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Development and Validation of a Duplex Real-Time PCR Method To Simultaneously Detect Potentially Allergenic Sesame and Hazelnut in Food

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The paper describes the development and validation of a duplex real-time PCR method allowing the simultaneous detection of traces of potentially allergenic sesame and hazelnut in food. For the detection of sesame and hazelnut, the genes coding for two major allergenic proteins, Ses i 1 and Cor a 1, were selected. The duplex real-time PCR assay did not show any cross-reactivity with 25 common food ingredients from sesame and/or hazelnut containing foods. Analysis of serially diluted sesame/hazelnut DNA resulted in good linearity up to a dilution of 1:10000 (corresponding to 10 pg μL^{-1} or 50 pg). Sesame and hazelnut could be detected in blank whole meal cookies which had been spiked with 0.005% sesame and 0.005% hazelnut. The applicability of the real-time PCR assay for determining sesame and hazelnut in different food matrices was investigated by analyzing 30 commercial foodstuffs comprising salty snacks, cookies, chocolates, creams, mueslis and muesli bars.

KEYWORDS: Sesame; hazelnut; allergen; duplex real-time polymerase chain reaction (duplex real-time PCR); food

INTRODUCTION

In industrialized countries about 3% of the adults and 6 to 8% of the children suffer from food allergies (*1*). Food allergy is—in contrast to other adverse food reactions such as food intolerance, toxic or pseudoallergic reactions—mediated by immunological mechanisms, most frequently by the production of immunoglobulin E (IgE) directed toward the food allergen. Clinical manifestations of food allergies often comprise symptoms such as the so-called oral allergy syndrome, gastrointestinal, skin or respiratory disorders (*2*). Some foods can induce severe life-threatening anaphylaxis, caused by the abrupt, massive release of mediators from mast cells and/or basophils (*3*). Among all foods known to be allergenic, particularly peanuts (*4*−*6*), tree nuts (*7, 8*) and sesame (*9*−*12*) have frequently been reported to cause anaphylactic reactions.

Most allergenic foods are known to contain more than one allergenic protein. In sesame some of them have been identified: a sulfur poor 2S albumin (molecular weight 10 kDa, Ses i 1), a sulfur rich 2S albumin (7 kDa, Ses i 2), a 7S vicillin-like globulin (45 kDa, Ses i 3) (*13, 14*), two oleosins (17 kDa, Ses i 4; 15 kDa, Ses i 5) (*15*) and two 11S globulins (Ses i 6 and Ses i 7) (*16*). Hazelnuts have been shown to contain Cor a 1, a homologue of the major birch pollen allergen (*17*), Cor a 2, a profilin (*18*), Cor a 8, a lipid transfer protein (*19*), Cor a 9, an 11S globulin-like seed storage protein (*20*) and Cor a 11, a vicilin (*21*).

For an allergic patient the only preventive strategy is to strictly avoid the food to which the patient is allergic. In order to facilitate the identification of allergenic ingredients in foodstuffs, the European Union legislation obliges producers to indicate 14 allergenic foods in the list of food ingredients, including sesame seeds and tree nuts and products thereof (*22*).

Although people are aware of their allergy and try to avoid the certain food, they can nevertheless sometimes be exposed to it. A retrospective study on adults in Spain showed that 119 from 530 allergic reactions were caused by the exposition to so-called “hidden” allergens, allergens which are not indicated in the list of ingredients (*23*). Contamination of food with hidden allergens can, for example, occur during food processing, e.g. due to insufficient cleaning of the production equipment. As a precautionary measure numerous food products are marketed with labels such as “may contain traces of ...” or “products containing ... are produced with the same equipment”.

In order to protect allergic patients from accidental intake of food allergens analytical measurements have to be carried out to verify if allergen containing products are labeled in compliance with the regulations and if there are maybe hidden allergens in nondeclared food products. Analytical methods developed so far can be divided into protein based and DNA based methods. Enzyme linked immunosorbent assays (ELISAs) which are based on the specific interaction between antigens and

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Table 1. Primers and Fluorescently Labeled Probes

primer/probe	sequence 5′→3′	T_m (°C)	product size (bp)
primer Ses i 1 forward	TGAGGAACGTGGACGAGAG	56.5	117
primer Ses i 1 reverse	CCCTAGCCCTCTGGTAAACC	57.1	
Taqman Ses i 1	FAM−CACCCTCCTGCTGCTGCTGCC−BHQ1	66.5	
primer Cor a 1 forward 2	ACTACATAAAGCAAAAGGTTGAAG	54.8	109
primer Cor a 1 reverse 2	TCGTAATTGATTTTCTCCAGTTTG	54.9	
Taqman Cor a 1	Cy5−CGGACAAAGCATCGCCTTCAATCA−BHQ2	63.1	
primer Cor a 1 forward 1	GGCTAGGCTGTTCAAGAGG	55.3	147
primer Cor a 1 reverse 1	AGAAATTAACCTTCATCGAAACAG	54.7	
primer Cor a 1 forward 3	GCAAACTTTTCATATCGCTACAG	55.4	80
primer Cor a 1 reverse 3	TTGATCTCGTAATTGATTTTCTCC	54.2	

antibodies play the most important role among the protein based methods (*24*).

Several papers report on the development of ELISAs to detect traces of hazelnut (*25*−*30*) in food. In addition, some authors described multiallergen ELISAs enabling the detection of hazelnut, together with either peanut and Brazil nut (*31*) or peanut, Brazil nut, almond and cashew nut (*32*). An ELISA allowing the determination of sesame in food has not been published in a peer-reviewed journal so far.

In DNA based methods specific DNA sequences (templates) are amplified by the polymerase chain reaction (PCR) and detected either by agarose gel electrophoresis or—in so-called real-time PCR—by using fluorescently labeled probes. PCR methods have already been presented for detecting either hazelnut (*33*−*36*) or sesame (*37*−*39*) in food.

The multiallergen ELISA methods mentioned above offer the possibility to detect several allergens in one run, but not in the same well of the microtiter plate (*32*) or on the same spot of the polyester cloth strip (*31*). In contrast, PCR methods have the potential of simultaneously amplifying multiple sequences (templates) in one well. Compared to common (singleplex) PCR, so-called multiplex PCR has the advantages of saving time, reducing reagent costs and lowering the probability of cross-contamination.

Recently, a duplex PCR assay was presented for simultaneously detecting hazelnut and peanut in foodstuffs (*40*). However, in this study the amplified DNA sequences were not detected in real-time but by post-PCR detection with peptide nucleic acid (PNA) probes. So far, the potential of multiplex real-time PCR for simultaneously detecting several food allergens has not been explored.

The aim of the present study was to develop a duplex real-time PCR method to simultaneously detect sesame and hazelnut in food. These two allergenic foods were selected not only because both are known to elicit life threatening anaphylactic reactions but also because an increasing number of foodstuffs are available on the market which may contain sesame and hazelnut in combination, either because they were added by intention or because they are present due to contamination.

MATERIALS AND METHODS

Chemicals and Food Products. Cetyltrimethylammonium bromide (CTAB), tris(hydroxymethyl)aminomethane (Tris), isopropanol and sodium chloride were obtained from Sigma (Vienna, Austria). Ethylendiaminetetraacetic acid (EDTA) and chloroform were purchased from VWR (Darmstadt, Germany). Agarose and ethidium bromide were obtained from BioRad (Hercules, USA). PCR reactions were carried out with water which had been bidistilled and subsequently autoclaved. Hazelnuts (four ground samples, three of them roasted and one unroasted) and sesame seeds (five white, three black and one golden (roasted) sesame) were purchased from different producers. Food products were bought in local food stores.

