

Effects of thermal treatment on walnut detection and allergenicity

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Abstract

BACKGROUND: Peanuts and tree nut allergies pose an increasing food safety problem. The aim of our study was to test the accuracy of different commercial enzyme-linked immunosorbent assay (ELISA) kits in the detection of the presence of walnuts in untreated and heat exposed food samples. The effects of thermal treatment of samples were evaluated by exposing walnuts to different heat treatments. All samples were first analysed by two different commercial ELISA assays. Then, we performed a skin prick test (SPT) on nine patients with proven nut allergy using small walnut pieces from raw and treated samples.

RESULTS: The presence of nuts proteins in thermally processed foods was not accurately detected by ELISA kits. All patients had a positive SPT reaction with raw walnut, while thermal treatments affected walnut allergenicity. The ELISA test gives a negative result in the case of strong thermal treatment, but at the same time allergic subjects react positively to stimulation with the same sample.

CONCLUSION: This study suggests that commercial ELISA kits may not be able to accurately determine the amount of proteins present in thermally processed foods due to changes in the solubility and immunoreactivity of the target proteins. Finally, the clinical results highlight that thermal treatment might induce a reduction in walnut allergenicity.

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Keywords: walnut; thermal treatment; ELISA; allergenicity; food allergies

INTRODUCTION

To date, it is estimated that 1–2% of adults and up to 5–7% of children suffer from some type of food allergy such as peanuts and tree nuts.¹ Different symptoms may appear in these patients, on the skin (urticaria, dermatitis, eczema, angioedema, itching), in the gastrointestinal tract (nausea, vomiting, diarrhoea, abdominal cramping) and in the respiratory system (rhinitis, asthma, laryngeal oedema).² Food-induced anaphylactic shock is a more severe reaction, occurring within minutes or hours, and may lead to death without prompt medical treatment.³ Oral food challenge (OFC) remains the gold-standard diagnostic test for food allergy but there is the possibility of inducing a systemic reaction. In addition, skin prick test (SPT) or serum allergen-specific immunoglobulin E (sIgE) level is used to identify food allergy.⁴

Tree nuts are an important cause of anaphylaxis and one of the most allergenic plant foods.^{1,5} There are different types of tree nuts, such as walnuts, almonds, pistachios and hazelnuts, that are mostly used in Europe.^{6,7} Different pastry products include walnuts because of their beneficial effects and pleasant taste.⁸ Nut allergens are present in seed storage proteins, such as vicilins (Jug r 2 in walnut, *Juglans regia*), legumins and 2S albumins, in structural proteins such as profilins and oleosins and in pathogenesis-related (PR) proteins such as chitinases, Bet v 1 homologues and lipid transfer proteins (LTP). In addition, manganese superoxide dismutase (MnSOD), 60S acidic ribosomal protein P2 and cytosolic small heat shock protein are new allergens in tree nuts under study.^{7,9} EU legislation requires mandatory labelling for allergenic food ingredients in order to protect the health of allergic individuals.

Regulation (EU) 1169/2011, Annex II, reports a list of 14 substances or groups of substances that cause allergies or intolerances.¹⁰ However, many processed foods contain allergens not correctly identified or are unintentionally cross-contaminated during production, shipping or storage (e.g. mustard as 'spices').^{11,12} In order to prevent problems, the food industry often declares 'may contain traces of tree nuts' as precautionary labelling, restricting the choice of processed foods.

Accordingly, suitable analytical methods are needed to verify the adequacy of allergen label statements and evaluate the potential risk to food-sensitive consumers.¹³

It is critical to have proper detection methods in food for these allergens. Moreover, thermal exposure may alter protein structure and influence the performance of the process aimed to identify specific antigens.¹⁴ Some allergenic foods are classified as heat stable (e.g. soya bean, cereals, celery, tree nuts and products derived) or labile (Rosaceae family fruits and carrots).¹⁴ Thermal processing can generate new allergenic epitopes or destroy existing ones.^{15,16} The aim of our study was to test the effect of thermal treatment on

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Table 1. The table describes the different thermal treatments performed on walnut samples to evaluate the effects of thermal treatment. Raw walnuts were subjected to nine different thermal treatments. Three kinds of procedures were applied: boiling, roasting and freezing at different times and temperatures

Treatment No.	Type of treatment
1	Boiling (10 min)
2	Roasting at 80 °C (10 min)
3	Roasting at 180 °C (10 min)
4	Roasting at 180 °C (30 min)
5	Freezing at –20 °C for 7 days
6	Boiling followed by freezing at –20 °C for 7 days
7	Roasting at 80 °C (10 min) then freezing at –20 °C for 7 days
8	Roasting at 180 °C (10 min) then freezing at –20 °C for 7 days
9	Roasting at 180 °C (30 min) then freezing at –20 °C for 7 days

walnut proteins, including allergenicity and performance of commercial enzyme-linked immunosorbent assay (ELISA) kits.

