Influence of Instant Controlled Pressure Drop (DIC) on allergenic potential of tree nuts

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Abstract: Pistachio and cashew contain allergenic proteins, which causes them to be removed from the diet of allergic people. Former evidences have demonstrated that food processing (thermal and non-thermal) can produce structural and/or conformational changes in proteins by altering their allergenic capacity. In this study, the influence of Instant Controlled Pressure Drop (DIC) on the pistachio and cashew allergenic capacity has been studied. Western blot was carried out using IgG anti-11S and anti-2S and IgE antibodies from sera of patients sensitized to pistachio and cashew. DIC processing causes changes in the electrophoretic pattern, reducing the number and intensity of protein bands, as the pressure and temperature treatment increment what results in a remarkable decrease of detection of potentially allergenic proteins. The harshest conditions of DIC (7bar, 120s) markedly reduce the immunodetection of allergenic proteins, not only by using IgG (anti 11S and anti 2S) but also when IgE sera from sensitized patients were used for Western blots. Such immunodetection is more affected in pistachio than in cashew nuts, but it not completely removed. Therefore, cashew proteins are possibly more resistant than pistachio proteins. According these findings, Instant Controlled Pressure Drop (DIC) can be considered a suitable technique in order to obtain hypoallergenic tree nuts flour to be used in food industry.

Keywords: Pistachio, Cashew, Allergens, DIC processing, pressure processing, thermal processing.

1. Introduction

According to the EAACI, food allergy is a public health problem that affects approximately 4-7% in young children in Europe [1]. More than 120 foods have been described as causing food allergies, however, according to the European Regulation 1169/2011, only 14 allergens have been classified of mandatory declaration on labeling: cereals containing gluten, crustaceans, eggs, fish, peanuts, soybeans, dairy products, nuts, celery, mustard, sesame grains, mollusks, lupines and sulphites.

The consumption of nuts has great health benefits and pistachio and cashew nuts have a high number of benefitial nutritional properties for human health [2]. Several trials have shown that pistachios promote blood lipid profiles favorable to the heart and can prevent cardiovascular disease. In addition, they help to maintain a healthy antioxidant and anti-inflammatory activity, glycemic control and endothelial function [3]. Cashew and its derivatives have been traditionally used for their

medical properties, such as antimicrobial, anticancer and anti-inflammatory capacity[4], reduction of hypertension or treatment of gastrointestinal disorders [5]. At the nutritional level both nuts have a high content of fatty acids, mainly oleic acid (18: 1) and linoleic acid (18: 2) (approximately 45-50% of the seed). Around 20% of the seed of these nuts is protein and highlights the presence of all essential amino acids. It also highlights the presence of carbohydrates and fiber, the latter being very notable in the case of pistachio with respect to other nuts. In addition, they are a source of vitamins, minerals and pigments [2].

Pistachio allergy is rare in the US. However, although there is no explicit data, it seems that the prevalence of pistachio allergy is increasing due to increased production and consumption, being the European Union the second largest consumer of pistachio, after Turkey. [6]. So far, five proteins have been identified and characterized as food allergens in pistachio Pis v 1, Pis v 2, Pis v 3 and Pis v 5, are seed storage proteins that belong to the prolamine and cupin superfamilies [7-9], while Pis v 4 is classified as a defense protein of the iron / manganese superoxide dismutase protein family [10].

Regarding cashew nut reactions and symptoms; several US studies have described that it is the second most common cause of nut allergy, together with the walnut, presenting a frequency of 20% [11]. Cashew allergy seems to be increasing epidemiologically in recent years, especially in northern Europe, where data ranges from 5 to 20% depending on the country [12]. So far, three allergenic proteins in cashew have been identified and characterized: two of them belong to the cupin superfamily (Ana or 1 and Ana or 2) and one to the prolamine superfamily (Ana or 3) [13-15]. It is estimated that more than 80% of patients with pistachio allergy have cross reactivity to other nuts, such as peanuts, walnuts, chestnuts, almonds, pine nuts and specially cashew given that the identity between Pis v 1 and Pis v 2 sequences with Ana o 3 and Ana o 2   (64% and 48% respectively) [7].

Many authors have documented the possibility of using technological food processing to modify the physical-chemical properties of proteins; the structure, function, digestibility and solubility could be altered, which could in turn alter its immunoreactivity. Any process that modifies the structure of a protein could be able to interfere with the ability of antibody binding. The degree of alteration of the allergenicity depends on many parameters: method used, processing conditions (time, intensity or environment), type of food and allergenic capacity [16].There are different types of processing: non-thermal treatments, as enzymatic processing performed, for example, for as the digestion of cow's milk using endo and exoproteases for consumption by allergic individuals or the digestion of cashew and pistachio flours, achieving a reduction on its reactivity against antibodies IgE [17]. Thermal treatments such as cooking, baking, frying and roasting among others. Cooking has little effect on allergens in the case of lentils, chickpeas, green beans, peas, soybeans, pistachio and cashew nuts, although recent studies have concluded that some peanut allergens are transferred from the seed to the cooking water during this type of processing. Baking has a high impact on the modification of milk and egg allergens, becoming used as a treatment in these foods to induce tolerance in allergic patients. Microwave heating generates little effect on allergenic capacity. Roasting and frying have variable effects depending on the type of allergen and the food studied, although the increase in allergenic capacity after roasting peanuts due to the Maillard reaction, which generates changes in the amino groups of proteins, increases the capacity of binding of IgE [16]. Another type of processing is the combination of heat and pressure, such as applied with autoclave or controlled instant depressurization (DIC). Regarding the former, it has been shown that conditions of 138 °C for 15 or 30min can reduce the immunoreactivity of IgE antibodies in peanut, pistachio, cashew, lentil or chickpea [17-19]. In the case of DIC treatment, similar reductions in IgE immunoreactivity have been observed to those obtained in the harshest autoclave treatments in different raw and roasted peanuts, lentils, chickpeas and soybeans [20, 21]. In the DIC processing, high pressures are applied, up to 8 bars, and high temperatures, up to 180 °C, for short periods of time (from a few seconds to a few minutes). This stage of exposure to high temperature and pressure for short times is followed by an instantaneous drop in pressure to 5mbar, which causes: an instantaneous cooling of the product, stopping thermal degradation and a self-vaporization of the water presented by the processed product [22, 23]. An advantage of DIC over other processing technologies is that pressures higher than other treatments are reached (up to 8bar while autoclave

is reached at most 3bar) and that the exposure to the treatment develops for very short periods of time, prevents damage caused by heat that can affect other properties, apart from allergenic capacity. However, there are no fixed rules on the effect of processing on the allergenic capacity of food, since, some epitopes can be eliminated, others can remain unchanged or even generate new ones (neo-allergens) of greater potency [16, 17, 24]. In that sense, the understanding of the potential effects of food processing on their allergenic properties constitutes an active area of investigation.

