JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

A Novel Approach for the Detection of Potentially Hazardous Pepsin Stable Hazelnut Proteins as Contaminants in Chocolate-Based Food

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Contamination of food products with pepsin resistant allergens is generally believed to be a serious threat to patients with severe food allergy. A sandwich type enzyme-linked immunosorbent assay (ELISA) was developed to measure pepsin resistant hazelnut protein in food products. Capturing and detecting rabbit antibodies were raised against pepsin-digested hazelnut and untreated hazelnut protein, respectively. The assay showed a detection limit of 0.7 ng/mL hazelnut protein or <1 μg hazelnut in 1 g food matrix and a maximum of 0.034% cross-reactivity (peanut). Chocolate samples spiked with 0.5−100 μg hazelnut/g chocolate showed a mean recovery of 97.3%. In 9/12 food products labeled "may contain nuts", hazelnut was detected between 1.2 and 417 μg hazelnut/g food. It can be concluded that the application of antibodies directed to pepsin-digested food extracts in ELISA can facilitate specific detection of stable proteins that have the highest potential of inducing severe food anaphylaxis.

KEYWORDS: ELISA; hazelnut; pepsin digestion; food matrix; allergens

INTRODUCTION

In Western countries, food allergy is estimated to affect 1−2% of the total population (*1*, *2*). Together with hen's egg, cow's milk, fish, crustacea, peanut, soybean, and wheat, tree nuts are most frequently identified (*3*−*5*) as foods causing severe allergic reactions. Among those, hazelnut (*Corylus avellana*) plays an important role. Most hazelnut allergic individuals are first sensitized to birch pollen. IgE against the major birch pollen allergens Bet v 1 (*6*) and Bet v 2 (birch profilin) can cross-react with their homologues found in hazelnut [Cor a 1 (*7*) and 2 (*8*), respectively]. Cor a 1 is extremely sensitive to processing and/or proteolytic attack. For that reason, Cor a 1 is generally regarded as an allergen that only causes mild symptoms in the oral cavity, referred to as the oral allergy syndrome (OAS) (*9*). Cor a 2 could be involved with OAS-like symptoms as well.

In general though, induction of severe systemic food allergy is thought to be a property limited to stable allergens that survive the proteolytic environment of the gastrointestinal tract, like 2S albumins, 7S vicilins, nonspecific lipid transfer protein (nsLTP), and thaumatin (*10*−*18*).

Although most allergic individuals with severe food allergy are aware of their allergic constitution and the causative food, accidental ingestion of allergens is frequent and potentially life-threatening (*19*). Several factors contribute to the occurrence of unwanted exposure to food allergens, including mislabeling or cross-contamination of food products during manufacturing. Double-blind placebo-controlled food challenges performed with peanut and hazelnut among peanut (*20*) and hazelnut allergic patients (*21*) revealed that objective reactions can occur at quantities ranging from 2 to 10 and 1 mg, respectively (*22*, *23*). In general, it is felt that food allergic patients are at risk after consumption of food products with allergenic contaminates at >10 μg/g food (*24*, *25*). Although the packaged food industry invests in changes in equipment design, extra cleaning, and sometimes precautionary labeling such as "may contain traces of...", unintended consumption is still possible. However, despite all precautions that can be taken to prevent contamination of food or unintended consumption of food allergens, absolute guarantees concerning the absence of unwanted contamination cannot be given, due to issues with incoming raw materials,

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10.1021/jf049278r CCC: $27.50
Published on Web 11/10/2004

etc. Therefore, the development of reliable immunoassays for the detection of residues of allergenic food ingredients is of the utmost importance.

Also, assays measuring reliably allergenic substances in compound food are urgently needed, since in both the European Union and the United States, mandatory labeling of allergenic foods will come into action. In the European Union, a list of 12 allergenic foods (including nuts) and products thereof was compiled. Starting November 2005, all food, containing food-(products) from this list, must be labeled accordingly (*26*).

Previous methods described for hazelnut residue detection (*27*−*31*) have not specifically addressed the issue of protein stability. This study aimed at developing a strategy to measure traces of contaminating hazelnut proteins insensitive to processing steps such as roasting.

MATERIALS AND METHODS

Chemicals. All chemicals were purchased from Merck (Darmstadt, Germany) unless stated differently.

