



Analytical Methods

High resolution TaqMan real-time PCR approach to detect hazelnut DNA encoding for ITS rDNA in foods



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ABSTRACT

A broad range of foods have been described as causing allergies, but the majority of allergic reactions can be ascribed to a limited number of food components. Recent extensive surveys showed how tree nuts, particularly hazelnut (*Corylus avellana* L.) seeds, rank amongst the most important sources of food allergy. In order to protect the allergic consumer, efficient and reliable methods are required for the detection of allergenic ingredients. For this purpose, we have developed a real-time polymerase chain reaction (PCR) for detection of hazelnut in commercial food products. In this way a specific hazelnut primer pair based on the ITS marker (70 bp) and a nuclease (TaqMan) probe labelled with FAM and BHQ were designed. Sensibility of real-time PCR was determined by analysis of raw and heat treated hazelnut-wheat flour mixtures with a range of detection of 0.1–100,000 ppm. Practical applicability of the real-time PCR assay developed for determining hazelnut in different food matrices was investigated by analyzing 179 commercial foodstuffs comprising snacks, biscuits, chocolates, bonbons, creams, nut bars, ice creams, precooked meals, breads, beverages, yogurts, cereals, meat products, rice cake and nougat. From the total of samples analyzed, 40 commercial food products that didn't declare hazelnut nor traces on the label were found to contain hazelnut. The real-time PCR method proposed herein due to its high sensitivity facilitates the detection of hazelnut traces in commercial food products and can also be useful for monitoring the effectiveness of cleaning processes and as consequence, can help to prevent the food allergic consumer from unintentional ingestion of hidden allergens.

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1. Introduction

Food allergies are a major health concern in industrialized countries and may affect up to 3% of the adult population and 6.8% of the children in Europe. The number of food allergen-induced life-threatening syndromes is increasing. The consumption of allergens by patients with hypersensitivity can trigger a variety of immunological reactions ranging from hives, pruritus, atopic dermatitis, swelling of the throat or facial tissues, vomiting, diarrhoea, asthmatic wheeze, difficulty in breathing, and hypotension to life-threatening anaphylaxis. A large number of anaphylactic reactions to food are treated in emergency departments each year, and it is estimated that food allergy causes several deaths annually (Mustorp, Dromtorp, & Holck, 2011). The level of exposure to provoke a reaction varies from food to food and from person to person. Most often, reactions are elicited after exposure of 0.1–100 ppm of an allergen, but sometimes, only minute amounts are required.

Treatment of food allergy is difficult, and avoiding the allergen-containing food is often the only option. This may sometimes be difficult, especially for processed foods, which may contain allergens either added deliberately, or unintentionally, when foods are contaminated during shipping and storage and from food production lines due to insufficient cleaning of the production equipment of shared processing equipment, or in reuse (rework) of allergen-containing products (Poms, Anklam, & Kuhn, 2004). As a precautionary measure numerous food products are marketed with labels such as “may contain traces of ...” or “products containing ... are produced with the same equipment”.

On the other hand, allergic consumers rely on accurate food labels to make informed choices to be able to avoid offending allergens. To minimize or eliminate the risk of cross-contamination, the industry needs to have in place an allergen control programme, which is usually integrated as part of its Hazard Analysis and Critical Control Point (HACCP) system. Analytical techniques can be used as tools to monitor potential errors before, during, and after manufacturing activities. In this way, sensitive analytical methods able to detect trace amounts of allergenic compounds in food products, are widely demanded to be used for screening, routine, and confirmatory analysis.

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Hazelnuts are one of the most nutritious nuts, with a flavour that makes them popular for use in food, especially in confectionery, where they are often found in pastries, chocolate spreads, ice cream, cereal bars, cookies, nougat, etc. Hazelnuts are consumed raw or roasted, intact, chopped, or processed into a praline paste. However, hazelnuts may be a threat to some consumers since they contain allergens, and consumption of food products containing hazelnuts may cause severe allergy in a part of population (Roux, Sathe, & Teuber, 2003; Sicherer & Sampson, 2000). In response to this fact, European regulation (EU) 1169/2011/EC (OJEU, 2011) requires labelling of food products in respect to the contents of hazelnuts and other allergenic components.

