Quantitative Sandwich ELISA for Determination of Traces of Hazelnut (*Corylus avellana*) Protein in Complex Food Matrixes

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A hazelnut-specific sandwich-type ELISA based on polyclonal antisera was developed for detection of hidden hazelnut protein residues in complex food matrixes. In the absence of a food matrix, extractable protein from different native and toasted hazelnuts was detected at rates of 94 ± 13 and 96 ± 7% applying standards prepared from native and toasted hazelnuts, respectively. From complex food matrixes, 0.001−10% of hazelnut was recovered between 67 and 132%, in average by 106 ± 17%. Depending on the food matrix, hazelnut protein could be detected down to the ppb (ng/g) level. Intraassay precision was <6% for hazelnut ≥ 0.001% and interassay precision was <15% for hazelnut ≥ 0.01%. In 12 of 28 commercial food products without labeling or declaration of hazelnut components, between 2 and 421 ppm of hazelnut protein was detected, demonstrating a remarkable presence of potentially allergenic hazelnut protein "hidden" in commercial food products.

Keywords: *Hazelnut (Corylus avellana); hidden allergens; ELISA; immunoblotting*

INTRODUCTION

Pollen-related allergy has a remarkable clinical impact, and the prevalence of allergic reactions induced by pollen is increasing. In central and northern areas of Europe, birch pollinosis is a very common allergy caused by sensitization due to inhalation of birch pollens (D'Amato et al., 1998). Patients with allergy to tree pollen, especially birch pollen, frequently show adverse reactions to fruits and vegetables, in particular to apples and hazelnuts (Hirschwehr et al., 1992). Only a very small proportion of allergy to nuts are independent of pollen allergy (Vieths, 1997). Among 167 patients that where preselected in a survey for pollen and food sensitization with at least one sensitization to one pollen species and to at least one kind of plant food, 151 (90%) were sensitized as demonstrated by the presence of specific IgE antibodies and 131 (78%) were allergic to hazelnuts (Janciewicz et al., 1996). Another clinical study showed that hazelnut was the most prevalent food allergen with 36.8% of 383 patients with a proved IgE-mediated food allergy and under special consideration of an oral allergy syndrome (OAS) (Etesamifar and Wüthrich, 1998). Oral allergies to plant foods in pollen allergic patients are based on cross-reactive IgE, and the immunogenic stimulus is most probably due to the inhalation of the pollen allergens (Vieths, 1997).

In hazelnut, an 18 kDa major allergen was found to be related to Cor a 1, the major hazel pollen allergen. Cor a 1, with a molecular mass of 17 kDa, is highly homologous with Bet v 1, the 17 kDa major birch pollen allergen. Also, a 14 kDa profilin known as cross-reacting plant pan-allergen was found in both hazel pollen and hazelnuts (Hirschwehr et al., 1992). Food profilins and food proteins related to Bet v 1 are relatively sensitive to heat and can easily be cleaved by proteases (Vieths, 1997). In addition, a heat-stable IgE binding capacity not related to birch pollen-specific IgE has been demonstrated (Vieths et al., 1998). However, little is known about thermostable and thermolabile allergens in hazelnut, even though hazelnuts are one of the most prevalent food allergens among birch pollen allergic individuals and may still elicit allergic reactions after heat treatment.

Hence, ingestion of the offending food has to be avoided by the sensitive individual. Problems may thus arise if the presence of the allergen may not be discernible due to mislabeling of the products or because of unknown contaminations resulting from the process of production.

Severe allergic reactions caused by hazelnut have already been reported (Brostoff, 1989; Malmheden Yman et al., 1994; Martín Muñoz et al., 1994; Ewan, 1996). In one case, a fragment of hazelnut in a teaspoon of muesli caused severe laryngeal oedema (Ewan, 1996). In another, ingestion of one piece (3−6 g) of chocolate containing 0.2% of undeclared hazelnut caused asthma in an allergic individual (Malmheden Yman et al., 1994). Therefore, 6−12 mg of hazelnut were sufficient to elicit a severe allergic reaction. Nondeclared traces of hazelnut protein in chocolate calendars between 0.002 and 0.3% have been determined by rocket immunoelectrophoresis (RIE) (Eriksson and Malmheden Yman, 1992) and allergenic hazelnut protein could also be detected in a blend of refined and unrefined commercial hazelnut oil by radioallergosorbent test (RAST) (Teuber et al., 1997).

With special regard to protection of highly sensitized consumers, sensitive detection methods are needed to specifically discover the presence of such hidden allergens at a level of ≤0.01% of the offending food in complex processed food matrixes and allowing more precise labeling of commercial food products.

Various electrophoretic and immunochemical techniques for detection of hazelnut protein in chocolate and nougat spreads as a quality characteristic have been reported (Mohr et al., 1983; Eichler and Rubach, 1985; Klein et al., 1985; Garrone et al., 1988) but unfortunately lack sufficient sensitivity concerning the detec-

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tion of traces of hidden allergens. One RIE application described (Eriksson and Malmheden Yman, 1992; Malmheden Yman et al., 1994) seemed to be sensitive enough but unfortunately lacks precise data on the preparation of sample extracts and standards as well as on validation of the assay. The mentioned RAST (Teuber et al., 1997) is unsuitable for routine analysis as it is based on potentially infectious human sera and radioisotopes.

The present study was undertaken to develop and validate a sensitive, specific, and reliable method for detection of traces of hazelnut protein in processed foods. The characteristics of the resulting ELISA procedure are described in this paper.

MATERIALS AND METHODS

Hazelnut Samples, Reference Samples, and Commercial Food Products. Hazelnuts of the variety Piemonte (Italy) and Nocciole Ordu (Turkey) toasted under industrial-like conditions at 140 °C for 20, 30, and 40 min as well as native reference material and different varieties of native hazelnuts (Italy) were provided by Dr. G. Malgarini, Sorematec, Arlon-Schoppach, Belgium. Samples of industrially manufactured whole-milk model chocolates containing either 0.1 or 0.5% of hazelnut, 0.1 or 0.5% of almond, 0.1 or 0.5% of peanut, and a reference model chocolate without addition of hazelnut, almond, or peanut as well as confectionary products containing hazelnut paste were provided by Professor Dr. R. Matissek, Institute of Food Chemistry of the German Confectionary Industry, Cologne, Germany. Chocolate samples taken at various intervals of a peanut-cleanup procedure due to switching between products were provided by Dr. W. Weber, Institut Kirchhoff, Berlin. Negative-control chocolates were self-made and consisted of hazelnut-free ingredients. Commercial food products were obtained at a local food store, including samples (3) with a warning "may contain hazelnut traces" or "not suitable for nut allergy sufferers", samples (17) with hazelnut or hazelnut components listed as an ingredient, and samples (28) without any declaration about the presence of hazelnut or hazelnut components listed in the list of ingredients or displayed within the description of the product. Food samples that did not have hazelnut listed as an ingredient were mainly selected from such companies that also produce foods containing hazelnuts.

Manufacture of Negative-Control Chocolates. Food ingredients of the self-prepared model chocolates were screened for traces of hazelnut prior to processing. Only hazelnut-free constituents were processed. The whole-milk chocolate consisted of 15% of cocoa mass simulated by a mixture of 54% of deoiled cocoa powder and 46% of cocoa butter, an additional 18% of cocoa butter, 47% of sucrose, 20% of full-fat milk powder, and 0.4% of protein-free lecithin Epikuron 145 F (Lucas Meyer, Hamburg, Germany). The half-bitter chocolate contained 45% of cocoa mass simulated by a mixture of deoiled cocoa powder and cocoa butter as described above, an additional 5% of cocoa butter, 50% of sucrose, and 0.4% of lecithin Epikuron 145 F. The processing of chocolates was done as described elsewhere (Matissek and Burghardt, 1991; Belitz and Grosch, 1992, pp 876−877).

