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Sandwich Immunoassays for the Determination of Peanut and Hazelnut Traces in Foods

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People suffering from food allergies are dependent on accurate food labeling, as an avoidance diet is the only effective countermeasure. Even a small amount of allergenic protein can trigger severe reactions in highly sensitized patients. Therefore, sensitive and reliable tests are needed to detect potential cross-contamination. In this paper two fast sandwich immunoassays are described for the determination of peanut (*Arachis hypogaea*) and hazelnut (*Corylus avellana*) traces in complex food matrices. Mouse monoclonal antibodies were used as capture antibodies, and labeled rabbit polyclonal antibodies were used as detection antibodies in both assays. The assay time was 30 min in total, and cross-reactivities against a variety of fruits and seeds were found to be in the low 10^{-4}% (ppm) level or in some cases not detectable. The recoveries in all tested food matrices ranged from 86 to 127%, and the limits of detection were in the range of 0.2−1.2 mg/kg (ppm) in food for both peanut and hazelnut, respectively.

KEYWORDS: Peanut; *Arachis hypogaea*; hazelnut; *Corylus avellana*; food allergy; hidden allergens; enzyme immunoassay; ELISA; Ara h1; Ara h2; immunoanalytical methods; immunochemistry

INTRODUCTION

The number of patients suffering from food allergies is constantly rising, especially in industrialized countries. In the United States, ∼3.7% of the adult population is affected, and among children the rate is up to 6% (*1*, *2*). Peanuts and tree nuts are consumed in high quantities, especially as ingredients in a variety of complex food products. Unfortunately, they are also known to contain very potent allergenic proteins and may provoke reactions in sensitized patients making up ∼1.1% of the population (*3*−*6*). Ara h1 and Ara h2 were identified as major peanut allergens, being recognized by IgE from >95% of peanut-sensitized patients. These allergens were characterized as 64.5 and 17.5 kDa glycoproteins belonging to the vicilin and conglutin families of seed storage proteins. Proteins of 18, 32, 35, and 47 kDa were identified as major hazelnut allergens, and the 18 kDa protein was found to be homologous to Bet v1 (*7*, *8*). Avoidance of allergen-containing food as the only measure for many patients is often limited by insufficient consumer knowledge on food compositions or accidental contaminations, for example, in food factories or via shared utensils during food preparation. Particularly, products in Asian style restaurants, bakeries, and ice cream parlors were reported as possibly containing hidden allergens (*9*, *10*). For the analysis of potentially contaminated food, either proteins or DNA fragments indicating the presence of allergens are used as targets. Detection of DNA after amplification via Polymerase Chain Reaction (PCR) is established in routine analysis. Widely known immunochemical techniques for the analysis of proteins are radio-allergosorbent tests (RAST), enzyme-allergosorbent tests (EAST), rocket immunoelectrophoresis (RIE), immunoblotting, and enzyme-linked immunosorbent assays (ELISA) (*11*−*14*). Similar methods are used for allergy diagnostic purposes. Therefore, binding of a patient's IgE to known allergen targets is investigated. Recently, a microarray-based method for the screening of allergen-specific IgE was described (*15*). Several ELISAs for the detection of peanut or hazelnut traces in foods have been published or are commercially available (*16*−*20*). However, the assay duration of most protocols is several hours,

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or the tests are not validated sufficiently. The aim of this study, therefore, was to develop and validate novel rapid ELISAs suitable for laboratory-based routine analysis.

MATERIALS AND METHODS

Standards and Food Samples. Standard peanut butter as peanut reference material (SRM 2387) was obtained from the National Institute of Standards and Technology (NIST, Gaithersburg, MD). Roasted hazelnut samples were provided by R. Fila from Masterfoods, Breitenbrunn, Austria. Cross-reactivity testing materials and food samples were bought at local stores and ground with an IKA A10 laboratory mill (IKA Werke, Staufen, Germany) before extraction.

Artificial Chocolates as Blank Material. Chocolates available in local stores have been shown to contain traces of nuts in almost all cases. Hence, the preparation of artificial chocolates was advisable. All of the ingredients were screened for peanut and hazelnut contaminations prior to use.

