



Novel approach based on single-tube nested real-time PCR to detect almond allergens in foods

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ARTICLE INFO

Article history:

Received 13 August 2012

Accepted 5 December 2012

Available online 20 December 2012

Keywords:

Almond detection

Single-tube nested real-time PCR

Tree nuts

Food allergens

ABSTRACT

Almond is a widely consumed nut due to its pleasant flavour and health benefits. However, since this nut is commonly present in many processed foods, it is regarded as a potential hidden allergen. In this work, the novel single-tube nested real-time PCR system, already successfully applied to hazelnut and peanut detection, was attempted to trace almond allergens in foods. The method consists of using two sets of primers and a hydrolysis probe specifically designed to target *Prunus dulcis* clone 276NPL *prunin* gene that encodes the Pru du 6 allergen. The system allowed lowering the limit of detection of the conventional real-time PCR from 100 mg/kg to 50 mg/kg of almond spiked in walnut. It also enabled an absolute sensitivity of 1.28 pg of almond DNA that corresponds to about 3.9 DNA copies. These results highlight the suitability of the newly developed method for the detection of almond allergens in processed foods. To our knowledge, these findings were never reported and represent a great achievement when considering the detection of this almond allergen in food products.

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1. Introduction

Almond (*Prunus dulcis* or *Amygdalus communis* L.) belongs to the tree nut group and it is considered one of the most appreciated and consumed nuts. Especially due to the potential health benefits attributed to this nut and to its recent recognition by the Food and Drug Administration (FDA), as “heart-protective” foods (FDA, 2003), the consumption of almond and other nuts has been increasing over the past years. Thus, almond can be found in all sorts of product formulations ranging from bakery foods to chocolates or confectionary foodstuffs (Alasalvar & Shahidi, 2008), which in some cases can be present as a hidden ingredient. Although almond can be safely ingested by the majority of the population, it can also represent a problem of public health to a certain percentage of sensitised consumers since it is a source of allergenic proteins. Until now, eight groups of allergenic proteins have been characterised in almond, being responsible to induce mild to potentially fatal reactions in sensitised individuals (Costa, Mafra, Carrapatoso, & Oliveira, 2012).

The actual prevalence of allergies related to tree nuts is not yet well established, though recent studies seem to indicate a clear increase on the incidence of tree nut allergies among young population. In the USA, the prevalence of allergy in children raised from 0.6% to 2.1% between 1997 and 2008 (Sicherer, Muñoz-Furlong, Godbold, & Sampson, 2010). Within the tree nut group, almond, walnut, cashew, pecan and Brazil nut are known to be responsible for food related allergies in the USA (Sicherer & Sampson, 2006). In Europe, allergy to hazelnut consumption

is more frequent and is often related to birch pollinosis (Burney et al., 2010). Therefore, it is mandatory to protect sensitised and allergic individuals from potentially life-threatening foods since the total avoidance of allergenic ingredients is rather difficult to achieve. In addition to this and mostly due to the excessively precautionary labelling practiced by food industry, allergic consumer's choices have become significantly restricted, affecting consequently their quality of life. Presently, food allergen management is considered as a major challenge, particularly to the clinical community and to the food industry (Rejeb, Abbott, Davies, Clérout, & Delahaut, 2005). Therefore, the development of proper analytical methodologies to help the industry managing the food allergens and to ensure consumer's safety is crucial.

Actually, most of the analytical tools used for the detection of food allergens target either proteins or DNA molecules (Holck et al., 2011). Regarding almond detection and quantification, some analytical approaches have been successfully applied. The immunochemical assays such as enzyme-linked immunosorbent assays (ELISA) or lateral flow devices (LFD) have been extensively used since they exhibited the advantage of directly detecting the offending ingredients (marker proteins) (Garber & Perry, 2010; Rejeb et al., 2005; Röder, Vieths, & Holzhauser, 2011; Scheibe, Weiss, Ruëff, Przybilla, & Görg, 2001). However, immunoassays present some major drawbacks that encompass the problems with the alteration of protein conformational structure as a result of food processing (van Hengel, 2007) or cross-reactivity with non-target proteins, especially with other fruits from the Rosaceae family. More recently, mass-spectrometry based techniques have also been successfully applied to the direct monitoring of almond proteins in food products (Bignardi, Elviri, Penna, Careri, & Mangia, 2010; Heick, Fischer, & Pöpping, 2011).

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Considering the availability of alternative analytical techniques, the DNA-based methods have attained a special emphasis since DNA molecules present relatively high stability upon food processing. Even though these techniques are regarded as indirect approaches for the detection of allergenic ingredients, the methods based on polymerase chain reaction (PCR) have been effectively used to detect almond in foods with good specificity and sensitivity (Costa, Mafra, & Oliveira, 2012; Pafundo, Gulli, & Marmiroli, 2009, 2010; Röder et al., 2011; Wang, Li, Zhao, Chen, & Ge, 2011). The referred studies present good sensitivity levels for almond detection, but only Pafundo et al. (2009, 2010) used a system targeting amandin (Pru 1 or Pru du 6) in foods. The method reported by Pafundo et al. (2010) was based on real-time PCR with a non-sequence specific binding DNA molecule (SYBR® GreenER™), reaching adequate sensitivity of almond DNA encoding Pru du 6 allergen, nevertheless lacked cross-reactivity assessment with related or non-related species.

In terms of molecular characterisation, Pru du 6 belongs to the cupin superfamily and is the most well studied allergen in almond (Albillos, Menhart, & Fu, 2009; Jin et al., 2009; Sathe et al., 2002), probably because it is a storage protein present in high amounts in almond kernel. Additionally, it is classified as a major allergen that is responsible for triggering severe allergic reactions (potentially life-threatening) in sensitised individuals (Roux, Teuber, & Sathe, 2003), which emphasises the importance of using Pru du 6 as a target almond allergen.

