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Sensitive and specific detection of almond (*Prunus dulcis*) in commercial food products by real-time PCR

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ABSTRACT

Almond has been widely used in all sorts of food products, mostly due to its pleasant flavor and health benefits. However almonds can become an important health problem since they are responsible for triggering adverse immune responses in allergic individuals, and since they are present in many processed foods they are considered as a potential hidden allergen. Consequently, it's important for food processors and regulatory agencies to be able to ensure accurate labeling of foods to protect the safety of the public and to avoid expensive recalls. We propose a simple and highly sensitive approach to detect almond in a wide range of processed foods. The method consists of a real-time PCR assay targeting the gene encoding for the ITS1 in almond, using a nuclease (TaqMan) probe labeled with FAM and BBQ. Sensitivity of real time PCR was determined by analysis of raw and heat treated almond-wheat flour mixtures with a range of detection of 0.1–100,000 mg/kg. The assay was successfully trialed on a total of 214 commercial foodstuffs allowing the detection of trace amounts of almond down to the level of 0.1 mg/kg, and is therefore proposed as a ready-to-use analytical tool to trace almond allergens in foods.

1. Introduction

Tree nuts have attained an important place in human diets because they are considered excellent foods, mainly due to their pleasant taste and potential health benefits. Particularly, the consumption of almonds has been associated with nutritional benefits, including cholesterol-lowering effects, protection against diabetes and potential prebiotic properties (Chen, Lapsley, & Blumberg, 2006; Jenkins et al., 2002, 2008; Mandalari, Nueno-Palop, Bisignano, Wickham, & Narbad, 2008). Almonds are used as an ingredient in a wide variety of food products (spreads, bakery, pastry, chocolates, and confectionary products) (Alasalvar & Shahidi, 2008), which are more or less related to the population habits and/or the type of tree nuts available in the geographical region. In the USA, almonds rank first in the per capita consumption among tree nuts (USDA, 2009). Between 1997 and 2007 the production capacity of the 20 countries growing most of almonds has approximately doubled from 0.993 to 2.019 million metric tons. Approximately 50% of the world's almond production originated from the USA (FAOSTAT, 2010).

However, this potential health benefits attributed to almonds do not apply to everyone since this tree nut is a known allergenic food. Almonds are known to be responsible for triggering abnormal immunological responses in allergic individuals, ranging from mild reactions to potentially fatal anaphylactic shocks, even after the ingestion of minimal doses of the allergenic food (Sampson, Mendelson, & Rosen, 1992; Yunginger et al., 1988). In the United States, by the use of random-calling telephone surveys, through an 11-year follow up study, there was an increase of almond allergy among other tree nut allergy prevalence in children, ranging from 0.6% in 1997 to 1.2% in 2002 and 2.1% in 2008, whereas in the adult population the same prevalence remained around 1.3% (Sicherer, Muñoz-Furlong, Godbold, & Sampson, 2010).

Food allergic individuals are strongly advised to manage their food allergy through strict avoidance of the allergenic food (Sampson, 2004, 2005; Sicherer & Sampson, 2006). Therefore, food labels that provide accurate ingredient information are of vital importance. Despite this, the ingestion of almonds often happens accidentally because of mislabeling of products, rework processes, which include almond-containing foods or cross-contamination during processing (Costa, Mafra, Carrapatoso, & Oliveira, 2012a).

Abbreviations: ASS, almond specific system; PAC, positive amplification control.

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To safeguard sensitized individuals with respect to the contents of almonds and other allergenic components in food products, legislation on food labeling has established clear guidelines in the European regulation (EU) 1169/2011/EC (OJEU, 2011), as well as in the USA (Food Allergen Labeling and Consumer Protection Act of 2004), Canada (Regulations Amending the Food and Drug Regulations, 2011) and other countries. Thus, appropriate analytical methods are needed for the sensitive and specific allergen detection to verify labeling requirements and to identify cross contact with allergenic foods.

The need for adequate methodology to detect food allergens has been rapidly increasing over recent years, especially in response to the demands imposed by current legislation. The food industry has been addressing with special interest the necessities of food allergic consumers, not only concerning the proper food labeling but also minimizing allergen cross-contamination among foodstuffs. Therefore, suitable analytical methods are required to detect allergenic proteins, as they are mostly present at trace levels (Baumgartner, Krska, & Welzig, 2007). The requirements needed for detecting allergenic ingredients in food involve appropriate specificity and sensitivity to trace minute amounts of the target allergens or the correspondent markers in complex food matrices, including processed foods.

Several methods for almond detection have been developed, mainly relying on immunochemical and DNA-based techniques. Immunochemical methods have been successfully applied for the detection of almond allergens in food with the specificity based on the specific interaction between the antibody and the antigen, which is the allergen or marker protein in the case of food allergen detection (Garber & Perry, 2010; Rejeb, Abbott, Davies, Cleroux, & Delahaut, 2005; Roux, Teuber, Robotham, & Sathe, 2001). Nevertheless, the use of immunoassays has numerous problems mainly due to the cross-reactivity with non target proteins and the low resistance of proteins to food processing during thermal processing, because it can cause conformational changes in the tridimensional structure of the epitopes (e.g., heat induced denaturation) and/or protein cleavage, affecting linear epitopes (e.g., fermentation).

