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Effect of thermal/pressure processing and simulated human digestion on the immunoreactivity of extractable peanut allergens

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A B S T R A C T

Peanut allergy is one of the most widespread types of food allergies especially affecting developed countries. To reduce the risk of triggering allergic reactions, several technological strategies have been devised to modify or remove allergens from foods. Herein we investigated the combination of high temperature and pressure on the modulation of peanuts immunoreactivity after simulated gastro-duodenal digestion. Extractable proteins of raw and autoclaved peanuts were separated on SDS-PAGE and immunogenicity was assessed by ELISA and Western Blot analyses. Proteins surviving the heat treatment and reacting towards allergic patients' sera were analysed and attributed to Ara h 3 and Ara h 1 proteins by untargeted LC-high resolution-MS/MS. A progressive reduction in the intensity of the major allergen proteins was also highlighted in the protein fraction extracted from autoclaved peanuts, with a total disappearance of the high molecular allergens when samples were preliminary exposed to 2 h hydration although the lower molecular weight fraction was not investigated in the present work. Furthermore, raw and processed peanuts underwent simulated digestion experiments and the IgE binding was assessed by using allergic patients' sera. The persistence of an immunoreactive band was displayed around 20 kDa. In conclusion, the synergistic effects of heat and pressure played a pivotal role in the disappearance of the major peanut allergens also contributing to the significant alteration of the final immunoreactivity. In addition, the surviving of allergenic determinants in peanuts after gastrointestinal breakdown provides more insights on the fate of allergenic proteins after autoclaving treatments.

1. Introduction

Food-induced allergy (FA) represents a public health problem affecting adults and children with a rising growth throughout the population especially in the developed countries. The current management of FA relies on the strict avoidance of the trigger food (Hebling, Ross, Callahan, & McFarland, 2012; Sicherer et al., 2010). Peanut allergy is one of the most widespread and life-threatening type of food allergy and is considered to be the major cause of anaphylactic shock (Al-Muhsen, Clarke, & Kagan, 2003; Pumphrey & Gowland, 2007).

Currently 16 peanut allergenic proteins have been registered by the IUIS Allergen Nomenclature Sub-Committee under the auspices of the World Health Organization (WHO) and the International Union of Immunological Societies (IUIS) in WHO/IUIS Allergen Nomenclature Database (http://www.allergen.org/). Among them, seed storage proteins Ara h 1, Ara h 2, Ara h 3 and Ara h 6 are considered the most important allergens and predictive of allergic reactions (Koppelman, Hefle, Taylor, & de Jong, 2010). These proteins display different chemical, physical and structural characteristics. Ara h 1 is a glycoprotein of 65-kDa belonging to the cupin family. It is the most abundant allergen of peanuts and naturally occurs as a symmetrical non-covalent trimer with a 3-fold axis running between the monomers. Each monomer is comprised of two cupin domains (known as a bicupin) with small cavities flanked by α-helices (Mueller, Maleki, & Pedersen, 2014; Shin et al., 1998; Van Boxtel, Van Beers, Koppelman, Van Den Broek, & Gruppen, 2006).

Ara h 3 also belongs to the cupin family and shares 21% sequence identity with Ara h 1. Despite the low sequence identity, the crystal

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structure of Ara h 3 is very similar to that of Ara h 1. Ara h 3 forms a heat- stable hexameric structure consisting of two Ara h 1-like trimers stacked head to head (Adachi et al., 2003; Boldt et al., 2005; Dodo, Viquez, Maleki, & Konan, 2004; Guo, Liang, Chung, & Maleki, 2008; Liang, Luo, Holbrook, & Guo, 2006). This allergenic protein is post-translationally modified by a proteolytic cleavage that occurs between the two cupin domains on a flexible loop. The processed protein consists of a triplet at approximately 42–45 kDa, another distinct band at approximately 25 kDa, and some less abundant isoforms banding between 12 and 18 kDa (Koppelman, Wensing, Ertmann, Knulst, & Knol, 2004; Piersma, Gaspari, Hefle, & Koppelman, 2005).

On the contrary, 2S albumins Ara h 2 and Ara h 6 together with a third low abundant 2S albumin Ara h 7, have a single chain precursor, proteolytically cleaved in peanut seeds into two subunits linked by intramolecular disulphide bonds (Bernard et al., 2007; Shewry, 1995). All members of this superfamily share a characteristic cysteine skeleton with at least 8 conserved cysteine residues (Shewry, 1995) and a three-dimensional structure comprising 5 α-helices arranged in a right-handed super helix that give the stability to thermal processing and proteolysis (Barre, Borges, Culerrier, & Rougé, 2005; Lehmann et al., 2006; Marsh et al., 2008).

Several strategies have been developed over the years aimed to reduce or prevent peanuts allergenicity and representing potential alternatives to a strict peanuts-free diet. The most interesting ones are based on enzymatic hydrolysis, physical approach or genetic modification methods. Among the physical methods, there are heat-based treatments which involve chemical modification such as denaturation or covalent bound of protein allergen with other nutrients including lipids and carbohydrates (Maillard reaction) (Jiménez-Saiz, Benedé, Molina, & López-Expósito, 2015). These modifications can produce an effect on the final allergenicity that might vary considerably depending on the temperature, type and duration of the treatment, the intrinsic characteristics of the protein and the physicochemical conditions of the food matrix under investigation (Nesbit et al., 2012; Sathe & Sharma, 2009; Schmitt, Nesbit, Hurlburt, Cheng, & Maleki, 2010). However, the effect of thermal treatments on peanuts has been questioned in the recent years. Whether roasting was reported to increase Ara h 1 and Ara h 2 allergenicity probably consequent to the formation of new epitopes, other hand treatments such as boiling or autoclaving were reported to effectively decrease peanut allergenicity (Blanc et al., 2011; Cabanillas et al., 2012). Herein we investigated the effect of autoclaving with or without preliminary hydration, performed at the temperature of 134 °C and the pressure of 2 atm, on peanut seeds in order to evaluate any alteration on the final immunoreactivity assessed on the soluble protein fraction by ELISA and western blot analysis by using allergic patients' sera. Furthermore, autoclaved peanuts were submitted to a standardized static *in vitro* digestion protocol in order to assess any change in allergen protein stability as a consequence of the technological process applied.

2. Material and methods

2.1. Chemicals

Trizma-base, sodium chloride, urea, ammonium bicarbonate (AMBIC), iodoacetamide (IAA), along with other chemicals for electrophoresis dithiothreitol (DTT), sodium dodecyl sulfate-SDS, glycine, glycerol, Coomassie brilliant blue-G 250 and methanol (HPLC grade) were purchased from Sigma-Aldrich (Milan, Italy). Bromophenol blue was provided by Carlo Erba Reagents (Cornaredo, Italia). Syringe filters in cellulose acetate (CA) from 1.2 μm were obtained from Labochem Science S.r.l. (Catania, Italy) whilst 0.45 μm filters in Polytetrafluoroethylene (PTFE) were purchased from Sartorius (Gottingem, Germania). Acetonitrile (Gold HPLC ultragradient), and trifluoroacetic acid (TFA) were purchased from Carlo Erba Reagents (Cornaredo, Milan, Italia) and ultrapure water was produced by a Millipore Milli-Q system (Millipore, Bedford, MA, USA). Formic acid (MS grade) was provided by Fluka (Milan, Italy) while trypsin (proteomic grade) for in gel protein digestion was purchased from Promega (Milan, Italy). As for *in vitro* digestion model, pepsin, trypsin, chymotrypsin, Tris-HCl, urea, guanidine chloride, phospholipids and p-toluene-sulfonyl-L-arginine methyl ester (TAME) were purchased from Sigma-Aldrich (St Louis, MO, USA). Egg lecithin was purchased from Lipid Products (Redhill UK).

