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Thermal processing effects on the IgE-reactivity of cashew and pistachio

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ABSTRACT

Thermal processing can modify the structure and function of food proteins and may alter their allergenicity. This work aimed to elucidate the influence of moist thermal treatments on the IgE-reactivity of cashew and pistachio. IgE-western blot and IgE-ELISA were complemented by Skin Prick Testing (SPT) and mediator release assay to determine the IgE cross-linking capability of treated and untreated samples. Moist thermal processing diminished the IgE-binding properties of both nuts, especially after heat/pressure treatment. The wheal size in SPT was importantly reduced after application of thermally-treated samples. For cashew, heat/pressure treated-samples still retain some capacity to cross-link IgE and degranulate basophils, however, this capacity was diminished when compared with untreated cashew. For pistachio, the degranulation of basophils after challenge with the harshest heat/pressure treatment was highly decreased. Boiling produced more variable results, however this treatment applied to both nuts for 60 min, led to an important decrease of basophil degranulation.

1. Introduction

Food allergy has a relevant impact on the quality of life of allergic people, and it is considered as an important lifelong persisting problem. Although the prevalence of food allergy varies depending on the geographical area, study populations analyzed and allergens studied, it is accepted that it affects up to 1–3% of the general population, reaching even 6–8% in children. Tree nuts are among the eight foods that cause the majority of allergic reactions to foods in Europe and the U.S. (Nwaru et al., 2014; Fernández Rivas, 2009). Furthermore, tree nuts are primarily responsible for fatal allergic reactions in the U.S and the U.K (Bock, Muñoz-Furlong, & Sampson, 2007; Pumphrey & Gowland, 2007). Tree nuts are included in the list of the most commonly allergenic ingredients (Regulation EU No 1169/2011/EC, OJEU 2011) and their presence in food must be indicated.

Consumption of tree nuts is on the rise due to their beneficial health effects, especially concerning risk reduction of coronary diseases and due to their rich nutritional composition. Particularly, pistachio nut contains a wide variety of healthy nutritional components, including high amounts of protein, antioxidants, minerals and low content of unhealthy fats (basically from MUFA and PUFA), among others (Bulló, Juanola-Falgarona, Hernández-Alonso, & Salas-Salvadó, 2015). Cashew nut, for its part, is highly energetic and rich in unsaturated fatty acids, fibre, amino acids and vitamins (Rico, Bulló, & Salas-Salvadó, 2015).

Typically, tree nuts allergens are identified as seed storage proteins, among others. In cashew, major allergens are characterized as a vicilin-like protein or 7S globulin (Ana o 1, 50 kDa), legumin-like protein or 11 S globulin (Ana o 2, 53 kDa) and 2S albumin (Ana o 3, 12 kDa) (Robotham et al., 2005; Wang et al., 2002; Wang, Robotham, Teuber, Sathe, & Roux, 2003). It seems that cashew allergy prevalence is in-

Abbreviations: BCA, Bicinchoninic acid assay; DBPCFC, double-blind placebocontrolled food challenge; FEIA, Fluorenzymeimmunoassay; HRP, horseradish peroxidase; MRA, mediator release assay; PVDF, polyvinylidene difluoride; SPT, Skin Prick Testing; TBS, Trisbuffered saline; TBST, TBS plus 0.5% Tween-20; TMB, tetramethylbenzidine.

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creasing over the years and it has been involved in severe anaphylaxis, even exceeding peanut allergy in severity (Clark, Anagnostou, & Ewan, 2007; Van der Valk, Dubois, Gerth van Wijk, Wichers, & de Jong, 2014). Pistachio is also a well-characterized tree nut whose allergens belong to 2S albumin (Pis v 1, 7 kDa), legumin-like proteins or 11S globulins (Pis v 2 and Pis v 5, 32 and 36 kDa), vicilin-like protein or 7S globulin (Pis v 3, 55 kDa) and superoxide dismutase (Pis v 4, 25.7 kDa) (Ahn, Bardina, Grishina, Beyer, & Sampson, 2009; Noorbakhsh, Mortazavi, Sankian, Shahidi, & Assarehzadegan et al., 2010; Willison et al., 2008). Cross-reactivity between cashew, pistachio and mango, all of them members of *Anacardiaceae* family, has been observed (García & Lizaso, 2011; Noorbakhsh et al., 2011). Currently, there is no treatment for cashew and pistachio allergy. Therefore, avoidance is the only effective "therapy" for allergic patients. However, cashew and pistachio presence as traces is sometimes difficult to eliminate, due to cross-contamination in food lines (Taylor & Baumert, 2010).

Thermal (moist heating, dry heating, dielectric heating) and non-thermal (mechanical, enzymatic, irradiation) treatments are mainly carried out in industry to improve food quality, preservation or safety. Moreover, certain thermal processing methods are also used by consumers in order to improve sensorial properties of foods. Food processing can modify the structure and function of food proteins and may alter (by increasing or decreasing) their allergenic properties (Cabanillas & Novak, 2017). In that sense, understanding the potential effects of food processing on the allergenic properties of foods constitutes an active area of research.

In the specific case of nuts, the influence on allergenicity after a wide variety of different treatments has been studied (Jiménez-Saiz, Benedé, Molina, & López-Expósito, 2015; Vanga & Raghavan, 2016; Verhoeckx et al., 2015). Thermal treatments in walnut (Cabanillas et al., 2014), HHP in hazelnut (Prieto et al., 2014), roasting, blanching, autoclaving and microwave heating in almond (Venkatachalam, Teuber, Roux, & Sathe, 2002) and several thermal processing conditions in peanut (Cabanillas et al., 2012, 2015; Maleki, Chung, Champagne, & Raufman, 2000) have been studied with different results, depending on the conditions of the treatments and the material analyzed. Knowledge about the effects of thermal processing on tree nuts such as cashew or pistachio is scarce and based on traditional *in vitro* immunoassays. Only a few studies have analyzed the influence of various treatments including autoclaving (at 121 °C), blanching, pH variation, microwave heating and γ-irradiation over cashew seeds. Although cashew proteins showed high stability to all processing methods used, autoclaving or a combination of γ-irradiation plus autoclaving seemed to cause some decrease in antigen detection (Su, Venkatachalam, Teuber, Roux, & Sathe, 2004; Venkatachalam et al., 2008). Mattison et al. (2014) found that sodium sulphite and heating treatment can modify the structure of specific cashew allergens, decreasing their IgE-binding (Mattison et al., 2014). Interestingly, the same authors also demonstrated by SDS-PAGE and LC-MS/MS that solubility of cashew proteins is modified by heat treatment and the relative amount of peptides from specific cashew allergens was also affected as well as IgE-binding capability of the soluble extracts (Mattison et al., 2016). Oleic acid has been found to bind cashew allergens, reducing the IgE-binding capacity (Chung, Mattison, Reed, Wasserman, & Desormeaux, 2015). In pistachio nuts, a reduced reactivity was observed by western blot and ELISA analysis after soaking in lemon water and steaming, without changes in sensory evaluation (Noorbakhsh, Mortazavi, Sankian, Shahidi, & Maleki et al., 2010).

