale ovens, based on different technologies, were used: hot air (HA) and infrared (IR). Two different temperature conditions were chosen for each oven: a low temperature (140 °C, LT) and a high temperature (170 °C, HT). Each drying cycle in the HA oven was set at 25, 30 and 35 min for the LT, and at 10, 13 and 17 min for the HT. The cycle times of the IR oven were set at 10, 12 and 14 min for the LT, and at 6, 8 and 10 min for the HT. The temperature of the ovens was monitored by means of a set of calibrated Pt100 temperature sensors placed in strategic positions inside the thermal process volume of each oven. Aliquots of 1.5 and 4.0 Kg of hazelnuts were used for the HA and IR ovens, respectively, and three separate replicates of the roasting experiments were performed (experimental plan in Fig. 1, panel A). The moisture content of the raw and roasted hazelnuts was assessed immediately after each roasting cycle by means of an Infrared Moisture Analyser (Sartorius, Gottinga, Germany).

2.2. Extraction of soluble and insoluble hazelnut protein

Soluble and insoluble proteins were extracted from raw and roasted hazelnuts in triplicate. One gram of chopped hazelnuts was defatted three times with 10 ml of hexane; the hexane phase was removed, at each step, after 30 min of shaking in ice. The samples were dried for 30 min in a Speedvac concentrator (Concentrator 5301, Eppendorf AG, Hamburg, Germany) at room temperature (RT). The defatted hazelnut powder was re-suspended in 1.6 ml of 25 mM Na₂HPO₄, 1.5 M NaCl (pH 7.5), containing 1 tablet of protease inhibitors (Complete, Roche, Basel, Switzerland). After sonication (4 cycles of 10 sec ON and 10 sec OFF), the samples were shaken for one hour and then centrifuged at $21,460 \times g$ for 20 min at 4 °C. The supernatants were filtrated through a 45 µm filter and collected as water-Soluble (S) samples. The pellets were extract over night (O/N) with 0.8 ml of 7 M urea, 2 M thiourea, 20 mM Tris-HCl and pH 8.8 (Urea buffer). After centrifugation at $21,460 \times g$ for 20 min at 4 °C, the supernatants were filtrated through a 45 µm filter and collected as water-Insoluble (I) samples. The S and I samples were quantified by means of a Bradford assay (Biorad, Hercules, California).

2.3. Extraction of the oil body associated protein

The oil bodies associated proteins were extracted as described in Nebbia et al. (unpublished results). Briefly, samples of chopped raw and roasted chopped hazelnuts were sonicated in a grinding medium (GM 1: 0.6 M sucrose, 10 mM sodium phosphate, pH 9.5) and filtered using a two-layer gauze. The filtrated sample was centrifuged at $21,460 \times g$ for 20 min at 4 °C and the pad floating on the top was collected. The oil bodies contained in the floating pad were re-suspended in different buffers: GM2 (GM 1 with the addition of 0.1% Tween 20), GM 3 (GM 2 with the addition of 2 M NaCl) and Urea 9 M (pH 11). The oil bodies were centrifuged and collected at a final concentration of 100 mg/ml. In order to remove any oil residues, the samples were incubated with a 2.5 vol of diethyl ether, and precipitated with methanol/chloroform/water, as described by Wessel and Flügge (1984). The samples were quantified by means of a Bradford assay (Biorad).

2.4. Lithium dodecyl sulfate-PAGE

Lithium dodecyl sulfate-PAGE (LDS-PAGE) was performed using precast gels (NuPAGE 4–12% Bis-Tris, Invitrogen Life Technologies Ltd., Paisley, UK) in an XCell SureLock Mini-Cell System (Invitrogen), according to the manufacturer's instructions. Each sample was diluted in a NuPAGE LDS Sample Buffer (Invitrogen), under a non-reducing condition, and loaded in equal volumes. Gels were stained with Colloidal Coomassie Blue (Candiano et al., 2004) and scanned with a ChemiDoc MP System densitometer (Bio-Rad).

2.5. Hazelnut allergic patients

Sera from 16 hazelnut allergic pediatric patients were retrospectively collected from the Paediatric Allergy Unit at the Regina Margherita Childrens' Hospital of Turin (Città della Salute e della Scienza, Turin, Italy). Patients with a convincing clinical history of hazelnut allergy were selected. The collected sera were grouped into five pools, according to ImmunoCAP (ThermoFisher Scientific, Waltham, Massachusetts), considering the hazelnut major allergens (Tab.S1 repository):

- pool A, 4 patients (ID1 to 4) with a positive ImmunoCAP to Cor a 1, Cor a 8, Cor a 9 and Cor a 14;
- pool B, 1 patient (ID 5) with a positive ImmunoCAP to Cor a 8, Cor a 9 and Cor a 14;
- pool C, 8 patients (ID 6 to 13) with a positive ImmunoCAP to Cor a 9 and Cor a 14;

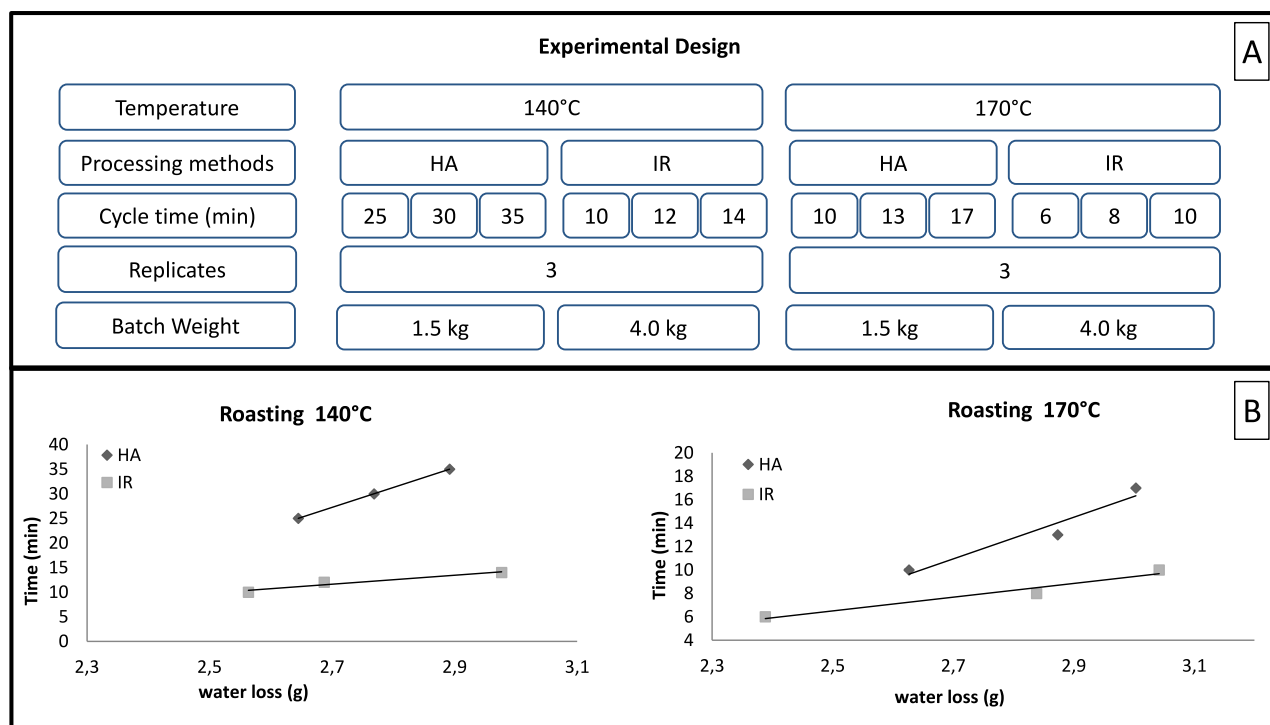


Fig. 1. Experimental design (A) and water loss correlation between Hot air (HA) and Infrared (IR) roasting, considering two different temperatures (140 °C and 170 °C) and 3 different processing times for each temperature (B).

- pool D, 3 patients (ID 14 to 16) with a positive ImmunoCAP to Cor a 8;
- pool E, all the patients (ID 1 to 16).

Three sera of non-nut sensitized non-allergic hazelnut consumers were pooled and used as a control. The study was reviewed and approved by the local ethics committee (approval no. 312 prot. no. 22050). All the families gave written informed consent before being enrolled in the study.

2.6. Immunoblotting analysis

The S and I hazelnut extracts were mixed (1:1) for the immunoblotting analysis. After LDS-PAGE, the protein bands were electrotransferred into Nitrocellulose Membranes (0.2 µm) with an XCell II Blot Module in a transfer buffer (Invitrogen) with 10% methanol (v/v). The membranes were blocked with TBS, containing 0.3% Tween 20, for 30 min and incubated O.N. at 4 °C with the patients' sera diluted 1:5 in the incubation buffer (TBS, 0.05% Tween 20, 0.05% vegetal gelatin). After incubation, the membranes were washed three times with a washing solution (TBS, 0.05% Tween 20) for 10 min, and incubated for 1 h at RT with an anti-Human IgE antibody (Sera Care Life Sciences Inc., Milford, Massachusetts) 1:5000 diluted in the incubation buffer. The membranes were washed three times and developed with an Alkaline Phosphatase Substrate Kit (Bio-Rad).

