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Detection of Potentially Allergenic Hazelnut (*Corylus avellana*) Residues in Food: A Comparative Study with DNA PCR-ELISA and Protein Sandwich-ELISA

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Allergen detection is of increasing interest for food labeling purposes. A comparative study with a commercial hazelnut-specific PCR-ELISA and a sandwich-type ELISA detecting hazelnut protein was performed to investigate to what extent immunochemical and DNA-based techniques would correlate in the detection of trace amounts of potentially allergenic hazelnut residues. Both methods were highly sensitive and allowed the detection of even <10 ppm of hazelnut in complex food matrixes. The protein-ELISA was highly specific for hazelnut. However, some foods could lead to false-positive results at the 10 ppm level. The PCR-ELISA did not show any cross-reactions with non-hazelnut foods, thus reducing the probability of having false positives at the trace level. Forty-one commercial food products with and without hazelnut components on their labels were analyzed for the presence of hazelnut. Of the 27 products in which hazelnut components were detected, two samples were not identified by the protein-ELISA, and only one sample, namely one white chocolate having <1 ppm of hazelnut protein, was not detected by PCR-ELISA. The good correlation of the results of PCR-ELISA and protein-ELISA suggested that both PCR-based and immunochemical techniques are suitable for reliable detection of potentially allergenic hazelnut residues in foods at the trace level.

KEYWORDS: Hazelnut (*Corylus avellana*); hidden allergens; PCR-ELISA; sandwich-ELISA

INTRODUCTION

In Western countries, up to 1−2% of the total human population suffer from clinically proven food allergies, and among children the prevalence is even higher, up to 8% (*1*−*4*).

The symptoms of food allergy range from mild urticaria to life-threatening anaphylactic shock. Indeed, several fatal episodes due to food allergy have already been reported and are summarized elsewhere (*5*−*9*). Burks et al. (*10*) estimated that approximately 120 deaths related to food allergy occur in the United States each year. Accordingly, Bock et al. (*8*) have calculated a rate of approximately 140 deaths owing to allergic reactions to hidden allergens in processed foods. In an English study, more than 50% of 172 documented severe anaphylactic reactions were related to foods (*11*). According to a status report of the Food and Agriculture Organization (FAO) (*12*), the most common foods responsible for >90% of severe allergic reactions are hen's egg, cow's milk, fish and crustacea, peanut, soybean, wheat, and tree nuts such as hazelnut, almond, walnut, and others.

To prevent possible life-threatening reactions, allergic individuals have to strictly avoid the consumption of the allergenic food. However, various studies demonstrated that anaphylactic reactions occurred even though people knew about their allergy and tried hard to avoid the intake of allergens (*8*, *13*−*16*). Hence, allergens may not always be identified by the consumer, for reasons such as product mislabeling or unintentional cross-contamination during food production. In Sweden, 51 of 77 documented severe allergic reactions due to an unintentional allergy intake could be related to insufficient food labeling, and 13 cases were caused by product-specific contamination (*17*).

To reduce the frequency of hidden allergens in foods, in the United States and Europe suggestions for the labeling of the most important food allergens as stated by the Codex Alimentarius Commission (*18*) of the FAO and the World Health Organization (WHO) were incorporated into drafts of future guidelines by the Food and Drug Administration (FDA) (*19*) and the European Commission (*20*). Accordingly, peanuts, soybeans, milk, eggs, fish, crustacea, tree nuts, and wheat, and additionally sesame and sulfite >10 mg/kg in the European Union, should be labeled in principle and independent from other labeling policies if the allergenic food or a product derived from it is an ingredient of the food product.

Therefore, analytical methods are required, not only to monitor the implementation of such labeling policies, but also to help manufacturers improve their food production in terms of hazard analysis of critical control points (HACCP) risk assessment and good manufacturing practice (GMP) (*21*−*23*).

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With more precise labeling, the composition of food products would become more transparent to allergic consumers, which would thus reduce the risk of reaction to hidden allergens.

Several double-blind placebo-controlled food challenge (DBPCFC) studies with food-allergic individuals indicated that even milligram amounts of the allergen could trigger an allergic reaction in highly sensitized subjects (*4*, *24*−*27*). Therefore, analytical methods should be able to specifically detect the corresponding allergenic food at the low parts-per-million (milligrams per kilogram) level.

Tree nuts are a frequent cause of severe food-related allergic reactions in Europe (*28*). In this study, hazelnut (*Corylus avellana*) as one important tree nut was chosen as an example because allergy to hazelnut is very common (*29*−*31*) and severe allergic reactions caused by hazelnut have been reported (*14*, *28*, *32*−*36*). Various immunochemical techniques, especially enzyme-linked immunosorbent assays (ELISA) and one application of the Polymerase Chain Reaction (PCR), for the detection of hazelnut traces have been reported (*34*, *37*−*40*). To our knowledge, none of these tests are available in a kit format for convenient application in the laboratories of food control agencies and the food industry. Here, we compare a new commercial hazelnut-specific PCR-ELISA with an already existing and extensively validated research ELISA to prove the applicability of such PCR tests in the sensitive and specific detection of hazelnut in complex food matrixes. As sequence verification is a necessary specificity confirmation in PCR (*41*), PCR products were detected in an ELISA-like technique using sequence-specific hybridization probes. The PCR-ELISA allowed a fast and simple sequence detection compared to other verification techniques.

