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Development of a Real-Time PCR and a Sandwich ELISA for Detection of Potentially Allergenic Trace Amounts of Peanut (*Arachis hypogaea*) in Processed Foods

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Hidden allergens in food products are, especially for peanut-allergic consumers, a serious problem because even low amounts (~200 μg) of peanut can elicit allergic reactions. Undeclared peanut traces can be found in processed food products, because contaminations with peanut during production processes are frequent. To minimize the risk of such cross-contaminations, it is necessary to develop sensitive analytical methods for the detection of hidden allergens in foods. For this approach we developed two peanut-specific assays based on the detection of peanut protein by specific antibodies (sandwich ELISA) and by the detection of peanut-specific DNA (part of the coding region of Ara h 2) by a real-time PCR. Both tests did not show any cross-reactivity with 22 common food ingredients (cereals, nuts, legumes), and the limit of detection is <10 ppm peanut in processed foods. Thirty-three random samples of food products were tested for the presence of peanut to compare both assay types with each other and to evaluate the percentage of foods on the German market that are contaminated with peanut traces. We found that four products (13.3%) without peanut in the list of ingredients contained peanut protein in a range from 1 to 74 ppm peanut protein and that the results of both tests correlated well. The real-time PCR was able to detect one more positive sample than the sandwich ELISA. In conclusion, both assays are sensitive and specific tools for the detection of hidden allergens in processed foods.

KEYWORDS: Peanut; *Arachis hypogaea*; real-time PCR; sandwich-ELISA; hidden allergen

INTRODUCTION

In Europe and in the United States the prevalence of food allergies is about 1−2% in adults and adolescents, and even up to 8% of children are affected (*1*−*6*). The clinical manifestation of food allergies varies from mild symptoms, such as oral allergy syndrome or mild urticaria, to severe anaphylactic reactions with fatal consequences. It is estimated that in the United States approximately 150 deaths and about 30000 cases of anaphylactic reactions are caused by food allergies each year (*7*, *8*). The majority of reported anaphylactic reactions could be traced back to an unintentional ingestion of peanuts or tree nuts (*9*−*11*). Especially peanut is a serious problem for allergic individuals for two reasons. First, one-third of all severe allergic reactions can be traced back to the ingestion of peanut (*7*, *8*), and second, very low amounts of peanut can trigger allergic reactions as described in two double-blind placebo-controlled food challenge (DBPCFC) studies (*12*, *13*). In these studies about 100 μg of peanut protein was sufficient to elicit mild reactions in peanut-sensitized persons. Another problem for peanut-allergic persons is the heat stability of peanut allergens. In a study by Maleki and co-workers it is described that the IgE reactivity of peanut allergens is even increased by roasting (*14*). Hence, it is not possible to reduce the allergenic potential of peanut by a heat treatment, as described for celery (*15*). Until now, the only possibility for peanut-allergic consumers to prevent allergic reactions is the strict avoidance of peanut-containing foods in combination with a peanut-management plan (*16*). Nevertheless, several studies show that an accidental ingestion of peanut and other food allergens is common. In 48% of peanut-allergic individuals a follow-up reaction occurs within a time period of one year after the last allergic event (*17*). A Swedish study reviewed 163 cases of severe anaphylactic reactions to foods with the result that in 62 cases an inadequate labeling and in 71 cases contaminations of the food product were responsible for the allergic reactions (*18*). In several surveillance studies of foods from the German market performed by our group we found between 28.6% and 62.5% of all analyzed foods contaminated with peanut or hazelnut (*19*−*23*). Such contaminations with hidden allergens can, e.g., occur due to insufficient cleaning procedures of the production equipment or the use of contaminated raw material, or labeling can be incorrect and incomplete (*24*).

To prevent the food-allergic consumer from unintentional ingestion of food allergens, it has been suggested to modify existing food labeling directives to commit food manufacturers

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to an obligate labeling of the most important food allergens if they are part of the recipe. Furthermore, it would be useful to avoid scientific terms for ingredients such as lecithin or casein, which are on the first view not distinguishable as ingredients with an allergenic potential (*25*). Within the European Union and Switzerland such labeling directives have been recently implemented in their regulations for food labeling (*26*, *27*). In addition the Swiss regulation demands that potentially allergenic foods, which are not part of the recipe, have to be declared in the list ingredients when their concentration is higher than 1 g/kg (1000 ppm). The development of analytical methods for allergen detection is necessary for monitoring the implementation of such guidelines, to protect the consumer from hidden allergens and to assist the manufacturers to establish concepts for hazard analysis and critical control points (HACCPs) to control the risk of contaminations. According to the threshold studies of peanut (*12*, *13*), such assays should be able to specifically detect the corresponding allergen at least at a level of 10 ppm.