As artificial matrices, dark chocolate, consisting of 50% cocoa mass, 45% sugar, 4.5% cocoa butter, and 0.5% lecithin, and milk chocolate, consisting of 30% cocoa mass, 35% sugar, 14.5% cocoa butter, 20% skim milk powder, and 0.5% lecithin, were chosen. The calculation of the recipe was carried out by assuming a content of 55% cocoa butter in cocoa mass and a content of 20% cocoa butter in deoiled cocoa powder. Hence, for the production of dark chocolate 45% of sucrose (Südzucker, Mannheim, Germany) was ground to a fine powder with the IKA A10 laboratory mill. Successively, 28% of deoiled cocoa powder (Bensdorp, Barry Callebaut, Norderstedt, Germany), 0.5% of lecithin (Spinnrad, Certus Handels GmbH, Norderstedt, Germany), and 26.5% of cocoa butter (Spinnrad) were added, and the mixture was homogenized thoroughly. Milk chocolate was simulated by homogenization of 35% sucrose, 16.5% of cocoa powder, 20% of skim milk powder (Fluka 70166, Buchs, Switzerland), 28% of cocoa butter, and 0.5% of lecithin. The mixtures were further processed for 24 h at 50 °C in a drying oven and stirred occasionally (*21*). For storage of the chocolates, a cool and dark place was chosen.

Materials and Instrumentation. Water for all solutions was taken from a Milli-Q plus 185 purification system (Millipore, Schwalbach, Germany). Phosphate-buffered saline (PBS; pH 7.6) consisted of 10 mM KH_2PO_4 (Merck 1.12034, Darmstadt, Germany), 70 mM K_2HPO_4 (Merck 1.05104), and 145 mM NaCl (Merck 1.06404). Substrate buffer contained 200 mM monobasic potassium citrate (Fluka 60214) and 0.01% potassium sorbate (Sigma S1751, Steinheim, Germany). Washing buffer concentrate consisted of 60 mM KH_2PO_4, 420 mM K_2HPO_4, 900 mM NaCl, and 3% Tween 20 (Merck 8.17072) and was diluted 1:60 before use. TMB stock solution contained 50 mg of 3,3′,5,5′-tetramethylbenzidine (TMB, Merck 1.08622) in 4 mL of dimethyl sulfoxide (Merck 1.16743). Substrate solution was prepared freshly by adding 240 μL of TMB stock solution and 80 μL of a 1% H_2O_2 solution (Merck 1.08597) to 20 mL of substrate buffer. Stop solution consisted of sulfuric acid (1.00731, Merck), diluted to 5%. Centrifugal devices were purchased from Pall Life Sciences (3 kDa molecular weight cutoff, Microsep 3k Omega, Ann Arbor, MI). Ninety-six-well, flat-bottom polystyrene ELISA microplates were obtained from Greiner (Microlon high-binding capacity 655061, Frickenhausen, Germany). Parafilm M (American National Can, Chicago, IL) was used for sealing the microplates.

Polyclonal and monoclonal anti-peanut and anti-hazelnut antibodies were obtained from CSL (York, U.K.) and RIKILT (Wageningen, The Netherlands) and were stored in 50% glycerol (Alfa Aesar 032450, Karlsruhe, Germany) at −18 °C until used. The antibodies Y70 (CSL) and 50-6B12 (RIKILT) both are monoclonal mouse IgG_1, and R695 (CSL) and R698 (CSL) both are rabbit polyclonal antibodies. For immunizations, Tris-buffered saline extracts (20 mM Tris, 1 M NaCl, pH 7.4) of roasted peanuts (Y70, R695) and extracts of roasted hazelnuts (50-6B12, R698) were used. Monoclonal antibodies were purified using protein G columns, and the processing of the polyclonal antibodies was done by ammonium sulfate precipitation.

In the peanut assay, the mouse monoclonal antibody Y70 was used as capture antibody and the rabbit polyclonal antibody R695 as detection antibody. In the hazelnut assay, the capture antibody was the monoclonal antibody 50-6B12 and the detection antibody was the rabbit polyclonal antibody R698. The respective antibody pairs were selected from 64 monoclonal and 28 polyclonal antibodies by several screening steps (not shown).

Dispensing of all the solutions into the microplate wells was done with an eight-channel pipet (Brand, Wertheim, Germany). Washing of microplates was performed three times with 400 μL in the overflow mode using a Columbus eight-channel washer (SLT, Crailsheim, Germany). Incubation of microplates was performed on a microplate shaker at a frequency of 500 × 1/min (Easyshaker EAS 2/4, SLT). Absorbances were measured at 450 nm using a 340 ATTC microplate reader (SLT).