Reagents, Buffers, and Instrumentation. For the sandwich-type ELISA, we applied a polyclonal antiserum from rabbit raised against the corylin fraction from native hazelnut protein (Riedel de-Haën, no. 45261, Seelze, Germany), a polyclonal antiserum from sheep raised against native and heated hazelnut corylin in a ratio of 1:1 (Dr. W. Weber, Institut Kirchhoff, Berlin, Germany) and horseraddish peroxidase (HRP)-labeled polyclonal anti-sheep IgG antibodies developed in rabbit (Dianova, no. 313-035-003, Hamburg, Germany). The rabbit anticorylin antiserum was immunoabsorbed against a variety of different nut proteins by the manufacturer. The rabbit antiserum was additionally immunoabsorbed against extracts from walnut and pumpkin seed as described below. The immunoreagents for ELISA were stored aliquoted in 50% glycerol at −20 °C until used. Immunoreagents at use could be stored at 4 °C for 2 weeks without reduction of immunoreactivity.

For immunoblotting experiments we used sera of hazelnut-allergic patients from our in-house serum collection (PEI 12, 17, 41, 65, 29, Bo 101) and one nonallergic control serum (PEI 22), alkaline phosphatase (AP)-labeled mouse monoclonal anti-human IgE (Pharmingen, no. 34613E, Hamburg, Germany), rabbit normal serum (Riedel de-Haën, no. 45263), sheep normal serum (Dianova, no. 013-000-001), immunoabsorbed rabbit anti-hazelnut antiserum (Riedel de-Haën), sheep anti-hazelnut antiserum (Dr. W. Weber), biotin-labeled goat anti-rabbit IgG (Dako, no. E0432, Hamburg, Germany), biotin-labeled rabbit anti-sheep IgG (Dianova, no. 313-065-003), and AP-labeled streptavidin (Caltag, obtained from Medac, no. SA1008, Hamburg, Germany).

All chemicals used were of analytical grade or as specified. The preparation of sample extraction buffer, coating buffer, blocking solution, incubation buffer, washing buffer, peroxidase substrate solution, and stopping solution for performing the ELISA are described elsewhere (Holzhauser and Vieths, 1999).

Buffers and reagents for immunoblotting were prepared as described elsewhere (Vieths et al., 1992; Holzhauser and Vieths, 1999) except for the use of tris-buffered saline (TBS), instead of phosphate-buffered saline (PBS). TBS, pH 7.4, consisted of 100 mM tris(hydroxymethyl)aminomethane (TRIS), 100 mM NaCl, 2.5 mM $MgCl_2 \cdot 6H_2O$, pH 7.4 adjusted with 10% HCl.

Dispensing of diluted sample extracts, standards, and immunoreagents in ELISA were performed at dispense mode with an eight-channel electronic pipet (Biohit Proline 50−1200 μL, Biohit, Helsinki, Finland). Washing of microwell plates was done using an eight-channel automatic microplate washer (MWG-Biotech, Ebersberg, Germany). ELISA incubations at 37 °C were carried out in a temperature-controlled horizontal shaker with heated bottom and lid (Thermostar, BMG Labtechnologies, Offenburg, Germany). ELISA readings of optical density (OD) were performed by a Spectramax 340 (Molecular Devices, Munich, Germany) controlled by data processing software (Softmax Pro, Molecular Devices).

Immunoabsorption of Hazelnut-Specific Antiserum from Rabbit. The rabbit anti-hazelnut antiserum was additionally in-batch immunoabsorbed against protein extracts from walnut and pumpkin seed. Extraction was carried out as described below. Extracts were additionally filtered through 0.45 μm cellulose nitrate filters (Sartorius, no. 11306, Göttingen, Germany), dialyzed overnight against distilled water in dialysis membranes with a molecular weight cutoff of 3500 Dalton (SpectraPor, no. 132590, Roth, Karlsruhe, Germany) and lyophilized. Lyophilized extracts were resolved in coupling buffer and coupled to CNBr-activated Sepharose 4 Fast Flow (Pharmacia, no. 17-0981-01, Freiburg, Germany) according to the manufacturer's instructions. Twenty-five milligram of pumpin seed protein or 10 mg of walnut protein were coupled to 5.25 mL of CNBr−Sepharose gel and 1 mL of rabbit anti-hazelnut antiserum was immunoabsorbed with 5.25 mL of ligand-coupled gel. Immunoabsorption was carried out for 2 h at room temperature. The gel suspension was centrifuged at 2000*g* and 4 °C for 5 min, and the supernatant was pooled with another two supernatants derived from subsequent washing of the gel with incubation buffer. The pooled supernatant was concentrated to a total volume of 1 mL in a microconcentrator (Centricon 10, Amicon, Beverly, MA).

Sample Homogenization, Microextraction, and Quantitative Extraction. Homogenization, microextraction, and quantitative extraction were performed as described elsewhere (Holzhauser and Vieths, 1999), except for time and temperature conditions. The microextraction was carried out at 37 °C for 45 min; the quantitative extraction was at 37 °C for 60 min. Sample extracts were not stable at −20 °C and had to be freshly prepared prior to use. For analysis by ELISA, extracts were diluted at least 1:5 in ELISA incubation buffer.

Preparation of Hazelnut Protein Standards. Hazelnuts of the variety Nocciole Ordu, toasted at 140 °C for 30 min,

were ground with an analytical mill (IKA M20, IKA Labortechnik, Staufen, Germany) and extracted with ELISA extraction buffer at 37 °C for 1 h according to the quantitative extraction procedure. The amount of protein in the hazelnut protein reference extract was determined according to a modified Bradford method as described elsewhere (Holzhauser and Vieths, 1999). Hazelnut protein reference extract was stable at −20 °C for several months without loss of activity. Hazelnut protein standards were derived from the hazelnut protein reference extract by a factor two serial dilution in incubation buffer, covering a concentration range between 5 and 1280 ng/mL. Diluted hazelnut protein standards were not stable at −20 °C and had to be freshly prepared prior to use.

Extracts for Cross-Reactivity Studies. Extracts of various foods and food constituents were prepared according to the quantitative extraction procedure and were derived from legumes (soybean; chick pea; green pea; lentil; kidney bean; peanut; white bean; red bean; pinto bean), nuts and stonefruits (almond; Brazil nut; cashew; coconut; macademia; pecan; pistachio; walnut), and various ingredients (cereal mix consisting of equal amounts of barley, corn, oats, rice, rye, and wheat; cocoa; coffee; hen's egg; pine seed; pumpkin seed; sesame seed; sugar; sunflower seed; skim-milk powder; whole-milk powder) as well as thickening and gelling agents (carob; guar flour; gum arabic; traganth; agar agar; carrageenan). Except for thickening and gelling agents, the extracts were tested at a 1:5 dilution in incubation buffer simulating a proportion of 100% of the food. Extracts leading to signals of ≥1 ppm of hazelnut protein were further investigated for determination of the detection limit in the corresponding food or food constituent (see results). Extracts of thickening and gelling agents were tested at a proportion of 20% simulated by dissolving 40 mg of sample in 20 mL of extraction buffer. These extracts were tested undiluted in ELISA.

