

# Effect of Instant Controlled Pressure Drop on IgE Antibody Reactivity to Peanut, Lentil, Chickpea and Soybean Proteins

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## Key Words

Peanut · Lentil · Chickpea · Soybean · Food allergy · IgE reactivity · Thermal processing · Instant controlled pressure drop · DIC<sup>®</sup>

## Abstract

**Background:** The use of legume seeds is being expanded in the food industry due to their excellent nutritional and technological properties. However, legumes have been considered causative agents of allergic reactions through ingestion. Previous studies indicated that processing methods combining heat and steam pressure, such as instant controlled pressure drop (DIC<sup>®</sup>), could decrease allergenicity. The aim of this study was to investigate the impact of DIC treatment on peanut, lentil, chickpea and soybean IgE antibody reactivity. **Methods:** Peanut, lentil, chickpea and soybean seeds were subjected to DIC treatment at different pressure and time conditions (3 and 6 bar for 1 and 3 min). Control (raw) and DIC-treated extracts were analyzed by SDS-PAGE and immunoblotting using a serum pool from sensitized patients. **Results:** DIC treatment did not affect the total protein content of legume seeds. Nevertheless, modifications of protein profiles after DIC showed a general decrease in IgE binding to legume proteins that was correlated to a higher steam pressure and longer treatment. The immu-

noreactivity of soybean proteins was almost abolished with treatment at 6 bar for 3 min. **Conclusions:** The results demonstrated that DIC treatment produces a reduction in the overall in vitro IgE binding of peanut, lentil and chickpea and a drastic reduction in soybean immunoreactivity.

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## Introduction

The use of legumes has been expanded in the food industry due to their excellent nutritional and technological properties. In fact, they are recommended as a staple food by health organizations [1], and dieticians are nowadays tending to encourage their consumption in counseling [2]. On the other hand, some typical constraints of legume seed proteins, including the antinutritional and allergenic activities of some of them, limit their wider use as raw food material or ingredients.

Among food allergies, peanut (*Arachis hypogaea*) allergy is one of the most common and severe IgE-mediated reactions to food and is typically lifelong [3]. Therefore, considerable effort has been spent in identifying and characterizing allergens from peanut. The 2 major peanut allergens, Ara h 1 and Ara h 2, are recognized by 70–90% of sensitized subjects [4] and correspond to the

vicilin (7S) and 2S families of storage proteins, respectively [5, 6]. Ara h 3 has been considered to play a lesser allergenic role and belongs to the 11S legumin-like proteins [7].

Lentils (*Lens culinaris*) and chickpeas (*Cicer arietinum*) have been reported as causes of IgE-mediated hypersensitivity reactions, particularly in pediatric patients [8–11]. A major lentil allergen, Len c 1, was identified as a 48-kDa vicilin [12, 13]. So far, no chickpea allergen has been identified but several IgE-binding bands (10–70 kDa) have been detected by immunoblotting [10, 11, 14].

To date, 33 soybean (*Glycine max*) proteins have been identified as allergens (from 7 to 71 kDa) [15]. The P34 protein, also referred to as Gly m Bd 30K (thiol protease), has been identified as an immunodominant allergen that shares homology sequence (70%) with Ara h 1. Moreover, the acidic and basic glycinin polypeptides (11S) are allergenic and resistant to processing [16, 17]. The 7S globulin fraction ( $\beta$ -conglycinin) includes the  $\alpha$  and  $\beta$  subunits, which are also allergenic proteins [18].

The effects of thermal processing on food protein allergenicity have been reviewed in several recent articles [19–21]. The molecular basis of changes in allergenic activity is the inactivation or destruction of epitope structures, the formation of new epitopes or enhanced access to cryptic epitopes by denaturation of the native allergen, which may increase or decrease the IgE immunoreactivity of a protein [22].

