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Development and Validation of a SYBR-Green I Real-Time PCR Protocol To Detect Hazelnut (*Corylus avellana* L.) in Foods through Calibration via Plasmid Reference Standard

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Many tree nuts are considered to be a serious problem in food safety, because of the presence of causative factors in IgE-mediated food allergies. Among these, hazelnut (*Corylus avellana* L.) seeds are largely used in a range of confectionery products and contain many well-characterized allergens. DNA-based methods and ELISA tests may prove to be useful to assess the presence of hidden ingredients in foods. The aim of this work was the development and validation of a species-specific SYBR Green I real-time PCR protocol for the detection of hazelnut in foods. A novel efficient primer pair on the Cor a 8 genomic coding region was designed by preparing a plasmid vector-based internal reference standard to calibrate the PCR. A good sensitivity, down to 20 (genomic) and 15 (plasmid) DNA copies, was established. All of the commercial samples considered in our study (containing hazelnut as ingredient or as a potential trace cross-contamination) were effectively amplified by PCR, showing a perfect correspondence with an ELISA commercial test, employed as a reference standard method.

KEYWORDS: Hazelnut; food allergy; real-time PCR; SYBR Green I; plasmid reference standard

INTRODUCTION

In Western countries up to 1−2% of the total human population suffer from clinically proven food allergies; among children, the prevalence is even higher, up to 8% for some allergens. The symptoms of food allergy range from mild urticaria to life-threatening anaphylactic shock (*1*). There is uncertainty regarding the prevalence of food allergy, particularly to fruits, vegetables, nuts, and other edible plants. The extent of the problem within the population remains unclear, affecting patients and their families, schools, the catering industry, food producers and retailers, health professionals, and policy makers. For all of these reasons, the control of the presence/absence of hidden or not declared allergens is a key topic in food safety.

Among different allergen-containing foods, tree nuts and peanuts are worldwide considered to be critical for a large part of the sensitized patients. Considering the research efforts based on double-blind placebo-controlled food challenge (DBPCFC) tests and skin prick tests (SPT), the highest estimated prevalence among tree nuts (> 4%) was recently highlighted by EuroPrevall for hazelnut (*2*). The allergen doses able to trigger objective symptoms in sensitized patients are difficult to calculate. Threshold doses for eliciting subjective reactions varied from a dose of 1 mg up to 100 mg of hazelnut protein (equivalent to 6.4−640 mg of hazelnut meal) (*3*).

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Currently, the only effective treatment for food allergy is avoidance of the allergen-containing food. Nevertheless, total avoidance is sometimes difficult to obtain by sensitized people, because processed commercial foods usually contain a variety of ingredients. Moreover, sensitive individuals may be inadvertently exposed to allergenic proteins by consumption of food products supposed to be free of a certain allergen. Food products can be contaminated with foreign food constituents during shipping, storage, and processing, from carry-over resulting from inadequate cleaning of shared processing equipment, or in reuse (rework) of allergen-containing products (*4*).

Hazelnuts seeds (*Corylus avellana* L.), belonging to widely consumed tree nuts, are used in a range of confectionery products (filled chocolates or wafers, cereal muesli mixtures, bakery products as a basic component of nut fillings in various cakes, and others) (*5*). Hazelnuts have been shown to contain different allergenic proteins (*6*). Cor a 1, the 18 kDa major hazel pollen allergen, displayed a high degree of homology with the major birch pollen allergen Bet v 1. Cor a 2, a 14 kDa profilin known as a cross-reacting plant pan-allergen, was found in both hazel pollen and hazelnuts (*7*); food profilins and Bet v 1 related food proteins are relatively sensitive to heat and can easily be cleaved by proteases (*8*). Cor a 8 is a lipid transfer protein (LTP) of 9 kDa; Cor a 9 is the first tree pollen-unrelated hazelnut allergen isolated, sequenced, and cloned. Moreover, hazelnut contains multiple isoforms of oleosin. The hazelnut vicilin Cor a 11 is a minor allergen, in regard to both prevalence and allergenic potency, whereas its glycan does not contribute to its allergenic activity (*6*).

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Recently, we suggested 2s albumin, Cor a 14, as a novel putative minor allergen in hazelnut (www.allergen.org).

With Directive 2006/142/EC the European Community created a list of potential allergenic ingredients requiring mandatory labeling on foods; this list comprises cereals containing gluten, crustaceans, eggs, fish, peanuts, soybeans, milk and dairy products (including lactose), tree nuts, celery, mustard, sesame seeds, lupine, molluscs, and sulfites of at least 10 mg/kg (*9*). Therefore, analytical methods for the determination of the hazelnut content in food are a necessary support for the legislation. The ELISA method is considered the "gold standard" for the direct allergen detection. Several sandwich ELISA-based methods are commercially available for the detection of hazelnuts in food (*10*). These immunochemical methods are highly sensitive; however, they may be biased by cross-reactivity with other tree nuts. Polymerase Chain Reaction (PCR) as alternative approach has been developed and tested, confirming its usefulness. PCR-based methods may promote an independent confirmation of immunochemical methods results (*5*). The real-time PCR technique, commonly used to detect and quantify genetically modified organisms (*11*,*12*) has been also recently suggested for the detection of allergens in food, such as peanut (*13*), celery (*14*, *15*), mustard (*15*), sesame (*15*−*18*), lupine (*19*), cashew nut (*20*−*22*), walnut (*23*), pistachio (*24*), pecan nut (*25*), almond (*26*), and macadamia nut (*27*). Our group proposed in 2007 a method to detect hazelnut in processed foods through the use of both Taqman and SYBR Green I chemistry, achieving a limit of detection of 0.1 ng of genomic DNA (*28*). More recently, Piknova and colleagues validated a real-time PCR protocol able to detect down to 27 hazelnut genome copies using a primer pair written on *hsp1* gene sequence, encoding for a low molecular weight heat shock protein (*5*). A duplex real-time PCR protocol for the simultaneous detection of hazelnut and sesame in foodstuffs has been recently developed (*29*). Real-time PCR requires more expensive laboratory equipment than conventional end-point PCR, allowing a more accurate and sensitive measurement. The largely used TaqMan chemistry is based on a fluorogenic probe to enable the detection of a specific PCR product as it accumulates during real-time PCR cycles. SYBR Green I chemistry is an alternative less expensive approach, useful to perform real-time PCR analysis. SYBR Green I is a dye able to bind the minor groove of double-stranded DNA. When SYBR Green I dye binds to double-stranded DNA, the intensity of the fluorescent emissions increases. As more double-stranded amplicons are produced, the signal intensity increases.