2.7. Mass spectrometry analysis

In gel digestion: bands were reduced in 10 mM DTT/50 mM NH_4HCO_3 for 45 min at 56 °C and alkylated in 55 mM IAA/50 mM NH_4HCO_3 for 30 min in the dark at RT. The bands were washed and destained three times with ACN 50%/50 mM NH_4HCO_3 , pure ACN and again with ACN 50%/50 mM NH_4HCO_3 . The samples were dried in a 5301 Eppendorf Concentrator (Eppendorf, Hamburg, Germany) and digested O/N. at 37 °C under shaking with modified porcine trypsin (Promega, Madison, Wisconsin) at 75 ng/µl of 25 mM NH_4HCO_3 /10%

formic acid.

ESI-Q-TOF: the peptide mixtures were desalted on a Discovery® DSC-18 solid phase extraction (SPE) 96-well Plate (25 mg/well) (Sigma-Aldrich Inc., St. Louis, MO) prior to the mass spectrometry analysis. The LC-MS/MS analyses were performed using a micro-LC system Eksigent Technologies (Dublin, California). The stationary phase was a Halo Fused C18 column (0.5 × 100 mm, 2.7 µm; Eksigent Technologies). The mobile phase was a mixture of 0.1% (v/v) formic acid in water (A) and 0.1% (v/v) formic acid in acetonitrile (B), eluting at a flow-rate of 15.0 µL/min and at an increasing concentration of solvent B, from 2% to 40%, over 30 min. The injection volume was 4.0 µL. The oven temperature was set at 40 °C. The LC system was interfaced with a 5600 + TripleTOF™ system (AB Sciex, Concord, Canada), equipped with a DuoSpray™ Ion Source and CDS (Calibrant Delivery System). The mass spectrometer worked in data dependent acquisition mode (DDA) (Cvijetic et al., 2017; Martinotti et al., 2016).

Protein database search: the DDA files were searched for, using Mascot v. 2.4 (Matrix Science Inc., Boston), in the UniProt *Corylus avellana* database. The following parameters were set for the search: an S-carbamidomethyl derivative on cysteine as a fixed modification, oxidation on methionine, Acetyl (N-term), Met-loss (Protein N-term M), as variable modifications and three missed cleavage sites for trypsin digestion. Peptide mass tolerance was set at 50 ppm and MS/MS tolerance at 0.1 Da. Only proteins with at least three peptides and with a peptide score > the peptide identity were considered for identification purposes. The list of the identified proteins is available as Table S2 (repository).

2.8. Microscopical analyses

Small pieces of hazelnut (Fig. S1) were fixed in 2.5% (v/v) glutaraldehyde in a 10 mM phosphate buffer (PB), pH 7.2, O.N. at 4 °C, rinsed in the same buffer and then post fixed in 1% OsO_4 in PB for 1 h, at RT. After rinsing in PB, the pieces were dehydrated in an ethanol series (30, 50, 70, 90 and 100%; 10 min each step) at room temperature. The samples were infiltrated in 2:1 (v/v) ethanol/London Resin White (EMS,

PA _ USA) for 1 h, 1:2 (v/v) ethanol/LRW for 2 h, and 100% LRW overnight at 4 °C, according to Moore, Swords, Lynch, and Staehelin (1991). Semi-thin sections (1 µm) were stained with 1% toluidine blue to check the quality of the sample, which was established using optical microscopy. Based on these observations, ultra-thin sections (70 nm) were then cut and stained with Uranyl Acetate Substituted (Agar Scientific, Stansted UK) and lead citrate before observation with a Philips CM10 transmission electron microscope.

2.9. Histochemistry

Carbohydrate staining with a periodic acid Schiff reagent (PAS) treatment: semi-thin sections (1 µm), obtained from the LR White embedded samples, were dipped into 1% (w/v) periodic acid for 30 min, rinsed in water for 5 min and stained in a Schiff reagent for 10 min, in the dark, and then were again rinsed in water for 10 min.

Protein staining with Coomassie Brilliant Blue (CBB): semi-thin sections (1 µm) were stained at RT for 30 min in a 0.25% (w/v) Coomassie Brilliant Blue R-250 dye in a methanol: acetic acid: water solution (MAW, 5:1:4). Semi-thin sections were rinsed in the MAW solution for 15 min at RT and then rinsed in water for 10 min.

Lipid staining with Nile Red: hazelnut sections (25 µm) were prepared using a cryostat device (Leica). The sections were incubated with 1 mg/ml of Nile Red in acetone and 1:100 diluted in PBS for 10 min at RT in the dark. The sections were mounted in 50% of glycerol. Observations were carried out using a confocal laser-scanning microscope (Leica TCS SP2). The specific yellow-gold fluorescence of lipids was measured at 488/550 nm.

3. Results and discussion

Although processing methods generally seem promising to reduce IgE reactivity toward tree nuts proteins, it is still uncertain whether roasting aggravates or mitigates hazelnut allergenicity (Vanga & Raghavan, 2017). In this scenario, we hypothesized that roasting may result in different changes in the structure of hazelnut proteins, which not only depend on the temperature level and cycle duration (time/temperature conditions), but also on the heat application method. We then designed an experiment aimed at obtaining roasted hazelnuts with similar moisture contents, by applying either HA or IR roasting, characterized by different heat transfer methods. The final objective of the study was to obtain new evidences on the effects of roasting on hazelnut allergens and to assess the implications pertaining to the detection of hazelnut allergens and the tolerance induction of hazelnut-allergic subjects. The experiments were designed to compare how two different roasting techniques (with temperature/time condition established in preliminary experiments, Fig. 1, panel A) can affect hazelnut protein solubility and allergenicity. A low temperature (LT, 140 °C) protocol was set up to simulate the production of soft aroma and light-colored nuts, while a high temperature (HT, 170 °C) protocol was set up to simulate the production of roasted hazelnut pastes with a strong flavor, color and texture. In order to reach the same final dry matter level, a longer roasting time was needed, using the same process temperature (Fig. 1 panel B). The two combinations of time and temperature that ensured comparable end products, in terms of residual moisture, between the two ovens were: 140 °C for 25 min (HA-LT) and 140 °C for 12 min (IR-LT); 170 °C for 17 min (HA-HT) and 170 °C for 10 min (IR-HT). These four experimental conditions (HA-LT, IR-LT, HA-HT and IR-HT) were considered for all the further protein aggregation, solubility and allergenicity determinations.

We observed a decrease in protein solubility for an increasing roasting temperature, for each oven, as already reported for peanuts (Kopper et al., 2005) and walnuts (Downs et al., 2016) (Fig. 2 panel A). Roasting at 140 °C, in particular for HA processing, resulted in similar water-soluble protein (S) and water-insoluble protein (I) profiles to those of raw hazelnuts. The S/I ratio in the samples processed at 170 °C,

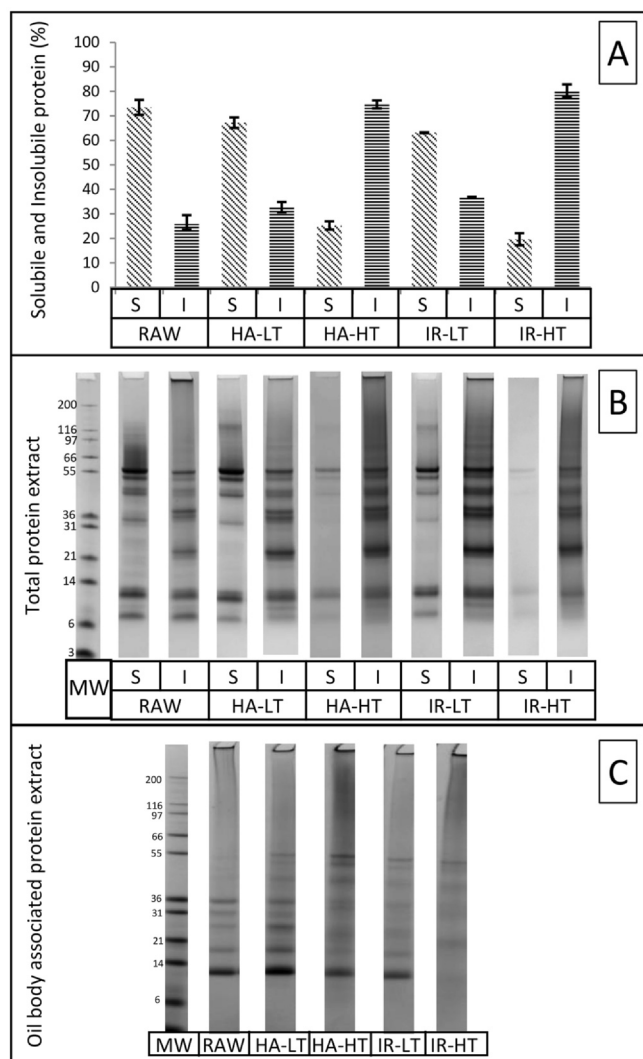


Fig. 2. Output of the hazelnut protein extraction. Protein quantification, by means of a Bradford assay, of RAW, HA-LT (Hot Air–Low Temperature), HA-HT (Hot Air–High Temperature), IR-LT (Infrared–Low Temperature) and IR-HT (Infrared–High Temperature) samples. The histograms represent the percentage of Soluble (S) and Insoluble (I) proteins in relation to the total amount of proteins (panel A). LDS-PAGE of the S and I fractions for each condition (panel B). LDS-PAGE of the OB (oil body) associated protein fractions for each condition (panel C). MW: molecular weight.

with both techniques, was reverted. The water-soluble protein component in the raw hazelnuts, and in the hazelnuts processed at 140 °C accounted for 63–73% of the total protein content, for both systems, while the water-soluble proteins in the samples treated at 170 °C represented only 19–26% (Fig. 2 panel A), regardless of the type of oven.