Previous studies have reported that boiling in an autoclave (2.6 bar for up to 30 min) markedly reduced lupin, lentil and chickpea allergenicity [23, 24]. Moreover, the use of instant controlled pressure drop (DIC®) treatment (6 bar for 3 min), combining heat and steam pressure as in autoclaving, greatly decreased IgE reactivity of the heat-resistant major lupin allergens (Lup-1 and Lup-2) [25, 26]. Taking into account these findings, the aim of this study was to investigate the impact of DIC treatment of raw and roasted peanuts, lentils, chickpeas and soybeans on IgE reactivity using a serum pool from sensitized patients.

## Materials and Methods

### Plant Material

Raw and roasted peanuts (*A. hypogaea* var. Virginia) were purchased from Aperitivos Medina (Madrid, Spain); lentil seeds (*L. culinaris* var. Magda) were provided by the Instituto Técnico Agronómico Provincial (Albacete, Spain), and chickpea seeds (*C. arietinum* var. Athenas) and soybean seeds (*G. max* var. Ostrumi) were obtained from the Instituto de Formación e Investigación

Agraria y Pesquera (Córdoba, Spain). All seeds were subjected to DIC treatment.

### DIC Treatment

DIC treatment was carried out following a factorial experimental design previously described [27, 28]. Briefly, the moistened product is 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 period of time (a 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 of  $\Delta P/\Delta t$  higher than 5 bar/s, simultaneously provokes autovaporization of part of the water in the product and an instantaneous cooling of the products, which stops thermal degradation. Whole seeds of raw and roasted peanuts, lentils, chickpeas and soybeans were treated at different pressures for different periods of time and with different initial water content conditions. For raw and roasted peanuts, a central composite design of 12 points was used with 4 repetitions of the central point, while for the other legumes, a 22-point central composite design with 10 repetitions of the central point was used. From the parameters used for all these DIC-treated samples, treatment at 3 and 6 bar for 1 and 3 min with constant initial water content of 50 g of water per 100 g of dry matter was selected for SDS-PAGE and immunoblotting analysis.

### Protein Extracts

Control (unprocessed) and DIC-treated seeds were milled to pass through a 1-mm sieve (Cyclotec 1093, Tecator, Sweden), and the flour was defatted with n-hexane (34 ml/g of flour) for 4 h, shaken and air dried after filtration. Defatted flour of raw and roasted peanut samples was extracted twice in a solution of 0.05 M Tris-HCl buffer (pH 8.0) with salt (0.5 M NaCl). The defatted flours of lentil, chickpea and soybean samples were extracted ( $\times 2$ ) with 0.1 M PBS buffer (pH 7.4) plus 0.15 M NaCl. All the extractions were carried out at 1:10 (w/v) for 1 h at 4°C with stirring. The extracts were clarified by centrifugation at 27,000 g for 30 min at 4°C, and the supernatants were dialyzed against distilled H<sub>2</sub>O for 24 h at 4°C using a dialysis membrane (Spectra/Por, Serva, Heidelberg, Germany) with a cut-off of 3.5 kDa and then freeze dried. The soluble protein content in each extract was measured by the Bradford dye binding assay (Bio-Rad, Hercules, Calif., USA) using bovine serum albumin (Sigma, St. Louis, Mo., USA) as standard. The total nitrogen was determined using the Kjeldahl procedure [29], and the total crude protein was calculated as N  $\times$  5.45 [30].

### Patient Sera

A serum pool with specific IgE to peanut (74.0 kU/l), lentil (31.3 kU/l), chickpea (29.0 kU/l) and soybean (13.9 kU/l), quantified by the CAP-FEIA system (Pharmacia Diagnostic, Uppsala, Sweden), was employed. There is no further clinical information available for this serum pool. Serum from a patient with specific IgE to *Anisakis* spp. (9.09 kU/l) and a total serum IgE value of 53.4 kU/l was used as a negative control.

