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Effects of Enzymatic Hydrolysis Combined with Pressured Heating on Tree Nut Allergenicity

Running title: Allergenic reactivity of processed tree nuts

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Abstract: Hazelnut, pistachio and cashew are tree nuts with health benefits but also with allergenic properties being prevalent food allergens in Europe. The allergic characteristics of these tree nuts after processing combining heat, pressure and enzymatic digestion were analyzed through in vitro (Western blot and ELISA) and in vivo test (Prick-Prick). In the analyzed population, the patients sensitized to Cor a 8 (nsLTP) were predominant over those sensitized against hazelnut seed storage proteins (Sprot, Cor a 9 and 14), which displayed higher IgE reactivity. The protease E5 effectively hydrolyzed proteins from hazelnut and pistachio, while E7 was efficient for cashew protein hydrolysis. When combined with pressured heating (autoclave and Controlled Instantaneous Depressurization (DIC)), these proteases notably reduced the allergenic reactivity. The combination of DIC treatment before enzymatic digestion resulted in the most effective methodology to drastically reduce or indeed eliminate the allergenic capacity of tree nuts.

Keywords: tree nut allergy; food processing; thermal treatment; pressure processing; enzymatic digestion

1. Introduction

In Europe, peanut and tree nuts are among the predominant foods involved in allergic reactions after fruits ((Lyons, et al. 2020). Meanwhile, in the last few years there has been an increase in nut and tree nut consumption because of their favorable health effects. The unique nutritional composition of tree nuts is likely the main reason for the increased consumption in recent years (Ros, 2010). Most of proteins causing tree nut allergy are member of protein families of vicilins, legumins, 2S albumins and nsLTPs. Profilins and pathogenesis-related (PR) proteins contributed to pollen associated tree nut allergy. In addition, oleosin and thaumatin-like proteins are relevant allergens. Most of tree nut allergens have high resistance to enzymatic degradation and denaturation. Currently, several allergens of hazelnut have been registered in the WHO-IUS list of allergenic proteins: Cor a 1 (Bet v1 homologue), Cor a 2 (profilin), Cor a 8 (LTP), Cor a 9 (11S legumin), Cor a 11 (7S vicilin), Cor a 14 (2S albumin) and Cor a 12, Cor a 13 and Cor a 15 (oleosins) (Costa, Mafra, Carrapatoso, & Oliveira, 2016)

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The five pistachio major allergens are Pis v 1 (2S albumin), two 11S legumins (Pis v 2 and 5), the 7S vicilin Pis v 3 and the superoxide dismutase Pis v 4 (Ayuso, Grishina, & Ahn, 2007; Noorbakhsh, Mortazavi, Sankian, Shahidi, Tehrani, Azad, et al., 2011; Willison, Tawde, Robotham, Penney, Teuber, Sathe, et al., 2008). Cashew can induce severe reaction even at minimal doses and three allergens are registered: Ana o 1 (7S vicilin), Ana o 2 (11S legumin) and Ana o 3 (2S albumin) (Robotham, Wang, Seamon, Teuber, Sathe, Sampson, et al., 2005). Most of epitopes of Pis v 1 and Pis v 3 are highly homologs with the epitopic regions of cashew allergens. This finding is considered the molecular base for the reported cross-reactivity between cashew and pistachio (Barre, Nguyen, Granier, Benoist, & Rougé, 2021).

The processing of foods is useful at industrial level to improve organoleptic characteristics and to ensure food safety. This processing can alter the food allergenicity because it can change the physicochemical characteristics of allergens. The level of alterations is related with several factors as the class of processing used, processing conditions, duration, food matrix, etc (Cabanillas & Novak, 2017). The alterations that food proteins can suffer during processing includes denaturation, aggregation, and chemical modifications. These modifications might alter IgE reactivity, increasing or reducing food allergenicity. Consequently, understanding how food processing can affect the protein characteristics, such as their resistance to pressure and heat, as well as, their mechanical and chemical activities is a relevant topic in food allergy management (Costa, Bavaro, Benedé, Diaz-Perales, Bueno-Diaz, Gelencser, et al., 2022; Fu, Cherayil, Shi, Wang, & Zhu, 2019; Valdelvira, Garcia-Medina, Crespo, & Cabanillas, 2022). The thermal processes include boiling, frying, roasting, microwave cooking and heating under pressure by autoclaving or DIC (Controlled Instantaneous Depressurization) treatment. Non-thermal treatments as HHP (high hydrostatic pressure) or enzymatic digestion might also be used on foods alone or in combinations. These treatments can produce changes in chemical properties of proteins or produce biochemical reactions inside the components of food matrix (Cuadrado, Sanchiz, & Linacero, 2021; Masthoff, Hoff, Verhoeckx, van Os-Medendorp, Michelsen-Huisman, Baumert, et al., 2013). There are an extensive number of studies on the modifications of tree nut allergenicity throughout different heat treatments (Jiménez-Saiz, Benedé, Molina, & López-Expósito, 2015; Masthoff, et al., 2013; Monaci, Lamonaca, Luparelli, Pilolli, & De Angelis, 2023; Vanga & Raghavan, 2017). Boiling at 100 ºC is not effective to reduce allergenicity of almond, cashew, pistachio, walnut (Cabanillas, Maleki, Rodriguez, Cheng, Teuber, Wallowitz, et al., 2014; Cuadrado, Sanchiz, Vicente, Ballesteros, & Linacero, 2020) as well as for peanuts (Cuadrado, Sanchiz, Arribas, Pedrosa, Gamboa, Betancor, et al., 2023). When the effect of processing on hazelnut is evaluated, an important reduction in immunoreactivity is observed upon roasting at 140ºC for 40 min (Hansen, Ballmer-Weber, Leuttkopf, Skov, Weuthrich, Bindslev-Jensen, et al., 2003) although this decrease is not of clinical relevance since 29% of the allergic patient still have allergic symptoms after ingestion of roasted hazelnuts.. The influence of autoclaving on tree nut immunoreactivity has been studied on almond, chestnut, cashew, pistachio, hazelnut, walnut, besides peanut (Cabanillas, et al., 2014; Cuadrado, et al., 2023; Cuadrado, Sanchiz, & Linacero, 2021; Cuadrado, Sanchiz, Vicente, Ballesteros, & Linacero, 2020; Lopez, Cuadrado, Burbano, Jimenez, Rodriguez, & Crespo, 2012; Sanchiz, Cuadrado, Dieguez, Ballesteros, Rodriguez, Crespo, et al., 2018). After autoclaving these tree nuts, their residual allergenicity is drastically diminished with a direct association with increased heat/pressure and time and even almost abolished at harsh conditions (138ºC for 30 min). The introduction of a hydration step (2h), before autoclaving, followed by drying enhanced the final efficacy of the treatment on almond and hazelnut allergenicity (De Angelis, Bavaro, Forte, Pilolli, & Monaci, 2018; De Angelis, Di Bona, Pilolli, Loiodice, Luparelli, Giliberti, et al., 2022). Moreover, the hazelnut allergen digestibility increased in prehydrated samples compared to the controls and none resistant peptides survive after both treatments suggesting high protein fragmentation (De Angelis, et al., 2022). The influence of other pressured heat treatment, such as DIC has been analyzed on tree nuts and peanuts allergenicity (Cuadrado, Cheng, Sanchiz, Ballesteros, Easson, Grimm, et al., 2018; Cuadrado, et al., 2023; Cuadrado, Sanchiz, & Linacero, 2021; Vicente, Sanchiz, Rodriguez-Perez, Pedrosa, Quirce, Haddad, et al., 2020). According to these results, DIC applied at harsh condition (7 bar, 120 s) produce a drastic reduction of the IgE immunoreactivity of tree nut and peanut allergenic

