



Analytical Methods

Real Time PCR to detect hazelnut allergen coding sequences in processed foods

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ABSTRACT

A quantitative RT-PCR method, employing novel primer sets designed on Cor a 9, Cor a 11 and Cor a 13 allergen-coding sequences has been setup and validated. Its specificity, sensitivity and applicability have been compared. The effect of processing on detectability of these hazelnut targets in complex food matrices was also studied. The DNA extraction method based on CTAB-phenol-chloroform was the best for hazelnut. RT-PCR using primers for Cor a 9, 11 and 13 allowed a specific and accurate amplification of these sequences. The limit of detection was 1 ppm of raw hazelnut. The method sensitivity and robustness were confirmed with spiked samples. Thermal treatments (roasting and autoclaving) reduced yield and amplifiability of hazelnut DNA, however, high-hydrostatic pressure did not affect. Compared with an ELISA assay, this RT-PCR showed higher sensitivity to detected hazelnut traces in commercial food-stuffs. The RT-PCR method described is the most sensitive of those reported for the detection of hazelnut traces in processed foods.

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1. Introduction

Food allergies are an emerging public health problem, especially in developed countries. Its prevalence is around 3% in adults and 6–8% of the child population. Clinical manifestations of food allergies can include gastrointestinal, cutaneous, systemic or respiratory symptoms. Some foods (including peanuts and tree nuts) can induce life-threatening anaphylaxis (Poms, Klein, & Anklam, 2004). Adverse reactions to hazelnuts are frequently severe or life-threatening, and consequently hazelnuts are regarded as one of the most dangerous allergenic foods by the FAO/WHO Codex Alimentarius Commission (1999) and the European Commission (2006). The estimated prevalence of hazelnut allergy in the European population is 0.1–4% (Zuidmeer et al., 2008). Hazelnut contains several allergenic proteins: Cor a 1 (18 kDa, PR protein, homologue of Bet v 1 birch pollen allergen), Cor a 2 (14 kDa, profilin), Cor a 8 (9 kDa, LTP), Cor a 9 (40 kDa, legumin-like), Cor a 11 (48 kDa, vicilin-like), oleosins Cor a 12 (17 kDa) and Cor a 13 (14–16 kDa) and 2S albumin Cor a 14 (15–16 kDa) (Akkerdaas et al., 2005; Garino et al., 2010; Pastorello et al., 2002).

Abbreviations: CT, cycle threshold; CTAB, hexadecyltrimethylammonium bromide; HHP, high hydrostatic pressure; NTC, non-template control; RT, real time.

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Food thermal processing can promote degradation of food constituents, modifications, or interactions with other components of the food matrix and therefore thermal processing has a potential to modify allergenic properties of proteins (Sathe & Sharma, 2009). Current data do not provide information for the identification of specific variables that could be used to reliably determine how processing will influence on protein allergenicity (Van Boekel et al., 2010). To date, there is scarce information on how high pressure can affect food allergens (Johnson et al., 2010).

The Directive 2006/142/EC (2006) provides a list of 14 groups of potential allergenic ingredients to be mandatorily labelled on foods which includes: cereal containing gluten, crustaceans, eggs, fish, peanuts, soybeans, milk (lactose included), tree nuts, celery, mustard, sesame seeds, lupine, molluscs and sulphite of at least 10 ppm. Several analytical approaches have been suggested to detect potential allergens in food products (Poms et al., 2004). These analytical methods are required to guarantee food safety for allergic consumers. A retrospective study on adults in Spain showed that 119 of 530 food allergic reactions were caused by the exposure to “hidden” allergens which are not indicated in the ingredients description label (Añíbarro, Seoane, & Múgica, 2007). Contamination of food with hidden allergens may occur during all the processing steps.