### Protein Electrophoresis and IgE Immunoblot Experiments

SDS-PAGE was performed according to Laemmli [31]. Samples (20  $\mu$ g of protein per lane) were mixed with Laemmli sample buffer (Bio-Rad) and 2-mercaptoethanol (Bio-Rad), heated at

90°C for 10 min and electrophoresed in 4–20% Tris-HCl linear gradient precast gel (Bio-Rad). Proteins were visualized with Coomassie Brilliant Blue R250 staining. Western blot was performed by electrophoretic transfer to a nitrocellulose membrane (Whatman, Dassel, Germany). After blocking with 3% (w/v) non-fat milk and 0.1% (v/v) Tween 20 in PBS (pH 7.4; blocking buffer), the membranes were incubated overnight with the serum pool or negative control serum (1:100 dilution), washed and then treated with mouse anti-human IgE monoclonal antibody HE-2 ascitic fluid (1:5,000 dilution for 1 h; ALK-Abello, Madrid, Spain). After washing, a goat anti-mouse IgG peroxidase-conjugated antibody (1:2,500 dilution for 1 h; Pierce, Rockford, Ill., USA) was added. Detection of IgE-binding components was achieved by means of enhanced chemiluminescence, according to the manufacturer's instructions (Amersham, UK).

## Results

### Protein Content

The total crude protein content was analyzed in the defatted legume flour, and the mean values were compared by Duncan's test with a significance level of  $p < 0.05$ . The results show that the crude protein content of untreated raw peanuts (42.96 g/100 g dry matter) was significantly lower than that in the DIC-processed samples, which ranged from 43.81 g/100 g at 3 bar for 1 min to 45.95 g/100 g at 3 bar for 3 min. Untreated roasted peanuts had 45.43 g/100 g, which was not significantly different from the samples treated with DIC at 3 bar (1 and 3 min) and 6 bar for 1 min (from 46.70 to 46.99 g/100 g), but was significantly lower than the sample treated with DIC at 6 bar for 3 min (49.43 g/100 g). There were no significant differences between the total protein content of lentil (22.85 g/100 g), chickpea (19.7 g/100 g) and soybean control samples (35.91 g/100 g) and the corresponding DIC-treated ones.

### Effect of DIC on Peanut Allergenic Proteins

The SDS-PAGE patterns of the protein extracts of raw and roasted peanuts before (control) and after DIC treatments are shown in figure 1. The extracts were also analyzed by IgE immunoblot using the serum pool. SDS-PAGE and IgE immunoblot band patterns of untreated raw and roasted peanuts were similar (fig. 1), showing multiple immunoreactive proteins, such as a protein around 65 kDa (putative Ara h 1), several bands ranging from 25 to 37 kDa, which very likely correspond to Ara h 3 fragments, and 1 band with a molecular weight around 17 kDa, presumably corresponding to Ara h 2 [4]. In addition, allergenic proteins of lower molecular weight (<16 kDa) were present.