Methods for detection of hazelnut in food are necessary to support this legislation. Enzyme linked immune sorbent assays (ELISAs), which are based on the specific interaction between antigens and antibodies play the most important role among the protein based methods employed for tree nut detection (Poms, Klein, & Anklam, 2004). ELISA approach is currently considered the “gold standard” for the direct allergen detection. Several sandwich ELISA-based methods are available for the detection of traces of hazelnuts in food (Akkerdaas et al., 2004; Ben Rejeb, Abbott, Davies, Cleroux, & Delahaut, 2005; Blais, Gaudreault, & Philippe, 2003; Blais & Philippe, 2001; Drs et al., 2004; Garber & Perry, 2010; Holzhauser & Vieths, 1999; Koppelman et al., 1999). However, specific detection of hazelnuts in food may be also carried out using alternative analytical methods based on genetic techniques (Germini et al., 2005; Herman, De Block, & Viane, 2003; Pafundo, Gulli, & Marmiroli, 2010; Piknova, Pangallo, & Kuchta, 2008) that, like ELISA, have the potential to be applied for quantitative purposes. In DNA based methods, specific DNA sequences (templates) are amplified by the polymerase chain reaction (PCR) and detected either by agarose gel electrophoresis or in so-called real-time PCRs by using fluorescently labelled probes. Herman et al. (2003) confirmed the possibility to detect hazelnut in complex food matrices, like chocolates, via end-point PCR using Nad1 mitochondrial primer pair, and achieving a LOD (Limit of detection) of 10 ppm. On the other hand, Holzhauser, Wangorsch, and Vieths (2000) developed an end-point PCR assay targeting the gene of the major hazelnut allergen Cor a 1. Conventional end-point PCR might be more accessible due to the less expensive equipment that is required compared to real-time cycling instrumentation and detection chemistries. However, its lower cost cannot compensate for the extreme sensitivity and quantitative properties of real-time PCR. Because of these adventitious properties, most laboratories are no longer investing in conventional cycling instruments but are rather switching to real-time PCR equipment. This trend has resulted in an increased reporting on real-time PCR methods for the detection of allergenic ingredients in recent years. Arlorio, Cereti, Coisson, Travaglia, and Martelli (2007) developed a real-time PCR assay targeting the Cor a 1 gene, which showed an LOD of 0.1 ng of genomic DNA. On the other hand, D'Andrea, Coission, Travaglia, Garino, and Arlorio (2009) developed a SYBR Green real-time PCR targeting the genomic region of Cor a 8 with a LOD of 10 ppm.

The aim of the present study was to develop a real-time PCR protocol, employing a specific primer set designed on ITS allergen coding sequence, useful to trace the presence of hidden hazelnut in food. This allergenic tree nut was selected not only because it is known to elicit life-threatening anaphylactic reactions but also because an increasing number of foodstuffs are available on the market which may contain hazelnut, either because it was added by intention or because it is present due to contamination. The applicability of the assay was evaluated through screening analysis with the ITS primer pair of a total of 179 different commercial food samples, consisting in a wide variety of brands and types of products.

2. Materials and methods

2.1. Sample selection

Hazelnuts, tree nuts, peanuts and various commercial brands of food were purchased from different local stores and several delicatessen markets, and stored at room temperature in the dark. A wide range of plant and animal species was also included in the assays for specificity control purposes (Table 1).

Hazelnuts were finely grounded and binary mixtures of raw and heat treated hazelnut (160 °C for 13 min) in wheat flour containing 0.1, 1, 10, 100, 1000, 10,000 and 100,000 ppm of hazelnuts were prepared to a final weight of 500 g using a kitchen robot (Thermomix, Vorwerk).

2.2. DNA extraction

Two hundred milligrams of each sample were homogenized with 860 µL of extraction buffer, pH 8.0 (10 mM Tris, 150 mM NaCl, 2 mM EDTA and 1% SDS), 100 µL of 5 M guanidine hydrochloride and 40 µL of 20 mg/mL proteinase K (Merck, Darmstadt Germany), and incubated overnight at 55 °C with shaking at 60 rpm. Then, the samples were left to cool at room temperature. Five hundred mL of chloroform (Sigma–Aldrich) were added to the lysate before centrifugation at 16,438g for 10 min.

Genomic DNA from the clear aqueous supernatant obtained after the centrifugation (500 µL) was purified using the Wizard DNA Clean-up System kit (Promega, Madison, WI, USA) as described by López-Calleja et al. (2007). The DNA was eluted in 50 µL of

Table 1
specify of the hazelnut real-time PCR system.

Common name	Scientific name	ITS H.S.S.	18S rRNA P.A.C.
Hazelnut	<i>Corylus avellana</i>	11.08 ± 0.01 ^a	14.53 ± 0.02
Peanut	<i>Arachis hypogaea</i>	–	16.61 ± 0.01
Nut	<i>Juglans regia</i>	–	15.02 ± 0.04
Pistachio	<i>Pistacia vera</i>	–	14.15 ± 0.06
Macadamia	<i>Macadamia intergrifolia</i>	–	16.17 ± 0.01
Cashew nut	<i>Anacardium occidentale</i>	–	15.13 ± 0.04
Almond	<i>Prunus dulcis</i>	–	14.79 ± 0.03
Brazil nut	<i>Bertholletia excelsa</i>	–	16.13 ± 0.01
Pecan	<i>Carya illinoensis</i>	–	16.20 ± 0.01
Soybean	<i>Glycine max</i>	–	16.52 ± 0.06
Tiger Nut	<i>Cyperus esculentum</i>	–	15.46 ± 0.04
Lupine	<i>Lupinus albus</i>	–	15.48 ± 0.03
Acorn	<i>Quercus ilex</i>	–	14.72 ± 0.02
Chestnut	<i>Aesculus hippocastanum</i>	–	16.38 ± 0.03
Sesame	<i>Sesamum indicum</i>	–	15.78 ± 0.01
Pine nut	<i>Pinus pinea</i>	–	16.34 ± 0.01
Barley	<i>Hordeum vulgare</i>	–	14.32 ± 0.06
Oat	<i>Avena sativa</i>	–	14.01 ± 0.03
Rye	<i>Secale cereale</i>	–	14.23 ± 0.02
Rice	<i>Oryza sativa</i>	–	14.56 ± 0.04
Sunflower	<i>Helianthus annuus</i>	–	14.11 ± 0.02
Maize	<i>Zea mays</i>	–	15.11 ± 0.06
Wheat	<i>Triticum aestivum</i>	–	13.55 ± 0.01
Cocoa	<i>Theobroma cacao</i>	–	15.05 ± 0.05
Orange	<i>Citrus Sinesis</i>	–	13.87 ± 0.01
Potato	<i>Solanum tuberosum</i>	–	14.02 ± 0.02
Olive	<i>Olea europaea</i>	–	16.26 ± 0.07
Cattle	<i>Bos taurus</i>	–	13.98 ± 0.00
Sheep	<i>Ovis aries</i>	–	14.12 ± 0.02
Goat	<i>Capra hircus</i>	–	15.44 ± 0.01
Swine	<i>Sus scrofa domestica</i>	–	14.34 ± 0.07