The aim of this work was to apply for the first time the new approach based on single tube nested real-time PCR system for the detection and semi-quantification of almond allergens in food products. The developed method was based on the same principle as reported by Bergerová, Brežná, and Kuchta (2011) and Costa, Mafra, Kuchta, and Oliveira (2012) for peanut and hazelnut detection, respectively, assembling the advantages of two techniques, namely, nested PCR and real-time PCR in one single reaction. The nested PCR technique allows producing PCR fragments with two different sizes, increasing the specificity of the reaction. The use of a fluorogenic probe during the real-time PCR assay enables the direct monitoring of the fragment production throughout the entire second phase of the reaction. The application of this new system aimed at enhancing the sensitivity and specificity of almond DNA detection in foods. This study also intends to compare the conventional real-time PCR technique with the proposed single-tube nested real-time PCR method regarding almond detection.

2. Materials and methods

2.1. Plant foods and sample preparation

Almonds were obtained from selected regional cultivars (“Duro Italiano”, “Orelha de mula”, “Casa Nova”, “Pegarinhos”, “Refego”, “Marcelina” and “Verdeal”) collected in orchards located in Southwest region of Trás-os-Montes (Northeast of Portugal) and commercial cultivars (“Gloriette”, “Ferragnes”, “Ferrastar”, “Ferradual”, and “Marcona”). Almond and other tree nuts that included walnut, macadamia nut, hazelnut, Brazil nut, chestnut, pine nut, cashew, pistachio and peanut, as well as different plant foods (soybean, lupine, fava bean, maize, oat, barley, rice, rye, wheat, pumpkin seeds, rapeseed, sunflower, tomato, peach, apricot, plum and cherry) were obtained at local markets.

In the absence of certified or testing reference standards for almond, binary model mixtures containing 10 mg/kg; 50 mg/kg; 100 mg/kg; 500 mg/kg; 1000 mg/kg; 5000 mg/kg; 10,000 mg/kg; 50,000 mg/kg and 100,000 mg/kg of almond in walnut matrix were prepared. The first sample containing 100,000 mg/kg of almond (10%) was prepared by adding 20 g of almond to 180 g of walnut. All the other model mixtures were serially diluted by successive additions of walnut material until 10 mg/kg (0.001%) in the equivalent proportion.

All plant food samples, as well as reference mixtures were ground and homogenised separately, into a fine powder of approximately 0.3 mm of diameter in a laboratory knife mill Grindomix GM200 (Retsch,

Haan, Germany) using different containers and material, previously treated with a DNA decontamination solution. To avoid accidental cross-contamination among samples, plant foods and standards, all materials were grounded in different days. The fruits, namely, tomato, peach, apricot, plum and cherry, were lyophilised before grinding.

After preparation, all samples and reference mixtures were immediately stored at -20°C until further DNA extraction.

2.2. DNA extraction

DNA was extracted from all samples by chaotropic solid-phase extraction using the commercial Nucleospin Food kit (Macherey-Nagel, Düren, Germany), according to the manufacturer's instructions with minor alterations, as described by Costa et al. (2012a).

Yield and purity of extracts were assessed by agarose gel electrophoresis and by UV spectrophotometry using a spectrophotometer UV1800 Shimadzu (Kyoto, Japan).

2.3. Target gene selection and oligonucleotide primers

The DNA sequence corresponding to *P. dulcis* clone 276NPL *prunin* gene encoding for the Pru du 6 allergen was retrieved from the GenBank database (accession no. EU919663). Two sets of primers with different annealing temperatures (T_a) were designed using the software Primer-BLAST designing tool (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>) (Table 1). The software parameters were set to design a first pair of primers (Prd6-1F/Prd6-1R) with an optimal T_a of 65°C . The second pair of primers was projected to have lower T_a (54°C), considering a difference of at least 10°C between the two sets of primers. For the application of real-time PCR systems a hydrolysis probe was also designed (Table 1). The basic local alignment search tool BLAST software (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) was also used to identify regions of local similarity between the chosen nucleotide sequence and homologue sequences of different species. In addition, the programme estimates the statistical significance of the matches, which confirmed the specificity of the designed primers for the selected *P. dulcis* clone 276NPL *prunin* gene (accession no. EU919663), *P. dulcis* prunin 1 precursor (accession no. GU059260) and *Prunus amygdalus* Batsch (Texas) pru1 mRNA (accession no. X78119) with 100%, 99% and 98% identity, respectively. Any other additional homology was found with plant species. Primers and probe were synthesised by Eurofins MWG Operon (Ebersberg, Germany).

2.4. Sequencing

For sequencing the region of interest, a third set of primers (Prd6-FS/Prd6-RS) was specifically designed to produce larger fragments with 274 bp, encompassing the target region of 121 bp amplified by the primers (Prd6-1F/Prd6-1R), using the end-point PCR conditions described below. The amplified fragments of five almond samples containing four different cultivars and the commercial almond used to

Table 1

Key data of primers and probe designed to target *Prunus dulcis* clone 276NPL *prunin* gene partially encoding for Pru du 6 protein (GenBank accession no. EU919663).

