

Comparison of different immunochemical methods for the detection and quantification of hazelnut proteins in food products

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Abstract

Hazelnuts are widely used in the food industry owing to their nutritive value and taste. The amount of hazelnut present in a recipe is usually considered as a mark of quality. On the other hand, contamination of foods that normally do not contain hazelnuts is a threat for patients with a hazelnut allergy. For this reason, the availability of a method for the detection and quantification of hazelnuts in foods would be desirable. The objective of this study was to develop a method for the detection and quantification of minor amounts of hazelnut protein in food products that is potentially applicable for the food industry. Several immunochemical methods, e.g., immunoblotting and enzyme-linked immunosorbent assay (ELISA), were developed with antibodies from both hazelnut-sensitized patient sera and the sera of rabbits hyperimmunized with hazelnut protein. Immunoblotting appeared to be non-specific when the sera of patients were used as a source of antibodies. Using immunopurified antibodies from rabbits immunized with hazelnuts, immunoblotting became specific, but the sensitivity of this method was limited. Inhibition of IgE binding is a generally used test in clinical laboratories to establish contamination with hazelnuts. This approach is sensitive and specific, but not readily accessible for the food industry since patient serum is needed. Similar results in terms of sensitivity and specificity were obtained with a sandwich ELISA constructed with an immunopurified antibody from rabbits sensitized to hazelnuts. No substantial cross-reactivity with other nuts, legumes or other food constituents was observed, and concentrations as low as 5 ng/ml, corresponding to 1 ppm in food products, were detected. In a field test, several consumer products regarded to be free of hazelnuts were shown to contain traces of hazelnut. This sandwich ELISA constructed with immunopurified antibodies from rabbits sensitized with hazelnut protein is a sensitive and specific method to detect and quantify hazelnut and is useful in detecting trace contamination with hazelnut in

Abbreviations: BSA, bovine serum albumin; DTT, dithiothreitol; ELISA, enzyme-linked immunosorbent assay; OAS, oral allergy syndrome; PBS, phosphate-buffered saline; ppm, parts per million; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SPTs, skin prick tests

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various consumer products. Since this test does not require serum from patients, it is appropriate for use in the food industry.
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1. Introduction

Hazelnut allergy affects a substantial part of the Western population and often coincides with allergies to tree pollen (Andersen and Lowenstein, 1978; Lowenstein and Eriksson, 1983). Approximately 50–70% of the patients with tree pollen allergy is sensitized to hazelnut and about half of them suffer from clinical symptoms of hazelnut allergy (oral allergy syndrome [OAS], in some cases combined with urticaria, angioedema, pharyngeal edema, asthma, vomiting, diarrhea, anaphylaxis), resulting in an estimated prevalence of 0.1–0.5% for hazelnut allergy in Europe (Groot et al., 1996). A similar prevalence of hazelnut sensitization was found in a birth cohort study reviewed at 1, 2 and 4 years of age (Tariq et al., 1996). Since treatment of hazelnut allergy is not yet available, patients need to exclude hazelnuts from their diets. Hazelnuts are used in confectionery products such as nougat and in chocolate products. Furthermore, recent product diversification has led to an increase in consumer products containing hazelnut. Many of these new products, such as cookies, muesli-bars and chocolate spreads, are produced in facilities where products that normally do not contain hazelnut are also formulated. This results in a risk of cross-contamination with hazelnut. With the growing public awareness of food allergy, the food industry is confronted with a significant problem. Although there is no legislation on labelling of food allergens on the ingredient list (Smith, 1997), most producers and retailers try to minimize the risk of cross-contamination. However, until now, the food industry has only limited access to tests for the detection of hazelnuts, in contrast to, e.g., peanut tests (Koppelman et al., 1996; Yeung and Collins, 1996). Several immunochemical tests for the detection of hazelnut in foods have been described (Mohr et al., 1983; Klein and Guenther, 1985; Klein et al., 1985; Gunther, 1986; Garrone et al., 1988). These tests were developed mainly for the quantification of hazelnut in hazelnut-containing products with respect to product adulteration and, therefore, special atten-

tion was paid to specificity and reproducibility. Also, the effect of roasting hazelnuts and the consequences thereof on the performance of these tests were extensively investigated. Because hazelnut-containing products such as chocolate and confectionery products contain substantial amounts of hazelnut (1–15%), a low detection limit was of less importance (Klein et al., 1985). In one report, the inadvertent presence of hazelnut in food was described. The test used was a rocket immunoelectrophoresis with a detection limit between 0.01 and 0.1% (100–1000 ppm) (Yman et al., 1994). No assays were described for the detection of minor amounts of hazelnut. This is, however, an important issue in the case of hazelnut allergy.

Little is known about the exact levels of hazelnuts leading to a reaction in allergic individuals and, most likely, these levels will differ from patient to patient. Taylor and Nordlee (1995) postulated that immunoassays for the detection of food allergens should have detection limits of at least 10 ppm since allergic individuals can react to traces of this order of magnitude. The aim of this study was to compare different immunological assays in their ability to detect and quantify hazelnut protein in foods with high sensitivity and specificity. We used both IgE antibodies from hazelnut allergic patients and polyclonal IgG antibodies from rabbits immunized with hazelnut protein and developed an assay with high sensitivity and specificity, which will be appropriate for use in the food industry.