Spiking Procedure. Spiking solutions were prepared by homogenizing peanut/hazelnut material with carboxymethylcellulose solution (*22*). Two grams of standard peanut butter (containing 90% roasted peanuts) and 2 g of hazelnut material each were blended with 18 g of sucrose to obtain a 1:10 blend and stored at −18 °C until used. Carboxymethylcellulose sodium salt (12 g, medium viscosity 21902, Fluka) was dissolved in 600 mL of water. Subsequently, 6 g of sodium azide (S2002, Sigma) and 1.5 g of bovine serum albumin (A3059, Sigma) were added. The peanut sucrose mixture (778 mg) and the hazelnut sucrose mixture (700 mg) were added to 70 mL of the carboxymethylcellulose solution and shaken thoroughly to obtain a 1 g/L concentration. The solution was diluted 1:10 with PBS, and an appropriate amount was added to 1 g of a ground food matrix weighed into a 50 mL polypropylene tube (62.548.004, Sarstedt, Nürnbrecht, Germany). Chocolate and ice cream were melted prior to spiking.

Extraction of Samples and Standards. Extraction buffer concentrate was obtained from R-Biopharm (RIDASCREEN Allergen extraction buffer, Darmstadt, Germany) and diluted 1:20 before use. The sample (5 g) was ground and mixed thoroughly, and 1 g was weighed into a 50 mL polypropylene tube and in the case of the chocolate-containing samples, 1 g of skim milk powder was added. Extraction buffer (20 mL) was added at a temperature of 60 °C, and the mixture was shaken vigorously. Extraction was performed at 60 °C in a water bath for 20 min. The mixtures were transferred in a 1.7 mL reaction tube (Roth 7080.1, Karlsruhe, Germany) and centrifuged at 17000 rpm and 20 °C using a Biofuge 28 RS (Heraeus, Hanau, Germany). Finally, the extracts were filtered using 0.45 μm syringe filters (Merck 512.2111).

For the preparation of the peanut standard solution, 2 g of the peanut butter/sucrose blend was extracted similarly with 20 mL of extraction buffer, resulting in a stock solution of 9 g/L whole peanut. A hazelnut standard solution was obtained by extracting 2 g of the hazelnut/sucrose blend with 20 mL of extraction buffer, yielding a concentration of 10 g/L whole hazelnut. The standards were aliquoted and stored at −18 °C.

Enzyme Labeling of Polyclonal Antibodies. Glycerol was removed from the antibodies using a PD-10 desalting column (Amersham Biosciences, Freiburg, Germany), following the manufacturer's instructions with PBS as chromatography buffer. The antibodies were concentrated to roughly 1 g/L using a centrifugal device in a Universal 30F centrifuge at 5000 rpm (Hettich, Tuttlingen, Germany). Conjugation of the antibodies with horseradish peroxidase (HRP) was done via periodate oxidation of the enzyme (*23*). HRP (814939, Roche, Basel, Switzerland) was dissolved at a concentration of 15 g/L in PBS. Ten microliters of a fresh sodium metaperiodate solution in water (88 mM, 1.06597, Merck) was added to 100 μL of HRP solution. The mixture was incubated for 20 min in the dark and afterward loaded immediately on a PD-10 column. Elution was done using PBS, and the sharp peak of HRP was collected with visual control. Antibody and activated HRP solutions were mixed in an equal mass ratio in a Microsep centrifugal device and incubated for 2 h at 2000 rpm, resulting in a concentration of roughly 2-fold. A 5 M sodium cyanoborohydride (71435, Fluka) solution was prepared in 1 M NaOH (1.06498, Merck), and 5 μL was added to 500 μL of the reaction solution. After 30 min of reaction time in the centrifuge at 1000 rpm, a 1 M Tris solution (25 μL, 154563, Aldrich) in water was added to the reaction solution. After another 30 min of reaction, the conjugate was purified using a PD-10 column and concentrated using a Microsep device to not less than 1 g/L. The conjugates were stored in 1% BSA and 50% glycerol until used.

Table 1. Cross-Reactivity Levels of Nuts and Seeds in Peanut and Hazelnut ELISA[a]