LDS-PAGE was performed on each sample under non-reducing conditions, in order to highlight any possible protein aggregation phenomena. The same amount of hazelnuts was extracted in order to point out any differences in the protein concentrations and the distribution between the S and I fractions, and between the different considered processing protocols. The protein profile of the HA hazelnuts processed at 140 °C showed the closest protein profile to raw samples, for both the S and I fractions (Fig. 2 panel B). Instead, the most severe treatment, in terms of protein shift from water-soluble to water-insoluble fraction (Fig. 2 panel A), was IR processing at 170 °C. The disappearance of the protein bands in the S lane, for HA-HT and for both IR treatments, was balanced by the appearance of some smearing at a high molecular weight (MW) in I lane, which probably indicates the

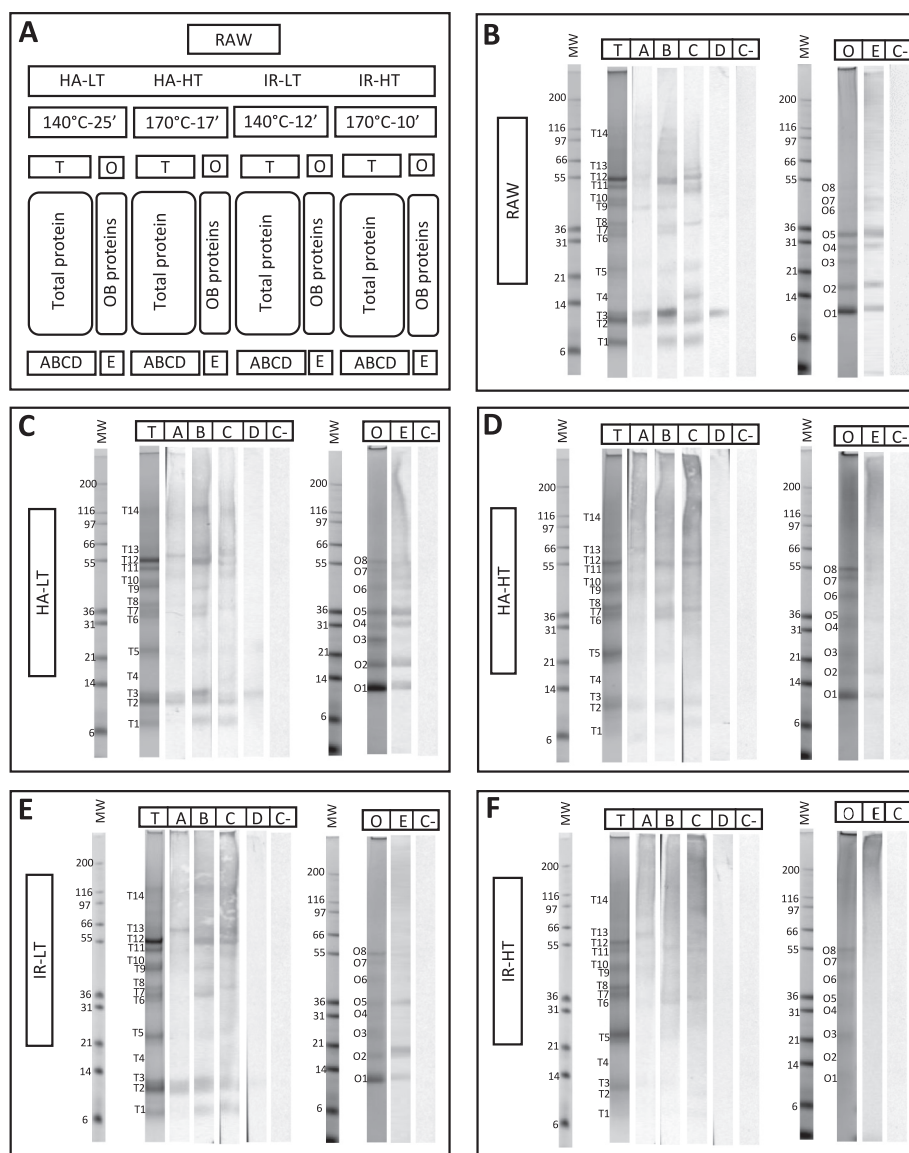


Fig. 3. Design of the protein extract analysis (panel A): the RAW (panel B), HA-LT (panel C), HA-HT (panel D), IR-LT (panel E) and IR-HT (panel F) samples considered for the LDS-PAGE and immunoblotting analysis. T: Total protein extract (soluble and insoluble proteins mixed 1:1); O: oil bodies associated protein extract; A, B, C, D: allergic patient pools characterized by different ImmunoCAP patterns (patients with positive IgE ImmunoCAP to Cor a 1, 8, 9 and 14 (A), to Cor a 8, 9 and 14 (B), to Cor a 9 and 14 (C), Cor a 8 (D); see Table S1 Repository) Pool E: patient pool pertaining to all the patients in the study. From T1 to T14: protein bands from total protein extracts (T) identified by means of LC-MS/MS. From O1 to O8: protein bands from oil bodies associated protein extracts (O); identified by means of LC-MS/MS. HA: Hot air; IR: Infrared; LT: Low Temperature; HT: High Temperature; MW: molecular weight.

presence of aggregated, insoluble protein complexes (Fig. 2 panel B), as already reported for peanuts by Schmidt et al. (2010) and for walnuts by Downs et al. (2016). The oil body protein extracts were also affected by the heat treatment, as previously reported (Nebbia et al., unpublished results). Unlike the total protein extract profile, the number of protein bands in the OB associated protein extract increased after roasting. Three new bands, which were barely visible in the raw samples, were detected between 45 and 60 kDa (Fig. 2, panel C). IR processing at 170 °C resulted in the appearance of protein smearing, without any detectable singular bands (Fig. 2, Panel C).

The changes in immune-reactivity induced in hazelnuts after processing were then investigated by conducting immunoblotting experiments with the sera of hazelnut pediatric allergic patients. A sample containing both soluble and insoluble proteins (total protein sample, T, obtained mixing the S and I fractions 1:1) and a sample containing the oil body enriched protein fraction (sample O) were incubated with serum pools of allergic patients for each processing condition (Fig. 3, panel A). The T samples were incubated with 4 pools of serum from allergic patients (A, B, C and D), while the O samples were incubated with a single pool (E) containing the sera of all the patients included in the study (Fig. 3 panel A, Table S1). Our experiment confirmed the findings pertaining to walnuts and peanuts (Downs et al., 2016; Kopper et al., 2005), regarding the effect of temperature on antigen-antibody

binding. It was found, for the total protein sample, that the higher the processing temperature was, the lower the overall intensity of the protein bands, and, therefore, the lower the immunoreactivity. As far as the OB enriched proteins are concerned, their immune-reactivity appeared to be affected more by the IR treatment than by the HA treatment, at both of the considered temperatures (Fig. 3).

The raw sample was the most immunoreactive of both the T and O protein samples (Fig. 3 panel B). Both of the processing methods performed at LT resulted in very similar immunoreactive protein patterns (Fig. 3 panel C and E), but these patterns were less intense than those of the raw samples (Fig. 3 panel B). The proteins from hazelnuts processed at 170 °C, although less immune-reactive, conserved a detectable reactivity, especially in HA in the 30 to 65 kDa molecular mass range (Fig. 3 panel D). Only the IR treatment at 170 °C resulted in an almost complete disappearance of immunoreactivity (Fig. 3 panel F). This evidence partially confirms what Masthoff et al. (2013) reported, and adds novel information about the times, temperatures and technology settings that allow a reduction in hazelnut immunoreactivity to be achieved. To the best of our knowledge, this is the first experiment that has made a direct comparison of the effects of different roasting conditions on the allergenicity of the same original raw hazelnut sample.

An important issue that should be taken into account is that different allergens show different responses to heat processing. The extent

Table 1

Protein identification of the total protein (T) and OB associated protein (O) extracts by means of LC-MS/MS. MW: molecular weight.