2.2. Sera of peanuts allergic patients

Sera were obtained from a total of 8 pediatric peanut allergic subjects with an age comprised between 3 and 8, according to the ethical requirements. The local Ethics Committee approved the study. The allergy symptoms in general ranged from urticaria to angioedema and anaphylaxis. The clinical features of the allergic individuals enrolled in this study are reported in Table S1. Since 2 out of 8 patients deemed allergic to peanuts did not show a meaningful reactivity to the SPT (wheal lower than 0.3 cm) and the specific IgE content was lower than 0.35 kUA/l, only a total of 6 reactive sera were pooled together and used for further analysis. Diagnosis of IgE-mediated allergy to peanut was confirmed by skin prick test (SPT) and oral food challenges. Either a SPT peanut extract or fresh peanut (prick-by-prick) was applied to the patients' volar forearm. Tests were performed using a 1-mm single peak lancet (ALK, Copenhagen, Denmark), with histamine dihydrochloride (10 mg/ml) and isotonic saline solution (0.9% NaCl) as the positive and negative controls, respectively. Reactions were recorded based on the largest diameter (in cm) of the wheal and flare at 15 min. A SPT result was considered “positive” if the wheal was 0.3 cm or larger, without a reaction to the negative control. The total serum IgE was quantified with the ImmunoCAP system (Phadia, Uppsala, Sweden) and was found to be ranging between 33 and 1836 kU/l. In particular, 6 out of the 8 patients enrolled in the study were found positive to the SPT and to the IgE assay with specific levels of IgE to peanuts higher than 0.35 kUA/l. All sera were stored at −20 °C before being used. Other details are reported in the manuscript from Di Stasio et al. (2017).

2.3. Autoclaving based treatments

Raw peanut seeds (*Arachis hypogaea* var. *Virginia*) analysed in the present study were provided from Besana s.p.a. (San GennaroVesuviano, NA, Italy). A total of 8 seeds (corresponding to approximately 10 g) were placed into a centrifuge tube and submitted to autoclaving treatments. Two processing schemes were applied including or not a preliminary hydration of the peanuts. The hydration of whole peanut seeds were performed for 2 h at room temperature in an orbital shaker (KS 4000 i-control shaker, IKA Works GmbH & Co. KG, Staufen, Germany) with ultrapure water, before autoclaving. Autoclave settings were: temperature at 134 °C at the pressure of 2 atm for 10 min and 20 min, respectively. The system took about 40 min to reach the final temperature of 134 °C.

2.4. Protein extraction and quantification

Ten gram of raw and thermally processed peanut seeds were milled by using an electric miller (Mulinex, Milan, Italy) and an aliquot was extracted by 7 M Urea (pH 8) containing TBS (50 mM Tris-HCl, 150 mM NaCl) buffer. Briefly, 10 ml of extraction buffer were added to 0,4 g of sample and left shaking for 1 h at room temperature in an orbital shaker (KS 4000 i-control shaker, IKA Works GmbH & Co. KG, Staufen, Germany). Afterwards, samples were centrifuged for 15 min at 1734*g* at 18 °C, the upper phase was discarded and the supernatant was carefully collected and filtered through 1.2 μm CA syringe filters. Protein concentration of raw and thermally processed peanuts was calculated as mg/albumin equivalent by Bradford assay (Quick Start™ Bradford Protein Assay). Samples were stored at −20 °C until use and filtered through 0.45 μm PTFE filters just before electrophoretic analysis.

2.5. ELISA assay

The decrease in the level of peanut allergens was evaluated by using a commercially available peanut ELISA kit (RidaScreen Fast, R-Biopharm, Germany), according to the instructions provided by the manufacturer. The R-Biopharm kit was directed to detect raw and roasted peanut proteins, although the antibodies immobilized in the kit were mainly raised against Ara h 1 and Ara h 2 proteins (as reported by the instructions). Samples were assayed at 1:10,000 dilution, to obtain test values within the standard calibration curve and analysed in three replicates. Plates were read at the wavelength 450 nm using a microplates reader (BioTek Instruments Inc. USA). Three extracts were analysed for each treatment under investigation and final results underwent statistical analysis according to the Tukey-Kramer test for multiple mean comparison.

2.6. SDS-PAGE analysis

Ten microgram of protein extracts from raw and treated peanuts, along with *in vitro* digested proteins, were separated, under reducing condition, by means of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 8–16% polyacrylamide pre-cast gels (8.6 cm × 6.7 cm × 1 mm) using a Mini-Protean Tetra Cell equipment (Bio-rad Laboratories, Segrate, MI, Italy). Samples were dissolved in a Laemmli buffer (62.5 mM TrisHCl, pH 6.8, 25% glycerol, 2% SDS, 0.01% Bromophenol Blue, in the presence or not of 100 mM DTT) (1:1 ratio) and denatured for 5 min at 100 °C. Running buffer was TGS (25 mM Tris, 192 mM Glycine, 0.1% SDS). Electrophoretic separation was performed at 100 V until the end. Gels were stained by using a Coomassie Brilliant Blue G-250 solution and the bands were detected on a Gel Doc EZ Imager system (Bio-Rad Laboratories, Segrate, MI, Italy). Precision Plus Protein™ all blue standards (10–250 kDa, Bio-Rad Laboratories) was used as protein molecular weight referencing.

2.7. In-gel protein digestion

Selected protein bands were cut from the polyacrylamide gel and destained by repeated washing (45 min, 37 °C) with 100 mM AMBIC/acetonitrile (1/1, v/v). Gel slices were further dehydratated in 100 μl of acetonitrile (5 min at room temperature) and dried in a "speed Vac" centrifuge (Christ RVC 2-18, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) for 10–15 min (room temperature). After drying, proteins were reduced by adding 10 mM DTT (prepared in 25 mM AMBIC) for 1 h at 60 °C and alkylated for 30 min (room temperature) with 55 mM iodoacetamide (prepared in 25 mM AMBIC). Digestion was carried out overnight at 37 °C with proteomic grade trypsin solution (0.1 μg/μl, enzyme: protein ratio 1:50) in 25 mM AMBIC. Successively, gel slices were incubated with 150 μl of MilliQ water for 10 min, with frequent vortex mixing. Then the liquid was removed and transferred into a new microcentrifuge tube. Peptides extraction from gel was accomplished by incubation with 50% acetonitrile/5% trifluoroacetic acid/ (1/1, v/v) for 60 min. This step was repeated twice. Peptide mixtures obtained from each extraction step were then pooled together and dried. Finally each sample was resuspended in 70 μl of H_2O/ACN, 95/5 + 0.1% formic acid (*v*/v) and 20 μl were further injected into LC/MS apparatus.

2.8. Protein identification by untargeted HR MS/MS analysis

Protein bands were analysed by using a Q-Exactive™ Plus Hybrid Quadrupole-Orbitrap™ Mass Spectrometer (Thermo Fisher Scientific, Bremen, Germany) coupled to a UHPLC pump systems (Thermo Fisher Scientific). Peptides mixture was separated on a reversed phase Aeris peptide analytical column (internal diameter 2.1 mm, length 150 mm, particle size 3.6 μm, porosity 100 Å, Phenomenex, Torrance, CA, US) at a flow rate of 200 μl/ml, using the following elution gradient: from: 0–55 min solvent B increased from 5% to 60%, 55–56 min further increase from 60% to 80%, then kept constant for 10 min, 66–85 min at a constant 5% for column conditioning before next injection. MS Spectra were acquired in positive ion mode. The HESI ion source setting are here reported: spray voltage at 3.4 kV, capillary temperature at 320 °C, sheath gas and auxiliary gas flow rates at 25 and 15 arbitrary units, respectively, S-lens at 55. MS analysis was carried out in data-dependent MS^2 acquisition mode (dd-MS^2). Up to 10 most intense ions in MS^1 were selected for subsequent fragmentation in MS/MS mode. A resolving power of 70,000 full width at half maximum (FWHM), a microscan of 1, an automatic gain control (AGC) target of 1 e6 and a maximum injection time (IT) of 30 ms were set to generate precursor spectra into the scan range 200–2000 m/z (full MS analysis). The parameters for MS^2 fragmentation experiments were set as following: resolving power 17,500 FWHM, microscan of 1, AGC target 1 e^5, maximum IT 60 ms, loop count 10, MSX count 1, isolation window of 2 m/z, isolation offset 0.4 m/z and normalized collision energy (NCE) at 27 eV; as for dd-setting maximum AGC target was set at 5.00 e^2, dynamic exclusion at 20 s, peptide match set to preferred and exclude isotopes enabled. All ions with charge equal to 1 and higher than 4 were excluded.