The main objective of this work is to evaluate the allergenic capacity of pistachio and cashew nuts, both control and subjected to a technological processing that combines heat and pressure (DIC), in order to analyze the effect of this processing on its protein profile and in its allergenic potential.

2. Results and Discussion

Food processing can modify the allergenic capacity by altering the physicochemical properties of its proteins [25]. Treatments that combine heat and pressure have proven effective in reducing the allergenic capacity of many animal and plant foods. According to previous studies [18, 26-30], processing by heat treatments (cooking for 30 and 60min) and combined with pressure (autoclave 121 °C, 15 and 30min and, 138 °C, 15 and 30min) applied in legumes and tree nuts as peanut, pistachio, cashew and walnut could reduce immunoreactivity in pistachio and cashew, although only in the most extreme conditions of pressure and heat are significant decreases achieved. Since food allergens are characterized by high stability to gastrointestinal digestion, enzymatic hydrolysis has been added to heat treatment to reduce immunoreactivity, such as roasted peanuts, walnuts, pistachios and cashews [17, 31, 32]. In the case of cashew nut, the application of autoclave combined with enzymatic hydrolysis and sonication was necessary to clearly reduce its allergenic potential. However, some allergens persist in this type of treatment [17]. In addition, it has been shown that heat treatment can alter other properties of food, which may affect its techno-functional properties [33]. That is why the application of DIC is proposed, which combines pressure and temperature at short times (1-2 min), as a treatment with great potential to reduce the allergenic capacity of these two nuts without affecting other properties.

3.1 Selection of DIC treatments

For the selection of the most effective DIC treatment conditions, an SDS-PAGE electrophoresis of 13 pistachio and cashew flour processed with different pressure and time conditions was carried out, from 3bar, 30s to 7bar, 75s (data not shown). From that electrophoresis, the 7 samples showing changes in the protein profile were selected. The SDS-PAGE electrophoretic profile of the control (ST) and these 7 treated pistachio and cashew nuts (DIC1-DIC7) samples is shown in Figure 1.
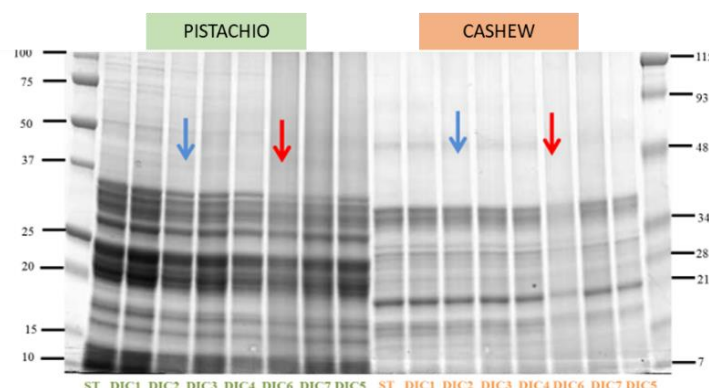
Figure 1. SDS-PAGE (12%) of pistachio flour (marked in green) and cashew nut (marked in orange). (20µg of protein per lane). The red and blue arrows indicate the selected treatments by presenting variations in the band pattern. Precision Plus (P+) weight marker was used (Bio-Rad).

The observed results allow us to deduce that the protein pattern, that is, the distribution of bands along the gel, hardly varies after applying the different DIC treatments. However, if the band

intensity is reduced in some of the treatments, it is the case of the DIC6 treatment (6.4bar, 107s) marked with a red arrow, in which the band intensity is reduced in both pistachio flour and cashew and, the DIC2 treatment (3,6bar, 107s) marked with a blue arrow, which reduces the band intensity only in the case of pistachio.

Other studies have also pointed out that thermal and pressure processing affects pistachio proteins more than cashew nuts. Sanchiz et al. [19] observed that most pistachio proteins disappeared in autoclave treatment. 138 °C, 15min, while cashew nuts did not disappear in the most intense treatment (138 °C, 30min). Cuadrado et al. [17] also observed that enzymatic digestion by sonication was more effective in pistachio than in cashew nuts, which required in addition to enzymatic hydrolysis treatment with temperature and / or pressure. In order to obtain a more effective DIC treatment in the reduction of protein intensity, a third treatment was developed with conditions of greater pressure and time (DIC8 7bar, 120s).

The first two treatments (DIC 2 and 6) have the same processing time (107s) and differ in the pressure applied (3.6 / 6.4bar), which could lead to deduce that the determining parameter in protein reduction is the applied pressure. However, when analyzing DIC2, DIC6, DIC7 and DIC8, a greater effect was observed when applying 6.4 bars during 107s (DIC6) than 7 bars during 75s (DIC7). While the decrease is clear when applying 7 bars for 120s (DIC8) compared to 7 bars and 75s (DIC7).(Figure 2). Similar results were observed by Cuadrado et al.[20], in the case of raw, roasted and soybean peanuts, in which at the same pressure, lower band intensity was observed to longer exposure times. Therefore, it can be deduced that both pressure and exposure time are necessary to favor protein modification.

3.2- Analytical composition

In order to know the effect that the different DIC treatments have on the protein content, different tests were carried out to assess the total and soluble protein content in each of them. The determination of the total protein was analyzed by RC-DC® Protein Assay (Bio-Rad) and by Dumas method (LECO® analysis) using 5.3 as nitrogen to-protein conversion factor (AOAC, 2003), whereas the soluble protein content was determined by Bradford method (Table 1)..

