

How Accurate and Safe Is the Diagnosis of Hazelnut Allergy by Means of Commercial Skin Prick Test Reagents?

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Key Words

DBPCFC · Hazelnut · Skin prick test

Abstract

Background: Allergy to tree nuts, like hazelnuts, ranks among the most frequently observed food allergies. These allergies can start at early childhood and are, in contrast to other food allergies, not always outgrown by the patient. Tree nut allergy is frequently associated with severe reactions. Diagnosis partially relies on in vivo testing by means of a skin prick test (SPT) using commercially available SPT reagents. **Methods:** Protein and allergen composition of nine commercial SPT solutions was evaluated using standard protein detection methods and specific immunoassays for measurement of five individual allergens. Diagnostic performance was assessed by SPT in 30 hazelnut-allergic subjects, of which 15 were provocation proven. **Results:** Protein concentrations ranged from 0.2–14 mg/ml. SDS-PAGE/silver staining revealed clear differences in protein composition. The

major allergen Cor a 1 was present in all extracts but concentrations differed up to a factor 50. An allergen associated with severe symptoms, Cor a 8 (lipid transfer protein), was not detected on immunoblot in three products, and concentrations varied by more than a factor 100 as was shown by RAST inhibition. Similar observations were made for profilin, thaumatin-like protein and a not fully characterized 38-kD allergen. Ratios of individual allergens were variable among the nine extracts. SPT showed significant difference, and 6/30 patients displayed false-negative results using 3/9 products. **Conclusion:** Variability in the composition of products for the diagnosis of hazelnut allergy is extreme. Sometimes, allergens implicated in severe anaphylaxis are not detected by immunoblotting. These shortcomings in standardisation and quality control can potentially cause a false-negative diagnosis in subjects at risk of severe reactions to hazelnuts.

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Introduction

Besides allergy to peanuts, eggs, milk, wheat, soy, fish and shellfish, allergy to tree nuts, like hazelnut, ranks among the most frequently observed food allergies. The prevalence of tree nut allergy is estimated to be about 0.7% (North America and Western Europe) [1–3]. Among tree nuts, hazelnut is most frequently involved in food-related symptoms. Adverse reactions to hazelnuts can be rather mild, i.e. the oral allergy syndrome (OAS), but can also result in more severe reactions like gastro-intestinal symptoms, wheezing, urticaria and even anaphylactic shock [4–6].

Mild symptoms, like OAS, are most frequently caused by cross-reactive IgE against the major hazelnut allergen Cor a 1 [7] as a result of sensitisation to the major birch pollen allergen Bet v 1. These related allergens belong to the so-called PR10 group of pathogenesis-related proteins in plants. Due to their labile properties [8], allergens from this family are rapidly degraded in the stomach and cannot easily reach the immune system of the gastro-intestinal tract in an IgE-binding conformation. It is now well accepted that more severe symptoms are caused by stable allergens like lipid transfer proteins (LTP) [9–11], 2S albumins [12, 13] and thaumatin-like protein (TLP) [14, 15] which survive the acidic and proteolytic environment of the gastro-intestinal tract. For identifying patients with type 1 allergy to hazelnut, a diagnostic tool should cover all relevant allergens. In order to minimise the risk of misdiagnosing patients with severe allergic reactions, it is of particular importance to have sufficient sensitivity for the stable allergens that are implicated in systemic reactions.

The double-blind placebo-controlled food challenge (DBPCFC) is considered the golden standard for the diagnosis of food allergy [16]. However, this method is not suitable for routine diagnosis in the daily practice of an outpatient clinic or private practice. Hence, a clinical history recorded by the clinician in combination with skin prick testing (SPT) with commercial food extracts or fresh foods (prick-to-prick) [17] is most widely applied as diagnostic approach. Reliable and reproducible SPT results can only be obtained when well-standardized diagnostics are used. It goes without saying that the prick-to-prick method cannot be standardized.

Different approaches in manufacturing and end-product formulation of SPT products may lead to discrepancies in diagnostic performance. Nine commercially available hazelnut extracts were immunochemically characterised, and their performance in skin testing was evaluated. For this purpose, allergen-specific antibody reagents were

developed and used to analyse the allergen composition. Furthermore, hazelnut allergic patients were skin tested to compare the diagnostic performance of different SPT products.