Minus sign indicates no positive signal after 50 PCR cycles. ITS H.S.S.: Hazelnut-specific system on the Internal Transcribed Spacer (*HazelnutITSdir* and *HazelnutITSinv*). 18S rRNA P.A.C.: Positive amplification control (*18Sdir/18Sinv* and *18SP*) for the hazelnut system on the 18S rRNA gene.

^a Average Cp value ± SD shown from triplicate PCR reactions from each DNA extraction.

sterile deionized water. DNA concentration was measured with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Montchanin, DE). Unless otherwise stated, three DNA replicates were extracted from each sample. A negative control sample was included in every DNA extraction.

2.3. Oligonucleotide primers and probes

The oligonucleotides used in the real-time PCR assay were designed upon the Internal Transcribed Spacer (ITS) and the nuclear 18S rRNA gene sequences from various plant and animal species available in the NCBI (National Center for Biotechnology Information) database.

Sequence alignment analysis of ITS1 sequences from hazelnut (HQ442261), peanut (HQ537458), Brazil nut (HE806059), macadamia (EU642799), pistachio (AY677201), cashew nut (AB071690), almond (HE806329), walnut (HE574850) and pecan (AF303825) allowed the design of hazelnut-specific primers: *HazelnutITSdir*/*HazelnutITSinv* for the amplification of a DNA fragment of 70 bp, respectively. Besides, a dual-labelled TaqMan probe *HazelnutITSP* with a reporter fluorophore at the 5' end (6-carboxyfluorescein, FAM) and a quencher fluorophore at the 3' end (Blackberry, BBQ), were designed to anneal within the ITS1 (Fig. 1) gene fragment generated by amplification of the corresponding target.

As a positive amplification control of real-time PCR experiments, a pair of universal primers (*18Sdir*/*18Sinv*) and a TaqMan probe (*18SP*) were designed on a conserved 18S rRNA gene fragment in all eukaryotic cells. This PCR system was expected to yield amplicons of the same length (approximately 77 bp) in all species analyzed in this work.

The EMMA programme included in the EMBOSS software package version 2.0 and the Primer Express 2.0 software (Perkin-Elmer/Applied Biosystems Division, Foster City, CA) were used for sequence alignment and primer design. TaqMan probes were designed and synthesized by TibMolBiol (Berlin, Germany). The sequences and description of every primer and probe used in this study are listed in Table 2.

2.4. Real-time PCR

Real-time PCR was run under generic cycling conditions. The optimum PCR concentrations of primers yielding the highest end-point fluorescence and the lowest C_p were experimentally determined for each set of primers: 300 nM for forward primers and 900 nM for reverse primers. The PCR reactions were carried out using the LightCycler® TaqMan® Master (Roche Diagnostics GmbH, Mannheim, Germany). 2 pmol of each TaqMan probe (TibMolBiol), and 2 µL of extracted DNA. Amplification reactions were done in a total reaction volume of 10 µL in a glass capillary tube and were run on the LightCycler 2.0 Instrument (Roche Applied Science, Pensberg, Germany) with the following programme: an amplification programme of 55 cycles at 95 °C for 5 s and 60 °C for 30 s. Samples were then cooled to 40 °C for 30 s. This programme was used to amplify the species-specific PCR system, along with the positive amplification control. Unless otherwise indicated, all real-time PCR reactions were carried out in triplicate for each DNA extract.

The crossing point value (C_p), which refers to the cycle number where the sample's fluorescence significantly increases above the background level, was calculated automatically by the LightCycler software as the first maximum of the second derivative of the curve. The continual measurement of fluorescence is related to the amount of amplicon in the real-time PCR, yielding a qualitative result on the presence of the target species.

2.5. Construction of standard curves and data analysis

To assess the efficiency, linear range and analytical sensitivity of the hazelnut-specific system (ITS1), a single standard curve was constructed using two arrays of binary mixtures of known hazelnuts' content (Fig. 2: raw and heat treated hazelnut/wheat flour) rendered under homogeneous conditions and containing increasing amounts of target material. The amount of target DNA in an unknown sample can be then measured by extrapolation of the C_p value obtained in the unknown sample in the corresponding standard curve of C_p values generated from known DNA concentrations of the target species. In addition, the correlation between the variables, crossing point (C_p) and concentration ($[]$) is semilogarithmic:

$$C_p = b \log [] + a$$

where b is the slope and a is the intercept.