Spiking of Samples and Recovery Studies. Hazelnut-free food matrixes were spiked with hazelnut of the variety Nocciole Ordu toasted at 140 °C for 30 min at levels of 10, 1, 0.1, 0.01, and 0.001%. Prior to spiking of samples, soluble protein from Nocciole Ordu (140 °C, 30 min) was quantified by the Bradford method for determination of a correlation factor (F_C) for calculation of the hazelnut content of a spiked sample from soluble hazelnut protein quantified by ELISA. One gram of hazelnut was added to 9 g of a food matrix, and the spiked sample was frozen with liquid nitrogen and ground with an analytical mill (IKA M20) for better sample homogeneity. Thereafter, 1 g of the spiked sample containing 10% of hazelnut was added to another 9 g of food matrix. The procedure was continued until a sample containing 0.001% of hazelnut was obtained. Each of the samples containing hazelnut at the concentration levels described was extracted according to the quantitative extraction protocol, and the amount of soluble hazelnut protein was determined by ELISA. The recovery was calculated as indicated:

$$\text{recovery} = \frac{\text{amount of hazelnut determined [ppm]}}{\text{amount of hazelnut added [ppm]}} \times 100\% =$$

$$= \frac{\text{amount of hazelnut protein determined [ppm]} \times F_C}{\text{amount of hazelnut added [ppm]}} \times 100\% =$$

$$= \frac{\text{amount of hazelnut protein determined [ppm]}}{\text{amount of hazelnut added [ppm]}} \times 12.75 \times 100\%$$

ELISA Procedure. Flat-bottom polystyrene microwell plates (Maxisorp F96, certified, Nunc, Wiesbaden, Germany) were coated with 150 μL/well of immunoabsorbed hazelnut-specific polyclonal rabbit antiserum diluted 1:10000 in coating buffer. After 16 h at room temperature, plates were washed twice of 4 min each with washing buffer. Unsaturated binding sites of the polystyrene surface were blocked by incubation with 200 μL/well of blocking solution at 37 °C for 1 h. Plates were washed twice of 4 min each with washing buffer, and emptied plates were subsequently stored in a vacuum-sealed plastic bags at −20 °C until use. Coated plates could be stored for several months without loss of catcher antibody activity.

Freeze-stored plates were washed once for 4 min with washing buffer prior to use. Thereafter, diluted food sample extracts and hazelnut protein standards were dispensed at 150 μL/well. Samples and standards were run in triplicates. After 1 h incubation at 37 °C, plates were washed twice of 2 min each with washing buffer, and the wells were subsequently filled with 150 μL each of hazelnut-specific polyclonal sheep antiserum diluted 1:1000 in incubation buffer. The sandwich was completed after 1 h incubation of the secondary antibody at 37 °C. Again, plates were washed twice of 2 min each with washing buffer. After 1 h incubation at 37 °C with 150 μL/well of 2.5 ng/mL of HRP-labeled rabbit anti-sheep IgG in incubation buffer, plates were washed 3 times of 2 min each. The enzymatic staining was performed in the dark after addition of 150 μL/well of HRP substrate solution. After 15−30 min, the reaction was stopped with 100 μL/well of stopping solution as to obtain maximal OD values of 1.2−1.8 for the most concentrated standard. The OD values were read bichromatically at 450 nm main wavelength and 630 nm reference wavelength. Plates were sealed with a plate sealing film (Rotilabo, Roth, Karlsruhe, Germany) during each incubation. For screening purposes, each sample was analyzed by one single determination (one triplet) of an extract prepared according to the microextraction protocol, and extracts were measured at a dilution of 1:5. The quantitative determination was based on the analysis of two independently prepared extracts according to the quantitative extraction protocol and with both determinations measured on separate microwell plates. Data processing included the reduction of mean OD values of samples and standards by the mean OD of the blank standard (incubation buffer only). Reduced ODs were plotted against the logarithm of the hazelnut protein standards. The resulting sigmoidal curve was fitted using a four-parameter logistic function.

Immunoblotting. The immunoblotting procedure was performed as described elsewhere (Vieths et al., 1992; Holzhauser and Vieths, 1999), except for the use of TBS instead of PBS during all blocking, washing and incubation steps. Briefly, 30 μg of protein/cm of polyacrylamide gel from native and toasted (140 °C, 30 min) hazelnuts of the variety Nocciole Ordu was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (T = 12.8%, C = 2.7%) and subsequently semi-dry-blotted onto a nitrocellulose membrane. The membrane was blocked with 0.3% Tween 20 in TBS. For total protein pattern, one strip of the membrane was stained with India ink. For detection of IgE-reactive hazelnut proteins, strips were incubated overnight with the sera of hazelnut-allergic patients diluted 1:6.7 and subsequently with AP-labeled mouse anti-human IgE diluted 1:750 for 4 h. Detection of IgG-reactive hazelnut proteins with either the immunoabsorbed hazelnut-specific antiserum from rabbit or the unabsorbed antiserum from sheep was done by overnight incubation of the hazelnut-specific antisera diluted 1:2500 and 1:4000, respectively. Bound hazelnut-specific IgG was detected by 1 h incubation of biotin-labeled goat anti-rabbit IgG (diluted 1:2500) and biotin-labeled rabbit anti-sheep IgG (diluted 1:12500), respectively. Finally, AP-labeled streptavidin diluted 1:3000 was incubated for 30 min. Enzymatic staining was performed for 10 min or as indicated with a commercial AP substrate kit (Bio-Rad, no. 170-6432, Munich, Germany) according to the manufacturer's instructions. Controls were incubated overnight and included a nonallergic human serum (diluted 1:6.7) for unspecific IgE detection, a rabbit normal serum (diluted 1:12000) and a sheep normal serum (diluted 1:4000) for detection of unspecific IgG reactions. Further incubations were identical with those described above. Between all incubation steps, strips were washed three times of 5 min each with 0.05% Tween 20 in TBS. All reactants described were diluted in incubation buffer (TBS, pH 7.4, 0.05% Tween 20, 0.1% BSA), and incubation reactions and enzymatic staining were carried out at room temperature.

RESULTS

Characterization of the Rabbit and Sheep Hazelnut-Specific Antisera. Both the rabbit and the sheep hazelnut-specific antiserum were tested for antiserum specificity in primary studies. The systems applied were indirect competitive ELISA techniques with hazelnut protein precoated to microtiter plates. Various legumes, nuts, and stonefruits as well as characteristic food ingredients were investigated for cross-reactivity (detailed results not shown). Thereafter, the rabbit antiserum which showed the higher specificity was chosen as the source of catcher antibody for an sandwich-type ELISA. The specificity of the ELISA increased significantly in comparison to the indirect competitive techniques but extracts of walnut and pumpkin seed still cross-reacted corresponding to some 10 ppm of hazelnut protein in a food matrix, when investigated at proportions of 100% in a food. After immunoabsorption of the rabbit antiserum against extracts from walnut and pumpkin seed, specificity could be improved 2-fold while the titer of the antiserum decreased 6-fold.

The immunoabsorbed rabbit anti-hazelnut and the sheep anti-hazelnut antiserum were further investigated by SDS-PAGE/immunoblotting for comparison of proteins detected by the antisera and by hazelnut-allergic patients. First, proteins from native and toasted hazelnuts of the varieties Nocciole Ordu and Piemonte, as well as proteins from various native hazelnuts were separated by SDS-PAGE and silver stained (results not shown). The protein pattern of the native varieties did not show any significant differences and the protein pattern from the varieties Nocciole Ordu and Piemonte toasted at 140 °C for 20, 30, and 40 min did not show any significant differences toward each other, either.

As an example for realistic conditions of toasting, protein from Nocciole Ordu toasted at 140 °C for 30 min was selected for further comparison with protein from native material by SDS-PAGE/immunoblotting (Figure 1).

Comparison of the total protein pattern (stained with India ink) revealed that toasting under industrial-like conditions reduces the minor protein components while the pattern of the major proteins remains almost unchanged. Both the immunoabsorbed rabbit antiserum (A) and the sheep antiserum (B) recognized about the same proteins of the corylin fraction from native (Figure 1a) and from toasted (Figure 1b) hazelnuts. Various IgE-reactive proteins from toasted and native hazelnut detected by the sera of 6 hazelnut-allergic patients (C) were also detected by the rabbit (A) and the sheep (B) antiserum, although the corylin fraction does not seem to represent the majority of IgE-reactive proteins and the detection with patients' IgE appears to be weaker. A protein of 18 kDa molecular mass, as strongly detected in native protein by patient serum no. 2, was not recognized by hazelnut-specific IgG of both the rabbit and the sheep antiserum. The IgE-reactivity of this 18 kDa protein appears to be not heat stable because no IgE-detection can be observed in toasted hazelnut with the serum of patient no. 2 who exclusively presented IgE specific for proteins belonging to the "Bet v 1- family". Competitive IgE-inhibition experiments revealed that this hazelnut protein cross-reacts with IgE specific for the major birch pollen allergen, Bet v 1 (not shown). All controls (*N*) were negative, demonstrating the specificity of the detection.

