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Detection of Allergenic Ingredients Using Real-Time PCR: A Case Study on Hazelnut (*Corylus avellena*) and Soy (*Glycine max*)

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ABSTRACT: Compliance with the European allergen labeling legislation (Directive 2007/68/EC) is only possible when coupled with appropriate methods to detect allergens in food. The aim of the current study was to develop new real-time PCR assays for the detection of hazelnut and soy and evaluate these assays via comparison with commercially available kits. Although the new assays were not as sensitive as the commercial qualitative assays, they proved to be more specific. Moreover, the cross-reactivity study indicated contamination of some of the food products used with either hazelnut or soy, which presents a risk for the allergic consumer. The assays were able to quantify as few as 5−15 genome copies. This unit, used to express analytical results for allergen detection by means of PCR, needs to be converted to a unit expressing the amount of allergenic ingredient in order to be informative. This study emphasizes that the use of real-time PCR for allergen quantification is complicated by the lack of appropriate reference materials for allergens.

KEYWORDS: food allergy, allergen detection, PCR, hazelnut, *Corylus avellana*, soy, *Glycine max*

INTRODUCTION

Food allergy is estimated to affect about 2−3% of the adult population and 4−8% of children.[1] The increasing focus on food allergies is caused by the rising number of diagnosed allergies, coupled with the ubiquitous presence of food allergens in the agri-food chain. A broad range of foods have been described as causing allergies, but the majority of allergic reactions can be ascribed to a limited number of food components. These foods or food groups, including hazelnut and soy, are contained within Directive 2007/68/EC concerning the labeling of allergens on prepackaged food products.[2] The food industry and the control bodies need sensitive analytical methods that are able to detect trace amounts of allergenic residues in food products to monitor production processes and (mis)labeling. The majority of analytical tools developed so far detect either proteins or DNA. The most frequently used protein-based format is ELISA, which detects allergenic ingredients based on the interaction of species-specific proteins/allergens with antibodies raised in animals. The detection of allergenic crops at the DNA level is performed by targeting species-specific nucleotide sequences. Up to now, allergen detection by means of PCR is still debated as it does not detect the allergenic actors, i.e. (the epitopes of) the allergens themselves. This argument cannot be denied because the presence of a gene encoding an allergen does not necessarily imply that the protein is expressed, nor does it provide information on the expression level. Consequently, the results obtained by DNA detection cannot be tied to the actual allergenic potential. However, some, if not most ELISA assays also do not necessarily detect the allergenic proteins themselves but rather detect species-specific marker proteins. This kind of ELISA assay cannot provide information on the allergenic potential of the food sample and from that viewpoint could be considered to be equally suited as PCR to detect allergenic commodities. Even if these tests do not provide direct information on the allergenic potential of the food product concerned, they are still able to provide information on the presence of the allergenic ingredient.

Herman et al.[3] developed a PCR for hazelnut detection in chocolate. This was an end-point PCR coupled to agarose gel electrophoresis, targeting a noncoding mitochondrial DNA sequence. Holzhauser et al.[4] developed an end-point PCR assay targeting the gene of the major hazelnut allergen *Cor a* 1. A similar semiquantitative assay was developed amplifying a sequence of the gene of the soybean *Gly m* Bd 30K allergen.[5] Hazelnut detection has been performed by means of various PNA-based detection platforms involving end-point PCR.[6,7] Hirao et al.[8] developed a PCR assay for soybean using an internal transcribed spacer region as target. Conventional end-point PCR might be more accessible due to the less expensive equipment that is required compared to real-time cycling instrumentation and detection chemistries. However, its lower cost cannot compensate for the extreme sensitivity and quantitative properties of real-time PCR, especially when coupled with its high-throughput potential. Because of these adventitious properties, most laboratories are no longer investing in conventional cycling instruments but are rather switching to real-time PCR equipment. This trend has resulted in increased reporting on qualitative real-time PCR methods for the detection of allergenic ingredients in recent years.[9−17] Piknová et al.[18] described a real-time PCR for hazelnut, detecting a low molecular weight heat-shock protein. Targeting the genomic coding region for allergens has been used previously to detect hazelnut and soy. Arlorio et al.[19] developed an assay targeting the *Cor a* 1 gene, which showed an LOD of 0.1 ng of genomic DNA. The SYBR Green real-time PCR of D'Andrea et al.[20] targeting the

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Table 1. Primer and Probe Sequences

target gene	designation	sequence (5′−3′)	amplicon size
Cor a 1	Cora1−F	AAGATAGTGGCATCCCCTCA	101 bp
	Cora1−R	CCAGCCTTAATCTGCTCGTC	
	Cora1−P	FAM−TGAAGAGCATCAGCAAGTACCACACCATA−TAMRA	
Cor a 8	Cora8−F	TGCGTGCTCTACCTGAAGAA	218 bp
	Cora8−R	GTGGAGGGGCTGATCTTGTA	
	Cora8−P	FAM−ACCGCCAGTCCGCTTGCAAC−TAMRA	
Gly m Bd 28K	Gly28−F	CGTTATCTGCAGCATTGACC	135 bp
	Gly28−R	CTTAGCCACAAGATGGCACA	
	Gly28−P	FAM−CCAGGTACATGCATGATGCATCCA−TAMRA	
Gly m Bd 30K	Gly30−F	CACATGCAATAGCAACAGGA	94 bp
	Gly30−R	TGCCATCCATTGTAACAACC	
	Gly30−P	FAM−CTGAACAAG-AACTCGTAGACTGTGTG-GAAGAAAGC−TAMRA	

genomic region of *Cor a* 8 could detect down to 20 genomic copies. Schöringhumer et al.[21] described a duplex real-time PCR for hazelnut (*Cor a* 1) and sesame using Taqman hydrolysis probes, while Pafundo et al.[22] have developed a multiplex real-time PCR using SYBR Green detection for allergen detection among which hazelnut (*Cor a* 1). A real-time PCR using a hydrolysis probe targeting the soy lectin gene has been described by Espineira et al.[23] having an LOD of 10 pg of genomic DNA. Recently, Mustorp et al.[24] developed a multiplex PCR by using ligation-dependent probes targeting among other allergens both hazelnut and soy. This assay was able to detect 1.4 ng and 13.6 ng of genomic hazelnut and soy DNA, respectively. Despite the continued increasing number of (real-time) PCR assays for allergen detection, certified reference standards which should serve as control and/or calibrator are currently still lacking.

The objective of the current research was to develop quantitative real-time PCR assays for hazelnut and soybean detection and compare them to commercial (qualitative) real-time PCR tests. The new assays were developed to target the genomic regions of the major hazelnut allergens *Cor a* 1 and *Cor a* 8 and the major soy allergens *Gly m* Bd 28K and *Gly m* Bd 30K. For each assay, we evaluated the analytical sensitivity and the specificity, as these are the most critical parameters in allergen detection.