Linearity test, sensitivity, accuracy and precision parameters of the species-specific real-time PCR systems were evaluated according to previously described methods (Camacho, Torres, Gil-Alegre, Obregón, & Ruz, 1993; International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use [ICH], 2005). To carry out the validation of the real-time PCR technique developed in this work, three separate DNA extractions of each hazelnut concentration were assayed in different days, using three replicates of each sample. Precision of the assay was evaluated by comparing the coefficient of variation (CV) values obtained in three different days versus those obtained in the same day, to assess the influence of random events (day of analysis, analyst, equipment, etc.) in the data obtained.

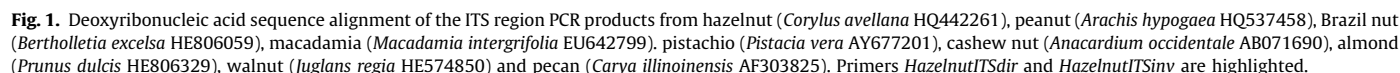
3. Results and discussion

3.1. Hazelnut-specific system on the ITS gene

The real-time PCR assay developed in this work was based on the Internal Transcribed Spacer 1 region. The ITS region results an ideal candidate for primer designing, due to the high copy number of rRNA genes easy to amplify even from small quantities of DNA which contribute to increase the sensitivity of the present PCR. Also, the presence of a multi-copy gene makes easier the recovery of at least a few copies when subjected to intense thermal processing conditions. Commercial production processes of tree nuts and peanuts involve heat treatment or roasting which degrades DNA, thereby the selection of a short ITS1 amplicon of 70 bp is relevant for real-time PCR analysis aimed to detection of hazelnut in commercial food products. Moreover, the ITS gene also presents a high degree of sequence variation required for identification of closely related species. In addition, the use of a conserved region in the nuclear 18S rRNA gene would provide a positive control as described before.

3.2. Specificity and sensitivity

Specificity and sensitivity are two important indices of performance of qualitative assays. The close phylogenetic relationships among hazelnut and tree nut species, together with the varied number of plants and animals components that can be present in different commercial food products indicates the need to check the cross-reactivity of the PCR against a wide range of species. Specificity of the hazelnut real-time PCR system (primers and probe) on the ITS1 was therefore assessed by analysis of the DNA extracted from 26 plant species and 4 animal species. As expected, the hazelnut-specific PCR system on the ITS1 suc-



Primers	Length (bp)	Sequence (5' → 3')	Description	Target gene	Fragment length (bp)
<i>HazelnutITSdir</i>	20	GAGACACTCGTGCCTTCTTG	Hazelnut-specific forward primer	ITS	70
<i>HazelnutITSinv</i>	17	GGAGCACTCTTTAGTTGAAGTTCC	Hazelnut-specific reverse primer		
<i>18Sdir</i>	16	TGGTGCCACGACGCCGC	Positive control forward primer	18S rRNA	77
<i>18Sinv</i>	25	TCCAACCTACGAGCTTTTAACTGCA	Positive control reverse primer		
Probes					
<i>HazelnutTSP</i>	18	6FAM-GCGCCGGGGTTCGTTGTT-BBQ	Hazelnut probe	ITS	
<i>18SP</i>	22	6FAM-CGCTATTGGAGCTGGAATTACC-BBQ	Universal probe	18S rRNA	

As shown in Fig. 2, the resulting slope of the linear equation is -3.44 for raw and heat treated hazelnut/wheat ITS1 hazelnut-spe-

cific system. A part from the demand for specificity, real-time PCR methods intended for species detection in commercial food products should be aimed at reaching a good sensitivity level when highly degraded DNA is present as consequence of high temperature treatments (Van Raamsdonk et al., 2007). Prior to consumption, hazelnuts usually undergo heat treatments like blanching or roasting. Therefore, it represents a material that might be comparable to the hazelnuts the consumer is exposed to. In this way, real-time PCR results indicate effective amplification of the desired DNA segments in all raw and heat-treated mixtures, confirming the ability of the PCR to amplify relatively short segments in highly damaged DNA.