Protein detection methods are commonly employed to detect the presence of food allergens in complex matrices. Enzyme-linked

immunosorbent assay (ELISA) is probably the most commonly used method in the food industry to assess the presence of allergens in food products. Several ELISA methods specific for hazelnuts are commercialised nowadays (Garber & Perry, 2010). However, the use of high-temperature or high-pressure could affect the protein extraction from food matrices and the integrity of the target allergenic proteins. Consequently, it could reduce the reliability of these techniques for processed foods (Johnson et al., 2010; Sathe & Sharma, 2009). An alternative for allergen detection are DNA-based methods in which the target molecules are specific DNA sequences amplified by the polymerase chain reaction (PCR). Although this technique represents an indirect investigation of the allergenic ingredients, DNA-based methods have advantages over protein methodologies: the target DNA is efficiently extracted under harsh denaturing conditions and could be less affected than proteins from food matrices. Another advantage is its stability vs. geographical and seasonal variations (Poms et al., 2004). In quantitative Real-Time PCR the increase in fluorescence is proportional to the amount of specific PCR product. The DNA target sequences could be a gene coding for an allergenic protein or a species-specific region in the genome of the allergenic food (Scaravelli, Brohée, Marchelli, & Van Hengel, 2009). Usually, consumers are exposed to processed ingredients in complex food matrices and therefore, it is very important to investigate the effects that food processing may exert on the detection of hazelnut-specific markers (Stephan & Vieths, 2004).

Cor a 1 has been used as a target sequence for the detection of hazelnut by RT-PCR (Arlorio, Cereti, Coisson, Travaglia, & Martelli, 2007; Holzhauser, Stephan, & Vieths, 2002; Holzhauser, Wangorsch, & Vieths, 2000; Stephan & Vieths, 2004). Cor a 8 and Cor a 14 allergen coding sequences have been also recently used to develop a new RT-PCR protocol (D'Andrea et al., 2011).

The objective of this work was to setup and to validate a SYBR Green Real-Time PCR protocol employing novel specific primer sets designed on Cor a 9, Cor a 11 and Cor a 13 allergen-coding sequences, and compare its specificity, sensitivity and applicability. Moreover, the effect of high temperature and/or high pressure treatments on the detectability of hazelnut DNA targets in complex food matrices was also studied.

2. Materials and methods

2.1. Samples

Four varieties of raw hazelnut (*Corylus avellana* L.) from two different geographical regions (var. Negreta and Pauetet from Spain and Tonda di Giffoni and San Giovanni from Italy) were used in this study. All of them were provided by the Germoplasm Bank of Institut de Recerca i Tecnologia Agroalimentàries (Batlle et al., 1999) (IRTA-Mas de Bover, Tarragona, Spain). Roasted hazelnuts (180 °C, 20–30 min) and a paste from Negreta roasted hazelnuts were supplied by Cooperativa COSELVA (Tarragona, Spain). This paste is commonly used as ingredient in the European chocolate industry. DNA from other plant samples (*Prunus dulcis* L., *Juglans regia* L., *Arachis hypogaea* L., *Oryza sativa* L., *Triticum aestivum* L., *Hordeum vulgare* L. and *Secale cereale* L.) were used in specificity studies. For the preparation of spiked samples, different amounts of defatted hazelnut flour were added to defatted peanut flour, obtaining a series of mixtures with 50%, 10%, 5%, 1%, 0.1%, 0.01%, 0.001% and 0.0001% w/w of hazelnut. Commercial foodstuffs (chocolates, biscuits, snacks, etc) purchased in local food stores were analysed for the method validation.

2.2. Heat and high pressured (HHP) treatments

Raw hazelnuts of Negreta variety were autoclaved in water (1:5 w/v) at 121 °C (120 kPa) and 138 °C (260 kPa) for 15 and

30 min (Compact 40 Benchtop Priorclave, UK). Raw and autoclaved samples were milled to pass through a 1 mm sieve (Tecator, Cyclotol 1093, Sweden) and the resulting meal was defatted with n-hexane (34 mL/g of flour).

Defatted flour from raw hazelnuts was soaked with distilled H₂O (1:4 w/v) (Kato, Katayama, Matsubara, Omi, & Matsuda, 2000) during 20 h at 4 °C, and then treated with high pressure (400, 500 and 600 MPa) for 15 min at 20 °C using a high hydrostatic pressure equipment (ACB, France). After high pressure treatment the samples were centrifuged (12,000 rpm, 10 min) and the pellets were freeze-dried and stored at –20 °C until use. Hazelnut paste was also treated with high pressure at the same conditions as defatted flour (HHP and roasted samples).