Raw data were processed *via* the commercial software Proteome Discoverer™ version 2.0 (Thermo-Fisher-Scientific, San Josè, US) and protein identification was achieved by Sequest^{HT} search against a peanut customized database extracted by Swiss Prot DB basing on the taxonomy code of *Arachis hypogaea* (ID: 3818) and containing about 1250 sequences. The identification of tryptic peptides originated by in gel digestion experiments was accomplished by setting at 5 ppm and 0.05 Da, respectively, the mass tolerance on the precursor and fragment ions. Only trustful peptide-spectrum matches were accepted and in particular a minimum of three peptides or higher were the minimum criteria for protein identification by selecting a high confidence (FDR < 1%).

2.9. Immunoblot for IgE-binding assay

SDS-PAGE of peanut protein extracts (corresponding to 5 μg of proteins loaded of both raw and treated peanuts) and SDS-PAGE of *in vitro* digested proteins (approximately 6 μg loaded onto the gel), under reducing and non-reducing conditions, were electroblotted onto nitrocellulose paper using a Trans-Blot Cell from BioRad (Bio-Rad Laboratories, Hercules, CA, USA) at 100 V and 4 °C for 1 h. Membranes were blocked for 1 h at room temperature with 5% bovine serum albumin (Sigma) in TBS containing 0.05% of Tween 20 (TBS-T). The membranes were incubated overnight at 4 °C with a pool of sera of young allergic patients (3–8 age) and healthy individuals were chosen as negative controls, by 1/20 dilution in TBS-T. After washing with TBS-T, monoclonal peroxidase-conjugated mouse anti-human IgE antibody (Sigma) diluted in blocking solution (1/10,000) was applied to the membrane for 1 h at room temperature. The membrane was extensively rinsed with TBS-T (3 × 10 min) and finally with TBS (1 × 10 min) before development. Chemiluminescence reagents (ECL Plus WB reagent, GE Healthcare) and X-ray film (Kodak, Chalons/Saône, France) were used to visualize the immunoreactive protein bands at various exposure times ranging from 0.5 to 10 min.

2.10. In vitro gastroduodenal digestion of raw and treated peanuts

Peanut seeds autoclaved for 10 min with and without pre-hydration, were submitted to *in vitro* gastro-duodenal digestion according to the protocol by Minekus et al. (2014). Raw peanuts were instead used as control. Simulated salivary fluid (SSF), simulated gastric fluid (SGF), and simulated intestinal fluid (SIF) were prepared according to the harmonized conditions. All digestion steps were carried out in a shaking incubator at 37 °C, at 170 rpm. For the oral phase, peanuts were grossly minced using a coffee grinder and 100 mg of the resulting coarse powder was suspended in 207 μl of SSF (included of 1500 U/ml of

human salivary amylase) and incubated for 2 min. Subsequently, the oral digest was mixed with 320 μl SGF containing 8 μl of phospholipids (10 mg/ml). The pH was adjusted to 2.7 with HCl 3 M and 40 μl of porcine pepsin (3000 U/mg) at a concentration of 12 mg/ml was added. Samples were then incubated for 2 h at 37 °C. Pepsin hydrolysis was stopped by raising the pH to 7.0 with 1 M sodium bicarbonate. The duodenal digestion was carried out for 2 h at 37 °C after incorporating 640 μl of SIF, bile salts (16 mg), porcine pancreatic lipase (1 mg), trypsin (0.7 mg, 100 U/mg as TAME activity), α-chymotrypsin (0.3 mg, 40 U/mg) and pancreatic α-amylase (1.1 mg, 10 U/ml). A final step of acidification with HCl was performed to stop the enzymatic reaction. After digestion, samples were subjected to a defatting step with diethyl ether under magnetic stirring (two steps of agitation for 10 min), followed by centrifugation at 10,000*g* (10 min). Large-sized polypeptides were precipitated with TCA up to a final concentration of 30% (w/v). After centrifugation, pellet was four-fold washed with 1 ml of cold acetone, to remove the residual TCA. The final digest was re-suspended in 50 μl of sample buffer before loading it onto the electrophoresis gel.

3. Results and discussion

3.1. Effect of autoclaving on the extractable peanut proteins/allergens as assessed by Bradford and ELISA assays

Raw and autoclaved peanuts were extracted with a 7 M urea extraction buffer, the extract was preliminary quantified by Bradford assay and subsequently analysed by SDS-PAGE. According to the result of the Bradford assay, a decrease in the protein levels was recorded in the extractable protein fraction, which extent varied in dependence of the processing conditions applied. In particular, a protein reduction down to 40 and 25% was pointed out in autoclaved peanuts strictly related to the extension of the autoclaving applied (10 or 20 min), as shown in Fig. 1. Our findings are in agreement with data obtained by Fu et al. that also found a decrease in protein content down to 38% according to the results of the BCA assay performed on peanut flour autoclaved for 10 min (Fue & Macs, 2013). This trend was even more remarked when seeds were hydrated (for 2 h) before autoclaving at 134 °C and 2 atm. In this case, a dramatic reduction in the extractable proteins down to nearly 15% in hydrated and autoclaved samples was highlighted, as pictured in Fig. 1. In order to investigate on the reduction of the main allergenic proteins recognized by the most common antibody-based kits, samples were analysed by ELISA kits. A general decrease in the IgG reactivity was observed after autoclaving. In particular, Fig. 2 reports in histograms the results of the ELISA tests carried out on raw and autoclaved peanuts with and without pre-incubation in water. Compared to the raw material, where a very high reactivity was recorded, in peanuts undergoing the autoclaving treatments preceded or not by hydration, a modulation of the immunoreactivity was observed.

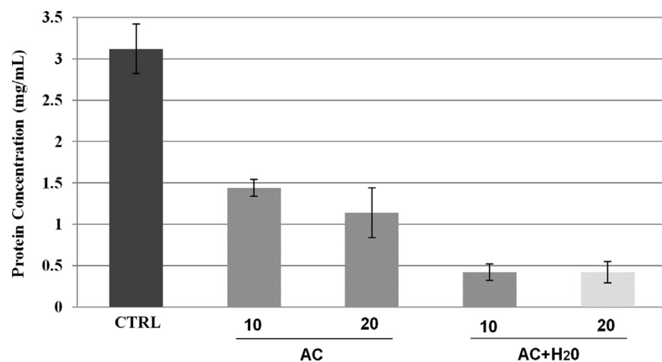

Fig. 1. Protein content in peanut extracts referred to raw (CTR), autoclaved (AC) samples for 10 and 20 min and pre-hydrated and autoclaved samples for 10 and 20 min at 134 °C.

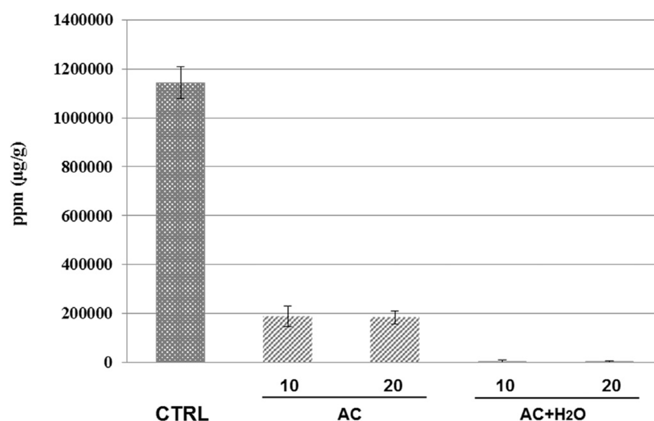

Fig. 2. Immunoreactivity of peanut proteins measured by ELISA referred to raw (CTRL), autoclaved (AC) samples for 10 and 20 min and pre-hydrated and autoclaved samples (AC + H_2O) for 10 and 20 min at 134 °C.

Similar results were also reported by Fu et al. indicating that despite protein quantification by BCA assay performed, the heat treatments resulted in a lower level of peanuts detected by using two different ELISA test kits (Fue & Macs, 2013). The degree of underestimation differed depending on the extent and type of heating applied and the specific test kit employed in the study (Fue & Macs, 2013).

