



Survey of undeclared allergenic pistachio (*Pistacia vera*) in commercial foods by hydrolysis probe real-time PCR



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ABSTRACT

Tree nut allergies represent an important health problem in industrialized countries. Among these, pistachio (*Pistacia vera*) kernels which are consumed as snack foods and used as ingredients in confectionery, chocolates, meat products, and ice-cream industries have been reported to cause IgE-mediated allergic reactions. Trace amounts of undeclared pistachio allergens can cause serious health risks for food-allergic consumers. In order to provide an appropriate method for the detection of pistachio in food products, a real-time polymerase chain reaction (PCR) system for the specific and sensitive detection of pistachio was developed. The sensitivity was investigated on spiked wheat flour samples with defined raw and heat-treated pistachio contents (0.1–100,000 mg kg⁻¹). The real-time PCR detected pistachio in these mixtures down to the lowest investigated spike level of 0.1 mg kg⁻¹. In addition, analysis of different retail samples from the market was performed to demonstrate the suitability of the assay in the food industry. The real-time PCR results obtained from the analysis of 229 commercial food products revealed 29 that didn't declare pistachio or traces on the label but were found to contain pistachio. The presented real time PCR method is useful for relatively fast, highly selective, and sensitive detection of pistachio in food samples.

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1. Introduction

Pistachio nuts, the fruits of the pistachio tree (*Pistacia vera*), are edible tree nuts highly valued for their organoleptic properties. The major pistachio producing countries of the world are, in decreasing order: Iran, United States (where all production occurs in California), Turkey and to a lesser extent, Syria, India, Greece and Pakistan. Nowadays, California has become one of the largest pistachio producers in the world, competing with other countries in the international market (Ahmad, Ferguson, & Southwick, 2003).

Because of its high nutritional value and split shell, pistachio is an increasingly important nut crop. Pistachio nuts are consumed as snack foods and used as ingredients in confectionery, chocolates, meat products, ice-cream industries and other gourmet products. However, pistachios may represent a threat to some consumers since they contain allergens that may cause severe allergy in a part of population (Fernández, Fiandor, Martínez-Garate, & Martínez

Quesada, 1995; Jansen, Lijster, Toorenen-Bergen, & Wijk, 1992; Parra et al., 1993; Roux, Teuber, & Sathe, 2003). The prevalence of clinical allergy to peanuts and nuts is estimated at about 0.4%–1.1% of the adult population, representing the allergy to pistachio the 7% of the cases of tree nut causing allergic reactions (EFSA, 2004; Sicherer, Furlong, Muñoz-Furlong, Burks, & Sampson, 2001). Undeclared pistachios may be present in food products as a result of adventitious contamination or fraudulent substitution. The former may occur by cross-contamination when various nuts are used in the same food processing facility and should be considered in the labeling. In this way, European legislation (EU) 1169/2011/EC (OJEU, 2011) requires labeling of food products in respect of the presence of allergenic components including pistachios among other tree nuts. Therefore, methods for the analysis of the pistachio content in food are required in order to enforce the legislation.

Enzyme-linked immunosorbent assay (ELISA) is the most frequently used analytical method to detect tree nut allergens (Akkerdaas et al., 2004; Kiening et al., 2005; Rejeb, Abbot, Davies, Cleroux, & Delahaut, 2005). However, up to date there has been no published work based on immunological techniques for pistachio nut allergen detection. Recently, ELISA kits for the detection of pistachios in food have become commercially available (Astori Tecnica; Bio-Check; Romer and Genon Laboratories).

Abbreviations: PSS, pistachio specific system; PAC, positive amplification control.