sample	cross-reactivity in peanut ELISA (%)	cross-reactivity in hazelnut ELISA (%)
peanut	**100**	$<5 \times 10^{-5}$
hazelnut	5×10^{-4}	**100**
almond	1.5×10^{-4}	1.2×10^{-4}
brazil nut	8×10^{-5}	$<5 \times 10^{-5}$
cashew	$<5 \times 10^{-5}$	1.2×10^{-4}
chickpea	8×10^{-5}	$<5 \times 10^{-5}$
coconut	1.1×10^{-4}	$<5 \times 10^{-5}$
green pea	8×10^{-5}	$<5 \times 10^{-5}$
lentil	1.6×10^{-4}	$<5 \times 10^{-5}$
macadamia	7×10^{-4}	1.1×10^{-4}
moth bean	$<5 \times 10^{-5}$	$<5 \times 10^{-5}$
pecan	8×10^{-5}	$<5 \times 10^{-5}$
pine nut	8×10^{-4}	$<5 \times 10^{-5}$
pistachio	1.8×10^{-4}	$<5 \times 10^{-5}$
pumpkin seeds	1.5×10^{-4}	$<5 \times 10^{-5}$
red bean	$<5 \times 10^{-5}$	$<5 \times 10^{-5}$
sesame	5×10^{-5}	$<5 \times 10^{-5}$
soy	2×10^{-4}	7×10^{-5}
sunflower seeds	1.3×10^{-4}	$<5 \times 10^{-5}$
walnut	$<5 \times 10^{-5}$	1.2×10^{-3}
white bean	1.2×10^{-4}	$<5 \times 10^{-5}$

[a] Values represent the mass ratios of whole peanut or whole hazelnut to cross-reactive matter generating equivalent signals ($n = 2$; 1 ppm = 10^{-4}%).

ELISA. Microplates were coated with monoclonal peanut or hazelnut antibodies diluted to 2.7 mg/L in PBS (100 μL per well) and incubated for 3 h at room temperature. The plates were sealed during all incubation steps with Parafilm. After washing, the plates were blocked with 200 μL per well PBS containing 3% skim milk powder for 2 h. After another washing step, 100 μL of a serial dilution of the peanut or hazelnut standard in extraction buffer and 100 μL of the sample extracts were pipetted quickly. The plates were incubated for 10 min and washed afterward; 100 μL/well of enzyme conjugate according to an antibody concentration of 0.8 mg/L in 1% skim milk powder containing PBS was added and incubated for 10 min. After washing, 100 μL/well of a freshly prepared substrate solution was added. Color development was stopped after 10 min by the addition of 100 μL/well stop solution. Data were evaluated using Origin 6.0 software (Microcal, Northampton, MA), and calibration curves were fitted using a four-parameter logistic (sigmoidal) function

$$y = \frac{(a - d)}{1 + (x/c)^b} + d \quad (1)$$

where x = concentration whole peanut/hazelnut (μg/L), y = absorbance at 450 nm, a = minimum absorbance (lower asymptote), b = slope parameter, c = inflection point (μg/L), and d = upper asymptote.

RESULTS

Assay Selectivities. Various nuts and seeds were selected for cross-reactivity studies, namely, almond, brazil nut, cashew, chickpea, coconut, green pea, lentil, macadamia, moth bean, pecan, pine nut, pistachio, pumpkin seeds, red bean, sesame, soy, sunflower seeds, walnut, and white bean. In the peanut assay also hazelnut was tested and vice versa (**Table 1**). Extracts were obtained according to the extraction protocol and measured undiluted in duplicate. In the case of a measurable cross-reactivity $>2 \times 10^{-4}$% (2 ppm), the extract was diluted 1:500 with extraction buffer to obtain a concentration of 100 ppm and the measurement was repeated. In the peanut assay, all extracts showed cross-reactivities of $<2 \times 10^{-4}$% (2 ppm) except macadamia, pine nut, soy, and hazelnut. At a 100 ppm level, the cross-reactivities were 7×10^{-5}% (0.7 ppm) for macadamia and $<5 \times 10^{-5}$% (0.5 ppm) for pine nut, soy, and hazelnut, respectively. Selectivities in the hazelnut ELISA proved to be even better, with only walnut showing a cross-reactivity of $>2 \times 10^{-4}$% (2 ppm). The cross-reactivity of the diluted walnut extract was found to be $\sim 7 \times 10^{-5}$% (0.7 ppm).