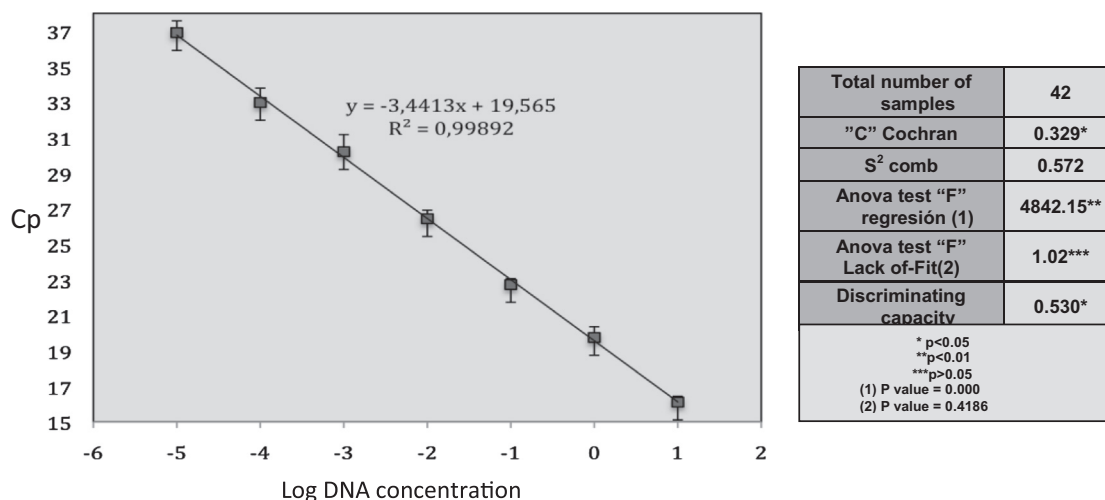


Fig. 2. Linearity test, regression line and sensitivity parameters of the hazelnut-specific TaqMan system on the ITS 1 region, using the mean value of three different DNA extractions of seven different hazelnut concentrations (100,000, 10,000, 1000, 100, 10, 1, 0.1 ppm) for both binary mixtures of raw and heat treated hazelnut/wheat flour analyzed together.

The mean Cp values obtained from the hazelnut calibration curve, plotted against the logarithm of the DNA concentrations, were used to test the sensitivity of the real-time PCR method.

Fig. 2 also shows the discriminating capacity, which is the least difference in logarithm of target DNA concentration in the sample that the analytical method can discriminate with a significant

Table 3

Results of the hazelnut real-time PCR analysis of 179 commercial products.

	Number of samples analyzed	ITS H.S.S. (Cp)	18S rRNA(Cp)
<i>Commercial food products that declared hazelnut in the labelling (30)</i>			
Chocolate	5	+	+
Bonbon	9	+	+
Biscuit	3	+	+
Nut bar	3	+	+
Cereal	5	+	+
Chocolate cream	2	+	+
Yogurt	1	+	+
Beverage	1	+	+
Bread	1	+	+
<i>Commercial food products that may contain traces of hazelnut (61)</i>			
Chocolate	15	+(9)/(–(6))	+
Bonbon	15	+	+
Biscuit	8	+(6)/(–(2))	+
Nut bar	9	+(4)/(–(5))	+
Cereal	6	+(4)/(–(2))	+
Ice cream	2	+(1)/(–(1))	+
Cashew cream	1	+	+
Rice Cake	2	+	+
Bread	2	+(1)/(–(1))	+
Meat products	1	+	+
<i>Commercial food products that not declared hazelnut nor traces on the label (88)</i>			
Chocolate	13	+(6)/(–(7))	+
Bonbon	5	+(4)/(–(1))	+
Biscuit	19	+(7)/(–(12))	+
Nut bar	10	+(4)/(–(6))	+
Cereal	1	–	+
Ice cream	6	+(2)/(–(4))	+
Bread	4	+(1)/(–(3))	+
Chocolate cream	1	–	+
Beverage	15	+(9)/(–(6))	+
Precooked meal	2	+(1)/(–(1))	+
Nougat	1	–	+
Rice cake	3	+(2)/(–(1))	+
Meat products	7	+(3)/(–(4))	+
Yogurt	1	+	+
Vegetable hamburger	1	–	+

(+) Plus sign indicates positive signal before 50 PCR cycles.

(–) Minus sign indicates no positive signal after 50 PCR cycles.

Table 4

Real-time PCR results obtained with the ITS hazelnut-specific system and 18S rRNA positive amplification control in the analysis of commercial food products that not declared hazelnut nor traces on the label.

