Indirect Competitive ELISA for Determination of Traces of Peanut (*Arachis hypogaea* L.) Protein in Complex Food Matrices

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An indirect competitive ELISA was developed allowing the detection of hidden peanut protein residues down to 2 ppm (micorgrams per gram) in various foods. The high-titer, peanut-specific polyclonal antiserum used recognized potentially allergenic proteins in both native and roasted peanuts. In the absence of a food matrix, extractable protein from roasted peanuts was detected at 104 ± 13%. From various food items, peanut protein at ≥13 ppm was recovered between 84 and 126%, and at 2 ppm of peanut protein recovery was 143 ± 6%. Intra- and interassay precision was <15%. In 5 of 17 commercial food products without declaration of peanut components, between 2 and 18 ppm of peanut protein was detected. This is the first assay based on commercially available reactants that allows the reliable determination of trace amounts of hidden peanut allergens in a variety of complex food matrices.

Keywords: *Peanut (Arachis hypogaea L.); hidden allergens; ELISA; immunoblotting*

INTRODUCTION

Peanuts are one of the most common causes of severe allergic reactions to foods and may lead to high rates of symptoms on even minimal contact (Hourihane et al., 1997a). Several case reports have been published about life-threatening anaphylaxis and even deaths due to the ingestion of peanuts (Yunginger et al., 1988; Sampson et al., 1992), which may account for up to one-third of all severe food-related allergic reactions as reviewed elsewhere (Vieths et al., 1994; Hourihane, 1997).

The prevalence of peanut allergy is estimated at just under 0.5% (MAFF, 1996) and up to 1.3% (Hourihane et al., 1996) of the British population, and it has been suggested that peanut allergic patients with severe symptoms may constitute ∼0.1−0.2% of the population (MAFF, 1996).

Various immunoglobulin E (IgE)-reactive peanut proteins and glycoproteins that initiate sensitization and trigger the allergic reaction have been identified and characterized (Sachs et al., 1981; Barnett and Howden, 1986; Burks et al., 1991, 1992a; Uhlemann et al., 1993). The major peanut allergens, Ara h 1 and Ara h 2, have been found to be strongly stable toward heat and simulated digestion in vitro (Burks et al., 1992b; Astwood et al., 1996; Becker, 1997).

For reasons of low-dose activity and highly persistent allergenicity of peanut proteins, even after food technological processing, and because of potentially severe allergic reactions, allergic patients are required to strictly avoid peanut-containing foods within their diet. By contrast, independent studies revealed that a high percentage of peanut-allergic individuals accidentally consume peanuts (Bock and Atkins, 1989; Hourihane et al., 1997a). This occurs, for example, if the presence of peanut allergens in a food may not be discernible due to mislabeling of the products, rework processes that include peanut-containing foods, or cross-contamination during processing.

For better protection of consumers, detection methods are required to specifically discover the presence of hidden allergens in a wide variety of food items. Such methods should be sufficiently sensitive and ideally independent from the food matrix because as little as 100 μg of peanut protein could still elicit allergic reactions in highly sensitized subjects (Hourihane et al., 1997b).

Several attempts have been made to detect hidden peanut allergens, including analytical techniques such as immunoblotting, radioimmunoassay (RIA), rocket immunoelectrophoresis (RIE), and enzyme-linked immunosorbent assay (ELISA). The published methods, their potential, and restrictions for routine analysis have been summarized elsewhere (Yeung and Collins, 1996; Mills et al., 1997; Holzhauser et al., 1998). Recently, two two-site ELISA techniques have been published (Koppelmann et al., 1996; Mills et al., 1997) but unfortunately lack precise validation data or adequate sensitivity, respectively. Additionally, we have developed an RIE application that was primarily designed for the analysis of chocolate and similar processed foods (Holzhauser et al., 1998). However, this in-gel precipitation technique cannot be automated in routine analysis. To date, only one ELISA for detection of peanut protein traces at a level of 1 ppm (μg/g) is commercially available (Peanut PAK, Pro-Lab diagnostic, Ontario, Canada), but again no data on validation as, for example, cross-reactivity, recoveries, or matrix effects are supplied by the manufacturer.

To overcome these limitations, we have developed and validated an indirect competitive ELISA based on the same commercially available antiserum as the mentioned RIE application. The characteristics and possible applications of this ELISA are described in this paper.

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MATERIALS AND METHODS

Peanut Samples, Reference Samples, and Commercial Food Products. Ground roasted peanuts for preparation of peanut reference protein and negative- and positive-control samples of industrially manufactured model chocolates containing 0 and 0.1% of peanut protein were provided by Professor Dr. R. Matissek, Institute of Food Chemistry of the German Confectionary Industry, Cologne, Germany. Different varieties of native peanuts were obtained from local markets in New Orleans, LA, and Leon, Nicaragua, and were also provided by Internut Handels GmbH, Hamburg, Germany, and by Dr. W.-M. Becker, Forschungszentrum Borstel, Germany. U.S. Medium Runner peanuts roasted under industrial-like conditions at 140 °C for 40, 45, and 50 min and at 160 °C for 10, 20, 30, and 40 min, as well as native reference material, were provided by Dr. G. Malgarini, Sorematec, Arlon-Schoppach, Belgium. Commercial food products were obtained at a local food store, including three samples with a warning "may contain traces of peanuts", 10 samples with peanut or peanut components listed as an ingredient, and 17 samples without any declaration about the presence of peanut or peanut components in the list of ingredients. The declaration was considered to be positive if peanut or any component of peanut including peanut paste, peanut oil, or peanut fat was listed as an ingredient, even though peanut protein may not be present in the food. Food samples that did not have peanut listed as an ingredient were mainly selected from such companies that also produce foods containing peanuts.