The possibility to provide a certified absolute quantification of genomic DNA via real-time PCR is strictly related to the commercial availability of certified reference standards. A complete series of certified reference standards for different genetically modified organisms (GMOs) is currently supplied, but their availability for food allergens is still lacking.

Few papers describe the use of an internal plasmid reference standard for the detection of hidden ingredients in food, such as allergens (*26*), or GMOs (*30*−*33*). Oguchi and collaborators (*30*) reported the production and use of a linearized modified plasmid (pSCM) in a duplex real-time PCR calibration to detect genetically modified maize. More recently, Pafundo and colleagues published a work based on the use of p-GEM vector for the calibration of a SYBR Green I real-time PCR method to detect almond (*Prunus dulcis* Mill.) in foods as hidden ingredients, although in that work the protocol for the preparation of the plasmid is not reported (*26*).

Our aim was to set up and validate a sensitive SYBR Green I real-time PCR protocol, employing a specific primer set designed on Cor a 8 allergen coding sequence, useful to trace the presence of hidden hazelnut in food. Our second aim was to create a novel plasmid vector, cloning the Cor a 8 sequence amplified from hazelnut. Finally, we considered an ELISA commercial kit to compare sensitivity and reliability.

MATERIALS AND METHODS

Samples. All hazelnut samples (*C. avellana* L.) used in this study are provided and certified by Istituto Nord Ovest Qualità (INOQ, Moretta, Piedmont, Italy). Other plant or seed samples (*Arachis hypogaea* L., *Juglans regia* L., *P. dulcis* Mill., *Carya illinoensis*, *Pistacia vera* L., *Lupinus albus* L., *Anacardium occidentale* L., *Triticum aestivum* L., *Oryza sativa* L., *Zea mays* L., *Avena sativa* L., *Cicer arietinum* L., *Glycine max* L., and *Theobroma cacao* L.) and some commercial foodstuffs (chocolates, biscuits, hazelnut creams, and wafers containing tree nuts) were purchased in local food stores.

Preparation of Spiked Samples. Hazelnuts were finely ground in a mortar with liquid nitrogen, then defatted by dichloromethane extraction in a Soxhlet apparatus, and sieved through 500 μm sieves. Then, fine hazelnut powder was to soft wheat flour: 5 g of commercial wheat flour was spiked with 0.5 g of defatted hazelnut flour and accurately mixed for 10 min (master mixture). Subsequently, we created a series of mixtures, using soft wheat flour, obtaining 1, 0.1, 0.01, and 0.001% hazelnut in soft wheat flour (w/w).

Genomic DNA Extraction and Quantification. A DNeasy Plant mini kit (Qiagen, Hilden, Germany) and a QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) were used for the extraction of both hazelnut seeds and commercial foods, comparing the extraction yield and cleanup of the genomic DNA. All extraction methods were used according to the manufacturer's protocols. The QIAamp DNA Stool Mini Kit (Qiagen) was finally selected to extract all of the samples. About 200 mg of each sample was used for the extraction, after the grinding in liquid nitrogen as previously described. DNA concentration was fluorometrically determined using Qubit (Invitrogen, Karlsruhe, Germany) and DNA HS (0−100 ng) reagents (Invitrogen, Karlsruhe, Germany) as reference.

Primer Design. All of the hazelnut-specific primers used in this work were created by Primer3 open source software (v. 0.4.0, http://primer3.sourceforge.net), on the basis of hazelnut LTP gene sequence (GenBank accession no. AF329829.1). The ITS1/ITS4 universal primer pair, used in end-point PCR for testing the quality and the amplification yield, was designed, respectively, on 18S and 28S rDNA gene sequences (*34*).

All primers (**Table 1**) were synthesized and purchased by Primm, Biotech customer service, Milan, Italy.

Qualitative End-Point PCR. All end-point PCRs were performed using iCycler [Bio-Rad Laboratories SrL Segrate (MI), Italy]. The optimal annealing temperature for each primer set was optimized through experimental observations and set at 55 °C, both for Cor a 8 M1/M2 and for ITS1/ITS4.

Concentrations of reagents (final volume = 20 μL, performed in 200 μL microtubes) were as follows: 10× PCR buffer with 15 mM $MgCl_2$ (Applied Biosystems, Monza, Italy), 200 μM of each dNTP (Promega Corp., Madison, WI), 0.25 μM of each primer, 1 unit of AmpliTaq Gold DNA polymerase 5 U/μL (Applied Biosystems), and ultrapure water (Millipore Milli-Q, Vimodrone, Italy), to reach the final volume. Two microliters of DNA (5 ng/μL) has been used as template. The thermal program was fixed as follows: initial denaturation, 95 °C for 5 min; denaturation, 95 °C for 30 s; annealing, 55 °C for 30 s; elongation, 72 °C for 40 s; repetition, 35 cycles. The last step was a final extension at 72 °C for 7 min. The PCR product was loaded on a 2% agarose gel (Euro clone, Italy) containing SYBR Safe (Invitrogen, Karlsruhe, Germany) dye and then analyzed by Fluor-S Multimager (Bio-Rad Laboratories SrL).

Real-Time PCR Protocol. Real-time PCR amplification reactions (22 μL) were run on Abi Prism 7900 HT SDS (Applied Biosystems) and on iQ iCycler Real-Time PCR Detection System (Bio-Rad Laboratories SrL), using SYBR Green I chemistry. All reactions were performed in ultrapure water, 1× Power SYBR Green I PCR Master Mix (Applied Biosystems), 120 nM Cor a 8 M1/M2 primer set, and 2 μL (5 ng/μL) of template DNA. The thermal cycling profile used for quantitative SYBR Green I assay was as follows: initial hold step at 95 °C for 10 min; denaturation at 95 °C for 15 s; annealing and extension at 60 °C for 1 min; repetition, 40 cycles. Dissociation stage allows the operator to check the melting temperature of

Table 1. Target Sequences Used for Primer Design[a]

primer	5′−3′ sequence	T_m (°C)	locus	gene index	amplicon (bp)
		Primers Specific for *Corylus avellana*			
Cor a 8 M1	patent pending	60	AF329829.1	13507261	78
Cor a 8 M2	patent pending	62			
		Primers for Internal Transcribed Spacer Sequences (Universal Control)			
ITS 1 (*34*)	TCCGTAGGTGAACCTGCGG	62	AB298970	134048943	∼700
ITS 4 (*34*)	TCCTCCGCTTATTGATATGC	57	EF565863	146738022	

[a] For each gene used the locus and the index are reported. Temperature of melting and length of ribosomal primers are reported for Cor a 8 M1 primers (patent pending).

the amplicon. The temperature program used for the melting curve analysis was 60 °C for 15 s, then 95 °C for 15 s and, finally, 60 °C for 15 swith a ramp rate of ±1.6 °C/s.