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proteins. The in vitro results obtained using IgE sera from sensitized patients (immunoblots and ELISA) indicated that the reduction in immunodetection affected more to pistachio (75%) than cashew, but it was not completely abolished. Consequently, pistachio allergens seems to be less resistant than cashew proteins.

Several investigations have reported the action of enzyme digestion on the digestibility and allergic potential of proteins of foods (Cabanillas, Crespo, Burbano, & Rodriguez, 2010; Cabanillas, Pedrosa, Rodriguez, Muzquiz, Maleki, Cuadrado, et al., 2012); Cabanillas et al. (2014); (Cuadrado, et al., 2018). Pistachio and cashew allergens were affected by enzymatic digestion under sonication and autoclaving separately producing a significant reduction of their allergenic potential. Nevertheless, to mitigate drastically IgE binding capacity of cashew allergens, heat combined with enzymatic hydrolysis was required. Simultaneous processing conditions (enzymatic digestion under sonication) have been proposed to eliminate effectively the allergic potential of cashew and pistachio (Cuadrado, et al., 2018). The enzyme digestion combined with pressured heating is more effective for mitigation of the IgE-binding capacity of nsLTP, since pressure modifies protein conformation resulting it more accessible to the enzymatic action and temperature (Costa, et al., 2022). More recent results confirmed that the combined action of pressured heating (mainly DIC treatment) with enzymatic digestion was the most effective strategy to drastically reduce or indeed eliminate main peanut allergens such as Ara h 1, 2, 3, 6, 8 and 9 (Cuadrado, et al., 2023).

The present research is aimed to analyze the IgE binding immunoreactivity and the allergic capacity of some tree nuts (hazelnut, pistachio and cashew) subjected to enzyme hydrolysis in combination with pressured heating (autoclave and DIC) by traditional in vitro immunoassays and in vivo skin prick tests (SPT).

2. Materials and Methods

2.1. Sera and Patients

Sera were collected from 25 patients, with 23 showing sensitization to hazelnut, 15 to pistachio, and 12 to cashew, as indicated by specific IgE and positive skin prick tests for these tree nuts. These sera were analyzed in this study. Patients were attended in any of the three Spanish Allergy Departments (HU Princesa, Fundación Jiménez Díaz, and HU Cruces) during 2021-2022 period (Table S1 supplementary).

Skin prick test (SPT) with raw tree nut extract (control unprocessed sample) and tree nut processed samples was performed according to Malling's method (1993) as follow: boiled for 60 min (B60), autoclaved at 256 kPa during 30 min (AU), DIC treated tree nut for 120 s (DIC), and enzymatically hydrolysed untreated and DIC tree nut samples. SPT was performed out by duplicate and positive (histamine dihydrochloride) and negative (phosphate buffered saline, PBS) controls were employed. SPT mean wheal size were estimated and positive results were those 3 mm greater than negative control.

Total IgE serum levels were determined with ImmunoCAP® (Thermo Fisher Scientific, Uppsala, Sweden). Specific IgE (sIgE) to hazelnut and cashew allergens was also measured (Cor a 1, Cor a 8, Cor a 9, Cora a 14 and Ana o 3). sIgE values > 0.35 kU/L were considered positive result as described in manufacturer's protocol.

A detailed anamnesis was made for assessment of the clinical relevance of positive sensitization. Tree nut allergy was diagnosed after suggestive clinical history of tree nut allergy together with positive specific IgE and/or SPT to tree nut. The allergic reactions symptoms were categorized into systemic or local symptoms. The analyzed patient population was classified into tree nut allergic (n=14 allergic to hazelnut, n=6 allergic to pistachio and n=6 allergic to cashew) and tree nut sensitized (n=9 sensitized to hazelnut, n=9 sensitized to pistachio and n=6 sensitized to cashew) with sIgE > 0.35 and positive skin prick test but who tolerate to ingest hazelnut, pistachio and cashew without clinical symptoms.

The investigation was authorized by the Ethics Committees of HU Princesa, HU Cruces and, Fundación Jiménez Díaz, in agreement with the regulations of the boards of their organizations (Permissions No. 3798, CBVI839/2M, PIC164-18, respectively).