MATERIALS AND METHODS

Sampling and study design

To evaluate the effects of thermal treatment, each collected walnut sample was exposed to different treatments. In particular: sample 1 was boiled for 10 min; sample 2 was roasted at 80 °C for 10 min; sample 3 was roasted at 180 °C for 10 min; sample 4 was roasted at 180 °C for 30 min; sample 5 was frozen at –20 °C for 7 days; sample 6 was boiled for 10 min and then frozen at –20 °C for 7 days; sample 7 was roasted at 80 °C for 10 min and then frozen; sample 8 was roasted at 180 °C for 10 min and then frozen; and sample 9 was roasted at 180 °C for 30 min and then frozen (Table 1). Subsequently, we collected 50 samples of pastry products (20 samples of backed dry biscuits, 20 cookies and 10 breakfast cereals) negative for walnut proteins; each sample was divided into 10 aliquots. The aliquots were spiked with 10 mg kg^{–1} of raw walnuts or walnut treated under different conditions as previously described.

Laboratory analyses

All samples were tested with two different commercial ELISA assays (kit A by Euroclone, Pero, Italy; kit B by Neogen, Lansing, MI, USA) according with the kit instructions. Five grams of sample was homogenized and then 1 g of the homogenized mixture was suspended in 20 mL pre-diluted extraction buffer. The suspension was incubated for 15 min in a preheated water bath at 60 °C to obtain the protein extraction for kit A. Kit B required different extraction conditions; indeed, samples were extracted using a Tris–high-salt–gelatine buffer at 60 °C for 20 min.

Kit A is an ELISA with an antibody directed against walnut proteins. Antibody bound on the surface of a microtiter plate. Samples or standards provided by the kit were incubated in the wells of the microtiter plate for 20 min at room temperature. The wells were then washed in order to eliminate unbound material. A peroxidase-conjugated second antibody directed against walnut proteins was placed in the wells for 20 min incubation, and the plate was washed. After the substrate solution step, the development of a blue colour was observed. Stop solution was necessary

to inhibit colour development; the colour turned yellow and was measured photometrically at 450 nm. The concentration of walnut proteins was directly proportional to the colour intensity of the test according to the standard curve (0, 2, 6, 20, 60 mg kg^{–1}). The limit of detection (LOD) was 0.35 mg kg^{–1}; the limit of quantification (LOQ) was 2 mg kg^{–1}.

The principle of kit B was similar to kit A but with a standard curve of 2.4–120 mg kg^{–1}, a LOD of 0.25 mg kg^{–1} and LOQ of 2.4 mg kg^{–1}.

Calculation of results was performed with software using the four-parameter method; concentration of walnut (mg kg^{–1}) was determined from the standard curve using the mean optical density of each sample.

Skin prick test (SPT)

Subjects

Patients ranging in age from 18 to 53 years old were recruited prospectively from a large outpatient population with histories of IgE-mediated reactions to food; in particular, the subjects with sensitivity to nuts were selected. This population was evaluated between 2014 and 2015 at the Hospital San Martino in Genoa (Italy).

Ethics

This study was not submitted to the local ethics and research committees of the Hospital San Martino in Genoa, as prick-by-prick tests for plant food are a common diagnostic test performed in the routine workout of allergic patients.

SPT

Nine patients with proven allergy were submitted to SPT. SPT were performed using a small piece of the raw walnut samples and of treated walnut. The samples used were the same as analysed by ELISA. A small amount of food sample was directly introduced into the patient's skin. Sodium chloride (0.9%) and histamine hydrochloride (10 mg mL^{–1}) served as negative and positive controls, respectively (Lofarma, Milan, Italy).

SPT were interpreted as positive if a wheal larger than 3 mm in diameter accompanied by erythema was present 20 min later, as recommended by the European Academy of Allergy and Clinical Immunology guidelines.¹⁷

Statistical analysis

For each ELISA assay, differences in terms of walnut detection (mg/kg) after thermal treatment were evaluated by one-way ANOVA (Prism graphPad 5.03). A *P*-value ≤ 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

This study evaluated the effect of different thermal treatments on walnut proteins including the allergenic response on patients and the performance of two commercial ELISA kits to detect walnut in food samples. The selection of kits to include in the study was made on the basis of technical characteristics of kits available on the market; in particular, LOD, LOQ, cross-reactivity and extraction methods were evaluated. This work highlighted that boiling (treatment 1, *P* < 0.05) and intense and prolonged roasting (treatment 4, *P* < 0.0001) showed a high influence on sensitivity of both kits (Fig. 1). Intense roasting (treatment 3) had significant effect only on the application of kit A, whereas mild