2.3. Genomic DNA isolation

Four methods were used for DNA extraction from raw hazelnuts of Negreta variety: (1) DNeasy Plant Mini Kit (Quiagen, Germany), (2) Power Plant Bread Solution (MoBio, CA USA), (3) Dellaporta method (Dellaporta, Wood, & Hicks, 1983) and (4) CTAB/phenol/chloroform-based extraction. All extraction methods were used according to the protocols instructions.

In protocol 4, DNA was extracted by incubating 100 mg of sample in 1 mL of CTAB (hexadecyltrimethylammonium bromide) buffer (1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl, pH 8) at 65 °C for 30 min. After extraction, 0.6 mL of phenol/chloroform/isoamyl alcohol 25:24:1 (v/v) was added. For undissolved material removal, the tubes were centrifuged at 14,000 rpm for 2 min. 0.8 mL of chloroform/isoamyl alcohol were added to the supernatant. This step was carried out twice. DNA was precipitated by the addition of 0.8 mL of isopropanol and it was recovered after 14,000 rpm centrifugation for 5 min. The pellet obtained was washed once by addition of 1.6 mL of ethanol 70%. DNA was cleaned using a silica membrane in a spin column from Power Plant DNA Isolation Kit (MoBio, CA USA) and finally eluted in 50 µL H₂O Milli-Q.

The CTAB/phenol/chloroform-based extraction was selected to obtain DNA from raw and processed hazelnuts (100 mg), as well as from spiked samples and complex food matrices (500–1000 mg).

The quantity and quality of the genomic DNA was determined with NanoDrop ND-1000 Spectrophotometer (ThermoFisher, MA USA) by measuring the absorbance at 230, 260 and 280 nm. DNA quality was also evaluated on 0.8% agarose gels. These parameters were compared in order to choose the best isolation method.

2.4. Conventional PCR, cloning, sequencing and RT-PCR primer design

Sets of primer pairs were designed with Oligo Primer Analysis Software on the target sequences of all the eight Cor a genes (data not shown). Only the three most sensitive and specific for measuring the hazelnut content were used: Cor a 9 (GenBank accession number AF449424) (GCCCATGGTGTATAACGAC/GTTGATGCGACCGACTTGTC), Cor a 11 (AF441864) (GCAACAAGAGGAGGGCAATAG/TGGGCTGGAGAAATGAACGTG) and Cor a 13 (AY224599) (TAGC CGGCACGGTCATTGCG/TCATGCGCGCGTCAAGT). These primers were used to carry out conventional PCRs.

PCR reactions were carried out using DNA isolated from the four varieties of raw hazelnut and the resulting amplicons were cloned in TOPO TA Cloning Kit (Invitrogen, Inc., UK) according to the manufacturer's indications. Plasmids were recovered from cultures using High Pure Plasmid Isolation Kit (Roche, Germany), and sequenced in an ABI PRISM 3700 sequencer (Applied Biosystems, CA, USA) from "Unidad de Genómica, Parque Científico de Madrid". DNA sequences were edited and analysed using Chromas Pro 1.22 software (Technelysium Pty, Ltd., Australia). These sequences were compared with those present in the GenBank (NCBI)

Table 1

Sequences, melting temperature (Tm), accession number for each locus, and amplicon size of specific primers used in Real-Time PCR studies.

Primer	Sequence 5'–3'	Tm	GenBank accession number	Amplicon (bp)
Cor a 9 f	GTCAAAGTGGGAAGGCAGGCTT	66.6	JN674437–	101
Cor a 9 r	TCCCGCTCTTGCTCACTCTC	67.1	JN674440	
Cor a 11 f	AACAGAGGAAGGCAGAGTGCA	66.3	JN674445–	101
Cor a 11 r	GATTTGCTTCAGAAATCGCC	65.7	JN674448	
Cor a 13 f	GCGGTCATCACAGTATCGCTT	66.3	JN674441–	101
Cor a 13 r	GTCACGTACCTGTAGATCCACGAC	65.6	JN674444	

non-redundant nucleotide (nr/nt) databases through BLASTn. Alignments between sequences of the same gene obtained from the four varieties were performed using the ClustalW algorithm (Thompson, Higgins, & Gibson, 1994).