Reagents, Buffers, and Instrumentation. For competitive ELISA, we applied a polyclonal antiserum raised against native peanut protein (Riedel de-Haën, No. 45262, Seelze, Germany) and horseradish peroxidase (HRP)-labeled polyclonal anti-rabbit IgG antibodies developed in goat (Sigma Chemical Co., No. A-0545, Deisenhofen, Germany). The peanut-specific antiserum was absorbed against soy protein, white bean, and marzipan by the manufacturer. For immunoblotting experiments we used a serum pool from sera of three peanut-allergic patients (Dr. W.-M. Becker) and one nonallergic control serum (PEI 75, in-house serum collection), alkaline phosphatase (AP)-labeled mouse anti-human IgE (Pharmingen, No. 34613E, Hamburg, Germany), rabbit negative-control serum (Riedel de-Haën, No. 45263), biotin-labeled goat anti-rabbit IgG (Dako, No. E0432, Hamburg, Germany), AP-labeled streptavidin (Caltag, No. SA1008, Hamburg, Germany), monoclonal antibody (mab) PN-t against Ara h 1 (Dr. W.-M. Becker), negative-control mab against mycobacterium paratuberculosis (Dr. A. Hoffmann, Paul-Ehrlich-Institut), and biotin-labeled rabbit anti-mouse IgG (Dianova, No. 315-065-003).

All chemicals used were of analytical grade or as specified. Sample extraction buffer consisted of 8 mM tris(hydroxymethyl)aminomethane (TRIS), 25 mM *N*-[tris(hydroxymethyl)-methyl]glycine (Tricine), and 2 mM calcium lactate, pH 8.6, adjusted with 10% HCl. Coating buffer, 50 mM carbonate, pH 9.6, contained 15 mM Na_2CO_3 and 35 mM $NaHCO_3$. Phosphate-buffered saline (PBS), pH 7.4, contained 10 mM $NaH_2PO_4 \cdot 2H_2O$, 70 mM $Na_2HPO_4 \cdot 2H_2O$), and 150 mM NaCl. Blocking solution contained 1.5% bovine serum albumin (BSA) (for enzyme immunoassay, Fluka, Neu-Ulm, Germany) in PBS, pH 7.4. Incubation buffer consisted of 0.5% BSA and 0.5% Tween 20 (Sigma) in PBS, pH 7.4. ELISA washing buffer consisted of 10-fold diluted PBS, pH 7.4, and 0.5% Tween 20. Peroxidase substrate reagent contained 1 mM 3,3′,5,5′-tetramethylbenzidine (TMB) (Merck, Darmstadt, Germany) and 3 mM H_2O_2 (Merck) in citrate buffer, pH 3.95, and was freshly prepared by addition of 4.95 μL of H_2O_2 (30%) and 750 μL of 21 mM TMB concentrate (126 mg TMB in 2.5 mL of acetone, made up to 25 mL with methanol) to 15 mL of citrate buffer, pH 3.95, which consisted of 210 mM citric acid monohydrate and 300 mM KOH. ELISA stopping solution was 3 M H_2SO_4.

Washing of microwell plates was done using an eight-channel automatic microplate washer (MWG-Biotech, Ebersberg, Germany). ELISA readings of optical density (OD) were carried out with a Titertek Multiscan Plus MK II (ICN Biomedicals, Eschwege, Germany) controlled by data processing software (EIA 3, ICN Biomedicals).

Buffers and reagents for immunoblotting were prepared as described elsewhere (Vieths et al., 1992), except for the replacement of streptavidin HRP conjugate by streptavidin AP conjugate or AP-labeled mouse anti-human IgE, and for the AP substrate solution, which was prepared from a commercial substrate kit (alkaline phosphatase conjugate substrate kit, Bio-Rad, No. 170-6432, Munich, Germany) according to the manufacturer's instructions.

Sample Homogenization. Prior to extraction, food samples for peanut protein analysis, cross-reactivity, and roasting studies were frozen with liquid nitrogen. Forty grams of sample was ground with an analytical mill (IKA M 20, IKA Labortechnik, Staufen, Germany).

Microextraction for Screening Purposes. One milliliter of extraction buffer was added to 50 mg of homogeneous sample powder in a 2 mL micro test tube (Eppendorf, Hamburg, Germany), and extraction was carried out in a temperature-controlled horizontal shaker (Thermomixer 5437, Eppendorf) at 45 °C and 900 rpm for 30 min. Samples were additionally vortexed in intervals of 5 min. Extracted samples were centrifuged at 20000*g* and 15 °C for 30 min, and the supernatants were analyzed by ELISA.

Quantitative Extraction. One gram of homogenized sample was suspended in extraction buffer and made up to a total volume of 20 mL. Extraction was carried out at 45 °C for 2−3 h. Samples were vortexed in intervals of 30 min. An aliquot of the extract was centifuged as described above, and the supernatant was analyzed by ELISA.

Preparation of Peanut Protein Standards. Ground roasted peanuts were extracted according to the quantitative extraction protocol, and the amount of protein in the peanut protein reference extract was determined according to a modified Bradford method (Zor and Selinger, 1996) using the Coomassie Plus Protein assay (Pierce, No. 23236, KMF Laborchemie, St. Augustin-Buisdorf, Germany) with BSA as a standard. Peanut protein reference extract was applied for coating of microwell plates (see below) and for preparation of eight peanut protein standards. The standards were prepared by serial dilution in incubation buffer covering a concentration range between 1280 and 10 ng/mL. Both protein reference extract and protein standards were stable at −20 °C for at least 6 months without loss of activity.

