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Single-Tube Nested Real-Time PCR as a New Highly Sensitive Approach to Trace Hazelnut

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Supporting Information

ABSTRACT: Hazelnut is one of the most commonly consumed tree nuts, being largely used by the food industry in a wide variety of processed foods. However, it is a source of allergens capable of inducing mild to severe allergic reactions in sensitized individuals. Hence, the development of highly sensitive methodologies for hazelnut traceability is essential. In this work, we developed a novel technique for hazelnut detection based on a single-tube nested real-time PCR system. The system presents high specificity and sensitivity, enabling a relative limit of detection of 50 mg/kg of hazelnut in wheat material and an absolute limit of detection of 0.5 pg of hazelnut DNA (1 DNA copy). Its application to processed food samples was successfully achieved, detecting trace amounts of hazelnut in chocolate down to 60 mg/kg. These results highlight the adequacy of the technique for the specific detection and semiquantitation of hazelnut as potential hidden allergens in foods.

KEYWORDS: *hazelnut detection, single-tube nested real-time PCR, tree nuts, food allergens*

INTRODUCTION

Food-induced allergic reactions are considered an emerging problem of public health with special impact in industrialized countries. They are defined as adverse, immune-mediated (IgE mediated) responses to the presence of offending food ingredients, namely, proteins and glycoproteins, in sensitized individuals.[1] In recent years, the prevalence of food-induced allergies seems to be rising and it is estimated to affect almost 3−4% of the adult population and 6% of young children.[1] A large range of foods are known to be responsible for triggering allergies, but the majority of the allergic reactions can be attributed to a specific number of foods, in which are included the tree nuts.[2]

Hazelnut (*Corylus avellana* L.) is one of the most commonly consumed tree nuts. It is well appreciated either raw or roasted and has a wide application, namely, in bakery and chocolate formulations. In Europe, hazelnut allergy is very frequent and is often related to birch pollinosis.[3] Clinical manifestations caused by hazelnut ingestion in allergic patients can vary from oral allergy syndrome and/or gastrointestinal symptoms, to fatal reactions (anaphylactic shock), mainly in children and adolescents.[4,5] Recently, in a study reported by EuroPrevall, involving a large multicountry sample, namely, USA, Australia, and 11 countries from Europe, hazelnut allergy was estimated with an overall prevalence of 7.2%.[6] Moreover, when birch allergic subjects were excluded from this test population, hazelnut remained the second most frequent food component inducing allergy with an overall incidence of 3.1%.[6] The allergen doses capable of inducing perceptible symptoms in sensitized patients are difficult to assess; however, threshold doses producing subjective reactions can range from 1 mg up to 100 mg of hazelnut protein that is equivalent to 6.4−640 mg of hazelnut meal.[4] Because these threshold levels are comparable to those potentially hidden in dietary food products, restriction labeling and more accurate strategies to prevent and detect contamination of foods with hazelnut traces should be implemented.

Currently, the majority of analytical tools for the detection and quantitation of allergens in foods target either proteins or DNA.[7] Regarding hazelnut, several analytical approaches have been used. The enzyme-linked immunosorbent assays (ELISA) are the most largely applied techniques due to their capacity to directly monitor the actual allergens or the marker proteins of the species.[8−11] The direct detection of the allergenic proteins in foods is regarded as one of the most commonly applied, so the immunochemical assays such as ELISA and lateral flow devices continue to be widely used. In general, this type of assays allows relatively low limits of detection (LOD) (10 ppm of hazelnut protein in cookies[8] and 1 ppm of hazelnut extract in doughs[9]) to be reached, being considered of rapid performance. However, the use of immunoassays faces numerous problems essentially related to cross-reactivity with nontarget proteins and their low resistance to food processing.[12] More recently, the mass spectrometry-based methods have also been used as protein identifying techniques.[13−15] Liquid chromatography coupled with the mass spectrometry approach has been recently applied to the detection and quantitation of proteins in foods with the clear advantage of directly monitoring the target peptides without depending on the antibody protein interaction such as in the case of immunoassays. Until now, this technique has been applied to detect hazelnut proteins in foods with high level of sensitivity (5 mg/kg of target peptide with 13 amino acid length in bread[14] and 30−35 mg/kg of Cor a 9 peptide with 5 amino acid length in a five nut mixture[15]). The high cost of the equipment and the complexity of data to be

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Table 1. Primers and Hydrolysis Probe Targeting *Corylus avellana* Low Molecular Weight Heat-Shock Protein (HSP1) mRNA Sequence

oligonucleotides	sequence (5′-3′)	amplicon (bp)	refs
	Outer Primers		
Hsp1F	AGC GTC GAG AGT GGC AAG TTC	126	this work[a]
Hsp1R	CCT GCT CGC CTC CGC TTT C		
Nocc1P	FAM-CCT GAC GAT GCG ATG CTC GAC CAG-BHQ2		Piknová et al.[21]
	Inner Primers		
Hsp2F	AGT TCG TGA GCA GGT TCA	97	this work[a]
Hsp2R	GCT TTC GGA ATA GTC ACA		
	Sequencing Primers		
Hsp3F	CAC GTG CTG AAG GCT TCT CTT C	323	this work[a]
Hsp3R	AGG AGC TCA CGA TAA CCT TCA ACA		

[a]Genbank accession no. AF021807.1.

analyzed are drawbacks associated with mass-spectrometry methods. Considering the higher stability of DNA to food processing and a lower probability of cross-reactivity, the techniques based on polymerase chain reaction (PCR) have also attained an essential role for the detection of allergens in foods. Species-specific PCR and real-time PCR approaches have been advanced as alternative tools for the indirect detection of hazelnut allergens in food products.[16−21] Until now, the reported methods for the detection and quantitation of hazelnut in foods by real-time PCR allow for attaining a relative LOD of 100 mg/kg of hazelnut in flour[18] and in walnut,[21] and an absolute LOD of 5 to 13 pg of hazelnut.[16,18,19] Concerning the low levels of hazelnut needed to induce an allergic reaction in sensitized consumers, the development of new and more sensitive methodologies is crucial.