An altered ability of food allergens to bind IgE using traditional *in vitro* immunoassays is not always directly related to a modified allergenic function (Shi et al., 2013). Therefore, physiologically relevant experiments, such as SPT and mediator release assays (MRA), in which the IgE cross-linking capacity of processed food proteins is analyzed in effector cells of allergy, should constitute an essential part on the research of allergenic properties of processed food. This kind of studies are important preliminary tests to ensure a possible reduction in IgE cross-linking capacity, before performing further clinical studies.

The aim of this work was to elucidate the influence of moist thermal treatments (boiling and heat/pressure) on the IgE-reactivity of cashew and pistachio proteins, by means of traditional *in vitro* immunoassays, and physiological relevant assays as SPT and MRA.

2. Materials and methods

2.1. Plant material, thermal processing and protein extraction

Cashew (*Anacardium occidentale,* type 320) obtained from Productos Manzanares S.L. (Spain) and raw pistachio (*Pistacia vera* Kerman) from the Germoplasm Bank of Institut de Recerca i Tecnología Agroalimentàries (IRTA-Mas de Bover, Tarragona, Spain) were used for this study. Cashew nuts were not purchased as raw, since they were industrially processed in order to remove harmful oils, shell and the skin.

Nuts were boiled in distilled water (1:5 w/v) for 30 and 60 min (named as "boiled 30′" and "boiled 60′" respectively), or subjected to heat and pressure treatments in distilled water (1:5 w/v) using a Compact 40 Benchtop autoclave (Priorclave, London, UK) at 121 °C (1.18 atm) for 15 min and 30 min (named as "AU 121 °C 15′" and "AU 121 °C 30′") or 138 °C (2.56 atm) for 15 and 30 min (named as "AU 138 °C 15′" and "AU 138 °C 30′").

Untreated and treated nuts were freeze-dried (Telstar Cryodos frezze-drier), ground using a kitchen robot (Thermomix 31-1, Vorwerk Elektrowerke, GmbH & Co. KG, Wüppertal, Germany), defatted with *n*-hexane (34 ml/g of flour) and milled with a sieve of 1 mm (Tecator, Cyclotec 1093, Höganäs, Sweden). The nitrogen contents of the pistachio and cashew flours were determined by LECO analysis, according to standard procedures based on Dumas method. The total protein content was calculated as N × 5.3 (AOAC, 2000).

Proteins from treated and untreated defatted flours from cashew were extracted in a solution of Borate Buffer Saline (BSB) 1:10 w/v (100 mM H_3BO_3, 25 mM $Na_2B_4O_7$ and 75 mM of NaCl, pH 8.4), overnight at 4 °C with constant shaking. After sonication (three times 15 s), centrifugation was carried out at 8250*g* (8500 rpm) at 4 °C for 20 min. Supernatant was collected and sterilized with 0.22 μm filters. The same buffer but adding 1% polyvinylpyrrolindone (PVP) was employed to obtain pistachio protein extract from untreated and treated defatted flours at 1:10 w/v, for 1 h at 4 °C and constant stirring. After centrifugation (27419*g* or 15000 rpm, 20 min at 4 °C), supernatants were dialyzed against distilled water using a membrane with a cut-off point of 3.5 kDa for 24 h at 4 °C, and then they were freeze-dried. Pistachio dry extracts were then resuspended in sterile PBS buffer and sterilized with 0.22 μm filters. The bicinchoninic acid assay (BCA) (Pierce Biotechnology, Rockford, IL, USA) was used for protein extracts quantitation.

2.2. Patients and sera

Sera from six patients with clinical allergy to pistachio and/or cashew, confirmed on the basis of either a convincing history of anaphylaxis with positive SPT and specific serum IgE levels to pistachio and/or cashew measured by means of fluorescent enzyme immunoassay (CAP-FEIA system, Phadia, Uppsala, Sweden), shown in Table 1, or a positive double-blind placebo-controlled food challenge (DBPCFC). The study was approved by the Ethics Committee of the Hospital Universitario 12 de Octubre, Madrid, Spain (Permission No. 0312150129).

Table 1
Immunological and clinical characteristics of the 6 patients allergic to pistachio and/or cashew included in this study.

Patient	Age/Sex	Allergen	IgE (kU/L)	Symptoms	Diagnostic challenge
#1	22/F	Pistachio	2.09	OAS	DBPCFC
		Cashew	1.38	Angioedema, urticaria, cough	a
#2	46/F	Pistachio	0.69	OAS, abdominal pain	DBPCFC
		Cashew	0.81	Urticaria, abdominal pain, vomiting	a
#3	42/F	Pistachio	1.05	OAS	DBPCFC
#4	23/M	Pistachio	12.1	Urticaria, Angioedema, cough.	a
		Cashew	8.75	Urticaria, cough, dizziness	a
#5	50/F	Pistachio	0.35	Urticaria, bronchospasm	a
#6	29/M	Pistachio	21.3	OAS, angioedema	DBPCFC
		Cashew	8.94	OAS, angioedema	DBPCFC

DBPCFC, double-blind, placebo-controlled food challenge; F, female; M, male; OAS, oral allergy syndrome. a, Not challenged because of a convincing history of anaphylaxis to cashew or pistachio.

Cashew and/or pistachio allergic patients underwent SPT with untreated and treated samples according to standard methods (Malling, 1993). The mean diameters of SPT reactions were expressed in millimetres, and calculated as the sum of the largest diameter and the perpendicular distance, divided by two. SPT was performed in duplicate and a positive (histamine dihydrochloride) and negative (PBS) control were applied. Positive results were considered when wheal size was at least 3 mm greater than that elicited by the negative control. Paired *t*-test was used for comparison of means from untreated with treated samples, and differences were considered significant with $p < .05$. The statistics software GraphPad Prism version 5 for Windows (GraphPAd Software, San Diego, CA, USA) was used.

2.3. Protein electrophoresis and IgE-western blot analysis

Cashew and pistachio proteins were separated by SDS-PAGE. Twenty micrograms of protein, calculated by BCA assay, were mixed with Laemmli sample buffer and β-mercaptoethanol and heated for 10 min at 95 °C and electrophoresed in a 12% SDS-polyacrylamide gels, employing a Mini-Protean Tetra Cell apparatus (Bio-Rad, Hercules, CA, USA). Proteins were visualized with Coomassie Brilliant Blue (Bio-Rad, Hercules, CA, USA) or transferred into a polyvinylidene difluoride (PVDF) membrane (Merck KgaA, Darmstadt, Germany) for IgE-western blot analysis, using a semi-dry system (Biometra GmbH, Göttingen, Germany). Blocking was carried out for 1 h at room temperature in Tris-buffered saline containing 0.5% of Tween-20 (TBST) and 5% w/v non-fat milk. Incubation with pooled sera from the patients with pistachio (patients No. 1–6) or cashew (patients No. 1, 2, 4 and 6) allergy at 1:10 dilution was performed overnight at 4 °C. Membranes were washed and incubated with an anti-human IgE antibody produced in mouse (clone 1A2, Abbiotec, San Diego, CA USA) (stock: 1 mg/ml, used at 1:1000) for three hours at room temperature. Membranes were washed and finally treated with HRP-conjugated goat anti-mouse IgG antibody (Santa Cruz Biotechnology, Dallas, Texas, USA) (stock: 0.4 mg/ml, used at 1:1000) for 1 h. Detection was achieved by means of enhanced chemiluminescence (SignalFire™ Elite ECL Reagent, Cell Signaling Technology Inc, Danvers, USA).