Oligonucleotides	Sequence (5'-3')	Amplicon (bp)
Outer primers		
Prd6-1F	CCGCAGAACCACTGCCAGCT	121
Prd6-1R	CCCCGGCACACTGGAAGTCCT	
Prd6-1P	FAM-GCTTCAAGCCCGCAACCCGACAAC-BHQ2	
Inner primers		
Prd6-1FN	AACCACTGCCAGCTCAA	88
Prd6-1RN	TGAAGTCCAGGTCTCGAT	
Sequencing primers		
Prd6-FS	CTCCTTCTCATTCTGTGTGAATGG	274
Prd6-RS	AGGAAGGCAAGTGTAGGCCGTT	

prepare the model mixtures were sequenced. Due to the usual problems of cross-reactivity between almond, peach and apricot, and since these two samples presented positive end-point PCR bands, they were also sequenced. All PCR products were purified with Jetquick PCR purification kit (Genomed, Löhne, Germany) to remove any possible interfering components and sent to a specialised research facility (STABVIDA, Lisbon, Portugal) for sequencing. Each target fragment was sequenced twice, performing the direct sequencing of both strands in opposite directions, which allowed the production of two complementary sequences with very good quality.

2.5. End-point PCR

PCR amplifications were carried out in 25 μ L of total reaction volume containing 2 μ L of almond DNA extract (100 ng), 670 mM of Tris–HCl (pH 8.8), 160 mM of $(\text{NH}_4)_2\text{SO}_4$, 0.1% of Tween 20, 200 μ M of each dNTP, 1.0 U of SuperHot Taq DNA Polymerase (Genaxxon Bioscience, Ulm, Germany), 3.0 mM of MgCl_2 and 200 nM of each primer Prd6-FS/Prd6-RS (Table 1). The reactions were performed in a MJ Mini thermal cycler (BioRad, Hercules, CA, USA) using the following programme: initial denaturation at 95 °C for 5 min; 40 cycles at 95 °C for 30 s, 65 °C for 45 s and 72 °C for 1 min; and a final extension at 72 °C for 5 min.

2.6. Real-time PCR assays

Real-time PCR assays were performed in 20 μ L of total reaction volume. For each reaction tube, 2 μ L of DNA (100 ng), 1 $\times$ of SsoFast Probes Supermix (BioRad, Hercules, CA, USA), 300 nM of each outer primer Prd6-1F/Prd6-1R and 150 nM of hydrolysis probe Prd6-1P (Table 1). For nested real-time PCR amplification, the mix included additionally 300 nM of the primers Prd6-1FN/Prd6-1RN, specifically designed for this assay (Table 1). All real-time PCR assays were made on a fluorometric thermal cycler CFX96 Real-time PCR Detection System (BioRad, Hercules, CA, USA). Real-time PCR amplifications based on the conventional technique were performed according to the following temperature protocol: 95 °C for 5 min, 50 cycles at 95 °C for 15 s and 65 °C for 45 s, with the fluorescence signal acquisition at the end of each cycle. Nested real-time PCR assays were carried out with two different programmes of temperature as presented in Table 2: phase 1, performed without collecting fluorescence signal; and phase 2, with collection of the fluorescence signal made at the end of each cycle. Data were collected and analysed using the software Bio-Rad CFX Manager 2.1 (BioRad, Hercules, CA, USA). Cycle threshold (Ct) values were calculated using the software at automatic threshold setting. Real-time PCR and nested real-time PCR trials were repeated two or three times using three replicates, respectively.

3. Results

For the proposed method, two sets of primers with different annealing temperatures were designed. The first set generating PCR fragments of 121 bp was used as the “outer” primers to delineate the chosen target sequence (Table 1, Fig. 1). Thus, for this system, primers Prd6-1F/Prd6-1R and probe Prd6-1P were selected to hybridise at high temperatures (65 °C), conferring great selectivity to the reaction. The second set of

primers (Prd6-1FN/Prd6-1RN), producing PCR fragments of 88 bp, was defined to act as “inner” primers at lower hybridisation temperatures (54 °C). The successful empirical rule for single-tube nested real-time PCR system, T_a (inner primers) < T_a (outer primers) < T_a (probe), used for Ara h 3 (Bergerová et al., 2011) and hsp1 (Costa et al., 2012a) detections, was also followed in this approach. In order to perform the single-tube nested real-time PCR system, two independent temperature phases have to be established (Table 2). During phase 1, PCR fragments of 121 bp were amplified to function as DNA template later on the reaction, with no fluorescence acquisition. Phase 2 was programmed to produce PCR fragments of 88 bp using the 121 bp fragments as template and the collection of fluorescence was performed at the end of each cycle. Therefore, the number of cycles used in each phase was defined considering the best performance in nested real-time PCR trials (data not shown). Phase 1 was set during 10 or 15 cycles, without fluorescence collection. In phase 2 fluorescence signal acquisition was carried out using 35 or 40 cycles, according to the initial number of cycles used in phase 1.

3.1. Sequencing of PCR products

An easy, simple and very reliable strategy for sequencing small regions is to use a third set of primers (Prd6-FS/Prd6-RS) to amplify a larger fragment (274 bp), encompassing the target sequence of 121 bp. This strategy was already successfully accomplished for the sequencing of hazelnut fragments in a previous report (Costa et al., 2012a). With this approach it was possible to amplify PCR fragments with adequate size for direct sequencing. The sequencing results for the confirmation of the identity of the PCR products are presented in Fig. 2. Regarding almond cultivars and the commercial sample used to prepare model standards, their sequences evidenced 100% homology with the sequence retrieved from GenBank (accession no. EU919663). However, the obtained sequences also highlighted the reasons for the existence of some cross-reactivity among species from the Rosaceae family. Though both apricot and peach DNA could be amplified with the designed primers, it was also possible to identify some differences between the sequences of almond and the other fruits (peach and apricot) in the target region of 121 bp. Peach and the respective variety of nectarine presented the same sequence, but with three nucleotide differences comparing with almond. Peach and nectarine sequences presented in the nucleotide position 161 a C base instead of a T, in the position 192 a G residue instead of a C and in position 209 a T instead of a C nucleotide. Apricot only presented a difference compared with almond in position 161. This difference corresponds to one of the three dissimilarities found in peach and nectarine sequences, where a C is observed instead of a T nucleotide. According to these results, apricot seems to be more closely related to almond than peach. Targeting these differences, a probe was designed in order to allow discrimination of almond from these fruits.