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On the other hand, DNA-based methods have been increasingly used as highly sensitive and specific alternatives for allergen detection, taking advantage of the greater thermal stability of DNA molecules compared to proteins (Poms, Anklaam, & Kuhn, 2004). In this way, real-time PCR technology has proved to be very effective for the detection of food allergens (Brezna, Dudánová, & Kuchta, 2008; Brezna, Hudecova, & Kuchta, 2006; Brezna & Kuchta, 2008; De la Cruz et al., 2013; López-Calleja et al., 2013a, 2013b; Pafundo, Gulli, & Marmiroli, 2009, 2010; Platteau, De Loose, De Meulenaer, & Taverniers, 2011; Poms, Klein, & Anklaam, 2004; Scaravelli, Brohee, Marchelli, & Van Hengel, 2008). Although, currently, PCR techniques described in bibliography for pistachio detection are scarce. Koppel, Velsen-Zimmerli, and Bucher (2012) developed a multiple-target assay based on a real-time multiplex PCR to detect milk, egg, sesame, walnut, almond and pistachio. This method enabled the detection of 1000 mg kg⁻¹ of pistachio (Pis v 1 gene). On the other hand, Ehlert, Demmel, Hupfer, Busch, and Engel (2009) reported a ligation-dependent probe amplification (LPA) system for the simultaneous detection of pistachio (COR gene) and other allergens with an LOD of 5 mg kg⁻¹. While Brezna et al. (2008) presented a real-time PCR system based on ITS region for the detection of pistachio allergen in foods, with an LOD of 4 mg kg⁻¹. Finally, Barbieri and Frigeri (2006) developed a PCR technique based on the COR gene to detect pistachio allergen traces in mortadella with an LOD of 100 mg kg⁻¹. On the other hand, Ahmad et al. (2003) reported the detection of pistachio with the use of microsatellite markers.

Here we describe the development of a real-time PCR method for the specific detection of traces of pistachio based on selective amplification of the ITS1 region. The applicability of the assay for pistachio mislabeling was evaluated through screening of 229 commercial food products.

2. Materials and methods

2.1. Sample selection

Pistachios, 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. Commercial pistachios from Iran, California and Turkey were considered for analysis. A wide range of plant and animal species was also included in the assays for specificity control purposes (Table 1).

Pistachios (California) were finely ground and two separate series of binary mixtures: raw in wheat flour and heat treated pistachio (160 °C for 13 min) in wheat flour containing 0.1, 1, 10, 100, 1000, 10,000 and 100,000 mg kg⁻¹ of pistachios were prepared for each series, to a final weight of 500 g using a kitchen robot (Thermomix, Vorwerk). Wheat flour samples containing 100,000 mg kg⁻¹ of pistachio were prepared by diluting 50 g of ground pistachios either raw or heat treated with 450 g of wheat flour by using the Thermomix at maximum speed (9000 rpm) during five minutes. 10-fold dilutions were repeated until 0.1 mg kg⁻¹ samples were obtained.

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⁻¹ 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

Table 1
Specificity of the pistachio real-time PCR system.

Common name	Scientific name	ITS P.S.S	18S rRNA P.A.C
Pistachio	<i>Pistacia vera</i>	12.36 ± 0.01 ^a	14.15 ± 0.06
Walnut	<i>Juglans regia</i>	–	14.79 ± 0.03
Hazelnut	<i>Corylus avellana</i>	–	14.53 ± 0.02
Peanut	<i>Arachis hypogaea</i>	–	16.61 ± 0.01
Almond	<i>Prunus dulcis</i>	–	15.02 ± 0.04
Macadamia	<i>Macadamia intergrifolia</i>	–	16.17 ± 0.01
Cashew nut	<i>Anacardium occidentale</i>	–	15.13 ± 0.04
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
Mango	<i>Mangifera indica</i>	–	15.87 ± 0.06
Green bean	<i>Phaseolus vulgaris</i>	–	16.01 ± 0.02
Green pea	<i>Pisum sativum</i>	–	15.01 ± 0.01
Chickpea	<i>Cicer arietinum</i>	–	14.87 ± 0.01
Lentil	<i>Lens culinaris</i>	–	15.21 ± 0.02
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
Grape	<i>Vitis vinifera</i>	–	15.04 ± 0.01
Pear	<i>Pyrus rosaceae</i>	–	14.81 ± 0.01
Apple	<i>Malus domestica</i>	–	14.43 ± 0.01
Apricot	<i>Prunus armeniaca</i>	–	15.34 ± 0.01
Cherry	<i>Prunus avium</i>	–	16.03 ± 0.01
Sour cherry	<i>Prunus cerasus</i>	–	15.21 ± 0.01
Peach	<i>Prunus persica</i>	–	15.03 ± 0.04
Plum	<i>Prunus domestica</i>	–	16.02 ± 0.02
Banana	<i>Musa cavendishii</i>	–	15.87 ± 0.01
Kiwifruit	<i>Actinidia deliciosa</i>	–	13.98 ± 0.01
Watermelon	<i>Citrullus lanatus</i>	–	14.67 ± 0.02
Orange	<i>Citrus sinensis</i>	–	13.87 ± 0.01
Potato	<i>Solanum tuberosum</i>	–	14.02 ± 0.02
Garlic	<i>Allium sativum</i>	–	15.09 ± 0.01
Onion	<i>Allium cepa</i>	–	14.73 ± 0.02
Eggplant	<i>Solanum melongena</i>	–	15.21 ± 0.01
Zucchini	<i>Cucurbita pepo</i>	–	16.34 ± 0.03
Tomato	<i>Solanum lycopersicum</i>	–	13.92 ± 0.01
Asparagus	<i>Asparagus officinalis</i>	–	14.87 ± 0.02
Carrot	<i>Daucus sativus</i>	–	15.45 ± 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