Extraction of DNA. Genomic DNA was extracted with the CTAB method. About 5 g of the food sample were ground in a mechanical mortar (type MM 2000, Retsch, Haan, Germany) for 20 min and stored at 4 °C until DNA extraction. A 50−100 mg aliquot of the ground sample was weighed in, mixed with 500 μL of the CTAB extraction solution (20 mg mL^{-1} CTAB, 1.4 M NaCl, 0.1 M Tris, 0.02 M EDTA, adjusted to pH 8 with concentrated HCl) and incubated at 65 °C for 30 min. After adding 15 μL of proteinase K (25 mg mL^{-1}) a further incubation step was carried out at 65 °C for 1 h. After centrifugation for 5 min at 8400*g* (model 5413, Laborpartner, Vienna, Austria) the supernatant was transferred into a new 1.5 mL microreaction tube. Then 200 μL of chloroform was added, and after vortexing for 1 min the mixture was centrifuged for 5 min. The aqueous phase was transferred into a new tube, mixed with 2 parts by volume of CTAB precipitation solution (5 g L^{-1} CTAB, 0.04 M NaCl) and incubated at room temperature for 60 min. After centrifugation for 5 min the precipitate was dissolved in 350 μL of 1.2 M sodium chloride solution. Then 350 μL of chloroform was added, and the mixture was vortexed for 1 min. The aqueous phase was transferred into a new tube, and 0.6 part by volume of isopropanol was added. After centrifugation for 5 min the pellet was mixed with 500 μL of ice cold ethanol. After a further centrifugation step (5 min) the supernatant was removed with a pipet. The pellet was dried in an oven (UE 500, Memmert, Schwabach, Germany) at 37 °C and dissolved in 100 μL of H_2O_{dd}. The extracted DNA was stored at −20 °C until analysis.

Determination of the Yield and Quality of DNA Extracts. DNA was quantified by measuring the absorbance at 260 nm (A_{260}) and 280 nm (A_{280}) in water with a spectrophotometer (Spectronic Genesys 10UV, Thermo Scientific, Waltham, MA, USA). The DNA concentration in the extracts was calculated according to the equation c [μg mL^{-1}] = $A_{260} \times 50 \times$ dilution factor. The A_{260}/A_{280} ratio was used to assess the quality of the isolated DNA.

PCR Analysis. *Design of Primers and Taqman Probes.* In order to allow the detection of sesame the gene coding for the Ses i 1 protein, one of the major allergenic proteins of sesame (NCBI accession number: AF240005), was selected. For the detection of hazelnut the gene coding for the major allergenic protein Cor a 1 (NCBI accession number: Z72440) was chosen. The design of the sesame primers had already been described in ref *37*. Hazelnut primers and Taqman probes for sesame and hazelnut were designed using the Beacon Designer 6.0 software from Premier Biosoft International (Palo Alto, CA, USA). Hazelnut primers were designed to meet the following requirements: to produce an amplicon 75−150 bp long, to have a length between 20 and 24 bases and an annealing temperature similar to that of the sesame primers. The Taqman probes were designed to have a melting temperature of ∼10 °C higher than that of the primers and a length between 20 and 24 bases. The sequences of the designed primer pairs and Taqman probes are given in **Table 1**. The Ses i 1 Taqman probe was labeled with the reporter dye FAM (6-carboxyfluorescein) on the 5′ end and the Black hole quencher BHQ 1 on the 3′ end. The Cor a 1 Taqman probe was labeled with Cy5 (Cyanine 5) and quenched with the Black hole quencher BHQ 2. Primers were synthesized by Sigma-Genosys (Steinheim, Germany), the Taqman probes by Metabion International AG (Martinsried, Germany).

Real-Time PCR Conditions. PCR reactions were carried out in 96 well PCR plates (BioRad) in a total volume of 25 μL using the iCycler

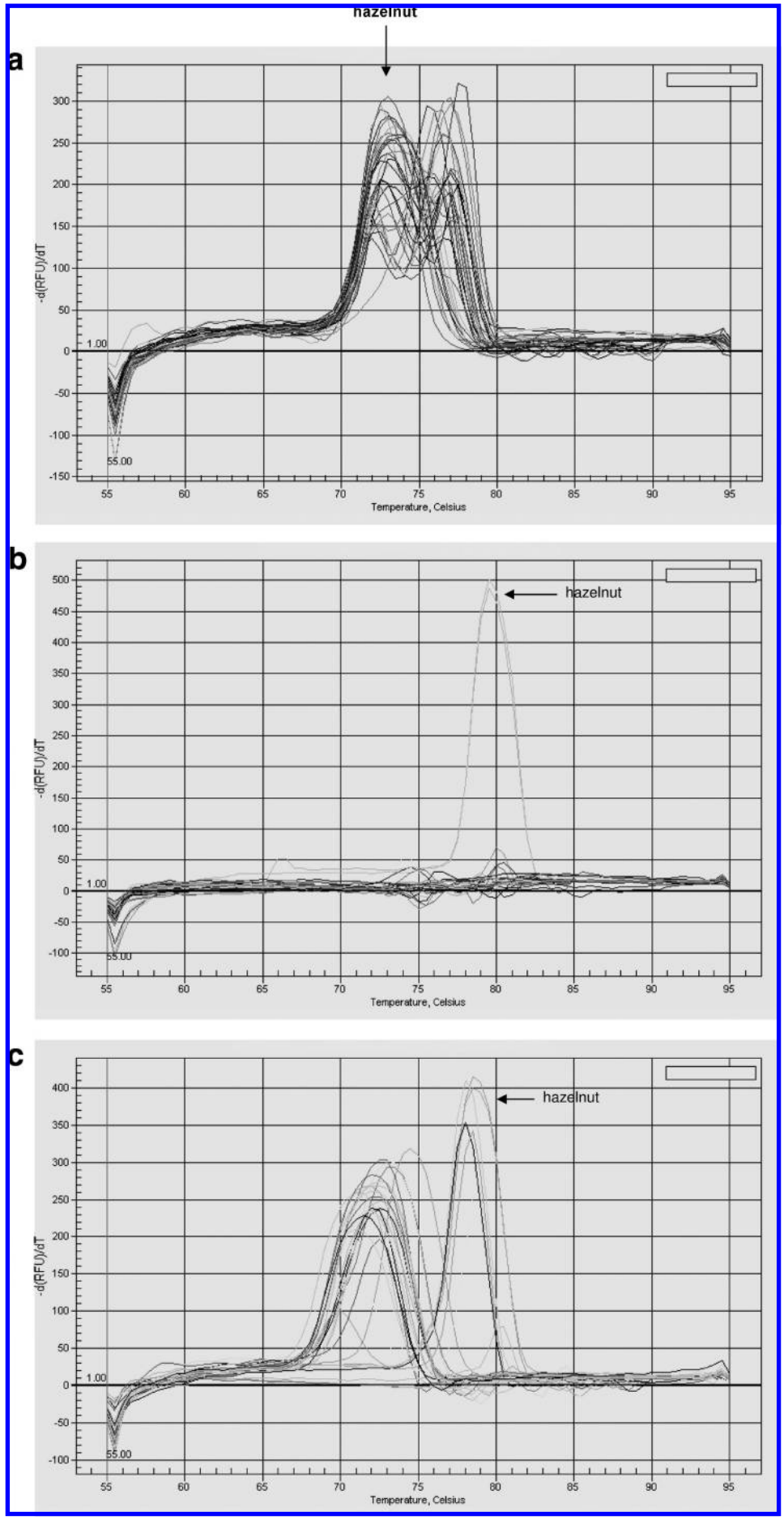

Figure 1. First derivatives of the melting curves of the PCR reaction mixtures obtained after Sybr Green PCR with the 3 hazelnut primer pairs listed in **Table 1**: (**a**) primer pair 1, (**b**) primer pair 2 and (**c**) primer pair 3.