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. These techniques rely on the use of polymerase chain reaction (PCR), either as qualitative end-point PCR, being distinguished on the basis of their differential migration through agarose gel electrophoresis, or by real-time PCR using fluorescent labeled probes or dyes. Nevertheless, real-time PCR has been so far the most widely applied PCR strategy to detect food allergens (Fajardo et al., 2009; López-Calleja et al., 2013; Platteau, De Loose, De Meulenaer, & Taverniers, 2011; Scaravelli, Brohee, Marchelli, & Van Hengel, 2008). Several real-time PCR approaches have been proposed to detect food allergens from almonds (Costa, Mafra, & Oliveira, 2012b; Koppel et al., 2010; Pafundo, Gullì, & Marmiroli, 2009, 2010; Röder, Vieths, & Holzhauser, 2011).

Table 1
List of almond varieties analyzed by the almond real-time PCR system.

Type of material	Almond variety	Scientific name	Country of origin	*ITS* A.S.S	*18S* rRNA P.A.C
Leaves	Wild Almond “Prunus webbi”	*Prunus webbii*	Unknown	+	+
Leaves	Wild Almond “Prunus vavilovi”	*Prunus vavilovi*	Unknown	+	+
Leaves	Wild Almond “Prunus kotchkii”	*Prunus kotchkii*	Unknown	+	+
Leaves	Wild Almond “Prunus orientalis”	*Prunus orientalis*	Unknown	+	+
Leaves	Almond “Gorki” cultivar	*Prunus dulcis*	Unknown	+	+
Leaves	Almond “Tuono” cultivar	*Prunus dulcis*	Italy	+	+
Leaves	Almond “Genco” cultivar	*Prunus dulcis*	Italy	+	+
Leaves	Almond “Lauranne” cultivar	*Prunus dulcis*	France	+	+
Leaves	Almond “Primorskii” cultivar	*Prunus dulcis*	Ucrania	+	+
Leaves	Almond “Nonpareil” cultivar	*Prunus dulcis*	EEUU	+	+
Leaves	Almond “Texas” cultivar	*Prunus dulcis*	EEUU	+	+
Leaves	Almond “Chellaston” cultivar	*Prunus dulcis*	Australia	+	+
Leaves	Almond “Ardechoise” cultivar	*Prunus dulcis*	France	+	+
Leaves	Almond “Penta” cultivar	*Prunus dulcis*	Tardona	+	+
Leaves	Almond “Tardona” cultivar	*Prunus dulcis*	Spain	+	+
Leaves	Almond “Achaak” cultivar	*Prunus dulcis*	Tuñez	+	+
Leaves	Almond “Marcona” cultivar	*Prunus dulcis*	Spain	+	+
Leaves	Almond “Desmayo Largueta” cultivar	*Prunus dulcis*	Spain	+	+
Leaves/Kernel	Almond “Antoñeta” cultivar	*Prunus dulcis*	Spain	+	+
Leaves/Kernel	Almond “Ferragnes” cultivar	*Prunus dulcis*	France	+	+
Leaves/Kernel	Almond “Marta” cultivar	*Prunus dulcis*	Spain	+	+
Kernel	Almond “Vairo” cultivar	*Prunus dulcis*	Spain	+	+
Kernel	Almond “Marinada” cultivar	*Prunus dulcis*	Spain	+	+
Kernel	Almond “Constanti” cultivar	*Prunus dulcis*	Spain	+	+
Kernel	Almond “Tarraco” cultivar	*Prunus dulcis*	Spain	+	+
Kernel	Almond “Masbovera” cultivar	*Prunus dulcis*	Spain	+	+
Kernel	Almond “Glorieta” cultivar	*Prunus dulcis*	Spain	+	+
Kernel	Almond “Francoli” cultivar	*Prunus dulcis*	Spain	+	+
Kernel	Almond “Guara” cultivar	*Prunus dulcis*	Spain	+	+
Kernel	Almond “Soleta” cultivar	*Prunus dulcis*	Spain	+	+
Kernel	Almond “Belona” cultivar	*Prunus dulcis*	Spain	+	+
Kernel	Almond “Bitter” cultivar	*Prunus dulcis*	Unknown	+	+
Kernel	Almond “Largueta”	*Prunus dulcis*	Spain	+	+
Kernel	Almond “Comuna”	*Prunus dulcis*	Spain	+	+
Kernel	Almond “Marcona”	*Prunus dulcis*	Spain	+	+
Kernel	Almond “California”	*Prunus dulcis*	EEUU	+	+

***ITS* A.S.S**: Almond-specific system on the Internal Transcribed Spacer (*AlmondITSdir/AlmondITSinv* and *AlmondITSP*).
***18S* rRNA P.A.C**: Positive amplification control (*18Sdir*/*18Sinv*, and *18SP*) for the almond system on the *18S* rRNA gene.

The aim of this study was to develop a DNA based qualitative real-time PCR method based on the use of a multicopy *ITS* genetic marker in order to improve the sensibility of PCR techniques for detection of traces of almonds in commercial products.

2. Materials and methods

2.1. Sample selection

Almonds, 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 total of 35 different almond cultivars (leaves and single kernels) provided by the IRTA (Institut de Recerca I Tecnologia Agroalimentaries, Tarragona, Spain) and the CEBAS-CSIC (Centro de Edafología y Biología Aplicada del Segura-Consejo Superior de Investigaciones Científicas, Murcia, Spain), and four commercial varieties (single kernels) obtained from different delicatessen markets, were considered for analysis in order to check the specificity of the almond real-time PCR system (Table 1). Moreover, a wide range of plant (flesh and kernels) and animal (flesh) species was also included in the assays for specificity control purposes (Table 2).

Almonds (Marcona) were finely grounded and binary mixtures of raw and heat treated almond in wheat flour, containing 0.1, 1, 10, 100, 1000, 10,000 and 100,000 mg/kg of almonds, were prepared to a final weight of 500 g using a kitchen robot (Thermomix, Vorwerk). Heat treatment, when applied, consisted of 160 °C for 13 min. This heat treatment was chosen to create samples homologous to certified IRRM materials for peanut.