According to Fig. 2, the reduction in immunoreactivity, in peanuts autoclaved for 10 and 20 min, was calculated to be approximately 78% compared to the control. By contrast, when a hydration step preceded the heating, a total absence of antibody reactivity was observed at both investigated times. All results underwent statistical Tukey-Kramer tests for multiple mean comparison and a statistically significant difference in the final immunoreactivity was found. In general, the efficacy of the treatment appeared to be enhanced when a preliminary incubation of the seeds in water was introduced along the procedure. It is worth noting that according to manufacturer instructions, the antibodies immobilized on the ELISA micro wells were directed towards Ara h 1 and Ara h 2; consequently, this kit can assess reactivity towards these only proteins.

3.2. Results of SDS PAGE and MS/MS analysis in peanuts subject to different processing

Peanut extracts were further subjected to SDS-PAGE analysis as shown in the Fig. 3. In addition, in order to deepen the knowledge on the stability of peanut allergens undergoing autoclaving treatments, in-gel tryptic digestions were carried out on selected bands detected in lanes 1–2–3-4 and labelled from *a* to *q* (see Fig. 3) and the resulting peptide pool was further analysed by HPLC and untargeted HR MS detection.

Protein identification was accomplished by means of a commercial software using HR MS and MS/MS spectra obtained for each individual protein band analysed and by putatively assigning each peptide detected to the corresponding peanut protein and/or subunit.

As pictured in the gel (Fig. 3), proteins 1 and 2 detected along lane 1 appeared unresolved, therefore they were pooled together, marked as band *b,* and further processed as a single spot. Table 1 summarizes the results retrieved by the software for each spot analysed. For more info on the list of peptides detected, please see Supplementary material Table S2. Due to the low resolution of the SDS-PAGE technique, several proteins were identified in the same band. As shown in Fig. 3, protein bands referred to raw peanuts (Fig. 3, lane 1) with molecular weight comprised between 60 and 150 kDa (Fig. 3, lane 1, band *a* and *b*) were mainly attributed to Ara h 1, while bands between *c* and *i*, with MW in the range 25–50 kDa were assigned to Ara h 3. Moreover, *c* and *g* bands also contained fragments of Ara h 1. Finally, protein bands at lower MW

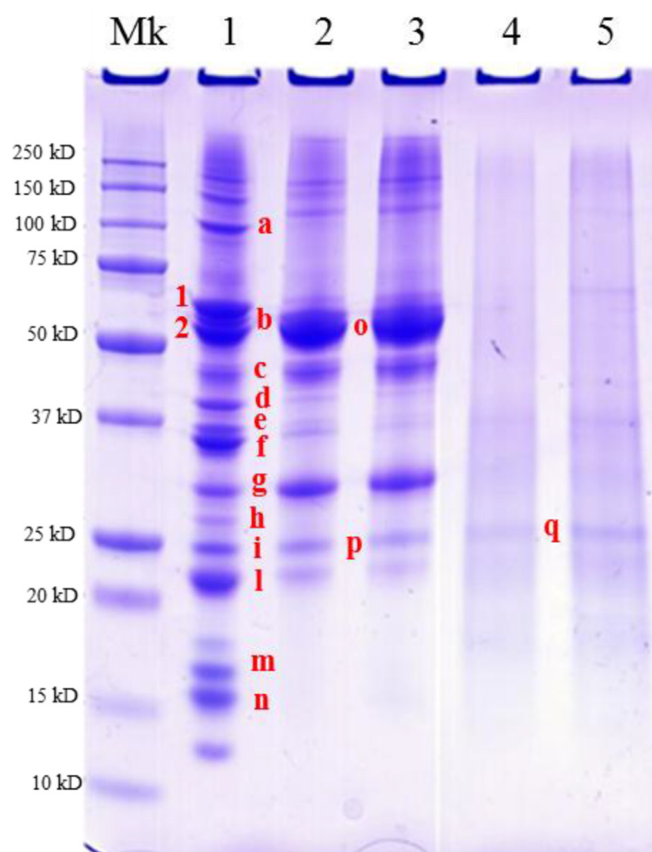

Fig. 3. SDS-PAGE of peanuts submitted to the different treatments: raw (lane 1), autoclaved at 134 °C, 2 atm for 10 min (lane 2) and for 20 min (lane 3), pre-hydrated and autoclaved at 134 °C, 2 atm for 10 min (lane 4) and for 20 min (lane 5).

(below 25 kDa, Fig. 3, lane 1, bands *l*, *m* and *n*) were mainly attributed to Ara h 2, Ara h 6 and Ara h 7, along with some subunits belonging to Ara h 3 group.

By comparing sample protein profiles of raw (Fig. 3, lane 1) and autoclaved (Fig. 3, lanes 2–3, 4–5) peanuts employing or not a pre-hydration, a significant difference in the electrophoretic bands was displayed. Protein bands detected in autoclaved samples at MW above 75 kDa (Fig. 3, band *a*, lane 1) in raw peanuts, showed a marked reduction when samples were autoclaved for 10 and 20 min at 134 °C (Fig. 3, lanes 2 and 3). Proteins banding around 65–60 kDa appeared to be composed by two close bands (*1* and *2* in lane 1): the higher (band 1) attributed to Ara h 1 disappeared after the treatment, whereas the lower showed to be resistant to the autoclaving (Fig. 3, bands *b* and *o* in the lanes 2 and 3). A previous study had already shown that the level of soluble Ara h 1 was greatly reduced in the boiled/autoclaved samples while remained unaffected in samples dry-heated at temperatures up to 176 °C (Fue & Macs, 2013). According to their results, authors demonstrated that the higher temperatures and pressure applied during autoclaving resulted in a similar decrease in protein yield and changes in the intensities of certain protein/peptide SDS-PAGE bands of what we found in our work. Dry-heat treatments also resulted in a decrease in protein solubility, although the decrease occurred at a much higher temperature (≥176 °C), suggesting that peanut proteins are more resistant to thermal denaturation under dry-heat conditions (Fue & Macs, 2013). In stark contrast with the bands at higher MWs, the band labelled as 1 in raw peanuts (at nearly 20 kDa) exhibited a much weaker intensity after autoclaving. Basing on bioinformatics tools for protein attribution, band *a* was assigned to Ara h 1, while band *b* (Fig. 3, lane 1, merge of band 1 and 2) and *o* were both assigned to Ara h 1 and to Ara h 3 proteins. The remarked decrease of band intensity after autoclaving, suggests a partial degradation or a rearrangement of the original protein. A thermal resistance, was observed also for bands *c* (with MW slightly below 50 kDa) and *g* (MW approximately 30 kDa), where the intensity was found nearly unaltered both in 10 min and 20 min autoclaved samples (Fig. 3, lanes 2 and 3) compared to raw peanuts. These protein bands were instead attributed both to Ara h 1 and Ara h 3 allergen proteins. By contrast, protein bands around 37 kDa (Fig. 3, lane 1, bands *d*, *e* and *f*) and protein banding at 26 kDa (Fig. 3, lane 1, band *h*), all assigned to Ara h 3 in untreated peanut, were not evidenced in autoclaved samples suggesting a susceptible behaviour of these proteins to the proposed treatment. Similar results were also displayed for proteins banding below 20 kDa (Fig. 3, lane 1, bands *m* and *n*), mainly assigned to Ara h 3. A different behaviour was observed for protein bands with MW between 20 and 25 kDa (Fig. 3, lane 1, bands *i* and *l*), where a partial reduction of intensity was displayed after autoclaving (Fig. 3, lane 2–3, bands *p*). Interestingly, while in the raw sample the protein bands *i* and *l* were mainly attributed to Ara h 2 and Ara h 7 along with Ara h 3 allergens, the corresponding band visualized in autoclaved samples (Fig. 3, lanes 2 and 3, band *p*) was putatively attributed only to Ara h 3; this suggests that Ara h 2 and Ara h 7 allergens are likely affected by thermal/pressure treatment. Concerning pre-hydrated and autoclaved peanuts at 134 °C for 10 and 20 min, a drastic reduction in the protein content of the extracts was highlighted by SDS-gel and it is worth noting that no protein bands were detectable in the corresponding electrophoretic patterns (Fig. 3, lanes 4 and 5 respectively), with the exception of a faint band displayed at approximately 25 kDa and putatively assigned to Ara h 3 group. Previous studies, already reported the resistance of some peanut proteins to the heating (Cabanillas et al., 2015; Kopper et al., 2005; Maleki & Hurlburt, 2004). Like other cupins, Ara h 1 is a thermostable protein and undergoes irreversible denaturation after heating at the temperatures above 80 °C causing a loss in the secondary and tertiary structures and an extensive aggregation (Koppelman, Bruijnzeel-Koomen, Hessing, & De Jongh, 1999). On this regard, the extreme heating like roasting at the temperatures higher than 140 °C was reported to produce an enhancement of IgE binding capacity of Ara h 1 (Mondoulet et al., 2011).