In this work, we applied a novel approach for hazelnut detection based on single-tube nested real-time PCR. The developed method was based on the same principle reported by Bergerová et al.[22] for peanut detection, assembling the advantages of two PCR techniques, namely, nested PCR and real-time PCR. The nested PCR technique has been widely used to increase sensitivity, reduce amplification of nonspecific DNA target, and to enable isolation or identification of a specific product.[23] The common procedure for its application is based on two sequential and distinct PCR amplifications, where in the first reaction the outer primers enable the production of fragments that will serve as the DNA template for the second reaction. However, the performance of conventional nested PCR to enhance the specificity and the production of target fragments also bears two major disadvantages: increased possibility of cross-contamination and higher number of manipulations than in one-round PCR. To overcome these drawbacks, the development of closed tube reactions containing both the outer (first PCR) and inner (second PCR) primers has been attempted.[23] One means of performing closed tube reactions consists of initial PCR cycles at high annealing temperatures followed by later cycles at low hybridization temperatures, combined in the same reaction. This approach has been applied for both end-point[23] and real-time PCR.[22−24] The technique consists of combining the use of two pairs of nested primers with different annealing temperatures coupled with real-time PCR technology in a single reaction. Thus, the two pairs of primers allow the production of two specific DNA fragments because they anneal at distinct temperatures. The second fragment produced with nested (inner) primers is directly monitored by the use of real-time PCR and a specific probe. This system eliminates the original problems of cross-contamination related with routine use of the nested reaction. It also introduces higher specificity to the method conferred by two pairs of primers and a real-time fluorogenic probe in a single reaction tube, which remains closed throughout the entire analysis. The application of a nested real-time PCR system to detect trace amounts of hazelnut in foods was aimed at enhancing the sensitivity of the technique by at least 1 order of magnitude, regarding the available methods described in the literature.

■ MATERIALS AND METHODS

Plant Foods and Sample Preparation. A total of 18 cultivars of hazelnut (Morell, Negret, Grossal, Buttler, Ennis, Pauetet, Fertile de Coutard, Segorbe, Sta María del Gésu, Tonda di Giffoni, Culplà, Merveille de Bollwiller, Camponica, Lunga di Spagna, Cosford, Gunslebert, Round du Piémont, and Lansing) were collected in an experimental orchard at Vila Real, in the north region of Portugal. Hazelnuts, other tree nuts that included walnuts, macadamia nuts, almonds, Brazil nuts, chestnuts, cashews, pistachios, and peanuts, and different plant foods (soybean, lupine, fava bean, maize, oat, barley, rice, pumpkin seeds, rapeseed, sunflower, tomato, peach, apricot, plum, cherry, strawberry, and raspberry) were obtained at local markets. Processed food samples were also obtained at local markets comprising 18 different chocolates containing hazelnuts and/or almonds as well as plain chocolates and a sample of breakfast cereals with nuts.

Because of the lack of reference or testing materials for hazelnut detection, binary model mixtures containing 0.001%, 0.005%, 0.01%, 0.05%, 0.1%, 0.5%, 1%, 5%, and 10% of commercial hazelnut in wheat material (triturated pasta) were prepared. The first sample spiked with 10% of hazelnut was prepared by adding 20 g of hazelnut to 180 g of pasta, performing a complete homogenization of the mixture. All the other model mixtures were serially diluted by successive additions of wheat material until 10 mg/kg (0.001%) in the equivalent proportion.

All plant and processed food samples, as well as reference mixtures were ground and homogenized separately, into a fine powder of approximately 0.3 mm of diameter, in a laboratory knife mill Grindomix GM200 (Retsch, Haan, Germany) using different containers and materials, previously treated with a DNA decontamination solution. The fruits, namely, tomato, peach, apricot, plum, cherry, strawberry, and raspberry, were lyophilized before grinding.

After preparation, all samples and reference mixtures were immediately stored at −20 °C until further DNA extraction.

DNA Extraction. DNA was extracted from all samples by chaotropic solid-phase extraction using the commercial Nucleospin Food kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions with minor alterations. Briefly, to 200 mg of sample, 700 μL of CF lysis solution preheated at 65 °C and 10 μL of proteinase K (20 mg/mL) were added. After an incubation period of 1 h at 65 °C with continuous stirring, 4 μL of RNase A (10 mg/mL) were added to each mixture, being submitted to new

incubation for 10 min at 37 °C with gentle stirring. The samples were centrifuged for 10 min (18,500*g* at 4 °C), and 550 μL of supernatant was transferred to a new sterile reaction tube. The supernatant was then submitted to a new centrifugation step for 10 min (18,500*g* at 4 °C). Approximately 450 μL of supernatant was removed to a new reaction tube, where C4 precipitation solution and ethanol 100% were added in equal volumes to the supernatant. Each mixture was homogenized by gentle inversion, and all the volume eluted through a one spin column by centrifugation (1 min, 13,000*g* at room temperature). The spin column was washed three times with 400 μL of CQW solution, 700 and 200 μL of C5 solution, followed by 1 min of centrifugation after the two first washes, and a 2 min final centrifugation (13,000*g* at room temperature). DNA was eluted from the column by adding 100 μL of CE solution at 70 °C, followed by 5 min of incubation at room temperature and centrifugation (1 min, 13,000*g*). All of the DNA isolates were kept at −20 °C until further analysis. The extractions were performed at least in duplicate for each sample.

Yield and purity of extracts were assessed by 1% agarose gel electrophoresis and by UV spectrophotometry using a UV1800 spectrophotometer (Shimadzu, Kyoto, Japan).