N° band	Entry	Name	MW experimental/MW theoretical	Protein Score	N° of matching peptides	Protein coverage (%)	Molar fraction (%)
T1	AHA36627.1	Cor a 9	8000/59200	465	7	20.4	100
T2	AHA36627.1	Cor a 9	10000/59200	702	8	26.7	32.6
	ACO56333.1	Cor a 14	100000/12600	173	4	23.1	67.3
T3	AHA36627.1	Cor a 9	11000/59200	297	4	8.9	20.60
	4XUW_A	Cor a 8	11000/9926	84	2	29.3	79.4
T4	AHA36627.1	Cor a 9	15000/59200	411	6	19.3	100
T5	AHA36627.1	Cor a 9	22000/59200	4667	14	32.5	95.62
	AAL86739.1	Cor a 11	22000/51110	69	2	11.6	4.38
T6	AHA36627.1	Cor a 9	34000/59200	1248	10	22	100
T7	AHA36627.1	Cor a 9	36000/59200	3242	18	44.4	100
T8	AHA36627.1	Cor a 9	38000/59200	1251	12	37.2	100
T9	AHA36627.1	Cor a 9	45000/59200	2684	13	31.9	66.17
	AAL86739.1	Cor a 11	45000/51110	842	10	27.5	33.83
T10	AHA36627.1	Cor a 9	47000/59200	2871	12	26.8	49.25
	AAL86739.1	Cor a 11	47000/51110	1984	15	35.5	50.75
T11	AHA36627.1	Cor a 9	50000/59200	2490	14	42.2	80.54
	AAL86739.1	Cor a 11	50000/51110	570	7	18.8	19.46
T12	AHA36627.1	Cor a 9	55000/59200	8359	22	56.4	95.17
	AAL86739.1	Cor a 11	55000/51110	319	6	15	4.83
T13	AHA36627.1	Cor a 9	60000/59200	4154	18	47.9	85.48
	AAL86739.1	Cor a 11	60000/51110	310	7	18.8	14.52
T14	AHA36627.1	Cor a 9	130000/59200	2429	14	37	79.6
	AAL86739.1	Cor a 11	130000/51110	623	7	19	20.40
<i>Oil bodies associated proteins (O)</i>							
O1	AAO65960.1	Cor a 13	12000/14723	2822	4	27.9	55.06
	AAO67349.2	Cor a 12	12000/16745	441	3	20.8	44.94
O2	MK737923	Cor a 15	17000/17741	935	7	42.6	70.27
	AAO67349.2	Cor a 12	17000/16745	172	2	23.3	20.71
	AAO65960.1	Cor a 13	17000/14723	179	2	17.9	9.02
O3	MK962827	Caleosin	27000/26912	1446	10	50.6	55.31
	AAO65960.1	Cor a 13	27000/14723	561	3	26.4	10.87
	AAO67349.2	Cor a 12	27000/16745	515	4	25.8	28.47
	AHA36627.1	Cor a 9	27000/59200	481	7	17.1	5.35
O4	AAO67349.2	Cor a 12	31000/16745	531	4	25.8	54.6
	MK737923	Cor a 15	31000/17741	175	3	32	24.6
	AAO65960.1	Cor a 13	31000/14723	114	3	26.4	20.8
O5	MK737923	Cor a 15	36000/17741	704	6	42.6	78.02
	AHA36627.1	Cor a 9	36000/59200	315	6	15.6	10.07
	AAO67349.2	Cor a 12	36000/16745	142	2	12.6	11.90
O6	AHA36627.1	Cor a 9	45000/59200	1843	9	20.2	44.02
	AAL86739.1	Cor a 11	45000/51110	174	5	13.2	24.88
	AAO67349.2	Cor a 12	45000/16745	90	2	12.6	31.10
O7	AHA36627.1	Cor a 9	50000/59200	765	8	17.7	42.86
	AAL86739.1	Cor a 11	50000/51110	166	4	8	21.69
	AAO67349.2	Cor a 12	50000/16745	60	2	15.1	35.45
O8	AHA36627.1	Cor a 9	55000/59200	2701	12	28.6	77.84
	AAL86739.1	Cor a 11	55000/51110	145	4	10.3	22.16

of the reduction of single immunoreactivity bands, caused by processing, depends on which allergen is present in the band and whether it is thermo-sensitive or not (Costa et al., 2016). Currently, eleven allergens from hazelnuts are reported in the WHO/IUIS Allergen Nomenclature Sub-committee database. Eight of them have been demonstrated to be involved in food allergies (Cor a 2, Cor a 8, Cor a 9, Cor a 11, Cor a 12, Cor a 13, Cor a 14, Cor a 15).

We found that Cor a 9 and Cor a 11, when subjected to electrophoretic separation in a non-reducing condition, were distributed in different subunits, ranging in mass from 10 to 65 kDa (Table 1), as already demonstrated by Rigby et al. (2008). Cor a 9 was identified in all the considered bands, either alone or in association with Cor a 11, Cor a 14 or Cor a 8 (Fig. 3, Table 1). Cor a 9 is a seed storage globulin that belongs to the cupin superfamily, and it is formed by an alkaline and an acidic chain. The polypeptides observed for a low MW mass (from 10 kDa to 40 kDa) are probably highly proteolyzed Cor a 9 subunits, as already pointed out by Rigby et al. (2008).

Cor a 11, a vicillin-like protein, is a glycosylated storage globulin, which also belongs to the cupin superfamily. According to the observed MW, a mature Cor a 11 subunit is likely to be contained in bands T9 and T10 (around 48 kDa), while the smaller polypeptide around 25 kDa

(band T5) could correspond to the subunit that is proteolyzed in the seed. The higher MW bands (from T11 to T14) probably contain the glycosylated form of unprocessed subunits (Rigby et al., 2008).

Cor a 14 (2S-albumin) is a small globular protein that is characterized by a 4 disulfide bonds, which belongs to the prolamin superfamily. It was identified in the T2 band with Cor a 9, and, according to the molar fraction data (Table 1), it contributed by 67% to the band.

In our experiment, Cor a 9, Cor a 11 and Cor a 14 immunoreactivity overall resulted to be stable after roasting at 140 °C, for both treatment types, while roasting at 170 °C caused a reduction in IgE binding, which was particularly noticeable after IR processing, that led to an almost complete disappearance of reactivity (Fig. 3). Cor a 9, Cor a 11 and Cor a 14 have already been reported as being stable after roasting treatments, although the roasting conditions in the different studies were not comparable (De Leon et al., 2003; Dooper et al., 2008; Müller et al., 2000; Pastorello et al., 2002; Pfeifer et al., 2015; Schocker et al., 2000; Wigotzki, Steinhart, & Paschke, 2000). In particular, it has been demonstrated that the beta-barrel-motif of Cor a 9 plays a key role in the retention of stability after thermal treatment as well as during digestion (Moreno & Clemente, 2008).

Cor a 8 is a non-specific lipid transfer protein. We found that Cor a

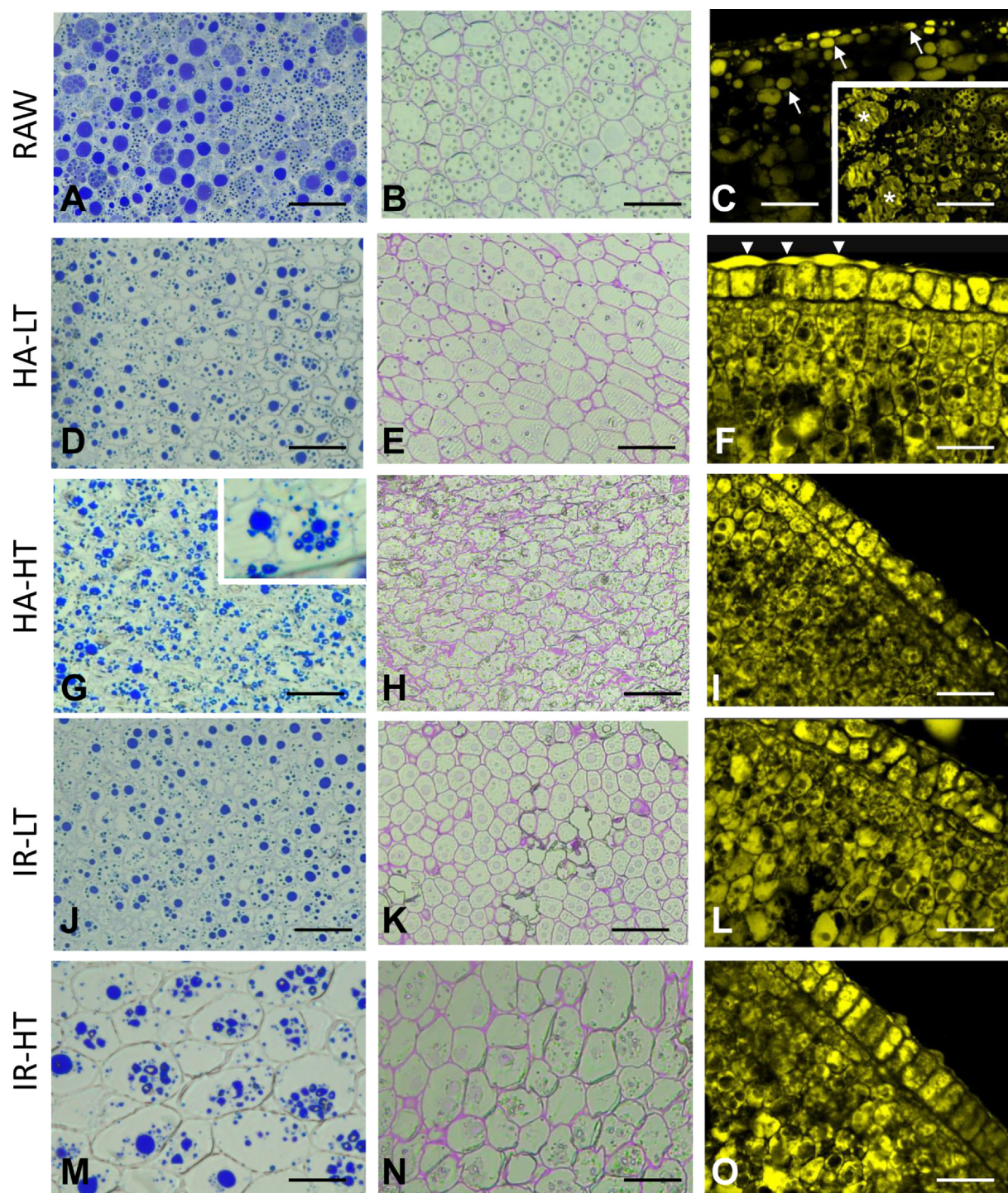


Fig. 4. Microscopical observations of raw and roasted (HA-LT, HA-HT, IR-LT and IR-HT) hazelnut seeds. (A-D-G-J-M) Coomassie Brilliant blue (CBB) staining of the total proteins; details of the protein body modifications after roasting are shown in the inset. (B-E-H-K-N) PAS staining. The cell walls appear to be stained pink. (C-F-I-L-O) Nile red staining. Localization of the oil bodies (in yellow) by means of confocal laser scanning microscopy; details of a specific lipid-rich region of the hazelnut seeds, marked with asterisks, are shown in the inset; oil bodies are indicated with arrows. Bars = 80 μ m in A, B, C, D, E, G, H, I, J, K and 50 μ m in F, L, M, O. HA: Hot air; IR: Infrared; LT: Low Temperature; HT: High Temperature.