MATERIALS AND METHODS

Hazelnut Samples and Commercial Food Products. Hazelnuts of the variety Nocciole Ordu (Turkey), both native and toasted at 140 °C for 30 min, were provided by Dr. G. Malgarini, Sorematec, Arlon-Schoppach, Belgium. Commercial food products were bought at a local food store.

Reagents, Buffers, and Instrumentation. All reagents and buffers used for the sandwich-type hazelnut protein-ELISA are described elsewhere in detail (*37*, *42*). Fish gelatin for protein extraction was purchased from Sigma (No. G7765, Taufkirchen, Germany). Except for the Platinum Taq hot-start polymerase (Invitrogen, No. 10966-034, Karlsruhe, Germany), all reagents and buffers needed to perform DNA amplification with PCR as well as reagents, buffers, and streptavidin-coated microtiterplates for DNA detection with DNA-ELISA were provided with the SureFood-Allergen Hazelnut Kit (CONGEN Biotechnology, No. S3002, Berlin, Germany), allowing amplification and detection of 192 reactions (two microtiterplates). The ready-to-use hazelnut-PCR premix contained PCR buffer, dNTPs, $MgCl_2$, and biotinylated hazelnut-specific primers derived from the cDNA sequence of the major hazelnut allergen, Cor a 1.0401 (*43*, *44*). One reaction volume of the hazelnut-PCR (master) mix for amplification was made by addition of 1.25 units of Platinum Taq polymerase to 45 μL of hazelnut-PCR premix. Liquid handling for the protein-ELISA and DNA-ELISA was performed with an eight-channel pipet or multistepper pipet (Eppendorf, Hamburg, Germany). Protein-ELISA incubations at 37 °C and DNA-ELISA incubations at 50 °C were carried out in a temperature-controlled horizontal shaker (Thermomixer comfort with microtiterplate frame, Eppendorf, Hamburg, Germany). Cell lysis for DNA preparation at 65 °C was performed in a temperature-controlled horizontal shaker (Thermomixer comfort with microtube frame, Eppendorf). PCR was run in thermocyclers with a heated lid (GeneAmp PCR System 9700, PE Applied Biosystems, Weiterstadt, Germany; Mastercycler, Eppendorf). Measurements of the optical density (OD) of protein-ELISA and PCR-ELISA were done with a Spectramax 340 (Molecular Devices, Munich, Germany) and a Fluostar (BMG Labtechnologies, Offenburg, Germany) microtiterplate reader controlled by data-processing software. DNA quantification of DNA preparations was done in a Hitachi U-2010 spectrophotometer (Binninger Analytik, Schwäbisch Gmünd, Germany).

Sample Preparation for Hazelnut Protein-ELISA. Prior to extraction, food samples of approximately 50−100 g were homogenized with an analytical grinder (Grindomix GM 200, Retsch, Haan, Germany). Samples with a high content of fat, such as chocolate, were frozen with liquid nitrogen prior to grinding. One gram of homogenized sample was extracted in a ratio of 1:20 with a Tris-Tricine sample extraction buffer, pH 8.6 (*42*), additionally containing 10% of fish gelatin, at 37 °C for 60 min. After centrifugation at 12000*g* for 15 min, the supernatant was diluted at least 1:5 with ELISA incubation buffer for protein-ELISA analysis.

Sample Preparation for Hazelnut PCR-ELISA. Samples were homogenized as described for hazelnut protein extraction. DNA preparation was done with the SureFood-PREP Plant X DNA-isolation kit (CONGEN Biotechnology, No. S1006) based on silica membrane technology with solvent-free reagents. In practice, 150 mg of the homogenized material was lysed in 580 μL of lysis buffer and in the presence of 20 μL of 20 μg/μL Proteinase K at 65 °C for 30 min. If the DNA concentration had to be measured spectrophotometrically, 400 μg of RNAse A was additionally added. The lysate was filtered through a spin filter fitted in a 2 mL microreaction tube by centrifugation at 12000*g* for 2 min. Optimal binding conditions of the clear lysate were adjusted with binding buffer, and the DNA was bound onto a new spin filter suited in a new 2 mL receiver tube by centrifugation. The bound DNA was washed twice with wash buffer and subsequently eluted with Elution Buffer X from the spin filter into a new reaction tube. Again, optimal binding conditions were set, and the DNA was bound onto a new spin filter, where it was washed twice. Finally, the purified DNA was eluted with 50 μL of elution buffer into a new receiver tube. Five microliters of the sample DNA was subjected to PCR. Alternatively, the purified DNA was stored at −20 °C until use. For determination of the concentration of genomic DNA, 5 μL of DNA was measured at 260 nm wavelength in a microcuvette in a spectrophotometer. One OD at 260 nm was equivalent to 50 ng/μL of genomic DNA.