Concerning the validation assessment of the real-time PCR protocol suggested in this work, we followed the most conventional guideline in the field of real-time PCR food analysis, "*The definition of the minimum performance requirements for analytical methods of GMO testing*", written by the European Network of GMO Laboratories (ENGL).

Preparation of Plasmid Template. First, the amplicon obtained using Cor a 8 primers was excised from the agarose gel and purified through Wizard SV Gel and PCR Clean-up system (Promega Corp.). The product was then ligated into pGEM-T Easy Vector (Promega Corp.) and cloned into *Escherichia coli* JM109 (DH5α) by using the pGEM-T Easy Vector System (Promega Corp.). Finally, the plasmid was extracted and purified by a Wizard *Plus* Minipreps DNA Purification System (Promega Corp.). An end-point PCR performed with a Cor a 8 specific primer set confirmed that the DNA fragment was correctly introduced into the plasmid.

The PCR product has been sequenced (Primm, Biotech customer service, Milan, Italy), and the result obtained was compared to the known sequence of Cor a 8 using MultiAlin online free software (http://bioinfo.genotoul.fr/multalin/multalin.html).

Creation of Standard Curves for Real-Time PCR Analysis. Standard curves for real-time PCR were created using both genomic and plasmid DNA. The concentration of genomic and plasmid DNA was previously checked using a fluorometer.

Starting from hazelnut genomic DNA, 10-fold serial dilutions were prepared to obtain samples containing down to 20 hazelnut genome copies. The whole hazelnut genome size is 0.48 pg (*35*).

The solution containing plasmid DNA was then serially diluted to prepare a series of 10-fold solutions, containing from 300,000 to 15 gene copies of plasmid.

ELISA Assay. Immunochemical hazelnut detection was performed using a commercial RidaScreen Fast hazelnut ELISA kit (R-Biopharm, Darmstadt, Germany). Protein extraction and detection procedures were carried out by following the instruction manual.

Mathematical and Statistical Calculations. All of the statistical analysis (amplification curves plotting, calculation of PCR parameters) were performed directly by the software of Abi Prism 7900 HT SDS (Applied Biosystems, 2002).

RESULTS AND DISCUSSION

DNA Extraction and Quantification. Optimization of the genomic DNA extraction from a fat-rich and polyphenol-rich food such as hazelnut was the first issue of this work. As is well-known, the preparation of high-quality template DNA is a key feature for the successful PCR. To achieve this, different extraction methods were tested (data not shown), and a DNeasy plant mini kit (Qiagen, Hilden, Germany) was initially selected for raw hazelnuts. This precast kit is specific for the extraction of DNA from plants and vegetables and allows the presence of some PCR inhibitors such as polyphenols to be reduced. Another commercial kit, DNA Stool kit (Qiagen, Hilden, Germany) specific for "complex matrices", was tested for comparison. Both extraction methods allowed us to obtain nondegraded genomic DNA, avoiding the presence of DNA polymerase inhibitors and permitting the following amplification. About 500 ng of genomic DNA starting from 100 mg of biological material was obtained, providing reliable results both for raw hazelnuts and for commercial samples. To make the process uniform, the DNA Stool kit was finally selected for further analyses. The majority of homemade DNA extraction protocols from plant, seed, and vegetable materials is based on the use of CTAB (*36*), with minor changes (e.g., the use of polyvinyl, PVP, to eliminate polyphenols). Recently, Pafundo and collaborators (*26*) published a paper focused on the detection of almond through SYBR Green I real-time PCR, using two separate DNA extraction approaches. A CTAB-based buffer was employed on leaves, whereas the "classical" protocol, described by Meyer and collaborators (*37*), was used to extract genomic DNA from food commercial samples. The use of different extraction protocols for the samples analyzed by the same PCR method could be critical from the analytical point of view with regard to the potential different extraction yields. The quality of genomic DNA was checked through agarose gel (0.8%) electrophoresis, showing well-defined bands for all of the samples considered in this study, including the commercial ones. For the preparation of the spiked samples, we chose to previously defat hazelnuts using dichloromethane using a Soxhlet apparatus. Moreover, the hazelnut-defatted powder is very fine, allowing the operator to homogeneously mix it together with commercial wheat flour, used as spiked matrix. An exhaustive analysis of this problem is still lacking in the scientific literature.

Qualitative End-Point PCR: ITS Region Amplification for Genomic Quality Control and Specificity Assessment. As a further quality control, the universal ribosomal primers ITS1/ITS4 (*34*) were usefully employed to verify the quality of genomic DNA. A universal PCR primers set, designed on highly conserved regions flanking the ITS sequence, enables easy amplification of ITS region, due to a high copy number of rDNA repeats (*38*). This makes the ITS region an interesting subject for evolutionary, phylogenetic, and biogeographic investigations (*39*), as well as a powerful tool for DNA quality checking. In our study, amplification with the ITS1/ITS4 primer pair, written, respectively, on 18S and 28S rDNA gene sequences, confirmed the high quality of genomic DNA, despite the relatively low yield of extraction (data not shown).