2. Materials and methods

2.1. Hazelnut protein extracts and food extracts

The procedure for preparing hazelnut protein extracts was similar to that described for peanuts (Barnett et al., 1983; de Jong et al., 1996; Clarke et al., 1998). In short, 1 g of ground hazelnut was mixed with 20 ml 20 mM Tris buffer (pH 8.2) and stirred for 2 h room temperature. The aqueous layer

was collected by centrifugation ($3000 \times g$, at room temperature) and this aqueous phase was subsequently centrifuged ($10\,000 \times g$ at room temperature) to remove residual traces of fat and insoluble particles. The clear extracts were dialysed against 20 mM Tris buffer (pH 8.2) at 4°C . This dialysis step did not alter the protein composition of the hazelnut extract as judged by Coomassie stained sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, not shown). Protein concentrations were determined using Bradford analysis with bovine serum albumin (BSA) as a standard. SDS-PAGE with extracts from ground hazelnuts showed a similar pattern as described by Hirschwehr et al. (1992). Hazelnut extracts usually contained 5 mg/ml protein as a yield of 1 g solids in 20 ml. This yield was used for calculations and the amounts of hazelnut in food products were subsequently expressed as percentage or in parts per million if more appropriate (1% corresponds with 10 000 ppm). To prepare food extracts, 1 g of homogenized sample was mixed with 20 ml 20 mM Tris buffer (pH 8.2) as described for hazelnuts in the upper part of this section. Extracts of nuts, legumes and other food constituents as used for cross-reactivity testing contained between 500 μg and 5 mg protein per milliliter. All extracts were stored at -20°C .

2.2. Preparation and characterization of rabbit antibodies directed against hazelnut protein

Two rabbits were immunized with 100 μg crude hazelnut protein dissolved in phosphate-buffered saline (PBS) and mixed with an equal volume of complete Freund's adjuvant. Two and four weeks after the initial immunization, booster injections with 100 μg crude hazelnut protein in PBS mixed with an equal amount of incomplete Freund's adjuvant were given. Ten days after the last booster injection, blood samples were taken and tested for hazelnut protein binding in a direct enzyme-linked immunosorbent assay (ELISA). Plasma was collected via plasmapheresis (3×50 ml in a time span of 5 days) and stored at -20°C . Two weeks after plasmapheresis, boosting and plasmapheresis were repeated for three times. After that, rabbits were sacrificed and serum was pooled with plasma (in total 400 ml) and stored at -20°C in aliquots. Specific IgG was isolated

using immunoaffinity chromatography. Hazelnut protein as present in a hazelnut protein extract (see above) was covalently coupled to CNBr-activated Sepharose (Pharmacia, Uppsala, Sweden) according to the manufacturer's instructions (final concentration 1 mg protein per milliliter of swollen Sepharose). Serum was applied onto hazelnut protein-column (10 ml serum/10 ml column volume) in PBS. The column was washed with PBS until the $A_{280\text{ nm}}$ of the flow-through was less than 0.01. The bound fraction was eluted with 0.1 M glycine (pH = 2.7) and fractions were immediately neutralised with a 10% volume of 1 M Tris, pH 9.0. Protein-containing fractions were pooled, dialysed against PBS, and stored at -20°C until use. On SDS-PAGE, one main band ($> 95\%$) was present at approximately 150 kDa and under reducing conditions, two bands were present at approximately 50 and 25 kDa, indicating that the preparation consisted mainly of IgG. Part of these immunopurified antibodies were covalently conjugated to horseradish peroxidase according to the manufacturer's instructions (Sigma, St. Louis, MO, USA) and stored in 50% glycerol at -20°C until use. During the immunization procedures, animal care was in accordance with the institutional guidelines.

2.3. Patients

Twenty-four patients with sensitization for hazelnuts, in most cases combined with tree pollen allergy, were tested for hazelnut-specific IgE by RAST (Cap System, Pharmacia, Uppsala, Sweden; results of ≥ 0.35 kIU/l were regarded as positive). Patients with a coinciding allergy for cow's milk, hen's egg, or peanut were excluded, resulting in a sub-population of six patients with RAST levels between 0.6 and 6 kIU/l (mean 3.6 kIU/l). This exclusion criterion was applied in order to obtain a serum pool with a relative high concentration of hazelnut-specific IgE compared to IgE directed to other food allergens. All six patients had a clinical history of atopy with symptoms such as atopic dermatitis, rhinoconjunctivitis, and asthma. Four patients were female, two male and their average age was 39 years, range 27–50. All had pollinosis at least for 1 year. Oral allergy syndrome and other symptoms of food allergy developed in all cases one or more years after

pollinosis. The patients experienced oral and pharyngeal pruritus, either or not associated with swelling within minutes after the intake of hazelnuts, apple and other fruits from the Rosaceae family (OAS). The most recent reactions, as far as registered, were between 3 months and several years ago. None of the patients had symptoms of cow's milk, hen's egg or peanut allergy. In five out of six patients, RAST investigations were negative for cow's milk, hen's egg and peanut. For one patient, RAST for peanut was not performed (RAST for cow's milk and hen's egg negative). Skin prick tests (SPTs) were performed on four out of six patients with a panel of food allergens including hazelnut, peanut, cow's milk and hen's egg (Alk-Abello, Madrid, Spain) on the volar surface of the forearms, following general recommendations (Malling, 1993). Labile food allergens such as apple were applied using the prick-to-prick method (Malling, 1993) with fresh materials. SPTs were positive (class 2 + and 3 +) for hazelnut and negative for cow's milk, hen's egg and peanut. In summary, all patients were sensitized for hazelnut with clinical symptoms of hazelnut allergy, and none of them was sensitized to cow's milk, hen's egg or peanut as demonstrated by RAST and/or SPT. Venous blood was withdrawn from the individuals and allowed to clot. Serum was centrifuged and stored in aliquots at -20°C until use. All studies were approved by the Medical and Ethical Committee of the University Medical Center Utrecht (Utrecht, the Netherlands) and informed consent was obtained from all patients.