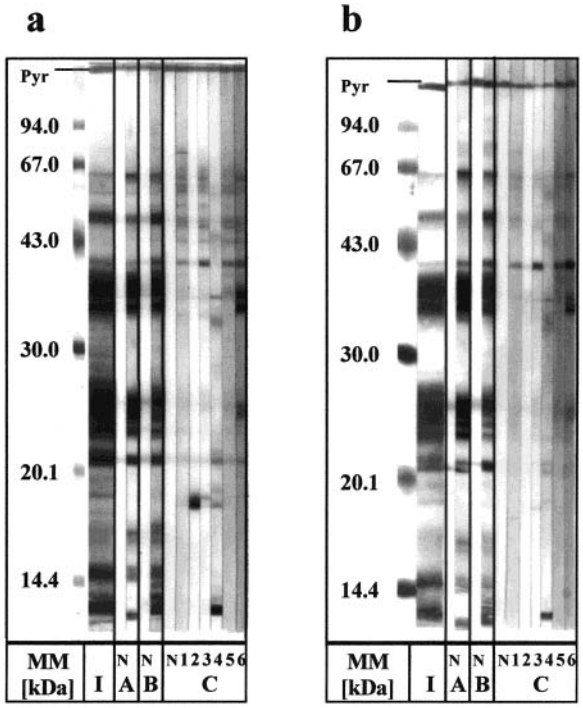

Figure 1. Immunoblotting of proteins from native hazelnut (a) and from hazelnut toasted at 140 °C for 30 min (b) of the variety Nocciole Ordu as detected by (A) rabbit hazelnut-specific antiserum, (B) sheep hazelnut-specific antiserum, and (C) patients' IgE from 6 hazelnut-allergic individuals (MM, molecular mass; Pyr, pyronine, unspecific blotting dye; I, India ink; patients' sera no. 1−6, PEI 12, PEI 17, PEI 41, PEI 65, PEI 29, Bo 101; *N*, left lanes of A−C indicate the control of nonspecific binding by normal rabbit serum (A), normal sheep serum (B), and nonallergic human serum (C); right lanes of A−C represent the specific detection).

ELISA Standard Curve and Precision Profile. A representative standard curve with the average characteristics of the standard curves from 44 different experiments performed on different days is shown in Figure 2a, displaying the point of inversion at 109 ± 25 ng/mL of hazelnut protein from Nocciole Ordu toasted at 140 °C for 30 min. The average slope at the point of inversion that characterizes the maximum assay precision was determined with 0.93 ± 0.05. The systems limit of detection and the limit of quantitative determination were evaluated with 0.6 ± 0.5 and 1.1 ± 0.9 ng/mL of hazelnut protein in incubation buffer, which corresponded to 60 ± 50 and 110 ± 90 ppb [ng/g] of hazelnut protein in a sample, respectively. These limits were defined as the protein concentration at a signal-to-noise ratio of 3:1 and 6:1, respectively, and were derived from the OD at zero dose of analyte (blank standard) increased by 3- or 6-fold the standard deviation of the mean OD of the blank standard.

A precision profile of the standard triplets based on the same data is given in Figure 2b. Standard precision, as expressed by the coefficient of variation (CV), is even ≤10% for quantification in the range of ∼3−360 ng/mL, and still ≤15% for quantification in the range of ∼1−600 ng/mL.

Antiserum Specificity in ELISA. Various legumes, nuts, and stonefruits as well as characteristic food ingredients were included in cross-reactivity studies. Except for thickening and gelling agents, extracts were tested at a dilution of 1:5 corresponding to a proportion of 100% in a food matrix. Thickening and gelling agents were tested at a simulated proportion of 20%. Food

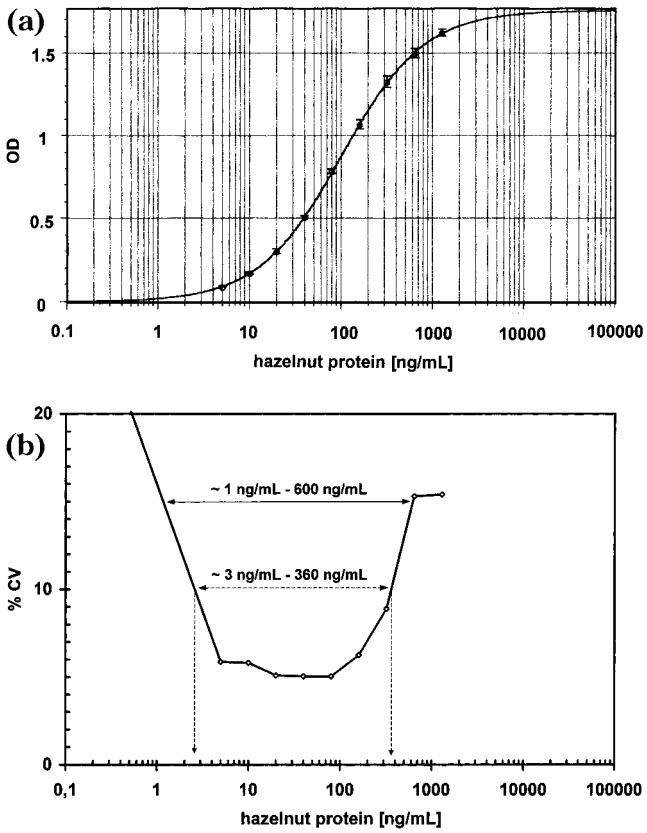

Figure 2. (a) Representative ELISA standard curve with the average characteristics derived from 44 different curves. Error bars indivate the standard deviation of the hazelnut protein standards. (b) Mean precision profile of the standard curve derived from 44 different curves.

Table 1. Potential Cross-Reactivity of the Most Cross-Reactive Foods Evaluated Depending on the Proportion in a Food Matrix[a]

	cross-reactivity (ppm hazelnut protein) at simulated proportions of			
food	100%	50%	20%	10%
walnut	4.2	nd	<1.0	<0.6
pumpkin seed	6.4	nd	1.0	0.6
cashew	2.1	<1.0	0.4	nd

[a] nd, not detected.

extracts that led to signals of ≥1 ppm of hazelnut protein at a simulated proportion of 100% in a food matrix were further diluted as to determine the proportion of the potentially cross-reactive food component that would not lead to signals equivalent to 1 ppm in a food matrix (Table 1). Only walnut, pumpkin seed, and cashew caused signals of ≥1 ppm. Further 2-to-10-fold dilution of the 1:5 diluted extracts led to signals equivalent to <1 ppm of hazelnut protein. Therefore, the assay tolerates 20% of walnut, 10% of pumpkin seed or 50% of cashew. All other investigated extracts showed no or only low signals of ≪1 ppm.

Detection Limit and Limit of Quantitative Determination in Various Complex Food Matrixes. Various blank samples of model chocolates and commercial food products that did not contain any detectable hazelnut protein as well as the most cross-reactive foods in this study were investigated for determination of the lower limits of detection (LLD) and of quantitative determination (LLQD) of hazelnut protein in these food matrixes. The limits were defined by a signal-to-noise ratio of the determined concentration of 3:1 and 6:1, respectively, and were derived from the mean concentration increased by 3- and 6-fold the standard deviation of the mean concentration. Each matrix was determined repeatedly on 6−8 different days (Table 2). In average, the LLD and LLQD of hazelnut protein in 11 different complex food matrixes was determined with 120 ± 110 and 200 ± 170 ppb, respectively. In 100% of either almond or chick pea, hazelnut protein ≥ 1.2 ppm may be specifically detected. In the case of either 100% of pumpkin seed or 100% of cashew or walnut, hazelnut protein may be detected at levels ≥10 or ≥5 ppm.