Table 2. Intra- and Inter-assay Variance Data in Peanut and Hazelnut ELISA (Intra-assay, $n = 7$; Inter-assay, $n = 21$)

sample	concn of whole peanut/ hazelnut	peanut ELISA		hazelnut ELISA	
		intra-assay CV (%)	inter-assay CV (%)	intra-assay CV (%)	inter-assay CV (%)
standard	100 μg/L	13.8	10.7	7.7	5.7
		5.5		4.8	
		3.6		4.4	
standard	300 μg/L	2.9	12.8	6.7	7.3
		14.6		3.8	
		4.4		1.9	
standard	1000 μg/L	6.6	7.0	7.2	10.5
		7.6		3.4	
		3.6		1.3	
standard	3000 μg/L	2.6	4.3	5.2	10.4
		5.1		4.4	
		2.9		10.0	
dark chocolate	250 μg/L (5 mg/kg)[a]	8.6		6.3	
		10.2	12.4	5.9	4.9
		8.0		2.1	
dark chocolate	500 μg/L (10 mg/kg)[a]	6.4		4.7	
		4.5	11.5	3.3	5.1
		9.5		4.1	
ice cream	250 μg/L (5 mg/kg)[a]	3.0		5.3	
		3.6	7.2	3.4	7.2
		7.8		1.2	
ice cream	500 μg/L (10 mg/kg)[a]	5.3		4.5	
		2.3	7.5	2.6	10.3
		4.5		1.4	

[a] The concentration of whole peanut/hazelnut in the extract, expressed as μg/L, corresponds to spiking level of food, expressed as mg/kg, based on an extraction proportion of 1 g of sample with 20 mL of extraction buffer according to the extraction protocol.

Inter-assay and Intra-assay Variance. Peanut and hazelnut standards and extracts of several spiked food matrices were measured in seven replicates in peanut and hazelnut assay, respectively. In the case of inter-assay variation, standard deviations (SD) and coefficients of variation (CV) were calculated on the basis of results on one plate and, in the case of inter-assay variation, results of three different plates were combined (**Table 2**). The peanut ELISA showed a high repeatability for all standards and for all samples as well with coefficients of variation of <15% in both intra- and inter-assay variance. Data for hazelnut ELISA displayed a maximum CV of 10% in intra-assay variance and <11% in inter-assay variance.

ELISA Standard Curve. Characteristic calibration curves showing the means and standard deviations were derived for the peanut and the hazelnut ELISA (**Figure 1**). All standards were measured in eight replicates, and results showed to be reproducible on different days. Detection limits were evaluated by calculation of the concentration according to the blank absorbance plus 3 times the standard deviation. For the peanut ELISA, the limit of detection (LOD) without food matrix was found at 7 μg/L whole peanut, theoretically corresponding to a sample contaminated with 0.14 mg/kg whole peanut. The matrix-free detection limit of the hazelnut ELISA turned out to be at 6 μg/L, according to a theoretical sample contamination of 0.12 mg/kg.

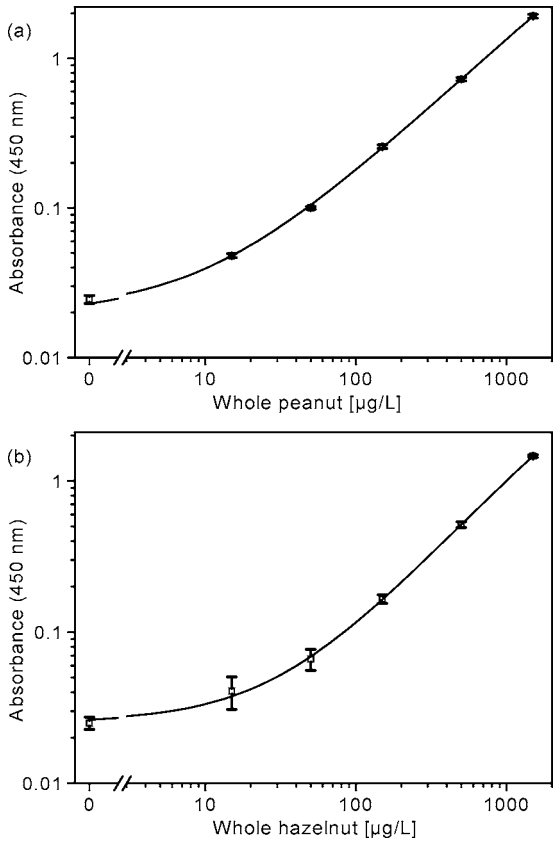

Figure 1. Characteristic ELISA standard curve (a) for the peanut ELISA, (b) for the hazelnut ELISA. Mean values are shown and standard deviations as error bars, respectively ($n = 8$).