Table 1. Total protein (LECO and RC-DC) and soluble (Bradford) in pistachio and cashew flours

Sample	Total Protein (LECO) (g/100g dm)	Total Protein (RC-DC) (g/100g dm)	Soluble Protein (Bradford) (g/100g dm)
Pistachio Raw	39.50 ± 0.14 [a]	54.86 ± 0.00 [a]	21.47 ± 1.24 [a]
Pistachio DIC2	39.13 ± 0.01 [a]	57.5 ± 3.17 [a]	10.69 ± 1.14 [b]
Pistachio DIC6	39.86 ± 0.28 [ab]	37.99 ± 0.97 [b]	5.84 ± 1.17 [c]
Pistachio DIC8	38.48 ± 0.26 [b]	30.91 ± 0.40 [c]	3.86 ± 0.67 [c]
Çashew Raw	35.43 ± 0.13 [a]	55.04 ± 3.35 [a]	23.24 ± 0.24 [a]
Cashew DIC2	35.59 ± 0.38 [a]	47.11 ± 1.06 [b]	14.54 ± 0.33 [b]
Cashew DIC6	33.74 ± 0.17 [b]	33.2 ± 0.40 [c]	2.80 ± 0.11 [c]
Cashew DIC8	37.50 ± 0.18 [c]	37.82 ± 1.50 [c]	6,50 ± 0.14 [d]

[a] Figures (means ± SE; n=3) followed by different superscripts in the same column were significantly different (P<0.05) compared by Duncan test.

Regarding the amount of total pistachio protein analyzed by LECO, hardly any changes are observed. Regarding the results of RC-DC, there is no significant difference between the control and the DIC2 treatment. Flour with more drastic treatments showed lower total protein values (43% reduction in DIC8 with respect to control). Data corresponding to pistachio soluble proteincontent are similar to those observed in total protein; as the treatment is more intense the amount of protein is reduced up to 82%.

The results of the evaluation obtained by means of LECO do not show a clear alteration in cashew. Regarding the results of RC-DC and Bradford, the control has the highest total and soluble protein values. Both values are reduced in the first treatment, there are significant differences. In the case of the second treatment, the values continue decreasing until reaching a reduction of almost 40% in the total protein and almost 90% in the soluble one. However, when applying the DIC8 treatment, the amount of total and soluble protein in cashew increases with respect to the previous treatment, although it is still less than in the control.

The determination of the total protein carried out by RC-DC method shows that pistachio and cashew proteins are susceptible to the treatments applied, causing a decrease in the protein content of the processed samples with a greater incidence in the DIC8 treatment in the case of pistachio and DIC6 in the case of cashew nut. No similar results were obtained with the LECO test, which may be due to the fact that, through this test, the total nitrogen content is measured, which does not have to be affected by the treatments, since, although the proteins lose their native conformation and undergo hydrolysis, the protein nitrogen content should remain stable. In the soluble protein content the same trend is observed, that is, it is affected by the treatment: as the pressure and time increase the soluble protein decreases. According to Cabanillas et al.,[31], the modifications produced by heat and pressure in the soluble protein fraction can be considered representative of the changes produced in the same treatments in the total protein. The value of soluble protein is also lower than that of total protein, because in most cases the solubility decreases after processing due to the formation of protein aggregates of reduced solubility[34]. Cuadrado et al.,[17] also observed similar values of soluble protein (around 33 and 42 g / 100g) in pistachio and cashew flours treated with cooking and with autoclave, in addition to little variation in the soluble protein content .

Another noteworthy fact is that the total protein reduction in DIC8 is greater in pistachio (44%) than in cashew (31%). Regarding soluble protein, DIC8 treatment causes a reduction of 82% in pistachio and 72% in cashew.

3.3- Electrophoretic analysis

Figure 2 shows the SDS-PAGE electrophoretic profile of treatedpistachio and cashew flours selected to carry out the immunoassays (DIC 2, DIC6 and DIC8) in addition to the control samples (ST).
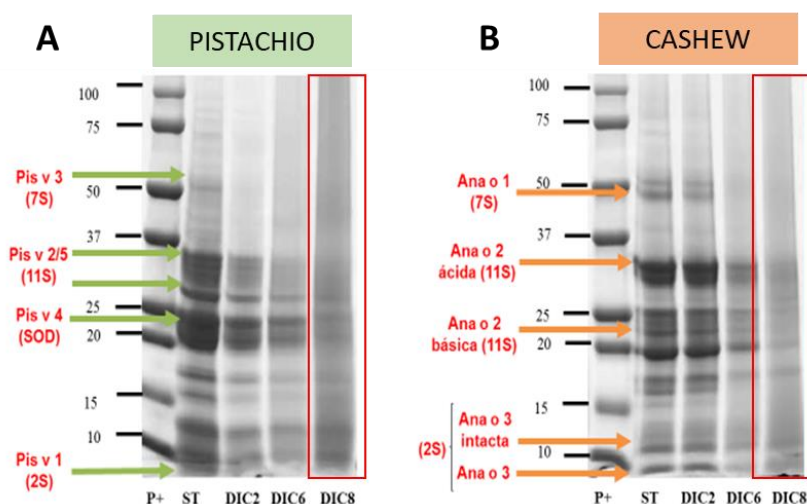
Figure 2. SDS-PAGE (4-20%) of control (ST) and DIC treated pistachio flour (A) and cashew (B) (20µg of protein/ lane). The green and orange arrows indicate the allergenic bands described in pistachio and cashew respectively.

The electrophoretic profile of the flours shows a band pattern that ranges between 10 and 100 kDa in the case of pistachio, the most intense bands being located around 10-15 kDa and 20-37 kDa.

In the case of cashew nuts, the band pattern ranges from 10 to 80kDa, finding the most intense bands around 20 kDa and 37 kDa.

The positions compatible with the allergens so far described are shown with arrows; in the case of pistachio (Pis v 1, Pis v 2, Pis v 3, Pis v 4 and Pis v 5), the bands located around 10-15 kDa stand out, which could correspond to Pis v 1 (2S), the bands around 25 and 37 kDa are compatible with the positions of the allergenic proteins Pis v 2 and Pis v 5, both 11S. Regarding Pis v 3 (7S) it is located around 50 kDa, although the band does not appreciate intensely and, finally, the bands observed between 20-25 kDa would correspond to the area where the allergen Pis v 4 (SOD) migrates.

Regarding the effect of the DIC treatment, and having as reference the control sample the number and intensity of bands, decreases as the treatment conditions become more drastic (higher pressure and time). In the weakest treatment (DIC2) significant differences in band intensity are already observed, which continues to decrease until almost disappear. In the DIC6 treatment, the same band pattern is still observed while the intensity is somewhat reduced. Like the results obtained from protein titrations, it follows that the most effective treatment is DIC8, in which both the number of bands and the intensity decrease. The most resistant bands are those located around 10-15 kDa, possibly a 2S albumin and those located around 20, 32 and 36 kDa, corresponding to SOD and 11S legumes, respectively.