2.4. SDS-PAGE and immunoblotting

SDS-PAGE was performed essentially according to Laemmli (1970) using a BioRad Mini Protean II system (BioRad, Hercules, CA, USA) with 15% acrylamide gels (15×10 cm). Pre-stained molecular weight markers with molecular weights of 14.3, 21.5, 30, 46, 66, 97.4 and 220 kDa were used as reference. Samples were mixed in a 1:1 ratio with 63 mM Tris buffer (pH 6.8) containing 1% dithiotreitol (DTT), 2% SDS, 0.01% bromophenol blue and 20% (v/v) glycerol and were subsequently boiled for 5 min. The final protein concentration of the samples applied on the gel ($20 \mu\text{l}$) was 500 $\mu\text{g}/\text{ml}$, unless

otherwise denoted. Gels were stained with Coomassie Brilliant Blue R-250 dissolved in de-staining solution (10% HAc (v/v), 5% methanol (v/v) in water). After de-staining, gels were scanned with an ImageMaster DTS (Pharmacia, Uppsala, Sweden). To study the immunoreactivity of the proteins, SDS-PAGE gels were prepared as described above and the separated proteins were transferred to polyvinylidene difluoride sheets (Immobilon-P, Millipore, Bedford, MA, USA) generally as described Towbin et al. (1979). Membranes were blocked with 3% BSA in wash buffer (50 mM Tris, pH 7.5, containing 0.1% BSA and 0.1% Tween 20) for 1 h at room temperature. A pool of serum of the six patients with a hazelnut allergy was diluted 1/10 in wash buffer and applied on the membrane (overnight at room temperature). Bound IgE was detected using a commercial anti-human IgE conjugated to peroxidase (Nordic, Tilburg, the Netherlands) and a subsequent staining reaction for peroxidase activity. When the reactivity towards polyclonal rabbit antibodies was studied, membranes were blocked, then incubated overnight with immunopurified antibodies conjugated to peroxidase (see above). Between each step, blot membranes were washed thoroughly with wash buffer for five times. Blots were dried and scanned with an ImageMaster DTS (Pharmacia, Uppsala, Sweden). Immunoreactivity was quantified by calculating the mathematical product of the intensity and surface of specific hazelnut-protein bands using known amounts of hazelnut protein as a standard. Non-specific binding of the anti-IgE antibody conjugate to proteins on the membrane was measured in a similar blotting procedure omitting the incubation step with patient sera, and appeared to be negligible.

2.5. IgE inhibition ELISA

Dilutions of extracts of hazelnuts or food products were incubated in a 1:30 dilution of patient serum in PBS containing 1% BSA and 0.1% Tween 20. In this fluid phase, IgE was allowed to bind to hazelnut proteins for 1 h at room temperature. In order to determine the unbound IgE fraction, the incubation mixtures were transferred to 96-well plates which previously were coated with 10 $\mu\text{g}/\text{ml}$ hazelnut protein in PBS and subsequently blocked with BSA

(1%) in PBS containing 0.1% Tween 20. IgE bound to the wells coated with hazelnut proteins was detected using an anti-human IgE antibody conjugated to horseradish peroxidase. Peroxidase activity was detected with an *ortho*-phenyldiamine staining procedure. Between each step, plates were washed five times with PBS containing 0.1% Tween 20. Extracts of native (not heat-treated) hazelnuts were used as a calibrator. To test the cross-reactivity, extracts of protein-rich raw materials were used in a 1:10 dilution.

2.6. Sandwich ELISA

A sandwich ELISA was constructed as follows. ELISA plates were coated with 1 µg/ml immunopurified anti-hazelnut IgG (overnight at room temperature). Plates were blocked with BSA (1%) in PBS containing 0.1% Tween 20. Samples were diluted in PBS containing 1% BSA and 0.1% Tween 20, and subsequently incubated for 2 h at room temperature. After washing, plates were incubated with peroxidase-conjugated immunopurified anti-hazelnut IgG conjugated to peroxidase, and bound peroxidase activity was detected with an *ortho*-phenyldiamine staining procedure. Between each step, plates were washed five times with PBS containing 0.1% Tween 20. Extracts of native (not heat-treated) hazelnuts were used as the calibrator. To test the cross-reactivity, extracts of protein-rich raw materials were used in several dilutions and the cross-reactivity was expressed as a percentage of the reactivity of hazelnuts.

3. Results

3.1. Detection using immunoblotting with patients' serum as a source for IgE

Serum from patients allergic to hazelnut is a natural source of antibodies directed against hazelnut proteins. Therefore, sera from six patients with hazelnut allergy were pooled and used for detection of hazelnut proteins on an IgE-immunoblot. To determine the sensitivity of this experimental setup, several dilutions of a hazelnut extract with a known protein concentration were tested. Fig. 1a shows different dilutions of a hazelnut extract. The pattern

observed in lane 1 shows distinct bands, indicating that a number of hazelnut proteins bind IgE and are therefore considered to be allergens. Main bands are found at 25 (doublet), 37 (doublet) and 55 kDa, whereas bands at 14, 18, 22, and 69 kDa show IgE binding to a lesser extent. Compared to the Coomassie-stained SDS-PAGE, the 6.5-kDa protein did not bind IgE. On the other hand, the 14-, 18- and 55-kDa proteins bind IgE potently while they are hardly visible on the Coomassie-stained SDS-PAGE. These observations demonstrate that IgE binds specifically to certain hazelnut proteins (both in a qualitative and quantitative manner) rather than to all proteins present in hazelnuts. The sensitivity of this experimental setup is poor since 0.5 µg appeared to be the lowest detectable amount of hazelnut protein. This value corresponds with a hazelnut protein concentration of 25 µg/ml. Taking into account the protein concentration of the hazelnut extract, this threshold corresponds to a hazelnut concentration of 0.5% in food products.

Most patients with a hazelnut allergy suffer from multiple food allergies. In order to obtain a serum pool with a relative high concentration of hazelnut-specific IgE, we have excluded hazelnut-allergic patients with allergies for cow's milk, hen's egg and peanut. To investigate the specificity of the IgE-immunoblot method, we analysed the possible cross-reactivity of cow's milk, hen's egg and peanut, since these food ingredients are commonly used in products with an increased risk of contamination with hazelnut. Fig. 1b shows that peanut and hen's egg cross-react significantly with hazelnut protein, while milk and soy show only a minor cross-reaction. This suggests that part of the IgE present in the serum of patients was directed against proteins not present in the hazelnut, although the main part was directed against hazelnut.