MATERIALS AND METHODS

Plant and Food Materials. Nine different commercial samples of hazelnuts (different commercial brands), including raw unpeeled hazelnuts and roasted peeled hazelnuts, were purchased in local supermarkets in Belgium. Soybeans were provided by Alpro (Wevelgem, Belgium) and Cargill (Mechelen, Belgium). The soybeans originated from Canada, Brazil, China, and Paraguay. A hazelnut mix and a soybean mix were made by combining equal amounts of each sample of hazelnut or soybean, respectively. All other food samples were purchased in local supermarkets. All samples were ground with a centrifugal mill applying a sieve with an aperture size of 0.75 mm (ZM 200, Retsch, Haan, Germany) after being frozen with liquid nitrogen and stored at −20 °C until use.

DNA Extraction. DNA was isolated using the DNeasy Plant mini kit (Qiagen, Hilden, Germany) and quantified using the Quantit Picogreen dsDNA Assay kit (Invitrogen Ltd., Paisley, UK). To avoid false negative PCR results, the presence of amplifiable DNA was tested with an in-house developed real-time PCR assay using universal primers and a 5′-FAM- and 3′-TAMRA-labeled oligonucleotide hydrolysis probe targeting a conserved eukaryotic sequence on the 18S rRNA gene. PCR reactions were performed in a volume of 25 μL containing 5 μL of template DNA ($\leq$10 ng μL^{-1}), 1× Real-Time PCR Mastermix (Diagenode, Liège, Belgium), 200 nM forward primer, 200 nM reverse primer, and 200 nM Taqman probe. PCR reactions were performed in an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Halle, Belgium). Each run was initiated by a decontamination reaction from dUTP-containing the template at 50 °C for 2 min, followed by deactivation of the uracil *N*-glycosylase and denaturation for 10 min at 95 °C. Each of the 45 cycles consisted of denaturation at 95 °C for 15 s and combined primer annealing and elongation at 60 °C for 60 s. Data were analyzed with 7000 System Sequence Detection Software, version 1.2.3 (Applied Biosystems).

Design of Primers and Probes. The genes encoding the allergens *Cor a* 1 and *Cor a* 8 of hazelnut (*Corylus avellana*) and *Gly m* Bd 28K and *Gly m* Bd 30K of soy (*Glycine max*) were used as marker genes to design primer pairs and 5′-FAM- and 3′-TAMRA-labeled hydrolysis probes. For each target, the available nucleotide sequences were assembled from public databases and, in the case of multiple sequences for a single target, aligned to each other using the ClustalW software (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The following accession numbers were used for the oligonucleotide design: Z72440 (*Cor a* 1, *Bet v* 1-related major hazelnut allergen), AF329829 (*Cor a* 8, lipid transfer protein, major allergen from hazelnut), EU493455, EU493458, EU493457, EU493460, EU493461 (*Gly m* Bd 28K, major soy allergen), EU883600, and DQ324851 (*Gly m* Bd 30K, major soy allergen). Primers and probes were designed on the conserved regions where possible using the Primer 3 tool available at http://frodo.wi.mit.edu/primer3//.[25] The software parameters were set to design primers with an optimal melting temperature (T_m) of 60 °C, to have a GC-content between 40 and 60%, to be at minimum 18 base pairs (bp) long and to produce an amplicon of 80−150 bp. Probes were designed to have an optimal T_m of 70 °C and a length of 18−30 bp. From the output, those primers and probes were selected which contained more C's than G's, no G at the 5′-end, and which preferably contained no repetitions of more than two equal bases. The resulting oligonucleotide sequences are listed in Table 1. For the *Cor a* 8 gene, it was not possible to design a primer pair that met all the conditions set, which eventually resulted in a primer pair producing an amplicon of 218 bp instead of <150 bp. Primers and probes were synthesized and RP-HPLC purified by Eurogentec (Liège, Belgium).

Real-Time PCR Assays. To verify if the designed primer pairs have the potential for primer dimer formation, a melting curve analysis of the PCR products was performed. PCR reactions were performed in a reaction volume of 25 μL containing 2.5 μL of template DNA (400 pg μL^{-1}, 160 pg μL^{-1}, 32 pg μL^{-1}, 6.4 pg μL^{-1}), 1× SYBR Green PCR Master

Table 2. Selected Food Ingredients for the Specificity Assessment

protein BLAST	primer BLAST	additional relevant ingredients
almond (*Prunus dulcis*)	apple (*Malus domestica*)	egg (chicken)
apple (*Malus domestica*)	grape (*Vitis vinifera*)	kamut (*Triticum turanicum*)
barley (*Hordeum vulgare*)	lettuce (*Lactuca sativa*)	milk (powder) (cow)
Brazil nut (*Bertholletia excelsa*)	maize (*Zea mays*)	spelt (*Triticum spelta*)
cashew (*Anacardium occidentale*)	oat (*Avena sativa*)	starch (potato)
grape (*Vitis vinifera*)	raspberry (*Rubus idaeus*)	sugar
hazelnut (*Corylus avellana*)	rice (*Oryza sativa*)	
macademia (*Macademia ternifolia*)	soy (*Glycine max*)	
maize (*Zea mays*)	spinach (*Spinacia oleracea*)	
oat (*Avena sativa*)	strawberry (*Fragaria ananassa*)	
olive (*Olea europaea*)	walnut (*Juglans regia*)	
pea (*Pisum sativum*)		
peanut (*Arachis hypogaea*)		
pecan (*Carya illinoinensis*)		
pistachio (*Pistacia vera*)		
rapeseed (*Brassica napus*)		
rice (*Oryza sativa*)		
soy (*Glycine max*)		
walnut (*Juglans regia*)		
wheat (*Triticum aestivum*)		

Mix (Applied Biosystems, Foster City, USA), 300 nM forward primer, and 300 nM reverse primer. Each run was initiated by a decontamination reaction from a dUTP-containing template at 50 °C for 2 min, followed by deactivation of the uracil *N*-glycosylase and denaturation for 10 min at 95 °C. Each of the 45 cycles consisted of denaturation at 95 °C for 15 s and combined primer annealing and elongation at 60 °C for 60 s. Upon completion of the cycling reaction, a dissociation stage was added in the program to construct melting curves of the PCR products formed.

The 5′-3′-exonuclease PCR reactions were performed in a volume of 25 μL containing 2,5 μL (1−10 ng) of template DNA, 1× Real-time PCR Mastermix (Diagenode). Concentrations of 50, 300, and 900 nM forward and reverse primers and 50, 100, 150, and 200 nM probe were used to optimize the PCR conditions. Cycling conditions were the same as those described for the runs with SYBR Green I detection.

The designed PCR assays were compared to existing commercial PCR assays for the detection of hazelnut and soy, respectively. The following kits were included in the study: First-Hazelnut and Soy PCR Kit (Gen-Ial, Troisdorf, Germany), and Surefood ALLERGEN Hazelnut and Soya (R-Biopharm, Darmstadt, Germany). For the commercial assays, runs were performed as described in the manuals.