Hazelnut Protein-ELISA Procedure. Details of hazelnut protein detection by sandwich-type ELISA are described elsewhere (*37*). Briefly, diluted extracts of samples and hazelnut protein standards were incubated at 150 μL/well in polystyrene 96-microwell plates coated with hazelnut-specific polyclonal antibodies from rabbit antiserum. After 1 h of incubation at 37 °C, the plates were further incubated with 150 μL/well of hazelnut-specific polyclonal sheep antiserum at 37 °C for 1 h, and subsequently with 150 μL/well of horseradish peroxidase (HRP)-labeled polyclonal anti-sheep IgG from rabbit for another 1 h at 37 °C. After each incubation step, the plates were washed at least twice with washing buffer and emptied. Solid-phase bound HRP was detected after addition of 150 μL/well of HRP substrate solution. The color development was stopped by addition of 100 μL/well of stop solution, and optical densities were read bichromatically at 450 nm main wavelength and 620 nm reference wavelength. ODs of the hazelnut protein standards were plotted against the logarithm of the hazelnut protein concentration covering a range between 10 and 1280 ng/mL. Using data-processing software, hazelnut protein concentrations of unknown food samples were derived from their sample ODs.

Hazelnut PCR-ELISA Procedure. The principle of the SureFood-Allergen Hazelnut test is displayed in **Figure 1**. In detail, the hazelnut PCR-ELISA was performed as follows: PCR was carried out in 200 μL thin-wall reaction tubes containing 50 μL of hazelnut reaction mix. The hazelnut reaction mix consisted of 45 μL of hazelnut-PCR mix and 5 μL of sample DNA. The PCR conditions were 95 °C for 1 min, followed by 45 cycles of 95 °C/20 s and 62 °C/20 s, with a final elongation step at 72 °C for 1 min. PCR performance was controlled by simultaneously investigating another two reactions with 5 μL of no-template control (NTC) and 5 μL of positive control DNA (PTC) added to 45 μL of hazelnut-PCR mix, respectively. For each sample, two DNA extracts were simultaneously amplified in separate reactions. For investigation of possible inhibitory effects caused by the sample matrix, one sample DNA was additionally analyzed in a corresponding reaction, with 5 μL each of the sample DNA and the PTC (sample

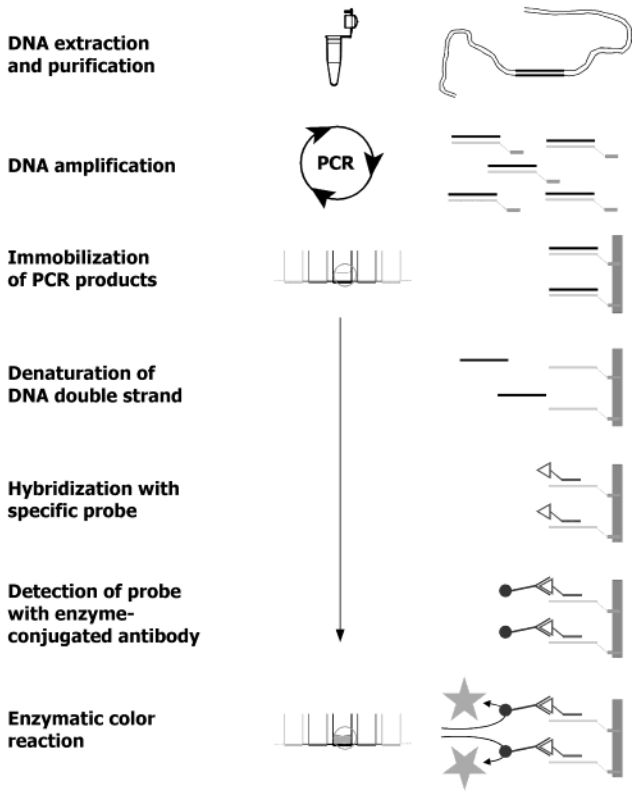

Figure 1. Principle of the SureFood-Allergen Hazelnut PCR-ELISA. Specific solid-phase detection of biotinylated hazelnut amplicons on a streptavidin-coated microtiterplate applying a sequence-specific hybridization probe.