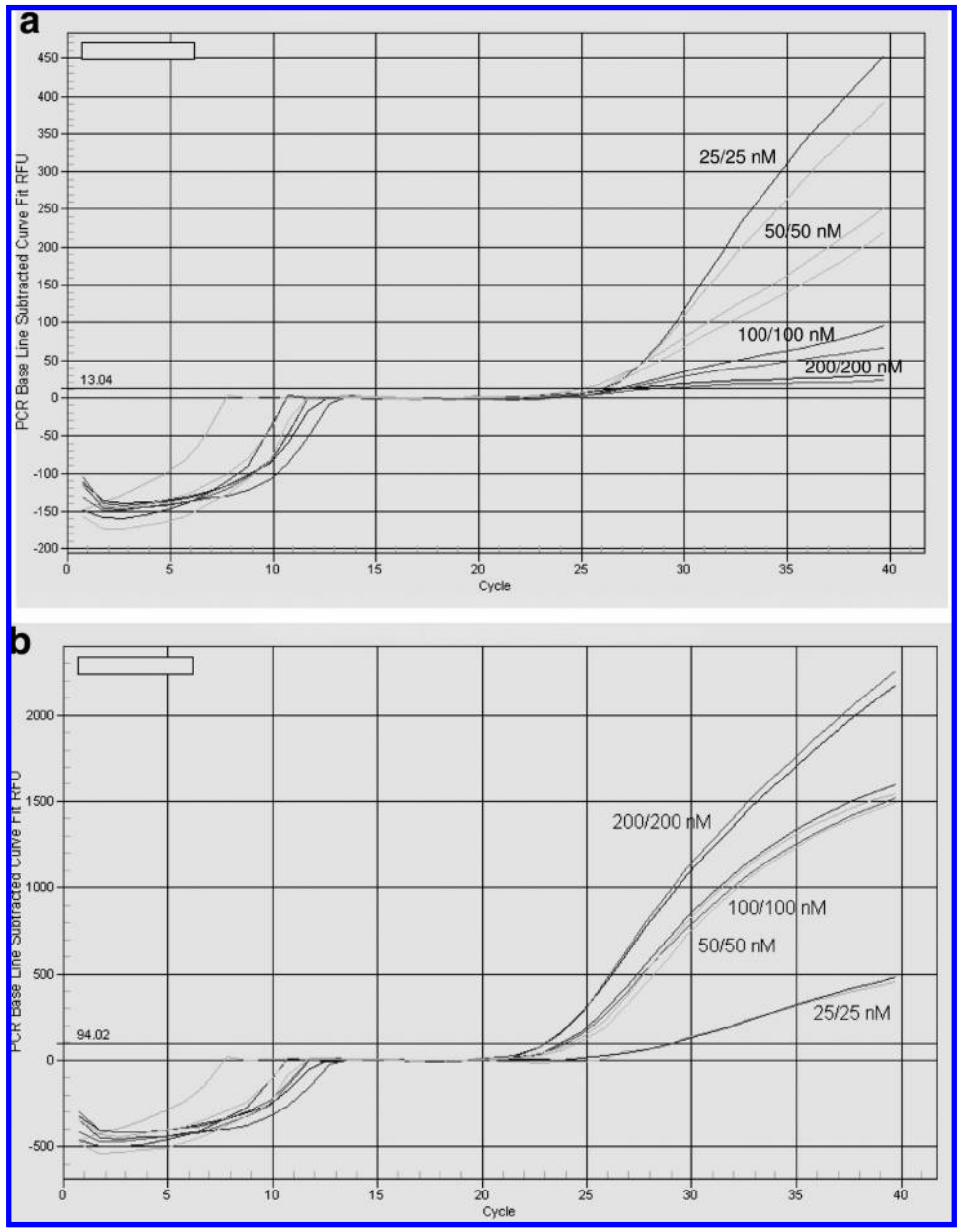

Figure 2. Influence of the sesame primer concentration in the duplex real-time PCR on the amplification of (**a**) hazelnut DNA and (**b**) sesame DNA.

thermocycler equipped with the IQ 5 multicolor real time PCR detection system (BioRad). All DNA samples were analyzed in duplicate. On each 96 well plate two nontemplate controls (NTC) were used to check the PCR performance.

Hazelnut Real-Time PCR with Sybr Green. Each reaction consisted of 12.5 μL of IQ Sybr Green Supermix (containing 100 mM KCl, 40 mM Tris-HCl, pH 8.4, 1.6 mM dNTPs, 50 units mL^{-1} iTaq DNA polymerase, 6 mM $MgCl_2$, SYBR Green I, 20 nM fluorescein) from BioRad, 300 nM primer Cor a 1 forward, 100 nM primer Cor a 1 reverse, 5 μL of template DNA (20 ng μL^{-1}) and H_2O_{dd}. The following PCR program was used: initial denaturation at 95 °C for 3 min followed by 40 cycles of denaturation at 95 °C for 30 s and primer annealing and elongation at 62 °C for 50 s.

Optimization of the Duplex Real-Time PCR Assay. Optimization experiments were carried out with mixtures of sesame and hazelnut DNA. DNA extracted from sesame was mixed with DNA extracted from hazelnut in the following ratios: 5:95, 10:90, 25:75, 75:25, 90:10 and 95:5, respectively. Duplex PCR assays were carried out with the commercial IQ Supermix (BioRad), adding either Taq polymerase (1.25 units), $MgCl_2$ (1, 2 or 3 mM) and/or dNTPs (0.2 or 0.4 mM). In addition, experiments were carried out by lowering the concentration of the sesame primers to either 25, 50, 100 or 200 nM.

Optimized Duplex Real-Time PCR Assay. Each reaction consisted of 12.5 μL of IQ Supermix (containing 100 mM KCl, 40 mM Tris-HCl, pH 8.4, 1.6 mM dNTPs, 50 units mL^{-1} iTaq DNA polymerase, 6 mM $MgCl_2$) from BioRad, 50 nM primer Ses i 1 forward, 50 nM primer Ses i 1 reverse, 300 nM primer Cor a 1 forward, 100 nM primer Cor a 1 reverse, 50 nM of each of the Taqman probes, 2 mM $MgCl_2$, 0.4 mM dNTPs, 5 μL of template DNA (20 ng μL^{-1}) and H_2O_{dd}.

The following PCR program was used: initial denaturation at 95 °C for 3 min followed by 40 cycles of denaturation at 95 °C for 10 s and primer annealing and elongation at 54 °C for 25 s.