In the case of the cashew nut (Ana o 1, Ana o 2 and Ana o 3) in the 50 kDa zone a band is observed that could correspond to Ana o 1. The bands located around 33 and 22 kDa are compatible with the positions of the subunits (acidic and basic) of Ana o 2. Finally, the band observed around 10 kDa coincides with the position of the intact subunit of the allergen Ana o 3 and the band around 7 kDa with that of the major subunit of this allergen. As in the case of pistachio, DIC treatment reduces band intensity as the pressure and time increase, it is noteworthy that there is hardly any difference between the control and the DIC2 treatment. The most effective treatment is DIC8 in which both the number and intensity of bands is reduced, although there are still 10-15 kDa proteins compatible with 2S albumin, around 16 kDa (protein not coinciding with any of the described as allergens in cashew nuts) and the possible acid and basic subunits of the 11S legumin located at 20 and 30 kDa.

Guillamón et al. [34] and Cuadrado et al. [20], also studied the effect of DIC at 3bar and 6bar for 1 and 3 minutes in flours of different plant species: lupine, chickpea, lentil, soybeans and raw and roasted peanuts. After the most drastic treatment, none of the lupine allergens was detected. In the case of peanut, the intensity of the electrophoretic pattern decreases without disappearing in the range of 15-65 kDa. In lentil and chickpea the band intensity decreased respect to the control, although changes were hardly observed between the different treatments, this fact leads to deduce that the conditions of the DIC8 treatment are more favorable for the decrease in the number and intensity of protein bands. However, even under these conditions, protein bands are still observed, especially those with low molecular weight (2S albumins, Pis v 1 and Ana o 3), it has been shown that this type of protein is able to maintain its integrity in drastic processing and digestion conditions [6, 35], which confirms its high stability and difficulty of elimination.

3.4- Immunodetection

3.4.1 Immunodetection with anti-2S and anti-11S IgG

As indicated above, several studies have shown that thermal processing with pressure produces a decrease in the immunoreactivity of some nuts. To deepen the study of the effect of the DIC treatment, at different pressures and times, immunodetection by Western blot (WB) of allergenic proteins was carried out in the defatted flours of the two nuts, using anti-2S and anti-11S IgG antibodies. The results of these analyzes are shown in Figure 3.

Figure 3 A.1 shows immunodetection with the anti-2S IgG antibody in control pistachio flours (ST) and treated with DIC. The binding of the antibody to bands of different molecular weights is appreciated, the one located in the 10 kDa zone being remarkable, since it could correspond to Pis v 1 (2S albumin). That protein band is still present in the DIC2 and DIC6 treatment, in DIC8 it does not disappear, but if it is significantly reduced, it becomes almost imperceptible. It also highlights the

band located around 25kDa, which would not correspond to any known 2S protein in pistachio, as was the case with Pis v 1, this band also disappears in the DIC8 treatment. The legumins are 11S globulins, belonging to the superfamily of the curtains, and are formed by two subunits, one acidic, 30-40kDa and another basic of about 20kDa, linked together by a disulfide bridge[11, 36]. The results of incubation with the anti-11S IgG antibody are observed in Fig. 3 A.2. In the control sample, three bands located around 50 kDa, 30kDa and 20kDa stand out, compatible with pistachio leguminous (11S) proteins: Pis v2 / 5 intact and the acid and basic subunits, respectively. In DIC2 treatment the bands continue to appear with an intensity similar to that of the control. In the case of DIC6 and DIC8, it can be seen that the bands that could correspond to the acid and basic subunits are almost imperceptible and that intact Pis v2 / 5 disappears. In the last treatment, a trail around 15kDa is observed, which could correspond to a protein hydrolysis product resulting from the applied processing.
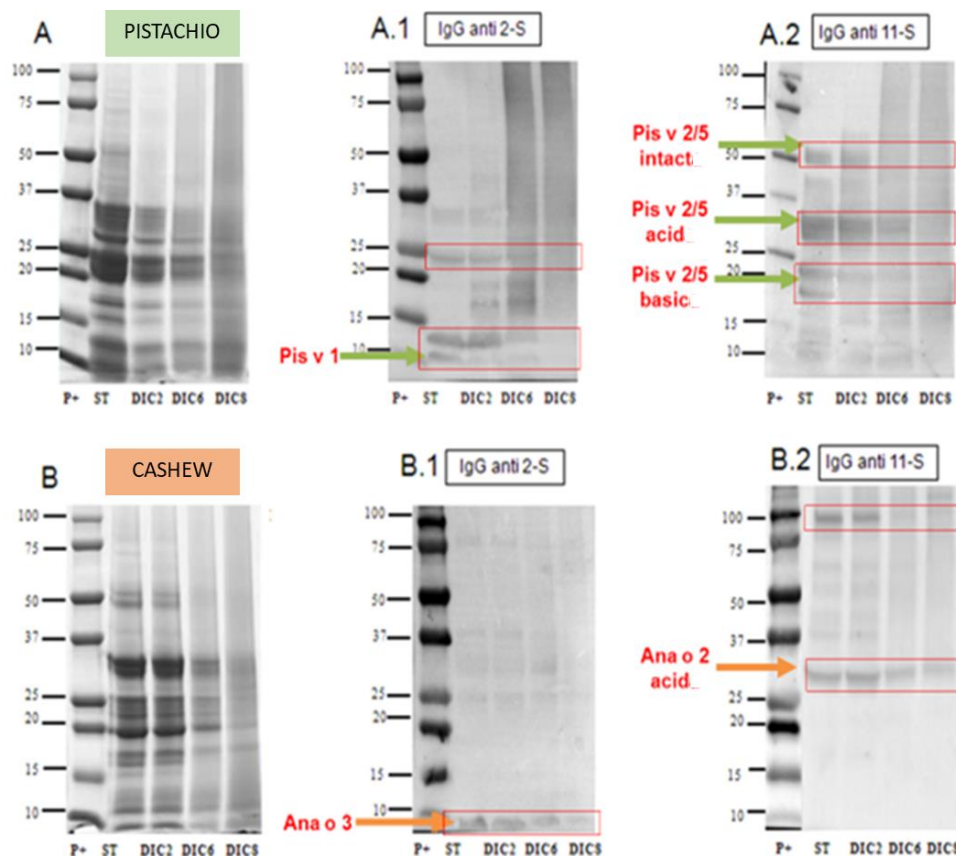
Figure 3: SDS-PAGE (4-20%) and IgG immunoblots of pistachio (A) and cashew (B). Immunoblots with IgG anti 2-S (A.1 y B.1) and anti 11-S (A.2 y B.2). Proteins recognized are marked in red and indicated by arrows. (20µg protein/lane)