inhibition control) added to 45 μL of hazelnut-PCR mix. After termination of the PCR, 5 μL of the PCR mix, potentially containing the 152 bp biotinylated amplicons, was added to a standard capacity streptavidin-coated microtiterplate prefilled with 100 μL of PCR binding buffer. Binding of PCR products was carried out at 50 °C for 15 min, followed by removal of the non-solid-phase-bound second DNA strand from the solid-phase-bound DNA strand by 5 min of incubation with 50 μL/well of denaturation buffer at ambient temperature. Sequence verification of the bound single-strand DNA was done by 15 min of incubation with 50 μL/well of hazelnut-specific hybridization probe at 50 °C. To ensure high stringency of the hybridization reaction, the wells were washed twice with stringency buffer at 50 °C for 5 min each. Thereafter, FITC-labeled hybridization probes were detected by 15 min of incubation with 50 μL/well of a FITC-specific antibody−enzyme conjugate at room temperature. After each incubation step, the plates were washed with washing buffer and emptied. Solid-phase-bound enzyme was detected by 10 min of incubation with 50 μL/well of substrate solution at room temperature. After addition of 50 μL/well of stop solution, ODs were read bichromatically at 450 nm main wavelenth and 620 nm reference wavelength. Parallel to the detection of all PCR products with the hazelnut-specific hybridization probe, the performance of the DNA-ELISA was controlled by detecting a synthetic biotinylated DNA fragment (hyb control) with a set of two sensitive hybridization probes (hyb-probe plus, hyb-probe minus). For correct interpretation of results, ODs of the negative PCR control (NTC) and of the hyb-probe minus had to be $\leq$0.2, whereas ODs of the positive PCR control (PTC) and of the hyb-probe plus had to be $>$0.2 and at minimum twice as big as those of the equivalent negative controls. Samples were interpreted to be positive when the ODs of samples and sample inhibition controls were $>$0.2. When the result of a sample DNA was negative, the OD of the corresponding sample inhibition control had to be at least 50% of the PTC to exclude inhibitory effects of the sample matrix. To minimize the possibility of having false-positive results, e.g., due to in-house cross-contamination, for each sample two independent DNA extractions were amplified and detected.

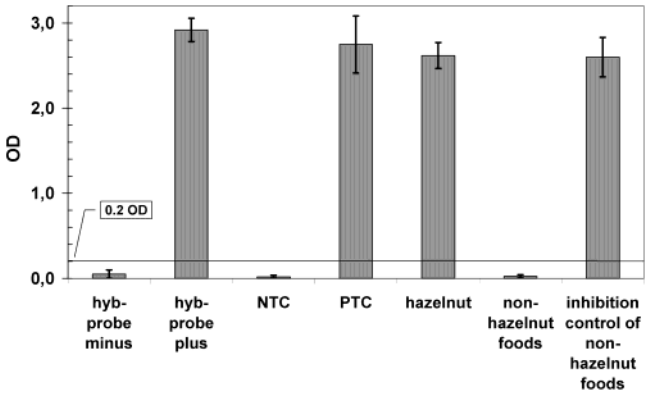

Figure 2. PCR-ELISA: mean optical densities (OD) of non-hazelnut foods investigated for possible cross-reactivities in comparison to hazelnut. The average ODs of 10 positive (PTC) and negative (NTC) controls for PCR as well as positive (hyb-probe plus) and negative (hyb-probe minus) controls for DNA-ELISA are displayed. Error bars indicate the standard deviation of ODs of either 10 corresponding controls or 35 different foods or food ingredients.

A sample was considered as being hazelnut positive only if both PCR reactions gave positive results.

Agarose Gel Electrophoresis. For a rough DNA size determination, 5 μL of amplified product was mixed with 1 μL of 6$\times$ concentrated gel loading buffer (15% (w/v) Ficoll, 0.025% (w/v) bromophenol blue, 0.025% (w/v) xylene cyanol in distilled water) and loaded onto 2% (w/v) agarose gels containing 0.005% (v/v) of 10000$\times$ GelStar nucleic acid stain (BioWhittaker Molecular Applications, via Biozym, No. 50535, Hess. Oldendorf, Germany). Gels were run in 1$\times$ TAE, pH 8.0, at 5 V/cm for 30 min, and PCR products were visualized on an UV transilluminator. The size of the PCR products was controlled by comparison with a 100 bp ladder (Invitrogen, No. 15628-019).

Preparation of Definite Copy Standards for PCR. Hazelnut DNA was amplified as described above, and 50 μL of the PCR product was purified from PCR reagents in a gel filtration column (CentriSpin-40, Princeton Separations, via EMP Biotech, Berlin, Germany) according to the manufacturer's instructions. The concentration of the 152 bp product was measured at 260 nm wavelength in a spectrophotometer. The copy number of amplicons per microliter was calculated from the measured DNA concentration, considering the molecular mass of the 152 bp product. Analysis was done after series dilution of the purified product in DNA-free water.

RESULTS

Specificity of Methods. The recently described hazelnut-specific sandwich-type protein-ELISA showed a high specificity for hazelnut protein. However, cross-reactivity with a signal of $>$1 ppm of hazelnut protein could be expected if either walnut, pumpkin seed, or cashew exceeding 20, 10, or 50%, respectively, or almond at a proportion of 100% was present in the investigated food matrix (*37*).