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2.2. Plant Material and Processing Conditions

Hazelnuts (*Corylus avellana,* Negreta variety) and pistachios (*Pistachia vera*, Kerman variety) obtained from the Germoplasm Bank of IRTA (Institut de Recerca I Tecnologia Agroalimentaries - Mas de Bover) and cashew (*Anacardium occidentale*, type 320) obtained from Productos Manzanares (Spain) were utilized in this research. Whole tree nut seeds rinsed in distilled water (1:5 w/v) were boiled (100 °C, 60 min) or autoclaved at 138 ºC, 256 kPa during 30 minutes in a Compact 40 Benchtop Autoclave (Priorclave, London, UK). Whole tree nut seeds were treated with Controlled Instantaneous Depressurization (DIC®) methodology, performed at La Rochelle University (LaSiE). DIC methodology was carried out following a factorial experimental design previously described (Haddad, Louka, Gadouleau, Juhel, & Allaf, 2001). In this experiment, the moistened whole tree nuts were subjected to steam pressure (7 bar) under up to 170 °C, during a short time (120 s) followed by an abrupt pressure drop towards vacuum at around 50 mbar. This instant pressure drop, at a rate $\Delta P/\Delta t$ higher than 5 bar/s, produced an auto-vaporization of part of the product water, and a simultaneous instant product cooling, which stopped thermal degradation. Untreated as well as treated (boil, autoclave and DIC) tree nuts were frezze-dried (Telstar Cryodos frezze-drier), ground using a Kitchen robot (Thermomix 31-1, Vorwerk Elektrowerke, GMBH & Co. KG, Wüppertal, Germany) and defatted with n-hexane (34 mL/g of flour, 4 h). Defatted flour from non-treated tree nuts were the controls for processed samples. The nitrogen amount of the samples was determined in duplicate by combustion instrument-based (Dumas) technique. according to standard methods (AOAC., 2003). The total content of protein was calculated as N x 5.3 (AOAC., 2003) and the results are summarized in Table S2 supplementary.

For enzymatic digestion of tree nut protein extracts, seven Amano food-grade proteases (Amano Enzyme Europe Ltd., Agno, Switzerland) were applied: E1 (Themoase PC10F, endoprotease), E2 (ProteAX, exoprotease), E3 (Protin SD – NY10, proprietary), E4 (Peptidase R, exopeptidase), E5 (Protin SD – AY10, alkalase-like), E6 (Protease M "Amano" SD, proprietary), E7 (Protease P "Amano" 3SD, proprietary). All were stable at 55°C and pH 7. Digestion of tree nut protein extracted with buffered saline borate (BSB, 0.1 M H_3BO_3, 0.025 M Na_2 B_4O_7, 0.075 M NaCl, 1% w/v PVP, pH 8.45) (2mg/mL) was carried out via incubation with each enzyme at 1 mg/mL in PBS pH 7.4 at 55°C for 19 hours and aliquots were taken at several time points (0, 1, 2, 3 and 19 hours). After SDS-PAGE analysis of hydrolysates (Figure S1 supplementary), E5 enzyme was chosen for successive assays in hazelnut and pistachio and E7 in cashew. Such experiments consisted of the enzyme hydrolysis of whole tree nut paste (as opposed to soluble extract) under ultrasonication as described by Cuadrado et al. (2018). All the assays were carried out at least twice and the figures are representative of these duplicate assays.

2.3. Protein Electrophoresis and Immunoblot

SDS-PAGE was carried out following Cuadrado et al. (2018) methodology. Briefly, samples (20 µg protein per lane) in Laemmli sample buffer (Bio-Rad, CA, USA) and 2-mercaptoethanol (Bio-Rad, CA, USA) were heated at 60ºC during 10 minutes, and electrophoresed in 4–20% Tris-HCl precast gels (Bio-Rad, CA, USA). Protein bands were visualized with Coomassie Brilliant Blue R250. IgE western blots were performed by transference to a polyvinylidene difluoride (PVDF) membrane (Millipore Corp., Bedford, MA, USA). Following a blocking step using 2% non-fat milk in PBS (1 h at 37ºC), the membranes were incubated overnight at 4 ºC with individual sera from 25 patients sensitized to hazelnut, pistachio and cashew (1:10, 1:20 dilutions), washed and then incubated with Horseradish peroxidase (HRP) conjugated mouse anti-human IgE (1:10,000 dilution during 30 min at room temperature) (Sigma, Saint Louis, MO, USA). IgE-bound proteins were detected by enhanced chemiluminescence using ECL substrate, as described in the manufacturer's protocol (Thermo Scientific, Waltham, MA, USA) in a ChemiDoc CCD camera (Bio-Rad, CA, USA).

 doi:10.20944/preprints202404.0693.v1

2.4. Detection of 7S Globulin, 2S Albumin, Bet v1 Homolog (PR-10) and LTP Allergens by Immunoblotting and ELISA

For western blot experiments, 20 µg of protein from whole pastes lane was applied into 12% SDS-PAGE gels and then transferred to nitrocellulose membranes (Amersham, Germany) in a semi-dry transfer system (Trans-Blot® TurboTM Transfer System, Bio-Rad, Hercules, CA, USA) at 0.5 A at room temperature during 30 minutes. Then, a blocking step was performed with 5% non-fat milk in PBS containing 0.5% Tween 20 (PBS-T) during 1 hour at room temperature with shacking. The membranes were then individually incubated with rabbit polyclonal antibodies as follow: anti-7S globulin (anti-Ara h 1), anti-2S albumin (anti-Ara h 2), anti-PR-10 (anti-Ara h 8) (Inbio, Cardiff, UK) (all used at 1:5,000 dilution), or anti-LTP (Mal d 3, Abcam, Amsterdam, Netherlands) (used at 1:10,000) at 4ºC overnight. The membranes were washed in PBS-T and incubated with a secondary antibody HRP-conjugated goa t anti-rabbit IgG (Enzo Life Sciences, Inc., Farmingdale, New York, USA) (stock: 0.4 mg/mL, 1:2,500 dilution) at room temperature during 1 hour with shaking. Enhanced chemiluminescence (ECL) was used for detection as described in the manufacturer's protocol (SupersignalTM West Pico Plus Chemiluminescent Substrate, Thermo Fisher Scientific, Madrid, Spain). To quantify the signal a ImageQuantTM LAS 4000 (GE Healthcare, Little Chalfont, UK) was emplyed.

For ELISA experiments, High-binding 96-well plates (Costar, Cambridge, MA, USA) were coated with protein extracts from hazelnut, pistachio and cashew (8 µg/mL) (overnight, at 4°C). Subsequently, the plates were washed with PBS-T and incubated using blocking solution (PBS-T with 3% non-fat milk) (Bio-Rad, Hercules, CA, USA) at room temperature during 1 hour. Following the blocking step, the plates were subjected to incubation with the previously mentioned polyclonal antibodies during 2 hours at room temperature, each antibody being diluted at a ratio of 1:5,000. After primary antibody incubation and subsequent washing, the secondary antibody HRP-conjugated goat anti-rabbit IgG (Enzo Life Sciences Inc., Farmingdale, New York, USA) (stock: 0.4 mg/mL, 1:5,000 dilution) was added and incubated during 1 hour with shaking. Following washing, the detection was conducted through a peroxidase-mediated reaction using tetramethylbenzidine (TMB) and hydrogen peroxide in a 1:1 v/v ratio (Substrate Reagent Pack, R&D Systems, Minneapolis, USA). The reaction was stopped using 2N H_2SO_4 (Thermo Fisher Scientific, Madrid, Spain), and then, the absorbance (O.D.) was determined at 450 nm. Negative control was well coated with blocking solution without any tree nut samples and then proceeding with both primary and secondary antibody incubations. The assays were carried out twice, and the mean optical density (OD) plus three times the standard deviation (SD) of the negative control was utilized to establish the threshold for positivity.