Extracts for Cross-Reactivity Studies. Extracts for investigation of cross-reactivity were prepared in the same manner (see Quantitative Extraction) from a wide range of foods and food constituents. Extracts were derived from legumes (soybean; chick pea; green pea; lentil; kidney bean; white bean; red bean; pinto bean), nuts and stone fruits (almond; Brazil nut; cashew; coconut; hazelnut; macademia; pecan; pistachio; walnut), and various ingredients (cereal mix consisting of equal amounts of barley, corn, oats, rice, rye, and wheat; coffee; hen's egg; pine seed; pumpkin seed; sesame seed; sunflower seed) as well as thickening and gelling agents (carob; guar flour; gum arabic; traganth; agar agar; carrageenan) and sugar. The protein concentration of extracts from legumes, nuts and stone fruits, and various ingredients varied between 0.2 and 16.7 mg/mL as determined by the Bradford method. These extracts were tested at dilutions simulating proportions of 100, 20, and 4% of the food. Providing a starting dilution of 1:4 for an unknown sample, these extracts were therefore tested at dilutions of 1:4, 1:20, and 1:100. Extracts of thickening and gelling agents corresponding to a proportion of 5% in a matrix were simulated by dissolving 50 mg of sample in 20 mL of extraction buffer, and a proportion of 100% of sugar was prepared by addition of 50 mg of sugar/mL of extraction buffer. Extracts of thickening and gelling agents were tested at simulated proportions of 5, 1, and 0.2%. The extract of sugar was tested undiluted so as to simulate a proportion of 100% in a food.

Spiking of Samples and Recovery Studies. Recovery experiments were conducted both on peanut-free food samples and on samples containing peanut protein. After grinding, the former were spiked with peanut protein at levels of 2, 20, and

	1	2	3	4	5	6	7	8	9	10	11	12
A	NSB	B_0	B_0	B_0	B_0	B_0	B_0	B_0	B_0	B_0	B_0	NSB
B	NSB	10	20	40	80	B_0	B_0	160	320	640	1280	NSB
C	NSB	10	20	40	80	B_0	B_0	160	320	640	1280	NSB
D	NSB	10	20	40	80	B_0	B_0	160	320	640	1280	NSB
E	NSB	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	NSB
F	NSB	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	NSB
G	NSB	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	NSB
H	NSB	B_0	B_0	B_0	B_0	B_0	B_0	B_0	B_0	B_0	B_0	NSB

Figure 1. Suggested plate layout of standards, samples, and controls (NSB) for optimized assay precision: samples and standards in triplets (standards 10−1280 ng/mL; samples S1−S10; B_0 maximal signal at zero dose of analyte; NSB nonspecific binding of the secondary antibody).

200 ppm. Samples that already contained peanut protein were treated as follows: After determination of the original peanut protein content, another 0.5 g sample was adjusted to the peanut protein content of a 1 g sample by adding reference protein. For the analysis of these samples the same dilution factors were chosen as for the original 1 g samples. All spiked samples were incubated for 90 min at room temperature prior to quantitative extraction. Recoveries of peanut-free samples spiked with peanut protein were expressed as the quotient of total peanut protein determined to protein added. Recoveries of samples containing peanut protein were derived by the quotient of the determined amount of peanut protein added to the actual amount of protein added. The calculation was done as indicated:

$$\text{recovery} = \frac{\text{determined amount of peanut protein added (4)}}{\text{added amount of peanut protein (2)}} \times 100\% =$$

$$\frac{\text{total peanut protein determined (3)} - \text{peanut protein determined in half-matrix (1)}}{\text{added amount of peanut protein (2)}} \times 100\%$$

Numbers in parentheses indicate the columns displayed in Table 2.

ELISA Procedure. Flat-bottom polystyrene microwell plates (Maxisorp F 96, certified, Nunc, Wiesbaden, Germany) were coated with 240 μL/well of 0.5 μg/mL peanut protein from roasted peanuts in coating buffer. After 16 h at room temperature, plates were washed three times of 4 min each with washing buffer. Unsaturated binding sites of the polystyrene surface were blocked by incubation with 250 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 vacuum-sealed plastic bags at −20 °C until used. Coated plates could be stored for at least 6 months without any decrease of peanut protein activity.

Freeze-stored plates were washed once for 4 min with washing buffer prior to use. The competitive step was performed by dispensing 100 μL/well of peanut protein standards or food sample extracts (minimal dilution 1:4 in incubation buffer) and afterward 100 μL/well of peanut-specific antiserum (diluted 1:200000 in incubation buffer). The competitive reaction was allowed to proceed for 3 h at 37 °C. Experiments were run in triplicate. To minimize edge effects, only the inner 60 wells were used for inhibitions according to the plate layout displayed in Figure 1. The outer wells were filled with 200 μL/well of incubation buffer for determination of nonspecific binding (NSB) of the secondary antibody or with 100 μL/well of incubation buffer and 100 μL/well of the diluted peanut-specific antiserum for determination of maximal signal (B_0) at zero dose of analyte, respectively. Thereafter, plates were washed three times of 4 min each. After incubation with 150 μL/well of HRP-labeled goat anti-rabbit IgG (diluted 1:40000 in incubation buffer) for 1 h at 37 °C, plates were washed twice with washing buffer and once with distilled water for 4 min each. After addition of 100 μL/well of HRP substrate solution, the enzymatic staining was performed for 15−30 min in the dark to obtain maximal OD values without inhibition of 1.4−1.6 when stopped by addition of 100 μL/well of stopping solution. The OD values were read bichromatically at 450 nm main wavelength and 630 nm reference wavelength. Plates were sealed with an adhesive 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 1:4. The quantitative determination was based on the analysis of two independently prepared extracts according to the quantitative extraction procedure and with both determinations measured on separate microwell plates. Data processing included the reduction of mean OD values of samples, standards, and B_0 values by the average OD of NSB. Reduced OD values were expressed as relative OD values (%OD = $B/B_0 \times 100\%$), and %OD values of standards were plotted against the logarithm of the concentration. The resulting sigmoidal curve was fitted using a four-parameter logistic function. If the outer B_0 values (wells A2−A11 and H2−H11) differed from the inner B_0 values (B6−D6 and B7−D7) by >5%, only the inner B_0 values were chosen for data analysis.