Sera. The serum was obtained from three hazelnut allergic patients (1−3) with clear positive radioallergosorbent assay (RAST) scores.

Antisera. Cor a 1 is a cross-reactive monoclonal antibody (mAb) 5H8 (400 μg/mL) (*32*) against Bet v 1. Radiolabeled (^{125}I) sheep-anti rabbit IgG (Sanquin, Amsterdam, The Netherlands) and ^{125}I-rat anti-mouse (RaM) κ monoclonal antibodies (M1482, Sanquin) were used.

Hazelnut Source Materials. For preparation of the hazelnut extract, a Turkish variety of hazelnuts was obtained from a local grocery store in Amsterdam. Raw and roasted hazelnut meals (Barcelona variety) were obtained from Westnut, Inc. (Dundee, OR). Westnut, Inc. has a dedicated facility for hazelnuts.

Plant Food and Pollen Extracts. Hazelnut, barley, almond, brazil nut, peanut, sesame seed, pine tree nut, walnut, and wheat extracts were prepared according to Björksten (*33*). In short, ground food was extracted (10% w/v) in polyvinylpolypyrrolidon (PVPP) buffer buffer containing PVPP and diethyldithiocarbamate. These reagents prevent the loss of allergenicity by inhibiting oxidative processes mediated by polyphenyloxidases and peroxidases upon disruption of the plant tissue. In particular, Bet v 1-related food allergens (i.e., Cor a 1 in the case of hazelnut) have been shown to be sensitive to these processes. After centrifugation [19700*g*, room temperature (RT)] to remove particulate matter, supernatants were dialyzed against deionized water at 4 °C and lyophilized. Birch pollen was purchased from ALK-Abelló (Hørsholm, Denmark) and extracted as described elsewhere (*34*).

Depletion of Hazelnut Extract for Cor a 1. Hazelnut extract was depleted for Cor a 1 using Sepharose-coupled mAb 5H8 against Cor a 1. A decrease of >99% was observed (data not shown).

Extraction of Food Products. Chocolate bars and cookies were ground in liquid nitrogen with a mortar and pestle and extracted using phosphate-buffered saline (PBS)/0.1% Tween 20 and extracted (10% w/v) for 2 h at 60 °C. Chocolate spreads were directly extracted under identical conditions. Undissolved material was removed by centrifugation at 3850*g* for 30 min at 10 °C. For reference purposes in enzyme-linked immunosorbent assay (ELISA), 1 g of ground Turkish hazelnuts was extracted at 10% w/v according to the same protocol. The mean protein yield ($n = 3$) was 3.9 ± 0.5 mg/mL, i.e., an average of 3.9%. This mean percentage was used to convert hazelnut protein concentrations measured in food products into μg hazelnut per g food. Hazelnuts have 12.7% protein on average (*35*).

Manufactured Hazelnut-in-Chocolate Standards. Individual reference milk chocolate standards were made in an industrial setting (Barry Callebaut, St. Hyacinthe, Quebec, Canada). Milk chocolate standards containing 0, 0.5, 1, 2, 5, 10, 25, and 100 μg hazelnut/g chocolate were prepared. In short, the chocolate was prepared using slightly heated chocolate liquor, butter oil, cocoa butter and sugar. Ground hazelnut (1 mg per g chocolate) was added to this, and subsequently, the resulting mixture was run through a refiner with a 34 μM cutoff. Standards were made by diluting this master batch with clean chocolate to make the standards indicated above. The batches of chocolate were then put in a conch (Hobart Corp., Troy, OH) and mixed for 12 h at 80 °C. Before they were molded, soy lecithin and vanillin were added and mixed to incorporate.

Two commercial hazelnut chocolate spreads were heated to 80 °C, then spiked with 10% w/w hazelnut meal (80 mg of hazelnut meal was thoroughly mixed with 0.8 g of hazelnut chocolate spread), and subsequently kept at 80 °C for 12 h and then chilled and kept at RT.