Table 3. Recoveries of Peanut and Hazelnut from Several Spiked Food Matrices ($n = 2$)

	conc	peanut ELISA		hazelnut ELISA	
sample	(mg/kg)	recovery (%)	CV (%)	recovery (%)	CV (%)
cookie	1	107	13.9	127	3.2
	1	106	11.1	118	1.7
	5	98	3.1	95	4.6
	5	99	0.6	102	3.7
	10	92	1.0	92	1.4
	10	93	1.1	100	6.4
cereals	1	105	4.5	100	5.4
	1	105	1.8	106	4.2
	5	114	2.0	95	2.1
	5	117	1.0	101	1.1
	10	112	1.6	95	0.7
	10	112	2.0	99	2.0
ice cream	1	110	8.7	108	2.3
	1	108	8.9	111	3.2
	5	94	2.3	93	3.0
	5	98	1.5	99	1.4
	10	97	1.6	95	0.9
	10	96	0.8	94	1.8
milk chocolate	1	113	3.0	<LOD	
	1	118	2.3	<LOD	
	5	123	2.7	109	2.6
	5	120	0.8	115	1.8
	10	114	2.0	95	2.6
	10	115	2.1	96	1.7
dark chocolate	1	92	2.5	86	6.2
	1	101	1.8	94	3.8
	5	89	1.1	94	3.4
	5	92	1.0	96	2.6
	10	87	1.4	91	2.5
	10	89	1.2	94	2.8

Sensitivity. LODs were studied in both assays for butter cookies, breakfast cereals, vanilla ice cream, milk chocolate, and dark chocolate. Ten blank samples were extracted, and extracts were measured in duplicate. LODs were calculated on the basis of the statistical scatter of the mean values of the blank matrix. Standard deviations were added three times to the value of the lower asymptote of the standard curve or to the mean matrix blank in case the latter was higher. LODs were defined as the standard concentrations corresponding to the absorbance values obtained.

The LOD for peanut in cookies was determined to 0.2 mg/kg, that in cereals and vanilla ice cream to be 0.4 mg/kg, that in dark chocolate to be 0.2 mg/kg, and that in milk chocolate to be 0.8 mg/kg. For hazelnut in cookie, the LOD was 0.4 mg/kg, that in cereals, 0.6 mg/kg, that in ice cream, 0.2 mg/kg, that in dark chocolate, 0.8 mg/kg, and that in milk chocolate, 1.2 mg/kg.

Recovery Studies. Several commercial food samples and artificial chocolates were spiked at different hazelnut and peanut levels, extracted, and measured in duplicate (**Table 3**). Recoveries for both peanut and hazelnut were calculated as the quotients of concentrations determined to the concentration estimated resulting from the spiking level. The recoveries using butter cookies as matrix ranged from 91 to 107% in the peanut assay and from 91 to 127% in the hazelnut assay, whereas the slight overestimations were observed only in the lowest spiking level. In breakfast cereals 95−117% of the peanut and hazelnut content were recovered. Spiked ice cream also yielded good results in the recovery study, with a range from 93 to 111%. Although chocolates are considered to be one of the most challenging matrices with regard to peanut and tree nut recovery, the recoveries were in the range from 95 to 123% for the milk chocolate and from 86 to 100% for the dark chocolate, respectively. The coefficients of variation in most cases were <10%, often even <5%.

Analysis of Commercial Food Products. Various commercial food samples including several chocolates, cereals, and cookies were investigated for the presence of peanut and hazelnut proteins (**Table 4**). Each product was homogenized before weighing, and shared utensils such as the mill were cleaned thoroughly each time to avoid cross-contaminations. To improve the reliability, every food sample was extracted twice. The deviation of the two mean concentrations was calculated as the CV, and when the CV exceeded 15%, both extractions and measurements were repeated. Although varying only little, LODs were determined on each microplate separately. To minimize false positive results, the detection of peanut or hazelnut was considered to be positive only if the found concentration exceeded both the plate-specific (matrix-free) and matrix-specific LOD. Also, the CVs of the single values were taken into account: typically higher CVs were observed at concentrations approaching the LOD.

In all food samples where peanut or hazelnut was mentioned on the ingredients list, the respective analyte was found. Products without labeling peanut or hazelnut either on the ingredients list or as potential contaminant in most cases indeed were found to be negative. However, some of them contained traces in the low parts per million range, and hazelnut contamination was found more often than peanut contamination. The highest finding in undeclared food was nearly 0.6% for hazelnut in a milk chocolate. All products analyzed by us and labeled as possibly containing peanut were found to be negative, whereas most possibly containing hazelnut products indeed proved to contain hazelnut. All results were regarded as reliable as the results of