2.5. ELISA Inhibition Assays

A competitive ELISA inhibition assay was carried out in accordance to Cuadrado et al. (2018) with some changes. Polystyrene microtiter plates (Immulon 4 HBX, Thermo Scientific, Waltham, MA, USA) were coated with 100 µL per well of raw hazelnut protein extracts (0.5 mg/mL in PBS pH 7) and incubated overnight at 4 ºC. In parallel, a serum pool from 7 hazelnut with sensitization to Sprot (patient no. #1, #3, #4, #5, #8, #22, #27; diluted 1:10) was preincubated with the following inhibitors: raw (control), boiled, autoclaved, DIC hazelnut, or 1 h enzymatically digested raw or DIC hazelnut samples (final concentrations: 0.1, 0.01, 0.001 mg/mL) overnight at 4 ºC with shaking. Un-inhibited serum was also included (serum preincubated with PBS). Then, plates were washed with PBS with 0.1 % Tween-20 and blocked with 100 µL per well of PBS containing 3 % non-fat milk (1h at 37 ºC). After washing, the serum preincubated with the different inhibitors, or un-inhibited serum, was added to the wells (100 µL per well) for 1 h and then washed again. 100 µL per well of Horseradish peroxidase (HRP) conjugated mouse anti-human IgE (1:1,000 dilution) (Sigma, Saint Louis, MO, USA) was added and incubated37 ºC during 1 h). After washing, 100 µL of peroxidase substrate (SureBlue TM, KPL, Gaithersburg, MD, USA) were added for peroxidase reaction (30 min) and the reaction was finished adding 100 µL of 1% HCl. The optical density (O.D.) was determined at 450 nm. The inhibition in IgE binding was calculated as a percentage with the formula: [(1 – (AI / AN)] x

 doi:10.20944/preprints202404.0693.v1

100, where AI is the absorbance value achieved from raw hazelnut samples preincubated with inhibited sera (raw or processed hazelnut samples), and AN is the absorbance value of raw hazelnut samples incubated with uninhibited sera. All assays were made out in triplicate and the graph (Figure 8) is representative of these assays. To statistically test any significant differences between untreated (control) and treated hazelnut samples, for each inhibitor concentration, the inhibition values were tested by one-way analysis of variance (ANOVA) and the means were compared by Duncan's multiple range test with the Statgraphic Centurion XVI.I software (Statpoint Tech. Inc., Warrenton, VA, USA). The table below the graph displays the comparisons.

3. Results

3.1. Effect of Pressured Heating on Patient SPT

Twenty five patients were studied in this research, 23 out of 25 patients were sensitized after hazelnut ingestion being 14 allergic with clinical symptoms (Table S1 supplementary). Specifically, eight (57.1%) reported oral allergy syndrome and 6 (42.8%) systemic symptoms. 15 patients were sensitized after pistachio exposure and 6 out of 15 were allergic, with systemic symptoms more frequently than oral allergy syndrome. Out of 25 patients, 12 were sensitized to cashew being 8 allergic with clinical symptoms, mainly with oral allergy syndrome. Because there not was significant differences in the SPT wheal size values between allergic patients and sensitized ones with neither hazelnut, pistachio nor cashew, they were considered as a single group of patients with sensitization to these tree nuts. Our studied population's average age is 27.2 ± 3.7 years, mostly females (14 namely 56%).

Total IgE mean was 1249.0 kU/L (± 481.1, ranging from 30.4 to 7600 kU/L) and hazelnut sIgE was 10.5 kU/L (± 3.9, ranging from 0.02 to 50.6 kU/L). Pistachio specific IgE was 6.23 kU/L (± 4.9, varying from 0.01 to 90.9 kU/L). Cashew sIgE was 4.49 kU/L (± 3.1, varying from 0 to 53.6 kU/L). Considering the allergen profile, the patients with sensitization to Cor a 8, nsLTP (68%) were predominant ensued by those with sensitization to Cor a 9 (11S), Cor a 1 (PR10) or Ana o 3 (2S) (36%, 20% and 20% respectively) and lastly Cor a 14, 2S (16%) (Table S1 supplementary). The total patient population (n=25) presented positive SPT, 23 of these were positive to raw hazelnut (control) (mean wheal 8.8 mm^2 ± 2.35) (Figure 1A). Additionally, in these 23 patients, hazelnut processed samples (boiled (B60), autoclaved (AU) and DIC treated (DIC)) were used in the SPT determinations. Moreover, in 14 out of the 23 patients, enzymatically digested raw and DIC hazelnut samples (Ctr+Enz and DIC+Enz) were assayed. The processed samples showed wheal diameter significantly lower than control raw hazelnut ($P<0.05$) (Figure 1A). A wheal size reduction of more than 50% was obtained after Ctr+Enz (3.0 mm^2 ± 0.74) and after B60 (4.1 mm^2 ± 1.33). Lesser values were achieved after pressured heating treatment: AU with 1.5 mm^2 (± 1.02), and DIC with 2.4 mm^2 (± 1.42). The minimum wheal diameter value was reached with DIC+Enz with 1.0 mm^2 (± 0.64). 15 out of 25 patients were sensitized to raw pistachio (control) and 12 to untreated raw cashew, showing a mean wheal of 8.6 mm^2 (± 1.36) and 9.1 (± 2.19), respectively (Figure 1B,C). The SPT of pistachio and cashew treated samples has significantly lesser wheal's diameter than untreated pistachio and cashew ($P<0.05$) (Figure 1B,C). The wheal size decreased more than 60% after Ctr+Enz (3.0 mm^2 ± 0.74 and 2.1 mm^2 ± 1.67) for pistachio and cashew, respectively, and after B60 to 4.1 mm^2 (± 1.33) for pistachio and 5.4 mm^2 (± 1.94) for cashew. After high-pressure procedures, lower values were obtained: AU to 1.5 mm^2 (± 1.02) for pistachio and 1.8 mm^2 (± 0.85) for cashew, and DIC to 2.4 mm^2 (± 1.42) for pistachio and 1.3 mm^2 (± 0.88) for cashew. For both pistachio and cashew, the minimum wheal diameter value was achieved with DIC+Enz to 0.0 mm^2 (Figure 1B,C).