In an additional experiment, untreated and treated cashew and pistachio flours were directly solubilized in SDS sample buffer as previously described to obtain total protein (Cabanillas et al., 2014). Electrophoretic analyses of cashew and pistachio total protein extractions were carried out as previously described (Cabanillas et al., 2014). Proteins were visualized with Coomassie Brilliant Blue or transferred onto PVDF membranes for IgE-western blot analysis as explained above.

2.4. IgE-ELISA and ELISA inhibition

Polystyrene 96-well plates (BD Falcon 353279, Heilderberg, Germany) were coated with 100 µl/well of cashew and pistachio extracts from untreated or treated samples (selected treatments: "boiled 60′", "AU 121 °C 30′" and "AU 138 °C 30′"), previously diluted at 50 µg/ml in PBS pH 7, and incubated overnight at 4 °C. Wells coated with blocking solution (PBST 0.1% (v/v) and 3% (w/v) of non-fat milk) instead of protein extracts were used as negative control. After washing with PBS-Tween 20 (PBST) at 0.5% (v/v), wells were blocked with blocking solution, for 1 h at room temperature. Plates were incubated with pooled sera or individual sera from the patients with pistachio and/or cashew allergy at 1:10 for 2 h at 37 °C, washed and treated with mouse anti-human IgE antibody (clone 1A2, Abbiotec, San Diego, CA USA, stock 1 mg/ml, used at 1:1000 dilution in blocking solution) for 1 h at 37 °C. After washing the wells, HRP-conjugated goat anti-mouse IgG antibody (Santa Cruz Biotechnology, Dallas, Texas, USA; Stock at 0.4 mg/ml, used at 1:1000 dilution) was added and incubated for 1 h at 37 °C. The reaction was developed with tetramethylbenzidine (TMB) and H_2O_2 substrate (R&D Systems, Minneapolis, USA), stopped with sulfuric acid 1M and OD was measured at 450 nm with 650 nm as a reference. All the tests were performed in duplicate. Cut-off point of positivity was calculated with the formula: mean OD + 3 × SD for the negative control, as described in previous studies (Palacin et al., 2007; Cabanillas et al., 2015); and it was represented as a horizontal line in the graphics. For the analysis of the results of ELISA performed with individual sera, paired *t*-test was used. Differences were considered as significant with $p < 0.05$. The statistics software GraphPad Prism version 5 for Windows was used.

For the ELISA inhibition experiment, polystyrene 96-well plates (BD Falcon 353279, Heidelberg, Germany) were coated with 100 µl/well of untreated pistachio or cashew at a final concentration of 250 µg/ml and incubated overnight at 4 °C. At the same time, in parallel, a pooled sera of pistachio or cashew allergic patients (final dilution 1:10) were pre-incubated with untreated or treated pistachio or cashew protein extracts as inhibitors (selected treatments: "boiled 60′", "AU 121 °C 30′" and "AU 138 °C 30′") at different final concentrations: 1, 0.1, 0.01 and 0.001 mg/ml, overnight at 4 °C and soft stirring. Pooled sera pre-incubated with PBS were also included (non-inhibited serum). Wells were washed and blocked with PBST 3% non-fat milk for 1 h. Incubation of the wells with the sera pre-incubated with the different inhibitors or the non-inhibited serum was carried out for 3 h at 37 °C. After washing the wells, incubations with mouse anti-human IgE antibody at 1:1000 for 1 h at 37 °C and HRP-conjugated goat anti-mouse IgG antibody at 1:1000 dilution were performed, and OD was measured at 450 nm with 650 nm as a reference. The percentage of inhi-

bition was calculated with the formula: $[1-(A_I/A_N)]\times 100$, where A_I is the absorbance value obtained in the wells incubated with inhibited serum and A_N the absorbance of the wells incubated with the non-inhibited serum (Cabanillas et al., 2015).

2.5. Rat basophil leukemia cell line (RBL-48) for MRA

RBL-48 cell line, transfected with the α chain from the high-affinity human IgE receptor FcεRI (a gift from Dr. J. Kochan) (Gilfillan et al., 1992), was used in order to analyze the release of allergic mediator β-hexosaminidase, induced by untreated and treated cashew and pistachio protein extracts. Cells were cultured in very low endotoxin RPMI 1640 Medium (Sigma-Aldrich, Saint Louis, MO, USA), supplemented with 10% heat-inactivated fetal calf serum, 1% antibiotic/antimycotics and 500 μg/ml of geneticin. The expression of the FcεRI-α chain was confirmed by flow cytometry, using an anti-human FcεRIα subunit antibody (eBioscience Inc, San Diego, CA, USA). Fifty μl of cells at 1×10^6/ml were plated in a 96-well tissue culture plate (Corning Inc, NY, USA) and sensitized with the pooled sera from allergic patients to cashew (patients No. 1, 2, 4 and 6) or pistachio (patients No. 1–6) at 1:10 final dilution, overnight at 37 °C, 5% of CO_2 and 95% humidity. Cells were washed with Tyrodes buffer and then stimulated for 1 h with sterile untreated and treated cashew or pistachio protein extracts at 1 mg/ml (selected treatments: "boiled 60′", "AU 121 °C 30′" and "AU 138 °C 30′"). Cells stimulated with Tyrodes Buffer instead protein extracts were used as a negative control. This negative control provides a measurement of the spontaneous release of mediator to the media alone. RBL-48 cells degranulation was measured by β-hexosaminidase release as previously described (Cabanillas et al., 2014). RBL-48 cells were lysed with 1% Triton X-100 for total mediator release. Percentage of β-hexosaminidase release was calculated as previously described (Cabanillas et al., 2014). Assays were performed in triplicate.

3. Results

3.1. SPT

SPT were carried out in cashew and pistachio allergic patients to determine the IgE cross-linking capability of untreated and all treated samples (boiled 30 and 60 min, heat/pressure 121 °C, 1.18 atm, 15 and 30 min and heat/pressure 138 °C, 2.56 atm, 15 and 30 min). Data are represented in Fig. 1. All patients had positive SPT with untreated cashew and pistachio protein extracts, and none of the patients had a positive result with AU 121 °C 30′, AU 138 °C 15′ and AU 138 °C 30′ extracts in both nuts. In pistachio, in which the statistical analysis using paired *t*-test was possible, a statistically significant decrease in the allergenic potential compared to untreated extracts was found for the mentioned treated samples. A decrease in the wheal size with boiled cashew and pistachio compared with the untreated samples was also found, although the decrease was not as strong as the one produced with heat-pressure samples. Even so, the decreased in the wheal size with boiled pistachio was significant, especially for the sample boiled for 60 min.