Most of the commercially available and published assays for allergen detection are based on the determination of potential allergenic proteins by ELISA techniques. PCR methods are well established and comprehensive tools for species differentiation and detection of GMO foods, but until now little has been known of whether the PCR technology is suitable for the detection of hidden allergens in processed foods, and of the correlation between protein- and DNA-based assays. Recently, we published a PCR−ELISA for the detection of hidden hazelnut traces in foods (*22*). In this study we analyzed 41 food samples for the presence of hazelnut with the PCR−ELISA and with a validated sandwich ELISA. The results obtained with both tests were in good concordance, indicating a comparability of both assay types.

To evaluate if the results of this hazelnut study are transferable to peanut-specific methods, we developed and validated a sandwich ELISA and a real-time PCR for the detection of peanut traces in processed foods. To compare the performance of both tests, several commercial food samples purchased from local food stores were analyzed with both methods.

MATERIALS AND METHODS

Buffers and Reagents. For the peanut sandwich ELISA a polyclonal sheep antiserum (Pharmacia, Uppsala, Sweden) and a polyclonal rabbit antiserum (Riedel de Haen, no. 45262, Seelze, Germany, out of sale) were used. Both were raised against extracts of peanuts. Furthermore, we applied a horseradish peroxidase labeled antiserum from goat raised against rabbit IgG (Sigma, A-5045, Munich, Germany). All antisera were aliquoted and stored in 50% glycerol at −20 °C. All chemicals used were of analytical grade or as specified. Sample extraction buffer consisted of 8 mM tris(hydroxymethyl)aminomethane (Tris) and 25 mM *N*-[tris(hydroxymethyl)methyl]glycine (Tricine) at pH 8.6 and additionally 6% fish skin gelatine (Sigma, no. G 7765, Deisenhofen, Germany). 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, 70 mM Na_2HPO_4, and 150 mM NaCl. Blocking solution was 1.5% bovine serum albumin (BSA) (enzyme immunoassay grade, Fluka, Neu-Ulm, Germany) in coating buffer. Incubation buffer (PBS, pH 7.4) consisted of 0.5% BSA and 0.5% Tween 20 (Sigma, Deisenhofen, Germany). PBS, pH 7.4, containing 0.5% Tween 20 was used as the washing buffer. For the preparation of the substrate buffer 1 mL of a 21 mM 3,3′,5,5′-tetramethylbenzidine (Merck, Darmstadt, Germany) solution was added to 20 mL of citrate buffer, containing 210 mM citric acid (Merck, Darmstadt, Germany) and 303 mM potassium hydroxide (Merck). Finally, 6.6 μL of 30% hydrogen peroxide (Merck) was added. The substrate reaction was stopped by adding stop solution consisting of 100 μL of 2.5 N H_2SO_4 (Merck).

Food Products. Commercial food products were purchased from local food stores. We selected 16 products without peanut in the list of ingredients, 14 products with a remark such as "may contain nuts", and 3 peanut-containing products. For recovery studies and evaluation of matrix effects we used industrially manufactured samples of milk and semisweet chocolates spiked with 10, 40, and 200 ppm peanut. Unspiked samples were used as negative controls.

Sample Homogenization. Sample homogenization for protein and DNA extraction was performed as described elsewhere (*20*). Briefly, 40 g of the food sample was frozen with liquid nitrogen and grounded three times for 15 s in an analytical mill (Grindomix GM 200, Retsch, Haan, Germany). After homogenization, the samples were stored at −20 °C.

Sample Preparation for Sandwich ELISA. Protein extraction from the food samples was performed as described elsewhere (*20*): Briefly, 1 g of homogenized sample was suspended in 18 mL of extraction buffer. Extraction of peanut protein was carried out for 1 h at 45°C, and the samples were vigorously shaken three times during extraction. Afterward the extraction volume was adjusted to 20 mL with extraction buffer. One milliliter aliquots of each sample were centrifuged for 30 min at 20000*g* and 15 °C. For further analysis 600 μL of the supernatants was transferred into fresh reaction tubes.