Figure 1. SPT reactivity (wheal median and mean values) of patients to the different samples (untreated raw (control), boiled at 60 min (B60), autoclaved at 256 kPa for 30 min (AU), DIC treated for 120 s (DIC) and control and DIC after 1h incubation under sonication with Enzyme (Crt+E and DIC+E). A) Hazelnut B) Pistachio and C) Cashew.

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3.2. Efficiency of Enzymes to Digest Tree Nut Proteins

For enzymatic hydrolysis of hazelnut, pistachio and cashew proteins, 7 enzymes (Amano) were applied to decrease the IgE binding potential. These seven enzymes were selected for their stability under high temperature and differential enzymatic characteristics. The seven enzymes were each incubated (E1-E7 at 1mg/mL each) with raw tree nut extracts for various amounts of time to try to identify the enzymes with the highest hydrolysis capacity. The digestion experiments with whole tree nut paste were performed in an ultra sonication bath for 1 h at 55 °C, in order to reduce the processing time and enhance digestion. Hydrolysis of hazelnut proteins is displayed in Figure 1 supplementary as an example. In the A panel is the SDS-PAGE analysis of hazelnut buffered saline borate extracts incubated for the longest digestion time (19 hours) for each enzyme. Enzyme 5 (Amano Protin SD – AY10) was found to be the most effective in hydrolyzing hazelnut proteins as well as for pistachio proteins and applied in the next experiments. The hydrolysis products for E5 at different times (1 h, 2 h, 3 h and 19 h, Figure S1B supplementary) were similar and consequently, 1 h treatment was chosen for incubation in further experiments. E7 was the most efficient for cashew proteins and 2 h treatment was selected.

3.3. Protein Electrophoresis and Immunoblots Experiments

Figure 2A shows the protein profiles from SDS-PAGE electrophoresis of hazelnut pastes made of untreated (control), boiled for 60 min (Boil 60), autoclaved at 256 kPa during 30 min (AU256) and DIC treated during 120 s (DIC), pre and post 1h of enzymatic digestion with Protease E5 under ultrasound. Electrophoretic profile of raw and boiled hazelnut samples (lanes 1 and 2) was almost the same with multiple bands distributed in a broad range of molecular weights (7 to 50 kDa). Hazelnut samples subjected to pressured heating (lanes 3 and 4: AU256 and DIC) displayed a reduction of sharp bands and more smearing that could be the result of protein degradation. Electrophoretic patterns of control and boiled hazelnut after enzymatic hydrolysis with E5 presented high protein fragmentation with an important reduction of bands higher than 25 kDa. The enzymatic digestion with E5 produced the strongest reduction with AU and DIC samples (lanes 7 and 8).

Figure 2. SDS–PAGE of control (untreated) (lane 1), Boil (boiled 100ºC, 60 min) (lane 2), AU (138ºC, 256 kPa, 30 min) (lane 3), DIC (170ºC, 7 bar, 120 s) (lane 4) and enzymatically treated (E5 or E7) tree nut samples (lanes 5-8). A) Hazelnut samples. B) Pistachio samples C) Cashew samples. Each lane was loaded with 20 μg of protein. 4-20% Tris-glycine gels were used and Precision Plus was used a MW marker (P+ lane).

Figure 2B shows the SDS-PAGE pattern of pistachio samples (untreated, boiled, autoclaved, DIC treated pre and after digestion with E5). Similarly to hazelnut, control and boiled pistachio samples were almost identical (lanes 1 and 2) with a broad band pattern from 10 to 75 kDa. Lanes 3 and 4 showed few defined bands below 20 kDa (lanes 3 and 4). The protease E5 produced an important fragmentation on raw and boiled pistachio samples (lanes 5 and 6) that was more important for samples treated with autoclave and DIC (lanes 7 and 8). The electrophoretic analysis of cashew samples (untreated, boiled, autoclaved, DIC treated pastes before and after enzymatic hydrolysis with E7) is shown in Figure 2C. As previously mentioned, control and boiled samples showed a wide range of stained bands (5 to 75 kDa) that almost disappeared after autoclave and DIC treatment (lanes 3 and 4). The enzymatic digestion with E7 reduced significantly the protein bands on untreated and boiled cashew (lanes 5 and 6) but almost eliminated protein bands on heat pressured cashew samples (lanes 7 and 8).

The IgE bound amount of these samples was evaluated by Western blot taking individual sera from 23 patients with sensitization to hazelnut (Figure S2 supplementary). For comparisons, all immunoblots contain control raw, boiled, autoclaved and DIC processed samples previous and post enzymatic digestion under sonication (lanes 1 to 8). In Figure 3A, the IgE blots of the 23 patients ordered according to the treatment applied to the hazelnut samples have been collected. The figure shows data of control hazelnut and the processed samples tested on patients for SPT analysis (boiled, autoclaved, DIC, Ctr+Enz and DIC+Enz). IgE binding profile was almost identical for untreated and

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boiled hazelnut before enzymatic digestion with the 23 individual sera. A global decrease in IgE immunoreactive bands was apparent with autoclaved and DIC hazelnut, with similar allergenic proteins recognition. IgE binding profile was similar to electrophoretic SDS-PAGE pattern (Figure 2).

Figure 3. IgE immunoblots of individual sera of patients sensitized to hazelnut, pistachio and cashew sorted according the processing applied: untreated (Control) and treated t samples B60 (boiled 100ºC, 60 min, AU (138ºC, 256 kPa, 30 min), DIC (170ºC, 7 bar, 120 s) and enzymatically treated raw (Control+E) and DIC (DIC+E). A) 23 patients sensitized to hazelnut. B) 15 patients sensitized to pistachio and C) 12 patients sensitized to cashew.