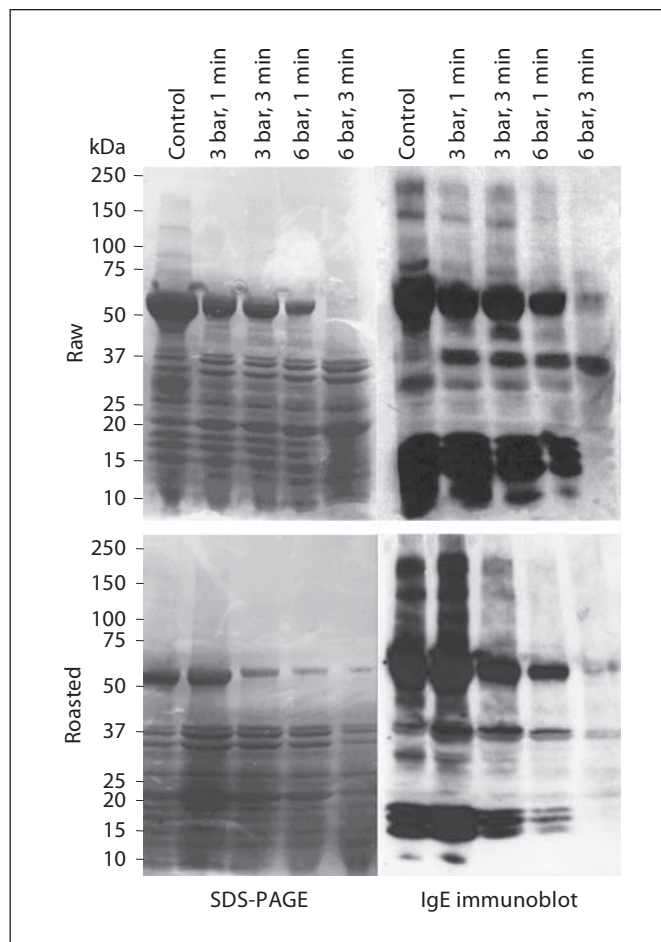


**Fig. 1.** SDS-PAGE patterns and IgE immunoblots of control and DIC-processed samples of raw and roasted peanuts (2 replicates were performed). The serum pool used was from sensitized subjects (specific IgE to peanut: 74 kU/l; 20 µg of protein per lane).

Following DIC treatment at 3 bar for 1 and 3 min, no changes in the SDS-PAGE protein band pattern and minimal changes (decrease/increase) in the immunoreactive band pattern of raw and roasted peanuts could be seen (fig. 1). At 6 bar for 1 and 3 min (fig. 1), a decrease in the band intensity in the range 15–65 kDa was observed; this change was more marked in roasted than in raw peanuts. More precisely, the 65- and 37-kDa protein bands (putative Ara h 1 and Ara h 3, respectively) were less intense in roasted than in raw peanuts, although they were still visible (fig. 5a, b).

### Effect of DIC on Lentil Allergenic Proteins

Figure 2 shows SDS-PAGE and Western blot patterns of control and DIC-processed lentil extracts. Control len-

**Fig. 2.** SDS-PAGE patterns and IgE immunoblots of control and DIC-processed samples of lentils (2 replicates were performed). The serum pool used was from sensitized patients (specific IgE to lentil: 31.3 kU/l; 20 µg of protein per lane).

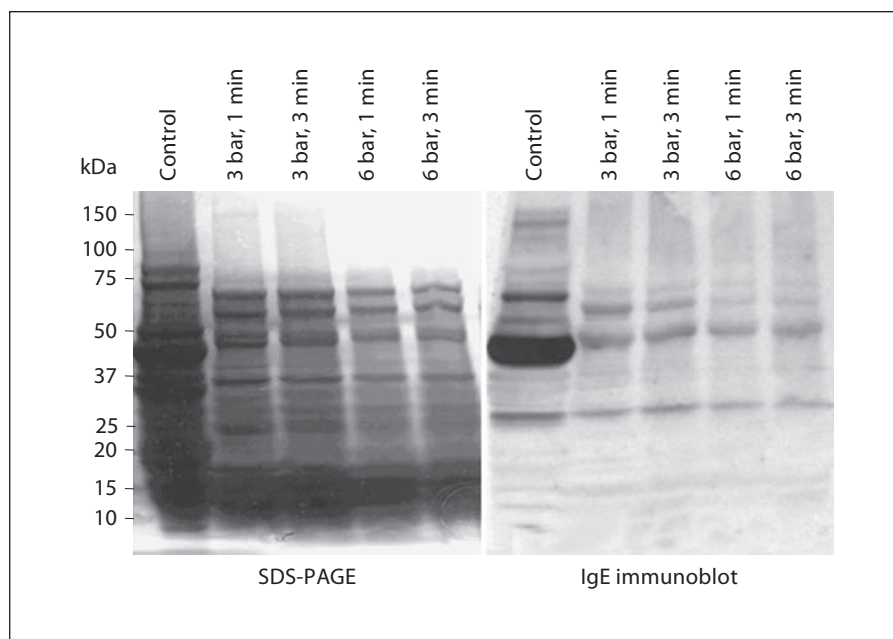
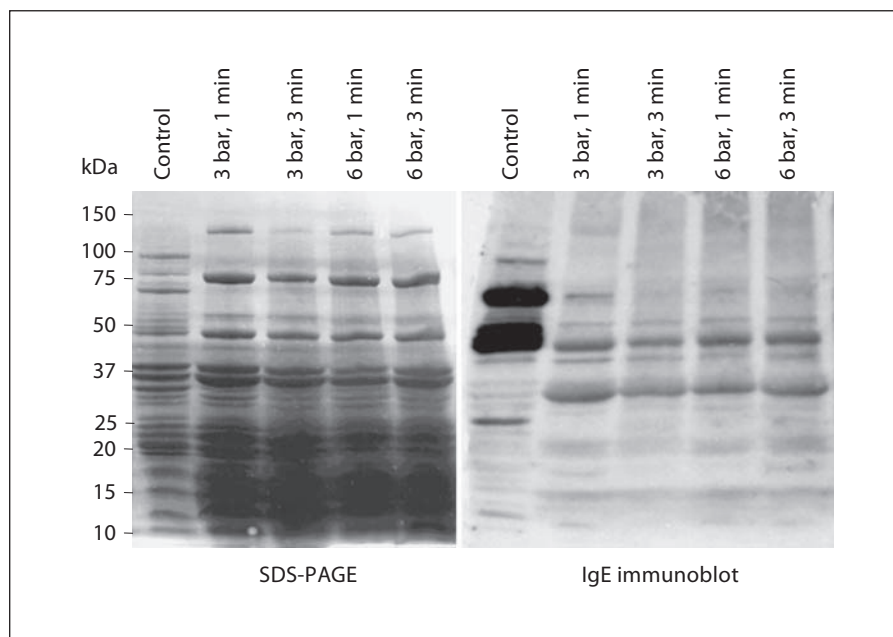