Analytical Sensitivity. The analytical sensitivity of the assays was established with dilutions of genomic DNA from hazelnut and soy. The concentrations ranged from 0.256 pg to 50 ng. Each dilution was first analyzed in duplicate. Subsequently, five dilutions were selected between the limit of amplification and no amplification and reanalyzed in six replicates. The limit of detection (LOD) was determined as the lowest concentration at which amplification occurred for all six replicates. The quantification range was assessed by analyzing the same dilution series in duplicate in two independent runs. The limit of quantification (LOQ) was set at the lowest concentration at which a linear relationship exists between the concentration and the quantification cycle (C_q) ($\log(\Delta conc) = (\Delta C_q)/3.32)$).[26]

Cross-Reactivity Study. To determine the specificity of the assays, a selection of different food species was made based on (i) the results of a protein BLAST of the known hazelnut/soy allergen amino acid sequences to search for plant species containing homologous proteins, (ii) the results of a primer BLAST with the designed primers (see Design of Primers and Probes) to assess their in silico specificity, and (iii) additional relevant food ingredients. For the protein BLAST, amino acid sequences of known hazelnut and soy allergens were retrieved from the Allergome database available at http://www.allergome.org.[27] The protein BLAST was performed with the Expasy Proteomics Server available at http://expasy.org/tools/blast/[28] against the Uniprot Knowledgebase database; this search was restricted to the Viridiplantae entries to limit the output. The primer BLAST was performed using the NCBI Primer BLAST tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The selected food products are presented in Table 2.

DNA was extracted from the different food products and quantified. DNA concentrations used in the PCR assays were ≤10 ng μL^{-1}. Samples returning a positive result were further examined to exclude contamination of the raw material by (1) sequencing the amplification products as described below (Confirmation of the Identity of PCR Products), (2) melting curve analysis, or (3) analyzing the DNA in an alternative PCR specific for hazelnut or soy. Melting curves were constructed by analyzing the positive samples in a PCR reaction containing 1× SYBR Green PCR Master Mix (Applied Biosystems), 300 nM forward primer, 300 nM reverse primer, and 2.5 μL of template in a final volume of 25 μL. Runs were performed as described above (Real-Time PCR Assays). The alternative hazelnut-specific PCR was derived from Herman et al.[3] and the soy-specific PCR targeting the lectin gene from Kuribara et al.[29]

Confirmation of the identity of PCR Products. PCR products amplified in the newly developed PCR assays were sequenced to confirm their identity. PCR amplification products were purified with the MSB Spin PCRapace kit (Invitek, Germany). The PCR sequencing reactions were performed in a final volume of 20 μL containing 4 μL of BigDye, version 1.1, Terminator Ready reaction mix, 2 μL of 5× Sequencing Buffer (Applied Biosystems), 0.16 μM primer, 2 μL of purified PCR product, and 11 μL of ultrapure water. PCR runs were performed in a DuoCycler (VWR International, Leuven, Belgium). To improve the reliability of the sequencing reaction, the sequencing primers contained a 60 bp 5′-nucleotide tail with the following sequence:

5′-CCCCCCCCCCCCCCCCCCCCAACTGACTAAACTAGGT-GCCACGTCGTGAAAGTCTGACAA-3′.[30]

The reaction mixture was subjected to 30 cycles of amplification. The first cycle was preceded by denaturation for 1 min at 96 °C. Each cycle

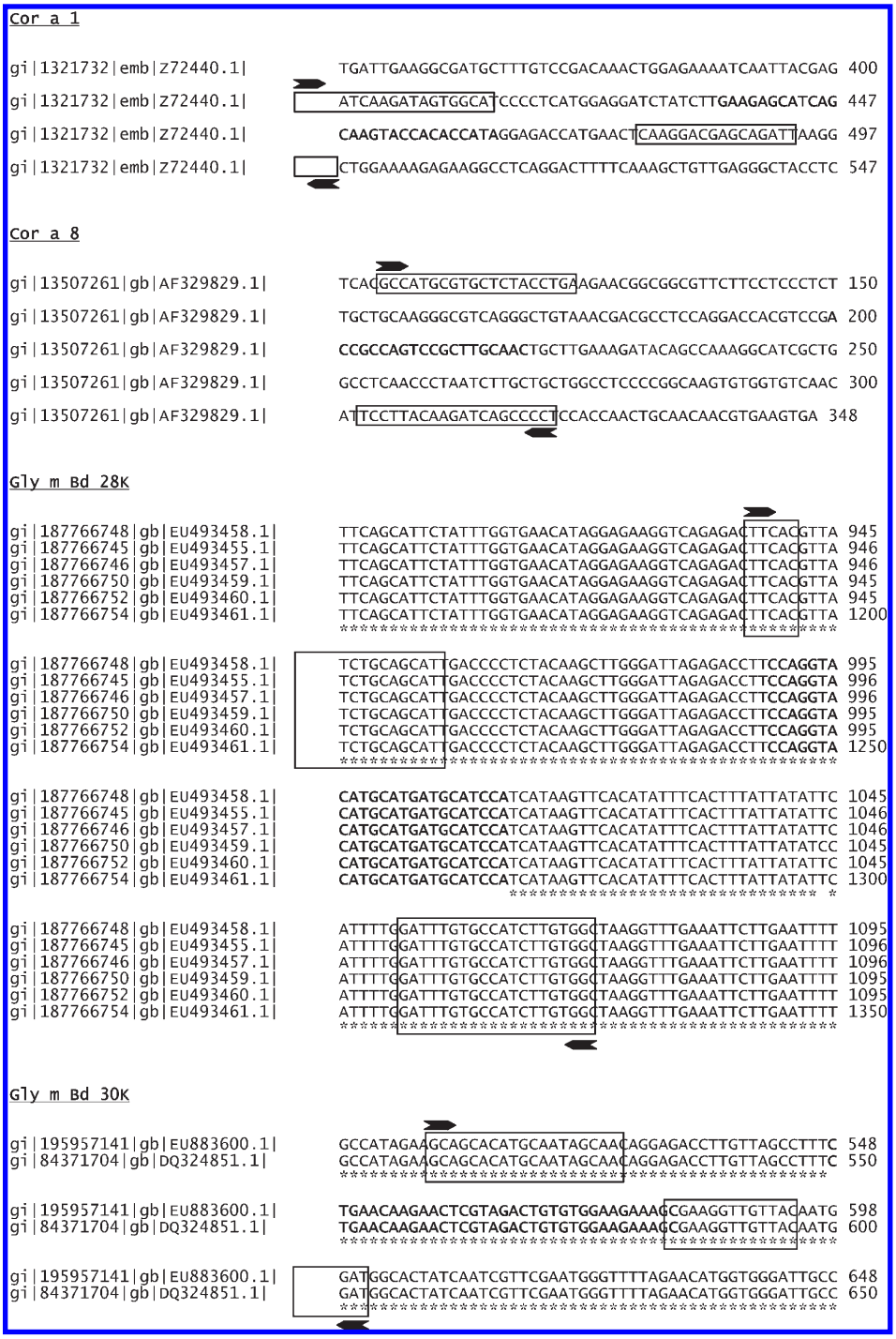

Figure 1. Sequence alignment of different accession numbers of the individual targets and location of the designed primers (forward arrow and reverse arrow) and probes (marked in bold).