Immunodetection with anti-2S in cashew flour is shown in Figure 3 B.1. In the case of the control and the three DIC treatments, a band around 10kDa was observed compatible with Ana or 3 albumin, it is remarkable that it is still present even in the most drastic treatment (DIC8). Immunodetection with anti-11S antibody in cashew flours is shown in Figure 3 B.2, highlighting the presence of a high molecular weight protein (100kDa), which would not correspond to any protein described in this tree nut, for which could be due to an aggregation of proteins due to heat and pressure treatment. Bands are detected at the height of 30kDa, which could correspond to the acid subunit of the legume Ana or 2, this band is maintained in all treatments, being less and less intense.

It should be noted that, although incubation in both nuts was done with the same antibodies, anti-2S and 11-S, and these belong to the same family (Anacardiaceae) electrophoretic patterns are

generated different from each other. In both immunodetection with anti-2S, a band around 10kDa that corresponds to the albumins can be seen in both cases, while the 25kDa band detected in pistachio does not appear in cashew. In the case of immunodetection with anti-11S antibodies, a different pattern is also observed, although in both cases 20 and 30 kDa bands are observed, corresponding to the acid and basic subunits of the legumins, however, the intact protein that is present in the case of pistachio does not appear in cashew nut. Although the treatment conditions are the same and the albumin and legume proteins have sequence homology although they belong to different plant species [36], it can be deduced that cashew allergens are more resistant to the treatment applied than those of pistachio, which are almost completely eliminated in DIC8, while cashew nuts, although they decrease in intensity, are still present.

3.4.2 Immunodetection with IgE from human sera

In order to deepen further the effect of DIC treatment on the allergenic capacity of pistachio and cashew, WB were carried out using individual sera from 11 Spanish patients sensitized to both nuts. Previous studies on immunodetection in pistachio and cashew flour were carried out using a mixture of sera (pool), instead of individual sera, which showed less variability in the band pattern [19]. The sera of the patients used in the present study belong to children with sensitivity to both pistachio and cashew nuts, some with a well-characterized clinical history that present from acute reactions (edema or hives) to serious adverse reactions (anaphylaxis), others present sensitization to both nuts, but without having consumed them (Table 2). The results of such immunodetection are shown in Figure 4.

In Figures 4 A.1-A.11 the WB with human sera in pistachio control and processed flours are showed. Patient 1 (Fig. 4 A.1) presents a pattern similar to that obtained in 2S and 11S, since, in the control bands around 10, 20 and 30kDa are detected, compatible with Pis v 1 and with the acid and subunits Basic Pis v 2 / 5, respectively. Other patients who have a pattern similar to P1 and therefore could detect both 2S and 11S proteins are P2, P3, P4, P5, P7, P9, P10 and P11 (Fig. 4 A.2-A.5, A.7, A.9, A.10 and A.11). In P7 the strong immunodetection around 40kDa that could be due to a non-specific binding. In the case of patient P6 and P11 is noticed (Fig.4 A.6 and A.11), the interaction with a protein of approximately 10 kDa, possibly Pis v 1 (similar to 2S). This recognition of a single 10 kDa band is similar to that observed in patient P8 (Fig. 4 A.8). In patients P6 and P8 the recognition of the 11S legumins is not excluded, but in the case that it occurred it would be scarce. Although individual differences are observed in the pattern recognized by the sera, all patients recognize 2S albumin (Pis v 1), and all except P6 and P8, recognize 11S legumin. Regarding the effect of DIC treatment, it is noteworthy that DIC8 significantly reduces the immunodetection of 2S with IgE from human sera, but without removing it completely, since a slight band around 10 kDa is still observed in all patients except P8 and P6. Regarding legumes, DIC8 treatment is able to reduce them, even disappearing as in P1-P4 and P7.