Target Gene Selection and Oligonucleotide Primers. The sequence corresponding to *Corylus avellana* low molecular weight heat-shock protein (HSP1) mRNA, complete cds was retrieved from the Genbank database (accession no. AF021807.1). Two sets of specific primers were designed using the software Primer-BLAST designing tool[25] (Table 1). The software parameters were set to design the first pair of primers (Hsp1F/Hsp1R) with an optimal annealing temperature (T_a) of around 66 °C. The second pair of primers was designed to anneal at a lower T_a (54 °C), considering a difference of at least 10 °C between the two sets of primers. The hydrolysis probe was the same as that in the original TaqMan system (Table 1).[21] All oligonucleotides were synthesized by Eurofins MWG Operon (Ebersberg, Germany).

Sequencing. For sequencing the region to be amplified, a third set of primers (Hsp3F/Hsp3R) was specifically designed to produce larger fragments (323 bp), encompassing the target region of 126 bp defined by the pair of primers Hsp1F/Hsp1R, using the end-point PCR conditions described below. The amplified fragments of 10 hazelnut samples, comprising nine different cultivars and the commercial hazelnut sample used to prepare the model mixtures were sequenced. All PCR products were purified with the Jetquick PCR purification kit (Genomed, Löhne, Germany) to remove interfering components and sequenced in a specialized research facility (STABVIDA, Lisbon, Portugal). Each target fragment was sequenced twice, performing the direct sequencing of both strands in opposite directions, which allowed the production of two high quality complementary sequences.

End-Point PCR. PCR amplification was carried out in 25 μL of total reaction volume containing 2 μL of DNA extract of hazelnut (100 ng), 670 mM of Tris−HCl (pH 8.8), 160 mM of $(NH_4)_2SO_4$, 0.1% of Tween 20, 200 μM of each dNTP, 1.0 U of SuperHot Taq DNA Polymerase (Genaxxon Bioscience, Ulm, Germany), 3 mM of $MgCl_2$, and 200 nM of each primer Hsp3F/Hsp3R (Table 1). The reactions were performed in an MJ Mini thermal cycler (BioRad, Hercules, CA) using the following program: initial denaturation at 95 °C for 5 min; 40 cycles at 95 °C for 30 s, 65 °C for 45 s, and 72 °C for 1 min; and a final extension at 72 °C for 5 min.

Real-Time PCR Assays. Real-time PCR assays were performed in 20 μL of total reaction volume. Each reaction tube comprised 2 μL of DNA (100 ng), 1× of SsoFast Probes Supermix (BioRad, Hercules, CA), 200 nM of each primer Hsp1F/Hsp1R, and 100 nM hydrolysis probe Nocc1P (Table 1). For nested real-time PCR amplification, the mix included additionally 200 nM of the primers Hsp2F/Hsp2R, specifically designed for this assay. All real-time PCR assays were made on a fluorometric thermal cycler CFX96 Real-time PCR Detection System (BioRad, Hercules, CA). Real-time PCR amplifications based on the conventional technique were performed according to the following temperature protocol: 95 °C for 5 min, 50 cycles at 95 °C for 15 s, and 66 °C for 45 s, with the collection of fluorescence signal at the end of each cycle. Nested real-time PCR assays were done in two different phases. Phase 1: 95 °C for 5 min, 10 or 14 cycles at 95 °C for 15 s, and 66 °C for 45 s. During phase 2, the collection of the fluorescence signal was made at the end of each cycle: 36 or 40 cycles at 95 °C for 15 s, 54 °C for 20 s, and 72 °C for 30 s. Data were collected and analyzed using the software Bio-Rad CFX Manager 2.1 (BioRad, Hercules, CA). Cycle threshold (*Ct*) values were calculated using the software at the automatic threshold setting. Real-time PCR and nested real-time PCR trials were repeated two and three times using four replicates, respectively.

Application of the Single-Tube Nested Real-Time PCR System to Commercial Foods. The amplifications by real-time PCR were carried out in 20 μL of total reaction volume containing 2 μL of DNA extract of hazelnut reference mixtures (100 ng) or commercial foods (20 ng) and the reaction components and temperature program described above for nested real-time PCR. The assays were performed in triplicate for standard reference mixtures and commercial samples.

RESULTS AND DISCUSSION

In order to answer to the increasing demand for more sensitive and accurate methods for allergen detection, we propose a new real-time PCR system based on the single-tube nested real-time PCR for the detection of hazelnut in food products. In this study, the *hsp1* gene encoding the heat shock protein hsp1 was the chosen target for the detection and semiquantitation of hazelnut. On the basis of a reported work applied to the detection of the microorganism *Crystosporidium parvum*[24] and, more recently, to the detection of peanut allergen Ara h 3,[22] a new single-tube nested real-time PCR system has been developed aiming to trace minute amounts of hazelnut.

For this novel technique, two sets of primers were designed. The first set producing PCR fragments of 126 bp were used as outer primers to define the chosen target sequence (Figure 1 and Table 1). In this system, primers Hsp1F/Hsp1R and probe Nocc1P were selected to anneal at relatively high hybridization temperatures (66 °C) that conferred great selectivity to the reaction. The second pair of primers (Hsp2F/Hsp2R), generating PCR fragments of 97 bp, were set to act as inner primers functional at lower hybridization temperatures (54 °C). The successful empirical rule for single-tube nested real-time PCR system for Ara h 3 detection[22] based on T_a (inner primers) < T_a (outer primers) < T_a (probe) was also followed