The results indicated that after 1h of enzymatic digestion with E5 under sonication many reactive bands were digested in untreated and heat-treated hazelnut samples. In spite of this, some resistant IgE binding proteins (mainly below 25 kDa) were reactive with sera of patients n. #2, 3, 4, 6, 8, 10, 11, 14, 18 and 27. Hazelnut samples DIC treated were more susceptible to enzymatic hydrolysis because no IgE-binding proteins were detected in most of 23 sera analyzed (Figure 3A).

In Figure 3 supplementary. are shown the IgE binding analysis with 15 individual sera sensitized to pistachio. In Figure 3B are these fifteen immunoblots grouped by processing. The control and boiled pistachio samples showed a similar IgE blot pattern with many reactive IgE bands, which were mostly not detected after heat pressure (autoclave and DIC). The enzymatic hydrolysis with E5 was also effective to digest pistachio allergenic proteins in Control samples and only few resistant bands appeared in sera n. # 2, 6, 10, 23 and 27. DIC treated pistachio samples were very susceptible to enzymatic digestion since the immunoblots did not detect barely reactive bands.

The same IgE binding study was applied to cashew samples with twelve individual sera from patients sensitized to cashew (Figure S4 supplementary). Figure 3C collected the immunoblots from the twelve sera grouped by processing. A wide range of reactive IgE bands were detected with control and boiled samples and few IgE binding proteins might be detected in autoclaved and DIC treated samples before digestion. The data showed that most of reactive bands were digested in control samples after 2 h of hydrolysis with E7, and only a few resistant bands were observed in sera n. # 3, 6, 8, 10, 27 and 31. Similarly to hazelnut and pistachio, DIC treatment increased digestion capacity of E7 and extremely few IgE reactive bands were hardly detected.

3.4. Detection of 7S Globulin, 2S Albumin, Bet v1 Homolog (PR-10) and LTP Allergens in Raw and Processed Samples

The electrophoretic SDS-PAGE analysis on hazelnut (Figure 4), pistachio (Figure 5), and cashew (Figure 6), subjected to boiling, autoclave, and DIC treatments before and after enzymatic hydrolysis (E5 enzyme for hazelnut and pistachio, E7 for cashew), revealed consistent patterns across the three tree nuts. Untreated samples displayed multiple stained bands across a broad molecular weight range of 10-110 kDa Boiling did not alter the protein profiles in hazelnut, cashew, and pistachio compared with untreated samples. However, autoclave and DIC treatments individually led to protein fragmentation in the three nuts, evidenced by an increase in smears. When the enzymatic treatments with E5 enzyme for hazelnut and pistachio, and E7 for cashew were considered, the protein patterns were affected, resulting in further reduction of bands above specific molecular weights (20 kDa for hazelnut, 37 kDa for cashew, and 25 kDa for pistachio), indicating increased fragmentation particularly in samples processed with both heat and pressure treatments in combination with enzyme treatments.

Figure 4. Protein electrophoresis and detection of hazelnut allergens in untreated and treated hazelnut samples using specific polyclonal antibodies anti 7S globulin, anti 2S albumin, anti Bet v1 homolog (PR10) and anti LTP. A: SDS–PAGE of control (untreated) (lane 1), Boil (boiled 100ºC, 60 min) (lane 2), AU (138ºC, 256 kPa, 30 min) (lane 3), DIC (170ºC, 7 bar, 120 s) (lane 4) and enzymatically treated (E5) hazelnut samples (lanes 5-8). B-E: Detection of 7S globulin (B), 2S albumin (C), PR10 proteins (D) and LTP proteins (E) by western blot (B1-E1) and ELISA (B2-E2). The Optical Density (O.D.) was measured at 450 nm. Graphs show the mean and the standard deviation of the assays

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(performed in duplicate). The horizontal lines indicate the cut-off points of positivity for ELISA results.

Figure 5. Protein electrophoresis and detection of pistachio allergens in untreated and treated pistachio samples using specific antibodies anti 7S globulin, anti 2S albumin, anti Bet v1 homolog (PR10) and anti LTP. A: SDS–PAGE of control (untreated) (lane 1), Boil (boiled 100ºC, 60 min) (lane 2), AU (138ºC, 256 kPa, 30 min) (lane 3), DIC (170ºC, 7 bar, 120 s) (lane 4) and enzymatically treated (E5) pistachio samples (lanes 5-8). B-E: Detection of 7S globulin (B), 2S albumin (C), PR10 proteins (D) and LTP proteins (E) by western blot (B1-E1) and ELISA (B2-E2). The Optical Density (O.D.) was measured at 450 nm. Graphs show the mean and the standard deviation of the assays (performed in duplicate). The horizontal lines indicate the cut-off points of positivity for ELISA results.

Figure 6. Protein electrophoresis and detection of cashew allergens in untreated and treated cashew samples using specific antibodies anti 7S globulin, anti 2S albumin, anti Bet v1 homolog (PR10) and anti LTP. A: SDS–PAGE of control (untreated) (lane 1), Boil (boiled 100ºC, 60 min) (lane 2), AU (138ºC, 256 kPa, 30 min) (lane 3), DIC (170ºC, 7 bar, 120 s) (lane 4) and enzymatically treated (E7) cashew samples (lanes 5-8). B-E: Detection of 7S globulin (B), 2S albumin (C), PR10 proteins (D) and LTP proteins (E) by western blot (B1-E1) and ELISA (B2-E2). The Optical Density (O.D.) was measured at 450 nm. Graphs show the mean and the standard deviation of the assays (performed in duplicate). The horizontal lines indicate the cut-off points of positivity for ELISA results.

Western blot and ELISA were carried out using polyclonal antibodies to study changes in the binding of the key allergens in raw and processed hazelnut, cashew, and pistachio samples. The reason to use these polyclonal antibodies anti-Ara h 1, anti-Ara h 2, anti-Ara h 8 and amti-Mal d 3 was the high homology between allergenic proteins from the same protein family (7S globulin, 2S albumin, PR10 and LTP)

In the case of hazelnut, western blot analysis revealed that the allergen 7S globulin (equivalent to Cor a 11) exhibited comparable recognition in both untreated and boiled hazelnut samples (Figure 4B1,B2). Conversely, hazelnut subjected to autoclave and DIC processing demonstrated a noticeable decrease in the 7S globulin detection. When enzymatic digestion with E5 was considered, both untreated and boiled hazelnut samples exhibited diminished allergen recognition, whereas samples processed with heat and pressure (AU and DIC) and the enzymatic digestion revealed a complete decrease in the presence of the allergen 7S globulin. ELISA experiments corroborated these findings, indicating that the combined action of heat, pressure, and enzyme hydrolysis reduced the detectability of 7S globulin. In the case of the allergen 2S albumin (Cor a 14) (Figure 4C1,C2), the detectability of this allergen in western blot was low, however AU treatment alone without the enzymatic treatment produced an important smear in the high molecular weight area with high detection. This effect was entirely abolished upon the application of enzymatic treatment with E5. For the allergens PR-10 and LTP in hazelnut (Cor a 1 and 8, respectively) (Figure 4D1–4E2), similar levels of recognition were observed in untreated and boiled samples, while a decrease in the detectability of these allergens were observed in the rest of the treatments in western blot. ELISA results confirmed that the samples that combine heat, pressure and enzymatic treatments showed a reduced detectability of both allergens.