Pepsin Digestion of Hazelnut Extract. The hazelnut extract was pepsin-digested (*36*) by incubating 1 mg of lyophilized hazelnut PVPP extract in 10 mmol/L HCl with agarose-linked pepsin (Sigma, St. Louis, MO) at a final concentration of 0.35% (w/v) pepsin for 1 h at 37 °C. Digestion was terminated by neutralization with 0.5 M K_2HPO_4. After centrifugation for 5 min at 10000*g* at RT, the supernatant solution was collected. As a control for digestion, the same protocol was followed without the addition of pepsin. Samples were dialyzed (Snake Skin, Pleated Dialysis Tubing, 3.5 kDa cutoff, Pierce, Rockford, IL) against 0.01 M PBS (pH 7.4) prior to administration to rabbits as immunogen.

Protein Determinations. Protein concentrations were determined using the BCA method according to the manufacturer's instructions with bovine serum albumin as a standard (Pierce).

Rabbit Antisera. Two female New Zealand white rabbits were immunized and boosted four times at 4 week intervals with 100 μg/mL pepsin-digested hazelnut or nondigested hazelnut extract. For each immunization, 1 mL of hazelnut extract was mixed with 1 mL of Montanide ISA-50 (Seppic, Paris, France). Serum pools of rabbit anti-hazelnut pepsin digest (from two rabbits) and rabbit anti-hazelnut (from three rabbits) were separately treated with caprylic acid in order to partly purify IgG antibodies (*37*). Precipitated serum proteins were removed by paper filtration (ashless 589[1] Black ribbon; Schleicher & Schuell, Dassel, Germany). The flow-through was dialyzed (Visking Dialysis Tubing, 10 kDa cutoff, Medicell Int., London, United Kingdom) against PBS for 2 days at 4 °C. The purity of IgG was monitored by automated agarose Hydragel 30 protein electrophoresis (Sebia, Issy-les-Moulineaux, France) followed by Coomassie Brilliant Blue 250 (Bio-Rad Laboratories, Hercules, CA) staining according to the manufacturer's instructions. The IgG content was quantified by measuring the absorbance at 280 nm.

Depletion of Rabbit Anti-Hazelnut Digest for Anti-Birch Pollen Reactivity. Rabbit antiserum against hazelnut pepsin digest was depleted for antibody cross-reactivity to structures in birch pollen, i.e., Bet v 1, Bet v 2, and carbohydrate determinants. To that end, 4 mg of birch pollen extract was coupled to 100 mg of CNBr-activated Sepharose (Amersham Pharmacia Biotech, Uppsala, Sweden) and subsequently incubated with 1 mL of caprylic acid-treated antiserum against hazelnut pepsin digest. As a control, the antiserum was also incubated with 100 mg of glycine-inactivated Sepharose. Supernatant solutions were collected after centrifugation (1400*g*) at RT. Efficacy of depletion was monitored by birch pollen and hazelnut RAST. To this end, Sepharose-coupled hazelnut extract (1.5 mg Sepharose/test) or Sepharose-coupled birch pollen extract (0.5 mg Sepharose/test) was incubated overnight with serial serum dilutions of control or depleted antiserum in a total volume of 300 μL PBS/0.3% BSA/0.1% Tween 20 (PBS-AT). After the unbound material was washed away, IgG bound was quantified using ^{125}I-radiolabeled sheep anti-rabbit IgG using a 1260 Multigamma II Gamma counter (Wallac LKB, Turku, Finland). Depletion resulted in a >99% decrease in reactivity with birch pollen extract, and reactivity to hazelnut extract was not significantly changed.

Biotinylation of the Detecting Antibody. Rabbit antibodies against hazelnut extract were biotinylated using sulfo-NHS-LC-biotin (Pierce) according to the manufacturer's instructions. A molar ratio of 1 to 450 was used for rabbit antibody and biotin, respectively.

Sandwich ELISA. Rabbit IgG antibodies directed to hazelnut pepsin digest were used as capturing antibodies. Step 1: NUNC-Immuno Plate Maxisorp microtiter plates (NUNC, Roskilde, Denmark) were coated with 1.1 μg of IgG (125 μL/well) in 0.1 mol/L $NaHCO_3$, pH 8.5 (coating buffer), overnight at 4 °C. Between each step, the plates were washed automatically with PBS/0.05% Tween-20. Step 2: The plate was subsequently blocked for 30 min with 150 μL/well 1% semiskimmed milk in coating buffer. Step 3: The plates were then incubated for 1 h with 100 μL/well of serial dilutions of reference or sample protein. The samples were diluted in conjugate buffer, consisting of 0.2%