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  1 GTCAATTTGGCAAAGATGTCGATCGTCCCAAACAATGAGCGAGAGCGCAGTGTCTCCAAT
 61 CCCTCCTCCAGAGACCTGTGGGACGTCTTCCGGAGCTTCAGAGAGAACCACCTTCAGGAC
121 CCATTCAGCGATTTACCTTTCGCTTCTACACTCTCCACGCTCTTCCCTCACTCCCCGTTC
181 GGGAGCTCGGTGAACACCAGGCTCGACTGGAGGGAGACCCCGAGAGCCCACGTGCTGAAG
241 GCTTCTCTTCCGGGGTTCGTGGACGAGGACGTGTTGGTGGAGCTCCAAGACGACCGAGTG
301 CTCCAGGTGAGCGTCGAGAGTGGCAAGTTCGTGAGCAGGTTCAAGGTCCCTGACGATGCG
361 ATGCTCGACCAGTTGAAGGCCTCGATGCACAATGGGGTTCTCACTGTGACTATTCCGAAA
421 GCGGAGGCGAGCAGGCCAACCGTTCGGACCATCGAGATCTCTGGCTAAATATGTGATCTT
481 CCTTTGCCCTGTCTGTCACTATGTTTATATGTTGTGTGTGTGTGGGTTGTTGAAGGTTAT
541 CGTGAGCTCCTCTGAGTTTGTTATGAAGGTGTTTGATAAAATGCGTCTAAAGTTTGTGAT
601 GTTTGTGTCGGTTCTTGTGATGTTGACCCTTTGCTTAGTGTGTCTTAAATGGTTGTTGGG
661 TTGAGAAGAAAATACACTATGTTGTTAACAACAAAAAAAAAAAAAAAAAAAAAAAAAAAA
721 AAA
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Figure 1. Region of the *hsp1* gene encoding the heat shock protein (hsp1) of hazelnut. Outer primers (Hsp1F/Hsp1R) are exhibited in bold, inner primers (Hsp2F/Hsp2R) are underlined, and the probe (Nocc1P) is displayed in bold and is double-underlined. Primers (Hsp3F/Hsp3R) used for sequencing are shaded in gray.

to propose a method for hazelnut detection. The temperature program was defined using two phases. Phase 1 was used to amplify PCR fragments of 126 bp without collection of fluorescence at the end of each cycle. This phase 1 aimed at producing fragments that would serve as the template for the second phase of the protocol. Therefore, phase 1 was optimized using different number of cycles ranging from 10 to 20 (data not shown), the best results being achieved using 10 and 14 amplification cycles in this phase. The entire protocol of reactions was always set to a total of 50 cycles; therefore, in phase 2, the number of cycles ranged from 36 to 40.

Sequencing of PCR Products for Identity Conformation. The fragments of 126 bp produced in PCR systems were rather short for accurate direct sequencing since the platform used often does not allow perfect resolution for the reading of the first 50 bp at the 5′-end of the sequence. To overcome this drawback, some strategies can be used, such as cloning amplicons into a vector. More recently, on the basis of the sequencing of highly degraded DNA fragments from fossil specimens,[26] the use of primers with a nonspecific tail of 60 nucleotide bases in the 5′-end was attempted to improve the quality of the sequencing of small hazelnut amplicons.[18] To avoid cloning PCR products into a vector or developing sequencing primers with long tails that are much more expensive, a third set of primers (Hsp3F/Hsp3R) was specifically designed to produce longer fragments encompassing the target region (Figure 1). This approach was considered easy, simple, and reliable since, by the production of 323 bp amplicons and direct sequencing, it was possible to obtain the complete and accurate information of the target 126 bp amplicon without any errors.

The results of sequencing the 10 hazelnut samples (9 different cultivars and a commercial sample) presented high resolution electropherograms, thus showing the adequacy of the chosen strategy. The method allowed for the sequencing of the fragments in all the extension of the target area, with no differences encountered among all the tested hazelnut samples (data not shown). The sequenced fragments were also aligned with the *Hsp1* gene sequence from GenBank, exhibiting 100% homology.

Specificity. Prior to the specific amplification of hazelnuts, the DNA extracts were evaluated for their amplifiability with universal eukaryotic primers 18SEUDIR/18SEUINV.[27] All samples tested positively with the universal primers, confirming the absence of false negative results that might occur due to the possibility of PCR inhibition or ineffective DNA extraction.

The specificity of the designed primers for the target sequence (*hsp1* gene) was extensively evaluated using eighteen hazelnut cultivars and a commercial hazelnut sample used to prepare the standards, other tree nuts and several plant species by PCR. The results for the specificity and cross-reactivity of the selected primers are presented in Supporting Information. Only the 19 hazelnut samples presented positive amplification with the designed primers. No positive amplification was observed for any other tree nuts or plant species. In contrast, cross-reactivity between hazelnut and other plant species such as strawberry, raspberry, and pistachio has been described by other authors for commercially available PCR assays.[18] In this study, samples of pistachio nuts, raspberry, and strawberry fruits were also included; however, no cross-reactivity was observed for those samples with the proposed primers for hazelnut identification. These data evidenced the adequacy of the chosen target sequence for the development of species-specific PCR methods regarding the detection of hazelnut in foods.

Analytical Method Evaluation. *Real-Time PCR System.* To optimize the conditions for real-time PCR amplification, DNA extracts from binary reference mixtures containing known amounts of hazelnut in wheat material were used in both systems (real-time PCR and single-tube nested real-time PCR). The prerequisites for the evaluation and comparison of the real-time PCR systems were based on the available document of the definition of minimum performance requirements for analytical methods of genetically modified organisms (GMO) testing[28] since no requirements are yet defined for allergen testing.