8, in association with Cor a 9, albeit only in band T3, accounted for 80% of the band, according to the molar fraction. By incubating the extract with the sera of patients with IgEs directed only toward Cor a 8 (Pool D), it was possible to highlight the changes in immunoreactivity of Cor a 8 after thermal processing (Fig. 3, lane D). Roasting at 140 °C, with both methods, caused a reduction in IgEs binding to Cor a 8, although to a greater extent in the IR treated samples (Fig. 3 panel E). The immunoreactivity of Cor a 8 was lost at 170 °C, for both conditions (Fig. 3

panel D and F). This observation is in agreement with the results of López et al. (2012), who demonstrated that IgE binding to Cor a 8 was affected to a great extent by high temperatures and wet processing (121 °C and 138 °C in an autoclave, for 15 and 30 min, respectively).

As for the OB associated proteins, we observed that immunoreactivity was increased after HA roasting at 140 °C, due to the additional immunoreactivity of newly generated high MW bands, in comparison to raw samples (Fig. 3, panel C). Instead, immunoreactivity

was reduced in the remaining treatments, following a IR-LT > HA-HT > IR-HT trend, until it completely disappeared.

The results demonstrate that hazelnut proteins IR processed at 170 °C almost completely lost their immunoreactivity, as far as both thermo-labile and thermostable allergens are concerned. Unlike HA roasting, IR roasting results in higher rate of heat transfer taking place from the core to the surface of the nut. IR radiation is an electromagnetic radiation transmitted as a wave and converted into heat, when it impinges on the food surface. It is known that exposure of food to electromagnetic radiation results in changes in electronic, vibrational and rotational states of molecules, including proteins (Rastogi, 2012). We, thus, speculate that the observed difference in immunoreactivity between IR and HA roasted hazelnuts, that were comparable in term of residual moisture, relies on effects of the different direction of heat transfer between the two technologies and on the physical changes induced by IR at protein molecular level.

Microscopical observations were then performed with the aim of verifying the impact of roasting on the internal organization of the cells in hazelnut seeds. CBB, PAS and Nile red stains were used to study the distribution of proteins, polysaccharides and storage lipids, respectively. After staining with CBB, protein bodies of different sizes were clearly evident inside the cells of mature seeds (Fig. 4A). These protein bodies have a high number of protein inclusions, varying in size and shape, which appeared as white dots after CBB staining. As reported by Dourado et al. (2003), these inclusions may consist of crystal globoids, protein crystalloids or calcium oxalate crystals. Broad and faint polysaccharide cytoplasmic staining was found after using PAS, while more intense staining was found in the cell walls (Fig. 4B), in agreement with the nature of this structure (Cosgrove, 2005). The same PAS staining pattern was also previously described in Chilean hazelnuts (*Gevuina avellana*) (Dourado et al., 2003). The neutral lipids stored in the OBs of oil seeds are mobilized to provide the carbon skeletons and energy necessary for their post-germinative growth (Serrano, Suárez, Olmedilla, Rapoport, & Rodríguez-García, 2008). Here, Nile Red staining shaped the OBs in raw hazelnuts (Fig. 4C), as already reported for olive cotyledons (Serrano et al., 2008). The same staining treatment led to a signal on structures that only fill the cell in specific regions of the hazelnut seed, confirming their lipidic nature (Fig. 4, inset). Roasting has already been reported to lead to changes in the microstructure of hazelnuts (Saklar, Ungan, & Katnas, 2003). These authors reported that changes in the microstructure of hazelnuts during a roasting process develop gradually for increasing of air temperatures, air velocities and roasting times (Saklar et al., 2003). Considering the roasted samples (Fig. 4D-O), CBB staining resulted in a major impact on the protein bodies after treatment with HA-HT and IR-HT. The protein bodies in these samples appeared to have partially lost their original structure, with respect to raw hazelnuts (Fig. 4G and M). The changes observed in the protein bodies are in agreement with those observed by Saklar et al. (2003), who reported the presence of swollen, granular and aggregated protein bodies in roasted hazelnuts (165 °C, 1 m/s, 25 min). As shown by the staining with PAS, major difference between the shape of the cell wall was evident after LT and HT (straight vs wavy), mainly for the HA process (Fig. 4E and H, respectively). Regarding lipid localization, the Nile Red staining is distributed throughout the cells, thus showing that lipids fill up most of the cell cytoplasm. They seemed to be mixed with the protein fraction, with a loss in the compartmentalization of the oil in OBs (Fig. 4F, I, L and O). This is in agreement with a partial damage of the sub-cellular organization, due to cytoplasmic network disruption, already reported during roasting (Saklar et al., 2003). Lipid vesicles attached to the external cell wall of the epidermis were also visible, in particular after the HA-LT treatment (Fig. 4F). Protein bodies (PBs) and OBs were observed, at an electron microscopy level, to occupy most of the cytoplasm of mature seeds, and OBs were found to be directly surrounding the PBs (Fig. 5A and B). After roasting, regardless of the type of oven, a loss of sub-cellular organization was evident, with the disappearance of the OBs (Fig. 5C and F); as a result of

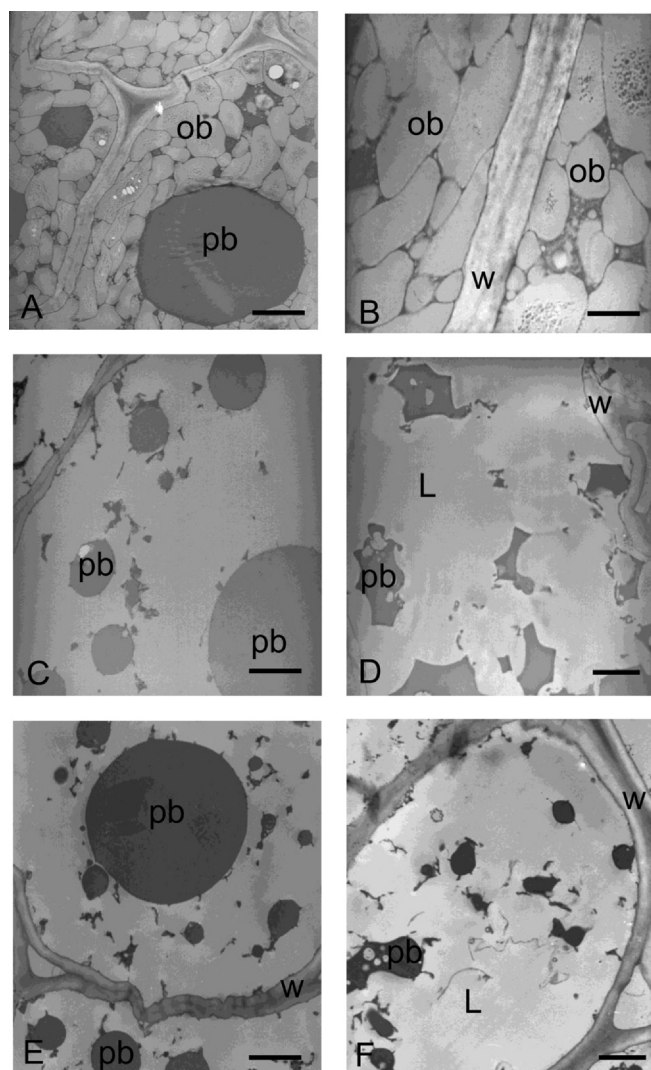


Fig. 5. TEM micrographs of the hazelnuts: A-B: raw sample; C: HA-LT sample; D: HA-HT sample E: IR-LT; F: IR-HT. ob: oil bodies; pb: protein bodies, L: large lipid droplet, W: cell wall. Bars = 3 µm in A, 1 µm in B, 2 µm in C, D, E, F. HA: Hot air; IR: Infrared; LT: Low Temperature; HT: High Temperature.

the cytoplasmic network disruption, the lipids had spread all over the cell, as also indicated by the Nile Red staining (Fig. 4). In some cases, e.g. in the HA-HT sample (Fig. 5D), the OBs may have fused to form a large lipid droplet, while the protein bodies may have lost their typical features. The protein bodies are clearly evident in the LT processed samples (Fig. 5C and F), in agreement with the optical microscopy observation, while they appear to have lost their original HT structure (Fig. 5D and F).

4. Conclusion

Thermal processing has been shown to have allergen-, temperature- and time-specific effects. High temperature roasting, regardless of the heat application method, reduced hazelnut protein immunorecognition by allergic patients, to different extents, for the different allergens, depending on their specific thermal stability. Infrared roasting, in particular when applied at a high temperature, resulted in an almost complete loss of hazelnut immunoreactivity.

Microscopical analyses have shown that both of the considered roasting methods markedly affected cell organization. Roasting caused a disruption of the cytoplasmic network with a loss of the compartmentalization of the lipids in OBs as well as an alteration of the protein

bodies and cell wall profile.