Amplification Efficiency and Limit of Detection (LOD). The amplification efficiencies of the singleplex and duplex real-time PCR assays were determined with DNA extracted from sesame seeds and hazelnut, serially diluted from 1:1 to 1:100000. In addition, the LOD of the duplex assay was determined by spiking commercially available blank whole meal cookies which, as we showed by PCR analysis, did not contain hazelnut and sesame. Spiked samples were prepared as follows: 98.0 g of ground cookies was mixed with 1.0 g of ground sesame and 1.0 g of ground hazelnut in a kitchen blender for 20 min. The resulting paste with a sesame/hazelnut concentration of 1% was further "diluted" in

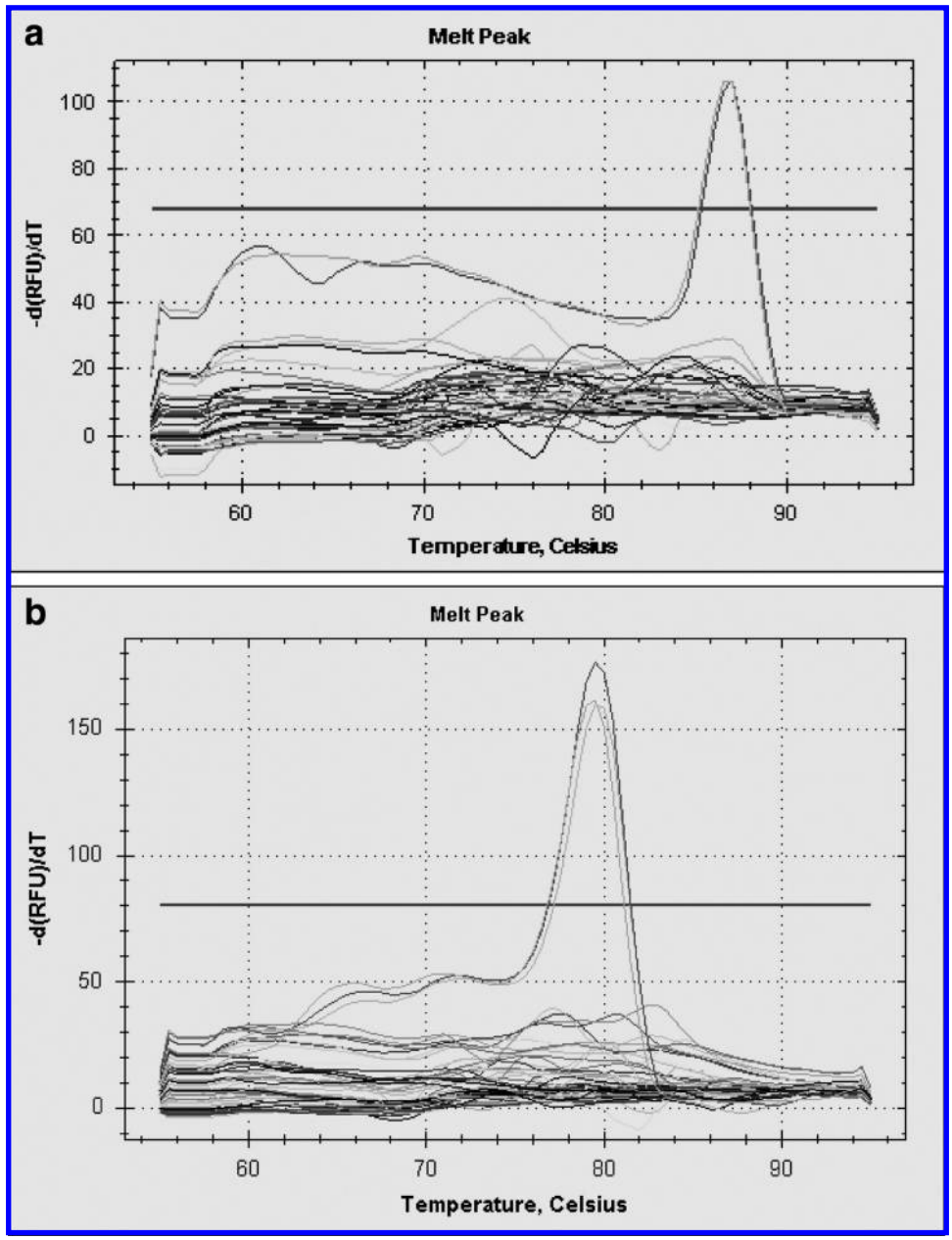

Figure 3. First derivatives of the melting curves of the PCR reaction mixtures obtained under the conditions of the optimized duplex assay: (**a**) sesame, (**b**) hazelnut.

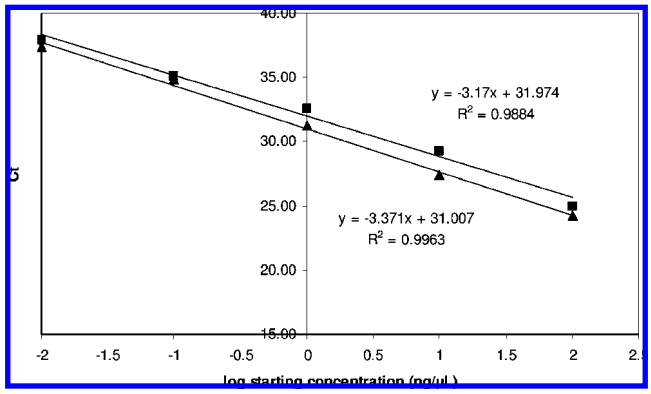

Figure 4. Standard curves obtained by amplifying sesame DNA (square) or hazelnut DNA (triangle) at different dilutions with the optimized duplex assay.

the same manner with an appropriate amount of blank cookies to obtain sesame/hazelnut concentrations of 0.5, 0.1, 0.05, 0.01, 0.005, and 0.001%.

Inhibition Control. Two microliters of DNA extract (containing 100 ng of sesame and 100 ng of hazelnut DNA) was applied to a well of a PCR plate and allowed to desiccate at room temperature overnight. The PCR plate was stored at 4 °C until use. After adding DNA from negative tested food samples the duplex assay was carried out as described above. The inhibition control reactions were carried out in duplicate.

Agarose Gel Electrophoresis. Ten microliter aliquots of the PCR products were mixed with 10 μL of a 1× nucleic acid sample loading buffer (1:5 diluted with water from a 5× stock solution) from BioRad. The products were loaded on a 3% (w/v) agarose gel containing 10 μg μL^{-1} ethidium bromide. The size of the amplicons was determined by comparison with a 20 bp ladder (BioRad). Electrophoresis was carried out with 1×TAE buffer at 150 V. The gels were visualized on a UVT-20 M transilluminator (Herolab, Wiesloch, Germany).

