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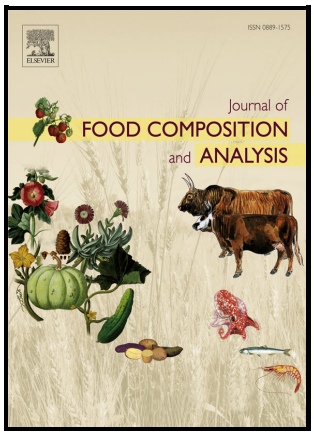

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Proteomic study of walnut oleosome and first evidence on oleosin sensitization in allergic patients

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Abstract

Walnuts are considered the healthiest of all nuts, mainly because of its polyunsaturated fatty acid content. As far as its protein component is concerned, a rather interesting class of oil-body (OB) associated proteins, which, together with lipids, generates the "oleosome", still needs to be explored in detail.This study aimed to characterize the walnut oleosome proteins by a double off-gel and in-gel approach, with a focus on the effect of processing on protein profile and oleosin immune recognition. The off-gel approach (label free quantification, LFQ) allowed the identification of all the three oleosome specific associated class of proteins: oleosins, caleosins, and steroleosins. The in-gel separation coupled with immunoblotting experiments proved that walnut oleosins were immune recognized by walnut allergic patients' IgEs, providing first evidence of their potential role in walnut allergenicity. Oleosin immune recognition seemed to be increased by boiling procedure compared to raw and roasted walnut, probably due to the increased solubility of oleosome associated proteins in this condition.

Keywords

Walnut, oleosome, oil body, sub-proteome, oleosin, processing, food allergy

1. Introduction

Walnuts, one of the most widely consumed tree nuts, are associated with a variety health benefits, including reduced cardiovascular and neurological disease risks (Hosseini Adarmanabadi et al., 2023; Simopoulos, 2002). These benefits are mainly attributed to their fatty acid (FA) composition, as they are rich in polyunsaturated fatty acids (PUFAs) (Kergomard et al., 2021). Among the PUFAs, walnuts contain 12-16% of α-linolenic acid (ALA, C18:3, ω3), which can very likely explain their cardioprotective effects (Hayes et al., 2016). Moreover, the walnut lipid fraction includes other interesting molecules, such as triacylglycerols (TAGs), phytosterols, phospholipids (PLs) and tocopherols, which are able to endow this fraction with high oxidative stability (Samdani et al., 2018), as well as other valuable minor components, including natural antioxidants and fat-soluble vitamins (Miraliakbari and Shahidi, 2007). In oleaginous seeds, these lipophilic molecules are stored in oil bodies (OBs), also known as oleosomes, which are spherical organelles (0.5-2.5 µm in diameter) that the plant uses to store the energy needed for germination and growth (Khor et al., 2013). In cotyledon plants, such as nuts, OBs surround much larger structures, called protein bodies (PBs), which contain the proteins involved in the embryogenic program of the seed (mainly legumins and vicilins) (Wang et al., 2012). Walnut OBs are formed by a TAG core (dominated, in order of abundance, by α-linolenic linoleic, oleic and palmitic acids) which is surrounded by a monolayer of PLs (mainly phosphatidylcholine and phosphatidylserine) with embedded proteins (oleosins, caleosins and steroleosins) (Napier et al., 2001; Nogales-Bueno et al., 2021). Oleosins are the most abounded OB proteins, and they are responsible for stabilizing OBs against aggregation and coalescence (Barre et al., 2018).

Oleosins, in addition to already characterized water-soluble allergens, have recently become relevant as nut allergens in both peanuts (Schwager et al., 2017) and hazelnuts (Nebbia et al., 2021; Zuidmeer-Jongejan et al., 2014). Among the various nut allergies, walnut allergy accounts for 16% of the known cases of anaphylaxis among children and adolescents in Europe. It is the tree nut that is most commonly responsible

for triggering allergies in the United States, where it accounts for 37% to 48% of all tree nut allergies (Borres et al., 2022). Currently, 8 walnut allergens, named *Jug r 1* to *Jug r 8*, have been formally acknowledged by the WHO/IUIS Allergen Nomenclature Sub-Committee (www.allergen.org), but no oleosins have yet been included.

At the molecular level, only English walnut (*Juglans regia*) has a fully sequenced genome, which was published in 2016 (Martínez-García et al., 2016), while a curated database was released in UniProt KB in February 2018. This knowledge at the DNA level has allowed a global proteomic analysis of raw English walnut to be performed (Xiong et al., 2019). However, because of their localization, their low abundance and the difficulty of their extraction, OB proteins have not yet been fully characterized. As regards OB proteins, 5 caleosins (A0A2I4DSD4, A0A2I4DH23, A0A2I4G1V2, A0A8333WSM5 and A0A833WXW0), 3 steroleosins (A0A2I4DWD2, A0A6P9F4P9 and A0A2I4HKD8) and 8 oleosins (A0A214DRG3, A0A2I4GNJ1, A0A2I4GES3, A0A2I4G1C3, A0A2I4GSQ3, G8H6H8, A0A833UQH7 and G8H6H9) have been indexed in the UniProt KB database (www.uniprot.org). Chen Y. et al. (2022) have recently confirmed the presence of all of these eight oleosins, one caleosin and one steroleosin at the protein level in the raw kernel of walnuts.

Considering the already demonstrated effect of processing on the OB structure (Lamberti et al., 2021), we decided to perform a proteomic investigation on raw, boiled and roasted walnuts. A comprehensive characterization of the OB-associated protein extracts was achieved by using the off-gel proteomic approach. Afterwards, the in-gel protein analysis was performed to characterize the electrophoretic mobility of the oleosome proteins and to understand how processing could affect protein extraction, denaturation and aggregation. Taking into account the role of oleosins as emerging allergens, the immunoblotting analysis with a pool of selected walnut-allergic patients provided a preliminary evaluation of the oleosin immune recognition, and allowed to understand whether processing could interfere with IgE binding.