3.2. Detection via IgE-binding inhibition techniques

Inhibition of IgE binding to immobilized allergens is a generally accepted tool for the detection of allergens in unknown samples. Fig. 2 shows the hazelnut-protein-dependent inhibition of IgE binding to plates coated with hazelnut protein. The sensitive range was from 30 ng/ml to 1 µg/ml, indicating that food products containing 6 ppm hazelnut protein

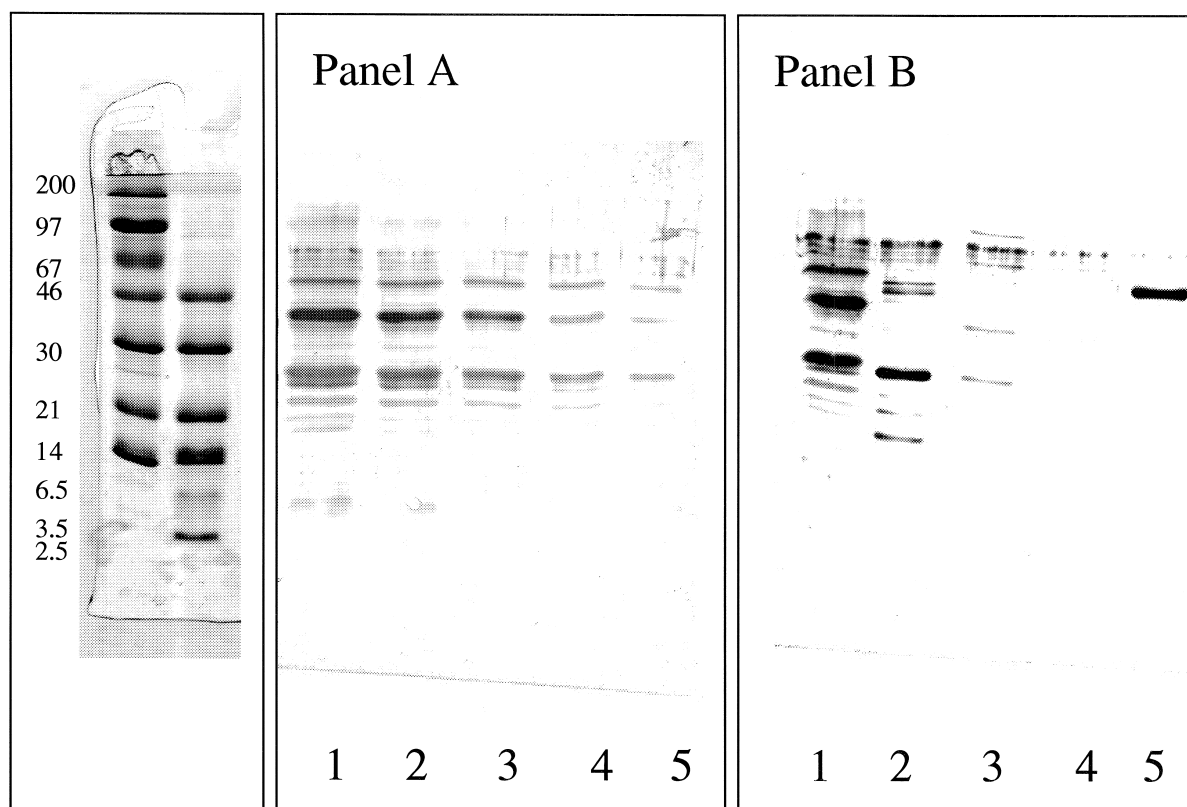


Fig. 1. Detection of hazelnut proteins by immunoblotting using sera from patients with a hazelnut allergy. Panel A: dilutions of a hazelnut extract; lane 1, 500 $\mu\text{g/ml}$; lane 2, 250 $\mu\text{g/ml}$; lane 3, 100 $\mu\text{g/ml}$; lane 4, 50 $\mu\text{g/ml}$; lane 5, 25 $\mu\text{g/ml}$. Panel B: cross-reactivity with other protein-rich food constituents (protein concentration: 500 $\mu\text{g/ml}$; 20 μl per lane); lane 1, hazelnuts; lane 2, peanuts; lane 3, soya; lane 4, milk; lane 5 hen's egg. Molecular weight distribution is shown at the left (kDa).

are recognized with this test. Roasted hazelnuts inhibit IgE binding similarly. An advantage of this assay, as compared to immunoblotting, is that the sensitivity is improved approximately 1000 times. Furthermore, only IgE directed to hazelnut proteins plays a role. Therefore, we expected the specificity to be increased compared to the immunoblotting method. Extracts of tree nuts and other common protein-rich food constituents were analysed in the inhibition ELISA. Fig. 3 (black bars at the left) shows that a hazelnut extract dose-dependently inhibits the IgE binding similar to the results of Fig. 2. Walnuts, and to a lesser extent cashew and pecan nuts, give rise to inhibition of IgE binding. This may be caused by cross-reaction at the IgE level. Alternatively, matrix effects from the tested sample may also influence the results. Taking into account the

dilution factors of the extracts, the cross-reactivity with walnuts was over 1000 times less than the reactivity of hazelnuts. Interestingly, peanuts do not cross-react in this assay, indicating that the cross-reactivity observed in the immunoblotting approach was due to multi-allergenicity rather than to cross-reactivity on epitope level.

3.3. Detection using polyclonal antibodies directed against hazelnuts

Immunopurified antibodies directed against hazelnut were used as detection antibodies in immunoblotting. An extract from hazelnuts was diluted to various concentrations and analysed on SDS-PAGE followed by immunoblotting as described in Section 2. Fig. 4a shows that bands at 25 (doublet), 37

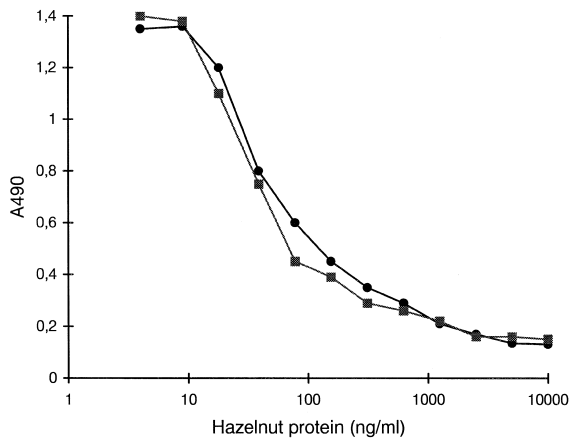


Fig. 2. Inhibition of IgE binding, sensitivity of the assay. Extracts of raw (circles) and roasted (squares) hazelnuts were incubated with diluted sera from patients and the non-bound IgE was quantified via binding to hazelnut-protein-coated microtiter plates.