RESULTS AND DISCUSSION

Optimization of the Singleplex Real-Time PCR Assays with Sybr Green. We aimed at developing the duplex PCR

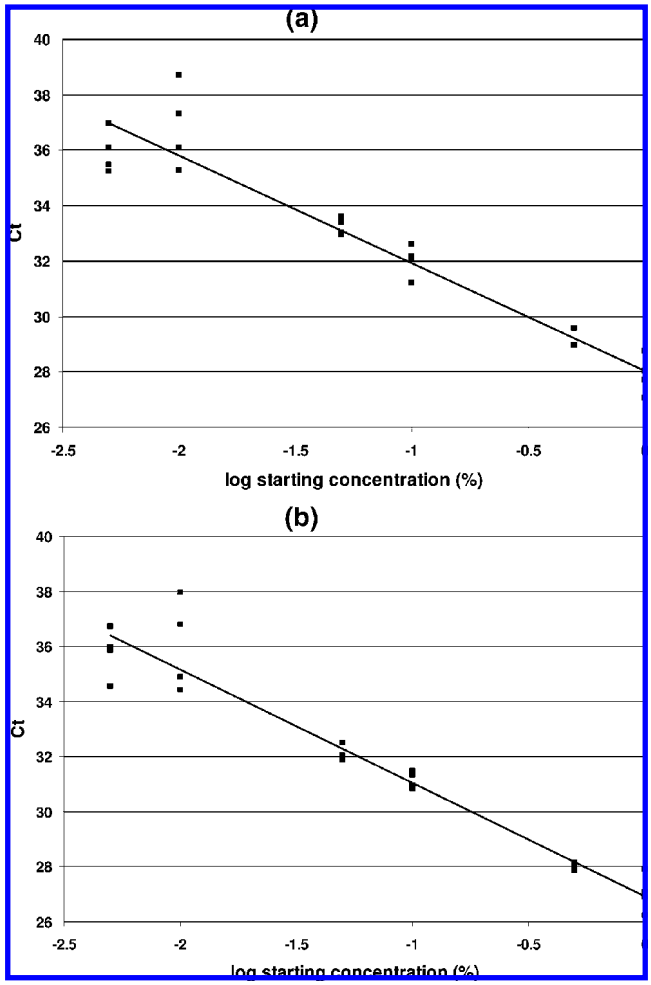

Figure 5. Standard curves obtained by amplifying DNA extracted from whole meal cookies which had previously been spiked with different amounts of sesame/hazelnut: (**a**) sesame, (**b**) hazelnut. Each DNA extract was analyzed four times.

method by using a sesame primer pair which had already been applied in a singleplex real-time PCR method enabling the detection of sesame in food (*37*). However, in contrast to the singleplex assay Taqman probes should be used in the duplex assay, mainly because of the lower costs of Taqman probes compared to Molecular Beacons. In the singleplex real-time PCR assay the optimal sesame primer concentrations had been found to be 800 nM for the primer forward and 400 nM for the primer reverse, and the optimal annealing temperature had been found to be 62 °C.

In the present study, PCR reactions with Sybr Green were carried out in order to optimize the concentration of the hazelnut primers. With each of the three primer pairs given in **Table 1** a primer matrix was carried out (100 nM, 200 nM, 300 nM, 400 nM), applying an annealing temperature of 62 °C, the optimal annealing temperature for the sesame primers in the singleplex assay. The experiments were carried out with a 2-step thermal program: 3 min at 95 °C followed by 45 cycles at 95 °C for 50 s and an annealing step at 62 °C for 30 s. The optimal reaction conditions were defined as those where the lowest Ct value and the highest ΔRn (which represents the background subtracted reporter fluorescence signal) were obtained. The optimal reaction conditions were found to be: primer pair 1, 200 nM primer forward, 300 nM primer reverse; primer pair 2, 300 nM primer forward, 100 nM primer reverse; and primer pair 3, 200 nM primer forward, 300 nM primer reverse.

Species Specificity of the Primer Pairs. In the singleplex assay the sesame primers proved to be specific for sesame and did not show any cross-reactivity with common food ingredients such as nuts, seeds and grains (*37*).

In order to investigate the species specificity of the 3 hazelnut primer pairs the DNA of different food samples and ingredients which might be present in sesame and hazelnut containing foods was subjected to PCR assays with Sybr Green. DNA extracts of Brazil nut, sunflower seeds, peanut, sesame, walnut, almond, potato, rice, soybean, poppy seed, oat, rye, wheat, maize, honey and chocolate were tested. **Figure 1** shows the first derivatives of the melting curves of the PCR reaction products obtained with each of the three primer pairs. **Figure 1a** and **Figure 1c** indicate that numerous unspecific products were obtained with the primer pairs 1 and 3. In contrast, hazelnut primer pair 2 proved to be specific for hazelnut (see **Figure 1b**). The following experiments were therefore carried out with hazelnut primer pair 2.

Real-Time PCR Reactions with Taqman Probes. Optimization of the real-time PCR reactions with Taqman probes was carried out in singleplex assays. For both sesame and hazelnut a temperature gradient was performed from 53 to 63 °C in order to determine the optimal annealing temperature. The concentration of the Taqman probes was either 50, 100 or 200 nM. In the case of sesame, in the range from 53 to 63 °C the annealing temperature did not have a strong influence on the Ct and ΔRn values. However, the lowest Ct and highest ΔRn values were obtained with an annealing temperature of 59 °C. In the case of hazelnut, the best results were obtained at 54 °C. Since in the case of hazelnut the annealing temperature did have a significant influence on the Ct and ΔRn values, the following experiments were carried out with an annealing temperature of 54 °C. For both sesame and hazelnut a TaqMan probe concentration of 50 nM proved to be optimal.

In order to save time, we tried to use a shortened PCR protocol: 3 min at 95 °C followed by 40 cycles at 95 °C for 10 s and an annealing step at 54 °C for 25 s. Since both the ΔRn and Ct values did not differ from those obtained with the former protocol, the following experiments were carried out with the shortened protocol.

Optimization of the Duplex Real-Time PCR Assay. In order to investigate if the presence of both primer pairs and both TaqMan probes has an influence on the amplification efficiency duplex real-time PCR assays were carried out with either serially diluted sesame or hazelnut DNA. These experiments showed that the amplification efficiency in the duplex assay was as high as in the singleplex assay when only one amplicon (either hazelnut or sesame DNA) was present.

In order to investigate if the amplification efficiency in the duplex assay is decreased when both templates are present, mixtures of sesame and hazelnut DNA (5:95, 10:90, 25:75, 75:25, 90:10 and 95:5) were subjected to the duplex real-time PCR assay. In contrast to sesame, which was detected in each of the mixtures, hazelnut could not be detected in a mixture containing 5% hazelnut DNA and 95% sesame DNA. The following experiments were therefore carried out to increase the detectability of hazelnut DNA by increasing the concentration of either the Taq polymerase, the dNTPs and/or of $MgCl_2$. The lowest Ct values were obtained by adding 2 mM of $MgCl_2$ and 0.4 mM of dNTPs to the IQ Supermix from BioRad, corresponding to $MgCl_2$ and dNTP concentrations of 5 and 1.2 mM, respectively. Increasing the concentration of the Taq polymerase did not have an influence on the Ct values. Under the optimized

Table 2. Data on the repeatability of the determination of sesame (Section **a**) and hazelnut (Section **b**) by the duplex real-time PCR method. DNA was extracted 4 times from cookies which had been spiked with 10% sesame and 10% hazelnut. Assays with Taqman probes were run in duplicates. The whole procedure was repeated on the next day. [a]: was regarded as an outlier.