3.2. Immunodetection assays of processed cashew

3.2.1. Electrophoretic pattern and IgE-western blot of cashew samples

The characterization of the electrophoretic profile of the soluble protein extracts from untreated and thermal-treated cashew samples is shown in Fig. 2A. IgE-binding proteins were analyzed by IgE-western blot, using pooled sera from the 4 cashew allergic patients (Fig. 2B). The results showed that the treatments of boiling during 30 min had no major effects in the SDS-PAGE profile and the IgE-binding proteins from cashew. High molecular weight proteins (around 50 kDa) were the first bands affected by treatments (boiling 60 min) in SDS-PAGE and IgE-western blot. Cashew subjected to heat and pressure treatments showed less distinctive stained bands in SDS-PAGE with an increased protein fragmentation that went along with a reduction in IgE-reactive bands (Fig. 2B).

We additionally studied the electrophoretic and IgE-binding patterns of total protein from cashew which were obtained by direct solubilization of untreated and treated cashew flours in SDS sample buffer as described in materials and methods. The results showed that the electrophoretic and IgE-binding pattern profiles of total protein were similar to soluble protein extract (Supplementary material Fig. 1), although a band below 15 kDa was especially immunoreactive as well as resistant to applied processing. The IgE reactive bands were strongly reduced in the samples treated with heat and pressure, but still detectable even in the sample AU 138 °C 15 min.

3.2.2. IgE-ELISA and ELISA inhibition with cashew samples

Untreated and the selected treated cashew samples: boiled 60 min, AU 121 °C 30 min and AU 138 °C 30 min were used for these experiments.

ELISA inhibition assay was carried out using the pooled sera from the 4 patients with cashew allergy. Untreated cashew proteins were used to coat the plate and the three thermally treated samples (boiled

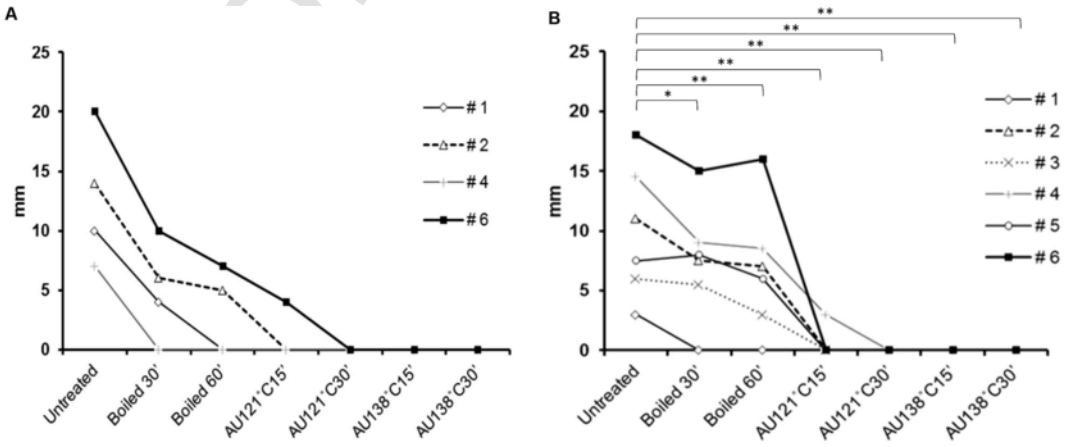

Fig. 1. SPT in patients with cashew and pistachio allergy. SPT with untreated and treated samples of cashew (A) and pistachio (B) in 4 and 6 patients with clinical allergy to cashew and pistachio, respectively. The mean diameters of the wheals in mm are shown. Significant differences with $^{*}p < .05$; $^{**}p < .005$ determined using paired *t*-test for pistachio.

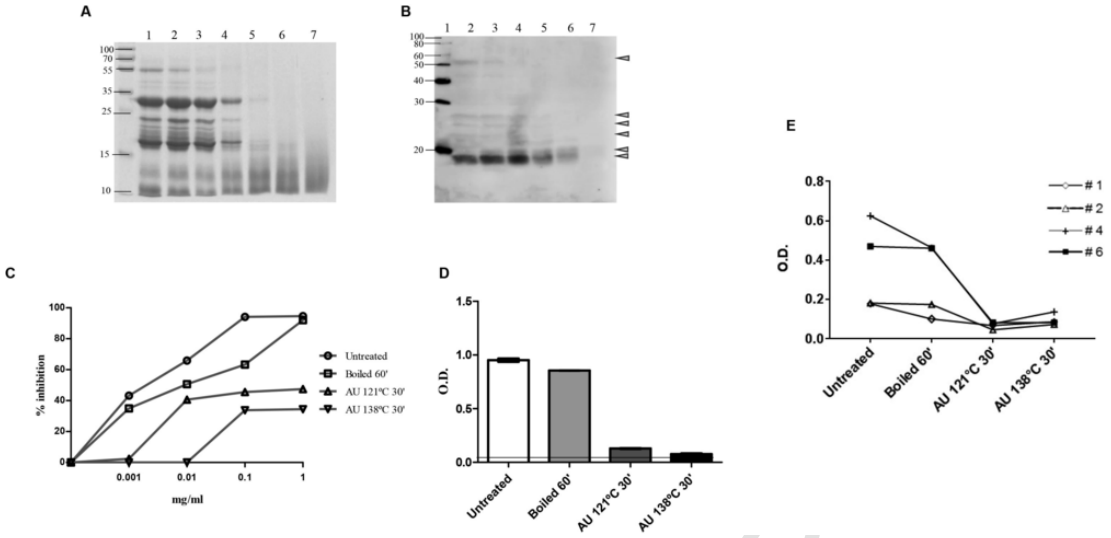

Fig. 2. Protein profile and IgE-immunodetection of cashew extracts. (A) SDS-PAGE and (B) IgE-western blot of cashew protein extract from untreated (lane 1) and treated nuts: boiled 30′ (lane 2), boiled 60′ (lane 3), AU 121 °C, 15′ (lane 4), AU 121 °C, 30′ (lane 5), AU 138 °C, 15′ (lane 6) and AU 138 °C, 30′ (lane 7). IgE-western blot was performed using pooled sera from four patients allergic to cashew (patients 1, 2, 4, and 6). IgE-reactive bands are marked with arrows. C. ELISA inhibition assay with untreated cashew extract for coating and untreated and treated cashew proteins (indicated in the legend) used as inhibitors. Pooled sera from four patients allergic to cashew was used (patients 1, 2, 4, and 6). D. IgE-ELISA of untreated and treated cashew incubated with the pooled sera. The cut-off point of positivity is indicated with a horizontal line. E. IgE-ELISA of untreated and treated cashew incubated with individual sera.

60 min, AU 121 °C 30 min and 138 °C 30 min) and untreated sample (control) were used as inhibitors mixed with the pooled sera at different concentrations. Results showed that proteins from untreated and boiled samples at 1 mg/ml inhibited 94% of the IgE-binding to untreated cashew proteins coated in the wells. The results also showed a decrease in IgE-binding capacity for heat/pressure treated proteins, showing a percentage of inhibition which became stagnant for AU 121 °C 30′ treatment at around 45% at 0.01, 0.1, and 1 mg/ml. For the treatment AU 138 °C 30′ the inhibition was about 0% up to 0.01 mg/ml and maximum of 37% at the highest concentration of inhibitor tested (Fig. 2C), which indicates a marked decrease in IgE-binding capacity for this specific treated cashew sample.