Immunoblotting. The immunoblotting procedure was performed as described elsewhere (Vieths et al., 1992) except for the use of AP as the marker enzyme and nitroblue tetrazolium (NBT)/5-bromo-4-chloro-3-indolyl phosphate (BCIP) as the substrate. Briefly, 25 μg of protein/cm of polyacrylamide gel from native and roasted (140 °C, 45 min) peanuts 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. Free binding sites of the membrane were blocked with 0.3% Tween 20 in PBS. For total protein pattern, one strip of the membrane was stained with India ink. For detection of IgE-reactive peanut proteins, strips were incubated overnight with a serum pool of peanut-allergic patients diluted 1:6.7 and subsequently with AP-labeled mouse anti-human IgE diluted 1:1000 for 4 h. Detection of IgG-reactive peanut proteins with the commercial peanut-specific antiserum was done by subsequent incubations of peanut-specific antiserum diluted 1:10000 (overnight), biotin-labeled goat anti-rabbit IgG diluted 1:2500 (1 h), and AP-labeled streptavidin diluted 1:3000 (30 min). The Ara h 1-specific mab PN-t was incubated overnight at a concentration of 1 μg/mL. Following incubation with biotin-labeled rabbit anti-mouse IgG diluted 1:2500 (1 h), AP-labeled streptavidin diluted 1:3000 was incubated for 30 min.

Controls were incubated overnight and included a nonallergic human serum (diluted 1:6.7) for unspecific IgE detection, a rabbit negative serum (diluted 1:10000) to check for the specificity of the peanut-specific rabbit IgG, and a nonrelevant mab against mycobacterium paratuberculosis (supernatant diluted 1:10) for testing of specificity of the Ara h 1 detection. 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 PBS. The reagents described were diluted in incubation buffer (PBS, pH 7.4; 0.05% Tween 20; 0.1% BSA), and incubation reactions and enzymatic staining were carried out at room temperature. The color was developed for 10 min or as indicated.

RESULTS

Characterization of the Rabbit Peanut-Specific Antiserum. Protein extracts of U.S. Medium Runner peanuts roasted under various conditions were sepa-

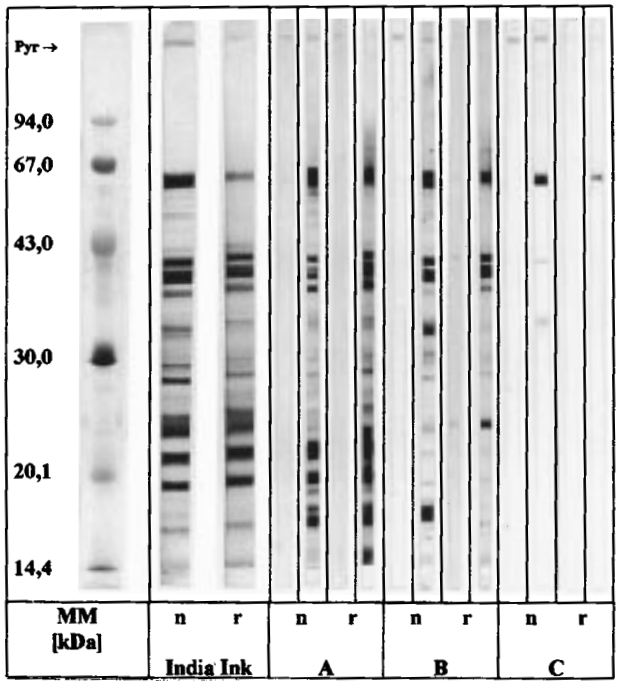

Figure 2. Immunoblotting of peanut proteins of the variety U.S. Medium Runner as detected by (A) patients' IgE, (B) rabbit peanut-specific antiserum, and (C) mab PN-t against Ara h 1 [MM, molecular mass; Pyr, pyronine, unspecific blotting dye; n, native; r, roasted at 140 °C for 45 min; left lanes of A−C indicate the control of nonspecific binding by nonallergic human serum (A), rabbit negative-control serum (B), and antimycobacterium paratuberculosis mab (C); right lanes of A−C represent the specific detection].

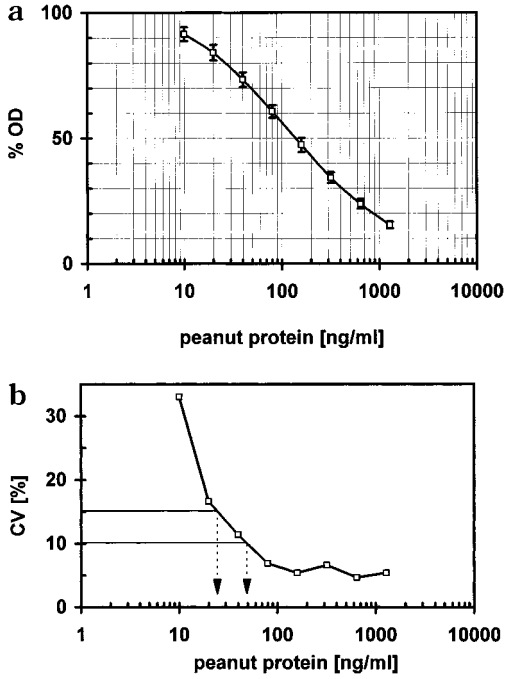

Figure 3. (a) Mean standard curve of the ELISA derived from 34 different curves. Error bars indicate the standard deviation of the peanut protein standards. (b) Mean precision profile of the standard curve derived from 34 different curves.

rated by SDS−PAGE and silver-stained. Except for peanuts roasted at 160 °C for 40 min, the protein patterns did not show any significant differences (results not shown). As an example for realistic conditions of roasting, protein from peanuts roasted at 140 °C for 45 min was selected for further comparison with protein from native material by SDS−PAGE and immunoblotting. Subsequently blotted proteins were subjected to immunodetection to compare IgE-reactive proteins with those detected by the peanut-specific antiserum developed in rabbits (Figure 2). Comparison of the total protein pattern (stained with India ink) from native and roasted peanuts revealed that roasting under industrial-like conditions hardly changes the protein composition of the derived extracts: Ara h 1, which is still present in roasted peanuts (C), is also detected by patients' IgE (A) and the rabbit antiserum (B), both in native material and after roasting. However, the content appeared to be reduced after roasting. Various IgE-reactive proteins from roasted and native peanuts (A) were also detected by the rabbit antiserum (B), and the potential allergenicity of peanut protein is not reduced in peanuts roasted under such conditions (A). All controls were negative, demonstrating the specificity of the detection.

