

D. Ibáñez Sandín
M. Martínez San Ireneo
F. Marañón Lizana
E. Fernández-Caldas
E. Alonso Lebrero
T. Laso Borrego

Specific IgE determinations to crude and boiled lentil (*Lens culinaris*) extracts in lentil-sensitive children and controls

Authors' affiliations:

D. Ibáñez Sandín, M. Martínez San Ireneo,
E. Alonso Lebrero, T. Laso Borrego, Allergy
Section, Hospital Niño Jesús, Madrid
F. Marañón Lizana, E. Fernández-Caldas,
Research and Development Laboratories C.B.F.
LETI, SA, Madrid, Spain

Correspondence to:

M^a Dolores Ibáñez Sandín
Allergy Section, Hospital Niño Jesús
Avenida Menéndez Pelayo 65
28009 Madrid
Spain

Key words: heat; hypersensitivity to legumes; *Lens culinaris*; lentil allergens; specific IgE.

Background: The aims of this study were to evaluate the allergenicity of boiled and crude lentil extracts and to compare specific IgE binding in tolerant and nontolerant lentil-allergic children.

Methods: Thirty-eight children were studied and divided into three groups. Group I comprised 24 children with a positive open oral challenge, or a convincing history of anaphylaxis after the ingestion of lentils; group II comprised nine children with a history of allergic reactions in the past, but currently tolerant of lentils; and group III comprised five children allergic to other legumes, but always tolerant of the ingestion of lentils. Specific IgE determinations and ELISA inhibitions were performed with the crude and boiled lentil extracts. The allergenic profile of both extracts was evaluated by SDS-PAGE and immunoblot.

Results: Mean specific IgE levels in group I were significantly higher than in groups II and III. The heating process caused a significant decrease in specific IgE binding. However, IgE-inhibition studies showed that the boiled lentil extract had a greater inhibitory capacity than the crude extract. Immunoblots revealed no important differences in IgE-binding patterns between the two extracts. Multiple allergens were detected in a wide range of molecular masses.

Conclusions: Boiled lentil extracts maintain strong allergenicity. Patients who have developed tolerance of lentil ingestion have lower specific IgE levels than symptomatic patients.

Date:

Accepted for publication 16 June 1999

To cite this article:

Ibáñez Sandín D., Martínez San Ireneo M., Marañón Lizana F., Fernández-Caldas E., Alonso Lebrero E. & Laso Borrego T. Specific IgE determinations to crude and boiled lentil (*Lens culinaris*) extracts in lentil-sensitive children and controls.
Allergy 1999; 54, 1209–1214.

Copyright © Munksgaard 1999

ISSN 0105-4538

In Spain, legumes are an important component of the diet and occupy the fifth place among foods that most frequently lead to an IgE-mediated reaction (1). Lentil (*Lens culinaris*) is a member of the Leguminosae family, a botanical group

which also includes peanuts, soybeans, garden peas, chickpeas, and several bean species. Peanuts and soybeans are important causes of food allergy in many countries, and their allergenic composition has been extensively studied. However, little information is currently available about lentil allergens. Isolated cases of IgE-mediated reactions to lentils (2–5) have been described. Pascual et al. (6) studied 22 children with hypersensitivity to lentils; six of these patients also had allergic reactions to chickpeas, two to peas, and one to green beans. The authors concluded that allergic reactions to lentils start early in life, usually below the age of 4 years.

Food allergy starts early in life and, in some cases, continues throughout life. It is not clear whether the persistence of food allergy is related to specific IgE levels, the type of food, or the age of onset. However, in most cases, the patients become tolerant. This is more frequent in children who are allergic to milk and egg.

There is little information on risk factors associated with the persistence of legume hypersensitivity. Reports on peanut allergy suggest that this sensitivity may persist for many years or indefinitely (7). A report suggests that children with a convincing history of reactions to peanuts may become tolerant of peanuts. These children have fewer other manifestations of atopy than children whose peanut allergy persists (8).

As legumes are a relatively common cause of allergic reactions among children in Spain (9), we have identified and selected a group of sensitized children who are truly allergic with symptomatic hypersensitivity to lentils, and a group of patients who, after an average of 5 years, became tolerant of the ingestion of this legume. The purposes of this study were to compare specific IgE binding in tolerant and nontolerant lentil-allergic children and to evaluate the allergenicity of boiled and crude lentil extracts.