**Fig. 3.** SDS-PAGE patterns and IgE immunoblots of control and DIC-processed samples of chickpeas (2 replicates were performed). The serum pool used was from sensitized subjects (specific IgE to chickpea: 29 kU/l; 20 µg of protein per lane).

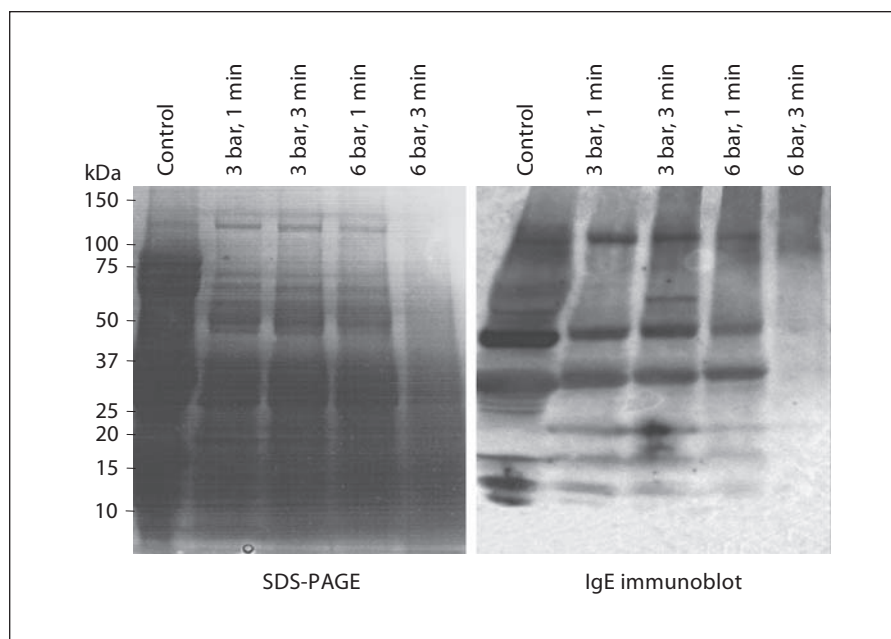


til was composed of numerous immunoreactive bands with molecular weights between 10 and 140 kDa, including a protein of 48 kDa (putative major allergen Len c 1). DIC processing at 3 and 6 bar for 1 and 3 min produced a marked decrease in overall immunoreactivity. Only 4 heat-stable bands were still present at harsh processing conditions of steam pressure and time (fig. 5c).