3.2. Specificity

After DNA extraction, all samples and reference mixtures were evaluated for their amplifiability using a set of eukaryotic primers 18SEUDIR/18SEUINV (Fajardo et al., 2008), in order to prevent any resulting false-negatives. All DNA extracts tested positively with the universal primers, confirming that they contained amplifiable DNA.

Table 2
Temperature protocol for nested real-time PCR system.

	Phase 1			Phase 2		
	Initial denaturation	Denaturation	Annealing/polymerisation	Denaturation	Annealing	Polymerisation
Temperature	95 °C	95 °C	65 °C	95 °C	54 °C	72 °C
Time	5 min	15 s	45 s	15 s	20 s	30 s
Number of cycles	10 or 15			35 or 40		

1 GGGGTGAGCTCGAATTACAAGTCAKCTATCTCCTTCTCATTCCTTGTGTAATGGCTARGG
 61 CTTTCGTTTTTTTCGCTTTGCTTGCTTCTGGTTTTCAATGGCTGCTTAGCGGCACGCCAGT
 121 CCCAGTTGAGTCCGCAGAACCCAGTGCCAGCTCAACCA**GCTTCAAGCCCGCAACCCGACA**
 181 **ACCGCATCCAGGCKGAGGCGGGTCAGATCGAGACCTGGAACCTCAACCAGGAGGACTTCC**
 241 **AGTGTGCCGGG**TSGCCGCTCTCGAATCACCATTACAGCGCAACGGCCTACACTTGCCCTT
 301 CCTACTCCAAC

Fig 1. Region of the *Prunus dulcis* clone 276NPL *prunin* gene partially encoding the Pru du 6 allergen in almond (GenBank accession no. EU919663). “Outer” primers (Prd6-1F/Prd6-1R) in bold, “inner” primers (Prd6-1FN/Prd6-1RN) underlined and probe (Prd6-1P) in bold and double-underlined. Primers (Prd6-FS/Prd6-RS) used for sequencing are shaded in grey.

Almond EU919663	69	TTTCGCTTTGCTTGCTTCTGGTTTTCAATGGCTGCTTAGCGGCACGCCAGTCCCAGTTGA
Almond Standard		TTTCGCTTTGCTTGCTTCTGGTTTTCAATGGCTGCTTAGCGGCACGCCAGTCCCAGTTGA
Almond “Marcelina”		TTTCGCTTTGCTTGCTTCTGGTTTTCAATGGCTGCTTAGCGGCACGCCAGTCCCAGTTGA
Almond “Gloriette”		TTTCGCTTTGCTTGCTTCTGGTTTTCAATGGCTGCTTAGCGGCACGCCAGTCCCAGTTGA
Almond “Ferragnes”		TTTCGCTTTGCTTGCTTCTGGTTTTCAATGGCTGCTTAGCGGCACGCCAGTCCCAGTTGA
Almond “Ferrastar”		TTTCGCTTTGCTTGCTTCTGGTTTTCAATGGCTGCTTAGCGGCACGCCAGTCCCAGTTGA
Apricot		-----
Peach var. Nectarine		-----
Peach		TTTCGCTTTGCTTGCTTCTGGTTTTCAATGGCTGCTTAGCGGCACGCCAGTCCCAGTTGA
Almond EU919663	129	GTCCGCAGAACCCAGTGCCAGCTCAACCAGCTTCAAGCCCGCAACCCGACAACCCGATCC
Almond Standard		GTCCGCAGAACCCAGTGCCAGCTCAACCAGCTTCAAGCCCGCAACCCGACAACCCGATCC
Almond “Marcelina”		GTCCGCAGAACCCAGTGCCAGCTCAACCAGCTTCAAGCCCGCAACCCGACAACCCGATCC
Almond “Gloriette”		GTCCGCAGAACCCAGTGCCAGCTCAACCAGCTTCAAGCCCGCAACCCGACAACCCGATCC
Almond “Ferragnes”		GTCCGCAGAACCCAGTGCCAGCTCAACCAGCTTCAAGCCCGCAACCCGACAACCCGATCC
Almond “Ferrastar”		GTCCGCAGAACCCAGTGCCAGCTCAACCAGCTTCAAGCCCGCAACCCGACAACCCGATCC
Apricot		--CCGCAGAACCCAGTGCCAGCTCAACCAGCTTCAAGCCCGCAACCCGACAACCCGATCC
Peach var. Nectarine		--CCGCAGAACCCAGTGCCAGCTCAACCAGCTTCAAGCCCGCAACCCGACAACCCGATCC
Peach		GTCCGCAGAACCCAGTGCCAGCTCAACCAGCTTCAAGCCCGCAACCCGACAACCCGATCC
Almond EU919663	189	AGGCGGAGGCGGGTCAGATCGAGACCTGGAACCTCAACCAGGAGGACTTCCAGTGTGCCG
Almond Standard		AGGCTGAGGCGGGTCAGATCGAGACCTGGAACCTCAACCAGGAGGACTTCCAGTGTGCCG
Almond “Marcelina”		AGGCGGAGGCGGGTCAGATCGAGACCTGGAACCTCAACCAGGAGGACTTCCAGTGTGCCG
Almond “Gloriette”		AGGCGGAGGCGGGTCAGATCGAGACCTGGAACCTCAACCAGGAGGACTTCCAGTGTGCCG
Almond “Ferragnes”		AGGCGGAGGCGGGTCAGATCGAGACCTGGAACCTCAACCAGGAGGACTTCCAGTGTGCCG
Almond “Ferrastar”		AGGCTGAGGCGGGTCAGATCGAGACCTGGAACCTCAACCAGGAGGACTTCCAGTGTGCCG
Apricot		AGGCGGAGGCGGGTCAGATCGAGACCTGGAACCTCAACCAGGAGGACTTCCAGTGTGCCG
Peach var. Nectarine		AGGGGGAGGCGGGTCAGATTGAGACCTGGAACCTCAACCAGGAGGACTTCCAGTGTGCCG
Peach		AGGGGGAGGCGGGTCAGATTGAGACCTGGAACCTCAACCAGGAGGACTTCCAGTGTGCCG
Almond EU919663	249	GGGTSGCCGCTCTCGAAT
Almond Standard		GGGTCGCCGCTCTCGAAT
Almond “Marcelina”		GGGTCGCCGCTCTCGAAT
Almond “Gloriette”		GGGTGGCCGCTCTCGAAT
Almond “Ferragnes”		GGGTCGCCGCTCTCGAAT
Almond “Ferrastar”		GGGTCGCCGCTCTCGAAT
Apricot		GGGTCGCCGCTCTCGAAT
Peach var. Nectarine		GGGTCGCCGCTCTCGAAN
Peach		GGGTCGCCGCTCTCGAAT