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 μL of chloroform (Sigma Aldrich) were added to the lysate before centrifugation at 16,438 g for 10 min at room temperature (Heraeus® Primo Centrifuge, United Kingdom).

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 sterile deionized water. DNA concentration was measured with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Montchanin, DE) and adjusted to a final concentration of 100 ng per reaction. 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 almond 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

Due to the fact that there weren't any almond *ITS* gene sequences available in the GenBank database at the beginning of the research, we purified and sequenced the *ITS* gene sequence for the target allergen. For this purpose, we used *ITS-1* and *ITS-2* oligonucleotides designed by Fernández et al. (2001) (with a slightly modified ITS-2 primer) for the amplification, purification and sequencing of an approximately 650 bp fragment from the *ITS-1*, *5.8S* rRNA and *ITS-2* region from three almond "cultivars" (Lauranne, Marcona and Nonpareil) as well as two plums (Chile and South Africa) and one apricot. Polymerase chain reaction products (120 μL) amplified with *ITS-1* and *ITS-2* oligonucleotides from almond were loaded in a 2% LM2 (Hispanlab S. A.) agarose gel containing 1 μg/mL of ethidium bromide in Tris-acetate buffer and electrophoresed at 90 V for 40 min. Each DNA fragment was excised from the agarose gel under UV light using a sterile scalpel. The gel

Table 2
Specificity of the almond real-time PCR system.

Common name	Scientific name	*ITS* A.S.S	*18S* rRNA P.A.C
Almond	*Prunus dulcis*	17.53 ± 0.33[a]	14.79 ± 0.03
Hazelnut	*Corylus avellana*	–	14.53 ± 0.02
Peanut	*Arachis hypogaea*	–	16.61 ± 0.01
Walnut	*Juglans regia*	–	15.02 ± 0.04
Pistachio	*Pistacia vera*	–	14.15 ± 0.06
Macadamia	*Macadamia intergrifolia*	–	16.17 ± 0.01
Cashew nut	*Anacardium occidentale*	–	15.13 ± 0.04
Brazil nut	*Bertholletia excelsa*	–	16.13 ± 0.01
Pecan	*Carya illinoinensis*	–	16.20 ± 0.01
Soybean	*Glycine max*	–	16.52 ± 0.06
Green bean	*Phaseolus vulgaris*	–	16.01 ± 0.02
Green pea	*Pisum sativum*	–	15.01 ± 0.01
Chickpea	*Cicer arietinum*	–	14.87 ± 0.01
Lentil	*Lens culinaris*	–	15.21 ± 0.02
Tiger Nut	*Cyperus esculentum*	–	15.46 ± 0.04
Lupine	*Lupinus albus*	–	15.48 ± 0.03
Acorn	*Quercus ilex*	–	14.72 ± 0.02
Chestnut	*Aesculus hippocastanum*	–	16.38 ± 0.03
Sesame	*Sesamum indicum*	–	15.78 ± 0.01
Pine nut	*Pinus pinea*	–	16.34 ± 0.01
Barley	*Hordeum vulgare*	–	14.32 ± 0.06
Oat	*Avena sativa*	–	14.01 ± 0.03
Rye	*Secale cereale*	–	14.23 ± 0.02
Rice	*Oryza sativa*	–	14.56 ± 0.04
Sunflower	*Helianthus annuus*	–	14.11 ± 0.02
Maize	*Zea mays*	–	15.11 ± 0.06
Wheat	*Tritucum aestivum*	–	13.55 ± 0.01
Cocoa	*Theobroma cacao*	–	15.05 ± 0.05
Grape	*Vitis vinifera*	–	15.04 ± 0.01
Pear	*Pryus rosaceae*	–	14.81 ± 0.01
Apple	*Malus domestica*	–	14.43 ± 0.01
Apricot	*Prunus armeniaca*	–	15.34 ± 0.01
Cherry	*Prunus avium*	–	16.03 ± 0.01
Sour cherry	*Prunus cerasus*	–	15.21 ± 0.01
Peach	*Prunus persica*	–	15.03 ± 0.04
Plum	*Prunus domestica*	–	16.02 ± 0.02
Banana	*Musa cavendishii*	–	15.87 ± 0.01
Kiwifruit	*Actinidia deliciosa*	–	13.98 ± 0.01
Watermelon	*Citrullus lanatus*	–	14.67 ± 0.02
Orange	*Citrus sinesis*	–	13.87 ± 0.01
Potato	*Solanum tuberosum*	–	14.02 ± 0.02
Garlic	*Allium sativum*	–	15.09 ± 0.01
Onion	*Allium cepa*	–	14.73 ± 0.02
Eggplant	*Solanum melongena*	–	15.21 ± 0.01
Zucchini	*Cucurbita pepo*	–	16.34 ± 0.03
Tomato	*Solanum lycopersicum*	–	13.92 ± 0.01
Asparagus	*Asparagus officinalis*	–	14.87 ± 0.02
Carrot	*Daucus sativus*	–	15.45 ± 0.02
Olive	*Olea europaea*	–	16.26 ± 0.07
Cattle	*Bos taurus*	–	13.98 ± 0.00
Sheep	*Ovis aries*	–	14.12 ± 0.02
Goat	*Capra hircus*	–	15.44 ± 0.01
Swine	*Sus scrofa domestica*	–	14.34 ± 0.07

***ITS* A.S.S**: Almond-specific system on the Internal Transcribed Spacer (*AlmondITSdir*/*AlmondITSinv* and *AlmondITSP*).