Commercial food product	Ingredients declared on the label	H.S.S. (Cp)	P.A.C. (Cp)	PPMS (parts per million)
Chocolate-1	Cocoa paste, maltitol, natural vanilla, soy lecithin	34.58 ± 0.01	15.82 ± 0.08	0.5
Chocolate-2	Milk chocolate (sugar, cocoa butter, milk ingredients, cocoa mass, lactose, soy lecithin, polyglycerol polyricinoleate, artificial flavour), enriched flour (flour, niacin, reduced iron, thiamine mononitrate, riboflavin, folic acid), sugar, hydrolyzed palm and palm kernel oil, corn syrup, milk ingredients, dextrose, salt, cocoa mass, sodium bicarbonate, soy lecithin, soybean oil, artificial flavour. May contain traces of peanuts	38.39 ± 0.02	15.92 ± 0.02	0.03
Chocolate-3	Sugar, wheat flour, vegetable fat, cocoa butter, skimmed milk powder, cocoa mass, whey powder, clarified butter, emulsifier: lecithin (soya), lactose, glucose syrup, powdered egg yolk, salt, whole milk powder, raising agents: sodium bicarbonate, flavouring, spices	41.97 ± 0.02	15.01 ± 0.01	0.003
Chocolate-4	Coverage of milk chocolate, black chocolate coverage, wheat flour, maltodextrin, glucomannan, fructose	30.47 ± 0.02	15.56 ± 0.02	6.7
Chocolate-5	Cocoa butter, sugar, skimmed milk powder, butter, emulsifier (soy lecithin) and vanilla	36.45 ± 0.01	15.22 ± 0.06	0.12
Chocolate-6	Organic milk chocolate, cane sugar, cocoa butter, whole milk powder, cocoa mass, emulsifier, soy lecithin, vanilla, organic ginger, organic black pepper, organic essential oil of grapefruit	40.78 ± 0.01	15.32 ± 0.02	0.007
Bonbon-1	50% Chocolate, cover 30%: sugar (sucrose) 43.3%, cocoa butter 30.1%, powdered milk 26.6%, viscosifier 0.7% (soybean lecithin), Tahitian vanilla), thickener (E414), anti-caking agent (E555), colourant (E172), 50% pistachio (Festucs)	28.98 ± 0.01	15.01 ± 0.02	18
Bonbon-2	Milk chocolate 50%: 34% of cocoa mass, whole milk powder, sugar, cocoa butter, vanilla, emulsifier, soy lecithin, macadamia 50%	30.03 ± 0.01	14.23 ± 0.01	9
Bonbon-3	Cream and pistachio truffle: Sugar, cocoa butter, whole milk powder, cocoa malt, vegetable oils and fats, pistachio, emulsifier, soy lecithin, ground coffee, milk chocolate, cocoa solids, 26% minimum milk solids, 26% minimum, chocolate cocoa solid. May contain traces of gluten and dairy	28.06 ± 0.01	15.86 ± 0.01	34
Bonbon-4	Walnuts with milk chocolate coating. May contain peanuts	26.20 ± 0.02	15.06 ± 0.01	>100
Biscuit-1	Sugar, enriched flour, high oleic canola oil, soybean oil, high fructose corn syrup, cornstarch, salt, soy lecithin, vanillin-an artificial flavour, chocolate, contains: wheat, soy and milk	35.04 ± 0.02	14.01 ± 0.01	0.3
Biscuit-2	Biscuit 35% with vanilla flavour cream 18% and covered with milk chocolate 47%. Sugar, enriched flour, high oleic canola oil, soybean oil, high fructose corn syrup, cornstarch, salt, soy lecithin, vanillin-an artificial flavour, chocolate, milk chocolate covering (sugar, cocoa paste, powder skim milk, lactose, soy lecithin). Contains: wheat, soy and milk	35.77 ± 0.01	15.83 ± 0.02	0.2
Biscuit-3	Unbleached enriched wheat flour (flour, niacin, reduced iron, thiamin mononitrate (vitamin B1), Riboflavin (vitamin B2), folic acid), sugar, semi-sweet chocolate (sugar, chocolate liquor, cocoa butter, dextrose, soy lecithin added as an emulsifier, vanilla extract), fructose, vegetable oils (palm oil), invert sugar, butter, cocoa processed with alkali (dutch), nonfat milk, whole eggs, contains 2% or less of: corn syrup solids, leavening (baking soda, cream of tartar, ammonium bicarbonate), rice starch (caramel colour added), salt, natural flavours, pectin, canola oil, wheat flour, wheat gluten, sodium stearoyl lactylate, calcium stearoyl lactylate and date (dough conditioner)	37.09 ± 0.01	16.78 ± 0.08	0.08
Biscuit-4	Semi-sweet chocolate (sugar, chocolate liquor, cocoa butter, dextrose, soy lecithin added as an emulsifier, vanilla extract), unbleached enriched wheat flour (flour, niacin, reduced iron, thiamin mononitrate (vitamin B1), riboflavin (vitamin B2), folic acid), sugar, (palm oil), butter (milk), whole eggs, brown sugar, contains 2 percent or less of: leavening (baking soda, ammonium bicarbonate, cream of tartar), butter oil, salt, natural flavour and caramel colour	37.52 ± 0.03	14.23 ± 0.01	0.06
Biscuit-5	Black chocolate coating 60%, cocoa mass, sugar, cocoa butter, emulsifier, soy lecithin, vanilla aroma, wheat flour, sugar, butter, white chocolate covering, almonds 4%. This product may contain traces of peanuts, egg and sesame	33.08 ± 0.07	14.81 ± 0.03	1.2
Biscuit-6	Cereal flour (wheat), sugar, vegetable fat (with antioxidant E-306), glucose and fructose syrup, vegetable fibre (4%), raising agents (E-550ii, E-503ii), salt, emulsifier (E-322) and colouring (E-150 a). May contain traces of egg, soy, milk and sulphites	38.65 ± 0.02	15.34 ± 0.03	0.02
Biscuit-7	Sunflower oil, fructose (18.8%), water, whole wheat flour (17.2%), potato maltodextrin, potato starch, egg albumin, flavouring, emulsifier (momo and diglycerides of fatty acids), whole egg powder gasifiers. May contain traces of milk and soy	36.63 ± 0.02	16.01 ± 0.03	0.1
Beverage-1	Water, almonds 8%, raw cane sugar, corn maltodextrin	36.73 ± 0.01	15.81 ± 0.03	0.1
Beverage-2 (Brand a)	Water, macadamia nuts 4.5%, raw cane sugar, corn maltodextrin, marine salt. It is manufactured in a facility that processes soy and oats	36.48 ± 0.01	14.82 ± 0.01	0.1
Beverage-2 (Brand b)	Water, macadamia nuts 4.5%, raw cane sugar, corn maltodextrin, marine salt. It is manufactured in a facility that processes soy and oats	37.12 ± 0.01	14.82 ± 0.01	0.08
Beverage-3	Water Kamut, khorasan wheat, agave syrup, inulin, high oleic sunflower oil, concentrated goji berry juice, sea salt	39.02 ± 0.01	15.45 ± 0.02	0.02
Beverage-4	Water, tiger nuts (12%) agave syrup, corn maltodextrin, tapioca starch and emulsifier: sunflower lecithin	35.12 ± 0.06	15.12 ± 0.06	0.30
Beverage-5 (Brand a)	Water, hulled soya beans, apple juice concentrate, tricalcium phosphate, sea salt	37.82 ± 0.01	15.72 ± 0.02	0.05
Beverage-5 (Brand b)	Water, hulled soya beans, apple juice concentrate, tricalcium phosphate, sea salt	38.93 ± 0.01	16.01 ± 0.02	0.02
Beverage-6 (Brand a)	Water, brown sugar cane, soya beans shelled corn maltodextrin, chocolate, cocoa degreasing, sea salt, vanilla extract	38.23 ± 0.01	15.93 ± 0.01	0.04
Beverage-6 (Brand b)	Water, brown sugar cane, soya beans shelled corn maltodextrin, chocolate, cocoa degreasing, sea salt, vanilla extract	39.01 ± 0.01	15.32 ± 0.01	0.02
Rice cake-1	Brown rice, sea salt	39.50 ± 0.01	15.14 ± 0.08	0.01
Rice cake-2	Dark chocolate 54%, sugar, cocoa mass, cocoa butter, cocoa powder, soy lecithin	36.31 ± 0.08	16.01 ± 0.08	0.14