ITS P.S.S: Pistachio-specific system on the Internal Transcribed Spacer 1 (PistITSdir/PistITSinv and PistITSF).

18S rRNA P.A.C: Positive amplification control (18Sdir/18Sinv and 18SP) for eukaryotes on the 18S rRNA gene.

^a Average Cp value ± SD shown from triplicate PCR reactions from each DNA extraction. Minus sign indicates no positive signal after 50 PCR cycles.

hundred µL of chloroform (Sigma Aldrich) were added to the lysate before centrifugation at 16,438 g for 10 min.

Genomic DNA was obtained from 500 µL of the clear aqueous supernatant 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 sterile deionized water. The 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. The DNA extracted from raw and heat-treated pistachio

was electrophoresed in a 1% D1 (Hispanlab S.A., Torrejón, Spain) agarose gel containing 1 µg/mL ethidium bromide in Tris–acetate buffer (0.04 M Tris–acetate and 0.001 M EDTA, pH 8.0) for 30 min at 110 V. Total DNA was visualized by UV transillumination using a Chemidoc x RS (Bio-Rad Laboratories, Hercules, CA).

2.3. Oligonucleotide primers and probes

The oligonucleotides used in the real-time PCR assay were designed on the *Internal Transcribed Spacer (ITS1)* region and the nuclear 18S rRNA gene sequences from various plant and animal species available in the NCBI (National Center for Biotechnology Information) database (<http://www.ncbi.nlm.nih.gov>).

Alignment and computer analysis of *ITS1* sequences allowed the design of pistachio-specific primers: *PistITSdir*/*PistITSinv* for the amplification of a DNA fragment of 72 bp. Besides, a dual-labeled hydrolysis probe *PistITSP* with a reporter fluorophore at the 5' end (6-carboxyfluorescein, FAM) and a quencher fluorophore at the 3' end (Blackberry, BBQ), was designed to anneal within the *ITS1* (Fig. 1) fragment generated by amplification of the corresponding target. Besides, *18Sdir* and *18Sinv* primers and a hydrolysis probe (*18SP*) labeled with FAM and BBQ, based on conserved 18S rRNA gene sequences available in the Genbank/EMBL database for various plant and animal species were used as positive control of the PCR assay (Pegels, González, García, & Martín, 2011). These primers were expected to amplify a 77 bp conserved fragment from the 18S rRNA gene.

The EMMA program included in the EMBOS 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. hydrolysis 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

The optimum PCR concentrations of primers yielding the highest endpoint fluorescence and the lowest crossing point value (Cp) 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 hydrolysis probe (TibMolBiol), and 100 ng of template 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 program: an amplification program 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 program was used to amplify the species-specific PCR system and subsequent amplification of the positive control. Unless otherwise indicated, all real-time PCR reactions were carried out in triplicate for each DNA extract.

The crossing point value (Cp), 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 fluorescence intensity is related to the amount of amplicon in the real time PCR. In addition, it provides quantitative information 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 *ITS1* pistachio-specific system, a standard curve was constructed using two separate series of binary mixtures of known pistachio content (raw pistachio/wheat flour & heat treated pistachio/wheat flour) and containing increasing amounts of target material.