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

[a] Average Cp value ± SD shown from triplicate PCR reactions from each DNA extracted from kernels from 18 almonds varieties. Minus sign indicates no positive signal after 50 PCR cycles.

```
                                        AlmondITSdir  →         AlmondITSP

                          30        40        50        60        70          80
                        ---|----:----|----:----|----:--...--|----:----|...----:----|
P. dulcis     (HF969276)  ACCTGCCTAGCCGAACGACCCGAGAACTAGT---TTCAAAGCGGGGG---CCGAGGGGTC
P. dulcis     (HF969274)  ...............................---.............---..........
P. dulcis     (HF969277)  ...............................---.............---..........
P. domestica  (HF969273)  ...........A...................---.............---A.......C.
P. domestica  (HF969272)  ...........A...................---.............---A.......C.
P. armeniaca  (HF969271)  ...........A...................---.............---A.......C.
P. pérsica    (JF978135)  ...........A...................---.............GG--.......C.
P. avium      (HM856805)  ...........A...................---.............G--AT........
P. cerasus    (FJ899099)  ...........A...................---.............G--AA........
P. vera       (AY677201)  .......G...A..........C....CT..--CA..C.T.......C--.T.C...C.T
A. occid      (AB071690)  .......CGC.A.G........C....AT..GCCC..CGT......AG--...C...CCT
M. interg     (EU642799)  .......C...G.---......T....AT.---.G.GTT..C.ATCA-AG.TA...CG
J. regia      (HE574850)  .......C...A..-.....T.T....AT..AATAA.CTTCT...T.GGGGT.TAAT.C.
C. illio      (AF303825)  .......C...A..........T.....T..AATAA.CTTCT...TTGGGGT.TCAT.C.
C. avell      (HQ442261)  .......C...A..........C.....T..ATAAA...CCG....CGGGGG.C---..T
A. hypog      (HQ537458)  ----------------...G..C....G...CCACAA.C.C.C.A..CG-GG..A...C.
B. excelsa    (HE806059)   ---GTTTCC.TA.GTGA...T.CAG.AAGA.-----..TT.TC.A.ATCCTG..C.ACC.


                                                            ←  AlmondITSinv

                                                       90        100         110
                          .--.....................--:.----|----:----|.----:----|----:-
P. dulcis     (HF969276)  -CT---------------------GCG-GCTCCTCGTCCCCTT-TATCTCGGGGGGGTTG
P. dulcis     (HF969274)  -..---------------------...-..............-................
P. dulcis     (HF969277)  -..---------------------...-..............-................
P. domestica  (HF969273)  -G.CTCG-----------------...T...........-..-................
P. domestica  (HF969272)  -G.CTCG-----------------...T...........-..-................
P. armeniaca  (HF969271)  -G.CTCG-----------------...T...........-..-....C..........
P. pérsica    (JF978135)  -..------------------...-......T.----.....T......C..........
P. avium      (HM856805)  -T.---------------------...-......T.....-..-.........A.....
P. cerasus    (FJ899099)  -T.---------------------...-......T.....-..-.........A.....
P. vera       (AY677201)  CG.GCCC-----------------.T.T..CT.CACC.GTGC.TCG..GG.T.TC...C.
A. occid      (AB071690)  AGCGCCC-----------------...T..C..C.AC.G.GC--CGG.GGCT..C..y.-
M. interg     (EU642799)  TGGGAGC-----------------.T.A......TCCTT..CGACCCT.GTAAAA...C.
J. regia      (HE574850)  C.CTCCCAAAAAACGGTTGGGAGG..AC.T.GAGATATG..CACCGCTC.TC.T.T..G.
C. illio      (AF303825)  C.CTCCCAAAAA-CGGTTGGGAGG..AC.A.GAAA.CTG..CACCGCTC.TC.T.T..G.
C. avell      (HQ442261)  CTCGCCC-------------------C.--------TG..C------C..AAC.-.CG.
A. hypog      (HQ537458)  -----------------------------------------------------------..CC.
B. excelsa    (HE806059)  ------------------------..AAA..TG.TCA.A.------------.AT.ACC-
```

Fig. 1. Deoxyribonucleic acid sequence alignment of the ITS region PCR products from almond (*Prunus dulcis*: Marcona/HF969276, Nonpareil/HF969274 and Lauranne/HF969277), plum (*Prunus domestica* HF969273 and HF969272), apricot (*Prunus armeniaca* HF969271), peach (*Prunus pérsica* JF978135), cherry (*Prunus avium* HM856805), sour cherry (*Prunus cerasus* FJ899099), pistachio (*Pistacia vera* AY677201), cashew nut (*Anacardium occidentale* AB071690), macadamia (*Macadamia intergrifolia* EU642799), walnut (*Juglans regia* HE574850), pecan (*Carya illinoinensis* AF303825), hazelnut (*Corylus avellana* HQ442261), peanut (*Arachis hypogaea* HQ537458) and Brazil nut (*Bertholletia excelsa* HE806059), available at the GenBank-EMBL database. Primers *AlmondITSdir*, *AlmondITSinv*, and *AlmondITSP* probe are highlighted.

slice was purified with the QIAquick Gel Extraction kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. The concentration of the PCR products was estimated by agarose gel electrophoresis using a standard as reference marker (REAL, Durviz S.L., Valencia, Spain). Purified PCR products were sequenced at Sistemas Genómicos S.L. (Parque Tecnológico de Valencia, Spain). Deoxyribonucleic acid sequencing was accomplished in an ABI Prism model 377 DNA sequencer (Perkin–Elmer/Applied Biosystems) using *ITS-1* and *ITS-2* primers with the dRhodamine Terminator Cycle Sequencing Ready Reaction kit (Perkin–Elmer/Applied Biosystems). The sequences obtained were submitted to GenBank-European Molecular Biology Laboratory database and were assigned with the accession numbers HF969277 (Lauranne), HF969276 (Marcona), HF969274 (Nonpareil), HF969273 (Plum-Chile), HF969272 (Plum-South Africa) and HF969271 (Apricot).