In Figures 4 B.1-B.11 are the WB with human sera in cashew control and treated flours. Patient 1 (Fig. 4 B.1) recognizes bands, around 10, 20, 30 and 50 kDa, compatible with the immunodetection of 2S and 11S proteins, Ana o 3, Ana o 2 acidic, basic and intact, although the 50 kDa could also be Ana o 1. A similar pattern is observed in Fig. 4 B.4, 4 B.5 and 4 B.10 (patients 4, 5 and 10). Regarding patient 2 (Fig. 4 B.2), he recognizes bands of approximately 10 kDa (possibly Ana o 3) and 30 kDa (acid subunit of Ana o 2). Patients P3, P6, P7 and P11 (Fig. 4 B.3, B.6, B.7, B.11) have an immunoreactivity similar to that of P2. The strong band detection around 37-50 kDa in all sera and especially in P2 and P3 is remarkable, but it is not compatible with either 11S or 2S proteins, therefore, it is unknown. Regarding P8 and P9, they have a different band pattern, just as the rest of the sera recognize a band of about 10 kDa compatible with 2S, a pair of bands around 25-37 kDa, which could correspond to the acid and basic subunits of Ana o 2 legumin and a 75 kDa protein, which does not correspond to any of the allergens described in cashew nuts and is therefore unknown.
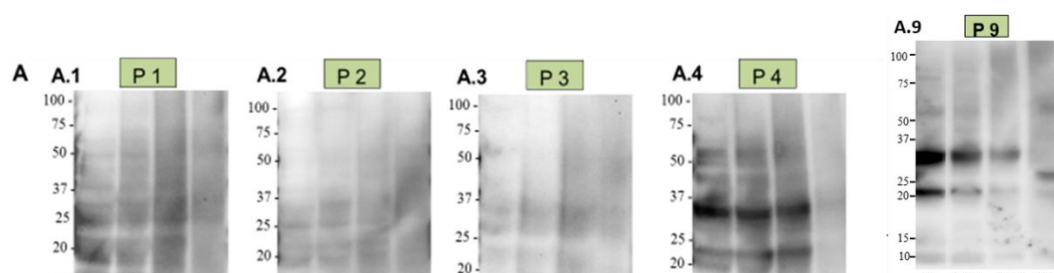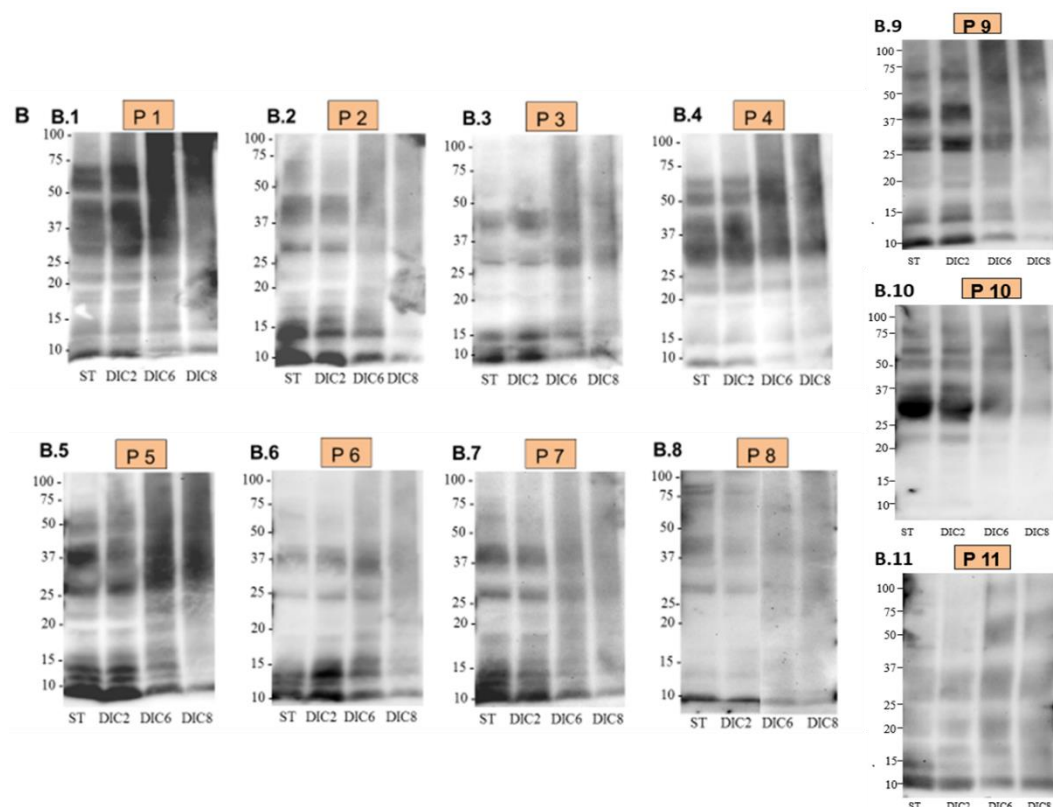
Figure 4: IgE immunoblot of proteins of pistachio (A) and cashew (B) of untreated control (ST) and DIC treated samples (20µg protein/lane). IgE immunoblots were carried out using individual sera from 11 patients allergic to pistachio and cashew (P1-P11).

As in the previous case, each patient has a different immunoreactive profile than the rest, however, all band patterns are similar to those of 2S albumin and 11S legumin, being able to recognize the two subunits of Ana o 2, and 4 out of the 11 patients (P1, P4, P5 and P10) might be recognizing

Ana o 2 intact, located around 50kDa, although this protein is also compatible with Ana o 1. Regarding the effect of treatments, the presence of protein degradation is remarkable in the area of high molecular weight of patients 1, 5 and 9, in the last two lanes. As the treatment is more intense the band intensity tends to decrease, but without eliminating the possible Ana o 3 albumin, even in the DIC8 treatment. Regarding the bands compatible with legumins, they tend to disappear in DIC8, although not in all cases, in patients 3, 4 and 11 the possible acid and basic subunits of Ana o 2 are still detected.

Patterns similar to those obtained in this study have been observed by Blanco et al. [37] also using sera from Spanish patients, in the case of pistachio 13-33 kDa proteins were detected and in the case of cashew nut around 19-50 kDa . However, the clear difference in immunoreactivity level and pattern of reactive bands against IgE in the different patients is remarkable. This may be due to the fact that the evaluation of immunoreactive capacity using human sera can lead to different results among individuals, populations or countries of origin thereof [38]. In previous studies, in which reactivity against IgE in pistachio and cashew flours was evaluated using individual sera of North American origin, individual differences were also observed in each of the immunological patterns [17]. In that case, the immunodetection of bands focused on areas of molecular weight ranging from 20 to 100 kDa), while in the results of the present study the detected proteins do not exceed 60 kDa. This indicates differences in immunoreactivity patterns not only among individuals of the same population, but also among populations based on their geographical origin (Spanish or North American).

It should also be noted that, although there are differences between individuals, they all recognize 2S albumins, both pistachio (Pis v 1) and cashew (Ana o 3), except P10. Previous studies have already described the high reactivity of the albumin of these two nuts, Pis v 1 is a major allergen that has been shown to be detected by more than 50% of sera from pistachio-allergic patients [6]. Regarding Ana o 3, it has been determined that it is detected by 93% of children allergic to cashew nuts, becoming a clinical indicator of cashew allergy, being even more specific than the whole extract [39]. Regarding the effect of DIC on this type of protein, its high resistance stands out, as already mentioned above and has been observed in the anti-11S IgG immunodetection (Figures 3 A.1 and B.1), since, although the recognition of these proteins decreases in all patients as the time and pressure of the treatment increases, the band does not disappear. In the case of immunodetection with human sera of pistachio flour if it is observed in patient 8 that in the most drastic treatment the Pis v 1 protein disappears. However, in the case of immunodetection of cashew nut proteins with human sera, although the band intensity decreases there is no such notable reduction compared to control. In addition, it should be noted that almost all patients recognize the 2S albumin of both nuts, probably due to the close botanical relationship between them; in the case of Pis v 1 and Ana o 3, 64% of the amino acid sequence is identical, so it is expected that individuals who react to the first also do so to the second, as observed. Recent studies by Bueno-Díaz et al. [40] prove the existence of a very high sensitivity to both allergens, without the appearance of cross-reactivity with other 2S albumins.