These results were confirmed by IgE-ELISA, in which the wells were coated with untreated and selected treated proteins and the pooled sera from cashew allergic patients were used. Results showed around 90% of reduction of IgE reactivity in heat/pressure treated samples and no marked effect was obtained after boiling treatment. The reduction of IgE reactivity after thermal treatments of cashew nuts was also analyzed by means of IgE-ELISA with the individual sera from the four patients with clinical allergy to cashew (1, 2, 4 and 6). IgE reactivity was strongly reduced after heat/pressure treatments (AU 121 °C 30 min and AU 138 °C 30 min). Boiling for 60 min, however, reduced IgE reactivity at a lesser extent in IgE-ELISA.

3.3. Immunodetection assays of processed pistachio

3.3.1. Electrophoretic pattern and IgE-western blot of pistachio samples

The protein profile, visualized by SDS-PAGE, of pistachio protein extract from untreated and boiling treated samples was very similar, and only a few high molecular weight bands were degraded. Moreover, some bands, mainly above 35 kDa, were reduced after the softest heat/pressure treatment (AU 121 °C, 15 min). The rest of heat/pressure treatments, especially AU 138 °C, 2.56 atm (15 min and 30 min) provoked a smear due to the degradation, rich in low molecular weight proteins. The strongest processing effect was obtained after harsh heat/pressure conditions (138 °C for 30 min) (Fig. 3A). The protein migration of pistachio flour directly solubilized in the electrophoretic sample buffer was also analyzed and no relevant differences compared to pistachio soluble protein extract were detected (Supplementary material Fig. 1).

IgE-western blot was performed with total and soluble protein extraction, using a pool of six patients' sera with pistachio allergy (1 − 6). IgE-reactivity was detected for several bands up to heat/pressure treatment at 121 °C for 15 min. Two bands were especially resistant (bands around 20 and 13 kDa), but also bands in the range from 30 to 55 kDa were easily observable in soluble protein extract western blot (Fig. 3B). The band around 13 kDa was detected in the IgE-western blot of pistachio total protein, even at 121 °C 30 min (Supplementary material Fig. 1). The soluble protein extracts from untreated and the selected treated samples: boiled 60′, AU 121 °C 30′ and AU 138° C 30′ were used for the rest of experiments.

3.3.2. IgE-ELISA and ELISA inhibition with pistachio samples

Inhibition of IgE-binding to immobilized untreated pistachio proteins augmented with increased concentrations of thermal-treated pistachio extracts (inhibitors). Untreated and boiled 60′ samples competed for IgE at 87% and 80% at 1 mg/ml respectively. Pistachio treated with heat/pressure at 138 °C for 30 min was a weaker competitor than boiled or soft heat/pressure treatment at all concentrations, reaching a maximum of 58% of inhibition at 1 mg/ml, which indicates that is the treatment that caused the major decrease in IgE-binding capacity (Fig. 3C).

A consistent IgE-reactivity decrease after boiling and heat/pressure treatments was observed in IgE-ELISA test with a pooled sera from the 6 patients allergic to pistachio (Fig. 3D). The results obtained with IgE-ELISA using individual sera from the six allergic patients (1–6) showed a significant decrease in IgE-reactivity for the three treatments compared with untreated pistachio (Fig. 3E).

3.4. MRA to assess thermal effect on cashew and pistachio allergens

The differential IgE cross-linking capability of untreated or treated cashew and pistachio allergens was analyzed by β-hexosaminidase release assay using the RBL-48 cell line. The cell line showed a consistent expression of the FcεRI-α chain (more than 90% of the cell population),

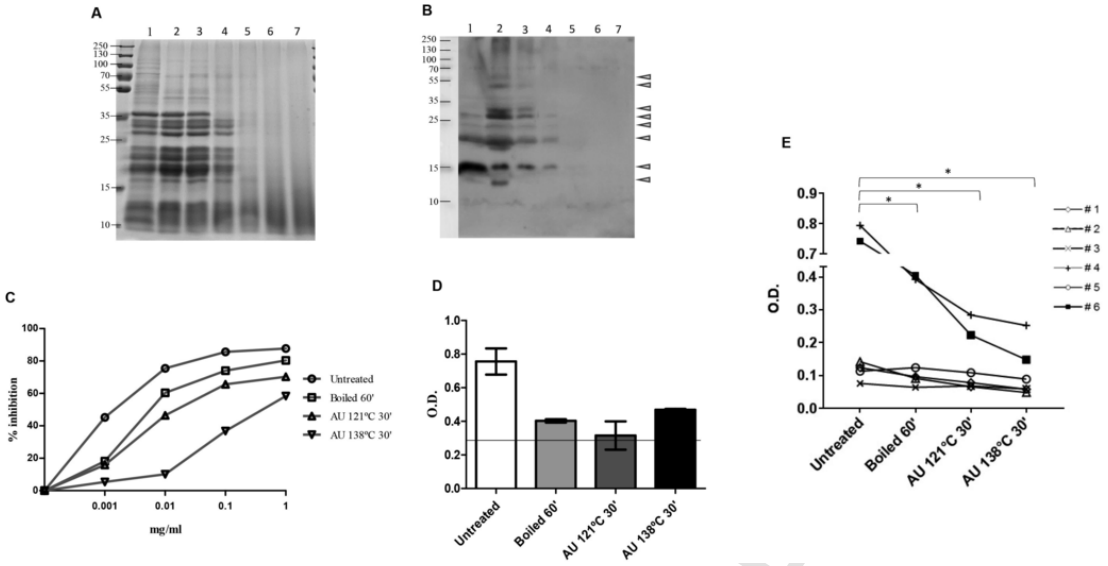

Fig. 3. Protein profile and IgE-immunodetection of pistachio extracts. (A) SDS-PAGE and (B) IgE-western blot of pistachio protein extract from untreated (lane 1) and treated nuts: boiled 30′ (lane 2), boiled 60′ (lane 3), AU 121 °C, 15′ (lane 4), AU 121 °C, 30′ (lane 5), AU 138 °C, 15′ (lane 6) and AU 138 °C, 30′ (lane 7). IgE-western blot was performed using pooled sera from six patients allergic to pistachio (patients 1–6). IgE-reactive bands are marked with arrows. C. ELISA inhibition assay with untreated pistachio extract for coating and untreated and treated pistachio proteins (indicated in the legend) used as inhibitors. A pooled sera from six patients allergic to pistachio was used. D. IgE-ELISA of untreated and treated pistachio incubated with pooled sera. The cut-off point of positivity is indicated with a horizontal line. E. IgE-ELISA of untreated and treated pistachio incubated with individual sera. Significant differences are determined with $^{*}p < .05$ using the paired *t*-test.

analyzed by flow cytometry before sensitization and mediator release assays (Fig. 4A). The cultured RBL-48 cell line was sensitized with a pooled sera from cashew or pistachio allergic patients, and afterwards, cells were stimulated with untreated or treated (boiled 60′, AU 121 °C, 30′, and AU 138 °C, 30′) cashew or pistachio protein extracts.