Materials and Methods

Patients

Thirty patients attending the outpatient clinic of the University Medical Centre Utrecht were recruited for this study. All patients had a convincing history of acute hazelnut-related symptoms. In 15 patients, symptoms were confirmed by DBPCFC with hazelnut [18]. All patients had hazelnut-specific IgE as confirmed by SPT ($\geq 2+$) with a commercial hazelnut SPT product (ALK-Abelló, Nieuwegein, The Netherlands; extract A in this study) and/or an IgE level ≥ 0.7 kU/ml (CAP system FEIA, Pharmacia & Upjohn, Uppsala, Sweden). Age varied between 18 and 69 years (median 29 years). Hazelnut-related symptoms consisted of typical oral symptoms like itching and tingling of the throat and lips (OAS) in all patients. In addition, 12 patients reported a feeling of swelling of the throat, a dry cough, and/or hoarseness, 2 patients had gastro-intestinal symptoms and 1 patient rhinoconjunctivitis. One patient developed generalised urticaria, nausea and faintness after initial symptoms of OAS. The study was reviewed and approved by a local ethical committee. Written informed consent was obtained from all patients before enrolment in the study.

Extracts

Commercially available hazelnut SPT extracts were purchased from nine manufacturers: (A) ALK-Abelló (Nieuwegein, The Netherlands), (B) Allergopharma (Reinbeck, Germany), (C) Allergy Laboratories of Ohio (Columbus, Ohio, USA), (D) Stallergènes, (Antony, France), (E) Artu Biologicals Europe, (Lelystad, The Netherlands), (F) Bayer (Elkhart, Ind., USA), (G) Greer Laboratories, (Lenoir, N.C., USA), (H) HAL Allergenen Lab. (Haarlem, The Netherlands) and (I) Nelco Laboratories (New York, N.Y., USA). Of all manufacturers two different batches were obtained. All reagents were purchased as ready-for-use products that did not require further dilution for SPT.

For all laboratory analyses, the extracts were extensively dialysed (<10 kD molecular weight cut-off membrane, Visking, type 8/32, Karlsruhe, Germany) against PBS, and changes in the volume were recorded. Protein concentrations were then determined in duplicate with the BCA reagent method according to the manufacturers' instructions (Pierce, Rockford, Ill., USA), with BSA as a standard. Results were adjusted for the observed changes in the volume.

An in-house hazelnut extract (IHE) was prepared according the method originally described for the extraction of apple allergens [19]. In short, ground hazelnut was extracted in a buffer containing polyvinylpyrrolidone and di-ethyldithiocarbamate. These reagents prevent loss of allergenicity by inhibiting oxidative processes mediated by polyphenyloxidases and polyphenols upon disruption of the plant tissue. In particular, Bet-v-1-related food allergens (i.e. Cor a 1 in case of hazelnuts) have been shown to be sensitive to these processes.

Purification of Hazelnut Allergens

Cor a 1 was purified from the IHE by means of affinity chromatography using monoclonal antibody (mAb) 5H8 against Bet v 1 [20] coupled to CNBr-activated Sepharose 4B (Amersham Pharmacia Biotech, Uppsala, Sweden). This mAb is cross-reactive with several plant food homologues of Bet v 1, e.g. Cor a 1. Cor a 2 (profilin) was purified from the same extract using poly-L-proline (PLP) coupled to Sepharose as affinity matrix, as described previously for other profilins [21].

Antibody Reagents

Three human serum samples were used for RAST inhibition and/or immunoblot screening. These three sera were selected on the basis of dominant IgE immunoblot recognition of Cor a 1 (No. 1), Cor a 8 (No. 2) and a 38-kD allergen (No. 3), respectively. The sera originated from patients with a convincing history of hazelnut allergy.

A fourth serum sample, No. 200, was used for measurement of profilin by indirect RAST. This serum is an in-house reference serum with a high titer of cross-reactive IgE antibodies against plant-derived profilins. Serum samples (14/30 on the basis of serum availability) of patients that had volunteered for the SPT analysis were used to evaluate the IgE-binding capacity of 7/9 SPT extracts in RAST. MAb 5H8 directed to Bet v 1 and cross-reactive to Cor a 1 was used to detect Cor a 1 [20].

Two polyclonal rabbit antisera were used for the detection of Cor a 8 and TLP. For these allergens, antisera were obtained after immunisation of New Zealand White rabbits with natural carrot LTP [22] and recombinant apple TLP [23], respectively.