Specific hazelnut primer pairs for Real-Time PCR (Table 1), were designed from the conserved sequences of Cor a 9, 11 and 13 by Primer Express 2.0 (Applied Biosystems, CA, USA). Primers meet the following requirements: length between 20 and 24 bases and to produce an amplicon between 75 and 150 bp. A conventional PCR was performed using these pairs of primers in order to amplify the genomic DNA sequences of Cor a genes. The reaction was carried out in 20 μ L volume, containing 10 ng of DNA, 0.25 μ M of each primer and 1 $\times$ Taq PCR Master Mix (Qiagen, Germany). SensoQuest LabCycler (Progen Scientific Ltd, Germany) was used and the following PCR program was used: an initial denaturation step at 95 $^{\circ}$ C, 4 min, followed by 35 cycles of denaturation at 94 $^{\circ}$ C for 45 s; annealing at 62–64 $^{\circ}$ C for 1 min; elongation at 72 $^{\circ}$ C for 1 min; last step at 72 $^{\circ}$ C for 6 min. The PCR products were electrophoresed on 2% agarose gels. The specificity of all primers was assessed by testing the amplification of non-hazelnut DNA extracts from a wide variety of plant samples.

2.5. Real-Time PCR conditions

Real-Time PCR reactions were carried out in a volume of 20 μ L, using 7900HT Fast Real-Time PCR System (Applied Biosystems, CA, USA). Each reaction consisted of 10 μ L of SYBR Premix Ex TaqTM (Takara, Japan) 2 μ mol of primer and variable amount of template DNA and H₂O Milli-Q. All samples were analysed in duplicate. On each plate, at least two non-template controls (NTCs) were used to check the PCR performance. The following PCR program was used: initial denaturation at 95 $^{\circ}$ C for 10 min followed by 40 cycles of denaturation at 95 $^{\circ}$ C for 15 s and primer annealing and elongation at 60–65 $^{\circ}$ C for 1 min. Dissociation studies were performed on PCR products at a constant temperature increase (60–95 $^{\circ}$ C), recording fluorescence each 10 s.

Standard curves for Real-Time PCR were generated using the cycle threshold (Ct) value obtained from 10-fold serial dilutions of hazelnut genomic DNA. The efficiency of each reaction was calculated from the slope of the standard curve (Ct vs. log DNA concentration) as indicated in the Applied Biosystem Manual.

2.6. ELISA assay

Immunochemical hazelnut detection was performed using a commercial hazelnut ELISA kit (RidaScreen Fast, R-Biopharm, Germany) according to the manufacturer's instructions. The kit includes monoclonal antibodies able to detect the main allergenic hazelnut proteins (14-, 18- and 42 kDa).

3. Results and discussion

3.1. DNA extraction

To setup a reliable Real-Time PCR protocol, the first step is to achieve a good DNA extraction considering yield and quality

values. Both are important requirements to optimise detection of the target sequences as well as other Real-Time PCR parameters (Ct values, efficiency and R² values). The fat material and polyphenol compounds of nuts could act as polymerase inhibitors (Venkatachalam & Sathe, 2006), therefore they should be eliminated from the DNA sample. As these components absorb at 230 nm and the proteins at 280 nm, the 260/230 and 260/280 ratios were used to test DNA quality.

CTAB-phenol-chloroform based extraction (protocol 4) produced a good yield of high quality DNA from both raw hazelnuts and processed foods, eliciting ratios of 260/280 = 2.09 $\pm$ 0.02 and 260/230 = 1.98 $\pm$ 0.06 for raw samples. The amount of DNA extracted with the traditional Dellaporta protocol (method 3) was the highest, but the 260/280 and 260/230 ratios were 1.90 $\pm$ 0.05 and 1.52 $\pm$ 0.16, respectively, probably due to the co-extraction of PCR-inhibitors. Moreover, using these DNA as template, it was not possible to obtain amplification by conventional PCR. Arlorio et al. (2007) obtained the best extraction results for hazelnut using commercial kits specifically designed for fatty foods. However, in our work the extraction with the commercial kits (1 and 2) did not provide good spectrophotometric values (ratio 260/230 = 0.77 $\pm$ 0.06 in protocol 1 and 0.67 $\pm$ 0.30 in protocol 2). The present results indicate that the CTAB-phenol-chloroform protocol seems to be the best extraction method for hazelnut as has been previously reported for other nuts (Holzhauser et al., 2000; Röder, Vieths, & Holzhauser, 2011). In addition, the quality of the extracted DNA was notably improved combining the CTAB-phenol-chloroform extraction with sample cleaning using a silica membrane from Power Plant DNA Isolation Kit. This combined method was used for all the DNA extractions: raw, treated hazelnuts and processed commercial foods.