consisted of denaturation for 10 s at 96 °C, annealing for 5 s at 50 °C, and elongation for 4 min at 60 °C. The PCR products of the sequencing reaction were purified by adding 1 μL of EDTA (250 mM), 2 μL of sodiumacetate (3 M, pH 4.6), and 50 μL of ethanol (95%). The 96-well plate was then sealed, vortexed, and incubated at room temperature for 15 min, then centrifuged at 3000*g* for 30 min. The seal was removed and the supernatant deposited onto absorbent paper. To remove any residual supernatant, the unsealed plate was centrifuged upside down for 1 min at 7000*g*. Then, 150 μL of 70% ethanol was added, the plate sealed again, vortexed, and centrifuged for 20 min at 2000*g*. The supernatant was removed as described above after which the plate was incubated without the seal at 90 °C for 2 min. Prior to sequencing, 15 μL of highly deionized formamide was added, the plate sealed again, and mixed by vortexing for 4 min. After a final incubation at 90 °C during 8 min, the samples were ready to be injected for sequencing. The purified products were sequenced on an ABI Prism 3130 (Applied Biosystems), and the sequences were analyzed using the Sequence Analysis software version 5.2 (Applied Biosystems).

RESULTS

Development of New Assays. *Primer and Probe Design.* For the development of new PCR assays to detect hazelnut (*Corylus avellana*) and soy (*Glycine max*), we chose to target genes encoding allergens of the respective commodities to ensure a high specificity of the assays. Amplification of small fragments (50−250 bp) was preferred with regard to the detection of the targets in processed food products and to ensure a short analysis time. Primers and probes were designed on the genes encoding *Cor a* 1 and *Cor a* 8 of hazelnut and *Gly m* Bd 28K and *Gly m* Bd 30K of soybean (Figure 1).

The specificity of the primer pairs for their target within the hazelnut or soybean genome was confirmed by conventional PCR and analysis through agarose gel electrophoresis (Figure 2). This also allowed us to verify if the amplicon formed had the expected length. The selected primers all amplified a single fragment with the expected length.

Primer dimers also bind SYBR Green I and will contribute to the fluorescent signal. On the basis of the amplification plot, it is not possible to distinguish these duplex molecules from the target amplicons. The potential of primer dimer formation was verified by evaluating the melting peaks of the amplification products. As the thermodynamic behavior of an oligonucleotide is dependent on its length and sequence, primer dimers will display a melting profile different from those of the PCR products. Primer dimers can also be detected in the negative control, not containing template DNA. Real-time analysis with SYBR Green I detection, combined with a dissociation step, showed that amplification plots were generated for the different samples in the individual assays (Figure 3). Fluorescent threshold values were set manually at the beginning of the exponential phase, and the baselines were set from cycle 3 to the third cycle before the smallest C_q. Melting curve analysis resulted in a clear single peak for the different tested primer pairs. No signal was observed in the negative control, which contained no template DNA. One can thus conclude that the selected primers do not possess self-complementarity. Successful amplification was also achieved with all assays in the 5′-3′-exonuclease format by applying the hydrolysis probes. To achieve the highest level of sensitivity, the PCR conditions were optimized. A primer−probe concentration optimization experiment was performed by assessing the PCR performance at different primer−probe concentration combinations. The best combination was determined by evaluating the amplification plots. The combination showing the lowest C_q value (most sensitive), the highest plateau (highest yield), and an optimal slope of −3.32 (best efficiency) is by definition the most optimal amplification reaction (ABI Prims 7000 Sequence Detection Systems manual). A combination of 300 nM primers and 200 nM probe yielded the best results in all assays, except for the Gly30-probe, where a concentration of 100 nM resulted in better amplification (results not shown). These combinations were used in all further runs.

Confirmation of the Identity of PCR Products. The amplicons of the different assays were sequenced to verify the identity of the products. The fragments to be analyzed were rather short; in some cases, they were only about 100 bp. The sequencing platform used in this study has the characteristic of poor performance when reading the first 50 bp at the 5′-end of the sequence. This means that short fragments of only 100 bp will not be sequenced in an appropriate way and would contain many errors. To overcome this problem, the amplicons can be cloned into a vector, or

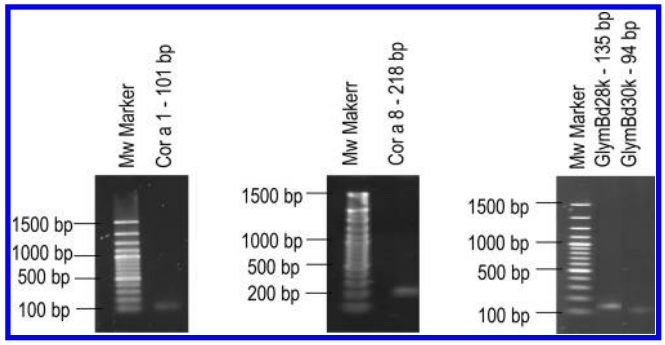

Figure 2. Agarosegel electrophoresis of PCR products obtained with the primer pairs designed on allergen encoding genes.

both strands could be sequenced in the opposite directions to produce complementary sequences with dual coverage in the middle and single coverage at the extremes. However, a more simple and easily accessible approach is described by Binladen et al.[30] and consists of adding a nonspecific nucleotide tail to the 5′-end of the sequencing primers. The author demonstrated that adding a 40- or 60-bp nucleotide tail to the sequencing primers enhanced the quality of the sequence obtained from fossil specimens. We compared sequencing of the amplification products with the original primers (Table 1) to using these primers with a 60 bp-tail. The quality of the electropherograms obtained with the tailed primers proved to be significantly better than when the original primers were used in the sequencing reaction. This allowed for determining the complete sequence of the amplicons with sufficient fidelity, where this was possible only for a part of the amplicon sequence with the original primers. The obtained sequences showed 100% homology with the respective sequences from hazelnut and soy, as proven by Nucleotide BLAST and confirmed the in silico determined amplicon sequence of the distinct assays. This sequencing approach could not be applied on the PCR products obtained with the commercial PCR assays, as the identity of the primers nor the target are known.

Evaluation of New and Existing Assays. *Analytical Sensitivity.* To protect the allergic consumer, allergen detection methods need to be sensitive enough to be able to detect contamination at trace levels. Therefore, we determined the analytical sensitivity of both the new and the commercial PCR assays by analyzing dilution series of genomic hazelnut or soy DNA. The concentrations ranged from 0.256 pg to 50 ng (total DNA content in the PCR reaction). The LOD was determined as 3.2 pg of genomic hazelnut DNA for the *Cor a* 1 and *Cor a* 8 assays and 1.28 pg of genomic soy DNA for the *Gly m* Bd 28K and *Gly m* Bd 30K assays. The commercial assays for both hazelnut and soy were all able to detect 1.28 pg of genomic hazelnut/soy DNA. When converting these amounts to the number of genome copies of the target that can be detected, taking into account the genome size of hazelnut (0.48 pg) [31] and soy (1.13 pg),[32] the commercial kits for soy detection showed to be the most sensitive, being able to detect as low as a 1.1 genome copy (Table 3). However, we must remark that for this calculation it is assumed that the targeted sequences are single copy genes.