In this work, we found an extensive reduction of Ara h 1 when peanuts underwent hydration (for 2 h) followed by autoclaving at 134 °C for 10 or 20 min, as depicted in Fig. 3 (Fig. 3, lane 4 and 5). Such results are in agreement with what reported by Cabanillas et al. (2015), although the technological treatment used by those authors slightly differed from that herein described. Specifically, in that paper authors investigated the influence of thermal/pressure processing on the IgE binding properties of raw, fried and roasted peanuts inferring that autoclaving samples at 138 °C and at 2.56 atm for 15 or 30 min in the presence of water produced a dramatic reduction of Ara h 1 levels. The same authors found a decrease in Ara h 1 content also when peanuts were subjected to mild thermal/pressure treatment, in contrast with our results instead showing a certain resistance of these proteins to autoclaving (134 °C, 2 atm, 10 or 20 min). These different results could be likely ascribed to the different treatment conditions applied to peanuts (presence/absence of water during autoclaving). Moreover, a drastic reduction of Ara h 3 proteins (proteins banding around 37 kDa in Fig. 3 lane 2–3 and 4–5) was observed in autoclaved peanuts submitted or not to preliminary hydration. The susceptible behaviour of this allergen group to thermal/pressure treatment was in line with what previously described by Cabanillas et al. (2015).

A similar trend was recorded for proteins banding in the range 20–25 kDa (mainly attributed to Ara h 2, Ara h 3 and Ara h 7) with a progressive disappearance of bands below 20 kDa in pre-hydrated autoclaved samples (Fig. 3, lanes 2–3 and 4–5). On the other hand, proteins banding below 20 kDa (attributed to Ara h 2, Ara h 3, Ara h 6 and Ara h 7) disappeared from the gel already after 10 min of autoclaving (Fig. 3, lanes 2, 3, 4, 5) demonstrating a high susceptibility of these proteins to the heating. On this regard, Johnson et al. have recently reported that Ara h 2/Ara h 6 exposition at the temperatures higher

Table 1
Identification of protein bands excised from the SDS gel and analysed by LC-HR-MS/MS through detection of the proteotypic peptides.

Sample	Band	Accession number	Allergenic protein	Coverage	Score	Filtered peptides (unique)
Raw peanut	*a*	Q6PSU4	Ara h 1	51,60	6,44	7(0)
		B3IXL2	Ara h 1	50,00	13,12	9(0)
		N1NG13	Ara h 1	48,10	10,22	8 (0)
	b	Q6PSU4	Ara h 1	69,16	131,64	23 (0)
		B3IXL2	Ara h 1	67,30	155,19	36 (0)
		Q6PSU6	Ara h 1	65,35	84,80	14 (0)
		E5G076	Ara h 1	50,56	93,38	27 (0)
		Q5I6T2	Ara h 3	33,14	2,76	5 (1)
	c	B5TYU1	Ara h 3	70,75	89,97	20 (0)
		Q5I6T2	Ara h 3	68,17	87,21	19 (1)
		Q9FZ11	Ara h 3	66,54	92,99	19 (1)
		Q8LKN1	Ara h 3	65,61	66,65	16 (0)
		Q6PSU4	Ara h 1	65,18	153,66	27 (0)
		Q647H3	Ara h 3	64,62	87,56	17 (1)
		A1DZF0	Ara h 3	61,06	81,39	17 (0)
		Q0GM57	Ara h 3	43,55	17,37	5 (0)
		B3IXL2	Ara h 1	59,93	148,54	33 (0)
		Q6PSU6	Ara h 1	58,75	85,21	14 (0)
		E5G076	Ara h 1	47,98	85,65	25 (0)
	d	Q647H3	Ara h 3	71,88	171,03	19 (0)
		Q8LKN1	Ara h 3	75,28	161,74	17 (0)
		Q9FZ11	Ara h 3	68,81	160,57	19 (1)
		B5TYU1	Ara h 3	65,09	150,06	17 (1)
		Q5I6T2	Ara h 3	68,74	142,35	19 (2)
		A1DZF0	Ara h 3	66,73	138,28	15 (1)
		Q9SQH7	Ara h 3	43,02	120,87	10 (0)
		O82580	Ara h 3	51,48	110,60	12 (0)
	e	Q9FZ11	Ara h 3	68,24	97,93	14 (0)
		Q5I6T2	Ara h 3	68,17	90,21	14 (2)
		B5TYU1	Ara h 3	65,28	92,08	12 (0)
		Q8LKN1	Ara h 3	60,04	78,33	9 (0)
		A1DZF0	Ara h 3	58,41	78,96	8 (0)
		Q9SQH7	Ara h 3	47,36	70,29	6 (0)
		E5G077	Ara h 3	40,63	13,65	6 (0)
	f	Q9FZ11	Ara h 3	50,47	23,28	8 (1)
		Q5I6T2	Ara h 3	49,53	23,20	8 (1)
		Q8LKN1	Ara h 3	41,64	17,07	6 (0)
		A1DZF0	Ara h 3	41,02	18,72	6 (0)
	g	A1DZF0	Ara h 3	48, 00	336,71	24 (2)
		N1NG13	Ara h 1	43,00	137,8	26 (1)
	h	Q9FZ11	Ara h 3	33,84	5,07	4 (0)
	i	A1DZF0	Ara h 3	37,62	3,91	4 (0)
		Q5I6T2	Ara h 3	34,65	1,83	3 (0)
	l	Q6PSU2	Ara h 2	72,09	59,52	15 (15)
		B4XID4	Ara h 7	53,05	9,84	7 (4)
		Q647H4	Ara h 3	46,83	242,25	20 (0)
		A1DZF0	Ara h 3	45,37	256,11	19 (0)
		Q647H3	Ara h 3	43,58	256,57	17 (0)
		B5TYU1	Ara h 3	43,40	249,89	18 (0)
		Q5I6T2	Arah 3	38,04	255,99	17 (0)
		Q9FZ11	Ara h 3	37,24	272,49	18 (1)
		O82580	Ara h 3	33,33	155,38	15 (0)
		Q0GM57	Ara h 3	30,08	60,24	7 (0)
		N1NG13	Ara h 1	28,91	0,00	3 (0)
		Q647H1	Ara h 1	24,92	24,30	7 (3)
	m	A5Z1R0	Ara h 6	64,83	17,76	6 (3)
		Q6PSU2	Ara h 2	55,81	11,40	6 (6)
		B4XID4	Ara h 7	46,95	13,07	9 (4)
		Q647G8	Ara h 7	46,20	9,62	5 (0)
		A1DZF0	Ara h 3	44,23	63,14	13 (0)
		A1DZE9	Ara h 6	42,07	17,76	3 (0)
		B5TYU1	Ara h 3	41,32	63,35	13 (0)
		Q8LKN1	Ara h 3	41,08	44,58	11 (0)
		Q647H3	Ara h 3	38,73	60,71	12 (1)
		Q9FZ11	Ara h 3	35,16	60,71	12 (1)
		Q0GM57	Ara h 3	28,52	8,05	4 (0)
	n	A5Z1R0	Ara h 6	72,41	28,08	12 (8)
		A1DZF0	Ara h 3	45,18	45,55	5 (0)
		Q9FZ11	Ara h 3	41,21	51,05	6 (0)
		A1DZE9	Ara h 6	40,00	20,64	4 (0)
		O82580	Ara h 3	36,49	51,05	6 (0)
		Q6PSU2	Ara h 2	34,88	0,00	4 (4)
		Q8LKN1	Ara h 3	33,83	47,15	5 (0)

(*continued on next page*)

Table 1 (*continued*)

Sample	Band	Accession number	Allergenic protein	Coverage	Score	Filtered peptides (unique)
		B4XID4	Ara h 7	28,05	0,00	5 (3)
Autoclaved 10'	*o*	Q9FZ11	Ara h 3	32,14	5,33	4 (0)
		Q5I6T2	Ara h 3	30,51	5,33	4 (0)
		N1NG13	Ara h 1	43,61	21,36	10 (0)
		Q6PSU3	Ara h 1	52,07	21,36	8 (0)
	p	B5TYU1	Ara h 3	41,89	83,46	12 (0)
		Q8LKN1	Ara h 3	41,64	68,84	11 (0)
		A1DZF0	Ara h 3	41,02	84,14	13 (0)
		Q647H3	Ara h 3	39,29	95,04	11 (1)
		Q9FZ11	Ara h 3	35,73	95,17	13 (1)
		Q0GM57	Ara h 3	27,34	34,46	6 (0)
Autoclaved 10' + H_2O	*q*	Q5I6T2	Ara h 3	25,99	4,21	3 (1)

than 110 °C induced a change in their secondary structure with a consequent transition from α-helix to random coil and, as a results, the formation of dimeric (MW 26–29 kDa) and tetrameric (MW 60–65 kDa) structures (Cabanillas et al., 2015; Johnson et al., 2010).