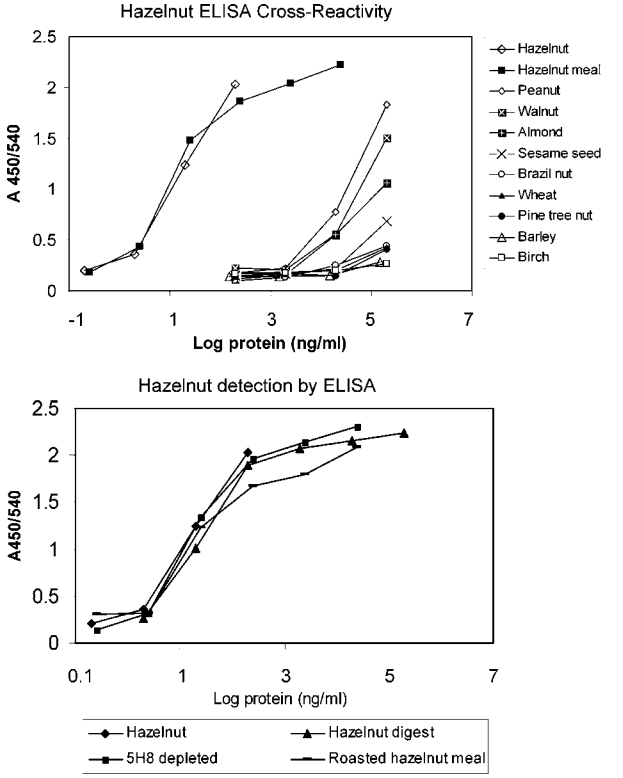

Figure 1. (**A**) Sensitivity and specificity of the hazelnut ELISA were determined by extracting various foods and evaluating them using the ELISA. The hazelnut ELISA showed 0.034, 0.017, 0.008, 0.002, and 0.001% cross-reactivity for peanut, walnut, almond, sesame seed, and Brazil nut, respectively. All other food materials showed <0.001% cross-reactivity. (**B**) This ELISA was developed to specifically measure stable hazelnut proteins. Roasting, pepsin digestion, or depletion of Cor a 1 showed no significant decrease in signal as compared to crude hazelnut extract when evaluated in PBS.

(w/v) bovine gelatin (Brocades, Maarssen, The Netherlands)/0.1% (w/v) BSA (Serva, Heidelberg, Germany)/PBS/0.02% Tween 20 (v/v). Step 4: The bound hazelnut protein was detected using 100 μL/well biotinylated rabbit anti-hazelnut (1:800 diluted in conjugate buffer). Step 5: After 1 h, the plate was subsequently incubated with 100 μL of streptavidin-HRP (Amersham Biosciences, Buckinghamshire, United Kingdom) diluted 1:1000 in PBS. Step 6: After 30 min, the plate was incubated with 100 μL/well substrate buffer [0.11 mol/L sodium acetate, pH 5.5, 0.01% TMB (tetramethylbenzidine dissolved in dimethyl sulfoxide), 0.003% v/v H_2O_2] for 10 min at RT. Step 7: Color development was stopped by the addition of 100 μL of 2 mol/L H_2SO_4.

To reduce incubation times, steps 2−5 were performed at 37 °C. The absorbance was measured at dual wavelengths 450 and 540 nm in a microtiter plate reader. The absorbance at 540 nm was subtracted as background.

RESULTS

A Sensitive and Specific ELISA for Pepsin Stable Hazelnut Proteins. A sandwich ELISA for the measurement of hazelnut protein was developed, using rabbit IgG directed to pepsin-digested hazelnut extract as capturing antibodies and against undigested hazelnut extract as detecting antibodies. The detection limit of the assay was found to be 0.7 ng/mL of hazelnut protein extracted in PVPP buffer (**Figure 1A**). This detection limit has been based on determining the mean of 12 blanks plus three times the standard deviation on four consecutive days. The coefficient of variation was determined using five standard curves. The precision of the assay expressed by this coefficient was <5% in the range of 0.6−150 ng/mL.