Sample Preparation for Real-Time PCR. For DNA preparation a commercial kit (SureFood Plant X, Congen, Berlin, Germany) was used according to the manufacturer's instructions. Briefly, the samples were lysed for 30 min with lysis buffer and proteinase K provided with the kit. After centrifugation the supernatant was cleaned up by filtration. For optimal DNA binding to the silica matrix binding buffer was added to the supernatant. After DNA binding to the silica column and subsequent washing steps, the prepurified DNA was eluted with elution buffer. Again, the DNA was bound to a new silica column, washed, and finally eluted with 50 μL of elution buffer. The purified DNA could be stored for 4 weeks at −20 °C.

Primers and Probes. All oligonucleotides for real-time PCR were synthesized by MWG Biotech, Ebersberg, Germany. Primers and probes were specific for the Ara h 2 gene (acc. no. L77197). Sequence information: forward primer AR-58 F, 5′-GCAGCACTGGGA ACTC-CAAGGAGACA-3′; reverse primer AR-143 R, 5′-GCATGAGATGT-TGCTCGCAG-3′; TaqMan probe, AR-103 T: 5′-CGAGAGGGC-GAACCTGAGGCC-3′ (modifications: 5′-FAM, 3′-TAMRA).

ELISA Standard Preparation. For preparing the protein standards for sandwich ELISA 1 g of peanut paste was extracted with 20 mL of extraction buffer without fish gelatin. The extraction procedure was performed as described above. The protein content was measured by a Bradford assay (Pierce Coomassie Plus, Perbio, Bonn, Germany). Serial dilutions in a range from 1280 to 5 ng/mL were prepared, and aliquots of 1 mL were stored at −20 °C for up to six months.

Real-Time PCR Procedure. A mastermix sufficient for 200 reactions was prepared by mixing the following compounds in a volume of 9 mL: 1× TaqMan buffer A, 20 μg/mL BSA, 5 mM $MgCl_2$, 200 μM dATP, dCTP, and dGTP, 400 μM dUTP, 300 nM oligonucleotides, 200 nM TaqMan probe. The mastermix was stored in 20 aliquots of 442.5 μL at 4 °C. One aliquot was sufficient for 10 reactions. A 2.5 μL sample of Taq-polymerase (5 U/μL) and 5 μL of uracil-*N*-glycosylase (UNG, 1 U/μL) were added immediately to each aliquot before the PCR mix was pipetted. Chemicals and enzymes used for the mastermix were part of the TaqMan PCR core reagent kit of Applied Biosystems. BSA (Calbiochem, Schwalbach, Germany) used in this assay was certified to be free of nuclease and DNAse. PCR reaction mixes were prepared by mixing 45 μL of mastermix (including Taq-polymerase and UNG) and a 5 μL sample of DNA in a 96-well optical plate (Applied Biosystems, Weiterstadt, Germany). Uracil-*N*-glycosylase was used to avoid false positive results due to carryover contaminations from previous amplification reactions. DNA samples were tested in duplicate. For sample analysis two separate DNA extractions were performed and amplified in two separate reactions. On each 96-well plate six nontemplate controls (NTCs) and two positive controls were used to control mastermix purity and PCR performance. The following PCR conditions (time release protocol) were used: primary incubation step at 50 °C for 2 min (UNG digest), second step at 95 °C for 2 min (activation of hot start Taq-polymerase), and 55 cyles of 95 °C for 30

s and 62 °C for 1 min. Fluorescence was read after each cycle in an ABI Prism 7700 (Applied Biosystems) thermocycler.

Sandwich ELISA Procedure. The cavities of a certified 96-well plate (Nunc, Wiesbaden, Germany) were coated overnight at 4 °C with sheep antiserum raised against peanut (dilution 1:50000 in 150 μL of coating buffer). The next day, the coating buffer was discarded. Blocking was performed with blocking buffer for 1 h at 37 °C followed by four washings with 200 μL of washing buffer. The coated 96-well plate was stable at −20 °C for several weeks. The protein extracts from the food samples (see above) were diluted 1:5 in incubation buffer, and 150 μL was applied to the plate. For sample analysis two independent protein extractions were performed and tested by sandwich ELISA. After incubation for 1 h at 37 °C, the ELISA plates were washed four times. A 150 μL sample of the rabbit antiserum (dilution 1:150000 in incubation buffer) was applied and incubated for 1 h at 37 °C followed by four washings. A 150 μL sample of the horseradish peroxidase labeled detection antibody from goat raised against rabbit IgG (dilution 1:20000) was added for 1 h at 37 °C. After washing, 150 μL of substrate buffer was applied and incubated for 15−30 min at room temperature. The substrate reaction was stopped by adding 100 μL of stop solution. The optical density of each well was read bichromatically at a 450 nm main wavelength and a 630 nm reference wavelength by a Spectramax 340 (Molecular Devices, Munich, Germany) plate reader. Logarithmic regression of standard curves was performed with a four-parameter logistic model. All washing steps were performed in a plate washer (BioTek Elx 405, Biotek, Neufahrn, Germany).