The developed hazelnut-specific PCR-ELISA that yields a 152 bp product from amplification of hazelnut DNA was tested for possible cross-reactions with DNA from various nuts and stone fruits, legumes, cereals, and other relevant food ingredients. More than 30 different foods or food ingredients were tested (apple, apricot, carrot, celery, cherry, pear, parsley, chickpea, green pea, kidney bean, lentil, peanut, soybean, white bean, almond, Brazil nut, coconut, cashew, macadamia nut, pecan, pistachio, walnut, pine seed, pumpkin seed, sesame seed, sunflower seed, corn, oats, rice, rye, wheat, coffee bean, cocoa, hen's egg, sugar). In PCR-ELISA, none of these sample DNAs yielded ODs of $>$0.2 (**Figure 2**), nor could amplified products be detected in the 152 bp range by agarose gel electrophoresis

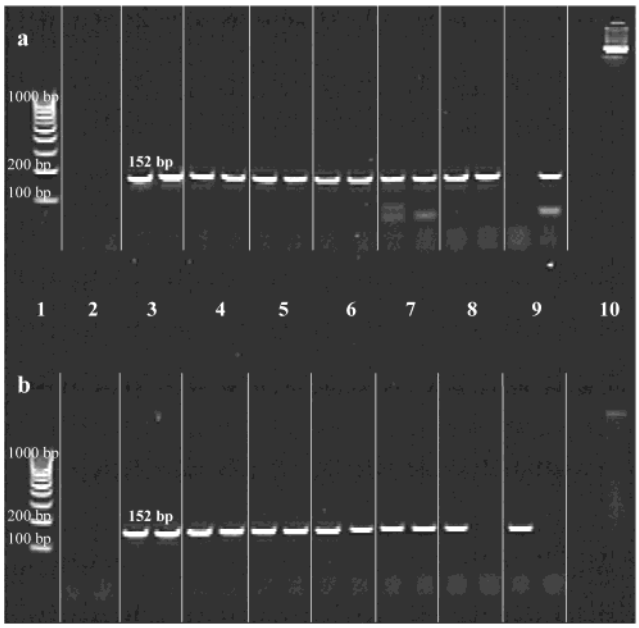

Figure 3. Sensitivity testing of the hazelnut PCR applying genomic DNA from either native hazelnut (a) or hazelnut toasted at 140 °C for 30 min (b) of the variety Nocciole Ordu (lane 1, DNA size marker, 100 bp ladder; lane 10, 300 ng of genomic DNA; lanes 2–9, PCR products from amplification of 0 (NTC)–100 pg of genomic hazelnut DNA (lane 2, NTC; lane 3, 100 pg; lane 4, 10 pg; lane 5, 8 pg; lane 6, 6 pg; lane 7, 4 pg; lane 8, 2 pg; lane 9, 1 pg)). PCRs were run in duplicate.

(electrophoresis results not shown). With the DNA preparation procedure described here, no inhibitory effects were observed in any of the tested samples, as shown in **Figure 2** (inhibition control). Even in 100% of the coffee bean and cocoa, which are difficult matrixes due to the presence of inhibitory compounds, hazelnut DNA as a positive control (inhibition control) was amplified successfully.

Sensitivity of Methods. For the sandwich-type protein-ELISA, a lower limit of detection of 1 ppm of hazelnut protein was assessed for unknown complex food matrixes, as was recently described in detail (*37*). At or above 1 ppm of hazelnut protein, potential cross-reactions with types of foods other than hazelnut could be excluded with a high probability, thus reducing the risk of false-positive results at very low concentrations.

Similarly, the PCR-ELISA was capable of detecting very small quantities of hazelnut DNA in a sample. For evaluation of the sensitivity of the PCR-ELISA, we used different approaches: one with diluted genomic hazelnut DNA and defined copy numbers as starting material to investigate the sensitivity of the PCR-ELISA method itself, and one with authentic food samples to determine the sensitivity of the whole PCR-ELISA application, from sample preparation of true food matrixes to results. **Figure 3** displays the gel detection of the 152 bp long PCR products after amplification of picogram amounts of hazelnut DNA. With DNA from native hazelnuts of the variety Nocciole Ordu, even 2 pg of genomic hazelnut DNA as template was suitable for amplification (**Figure 3a**, lane 8) and detected reproducibly in two independent reactions. DNA of native hazelnut mainly showed fragment sizes of $\gg$1000 bp (**Figure 3a**, lane 10). Even though toasting of hazelnut at 140 °C for 30 min obviously degrades the genomic DNA to fragments of $<$1000 bp (**Figure 3b**, lane 10), $\geq$4 pg of DNA from toasted hazelnut was amplified in a reproducible way (**Figure 3b**, lane 7). When the PCR products were detected with DNA-ELISA, the results of the gel detection and the ELISA detection with specific hybridization probes were in complete accord, with ODs of positive reactions ranging between 2.3 and 2.9 and ODs of negative reactions, including NTCs, at $<$0.05, respectively.

Table 1. Sensitivity of the PCR-ELISA As Determined with Defined Copy Numbers of the 152 bp Target

no. of 152 bp copies	PCR 1[b]	PCR 2
0 (NTC[a])	–	–
1	++	+++
2	+++	+++
4	+++	+++
6	+++	+++
8	+++	+++
10	+++	+
100	+++	++
hyb-probe minus	–	–
hyb-probe plus	+++	+++

[a] NTC, no template control. [b] PCR 1, PCR 2, two independent PCR investigations: –, OD $\leq$0.2, negative; +, OD $>$0.2, positive; ++, OD $>$1.0, positive; +++, OD $>$2.0, positive.

Using defined copy numbers of the 152 bp product in PCR, 1−100 copies as starting template were amplified. Even though one single copy may not always be detectable due to variable statistical distribution, still less than 10 copies were detected with PCR-ELISA (**Table 1**), thus demonstrating a highly efficient amplification and detection.