Fig 2. Alignment of PCR products of 4 almond cultivars, almond used in reference mixtures, apricot and peach obtained by sequencing. The shadowed region of 121 bp corresponds to the PCR fragments using “outer” primers Prd6-1F/Prd6-1R.

To assess the specificity of the designed primers for the target sequence, twelve almond cultivars, other tree nuts and several plants were tested by PCR. Specificity and cross-reactivity results for the designed primers are presented in Table 3. All twelve almond cultivars and the almond sample used for reference mixture preparation tested positively by qualitative PCR. The similarity among almond and other fruits from the Rosaceae family was patent, since the samples apricot and peach tested positively with this set of primers when submitted to end-point PCR (data not shown), though with more faint bands. These results evidence the homology among these fruits and consequently affect the specificity of the existing methods for almond amplification. All the other species did not amplify with the selected primers. Though the use of these primers was found to be adequate for Pru du 6 identification, some caution should be considered when analysing foods susceptible of containing peach or apricot.

3.3. Development of analytical method

Both conventional real-time PCR and single-tube nested real-time PCR systems were optimised using model standards of walnut spiked with known amounts of almond. Since no requirements for allergen

Table 3

Results of PCR amplifications of *Prunus dulcis* clone 276NPL *prunin* gene applied to 12 almond cultivars and other plant species (26), including other tree nuts (8).

Sample name	Country of origin	Scientific denomination	PCR
12 Almond cultivars ("Orelha de mula", "Casa Nova", "Pegarinhos", "Refego", "Marcelina", "Verdeal", "Gloriette", "Duro Italiano", "Ferragnes", "Ferrastar", "Ferradual" and "Marcona")	Portugal	<i>Prunus dulcis</i>	+
Almond (commercial)	Portugal	<i>Prunus dulcis</i>	+
Hazelnut	Portugal	<i>Corylus avellana</i>	—
Walnut	France	<i>Juglans regia</i>	—
Macadamia nut	Austria	<i>Macadamia tetraphylla</i>	—
Pine nut	Portugal	<i>Pinus pinea</i>	—
Brazil nut	Bolivia	<i>Bertholletia excelsa</i>	—
Pistachio	USA	<i>Pistacia vera</i>	—
Cashew	India	<i>Anacardium occidentale</i>	—
Chestnut	Portugal	<i>Castanea sativa</i>	—
Peanut	USA	<i>Arachis hypogaea</i>	—
Lupine	Portugal	<i>Lupinus albus</i>	—
Fava bean	Greece	<i>Vicia faba</i>	—
Soybean	USA	<i>Glycine max</i>	—
Maize	USA	<i>Zea mays</i>	—
Wheat	Portugal	<i>Triticum aestivum</i>	—
Rice	Portugal	<i>Oryza sativa</i>	—
Oat	Portugal	<i>Avena sativa</i>	—
Barley	Spain	<i>Hordeum vulgare</i>	—
Pumpkins seeds	Greece	<i>Cucurbita mixta</i>	—
Rapeseed	Brazil	<i>Brassica napus</i>	—
Sunflower	Brazil	<i>Helianthus annuus</i>	—
Rye	Portugal	<i>Secale cereale</i>	—
Tomato	Spain	<i>Solanum lycopersicum</i>	—
Peach	Portugal	<i>Prunus persica</i>	+
Apricot	Turkey	<i>Prunus armeniaca</i>	+
Plum	Chile	<i>Prunus cerasifera</i>	—
Cherry	Chile	<i>Prunus avium</i>	—

testing are yet defined, the fundamentals for the assessment and comparison of the real-time PCR systems were based on the existing documentation of the definition of the minimum performance requirements for analytical methods of genetically modified organism testing. The real-time PCR systems were also developed and evaluated considering the requisites defined by MIQE Guidelines (Bustin et al., 2009).