day 1	Ct	mean Ct	*S*	RSD (%)	day 2	Ct	mean Ct	S	RSD (%)
(**a**) Repeatability of the Determination of Sesame									
1. extraction					1. extraction				
1. replicate	24.64				1. replicate	25.46			
2. replicate	24.64				2. replicate	24.57			
		24.64					25.02		
2. extraction					2. extraction				
1. replicate	24.11				1. replicate	25.12			
2. replicate	24.2				2. replicate	25.03			
		24.16					25.08		
3. extraction					3. extraction				
1. replicate	24.03				1. replicate	24.46			
2. replicate	23.91				2. replicate	>35[a]			
		23.97					24.46		
4. extraction					4. extraction				
1. replicate	24.83				1. replicate	25.77			
2. replicate	24.44				2. replicate	25.48			
		24.64					25.63		
all replicates		24.35	0.33	1.37	all replicates		25.13	0.49	1.93
					interday		24.71	0.56	2.28
(**b**) Repeatability of the Determination of Hazelnut									
1. extraction					1. extraction				
1. replicate	24.91				1. replicate	24.66			
2. replicate	24.78				2. replicate	24.52			
		24.85					24.59		
2. extraction					2. extraction				
1. replicate	24.41				1. replicate	24.22			
2. replicate	24.35				2. replicate	24.24			
		24.38					24.23		
3. extraction					3. extraction				
1. replicate	23.95				1. replicate	24.2			
2. replicate	23.82				2. replicate	>35[a]			
		23.89					24.20		
4. extraction					4. extraction				
1. replicate	24.88				1. replicate	25			
2. replicate	24.26				2. replicate	24.92			
		24.57					24.96		
all replicates		24.42	0.41	1.69	all replicates		24.54	0.34	1.37
					interday		24.47	0.37	1.51

conditions it was possible to detect hazelnut in a mixture consisting of 5% hazelnut DNA and 95% sesame DNA.

In the experiments described above the concentration of the sesame primers (800 nM primer forward, 400 nM primer reverse) was significantly higher than that of the hazelnut primers (300 nM primer forward, 100 nM primer reverse). Next we investigated the influence of the sesame primer concentration (in the range from 25 to 200 nM) on the detectability of sesame and hazelnut. In the case of hazelnut, the lowest Ct values and the highest ΔRn values were observed at a sesame primer concentration of 25 nM (see **Figure 2a**). However lowering the sesame primer concentration to 25 nM significantly increased the Ct values for sesame (see **Figure 2b**). For this reason a sesame primer concentration of 50 nM was used in the following experiments.

Selectivity of the Duplex Assay. As mentioned above, in the sesame and hazelnut singleplex assays the primers proved to be specific for sesame and hazelnut, respectively. Since in optimizing the duplex assay several parameters, e.g. sesame primer concentration, Mg^{2+} concentration, dNTP concentration and annealing temperature, had to be changed, we therefore repeated cross-reactivity tests applying the optimized conditions of the duplex assay. DNA extracts of Brazil nut, sunflower seeds, peanut, walnut, pecan, cashew nut, almond, potato, rice, soybean, poppy seed, oat, rye, wheat, barley, spelt, apple, pear, strawberry, raspberry, cherry, sour cherry, maize, honey and chocolate were tested. The first derivatives of the melting curves shown in **Figure 3a** and **Figure 3b** indicate the specificity of the duplex assay developed in the present paper.

In addition, the duplex assay was applied to sesame and hazelnut samples from different producers: five white, three black and one golden (roasted) sesame samples and four ground hazelnut samples, three of them roasted and one unroasted. Ct values for the five white and three black sesame samples were in the range from 22.1 to 24.5; for the golden (roasted) sesame the Ct value was found to be 26.8. The Ct values for the four hazelnut samples were in a narrow range from 22.8 to 24.0.

Amplification Efficiency and LOD of the Duplex Assay. Amplification efficiency and limit of detection of the duplex assay were determined in two ways, by analyzing serially diluted sesame/hazelnut DNA and by analyzing whole meal cookies spiked with certain amounts of sesame/hazelnut.

Analysis of serially diluted sesame/hazelnut DNA resulted in good linearity up to a dilution of 1:10000 (corresponding to 10 pg μL^{-1} or 50 pg) (see **Figure 4**). The correlation coefficients (r^2) of the calibration curves for sesame and hazelnut were 0.988 and 0.996, respectively, indicating a high correlation between the sesame and hazelnut DNA amounts and their corresponding Ct values. The slopes of the calibration curves for sesame and hazelnut were −3.170 and −3.371, indicating high amplification efficiencies of 106.7 and 98.0%, respectively.

In order to determine the LOD in samples spiked with sesame/hazelnut blank whole meal cookies were spiked with sesame and hazelnut to obtain sesame/hazelnut concentrations of 1, 0.5,

Table 3. PCR Results Obtained by Analyzing Different Food Products

	sesame		hazelnut	
sample	declaration[a]	result	declaration[a]	result
cereal snack	+	+	+ (7% hazelnut)	+
cereal snack with chocolate	+	+	+ (6% hazelnut)	+
crispy cookies	+ (10−15%)	+	+ (4% hazelnut)	+
salty snack with sesame 3	+ (12%)	+	−	−
crispbread rye with sesame	+ (10%)	+	−	−
salty snack with sesame 2	+ (9%)	+	−	−
salty snack with sesame 1	+ (8%)	+	−	−
cookies with sesame	+	+	−	−
sesame balls	+	+	−	−
sesame cracker	+	+	−	−
muesli bar 2	±	−	+ (7% hazelnut)	+
whole meal cookies	±	−	+ (hazelnut)	+
nut muesli	±	−	+ (hazelnut)	+
cookies	±	−	± (nuts)	−
waffel rolls	±	−	± (nuts)	+
peanut bar	±	−	± (nuts)	−
muesli bar 1	−	−	+ (29% hazelnut)	+
chocolate nut bar	−	−	+ (17% hazelnut)	+
nut-almond muesli	−	−	+ (11% hazelnut)	+
chocolate with hazelnut	−	−	+ (11% hazelnut)	+
nut nougat cookies	−	−	+ (8% hazelnut)	+
almond-nut cornflakes	−	−	+ (hazelnut)	+
yogurt crisp muesli	−	−	+ (hazelnut)	+
ginger bar	−	−	+ (hazelnut)	+
hazelnut cream	−	−	+ (hazelnut)	+
coconut cookies	−	−	± (nuts)	+
whole milk chocolate	−	−	± (nuts)	+
shortbread	−	−	± (hazelnut)	−
almond bar	−	−	± (nuts)	−
peanut butter	−	−	−	−

[a] Declaration: + sesame/hazelnut listed, − sesame/hazelnut not listed, ± may contain sesame/hazelnut (nuts).

0.1, 0.05, 0.01, 0.005, and 0.001%. Analysis of DNA extracted from the cookies resulted in the standard curves shown in **Figure 5**. The correlation factors, r^2, were 0.9229 for sesame and 0.9413 for hazelnut. The slopes of the calibration curves for sesame and hazelnut were −3.867. and −4.112, indicating amplification efficiencies of 81.4% and 75.1%, respectively. The figures indicate that sesame and hazelnut could be detected in whole meal cookies containing 0.005% (50 ppm) sesame/hazelnut. According to Poms et al. (*24*) the LOD of an analytical method suitable for the detection of allergenic foods should be between 1 and 100 ppm. The duplex assay we developed meets this requirement.

Reproducibility of the Duplex Real-Time PCR Assay. The reproducibility of the duplex real-time PCR assay was investigated by preparing 4 DNA extracts from cookies which had been spiked with 10% sesame and 10% hazelnut. PCR assays were carried out with each DNA extract in duplicate. The whole procedure was repeated on the next day. The Ct values listed in **Table 2a**,**b** demonstrate the high reproducibility of both the extraction and the duplex real-time PCR assay.