Agarose Gel Electrophoresis. For analyzing the PCR products by gel electrophoresis 5 μL of the PCR reaction mixture was mixed with 1 μL of sample buffer, containing 15% ficoll, 0.06% bromophenol blue, and 0.06% xylencyanol. The samples were loaded onto a 3% agarose gel. The gels were run with 1× TAE in a RAGE RGX-60 chamber (TEBU, Frankfurt/Main, Germany) for 10 min at 200 V. The gels were stained with ethidium bromide (20 μg/mL) and visualized on a UV transilluminator.

RESULTS

Cross-Reactivity Studies. For determining cross-reactivities of both assays several nuts, legumes, and cereals (almond, barley, Brazil nut, chickpea, coconut, hazelnut, kidney bean, oat, peas, pine seed, pinto bean, pistachio, pumpkin seed, rice, rye, sesame seed, soy bean, walnut, wheat, white beans) were analyzed twice by sandwich ELISA and real-time PCR.

The rational for choosing these foods for cross-reactivity testing was that both assays are mainly intended for the analysis of confectionary products. Therefore, we did not test for cross-reactions with spices such as curry. For testing by sandwich ELISA extracts were diluted 1:5 with incubation buffer corresponding to 100% of tested food, and for real-time PCR 5 μL of extracted DNA was used as the template. No cross-reactions were observed with the examined foods.

Detection Limit and Limit of Quantitative Detection. The evaluation of the limit of detection (LOD) for the sandwich ELISA was based on the assumption that a peanut-specific signal is generated when the measured optical density is higher than the background signal (generated by incubation buffer) plus 3-fold the standard deviation (3σ). For calculation 24 sandwich ELISAs were analyzed, resulting in an LOD of 0.07 ± 0.04 ppm peanut protein. The limit of quantification (LOQ) was defined as above, but calculated with the 6-fold standard deviation (6σ) of the background signal, resulting in an LOQ of 0.15 ± 0.12 ppm peanut protein. For practical reasons we defined the LOQ at 0.5 ppm peanut protein corresponding to the lowest standard concentration used for the sandwich ELISA. Furthermore, we analyzed the precision profile of the standard curve to evaluate the range of highest precision. Therefore, the mean value of the reversal points of the 24 standard curves, derived by the four-parameter logistic (4-PL) model, was calculated, indicating the highest assay precision at a protein concentration of 78.8 ± 18.7 ng/mL. A typically shaped standard curve is shown in **Figure 1**. To evaluate the sensitivity of the real-time PCR, industrially manufactured chocolate samples (whole milk and semisweet chocolate) that contained defined amounts of peanut (200 and 10 ppm) were analyzed. Peanut was clearly detectable at both concentrations in whole milk chocolate as well as in semisweet chocolate. (**Figure 2**). Control samples without spiked peanut of both model matrices gave negative results.

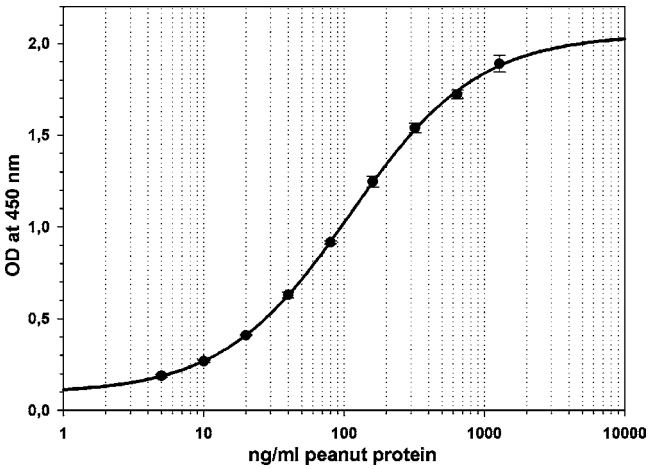

Figure 1. Representative standard curve of the peanut sandwich ELISA. The LOD of this assay was determined at 0.07 ± 0.04 ppm peanut protein. The LOQ was calculated at 0.15 ± 0.12 ppm. The highest assay precision was found at 78.8 ± 18.7 ng/mL peanut protein.