Fig. 1. Deoxyribonucleic acid sequence alignment of the ITS region PCR products from pistachio (*Pistacia vera* AY677201), walnut (*Juglans regia* HE574850), pecan (*Carya illinoensis* AF303825), cashew nut (*Anacardium occidentale* AB071690), almond (*Prunus dulcis* HE806329), hazelnut (*Corylus avellana* HQ442261), peanut (*Arachis hypogaea* HQ537458), Brazil nut (*Bertholletia excelsa* HE806059) and macadamia (*Macadamia integrifolia* EU642799) available at the GeneBank-EMBL database. Primers *PistITSdir*, *PistITSinv*, and *PistITSP* probe are highlighted.

Table 2
DNA sequences and description of the primers and probes used in this study.

Primers	Length (bp)	Sequence (5'→3')	Description	Target gene	Fragment length (bp)
<i>PistITSdir</i>	20	GGCGTTCGAACAGAACCTA	Pistachio-specific forward primer	ITS1	72
<i>PistITSinv</i>	20	CGAGGGTCAAAGAGTCCGTA	Pistachio-specific reverse primer		
<i>18Sdir</i>	16	TGGTGCCAGCAGCCGC	Positive control forward primer	18S rRNA	77
<i>18Sinv</i>	25	TCCAACCTACGAGCTTTTAACTGCA	Positive control reverse primer		
Probes					
<i>PistITSP</i>	22	6FAM-TCGTGCGGTTGCGTTCTACTGT-BBQ	Pistachio probe	ITS1	
<i>18SP</i>	22	6FAM-CGCTATTGGAGCTGGAATTACC-BBQ	Universal probe	18S rRNA	

The pistachio concentration in an unknown sample (mg kg^{-1}) can be then measured by extrapolation of the Cp value obtained in the “unknown sample” in the standard curve of Cp values generated from known pistachio DNA concentrations. In addition, the correlation between the variables, crossing point (Cp) and concentration ($[\cdot]$) is semilogarithmic:

$$\text{Cp} = b \log[\cdot] + a$$

where b is the slope and a is the intercept.

Linearity test, sensitivity, accuracy and precision parameters of the species-specific real-time PCR system were evaluated according to previously described methods (International Conference on Harmonization 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 pistachio concentration were assayed on different days, using three replicates of each sample. Precision of the assay was evaluated by comparing the coefficient of variation (CV) values obtained on 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 (Statgraphics Centurion XVI).

3. Results and discussion

3.1. Pistachio-specific system on the ITS1 region

3.1.1. Hydrolysis probe-based real-time PCR

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 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 (Fernández et al., 2001). 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 72 bp is relevant for real-time PCR analysis aimed at the detection of pistachio in commercial food products. Moreover, the ITS region 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 eukaryotic nuclear 18S rRNA gene would provide a positive control as described before.

3.1.2. Specificity and sensitivity

Specificity and sensitivity are two important indices of performance of qualitative assays. The close phylogenetic relationships among pistachio 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 pistachio real-time PCR system (primers and probe) on

the ITS1 was therefore assessed by analysis of the DNA extracted from 49 plant species and 4 animal species. No other *Pistacia* species were included because *P. vera* is the only species of commercial importance. The other *Pistacia* species grow in the wild and their seeds are used mainly as rootstock seed source (Kafkas & Perl-Treves, 2002). The pistachio specific PCR system on the ITS1 successfully detected a DNA fragment of 72 bp from pistachio (medium Cp value 12.36 ± 0.09), while no positive amplification signal was obtained on the animal and plant species tested. No differences in Cps were obtained among the varieties of pistachios analyzed. The 18S rRNA eukaryotic system amplified a 77 bp fragment from all samples tested (Table 1).

Two separate binary mixtures of raw California pistachio in wheat flour and heat treated pistachio (160°C for 13 min) in wheat flour, containing increasing amounts of the target pistachio were used to construct the calibration curve using a simple regression model with the log input DNA concentration versus the Cp. Mean values and standard deviation of all the Cp values are plotted in Fig. 2.