(doublet) and approximately 55 kDa bind IgG. IgG binding to a number of other proteins (14, 18, 22,

and 69 kDa) is recognized to a lesser extent. This band pattern is very similar to that obtained with immunoblotting using patients' serum (IgE blot) except for the band between 46 and 69 kDa which is, in this assay, more pronounced. This similarity indicates that the proteins recognized by our rabbit serum are indeed allergens as visualized on immunoblot using IgE from patients. Concentrations between 15 and 500 $\mu\text{g}/\text{ml}$ can be detected in a dose-dependent way, corresponding with 0.3 and 10% hazelnuts in food products, respectively. The most intense band at approximately 55 kDa was quantified for all samples using a gel scanner, expressed as the mathematical product of intensity and surface of each band, and a calibration curve was fitted (not shown). Similar calibration curves were found when the other main bands of the blot were quantified, although the reproducibility was inferior compared to the calibration curve obtained with the 55-kDa band. Using this calibration curve, the amount of hazelnut protein in a complaint sample of chocolate spread could be deter-

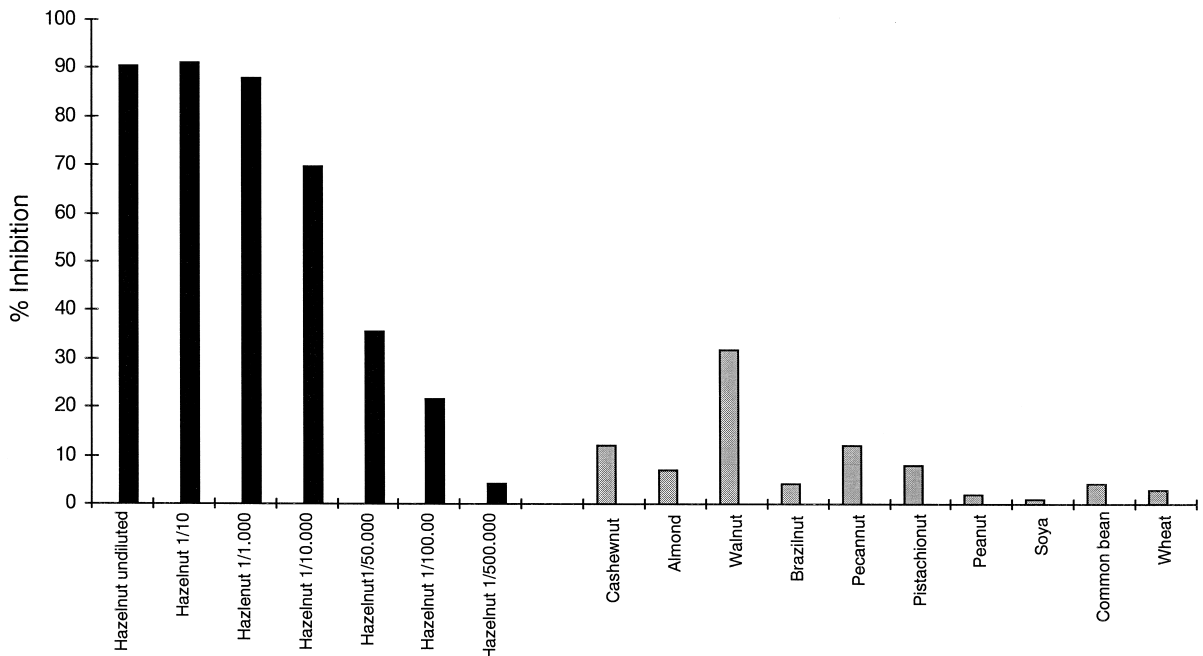


Fig. 3. Inhibition of IgE binding, specificity of the assay. Extracts were incubated with diluted sera from patients and the non-bound IgE was quantified following binding to hazelnut-protein-coated microtiter plates. Black bars: extracts of hazelnuts. Gray bars: extracts of food constituents (diluted 10 times).