2. Materials and methods

2.1 Chemicals

The methanol (for HPLC plus gradient ,99,9%), chloroform, absolute ethanol, ortophosphoric acid (> 85%), HCl (99,8%), NaCl, KCl (99,5%), Na_2HPO_4 (97%), KH_2PO_4 (99%), NH_4CHO_3 (> 99,5%), urea (>99,5%), Coomassie Blue, Tween 20, Trizma base, vegetal gelatin, as well as the acetonitrile and formic acid (98-100%) MS grade used in the experiment were obtained from Merck KGaA (Darmstadt, Germany). The Solid Phase Extraction Tubes were Strata X from Phenomenex (Torrance, California, USA). The Alkaline Phosphatase Substrate Kit was from Biorad (Hercules, California, USA). The 2DQuant-kit was from (GE Healthcare, Chicago, IL, USA). The goat anti-Human IgE antibody was from SeraCare Life Sciences Inc. (Milford, Massachusetts, USA). The NuPAGE Lithium dodecyl sulfate (LDS) Sample Buffer, Low Molecular Weight (LMW) standards, NuPAGE Sample Reducing Agent, 10% NuPAGE mini gels, 4-Morpholineethanesulfonic acid, 2-(N-Morpholino) ethanesulfonic acidhydrate (MES) Running Buffer, Transfer buffer and Nitrocellulose Membranes were all from Invitrogen, Life Technologies Ltd. (Paisley, UK). The sequence grade modified trypsin was acquired from Promega (Madison, WI, USA).

2.2 Thermal processing of walnuts

Raw walnut kernels (*Juglans regia* cv. Chandler) were supplied by an Italian company. To obtain the processed walnuts, the boiling and roasting procedures were chosen since they represent the most common processing for walnuts in the food production chain. The processed walnuts were obtained by boiling the walnut kernels at 100 °C for 5 minutes or roasting them at 160 °C for 15 minutes using the convection oven mode (Ghibli 50, Fratelli Galli Laboratory and Industrial Solutions) . All the samples were stored at -20 °C until use.

2.3 Extraction of proteins from walnut OB

The OB-associated proteins were extracted as described in Nebbia et al. (2021). Briefly, 10 g of raw, boiled and roasted chopped walnuts were sonicated in a 30 mL grinding medium (GM1: 0.6 M sucrose, 10 mM sodium phosphate, pH 9.5) and filtered using two-layer gauze. The filtrated sample was then centrifuged at 21460×g for 20 min at 4 °C, and the resulting pad floating on the top was collected. The oil pad was

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subjected to three resuspension and centrifugation steps during which the oil bodies contained in the floating pad were resuspended progressively in three different buffers: firstly, 10 mL of GM2 (GM1 with the addition of 0.1% Tween 20), secondly, 5 mL GM3 (GM2 with the addition of 2 M NaCl) and lastly, Urea 9 M (pH 11). Finally, the oil bodies were centrifuged, collected and re-suspended in GM1 at a final concentration of 100 mg/mL. In order to remove any oil residues, the samples were 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 Off-gel analysis

2.4.1 In solution tryptic digestion

An aliquot of fifty µg of OB protein extracts from raw, boiled and roasted walnuts was suspended in 180 µL of 25 mM ammonium bicarbonate with 0.1 % SDS. The samples were reduced with DTT to the final concentration of 20 mM, kept for 1 hour at 37 °C, and alkylated with IAA at the final concentration of 55 mM, for 1 hour in the dark at room temperature (RT). The residual IAA was quenched in a second incubation step with DTT at the final concentration of 30 mM for 15 minutes at RT. The protein mixtures were digested O/N at 37 °C under shaking with modified porcine proteomic grade trypsin (1 µg/µL) at a 1:20 trypsin-protein ratio. An aliquot of five µL of pure formic acid (FA) was added to stop the digestion. The tryptic peptide mixtures were desalted using Strata-X Polymeric SPE tubes. Briefly, the tubes were conditioned with 2 mL of acetonitrile (ACN), then equilibrated with 2 mL of 0.1% FA. The samples, previously diluted with 2 mL of 0.1% FA, were loaded in the Strata-X tubes. The tubes were then washed with 2 mL of 0.1% FA, and the peptides were eluted with 2 mL of 0.1% (v/v) FA/70% ACN. After desalting, the samples were dried in a 5301 Eppendorf Concentrator (Eppendorf, Hamburg, Germany) and suspended in 30µL of 0.1% (v/v) FA/5% ACN before the analysis. Three biological replicates for each condition (raw, boiled and roasted) were prepared and analysed by means of LC-MS/MS.

2.4.2 LC-MS/MS analysis

The analyses were performed using an Orbitrap Q Exactive Plus, coupled to a UHPLC binary pump system (Vanquish Thermo Fisher Scientific, Waltham, Massachusetts, USA). The stationary phase was a BioBasic™ C18 HPLC Column (1 × 150 mm, 5 µm; Thermo Scientific). The mobile phases were 0.1% (v/v) FA in MilliQ water (A) and 0.1% (v/v) FA in ACN (B), and they were eluted at a flow-rate of 68.0 µL/min at increasing concentrations of solvent B, that is, from 5% to 45%, over 57 min, and from 45% to 50% in 2 min. The injection volume was 2.0 µL. The oven temperature was set at 55 °C. The autosampler was set at 6 °C. Mass spectra were acquired in Full MS-ddMS2 mode. The instrument was set up so that Full MS spectra were acquired in an m/z scan range of 150-1600, with an isolation window of 2 m/z, while the resolution was set at 70,000, the AGC target was $1x10^6$, and the maximum IT was 256 ms. Up to 12 of the most intense ions in MS1 were selected for fragmentation in MS/MS mode. The fragmentation spectra resolution was set at 17,500 for the MS/MS spectra, with a dynamic exclusion of 40 s, the NCE was set at 28%, the AGC target at $2x10^4$, and the maximum IT at 200 ms. The allowed charge state was 2-6. The experiments were performed in triplicate for each condition.

2.5 In-gel analysis

2.5.1 Separation of the OB-associated proteins by means of LDS PAGE

Lithium dodecyl sulfate-PAGE (LDS-PAGE) was performed using precast gels (NuPAGE 4–12% Bis–Tris) in an XCell SureLock Mini-Cell System (Invitrogen), according to the manufacturer's instructions. Each sample was diluted in the NuPage LDS Sample Buffer, under reducing conditions (NuPAGE Sample Reducing Agent), and loaded with an equal amount of proteins (5 µg). The gels were stained with Colloidal Coomassie Blue (Candiano et al., 2004) and scanned with a ChemiDoc MP System densitometer (Bio-Rad) at a resolution of 600 dpi.