In the present work, we were able to obtain processed hazelnuts with modified protein structure, that resulted in reduced IgE binding by sera of hazelnut allergic patients. The obtained results may give an answer to the need of food matrices with reduced allergenicity that are suitable for oral immunotherapy.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors gratefully acknowledge the financial support provided by the EURAMET EMPIR (Call 2014 – Industry/Project n. 14IND11HIT) and Marta Vallino for her help in the TEM observations.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodchem.2020.128174>.

References

- Alasalvar, C., & Shahidi, F. (2008). *Tree Nuts Composition, Phytochemical and Health Effects*. CRC Press.
- Belviso, S., Dal Bello, B., Giacosa, S., Bertolino, M., Ghirardello, D., Giordano, M., ... Zeppa, G. (2017). Chemical, mechanical and sensory monitoring of hot air- and infrared-roasted hazelnuts (*Corylus avellana* L.) during nine months of storage. *Food Chemistry*, 217, 398–408. <https://doi.org/10.1016/j.foodchem.2016.08.103>.
- Binello, A., Giorgis, M., Cena, C., Cravotto, G., Rotolo, L., Oliveri, P., ... Casale, M. (2018). Chemical modifications of Tonda Gentile Trilobata hazelnut and derived processing products under different infrared and hot air roasting conditions: A combined analytical study. *Journal of the Science of Food and Agriculture*, 98(12), 4561–4569. <https://doi.org/10.1002/jsfa.8984>.
- Candiano, G., Bruschi, M., Musante, L., Santucci, L., Ghiggeri, G. M., Carnemolla, B., ... Righetti, P. G. (2004). Blue silver: A very sensitive colloidal Coomassie G-250 staining for proteome analysis. *Electrophoresis*, 25(9), 1327–1333. <https://doi.org/10.1002/elps.200305844>.
- CODEx STAN1. (1985). General Standard for the Labelling of Pre-packaged Foods, FAO/WHO Standards, a mended in 1991, 1999, 2001, 2003, 2005, 2008, and 2010.
- Cosgrove, D. J. (2005). Growth of the plant cell wall. *Nature Reviews Molecular Cell Biology*, 6, 850–861.
- Costa, J., Mafra, I., Carrapatoso, I., & Oliveira, M. B. P. P. (2016). Hazelnut allergens: Molecular characterization, detection, and clinical relevance. *Critical Reviews in Food Science and Nutrition*, 56(15), 2579–2605. <https://doi.org/10.1080/10408398.2013.826173>.
- Cvijetic, S., Bortolotto, V., Manfredi, M., Ranzato, E., Marengo, E., Salem, R., ... Grilli, M. (2017). Cell autonomous and noncell-autonomous role of NF- κ B p50 in astrocyte-mediated fate specification of adult neural progenitor cells. *Glia*, 65(1), 169–181. <https://doi.org/10.1002/glia.23085>.
- De Leon, M. P., Glaspole, I. N., Drew, A. C., Rolland, J. M., O'Hehir, R. E., & Suphioglu, C. (2003). Immunological analysis of allergenic cross-reactivity between peanut and tree nuts. *Clinical and Experimental Allergy*, 33(9), 1273–1280. <https://doi.org/10.1046/j.1365-2222.2003.01761.x>.
- Dooper, M. M. B. W., Plassen, C., Holden, L., Moen, L. H., Namork, E., & Egaas, E. (2008). Antibody binding to hazelnut (*Corylus avellana*) proteins: The effects of extraction procedure and hazelnut source. *Food and Agricultural Immunology*, 19(3), 229–240. <https://doi.org/10.1080/09540100802243325>.
- Dourado, F., Vasco, P., Barros, A., Mota, M., Coimbra, M. A., & Gama, F. M. (2003). Characterisation of Chilean hazelnut (*Gevuina avellana*) tissues: Light microscopy and cell wall polysaccharides. *Journal of the Science of Food and Agriculture*, 83(3), 158–165. <https://doi.org/10.1002/jsfa.1287>.
- Downs, M. L., Simpson, A., Custovic, A., Semic-Jusufagic, A., Bartra, J., Fernandez-Rivas, M., ... Mills, E. N. C. (2016). Insoluble and soluble roasted walnut proteins retain antibody reactivity. *Food Chemistry*, 194, 1013–1021. <https://doi.org/10.1016/j.foodchem.2015.08.119>.
- Göncüoğlu Taş, N., & Gökmen, V. (2017). Maillard reaction and caramelization during hazelnut roasting: A multiresponse kinetic study. *Food Chemistry*, 221, 1911–1922. <https://doi.org/10.1016/j.foodchem.2016.11.159>.
- Hansen, K. S., Ballmer-Weber, B. K., Lüttkopf, D., Skov, P. S., Wüthrich, B., Bindslev-Jensen, C., ... Poulsen, L. K. (2003). Roasted hazelnuts-allergenic activity evaluated by double-blind, placebo-controlled food challenge. *Allergy*, 58(2), 132–138. <https://doi.org/10.1034/j.1398-9955.2003.23959.x>.
- Klockmann, S., Reiner, E., Bachmann, R., Hackl, T., & Fischer, M. (2016). Food fingerprinting: Metabolomic approaches for geographical origin discrimination of hazelnuts (*Corylus avellana*) by UPLC-QTOF-MS. *Journal of Agricultural and Food Chemistry*, 64(48), 9253–9262. <https://doi.org/10.1021/acs.jafc.6b04433>.
- Kopper, R., Odum, N., Sen, M., Helm, R., Stanley, J., & Burks, A. (2005). Peanut protein allergens: The effect of roasting on solubility and allergenicity. *International Archives of Allergy and Immunology*, 136(1), 16–22. <https://doi.org/10.1159/000082580>.
- López, E., Cuadrado, C., Burbano, C., Jiménez, M., Rodríguez, J., & Crespo, J. F. (2012). Effects of autoclaving and high pressure on allergenicity of hazelnut proteins. *Journal of Clinical Bioinformatics*, 2(1), 12. <https://doi.org/10.1186/2043-9113-2-12>.
- Martinotti, S., Patrone, M., Manfredi, M., Gosetti, F., Pedrazzi, M., Marengo, E., & Ranzato, E. (2016). HMGB1 osteo-modulatory action on osteosarcoma SaOS-2 cell line: An integrated study from biochemical and -omics approaches. *Journal of Cellular Biochemistry*, 2559–2569. <https://doi.org/10.1002/jcb.25549>.
- Marzocchi, S., Pasini, F., Verardo, V., Cierniewska-Zytkiewicz, H., Caboni, M. F., & Romani, S. (2017). Effects of different roasting conditions on physical-chemical properties of Polish hazelnuts (*Corylus avellana* L. var. Kataloński). *LWT - Food Science and Technology*, 77, 440–448. <https://doi.org/10.1016/j.lwt.2016.11.068>.
- Masthoff, L. J., Hoff, R., Verhoeckx, K. C. M., Van Os-Medendorp, H., Michelsen-Huisman, A., Baumert, J. L., ... Knulst, A. C. (2013). A systematic review of the effect of thermal processing on the allergenicity of tree nuts. *Allergy: European Journal of Allergy and Clinical Immunology*, 68(8), 983–993. <https://doi.org/10.1111/all.12185>.
- Moore, P. J., Swords, K. M., Lynch, M. A., & Staehelin, L. A. (1991). Spatial organization of the assembly pathways of glycoproteins and complex polysaccharides in the Golgi apparatus of plants. *The Journal of Cell Biology*, 112(4), 589–602. <https://doi.org/10.1083/jcb.112.4.589>.
- Moreno, F. J., & Clemente, A. (2008). 2S Albumin storage proteins: What makes them food allergens? *The Open Biochemistry Journal*, 2, 16–28. <https://doi.org/10.2174/1874091x00802010016>.
- Müller, U., Lüttkopf, D., Hoffmann, A., Petersen, A., Becker, W. M., Schocker, F., ... Vieths, S. (2000). Allergens in raw and roasted hazelnuts (*Corylus avellana*) and their cross-reactivity to pollen. *European Food Research and Technology*, 212, 2–12.
- Nebbia, S., Lamberti, C., Giorgis, V., Giuffrida, M. G., Manfredi, M., Marengo, E., ... Rolla, G. (2019). The Cockroach Allergen-like protein is involved in primary respiratory and food allergy to yellow mealworm (*Tenebrio molitor*). *Clinical & Experimental Allergy*, May, 1–4. <https://doi.org/10.1111/cea.13461>.
- Özdemir, M., Açıktur, F., Yildiz, M., Biringen, G., Gürcan, T., & Löker, M. (2001). Effect of roasting on some nutrients of hazelnuts (*Corylus Avellena* L.). *Food Chemistry*, 73(2), 185–190. [https://doi.org/10.1016/S0308-8146\(00\)00260-0](https://doi.org/10.1016/S0308-8146(00)00260-0).
- Pastorello, E. A., Vieths, S., Pravettoni, V., Farioli, L., Trambaioli, C., Fortunato, D., ... Conti, A. (2002). Identification of hazelnut major allergens in sensitive patients with positive double-blind, placebo-controlled food challenge results. *Journal of Allergy and Clinical Immunology*, 109(3), 563–570. <https://doi.org/10.1067/MAI.2002.121946>.
- Perren, R., & Escher, F. (2007). Nut roasting the oxidative stability of nuTechnology and product quality. *The Manufacturing Confectioner*, 87(6), 65–75.
- Pfeifer, S., Bublin, M., Dubiel, P., Hummel, K., Wortmann, J., Hofer, G., ... Hoffmann-Sommergruber, K. (2015). Cor a 14, the allergenic 2S albumin from hazelnut, is highly thermostable and resistant to gastrointestinal digestion. *Molecular Nutrition and Food Research*, 59(10), 2077–2086. <https://doi.org/10.1002/mnfr.201500071>.
- Rastogi, N. K. (2012). Recent trends and developments in infrared heating in food processing. *Critical Reviews in Food Science and Nutrition*, 52(9), 737–760. <https://doi.org/10.1080/10408398.2010.508138>.
- Rigby, N. M., Marsh, J., Sancho, A. I., Wellner, K., Akkerdaas, J., Van Ree, R., ... Mills, E. N. C. (2008). The purification and characterisation of allergenic hazelnut seed proteins. *Molecular Nutrition and Food Research*, 52(SUPPL. 2), 251–261. <https://doi.org/10.1002/mnfr.200800083>.
- Saklar, S., Urgan, S., & Katnas, S. (2003). Microstructural changes in hazelnuts during roasting. *Food Research International*, 36(1), 19–23. [https://doi.org/10.1016/S0963-9969\(02\)00103-5](https://doi.org/10.1016/S0963-9969(02)00103-5).
- Schmidt, H., Krause, S., Gelhaus, C., Petersen, A., Janssen, O., & Becker, W. M. (2010). Detection and structural characterization of natural Ara h 7, the third peanut allergen of the 2S albumin family. *Journal of Proteome Research*, 9(7), 3701–3709. <https://doi.org/10.1021/pr1002406>.
- Schocker, F., Lüttkopf, D., Müller, U., Thomas, P., Vieths, S., & Becker, W. M. (2000). IgE binding to unique hazelnut allergens: Identification of non pollen-related and heat-stable hazelnut allergens eliciting severe allergic reactions. *European Journal of Nutrition*, 39(4), 172–180. <https://doi.org/10.1007/s003940070021>.
- Serrano, I., Suárez, C., Olmedilla, A., Rapoport, H. F., & Rodríguez-García, M. I. (2008). Structural organization and cytochemical features of the pistil in Olive (*Olea europaea* L.) cv. Picual at anthesis. *Sexual Plant Reproduction*, 21(2), 99–111. <https://doi.org/10.1007/s00497-008-0075-y>.
- Vanga, S. K., & Raghavan, V. (2017). Processing effects on tree nut allergens: A review. *Critical Reviews in Food Science and Nutrition*, 57(17), 3794–3806. <https://doi.org/10.1080/10408398.2016.1175415>.
- Wessel, D., & Flüge, U. I. (1984). A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. *Analytical Biochemistry*, 138(1), 141–143. [https://doi.org/10.1016/0003-2697\(84\)90782-6](https://doi.org/10.1016/0003-2697(84)90782-6).
- Wigotzki, M., Steinhart, H., & Paschke, A. (2000). Influence of varieties, storage and heat treatment on IgE-binding proteins in hazelnuts (*Corylus avellana*). *Food and Agricultural Immunology*, 12(3), 217–229. <https://doi.org/10.1080/09540100050140759>.
- Worm, M., Hompes, S., Fiedler, E. M., Illner, A. K., Zuberbier, T., & Vieths, S. (2009). Impact of native, heat-processed and encapsulated hazelnuts on the allergic response in hazelnut-allergic patients. *Clinical and Experimental Allergy*, 39(1), 159–166. <https://doi.org/10.1111/j.1365-2222.2008.03143.x>.
- Yang, J., Bingol, G., Pan, Z., Brandl, M. T., McHugh, T. H., & Wang, H. (2010). Infrared heating for dry-roasting and pasteurization of almonds. *Journal of Food Engineering*, 101(3), 273–280. <https://doi.org/10.1016/J.JFOODENG.2010.07.007>.