SDS-PAGE/Immunoblotting

For evaluation of protein composition by SDS-PAGE/silver staining, hazelnut SPT extracts were run on an Excelgel SDS, gradient 8–18%, (Amersham Pharmacia Biotech). Sample preparation and silverstaining was done according to the manufacturers instructions. Per lane 3.75 µg of hazelnut protein was loaded, with the exception of extracts B and E of which 3.23 and 3.45 µg were used, respectively.

For immunoblotting, the same quantities of each extract were separated by SDS-PAGE on a Novex XCell II system using a 4–12% gel (Novex, San Diego, Calif., USA), followed by immunoblotting to nitrocellulose according to the manufacturers instructions.

Allergen Measurements

Competitive RIA. A competitive RIA [24] was used to monitor Cor a 1. To this end, 50 µl cross-reactive mAb 5H8 (133 ng/ml) against Bet v 1 was incubated with serial dilutions of hazelnut SPT extract in 100 µl PBS/AT (PBS/AT: phosphate-buffered saline, 10 mM EDTA, 0.3% bovine serum albumin, 0.1% Tween-20). After 2 h, Sepharose-coupled goat antibodies against mouse IgG (GM 17D, Sanquin, Amsterdam, The Netherlands), ¹²⁵I-radiolabeled Bet v 1 and 500 µl PBS/AT were added resulting in a final volume of 850 µl. After overnight incubation and washing, bound radioactivity was measured on a γ-counter. Cor a 1 concentrations were calculated using a standard curve of affinity-purified Cor a 1.

Indirect RAST. Cor a 2 (profilin) was measured using its high affinity for PLP [21]. Sepharose-coupled PLP (0.5 mg) was incubated overnight with serial dilutions of hazelnut extract (50 µl). After washing, 50 µl serum No. 200 was added followed by another overnight incubation. After washing, bound IgE was detected with ¹²⁵I-radiolabeled sheep anti-human IgE (SH25-1-p7, Sanquin). A standard curve

of affinity-purified hazelnut profilin was used to calculate the Cor a 2 content. Previous plant-derived profilin purifications using PLP-Sepharose have proven to produce almost pure profilin (data not shown). This justifies the choice of using human serum with polyclonal IgE to several allergens.

RAST (Inhibition)

RAST was performed as described previously [25]. In short, 0.5 mg Sepharose (2 mg of SPT extract protein was coupled to 100 mg Sepharose) was incubated overnight with 50 µl human serum in a final volume of 300 µl. After washing away unbound antibodies, bound IgE was detected using ¹²⁵I-radiolabeled sheep anti-human IgE.

In order to compare the IgE binding potency of SPT extracts with respect to Cor a 1 or hazelnut LTP, SPT extracts were analysed by means of RAST inhibition [25]. To this end, 50 µl of serial dilutions of hazelnut SPT extracts were incubated with 50 µl human serum (serum No. 1 for Cor a 1 and serum No. 2 for LTP). After 2 h, Sepharose-coupled hazelnut SPT extract (0.5 mg Sepharose/10 µg hazelnut protein/test) was added to this mixture and incubated overnight in a final volume of 350 µl PBS/AT. The next steps were identical to those for RAST. Depending on the allergen analysed, SPT extracts with the highest Cor a 1 or LTP concentrations (per mg total protein) were used for solid-phase immobilization. For Cor a 1, this was extract E, for LTP extract I. In both cases, 2 mg of protein was coupled to 100 mg Sepharose.

Parallel line analysis was applied on the obtained inhibition curves. From this analysis, the relative amount of Cor a 1 or LTP could be calculated.

Skin Prick Tests

SPTs were performed using the first batch of six extracts (A, B, D, G, H and I) on the flexor aspect of the patients forearm with a standardized prick needle (ALK Lancet) according to Dreborg and Frew [26]. All extracts were used as delivered and advised by the manufacturer without taking potentially different protein and allergen concentrations into account.

Histamine dihydrochloride (10 mg/ml) was used as a positive control, and the glycerol diluent of the SPT extracts as a negative control (ALK-Abellø). SPT wheal sizes were expressed relative to the wheal induced by histamine, the so-called histamine equivalent intracutaneous test (HEIC). The mean diameter of the allergen wheal is divided by that of the histamine wheal. All patients fulfilled the criteria of a wheal <3 mm for the negative control and ≥5 mm for histamine. A HEIC of 0.25 or less was regarded as negative. Prior to the skin tests, patients discontinued antihistamines for 1 week.