In addition, it has been demonstrated that like in the case of high pressure treatments applied up to 180 MPa, Ara h 2 unfolding can occur with consequent exposition of hydrophobic residues (Hu et al., 2011). Similar results were obtained by Cabanillas et al. (2015) that ascertained reduced levels of Ara h 2/Ara h 6 allergens in autoclaved raw, roasted and fried peanuts.

It can be speculated that exposition of peanuts to water before autoclaving could alter protein stability also inducing extensive protein denaturation. Water absorption by seeds might on one hand facilitate heat propagation in the inner part of the seed and on the other hand exert a mechanical effect while autoclaving at the higher pressure thus causing protein disgregation and a decrease in spot intensity. Several reasons might account for such behaviour *e.g.* conformational changes in the protein, formation of intra and/or inter-molecular covalent and non-covalent interactions, *etc.* Some authors hypothesized that, in general, structural changes caused by heating can alter protein solubility consequently lowering the extraction efficiency of the containing proteins or in other cases promote protein aggregation thus preventing the protein complex from entering the polyacrylamide gel (Comstock, Maleki, & Teuber, 2016).

In this study our investigation was only addressed to the extractable proteins with TBS also containing 7 M urea. However, taking into account the solubility issues, targeted analyses on the insoluble fraction of raw and treated peanuts were carried out (data not shown). Specifically, a sequential extraction procedure was followed based on a first step with TBS buffer also containing 7 M Urea, and a subsequent extraction on the remaining pellet with harsher conditions using the SDS-PAGE sample buffer. Protein pools sequentially extracted, presumably composed by most soluble (first fraction) and partly insoluble proteins (second fraction), were analysed by SDS-PAGE. Electrophoretic patterns showed that the profiles of pellets undergoing a harsher extraction were similar to those obtained by the first extraction (data not shown). Similar results were also described by Sanchiz et al. (2018), the did not report any difference in the electrophoretic pattern nor in the IgE reactivity of cashew and pistachio extracts subjected to heat and pressure treatments, applying strong conditions for protein solubilization (flours directly solubilized in SDS-PAGE sample buffer) compared to the first extraction.

In conclusion, our results demonstrated a certain decrease for certain allergenic proteins after autoclaving peanut seeds, according to the results provided by the Bradford assay and ELISA analysis. In addition, the remarked reduction in the intensity of the protein bands along the individual lanes after a prolonged heating, could be attributed either to a reduced extraction yield from the processed food material (Walczyk, Smith, Tovey, & Roberts, 2017) or to protein fragmentation caused by the thermal treatment applied, that could finally lead to a lower IgE binding.

3.3. Immunoblot analysis to assess IgE binding reduction in the extractable fraction of autoclaved peanuts

In order to compare the efficacy of autoclaving for different time extents including or not an incubation step in water, the change in the final immunoreactivity was assessed by Immunoblot analysis using a pool of sera from allergic young patients (3–8 age). A picture reporting the western blot analysis, under reducing conditions, performed with sera of patients allergic to peanuts is shown in Fig. 4.

As for autoclaved peanuts, while band at 120 kDa MW (Fig. 4, lane 1, band *a*, assigned to Ara h 1) lose its immunoreactivity after treatment, bands with MW of approximately 60 kDa (Fig. 4, lanes 2 and 3 corresponded to band *o*, experimentally assigned to Ara h 1 and Ara h 3), confirmed the reduction of their IgE binding after autoclaving (Fig. 4, lane 2, 3). By contrast, no difference in IgE reactivity was observed for protein bands with MW of approximately 50 kDa (band *c* in untreated sample), that dd-MS^2 experiments putatively attributed to Ara h 3 and Ara h 1, and of band at 25 kDa (Fig. 4, lane 2 and 3, band *p*) belonging to Ara h 3 (Table 1). On the contrary, the general IgE reactivity of these proteins appeared to be drastically reduced when peanuts were hydrated for 2 h prior to autoclaving (10 or 20 min). In this case, only proteins banding at 25 kDa (previously attributed to Ara h 3 in raw samples) still displayed a IgE binding and assigned to Ara h 3 subunit. On the other hand some other bands displayed in lane 4 and 5 at the lower MW around 25 kDa, might appear also more reactive compared to the control giving rise to infer that a change in the immunoreactivity (reduction for certain proteins and enhancement for others) may occur under specific processing conditions applied (Guillon, Bernard, Drumare, Hazebrouck, & Adel-Patient, 2016).

It is well known that food processing can induce conformational changes on the allergenic protein, influencing its allergenicity by disruption of conformational or linear epitopes spread along the moiety. As a result, conformational epitopes can be exposed or hidden by unfolding or aggregation of proteins (Rahaman, Vasiljevic, & Ramchandran, 2016). On this regard, a recent study have reported that thermal processing of peanuts induced a major decrease in Ara h 1 immunoreactivity compared to Ara h 2. This different behaviour could be due to a higher degree of denaturation and/or aggregation of Ara h 1 (Montserrat et al., 2015). Blanc et al. (2011) showed that after boiling, Ara h 1 formed branched rod-shaped aggregates with a loss of some secondary structures and consequently a reduction of IgE binding ability. In general, the loss or change in the conformational or linear

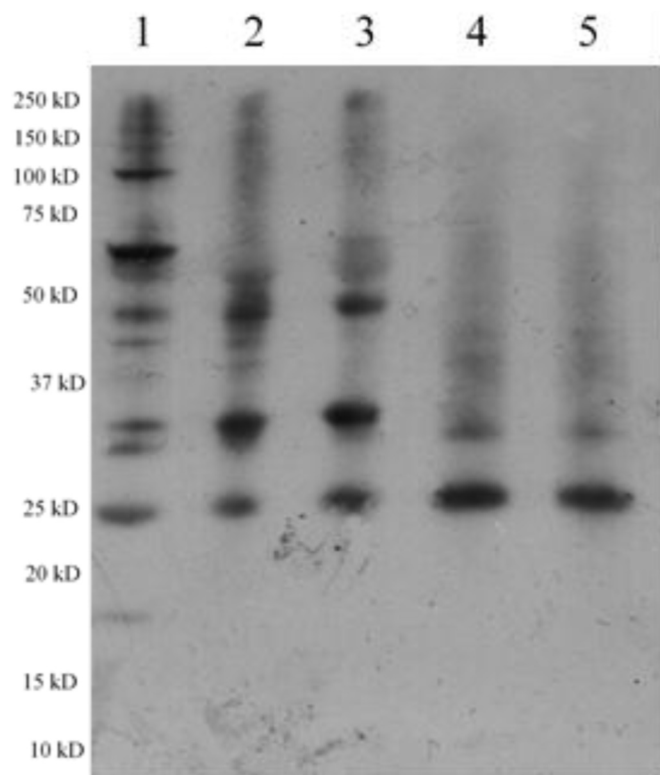

Fig. 4. Immunoblot of peanuts under reducing conditions referred to raw (lane 1), autoclaved for 10 min (lane 2) and for 20 min (lane 3); pre-hydrated and autoclaved for 10 min (lane 4), pre-hydrated and autoclaved for 20 min (lane 5) at 134 °C and 2 atm. The immunoblot was carried out on a pool of sera of young patients (3–8 age) with a clinical allergy to peanuts.