The LOQ was determined by constructing a standard curve for each assay. Table 4 shows the parameters of the standard curves, which are the mean values obtained after duplicate analysis of the samples in two independent runs. As to date no requirements are available for PCR assays detecting food allergens, evaluation of the curves was done based on the Minimum

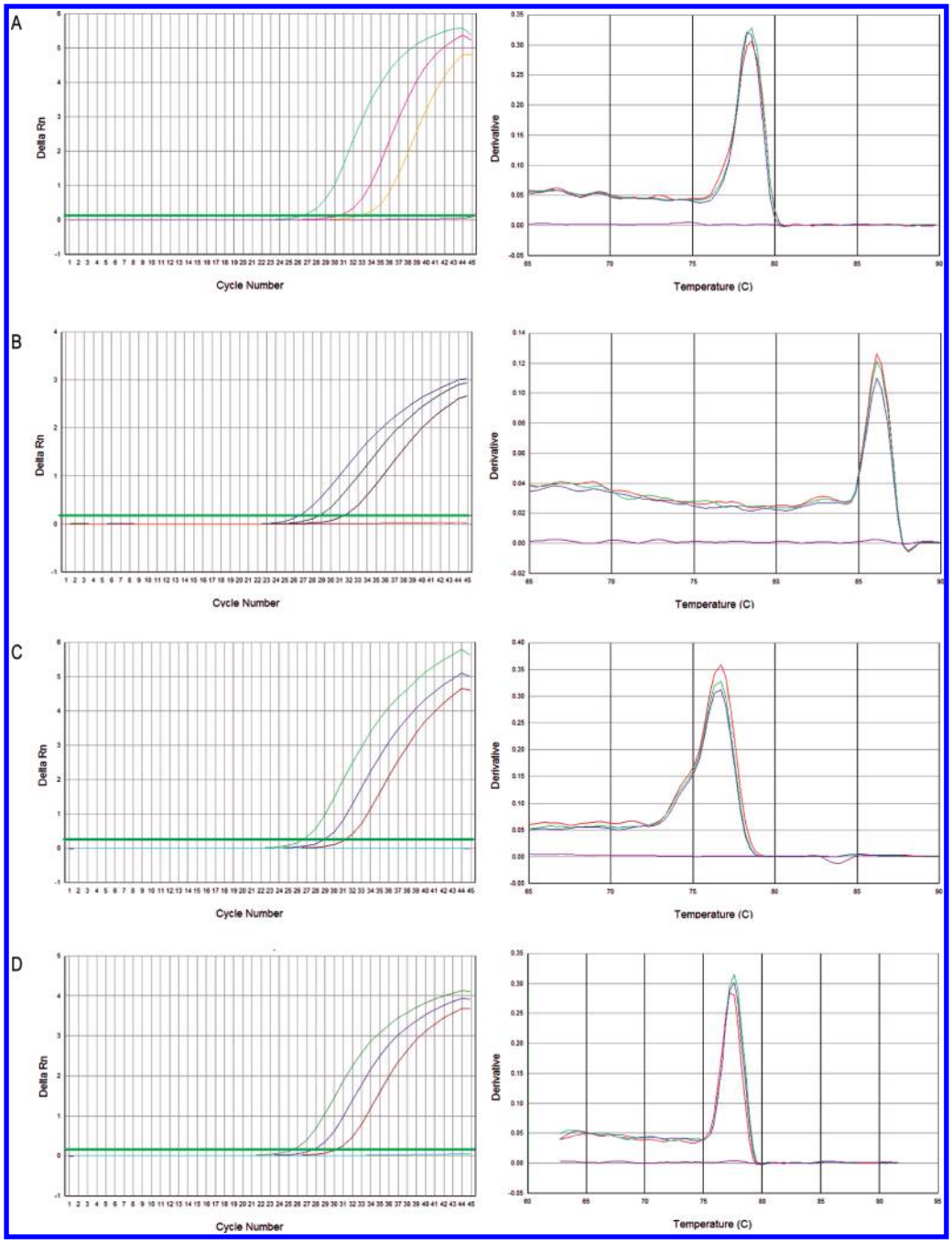

Figure 3. Amplification plots and melting peaks resulting from real-time PCR with SYBR Green I detection with the selected primers designed on the gene coding for *Cor a* 1 (A), *Cor a* 8 (B), *Gly m* Bd 28K (C), and *Gly m* Bd 30K (D) applying different dilutions of genomic DNA of hazelnut and soybean.

Requirements of Analytical Methods for GMO Testing.[33] This document prescribes that the correlation coefficient (R^2) has to be ≥0.98 and that the slope of the curve should be equal to or between −3.1 and −3.6, resulting in an PCR efficiency of 110% and 90%, respectively. Each assay evaluated fulfilled these requirements. For each assay, the lowest point of the standard curve, i.e., the quantification limit, was 6.4 pg of DNA.

Specificity. The examined assays are intended to analyze food products containing a broad variety of ingredients. Hence, it is important that the assays will only amplify and consequently detect the intended target and will not cross-react with DNA sequences from other possible ingredients present. The specificity of the designed assays was first determined in silico by searching for homologies with DNA sequences present in the NCBI database. We did not search for entries containing a nucleotide sequence similar to the whole PCR product, as cross-reactivity could result from the primers binding to another DNA sequence than the target of interest with a differing interprimer sequence. Instead, a search for sequences which the primers could anneal with was performed using the NCBI Primer BLAST tool. For the same reason, a BLAST with the complete gene sequence of the respective targets has not been performed. This approach was not possible

Table 3. Limit of Detection (LOD) and Quantification (LOQ) of the Different Hazelnut and Soy PCR Assays Expressed As Amount of Genomic DNA (pg DNA) or Number of Haploid Genome Copies (Copies) (Genomic Weight of Hazelnut = 0.48pg; soy = 1.13 pg) (n = 6)

	LOD		LOQ	
assay	pg DNA	copies	pg DNA	copies
Cor a 1	3.2	6.6	6.4	13.3
Cor a 8	3.2	6.6	6.4	13.3
First Hazelnut	1.28	2.7	6.4	13.3
Surefood Hazelnut	1.28	2.7	6.4	13.3
Gly m Bd 28K	3.2	2.8	6.4	5.7
Gly m Bd 30K	3.2	2.8	6.4	5.7
Surefood soy	1.28	1.1	6.4	5.7
First soy	1.28	1.1	6.4	5.7