Material and methods

Patient population

Serum samples were obtained from 38 patients allergic to legumes; the age range was 3–19 years (mean age 8.3 years). Patients were divided into three groups. Group I comprised 24 patients (mean age 7.8 years) with symptomatic type I hypersensitivity to lentils (urticaria, angioedema, vomiting, rhinitis, and/or asthma), confirmed by open oral challenges, as previously described (10), or a recent, convincing history of anaphylaxis; all patients had a positive prick test to a

commercial lentil extract (10 mg/ml w/v, C.B.F. LETI, Spain). Group II comprised nine patients (mean age 9 years) with a history of allergic reactions to lentil in the past, but good tolerance at the time of inclusion in the study, verified by clinical history and/or provocation; in this group, 77% of the children had a positive prick test to the same lentil extract. Group III comprised five patients (mean age 10 years) allergic to other legumes (two to peanut, two to vetchling, and one to white bean), but always tolerant of lentil; only one of these patients had a positive prick test to lentil. A serum pool containing sera of grass-pollen-sensitive children was used as a control. Serum samples were collected from each patient and frozen in small aliquots until use.

Lentil extracts

Two types of Castilian lentil extracts were prepared. Crushed lentils were extracted 1:20 w/v in 0.85% saline solution for 16 h at 4°C. Half of this extract was heated for 15 min at 100°C. Both extracts were centrifuged at 10 000 rpm for 30 min and finally passed through a filter of 0.22-µm pore size. Extracts were kept at –20°C until use. Protein concentration was determined by the Lowry-Biured method (Sigma Diagnostics, St Louis, MO, USA) according to the instructions of the manufacturer. Both extracts had a similar protein concentration; 2.5 mg/ml for the crude extract and 2.8 mg/ml for the boiled extract.

Specific IgE determination

Specific IgE binding to the crude and boiled lentil extracts and inhibition experiments were conducted by methods previously published (11, 12). A result was considered positive when a serum bound four times more than a negative control. Briefly, 100-µl aliquots of lentil extracts (crude or boiled), at a protein concentration of 20 µg/ml in sodium bicarbonate buffer (0.2 M, pH 9.2), were coated on the surface of plastic microtiter plates (Immulon 4, Chantilly, USA) and, after overnight incubation in a humid chamber, were blocked with 1% HSA in PBS for 1 h at room temperature. After three washings, 100 µl of the serum samples, in duplicate, were incubated overnight. After this incubation period and further washings, 100 µl of antihuman IgE (Ingenasa, Madrid, Spain) labeled with horseradish peroxidase (HRP) (1 µg/ml in blocking solution) was added and left to stand for 2 h at room temperature. After five washings, the HRP was evaluated by incubation with a development solution containing 0.16 M acetate

buffer (pH 6) (1 ml), tetramethylbenzidine (TMB; Boehringer Mannheim, Germany) in DMSO (Merck, Germany) (20 µl), and 1 M H₂O₂ (2 µl). The enzymatic reaction was stopped by the addition of 100 µl of 2 N sulfuric acid. Optical densities (OD) were read at 450 nm in a microtiter plate reader (Titertetek Multiskan, Finland).

Specific IgE inhibition experiments were conducted in a similar manner; 125 µl of serial dilutions of crude or boiled lentil extracts, respectively, was mixed with 125 µl of a serum pool containing high titers of specific IgE to lentil allergens. These mixtures were incubated for 2 h at room temperature. After the inhibition period, 100 µl of these mixtures was added to the coated wells (as previously described) for overnight incubation. Afterward, the assay was completed as for the specific IgE determinations.

SDS-PAGE and immunoblots

Separation by electrophoresis was accomplished in acrylamide gels by the method of Laemmli (13). Crude and boiled extracts were used under reduced and nonreduced conditions; 14% separating and 4% packing gels were used. The samples were dissolved in Tris-HCl (pH 6.8) buffer containing 10% glycerol and 2% SDS. The gel was run in Tris-HCl 62.5 mM buffer with 0.1% SDS; commercial standards of low-range molecular size (Bio-Rad (Richmond, VA, USA) were used. The electrical field conditions were 250 V, 35 mA, and 8 W. Gels were dyed with 0.1% Coomassie brilliant blue (R-250) and undyed in a water:methanol:acetic acid (50:40:10) solution.