Results

Protein Composition

Two batches of hazelnut SPT products of nine manufacturers were tested for total protein content. Inter-company variation was considerable up to a maximum difference of a factor 65 (table 1). Variation between batches of the same manufacturer was limited (< factor 3). All extracts were also analysed by SDS-PAGE/silver staining,

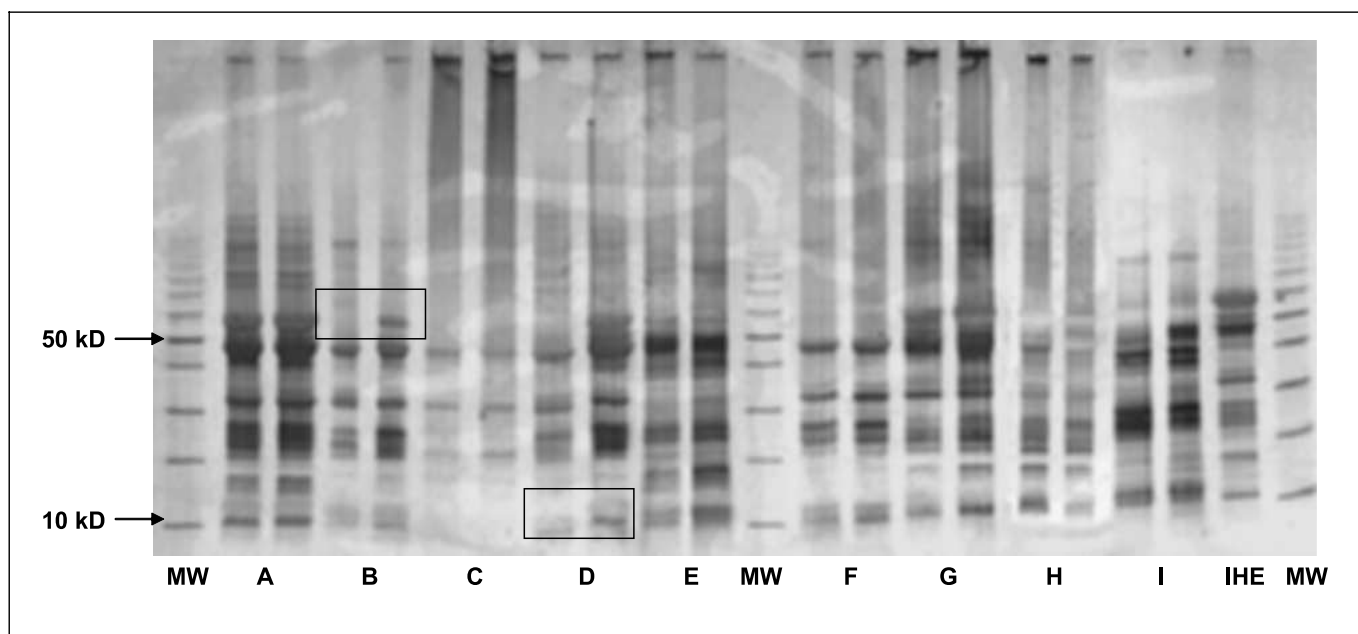


Fig. 1. SDS-PAGE followed by silver staining of nine hazelnut SPT extracts. A–I = Hazelnut SPT extracts (for each extract: left lane is batch 1, and right lane is batch 2); MW = molecular weight marker. Two examples of qualitative differences are highlighted by rectangles.

Table 1. Analysis of nine hazelnut SPT extracts

SPT manufacturer	Label	Indicated concentration	Diluent preservative	Protein B1 $\mu\text{g/ml}$	Protein B2 $\mu\text{g/ml}$	Protein ratio B1/B2
ALK-Abelló	A	10,000 BU/ml	NI	751	1,003	0.75
Allergopharma	B	5,000 PNU/ml	G, NaCl, P	250	217	1.15
Allergy Laboratories	C	10% w/v	50% v/v G	2,700	1,904	1.42
Alyo-Stallergènes	D	1,000 IC/ml	NI	3,340	3,360	0.99
ARTU	E	1 mg/ml	P	230	389	0.59
Bayer	F	10% w/v	50% v/v G	4,800	4,995	0.96
Greer	G	20% w/v	0.2% P	3,250	4,810	0.68
HAL	H	50% w/v	5 mg/ml P	14,091	13,440	1.05
Nelco	I	10% w/v	0.4% P	715	970	0.74

B1 = Batch 1; B2 = batch 2; NI = not indicated on vial; G = glycerin; P = phenol.

showing that qualitative differences exist between manufacturers but also between batches of a single company (fig. 1).