epitopes, can play an important role in modulating the allergenic potential of a food by altering the IgE binding capacity, the activation of basophils and mast cells that causes a reduction in histamine release (Nesbit et al., 2012). In addition, it has been shown that an extensive protein fragmentation can lead to a reduced allergenicity (Fue, 2002). As found in this work, when autoclaving was preceded by a hydration step, a strong reduction in the final immunoreactivity was observed. According to our findings, only a protein banding at MW 25 kDa showed to retain some reactivity, giving rise to exclude any aggregation phenomena occurring or an eventual decrease in protein solubility; instead, a probable fragmentation of proteins might have occurred as a consequence of the prolonged exposition to water. In support of this, Cabanillas et al. (2015) reported that both autoclaving and boiling caused protein fragmentation. In the same work, they also reported that after solubilising peanut flour directly in the sample buffer, proteins showed a high extent of fragmentation, that also reflected a decreased capacity to bind IgE. In addition, another study demonstrated that boiling peanut seeds in closed vessels resulted in a loss of Ara h 2, Ara h 6 and Ara h 7 proteins due to a probable leaching of these allergens into the cooking water (Turner et al., 2014). However, those data demonstrated that boiling reduced IgE reactivity but did not vanish the capacity to stimulate antigen-specific T cells, as shown by activation and proliferation tests (Tao et al., 2016). On the other hand, study on structural alterations induced by heating Ara h 2 and carried out by CD spectroscopy revealed that Ara h 2 did not refold upon temperature decrease but remained in this partially unfolded state with a significantly increased of protein oligomers (Starkl et al., 2011).

The global reduction of peanuts allergenic potential recorded in the present study proved that the implementation of wet heat and high-pressure treatments is essential to significantly decrease the IgE response. However, it is worthy to be underlined that this does not confirm the total abolishment of allergenicity and an antibody reactivity cannot be excluded. According to what described in other studies a persistent allergenicity can be displayed after heat treatments applied depending on the type of nut under study or the specific cultivar/variety (Downs et al., 2016; Noorbakhsh et al., 2010). In addition, the effect of these treatments cannot be uniquely associated with structural modifications of proteins, but also with the generation of protein fragments as also confirmed by other studies (Cabanillas et al., 2012, 2014; Cabanillas et al., 2015).

3.4. Digestibility and IgE binding capacity of autoclaved peanuts after in vitro gastro-intestinal digestion

Food allergens display the typical characteristics to resist to the proteolytic activity of the enzymes occurring along the gastrointestinal tract (GI), being able to reach the intestinal mucosa such as large immunologically active fragments and capable of inducing sensitization after their absorption. Several studies demonstrated that digestion of Ara h 2 and Ara h 6 by pepsin and/or trypsin/chymotrypsin can originate large residual peptides (Koppelman et al., 2010) endowed with unmodified immunological potential (Apostolovic et al., 2016). In addition, these proteins also proved to be resistant to gastro-intestinal digestion even after heating (Koppelman et al., 2010; Maleki & Hurlburt, 2004; Sen et al., 2002; Suhr, Wicklein, Lepp, & Becker, 2004). In stark contrast with that, Ara h 1 and Ara h 3 were rapidly hydrolyzed by pepsin (Koppelman et al., 2010). Nevertheless, peptides obtained after gastro-duodenal digestion of Ara h 1 still retained sensitizing capability and IgE-binding properties. The limits of these studies lied in the fact that they were carried out on single purified proteins (Bøgh & Madsen, 2016) or by employing very simple digestion fluids (Apostolovic et al., 2016) not taking into account the overall complexity of the real physiological conditions. In a recent study, a standardized *in vitro* digestion protocol has been utilised to assess stability of the major peanut allergens by simulating human digestion, also evaluating the residual immunoreactivity of the generated peptide mixture (Di Stasio et al., 2017). In order to assess the effect of the digestion on the major peanut allergens, autoclaved peanuts were subjected to *in vitro* digestion experiments and the residual immunoreactivity of the digests was finally assessed. To this purpose, in this work peanuts autoclaved for 10 min, including or not a pre-hydration phase, underwent a standardized *in vitro* digestion protocol (Minekus et al., 2014) where chew, gastric and duodenal phases simulated physiological conditions (as for enzymes and fluids composition).

Digestibility of raw peanut proteins, undergoing or not digestion, is shown in the SDS-PAGE gel obtained under reducing conditions, pictured in Fig. 5A. As appearing in the figure, the prominent band with MW at approximately 60 kDa detected in undigested raw peanuts was lost after simulated digestion (Fig. 5A, lane 2).

When autoclaved peanuts underwent digestion, an additional protein banding between 37 and 50 kDa were displayed along the gel (Fig. 5A, lane 3), despite its absence in digested raw peanuts (Fig. 5A, lane 2) with special regard to a defined spot nearly 42 kDa. On the contrary, the high intense band detected between 20 and 25 kDa in undigested raw samples proved to be resistant throughout digestion (Fig. 5A, lanes 1, 2). New smeared bands in the lower MW range below 15 kDa were also highlighted in all digests (Fig. 5A, lane 2, 3). On the base of the MS/MS identification accomplished in undigested and digested raw peanuts, polypeptides banding at 50 kDa in the raw digests were mainly attributed to Ara h 1 digestion, whereas the protein at 37 kDa attributed to Ara h 3 appeared completely degraded. Moreover, the protein banding at 22 kDa, showed to persist throughout digestion, suggesting a good resistance of this protein towards digestive enzymes. Notably, smeared bands below 15 kDa might represent some digest products of several. Ara h proteins. However, further investigations will be directed to give more insights on these polypeptides and on the