2.5.2 In-gel tryptic digestion

The selected bands were cut and 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. They 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 the 5301 Eppendorf Concentrator (Eppendorf, Hamburg, Germany) and digested O/N at 37 °C under shaking with 7µl of modified porcine proteomic grade trypsin at 75 ng/µL concentration in 25 mM NH_4HCO_3/10% ACN. One µL of 5% FA was added to stop the digestion.

2.5.3 LC-MS/MS analysis

The analyses were performed using the same previously described LC-MS system (2.4.2), albeit with some changes. The flow rate was set at 50.0 µL/min, with an increasing concentration of solvent B, from 5% to 70%, over 50 min and with 80% for 5 min. The injection volume was 4.0 µL. Mass spectra were acquired in Full MS-ddMS^2 mode. The instrument was set up so that Full MS spectra were acquired in an *m/z* scan range of 150-1800, the resolution was set at 70,000, the maximum IT was 200 ms, the AGC target was $5x10^5$, and the charge exclusion was unassigned. Up to 12 of the most intense ions in MS1 were selected for fragmentation in the MS/MS mode. The fragmentation spectra resolution was set at 17,500 for the MS/MS spectra, with a dynamic exclusion of 20 s and an isolation window of 2.0 m/z, the normalized collision energy was set at 28, the maximum IT at 200 ms and the AGC target at $2x10^4$.

Both the off-gel and in-gel mass spectrometry proteomic data were deposited with the ProteomeXchange Consortium via the PRIDE partner repository with dataset identifier PXD038199 (to revise the proteomic data log in to the account with Username: reviewer_pxd038199@ebi.ac.uk and Password: xmmt2Q84).

2.6 Protein identification strategy

All the Data Dependent Analysis (DDA) files were searched using MaxQuant (https://maxquant.org) v. 2.0.3.0 against the UniProt *Juglans* database (reviewed and unreviewed, 63749 entries). The amino acid sequences of the mature forms of 24 proteins (known as allergens), were added to the database to match the information of the protein Molecular Weights (MWs) derived from the electrophoretic run.

The search parameters were set as follow: S-carbamidomethyl derivate on cysteine as a fixed modification, oxidation on methionine, Acetyl (N-term), as variable modifications and two missed cleavage sites for trypsin digestion. The MS/MS fragment mass tolerance was 20 ppm. A minimum of 2 peptides, FDR of 0.01% for both protein and peptides, and a score of 20 for unmodified and modified peptides were set for

the protein identification. A Label Free Quantification (LFQ) analysis was set up to compare the results of the shotgun proteomic analyses of the three biological replicates of the walnut samples (raw, boiled and roasted). The lists of the identified proteins obtained following the two different approaches are available as Table S1 and S2.

2.7 Patient characterization

Sera from 10 walnut allergic pediatric patients with a convincing clinical history of walnut allergy were retrospectively collected from the Pediatric Allergy Unit at the Regina Margherita Children Hospital of Turin (Città della Salute e della Scienza, Turin, Italy). The collected sera were grouped into a single pool and three sera of non-nut sensitized non-allergic walnut 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.

2.8 Immunoblotting analysis

After LDS-PAGE, the protein bands were electro transferred 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 a pool of the sera of walnut allergic patients 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 then incubated for 1 h, at RT, with an anti-Human IgE antibody 1:5000 diluted in the incubation buffer. The membranes were washed three times and the immune reactivity was highlighted with the Alkaline Phosphatase Substrate Kit (Bio-Rad) following the manufacture's instruction. The membranes were then acquired by means of the ChemiDoc MP System densitometer (Bio-Rad) at a resolution of 600 dpi.

2.9 Statistical and in silico analysis

All the statistical tests were performed using GraphPad Prism, version 8.4.3. UniProt tools were used (https://www.uniprot.org/) for the analysis of the protein sequences, the protein mapping and the Gene Ontology annotation.

3. Results and discussion

3.1 Walnut OB-associated protein characterization by means of the off-gel approach

The off-gel sub-proteome analysis allowed 82, 256 and 119 proteins to be identified in the raw, boiled and roasted walnuts, respectively. (the 60 shared proteins (Fig. 1), among the three conditions, include the oleosome characterizing proteins :. :one caleosin (A0A2I4DSD4), one steroleosin (A0A2I4DWD2) and eight oleosins (corresponding to all those indexed in the UniProt KB walnut database) were identified. However, since the A0A833UQH7 oleosin was not unambiguously identified by the unique peptides (it is listed as a second hit in the G8H6H8 identification), we did not consider A0A833UQH7 for the subsequent data processing or comments. The higher LFQ intensities observed for the caleosins, the steroleosin and the oleosins after boiling, compared to the raw and roasted samples, suggested that this processing was able to promote the disaggregation phenomena thus improving the protein recovery (Fig. 2). Moreover, when considering proteins exclusively present in one condition, the boiled sample is the richest with 149 proteins being identified. Most of these proteins do not specifically belong to the oleosome, as they are characterized by different seed sub-localization. They were probably identified because they belong to seed structures closely associated with OB, called protein bodies (PB) (Wang et al., 2011). A Gene Ontology analysis was carried out to cluster the proteins by three functional categories: biological process (BP), cellular component (CC) and molecular function (MF) (Fig. 3). This analysis highlighted a lack of the Gene Ontology annotation for the walnut proteins, Indeed, only ranges of 59-65%, 50-57% and 68-76% of the identified proteins showed to be annotated for BP, CC and MF, respectively. Considering the Gene Ontology annotations common to the three samples, the BP category was assigned to 23 subcategories, in which “lipid storage” and “lipid metabolic process” were predominant. These results are consistent with the finding that the walnut kernel is an energy reservoir (Chenevard et al., 1994). The CC category was further

classified into 12 subcategories, and according to the specific lipophilic protein extraction protocol that was used, the largest was the "integral component of the membrane", which contained 11, 16 and 9 proteins, in the raw, boiled and roasted samples, respectively. As regards the boiled sample, a large number of proteins belonging to "cytoplasm" and "cytosol" subcategories (43 and 23, respectively) were found; this is probably correlated with the thermal processing, as already demonstrated for hazelnut roasting by Lamberti et al. (Lamberti et al., 2021). As microscopic analyses have already demonstrated for hazelnuts, the processing may cause a disruption of the cytoplasmic network, with a consequent loss of the compartmentalization of the lipids in OBs as well as an alteration of the PB and cell wall profile (Lamberti et al., 2021). Lastly, the MF category was mapped onto 24 Gene Ontology terms, and the most abundant subcategories were "ATP binding" for the boiled samples, "nutrient reservoir activity" for the boiled and roasted samples, and "osmosensor activity" for the raw sample. The annotation of the protein in these categories is not surprising, since OBs are the storehouses of energy that a plant needs for germination and growth (Kergomard et al., 2021).