Immunoblots were carried out with the crude and boiled extracts under nonreduced conditions with a serum pool, diluted 1:3, of group I patients. Gel proteins were transferred to Immobilon membranes (Millipore, Bedford, MA, USA) in a Trans Blot (Bio-Rad) tank in basic media. After transfer, the membranes were allowed to dry and incubated overnight with the serum pool. Afterward, the strips were washed with 0.1% PBS Tween and incubated for 2 h at room temperature with peroxidase-labeled monoclonal human anti-IgE (Ingenasa, Madrid, Spain) at a concentration of 2 µg/ml. After the corresponding washes, the enzymatic reaction took place in a solution composed of a 0.1 M citrate tampon (pH 5.0) containing 10 mM EDTA and 1% dextran sulfate (1 ml), TMB in DMSO (20 µl), and 1 M H₂O₂ (2 µl).

Immunoblots, only to the crude extract, using individual sera of groups I, II, and III patients, diluted 1:3, were carried out by the same method.

Results

The results of the oral challenges and other clinical features are shown in Table 1. Wheal sizes to lentil were significantly greater in group I (mean diameter 6.9 mm) than in group II (3.5 mm) ($P < 0.01$). Only one group II patient had a wheal greater than 6 mm. A history of atopy, as described as positive skin tests to other allergens and the presence of allergic diseases, was similar in both groups; 21 (80%) and 13 (54%) of group I patients were clinically sensitive to chickpea and pea, respectively, as confirmed by oral challenges; only one group II patient was sensitized to chickpea; other characteristics were not significantly different (Table 1).

Significant differences in specific IgE-binding levels to lentil allergens were observed between group I (mean OD: 2.35), group II (mean OD 0.56) ($P < 0.001$), and group III (mean OD: 0.13), as well as between groups II and III ($P < 0.001$) (Wilcoxon signed-rank test).

Specific IgE-binding experiments to the crude and boiled lentil extracts, using sera from groups I and II, demonstrated a higher binding capacity to the crude than to the boiled extract ($P = 0.0001$). Specific IgE-binding levels in group I

Table 1. Clinical features and laboratory findings in group I and group II

Features	Group I (n = 24)	Group II (n = 9)
History of atopy (%)‡	22/24 (91.7)	9/9 (100)
Mean age (years) at first reaction (range)	2.2 (0.3–12)	4 (1.2–12)
Mean age (years) at tolerance (range)	–	9.2 (3–19)
Symptoms (%)*		
Skin	17/24 (70.8)	7/9 (77.7)
Respiratory	11/24 (45.8)	2/9 (22.2)
Gastrointestinal	5/25 (20.8)	3/9 (33.3)
No. of reactions (mean)	3.4	5
Clinical allergy to other legumes (%)		
Chickpea	21/24 (80)	1/9 (11)
Pea	13/24 (54)	0/9 (0)
Peanut	1/24 (4)	1/9 (11)
Mean total IgE (kU/l) (range)	504.4 (25–1878)	719.4 (123–1346)
Mean wheal size (mm) on skin prick testing (range)	6.9 (3–13)**	3.5 (2–7)**

‡Described as concomitant presence of other positive skin tests and allergic diseases.
 *As confirmed by oral challenges.
 Skin: urticaria and/or angioedema; respiratory: cough, wheezing, rhinitis, conjunctivitis, and/or laryngeal edema; gastrointestinal: vomiting.
 ** $P = 0.0016$.

were 2.35 OD to the crude extract vs 1.7 OD to the boiled extract, and in group II, 0.56 OD vs 0.24 OD.

ELISA-inhibition experiments were also conducted with both extracts. Surprisingly, the boiled lentil extract had a higher specific IgE-inhibition capacity on both solid phases than the crude extract (Fig. 1A and B). Parallel lines were obtained when the boiled extract was used in the solid phase, but not when the crude extract was used, suggesting some allergenic discrepancies.

Immunoblots demonstrated less intense specific IgE binding to the boiled than to the crude extract with a serum pool of group I patients. Although the binding pattern was similar, less binding was observed, especially in the 20–70-kDa molecular mass range. These results confirm the data obtained from direct specific IgE-binding experiments (data not shown). The crude lentil immunoblot exhibited approximately 30 bands. The mean number of bands recognized by group I sera was approximately 16 (range 3–30), group II recognized a mean number of 7 (range 0–15), and group III, a mean of 2 (range 0–6). No bands were detected by a serum pool containing sera from grass-allergic individuals (Fig. 2). A band of approximately 53 kDa was recognized by 92% of group I patients. Other bands were recognized by more than 50% of sera from patients with clinical sensitivity to lentil (69.8, 51.8, 39.8, 20.7, and 18.4 kDa).