The design of a novel hazelnut-specific primer pair was performed on a Cor a 8 coding region sequence (GenBank accession no. AF329829.1). The specific primer set was analyzed on BLAST to verify its specificity (absence of genetic homologies with other related species or other species currently employed as food ingredients). Although the selection of an allergen-coding genomic region as PCR target is neither indispensable nor strategic to assess the presence of hazelnut (i.e., specific ITS region easily leads to a high yield of amplified product), we chose Cor a 8 as target sequence to improve the hazelnut specificity. For

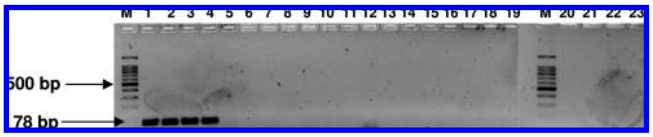

Figure 1. Electrophoretic profile (2% agarose) of end-point PCR with Cor a 8 M1/M2 primer set (lower arrow, hazelnut-specific 78 bp band). Lanes: M, molecular marker 100 bp; 1, hazelnut (Tonda Gentile delle Langhe cultivar); 2, hazelnut (Tonda Romana cultivar); 3, hazelnut (Giffoni cultivar); 4, hazelnut (Tombul cultivar); 5, peanut; 6, walnut; 7, almond; 8, macadamia nut; 9, pecan nut; 10, pistachio; 11, lupine; 12, cashew nut; 13, soft wheat; 14, rice; 15, oat; 16, chickpea; 17, soy; 18, potato; 19, basil; 20, sage; 21, mint; 22, cocoa; 23, no template control.

the same reason, in their recent work Pafundo and colleagues designed a specific primer set for the specific detection of almond on the Pru du 1 allergen coding region. In this work Pru du 1 primers were shown to be able to detect almond both as main ingredient and as a contaminant in small traces (*26*).

The Cor a 8 primer set, tested on 4 different hazelnut cultivars and 18 other plant species (genetically related to hazelnut or usually employed as food ingredients in industrial procedures), showed a reliable specificity on hazelnut, allowing a 78 bp amplicon to be obtained, as reported in **Figure 1**. To control the real applicability of the suggested test, we considered for amplification some samples of genomic DNA obtained from hazelnut from different geographical regions, belonging to several cultivars (Tonda Gentile delle Langhe, from Piedmont, Italy; Tonda di Giffoni, from Campania, Italy; Tonda Gentile Romana, from Lazio, Italy; and Tombul, from Tombul region, Turkey). The intraspecific polymorphism is another potential source of trouble in PCR tests (false-negative responses), due to site-specific point mutations. The intervarietal polymorphisms are largely underestimated in the phase of the primer design.

The specificity was further assessed using real-time PCR, as described below.

Development and Validation of Real-Time PCR Protocols Using SYBR Green I Chemistry. The setting up and optimization of real-time PCR protocol was performed using SYBR Green I (2-[*N*-(3-dimethylaminopropyl)-*N*-propylamino]-4-[2,3-dihydro-3-methyl-(benzo-1,3-thiazol-2-yl)methylidene]-1-phenylquinolinium), a green-emitting dye belonging to the family of the cyanine dyes. We decided to exploit the SYBR Green I chemistry because this dye is less expensive (no specific probes are required), requiring less time and having easier optimization steps than TaqMan chemistry. SYBR Green I is a highly specific double-strand DNA binding dye that allows the detection of products accumulated during amplification. The most critical disadvantage of SYBR Green I dye chemistry is the generation of false-positive signals, because it binds to any double-stranded DNA, including non-specific double-stranded DNA sequences. All of these facts make the evaluation of specific dissociation curves and melting temperatures critical for the identification of specific amplicons from other unspecific products (false positives). As a matter of fact, few papers focusing on "hidden ingredients detection" have been developed using SYBR Green I so far (*26*). Moreover, previous papers on GMO detection reported an equivalence on sensitivity and equal performance of SYBR Green I, TaqMan-based, and molecular beacon-based techniques (*40*). Another key point in real-time PCR method development is the use of certified standards as reference templates to generate a standard curve. The availability of certified reference standards is still lacking for allergens, and the use of genomic DNA extracted from raw material can be biased by many factors, such as the physical or chemical composition of the examined matrix or the thermal processing. A standard curve for the detection of hazelnut in samples has been prepared using a plasmid template as internal reference. The advantages of this approach are that large amounts of standard can be produced; the identification of the target can be verified through sequencing, and the DNA can be easily quantified. Our optimized protocol allowed us to produce a large quantity of reference plasmid. After the purification of plasmid from an *Escherichia coli* strain, our target sequence was checked by PCR, and standard curves were generated using a 10-fold dilution of plasmid DNA. Using this approach, we accurately prepared different reference standards with different dilution factors, containing from 3×10^5 to 3×10^1 copies of Cor a 8 PCR product (fluorometric quantification).

As previously reported in our paper, despite the availability of modified plasmids as reference material standards (we observed how some commercially available real-time PCR kits for the detection of allergenic ingredients are based on this approach), few papers have reported the use of this system in real-time PCR for hidden ingredients detection.

Specificity, amplification efficiency, sensitivity, robustness, and applicability of our protocol were determined, as suggested by the European Network of GMO Laboratories' guideline (see Materials and Methods).

Specificity. The specificity level of the designed hazelnut primer set observed in end-point PCR (**Figure 1**) was confirmed by real-time assay, displaying no amplification on nonhazelnut samples used as negative controls (data not shown). The obtained hazelnut-specific 78 bp amplicons showed constant melting temperatures both for plasmid and genomic amplified DNA (79.1 ± 0.5 and 79.6 ± 0.5 °C, respectively, **Figure 2**).

Amplification Efficiency and Correlation Coefficient (R^2). The obtained standard curves are in accord with ENGL acceptance criteria (acceptable slope values between -3.1 to -3.6 and $R^2 > 0.98$). The same parameters for plasmid standard curve were -3.336 ± 0.111 and 0.99 ± 0.001 (mean of 10 different determinations). Also, a 10-fold serial dilution standard curve prepared with hazelnut genomic DNA was in accordance with ENGL acceptance criteria. The mean value of slope obtained was -3.353 ± 0.27 with an R^2 of 0.99 ± 0.01. Standard curves prepared with serial dilution of plasmid DNA showed a standard deviation lower than those prepared with genomic DNA, attesting to the robustness and reliability of the method.

Sensitivity. Sensitivity of the method has been assessed using both genomic DNA and plasmid serial dilutions. Hazelnut genomic DNA has been extracted from *C. avellana* seeds and quantified by fluorometer, creating 5-fold serial dilutions. With the Cor a 8 specific primer pair a limit of detection (LOD) of 9.6 pg of hazelnut DNA was obtained. Hazelnut genome weight is estimated as about 0.48 pg (*35*), so this limit corresponds to 20 hazelnut genome copies. The same approach has been performed using plasmid 10-fold serial dilutions, to obtain the calibration curve. The Cor a 8 primer set in this case was able to detect down to 15 genome copies. In a previous work (*26*), almond genomic and plasmid DNAs could be detected down to 1 copy, using the Pru du 1 (11S globulin) gene as target. This apparently can be explained through the significant difference in genome weight between hazelnut and almond (0.48 pg versus 0.3 ng). This parameter significantly affects the sensitivity of the method (20 copies for hazelnut versus 1 copy for almond).