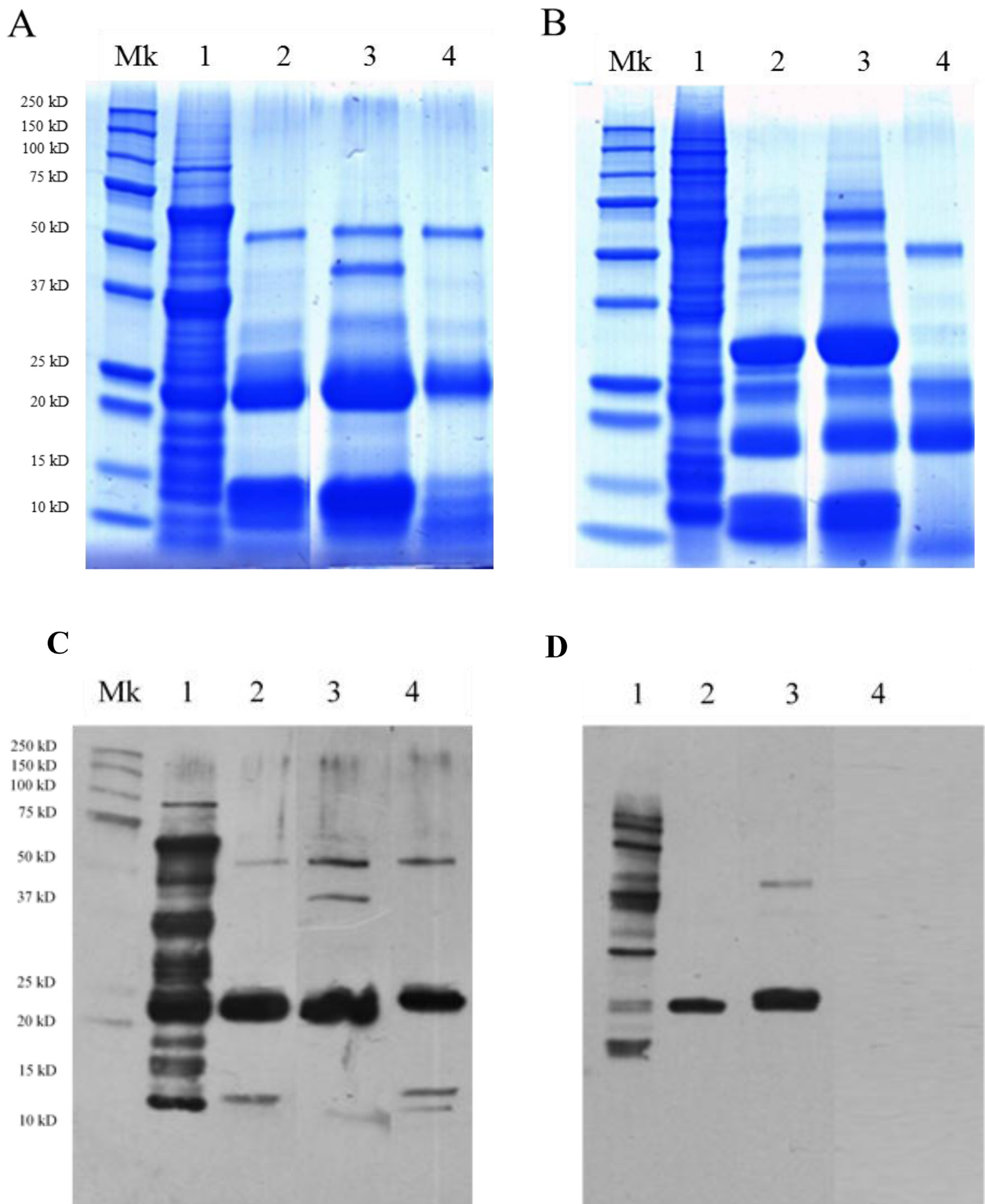

Fig. 5. SDS-PAGE analysis under reducing **(A)** and non-reducing **(B)** conditions of peptides mixture generated from untreated and treated peanuts submitted to simulated gastro-duodenal digestion. Undigested raw peanuts (lane 1), digested raw peanuts (lane 2), autoclaved (at 134 °C, 2 atm for 10 min) and digested peanuts (lane 3), pre-hydrated, autoclaved (at 134 °C and 2 atm for 10 min) and digested peanuts (lane 4). In the lower panel are shown immunoblot with a pool of 6 sera of young patients allergic to peanuts, under reducing **(C)** and non-reducing **(D)** conditions relative to peptides mixture generated from untreated and treated peanuts submitted to simulated gastro-duodenal digestion along with undigested control. Undigested peanut (lane 1), digested raw peanut (lane 2), autoclaved at 134 °C, 2 atm for 10 min (lane 3), pre-hydrated and autoclaved at 134 °C and 2 atm for 10 min (lane 4).

fraction lower than 10 kDa. By inspecting the protein pattern of autoclaved and digested peanuts (Fig. 5A, lanes 3, 4), slight differences were found compared to digested raw samples. Specifically, the electrophoretic profiles of digested autoclaved peanuts without pre-hydration (Fig. 5A, lane 3) showed the appearance of an additional band at approximately 42 kDa. This new emerging band could be the result of an incomplete digestion of Ara h 1, likely due to conformational modifications occurring during the treatment that contributed to hide enzyme cleavage sites. Whereas, the digestion of hydrated and autoclaved peanuts produced an electrophoretic profile (Fig. 5A, lane 4) with a general decrease of protein bands intensity especially noticed in the MW ranging from 15 and 45 kDa.

Different results were obtained for peanut digests analysed under non-reducing conditions as shown in Fig. 5B. Again a change in the protein pattern was noticed in the autoclaved samples (see Fig. 5B, lane 3), where a protein banding at approximately 60 kDa was detected By contrast, a high resistance of proteins comprised in the range from 37 to 20 kDa was highlighted in the lanes 2, 3 of Fig. 5 B with special regard to band at 25 kDa.

Conversely, in hydrated and autoclaved peanuts digests (Fig. 5B, lane 4), a protein banding at 50 kDa was highlighted with a concomitant disappearance of bands between 50 and 37 kDa. Moreover, a resistance of bands with MW in the range 25–15 kDa, along the electrophoretic profile of raw and autoclaved digested samples, was also observed, with a significant reduction in the intensity of bands below 15 kDa.

These results, in both cases, point out that pre-hydrating peanuts before autoclaving is likely to extensively promote digestion of peanut allergens thus facilitating proteolysis of the major protein allergens. Nonetheless, some protein bands showed to persist throughout digestion.

Finally, due to the scarce knowledge of the residual immunoreactivity still retained from autoclaved peanuts after human simulated digestion, digests obtained from raw and autoclaved peanuts were submitted to final immunoblot analysis, under reducing and non-reducing conditions, by using sera of allergic patients.

Under reducing conditions (Fig. 5C), only peptide fragments banding at approximately 20 kDa determined an IgE binding in both raw and autoclaved samples, while the two signals at approximately 50 and 15 kDa were weakly detected in untreated and pre-hydrated autoclaved samples. In addition, a high intense signal was observed for the proteins at 20 kDa in untreated and treated digested peanuts.

On the contrary, under non-reducing condition (Fig. 5D), raw and autoclaved samples showed IgE reactivity of proteins at approximately 20 kDa, despite hydrated and autoclaved samples were none antibody response was recorded.

The loss of IgE response under non-reducing condition, would suggest that the combination of heat and pressure coupled with a preliminary hydration step might account for a structural change in peanut proteins, hiding the allergen structure with a probable aggregation phenomenon. These new protein structures could inhibit the action of trypsin as well as other digestive enzymes, leading to a decrease in protein digestibility, and consequently masking some IgE reactive determinants. Only the action of a strong reducing agent such as DTT or β-mercaptoethanol (β-ME) might induce reduction of aggregates and the consequent exposure of epitopes to IgE binding.

To the best of our knowledge, this is the first time that the stability and the residual immunoreactivity of peanut allergens submitted to autoclave process and simulated gastro-intestinal *in vitro* digestion was studied. Compared to the existing literature, and in order to have more realistic data about the digestibility of processed peanut proteins, the whole peanut commodity was submitted to digestion accounting then for the influence of the matrix on the proteolytic degradation of the contained allergens. A similar approach has been recently described by Di Stasio et al. (2017). The authors investigated the digestion stability of the major peanut allergens directly in the natural matrix using the standardized Infogest digestion protocol and the residual immunoreactivity of the resulting digest fragments was finally assessed. It was found that only large sized fragments of Ara h 2, Ara h 6 and Ara h 3 survived the hydrolysis. In the same paper authors finally identified by LC-MS/MS analyses smaller resistant peptides mainly arising from Ara h 3 and Ara h 1. Concerning untreated peanuts, our results are in agreement with what described by Di Stasio and co-workers (2017). We also found a thermostable and an immunoreactive protein at approximately 22 kDa in the SDS-gel of gastro-duodenal digest of unprocessed peanuts that was attributed to Ara h 3 in line with the MS/MS analysis of the corresponding band done by Di Stasio et al.

Concerning samples submitted to autoclaving treatment without pre-hydration, we observed that protein fragments/subunits arising from gastro-duodenal digestion still retained their allergenic potential because no significant differences were highlighted in Western-blotting profiles of raw and autoclaved peanuts samples under reducing and non-reducing condition.

By contrary, immunoblot analysis carried out on gastro-duodenal digests of pre-hydrated and autoclaved samples (Fig. 5D, lane 4) demonstrated that the allergenic potential was lost after digestion by working under non reducing condition.

However, it is worthy to be said that in this paper we did not investigate peptides lower than 6 kDa that escaped the electrophoretic detection. Work is in progress to identify the reactive band visualized around 20 kDa as well as to characterize the lower MW fraction.

Our findings confirm that when a more drastic processing was applied (*e.g.* hydration followed by autoclaving), a different result in the final immunoreactivity was displayed strictly depending on the operative conditions adopted during SDS PAGE experiments (under reducing or non reducing conditions).

4. Conclusions

In the light of our results, thermal/pressure treatment has demonstrated to modulate peanuts immunoreactivity. In particular, hydration prior to autoclaving proved to increase the efficacy of the thermal treatment contributing to the disappearance of the main allergenic protein bands and altering significantly the final immunoreactivity as assessed by immunoblot experiments. Furthermore, attention was placed on the residual immunoreactivity detected after gastro-intestinal digestion, thus demonstrating that the combination of hydration and autoclaving may induce a drastic reduction of peanuts immunoreactivity especially displayed when working under non reducing conditions.

However, further studies will be necessary to better investigate the decrease in IgE crosslinking capacity of heat/pressure treated samples in *in vivo* models.

Understanding the fate of allergenic proteins subjected to novel processing techniques can help to develop useful strategies for food tolerance induction and/or to establish threshold levels of sensitization/elicitation for hypoallergenic foods. In order to confirm these results, a deeper investigation should be undertaken by using individual sera of allergic patients and designing a food oral challenge test study.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2018.04.021.

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