ELISA Standard Curve and Precision Profile. A mean standard curve was derived from 34 different curves measured on different days (Figure 3a). The standard deviation of the peanut protein standards ranged between 1.4% OD and 3.1% OD. The system's technical detection limit and the limit of a reliable peanut-specific detection were evaluated with 10 and 24 ng/mL of peanut protein in incubation buffer, which corresponded to 800 ppb and 2 ppm of peanut protein in a sample, respectively. These limits were defined as the protein concentration derived from the OD at zero inhibition (B_0) reduced by 3-fold or 6-fold the standard deviation of the mean B_0 value, expressed as %OD. Greatest mean precision of quantification, as defined at 50% inhibition, was obtained by 140 ng/mL peanut protein, corresponding to 11.2 ppm of peanut protein in a sample, providing a minimal extract dilution of 1:4.

A precision profile of the standard triplets based on the same data is given in Figure 3b. Signal precision, as expressed by the coefficient of variation (CV), increased for quantification above 24 ng/mL of peanut protein, and mean CVs of ≤15% were obtained.

Antiserum Specificity in ELISA. More than 30 different legumes, nuts, and stone fruits as well as various food ingredients were included in cross-reactivity studies. Extracts from characteristic and potentially cross-reactive foods and food ingredients were tested at dilutions simulating proportions of 100, 20, and 4% except for thickening and gelling agents, which were applied at simulated proportions of 5, 1, and 0.2% in a food matrix.

At a dilution of 1:4 corresponding to 100% of the food, only extracts of walnut and pinto bean caused an inhibition of 22%, equivalent to 3.2 ppm of peanut protein. However, inhibition was far below the limit of detection (2 ppm of peanut protein in the food) for both extracts when diluted 1:20. All other investigated extracts showed no or little inhibition of maximal 5−10% equivalent to ≪1 ppm. The extraction buffer did not cause any inhibition either.

Detection Limit and Limit of Quantitative Determination. For evaluation of the limits of detection and quantitative determination, data on antiserum specificity and standard curve precision were taken into consideration. The limit of detection was defined by the limit of a reliable peanut-specific signal with a mean precision of standard triplets to be ≤15% (Figure 3b). Therefore, the average limit of detection was given at 24 ng/mL of peanut protein and resulted from ∼18−

Table 1. Extractable Peanut Protein from Native Peanuts and from Peanuts Roasted under Various Conditions As Determined by the Bradford Method and by ELISA[a]

source of peanut/variety	native/roasted	Bradford protein (mg/mL)	Bradford CV (%)	ELISA protein (mg/mL)	ELISA CV (%)	ELISA/ Bradford (%)
U.S. (New Orleans)/ Virginia	native	10.19	4.2	6.24	0.9	61.3
U.S. (Virginia)/Jumbo	native	8.42	5.7	4.95	1.4	58.8
U.S. (Virginia)/Giant	native	11.81	3.5	7.23	3.4	61.2
U.S. (unknown)/Giant	native	13.28	2.0	8.32	7.0	62.7
China (unknown)/Jumbo	native	11.99	3.2	6.75	0.4	56.3
China (unknown)	native	11.22	4.2	7.06	3.7	62.9
Nicaragua (Leon)/unknown	native	11.42	0.8	8.20	6.7	71.8
U.S./Medium Runner	native	11.56	7.3	9.95	0.03	86.1
U.S./Medium Runner	140 °C, 40 min	5.91	1.0	5.67	4.1	95.8
U.S./Medium Runner	140 °C, 45 min	4.56	1.8	4.97	2.2	109.0
U.S./Medium Runner	140 °C, 50 min	3.43	0.5	3.62	1.3	105.6
U.S./Medium Runner	160 °C, 10 min	9.30	0.3	8.19	5.2	88.1
U.S./Medium Runner	160 °C, 20 min	5.44	1.6	6.70	2.3	123.3
U.S./Medium Runner (OR)[b]	160 °C, 30 min	2.08	1.4	1.46	14.6	70.3
U.S./Medium Runner(OR)[b]	160 °C, 40 min	1.24	1.1	0.33	5.3	26.8

[a] Values are the average of duplicate determinations. [b] OR, apparently overroasted as was assessed by appearance and taste.

Table 2. Recovery of Peanut Protein from Commercial Food Samples Containing Various Concentrations of Peanut Protein[a]

sample	peanut protein in whole matrix ppm	peanut protein in whole matrix CV (%)	peanut protein in half-matrix (1) (ppm)	peanut protein added (2) (ppm)	total peanut protein determined (3) (ppm)	added peanut protein determined (4) (ppm)	recovery of peanut protein %	recovery of peanut protein CV (%)
whole-milk chocolate	13.4	5.7	6.7	6.7	14.7	8.4	126	27.4
chocolate cornballs	16.6	6.5	8.3	8.3	19.1	10.4	125	6.6
model chocolate[b]	70.8	3.1	35.4	35.4	70.1	35.0	99	23.0
crunchy chocolate flakes	246.0	3.9	123.0	123.0	262.0	139.0	113	10.5
mixed cereals	290.0	0.5	145.0	145.0	299.0	154.0	106	8.1
corn flakes	1094.0	5.5	547.0	547.0	1207.0	662.0	121	3.1
peanut candy	1458.0	2.8	729.0	729.0	1589.0	863.0	118	6.2

[a] Values are the average of duplicate determinations. [b] Positive-control sample.