To assess the sensitivity on a spiked sample, we performed PCRs using wheat flour spiked with different known amounts of hazelnut defatted powder. The measured LOD was 0.001%

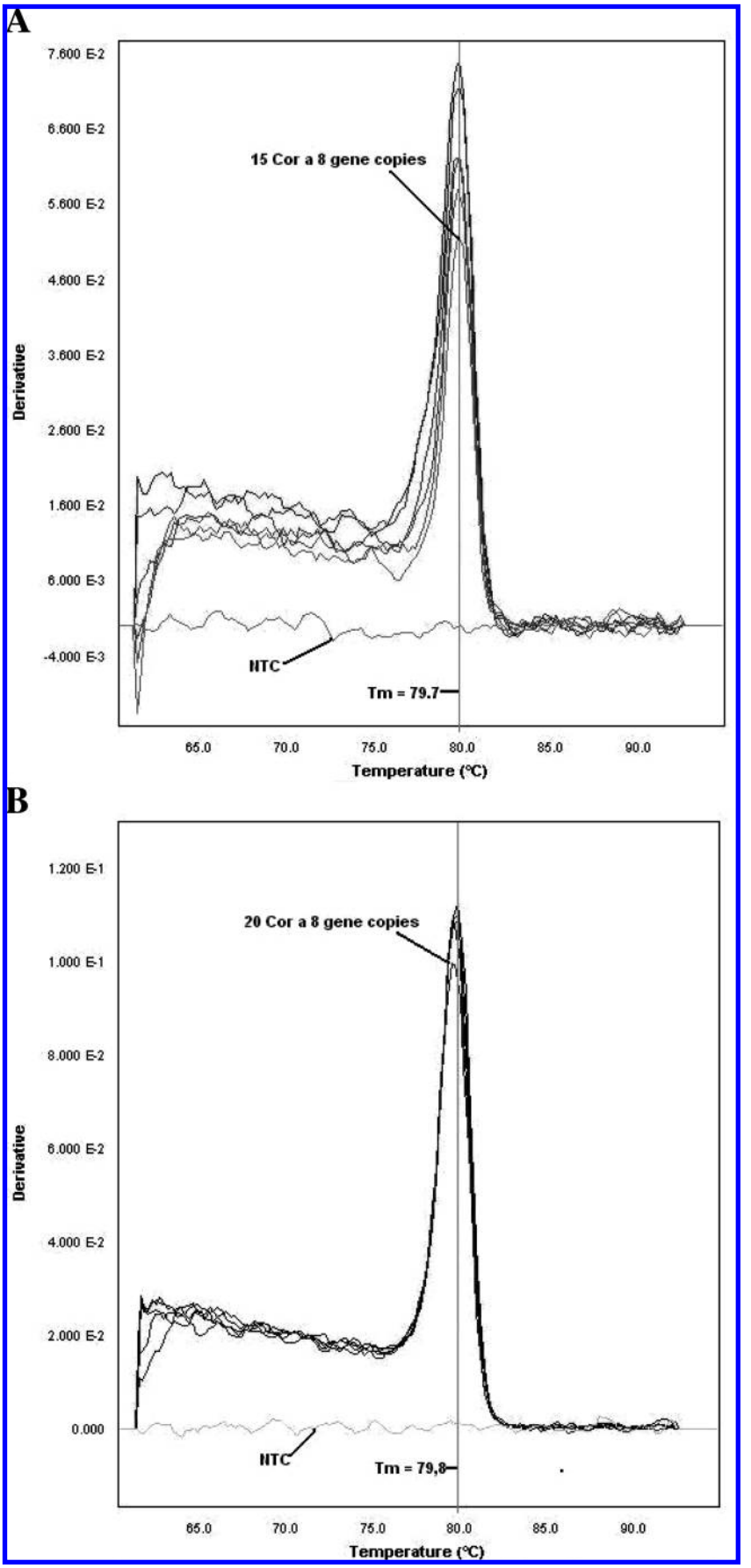

Figure 2. Derivates of melting curves of Cor a 8 M1/M2 specific amplicon obtained by amplifying plasmid (**A**) and genomic DNA (**B**), respectively. Temperatures of melting are reported; NTC, no template control.