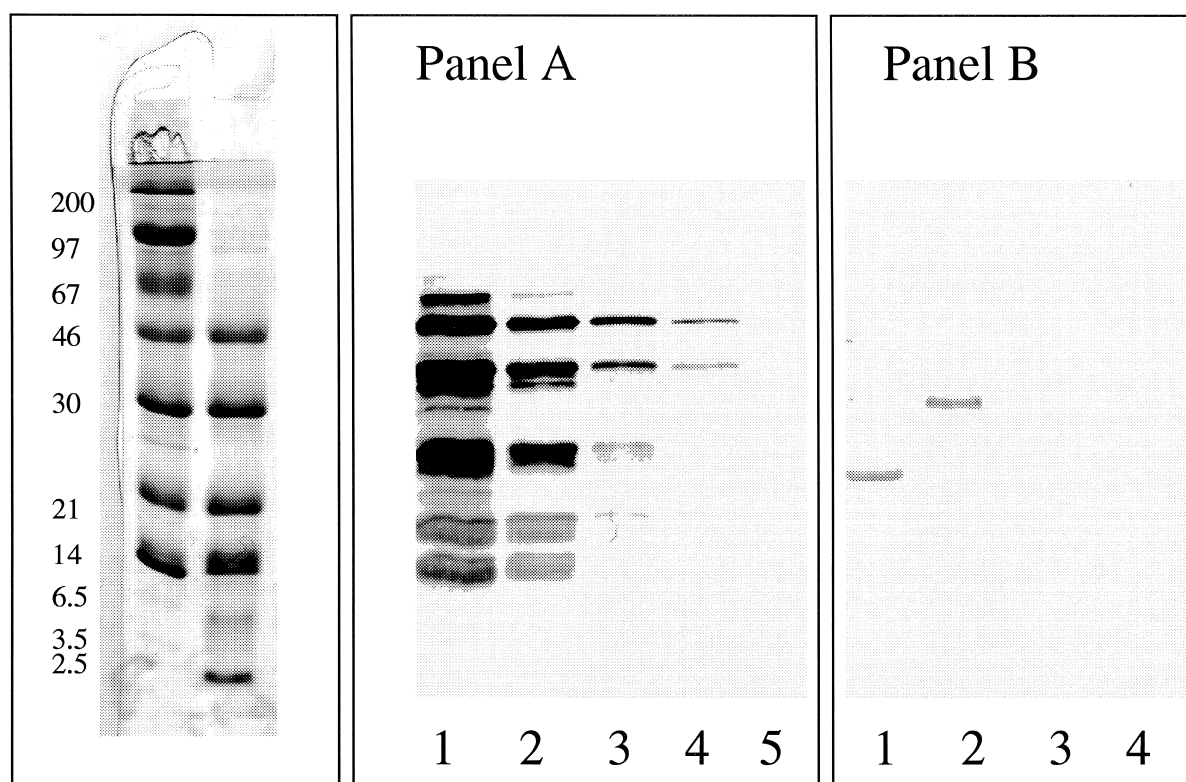


Fig. 4. Detection of hazelnut proteins by immunoblotting using immunopurified rabbit polyclonal antibodies. Panel A: dilutions of a hazelnut extract; lane 1, 500 $\mu\text{g/ml}$; lane 2, 150 $\mu\text{g/ml}$; lane 3, 50 $\mu\text{g/ml}$; lane 4, 15 $\mu\text{g/ml}$; lane 5, 5 $\mu\text{g/ml}$. Panel B: cross-reactivity with other protein-rich food constituents (protein concentration: 500 $\mu\text{g/ml}$; 20 μl per lane); lane 1, peanuts; lane 2, soya; lane 3, milk; lane 4, hen's egg. Molecular weight distribution is shown at the left (kDa).

mined as 0.4% (4000 ppm). The specificity of this method was investigated using extracts of common protein-rich raw materials and ingredients (Fig. 4b). Faint bands were visible for peanut and soy, whereas cow's milk and hen's egg did not exhibit any cross-reaction. For food products with a known composition, excluding the cross-reacting proteins, this assay was applied successfully. Since the band pattern for hazelnut is clearly different from that of soya and peanut (main bands at 22 and 30 kDa, respectively), products containing limited amounts of these legumes could be analysed as well, although the interpretation requires great care. Hazelnut was detected both in samples thought to be contaminated obtained via patients with a hazelnut allergy, and in products at an increased risk of contamination with traces of hazelnut (not shown).

A sandwich ELISA was constructed using immunopurified antibody for capturing and a peroxidase-conjugate of the same antibody for detection. An advantage of this setup, as compared to immunoblotting, is that larger number of samples can be tested in one cycle. Fig. 5 shows the calibration curve of this sandwich ELISA. The sensitive range was from 5 ng/ml to 1 $\mu\text{g/ml}$, which is in the same order of magnitude as found for the IgE inhibition test. Extracts of roasted hazelnuts show a similar calibration curve (Fig. 5), indicating that this assay is able to detect roasted hazelnuts as well. Since this test is the most promising for application in the food industry, an extensive study of possible cross-reactions was performed. Table 1 shows several nuts, legumes and other protein-rich raw materials and ingredients commonly used in the food industry.

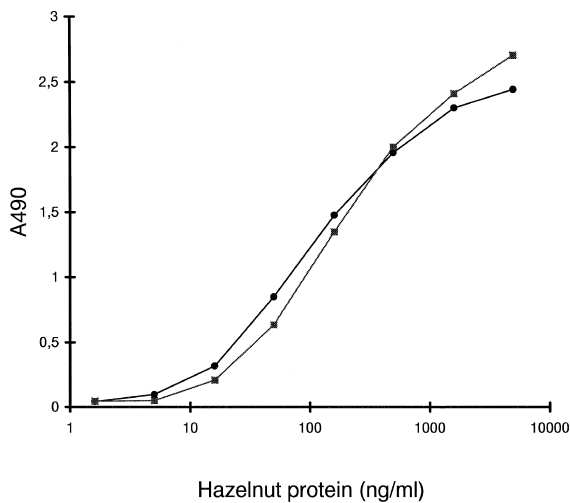


Fig. 5. Sandwich ELISA, sensitivity of the assay. Circles: dilutions of an extract of raw hazelnuts. Squares: dilutions of an extract of roasted hazelnuts.

Only walnut shows a significant cross-reactivity, although it reacts approximately 1300 times less than hazelnut. Cashew nut, brazil nut and almond show a detectable, but minor, cross-reaction.

3.4. Analysis of consumer products

The sandwich ELISA constructed with the immunopurified polyclonal antibodies, as described in Section 3.3, was used to investigate possible contamination of various consumer products with traces of hazelnut. Consumer products were purchased at a local retailer and extracts were made as described in Section 2. At least four serial dilutions of each sample were measured and the results were calculated using a calibration curve as shown in Fig. 5, taking into account the dilution factor used. The calculated results were independent of the dilution factor, demonstrating that in none of the samples matrix did effects influence the analysis. Based on the analytical results of this survey (Table 2), the tested consumer products can be divided in three groups. The first group consists of products with hazelnut listed on the label. These products indeed contained hazelnut and the concentration of hazelnut that we have found was in agreement with the percentage labelled in the ingredient declaration (Ta-

ble 2, columns 5 and 4, respectively). The second group consists of products assumed to be free of hazelnut but which did contain traces of hazelnut. These products are all produced by manufacturers that also process hazelnut (manufacturers A, B, D, E, G, H and J). The third group consists of products assumed to be free of hazelnut and were indeed free of hazelnuts (applying a detection limit of 1 ppm under the present experimental conditions). These products were from manufacturers that are known not to process hazelnuts in their facilities (C, F, K, L, N and O). However, one manufacturer (I) producing chocolate cookies with hazelnut produced similar chocolate cookies without hazelnut that were indeed negative in our analysis. Finally, a sample that was thought to be contaminated with hazelnut obtained