Table 4. Analysis of Various Commercial Food Samples for Peanut and Hazelnut Using Sandwich ELISAs ($n = 2$)

	peanut			hazelnut		
sample	declaration[a]	found (mg/kg)	CV (%)	declaration[a]	found (mg/kg)	CV (%)
milk chocolate, manufacturer I	±	<LOD		±	1.5	6.8
dark chocolate, manufacturer I	±	<LOD		±	50	8.5
dark chocolate, manufacturer II	±	<LOD		±	1200	7.5
milk chocolate, manufacturer II	±	<LOD		±	1100	6.3
milk chocolate, manufacturer III	–	1.1	1.9	–	5800	1.7
dark chocolate, manufacturer III	–	5.9	0.6	–	630	3.0
milk chocolate, manufacturer IV	±	<LOD		+	7100	0.8
butter cookie	–	<LOD		–	<LOD	
breakfast cereals	±	<LOD		–	<LOD	
candy, coconut almond	–	<LOD		–	<LOD	
fruit granola	±	<LOD		±	0.8	8.6
chocolate bar, caramel candy	±	<LOD		–	<LOD	
chocolate bar, candy cream	±	<LOD		–	<LOD	
cookie, milk cream	–	0.8	6.3	–	87	2.2
wafer, lemon cream	–	<LOD		–	<LOD	
wafer, milk and hazelnut	–	<LOD		+	59000	2.2
cookie, chocolate filling	–	<LOD		+	550	0.6
candy, milk hazelnut	–	<LOD		+	150000	1.2
cookies, chocolate containing	±	<LOD		±	5.0	2.1
toffee	–	<LOD		–	<LOD	
cookie stick, chocolate coated	±	<LOD		±	<LOD	
chocolate hazelnut spread	–	<LOD		+	66000	3.7
chocolate bar, milk cream filling	–	<LOD		–	<LOD	
marzipan bar, chocolate coated	–	<LOD		–	59	1.5
nougat bar	–	<LOD		+	210000	1.4
wafer, milk cream	±	<LOD		±	<LOD	
chocolate bar, cereals	–	<LOD		–	<LOD	
wafer, nougat chocolate	–	<LOD		+	29000	0.8
cookie, chocolate coated	±	<LOD		±	1.7	1.3
chocolate bar, peanut	+	68000	1.4	–	<LOD	
wafer, hazelnut chocolate	±	<LOD		+	53000	1.6
cereal bar, yogurt and berries	±	<LOD		±	1.0	5.8

[a] Declaration on food packaging: +, peanut/hazelnut declared as ingredient; ±, may contain peanut or hazelnut; –, no declaration.

separate sample preparation were consistent and all CVs of the positive tested foods were <9%.

DISCUSSION

IgE-mediated allergies are a health issue of rising relevance, especially in industrialized countries. Symptoms after getting into contact with the allergen may be of mild character such as sneezing or atopic dermatitis but also may be severe such as anaphylaxis, mainly depending on the degree of sensitization and the amount of allergen. Food-allergic patients are advised to follow avoidance diets facing restrictions with regard to food in restaurants or industrially processed food. In most food factories many different products are manufactured with various ingredients; sometimes they even run on the same production line. Under such conditions it is practically impossible to eliminate the risk of cross-contamination completely, when products containing and not containing the particular ingredient are processed in the same building. A zero-threshold therefore cannot be guaranteed. Shared utensils are considered to be critical as well as raw materials. To meet demands of producing safe food for allergic patients, risk assessment is required, including the analysis of products and raw materials at regular intervals as part of quality management. The laboratory-based ELISA presented in this paper was developed to offer a tool for routine analysis with the focus particularly on rapidity and high sensitivity.

To estimate threshold doses for allergenic substances, double-blind, placebo-controlled food challenges are the method of choice. Numerous studies have been published dealing with peanut, and the lowest provoking doses were found to be in the low milligram range of whole peanut. Threshold doses for hazelnut, however, are more difficult to find in the literature. However, the order of magnitude seems to be similar. It was reported that in one case a dose of 6 mg of hazelnut in chocolate led to allergic reactions that required medication (*24*, *25*). On the basis of these values, the ELISAs presented here are of adequate sensitivity. The LODs range from 0.2 to 1.2 mg/kg (ppm) whole peanut/hazelnut in food. No severe reactions are expected after the consumption of food at this contamination level. Our tests also meet the general agreement that the detection limit for allergens in food products should be between 1 and 100 mg/kg (*12*).