3.2 Walnut OB-associated protein characterization by means of the in-gel approach

The electrophoretic profiles of the raw, boiled and roasted walnut proteins are shown in Fig.4, and the protein identifications are summarized in Table S2.

The roasted walnut OB-proteins show the profile that differs the most among all the samples. This profile is characterized by medium MW bands (nos. 23, 24, 25), which are more intense than the corresponding oleosin-containing band (no. 26). On the other hand, the raw and boiled walnuts show the most intense oleosin-containing bands (nos. 7, 8, 17 and 18), and thus are those that characterize the protein profile. Unlike the other two samples, the oleosins in the boiled walnuts were identified in bands nos. 9, 10, 11, 13, 14, and 16, in addition to those corresponding to their theoretical MW (nos. 17 and 18). Moreover, a larger number of proteins *per* band was identified in the boiled sample (thus confirming the results of the off-gel analysis) for both the specific and unspecific OB-associated proteins. The unspecific OB-associated proteins probably derive from seed structures closely associated with OB. Considering the previously mentioned

proteins, 11S globulin Jug r4 (Q2TPW5), legumine B-like proteins (A0A2I4GEH1 and A0A2I4GEI2) and 11S globulin-like (A0A833YB29 and A0A2I4EG83) ones were the more frequently identified (Table S2). These proteins, which belong to PB (Wang et al., 2012), appeared to be distributed across a large range of MW, probably because of aggregation and disaggregation phenomena, which are particularly frequent when a high temperature processing occurs (Downs et al., 2016; Lamberti et al., 2021).

3.3 Comparison of the oleosin sequences already known to be allergens with the newly discovered walnut oleosins

All the walnut oleosins identified by both approaches were aligned, using the UniProt Align tool, with the oleosins of peanuts (*Arachis hypogaea*), hazelnuts (*Corylus avellana*) and sesame (*Sesamum indicum*) which are known to be allergenic. This analysis was aimed at highlighting the possible sequence similarities among oleosins from different plants, and at obtaining a preliminary evaluation of the potential allergenicity of walnut oleosins. The phylogenetic analysis (Fig. 5) showed five clearly divided clusters.

However, apart from the cluster made up of two peanut oleosins (*Ara h 10* and *Ara h 14*), the other four clusters grouped oleosins from different organisms, thus suggesting the absence of a strong genetic separation. The oleosins that appeared to be genetically closest are: A0A2I4GES3 with *Cor a 12*, G8H6H8 and G8H6H9 with *Cor a 13* (Q84T91), A0A2I4GNJ1 with *Ara h 15*, and A0A2I4GEC3 and A0A2I4DRG3 with *Cor a 15* (C0HM28) which has recently been discovered (Nebbia et al., 2021). The alignments of these specific oleosins are reported in Fig. 6. Cor a 13 and G8H6H9, with a similarity of 86.3 %, were the best matched oleosins, while the similarity decreased to 80.8% when G8H6H8 was considered. Cor a 12 and A0A2I4GES3 showed a similarity of 80.5%, while Cor a 15 and the two walnut oleosins A0A2I4GEC3 and A0A2I4DRG3 showed a similarity of 69.6% and of 62.6%, respectively. Lastly, *Ara h 15* and A0A2I4GNJ1 showed the lowest level of similarity (61.4%). As already observed, the transmembrane domains appeared to be highly conserved regions for all the alignments, while the N-terminal and C-terminal regions presented higher variability. This evidence suggests that the amino acid exchanges, which mostly involve

the outer-membrane regions, might give rise to new or modified epitopes with a different patients' IgE immune recognition (Schwager et al., 2017).

3.4 Processing affects the IgE recognition of walnut oleosins in allergic patients

The identification of all the oleosins indexed by UniProt was achieved by means of the off-gel approach. When analyzing the proteins by means of electrophoretic separation, only the boiled walnuts appeared to contain all the indexed oleosins. This result could suggest that the in-gel approach was less sensitive and it was more affected by processing than the off-gel approach. However, such an approach is needed to verify the recognition of allergenic proteins by the IgE of patients. Moreover, it is important to consider that, from an allergological point of view, the more abundant proteins are those that are more likely to be relevant in the phase of sensitization to the offending food (Bannon, 2004). This means that, even though the in-gel approach may be less suitable for proteome characterization, its limitations, in terms of sensitivity, may not be crucial when the goal is to discover the proteins involved in antibody recognition.

In order to preliminary investigate the possible allergenic nature of walnut oleosins, the sera of ten walnut allergic patients were pooled and used for IgE immune recognition against walnut oleosin extracts (Fig. 7). Interestingly, three bands that exclusively contained walnut oleosins (nos. 8, 17, and 18) were immune reactive to the IgE from walnut allergic patients. This is the first time that a possible involvement of oleosins in walnut allergy has been demonstrated.

A clear pattern correlating the IgE binding of oleosins with the different applied processing has been highlighted: the patients' IgEs showed a lower immune reactivity toward raw oleosins (bands 7 and 8) and roasted ones (band 26), while the boiled walnuts showed not only a higher number of oleosin containing bands, but also more immune reactive bands (bands 14, 16, 17 and 18). Differently from what reported by Downs et al (Downs et al., 2016) we demonstrated that the boiling procedure makes oleosin extraction more effective, this resulting in an enhancement of oleosin binding by patients' IgE. When considering the intensity values, the most abundant oleosins in the immune reactive bands are G8H6H8, G8H6H9, and

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A0A2I4GES3. As shown in the phylogenetic tree, and in the alignments with the oleosins already known as allergen in other seeds (sesame, peanut and hazelnut), the G8H6H9 oleosin appears to be the best candidate for further allergological characterization. In fact, it is similar to *Cor a 13,* which has already been demonstrated to be a risk factor for severe hazelnut allergies (Zuidmeer-Jongejan et al., 2014).