3.2. Primer design and sequence analyses

Primers designed from Cor a 9, Cor a 11 and Cor a 13 cDNAs sequences deposited in the GenBank were used to amplify partial allergen-coding sequences of four hazelnut varieties from different geographical origins. This allows to assess the intra-specific polymorphism which could generate false negatives. The sequences obtained from these varieties (GenBank accessions numbers JN674437–JN674448) were aligned giving a high similarity degree among varieties for the three cases (Cor a 9 = 96.7%; Cor a 11 = 99.7%; Cor a 13 = 98.8%). These sequences were used to design the specific Real-Time PCR primers (Table 1).

3.3. Optimisation of Real-Time PCR assays with SYBR Green

The coding sequences of Cor a 9, Cor a 11 and Cor a 13 proteins were selected as target sequences because the primer pairs designed for them were the most sensitive and specific for measuring hazelnut content and, as far as we know, there is not a RT-PCR method to specifically detect them. Moreover, Cor a 9 and Cor a 11 have been described as major hazelnut allergens by Pastorello et al. (2002) and Beyer, Grishina, Bardina, Grishin, and Sampson (2002). According to previous investigations, different RT-PCR protocols for the specific detection of hazelnut allergens have been developed for Cor a 1 (Arlorio et al., 2007; Holzhauser et al., 2000; Holzhauser et al., 2002; Stephan & Vieths, 2004) and more recently for Cor a 8 and Cor a 14 (D'Andrea et al., 2011). Single RT-PCR reactions for each sequence were chosen, instead of a multiple strategy, because this method enables to study the effect of each sequence on important parameters of RT-PCR (specificity, amplification efficiency and sensitivity) and to establish valid comparisons. The election of the SYBR Green I methodology, versus the TaqMan, is based on its lower cost; nevertheless it gives equal

sensitivity and performance (Andersen, Holst-Jensen, Berdal, Thorstensen, & Tengs, 2006).

3.3.1. Specificity

To assess specificity, DNA from hazelnut as well as other plants commonly used as food ingredients (rice, wheat, barley, rye, peanut, almond, and walnut), were amplified by conventional PCR and the amplification results were electrophoresed on agarose. Primers for Cor a 9, Cor a 11 and Cor a 13 genes were 100% specific for hazelnut (data not shown).

By means of genomic DNA amplification from raw hazelnuts in RT-PCR a 101 bp amplicon has been obtained for each primer pair tested (Table 1). Their specificity was confirmed by the evaluation of the dissociation curves (Fig. 1). The curves showed a single amplicon when DNA from the four varieties of hazelnut was used as template and no amplification was observed from other plant samples. These results proved that primer pairs for Cor a 9, Cor a 11 and Cor a 13 allow a specific detection of the varieties of hazelnut tested without amplification in other species.

3.3.2. Amplification efficiency and R^2 coefficient

In a RT-PCR procedure, the slope of the standard curve is used to estimate the amplification efficiency of the reaction. A standard curve slope of -3.32 indicates a 100% efficient PCR reaction according to the supplier indications. The R^2 describes the correlation between the cycle threshold value (C_t) and the logarithm of the amount of DNA in the standard curve. To generate the standard curves, three separate DNA extractions of raw hazelnut were performed, DNA was 10-fold serially diluted and 5 μ l of each dilution was added to each reaction. All the standard curve values obtained were optimal, in accordance to ENGL acceptance criteria (acceptable slope between -3.1 and -3.6 and correlation coefficient greater than 0.98) (European Network of GMO Laboratories, 2005). The efficiency values were 98.65% for Cor a 9, 103.18% for Cor a 11, and 99.03% for Cor a 13.