Results showed that untreated cashew and pistachio provoked a β-hexosaminidase release of 9 and 21%, respectively (Fig. 4B and C). In both cases, boiling for 60 min and heat/pressure processing showed a relevant lower capacity to trigger degranulation of RBL-48 cells, reducing the percentage of mediator release around 60% compared to the untreated samples. In pistachio, the degranulation of RBL-48 cells after the challenge with the harshest heat/pressure treatment (AU 138 °C 30′) was highly decreased, effect that was more attenuated in cashew treated with the same thermal conditions. Heat/pressure treated-cashew seemed to be able to cross-link IgE on basophils and to induce the β-hexosaminidase release to the media, although this capacity was diminished when compared with untreated cashew.

4. Discussion

In this study, the influence of moist thermal treatments on the IgE-reactivity of cashew and pistachio has been evaluated by traditional immunoassays such as IgE-ELISA, inhibition ELISA or IgE-western blot and by *in vivo* and physiologically relevant assays, as SPT and MRA that evaluate the IgE cross-linking capacity of untreated and treated proteins on effectors cells of allergy. All the results corroborated that heat and pressure treatment at the harshest conditions considered (AU 2.56 atm, 138 °C 30 min) produced an overall decrease in IgE-binding of both tree nuts, analyzed by IgE-western blot and IgE ELISA, using pooled or individual sera. Interestingly, applied treatments of heat and pressure seemed to affect cashew allergens to a greater extent than pistachio allergens, in regard to the IgE-binding capacity (evaluated by IgE-ELISA, indirect and by inhibition). Results went along with a marked decrease in the wheal size in SPT due to heat and pressure treatments in both tree nuts. Higher sensitivity of

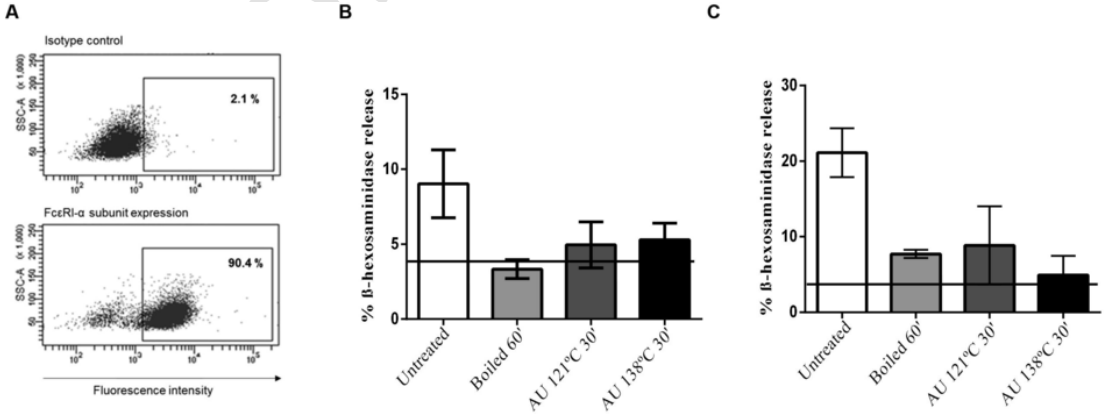

Fig. 4. Mediator release assay. Analysis of the surface expression of the human receptor FcεRI (α subunit) by means of flow cytometry on RBL-48, performed before serum sensitization and mediator release assay (A). Percentages of β-hexosaminidase release from RBL-48 sensitized with pooled sera of 4 cashew (B) or 6 pistachio (C) allergic patients. Sensitized cells were challenged with untreated and treated cashew (B) or pistachio (C). The mediator release value from negative control is indicated with a horizontal line in the graphs.

cashew proteins to boiling processing compared to pistachio was observed by this test. SPT showed negative reactions in all patients after applying cashew and pistachio protein extracts from AU 121 °C 30 min and AU 138 °C for 15 and 30 min samples. The effects of thermal processing on the allergenic properties of cashew and pistachio have been addressed by a limited number of studies, especially in the case of pistachio, based on assays that evaluated IgE-binding in western blot or ELISA (Masthoff et al., 2013). In 2008, Venkatachalam et al., found that cashew allergens had high stability to a wide variety of treatments assayed, including pressure cooking at 121 °C (during 5, 10, 20, and 30 min), boiling (during 1, 4, 7, and 10 min), microwave heating, dry roasting, and non-thermal treatments such as γ-irradiation or pH variation. Interestingly, although high stable, cashew allergens seem to be affected at some extent only by the treatment that applied heat and pressure (autoclaving) at 121 °C for the longest period of time from the plethora of treatments analyzed. Mattison et al. (2016) applied dry heat treatment to cashew (149 °C for 12, 20 and 24 min, and 177 °C for 24 min) and found that the soluble protein profile of cashew was altered after roasting. Consequently, processing seemed to change the relative amount of specific allergenic peptides, and IgE-binding capability was reduced to a less than 60% after dark roasting at 149 °C for 24 min. There is less information about thermal effect on pistachio immunoreactivity or IgE-binding capability. Noorbakhsh et al. found lower IgE-binding capacity for the protein extract prepared from steam-roasted than from raw and dry-roasted pistachio nuts (Noorbakhsh, Mortazavi, Sankian, Shahidi, Maleki, et al., 2010). In our study, we have applied thermal treatments to cashew and pistachio that included not only boiling without pressure and heat/pressure treatments at soft conditions, but also harsher conditions of heat and pressure (138 °C, 2.56 atm, during 15 and 30 min), which turned to be the most efficient treatment to decrease the allergenic properties of both tree nuts considered in our study.

IgE cross-linking capability of proteins from cashew and pistachio was also affected by all the applied treatments. However, the results of MRA using the cell line RBL-48, seemed to indicate that cashew proteins treated with heat and pressure, although importantly diminished compared to untreated cashew protein extract, still retained some capacity to cross-link IgE. This result indicates that an altered ability of food allergens to bind IgE using traditional *in vitro* immunoassays, such as IgE-western blot and IgE-ELISA for cashew, is not always correlated to an equal alteration of the IgE cross-linking capacity (Shi et al., 2013). In pistachio, however, the degranulation of RBL-48 cells after challenge with the harshest heat/pressure treatment was highly decreased, with a value similar to the negative control (less than 5% of mediator release). Recent studies have also found some discrepancies in the effect of processing (thermal, enzymatic, etc) on food allergenicity when using traditional immunoassays and assays that analyze the capacity of treated proteins to trigger the release of allergic mediators (Panda, Tetteh, Pramod, & Goodman, 2015; Shi et al., 2013). As observed with cashew in our study, the residual degranulation of effector cells obtained after the challenge with cashew proteins treated with heat and pressure may be explained by the survival of part of IgE-binding epitopes, which are able to cross-link IgE although in a less efficient way than untreated cashew extract. Other studies, however, have found a good correlation between variations on IgE-binding capacity of thermal treated nuts in IgE-ELISA or IgE-western blot and an altered capacity to cross-link IgE in MRA (Cabanillas et al., 2014, 2015).