Allergen Composition

Cor a 1 was measured by competitive RIA in batch 2 of all manufacturers (table 2). Concentrations varied between 0.6 and 32 $\mu\text{g/ml}$, but relative to the total protein the Cor a 1 content was very similar for 7/9 extracts

(0.17–0.32%). The remaining two extracts contained <0.05 and 1.57% Cor a 1, respectively. Profilin (Cor a 2) was measured by indirect RAST. Concentrations ranged from <0.4 to 13.2 $\mu\text{g/ml}$ (0.01–1.3%). Ratios of Cor a 1 and Cor a 2 differed by almost a factor 35 (mean $\pm$ SEM 3.03 ± 0.81).

Three additional allergens were monitored by immunoblotting. Polyclonal rabbit antibodies against carrot LTP detected variable levels of hazelnut LTP in the nine

Fig. 2. Hazelnut SPT extract immunoblot with rabbit anti-carrot LTP (**A**). Batch 1 and 2 were monitored for hazelnut LTP. **B** Rabbit anti-TLP on batch 2. **C** Human serum IgE No. 3.

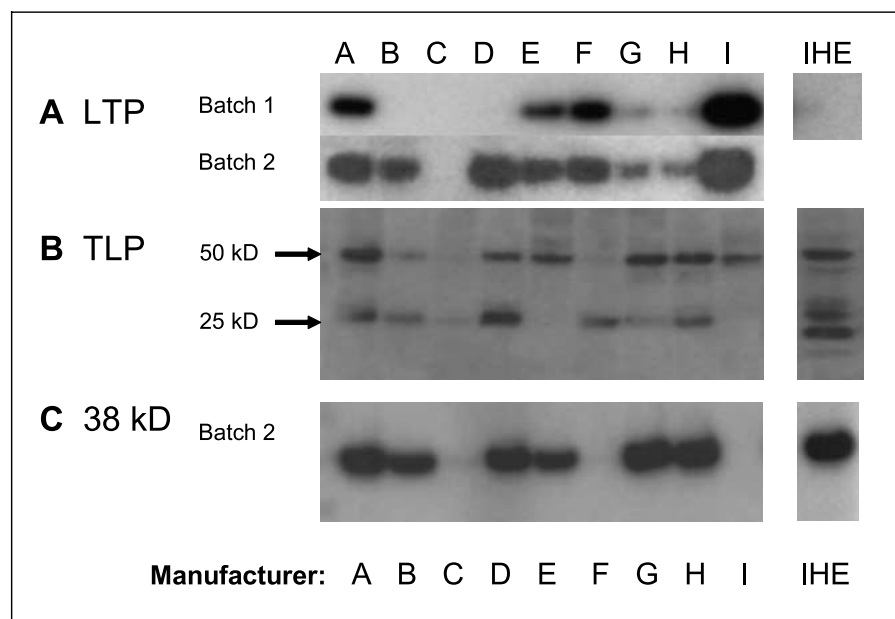


Table 2. Measurement of Cor a 1 and profilin in nine hazelnut SPT extracts and the IHE

	Cor a 1 µg/ml	Cor a 1/total protein, % w/w	Profilin µg/ml	Profilin/total protein, % w/w	Ratio Cor a 1/profilin
A: ALK	3.2	0.32	13.2	1.75	0.2
B: Allergopharma	0.6	0.28	0.4	0.16	1.5
C: Allergy Laboratories	3.3	0.17	0.6	0.02	5.5
D: Alyo-Stallergènes	1.5	0.05	0.4	0.01	3.8
E: Artu	6.1	1.57	1.0	0.43	6.1
F: Bayer	11.0	0.22	5.0	0.10	2.2
G: Greer	10.9	0.23	5.0	0.15	2.2
H: HAL	32.0	0.24	4.5	0.03	7.1
I: Nelco	2.4	0.25	<0.4	<0.06	6.0
IHE (5 mg/ml protein)	16.0	0.32	17.5	0.35	0.9

Measurement of Cor a 1 and profilin (batch 2 of the SPT extracts and the IHE). Due to insufficient quantity of batch 1, allergens were only measured in batch 2. Cor a 1 concentrations were measured by competitive RIA. Profilin concentrations were measured by indirect RAST.

extracts (fig. 2A). Batch 1 of three manufacturers contained no detectable Cor a 8. This was also true for our IHE extract. For two manufacturers, batch 2 was a clear improvement, illustrating variability between different production charges again.