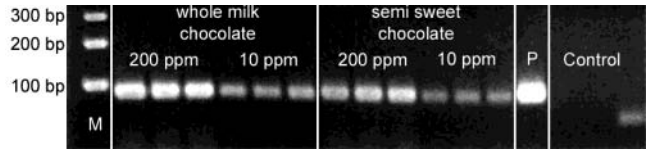

Figure 2. Determination of PCR sensitivity by analyzing peanut spiked-samples of whole milk and semisweet chocolates. The chocolates were spiked with 200 and 10 ppm whole peanut and analyzed in triplet. The PCR products with a size of 86 base pairs are peanut-specific, whereas the PCR product appearing in the negative control (C) has a size of approximately 40 base pairs and is therefore not specific for peanut. This band is presumably the result of an occasionally occurring primer dimer formation. M = 100 bp marker, P = positive control (template = 5 ng of peanut DNA), and control = negative control.

For determining the sensitivity of the PCR method we prepared a DNA standard with defined copy numbers as previously described (*22*). Positive PCR results were obtained even with a DNA standard containing 10 copies per reaction (data not shown).

Recovery Studies and Matrix Effects. The recovery rates of the sandwich ELISA were evaluated by analyzing the industrially manufactured chocolates, which were used to determine the sensitivity of the real-time PCR. Therefore, five extractions of each spiked chocolate were performed, and the extracts were analyzed by the peanut-specific sandwich ELISA (**Figure 3**). To calculate the correlation factor from peanut protein to whole peanut, we extracted protein from the authentic peanut paste, used for spiking the industrially manufactured chocolates. In five independent protein extractions we found a correlation factor of 6.3, corresponding to 15.9% extractable protein. Protein determination was done by a commercially

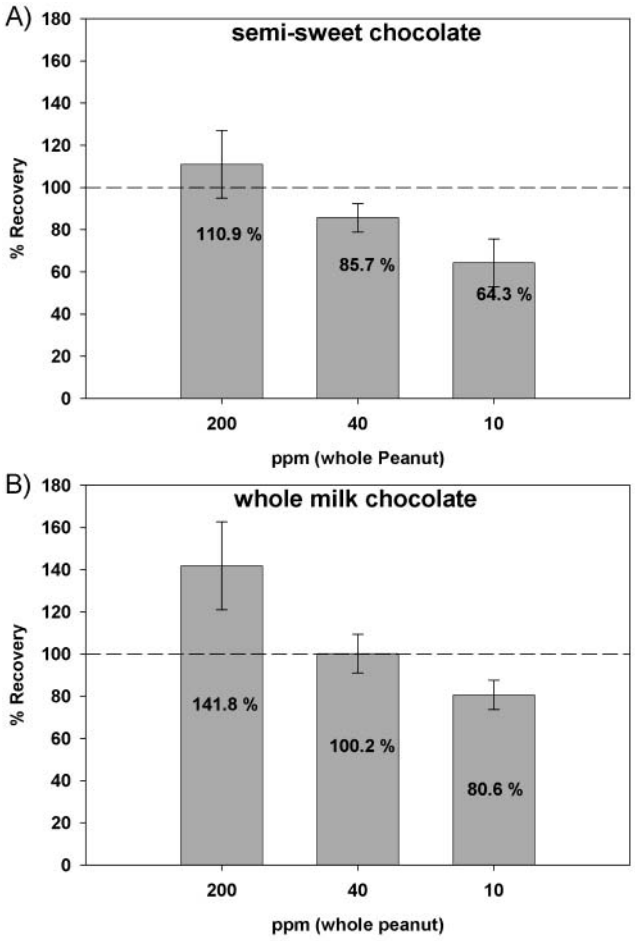

Figure 3. Recovery rates for the sandwich ELISA determined in semisweet (**A**) and in whole milk (**B**) chocolate. Both chocolates were spiked with 200, 40, and 10 ppm peanut. For determining the recovery rates, five analyses of each spiked chocolate were performed and the average recovery was calculated.

Table 1. Inter- and Intraassay Variances Determined for the Sandwich ELISA

peanut concn (ppm)	interassay variance ($n = 5$) (%)	intraassay variance ($n = 10$) (%)
	Whole Milk Chocolate	
200	14.7	6.0
40	9.2	4.4
10	8.6	8.0
	Semisweet Chocolate	
200	14.5	5.3
40	7.9	4.6
10	17.5	11.0

available Bradford assay. In whole milk chocolate and semisweet chocolate we found recovery rates from 80.6% to 141.8% and from 64.3% to 110.9%, respectively. Control samples without peanut gave results below 0.5 ppm.