The harshest conditions of heat and pressure applied in our study produced a degradation of cashew and pistachio proteins, with an increased protein fragmentation seen as an intense smear in the low molecular weight area in the SDS-PAGE. Such alteration in the electrophoretic and IgE-binding patterns after heat and pressure treatments cannot be explained by a potential loss of solubility of proteins due to the thermal treatments, since the experiments carried out with strong conditions of protein solubilization (flours directly solubilized in SDS-PAGE sample buffer), showed the same pattern of protein degradation for heat and pressure-treated samples. The degradation of proteins after harsh heat/pressure treatments obtained in our study is similar to the degradation produced by some enzymatic treatments. In that sense, Kulis et al. (2012), showed a drastic difference in the electrophoretic pattern after 30 min of pepsin digestion in cashew proteins, with an evident degradation of the main cashew proteins, translated into a strong increase in protein fragments around 3–6 kDa, similar to the results obtained in our study. Interestingly, the authors found that such hydrolyzed cashew sample significantly decreased the allergenicity in a mouse model of cashew allergy. Furthermore, immunotherapy with such pepsinized cashew in orally sensitized mice induced IgG production and decreased Th2 cytokine responses (Kulis et al., 2012). In our study, we have found a marked decrease in the IgE-binding properties of cashew and pistachio proteins, with a reduced capacity to cross-link IgE in effectors cells of allergy, especially in the case of pistachio. However, the potential use for immunotherapy of cashew and pistachio subjected to harsh conditions of heat and pressure will be a matter of future studies. Several studies have proposed the use of peptides with reduced IgE cross-linking capacity as an attractive strategy for immunotherapy (Novak, Haberstok, Bieber, & Allam, 2008). Recently, it has been demonstrated that peanut boiled during 12 h showed a protein fragmentation with an increased number of peptides with a diminished capacity to bind IgE, but with the ability to activate antigen-specific T cells, an essential step for successful oral immunotherapy (Tao et al., 2016). In other foods, such as milk or egg, it has been demonstrated in clinical trials that around 70% of the children with milk and egg allergies included in the study tolerated heated milk or egg products, with decreased wheal sizes in SPT and increased levels of specific IgG4 antibodies (Lemon-Mulé et al., 2008; Nowak-Wegrzyn et al., 2008).

The limitation of our study includes the use of a relative small study population of cashew and/or pistachio allergic patients. However, the patients included here were not only sensitized to cashew and/or pistachio, but also had well-characterized clinical allergies to cashew and/or pistachio.

In conclusion, the results of our study indicate that heat/pressure treatments were able to decrease the IgE-binding properties of cashew and pistachio protein extracts evaluated in IgE-ELISA and IgE-western blot. SPT and MRA assays confirmed a diminished capacity to cross-link IgE for pistachio samples. In cashew, although heat and pressure treated samples still retain some capacity to trigger the release of allergic mediators in cells implicated in the allergic response, this capacity was diminished when compared with untreated sample. Boiling produced more variable results, however this treatment applied to both nuts for 60 min led to an important decrease of basophil degranulation. Further studies will be necessary to analyze the decreased IgE cross-linking capacity of heat/pressure treated samples in *in vivo* models of food allergy. Furthermore, the potential capacity of such treated samples in the induction of T cell reactivity for a potential use in oral immunotherapy should be also addressed in future studies.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.foodchem.2017.10.132.

References

Ahn, K., Bardina, L., Grishina, G., Beyer, K., Sampson, H.A., 2009. Identification of two pistachio allergens, Pis v 1 and Pis v 2, belonging to the 2S albumin and 11S globulin family. Clinical & Experimental Allergy 39, 926–934.

AOAC, 2000. Official methods of analysis, 17th ed. Association of Analytical Chemists, Gaithersburg, MD, USA.

Bock, S.A., Muñoz-Furlong, A., Sampson, H.A., 2007. Further fatalities caused by anaphylactic reactions to food, 2001–2006. Journal of Allergy Clinical Immunology 119, 1016–1018.

Bulló, M., Juanola-Falgarona, M., Hernández-Alonso, P., Salas-Salvadó, J., 2015. Nutrition attributes and health effects of pistachio nuts. British Journal of Nutrition 113, S79–93.

Cabanillas, B., Cuadrado, C., Rodriguez, J., Hart, J., Burbano, C., Crespo, J.F., Novak, N., 2015. Potential changes in the allergenicity of three forms of peanut after thermal processing. Food Chemistry 183, 18–25.

Cabanillas, B., Maleki, S.J., Rodríguez, J., Burbano, C., Muzquiz, M., Jiménez, M.A., et al., 2012. Heat and pressure treatments effects on peanut allergenicity. Food Chemistry 132, 360–366.

Cabanillas, B., Maleki, S.J., Rodríguez, J., Cheng, H., Teuber, S.S., Wallowitz, M.L., et al., 2014. Allergenic properties and differential response of walnut subjected to processing treatments. Food Chemistry 157, 141–147.

Cabanillas, B., Novak, N., 2017. Effects of daily food processing on allergenicity. Critical Reviews in Food Science and Nutrition 1–12. https://doi.org/10.1080/10408398.2017.1356264.

Chung, S.Y., Mattison, C.P., Reed, S., Wasserman, R.L., Desormeaux, W.A., 2015. Treatment with oleic acid reduces IgE binding to peanut and cashew allergens. Food Chemistry 180, 295–300.

Clark, A.T., Anagnostou, K., Ewan, P.W., 2007. Cashew nut causes more severe reactions than peanut: Case-matched comparison in 141 children. Allergy 62, 913–916.

Fernández Rivas, M., 2009. Food allergy in Alergológica-2005. The Journal of Investigational Allergology and Clinical Immunology 19, 37–44.

García, B.E., Lizaso, M.T., 2011. Cross-reactivity syndromes in food allergy. The Journal of Investigational Allergology and Clinical Immunology 21, 162–170.

Gilfillan, A.M., Kado-Fong, H., Wiggan, G.A., Hakimi, J., Kent, U., Kochan, J.P., 1992. Conservation of signal transduction mechanisms via the human Fc epsilon RI alpha after transfection into a rat mast cell line, RBL 2H3. The Journal of Immunology 149, 2445–2451.

Jiménez-Saiz, R., Benedé, S., Molina, E., López-Expósito, I., 2015. Effect of processing technologies on the allergenicity of food products. Critical Reviews in Food Science and Nutrition 55, 1902–1917.

Kulis, M., Macqueen, I., Li, Y., Guo, R., Zhong, X.P., Burks, A.W., 2012. Pepsinized cashew proteins are hypoallergenic and immunogenic and provide effective immunotherapy in mice with cashew allergy. Journal of Allergy Clinical Immunology 130, 716–723.

Lemon-Mulé, H., Sampson, H.A., Sicherer, S.H., Shreffler, W.G., Noone, S., Nowak-Wegrzyn, A., 2008. Immunologic changes in children with egg allergy ingesting extensively heated egg. Journal of Allergy Clinical Immunology 122, 977–983.

Maleki, S.J., Chung, S.Y., Champagne, E.T., Raufman, J.P., 2000. The effects of roasting on the allergenic properties of peanut proteins. Journal of Allergy Clinical Immunology 106, 763–768.

Malling, H.J., 1993. Methods of skin testing. Allergen standardization and skin test (position paper). Allergy 48, 55–56.