#### *Effect of DIC on Chickpea Allergenic Proteins*

SDS-PAGE patterns of protein extracts from untreated (control) and DIC-treated chickpeas are shown in figure 3, which also shows IgE immunoblotting analysis of these extracts using a serum pool from subjects with specific IgE to chickpea. Untreated chickpeas showed numerous IgE-binding proteins with molecular weights between 12 and 82 kDa. The immunoreactive band pattern

**Fig. 4.** SDS-PAGE patterns and IgE immunoblots of control and DIC-processed samples of soybeans (2 replicates were performed). The serum pool used was from sensitized patients (specific IgE to soybean: 13.9 kU/l; 20 µg of protein per lane).



after DIC treatments showed a marked decrease in the number and intensity of IgE-binding proteins; however, there were no apparent distinctions between the different DIC conditions applied, and only a few heat-stable immunoreactive proteins were still present (fig. 5d).

#### *Effect of DIC on Soybean Allergenic Proteins*

SDS-PAGE and Western blot using a serum pool with specific IgE to soybean were performed with control and DIC-processed soybean samples (fig. 4). In untreated soybeans, the major allergens and other minor immunoreactive proteins between 13 and 119 kDa were detected. The DIC process applied at 3 bar for 1 and 3 min and at 6 bar for 1 min produced a similar reduction of IgE-immunoreactive bands (fig. 4). However, after DIC at 6 bar for 3 min, almost all immunoreactive proteins were eliminated (fig. 5e).

#### **Discussion**

DIC processing can retexture biological products, improving their functional properties for use in industrial processes [32], but usually does not affect the total crude protein content. In the present study, as expected [25, 32], there was no reduction in the total protein content in DIC-treated legume seeds compared with the control samples. However, the soluble protein content values

measured in each legume extract (data not shown) indicated that more protein remained in the residue as unextractable material after DIC processing than in the control legume samples, a fact which is in agreement with previous experiments with lupins [25] and with reports indicating that heating promotes protein denaturation and aggregation and therefore can make some seed proteins unextractable and causes them to remain in the residue [33].

Significant alterations in protein structure may occur during heat treatment, the nature and extent of which depend on the temperature and duration of the thermal processing. Heat treatment can destroy epitopes by denaturation of proteins, with the result that only linear epitopes are available for binding to antibodies [33]. Most investigations have focused on the influence of thermal processing on peanut [6, 33–35]. Maleki and Hurlburt [36] showed that roasting, in addition to the effect of the Maillard reaction products, induces structural modifications reducing the solubility of the peanut allergens Ara h 1 and Ara h 2 and inducing their resistance to digestion, a fact which could contribute to the enhanced IgE-binding capacity of roasted peanut.

At present, little is known about the effect of thermal treatment under pressure, such as autoclaving and DIC, on allergenic proteins. According to our previous studies, lupin allergenicity was abolished by autoclaving at 2.6 bar for 30 min [23] or by DIC treatment at 6 bar for 3 min [25],