20% of signal inhibition. The limit of quantitative determination was set at the concentration of standard protein that resulted in a mean precision of ≤10% at an average of 30% inhibition (Figure 3b) and arose from 50 ng/mL peanut protein. In comparison, the average inhibition caused by the most significant cross-reactivities or by matrix effects was between 5 and 10%. Providing 1:4 dilution of a sample extract, the limit of detection for a given foodstuff would be 2 ppm and the limit of quantitative determination would be 4 ppm of peanut protein.

Detection of Proteins from Native and Roasted Peanuts. Quantitative detection of extractable peanut protein, ideally independent from the degree of roasting, is the prerequisite of accurate analysis. Therefore, extracts of native peanuts from different origins and of peanuts roasted under industrial-like conditions were analyzed for extractable peanut protein using the Bradford method with BSA as the standard. Following protein quantification of the extracts by ELISA, results of ELISA were correlated to those of the Bradford method (Table 1). Extractable protein of eight different sources of native peanuts was detected by ELISA at a mean level of 65 ± 10%. Quantification of peanuts roasted under realistic conditions (all samples except those marked OR) revealed that extractable protein from roasted peanuts could be determined at 104 ± 13%. Additionally, the absolute amount of extracted protein was highly dependent on the degree of roasting; thus, the proportion of peanut in a food sample cannot be calculated from the amount of detectable peanut protein. Roasting at 160 °C for 30 or 40 min resulted in dark brownish kernels that were clearly overroasted. In these samples the detectability of protein decreased significantly, most likely due to thermal degradation of the epitopes.

Recovery Studies. Recovery experiments were carried out on commercial samples containing peanut protein (Table 2) and on commercial blank samples that were artificially contaminated with peanut protein (Table 3). Recoveries of samples containing peanut protein in a range between 13 and 1458 ppm were determined at a mean of 115 ± 10% ($N = 7$).

Blank samples spiked prior to extraction with 20 or 200 ppm of peanut protein showed similar recoveries at a mean level of 103 ± 15% ($N = 6$). At the limit of detection, recoveries increased up to 143 ± 6% ($N = 3$). However, if accurate data are needed, determination of recoveries is always essential. Nevertheless, recoveries were reproducible and almost independent from the type of matrix in all investigated samples, with only little overestimation of the amount of peanut protein present in a sample. To distinguish between matrix effects on the immunoassay and extraction-related effects, blank extracts from blank samples were additionally spiked with peanut protein at the indicated levels (Table 3). The determined recoveries were comparable to those observed on the spiked samples that went through the entire extraction procedure. Extracts of blank samples that were subsequently spiked with peanut protein equivalent to 20 or 200 ppm after extraction were recovered by an average of 102 ± 11% ($N = 6$), and the equivalent to 2 ppm was recovered by an average of 145 ± 16% ($N = 3$).

Table 3. Recovery of Peanut Protein from Blank Commercial Food Samples That Were Artificially Contaminated with Peanut Protein (A) prior to and (B) after the Extraction Procedure[a]

		(A) addition of peanut protein prior to extraction			(B) addition of peanut protein after extraction	
			recovery of samples spiked prior to extraction		recovery of samples spiked after extraction	
blank samples	peanut protein added (ppm)	peanut protein determined (ppm)	%	CV (%)	%	CV (%)
buffer	2	2.8	140	14.0	nd[b]	nd
	20	20	100	6.0	nd	nd
	200	204	102	2.9	nd	nd
whole-milk chocolate	2	2.8	140	4.3	161	4.7
	20	18	90	2.2	118	1.7
	200	168	84	3.0	92	2.2
rice cracker	2	3	150	14.0	145	3.8
	20	24	120	7.0	104	1.9
	200	230	115	5.2	89	1.7
hazelnut chocolate candy	2	2.8	140	12.5	129	0.1
	20	22	110	13.0	110	2.8
	200	192	96	2.0	100	3.6

[a] Values are the average of duplicate determinations. [b] nd, not determined.

Intra- and Interassay Precision. For estimation of the intraassay precision, the peanut protein concentrations of five different commercial food samples that contained peanut protein in a range between 2 and 1150 ppm were determined in 10 triplet replicates on one separate microwell plate for each sample. The mean CVs were determined as 11.3, 9.6, and 4.7% for peanut protein at levels of 2, >15, and ≥260 ppm, respectively.

The interassay precision was determined for 10 different commercial foods that contained peanut protein in a range between 2 and 1700 ppm on five different days each. The interassay precision was calculated for each sample and resulted in averages of 14.0, 9.0, and 6.4% for peanut protein at levels of 2, >15, and >220 ppm, respectively.

Investigation of Commercial Food Products. To test the applicability of the assay, 30 different commercial food products and 3 industrially manufactured negative-control samples were analyzed (Table 4). Samples were considered as positive if peanut protein ≥2 ppm could be detected. No positives could be detected in our three negative-control samples, and the three samples that "may contain traces of peanuts" were negative, too. Five samples having peanut or peanut paste declared were found to contain peanut protein in the range between 4.4 and 13552 ppm. Two samples containing peanut fat or peanut oil as well as the refined oil sample did not contain detectable peanut protein. No peanut protein could be detected in either of two samples, "chocolate bar, candy creme II" and "wafer, chocolate and cereal crisp", of which peanut was listed as the last component in the list of ingredients. Twenty-nine percent (5 of 17) of the samples whose labeling did not have peanut or peanut components listed contained 2−18 ppm of peanut protein. The results were independently confirmed following the protocol of our RIE application (Holzhauser et al., 1998). No contradictionary results were obtained.