Discussion

Although peanuts and soybeans are the best-known causes of allergic reactions in children with legume sensitivity (14), lentils and chickpeas are the legumes most frequently involved in allergic reactions in Spain (6, 15).

There are confounding reports in the literature on the role of specific IgE in legume hypersensitivity. Hourihane et al. (8) investigated whether there were any differences between children who remained allergic to peanut and children with similar histories but a negative reaction after a peanut challenge. The authors concluded that the differences in total and specific IgE levels did not reach statistical significance, although in the nontolerant group, the levels were higher. Skin test results were also evaluated in this study. The authors determined that 61.5% of the tolerant children had negative skin tests, and none had a wheal greater than 6 mm. However, in the nontolerant group, 78.5% had a wheal size greater than 6 mm (8). In our study, specific IgE levels and wheal sizes were smaller in group II than in group I patients. These findings are in accordance with the data of Hourihane et al. (8) and suggest that a

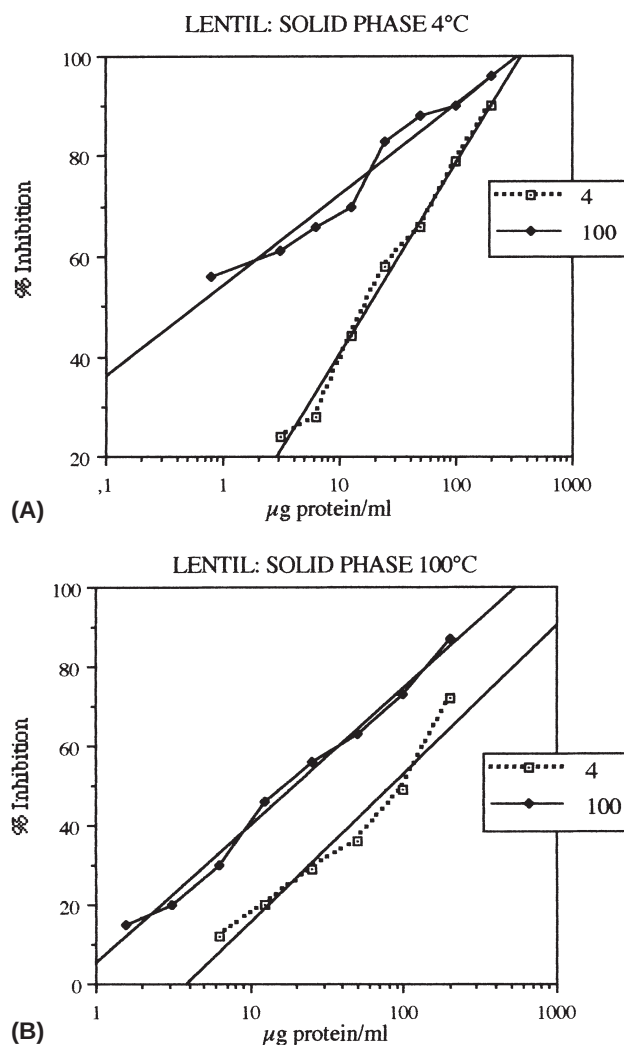


Figure 1A and B. ELISA-inhibition assays using crude and boiled lentil extracts in solid phases, respectively.

reduction in specific IgE levels to this legume is associated with the appearance of tolerance of lentils.

There is little information available about the allergenicity of lentils after heating. Our results demonstrate that lentil extracts contain thermostable allergens, since their allergenicity is maintained after boiling for 15 min. Other authors have also suggested that lentil extracts contain heat-stable allergens which are capable of inducing positive skin tests and specific IgE determinations (2–5). None of the previous studies compared both types of extracts by specific IgE-inhibition assays. Our results from the inhibition assays demonstrated that the boiled extract had an inhibition capacity greater than the crude extract on both solid phases, confirming its strong allergenicity. This fact is probably due to the formation of neoallergens (16–18). This possibility cannot be excluded in the present study and should be further evaluated. Thus, it appears to be important that studies on legume sensitivity clearly indicate whether the