3.3.1. Real-time PCR system

The application of real-time PCR based on the conventional system to binary mixtures ranging from 100,000 mg/kg to 10 mg/kg (10–0.001%) enabled a relative limit of detection (LOD) of 100 mg/kg of almond in walnut (Table 4, Fig. 3a). According to the minimum performance requirements defined by the European Network of GMO Laboratories (ENGL, 2008), the LOD should be determined considering a positive identification of the analyte at least 95% of the times, thus assuring less than 5% of false negative results. In this study, the LOD was determined assuming the lowest amount of almond DNA with positive amplification in all replicates. The limit of quantification (LOQ) achieved was equal to the LOD (100 mg/kg of almond in walnut) since the lowest amplified level was within the linear range of the calibration curve. In order to correctly evaluate a real-time PCR run, several parameters have to comply with the acceptance criteria defined for these assays. The parameters encompass the slope, the correlation coefficient and the PCR efficiency. A real-time PCR assay is considered of good performance when the correlation coefficient is above 0.98, the PCR efficiency ranging from 90% to 110% and the slope between -3.6 and -3.1 . All the assays performed with real-time PCR system presented high values of correlation and efficiency, exhibiting a correlation factor (R^2) of 0.9989, slope of -3.5032 and PCR efficiency of 93.0% (Table 4, Fig. 3c.). Thus, the referred parameters are in good agreement with the acceptance criteria defined for real-time PCR method performance (Bustin et al., 2009; ENGL, 2008). The Ct mean value established for the lowest amplified standard of walnut spiked with almond (100 mg/kg) was 37.04 cycles (Table 4).

In order to establish the dynamic range and the absolute sensitivity of the method, almond DNA extracts 5-fold serially diluted ranging from 20 ng to 1.28 pg were evaluated using the conventional real-time PCR system developed for this study (Fig. 4a). The set of primers and probe presented 100% PCR efficiency with approximately two additional cycles (~ 2 Ct) for a 5-fold dilution of almond template. Real-time PCR assays exhibited high performance, with average values of PCR efficiency of 99.5%, slope of -3.333 and R^2 of 0.9968. The real-time PCR system allowed amplifying almond DNA until a dilution factor of 625, with a LOD of 32 pg that corresponded to 97 DNA copies (Table 5, Fig. 4a). The number of almond DNA copies was estimated according to the genome size of almond (0.33 pg) and assuming that the target sequences are single copy genes. The value for almond genome size was retrieved from the Plant DNA C-values database (RBC, Kew). This

Table 4

Real-time PCR and nested real-time PCR results for the relative detection of spiked almond in reference model mixtures.

Spiked level (mg/kg)	Real-time PCR system	Nested real-time PCR system
	Ct $\pm$ SD ^a	Ct $\pm$ SD ^a
10	nd ^b	nd
50	nd	26.89 $\pm$ 1.20 (9)
100	37.04 $\pm$ 0.70 (6)	26.18 $\pm$ 1.39 (9)
500	34.58 $\pm$ 0.68 (6)	23.18 $\pm$ 0.51 (9)
1000	33.52 $\pm$ 0.30 (6)	21.84 $\pm$ 0.74 (9)
5000	30.82 $\pm$ 0.20 (6)	19.58 $\pm$ 0.71 (9)
10,000	29.84 $\pm$ 0.32 (6)	18.90 $\pm$ 0.72 (9)
50,000	27.56 $\pm$ 0.16 (6)	16.35 $\pm$ 0.81 (9)
100,000	26.61 $\pm$ 0.64 (6)	15.92 $\pm$ 0.59 (9)
Correlation coefficient (R^2)	0.9989	0.9933
Slope	-3.503	-3.433
PCR efficiency (%)	93.0	95.6

^a Mean cycle threshold (Ct) values $\pm$ standard deviation (SD) ($n=6$) and ($n=9$).

^b nd, not detected.

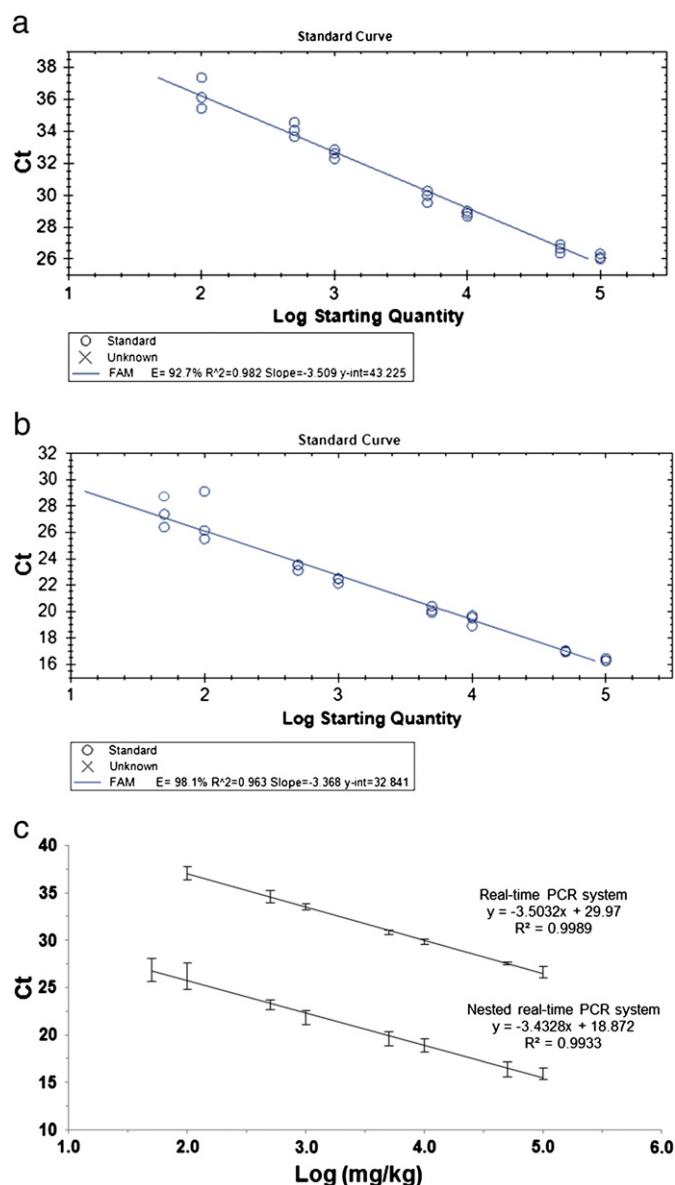


Fig. 3. Calibration curves obtained by real-time PCR (a) and nested real-time PCR (b) of reference mixtures containing 100,000; 50,000; 10,000; 5000; 1000; 500; 100; 50 and 10 mg/kg of almond in walnut. Average values and corresponding standard deviations of $n=6$ and $n=9$ replicates for real-time PCR and nested real-time PCR, respectively (c).

database compiles relevant information regarding several plant species such as genome size (C-value), estimation method, ploidy level, chromosome number and original references.