As is shown in the section Investigation of Commercial Food Samples (below), the overall sensitivity of PCR-ELISA in true food matrixes was similar to that of protein-ELISA. With PCR-ELISA, samples containing $<$1 ppm of hazelnut protein were detected positive. This corresponded to $<$10 ppm of whole hazelnut, assuming a rough correlation of 1:10 between protein content and whole hazelnut (*37*, *38*).

Investigation of Commercial Food Samples. Forty-one different commercial food samples from local food stores were analyzed for hazelnut components with both hazelnut-specific protein-ELISA and PCR-ELISA. The samples investigated were white, milk, and bitter chocolates with or without various types of food ingredients such as tree nuts (hazelnut, almond), peanuts, fruits, and cereals. Moreover, nougat, caramel, and milk products as well as cereal products were investigated (**Table 2**). Fifteen of the samples had hazelnut in the list of ingredients, 12 samples did not have any hazelnut components declared, and 14 samples had a warning for a potential presence of hazelnut traces.

For investigation of the food samples with protein-ELISA, two determinations on two independent sample extracts according to the hazelnut protein extraction protocol were performed, and the average of the two determinations is displayed in **Table 2**. Similarly, two independent DNA preparations of each sample were analyzed with PCR-ELISA, and samples were considered as hazelnut positive only if both reactions were positive.

With PCR-ELISA, in all of the 15 samples with hazelnut listed in the ingredients, hazelnut was detected. Thirteen of these samples were confirmed with the protein-ELISA, but in 2 cream desserts, hazelnut protein could not be detected even though hazelnut was added as an ingredient, as stated by the manufacturer.

With the protein-ELISA, 7 of 14 samples having precautionary labeling were detected positive, with hazelnut protein ranging between 1.5 and 37 ppm. The 7 positive samples were confirmed by PCR-ELISA. Although the protein-ELISA had a

Table 2. Investigation of Commercial Food Samples with Hazelnut-Specific Protein-ELISA and PCR-ELISA

			protein-ELISA	PCR-ELISA		
no.	sample	D[a]	ppm HN-protein[b]	PCR 1[c]	PCR 2[c]	result
1	chocolate, hazelnut	+	15000	+++	+++	positive
2	milk chokolate, biscuit I	+	486	+++	+++	positive
3	milk chokolate, biscuit II	+	171	+++	+++	positive
4	milk chocolate, biskuit III	+	47	+++	+++	positive
5	milk chocolate, coconut	+	348	+++	+++	positive
6	white chocolate, cereal crisp & brittle	+	1006	+++	+++	positive
7	nougat	+	15000	+++	+++	positive
8	cream dessert, hazelnut pudding	+	–	++	++	positive
9	cream dessert, chocolate & hazelnut	+	–	++	++	positive
10	breakfast flakes, wheat & fruit	+	5645	+++	+++	positive
11	cereal bar, chocolate	+	1918	+++	+++	positive
12	cereal bar, peanut	+	708	+++	+++	positive
13	cereal bar, cereal mix	+	13	++	++	positive
14	cereal bar, mixed berries	+	23	+++	+++	positive
15	cookies, hazelnut	+	3929	+++	+++	positive
16	milk chocolate, plain I	±	18	+++	++	positive
17	milk chocolate plain II	±	<1	+	+	positive
18	white chocolate, plain I	±	–	+	–	negative
19	white chocolate, plain II	±	<1	–	–	negative
20	bitter chocolate	±	<1	++	++	positive
21	chocolate, cappucino & cream	±	45	+++	+++	positive
22	chocolate, cherry	±	1.5	++	++	positive
23	chocolate, lemon	±	–	–	–	negative
24	chocolate, marzipan	±	8.6	++	++	positive
25	chocolate sticks	±	30	+++	+++	positive
26	chocolate, strawberry	±	30	+++	+++	positive
27	chocolate, strawberry & buttermilk	±	–	–	–	negative
28	chocolate, strawberry & yogurt	±	37	+++	+++	positive
29	caramel bar	±	–	–	–	negative
30	white chocolate, oat flakes	–	–	–	–	negative
31	cream pudding, plain	–	–	–	–	negative
32	milk drink, cappucino	–	–	–	–	negative
33	cookies, plain	–	–	+	–	negative
34	biscuit, organic	–	–	–	–	negative
35	cornflakes, plain	–	–	–	–	negative
36	cornflakes, peanuts	–	–	–	–	negative
37	breakfast flakes, wholemeal	–	–	–	–	negative
38	breakfast flakes, wheat & honey	–	–	–	–	negative
39	breakfast flakes, rice & wheat	–	–	–	–	negative
40	breakfast spread, almond & chocolate	–	1348	++	++	positive
41	muesli, honey	–	<1	++	++	positive