As shown in Fig. 2, the resulting slope of the linear equation is -3.21 for raw and heat treated pistachio in wheat flour using the ITS1 pistachio-specific real-time PCR system. Apart 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 even when these food products are submitted to high temperature treatments (Van Raamsdonk et al., 2007). Prior to consumption, pistachios usually undergo heat treatments like blanching or roasting. In this way, the 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 fragments.

The mean Cp values and standard deviations obtained from the 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 level. Linearity of the pistachio 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 pistachio DNA in the reference mixtures were homogeneous, (b) regression analysis and (c) variance analysis with lack of-fit. All parameters were evaluated following previously described validation protocols (Camacho, Torres, Gil-Alegre, Obregón, & Ruz, 1993).

In order to determine the accuracy of the ITS1 pistachio-specific system, seven concentrations (0.1 , 1 , 10 , 100 , 1000 , $10,000$ and $100,000 \text{ mg kg}^{-1}$) 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 mg kg^{-1} . 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

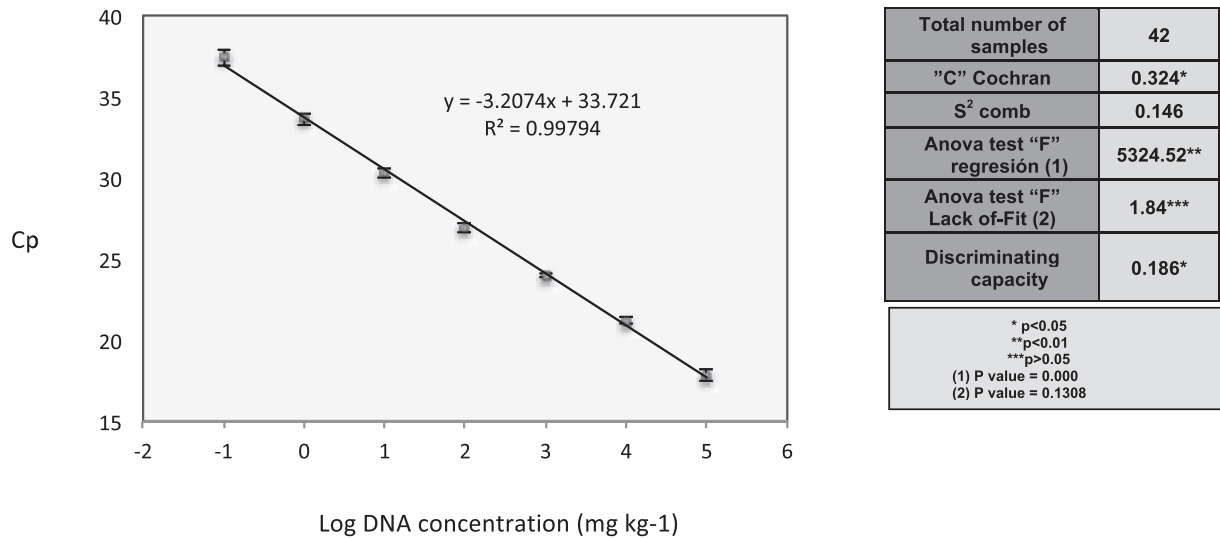


Fig. 2. Linearity test, regression line, sensitivity parameters and standard deviation of the pistachio specific TaqMan system on the *ITS 1* region, using three different DNA extractions of seven different pistachio concentrations (100,000, 10,000, 1000, 100, 10, 1, 0.1 mg kg⁻¹) for both binary mixtures of raw and heat treated pistachio/wheat flour.

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 mg kg⁻¹, three times on the same day, versus the values obtained for the same samples on three different days. Results showed no significant differences.

3.1.3. Commercial food products analysis

The method was also tested with regard to its suitability for the detection of pistachio DNA in commercial food products. In this way, the *ITS* pistachio-specific system was applied to the analysis of a collection of 229 commercial food products of different brands with different label declaration: 11 containing pistachio as an ingredient, 69 carrying the precautionary statement "may contain traces of nuts" and 149 "without pistachios or nuts mentioned on the label". Pistachio DNA was detected in all 11 food samples with pistachio declared as an ingredient, in 9 carrying the precautionary statement "may contain traces of nuts" and in 29 food samples that were tested positive, from the 149 commercial food products which did not carry any labeling referring to pistachios or nuts (Table 3).