In pistachio, the allergens 7S globulin (Pis v 3), 2S albumin (Pis v 1), PR-10, and LTP were recognized in raw and boiled samples in an almost similar way (Figure 5B1–5E2). Upon exposure to pressured heating treatments (AU 256 and DIC) alone, a decrease in the recognition of 7S globulin, PR-10, and LTP was observed via western blot analysis. However, for 2S albumin, an intensified high molecular weight band appeared prominently in AU-treated samples. Nevertheless, when all these samples were treated enzymatically, a general reduction in the content of the four analyzed allergens was observed, especially relevant for samples autoclaved and DIC treated in combination with the enzymatic treatment with E5. ELISA results displayed a reduction of the detection of 7S globulin, 2S albumin, PR-10, and LTP in the pistachio processed with pressured heating in combination with the enzymatic treatment with E5, however, in the case of PR-10, the signal was still positive.

 doi:10.20944/preprints202404.0693.v1

In the case of cashew, western blots showed that the allergens 7S globulin (Ana o 1), 2S albumin (Ana o 3), PR-10, and LTP were detected in similar levels in both untreated and boiled cashew samples (Figure 6B1–E1). However, the application of AU and DIC treatments alone led to a decrease in all analyzed allergens' detectability. Notably, when the samples were subjected to enzymatic hydrolysis with E7, the detection of 7S globulin, 2S albumin, and PR-10 was high in untreated and boiled samples compared with those without enzymatic treatment. Yet, the combined action pressured heating, and enzyme treatment with E7 completely eliminated the recognition of all four allergens in immunoblots assays. Similarly, ELISA results indicated a reduction in the detectability of the four allergens in cashew processed with AU and DIC, followed by enzymatic hydrolysis with E7.

3.5. IgE Binding to Hazelnut ns LTP vs Seed Storage Proteins

As reported in section 3.1 of results, there was a predominance of individuals sensitized to hazelnut in our studied population (23 out of 25 patients). In relation to the sensitization profile, inside this subgroup, were predominant the patients with sensitization against nsLTP, Cor a 8 (15 out of 23) vs seed storage protein (Sprot) Cor a 9 (11S) and Cor a 14 (2S) (7 out of 23) (Table S1 supplementary).

The wheal diameter of patients with LTP profile was significantly different to those of Sprot sensitized patients (Figure 7A). The SPT values were significantly lower when raw hazelnut (control) was tested with LTP patients (6.80 ± 0.85) than with Sprot patients (14.50 ± 4.53) and when the wheal size means were contrasted after prick of processed hazelnut samples (boiling, autoclave, DIC or enzymatically digested control and DIC). In Figure 7B, the IgE western blots of the 23 individual sera are ordered by the protein profile of sensitization (Sprot or LTP) and the processing applied. As can be observed, these groups showed different immunoreactive band pattern. However, in both control and boiled hazelnut samples, the IgE blots were with high number of immunoreactive bands and additionally, for both Sprot and LTP groups, a reduction of immunoreactive bands was observed after autoclaving and DIC treatment and even higher reduction was achieved after enzymatical treatment. Figure 7C,D showed the IgE blots of pool sera of patients sensitized against Sprot (n=7) and LTP (n=15), respectively. Although higher immunoreactivity was observed for Sprot sera pools, in both there is a clear reduction of IgE binding after heat pressured processing (autoclave and DIC) and enzymatically digested hazelnuts in concordance with SPT results (Figure 7A).

Figure 7. A: SPT reactivity of patients sensitized to hazelnut storage proteins (11S globulin Cor a 9 and 2S albumin Cor a 14) or hazelnut nLTP (Cor a 8). B: IgE immunoblots of 23 individual sera of patients sensitized and reactive to hazelnut ordered according processing applied to hazelnut and the type of hazelnut protein sensitized profile (Sprot or LTP). C: IgE immunoblots of pool sera (n=7) of patient sensitized to Sprot (patient no.#1, #3, #4, #5, #8, #22, #27). D: IgE immunoblots of pool sera (n=15) of patient sensitized to LTP (#2, #6, #10, #11, #13, #14, #16, #18, #19, #20, #23, #24, #26, #30, #33).

3.6. ELISA Inhibition

A competitive ELISA inhibition assay was made to evaluate the IgE binding reactivity of hazelnut raw and processed samples using pool sera from 7 patients with sensitization against Sprot hazelnut allergens. As solid phase, untreated hazelnut (control) was preincubated with the following inhibitors: raw hazelnut as control, boiled, autoclaved, DIC treated hazelnut, or E5 enzymatically digested raw and DIC hazelnut samples. The inhibition of IgE binding was calculated as described in section 2.5 of Material and Methods and represented in Figure 8. The contrast via Duncan's test of mean values of raw and processed hazelnut samples for each inhibitor concentration is shown in the table below the graph. Untreated hazelnut competed for IgE binding without and with hydrolysis with E5 (63.5% and 44.6%, respectively). Meanwhile, the pressured heated hazelnut samples had significant lower IgE binding inhibition capacity compared to raw hazelnut. Autoclaved and DIC treated hazelnut samples pre and post E5 digestion were the weakest competitors for IgE binding. These findings are consistent with the decrease in IgE binding revealed by immunoblotting for these hazelnut treated samples (Figure 3A).

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Figure 8. Competitive ELISA inhibition of the IgE binding to immobilized raw hazelnut by increasing concentration of untreated raw (control), boiled, autoclaved, DIC treated hazelnut, or 1h E5 enzymatically treated raw or DIC treated hazelnut samples, as inhibitors. Means in the same column followed with the same superscript are not significantly different (P>0.05).