4. Conclusion

TThis study showed for the first time that walnut oleosins can be immune recognized by the sera of walnut allergic patients, thereby highlighting their possible involvement in walnut allergy. The shotgun approach showed an unexpected complexity of the oleosome sub-proteome suggesting that the different types of processing may affect the extraction and separation of OB associated proteins, even though to different extents. The effect of processing is particularly relevant when the oleosin allergenic potential is considered. When walnuts underwent a boiling process, a higher level of immune reactivity of the sera of allergic patients was observed. This effect may be due to either an increased oleosin extraction or a change in the epitope structures. Further investigations involving a larger number of patients and including digestomic experiments may help to clarify the allergenic potential of reacting oleosins and to what extent processing may permanently modify the existing epitopes.

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Figure captions

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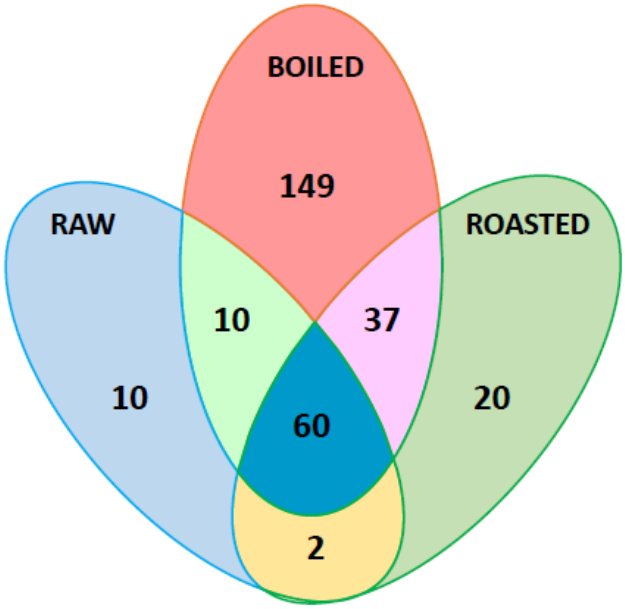

Fig. 1. Venn diagram showing the distribution of the proteins identified in the shot-gun proteomic analysis in the raw, boiled and roasted walnuts.

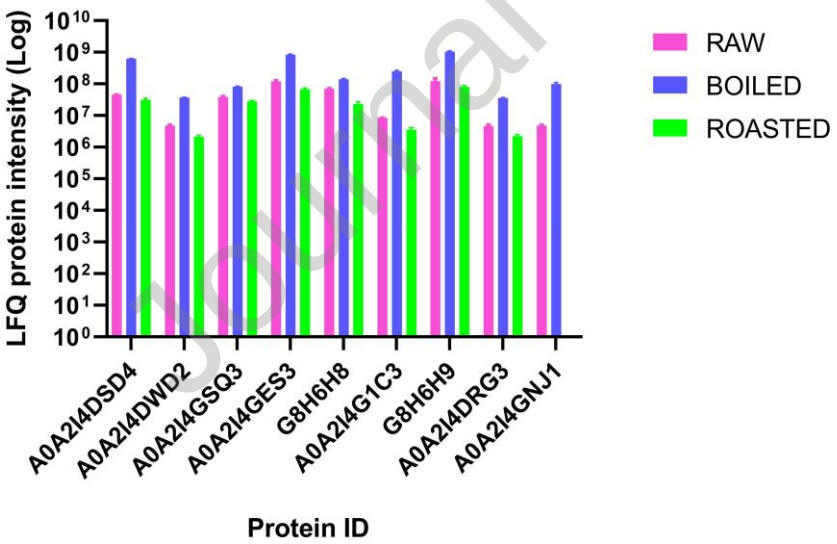

Figure 2

Fig. 2. LFQ proteomic comparison of the samples (raw, boiled and roasted). Relative abundance of the caleosin (A0A2I4DSD4), steroleosin (A0A2I4DWD2) and oleosins (A0A2I4GSQ3, A0A2I4GES3, G8H6H8, A0A2I4G1C3, G8H6H9, A0A2I4DRG3 and A0A2I4GNJ1) identified in the shotgun analysis (Table S1).