The real-time PCR assays were performed using reference mixtures ranging from 10% to 0.001% of hazelnut in wheat material. The application of this system to model mixtures allowed one to establish a relative limit of detection (LOD) of 0.01% (100 mg/kg) of hazelnut in wheat material (Figure 2A and Table 2). This sensitivity value was determined accounting for the total number of positive replicates in all the performed real-time PCR assays since analytical methods should detect the presence of the target analyte at least 95% of the time at the LOD, thus ensuring less than 5% of false negative results.[28,29] According to the definition of minimum performance requirements, the correlation coefficient (R^2) of standard curves should be above 0.98 and PCR efficiency between 90% and 110%, which implicates slopes ranging from −3.6 and −3.1, respectively.[28] All the assays performed with the real-time PCR system presented high values of correlation and efficiency. The parameters of the reactions exhibited average values for R^2 of 0.9926, slope of −3.302, and PCR efficiency of 100.8% (Figure 2C and Table 2), being in good accordance with the acceptance criteria for method performance.[28,29] The mean value of *Ct* established for the lowest amplified standard (0.01%) corresponded to 37.17 cycles (Table 2).

To establish the dynamic range and the absolute limit of detection, 10-fold serial dilutions of hazelnut DNA extracts from 50 ng down to 0.5 pg were tested by both real-time PCR systems (Figure 3). The primer and probe set worked at 100% PCR efficiency with approximately three additional cycles (3 *Ct*) for a 10-fold dilution of the template. The method allowed for amplification until the dilution factor of 10,000 of the template DNA (corresponding to 5 pg of hazelnut) and showed a high correlation coefficient (R^2 = 0.9995) and PCR efficiency (102.6%) (Figure 3C and Table 3). The number of DNA copies was calculated according to the genome size of hazelnut (0.48 pg) retrieved from the Plant DNA C-values database.[30] This database compiles relevant information regarding several plant species such as genome size (C-value), estimation method, ploidy level, chromosome number, and original references. The reference value of 0.48 pg for hazelnut genome size is the most widely used in the literature since it is considered the prime estimate value for this species. Real-time PCR allowed the amplification of 5 pg of hazelnut corresponding to 10 DNA copies, assuming that the targeted sequences are single copy genes (Figure 3A and Table 3). The limit of quantitation (LOQ) is, by definition, the lowest amount of analyte in a sample, which can be reliably quantitated with an acceptable level of precision and accuracy.[28,29] In this real-time PCR system, the LOQ equaled the LOD, as the lowest amount of DNA target (5 pg) was within the linear range of the calibration curve.

Nested Real-Time PCR System. To evaluate the newly developed single-tube nested real-time PCR system for

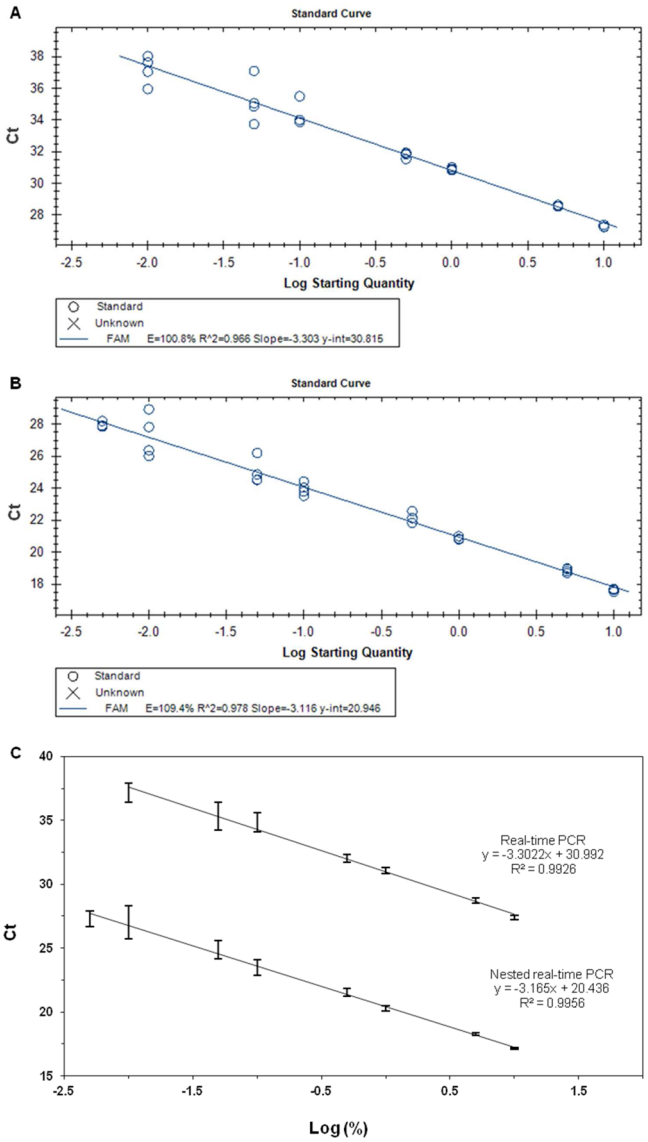

Figure 2. Calibration curves for real-time PCR (A) and nested real-time PCR (B) systems of reference binary mixtures containing 10%, 5%, 1%, 0.5%, 0.1%, 0.05%, 0.01%, 0.005%, and 0.001% of hazelnut in wheat material. Average values and corresponding standard deviations of $n = 8$ and $n = 12$ replicates for real-time PCR and nested real-time PCR, respectively (C).

hazelnut detection, the same set of binary reference mixtures (10% to 0.001%) was used. The protocol defined for this system involved two distinct PCR phases with different number of cycles. In phase 1, the amplification was carried out with outer primers, where 10 cycles were found optimal in terms of highest relative sensitivity at best linearity (data not shown). Using the above conditions, the system permitted one to establish a relative LOD of 0.005% (50 mg/kg) of hazelnut in wheat material (Figure 2B and Table 2). In comparison to the initial TaqMan real-time PCR, the former limit of 100 mg/kg was lowered to 50 mg/kg of hazelnut in wheat material. This sensitivity value was also defined regarding the total number of positive replicates in all the performed assays, as it is recommended by the criteria for method performance.[28,29] All assays performed with the nested real-time PCR system presented high correlation coefficients ($R^2 = 0.9956$), adequate