Table 1. Hazelnut Content of Selected Commercial Hazelnut-Containing Food Products[a]

food product	labeled g/ 100 g food	measured g/ 100 g food	ratio measured/ labeled
hazelnut chocolate spread 1	13	9.3	0.72
hazelnut chocolate spread 2	4	8.8	2.20
hazelnut chocolate spread 3	13	14.3	1.10
hazelnut cookie	7	6.6	0.94

[a] Food products ($n = 4$) containing hazelnut were analyzed using ELISA. Results are expressed in g hazelnut per 100 g food. The average ratio of measured to labeled hazelnut content was 1.2.

The specificity of the ELISA was assessed by testing extracts of various plant foods ($n = 10$) and of birch pollen. The maximum cross-reactivity was observed for peanut (0.034%), walnut (0.017%), almond (0.008%), and sesame seed (0.002%). For Brazil nut, wheat, pine tree nut, barley, and birch pollen, less than 0.001% cross-reactivity was observed (**Figure 1A**).

To evaluate whether the ELISA was insensitive to food processing methods, a comparison was made between extracts of raw hazelnut meal and of roasted hazelnut meal. Roasting did not alter the reactivity in ELISA significantly (**Figure 1B**). The ELISA was also insensitive to extensive pepsin digestion of hazelnut extract (**Figure 1B**). Depletion of Cor a 1 from the hazelnut extract did not alter the reactivity by ELISA.

Detection of Hazelnut Protein in Food Matrixes. To initially assess the performance of the ELISA with respect to measuring hazelnut protein contained in food matrixes, three brands of hazelnut-containing chocolate spreads and a single brand of hazelnut cookies were evaluated using the ELISA. The hazelnut protein concentrations measured were converted to % hazelnut (w/w). The mean ratio of these percentages relative to the percentages listed on the food labels was 1.2 (see **Table 1**). For example, an indicated hazelnut concentration of 10% (v/v) and a measured hazelnut concentration of 12% (v/v) result in a ratio of 1.2. An explanation for a calculated mean ratio >1.0 might be due to the measurement of hazelnut protein in "hazelnut chocolate spread 2", which resulted in a 2.2 ratio measured/labeled hazelnut. This specific ratio could be due to incorrect labeling, nonrepresentative sampling, or incorrect hazelnut protein measurement.

To further assess the efficacy of hazelnut protein extraction recovery, hazelnut-in-chocolate standards manufactured under industrial conditions and chocolate spreads spiked with hazelnut meal were evaluated (**Table 2**). Reference hazelnut material from 0.5 to 100 μg hazelnut/g chocolate was recovered in milk chocolate between 53 and 120% (mean 97.3%).

When 10% (w/w) hazelnut meal was spiked into two different hazelnut chocolate spreads, 7.4 and 10.5% were the elevated concentrations calculated on the basis of ELISA evaluation, with a recovery of 81.4 and 103.2%, respectively. Taken together, the data from **Tables 1** and **2**, the ratio indicated vs measured concentration hazelnut varies from 0.4 to 2.2.

Foods Labeled as "May Contain Traces of Nuts". Twelve samples of European retail food products precautionary labeled with "may contain traces of nuts" (10 different chocolate bars and two batches of a single brand of cookies) were evaluated using the ELISA. In addition, two chocolate products not mentioning nuts as an ingredient on the label and two explicitly listing hazelnut were included in the analysis. In the two products without hazelnut on their labels, ELISA readings registered below the detection limit (**Table 3**). The hazelnut-containing chocolate samples showed 89992 and 21250 μg

Table 2. Milk Chocolate Standards Manufactured (According to Standard Production Recipes) to Contain Specific Amounts of Hazelnut Meal ($n = 8$) and Hazelnut Chocolate Spreads Spiked with Hazelnut ($n = 2$) Were Spiked with the Indicated Quantities of Hazelnut[a]

milk chocolate standards	hazelnut meal (μg/g food)	measured (μg/g food)	recovery (%)
1	0.0	0.0	
2	0.5	0.6	120
3	1.0	1.0	100
4	2.0	2.0	100
5	5.0	5.4	108
6	10.0	8.0	80
7	25.0	13.2	53
8	100.0	120.0	120

hazelnut-spiked chocolate spreads	labeled g/ 100 g food	measured g/ 100 g food	spiked 10 g/ 100 g food	measured g/ 100 g food	recovery (%)
1	10.5	4.0	14.0	11.4	81.4
2	13.0	5.5	15.5	16.0	103.2

[a] The average recovery of hazelnut protein from the chocolate standards was 97.3%. Hazelnut chocolate spreads spiked with 10 g/100 g food hazelnut showed an average recovery of 92.3%.