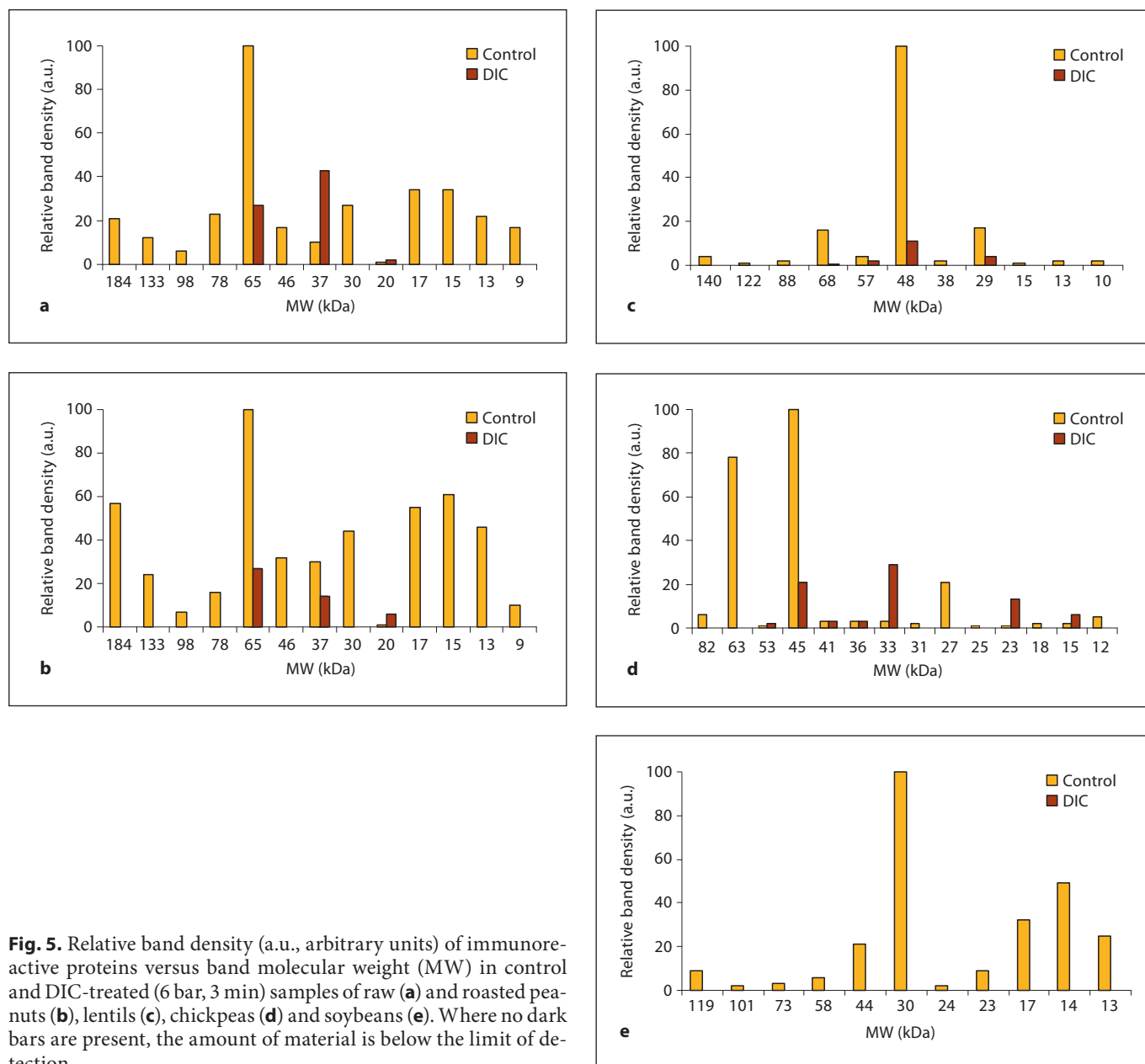


**Fig. 5.** Relative band density (a.u., arbitrary units) of immunoreactive proteins versus band molecular weight (MW) in control and DIC-treated (6 bar, 3 min) samples of raw (a) and roasted peanuts (b), lentils (c), chickpeas (d) and soybeans (e). Where no dark bars are present, the amount of material is below the limit of detection.

whereas it was only slightly affected by boiling and microwave and extrusion cooking [23]. These results support the high thermal resistance of the lupin allergens Lup-1 (34.5 kDa) and Lup-2 (20 kDa) [26].