Determination of Sesame and Hazelnut in Food Samples. The applicability of the real-time PCR assay for determining sesame and hazelnut in food was investigated by analyzing 30 commercial foodstuffs comprising salty snacks, cookies, chocolates, creams, mueslis and muesli bars. Part of the food products were declared to contain either sesame or hazelnut, part of them had a note that sesame and/or hazelnut (nuts) could be contained in the product and part of them did not have any information about containing sesame or hazelnut. All samples were tested in duplicate.

DNA was extracted using the CTAB method. For most samples, the A_{260}/A_{280} was within 1.7 and 1.9, which indicates a high DNA purity. $A_{260}/A_{280} < 1.5$ were obtained for yogurt crisp muesli, hazelnut cream, chocolate with hazelnut and a ginger bar. Extraction of DNA from nut nougat cookies, whole meal cookies and whole milk chocolate resulted in $A_{260}/A_{280} > 2.0$. DNA yields >300 μg/g were obtained for cereal snack with chocolate, cookies with sesame, crispbread rye with sesame, nut-almond muesli, peanut bar and peanut butter, and very low DNA yields (<50 μg/g) for nut nougat cookies, whole milk chocolate, coconut cookies, yogurt crisp muesli, hazelnut cream and chocolate with hazelnut.

For all samples labeled as containing either sesame or hazelnut or both, the duplex real-time PCR assay gave positive results (Ct < 40), indicating the presence of sesame or hazelnut (see **Table 3**). Sesame was, however, not detected in samples precautiously labeled with "might contain sesame". An analogous negative result indicating the absence of hazelnut was obtained for a sample with the label "might contain hazelnut". Six samples were labeled with "might contain nuts". Hazelnuts could be detected in three of them, the others giving negative results. Hazelnut/sesame could not be detected in foodstuffs which did not have any information about containing sesame or hazelnut.

With negative tested food samples inhibition control reactions were carried out in order to ensure that the negative results were not caused by polymerase inhibiting compounds. In all samples, sesame and hazelnut DNA were amplified successfully, indicating that the negative results were not caused by enzyme inhibition.

Conclusions. The duplex real-time PCR method developed in the present study allows the simultaneous detection of sesame and hazelnut, two highly potent allergenic foods. Compared to singleplex PCR, multiplex PCR methods have the advantages of saving time, reducing costs and lowering the probability of cross-contamination. In contrast to multiallergen ELISA methods where several allergens can be detected in one run, but not in the same well of the microtiter plate, the duplex PCR method offers the possibility to simultaneously amplify sesame and hazelnut DNA in one well.

LITERATURE CITED

(1) Burks, W.; Ballmer-Weber, B. K. Food allergy. *Mol. Nutr. Food. Res.* **2006**, *50*, 595–603.

(2) Ring, J.; Brockow, K.; Behrendt, H. Adverse reactions to foods. *J. Chromatogr.* **2001**, *756*, 3–10.

(3) Asero, R.; Ballmer-Weber, B. K.; Beyer, K.; Conti, A.; Dubakiene, R.; Fernandez-Rivas, M.; Hoffmann-Sommergruber, K.; Lidholm, J.; Mustakov, T.; Oude Elberink, J. N. G.; Pumphrey, R. S. H.; Stahl Skov, P.; Van Ree, R.; Vlieg-Boerstra, B. J.; Hiller, R.; Hourihane, J. O.; Kowalski, M.; Papadopoulos, N. G.; Wal, J.-M.; Mills, E. N. C.; Vieths, S. IgE-mediated food allergy diagnosis: current status and new perspectives. *Mol. Nutr. Food Res.* **2007**, *51*, 135–147.

(4) Sicherer, S. H.; Munoz-Furlong, A.; Burks, A. W.; Sampson, H. A. Prevalence of peanut and tree nut allergy in the US determined by a random digit dial telephone survey. *J. Allergy Clin. Immunol.* **1999**, *103*, 559–562.

(5) Sicherer, S. H.; Munoz-Furlong, A.; Sampson, H. A. Prevalence of peanut and tree nut allergy in the United States determined by means of a random digit dial telephone survey: a 5-year follow-up study. *J. Allergy Clin. Immunol.* **2003**, *112*, 1203–1207.

(6) Al-Muhsen, S.; Clarke, A. E.; Kagan, R. S. Peanut allergy: an overview. *Can. Med. Assoc. J.* **2003**, *168*, 1279–1285.

(7) Asero, R.; Mistrello, G.; Roncarolo, D.; Amato, S. Walnut-induced anaphylaxis with cross-reactivity to hazelnut and Brazil nut. *J. Allergy Clin. Immunol.* **2004**, *113*, 358–360.

(8) Pastorello, E. A.; Vieths, S.; Pravettoni, V.; Farioli, L.; Trambaioli, C.; Fortunato, D.; Lüttkopf, D.; Calamari, M.; Ansaloni, R.; Scibilia, J.; Ballmer-Weber, B. K.; Poulsen, L. K.; Wütrich, B.; Skamstrup Hansen, K.; Robino, A. M.; Ortolani, C.; Conti, A. Identification of hazelnut major allergens in sensitive patients with positive double-blind, placebo-controlled food challenge results. *J. Allergy Clin. Immunol*. **2002**, *109*, 563–570.

(9) Dalal, I.; Binson, I.; Reifen, R.; Amitai, Z.; Shohat, T.; Rahmani, S.; Levine, A.; Ballin, A.; Somekh, E. Food allergy is a matter of geography after all: sesame as a major cause of severe IgE-mediated food allergic reactions among infants and young children in Israel. *Allergy* **2002**, *57*, 362–365.

(10) Kägi, M. K.; Wüthrich, B. Falafel burger anaphylaxis due to sesame seed allergy. *Lancet* **1991**, *338*, 582.

(11) Asero, R.; Mistrello, G.; Roncarolo, D.; Antoniotti, P. L.; Falagiani, P. A case of sesame seed-induced anaphylaxis. *Allergy* **1999**, *54*, 526–527.

(12) Kanny, G.; De Hauteclocque, C.; Moneret-Vautrin, D. A. Sesame seed and sesame oil contain masked allergens of growing importance. *Allergy* **1996**, *51*, 952–957.

(13) Pastorello, E. A.; Varin, E.; Farioli, L.; Pravettoni, V.; Ortolani, C.; Trambaioli, C.; Fortunato, D.; Gabriella Giuffrida, M.; Rivolta, F.; Robino, A.; Calamari, A. M.; Lacava, L.; Conti, A. The major allergen of sesame seeds (*Sesamum indicum*) is a 2S albumin. *J. Chromatogr. B* **2001**, *756*, 85–93.

(14) Beyer, K.; Bardina, L.; Grishina, G.; Sampson, H. A. Identification of sesame seed allergens by 2-dimensional proteomics and Edman sequencing: Seed storage proteins as common food allergens. *J. Allergy Clin. Immunol*. **2002**, *110*, 154–159.

(15) Leduc, V.; Moneret-Vautrin, D. A.; Tzen, J. T. C.; Morisset, M.; Guerin, L.; Kanny, G. Identification of oleosins as major allergens in sesame seed allergic patients. *Allergy* **2006**, *61*, 349–356.