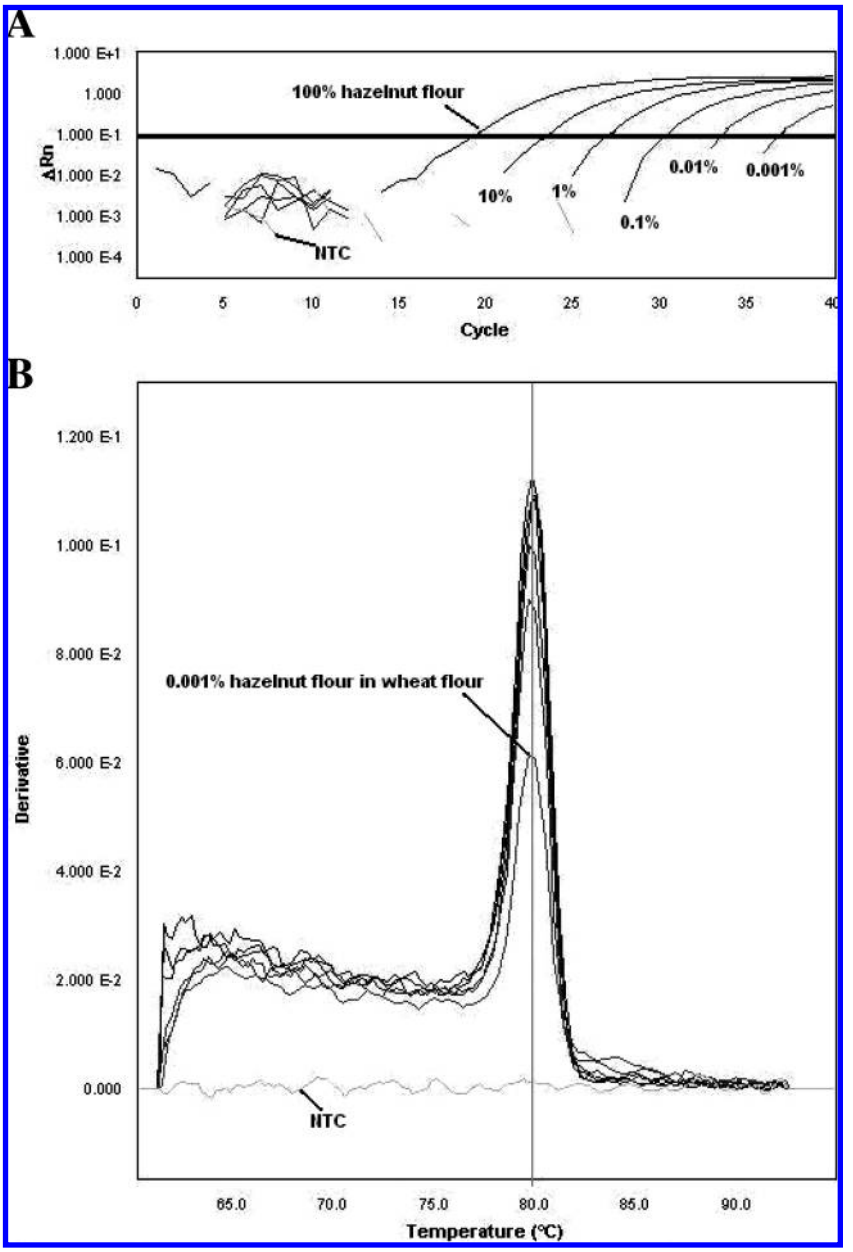

Figure 3. Amplification plot (**A**) and derivatives of melting curves (**B**) of real-time PCR analysis performed on wheat flour spiked with serial dilutions of hazelnut flour (see Materials and Methods for preparation). Arrows indicate the different spiking percentages on different amplification curves. NTC, no template control.

hazelnut in wheat, corresponding to 10 ppm (with a mean Ct of 34.56 ± 0.55, **Figure 3**). The mean value of the slope obtained was −3.435 ± 0.32 with R^2 of 0.99 ± 0.01, comparable to the amplification efficiency obtained on plasmid and genomic DNA. Every experiment was performed 10 times.

Robustness. The robustness of the two reactions (genomic DNA-based and plasmid-based PCRs) was assessed by performing hazelnut detection on commercial food products, using the same hazelnuts and commercial food sample, performing amplification on different thermocyclers (Abi Prism 7900 HT SDS, Applied Biosystems, and iQ iCycler Real-Time PCR Detection System; Bio-Rad Laboratories SrL), obtaining the same results displayed in **Table 2**.

Applicability on Commercial Food Samples. All 13 commercial food samples analyzed through real-time PCR showed low values of standard deviations and coefficients of variation (**Table 2**), confirming the reliability of our method. The presence of hazelnut in food samples was assessed in accord with the declaration of the presence/absence on the label. Therefore, the Cor a 8 primer pair proved to detect hazelnut also in traces, without the production of false-positive or -negative results. With regard to the quantitative aspect, we highlighted the connection between the increasing content of hazelnut in creams and its corresponding increase in Ct values, probably depending on the different impacts of the roasting process. This fundamental aspect should be thoroughly investigated in the future.

Comparison with Hazelnut-Specific ELISA. The same commercial food samples were also analyzed using a commercial hazelnut-specific ELISA kit. For all samples analyzed, a perfect correspondence of results has been verified (**Table 2**). Undetermined samples reported in the table displayed no amplification (**Figure 4**). The positive reaction of the ELISA test confirmed the presence of hazelnut proteins also in samples declaring "traces" on the label. These results confirm the reliability and sensitivity of the real-time PCR protocol in the detection of hazelnut in commercial food samples.

Table 2. Commercial Food Products Analyzed with Real-Time PCR and ELISA[a]

food sample	hazelnut (% declared)	mean Ct[b]	SD[b]	CV[b] (%)	Cor a 8 M1-M2 primers	ELISA[c]
bitter dark chocolate 1	no declaration	undetermined			−	−
bitter dark chocolate 2	Th	31.15	0.5	1.61	+	+
milk chocolate	Th	32.6	0.7	2.17	+	+
white chocolate	Th	32.94	0.7	2.11	+	+
gianduja chocolate	14	28.37	0.55	1.95	+	+
hazelnut cream 1	10	30.2	0.54	1.82	+	+
hazelnut cream 2	13	28.04	0.62	2.2	+	+
hazelnut cream 3	16	30.35	0.6	1.99	+	+
wafer filled with cocoa	Tt	undetermined			−	−
wafer filled with hazelnut	9	28.62	0.5	1.77	+	+
biscuit 1	Tt	31.75	0.57	1.81	+	+
biscuit 2	Th	29.23	0.52	1.79	+	+
roll containing hazelnut	2.80	31.1	0.78	2.53	+	+

[a] ELISA was carried out on total protein extract purified from the food sample. Th, traces of hazelnut; Tt, traces of tree nuts; undetermined, no amplification within 40 cycles; SD, standard deviation; CV, coefficient of variation; +, positive result (corresponding to a peak of predicted specific T_m in PCR and positive response in ELISA); − negative result (corresponding to the absence of a peak in PCR and a negative response in ELISA). Any peaks with different T_m or false-positive results for ELISA were detected. [b] Mean Ct, standard deviation, and coefficient of variation have been calculated by mean of 10 repetitions. [c] R-Biopharm declares for its RIDASCREEN FAST Hazelnut ELISA test a LOD of 1.5 mg/kg (ppm) and a LOQ of 2.5 mg/kg (ppm).