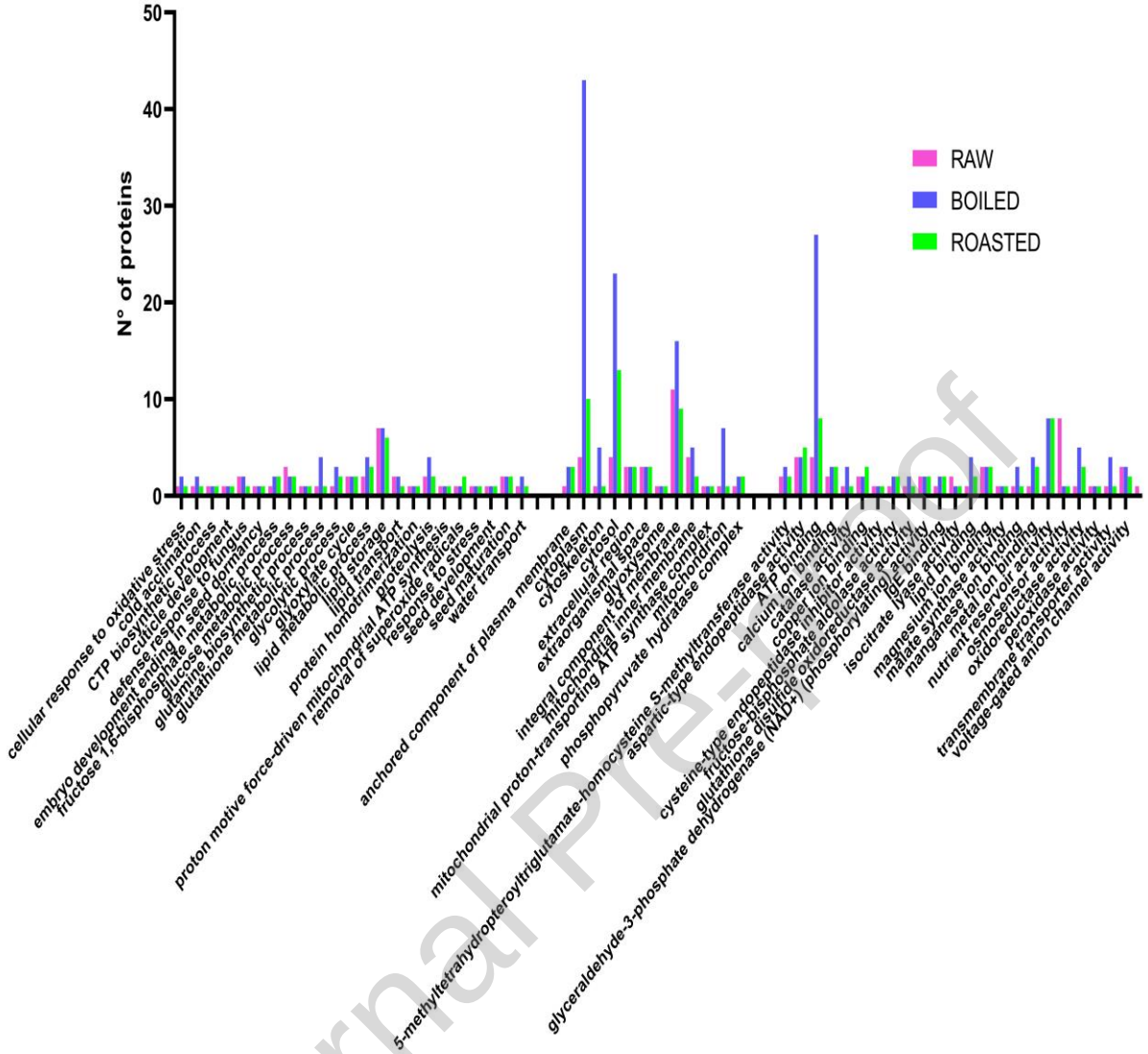

Figure 3

Fig. 3. Gene Ontology classification (biological process, cellular compartment and molecular function) of proteins identified through the shotgun analysis in the raw, boiled and roasted walnuts.

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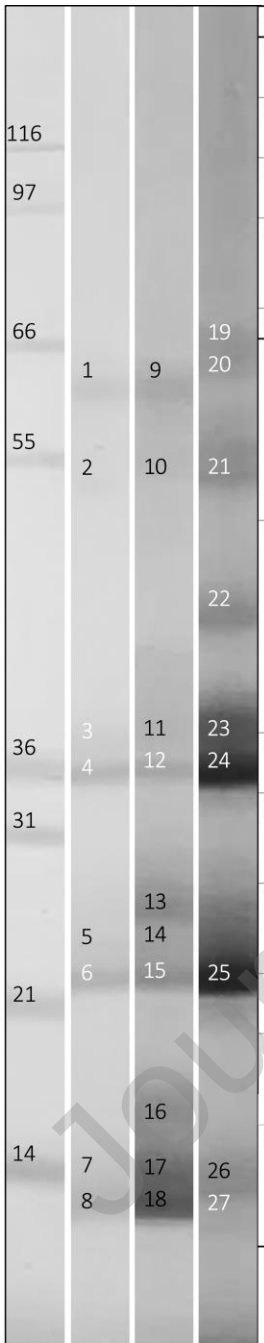

BAND	PROCESSING	ID	PROTEIN	RELATIVE MQ INTENSITY
1	raw	A0A2I4DSD4	caleosin	23.3%
			other proteins	76.7%
2	raw	A0A2I4DSD4	caleosin	6.9%
			other proteins	93.1%
5	raw	A0A2I4DSD4	caleosin	56%
			other proteins	44%
7	raw	G8H6H9	oleosin	59.1%
		A0A2I4GES3	oleosin 5-like	35.1%
			other proteins	5.8%
8	raw	G8H6H9	oleosin	100%
9	boiled	A0A2I4GES3	oleosin 5-like	35.5%
		G8H6H9	oleosin	21.3%
		A0A2I4DSD4	caleosin	17.9%
		G8H6H8	oleosin	7.5%
		A0A2I4G1C3	oleosin 18.2 kDa-like	3.7%
			other proteins	14.1%
10	boiled	G8H6H9	oleosin	26%
		A0A2I4GES3	oleosin 5-like	22.8%
		G8H6H8	oleosin	18.4%
		A0A2I4DSD4	caleosin	3.8%
		A0A2I4GSQ3	oleosin-like	3.3%
		A0A2I4G1C3	oleosin 18.2 kDa-like	1.5%
			other proteins	24.2%
11	boiled	A0A2I4GES3	oleosin 5-like	16.3%
			other proteins	83.7%
13	boiled	A0A2I4DSD4	caleosin	69.3%
		A0A2I4GES3	oleosin 5-like	25%
			other proteins	5.7%
14	boiled	A0A2I4DSD4	caleosin	5.6%
		A0A2I4GES3	oleosin 5-like	3.7%
			other proteins	90.7%
16	boiled	A0A2I4G1C3	oleosin 18.2 kDa-like	13.8%
			other proteins	86.2%
17	boiled	G8H6H9	oleosin	56.9%
		A0A2I4GES3	oleosin 5-like	39.1%
		A0A2I4GNJ1	oleosin	4.0%
18	boiled	A0A2I4GES3	oleosin 5-like	72.8%
		G8H6H9	oleosin	16.0%
		G8H6H8	oleosin	10.8%
		A0A2I4DRG3	oleosin 18.2 kDa-like	0.4%
26	roasted	G8H6H9	oleosin	17.3%
		G8H6H8	oleosin	16.7%
		A0A2I4GES3	oleosin 5-like	16.4%
		A0A2I4DRG3	oleosin 18.2 kDa-like	0.5%
			other proteins	49.1%

Figure 4

Fig. 4. LDS page of the raw (R), boiled (B) and roasted (S) walnuts. The bands containing oleosome proteins are in black. M: molecular weight (MW) markers. The table summarizes the OB associated proteins

identified by means of the in-gel approach. Relative intensity was calculated according to the formula: MQ intensity of the protein/total MQ intensity of all the proteins in the * 100 band.