The *ITS* gene sequences obtained from Lauranne, Marcona and Nonpareil almonds were aligned together with the sequences from plum and apricot and other plants and animal *ITS* gene sequences available in the GenBank database, in order to visualize similarities and differences among the sequences of different almond cultivars and other close related plant species. Information obtained after alignment and comparison of almond and the other *ITS* gene sequences was used to design the almond-specific primer pair *AlmondITSdir*/*AlmondITSinv*, potentially suitable for the amplification of a specific 76-bp DNA fragment.

Primers *18Sdir* and *18Sinv*, based on conserved *18S* rRNA gene sequences available in the Genbank/EMBL database for various plant and animal species, were used as positive amplification control of the PCR assay (Pegels et al., 2011, 2012; Pegels, González, Fernández, García, & Martín, 2012; Pegels, González, García, & Martín, 2011; Pegels, González, López-Calleja, García, & Martín, 2013). These primers were expected to amplify a conserved fragment of 77 bp from the *18S* rRNA gene in eukaryotes.

The EMMA program 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.

To accomplish the detection procedure, specific fluorescent hybridization TaqMan probe for almond (*AlmondITSP*) was used. Besides, another TaqMan probe (*18SP*) based on conserved *18S* rRNA gene sequences available in the Genbank/EMBL database for various plant and animal species was used as a positive control in the real-time PCR system. TaqMan probes were designed and synthesized by TibMolBiol (Berlin, Germany). The TaqMan probes were aimed to anneal within the gene fragment generated by amplification of the corresponding target (Fig. 1) and were dual labeled on the 5′ end with the fluorescent reporter dye 6-carboxyfluorescein (FAM) and on the 3′ end with the blackberry (BBQ) fluorescent quencher.

The sequences and description of the primers and probes used in this work are summarized in Table 3.

Table 3
DNA sequences and description of the primers and probes used in this study.

	Length (bp)	Sequence (5′→3′)	Description	Target gene	Fragment length (bp)
Primers					
ITS-1	19	GTTTCCGTAGGTGAACCTG	Sequencing forward primer	*ITS*	650
ITS-2	19	CGTCTGAYCTGRGGTCG	Sequencing reverse primer		
AlmondITSdir	19	CTAGCCGAACGACCCGAGA	Almond-specific forward primer	*ITS*	76
AlmondITSinv	19	CCGAGATAAAGGGGACGAG	Almond-specific reverse primer		
18Sdir	16	TGGTGCCAGCAGCCGC	Positive control forward primer	*18S* rRNA	77
18Sinv	25	TCCAACTACGAGCTTTTTAACTGCA	Positive control reverse primer		
Probes					
AlmondITSP	22	6FAM-ACTAGTTTCAAAGCGGGGG-BBQ	Almond probe	*ITS*	
18SP	22	6FAM-CGCTATTGGAGCTGGAATTACC-BBQ	Universal probe	*18S* rRNA	

2.4. Real-time PCR

Real-time PCR was run under generic cycling conditions. The optimum PCR concentrations of primers yielding the highest endpoint fluorescence and the lowest Cp (crossing point value) 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 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, 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 (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 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 *ITS1* almond-specific system, a standard curve were constructed using two binary mixtures of known almonds content (raw & heat treated almond/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 Cp value obtained in the unknown sample in the standard curve of Cp values generated from known DNA concentrations of the target species. In addition, the correlation between the variables, crossing point (Cp) and concentration ($[\cdot]$) is semilogarithmic:

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

where b is the slope and a is the intercept.

Linearity test, sensitivity, accuracy and precision parameters of the species-specific real-time PCR 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 almond 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. Almond-specific system on the ITS1 gene

3.1.1. TaqMan 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 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 such as roasting, which degrades DNA, thereby the selection of a short *ITS1* amplicon of 76 bp is relevant for real-time PCR analysis aimed to detection of almond 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.

3.1.2. Specificity and sensitivity

Specificity and sensitivity are two important indices of performance of qualitative assays. The close phylogenetic relationships among almond 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. In this sense, and even though a Blast evaluation of the almond sequences in the NCBI database suggested a possible cross reactivity with some other *Prunus* species, a total of 35 samples of these "stone fruits" (plums, apricots, peaches, cherries and sour cherries) where tested and no amplification was achieved, proving that the system is almond-specific. Additionally to this the specificity of the almond real-time PCR system (primers and probe) on the *ITS1* gene was assessed by analysis of the DNA extracted from 48 plant species (including the latter ones) and 4 animal species. As expected, the almond specific PCR system on the *ITS1* gene successfully detected a DNA fragment of 76 bp from almonds, while no positive amplification signal was obtained on the animal and plant species tested (Table 2). Moreover, leaves and kernels from a total of 35 almond cultivars and 4 almond commercial varieties were analyzed and successfully amplified, proving the capability of amplification in any almond variety (Table 1). Also, it should be stressed that, because pollination is not controlled, the seeds from trees of a defined cultivar can in fact contain DNA from different pollinators. Nevertheless, we would like to point out that in the case of the seeds we analyzed single kernels, what minimizes the complexity of the sample to just one possible DNA pollinator per kernel. The *18S* rRNA eukaryotic system amplified a 77 bp fragment from all samples tested. The Cps obtained from the analysis of the "individual kernels" from 18 almond varieties oscillated between 17 and 18 (17.53 ± 0.33). Thus, the target DNA copy number is similar in all varieties analyzed despite their origin. This result differs from that described by Rogers et al. (1987) in *Vicia faba*, who reported up to a 95-fold difference of copy numbers within the ribosomal RNA genes of different individuals of this species.