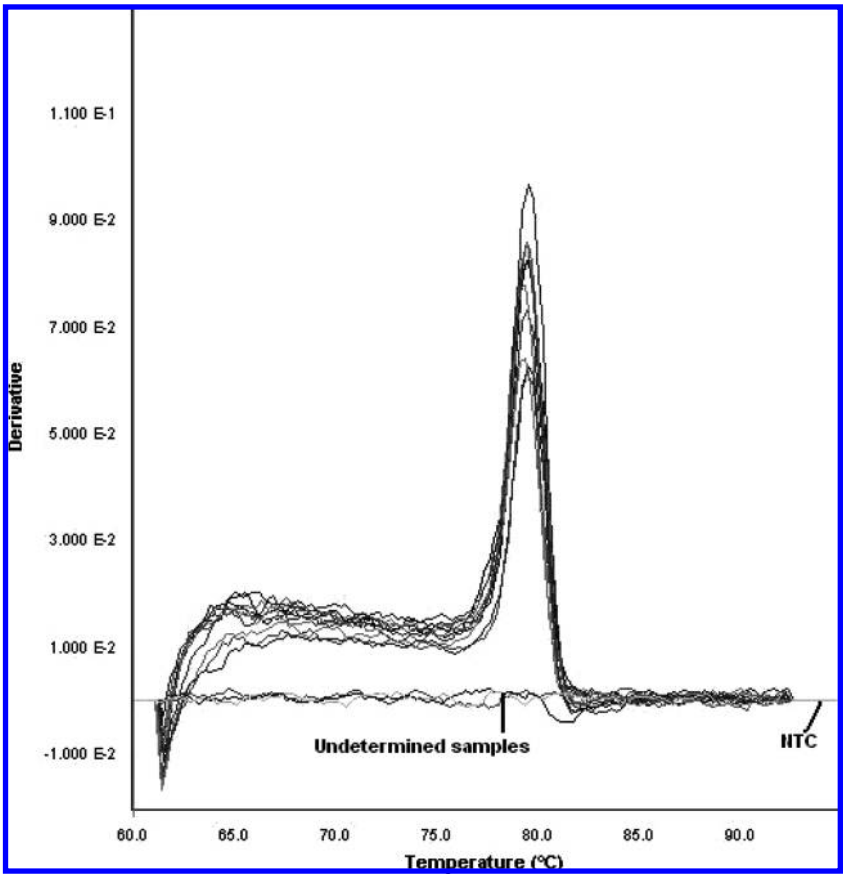

Figure 4. Derivates of melting curves of Cor a 8 M1/M2 specific amplicon obtained by amplifying commercial food products. Undetermined samples (bitter chocolate 1 and wafer filled with cocoa) displayed no amplification (baseline).

In conclusion, we suggest the use in combination of SYBR Green I real-time PCR and ELISA tests to provide complete information on the presence or absence of hidden hazelnut allergen in complex foods.

LITERATURE CITED

(1) Holzhauser, T.; Stephan, O.; Vieths, S. Detection of potentially allergenic hazelnut (*Corylus avellana*) residues in food: a comparative study with DNA PCR-ELISA and protein sandwich-ELISA. *J. Agric. Food Chem.* **2002**, *50*, 5808–5815.

(2) Zuidmeer, L.; Goldhahn, K.; Rona, J. R.; Gislason, D.; Madsen, C.; Summers, C.; Sodergren, E.; Dahlstrom, J.; Lindner, T.; Sigurdadottir, S. T.; McBride, D.; Keil, T. The prevalence of plant food allergies: a systematic review. *J. Allergy Clin. Immunol.* **2008**, *121*, 1210–1218.

(3) Wensing, M.; Penninks, A. H.; Hefle, S. L.; Akkerdas, J. H.; Van Ree, R.; Koppelman, S. J.; Bruijnzeel-Koomen, C. A.; Knulst, A. C. The range of minimum provoking doses in hazelnut-allergic patients as determined by double-blind, placebo-controlled food challenges. *Clin. Exp. Allergy* **2002**, *32*, 1757–62.

(4) Poms, R. L.; Anklam, E.; Kuhn, M. Polymerase Chain Reaction techniques for food allergen detection. *J. AOAC Int.* **2004**, *87*, 1391–1397.

(5) Piknová, L.; Sangallo, D.; Kuchta, T. A novel real-time Polymerase Chain Reaction (PCR) method for the detection of hazelnuts in food. *Eur. Food Res. Technol.* **2008**, *226*, 1155–1158.