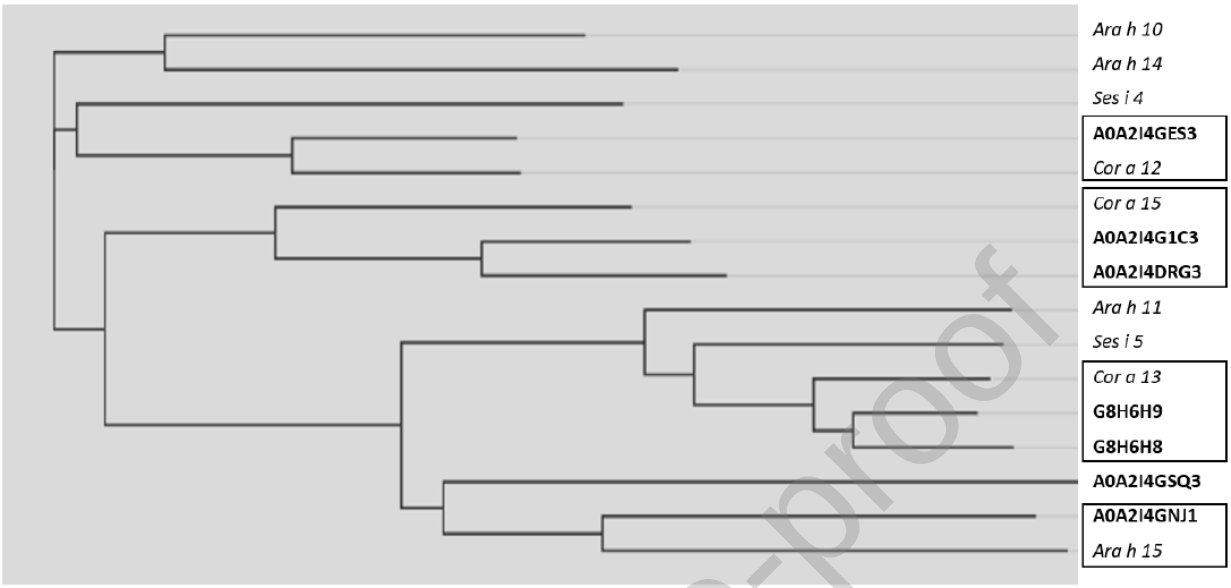

Fig. 5. Phylogenetic tree of the identified walnut oleosins (A0A2I4GES3, A0A2I4G1C3, A0A2I4DRG3, A0A2I4GNJ1, A0A2I4GSQ3, G8H6H8 and G8H6H9) and the allergenic oleosins of peanuts (Ara h 10, Ara h 11, Ara h 14 and Ara h 15), hazelnuts (Cor a 12, Cor a 13 and Cor a 15) and sesame (Ses i 4 and Ses i 5). Alignment was performed using the UniProt Align tool.

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A
A0A2I4GES3      MTDRPHQLLVHPQR---HYNGGPKSQR--GPSATKILAVLGGLPVGGTLLALAGLTLVGS    55
cor-a-12        MADRPQQLQVHPQRGHGHYEGGIKNQRGGGPSAVKVMAVLAALPVGGTLLALAGLTLAGS    60
                *:***:** *****   **:** *.**  ****.*..***..***************.**

A0A2I4GES3      LIGLAITTPLFIICSPVLVPAAIAIGLAIIGFLCSGALGLTGLVSLSWVLNYLRQASRSL    115
cor-a-12        VIGLLVTSPLFIIFSPVLVPAAIVVGLAVASFLSSGALGLTGLSSLSWVLNYLRCASQSL    120
                .*** .*:***** *********.***:  .**.********* ********** **:**

A0A2I4GES3      PQEMDQAKRRMQDMAAYVGQKTKEVGQEIQSKAQEGRRT 154
cor-a-12        PREMDQAKRRMQDMAAFVGQKTREVGQEIQSRAQEGRRT 159
                *:**************:*****:********:*******

B
cor-a-13        MAEHPRQLQDPAHQPRSHQVVKAATAATAGGSLLVPSGLILAGTVIALTLATPLFVIFSP     60
G8H6H8          MAEHQQQSQHPAQQPRSHQVVKAATAATAGGSLLLLSGLILASTVIALTVVTPLLVIFSP     60
G8H6H9          MAEHQQQSQRPDLQPRSHQVVKAATAATAGGSLLVLSGLILTGTVISLTVATPLFVIFSP     60
                **** :* * *  *********************: *****:.***:**:.***:*****

cor-a-13        VLVPAVITVSLIIMGFLASGGFGVAAVTVLSWIYRYVTGRHPPGADQLDHARMKLASKAR     120
G8H6H8          VLVPAVITVCLIIFGFLASGGFGVAALTVLSWIYRYMTGRQPPGTEQIDQARMKLASKAR     120
G8H6H9          VLVPAVITVALIILGFLASGGFGVAAVTVLSWIYRYVTGRQPPGAEQIDQARMRLASKAR     120
                ********.***:************:*********:***:***::*.*:***:******

cor-a-13        EMKDRAEQFGQQHVTGSQGS      140
G8H6H8          EMKDKAEQFGQQHVSGAQSS      140
G8H6H9          EMKDRAEQFGQQHVSGAQG-      139
                ****:*********:*:*.

C
cor-a-15        MADYQHQQQHQRPADA-FKGMFPEKGQAQVQGPSASKVIAVVTLLPLGGFLLLLAGLTFA     59
A0A2I4G1C3      ---MAEQYQQQRPTDTGMKGMFSG--KGQGQGPPTSKVIAVVTLLPLAGFFLLLSGLTLV     55
A0A2I4DRG3      ---MADQSHQQRPTDA-MKGMFAGQ-KGQAQGPPTSKVLAVVTLVPVAGFLLLLSGLTLA     55
                     .* ::***:*: :****    :.* *** :***:*****:*:.**:***:***:.

cor-a-15        GTLIGLALSTPLFVLCSPVLVPAAIVIGLAVTGFLTSGAFGITGISSLSWILKYLRGTSV     119
A0A2I4G1C3      ATLIGLAVSTPLFVICSPVLVPAAVVIALAVTGFLASGAFGITGLSSLSWIANYLRRTRV     115
A0A2I4DRG3      ATFIGLAVSTPLLVMCSPVLVPAAALIGLAMTGFLASGAFGITGISSLSWIANYLRRARL     115
                .*:****:****:*:********  :*.**:****:*******:******  :*** : :

cor-a-15        PEQMEHAKRRAQDTAGHLGQKARETGQTVTGKGQEAGKTLEGGRGEEKKT       169
A0A2I4G1C3      PEQLEHAKRRAHEAAGQMGQTVTGKAQETAGKAQEAG------RGEEKRT       159
A0A2I4DRG3      PEHLEHAKQRAQETAGQMGQTVTGKAQETAGRAQEAA------GTG----       155
                **:****:**:::**::**..  ..* .:*:.***.