With the screening protocol based on a single determination, the peanut protein concentration of positive samples was determined in the correct order of magnitude and no detectable peanut protein was found in any negative samples.

DISCUSSION

The polyclonal antiserum used in competitive ELISA reacts with proteins from both native and roasted peanuts as was demonstrated by immunoblotting and by analyzing extracts from different peanuts with the developed ELISA procedure. Various IgE-reactive and thus potentially allergenic proteins of native and roasted peanuts were detected by the rabbit antiserum, as was Ara h 1, a heat-stable major peanut allergen of 63.5−66.0 kDa, which is recognized by the IgE of at least 90% of peanut-sensitive patients (Burks et al., 1991, 1995; Uhlemann et al., 1993).

The ELISA correctly quantified extractable protein from peanuts roasted under realistic conditions by an average of 104 ± 13% when compared to protein quantification by the Bradford method. Accurate detectability was mainly independent from roasting conditions as long as overroasting (160 °C, 30 or 40 min) was prevented. By contrast, native material was underestimated by ∼35%. If quantification of native protein was crucial, the underestimation may be compensated by the use of native material for coating of the plates and for preparation of the standards. However, roasted peanuts are used in the majority of peanut-containing foods. The amount of extractable and detectable protein decreased with increasing time and temperature of the roasting process. Thus, exact calculation of the amount of peanut present in a sample is not possible. Depending on the degree of roasting, the proportion of peanut in a sample may be estimated by the 5−15-fold (on average 10-fold) concentration of peanut protein. Hence, this ELISA and any other technique based on the detection of extractable peanut protein are unsuitable for quantitative determination of peanut in a foodstuff as a quality characteristic.

The antiserum applied showed high specificity for peanut protein, most probably due to its immunoabsorption against potentially cross-reactive foods by the manufacturer. Moreover, our optimized assay conditions at 37 °C further reduced the cross-reactivity. Only walnut and pinto bean caused a signal of ∼3 ppm when investigated at a protein concentration that was equivalent to 100% of a foodstuff. Further 5-fold dilution led to signals equivalent to <1 ppm. Therefore, the assay tolerates 20% of walnut or pinto bean in a food without producing false-positive results. In contrast to other cross-reactivity studies of published peanut-specific assays (Yeung and Collins, 1996; Koppelmann et al., 1996; Mills et al., 1997), extracts of potentially cross-

Table 4. Analysis of Various Commercial Food Commodities for Peanut Protein According to (A) the Screening Protocol and (B) the Quantitative Procedure

	(A) screening		(B) quantitative determination		
sample	D[a]	ppm	ppm	CV (%)	result
cashews, roasted	−	4.3	4.2	11.9	positive
almonds, roasted	−	0[e]	0		negative
white chocolate I	±	0	0		negative
white chocolate II	−	0	0		negative
whole-milk chocolate I	±	0	0		negative
whole-milk chocolate II	−	0	0		negative
chocolate, plain	−	4.5	3.3	14.4	positive
chocolate, marzipan	−	2.1	2.0	7.2	positive
chocolate, whole-milk and nut	−	29	18	12.6	positive
chocolate, hazelnut	−	0	0		negative
chocolate, for children	−	0	0		negative
chocolate bar, almond candy cream	−	0	0		negative
chocolate bar, candy cream I	−	0	0		negative
chocolate bar, candy cream II	+	0	0		negative
chocolate bar, caramel	±	0	0		negative
chocolate candy	−	0	0		negative
chocolate chips[b]	+	0	0		negative
chocolate and cookie	−	0	0		negatice
nut and chocolate	+	99	104	4.8	positive
raisin and chocolate	+	35	34	11.8	positive
wafer, chocolate and cereal crisp	+	0	0		negative
chocolate sponge cake[c]	+	0	0		negative
nougat bar	−	0	0		negative
cookie, plain	−	nd[f]	0		negative
cookie, coconut	−	2.7	3.6	9.8	positive
amarettini	+	nd	13552	1.9	positive
breakfast cereal bar	−	nd	0		negative
cereal bar, yoghurt	+	3.5	4.4	1.7	positive
cereal bar, chocolate	+	1467	7193	4.3	positive
peanut oil, refined	+	0	0		negative
chocolate, coffee creme[d]		0	0		negative
chocolate, nougat[d]		0	0		negative
chocolate, whole-milk[d]		0	0		negative

[a] D, declaration of peanut or peanut components: −, no declaration; +, positive declaration; ±, may contain traces of peanuts. [b] Contains peanut oil. [c] Contains peanut fat. [d] Negative-control sample. [e] 0, no detectable peanut protein. [f] nd, not determined.

reactive food components were investigated at the highest protein concentration possible. The average inhibition of the most significant cross-reactivities observed was between 5 and 10%, whereas specific inhibitions were defined by a minimal inhibiton of ∼18−20%, the limit of detection. False-positive results at the lower parts per million range are only to be expected if walnut or pinto bean exceeding 20% of the whole food is present.

The antiserum used was of very high titer and showed only little variation in batch-to-batch consistency. Even though the antiserum had to be diluted 1:200000, performance of the immunoassay was very reproducible.

One aim of this work was to develop a peanut-specific detection system for both screening and quantification purposes. Thus, the standard curve of this assay represents a compromise between maximal slope for precise quantification and a wide dynamic range to minimize the number of sample dilutions. Samples containing 2−102 ppm of peanut protein or ∼10 (0.001%)−1000 ppm (0.1%) of peanut could be quantified at one single step of dilution (1:4). Two parts per million of peanut protein was the limit of detection which resulted from a minimal inhibition of the order of 6-fold the standard deviation of the B_0 signal and from a mean precision of the standard triplets to be ≤15%. The precision was even better than 10% for peanut protein concentrations of ≥50 ng/mL (equivalent to 4 ppm), the limit of quantitative determination. Intra- and interassay precisions were <10% for samples containing >15 ppm of peanut protein and still <15% at the limit of detection. For best assay precision we suggest following the described assay layout.