A polyclonal rabbit antiserum against recombinant apple TLP was used to evaluate the TLP content of batch 2 of all nine manufacturers (fig. 2B). Extract C contained hardly any detectable TLP, the others (including the IHE) contained either monomeric or dimeric TLP or a combi-

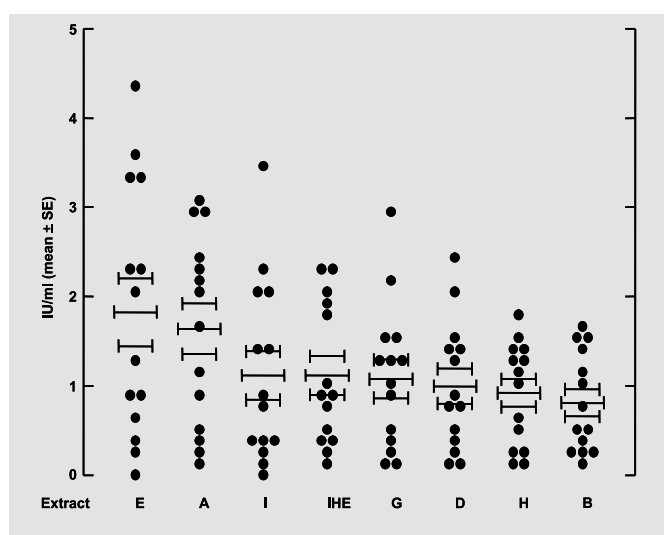
nation of both. A third as yet unidentified allergen of 38 kD recognized by IgE immunoblot in serum No. 3 was not detected in three extracts (fig. 2C).

In vitro IgE-Binding Potency

RAST performed with identical protein quantities of 7/9 SPT extracts and the IHE demonstrated significantly higher IgE-binding capacity for extracts E and A as compared to the other extracts (mean difference between extracts A and I: 0.55 IU/ml, 95% confidence interval:

0.05–1.0). No significant differences were found between extracts E and A, and between the extracts I, G, D, H, B and the IHE (fig. 3).

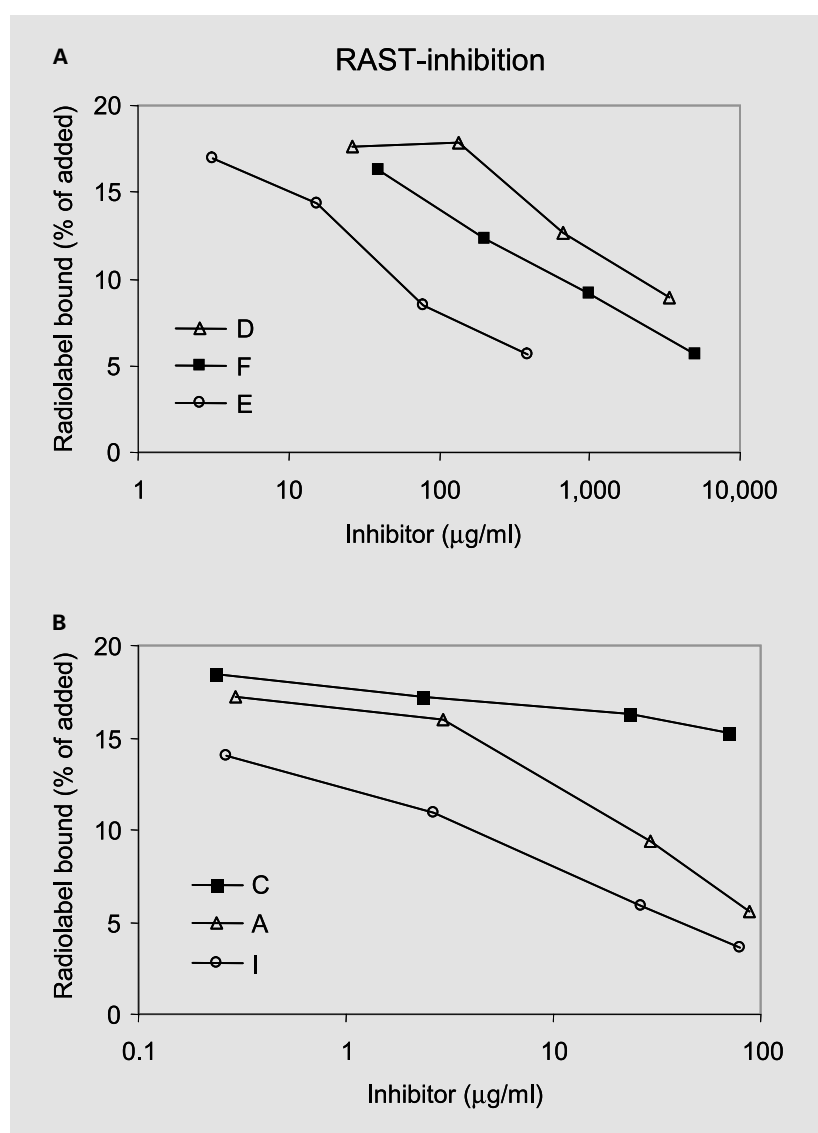
Differences observed with allergen-specific hyperimmune animal antibody reagents were confirmed by means of RAST inhibition. Using Cor-a-1-specific serum No. 1, similar differences were found as detected by competitive RIA (fig. 4A). Extracts with the lowest and highest Cor a 1 content (relative to total protein) differed by a factor 41, which is close to the 30-fold difference found in competitive RIA (fig. 4A). RAST inhibition using serum No. 2 with LTP-specific IgE confirmed the virtual absence of LTP in extract C (fig. 4B) as also observed by immunoblotting (fig. 2A).