The inter-assay reproducibility was investigated overlapping the three standard curves obtained from different samples in separate experiments. The mean slope and the C_t -values were similar

for the four varieties used, all of which allowed to conclude that this RT-PCR method is highly reproducible.

3.3.3. Sensitivity

The sensitivity of this RT-PCR protocol has been assessed by at least three separate DNA extractions of raw hazelnut. Similarly to the efficiency experiments, the DNA was 10-fold serially diluted and 5 μ l of each dilution was added to each reaction. The Limit of Detection (LOD), defined as the amount of sample giving a signal strength four to six times higher than the blank signal strength, was 2.16 pg for Cor a 9, Cor a 11 and Cor a 13. The present procedure allowed to detect a 1:10,000 dilution with the three specific primer pairs, being a more sensitive RT-PCR protocol than that validated by D'Andrea et al. (2011), also based on SYBR chemistry (<9.6 pg vs 2.16 pg of hazelnut DNA).

To evaluate the sensitivity of this method on food matrices, we performed PCRs using defatted peanut flour spiked with known amounts of defatted hazelnut flour (from 1% to 0.0001% w/w). For each spiking level the DNA was extracted and analysed by RT-PCR in duplicate. The LOD measured was 0.0001% w/w of hazelnut in peanut, corresponding to 1 ppm for the three allergens sequences. Their C_t values were: 31.79 ± 0.21 for Cor a 9, 35.42 ± 0.30 for Cor a 11 and 31.56 ± 0.32 for Cor a 13. Previous works on the detection of hazelnut using Real-Time PCR obtained a limit of detection of 100 ppm or 10 ppm (D'Andrea et al., 2011; Píknová, Sangallo, & Kuchta, 2008). Therefore, the present method showed higher sensitivity than the previous ones which is its main advantage compared with them.

3.4. Effect of roasting, high-pressure and autoclave treatments

In order to study the effect of treatments on the detection ability of the proposed method, a calibration curve was performed for each primer (Cor a 9, Cor a 11, Cor a 13) from DNA extracted from spiked samples prepared with peanut and known amounts of hazelnut flour (from 50% to 1%, w/w). By plotting the C_t values against the logarithm of the corresponding hazelnut quantity a linear regression was obtained in all cases. The equations had R^2 values higher than 0.99. These curves were used to determine the amount of hazelnut DNA in raw and processed samples. Three DNA extracts were obtained from each treatment and analysed in duplicate. The average values were normalised to the DNA hazelnut content determined in the raw samples. The results are represented in Fig. 2.

The roasting and autoclaving processes applied dramatically reduced the ability to detect hazelnut DNA. This reduction was not observed in the samples treated only with high pressure (7, 8 and 9 samples). Similar results have been reported by Scaravelli, Brohée, Marchelli, and Van Hengel (2009) who found that heat processing causes a marked drop in peanut DNA detectability when PCR methods are employed. They conclude that peanut DNA extraction yields considerably decreases as thermal treatment increases but the DNA integrity or quality is not affected. As far as we now, there are no data on the effect of HHP treatments on DNA yield, quality and integrity.

To determine if processing affects the amount and/or integrity of hazelnut DNA, both parameters were evaluated in the present study. DNA quantification of raw and treated hazelnut samples indicated that the extraction yield progressively decreased as heat treatment increased (Table 2). The DNA yield was reduced from 13.39 ng/mg in raw hazelnut to 1.83 ng/mg in autoclaved samples at harsh conditions (260 kPa, 30 min). These results could explain that the detection levels shown in Fig. 2 were due to lower amounts of DNA in the PCR reactions when roasted and autoclaved hazelnut samples were analysed.