Detection of Proteins from Native and Toasted Hazelnuts. For accurate quantitative determination of extractable hazelnut protein it had to be assessed to which degree the ELISA was able to determine extractable hazelnut protein. Extracts from native hazelnuts of different origin and from hazelnuts toasted under industrial-like conditions were therefore analyzed for extractable hazelnut protein using the Bradford method with BSA as the standard. The extracts were additionally analyzed for hazelnut protein by ELISA using either standard curves prepared with protein from native or from toasted (140 °C, 30 min) hazelnuts of the variety Nocciole Ordu and results were correlated to those of the Bradford method (Table 3). On the basis of the detection with standards prepared from native hazelnut, protein from native hazelnuts was detected by ELISA at a mean level of 94 ± 13% (N = 6), and protein from toasted hazelnuts was quantified at a level of 115 ± 12% (N = 6). Using protein from toasted hazelnuts for preparation of the standards, protein from native hazelnuts could be determined by ELISA at a level of 77 ± 7% (N = 6) and protein from toasted hazelnuts at 96 ± 7% (N = 6).

The amount of extractable hazelnut protein varied depending on the analyzed variety and on the degree of toasting. The exact proportion of hazelnut in a food sample may therefore not be calculated from the amount of detectable hazelnut protein unless authentic reference material is available. With a standard curve derived from native hazelnut a correlation factor of 12.7 ± 3.4 may be applied. On the basis of the quantification with standards prepared from toasted hazelnuts, a correlation factor of 17.7 ± 5.7 was determined for evaluation of the amount of toasted hazelnut in a food sample.

Recovery Studies. Three blank commercial food samples and one self-prepared negative-control whole-milk chocolate were spiked with hazelnut of the variety Nocciole Ordu toasted at 140 °C for 30 min and at decreasing concentration levels (Table 4). Extractable hazelnut protein was determined by ELISA and the corresponding amount of detectable hazelnut was calculated from the amount of hazelnut protein determined applying a correlation factor of 12.75 that was predetermined from 3 independently prepared extracts of Nocciole Ordu (140 °C, 30 min).

Depending on the food matrix, 0.001% of hazelnut were recovered between 67 and 132%, and 10% of hazelnut could be determined at rates between 90 and 120%. The overall recoveries were good with an average of 106 ± 17% independent from the type of food matrix and the concentration level investigated.

Intra- and Interassay Precision in Chocolate Samples. For estimation of the intrassay precision, the hazelnut protein concentrations of four different self-

Table 2. Lower Limit of Detection (LLD) and Lower Limit of Quantitative Determination (LLQD) of Hazelnut Protein in Various Complex Food Matrixes and in the Most Cross-Reactive Foods[a]

		corresponding to ppm of hazelnut protein in a food matrix			
food	N	X	σ_{n-1}	LLD, $X + 3^*(\sigma_{n-1})$	LLQD, $X + 6^*(\sigma_{n-1})$
whole-milk chocolate[b]	8	0.019	0.018	0.07	0.13
half-bitter chocolate[b]	8	0.032	0.026	0.11	0.19
chocolate dragees	7	0.020	0.020	0.08	0.14
chocolate bar, candy cream I	7	0.044	0.044	0.18	0.31
chocolate bar, candy cream II	7	0.008	0.007	0.03	0.05
white chocolate I	6	0.024	0.010	0.06	0.09
breakfast cereal bar	7	0.022	0.010	0.05	0.08
ice cream, vanilla-cinnamon-peanut	6	0.014	0.018	0.07	0.12
puffed corn, peanut-flavored	7	0.179	0.082	0.43	0.67
potato snack, paprika-flavored	6	0.038	0.016	0.09	0.14
snack, asian nuts	7	0.097	0.023	0.17	0.24
almond[c]	6	0.696	0.118	1.1	1.4
cashew[c]	7	2.141	0.799	4.5	6.9
chick pea[c]	6	0.887	0.103	1.2	1.5
pumpkin seed[c]	7	6.423	1.285	10.3	14.1
walnut[c]	6	4.184	0.449	5.5	6.9

[a] N, number of independent determinations on different days; X, mean concentration; σ_{n-1}, standard deviation of the mean concentration. [b] Self-prepared negative-control samples. [c] Most cross-reactive foods determined.

Table 3. Extractable Hazelnut Protein from Native Hazelnuts and from Hazelnuts Toasted under Various Conditions As Determined by the Bradford Method and by ELISA[a]

		Bradford		ELISA[b]		ELISA[c]			
source of hazelnut/ variety	native/ toasted	protein (mg/mL)	CV (%)	protein (mg/mL)	CV (%)	protein (mg/mL)	CV (%)	ELISA[b]/ Bradford (%)	ELISA[c]/ Bradford (%)
Italy, Mortarella	native	4.69	0.5	4.20	6.1	3.56	5.1	90	76
Italy, San Giovanni	native	6.17	2.5	6.59	25.2	4.18	8.5	107	68
Italy, Tonda di Giffoni	native	3.98	3.9	3.52	17.1	3.16	2.2	88	79
Italy, Tonda Romana	native	4.91	2.9	3.92	16.8	3.43	1.0	80	70
Italy, Piemonte	native	3.25	1.1	2.76	3.4	2.58	7.9	85	79
Italy, Piemonte	140 °C, 20 min	3.48	3.8	3.55	4.5	3.00	7.4	102	86
Italy, Piemonte	140 °C, 30 min	3.24	5.0	3.53	5.5	3.01	1.3	109	93
Italy, Piemonte	140 °C, 40 min	1.91	5.3	1.95	1.7	1.73	5.7	102	91
Turkey, Nocciole Ordu	native	3.70	0.9	4.21	0.5	3.25	2.3	114	88
Turkey, Nocciole Ordu	140 °C, 20 min	3.48	2.3	4.47	4.9	3.41	2.5	128	98
Turkey, Nocciole Ordu	140 °C, 30 min	3.78	2.3	4.83	2.3	3.93	4.6	128	104
Turkey, Nocciole Ordu	140 °C, 40 min	2.92	0.5	3.45	0.7	3.05	1.1	118	104

[a] Values are the average of duplicate determinations. [b] Standard curve prepared with protein from native hazelnut of the variety Nocciole Ordu. [c] Standard curve prepared with protein from toasted (140 °C, 30 min) hazelnut Nocciole Ordu.

Table 4. Recovery of Hazelnut from Blank Commercial and Model Food Samples That Were Artificially Contaminated with Hazelnut at Levels of 10, 1, 0.1, 0.01, and 0.001%[a]

	% recovery of hazelnut/% CV of duplicate determinations					
blank samples spiked with hazelnut	10%	1%	0.1%	0.01%	0.001%	mean % recovery
cookie, coconut	90.2/9.8	95.5/7.9	112.0/5.4	126.8/21.2	116.4/38.9	108 ± 15
cereal bar, yoghurt	119.7/6.2	127.1/2.5	98.0/4.6	87.2/9.7	67.0/17.6	100 ± 24
chocolate bar, almond candy cream	102.8/0.3	111.9/7.8	114.1/8.5	103.9/32.2	132.0/31.4	113 ± 12
whole-milk chocolate[b]	111.8/11.3	113.7/1.3	117.5/5.2	90.6/3.2	82.8/6.5	103 ± 16

[a] Values are the average of duplicate determinations. [b] Self-prepared negative-control sample.

prepared chocolates that contained hazelnut between 0.001 and 1% were determined in 21 triplet replicates on one separate microwell plate for each sample. The mean CVs were determined as 2.9, 4.8, 4.4, and 5.8% for hazelnut at levels of 0.001, 0.01, 0.1, and 1%, respectively.

The interassay precision was determined for self-prepared chocolates that contained hazelnut in a range between 0.001 and 10% on five different days each. The interassay precision resulted in averages of 18.4, 3.8, 5.5, 13.1, and 5.9% for hazelnut at levels of 0.001, 0.01, 0.1, 1, and 10%, respectively.

Investigation of Industrially Manufactured Model Foods and Commercial Chocolates from a Peanut Cleanup Procedure. Industrially manufactured model chocolates with the addition of definite amounts of hazelnut, almond, or peanut as well as one model chocolate without addition of any nut components and one peanut paste were analyzed for hazelnut protein (Table 5). Additionally, samples of a chocolate manufacture taken at several intervals of a cleanup procedure for peanut components were investigated for hazelnut protein.