Table 4 shows the average Cp values and the content estimated in mg kg⁻¹ for commercial samples with a declared pistachio percentage and those samples that didn't indicate the presence of pistachios or traces on the labeling, but were found to contain pistachio traces. From the extrapolation of the different Cps obtained for the list of products that didn't indicate the presence of pistachios or traces on the labeling, we estimated that 19 of the 29 samples with an undeclared pistachio content contained less than 10 mg kg⁻¹ of pistachio, whereas 3 contained between 10 mg kg⁻¹ and 100 mg kg⁻¹ and the other 7 contained more than 100 mg kg⁻¹ of pistachio. On the other hand, the pistachio estimation content obtained by substitution of the Cps in the corresponding Pistachio Specific System (P.S.S.) equation for seven out of eight commercial food samples with declared pistachio content, were slightly lower to the one indicated in the labeling. This could be because it might actually contain a smaller amount of pistachios than the percentage stated in the list of ingredients or due to a number of other factors, which affect DNA yield such as the extent of DNA degradation due to processing treatments and the presence of different matrices in the samples potentially containing different type and amounts of DNA-yielding cells (milk, fat, seeds, etc.), which might interfere with the PCR reaction. As consequence, quantitative estimation of

target allergen content in a food product would be only useable if samples with identical terms of composition and processing treatment than the analyzed sample are available for the preparation of suitable calibration standards. Nevertheless, considering the great variety of ingredients used in the preparation of commercial food products (chocolate, lecithin, oil, milk, butter, vegetables, etc.) as well as the diversity of processing treatments applied by the food industry, the preparation of calibration standards for each type of product is not feasible from a practical point of view. Some approaches have been recently purposed to compensate for the variability in DNA extraction and amplification in food materials (Demmel, Hupfer, Busch, & Engel, 2012; Hirao, Hiramoto, Imai, & Kato, 2006), quantification of pistachio in commercial products is affected by the food processing and food matrices analyzed. Thus, accurate quantification would require different standard curves for different products, according to their composition and processing conditions as has been remarked by other authors in previous studies (Fumière, Dubois, Baeten, von Holst, & Berben, 2006; Koppelman & Hefle, 2006; Koppel et al., 2012; Pegels, González, Martín, et al., 2011; Píknova, Pangallo, & Kuchta, 2008; Prado et al., 2007, 2009; Röder, Filbert, & Holzhauser, 2010; Rojas et al., 2010, 2011). The main limiting factor pointed out by these authors is the lack of knowledge of the food product composition, and processing conditions of the analyzed samples, which prevents the construction of appropriate calibration standards. Therefore, although in theory real-time PCR techniques have the potential for quantitative measurements, the reality is that their application for the analysis of real commercial food products would be limited to a qualitative detection of the target "tree nut". Regardless to this fact, for allergic sensitized individuals to determine the presence of the tree nut in a food product that doesn't declare it on the labeling is what is more relevant.

For those commercial food products "without pistachios or nuts" mentioned on the label, that gave positive to the presence of pistachio, it may probably be due to contamination during the production, since they were produced by companies that also process pistachios. The degree of contamination of commercial food samples with pistachios, whether fraudulent or accidental, can cause a serious public health problem in sensitive individuals and, therefore, detection methods should be selective and sensitive detection level. One of the advantages of the assay described in this work is the ability to specifically detect down to 0.1 mg kg⁻¹ of the

Table 3
Results of the pistachio real time PCR analysis of 229 commercial products.