Table S1. Hazelnut allergic patients' characterization by means of ImmunoCAP analysis. Pool A: 4 patients (ID from 1 to 4) with ImmunoCAP positive to Cor a 1, Cor a 8, Cor a 9 and Cor a 14. Pool B: 1 patient (ID 5) with ImmunoCAP positive to Cor a 8, Cor a 9 and Cor a 14. Pool C: 8 patients (ID from 6 to 13) with ImmunoCAP positive to Cor a 9 and Cor a 14. Pool D: 3 patients (ID from 14 to 16) with ImmunoCAP positive to Cor a 8. Pool E: pool of all the patients in the study (ID from 1 to 16).

POOL ID		SUBJECTED ID	SEX	AGE	IgE ImmunoCAP (KU/l)				
					Cor a 1	Cor a 8	Cor a 9	Cor a 14	Total hazelnut IgE
E	A	1	M	13	49,70	0,50	1,71	15,70	50,70
		2	M	18	40,40	0,73	11,70	31,20	48,10
		3	M	6	74,30	13,90	6,67	21,90	n.d.
		4	F	3	0,26	0,16	8,89	1,75	7,76
	B	5	M	9	<0,1	>100	18,40	68,00	46,70
	C	6	F	3	<0,1	<0,1	2,43	1,53	2,34
		7	M	6	<0,1	<0,1	1,13	1,70	n.d.
		8	F	11	<0,1	<0,1	>100	>100	>100
		9	F	12	<0,1	<0,1	>100	29,30	>100
		10	M	8	<0,1	<0,1	6,00	6,34	13,50
		11	M	3	<0,1	<0,1	27,20	7,42	11,00
		12	M	6	<0,1	<0,1	5,17	1,42	2,93
		13	F	6	<0,1	0,58	4,01	2,35	2,52
	D	14	F	5	n.d.	2,46	n.d.	<0,1	7,43
		15	F	4	n.d.	1,85	<0,1	<0,1	n.d.
		16	M	9	n.d.	5,64	<0,1	<0,1	n.d.

Table S2. List of the identified proteins from total protein (T) and oil bodies associated protein (O) extracts, including the amino acid sequence of the detected peptides.

Bands	ID (NCBI)	Description	Prot_score	Matching peptides	Peptide sequences
Hazelnut total proteins extract (T)					
T1	AHA36627.1	Cor a 9	465	7	ADIYTEQVGR AESEGFVWVAFK INTVNSNTLPVLR QGQVLTIPQNFVAVAK ALPDDVLANAFQISR VQVVDDNGNTVFDDELRL TIEPNGLLLPQYSNAPELIYIER
T2	AHA36627.1	Cor a 9	702	8	WLQLSAER ADIYTEQVGR TNDNAQISPLAGR AESEGFVWVAFK INTVNSNTLPVLR QGQVLTIPQNFVAVAK ALPDDVLANAFQISR EGEQEQQGEGNNVFSGFDAEFLADAFNVDVDTAR
	ACO56333.1	Cor a 14	173	4	EVMETAR-Oxidation (M) DLPNQCR QQNLNQCCR QAVMQQGGEMR
T3	AHA36627.1	Cor a 9	297	4	RLQSNQDK WLQLSAER INTVNSNTLPVLR VQVVDDNGNTVFDDELRL
	4XUW_A	Cor a 8	84	2	GNLTPCVLYLK GIAGLNPNLAAGLPGK
T4	AHA36627.1	Cor a 9	411	6	WLQLSAER AESEGFVWVAFK INTVNSNTLPVLR QGQVLTIPQNFVAVAK ALPDDVLANAFQISR GKEGEQEQQGEGNNVFSGFDAEFLADAFNVDVDTAR
T5	AHA36627.1	Cor a 9	4667	14	QETTLAR LMENIGSR WLQLSAER LNALEPTNR ADIYTEQVGR TNDNAQISPLAGR SRADIYTEQVGR AESEGFVWVAFK INTVNSNTLPVLR RAESEGFVWVAFK QGQVLTIPQNFVAVAK ALPDDVLANAFQISR VQVVDDNGNTVFDDELRL IEAEAGQIESWDHNDQQFQCAGVAVIR
	AAL86739.1	Cor a 11	69	2	VQVLENFTK IPAGTPVYMINR
T6	AHA36627.1	Cor a 9	1248	10	WLQLSAER LNALEPTNR ADIYTEQVGR TNDNAQISPLAGR AESEGFVWVAFK INTVNSNTLPVLR RAESEGFVWVAFK QGQVLTIPQNFVAVAK ALPDDVLANAFQISR VQVVDDNGNTVFDDELRL
T7	AHA36627.1	Cor a 9	3242	18	QETTLAR LMENIGSR