Each sample with a declaration of hazelnut contained detectable hazelnut protein. Surprisingly, each of the industrially manufactured model samples without addition of hazelnut but with the addition of definite amounts of almond or peanut as well as one chocolate without addition of any nut components contained detectable hazelnut protein in the order of 74−141 ppm. Even the peanut paste contained 119 ppm of extractable hazelnut protein.

Table 5. Analysis of Industrially Manufactured Model Foods and Chocolates at Various Steps of a Peanut Cleanup Procedure for Hazelnut Protein According to (A) the Screening Protocol and (B) the Quantitative Procedure[a]

		(A) screening	(B) quantitative determination[b]	
sample	D[a]	ppm	ppm	CV (%)
chocolate, nougat[d]	hazelnut paste	>128	16046	nd[c]
chocolate, coffee creme[d]	hazelnut paste	>128	966	nd
chocolate bar, nougat crisp[d]	hazelnut and almond paste	>128	8636	nd
chocolate, whole-milk[d]	without addition of 'nuts'	56	99	13.7
chocolate, whole-milk[d]	0.1% almond	56	74	2.9
chocolate, whole-milk[d]	0.5% almond	56	140	1.9
chocolate, whole-milk[d]	0.1% hazelnut	>128	195	nd
chocolate, whole-milk[d]	0.5% hazelnut	>128	499	nd
chocolate, whole-milk[d]	0.1% peanut	99	126	5.9
chocolate, whole-milk[d]	0.5% peanut	109	141	3.3
peanut paste[d]	−	>128	119	1.1
chocolate, cleanup 1	−	34	35	6.2
chocolate, cleanup 2	−	37	35	0.9
chocolate, cleanup 3	−	34	34	3.4
chocolate, cleanup 4	−	37	34	3.8
chocolate, cleanup 5	−	34	34	3.9

[a] D, declaration of hazelnut or hazelnut compounds; − no declaration. [b] Values are the average of duplicate determinations or as specified. [c] nd, not determined: only one determination. [d] Industrially manufactured model samples.

It was assumed that the model chocolates were prepared from one or more chocolates that were basically contaminated with hazelnut because the self-prepared model chocolates of this study did not cause any signal. Even the chocolates with addition of 0.1 and 0.5% of hazelnut showed elevated amounts of detectable hazelnut protein when compared to the reproducible results of the recovery studies. Hence, a basic contamination of these two chocolates at a level of some 120 ppm had to be assumed. Because peanut did not cause any signal in cross-reactivity studies, the peanut paste definetly contained hazelnut in a range between 0.1 and 0.2%. The cleanup samples were also contaminated with hazelnut. With our previously described peanut-specific ELISA (Holzhauser and Vieths, 1999), the amount of peanut protein was determined in these samples and decreased from 196 to 25 ppm of peanut protein from sample 1 to sample 5. As all cleanup samples showed a constant level of some 35 ppm of hazelnut protein, a virtually hazelnut-contaminated chocolate must have been used for dilution of the amount of peanut in this lot of chocolate.

Investigation of Commercial Food Products. Forty-eight different commercial food products were analyzed for the presence of hazelnut protein (Table 6). Samples were considered as positive if hazelnut protein ≥ 1 ppm could be detected. Even though studies on the lower limit of detection in various complex food matrixes demonstrated that analysis may be carried out at the ppb level, a threshold of 1 ppm of hazelnut protein was chosen to minimize the probability of false-positive results from unknown food matrixes and to increase the accuracy and precision of the results. Samples were analyzed according to both the screening and the quantitative protocols. Only one quantitative determination was carried out if samples having hazelnut or hazelnut components declared, and samples without such declaration were experimentally confirmed by ELISA according to the screening procedure. Results of the screening trial contradictionary to the labeling of products were checked with two independent determinations according to the quantitative assay protocol.

Any of the 17 products having hazelnut or hazelnut components declared contained hazelnut protein between 45 and 20028 ppm equivalent to ∼0.06−25% of hazelnut, calculated as Nocciole Ordu (140 °C, 30 min). No hazelnut protein could be detected in the three samples that "may contain nut or hazelnut traces" or are "not suitable for nut allergy sufferers". Forty-three percent (12/28) of the samples without labeling or declaration of hazelnut components within the list of ingredients contained hazelnut protein between 1.8 and 421 ppm equivalent to ∼0.002−0.5% of hazelnut of the variety Nocciole Ordu (140 °C, 30 min).

With the simplified screening protocol, no contradictionary results were obtained and traces of hazelnut protein between 1 and 128 ppm could be determined at a rate of 97 ± 23% (N = 11) when compared to the quantitative determination.

DISCUSSION

The variety Nocciole Ordu, grown in Turkey, was chosen as reference material for immunoblotting studies and the preparation of standards for ELISA. Because, on one hand, Turkish hazelnuts are the most abundant among the world's crop (Belitz and Grosch, 1992, p 730), and on the other, there seemed to be no significant differences between the varieties as was demonstrated elsewhere (Garone et al., 1988) and in this study.

Both polyclonal antisera used in the two-site ELISA reacted with proteins from native and toasted hazelnuts as was demonstrated by immunoblotting and by analyzing extracts from different hazelnuts with the developed ELISA technique. The immunoblotting revealed that the rabbit and sheep polyclonal antisera recognize about the same proteins of the corylin fraction in native and toasted hazelnuts. Comparison with IgE-reactive proteins, thus allergens, detected by selected patients' sera, showed that the polyclonal antisera detected some of the allergens but the pattern of the IgE- and IgG-reactive proteins differed. The main criteria for the selection of patients' sera was to display the variety of allergenic proteins rather than to give a representative distribution of the most prevalent allergens recognized by patients' sera. Most of the IgE-reactive proteins were not stable toward heat and seemed to lose their allergenicity. The IgE-detection of patient no. 2 who is exclusively sensitized to proteins of the "Bet v 1-family" was chosen as an example: An 18 kDa allergen that is related to Cor a 1, a 17 kDa Bet v 1-related major allergen in hazel pollen (Hirschwehr et al., 1992), cannot

Table 6. Analysis of Various Commercial Food Commodities for Hazelnut Protein According to (A) the Screening Protocol and (B) the Quantitative Procedure[a]

		(A) screening	(B) quantitative determination		
sample	D[a]	ppm	ppm	CV (%)	result
amarettini	+	>128	2968		positive
breakfast muesli	+	>128	701		positive
cereal bar, chocolate	+	>128	1373		positive
cornflakes, honey and nuts	+	>128	436		positive
chocolate bar, caramel	+	>128	572		positive
chocolate candy, plain	+	>128	3852		positive
chocolate candy, hazelnut	+	>128	17123		positive
chocolate and cookie	+	>128	459		positive
chocolate, whole milk II	+	>128	1351		positive
chocolate, whole milk and nut I	+	>128	15183		positive
chocolate, whole milk and nut II	+	>128	9788		positive
marzipan bar	+	33	45		positive
nougat bar	+	>128	16120		positive
nougat spread	+	>128	679	5.9	positive
nougat spread	+	>128	1449	3.9	positive
nougat spread	+	>128	4311	6.2	positive
nut and chocolate	+	>128	20028		positive
asian nut snack	−	0[b]	0		negative
asian rice cracker	−	0	0		negative
breakfast cereal bar	−	0	0		negative
cereal bar, yoghurt	−	0	0		negative
chocolate bar, candy cream I	−	0	0		negative
chocolate bar, candy cream II	−	0	0		negative
chocolate bar, candy cream and almond	−	0	0		negative
chocolate chips	−	3.8	3.0	3.2	positive
chocolate corn balls	−	27	35	11.3	positive
chocolate dragees	−	0	0		negative
chocolate, for children	−	0	0		negative
chocolate, half-bitter	−	0	0		negative
chocolate, marzipan	−	7.7	5.8	1.5	positive
chocolate, plain	−	~128	111	0.4	positive
chocolate, sponge cake I	−	18	27	1.0	positive
chocolate, sponge cake II	−	2.0	1.8	3.6	positive
chocolate spread	−	>128	169	9.5	positive
chocolate, white II	−	0	0		negative
chocolate, whole milk I	−	4.6	4.2	3.5	positive
chocolate, whole milk III	−	67	76	2.8	positive
cookie, coconut	−	0	0		negative
crunchy chocolate flakes	−	19	26	4.7	positive
ice-cream, vanilla- peanut	−	0	0		negative
muesli, fruit[d]	−	>128	421	3.6	positive
potato snack, paprika-flavored	−	0	0		negative
puffed corn, peanut-flavored	−	0	0		negative
raisin and chocolate	−	4.5	4.9	0.6	positive
wafer, chocolate and cereal crisp	−	0	nd[c]		negative
candy bar, caramel	±	0	0		negative
chocolate mints	±	0	0		negative
chocolate, white I	±	0	0		negative