Table 1

Cross-reactivities of several nuts, legumes and other food constituents in the sandwich ELISA using immunopurified antibodies from rabbits sensitized with hazelnut protein

Sample	Cross-reactivity (%) ^a
Cashew nut	0.0034
Walnut	0.0787
Brazil nut	0.0028
Pecan nut	<
Pine nut	0.0010
Pistachio nut	<
Peanut	0.0011
Soya	<
Pea	<
<i>Phaseolus vulgaris</i>	<
<i>Vicia faba</i>	<
Lentil, black	<
Lentil, red	<
White bean	<
Black bean	<
Kidney bean	<
Aduki bean	<
Katjang idju	<
Almond	0.0028
Sesame seed	<
Sunflower seed	<
Pumpkin seed	<
Line seed	<
Wheat	<
Oats	<
Rice	<
Maize	<

^a(<) Means cross-reactivity below the detection limit (0.0010%).

Table 2
Analysis of consumer products

Product description		Manufacturer	Hazelnuts	
			Declared (%)	Measured ^a
Chocolate spread	Natural	A	—	752 ± 59 ppm
	Hazelnut	A	13	7.1 ± 1.9%
	Banana flavor	A	—	115 ± 15 ppm
	Milk flavor	A	—	11 ± 1.8 ppm
	Hazelnut	B	+	2.5 ± 0.5%
Chocolate bar	Caramel	C	—	<
	Peanut	C	—	<
	Biscuit	C	—	<
	Cocos	C	—	<
	Milk flavor	D	—	3.4 ± 0.4 ppm
	Hazelnut	D	+	13.5 ± 0.5%
	Caramel + biscuit	D	—	11 ± 10 ppm
	Natural	A	—	<
	Natural	E	—	14 ± 0.4 ppm
	Hazelnut	E	25	23 ± 1.0%
	Natural	F	—	<
Chocolate cookie	Hazelnut	A	5	5 ± 0.7%
	Whole wheat	A	—	6.2 ± 2 ppm
	Natural	A	—	557 ± 74 ppm
	Hazelnut	G	5	5 ± 0.4%
	Natural	G	—	18 ± 1.4 ppm
	Hazelnut	D	+	1.6 ± 0.2%
	Caramel	D	—	664 ± 74 ppm
	Cocoa	D	—	125 ± 2 ppm
	Hazelnut	H	3	1.9 ± 0.1%
	Natural	H	—	187 ± 13 ppm
	Hazelnut	I	3	2.9 ± 0.3%
	Natural	I	—	<
Muesli cookie	Nut	J	2.5	1.1 ± 0.1%
	Apple	J	—	5 ± 0.2 ppm
	Fruit	J	—	27 ± 1.2 ppm
Cake	Ginger	K	—	<
	Ginger	L	—	<
	Ginger + nuts	M	+	3.8 ± 0.6%
	Ginger + spices	N	—	<
	Caramel	O	—	<
Complaint sample	Milk flavor	B	—	4000 ± 100 ppm
Chocolate spread				

^a(<) Means below the detection limit (1 ppm under the used conditions).

from a chocolate spread manufacturer was shown to contain a considerable amount of hazelnut (0.4%)

4. Discussion

In this study, we have compared different immunochemical methods for the detection and quantification of hazelnut protein in food products. In

contrast with earlier described tests for the quantification of hazelnuts (Mohr et al., 1983; Klein and Guenther, 1985; Klein et al., 1985; Gunther, 1986; Garrone et al., 1988; Yman et al., 1994), our primary goal was to reach a high sensitivity in order to detect minor amounts of hazelnut protein as they play a role in the provocation of reactions in patients with hazelnut allergy. The amount of hazelnut protein required to trigger an allergic in vivo reaction is not

known. Such threshold levels were determined for peanuts recently by Hourihane et al. (1997) who demonstrated that 100 µg peanut in some peanut-allergic individuals caused reactions. This corresponds with 1 ppm in 100 g of food. Recently, Taylor and Nordlee (1995) postulated that immunoassays for the detection of food allergens should have detection limits of less than 10 ppm. Tests for peanut allergens that are presently commercially available have far lower detection limits (0.1 ppm, Koppelman et al., 1996; and 0.4 ppm, Yeung and Collins, 1996). The detection limit of an assay for the quantification of residual whey protein in hydrolysed milk formulas is as low as 0.01 ppm (R. Dreher, PhD, r-Biopharm, Darmstadt, Germany, personal communication). The detection limit of a previously described test for hazelnut protein was 0.01% or 100 ppm (Klein et al., 1985), demonstrating the necessity of a test method with a low detection limit (10 ppm or less). To make such a test applicable for food ingredients and consumer products, the cross-reactivity with other food components should be negligible.

4.1. Detection of hazelnut protein using patients' serum

First, we used serum from six patients with a hazelnut allergy as a source for antibodies against hazelnut proteins. These patients have been selected from a group of 26 hazelnut-sensitized individuals using allergy for cow's milk, hen's egg or peanut as an exclusion criterion. This selection was made because these proteins are often used in products with an increased risk of contamination with hazelnut such as cookies, confectionary products, and chocolates. The presence of IgE towards other ingredients of food products in the test methods would give rise to false-positive test results. In an immunoblotting experiment, we showed that a number of hazelnut proteins bind IgE similar to previously published observations (Vocks et al., 1993) based on a serum pool from hazelnut-allergic individuals to identify hazelnut allergens. In that study, bands similar in molecular weight to those described here were found. Furthermore, the relative intensities were in agreement with our observations. However, Hirschwehr et al. (1992) identified a protein of 18 kDa, similar to *Cor a 1* from hazel pollen, as a major IgE binding