					SRQEWER WLQLSAER LNALEPTNR QGQQQFGQR HFGECNDR ADIYTEQVGR TNDNAQISPLAGR AESEGF EWVAFK INTVNSNTLPVLR RAESEGF EWVAFK QGQVLTIPQNFAVAK ALPDDVLANAFQISR HFYLAGNPDDDEHQR VQVVDDNGNTVFDDDEL IEAEAGQIESWDHNDQQFQCAGVAVIR EGEQEQQGEGNNVFSGFDAEFLADAFNVDVDTAR
T8	AHA36627.1	Cor a 9	1251	12	WLQLSAER LNALEPTNR QGQQQFGQR ADIYTEQVGR TNDNAQISPLAGR AESEGF EWVAFK INTVNSNTLPVLR QGQVLTIPQNFAVAK ALPDDVLANAFQISR VQVVDDNGNTVFDDDEL IEAEAGQIESWDHNDQQFQCAGVAVIR EGEQEQQGEGNNVFSGFDAEFLADAFNVDVDTAR
T9	AHA36627.1	Cor a 9	2684	13	QETTLAR LMENIGSR WLQLSAER LNALEPTNR HFGECNDR ADIYTEQVGR TNDNAQISPLAGR AESEGF EWVAFK INTVNSNTLPVLR RAESEGF EWVAFK QGQVLTIPQNFAVAK ALPDDVLANAFQISR VQVVDDNGNTVFDDDEL
	AAL86739.1	Cor a 11	842	10	ELAFNLPSR LLSGIENFR GNIVNEFER VQVLENFTK ALSQHEEGPPR IPAGTPVYMINR AFSWEVLEAALK ESFNVEHGDIIIR NQDQAFFFPKPNK ILQPVSAPGHFEAFYGAGGEDPESFYR
T10	AAL86739.1	Cor a 9	2871	12	QETTLAR LMENIGSR WLQLSAER LNALEPTNR ADIYTEQVGR TNDNAQISPLAGR AESEGF EWVAFK INTVNSNTLPVLR RAESEGF EWVAFK QGQVLTIPQNFAVAK ALPDDVLANAFQISR VQVVDDNGNTVFDDDEL
	AHA36627.1	Cor a 11	1984	15	ATITMVR VFGEQSK QEEGGR ELAFNLPSR LLSGIENFR GNIVNEFER VQVLENFTK HPSQSNQFGR ALSQHEEGPPR

					IPAGTPVYMINR AFSWEVLEAALK ESFNVEHGDIIIR NQDQAFFFGPNK IPAGTPVYMINRDENEK ILQPVSAPGHFEAFYGAGGEDPESFYR
T11	AHA36627.1	Cor a 9	2490	14	LMENIGSR WLQLSAER LNALEPTNR ADIYTEQVGR TNDNAQISPLAGR AESEGF EWVAFK INTVNSNTLPVLR RAESEGF EWVAFK QGQVLTIPQNF AVAK ALPDDVLANAFQISR VQVVDDNGNTVFDDLR TIEPNGLLLPQYSNAPELIYIER IEAEAGQIESWDHNDQQFQCAGVAVIR GKEGEQQQEGGNNVFSGFDAEFLADAFNVDVDTAR
	AAL86739.1	Cor a 11	570	7	ELAFNLPSR LLSGIENFR GNIVNEFER VQVLENFTK IPAGTPVYMINR AFSWEVLEAALK NQDQAFFFGPNK
T12	AHA36627.1	Cor a 9	8359	22	QETTLAR LMENIGSR RLQSNQDK SRQEWER WLQLSAER LNALEPTNR QGQQQFGQR HFGECNLDR ADIYTEQVGR TNDNAQISPLAGR SRADIYTEQVGR AESEGF EWVAFK INTVNSNTLPVLR RAESEGF EWVAFK QGQVLTIPQNF AVAK ALPDDVLANAFQISR HFYLAGNPDDQHQR VQVVDDNGNTVFDDLR IEAEAGQIESWDHNDQQFQCAGVAVIR GITGVLPFGCPETFEDPQQSQGQGGQGSQR EGEQQQEGGNNVFSGFDAEFLADAFNVDVDTAR GKEGEQQQEGGNNVFSGFDAEFLADAFNVDVDTAR
	AAL86739.1	Cor a 11	319	6	ELAFNLPSR LLSGIENFR GNIVNEFER VQVLENFTK IPAGTPVYMINR AFSWEVLEAALK
T13	AHA36627.1	Cor a 9	4154	18	QETTLAR LMENIGSR WLQLSAER LNALEPTNR QGQQQFGQR ADIYTEQVGR TNDNAQISPLAGR AESEGF EWVAFK INTVNSNTLPVLR RAESEGF EWVAFK QGQVLTIPQNF AVAK ALPDDVLANAFQISR HFYLAGNPDDQHQR VQVVDDNGNTVFDDLR IEAEAGQIESWDHNDQQFQCAGVAVIR GITGVLPFGCPETFEDPQQSQGQGGQGSQR EGEQQQEGGNNVFSGFDAEFLADAFNVDVDTAR

					GKEGEQEQQGEGNNVFSGFDAEFLADAFNVDVDTAR
	AAL86739.1	Cor a 11	310	7	ELAFNLPSR LLSGIENFR GNIVNEFER VQVLENFTK IPAGTPVYMINR AFSWEVLEAALK NQDQAFFFPGPKNK
T14	AHA36627.1	Cor a 9	2429	14	LMENIGSR WLQLSAER LNALEPTNR QGQQQFGQR ADIYTEQVGR TNDNAQISPLAGR AESEGFVWVAFK INTVNSNTLPVLR RAESEGFVWVAFK QGQVLTIPQNFVAVK ALPDDVLANAFQISR VQVDDNGNTVFDDEL IEAEAGQIESWDHNDQQFQCAGVAVIR GITGVLPFGCPETFEDPQQQSQQGQGGQSQR
	AAL86739.1	Cor a 11	623	7	ELAFNLPSR LLSGIENFR GNIVNEFER VQVLENFTK IPAGTPVYMINR AFSWEVLEAALK NQDQAFFFPGPKNK
Oil bodies associated proteins (O)					
O1	AAO65960.1	Cor a 13	2822	4	QLQDPAHQPR HPPGADQLDHAR AEQFGQQHVTGSQGS DRAEQFGQQHVTGSQGS
	AAO67349.2	Cor a 12	441	3	EVGQEIQSR MQDMAAFVGQK ADRPQQLQVHPQR
O2	MK737923	Cor a 15	935	7	ETGQTVTGK TLEGGRGEEK AQDTAGHLGQK GQAQVQGPSASK RAQDTAGHLGQK GTSVPEQMEHAK ADYQHQQQHQRPADAFK
	AAO67349.2	Cor a 12	172	2	QLQDPAHQPR AEQFGQQHVTGSQGS
	AAO65960.1	Cor a 13	179	2	EVGQEIQSR MQDMAAFVGQK
O3	MK962827	Caleosin	1446	10	EALATEAR YAPVTSER MEWGILYVLAR VAFDIFGWIASK HGSDSGTYDTEGR YVPVNLENMFSK CFDGSLFEYCAK LTLGELWAMTQGNR EDLEVSLPKPYLAR ALVAPDTNYPTGTPGHK
	AAO65960.1	Cor a 13	561	3	QLQDPAHQPR HPPGADQLDHAR AEQFGQQHVTGSQGS
	AAO67349.2	Cor a 12	515	4	CASQSLPR EVGQEIQSR MQDMAAFVGQK ADRPQQLQVHPQR
	AHA36627.1	Cor a 9	481	7	WLQLSAER ADIYTEQVGR TNDNAQISPLAGR AESEGFVWVAFK INTVNSNTLPVLR ALPDDVLANAFQISR

					VQVDDNGNTVFDDEL
O4	AAO67349.2	Cor a 12	531	4	CASQSLPR EVGQEIQSR MQDMAAFVGQK ADRPQQLQVHPQR
	MK737923	Cor a 15	175	3	ETGQTVTGK AQDTAGHLGQK GTSVPEQMEHAK
	AAO65960.1	Cor a 13	114	3	QLQDPAHQPR HPPGADQLDHAR AEQFGQQHVTGSQGS
O5	MK737923	Cor a 15	704	6	ETGQTVTGK TLEGGRGEEK AQDTAGHLGQK RAQDTAGHLGQK GTSVPEQMEHAK ADYQHQQQHQRPADAFK
	AHA36627.1	Cor a 9	315	6	WLQLSAER LNALEPTNR AESEGFVWVAFK INTVNSNTLPVLR QGQVLTIPQNFAVAK ALPDDVLANAFQISR
	AAO67349.2	Cor a 12	142	2	EVGQEIQSR MQDMAAFVGQK
O6	AHA36627.1	Cor a 9	1843	9	WLQLSAER ADIYTEQVGR TNDNAQISPLAGR AESEGFVWVAFK INTVNSNTLPVLR RAESEGFEWVAFK QGQVLTIPQNFAVAK ALPDDVLANAFQISR VQVDDNGNTVFDDEL
	AAL86739.1	Cor a 11	174	5	ELAFNLPSR LLSGIENFR GNIVNEFER VQVLENFTK AFSWEVLEAALK
	AAO67349.2	Cor a 12	90	2	EVGQEIQSR MQDMAAFVGQK
O7	AHA36627.1	Cor a 9	765	8	WLQLSAER ADIYTEQVGR AESEGFVWVAFK INTVNSNTLPVLR RAESEGFEWVAFK QGQVLTIPQNFAVAK ALPDDVLANAFQISR VQVDDNGNTVFDDEL
	AAL86739.1	Cor a 11	166	4	ELAFNLPSR LLSGIENFR GNIVNEFER VQVLENFTK
	AAO67349.2	Cor a 12	60	2	MQDMAAFVGQK ADRPQQLQVHPQR
O8	AHA36627.1	Cor a 9	2701	12	QETTLAR WLQLSAER LNALEPTNR ADIYTEQVGR TNDNAQISPLAGR AESEGFVWVAFK INTVNSNTLPVLR RAESEGFEWVAFK QGQVLTIPQNFAVAK ALPDDVLANAFQISR VQVDDNGNTVFDDEL IEAEAGQIESWDHNDQQFCAGVAVIR
	AAL86739.1	Cor a 11	145	4	ELAFNLPSR LLSGIENFR GNIVNEFER AFSWEVLEAALK