Table 4. Parameters of the Standard Curves of the Different PCR Assays

assay	R^2	slope	efficiency
Cor a 1	0.9979	−3.4127	96.3%
Cor a 8	0.9946	−3.2254	104.2%
First Hazelnut	0.9991	−3.2183	104.5%
Surefood Hazelnut	0.9968	−3.5249	92.2%
Gly m Bd 28K	0.9989	−3.4148	96.3%
Gly m Bd 30K	0.9995	−3.2791	101.8%
Surefood soy	0.9982	−3.4597	94.6%
First soy	0.9984	−3.1173	109.3%

for the commercial assays as the primer sequences are the intellectual property of the kits' manufacturers. The *Cor a* 8-primers demonstrated homologies with nucleotide sequences within the genome of apple (*Malus domestica*), lettuce (*Lactuca sativa*), oat (*Avena sativa*), raspberry (*Rubus idaeus*), strawberry (*Fragaria ananassa*), walnut (*Juglans regia*), grape (*Vitis vinifera*), and soy (*Glycine max*). Although the alignment of the primers with these sequences contained single or multiple mismatches, successful annealing cannot be ruled out. In practice, a false-positive signal could be obtained only if the hydrolysis probe is also able to bind to the interprimer sequence within the genome of these species. To elucidate this, a homology search by means of a nucleotide BLAST was performed for the individual *Taqman* probes. For a 5′-3′-exonuclease assay to be successful, it is important that the probe anneals with the target sequence over its complete length without any mismatches. Although the BLAST search revealed that the probe sequences were not unique for the hazelnut or soy genome, no entries were found containing a stretch of nucleotides with 100% equal identity covered over the complete probe sequence. Nevertheless, the possible cross-reacting species identified in the Primer BLAST were included in our experimental specificity assessment to confirm the in silico findings. In-house developed primer pairs to amplify a fragment of the *Cor a* 9 and *Cor a* 10 genes of hazelnut (unpublished) showed in silico cross-reactivity with spinach (*Spinacia oleracea*), maize (*Zea mays*), and rice (*Oryza sativa*), respectively. Even though these primers are not described further in this study, these plant species were also included in the specificity test, if they were not included yet based on the protein BLAST selection (see below).

In addition to the food species addressed above, a selection was made of foods containing proteins with an amino acid sequence similar to that of known hazelnut and soy allergens and foods known to be present as bulk ingredients in products containing hazelnut and soy (Table 1).

DNA was extracted from all the foodstuffs. As a control to avoid false-negative results, the presence of PCR inhibitors and the integrity of the DNA in the samples were assessed first. This was done by amplification of a conserved eukaryotic sequence on the 18S rRNA gene. Successful amplification was achieved for all samples.

A sample was considered as positive if a good amplification plot and a C_q value were obtained after data processing. If no amplification was observed in the first analysis, the concerned species was assumed to be not cross-reacting in the hazelnut or soy PCR assays. Positive signals in the first analysis were confirmed by analyzing a second independent DNA sample from the food product and were an indication that the concerned species presents cross-reactivity. However, possible contamination of the raw material with hazelnut or soy should also be considered, as we are working with retail food samples. Therefore, further investigation was performed to elucidate what the underlying cause of the positive signal was. For the in-house developed PCR assays, contamination of the sample could be examined by melting curve analysis by repeating the PCR reaction with two independent DNA samples with SYBR Green I detection instead of the hydrolysis probe. The formed PCR product could also be further identified by sequencing the fragment. The sequencing approach used in this study and the melting curve analysis both require knowing the identity of the primers. This means that these approaches were not possible with the commercial PCR tests. Moreover, the primers are contained in a mix with other PCR components in the kits, whose exact composition is not known. To elucidate whether the positive signals obtained in the commercial PCR assays were due to contamination of the raw material or whether they could be ascribed to cross-reactivity, an alternative approach was used. Two independent PCR assays detecting hazelnut or soy were used to analyze the positive samples. A negative result with two independent DNA samples in these independent assays indicated that the positive amplification in the new or commercial PCR tests was caused by cross-reactivity of the concerned species, causing a false-positive result. If the DNA samples returned a positive signal in these independent assays, it could then be concluded that the food sample was contaminated with either hazelnut or soy. To finally confirm whether or not the concerned food species shows cross-reactivity, a new sample of the food product was purchased and analyzed as above. Only when this new sample returned a negative result in the independent PCR and positive amplification in the allergen detection test, could it be defined as cross-reactive.

Hazelnut PCR Assays. In the first analysis of the DNA samples from the selected food species with the different hazelnut PCR assays, a positive amplification signal was obtained only with the commercial assays (Table 5). Further investigation of these samples was only possible by analysis using the independent hazelnut PCR. This assay amplifies a noncoding mitochondrial sequence of 294 bp and has been developed for the detection of hazelnut in chocolate.[3] Only the pistachio sample returned a positive signal using this assay, although very faint (Table 5, second analysis of First Hazelnut and Figure 4, pistachio 1). This indicated that the pistachio nuts were contaminated by hazelnut. These results were also confirmed by melting curve analysis by performing this

Table 5. Specificity of the Hazelnut PCR Assays[a]

		Cor a 1	*Cor a* 8	First Hazelnut			Surefood Hazelnut		
	species	1	1	1	2	3	1	2	3
protein BLAST	almond	−	−	−			−		
	barley	−	−	−			−		
	brazil nut	−	−	−			−		
	cashew	−	−	−			−		
	macademia	−	−	−			−		
	olive	−	−	−			−		
	pea	−	−	−			−		
	peanut	−	−	−			−		
	pecan	−	−	−			−		
	pistachio	−	−	+	+	−	−		
	rapeseed	−	−	−			−		
	wheat	−	−	−			−		
prime r BLAST	lettuce	−	−	+	−		−		
	raspberry	−	−	+	−		+	−	
	spinach	−	−	−			−		
	strawberry	−	−	+	−		+	−	
protein + primer BLAST	apple	−	−	−			−		
	grape	−	−	−			−		
	maize	−	−	−			−		
	oat	−	−	−			−		
	rice	−	−	−			−		
	soy	−	−	−			−		
	walnut	−	−	−			−		
bulk ingredients	egg	−	−	−			−		
	Kamut	−	−	−			−		
	milk powder	−	−	−			−		
	spelt	−	−	−			−		
	starch	−	−	−			−		
	sugar	−	−	−			−		

[a] Test results of the analysis of two independent DNA samples in (1) new/commercial hazelnut PCR, (2) independent hazelnut PCR, and (3) second food sample in new/commercial hazelnut PCR.

PCR reaction in real-time format with SYBR Green I detection (Figure 4). The results from this independent PCR prove that the positive signal with strawberry, raspberry, and lettuce results from cross-reactivity of the primers of both commercial assays with these food/plant species (only First Hazelnut kit for lettuce). Interestingly, although these species were selected based on the results of the primer BLAST with the *Cor a* 8 primers, no cross-reactivity was observed in this assay. Subsequently, a new sample of pistachio nuts was purchased, which indicated clearly on the package label that the product was free from other nuts. Nevertheless, the sample was first checked for contamination with the independent hazelnut PCR, returning a negative result (Figure 4, pistachio 2 and Table 5, third analysis). Analyzing the sample using the First Hazelnut PCR also returned a negative result, indicating that the positive signal in the first analysis was due to the contamination of the pistachio sample with hazelnut. In Figure 4, the small peaks that can be seen between 76 and 78 °C originate from the reaction with DNA from the second pistachio sample. This signal could be assigned to primer dimers, which are typically seen at temperatures below the melting temperature of the amplicon. However, no primer dimers were observed in the negative control reaction, containing no DNA. Therefore, these signals are probably background noise.