Masthoff, L.J., Hoff, R., Verhoeckx, K.C., van Os-Medendorp, H., Michelsen-Huisman, A., Baumert, J.L., et al., 2013. A systematic review of the effect of thermal processing on the allergenicity of tree nuts. Allergy 68, 983–993.

Mattison, C.P., Bren-Mattison, Y., Vant-hull, B., Vargas, A.M., Wasserman, R.L., Grimm, C.C., 2016. Heat-induced alterations in cashew allergen solubility and IgE binding. Toxicology Reports 3, 244–251.

Mattison, C.P., Desormeaux, W.a., Wasserman, R.L., Yoshioka-Tarver, M., Condon, B., Grimm, C.C., 2014. Decreased immunoglobulin E (IgE) binding to cashew allergens following sodium sulfite treatment and heating. Journal of Agricultural and Food Chemistry 62, 6746–6755.

Noorbakhsh, R., Mortazavi, S.A., Sankian, M., Shahidi, F., Assarehzadegan, M.A., Varasteh, A., 2010. Cloning, expression, characterization, and computational approach for cross-reactivity prediction of manganese superoxide dismutase allergen from pistachio nut. Allergology International 59, 295–304.

Noorbakhsh, R., Mortazavi, S.A., Sankian, M., Shahidi, F., Maleki, S.J., Nasiraii, L.R., Varasteh, A., 2010. Influence of processing on the allergenic properties of pistachio nut assessed in vitro. Journal of Agricultural and Food Chemistry 58, 10231–10235.

Noorbakhsh, R., Mortazavi, S.A., Sankian, M., Shahidi, F., Tehrani, M., Jabbari Azad, F., Varasteh, A., 2011. Pistachio allergy-prevalence and in vitro cross-reactivity with other nuts. Allergology International 60, 425–432.

Novak, N., Haberstok, J., Bieber, T., Allam, J.P., 2008. The immune privilege of the oral mucosa. Trends in Molecular Medicine 14, 191–198.

Nowak-Wegrzyn, A., Bloom, K.A., Sicherer, S.H., Shreffler, W.G., Noone, S., Wanich, N., Sampson, H.A., 2008. Tolerance to extensively heated milk in children with cow's milk allergy. Journal of Allergy Clinical Immunology 122, 342–347.

Nwaru, B.I., Hickstein, L., Panesar, S.S., Roberts, G., Muraro, A., Sheikh, A., et al., 2014. Prevalence of common food allergies in Europe: A systematic review and meta-analysis. Allergy 69, 992–1007.

Palacin, A., Quirce, S., Armentia, A., Fernández-Nieto, M., Pacios, L.F., Asensio, T., Salcedo, G., 2007. Wheat lipid transfer protein is a major allergen associated with baker's asthma. Journal of Allergy and Clinical Immunology 120, 1132–1138.

Panda, R., Tetteh, A.O., Pramod, S.N., Goodman, R.E., 2015. Enzymatic hydrolysis does not reduce the biological reactivity of soybean proteins for all allergic subjects. The Journal of Agricultural and Food Chemistry 63, 9629–9639.

Prieto, N., Burbano, C., Iniesto, E., Rodriguez, J., Cabanillas, B., Crespo, J.F., et al., 2014. A novel proteomic analysis of the modifications induced by high hydrostatic pressure on hazelnut water-soluble proteins. Foods 3, 279–289.

Pumphrey, R.S., Gowland, M.H., 2007. Further fatal allergic reactions to food in the United Kingdom, 1999–2006. Journal of Allergy Clinical Immunology 119, 1018–1019.

Rico, R., Bulló, M., Salas-Salvadó, J., 2015. Nutritional composition of raw fresh cashew (Anacardium occidentale L.) kernels from different origin. Food Science & Nutrition 4, 329–338.

Robotham, J.M., Wang, F., Seamon, V., Teuber, S.S., Sathe, S.K., Sampson, H.A., 2005. Ana o 3, an important cashew nut (Anacardium occidentale L.) allergen of the 2S albumin family. Journal of Allergy Clinical Immunology 115, 1284–1290.

Shi, X., Guo, R., White, B.L., Yancey, A., Sanders, T.H., Davis, J.P., et al., 2013. Allergenic properties of enzymatically hydrolyzed peanut flour extracts. International Archives of Allergy and Immunology 162, 123–130.

Su, M., Venkatachalam, M., Teuber, S.S., Roux, K.H., Sathe, S.K., 2004. Impact of γ-irradiation and thermal processing on the antigenicity of almond, cashew nut and walnut proteins. Journal of the Science of Food and Agriculture 84, 1119–1125.

Tao, B., Bernardo, K., Eldi, P., Chegeni, N., Wiese, M., Colella, A., et al., 2016. Extended boiling of peanut progressively reduces IgE allergenicity while retaining T cell reactivity. Clinical & Experimental Allergy 46, 1004–1014.

Taylor, S.L., Baumert, J.L., 2010. Cross-contamination of foods and implications for food allergic patients. Current Allergy and Asthma Reports 10, 265–270.

Van der Valk, J.P., Dubois, A.E., Gerth van Wijk, R., Wichers, H.J., de Jong, N.W., 2014. Systematic review on cashew nut allergy. Allergy 69, 692–698.

Vanga, S.K., Raghavan, V., 2016. Processing effects on tree nut allergens: A review. Critical Reviews in Food Science and Nutrition https://doi.org/10.1080/10408398.2016.1175415.

Venkatachalam, M., Monaghan, E.K., Kshirsagar, H.H., Robotham, J.M., O'Donnell, S.E., Gerber, M.S., et al., 2008. Effects of processing on immunoreactivity of cashew nut (Anacardium occidentale L.) seed flour proteins. The Journal of Agricultural and Food Chemistry 56, 8998–9005.

Venkatachalam, M., Teuber, S.S., Roux, K.H., Sathe, S.K., 2002. Effects of roasting, blanching, autoclaving, and microwave heating on antigenicity of almond (Prunus dulcis L.) proteins. The Journal of Agricultural and Food Chemistry 50, 3544–3548.

Verhoeckx, K.C., Vissers, Y.M., Baumert, J.L., Faludi, R., Feys, M., Flanagan, S., et al., 2015. Food processing and allergenicity. Food and Chemical Toxicology 80, 223–240.

Wang, F., Robotham, J.M., Teuber, S.S., Sathe, S.K., Roux, K.H., 2003. Ana o 2, a major cashew (Anacardium occidentale L.) nut allergen of the legumin family. International Archives of Allergy and Immunology 132, 27–39.

Wang, F., Robotham, J.M., Teuber, S.S., Tawde, P., Sathe, S.K., Roux, K.H., 2002. Ana o 1, a cashew (Anacardium occidental) allergen of the vicilin seed storage protein family. Journal of Allergy Clinical Immunology 110, 160–166.

Willison, L.N., Tawde, P., Robotham, J.M., Penney, R.M., Teuber, S.S., Sathe, S.K., Roux, K.H., 2008. Pistachio vicilin, Pis v 3, is immunoglobulin E-reactive and cross-reacts with the homologous cashew allergen, Ana o 1. Clinical & Experimental Allergy 38, 1229–1238.