Two binary mixtures of raw and heat treated (160 °C for 13 min) Marcona almond in wheat flour, containing increasing amounts of the target almond, were used to construct a 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 can be observed in Fig. 2, the resulting slope of the linear equation is −3.53 for raw and heat treated almond/wheat *ITS1* almond-specific 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 submitted to high temperature treatments (Van Raamsdonk et al., 2007). Prior to consumption, almonds usually undergo heat treatments like blanching or roasting. 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.

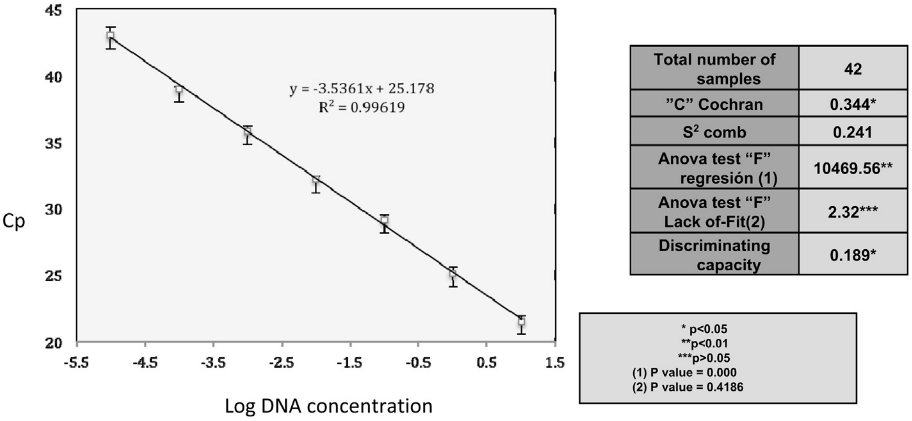
The mean Cp values and standard deviation obtained from almond calibration curve, plotted against the logarithm of the DNA concentrations, were used to test the sensitivity of the real-time PCR method. Fig. 2 also shows the discriminating capacity, which is the least difference in logarithm of target DNA concentration in the sample that the analytical method can discriminate with a significant level. Linearity of the almond real-time PCR response was also analyzed. In this PCR system, the following parameters were evaluated: (a) Cochran's test, which determined whether the variances of the responses obtained for each concentration of almond DNA in the reference samples were homogeneous, (b) regression analysis and (c) variance analysis with lack of-fit. All parameters were evaluated following previously described validation protocols.

Table 4
Results of the almond real time PCR analysis of 214 commercial products.

	Number of samples analyzed	*ITS* A.S.S. (Cp)	*18S* rRNA (Cp)
Commercial food products that declared almond in the labeling (28)			
Chocolate	3	+	+
Bonbon	3	+	+
Biscuits	4	+	+
Nut bar	6	+	+
Cereal	8	+	+
Yogurt	1	+	+
Ice cream	1	+	+
Nougat	1	+	+
Beverage	1	+	+
Commercial food products that declared traces of nuts (63)			
Chocolate	17	+(10)/−(7)	+
Bonbon	18	+(1)/−(17)	+
Rice/corn cake	2	−(2)	+
Biscuits	11	+(7)/−(4)	+
Nut bar	6	+(3)/−(3)	+
Cereal	1	−(1)	+
Ice cream	2	+(1)/−(1)	+
Bread	5	+(4)/−(1)	+
Precooked meal	1	−(1)	+
Commercial food products that not declared almond nor traces in the labeling (123)			
Chocolate	19	+(9)/−(10)	+
Bonbon	14	+(4)/−(10)	+
Biscuits	19	+(8)/−(11)	+
Nut bar	9	+(4)/−(5)	+
Cereal	4	+(2)/−(2)	+
Ice cream	10	+(4)/−(6)	+
Bread	9	+(3)/−(6)	+
Chocolate cream	3	+(1)/−(2)	+
Peanut cream	1	−(1)	+
Cashew cream	1	−(1)	+
Beverage	17	+(9)/−(8)	+
Sauce	1	−(1)	+
Precooked meal	1	+(1)	+
Meat products	11	+(3)/−(8)	+
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.

In order to determine the accuracy of the *ITS1* almond-specific system seven concentrations (0.1, 1, 10, 100, 1000, 10,000 and 100,000 mg/kg) 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. Using the Snedecor F-test, it was verified



Total number of samples	42
"C" Cochran	0.344*
S^2 comb	0.241
Anova test "F" regresión (1)	10469.56**
Anova test "F" Lack of-Fit(2)	2.32***
Discriminating capacity	0.189*

* $p<0.05$
** $p<0.01$
*** $p>0.05$
(1) P value = 0.000
(2) P value = 0.4186

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

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

Table 5
Real-time PCR results obtained with the *ITS* almond-specific system and *18S* rRNA positive amplification control in the analysis of commercial food products with a declared almond percentage and those samples that didn't indicate the presence of almonds or traces on the labeling, but were found to contain almond traces.