The present results indicated that DIC treatment at 3 bar for 1 and 3 min and 6 bar for 1 min produced little modification of SDS-PAGE patterns or IgE-binding capacity of raw and roasted peanut proteins. DIC treatment at harsh conditions (6 bar for 3 min) produced in both samples a remarkable decrease in the protein bands of 65

kDa (Ara h 1), and no immunoreactive bands lower than 20 kDa were recognized. However, some bands (e.g. 37 kDa, Ara h 3) behave differently in raw and roasted peanuts (fig. 5a, b). Taking into account the reduction of in vitro immunoreactivity obtained at the highest pressure and time applied, the DIC technique seems to have more effect on the immunoreactivity of roasted peanut proteins, which seem to be even less immunoreactive than DIC-treated raw peanuts.

Some data also exist on adverse reactions to temperate legumes, including lentils and chickpeas. Boiling lentils did not reduce immunoreactivity [12, 37]. Chickpea allergy has been reported in Spain, where this legume is commonly consumed [10], and recently a high degree of cross-reactivity among lentils, chickpeas and peas has been observed [38].

Regarding the effect of thermal treatment on lentil and chickpea seeds, our previous studies show that autoclaving at 2.6 bar for 30 min produced a significant decrease in the IgE-binding capacity of lentil and chickpea allergens, although extremely resistant immunoreactive proteins still remained in both legumes even after this heat and pressure process [24]. In this study, extreme DIC treatments produced a similar pattern of IgE-binding proteins to that observed with autoclave at harsh conditions and, therefore, a parallel reduction in the overall IgE immunoreactivity. However, similarly, some heat-stable allergenic proteins were still detected in the Western blot analysis. These results confirmed the thermostability of some lentil and chickpea allergens.

Due to the commercial use of soybean protein-based food products, which frequently involves thermally processed proteins, it is important to investigate the effect of heat treatment on the main allergens of this legume. Burks et al. [39] did not find a significant decrease in IgE binding after heating soy proteins at various temperatures and time intervals. Wilson et al. [15] concluded that several procedures are needed to eliminate soy allergenicity, particularly of P34, the major allergenic protein. Our present results showed that although DIC treatment at 3 bar for 1 and 3 min resulted in a slight reduction of the soybean immunoreactive bands, when pressure was increased up to 6 bar and applied for 3 min, the overall *in vitro* IgE reactivity was markedly decreased, similarly to the effect of autoclaving (2.6 bar, 30 min) on this legume [40]. The DIC technique employed has a strong effect on the immunoreactive proteins of soybean with a short processing time (3 min), a fact which might represent an advantage for future potential applications in the food in-

dustry. Elimination of allergenic proteins via processing could eventually enhance the safe use of soybean products, making them available to soy-sensitized individuals. According to Thomas et al. [41], food processing may impact the allergenicity potential of proteins; however, there are no general rules regarding how different allergenic foods respond to physical, chemical or biochemical processing methods. In some, the epitopes are destroyed, but in others they are unaltered. The modifications eventually result in loss of organized structure and protein denaturation. However, it has been demonstrated that the degree of processing can dramatically affect the results of digestibility, solubility and other parameters [42]. During processing, proteins can form oligomers, become denatured, degraded, aggregated, cross-linked, fragmented and reassembled, and these changes most often cause a reduction in solubility [42]. In this way, processing can alter the overall IgE-binding profiles of a particular extract [33]. Therefore, further studies would be needed to evaluate the effects of these treatments on the insoluble protein fraction of these legumes.

This study supports our previous findings regarding the effect of DIC treatment (steam pressure and high temperature) on heat-stable lupin allergens. The present results show that DIC treatment is able to produce an important decrease in the overall immunoreactivity of peanut, lentil and chickpea extracts and a marked reduction in IgE recognition of soybean protein extract. These DIC-treated protein extracts could constitute an alternative to intact proteins in the development of different food products.

## Acknowledgements

We are grateful to Dr. M. Delgado for nitrogen analysis and B. Cabellos and A. Varela for technical assistance (Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria, Spain). This study was supported by the AGL2004-07971 research project funded by the National Program I+D+i. The DIC technology is supported by innovation and the SME EU Program, coordinated by Abcar-DIC Process ([www.abcar-dic.com](http://www.abcar-dic.com)).

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