protein, while we found limited IgE reactivity for this protein. Differences between our study and Vocks's study on one hand, and Hirschwehr's study on the other hand, may be explained by differences between patient populations, although all came from Western Europe. Alternatively, different methods to select patients may also explain the observed differences. The sensitivity of the IgE-immunoblotting method was poor. The detection limit (0.5% or 5000 ppm) was far above the 10 ppm set by Taylor and Nordlee (1995) (Fig. 1A). Peanut and hen's egg cross-reacted significantly in this test, while cow's milk and soy protein did not give rise to cross-reactions. The observed cross-reactivity can be explained in two ways. First, epitopes on the tested proteins may resemble allergic epitopes as found on hazelnut proteins. The denaturing SDS character of the immunoblotting procedure contributes to the exposure of epitopes that are buried in the interior of the protein under native conditions, thereby increasing the possibility of epitope cross-reactivity (Towbin et al., 1979). However, there are no reports describing the epitope or sequence similarity of hazelnut proteins and egg proteins. An alternative explanation for the observed cross-reactivity is that the serum of the patients may contain IgE directed against the tested allergens, since allergy to hazelnuts often coincides with other (food) allergies (Andersen and Lowenstein, 1978; Lowenstein and Eriksson, 1983). This hypothesis is more likely since the cross-reactivity of peanuts and hen's egg was considerable. Although RAST data from the six patients used in this study do not show IgE toward hen's egg or peanut, some IgE may have been present since RAST is considered to be less sensitive compared to immunoelectrophoretic techniques (Barnett et al., 1983; Hoffman, 1983).

To exclude cross-reactions based on the presence of IgE directed against other proteins, inhibition of IgE binding to immobilized hazelnut protein was studied. In this experimental design, only IgE directed against hazelnut protein plays a role. IgE that cross-reacts with other components due to epitope similarity can still occur. This approach results in a very high specificity; only walnuts and, to a lesser extent, cashew nuts and pecan nuts were recognized. This cross-reactivity was 1000 times less than the reactivity of hazelnuts. The cross-reacting samples

are phylogenetically related, indicative of epitope similarity. Peanut did not give rise to cross-reaction in this assay, indicating that, in contrast with immunoblotting, IgE directed against proteins other than hazelnut are excluded in this experimental procedure. The 17-kDa pollen allergen shares some biochemical and immunochemical characteristics with the 18-kDa allergen from hazelnuts (Hirschwehr et al., 1992) and may therefore cross-react at the epitope level in this test. However, considering the manufacturing procedures in the food industry, it is unlikely that food products are contaminated with pollen allergens. The high specificity of the IgE-inhibition ELISA permits the use of relative high concentrations of patient serum, resulting in an approximately thousand-times improved sensitivity compared to the immunoblot analysis. Concentrations as low as 30 ng/ml, corresponding to 6 ppm in food products, were detected and suggested that this assay was suitable for the detection of minute traces of hazelnut proteins.

4.2. Detection of hazelnut protein using rabbit antibodies

A major drawback for the food industry for the application of this test is the dependence on patient serum. Therefore, rabbits were immunized with hazelnut protein in order to obtain polyclonal rabbit antibodies directed against hazelnut. Immunopurification was applied to select specific antibodies. The immunopurified antibodies were used in immunoblotting and compared well with serum of patient, indicating that in the latter approach, the presence of IgE against other components was indeed the cause of the observed cross-reactivity. The rabbit antibodies on immunoblotting showed a band pattern similar to that obtained with IgE from hazelnut-allergic patients. The similarity in molecular weights of proteins recognized by the IgG from the rabbit serum compared to those recognized by patients' IgE suggests that physiologically relevant allergens are detected with this approach. The sensitivity of this test was 50 µg/ml, corresponding with 0.1% or 1000 ppm in food products. Attempts to improve the sensitivity by increasing the concentration of rabbit antibodies or the incubation times led to a higher cross-reactivity and higher backgrounds, respec-

tively, and were therefore not appropriate. A sandwich ELISA constructed with the rabbit antibodies showed a specificity comparable with the assay based on inhibition of IgE binding as illustrated by the fact that only walnuts cross-reacted. This cross-reactivity was more than 1300 times less than the reactivity of hazelnuts, indicating that this assay is suitable for the detection of hazelnuts in miscellaneous consumer products. The sensitivity of this assay was 1 ppm, which is considered to be safe to avoid provocation of an allergic reaction (Taylor and Nordlee, 1995), and is of the same order of magnitude as for a sandwich ELISA that we developed earlier for the detection of peanut proteins (Koppelman et al., 1996). Furthermore, roasted hazelnuts were recognised to the same extent as their raw counterparts, which is an important requirement since roasted hazelnuts are commonly used in many consumer products.

4.3. Analysis of consumer products

In a field test, we found that several consumer products that are assumed to be free of hazelnut do contain traces of hazelnut. The values that we have found range between 1 and 4000 ppm. In products from seven out of eight manufacturers that are known to process hazelnuts, contamination with traces of hazelnut was established. This indicates that contamination with hazelnuts of products that are generally regarded not to contain hazelnut may be a widespread problem among consumer product manufacturers. A survey including more product types from a wide range of different manufacturers is needed to evaluate the true extent of this problem. There are no clinical data on threshold levels of hazelnut for the provocation of an allergic reaction in patients sensitive to hazelnut. Therefore, it is not known what the risks are for hazelnut-allergic patients exposed to products containing these levels of hazelnut. Risk assessment studies including oral challenges to establish threshold values and epidemiological surveys may provide insight into this question.

5. Conclusion

In this study, we have compared different immunochemical approaches to detect hazelnut in foods.

In contrast to earlier described tests, we paid special attention to obtain a high sensitivity. We found that a sandwich ELISA constructed with immunopurified antibodies obtained from rabbits sensitized with hazelnut protein was able to detect traces of physiologically relevant hazelnut allergens as low as 1 ppm. The high specificity suggests that this assay is applicable to miscellaneous consumer products, and we demonstrated in several cases traces of hazelnut in products that are generally regarded as free of hazelnut. This assay does not use the serum of patients and would therefore be attractive to the food industry. The availability of tests for the detection of traces of food allergens (Koppelman et al., 1996; Yeung and Collins, 1996) and the growing interest in food allergy, from both clinicians and the food industry, may lead to safer consumer products in the future.

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