Inter- and Intraassay Precision in Chocolate Samples. Intraassay precision and interassay precision of the sandwich ELISA were determined by analyzing extracts of the industrially manufactured whole milk and semisweet chocolates with defined contents of peanut (**Table 1**). For intraassay precision 30 replicates of each extract were analyzed on separate microwell plates. The mean coefficients of variation (CVs) were determined as 6.0%, 4.4%, and 8.0% for 200, 40, and 10 ppm peanut in whole milk. For semisweet chocolate spiked with 200, 40, and 10 ppm the mean CVs were determined as 5.3%, 4.6%, and 11%.

For interassay precision five independent extracts of each chocolate were analyzed on five different days. The mean CVs were determined at levels of 14.7%, 9.2%, 8.6% in whole milk chocolate and at levels of 14.5%, 7.9%, and 17.5% in semisweet chocolate, each containing 200, 40, and 10 ppm peanut.

Investigation of Commercial Food Products. To compare the results of the sandwich ELISA and the real-time PCR, we purchased 33 food samples from local food stores and tested them for the presence of peanut with both tests. Three of the purchased samples contained peanut as indicated in the list of ingredients. Sixteen samples were labeled with an advisory remark such as "may contain traces of nuts", and 14 samples did not have peanut in the list of ingredients. For analyzing, the DNA and protein of all samples were isolated in two independent extractions and tested for the presence of peanut protein and DNA, respectively. To minimize the risk of false positive results by the ELISA, samples were considered as positive if peanut protein >0.5 ppm was detected even if the limit of detection was determined at 0.07 ± 0.04 ppm peanut protein. For quantification of the peanut content the mean value of two determinations on two independent sample extracts was calculated. Samples analyzed by real-time PCR were considered positive for the presence of peanut traces if both independent DNA extracts tested positive. All results are displayed in **Table 2**, and in **Figure 4** amplification plots of selected foods are displayed. The C_t values were compared with the corresponding amount of peanut protein measured with the sandwich ELISA.

In samples 1−3 which contained peanut in the list of ingredients, peanut was detected with both methods. The content of peanut protein measured by the protein ELISA ranged from 1727 ppm (sample 2) to 7512 ppm (sample 1). By analyzing these samples with the real-time PCR it was confirmed that the peanut content of sample 2 ($C_t = 32.52 \pm 0.11$) was lower than the peanut content of sample 1 ($C_t = 28.58 \pm 0.11$). From the 16 samples with a precautionary labeling samples 17 and 19 tested positive by the protein ELISA. These results were confirmed by the real-time PCR.

Two (samples 32 and 33) of the sixteen samples, which were not labeled for the presence of peanut, tested positive with both assays. Additionally, sample 24, a whole milk chocolate with hazelnut, tested positive by the real-time PCR. The obtained C_t was at 45.75 ± 2.89.

DISCUSSION

The majority of published and commercially available peanut-specific assays are based on the detection protein with peanut-specific antibodies. The first PCR method for the detection of peanut was published in 2003 (*28*). With this real-time PCR the authors were able to detect peanut down to 2 mg/kg in spiked biscuits. However, the PCR results were not compared to a validated ELISA.

Within this study we developed and validated a sandwich ELISA and a semiquantitative real-time PCR for the detection of trace amounts of peanut in processed foods. Both assays were able to detect peanut traces down to 10 ppm in whole milk and semisweet chocolate. No cross-reactivities with a variety of legumes, nuts, and cereals were observed, indicating a high specificity of both detection systems.

Peanut-specific polyclonal sheep and rabbit antisera, which were used for the sandwich ELISA, were generated by immunization against peanut protein extracts. For protein standard