Commercial food products that declared pistachio in the labeling (11)	Number of samples analyzed	ITS P.S.S. (Cp)	18S rRNA (Cp)
Chocolate	1	+	+
Bonbon	2	+	+
Biscuit	1	+	+
Cereal	1	+	+
Meat products	5	+	+
Ice cream	1	+	+
Commercial food products that declared traces of nuts (69)	Number of samples analyzed	ITS P.S.S. (Cp)	18S rRNA (Cp)
Chocolate	8	+(1)/–(7)	+
Bonbon	21	–(21)	+
Rice/corn cake	2	–(2)	+
Biscuit	12	+(1)/–(11)	+
Nut bar	8	–(8)	+
Cereal	6	–(6)	+
Ice cream	3	+(1)/–(2)	+
Yogurt	1	–(1)	+
Bread	7	+(6)/–(1)	+
Precooked meal	1	–(1)	+
Commercial food products that not declared pistachio nor traces on the label (149)	Number of samples analyzed	ITS P.S.S. (Cp)	18S rRNA (Cp)
Chocolate	33	+(6)/–(27)	+
Bonbon	15	+(13)/–(2)	+
Biscuits	23	+(2)/–(21)	+
Nut bar	13	–(13)	+
Cereal	7	+(2)/–(5)	+
Ice cream	10	+(3)/–(7)	+
Bread	10	–(10)	+
Chocolate cream	3	–(3)	+
Peanut cream	1	–(1)	+
Cashew cream	1	–(1)	+
Pecan syrup	1	–(1)	+
Nougat	1	–(1)	+
Beverage	18	–(18)	+
Sauce	1	–(1)	+
Precooked meal	1	–(1)	+
Meat products	7	+(3)/–(4)	+
Rice cake	2	–(2)	+
Yogurt	2	–(2)	+

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

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

target in food samples, which is the lowest detection limits achieved to the moment for pistachio detection. While methods for analysis of the pistachio content in food are necessary to support legislation, no immunoassay-based research works have been published up to date by any author for its detection. However recently, several commercial allergy-testing kits are available for pistachio detection on food. Such as Romer and Genon Laboratories, which have ELISA kits available for pistachio detection with an LOD of 1 mg kg^{–1} and 10 mg kg^{–1}, respectively. While Bio-Check laboratories achieved an LOD of 0.2 mg kg^{–1}.

In this way, alternative methods based on the polymerase chain reaction (PCR) have been developed for the specific identification of this tree nut (Ahmad et al., 2003; Barbieri & Frigeri, 2006; Brezna et al., 2008; Ehlert et al., 2009; Koppel et al., 2012). Nevertheless, none of the published work done so far for pistachio detection have reached a limit of detection so low as the one we present herein.

These observations suggest that the real-time PCR method proposed herein provides an effective method for detecting traces of pistachio in food, and so can be utilized as a screening test of their presence/absence in foodstuffs. In all, these results

Table 4

Real-time PCR results obtained with the ITS pistachio-specific system and 18S rRNA positive amplification control in the analysis of commercial food products with a declared pistachio percentage and those samples that didn't indicate the presence of pistachios or traces on the labeling, but were found to contain pistachio traces.