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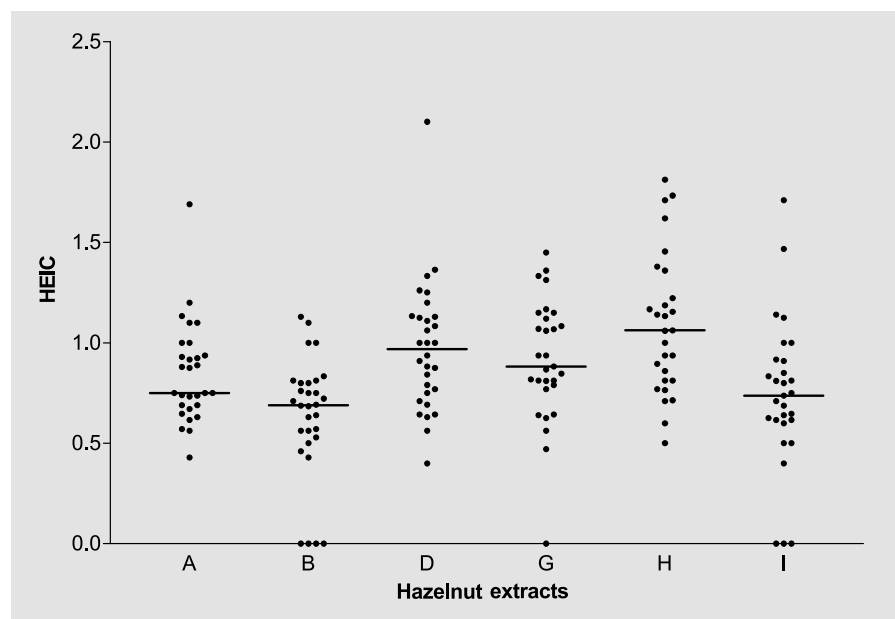
Fig. 3. Direct RAST performed with seven hazelnut SPT extracts and the IHE. 14/30 serum samples from the hazelnut SPT panel were used. Extracts E and A bound significantly more IgE compared to the other extracts (mean difference between extracts A and I: 0.55 IU/ml, 95% confidence interval: 0.05–1.0). Means $\pm$ SE are indicated by horizontal bars.

Fig. 4. The IgE binding potency of SPT extracts with respect to Cor a 1 or Cor a 8 (LTP) were analysed by means of RAST inhibition. **A** Cor a 1 was monitored with serum No. 1. Extract E was coupled to Sepharose. Extracts D, E and F were used as inhibitors. **B** LTP was monitored with serum No. 2. Extract I was coupled to Sepharose. Extracts A, C and I were used as inhibitors.