[a] D declaration of hazelnut or hazelnut components: –, none declared; +, positive declaration; ±, may contain hazelnut traces or not suitable for nut allergy sufferers. [b] Quantitative result of hazelnut protein determination with protein-ELISA: <1 ppm for samples ≥0.4 ppm and <1 ppm. –, no detectable hazelnut protein <0.2 ppm. [c] PCR 1, PCR 2, two independent PCR investigations on two independent DNA preparations of one sample: –, OD ≤0.2, negative; +, OD >0.2, positive; ++, OD >1.0, positive; +++, OD >2.0, positive.

general detection limit of 1 ppm of hazelnut protein, another 4 samples (nos. 17, 19, 20, and 41) having between 0.4 and 1 ppm of hazelnut protein were considered as being hazelnut positive, because previous investigations demonstrated a lower detection limit of 0.2 ppm of hazelnut protein in these types of matrixes (*37*). Of these products, 3 were detected positive by the PCR-ELISA. Sample No. 19, "white chocolate, plain II", which was quantified at 0.5 ppm of hazelnut protein with protein-ELISA, was not confirmed with PCR-ELISA.

Two of 12 samples not labeled as having hazelnut components were identified as being hazelnut positive by both independent methods. One of these, "breakfast spread, almond & chocolate" (no. 40), contained hazelnut at the 1% level.

DISCUSSION

The detection of hazelnut protein was based upon the principle of sandwich-type ELISA using polyclonal antisera from rabbit and sheep as the source of either capture or detector antibody. In a recent study, we demonstrated a high specificity of the protein-ELISA for hazelnut protein (*37*). However, considerable proportions of walnut, pumpkin seed, cashew, almond, and chickpea produced false-positive signals of ≥1 ppm of hazelnut protein, which was the general limit of detection for the investigation of unknown sample matrixes. Similar findings were observed by Koppelman et al. (*38*), whose hazelnut-specific sandwich-ELISA also showed cross-reactivities to walnut and cashew, with walnut being detected at about some 79-fold of the detection limit of 10 ppm of hazelnut or 1 ppm of hazelnut protein, respectively.

For hazelnut detection by PCR-ELISA, the cDNA of the recombinant major hazelnut allergen, rCor a 1.0401 (*43*, *44*), was chosen as template, as was recently described (*39*), but primer pairs had to be optimized since the formerly described 182 bp PCR tended to form artifacts which might reduce the sensitivity of the system. With our new 152 bp-PCR, artifacts as seen in **Figure 3** were rarely generated and did not give any signal in PCR-ELISA. Furthermore, positive signals detected with our ELISA-like technique were exclusively obtained from hazelnut. No cross-reactions with other foods were observed, thus reflecting a very specific detection system. The high

specificity that is crucial to avoid false positives, especially at the limit of detection, was achieved by verification of the DNA sequence of the PCR products using a sequence-specific hybridization probe in combination with specific primers. In contrast, detection of PCR products by simple agarose gel electrophoresis without further sequence verification would not be specific enough, as it is too imprecise for accurate size determination of amplicons and because artifacts similar in size to the specific product could lead to false-positive results. According to a standard developed by the German Institute of Standardization (DIN) in collaboration with the American Society for Testing and Materials (ASTM), sequence verification of PCR products is a general requirement for specificity confirmation of the amplification reaction (*41*). The PCR-ELISA described is much simpler and faster to perform than other sequence verification techniques, such as restriction endonuclease digestion combined with gel electrophoresis, gel electrophoresis combined with Southern blotting, or nucleotide sequencing in a sequencer. In contrast to real-time PCR with sequence verification, no expensive equipment is needed in PCR-ELISA. Additionally, toxic staining of agarose gels is avoided.

To increase food safety for hazelnut-allergic individuals, detection of hazelnut in foods should take place at a level where at least mild or no allergic reactions are to be expected. Unfortunately, there are no data available that consider a no-effect level (NOEL) for hazelnut-sensitive subjects (*27*). However, case reports of unintentional hazelnut intake with subsequent allergic reactions demonstrated that even 6 mg of hazelnut (~0.7 ppm of hazelnut protein) in one episode and 50 mg of hazelnut protein in another elicited severe allergic reactions (*17*, *28*, *34*). In the case of 6 mg of hazelnut leading to an adverse reaction, approximately 3 g of a chocolate containing 2000 ppm (mg/kg) of hazelnut was consumed. Assuming that up to 500 g of a food may be ingested at once, a total dose of 6 mg of hazelnut would be consumed with hazelnut present at a concentration as low as 12 ppm. Thus, analytical methods should be able to detect at least 10 ppm of hazelnut or 1−2 ppm of hazelnut protein in a foodstuff. The hazelnut-specific protein-ELISA had a general detection limit of 1 ppm of hazelnut protein for unknown food matrixes. Nonetheless, in food samples investigated in this study, the ELISA's lower limit of detection was determined to be 0.2 ppm of hazelnut protein (*37*). Here, samples were identified having ≥0.4 ppm of hazelnut protein or ≥4 ppm of hazelnut. The DNA-based PCR-ELISA was also able to detect as little as 4 ppm of hazelnut. At this concentration level, more than 1000 g of a food would have to be consumed to reach a dose of 6 mg of hazelnut. We believe that if raw materials and end products were screened at a level of ≤10 ppm of hazelnut, a substantial increase in food safety would be achieved for the majority of hazelnut-allergic consumers.