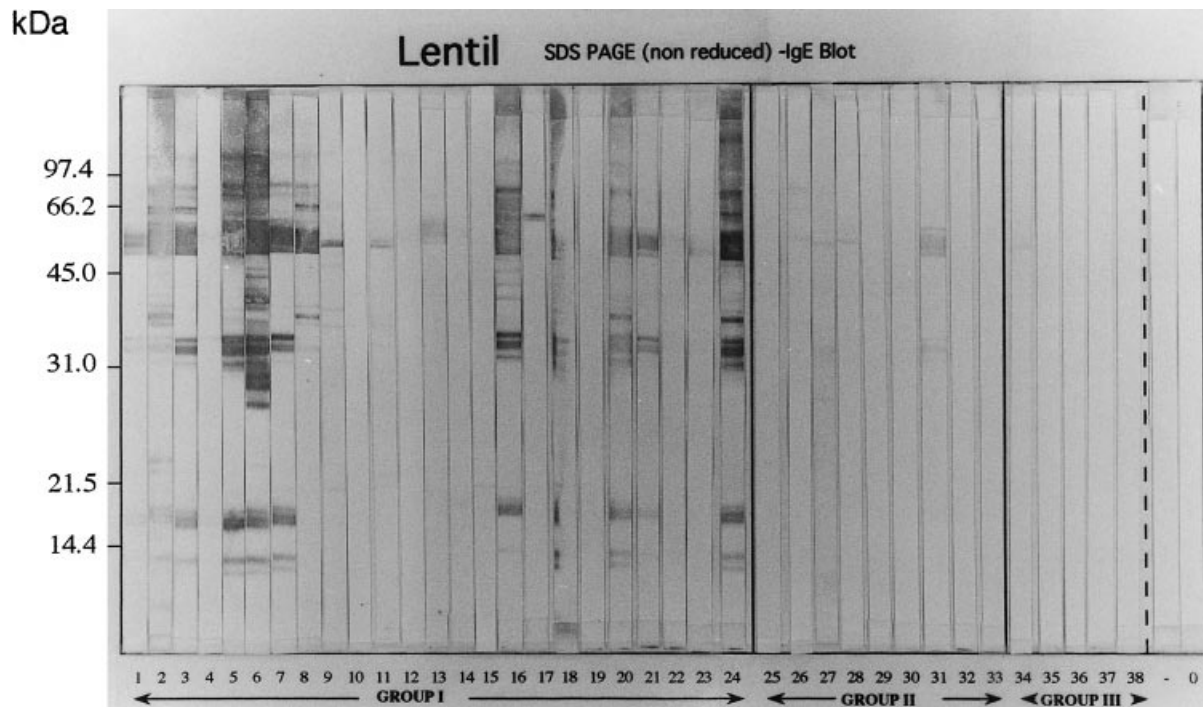


Figure 2. SDS-PAGE immunoblots of crude lentil extract under nonreduced conditions with sera of group I, group II, and group III patients. Test included serum pool from patients sensitive to grasses, but negative to legumes (–), and one lane incubated with labeled anti-IgE (o).

extracts were heated or not when used for skin testing and/or *in vitro* determinations.

The lentil allergens identified in our study were multiple and distributed throughout a wide molecular mass range. Pascual et al. (6) also detected multiple IgE-binding bands in a molecular mass range of 14–84 kDa, including a band of 54 kDa with a 77% binding prevalence. In our study, a band of approximately 53 kDa was recognized by 92% of group I patients. A slight recognition of a few bands was observed in some sera of group III patients who never had clinical allergy to lentil, but who had experienced clinical reactions to other legumes. This finding suggests the existence of a certain degree of cross-reactivity *in vitro* among these legumes, as has been demonstrated in other studies (19, 20). Although other authors have suggested that clinical sensitivity to more than one legume species is rare (21), we found that 80% of our group I patients were also clinically sensitive to chickpeas.

Several conclusions can be drawn from this study.

- 1) Patients who have developed tolerance of lentils have lower specific IgE levels than nontolerant patients.

- 2) Lentil allergens, which may have clinical importance, retain their allergenicity after boiling for 15 min. However, the boiling process seems to denature several allergens which may be important for the *in vitro* diagnosis of lentil sensitivity. After boiling, the inhibitory capacity of lentil allergens is increased.
- 3) Immunoblots demonstrate that the heated extract retains a similar IgE-binding pattern, although the intensity of binding is reduced.
- 4) Lentil extracts contain multiple allergens distributed over a wide molecular mass range. A band of approximately 53 kDa was recognized by 92% of group I patients and several other bands by more than 50% of the patients with clinical sensitivity to lentils. Group II patients recognized fewer allergenic bands than those patients who maintained clinical sensitivity to lentil.