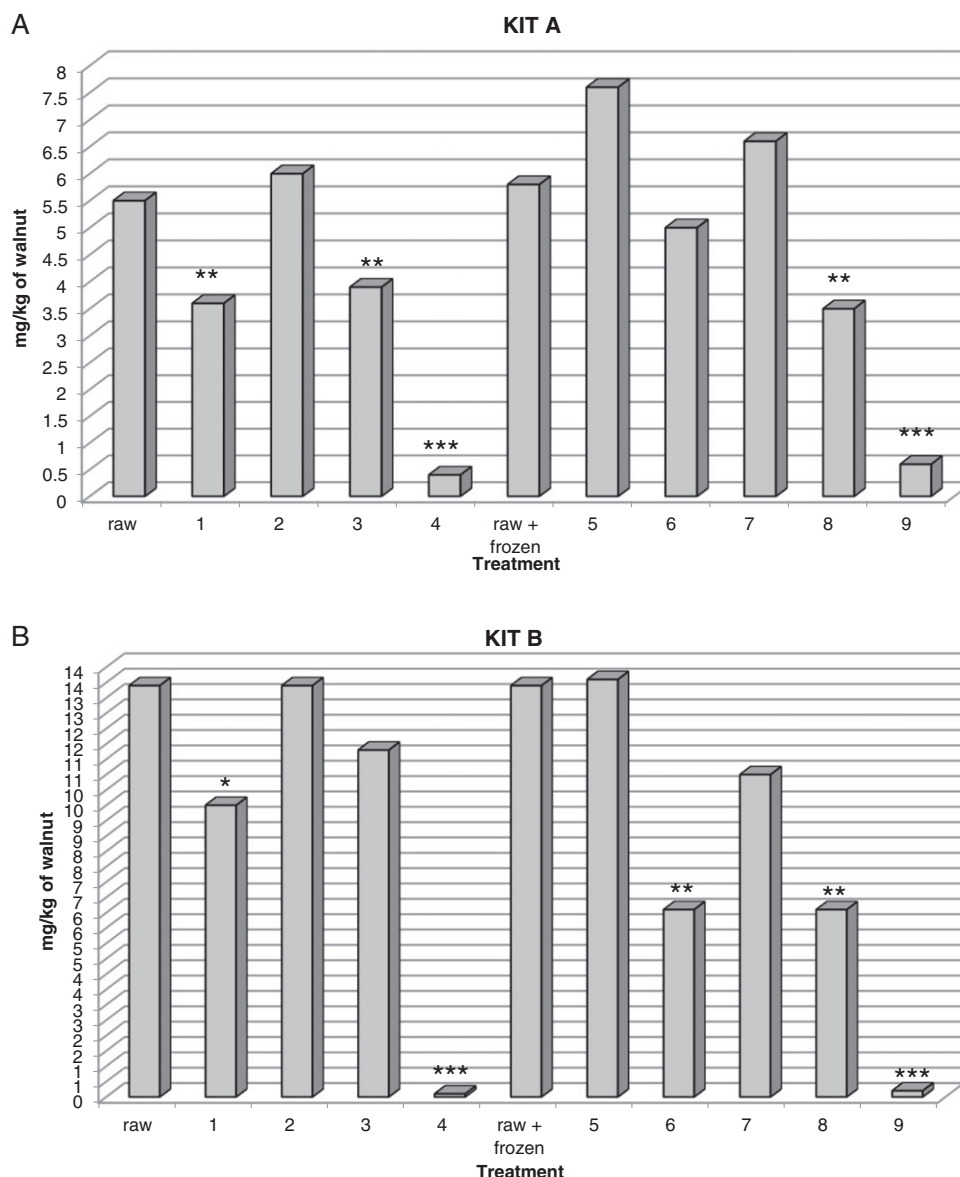


Figure 1. Panel A: effects of thermal treatment on ELISA kit A performance. Panel B: effects of thermal treatment on ELISA kit B performance. Thermal treatment (T) 1: boiling (10 min); T2: roasting at 80 °C (10 min); T3: roasting at 180 °C (10 min); T4: roasting at 180 °C (30 min); T5: frozen at –20 °C for 7 days; T6: boiling followed by freezing at –20 °C for 7 days; T7: roasting at 80 °C (10 min) then freezing at –20 °C for 7 days; T8: roasting at 180 °C (10 min) then freezing at –20 °C for 7 days; T9: roasting at 180 °C (30 min) then freezing at –20 °C for 7 days. Data are expressed as mg kg^{–1} of walnut proteins compared with mg kg^{–1} of raw walnut proteins. ****P*-value < 0,0001; ***P*-value < 0,001; **P*-value < 0,05.

roasting did not significantly influence detection ability ($P > 0.05$) of ELISA kits (Fig. 1). Cooking in combination with freezing caused a significant decrease ($P < 0.001$) in test performance on kits A and B (Fig. 1), whereas boiling in combination with freezing did not determined any significant effects on kit A (treatment 6); however, it caused a decrease in kit B performance. This commercial kit showed major sensitivity in detecting allergens in frozen food compared to kit A.