It should be noted that the other PCR assays were not able to detect this contamination. The amount of hazelnut genomic DNA that could be quantified in the pistachio sample with the First Hazelnut PCR using the calibration curve constructed by analyzing a dilution series of hazelnut DNA, was either just above or just below the LOD of the assay. This indicates that the level of contamination was close to the LOD of this assay and the Surefood Hazelnut assay and below the LOD of the *Cor a* 1 and *Cor a* 8 assays. Nonetheless, it could be confirmed by analyzing the pistachio sample in commercial ELISA kits for hazelnut detection (data not shown).

Soy PCR Assays. To assess whether the positive signal obtained with the kamut sample in both the new and commercial soy PCR tests (Table 6) was related to soy-contamination of the product, the *Gly m* Bd 28K and *Gly m* Bd 30K assays were repeated with SYBR Green I detection and melting curve analysis. However, the results obtained by this approach were not unequivocal and could not be used to elucidate this question. This was done by sequencing the amplification products applying the 60-bp tailed primers, as described above, to reveal their identity. Nucleotide BLAST of the

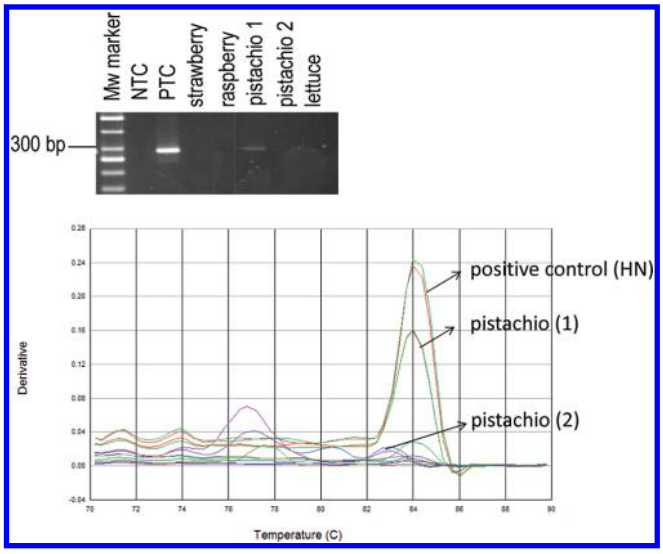

Figure 4. Independent hazelnut PCR. Top: conventional PCR with detection of PCR products by agarosegel electrophoresis and ethidium bromide staining. Bottom: melting curves obtained after amplification of DNA obtained from hazelnut (positive control), first pistachio sample (1), pistachio without contamination (2), raspberry, strawberry, and lettuce.

PCR products obtained in the *Gly m* Bd 30K PCR returned the best similarity match with multiple database entries for the *Glycine max* Bd 30K gene. This result suggested that the kamut sample was contaminated with soy as the *Gly m* Bd 30K target could be detected. However, the Nucleotide BLAST of the *Gly m* Bd 28K PCR products showed highest similarity with a sequence from *Triticum monococcum* (wild einkorn), an ancient wheat variety like kamut itself. Similarity hits with the *Gly m* Bd 28K allergen gene were also found but had less good scores. Based on this result, one could conclude that the *Gly m* Bd 28K PCR shows cross-reactivity with wheat varieties. However, no cross-reactivity could be observed for the wheat variety *Triticum spelta* (spelt) in this study. Analysis of the kamut sample in the independent soy PCR confirmed the soy contamination. This assay is used in GMO detection and targets the lectin gene.[29] However, the cross-reactivity could not be confirmed with a newly purchased kamut sample. This sample was first verified for soy contamination in the lectin PCR (result negative, see Table 6, third analysis) and returned negative results upon analysis in both the *Gly m* Bd 28K and *Gly m* Bd 30K PCR as well as in the commercial soy PCR tests. A possible explanation could be that both kamut samples did not contain the same wheat variety, with the one variety showing cross-reactivity with the *Gly m* Bd 28K PCR and the other not. Kamut is actually a trademark for the wheat variety *Triticum Korasan*, which originated in ancient Egypt. The second sample was labeled to contain Kamut, *Triticum polonicum*. The question is whether these two grains are the same.

The Brazil nuts also proved to be contaminated with soy as demonstrated with the independent PCR test (Table 6). Also here, the level of contamination was proven to be low, given that the amount of genomic soy DNA that could be quantified with the First Soy PCR was either just above or just below the LOD of the assay. This could again explain why the contamination has not been detected with the other soy PCRs. The PCR products that were formed could not be sequenced by the approach applied in this study due to the lack of the primer sequences used in the commercial assays. Analysis of the newly purchased Brazil nut samples showed no cross-reactivity in all PCR assays.

The soy contamination of the kamut and the Brazil nut samples could be confirmed by analysis with commercial ELISA assays for soy detection (data not shown).

DISCUSSION

In this study, quantitative PCR assays for the detection of hazelnut and soy were based on the 5′-3′-exonuclease assay using *Taqman* probes with primers specific for an allergen coding gene. The identity of the target does not play a pivotal role in PCR-based allergen detection, as detection of the DNA only provides information on the presence of the plant species it resides in and not of the protein the DNA target encodes. Nevertheless, we chose to target these genes in an attempt to obtain highly specific assays, which proved to be successful. The use of *Taqman* probes as detection chemistry had the same objective. Of the primers designed with the applied software tool, only those were selected which proved to be specific within the species. This means that the primer pair should produce a single amplicon having the expected length as confirmed by analysis of the amplification products by agarose gel electrophoresis. SYBR Green I real-time PCR provided a convenient way to verify whether the selected primer pairs had the potential to form hairpin structures due to self-complementarity or to anneal with each other. The single peaks of the obtained dissociation curves and the absence of a peak in the negative controls demonstrated that the primers do not form double helix structures. Moreover, these results confirmed the specificity of the primers for their specific target within the hazelnut or soybean genome. After the primers had been tested in real-time PCR with SYBR Green I detection chemistry, the performance of the 5′-3′-exonuclease assays with the *Taqman* hydrolysis probes was evaluated. Four commercial real-time PCR tests, two for hazelnut detection and two for soy detection, were also included in this study. These commercial kits are sold as qualitative tests for the detection of hazelnut and soy in raw materials and food products. While they are described to have a detection limit of 5 genome copies, the values determined in this study were even lower: 2.7 copies for both commercial hazelnut kits and 1.1 copies for the two soy PCR tests. The in-house developed hazelnut and soy PCR assays were less sensitive than the commercial kits, but the quantification limit was equal for both. The assays developed and investigated were proven to be more sensitive than similar assays previously described detecting *Cor a* 1 (0.1 ng genomic hazelnut DNA[19]), *Cor a* 8 (20 genomic copies[20]), or soy lectin (10 pg genomic DNA[23]). All PCR assays examined in this study have a dynamic range of at least 10^4 orders of magnitude in terms of detection of quantities of genomic DNA, which is comparable with generally reported values for real-time PCR.[34,35] As 50 ng was the highest DNA quantity tested, it is possible that the actual dynamic range is even one or several orders of magnitude higher. The efficiency calculated from the slope of the calibration curve was within the limits required by the ENGL[33] for all PCR assays investigated. The different parameters of the calibration curves also show that their quality cannot be solely determined based on the R^2 values; minor differences among the R^2 values do not reflect the more pronounced variations in the PCR efficiencies.