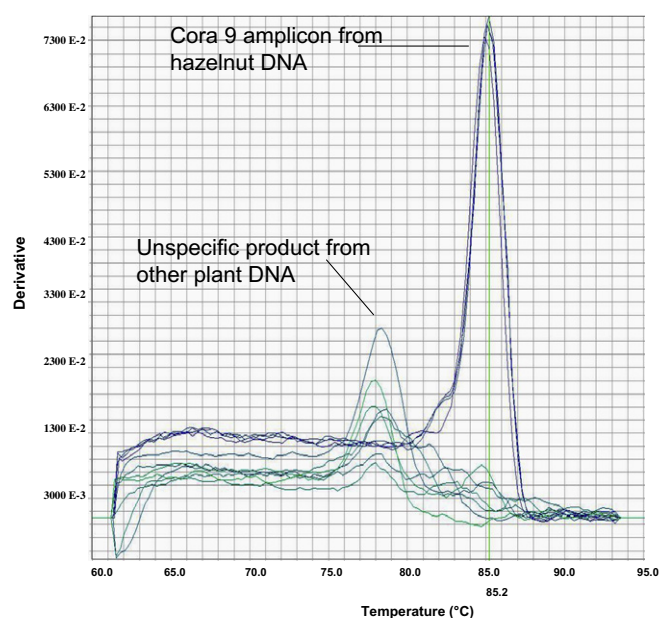


Fig. 1. Derivates of melting curves of Cor a 9 assays. The curves show a specific amplicon (101 bp) obtained from hazelnut DNA and an unspecific product from other plant DNAs.

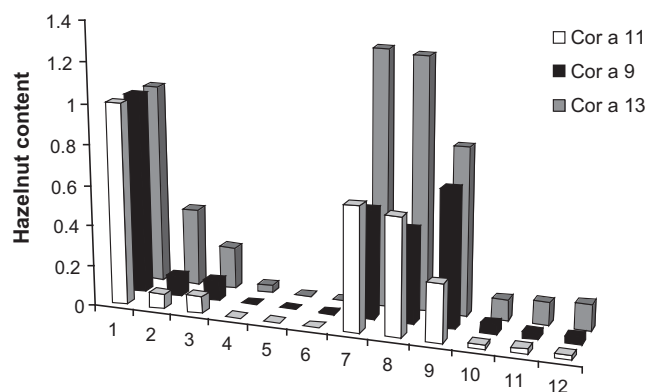


Fig. 2. Estimation of hazelnut content in different raw and treated hazelnut samples. 1: Raw; 2: Roasted; 3: Autoclaved (120 kPa, 15'); 4: Autoclaved (120 kPa, 30'); 5: Autoclaved (260 kPa, 15'); 6: Autoclaved (260 kPa, 30'); 7: High-pressured (400 MPa); 8: High-pressured (500 MPa); 9: High-pressured (600 MPa); 10: High-pressured and roasted (400 MPa); 11: High-pressured and roasted (500 MPa); 12: High-pressured and roasted (600 MPa).

Table 2

DNA extraction yields obtained from raw and processed hazelnut samples.

Hazelnut samples	Yields (ng mg ⁻¹)
Raw	13.39 ± 1.69
Roasted (180 °C, 20 min)	3.71 ± 0.39
Autoclaved (120 kPa, 15 min)	6.60 ± 1.40
Autoclaved (120 kPa, 30 min)	3.57 ± 0.81
Autoclaved (260 kPa, 15 min)	3.53 ± 1.01
Autoclaved (260 kPa, 30 min)	1.83 ± 0.66
HHP (400 MPa)	15.67 ± 1.93
HHP (500 MPa)	18.40 ± 4.71
HHP (600 MPa)	20.35 ± 2.40
HHP and roasted (400 MPa)	2.83 ± 0.47
HHP and roasted (500 MPa)	4.34 ± 0.81
HHP and roasted (600 MPa)	3.78 ± 1.25

On the other hand, the quality or integrity of DNA was investigated by studying its amplifiability in raw and processed samples. For this purpose the DNA amount was standardised to 25 ng and analysed in duplicate. The Ct values so obtained from raw and some treated hazelnut samples are represented in Fig. 3. Raw and high pressure-treated hazelnuts had both low Ct values, but thermally-treated hazelnuts (autoclaved and high-pressured and roasted) had similar higher Ct values. This data can be interpreted as a decrease of the hazelnut DNA quality due to thermal treatment. DNA integrity analysed by agarose gel electrophoresis showed results compatible with degradation of DNA in the treated samples. In contrast to the results above mentioned (Scaravelli et al., 2009), our data demonstrated that the heat treatments used in this study not only decreased the DNA yield but also affected hazelnut DNA integrity or quality.