3.3.2. Single-tube nested real-time PCR system

The proposed single-tube nested real-time PCR system for the detection of almond allergen was optimised similar to the conventional system using the same set of reference mixtures ranging from 100,000 mg/kg to 10 mg/kg of almond in walnut. In the case of the nested assay, the two distinct phases of amplification need to be adjusted (Table 2). The first phase was pre-established to amplify PCR fragments with 10 amplification cycles using the outer primers. Additional experiments using higher number of cycles were attempted (data not shown), selecting the value of 10 cycles in phase 1 as it presented improved relative sensitivity at best linearity.

The nested real-time PCR system achieved a relative LOD of 50 mg/kg, which was $2\times$ lower than the value obtained with the conventional

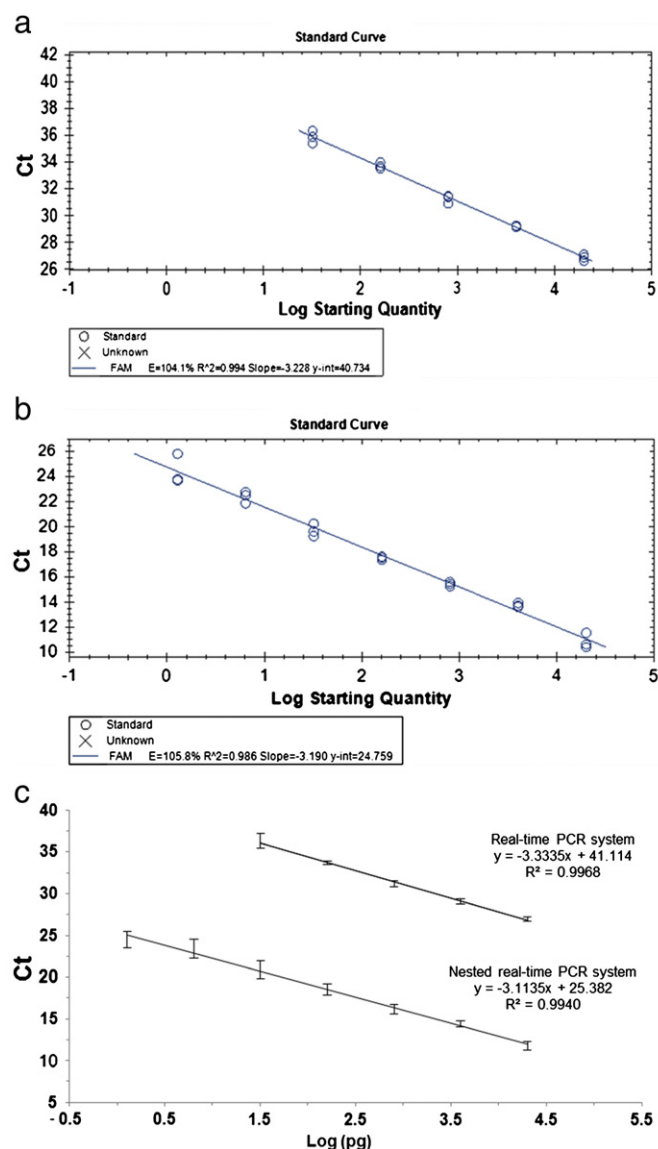


Fig. 4. Calibration curves obtained by real-time PCR (a) and nested real-time PCR (b) of almond DNA serially diluted (5-fold) from 20 ng to 1.28 pg. Average values and corresponding standard deviations of $n=6$ and $n=9$ replicates for real-time PCR and nested real-time PCR, respectively (c).

real-time PCR system (Table 4, Fig. 3b). The LOD was the same as the LOD, considering that the lowest amplified standard was within the linear range of the calibration curve. The mean value for all the assays obtained with the new system exhibited high correlation coefficient ($R^2 = 0.9933$), appropriate slope (-3.433) and PCR efficiency (95.6%) (Table 4, Fig. 3c). The absolute sensitivity of the nested real-time PCR system was also determined using identical almond DNA dilutions ranging from 20 ng to 1.28 pg. For these set of experiments the protocol for nested real-time PCR system was defined with 15 cycles for phase 1. The mean value for all the nested real-time PCR assays exhibited high PCR efficiency (109.5%) and correlation coefficient ($R^2 = 0.9940$) (Table 5, Fig. 4c). With the novel system, it was possible to increase the absolute sensitivity to 1.28 pg of almond DNA (corresponding to 3.9 DNA copies), which is $25\times$ lower than the LOD obtained with the conventional real-time PCR system. The nested real-time PCR system allowed amplifying almond DNA until a dilution factor of 15,625. As in the previous relative quantification, the LOD was the same as the LOD.

The nested real-time PCR system was further tested to assess any possible cross-reactivity that might occur with the closely related species of apricot and peach, leading to false positive results to

Table 5

Real-time PCR and nested real-time PCR results for the absolute detection of almond DNA.