Regarding the 11S legumes, they are not detected in all patients: in the case of pistachio in 8 out of 11 patients and in all patients in the case of cashew. In addition, the detected band intensity is quite lower compared to those of 2S albumins, with the exception of patient 4 and 10 (Fig. 4 A.4 and A.10 and B.4 and B.10), which has a   higher band intensity in the 20 and 30 kDa zone than in 10 kDa. Regarding the effect of DIC on this type of protein, it is noted that they are less resistant than 2S albumins, as has also been observed in the anti-11S IgG blots (Figures 3 A.2 and B.2), since, in DIC8 treatment the detection of the corresponding bands was not possible for the majority of patients. Previous studies have shown that 11S legumes are denaturalized at 94 °C, leading to the conclusion that DIC treatment interferes with antibody binding capacity [17, 41]. In addition, the effect of DIC8 treatment is more effective in pistachio legumins (Pis v 2/5) than in cashew legumins, which although their intensity decreases greatly, they are still detected.

Currently, once the food allergy has been diagnosed, the only treatment is the elimination of the diet from the causative foods, which leads to nutritional and therefore health problems [42]. Several studies have proposed the use of food peptides with reduced immunoreactivity as a strategy to

develop immunotherapy against allergies [43], so it is interesting to apply processing techniques other than those already studied. Although there is little information on the effect of DIC in food allergens of nuts, other authors have described the high efficacy of this treatment in the elimination of in vitro immunoreactivity of other plant species, such as lupine [34], in which the DIC treatment at 6bar, 3min is able to eliminate IgE immunodetection almost completely. In the case of peanuts, the DIC treatment at the same pressure and time conditions generates a remarkable reduction in 65 kDa protein bands and eliminates the immunoreaction of bands less than 20 kDa. In soybeans, all immunoreactive proteins were eliminated and in chickpea immunoreaction with human sera detected a lower number of bands than the control treatment, and the intensity of them also decreases. Regarding the effect of DIC treatment on pistachio and cashew nut allergens, it seems to be effective, although it is not capable of abolishing its allergenic capacity. Previous studies [19] have shown that heat treatment by cooking for 30 and 60 minutes does not reduce the detection of allergenic proteins by human sera, using a pool of 6 human sera from Spanish patients. However, in the same study it is observed how the treatment of pressure and temperature reduces the detection of allergenic proteins, even eliminating the detection completely under the strongest conditions of time and temperature (138 °C, 30 min). Similar results were observed by Cuadrado et al. [17] when applying the same treatment together with an enzymatic hydrolysis with proteases, using 7 individual sera from North American patients. Date suggest, therefore, that the conditions of DIC treatment applied are less effective for the reduction of immunoreactivity in allergic patients than autoclave treatments. However, the fact that the in vitro detection of allergens decreases as a consequence of food processing not always implies a reduction on the in vivo allergenicity . To complete the analysis, would be necessary to perform tests using in vitro allergy models as human basophil activation test (BAT) or humanized basophil cell lines (RBL), animal models or clinical trials, using skin tests (SPT). The results of this study could be relevant, since the use of peptides with hypoallergenic properties has been proposed as a strategy to develop immunotherapy against food allergies. After a mass spectrometry characterization, these peptides might have the ability to activate the immune system but without triggering the allergic response [43].

4. Materials and Methods

4.1 Plant material

Pistachio (*Pistachia vera* L. var. Kerman) and cashew nuts (*Anacardium occidentale* L. type 320) were obtained from the germplasm bank of IRTA Mas Bover (Institute of Agrifood Research Technology, Tarragona, Spain) and Productos Manzanares SL (Cuenca, Spain) respectively.

4.2. Human sera

Anonym individual sera were chosen from 11 patients from the Hospital La Paz in Madrid sensitized to both nuts, positive IgE-CAP (>0.35 kU/ml) whose IgE values ranged from 15.4> 100 kU/ml for pistachio and 10.1> 100 kU/ml for cashew nuts (Table 2).

Table 2. Immunological and clinical information from the 11 allergic patients included in this study.

Patient	Age/Sex	IgE Pistachio (kU/l)	IgE Cashew (kU/l)	Symptoms
P1	9/M	>100	>100	Anaphylaxis
P2	19/F	>100	>100	SWPC
P3	6/F	71	70.1	SWPC
P4	10/M	76.2	75.4	TS
P5	4/M	74.4	74.5	SWPC
P6	5/M	33.2	39,9	Urticaria, ES, C , D
P7	11/F	16.4	10,1	SWPC
P8	7/F	15.4	10.6	SWPC *
P9	5/F	29.3	23.4	Pruritus
P10	7/F	19.9	13.1	Anaphylaxis
P11	6/F	18.0	11.3	Urticaria, LS, ES

ES, eye swell; TS, tongue swell; D, dysphonia; C, cough; LS, lip swell; SWPC Sensitization without previous consumption * without access to clinical information

4.3. Controlled Instant Depressurization Treatments (DIC)

Both nuts were subjected to controlled Instant Depressurization (DIC) treatments carried out at the University of La Rochelle (Laboratoire Maîtrise des Technologies Agro-Industrielles, La Rochelle, France). DIC treatment was carried out following a factorial experimental design previously described [22, 23]. Briefly, the moistened whole nuts are placed in a processing chamber and exposed to steam pressure (up to 8 bar) at high temperature (up to 170°C), over a relatively short time (few seconds to some minutes). This high-temperature-short time stage is followed by an instant pressure drop towards a vacuum at about 50 mbar. This abrupt pressure drop, at a rate $\Delta P/\Delta t$ higher than 5 bar s-1, simultaneously provokes an auto-vaporization of a part of the water in the product, and an instantaneous cooling of the products, which stops thermal degradation. After the DIC treatment of pistachio and cashew nuts, a total of 13 samples were obtained that include treatments at different pressure and time conditions: from a minimum point of 3bar, 30s; up to a maximum of 7bar, 75s; being the central point of 5bar, 75s. The electrophoretic profile of these 13 samples was analyzed and the 7 that presented changes in the protein pattern were selected (DIC1-DIC7, Table 3).

Table 3. Conditions (pressure and time) of the DIC treatments initially selected from those applied in pistachio and cashew nuts. The treatments in bold are those selected for subsequent electrophoretic and immunodetection analysis.