Concerning SPT tests, no systemic allergic reactions were observed. All patients had a positive SPT reaction with raw walnut; while thermal treatments affected walnut allergenicity, prolonged roasting (treatment 4) and resulted in a reduction in walnut immunogenicity (7/9 patients); however, only three subjects showed a total loss of reaction. The association between intense roasting and freezing (treatment 9) induced a decrease in

walnut allergenicity in 8/9 patients (Table 2). These results highlight that thermal treatment might induce a reduction in walnut allergenicity. Furthermore, it is suggested that technological processing may act as means a toll to reduce the risk associated with food consumption, ensuring consumer safety.

Many studies were performed to evaluate the effects of thermal treatment on walnut immune reactivity; these studies showed a low sensitivity of walnut protein to these treatments. Recent evidence suggests a different effect of technological treatment on different proteins. In particular, Hansen and co-workers reported, in a double-blind placebo-controlled food challenges study, a decreasing allergenicity of hazelnut after roasting; however, clinical symptoms were not entirely relieved in all patients.¹⁸ Other hazelnut studies have indicated a reduction in allergenicity of PR-10 proteins and profilins after roasting^{19–21}; the same effect was not

Table 2. Effects of different thermal treatments on walnut allergenicity. Nine patients in care at the Hospital San Martino in Genoa (Italy), with proven allergy were evaluated by prick test using a small piece of the raw walnut samples and of treated walnuts. Data are expressed as + (positive reaction) or – (negative reaction) based on size of the wheal after walnut exposure (–, <3 mm; +/-, 3–4 mm; +, 5–6 mm; ++, 7–8 mm; +++, ≥9 mm)

Patient		Prick test response to different thermal treatments									
ID	Born	Raw walnut	1	2	3	4	5	6	7	8	9
1	1972	+/-	–	–	–	–	–	–	–	–	–
2	1969	+++	++	+	+/-	–	+++	+	+	++	–
3	1998	+/-	+/-	+/-	–	–	++	++	++	++	–
4	1991	++	+	++	+	+/-	+++	+++	+++	+	+/-
5	1971	+++	++	+++	+++	++	+++	+++	+++	++	+
6	1970	++	+	+/-	+/-	++	++	+	+	+	–
7	1970	+++	+++	+++	++	++	+++	+++	+++	++	+
8	1963	+/-	+/-	+/-	+/-	+/-	+/-	+	–	–	–
9	1991	+++	+++	+++	+++	++	+++	+++	+++	+++	+++

reproduced on nonspecific lipid transfer proteins (nsLTP) and or storage proteins.^{19,22,23} Concerning the allergenicity of proteins under study, in walnut five allergenic proteins have been characterized: Jug r 1 (2S albumin), Jug r 2 (7S globulin), Jug r 3 (LTP), Jug r 4 (11S globulin) and Jug r 5 (profilin), with different sensitivity to thermal treatments.²⁴ Two studies on walnut allergens (Jug r 4, Jug r 2) have shown a partial effect of different thermal treatments (blanching, roasting, frying and microwave heating) on immunoreactivity by using circular dichroism spectra and polyclonal immunoglobulin G detection.^{25,26} In our study, we demonstrated a decreasing allergenicity of walnuts after treatment in a few patients. This suggests a limited effect of these treatments on allergenicity of walnuts. In this respect, it is important to remember that there are few studies currently available on thermal treatment effects on ELISA test performance. Regarding this topic, our results suggest different sensitivities of kits A and B concerning cooking and freezing processes, while the performance of both commercial kits decreased after boiling, which could be due to the loss of protein or their denaturation. The association of different treatments caused an increased ability of kit A to detect walnut traces; this is probably due to different antibodies coated in the ELISA plate. Moreover, ELISA test results after prolonged roasting highlight the unsuitability of these kits for walnut detection in some processed food.

CONCLUSION

This study suggests that commercial ELISA kits may not be able to accurately determine the amount of proteins present in thermally processed foods due to changes in the solubility and immunoreactivity of the target proteins. In particular, the ELISA test gives a negative result in the case of strong treatment (No. 4), but at the same time allergic subjects react positively to stimulation with the same sample. These data underline the importance of evaluating the most suitable detection method for allergen contaminants in food, also considering the effect of thermic treatments, to ensure consumer safety.

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