Table 2. Analysis of 33 Prepacked Foods for the Presence of Peanut

sample			sandwich ELISA			real-time PCR
no.	description	D[a]	protein concn (ppm)	% CV	result	result (C_t)
1	cereal bar, peanut	+	7512	7.2	positive	positive (28.58 ± 0.11)
2	chocolate lens, peanut	+	1727	6.9	positive	positive (32.52 ± 0.19)
3	cookies, peanut	+	2783	14.2	positive	positive (29.77 ± 1.99)
4	yogurt, straciatella	±			negative	negative
5	cookies, whole milk chocolate I	±			negative	negative
6	cookies, cereals	±			negative	negative
7	pudding, caramel	±			negative	negative
8	pudding, whole milk chocolate	±			negative	negative
9	dessert, whole milk chocolate	±			negative	negative
10	candy bar I	±			negative	negative
11	cookies, whole milk chocolate II	±			negative	negative
12	chocolate, semisweet I	±			negative	negative
13	cookies, whole milk chocolate III	±			negative	negative
14	cereals, nougat	±			negative	negative
15	cereals I	±			negative	negative
16	chocolate lens	±			negative	negative
17	chocolate, semisweet II	±	0.9	9.6	positive	positive (42.36 ± 0.37)
18	chocolate, whole milk—strawberry	±			negative	negative
19	chocolate, whole milk—hazelnut I	±	24.1	10.4	positive	positive (41.52 ± 2.10)
20	chocolate, whole milk—yogurt	–			negative	negative
21	cereals II	–			negative	negative
22	cereals III	–			negative	negative
23	chocolate, white	–			negative	negative
24	chocolate, whole milk—hazelnut II	–			negative	positive (45.75 ± 2.89)
25	cookies, whole milk chocolate IV	–			negative	negative
26	chocolate, bitter	–			negative	negative
27	chocolate, whole milk—coconut	–			negative	negative
28	chocolate, whole milk I	–			negative	negative
29	chocolate, semisweet—hazelnut	–			negative	negative
30	chocolate, semisweet—mint	–			negative	negative
31	chocolate, whole milk nougat	–			negative	negative
32	chocolate, semisweet III	–	74	16.8	positive	positive (42.05 ± 0.86)
33	chocolate, whole milk II	–	6.3	5.8	positive	positive (39.63 ± 0.36)

[a] Labeling of peanut components: +, positive declaration; –, negative declaration; ±, labeled with a remark such as "may contain traces of peanuts or nuts".

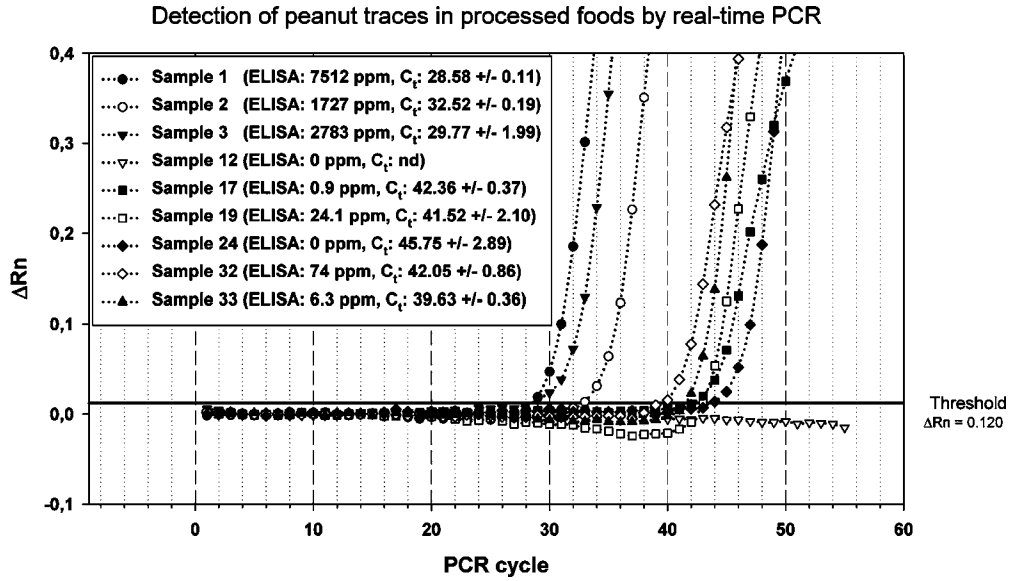

Figure 4. Amplification plot of some exemplary foods analyzed by real-time PCR in comparison with quantitative results obtained by the sandwich ELISA.

preparation we used extracts of peanut paste, which is used by food manufacturers as an ingredient. In recovery studies with industrially manufactured chocolates with defined amounts of peanut we found for whole milk chocolate recovery rates in a range between 80.6% and 141.9%, and for semisweet chocolate recovery rates in a range between 64.3% and 110.9%. As recommended by Keck-Gassenmeier and co-workers (*29*) we added fish gelatin to the extraction buffer to minimize the effect of inhibitory substances on the ELISA. Nevertheless, the findings of the recovery experiments indicate that inhibitory substances such as tannins or other polyphenolic components may still interfere with the extraction or the immunoassay procedure, resulting in a decreased recovery rate in chocolates with a low peanut content. For peanut detection by real-time PCR the used primers and probes were specific for a section of the coding region of the Ara h 2 gene from peanut, and