Sample	Pressure (bar)	Time (s)
ST	--	--
DIC1	3.6	43
DIC2	**3.6**	**107**
DIC3	5	75
DIC4	5	120
DIC5	6.4	43
DIC6	**6.4**	**107**
DIC7	7	75
DIC8	**7**	**120**

Two treatments (DIC2 and DIC6) out of seven were selected to compare the possible reduction in the number and intensity of protein bands. A posteriori, another DIC treatment was carried out at more pressure and time (DIC8, 7bar and 120s), in order to check if the effectiveness increased and a greater protein modification was obtained, which was also selected. Therefore, three samples, DIC2, DIC6 and DIC8, were selected to carry out the electrophoretic and immunodetection analysis.

All processed and control samples were grounded using a Thermomix cooking robot (Vorkwek) for approximately 10s, thus obtaining cashew and pistachio flours, both treated and untreated. The flours were defatted with n-hexane (34 ml / g dm). The defatted flour was passed through a 1mm mesh. This flour was used in subsequent experiments and assessments.

4.4. Protein separation and immunoblot

Defatted and milled flours of untreated (control) and DIC-treated tree nuts were used for these analyses. SDS-PAGE was performed according to Laemmli protocol [44] using 12% polyacrylamide gels (Criterium, BioRad) and 4-20% gradient polyacrylamide gels (Miniprotean, BioRad). Staining was performed with Coomassie Brilliant Blue R-250. The gels were analyzed with Quantity One (BioRad) program. Total protein content was determined by RC-DC (Reducing Agent and Detergent Compatible, BioRad) method, based on the method of Lowry et al. [45] and the nitrogen contents of the samples were determined by LECO analysis according to standard procedures based on Dumas method [46] (The total protein content was calculated as N x 5.3 [46]. The analyses were carried out in duplicate and the results summarized in Table 3. The protein content of processed samples was higher than in the raw ones. This could be related to the reduction of dry matter in these processed samples.

4.4.1. Immunodetection with IgG antibodies.

For western blot, proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore Corp., Bedford, MA, USA) using a semi-dry transfer system (iBlot 2Dry Blotting System, Invitrogen). Blocking was carried out for 1 h, at room temperature in PBS plus 0.5 % Tween-20 (PBST) containing 3 % milk (blocking solution). IgG mouse anti-11S (dilution 1:10000) and anti-2S (1:25000)were diluted in blocking solution and incubated with the PVDF membranes for 1 h (Membranes were washed and then treated with alkaline phosphatase (AP) conjugated goat anti mouse antibody (1:5000) (Sigma, Saint Louis, MO, USA) diluted in blocking solution. Detection was achieved by means of BCIP/NBT substrate (Sigma, Saint Louis, MO, USA). The signal was measured using ChemiDoc (Bio-Rad, Hercules, CA, USA). For IgE western blot, proteins were transferred to a polyvinylidene difluoride (PVDF) membrane. After that, membranes were incubated overnight at 4 ºC with pool sera from 11 patients with tree nuts allergy, washed and then treated with Horseradish peroxidase (HRP) conjugated mouse anti-human IgE (1:10000 dilution for 30 min at RT) (Sigma, Saint Louis, MO, USA). Detection of IgE-binding proteins was achieved by means of enhanced chemiluminescence, according to the manufacturer's instructions (Thermo Scientific, Waltham, MA, USA). The signal was measured using CCD camera system of ChemiDoc (Bio-Rad, Hercules, CA, USA).

4.4.2. Immunodetection with IgE of human sera.

After electrophoresis, the gels were transferred to PVDF membrane (Millipore Corp., Bedford, MA, USA) at 20V, 7min, using a semi-dry transfer system (iBlot 2Dry Blotting System, Invitrogen). After the transfer, the membrane was blocked with 2% blocking solution of milk in PBST for 30 minutes with stirring. To remove excess blocking solution, the membranes were washed three times for 5min in PBST. Then, the membranes were incubated with the 11 individual sera (dilution 1:10 or 1:20 in PBST), for 16h, at 4 °C. After washing three times with PBST were incubated with the secondary antibody (Mouse Anti-Human IgE Fc-HRP, Southern Biotech) (diluted 1: 10,000 in 2% blocking solution and DynaLight (1: 62500) (Precision protein Strep Tractin-HRP Conjugate, Invitrogen). The membranes were washed three times with PBST and a final wash with PBS was given. In this case, chemiluminescence was used for membrane development. The substrate Pierce

ECL 2 (Thermo Scientific) was used under the conditions indicated by the manufacturer. The membrane was also scanned in ChemiDoc (BioRad) and the images were analyzed with ImageLab software (BioRad) at different exposure times.

4.5. Statistical analysis

To compare the total and soluble protein content of treated and untreated (control) samples, both cashew and pistachio, a simple variance analysis (ANOVA) was carried out. Subsequently, a comparison of means was carried out using the Duncan test and significant differences were considered when $P < 0.05$. All analyzes were performed using the StatGraphic Centurion version XVII program.

5. Conclusions

Thermal and pressure processing through Controlled Instant Depressurization (DIC) hardly influences the total protein content measured with LECO, it does affect the total content measured by RC-DC and considerably reduces the soluble protein content in both pistachio and cashew. The analysis of the electrophoretic profile of the control and treated flours of pistachio and cashew nut indicates that the number and intensity of bands is reduced in both as the pressure and DIC treatment time is increased. The greatest decrease is achieved with the most drastic treatment (DIC 7bar, 120s), although without reaching complete elimination in any of them. Immunodetection of potentially allergenic proteins with anti-2S and anti-11S IgG, also decreases markedly as a result of DIC processing as the treatment becomes more severe, especially after treatment at 7 bars and 120 seconds. Such treatment is more effective in pistachio than in cashew nut, with 11S legumes being more susceptible to degradation. Immunodetection with IgE of human sera reveals a clear individual variability at the level of the pattern of reactive bands. However, almost all sera recognize 2S albumin of both pistachio and cashew nuts (Pis v 1 and Ana or 3) and some of them also 11S legumes. Although each patient has similar levels of IgE against both nuts, recognize with more intensity the cashew proteins.

Therefore, pistachio and cashew have similar total and soluble protein but show differences in the electrophoretic and immunoreactive pattern against IgG and IgE antibodies. The DIC treatment at 7 bars during 120s clearly reduces the allergenic capacity of both nuts being pistachio more susceptible than cashew.

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