Table 2. Results of Detection of Spiked Hazelnut in Reference Model Mixtures by Two Different PCR Systems (Real-Time PCR and Nested Real-Time PCR)

	real-time PCR system	nested real-time PCR system
spiked level (mg/kg)	$Ct \pm SD^a$	$Ct \pm SD^a$
10	nd[b]	nd
50	nd	27.29 ± 0.59 (12)
100	37.16 ± 0.78 (8)	27.02 ± 1.28 (12)
500	35.32 ± 1.06 (8)	24.90 ± 0.72 (12)
1,000	34.84 ± 0.76 (8)	23.50 ± 0.60 (12)
5,000	32.03 ± 0.28 (8)	21.56 ± 0.31 (12)
10,000	31.07 ± 0.22 (8)	20.28 ± 0.23 (12)
50,000	28.72 ± 0.19 (8)	18.28 ± 0.10 (12)
100,000	27.40 ± 0.15 (8)	17.14 ± 0.05 (12)
correlation coefficient (R^2)	0.9926	0.9956
slope	−3.3022	−3.1650
PCR efficiency (%)	100.8	107.0

[a]Mean cycle threshold (Ct) values ± standard deviation (SD) ($n = 8$) and ($n = 12$). [b]nd, not detected.

slopes (−3.165), and PCR efficiencies (107.0%) (Figure 2C and Table 2).

The absolute sensitivity of the nested real-time PCR system was also assessed with the same range of hazelnut DNA dilutions (50 ng−0.5 pg) and using 14 cycles in phase 1 of the program temperature protocol. The profile of the standard curves for single-tube nested real-time PCR assays evidenced behavior similar to that of the initial real-time PCR system, with a high correlation coefficient ($R^2 = 0.9988$), slope of −3.2431, and good PCR efficiency (103.4%) (Figure 2C and Table 3). Single-tube nested real-time PCR assays enabled one to amplify 0.5 pg of hazelnut, which corresponds to 1 DNA copy. This absolute LOD for hazelnut was 10× lower in the new method when compared to that in the above real-time PCR, revealing that this system allows for increased sensitivity by 1 order of magnitude. The LOQ obtained with the nested real-time PCR system was similar to the LOD value since the lowest amount of diluted hazelnut (0.5 pg) was within the linear range of the calibration curve for all the assays.

Application of the Nested Real-Time PCR System. In order to evaluate the application of the developed method to real foods, several chocolate samples and breakfast cereal samples were tested. The commercial chocolates were carefully chosen to include samples containing hazelnut, other nuts such as almond, and also plain chocolate. The results obtained using the single-tube nested real-time PCR system, together with the corresponding label information, are presented in Table 4. The food samples were analyzed in parallel amplifications with the reference mixtures containing known amounts of hazelnut for calibration curves. All of the single-tube nested real-time PCR assays presented high efficiency (109.5% to 112.9%), slopes ranging from −3.114 to −3.087, and with correlation coefficients of approximately 0.980. In the case of nine chocolate samples containing hazelnut, the results showed that the percentage found for the presence of this nut was always in accordance with the respective label information. The two plain chocolates declaring the information "may contain traces of tree nuts" tested negatively with the proposed nested real-time PCR system. Regarding the potential presence of hazelnut in chocolate samples labeled as "may contain traces of

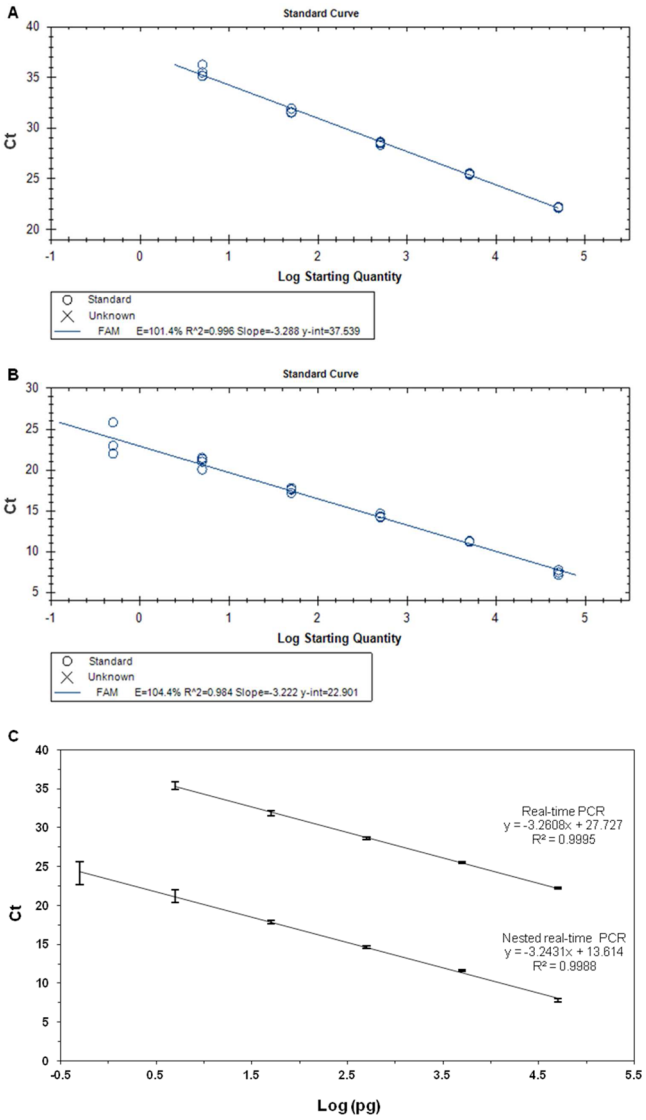

Figure 3. Calibration curves for real-time PCR (A) and nested real-time PCR (B) systems of hazelnut DNA serially diluted (10-fold) from 50 ng to 0.5 pg. Average values and corresponding standard deviations of $n = 8$ and $n = 12$ replicates for real-time PCR and nested real-time PCR, respectively (C).

other tree nuts," four of the chocolates containing known amounts of almond tested positive for hazelnut, while one almond chocolate with the same label information tested negatively.