The specificity assessment revealed that both commercial hazelnut tests cross-react with strawberry and raspberry, and one of these tests showed an additional cross-reactivity for lettuce. While these species were selected based on the in silico cross-reactivity of the *Cor a* 8 primer pair, surprisingly, no amplification

Table 6. Specificity of the Soy PCR Assays[a]

		Gly m Bd 28K			*Gly m Bd* 30K			First Soy			Surefood Soy		
	species	1	2	3	1	2	3	1	2	3	1	2	3
protein BLAST	almond	−			−			−			−		
	barley	−			−			−			−		
	Brazil nut	−			−			+	+	−	−		
	cashew	−			−			−			−		
	hazelnut	−			−			−			−		
	macademia	−			−			−			−		
	olive	−			−			−			−		
	pea	−			−			−			−		
	peanut	−			−			−			−		
	pecan	−			−			−			−		
	pistachio	−			−			−			−		
	rapeseed	−			−			−			−		
	wheat	−			−			−			−		
prime r BLAST	lettuce	−			−			−			−		
	raspberry	−			−			−			−		
	spinach	−			−			−			−		
	strawberry	−			−			−			−		
protein + primer BLAST	apple	−			−			−			−		
	grape	−			−			−			−		
	maize	−			−			−			−		
	oat	−			−			−			−		
	rice	−			−			−			−		
	walnut	−			−			−			−		
bulk ingredients	egg	−			−			−			−		
	kamut	+	+	−	+	+	−	+	+	−	+	+	−
	milkpowder	−			−			−			−		
	spelt	−			−			−			−		
	starch	−			−			−			−		
	sugar	−			−			−			−		

[a] Test results of the analysis of two independent DNA samples in (1) new/commercial soybean PCR, (2) independent soybean PCR, (3) second food sample in new/commercial soybean PCR.

of their DNA was seen in this PCR. Either these commercial kits amplify the same gene sequence with less specific primers or the target is different but more related to these three species. As the primer and probe sequences are the intellectual property of the kit developer, these findings could not be further investigated with the approaches applied in this study. No cross-reactivity with any of the tested species was found in either of the in-house developed PCR assays for both hazelnut and soy. This illustrates the suitability of allergen encoding genes as targets for specific PCR-based allergen detection. The indication that some of the purchased food products to perform this assessment seemed to be contaminated was not completely unexpected. Although allergen control should be part of the HACCP plan in every production plant, accidental cross-contamination still occurs, or products are mislabeled as not indicating the presence of an allergen. The product recalls by the Belgian Federal Food Safety Agency (FAVV) prove this regularly. This illustrates that the risk for allergic patients due to cross-contamination is still real. However, the false-positive results of our specificity assessment also illustrate that these recalls might not always be justified. Still, we were surprised to find the soy contamination in the Brazil nuts.

At this time, only qualitative PCR assays for allergen detection are commercially available, although we have proven that their application for quantitative purposes is achievable by the construction of a calibration curve. Detection of allergens by means of quantitative real-time PCR is also extensively described in the literature.[11,12,14,17−21] Even though these studies proved to be able to detect the particular allergenic component in food products, no quantitative data have been reported. The explanation can be found in the unit to express the analytical results. Quantitative real-time PCR determines the amount of a certain DNA fragment, and the result is expressed as a weight (e.g., pg DNA), a concentration (e.g., pg DNA μL^{-1}), or a copy number. To use these data for quantification of an allergenic ingredient, they have to be converted to the corresponding amount of the allergenic commodity. Such a conversion is also made with data obtained by ELISA, where the concentration of the allergenic ingredient in the sample can be calculated from the amount of proteins detected with the test, based on the (total or specific) protein content of the food ingredient. However, there are no data available on the total DNA content of investigated species. Applying DNA-solution-based calibrators makes it hence not evident to quantify allergenic ingredients through real-time PCR.

A possible solution could be to use matrix-based calibrators: a food matrix incurred with known amounts of the allergenic ingredient to be quantified. From these calibrators, DNA would be isolated with the same protocol used for the samples and analyzed simultaneously. By applying this kind of calibrators, a certain amount of DNA detected from the allergenic ingredient could be linked to its concentration in a food sample. However, using such calibrators is accompanied by some practical issues. An appropriate matrix to incur the allergenic ingredient of interest would hence have to be selected. A calibrator has to be representative of the samples to be analyzed. This representativeness is important given that the food matrix can influence the analytical results; PCR inhibitors derived from the matrix can affect the PCR efficiency; different DNA extraction efficiencies can be expected from different matrices or identical matrices subjected to different food processing steps. Because allergens are contained in a broad range of food products, it is hence clear that defining a single representative food matrix-based calibrator will be a very challenging task to tackle.

An alternative strategy could be using internal standards, where a known quantity of the allergenic ingredient is added to the sample to be analyzed. This overrules the complicated selection of an appropriate food matrix as described above. Practical issues that should be considered in this case are selection of the nature of the allergenic ingredient used. Should the raw material or a processed form of the food be used? As food processing can substantially influence both the intactness of the analyte of interest, in this case DNA, and its extractability, this selection will fundamentally dictate the correctness of the analytical results.

In some ways, allergen detection and GMO detection are very similar: both want to quantify an ingredient of a food product. In GMO detection, relative quantification is applied for this purpose. Let us consider this as a possibility for allergen quantification. The allergenic ingredient would then be quantified relative to another reference ingredient whose concentration in the food product is known. This makes any form of incurring and its associated complications unnecessary. The amount of DNA measured from the reference ingredient can then be coupled to its concentration in the product. Is it then justified to use the relationship between these two values of the reference ingredient to calculate the amount of allergenic ingredient based on its detected DNA amount? It is actually not correct to make this conversion due to natural differences in the absolute DNA content and/or genome size of species. The relationship between the quantity of DNA detected and the ingredient concentration is thus not always constant. Moreover, the choice for a suited reference ingredient requires that it is present in all food products that have to be analyzed (i.e., products the allergen can be contained in) and that its exact concentration is known. Given the broad range of food products, the choice would again not be evident. Also, the exact concentration of an ingredient in a food product is often not known.

In conclusion, the PCR-based allergen quantification issue is actually a reflection of the whole allergen reference material matter. The fact that these are still lacking is just because it is so complicated to define them. The first step toward it should be official guidelines on what allergen detection methods have to detect (specific protein, total protein, and DNA) and how results should be expressed (protein concentration, DNA concentration, and ingredient concentration). Only then will it be possible to determine whether and how real-time PCR can be applied for allergen quantification.

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