generated and detected an amplicon with a size of 86 bp. Cross-reactivities were tested with the same samples as used for validation of the sandwich ELISA. Again we could not find any cross-reactions with other food products, indicating the same high specificity as for the sandwich ELISA. For testing sensitivity we used industrially manufactured semisweet and whole milk chocolates as described above. The PCR method detected peanut at a level of 10 ppm in both matrices, but we recommend to use this PCR method only as a semiquantitative tool due to the high CVs, and because the influence of PCR inhibitors and thermal degradation of peanut DNA were not specifically addressed in this study. Despite this limitation, we think that the presented PCR method is still useful, because institutions such as the German Institute for Standardization (DIN) and the American Society for Testing and Materials (ASTM) demanded to verify the sequence of a PCR product, if a PCR is used for analytical approaches. Common verification techniques (southern blot, sequencing, restriction cuts) are time-consuming and expensive and can be overcome by the use of a real-time PCR because the sequence verification of the generated PCR product is guaranteed by the FAM/TAMRA labeled probe, which binds specifically to the amplified sequence. Also, the use of carcinogenic ethidium bromide for staining of agarose gels can be avoided by this PCR technique. In general, the real-time PCR technology has the potential to become a tool for quantitative analysis of hidden allergens in processed foods, if standardized reference materials become available. Such materials are essential for standardization of DNA- and also protein-based assays. Even if such reference materials would be available, other factors such as a different degree of thermal degradation of template DNA in real food products would still affect quantitative evaluation of real-time PCR results.

After in -house-validation of both assay formats we tested 33 commercial food products that were purchased at local food markets. The aim of this part of the study was first to evaluate whether the results of a protein-based and a DNA-based detection method are comparable, and second to determine the percentage of peanut-contaminated foods of the German market. Three of the purchased foods contained peanut in the list of ingredients, sixteen samples were labeled with a precautionary advice such as "may contain nuts", and fourteen products did not contain peanut as stated by the manufacturer. For analysis we isolated protein and DNA from the food samples and tested these isolates by ELISA and real-time PCR, respectively. Two independent extractions were performed, and each sample was analyzed twice with both tests.

Two of the food samples without precautionary labeling and without peanut in the list of ingredients were contaminated with 6.3 ppm (sample 33) and 74 ppm (sample 32) peanut protein, which corresponds to 630 μg and 7.4 mg of protein in 100 g of chocolate. In highly sensitized persons these amounts probably have the potential to elicit allergic reactions. By analysis with real-time PCR samples 32 and 33 tested positive as well. Additionally, sample 24 was found positive at a very low concentration. This finding may indicate a slightly higher sensitivity of the real-time PCR in comparison to the sandwich-ELISA. Our results demonstrated that the contamination of foods, especially in chocolates, with potential allergens is still a common problem. Furthermore, the relative C_t values obtained by the real-time PCR for all samples that tested positive reflected the amount of protein measured by the sandwich ELISA. This emphasizes that the PCR can be used as a semiquantitative method for the detection of peanut traces in processed foods if reference materials with defined amounts of peanut are available. In comparison with other market studies performed in the last few years, the percentage of contaminated foods seems to be constant.

Although the results of both assays are not in complete concordance, our data indicate that DNA-based and immunological assays give comparable results for the detection of peanut traces in processed foods, and that both assay types are suitable for analyzing foods for the presence of hidden allergens. This conclusion is supported by the results of a further study where a PCR−ELISA and a sandwich ELISA for the detection of hazelnut were compared (*22*). Similar to the results of this study, divergent results between DNA and protein analysis were only found at the level below 10 ppm hazelnut.

Despite the good qualitative correlation observed with hazelnut and peanut assays, it is not clear whether quantitative PCR methods are suitable to determine the allergen content of any matrices. Especially in highly processed matrices such as vegetable oils, pickled products, and canned foods, it is likely that the results of immunological and PCR assays will not be comparable due to DNA degradation during manufacturing. Moreover, divergent results are to be expected in cases were isolated protein fractions are used as a food ingredient.

Nevertheless, we have shown that DNA-based assays are suitable for analyzing confectionary and dairy products for the presence of hidden peanut and hazelnut allergens. These methods can also be used for monitoring the effectiveness of cleaning processes from production units of the food industry, and as a consequence, they can help to prevent the food-allergic consumer from unintentional ingestion of hidden allergens.

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