Since hazelnut is a common ingredient in chocolate, cookies, and mixed cereal products, successful detection of hazelnut in these types of food matrixes is crucial. Unfortunately, cocoa and cereal products contain matrix compounds such as polyphenols and heteropolysaccharides that may disturb or fully inhibit antibody−antigen and enzymatic reactions of ELISA and PCR, respectively (*45*−*47*). To increase the detectability of hazelnut by protein-ELISA in difficult matrixes such as chocolate, 10% of fish gelatin was incorporated into the sample extraction buffer, as recommended by Keck-Gassenmeier et al. (*47*). Therewith, <1 ppm of hazelnut protein corresponding to <10 ppm of hazelnut could be detected successfully even in dark chocolate in this study. With PCR-ELISA, hazelnut DNA, applied as an inhibition control, was amplified in all of the samples, and successful detection of <10 ppm of hazelnut in dark chocolate was achieved. Even in 100% of the coffee bean and cocoa, hazelnut DNA as a positive control was amplified successfully. Thus, the described DNA extraction procedure yielded amplifiable DNA of high purity, without any inhibitors. The extracted DNA could be stored at −20 °C for several months and was accessible for analysis with other allergen-specific PCR-ELISA systems, such as for the detection of peanut, soybean, almond, or walnut. By contrast, hazelnut protein showed a relatively low stability in solution (*37*) and had to be analyzed by the sandwich-ELISA immediately after extraction.

When 41 commercial food products were investigated with protein-ELISA and PCR-ELISA, the results of the two methods were in good agreement. Of the 27 products in which hazelnut components were detected, only 2 cream desserts were negative in the protein-ELISA. Both samples were "hazelnut desserts" with hazelnut added as an ingredient, as stated by the manufacturer in the list of ingredients. Probably due to the acidic conditions or microbial enzymatic activity in these two milk products, hazelnut protein was already degraded or denatured and therefore was not accessible for detection with antibodies. Furthermore, previous studies with our protein-ELISA indicated a lower stability of hazelnut protein in solution than that of, e.g., peanut protein (*37*). With PCR-ELISA, all but one sample with detectable hazelnut components were detected positive. The negative sample was a white chocolate having only 0.5 ppm of hazelnut protein, as determined by protein-ELISA. All other samples having between 0.4 and 1 ppm of hazelnut protein were clearly confirmed by PCR-ELISA. Of the 12 samples without hazelnut identified on their labels, 2 samples contained hazelnut: in "breakfast spread, almond & chocolate", 1348 ppm of hazelnut protein, corresponding to 1% of hazelnut, was present. Such high amounts of hidden hazelnut undoubtedly pose a tremendous risk to allergic individuals. All of the 13 investigated chocolate products and one caramel bar had a precautionary warning that nut traces may be present. Of these samples, 71% (10 samples) contained hazelnut, demonstrating a remarkable presence of potentially allergenic hazelnut residues. In 1999, when similar products were analyzed for hidden hazelnut (*37*), only 3 products displayed a precautionary allergen warning, and 16 chocolate samples did not have any hazelnut declared on the label. In contrast to the present study, the samples with a warning did not contain hazelnut, whereas 9 of 16 (56%) chocolates contained undeclared hazelnut. Even though the two sets of data are not fully representative of the German market, it was obvious that food manufacturers had changed their labeling strategies, whereas the problem of hazelnut residues in these types of foods still persists. In our opinion, an overall increase in precautionary labeling is not very helpful for the allergic consumer, as industrially manufactured foods are widely used in Western societies, and therefore a large number of products would have to be excluded from the daily diet.

With this study, we have demonstrated that both enzyme immunoassays and DNA-based techniques such as PCR-ELISA are powerful tools for allergen monitoring of foods at a level of ≤10 ppm. Due to the higher stability of DNA in comparison to that of hazelnut protein, the PCR-ELISA seemed to have some advantage over protein-ELISA, e.g., when detecting hazelnut in dairy products. Therefore, additional studies on such matrixes are in progress. Furthermore, the PCR-ELISA showed an extraordinary specificity for hazelnut. To our knowledge, the described PCR-ELISA is the first commercially available hazelnut-specific test kit that is fully accessible to all analytical

facilities. Hence, foods may be investigated in the laboratories of food manufacturers and food control agencies. Finally, reagents used for PCR-ELISA are available in constant quality and defined consistency, as the chemistry is reproducible and the polymerase is generated by means of genetic engineering. Based on common ELISA equipment, the PCR-ELISA can be performed with a thermocycler as the only additional equipment needed when ELISA techniques are already set up. The time required for sample preparation and analysis is comparable to that of ELISA methods, and results can be obtained in less than one working day. With an accessible ready-to-use test kit, we hope to encourage manufacturers to adopt new strategies in HACCP concepts and GMP in combination with analytical testing of raw materials and end products to reduce the presence of potentially allergenic residues in foods rather than to imply a general precautionary labeling practice that would not support the needs of allergic subjects for more precise food labeling.

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