Commercial products (percentage of pistachios declared on the label)	P.S.S. (Cp)	P.A.C. (Cp)	mg kg ^{–1} (estimated pistachio percentage)
Biscuit-1 (7%)	18.12 ± 0.01	14.92 ± 0.01	73,125 mg kg ^{–1} (7.3%)
Cereal-1 (3%)	20.12 ± 0.01	14.34 ± 0.01	17,398 mg kg ^{–1} (1.74%)
Ice Cream-1 (2%)	20.27 ± 0.01	14.92 ± 0.01	15,622 mg kg ^{–1} (1.56%)
Meat Product-1 (1%)	22.57 ± 0.01	16.21 ± 0.01	2997 mg kg ^{–1} (0.30%)
Meat Product-2 (0.5%)	22.61 ± 0.01	15.33 ± 0.01	3045 mg kg ^{–1} (0.30%)
Meat Product-3 (0.5%)	23.12 ± 0.01	14.92 ± 0.01	2019 mg kg ^{–1} (0.20%)
Meat Product-4 (2%)	20.34 ± 0.01	16.22 ± 0.01	14,856 mg kg ^{–1} (1.48%)
Meat Product-5 (1%)	21.12 ± 0.01	15.01 ± 0.01	8486 mg kg ^{–1} (0.85%)
Commercial products (not declared pistachio nor traces on the label)	P.S.S. (Cp)	P.A.C. (Cp)	mg kg ^{–1}
Bonbon-1	32.34 ± 0.06	14.01 ± 0.02	3 mg kg ^{–1}
Bonbon-2	26.90 ± 0.06	14.23 ± 0.02	134 mg kg ^{–1}
Bonbon-3	25.74 ± 0.06	15.45 ± 0.02	308 mg kg ^{–1}
Bonbon-4	24.54 ± 0.06	16.03 ± 0.02	728 mg kg ^{–1}
Bonbon-5	24.03 ± 0.06	15.78 ± 0.02	1050 mg kg ^{–1}
Bonbon-6	28.45 ± 0.06	16.45 ± 0.02	44 mg kg ^{–1}
Bonbon-7	25.13 ± 0.06	15.98 ± 0.02	477 mg kg ^{–1}
Bonbon-8	25.23 ± 0.01	16.01 ± 0.02	444 mg kg ^{–1}
Bonbon-9	24.71 ± 0.06	15.56 ± 0.02	645 mg kg ^{–1}
Bonbon-10	27.52 ± 0.06	15.06 ± 0.01	86 mg kg ^{–1}
Bonbon-11	33.01 ± 0.06	14.23 ± 0.01	1.7 mg kg ^{–1}
Bonbon-12	33.21 ± 0.01	14.98 ± 0.02	1.4 mg kg ^{–1}
Bonbon-13	30.73 ± 0.02	15.11 ± 0.02	8.6 mg kg ^{–1}
Chocolate-1	33.43 ± 0.01	16.22 ± 0.02	1.32 mg kg ^{–1}
Chocolate-2	35.06 ± 0.01	15.02 ± 0.08	0.4 mg kg ^{–1}
Chocolate-3	34.67 ± 0.01	14.34 ± 0.08	0.5 mg kg ^{–1}
Chocolate-4	36.22 ± 0.01	15.23 ± 0.08	0.17 mg kg ^{–1}
Chocolate-5	35.82 ± 0.01	16.03 ± 0.08	0.2 mg kg ^{–1}
Chocolate-6	29.70 ± 0.01	15.08 ± 0.02	18 mg kg ^{–1}
Ice cream-1	33.78 ± 0.01	14.26 ± 0.01	0.96 mg kg ^{–1}
Ice cream-2	35.21 ± 0.01	16.23 ± 0.01	0.3 mg kg ^{–1}
Ice cream-3	38.60 ± 0.01	14.78 ± 0.01	0.03 mg kg ^{–1}
Biscuit-1	34.02 ± 0.02	15.21 ± 0.02	0.8 mg kg ^{–1}
Biscuit-2	33.12 ± 0.03	14.23 ± 0.01	1.5 mg kg ^{–1}
Cereal-1	32.75 ± 0.01	15.21 ± 0.03	2 mg kg ^{–1}
Cereal-2	32.06 ± 0.01	14.87 ± 0.03	3.3 mg kg ^{–1}
Meat Product-1	37.12 ± 0.01	16.67 ± 0.02	0.08 mg kg ^{–1}
Meat Product-2	34.21 ± 0.01	15.56 ± 0.02	0.7 mg kg ^{–1}
Meat Product-2	35.23 ± 0.01	14.21 ± 0.02	0.33 mg kg ^{–1}

demonstrate that labeling may not always be reliable for allergic individuals. Moreover, this real-time PCR could be applicable for routine screening purposes in product quality control, in order to avoid the unintentional presence of pistachios in food products and therefore preventing the food allergic consumer from unintentional ingestion of hidden allergens.

4. Conclusions

The ITS pistachio specific system was applied to the analysis of a collection of 229 commercial food products, and PCR results conformed to the labeling in all but 29 samples which contained undeclared pistachios. This demonstrates the potential of the real-time PCR-based method to detect the presence of pistachio in commercial products. As demonstrated, the presented real-time PCR fulfills the requirements for highly specific and relatively rapid detection of small amounts of pistachios in food samples. The method is relatively straightforward and it can be easily implemented in any analytical laboratory routinely performing real-time PCR. Consequently, this methodology could be used in inspection programs to enforce accurate labeling of commercial food products, thereby protecting both producers and consumers against hidden allergens.

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