Peanut butter from NIST was chosen as reference material for availability and comparability reasons. Unfortunately, no such standard is available for hazelnut. Therefore, hazelnut samples as standard material were obtained from the food industry. Peanuts and hazelnuts processed in the food industry are roasted in most cases. In several studies it was shown that roasting conditions and peanut/hazelnut varieties affect the extractable protein content and the immunoanalytically recognized antigen content. The maximum range may vary about the factor 2−3. In highly roasted peanuts and hazelnuts, the amount of detectable protein decreased very strongly in both ELISAs and in methods measuring the total protein content. However, these samples already looked and tasted apparently overroasted (*16*, *17*, *26*). The influence of natural variations and roasting conditions thus may lead to a certain degree of variation, which has to be borne in mind for the interpretation of results. To improve the accuracy, calibration with material suspected as contamination source is recommended, if possible. On the other hand, in most cases only qualitative or semiquantitative results may suffice.

All combinations of antibodies available to us were tested for the setup of the sandwich ELISAs (data not shown). Under our conditions it turned out that the sensitivity was generally higher when monoclonal antibodies were used as capture antibodies. This is not surprising and can be explained by the enrichment of one particular protein. Screening was performed to find matching pairs of antibodies with regard to highest sensitivity and minimal cross-reactivity. A combination of mouse monoclonal antibodies as capture antibody and rabbit polyclonal antibodies as detection antibody finally proved to be most suitable for both the peanut ELISA and the hazelnut ELISA, respectively. The selectivity of the peanut ELISA is quite high; cross-reactivities turned out to be in the 10^{-4}% (ppm) or even in the 10^{-5}% (100 ppt) range. Low cross-reactivities of 1.2×10^{-3}% (12 ppm) were detected for the hazelnut ELISA using walnut extract, but were not detectable for most other nuts and seeds. In summary, for the analysis of nearly all food samples, no problems with cross-reactivities are expected. In some very special cases, however, false positive results might possibly be obtained, for example, if traces of peanut in pine nut as matrix or traces of hazelnut in walnut as matrix should be detected. In such cases, matrix calibration is recommended to be carried out instead of external calibration, considering the higher blank value. In our studies, though, no false positive results were observed, neither, for example, in the peanut ELISA for a marzipan bar, containing almond as major component, nor for a coconut almond candy in the hazelnut ELISA.

Cookies, ice cream, dark chocolate, milk chocolate, and breakfast cereals were chosen as matrices for the validation of both assays. These foods are highly relevant in terms of possible peanut or tree nut contamination. Of course, there are other applications imaginable for our tests. In some kinds of yogurts, puddings, and soft cheese, hazelnut is contained and cross-contamination therefore is considered as possible under certain conditions. One could also think of applying the ELISAs for the analysis of instant soups or Chinese style food. However, for all of these matrices the reliability has to be ensured by individual validation to prevent false positive results due to high background effect or, even more important, to prevent false negative results due to insufficient recoveries.

For some food matrices it is difficult to get blank samples. Therefore, artificial chocolates had to be prepared in this study. To measure recoveries in complex foods, alternatively it is even possible to use contaminated samples, provided that the contamination level is in the lower range of the calibration curve (*17*).

After the selection of the most suitable antibodies, our assays were optimized with respect to high speed. The extraction time is 20 min, and the total assay duration is 30 min. Our ELISAs therefore are suitable for measuring a large number of samples at high throughput. As expected, we observed that the assay sensitivities can be improved by extending the incubation time of the sample and the detection antibody. Thus, if higher sensitivity is desired, we recommend extending both the sample and the detection antibody incubation times to each 30 min or even 60 min, by which the sensitivity may be increased up to 10-fold. In this context it also has to be mentioned that it is mandatory to keep the incubation times constant. The incubation time of the detection antibody and the substrate is considered to be less critical, because the solution is identical for all of the wells. In contrast, pipetting of the standards and samples should be finished within 1 min to avoid unacceptable deviations. If many different solutions have to be pipetted, it is advised to subdivide the microplate into two or three subsections and to process them one after another.

In summary, the ELISAs presented here offer new alternatives for efficient screening of foods possibly contaminated with peanut or hazelnut traces. On the basis of available data, the sensitivity is high enough to meet the needs of allergic patients. Both selectivity and recoveries for all tested food matrices ensure reliable results, and the assays therefore should be useful for both the food industry and consumer protection agencies.

ABBREVIATIONS USED

BSA, bovine serum albumin; CV, coefficient of variation; ELISA, enzyme-linked immunosorbent assay; HRP, horseradish peroxidase; LOD, limit of detection; PBS, phosphate-buffered saline; TMB, 3,3′,5,5′-tetramethylbenzidine; Tris, tris(hydroxymethyl)aminomethane.

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