The antigens of the standard protein solution and of the food extracts should ideally behave identically for accurate analysis of peanut protein in a sample. In contrast to other methods (Yeung and Collins, 1996; Holzhauser et al., 1998), which required the application of standards in modified sample matrices, our ELISA yielded good overall recoveries with standards prepared in buffer only. Thus, our ELISA is suitable for analysis of a wide range of food items without restrictions. Recoveries were good with little overestimation when peanut protein ≥20 ppm was present. Two parts per million of peanut protein was overestimated by an average of 43%, but it has to be kept in mind that analysis of 2 ppm was below the limit of quantitative determination. Even in the absence of matrix (see buffer), 2 ppm of peanut protein was recovered at 140%. We therefore assume that an overestimation at the limit of detection does not occur due to considerable effects caused by the matrix but may depend on the quality of the curve fitting procedure at low slope. For spiking of samples that already contained peanut protein, it was assumed that half of the matrix would have a similar effect on the extractability and the detectability of the analyte as the whole matrix. However, similar results were obtained for both types of recovery experiments. Comparison of recoveries from blank samples spiked prior to or after extraction revealed that the food matrix had only little effect on the effectiveness of the extraction and on the antibody−antigen interaction. Except

for analysis at the limit of detection, overestimation may rank within the order of variation of the detectability that resulted from different conditions of roasting. For most accurate quantification, performance of recovery experiments on the analyzed samples should be imperative.

The applicability of the assay was further demonstrated by the investigation of a wide variety of food products purchased from a local food store. About 30% (5 of 17) of all samples that did not have peanut listed as an ingredient contained peanut protein between 2 and 18 ppm. Even though this study may not be representative for the German market, because only a restricted number of samples was picked at one time and batch-to-batch variations were not considered, the investigation clearly demonstrated that there is a problem of known or unknown contamination with peanut protein which cannot be identified by the consumer. Similar results on different food samples were obtained in former studies with our rocket method (Holzhauser et al., 1998). The negative-control samples of this study were confirmed by ELISA, and samples having peanut fat or peanut oil as an ingredient as well as one refined peanut oil did not contain any detectable peanut protein. Samples having labeling of peanut or peanut paste indicating the presence of peanut protein were confirmed by ELISA, too. Several samples had peanut labeled at the last position of the ingredients list, indicating that peanut constituted the lowest percentage of all ingredients. However, in two of these samples peanut protein could not be detected. This may be explained by two reasons. Either the amount of peanut protein was beyond the limit of detection or no peanut protein was present at all. The possibility that protein at a level below 2 ppm was added as an ingredient may be negligible because addition of a food at such a low level would not make any sense in terms of food technological processing. Consequently, peanut might have been labeled because cross-contamination of the product could not be excluded with certainty. This may not be surprising as some products that are considered to have a potential presence of any nut material or are at risk from cross-contamination have already been labeled by the manufacturer (Craddock, 1997). In three other samples that were labeled as "may contain traces of peanuts", no peanut protein could be detected, either. The short screening protocol with a simplified extraction procedure was suitable to detect peanut protein in all samples in which peanut protein was present and vice versa, even at the limit of detection.

False-negative results could be excluded because even trace amounts of peanut protein were recovered from various types of food matrices (Tables 2 and 3). In-house contamination of samples was excluded because sample preparation of food items having peanut components declared and of samples without such declaration were separated from each other. Sample preparation was further controlled by routinely grinding blank samples after selected samples that apparently contained a high amount of peanut.

With this work we could demonstrate that reliable and reproducible quantitative analysis of peanut protein at trace levels and in complex food matrices can be achieved. We feel that our ELISA is sufficiently sensitive to detect peanut protein at levels where only mild or no allergic reactions are to be expected in sensitive individuals. As little as 200 μg of peanut protein in a sample of 100 g could still be detected, about the same absolute amount of peanut protein that could still elicit mild subjective reactions in a peanut-sensitive patient (Hourihane et al., 1997b), whereas a systemic reaction was triggered by an estimated amount of 45 mg of undeclared peanut protein in a dry soup mix (McKenna and Klontz, 1997). However, on the one hand, it has to be kept in mind that the food challenge experiments (Hourihane et al., 1997b) were done with peanut protein in one simple food matrix (flavored rice pudding) and at a not specified concentration level. On the other hand, we do not know for certain if highly sensitive subjects could still react to peanut protein at levels far below the ELISA's limit of detection.

Combining a rapid extraction procedure with the use of precoated microwell plates and standards stored at −20 °C, the screening variant of the developed ELISA allows analysis of peanut protein within <1 working day. The assay is reliable, sensitive, and specific and may be automated. Because of the commercially available antiserum and due to extensive validation data, to our knowledge, this assay is the first commonly available ELISA having characteristics that are also sufficiently transparent. Manufacturers are now able to monitor their products at reasonable expenditure. The benefits would be more precise labeling of food products and increased food safety for the majority of peanut-allergic subjects.

ABBREVIATIONS USED

RIA, radioimmunoassay; ELISA, enzyme-linked immunosorbent assay; Tris, tris(hydroxymethyl)aminomethane; Tricine, *N*-[tris(hydroxymethyl)methyl]glycine; PBS, phosphate-buffered saline; BSA, bovine serum albumin; Tween 20, polyethylene-sorbitan monolaurate; TMB, 3,3′,5,5′-tetramethylbenzidine; HRP, horseradish peroxidase; AP, alkaline phosphatase; OD, optical density; IgE/IgG, immunoglobulin E/G; NSB, nonspecific binding; B, signal at definite dose of analyte; B_0, maximal signal at zero dose of analyte (zero inhibition); mab, monoclonal antibody; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; CV, coefficient of variation; NBT, nitroblue tetrazolium; BCIP, 5-bromo-4-chloro-3-indolyl phosphate.

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