4. Discussion

In the present research, the influence that thermal treatments (boiling, autoclave or DIC technology), applied individually or in combination with enzymatic digestion, on the in vitro allergenic reactivity of hazelnut, pistachio and cashew have been studied. Pressured heating treatments, selected in the present research, were chosen on the basis of former works concluding that autoclave processing (138 °C, 256 kPa during 30 minutes) decrease notably IgE binding in legumes and tree nuts (Álvarez-Álvarez, Guillamón, Crespo, Cuadrado, Burbano, Rodríguez, et al., 2005; Cabanillas, Cuadrado, Rodriguez, Hart, Burbano, Crespo, et al., 2015; Cabanillas, Maleki, Rodriguez, Burbano, Muzquiz, Jimenez, et al., 2012; Cuadrado, Cabanillas, Pedrosa, Varela, Guillamon, Muzquiz, et al., 2009; Cuadrado, et al., 2018; Cuadrado, et al., 2023; Cuadrado, Sanchiz, Vicente, Ballesteros, & Linacero, 2020; De Angelis, et al., 2022; Lopez, Cuadrado, Burbano, Jimenez, Rodriguez, & Crespo, 2012; Sanchiz, et al., 2018; Vicente, et al., 2020). With regard to DIC treatments, steam pressure of 7 bar for 120 s has been previously established as the most efficient condition for IgE binding reduction (Vicente, et al., 2020; Cuadrado, et al., 2023)

This investigation revealed that heat pressured processing (AU and DIC treatments) applied individually effectively caused protein degradation in hazelnut, cashew, and pistachio as evidenced by the smearing patterns observed on SDS-PAGE analyses for each of the three types of nuts. The boiling method, on the other hand, did not exhibit any notable differences in protein profiles when contrasted to the untreated control samples. The inclusion of enzymatic treatments, utilizing enzyme E5 for hazelnut and pistachio and enzyme E7 for cashew, subsequent to the thermal treatments, resulted in even more pronounced fragmentation of proteins. Immunoassays, conducted with specific polyclonal antibodies targeting main allergens in hazelnut, cashew, and pistachio, showed a marked reduction in the levels of the 7S globulin, 2S albumin, PR-10, and LTP allergens in the nut samples that underwent the combined action of pressured heating (AU and DIC treatments), and enzymatic treatments. This reduction in the detectability of the 7S globulin, 2S albumin, PR-10, and LTP key allergens was further corroborated by ELISA tests, indicating a decrease in allergenic content post-treatment.

Our results are in agreement with former findings reporting that the treatments combination, such as pressure heating and enzyme digestion, is necessary to achieve a strong reduction of IgE immunoreactivity in pistachio and cashew pastes by in vitro testing using sera from 7 patients from USA (Cuadrado, et al., 2018). In that study, autoclave applied before enzymatic digestion with different proteases was the most effective combination to reduce the IgE immunoreactivity of pistachio and cashew (Cuadrado, et al., 2018). In the present research, we have included DIC treatment (7 bar, 120 s) on pistachio and cashew samples, by itself or with the combination of a commercial protease hydrolysis. More recently, we have demonstrated in peanut that the combination of enzymatic digestion with another pressured heating treatment, DIC at 7 bar for 120 s is as effective as the combination with autoclave (at 256 kPa for 30 min) for inducing protein fragmentation and an almost complete elimination of the allergic reactivity of peanut but viaa faster way (120 s vs 30 min). Only a few peptides could be characterized post enzymatic digestion of DIC peanut samples, all belonging to Ara h 1 and Ara h 3 (Cuadrado, et al., 2023).The present research intends to advance in that line and to analyze the combined action of heat, pressure, and enzyme digestion on allergenic reactivity of tree nuts prevalent in Europe, such as hazelnut, pistachio and cashew.

The evaluation of the effect of enzymatic digestion, in order to check the potential reduced allergenicity, should be carried out through in vitro assays with sera from well-studied allergic subjects and in vivo allergenicity test to corroborate that these processing methods can produce a drastic reduction of the in vivo allergenic reaction of three nuts (Lucas & Atkinson, 2008). For

 doi:10.20944/preprints202404.0693.v1

allergenicity assessment the most reliable tests involve oral food challenge, (Costa, et al., 2022). Moreover, a good parameter for the characterization of allergens is their stability under gastric conditions (Moreno, 2007; Pali-Schöll, Untersmayr, Klems, & Jensen-Jarolim, 2018), and in vitro tests of pepsin digestion was incorporated since 2001 into a FAO/WHO procedure for the allergenicity assessment of novel food proteins (FAO, 2001). Enzymatic protein hydrolysates are a good alternative to intact proteins for obtaining special formulations to provide nutritive support to specific population groups such as elderly, infants, and food-allergic patients. Additionally, protein hydrolysates have technological improvements. The extensive enzymatic processing in combination with other food processing, such as heat, pressure and ultrasonication, is considered very efficient to develop hypoallergenic protein products with an high added value for allergic populations (Cuadrado, et al., 2018; Sanchiz, Pedrosa, Guillamón, Arribas, Cabellos, Linacero, et al., 2019).

In conclusion, these findings clearly demonstrate that the combined action of heat/pressure processing (autoclave and DIC) followed by enzyme hydrolysis was the most effective methodology to drastically reduce or indeed eliminate the allergenic reactivity of hazelnut, pistachio and cashew. Heat/pressure processing (autoclave and DIC) and enzyme digestion under ultrasonication individually induced an important decrease of the IgE binding capacity of pastes made from treated hazelnut, resulting with higher effect on nsLTP (Cor a 8) than on Sprot (Cor a 9 and Cor a 14) hazelnut allergens. Nevertheless, DIC technology at 7 bar during 120 s combined with enzyme hydrolysis is required to drastically decrease IgE immunoreactivity against Sprot hazelnut allergens. For pistachio and cashew, the combination of DIC treatment before enzymatic digestion resulted in the most effective methodology to drastically reduce or indeed eliminate the allergenic capacity of these tree nuts.

In the future, the decreased IgE crosslinking capacity of these tree nuts caused by these combined treatments using in vivo models of food allergy should be addressed. The characterizations such as FTIR (fourier transform infrared spectroscopy), SEM (scanning electronic microscopy), and EDX (X-ray spectroscopy), which could be useful tools for the design of specific methods for the removal of allergic compositions without damaging the protein components of the nuts.

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