The selection of a certain method over other alternatives is highly dependent on the matrix and/or the allergens present in foods, the required sensitivity and specificity, and the time/cost rate, among other factors. Regarding the protein- and the DNA-based methods, the choice of the technique also must rely on the availability of good antibody/protein interactions or specific DNA primers/probe and/or on food processing. In the present work, a novel system based on single-tube real-time PCR was developed aiming to achieve higher levels of sensitivity towards the detection of trace amounts of hazelnut in foods, in comparison to the molecular methods available so far. The application of the described nested real-time PCR system as a highly effective molecular approach for the detection of hazelnut encompassed the advantages of the nested PCR and

Table 3. Results of Absolute Detection of Hazelnut DNA by Two Different PCR Systems (Real-Time PCR and Nested Real-Time PCR)

	real-time PCR system		nested real-time PCR system	
absolute quantity (pg)	$Ct \pm SD^a$	DNA copies[b]	$Ct \pm SD^a$	DNA copies[b]
0.5	nd[c]		24.14 ± 1.46 (12)	1.0
5	35.37 ± 0.50 (8)	10.4	21.21 ± 0.84 (12)	10.4
50	31.84 ± 0.30 (8)	104	17.83 ± 0.21 (12)	104
500	28.60 ± 0.14 (8)	1042	14.65 ± 0.18 (12)	1042
5,000	25.50 ± 0.07 (8)	10,417	11.64 ± 0.12 (12)	10,417
50,000	22.24 ± 0.08 (8)	104,167	7.81 ± 0.23 (12)	104,167
correlation coefficient (R^2)	0.9995		0.9988	
slope	−3.2608		−3.2431	
PCR efficiency (%)	102.6		103.4	

[a]Mean cycle threshold (Ct) values ± standard deviation (SD) ($n = 8$) and ($n = 12$). [b]Number of hazelnut haploid genome copies (0.48 pg).[30]. [c]nd, not detected.

real-time PCR techniques in a single-tube reaction, thus eliminating the problems of cross-contamination frequently associated with nested PCR. Results from the real-time PCR system using the outer primers, i.e., the conventional assay, allowed for the amplification of 100 mg/kg of hazelnut in wheat material, which is in good agreement with the relative LOD of hazelnut in walnut and hazelnut in flour reported by Piknová et al.[21] and Platteau et al.,[18] respectively. The introduction of a new pair of inner primers and a different program of temperatures permitted one to enhance the sensitivity of the developed method. Therefore, the single-tube nested real-time PCR approach enabled one to lower 2× the relative LOD down to 50 mg/kg of hazelnut in wheat material, with 100% of positive replicates in all the performed assays. Regarding the relative LOQ, it was found to be equal to the LOD since the lowest hazelnut standard (0.005%) was within the linear range of the calibration curve, which constitutes an additional advantage on the quantitation of food samples. In terms of absolute LOD, the conventional real-time PCR system tested in this work allowed tracing 5 pg of diluted hazelnut DNA that was lower than the LOD of 9.6 pg and 13 pg of hazelnut reported.[17,21] The proposed nested real-time PCR system was able to increase the sensitivity by 1 order of magnitude, allowing the detection of 0.5 pg of hazelnut DNA. To our knowledge, this is the lowest LOD ever reported in the literature for the absolute detection of hazelnut DNA.[18,19,21] Even when compared to the LOD ≤ 5 pg of the commercial kit SureFood allergen Hazelnut (R-Biopharm, Darmstadt, Germany), the single-tube nested real-time PCR presented the best performance regarding the sensitivity level for hazelnut detection. According to the achieved LOD using the nested real-time PCR system, it was possible to detect down to 1 DNA copy of hazelnut.

The optimization of the single-tube nested real-time PCR system enabled detecting hazelnut at trace amounts with high performance criteria and apparent robustness since the system was not affected by additional temperature and time shifts,

Table 4. Results of the Application of Single-Tube Nested Real-Time PCR to Commercial Food Samples

sample	label information	$Ct \pm SD^a$	estimated hazelnut (%)
breakfast cereals with almonds	18% almonds and 8.4% of other crispy nuts (hazelnuts, walnuts, and pecan nuts)	19.77 ± 0.28	0.25 ± 0.05
chocolate crispy nuts	2% hazelnuts, may contain traces of other tree nuts	14.76 ± 0.20	1.95 ± 0.29
chocolate with hazelnuts and almonds	5% hazelnuts, 5% almonds, may contain traces of other tree nuts	13.84 ± 0.31	3.96 ± 0.89
black chocolate	may contain traces of tree nuts	nd[b]	nd
black chocolate filled with tiramisu	may contain traces of tree nuts	nd	nd
milk chocolate with almonds	25% almonds, contains other tree nuts	23.92 ± 0.96	0.013 ± 0.009
milk chocolate with almonds	15% almond, may contain traces of other tree nuts	nd	nd
milk chocolate with almonds	11% almonds, may contain other tree nuts	24.95 ± 0.15	0.006 ± 0.001
milk chocolate with hazelnuts	10% hazelnuts + hazelnut filling (paste), may contain traces of other tree nuts	11.70 ± 0.15	19.6 ± 2.2
milk chocolate with hazelnuts	12% hazelnuts, may contain traces of other tree nuts	15.22 ± 0.13	7.27 ± 0.72
milk chocolate with hazelnuts	27% hazelnuts	13.55 ± 0.01	24.4 ± 0.1
white chocolate with hazelnuts	14% hazelnuts, may contain traces of other tree nuts	13.88 ± 0.24	19.6 ± 3.5
chocolate with hazelnuts	25% hazelnuts, may contain traces of other tree nuts	13.28 ± 0.09	30.4 ± 1.9
milk chocolate with hazelnut filling	5% hazelnut filling (paste), may contain traces of other tree nuts	13.65 ± 0.19	4.51 ± 0.66
black chocolate with tree nuts	24.5% of almonds, hazelnuts, and raisins, may contain traces of other tree nuts	17.80 ± 0.18	1.09 ± 0.15
nougat chocolate	7.5% almonds, may contain other tree nuts	24.42 ± 0.57	0.009 ± 0.003
milk chocolate fruit and nuts	7% almonds, contains other tree nuts	24.02 ± 0.39	0.011 ± 0.003
truffle chocolate with almonds	8% almonds, 4% almond filling and hazelnut filling (% not mentioned), may contain traces of other tree nuts	18.20 ± 0.08	0.816 ± 0.005
chocolate with noisettes	11% hazelnuts, may contain traces of other tree nuts	15.26 ± 0.03	7.16 ± 0.15