Table 3. Food Products ($n = 16$) Were Analyzed by ELISA for Hazelnut Protein[a]

		labeling			
sample	food description	may contain nuts	no hazelnut on label	hazelnut on label	measured (μg/g)
1	chocolate–strawberry 1	×			417
2	chocolate–strawberry 2	×			3.5
3	chocolate–diet	×			1.2
4	chocolate–white 1	×			3.7
5	chocolate–white with coconut	×			4.1
6	chocolate–marzipan	×			16.2
7	chocolate–semisweet	×			BLD
8	chocolate–pineapple	×			26.3
9	chocolate–milk	×			5
10	chocolate–cherry	×			22.8
11	cookie lot 1	×			BLD
12	cookie lot 2	×			BLD
13	chocolate–cranberry		×		BLD
14	chocolate–white 2		×		BLD
15	chocolate–nougat			×	>80000
16	chocolate–brittle			×	>21000

[a] BLD: Below the limit of detection. Results (mean values from duplicate analysis) are expressed in hazelnut (μg/g food).

hazelnut per g food, respectively. Both batches of cookies and one of the chocolate samples labeled as "may contain traces of nuts" gave readings below the detection limit. However, these samples could have had other tree nut residues in them, which would not have been detected by the hazelnut ELISA. The remaining nine chocolate samples all had traces of hazelnut, four of them exceeding 10 μg/g food (**Table 3**). The highest hazelnut concentration found was 417 μg/g food.

Sodium Dodecyl Sulfate/Polyacrylamide Gel Eelectrophoresis Immunoblotting. Pepsin digestion of hazelnut proteins (as compared to control PVPP extract) resulted in a decrease of antibody binding with respect to both intensity and specificity (**Figure 2**). Digestion of Cor a 1 was almost complete as was detected with mAb 5H8 and IgE from serum 2. Rabbit anti-hazelnut digest recognized stable proteins at ~10 and 25−65 kDa. Pepsin resistant IgE binding was observed at ~14 and 35−75 kDa by serums 1 and 3.

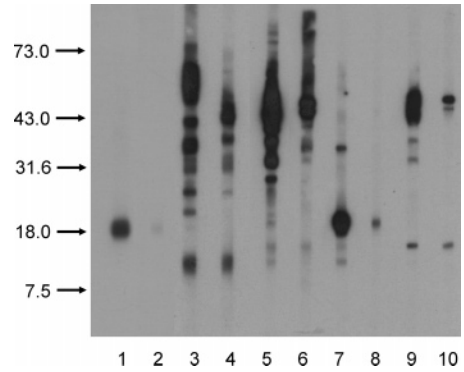

Figure 2. Blot strips of hazelnut PVPP extract (odd numbers) and pepsin-digested hazelnut PVPP extract (even numbers) were incubated with monoclonal 5H8 (lanes 1 and 2), rabbit anti-hazelnut pepsin digest (lanes 3 and 4), and human serums 1 (lanes 5 and 6), 2 (lanes 7 and 8), and 3 (lanes 9 and 10). Radiolabel controls RaM, sheep anti-rabbit, and sheep anti-human IgE were all negative (data not shown).

DISCUSSION

A novel approach to trace unintended protein contamination of food products was successfully developed. The described sandwich ELISA for hazelnut protein can be applied to evaluating the allergenic risk of undeclared hazelnut residues in foods. The novelty of this immunoassay lies in its focus on measuring stable proteins. The measurement of stable proteins is important because many food products undergo some sort of processing. After processing, the major hazelnut allergen Cor a 1 (*38*) is rapidly degraded while immunoreactive stable allergens such as LTP (Cor a 8) (*16*) or vicilin (Cor a 11) (*13*) could still be present. Immunoblotting experiments showed antibody binding to pepsin resistant hazelnut proteins at Cor a 8 and Cor a 11 molecular weight levels, respectively (**Figure 2**), while antibody binding to Cor a 1 was abolished.