(continued on next page)

Table 4 (continued)

Commercial food product	Ingredients declared on the label	H.S.S. (Cp)	P.A.C. (Cp)	PPMS (parts per million)
Nut bar-1	Sesame, sugar, glucose syrup	35.54 ± 0.01	16.23 ± 0.08	0.23
Nut bar-2	Ground whole soybeans, raisin, sugar, butter, egg, dried coconut, maltodextrin, dried wild blueberries, white chocolate chips, dried cranberries, salt, nonfat milk, mutual flavours, canola oil, fermented milk powder, powdered whole milk	40.12 ± 0.01	15.12 ± 0.08	0.01
Nut bar-3	Ground whole soybeans, raisin, sugar, butter, egg, dried coconut, dried strawberry, dried pineapple, maltodextrin, chocolate chips, dried cranberries, salt, nonfat milk, mutual flavours, canola oil, fermented milk powder, powdered whole milk	39.78 ± 0.01	16.42 ± 0.08	0.01
Nut bar-4	Ground whole soybeans, raisin, sugar, butter, egg, dried coconut, dried orange, dried pineapple, maltodextrin, white chocolate chips, dried cranberries, salt, nonfat milk, mutual flavours, canola oil, fermented milk powder, powdered whole milk	39.12 ± 0.01	16.22 ± 0.08	0.02
Ice cream-1	Rehydrated skimmed milk powder, sugar, cocoa butter, glucose syrup – fructose, skimmed milk powder, vegetable fat, concentrated butter, cocoa paste, wheat flour, lactose and milk proteins, peanuts, stabilizers, emulsifiers, salt, raising agent, flavourings, colourings	40.12 ± 0.01	15.57 ± 0.02	0.01
Ice cream-2	Milk, cream, sugar, whey, mono and diglycerides, locust bean gum, vanilla bean specks, natural flavour, annatto extract, Belgian white chocolate coating, sugar, cocoa butter, milk, milk fat, soy lecithin	39.97 ± 0.01	16.12 ± 0.02	0.01
Precooked meal-1	Potato 91%, vegetable oil, onion extract, rice and wheat starch, salt, milk powder, celery, May contain traces of peanuts, eggs and soy	33.71 ± 0.01	13.78 ± 0.02	0.7
Yogurt-1	Milk, sugar (6.4%), cream, gelatin, lactase and lactic ferments. May contain traces of soy	35.62 ± 0.02	16.45 ± 0.08	0.23
Bread-1	Wheat flour, yeast, vegetable oil, sesame seeds, corn extract, salt, yeast from beer. Produced in a fabric that also uses sesame and milk	39.00 ± 0.01	15.85 ± 0.08	0.02
Meat product-1	Chicken breast, salt, potato starch, stabilizers, preservatives, glucose syrup, flavourings, antioxidants and sunflower oil. May contain traces of celery, lactose, soy/soy protein, milk protein, mustard, sesame and sulphites	37.52 ± 0.02	15.02 ± 0.02	0.06
Meat product-2	Ham, water, salt, dextrose, sugar, antioxidants, stabilizers and preservative. Contains soy and milk	38.27 ± 0.01	15.67 ± 0.02	0.04
Meat product-3	Mechanically recovered meat form chicken and turkey, skim milk (20%), bacon, pork rind, soy protein, starch, salt, stabilizers, flavourings, antioxidants and preservatives	37.81 ± 0.01	16.01 ± 0.02	0.05

H.S.S.: Hazelnut-specific system on the Internal Transcribed Spacer; P.A.C.: Positive amplification control for the peanut system on the 18S rRNA gene.

level. Linearity of the hazelnut real-time PCR response was also analyzed. In each PCR system, the following parameters were evaluated: (a) Cochran's test, which determined whether the variances of the responses obtained for each concentration of hazelnut DNA in the reference feeds were homogeneous, (b) regression analysis and (c) variance analysis with lack of-fit. All parameters were evaluated following previously described validation protocols.