Commercial products (percentage of almonds declared on the label)	A.S.S. (Cp)	P.A.C. (Cp)	mg/kg (Estimated almond percentage)
Nut bar-1 (2%)	25.60 ± 0.01	16.01 ± 0.02	7600 mg/kg (0.76%)
Biscuit-1 (4%)	23.14 ± 0.01	16.34 ± 0.02	37,701 mg/kg (3.7%)
Cereal-1 (3%)	24.73 ± 0.01	14.92 ± 0.01	13,000 mg/kg (1.3%)
Cereal-2 (1.4%)	29.12 ± 0.01	14.34 ± 0.01	800 mg/kg (0.08%)
Cereal-3 (2.8%)	26.87 ± 0.01	14.01 ± 0.01	3323 mg/kg (0.3%)
Beverage-1 (7%)	25.14 ± 0.02	15.02 ± 0.02	10,250 mg/kg (1.02%)
Commercial products (not declared almond nor traces on the label)	A.S.S. (Cp)	P.A.C. (Cp)	mg/kg
Bonbon-1	37.28 ± 0.01	14.01 ± 0.02	3.8 mg/kg
Bonbon-2	33.12 ± 0.02	15.06 ± 0.01	56 mg/kg
Bonbon-3	37.01 ± 0.01	15.01 ± 0.02	4.5 mg/kg
Bonbon-4	35.02 ± 0.01	14.23 ± 0.01	16 mg/kg
Chocolate-1	41.05 ± 0.01	14.45 ± 0.02	0.3 mg/kg
Chocolate-2	40.01 ± 0.02	15.08 ± 0.02	0.6 mg/kg
Chocolate-3	39.34 ± 0.02	16.78 ± 0.08	1 mg/kg
Chocolate-4	36.12 ± 0.01	15.08 ± 0.02	8 mg/kg
Chocolate-5	37.21 ± 0.01	15.88 ± 0.02	3.9 mg/kg
Chocolate-6	38.01 ± 0.01	15.02 ± 0.08	2.3 mg/kg
Chocolate-7	37.83 ± 0.01	14.34 ± 0.08	2.6 mg/kg
Chocolate-8	38.01 ± 0.01	15.12 ± 0.08	2.3 mg/kg
Chocolate-9	37.34 ± 0.01	14.89 ± 0.02	4 mg/kg
Chocolate Cream-1	33.41 ± 0.02	14.26 ± 0.01	46.3 mg/kg
Ice cream-1	39.23 ± 0.01	14.26 ± 0.01	1 mg/kg
Ice cream-2	40.12 ± 0.01	16.23 ± 0.01	0.6 mg/kg
Ice cream-3	40.01 ± 0.01	14.78 ± 0.01	0.6 mg/kg
Ice cream-4	39.71 ± 0.01	14.87 ± 0.01	0.7 mg/kg
Yogurt-1	36.34 ± 0.01	14.92 ± 0.01	6.9 mg/kg
Yogurt-2	37.12 ± 0.01	14.34 ± 0.02	4.1 mg/kg
Nut bar-1	39.81 ± 0.01	14.12 ± 0.01	0.7 mg/kg
Nut bar-2	40.32 ± 0.01	14.98 ± 0.05	0.5 mg/kg
Nut bar-3	38.03 ± 0.01	14.12 ± 0.01	2.3 mg/kg
Nut bar-4	38.51 ± 0.01	16.01 ± 0.01	1.7 mg/kg
Biscuit-1	36.12 ± 0.01	14.01 ± 0.01	8 mg/kg
Biscuit-2	38.23 ± 0.02	15.83 ± 0.02	2 mg/kg
Biscuit-3	37.87 ± 0.01	16.78 ± 0.08	2.5 mg/kg
Biscuit-4	35.49 ± 0.03	14.23 ± 0.01	12 mg/kg
Biscuit-5	37.12 ± 0.03	14.23 ± 0.01	4 mg/kg
Biscuit-6	38.65 ± 0.01	15.01 ± 0.02	1.5 mg/kg
Biscuit-7	36.63 ± 0.01	13.71 ± 0.02	5.7 mg/kg
Biscuit-8	35.14 ± 0.01	16.56 ± 0.01	15.3 mg/kg
Cereal-1	36.66 ± 0.01	15.11 ± 0.04	5.6 mg/kg
Cereal-2	36.02 ± 0.01	14.87 ± 0.03	8 mg/kg
Bread-1	39.12 ± 0.01	16.01 ± 0.03	1.1 mg/kg
Bread-2	33.02 ± 0.01	13.34 ± 0.03	60 mg/kg
Bread-3	33.87 ± 0.01	14.12 ± 0.01	>100 mg/kg
Beverage-1	38.13 ± 0.01	14.45 ± 0.02	2.1 mg/kg
Beverage-2	34.82 ± 0.01	16.23 ± 0.08	18.5 mg/kg
Beverage-2a	35.12 ± 0.01	16.23 ± 0.08	15.3 mg/kg
Beverage-3	36.67 ± 0.01	14.56 ± 0.03	5.5 mg/kg
Beverage-4	38.01 ± 0.01	13.93 ± 0.01	2.3 mg/kg
Beverage-4a	37.71 ± 0.01	13.78 ± 0.01	2.8 mg/kg
Beverage-5	37.01 ± 0.01	15.21 ± 0.01	4.5 mg/kg
Beverage-5a	38.22 ± 0.02	16.21 ± 0.01	2 mg/kg
Beverage-6	36.32 ± 0.06	16.23 ± 0.08	7 mg/kg
Meat Product-1	38.91 ± 0.01	16.67 ± 0.02	1.3 mg/kg
Meat Product-2	37.01 ± 0.01	15.56 ± 0.02	4.5 mg/kg
Meat Product-3	38.01 ± 0.01	14.21 ± 0.02	2.3 mg/kg
Precooked meal-1	35.01 ± 0.01	13.78 ± 0.02	16.4 mg/kg

A.S.S: Almond-specific system on the *Internal Transcribed Spacer.*
P.A.C: Positive amplification control for the almond system on the *18S* rRNA gene.