Acknowledgment This study was supported, in part, by a grant of the Fundación de la Sociedad Española de Alergia e Inmunología Clínica.

References

1. Alergia a alimentos. In: SEAIC ALK-Abelló, editors. *Alergológica: Factores epidemiológicos, clínicos y socioeconómicos de las enfermedades alérgicas en España*. Madrid: NILO Industria Gráfica, 1995:165–183.
2. Martín JA, Compaired JA, de la Hoz B, Quirce S, Alonso MD, Igea JM, Losada E. Bronchial asthma induced by chickpea and lentil. *Allergy* 1992;**47**:185–187.
3. Carrillo T, Cuevas M, Díez ML, Losada E, Moneo I. Diagnóstico inmunológico *in vitro* en la hipersensibilidad a legumbres. *Allergol Immunopathol (Madr)* 1986;**14**:139–146.
4. García JC, López-Asunsolo A, Cosmes P, Martín A. Bronchial asthma induced by hypersensitivity to legumes. *Allergol Immunopathol (Madr)* 1995;**23**:38–40.
5. Kalogeromitros D, Armenaka M, Galatas I, Capellou O, Katsarou A. Anaphylaxis induced by lentils. *Ann Allergy Asthma Immunol* 1996;**77**:480–482.
6. Pascual CY, Fernández-Crespo J, Sánchez-Pastor S, et al. Allergy to lentils in Mediterranean pediatric patients. *J Allergy Clin Immunol* 1999;**103**:154–158.
7. Bock SA, Atkins FM. The natural history of peanut allergy. *J Allergy Clin Immunol* 1989;**83**:900–904.
8. Hourihane JO'B, Roberts SA, Warner JO. Resolution of peanut allergy: case-control study. *BMJ* 1998;**336**:1271–1275.
9. Crespo JF, Pascual C, Burks AW, Helm RM, Esteban MM. Frequency of food allergy in a pediatric population from Spain. *Pediatr Allergy Immunol* 1995;**6**:39–43.
10. Martínez M, Ibáñez MD, Elices A, Marañón F, Fernández-Caldas E, Laso MT. Alergenicidad de la almorta (*Lathyrus sativus*). *Rev Esp Alergol Clin* 1998;**13**:282–286.
11. Boluda L, Alonso C, Fernández-Caldas E. Purification, characterization and partial sequencing of 2 new allergens of *Olea europaea*. *J Allergy Clinical Immunol* 1998;**101**:210–216.
12. Boluda L, Casanovas M, Prieto JL, Fernández-Caldas E. Determinations of Par j 1 by a competitive enzyme immunoassay using human specific IgE and IgG. Validation by skin prick testing. *J Invest Allergol Clin Immunol* 1998;**8**:207–213.
13. Laemmli UK. Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* 1970;**227**:680–685.
14. Sampson HA. IgE-mediated food intolerance. *J Allergy Clin Immunol* 1988;**81**:495–504.
15. Ibáñez MD, Martínez M, Fernández-Caldas E, et al. Allergy to legumes. Clinical and immunological studies. *Cuadernos de Immuno-Alergología Pediátrica* 1997;**12**:57–58.
16. Codina R, Oehling AG, Lockey RF. Neoallergens in heated soybean hull. *Int Arch Allergy Immunol* 1998;**117**:120–125.
17. Malanin K, Lundberg M, Johansson SGO. Anaphylactic reaction caused by neoallergens in heated pecan nut. *Allergy* 1995;**50**:988–991.
18. Berrens L. Neoallergens in heated pecan nut: products of Maillard-type degradation? *Allergy* 1996;**51**:277–278.
19. Bernhisel-Broadbent J, Taylor S, Sampson HA. Cross-allergenicity in the legume botanical family in children with food hypersensitivity. II. Laboratory correlates. *J Allergy Clin Immunol* 1989;**84**:701–709.
20. Barnett D, Bonham B, Howden M. Allergenic cross-reactions among legume foods – an *in vitro* study. *J Allergy Clin Immunol* 1987;**79**:433–438.
21. Bernhisel-Broadbent J, Sampson HA. Cross-allergenicity in the legume botanical family in children with food hypersensitivity. *J Allergy Clin Immunol* 1989;**83**:435–440.