(6) Pastorello, E. A.; Vieths, S.; Pravettoni, V.; Farioli, L.; Trambaioli, C.; Fortunato, D.; Lüttkopf, D.; Calamari, M.; Ansaloni, R.; Scibilia, J.; Ballmer-Weber, B. K.; Poulsen, L. K.; Wütrich, B.; Skamstrup Hansen, K.; Robino, A. M.; Ortolani, C.; Conti, A. Identification of hazelnut major allergens in sensitive patients with positive double-blind, placebo-controlled food challenge results. *J. Allergy Clin. Immunol.* **2002**, *109*, 563–570.
(7) Hirschwehr, R.; Valenta, R.; Ebner, C.; Ferreira, F.; Sperr, W. R.; Valent, P.; Rohac, M.; Rumpold, H.; Scheiner, O.; Kraft, D. Identification of common allergenic structures in hazel pollen and hazelnuts: a possible explanation for sensitivity to hazelnuts in patients allergic to tree pollen. *J. Allergy Clin. Immunol.* **1992**, *90*, 927–936.
(8) Vieths, S. Allergenic cross-reactivity, food allergy and pollen. *ETAP−Environ. Toxicol. Pharmacol.* **1997**, *4*, 61–70.
(9) Directive 2006/142/EC of the European Parlament and of the Council. *Off. J. Eur. Union* **2006**, *L 368*, 110.
(10) Holzhauser, T.; Vieths, S. Quantitative sandwich ELISA for determination of traces of hazelnut (*Corylus avellana*) protein in complex food matrixes. *J. Agric. Food Chem.* **1999**, *47*, 4209–4218.
(11) Pardigol, A.; Guillet, S.; Pöpping, B. A simple procedure for quantification of genetically modified organisms using hybrid amplicon standards. *Eur. Food Res. Technol.* **2003**, *216*, 412–420.
(12) Rønning, S. B.; Vaïtilingom, M.; Berdal, K. G.; Holst-Jensen, A. Event specific real-time quantitative PCR for genetically modified Bt11 maize (*Zea mays*). *Eur. Food Res. Technol.* **2003**, *216*, 347–354.
(13) Scaravelli, E.; Brohe, M.; Marchelli, R.; Van Hengel, A. J. Development of three real-time PCR assays to detect peanut allergen residue in processed food products. *Eur. Food Res. Technol.* **2008**, *227*, 857–869.
(14) Hupfer, C.; Waiblinger, H. U.; Busch, U. Development and validation of a real-time PCR detection method for celery in food. *Eur. Food Res. Technol.* **2007**, *225*, 329–335.
(15) Mustorp, S.; Engdahl-Axelsson, C.; Svensson, U.; Holck, A. Detection of celery (*Apium graveolens*), mustard (*Sinapis alba*, *Brassica juncea*, *Brassica nigra*) and sesame (*Sesamum indicum*) in food by real-time PCR. *Eur. Food Res. Technol.* **2008**, *226*, 771–778.
(16) Schoringhumer, K.; Cichna-Markl, M. Development of a real-time PCR method to detect potentially allergenic sesame (*Sesamum indicum*) in food. *J. Agric. Food Chem.* **2007**, *55*, 10540–10547.
(17) Hupfer, C.; Demmel, A.; Busch, U. Validation of real-time PCR methods as proof of sesame in food. *Dtsch. Lebensm.-Rundsch.* **2009**, *105*, 95–100.
(18) Brzezeinski, J. L. Detection of sesame seed DNA in foods using real-time PCR. *J. Food Prot.* **2007**, *70*, 1033–1036.
(19) Demmel, A.; Hupfer, C.; Hampe, E. L.; Busch, U.; Engel, K. H. Development of a real-time PCR for the detection of lupine DNA (*Lupinus* species) in foods. *J. Agric. Food Chem.* **2008**, *56*, 4328–4332.
(20) Brzezinski, J. L. Detection of cashew nut DNA in spiked baked goods using a real-time Polymerase Chain Reaction method. *J. AOAC Int.* **2006**, *89*, 1035–1038.
(21) Piknova, L.; Kuchta, T. Detection of cashew nuts in food by real-time Polymerase Chain Reaction. *J. Food Nutr. Res.* **2007**, *46*, 101–104.
(22) Ehlert, A.; Hupfer, C.; Demmel, A.; Engel, K. H.; Busch, U. Detection of cashew nut in foods by a specific real-time PCR method. *Food Anal. Methods* **2008**, *2*, 136–143.
(23) Brezna, B.; Hudecova, L.; Kutcha, T. A novel real-time Polymerase Chain Reaction (PCR) method for the detection of walnuts in food. *Eur. Food Res. Technol.* **2006**, *223*, 373–377.
(24) Brezna, B.; Dudasova, H.; Kuchta, T. A novel real-time Polymerase Chain Reaction method for the qualitative detection of pistachio in food. *Eur. Food Res. Technol.* **2008**, *228*, 197–203.
(25) Brezna, B.; Kuchta, T. A novel real-time Polymerase Chain Reaction method for the detection of pecan nuts in food. *Eur. Food Res. Technol.* **2008**, *226*, 1113–1118.
(26) Pafundo, S.; Gullì, M.; Marmiroli, N. SYBR GreenER real-time PCR to detect almond in traces in processed food. *Food Chem.* **2009**, *116*, 811–815.
(27) Brezna, B.; Piknova, L.; Kuchta, T. A novel real-time Polymerase Chain Reaction method for the detection of macadamia nuts in food. *Eur. Food Res. Technol.* **2009**, *229*, 397–401.
(28) Arlorio, M.; Cereti, E.; Coisson, J. D.; Travaglia, F.; Martelli, A. Detection of hazelnut (*Corylus* spp.) in processed foods using real-time PCR. *Food Control* **2007**, *18*, 140–148.
(29) Schoringhumer, K.; Redl, G.; Cichna-Markl, M. Development and validation of a duplex real-time PCR method to simultaneously detect potentially allergenic sesame and hazelnut in food. *J. Agric. Food Chem.* **2009**, *57*, 2126–2134.
(30) Oguchi, T.; Onishi, M.; Minegishi, Y.; Kurosawa, Y.; Kasahara, M.; Akiyama, H.; Teshima, R.; Futo, S.; Furui, S.; Hino, A.; Kitta, K. Development of quantitative duplex real-time PCR method for screening analysis of genetically modified maize. *J. Food Hyg. Soc. Jpn.* **2009**, *50* (3), 117–125.
(31) Nakajima, O.; Akiyama, H.; Teshima, R. Real-time Polymerase Chain Reaction method for detecting contamination of beef by material from genetically engineered cattle. *Biol. Pharm. Bull.* **2009**, *32*, 1313–1316.
(32) Chaouachi, M.; Fortabat, M. N.; Geldreich, A.; Yot, P.; Kerlan, C.; Kebdani, N.; Audeon, C.; Romaniuk, M.; Bertheau, Y. An accurate real-time PCR test for the detection and quantification of cauliflower mosaic virus (CaMV): applicable in GMO screening. *Eur. Food Res. Technol.* **2008**, *227*, 789–798.
(33) Taverniers, I.; Windels, P.; Vaïtilingom, M.; Milcamps, A.; Van Bockstaele, E.; Van den Eede, G.; De Loose, M. Event-specific plasmid standards and real-time PCR methods for transgenic Bt11, Bt176, and GA21 maize and transgenic GT73 canola. *J. Agric. Food Chem.* **2005**, *53*, 3041–3052.
(34) White, J. T.; Bruns, T.; Lee, S.; Taylor, J. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR Protocols: A Guide to Methods and Application*; Academic Press: New York, 1990; pp 315−329
(35) Bennett, M. D.; Smith, J. B. Nuclear DNA amounts in angiosperms. *Philos. Trans. R. Soc. London Ser. B* **1991**, *334*, 309–345.
(36) Doyle, J. J.; Doyle, J. L. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem. Bull.* **1987**, *19*, 11–15.
(37) Meyer, R.; Chardonnens, F.; Hübner, P.; Lüthy, J. Polymerase Chain Reaction (PCR) in the quality and safety assurance of food: detection of soya in processed meat products. *Z. Lebensm. Unters. Forsch.* **1996**, *203*, 339–344.
(38) Arlorio, M.; Coisson, J. D.; Martelli, A. Identification of Saccharomyces cerevisiae in bakery products by PCR amplification of the ITS region of ribosomal DNA. *Eur. Food Res. Technol.* **1999**, *209*, 185–191.
(39) Sharma, S.; Rustgi, S.; Balyan, H. S.; Gupta, P. K. Intraspecific sequence variation in the internal transcribed spacer (ITS) region of ribosomal DNA in common wheat and wild barley. *Barley Genet. Newsl.* **2002**, *32*, 38–45.
(40) Andersen, C. B.; Holst-Jensen, A.; Berdal, K. G.; Thorstensen, T.; Tengs, T. Equal performance of TaqMan, MGB, molecular beacon, and SYBR green-based detection assays. *J. Agric. Food Chem.* **2006**, *54*, 9658–9663.

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