D
ara-h-15        MSDQTRTGYGGGGSYGSSYGGGGTYGSSYGTSYDPSTNQPIRQAIKFMTASTIGVSFLIL     60
A0A2I4GNJ1      MSDHS--------------------RPVSQALYDPS-STSSRQAVKFLTAVTIGATLLIL     39
                ***::                       : **** .   ***:**:** ***.::***

ara-h-15        SGLILTGTVIGLIIATPLLVIFSPILVPAAITLALAAGGFLFSGGCGVAAIAALSWLYSY     120
A0A2I4GNJ1      SGLTLTGTVIALILATPVLVLFSPILVPAGIVLFLVAAGLVVSGGCGVVAMTALSWIYNY     99
                *** ******.**:***:**:********.*.* *.*.*::.******.*::****:*.*

ara-h-15        VTGKHPAGSDRLDYAKGVIADKARDVKDRAKDYAGAGRAQ--EGTPGY        166
A0A2I4GNJ1      VTGKHPMGADRLDYARMRIADKARDMKEKAKEYGQFVQHKAREATQGA        147
                ****** *:******:  *******:*::**:*.   : :  *.* *
```

Fig. 6. Amino acid sequence alignments (performed using the UniProt Align tool) of A0A2I4GES3 and Cor a 12 (A), G8H6H8, G8H6H9, Cor a 13 (B), A0A2I4GEC3, A0A2I4DRG3, Cor a 15 (C), A0A2I4GNJ1, and Ara h 15 (D). The black lines indicate the transmembrane domains. (*) ndicates positions which have a single, fully

conserved residue; (:) indicates conservation between groups of strongly similar properties; (.) indicates conservation between groups of weakly similar properties. A blank space signifies none of the above.

Alignment was performed using the UniProt Align tool.

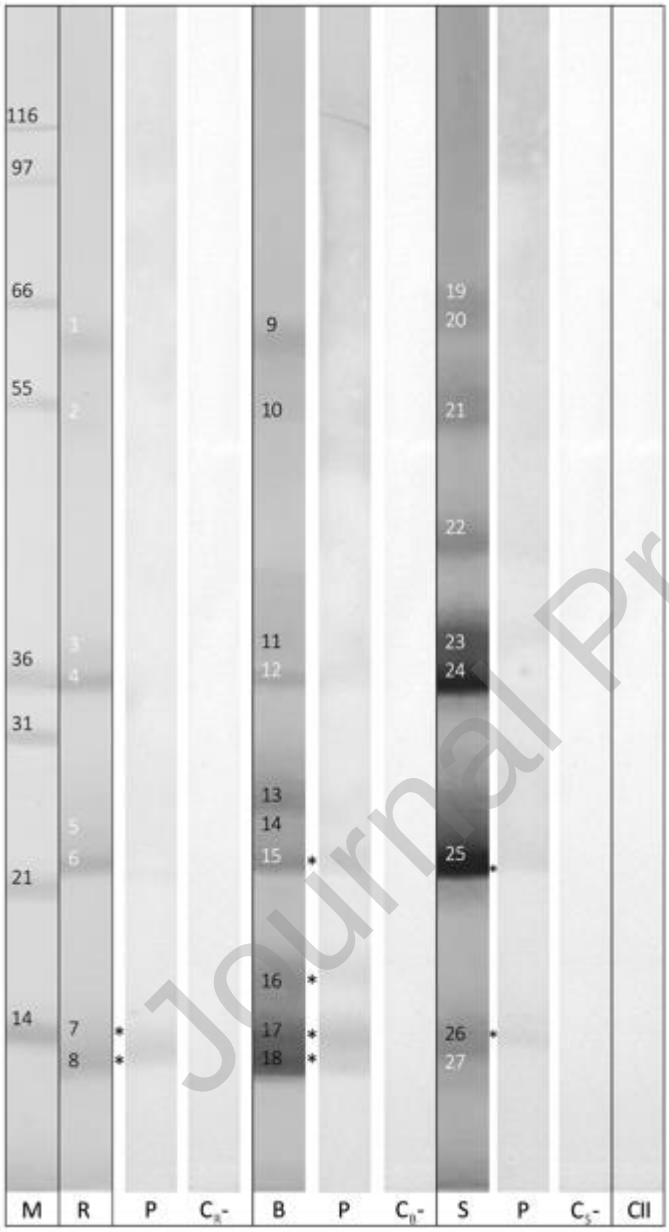

Fig. 7. Immunoblotting of raw (R), boiled (B) and roasted (S) walnuts performed with a pool of ten sera of walnut allergic pediatric patients (P). C-: pool of ten sera of non-nut-allergic pediatric subjects used as a negative control. CII: secondary antibody control. M: molecular weight markers. * Immunoreactive bands. On the LDS-PAGE of each extracts (R, B and S) the oleosins containing bands are numbered in black.

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Highlights

- Eight oleosins were identified from the oil body extracts by the shotgun approach
- Most of the identified proteins belong to the lipid storage and lipid metabolic process
- Sera of walnut allergic patients' immune recognized walnut oleosins
- Processing affects oleosin extraction and immune recognition by walnut allergic patients' sera

Declaration of Competing Interest

The authors have no conflicts of interest to disclose.

CRediT Author statements

Cirrincione Simona: Conceptualization, Methodology, Investigation, Writing - Original Draft, Writing-review & editing. **Aiuto Beatrice**: Investigation, Methodology, Validation. **Gosso Elena:** Investigation, Metodology, Validation. **Schiavone Consolato:** Investigation, Validation. **Portesi Chiara:** Writing-review & editing, Funding acquisition. **Rossi Andrea Mario:** Writing-review & editing, Project administration. **Monti Giovanna**: Conceptualization, Resources. **Cavallarin Laura**: Conceptualization, Writing - Original Draft, Project administration, Funding acquisition. **Lamberti Cristina**: Conceptualization, Investigation, Supervision, Writing - Original Draft, Writing-review & editing. **Giuffrida Maria Gabriella**: Conceptualization, Supervision, Writing - Original Draft.

All authors read and approved the manuscript.

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