For tracing contamination of food products with undeclared allergenic residues, immunoassays should therefore measure processing insensitive proteins as markers for contamination. By applying rabbit antibodies directed to pepsin-digested hazelnut extract, such focus on stable proteins was accomplished. Another justification for the approach is that food allergens with the potential to cause severe food allergy are generally known to fall in the category of pepsin resistant proteins (*36*).

Cross-reactivity of the assay is limited due to the depletion for birch pollen cross-reactive antibodies. The highest degree of cross-reactivity was observed for peanut (0.034%). Because the focus of the ELISA was on pepsin resistant proteins such as the 2S (*39*), 7S, and 11S seed storage proteins and LTPs that could also be present in peanut extract, cross-reactivity could not completely be prevented. Because of this crossreactivity, it cannot be ruled out that a level of hazelnut contamination of ~3.4 μg/g chocolate is in fact (partly) due to 1% peanut contamination (*40*, *41*). However, additional adsorption of the antiserum against peanut could remove this residual cross-reactivity and this approach is being investigated.

The ELISA detection limit for measuring hazelnut protein in hazelnut PVPP extract was 0.7 ng hazelnut protein/mL. Efficient solubilization of hazelnut protein present in food products is essential to reach sensitivities in the range of 1−2.5 μg hazelnut/g food (considered appropriate for allergenic residue

monitoring). Indeed, the described ELISA could detect the lowest manufactured hazelnut-in-milk chocolate, 0.5 μg hazelnut/g chocolate.

Unwanted contamination of food products, due to incoming raw materials or other causes, can, despite changes in equipment design and/or extra cleaning, not always be prevented. Therefore, labeling of food products with the phrase "may contain traces of nuts" is still common practice for many food producers. This study has shown that 4/12 products labeled in this way indeed contained significant amounts of hazelnut, ranging from 10 to 400 μg hazelnut/g food.

While there may be occasions where use of this labeling is prudent, widespread use of this type of labeling should be avoided if possible, because it causes a lot of confusion among food allergic patients. Labeling foods with true levels of all ingredients, as could be determined by using international approved standards, is preferable.

Specific immunoassays such as the one reported here would be useful to the food manufacturer in evaluating finished products and raw materials and to assess the effectiveness of sanitation and scheduling strategies in the development of an allergen control program. This analytical technique would be useful in developing risk assessment strategies for dealing with allergens in food production and manufacturing environments. In addition, this technique would be useful to food regulatory agencies in investigating hazelnut allergic consumer complaints. It is very important that immunochemical methods for allergenic food residues are validated using in-house reference standards (such as the hazelnut-in-milk chocolate standards used in this study) made in actual industrial or pilot plant settings to ascertain the true extraction efficiencies, usefulness, and robustness of the method. However, each food matrix permits a unique recovery of hazelnut after extraction and therefore still needs its own in-house reference standards (*30*).

This newly developed ELISA overcomes the problem of decreased sensitivity of the measurement of hazelnut after the processing of food. What remains is the development of improved extraction methods and standards that could be used for a wider range of hazelnut-containing food stuffs.

ABBREVIATIONS

mAb, monoclonal antibody; nsLTP, nonspecific lipid transfer protein; OAS, oral allergy syndrome; PBS-AT, phosphate-buffered saline/bovine serum albumin (0.3% v/v)/Tween 20 (0.2% v/v); RaM, Rat anti-mouse; RT, room temperature; TMB, 3,3′,5,5′-tetramethylbenzidine.

ACKNOWLEDGMENT

We acknowledge Julie Nordlee, Lynn Niemann, and Debra Lambrecht of the University of Nebraska and Barry-Callebaut, St. Hyacinthe, Quebec, Canada, for manufacturing of the hazelnut-in-chocolate standards.

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Received for review May 6, 2004. Revised manuscript received September 10, 2004. Accepted September 22, 2004. This work was supported (in part) by a grant from the Allergy and Immunology Institute of the International Life Sciences Institute Research Foundation (Washington DC). The opinions expressed herein are those of the author(s) and do not necessarily represent the views of ILSI. This research was also financially supported by the Food Allergy Research and Resource Program of the University of Nebraska, Lincoln, NE, a contribution of the University of Nebraska Agricultural Research Division, Lincoln, Nebraska; Journal Series 14550.

JF049278R