3.1.3. *Commercial food products analysis*

The method was also tested with regard to its suitability for the detection of almond DNA in commercial food products. In this way, the *ITS* almond-specific system was applied to the analysis of a collection of 214 commercial food products of different brands with different label declaration: 28 containing almond as an ingredient, 63 may contain traces of nuts and 123 did not declare the presence of almonds. PCR results were found to conform to the labeling in all samples except for 50 food samples with undeclared almonds in the labeling (Table 4). 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 equation of the reference curve in order to determine whether the detected almond content (%) of each sample fitted with the declared one. Table 5 shows the average Cp values and the content estimated in mg/kg for commercial samples with a declared almond percentage, and those samples that didn't indicate the presence of almonds or traces on the labeling but were found to contain almond traces. The almond estimation content obtained by substitution of the Cps in the corresponding A.S.S. equation for those commercial food samples with declared almond content, resulted to be lower than the percentage indicated in the labeling. This underestimation of the amount of allergen could be due to a great number of factors which affect DNA yield, such as the extent of DNA degradation due to processing treatments and the presence of different food matrices potentially containing different amounts of DNA (milk, fat, seeds, etc.) which might interfere with the PCR reaction. As a consequence, quantitative estimation of target allergen content in a food product would be only reliable if samples with identical composition and processing treatment are used for the preparation of 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. 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 it is difficult to determine the exact amount of allergen (percentage) necessary to trigger symptoms, as this may vary from one person to the other. In this way, 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.

On the other hand, from the extrapolation of the different Cps obtained for the list of products that didn't indicate the presence of almonds or traces on the labeling, we estimated that 40 of the 50 samples with an undeclared almond content contained less than 10 mg/kg of almond, whereas 7 contained between 10 and 50 and the other three contained more than 50 mg/kg of almond. As mentioned before this calculated almond concentration given is a mere orientation of the tree nut content. However, what really matters to individuals who are susceptible of allergic reactions is to determine the presence of allergens in commercial food products that don't declare it. For these commercial food products that gave positive for the presence of almond, contamination probably occurred during the production, since they were produced by companies that also process almonds.

The degree of contamination of commercial food samples with almonds, 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 mg/kg of the target in food samples, which is one of the lowest detection limits achieved to the moment.

Methods for analysis of the almond content in food are necessary to support legislation. Several immunoassay based methods are available for the detection of almonds in food (Acosta, Rouy, Teuber, & Sathe, 1999; Hlywka, Hefle, & Taylor, 2000). Roux et al. (2001) reported the development of a competitive ELISA for the detection of the major allergen of almond, amandin with an LOD of 5–37 mg/kg in several spiked foods presenting minor cross-reactivity with other nuts and legumes. Rejeb et al. (2005) developed a competitive indirect ELISA, which allowed the simultaneous determination of almond, peanut, hazelnut, Brazil nut, and cashew nut with an LOD of 1 mg/kg of target protein in chocolate samples. On the other hand, Garber and Perry (2010) compared three commercial sandwich ELISA test kits for the detection of hazelnuts and almonds. The determined LOD and dynamic ranges for almonds spiked into cooked oatmeal, dipping chocolate, and muffins (baked) varied from 3 to 39 mg/kg, depending on the food matrix and the tested ELISA kit (Garber & Perry, 2010). These immunochemical methods although highly sensitive, 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 almond such as Pafundo et al. (2009) who developed two systems for the detection of almond allergens using SYBR Green real-time PCR. The development of the two systems allowed the detection of almond in biscuits containing processed almond down to 1 mg/kg. In another work, the same authors reported the development of a multiple-target assay based on SYBR Green real-time multiplex PCR to detect sesame, peanut, cashew nut, hazelnut, walnut, and almond (Pafundo, Gullì, & Marmiroli, 2010). This method enabled the detection of low quantities of almond DNA (5 pg), with LOD ranging from 1 to 100 mg/kg of almond in spiked biscuits (Pafundo et al., 2010). On the other hand, Koppel et al. (2010) presented two tetraplex real-time PCR systems for the detection of eight allergens in foods based on the application of TaqMan probes. The assays exhibited good specificity and sensitivity in the range of 100 mg/kg of target ingredient in rice cookies. Concerning the specific detection of almond, an LOD of 10 mg/kg was obtained for almond spiked in rice cookies. Röder et al. (2011) also developed a method based on real-time PCR system with a TaqMan probe to detect almond allergen Pru du 3 down to an LOD of 5 mg/kg of almond in a variety of food matrices, while Costa et al. (2012b) reported the detection of the gene encoding for Pru du 5 allergen with a relative LOD of 50 mg/kg of almond and an absolute LOD of 10 pg of almond DNA. These same authors describe a single-tube nested real-time PCR to detect almond allergen Pru du 6 with the same LOD of 50 mg/kg and an absolute LOD of 1.28 pg (Costa, Oliveira, & Mafra, 2013).

This observation suggests that the real-time PCR method proposed herein is beneficial because, due to its high sensitivity, it facilitates the detection of almond traces in commercial food products. In all, these results demonstrate that labeling may not always be reliable for almond 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 almonds in food products and therefore preventing the food allergic consumer from unintentional ingestion of hidden allergens.

4. Conclusions

The *ITS* almond specific system was applied to the analysis of a collection of 214 commercial food products, and PCR results conformed to the labeling in all but 50 samples, which contained undeclared almonds. This demonstrates the potential of the real-time PCR-based method to detect the presence of almond in commercial products. As demonstrated, the presented PCR method is highly sensitive and selective, which makes it suitable for the detection of small amounts of almonds 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 programs to enforce accurate labeling of commercial food products, thereby protecting both producers and consumers against hidden allergens.

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