(16) Beyer, K.; Grishina, G.; Bardina, L.; Sampson, H. A. Identification of 2 new sesame seed allergens: Ses i 6 and Ses i 7. *J. Allergy Clin. Immunol*. **2007**, *119*, 1554–1556.

(17) Hirschwehr, R.; Valenta, R.; Ebner, C.; Ferreira, F.; Sperr, W. R.; Valent, P.; Rohac, M.; Rumpold, H.; Scheiner, O.; Kraft, D. Identification of common allergenic structures in hazel pollen and hazelnuts. A possible explanation for sensitivity to hazelnuts in patients allergic to tree pollen. *J. Allergy Clin. Immunol*. **1992**, *90*, 927–936.

(18) Skamstrup Hansen, K.; Ballmer-Weber, B. K.; Lüttkopf, D.; Skov, P. S.; Wüthrich, B.; Bindslev-Jensen, C.; Vieths, S.; Poulsen, L. K. Roasted hazelnuts - allergenic activity evaluated by double-blind, placebo-controlled food challenge. *Allergy* **2003**, *58*, 132–138.

(19) Schocker, F.; Lüttkopf, D.; Scheurer, S.; Petersen, A.; Cistero-Bahima, A.; Enrique, E.; San Miguel-Moncin, M.; Akkerdaas, J.; Van Ree, R.; Vieths, S.; Becker, W.-M. Recombinant lipid transfer protein Cor a 8 from hazelnut: a new tool for in vitro diagnosis of potentially severe hazelnut allergy. *J. Allergy Clin. Immunol*. **2004**, *113*, 141–147.

(20) Beyer, K.; Grishina, G.; Bardina, L.; Sampson, H. A. Identification of an 11S globulin as a major hazelnut food allergen in hazelnut-induced systemic reactions. *J. Allergy Clin. Immunol*. **2002**, *110*, 517–523.

(21) Lauer, I.; Foetisch, K.; Kolarich, D.; Ballmer-Weber, B. K.; Conti, A.; Altmann, F.; Vieths, S.; Scheurer, S. Hazelnut (Corylus avellana) vicilin Cor a 11: molecular characterization of a glycoprotein and its allergenic activity. *Biochem. J*. **2004**, *383*, 327–334.

(22) Directive 2006/142/EC of the European Parliament and of the Council. Official Journal of the European Union L 368/110, 23.12.2006.

(23) Anibarro, B.; Seoane, F. J.; Mugica, M. V. Involvement of hidden allergens in food allergic reactions. *J. Invest. Allergol. Clin. Immunol*. **2007**, *17*, 168–172.

(24) Poms, R. E.; Klein, C. L.; Anklam, E. Methods for allergen analysis in food: a review. *Food Addit. Contam*. **2004**, *21*, 1–31.

(25) Holzhauser, T.; Vieths, S. Quantitative sandwich ELISA for determination of traces of hazelnut (*Corylus avellana*) protein in complex food matrixes. *J. Agric. Food Chem*. **1999**, *47*, 4209–4218.

(26) Koppelman, S. J.; Knulst, A. C.; Koers, W. J.; Penninks, A. H.; Peppelman, H.; Vlooswijk, R.; Pigmans, I.; Van Duijn, G.; Hessing, M. Comparison of different immunochemical methods for the detection and quantification of hazelnut proteins in food products. *J. Immunol. Methods* **1999**, *229*, 107–120.

(27) Blais, B. W.; Philippe, L. Detection of hazelnut proteins in foods by enzyme immunoassay using egg yolk antibodies. *J. Food Prot*. **2001**, *64*, 895–898.

(28) Drs, E.; Baumgartner, S.; Bremer, M.; Kemmers-Voncken, A.; Smits, N.; Haasnoot, W.; Banks, J.; Reece, P.; Danks, C.; Tomkies, V.; Immer, U.; Schmitt, K.; Krska, R. Detection of hidden hazelnut protein in food by IgY-based indirect competitive enzyme-immunoassay. *Anal. Chim. Acta* **2004**, *520*, 223–228.

(29) Akkerdaas, J. H.; Wensing, M.; Knulst, A. C.; Stephan, O.; Hefle, S. L.; Aalberse, R. C.; Van Ree, R. A novel approach for the detection of potentially hazardous pepsin stable hazelnut proteins as contaminants in chocolate-based food. *J. Agric. Food Chem*. **2004**, *52*, 7726–7731.

(30) Kiening, M.; Niessner, R.; Drs, E.; Baumgartner, S.; Krska, R.; Bremer, M.; Tomkies, V.; Reece, P.; Danks, C.; Immer, U.; Weller, M. G. Sandwich immunoassays for the determination of peanut and hazelnut traces in foods. *J. Agric. Food Chem*. **2005**, *53*, 3321–3327.

(31) Blais, B. W.; Gaudreault, M.; Philippe, L. M. Multiplex enzyme immunoassay system for the simultaneous detection of multiple allergens in foods. *Food Control* **2003**, *14*, 43–47.

(32) Ben Rejeb, S.; Abbott, M.; Davies, D.; Cleroux, C.; Delahaut, P. Multi-allergen screening immunoassay for the detection of protein markers of peanut and four tree nuts in chocolate. *Food Addit. Contam*. **2005**, *22*, 709–715.

(33) Holzhauser, T.; Wangorsch, A.; Vieths, S. Polymerase chain reaction (PCR) for detection of potentially allergenic hazelnut residues in complex food matrixes. *Eur. Food Res. Technol*. **2000**, *211*, 360–365.

(34) Germini, A.; Scaravelli, E.; Lesignoli, F.; Sforza, S.; Corradini, R.; Marchelli, R. Polymerase chain reaction coupled with peptide nucleic acid high-performance liquid chromatography for the sensitive detection of traces of potentially allergenic hazelnut in foodstuffs. *Eur. Food Res. Technol*. **2005**, *220*, 619–624.

(35) Arlorio, M.; Cereti, E.; Coisson, J. D.; Travaglia, F.; Martelli, A. Detection of hazelnut (*Corylus* spp.) in processed foods using real-time PCR. *Food Control* **2007**, *18*, 140–148.

(36) Piknova, L.; Pangallo, D.; Kuchta, T. A novel real-time polymerase chain reaction (PCR) method for the detection of hazelnuts in food. *Eur. Food Res. Technol*. **2008**, *226*, 1155–1158.

(37) Schöringhumer, K.; Cichna-Markl, M. Development of a real-time PCR method to detect potentially allergenic sesame (*Sesamum indicum*) in food. *J. Agric. Food Chem*. **2007**, *55*, 10540–10547.

(38) Brzezinski, J. L. Detection of sesame seed DNA in foods using real-time PCR. *J. Food Prot*. **2007**, *70*, 1033–1036.

(39) Mustorp, S.; Engdahl-Axelsson, C.; Svensson, U.; Holck, A. Detection of celery (*Apium graveolens*), mustard (*Sinapis alba*, *Brassica juncea*, *Brassica nigra*) and sesame (*Sesamum indicum*) in food by real-time PCR. *Eur. Food Res. Technol*. **2008**, *226*, 771–778.

(40) Rossi, S.; Scaravelli, E.; Germini, A.; Corradini, R.; Fogher, C.; Marchelli, R. A PNA-array platform for the detection of hidden allergens in foodstuffs. *Eur. Food Res. Technol*. **2006**, *223*, 1–6.

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