In order to determine the accuracy of the ITS1 hazelnut-specific system seven concentrations on the ITS1 (0.1, 1, 10, 100, 1000, 10,000 and 100,000 ppm) of reference samples were elaborated and analyzed. Accuracy is reported as percentage recovery by the assay of known added amounts of analyte. ITS1 primers were able to detect up to 0.1 ppm. Using the Snedecor *F*-test, it was verified that the concentration of DNA present in a sample did not affect the variation of the results. The values for Student's *t*-test obtained demonstrated that the method was accurate, since no significant differences between 100% and the mean recovery values were detected. The influence of day of analysis on the precision of the assay was also tested by comparing the coefficients of variation (CV) of three separate DNA extractions performed on the 0.1 ppm ITS1, analyzed in duplicate three times on the same day, versus the values obtained for the same samples on three different days. Results showed significant differences with higher CV values when the assay was done on different days with respect to the same day. Thus, a standard curve should be determined on each day of analysis.

3.3. Commercial food products analysis

The method was also tested with regard to its suitability for the detection of hazelnut DNA in commercial food products. In this way, the ITS hazelnut-specific system was applied to the analysis of a collection of 179 commercial food products of different brands with different label declaration: 30 containing hazelnut as an ingredient, 61 may contain traces of hazelnut and 88 that had no traces of hazelnuts. PCR results were found to conform to the labelling in all samples except for 40 food samples with undeclared hazelnut in the labelling (Table 3).

To assess the quantification ability of the assay, extrapolation analysis of the Cp data obtained for different commercial products (chocolates, bonbon, biscuits, cereal, nut bars, ice cream, chocolate cream, yogurt, beverage, bread, precooked meal, nougat and rice cake) was carried out using the linear regression equations of the reference curves in order to determine whether the detected hazelnut content (%) of each sample fitted with the declared (real) one. Table 4 shows the average Cp values obtained for all the food commercial samples and the content estimated for these food products by substitution of the Cps in the corresponding P.S.S. equations. From the extrapolation of the different Cps obtained for this food samples, we estimated that: 37 of the 40 samples with an undeclared hazelnut content contained less than 10 ppm of hazelnut, whereas from the other three: 1 contained 18 ppm, another 34 ppm and the other one contained more than 100 ppm of hazelnut. For those commercial food products that gave positive the presence of hazelnut, it was probably due to contamination during the production, as they were produced by companies which also process hazelnuts.

The degree of contamination of commercial food samples with hazelnuts, whether fraudulent or accidental, can cause a serious public health problem in sensitive individuals and, therefore, detection methods should provide a selective and sensitive detection level. One of the achievements of the assay described in this work is the ability to specifically detect down to 0.1 ppm of the target in food samples, which is one of the lowest detection limits achieved to the moment. Methods for analysis of the hazelnut

content in food are necessary to support legislation. Several sandwich ELISA-based methods are available for the detection of hazelnuts in food (Ben Rejeb et al., 2003; Kiening et al., 2005). Holzhauser and Vieths (1999) developed a hazelnut-specific sandwich-type ELISA based on polyclonal antibody with a 2 ppm LOD. On the other hand, Pele, Brohee, Anklam, and van Hengel (2007) reported an ELISA kit for hazelnut detection with a 1.5 ppm LOD. These immunochemical methods are highly sensitive, however, they may suffer of cross-reactivity with other tree nuts.

In this way, alternative methods based on the polymerase chain reaction (PCR) have been developed for the specific identification of hazelnut: Píknova et al. (2008) reported a real-time PCR based on the Cora 11 gene with a detection limit of 10 ppm. Whereas Schöringhumer, Redl, and Cichna-Markl (2009) described a duplex real-time PCR for the detection of sesame and hazelnut, based on the Cora1 gene for hazelnut detection with a LOD of 5 ppm. Koppel et al. (2010), developed two tetraplex real-time PCR assays for the detection of several allergens, with a detection limit of 100 ppm on hazelnut, based on Cora1 gene. On the other hand, Ehlert, Demmel, Hupfer, Busch, and Engel (2009) and Mustorp et al. (2011) reported a LOD assay for the simultaneous detection of several allergens based on Cora1 gene for hazelnut detection with a LOD of 5 and 1 ppm, respectively.

This observation suggests that the real-time PCR method proposed herein is beneficial because due to its high sensitivity it facilitates the detection of hazelnut traces in commercial food products. Moreover, this real-time PCR can also be useful for monitoring the effectiveness of cleaning processes from production units of the food industry, and as consequence, they can help to prevent the food allergic consumer from unintentional ingestion of hidden allergens.

4. Conclusions

The ITS hazelnut-specific system was applied to the analysis of a collection of 179 commercial food products, and PCR results were found to conform to the labelling in all samples but 40 which gave positive results by real-time PCR, and didn't declare the presence of hazelnut nor its traces in the labelling. This demonstrates the potential of the real-time PCR-based method to produce accurate results at the analysis of commercial products. As demonstrated, the presented PCR method is highly sensitive and selective, which makes it suitable for the detection of small amounts of hazelnuts in food samples. The method is relatively straightforward, it can be easily implemented in any analytical laboratory routinely performing real-time PCR. Consequently, this methodology could be used in inspection programmes to enforce accurate labelling of commercial food products, thereby protecting both producers and consumers against hazelnut adulteration and misrepresentation.

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