[a]Mean cycle threshold (Ct) values ± standard deviation (SD) ($n = 3$) of three independent runs. [b]nd, not detected.

considering the occurrence of two different reaction protocols within the same real-time PCR run. In all the assays, the parameters were always within the criteria defined by the European Network of GMO Laboratories,[28] which highlights the suitability of the developed method for the identification and quantitation of trace amounts of hazelnut as hidden ingredients in foods.

The successful application of the proposed technique was further demonstrated in real commercial food samples with and without hazelnuts. The estimated percentages of hazelnut in chocolate samples were always in the same order of magnitude as the labeled information, the greater differences being found for samples containing large amounts of hazelnut. It is important to highlight that most of the chocolates contained entire or wrapped hazelnuts, meaning that the values obtained from single chocolate samples (100−200 g) might not be representative of the production lots. Thus, differences found in the semiquantitation of hazelnut in chocolates are probably due to low size sampling rather than to mislabeling. Regarding the results of two plain chocolates and the sample containing 15% of almond, hazelnut was not detected indicating that the result agreed with the labeled ingredients and that the precautionary labeling was probably unnecessary for nuts/other nuts. However, in the other four almond chocolates with no declared hazelnut, the estimated amounts ranging from 60 mg/kg to 130 mg/kg justifies the use of precautionary labeling in these cases, which might be the result of cross-contamination during production. The breakfast cereals revealed the presence of 250 mg/kg of hazelnut, which was a rather low value for hazelnut as an ingredient, but no further conclusion could be drawn since the label indicated the presence of several crispy nuts, without specifying the hazelnut amount.

Taking into account the performance and applicability to the analysis of commercial food samples, the single-tube nested real-time PCR system proved to be a highly specific and sensitive technique for the detection of hazelnut when compared to those of the other methods proposed so far.[17−19,21] However, to validate and correctly implement this and/or other methodologies for monitoring the presence of allergens in foods, it is essential that certified reference materials are developed, such as those in the case of GMO. Official guidelines should also be made available shortly, regulating limits for the presence of potentially allergenic ingredients in food and recommending methodology for their monitoring.

In summary, with this work we were able to present a novel and effective alternative method to detect hazelnut traces in foods, namely, in complex food samples such as the case of chocolates. In addition to the simple and highly sensitive real-time PCR assay, we developed for the first time a single-tube nested real-time PCR system as a cost-effective and powerful tool for high-throughput DNA-based identification of hazelnut allergens in foods.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Results of PCR amplifications of the *hsp1* gene applied to 18 hazelnut cultivars and several additional plant species. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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Supportting information. Results of PCR amplifications of *hsp1* gene applied to 18 hazelnut cultivars and several additional plant species (28), including other tree nuts (8).

Name	Scientific denomination	Country of Origin	PCR
18 Hazelnut cv. (“Morell”, “Negret”, “Grossal”, “Buttler”, “Ennis”, “Pauetet”, “Fertile de Coutard”,” Sta María del Gesú”, “Segorbe”, “Tonda di Giffoni”, “Culplà”, “Merveille de Bollwiller”, “Camponica”, “Lunga di Spagna”, “Cosford”, “Gunslebert”, “Round du Piémont” and “Lansing”)	C*orylus avellana*	Portugal	+
Hazelnut (commercial)	C*orylus avellana*	Portugal	+
Almond	*Prunus dulcis*	Portugal	-
Walnut	*Juglans regia*	France	-
Macadamia nut	*Macadamia tetraphylla*	Austria	-
Pine nut	*Pinus pinea*	Portugal	-
Brazil nut	*Bertholletia excelsa*	Bolivia	-
Pistachio	*Pistacia vera*	USA	-
Cashew	*Anacardium occidentale*	India	-
Chestnut	*Castanea sativa*	Portugal	-
Peanut	*Arachis hypogaea*	USA	-
Lupine	*Lupinus albus*	Portugal	-
Fava bean	*Vicia faba*	Greece	-
Soybean	*Glycine max*	USA	-
Maize	*Zea mays*	USA	-
Wheat	*Triticum aestivum*	Portugal	-
Rice	*Oryza sativa*	Portugal	-
Oat	*Avena sativa*	Portugal	-
Barley	*Hordeum vulgare*	Spain	-
Pumpkins seeds	*Cucurbita mixta*	Greece	-
Rapeseed	*Brassica napus*	Brazil	-
Sunflower	*Helianthus annuus*	Brazil	-
Rye	*Secale cereale*	Portugal	-
Tomato	*Solanum lycopersicum*	Spain	-
Peach	*Prunus persica*	Portugal	-
Apricot	*Prunus armeniaca*	Turkey	-
Plum	*Prunus cerasifera*	Chile	-
Cherry	*Prunus avium*	Chile	-
Strawberry	*Fragaria virginiana*	Spain	-
Raspberry	*Rubus idaeus*	Portugal	-