[a] D, declaration of hazelnut or hazelnut components; − no declaration; + positive declaration; ±, may contain hazelnut traces or not suitable for nut allergy sufferers. [b] 0, no detectable hazelnut protein. [c] nd, not determined: only one determination. [d] Sample without labeling.

be detected in toasted hazelnut. The birch pollen-related 18 kDa allergen in hazelnut was sequenced, and the corresponding recombinant non fusion allergen was cloned in our group (Lüttkopf et al., 1999). It is likely that this protein is identical with the major allergenic structure in hazelnut as described elsewhere (Hirschwehr et al., 1992; Lüttkopf et al., in preparation). By contrast, IgE detection with the sera of patients nos. 1 and 3−6 who presented IgE to both native and toasted hazelnuts showed that heat-stable IgE-reactive protein structures at higher molecular mass and around 12 kDa are probably responsible for a persistent allergenicity of hazelnut, even after food technological processing such as toasting. Comparing our results of immunoblotting with those obtained by SDS-PAGE immunoblotting (Hirschwehr et al., 1992) and by RBL cell mediator-release assay (Vieths et al., 1998), the heat-stable IgE-reactive proteins in hazelnut do not seem to be related to known birch pollen allergens. By use of recombinant rBet v 1 and rBet v 2 (birch pollen profilin), only IgE-binding to a Cor a 1-related 18 kDa and a 14 kDa allergen could be inhibited, respectively (Hirschwehr et al., 1992). With the RBL cell assay that mimics a main event of the allergic type-I reaction (Hoffmann et al., 1997) the degree of IgE cross-linking of passively sensitized RBL cells by protein from native and toasted hazelnut was monitored in vitro. It was demonstrated that heat treatment of hazelnuts resulted in a reduced immunoreactivity of the extracted allergens when the cells were passively sensitized by murine anti-birch pollen IgE while a considerable potential for specific mediator release persisted independently of toasting conditions when cells were passively sensitized with murine anti-hazelnut IgE prior to cross-linking (Vieths et al., 1998). We therefore conclude that sensitization to heat-stable structures is initiated by hazelnut

proteins, whereas cross-reactivity to heat-labile hazelnut proteins is related to tree pollen-specific IgE. The former structures seem to be responsible for a persistent allergenicity of even heat-treated hazelnut in susceptible individuals. Hence, a capability of the ELISA to detect potentially allergenic proteins in both native and toasted hazelnuts is of great importance.

Applying standards from native hazelnuts, the ELISA could quantify, thus recognize, extractable protein from various native hazelnuts at a level of 94 ± 13% when compared to protein quantification by the Bradford method. Proteins from hazelnuts toasted under realistic conditions were quantified by an average of 96 ± 7% using a standard curve prepared from toasted hazelnut. Depending on the source of hazelnut, correct quantification of extractable hazelnut protein can be achieved by simply switching between standards from native or toasted hazelnuts. Accurate detectability was mainly independent from the conditions of toasting. Most of the food matrixes of interest are among confectionary and confectionary-related products, and because of improved characteristics in flavor, especially toasted hazelnuts are to be expected in these types of food. In general, quantification may therefore be done by the use of standards derived from toasted hazelnuts. However, underestimation of native nut protein in the order of 20−30% has to be considered. The amount of extractable and detectable protein varied depending on the type of nut and the toasting conditions. Therefore, the exact calculation of the amount of hazelnut present in a sample is not possible, unless authentic reference material is available as was also demonstrated elsewhere (Eichler and Rubach, 1985; Klein and Günther, 1985). In our study, the amount of native hazelnut could be estimated from detectable hazelnut protein by a mean correlation factor of 12.7 (%CV = 27) while a mean correlation factor of 17.7 (%CV = 32) had to be applied for toasted material. On the basis of the analysis with a rocket immunoelectrophoresis, Klein and Günther (1985) suggested a mean correlation factor of 12.1, deduced from values ranging between 8.3 and 17.7, for an unknown sample of toasted hazelnuts. However, conditions of toasting were different to ours and it has to be mentioned that correlation factors depend on the detectability of extractable protein by the method applied and on the material investigated.

The combination of two different hazelnut-specific antisera and immunoabsorption of the rabbit antiserum led to a high specificity of the ELISA for hazelnut protein. Cross-reactivities with a signal of >1 ppm of hazelnut protein should only be expected if either walnut, pumpkin seed, or cashew exceeding 20, 10, or 50%, respectively, were present in the investigated food stuff. This should only be the case for pastes of or nut mixtures with the mentioned foods. These types of foods shall anyway be avoided by allergic individuals.

In selected food matrixes, statistically derived detection limits of <100 ppb of hazelnut protein could be demonstrated. If even less hazelnut protein is to be detected, the use of affinity-purified specific antibodies may be considered. In practice, a lower limit of detection of 1 ppm of hazelnut protein in an unknown complex food matrix may be applied, resulting in an improved signal precision and a very low probability of false-positive results at trace levels. For accurate analysis of ≥1 ppm of hazelnut protein corresponding to ≥10 ng of protein/mL of the 1:5 diluted sample extract, a mean CV of <10% may be expected. In chocolate samples, intraassay precision was <6% for hazelnut ≥0.001% and interassay precision was <15% for hazelnut ≥0.01%.

In our studies, extracted hazelnut protein at trace levels was less stable than peanut protein that could even be stored diluted at −20 °C for several months (Holzhauser and Vieths, 1999). If soluble hazelnut protein at low concentration levels was lyophilized or overnight freeze-stored at −20 °C, detectability of hazelnut protein was reduced by up to ∼40−50%. By contrast, the undiluted extract from pure hazelnut could be stored at −20 °C for at least 4 months. Therefore, extracts were always prepared immediately prior to use and standards were also prepared freshly from freeze-stored hazelnut protein reference extract. In this context, recovery experiments had to be done with hazelnut spiked to the food because protein once extracted and spiked to a matrix would degrade to a certain degree when extracted for a second time and in the presence of a matrix. The recoveries would thus be underestimated. Hazelnut between 0.001 and 10% added to four different types of complex confectionary and muesli/cereal matrixes was recovered in average by 106 ± 17%. Even though the amount of hazelnut detected from the 0.001% hazelnut-spikes was <1 ppm, protein could be recovered between 67 and 132%. Considering the data on assay precision and recovery, the accuracy of the determination of hazelnut protein from an unknown sample may rank within the order of variation of the detectability that resulted from different sources of hazelnuts and from different conditions of toasting. For most accurate quantification, especially <1 ppm of hazelnut protein and in an unknown food matrix, recovery studies on the analyzed sample should nevertheless be performed.

The investigation of a wide variety of commercial food items and also of industrially manufactured model foods made clear that, especially within snacks, sweets, chocolates, and cereals, contamination with hazelnut traces likely occurs. Almost 43% (12 of 28) of all commercially purchased samples that did not have hazelnut listed as an ingredient or that did not have any labeling contained between 1.8 and 421 ppm of hazelnut protein corresponding to some 0.002−0.5% of hazelnut. The prevalence of hidden hazelnut protein was even greater than the rate of peanut contamination previously described (Holzhauser and Vieths, 1999). Although the samples were selected at one time and no batch-to-batch variations were considered, the study clearly shows a tendency that hidden hazelnut protein, thus potential allergen, may be found to a considerable extend in the food matrixes discussed. Even the industrially manufactured model chocolates without addition of hazelnut and one peanut paste contained between 74 and 141 ppm of hazelnut protein.