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Fig. 5. Skin test responses to extracts from six different companies. Median HEIC are indicated by horizontal bars. A = ALK; B = Allergopharma; D = Alyo-Stallergènes; G = Greer; H = HAL; I = Nelco.



Skin Prick Test

A panel of 30 patients was skin prick tested with 6/9 SPT extracts. Figure 5 shows the results with median skin test scores. Statistics (using the non-parametric Friedman test in SPSS 10.0) revealed that the skin test responses yielded by the different extracts differed significantly mutually. In other words, some skin test extracts give consistently higher or lower wheal sizes than others. Extract B, with the lowest protein concentration (217 µg/ml), gave the lowest skin test response and extract H, with the highest protein concentration (14 mg/ml), gave the highest skin test response. In 6 patients, one or more of the extracts used were negative. Application of extract B resulted in four false-negative SPTs, extract G in one and extract I in three. Of the 6 patients with a false-negative SPT, 4 were tested using the DBPCFC. All 4 had a positive DBPCFC, ranging from OAS to rhinoconjunctivitis, angioedema and urticaria.

Discussion

In this study, products marketed for the in vivo diagnosis of hazelnut allergy were analysed in depth for their protein and allergen composition. Five different allergens were monitored. The major birch-pollen-related hazelnut allergen Cor a 1 was shown to be present in concentrations that greatly differed but were adequate to correctly diagnose patients with Cor-a-1-specific IgE. Overall, there was

a strong correlation ($R_{\text{Pearson}} = 0.87$) between the protein concentration of the SPT extract and the skin reactivity. False-negative SPT results were only found for extracts with the lower protein concentrations (extracts B and I) with one exception for extract G. Since most extracts contained comparable ratios of Cor a 1/total protein, it was not surprising to find these results in Dutch hazelnut-allergic patients who have predominantly IgE against Cor a 1. However, firm conclusions related to the exact cause of false-negative SPTs cannot be drawn, since SPTs were performed with batch 1 and Cor a 1 and some of the other allergens were only measured in batch 2.

Extracts E and A were the most potent IgE-binding SPT extracts in RAST, when identical concentrations were used of each extract. Taking the predominance of Cor a 1 among Dutch patients into account, it is therefore not surprising that these extracts ranked highest in Cor a 1/total protein ratios (table 2). Since the SPT evaluation was performed using identical volumes instead of protein quantities, no significant correlation was found with the RAST results. Here the ranking roughly followed the Cor a 1 concentrations in µg/ml.

Cor a 2 (profilin), the second pollen-related allergen, was also present in variable quantities. Cor a 8 (LTP) was virtually absent in 3/9 extracts marketed for SPT. LTP has been reported to be particularly relevant for severe plant food allergy [8, 11]. This has been attributed to its extreme stability to processing and proteolysis in the gastro-intestinal tract. In countries of the Northern Hemi-

sphere like The Netherlands, Austria or Germany, LTP reactivity is rare [27]. This study was performed among Dutch patients. The observed false-negative SPT results are therefore most likely not due to the absence of LTP. For countries around the Mediterranean Sea, LTP reactivity was reported to be much more common [28]. In these countries, application of SPT products without LTP could result in a false-negative diagnosis that could potentially endanger a patient's life.

Some hazelnut allergic patients in our studies have IgE antibodies against Ber e 1 [29] and a 10-kD allergen in hazelnut. Preliminary analysis of the nine skin prick test reagents revealed that this putative hazelnut 2S-albumin is also present at highly variable concentrations. Again, such diagnostic tools might lead to potentially dangerous false-negative test results.

Lack of standardisation and comparability was also confirmed by the variable content of two additional allergens: hazelnut TLP and an as yet unidentified 38-kD allergen. The cause of this undesirable situation most likely lies in a complex combination of factors: differences in source material like hazelnut cultivars and ripeness of hazelnuts, differences in post-harvest treatment of hazelnut source materials, in extraction methods including defatting procedures, and in additives aiming to stabilise the products.

The complex nature of the problem stresses the need of well-defined and stable reagents to replace the current generation of diagnostics. A suitable alternative is now being offered by recombinant food allergens. Several of these reagents are now available for various foods including hazelnuts [30, 31]. In conclusion, currently available hazelnut extracts for SPT are not reliable if test results are negative. This does however not imply that a positive SPT is always associated with clinical food allergy [32]. Convincing clinical histories should complement SPT results. When doubt remains, a DBPCFC is the best solution.

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