3.5. Applicability on commercial food samples

The actual applicability of this RT-PCR method for determining hazelnut allergens in foods was investigated by analysing nine commercial food stuffs comprising snacks, chocolates, cookies, nougat, etc. Three of these products labelled the percentage of contained hazelnut, four of them indicated that the product might contain traces of hazelnut or tree nuts and two did not provide any information about hazelnut content. All commercial food samples were also analysed by ELISA and the results are shown in Table 3.

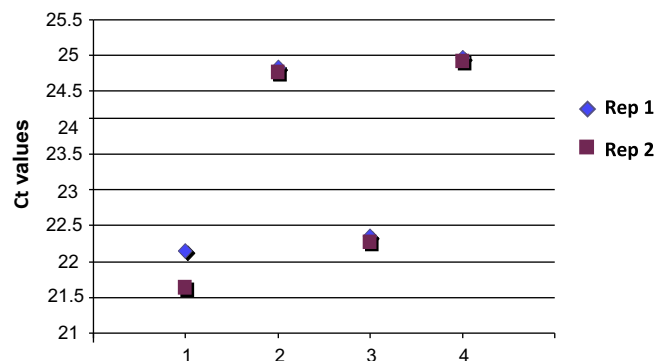


Fig. 3. DNA amplifiability of different hazelnut samples measured by dispersion of Ct values. 1: Raw; 2: Autoclaved (120 kPa, 30'); 3: High-pressured (600 MPa); 4: High-pressured and roasted (600 MPa).

Table 3

Results of hazelnut presence or absence in commercial food products analysed through Real-Time PCR and ELISA. + = detected; – = non-detected.

Commercial foods	Hazelnut declaration	Cor a 9	Cor a 11	Cor a 13	ELISA
Cereal snack	Contain hazelnut	+	+	+	+
Chocolate with hazelnut	Contain hazelnut	+	+	+	+
Cereal snack II	Contain hazelnut	+	+	+	+
Chocolate	Might contain hazelnut	+	+	+	+
Biscuit	Might contain hazelnut	+	+	+	+
Cereal snack with chocolate	Might contain nuts	+	+	+	+
Nougat	Might contain nuts	+	+	+	+
Chocolate snack with cookie	Not declared	+	+	+	–
Chocolate cookie	Not declared	–	–	–	–

The three primer pairs detected the presence of hazelnut according to the declaration on the label even at trace levels. The utilisation of this validated Real-Time protocol based in three different target genes ensure more reliable results, reducing the probability of false negatives or false positives results according to D'Andrea et al. (2011). A perfect correspondence between ELISA results and those obtained by RT-PCR for 8 out of 9 foods products analysed confirmed the reliability of this method. In the chocolate snack with cookie sample the signal obtained with the ELISA was below the limit of detection of RidaScreen Fast (1.5 ppm) but, traces of hazelnut DNA were detected in this sample by the three target allergen PCRs. According to Stephan and Vieths (2004), the detection of hazelnut DNA in a non-declared sample, whose result in ELISA analysis were negative, confirms the higher sensitivity of the RT-PCR method developed. The procedure here described seems to be more reliable than others and in addition, the dye used (SYBR Green I) detects the food traces at a cheaper analysis cost.

4. Concluding remarks

In this work a specific and sensitive RT-PCR method to detect hazelnut DNA has been developed. The novel primers designed allow the DNA amplification of the coding region of three hazelnut allergens (Cor a 9, 11 and 13). This method seems to be the most sensitive of those described so far for the detection of hazelnut by RT-PCR due to its LOD (1 ppm). Additionally, when compared with ELISA assays it shows higher sensitivity to detect hazelnut

traces not only because of its lower LOD but also because of its ability to detect the presence of hidden hazelnut allergens in commercial foods where ELISA gives negative results. The effect of thermal treatments must be taken into account when processed samples or complex food matrix are analysed. Despite the reduction in DNA quality and quantity elicited by heat processing, in the present study the detection has always been possible and accurate.

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