Little is known about the minimal dose of hazelnut protein that would still elicit allergic reactions in sensitive individuals. As was demonstrated for peanut, as little as 100 μg of peanut protein could still elicit mild reactions in a peanut-sensitive patient (Hourihane et al., 1997). In the case of hazelnut, it may be expected that <0.01% of hazelnut in processed foods could still trigger severe allergic reactions (Eriksson and Malmheden Yman, 1992). The developed ELISA is able to specifically detect and quantify hazelnut protein in an unknown matrix at a level of 1 ppm corresponding to

~0.001% of hazelnut. Even less may be detected if the matrixes of interest are further investigated and validated. With the screening extraction procedure, a large number of samples could be treated at once and within <1 working day, and the accurate amount of hazelnut protein could be determined at a rate of 97 ± 23% when compared to the quantitative procedure. The sensitivity of the ELISA should be sufficient to increase the food safety for the majority of hazelnut-allergic individuals if used for screening of food ingredients and final processed products, and in return, to obtain a more precise labeling of processed foods. However, we cannot exclude that highly sensitized individuals may still react to hazelnut protein below the detection threshold of the ELISA.

ABBREVIATIONS USED

AP, alkaline phosphatase; BSA, bovine serum albumin; CV, coefficient of variation; ELISA, enzyme-linked immunosorbent assay; F_C, correlation factor; HRP, horseradish peroxidase; IgE/IgG, immunoglobulin E/G; I, India ink; kDA, kilo Dalton; LLD, lower limit of detection; LLQD, lower limit of quantitative determination; MM, molecular mass; OAS, oral allergy syndrome; OD, optical density; PBS, phosphate-buffered saline; ppb, part per billion; ppm, part per million; pyr, pyronine; RAST, radioallergosorbent test; RIE, rocket immunoelectrophoresis; SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; Tris, tris-(hydroxymethyl)aminomethane; TBS, tris-buffered saline; Tween 20, polyethylene-sorbitan monolaurate.

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LITERATURE CITED

Belitz, H.-D.; Grosch, W. Kakao und Schokolade. In *Lehrbuch der Lebensmittelchemie*, 4th ed.; Springer, Berlin, Germany, 1992; pp 876−877.

Brostoff, J. Anaphylactic reaction to injected hazelnut. *Lancet* **1989**, *2*, 459.

D'Amato, G.; Spieksma, F. Th. M.; Liccardi, G.; Jäger, S.; Russo, M.; Kontou-Fili, K.; Nikkels, H.; Wüthrich, B.; Bonini, S. Pollen-related allergy in Europe. *Allergy* **1998**, *53*, 567−578.

Eichler, D.; Rubach, K. Bestimmung von Haselnussmark in Nussnougat-Cremes mittels ELISA. *Dtsch. Lebensm-Rundsch.* **1985**, *81*, 137−139.

Erikkson, A.; Malmheden Yman, I. Quantitative analysis of casein, egg proteins and hazelnut in food by rocket immunoelectrophoresis on PhastSystem. In *Food safety and quality assurance. Applications of immunoassay systems*; Morgan, M., Smith, C., Williams, P., Eds.; Elsevier: Barking, U.K., 1992; pp 65−68.

Etesamifar, M.; Wüthrich, B. IgE-mediated food allergies including oral allergy syndrome in 383 patients. *Allergologie* **1998**, *21*, 451−457.

Ewan, P. W. Clinical study of peanut and nut allergy in 62 consecutive patients: new features and associations. *Br. Med. J.* **1996**, *312*, 1074−1078.

Garrone, W.; Antonucci, M.; Bona, U.; Clementi, S. Determination of hazelnut content by means of their protein fraction in chocolate bars, chocolates and milk containing spreads. *Lebensm.-Wiss. -Technol.* **1988**, *21*, 76−82.

Hirschwehr, R.; Valenta, R.; Ebner, C.; Ferreira, F.; Sperr, W. R.; Valent, P.; Rohac, M.; Rumpold, H.; Scheiner, O.; Kraft, D. Identification of common allergenic structures in hazel pollen and hazelnuts: A possible explanation for sensitivity to hazelnuts in patients allergic to tree pollen. *J. Allergy Clin. Immunol.* **1992**, *90*, 927−936.

Hoffmann, A.; Vieths, S.; Haustein, D. Biologic allergen assay for *in vivo* test allergens with an *in vitro* model of the murine type I reaction. *J. Allergy Clin. Immunol.* **1997**, *99*, 227−232.

Holzhauser, T.; Vieths, S. Indirect competitive ELISA for determination of traces of peanut (*Arachis hypogaea* L.) protein in complex food matrixes. *J. Agric. Food Chem.* **1999**, *47*, 603−611.

Hourihane, J.; Kilburn, S.; Nordlee, J.; Hefle, S.; Taylor, S.; Warner, J. An evaluation of the sensitivity of subjects with peanut allergy to very low doses of peanut protein: A randomized, double-blind, placebo-controlled food challenge study. *J. Allergy Clin. Immunol.* **1997**, *100*, 596−600.

Janciewicz, A.; Aulepp, H.; Baltes, W.; Bögl, K. W.; Dehne, L. I.; Zuberbier, T.; Vieths, S. Allergic sensitization to native and heated celery root in pollen-sensitive parients investigated by skin test and IgE binding. *Int. Arch. Allergy Immunol.* **1996**, *111*, 268−278.

Klein, E.; Baudner, S.; Günther, H. Immunchemische Bestimmung des Haselnussproteins mit Hilfe der Elektroimmundiffusion nach Laurell. *Z. Lebensm. Unters. Forsch.* **1985**, *180*, 30−35.

Lüttkopf, D.; Scheurer, S.; Haustein, D.; Vieths, S. NCBI GenBank database Acc.: AF136945.

Malmheden Yman, I.; Eriksson, A.; Everitt, G.; Yman, L.; Karlsson, T. Analysis of food proteins for verification of contamination or mislabeling. *Food Agric. Immunol.* **1994**, *6*, 167−172.

Martín Muñoz, F.; López Cazaña, J. M.; Villas, F.; Contreras, J. F.; Díaz, J. M.; Ojeda, J. A. Exercise-induced anaphylactic reaction to hazelnut. *Allergy* **1994**, *49*, 314−316.

Matissek, R.; Burkhardt, H. G. Süsswaren und Honig. In *Taschenbuch für Lebensmittelchemiker und- technologen 1*; Frede, W., Ed.; Springer: Berlin, Germany, 1991; pp 433.

Mohr, E.; Wichmann; Penkala, M.; Winoto, S. Der quantitative Nachweis des Haselnussgehaltes in Nuss-Nougat-Cremes. *Gordian* **1983**, *10*, 193−199.

Teuber, S.; Brown, R.; Haapanen, L. Allergenicity of gourmet nut oils processed by different methods. *J. Allergy Clin. Immunol.* **1997**, *99*, 502−507.

Vieths, S.; Schöning, B.; Baltes, W. Allergy to fruits and vegetables in pollen-sensitive patients: Allergen characterization by IgE immunoblotting and peroxidase staining. *Food Agric. Immunol.* **1992**, *4*, 181−197.

Vieths, S. Allergenic cross-reactivity, food allergy and pollen. *ETAP-Environ. Toxicol. Pharmacol.* **1997**, *4*, 61−70.

Vieths, S.; Hoffmann, A.; Holzhauser, T.; Müller, U.; Reindl, J.; Haustein, D. Factors influencing the quality of food extracts for *in vitro* and *in vivo* diagnosis. *Allergy* **1998**, *(Suppl. 46) 53*, 65−71.

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