Absolute quantity (pg)	Real-time PCR system		Nested real-time PCR system	
	Ct $\pm$ SD ^a	DNA copies ^b	Ct $\pm$ SD ^a	DNA copies ^b
1.28	nd ^c	–	24.48 $\pm$ 0.98 (9)	3.9
6.4	40.31 $\pm$ 1.65 (2)	19	23.42 $\pm$ 1.11 (9)	19
32	36.31 $\pm$ 0.90 (6)	97	20.91 $\pm$ 1.06 (9)	97
160	33.69 $\pm$ 0.23 (6)	485	18.53 $\pm$ 0.65 (9)	485
800	31.14 $\pm$ 0.36 (6)	2424	16.15 $\pm$ 0.60 (9)	2424
4000	29.09 $\pm$ 0.28 (6)	12,121	14.38 $\pm$ 0.37 (9)	12,121
20,000	26.96 $\pm$ 0.26 (6)	60,606	11.78 $\pm$ 0.56 (9)	60,606
Correlation coefficient (R^2)	0.9968		0.9940	
Slope	–3.333		–3.114	
PCR efficiency (%)	99.5		109.5	

^a Mean cycle threshold (Ct) values $\pm$ standard deviation (SD) ($n=6$) and ($n=9$).^b Number of almond haploid genome copies (0.33 pg)²⁹.^c nd, not detected.

almond. According to the sequencing results apricot and peach presented a very close genetic relation towards almond, with 1 and 3 nucleotide differences encountered for apricot and peach, respectively (Fig. 2). Application results of nested real-time PCR system to pure DNA extracts of almond, apricot, peach and nectarine 10-fold serially diluted, ranging from 20,000 pg to 20 pg are presented in Table 6 and Fig. 5. The results evidenced a different real-time PCR profile for peach, which is in good agreement with sequencing results. Thus, for the same dilution factor for almond and peach/peach var. nectarine, amplifications presented a shift in the Ct value of approximately 8 (Table 6, Fig. 5.). Additionally, for amounts lower than 2000 pg for peach, the nested real-time PCR system did not produce positive amplifications. Comparing almond with apricot amplification, the shift in Ct value is approximately 3, with positive amplifications until 20 pg of apricot.

4. Discussion

The correct evaluation of the potential presence of offending ingredients using molecular methodologies is highly dependent on numerous factors, such as: the type of food matrix, the allergens/DNA markers, and the chosen methodology, among others. In this work, we intended to present an alternative method based on the assembly of two DNA-based techniques (nested PCR and real-time PCR) for the detection of almond DNA encoding Pru du 6 allergen. This protein is one of the most severe allergens in almond, which highlights the importance of determining its presence, even if indirectly. In spite of the demonstrated relevance of Pru du 6 allergen, few studies have targeted its detection in foods by DNA-based methods.

Table 6

Comparison of cycle threshold values obtained by nested real-time PCR applied to almond, apricot and peach.

Absolute quantity (pg)	Almond	Apricot	Peach	Peach var. nectarine
	Ct $\pm$ SD ^a	Ct $\pm$ SD	Ct $\pm$ SD	Ct $\pm$ SD
20	20.57 $\pm$ 0.49 (3)	24.50 $\pm$ 0.23 (3)	nd ^b	nd
200	17.24 $\pm$ 0.27 (3)	20.47 $\pm$ 0.55 (3)	nd	22.83 $\pm$ 0.80 (2)
2000	14.03 $\pm$ 0.20 (3)	16.99 $\pm$ 0.08 (3)	20.63 $\pm$ 1.17 (3)	19.77 $\pm$ 0.29 (3)
20,000	11.26 $\pm$ 0.18 (3)	13.99 $\pm$ 0.07 (3)	18.29 $\pm$ 0.25 (3)	17.18 $\pm$ 0.20 (3)

^a Mean cycle threshold (Ct) values $\pm$ standard deviation (SD) ($n=3$).^b nd, not detected.

Regarding the two real-time PCR systems developed in this work, the results of the conventional real-time PCR using the “outer” primers enable the detection of 100 mg/kg of almond. This sensitivity level is in good agreement with the relative LOD for the Pru du 6 allergen in a mix matrix of 5 plant species (hazelnut, walnut, peanut, sesame and cashew) reported by Pafundo et al. (2010). By the introduction of the nested approach, it was possible to enhance the performance of the real-time PCR. Concerning the relative detection of almond, the nested real-time PCR allowed lowering 2 $\times$ the LOD down to 50 mg/kg with 100% of positive replicates in all the performed assays. This LOD is comparable to the one reported by Costa et al. (2012a) regarding the detection of a different almond allergen (Pru du 5). The proposed new system presents the advantage of high specificity conferred by the use of two pairs of primers and a hydrolysis probe instead of DNA intercalating dyes such as Evagreen Costa et al. (2012b) or SYBR GreenER (Pafundo et al., 2009, 2010). Köppel et al. (2010) reported the detection of 50 mg/kg of almond, although the estimated value presented a rate of 21% of false-negatives. For quantification purposes, the estimated LOQ of 50 mg/kg establishes a supplementary improvement regarding the quantification of almond in food samples. Using the novel nested real-time PCR system, it was possible to decrease the absolute level of detection to 1.28 pg of almond representing 3.9 DNA copies. Pafundo et al. (2009) reported an apparently lower level of almond detection (1 DNA copy) on the basis of an almond genome size of 0.30 ng, which is approximately 1000 times higher than the 0.33 pg, reported by Baird, Estager, and Wells (1994) and the Plant DNA C-values database (RGB, Kew). Thus, taking this fact into consideration, the proposed method is able to detect a much inferior level of almond than the reported value of Pafundo et al. (2009).

Besides tracing small amounts of Pru du 6 allergen, the single-tube nested real-time PCR presents high performance criteria and apparent robustness. The developed approach was not affected by shifts in temperature, time and cycle number, considering the existence of two different reaction protocols within the same assay. In both real-time PCR systems, the tested parameters complied with the criteria established by the ENGL (2008) and with the MIQE guidelines (Bustin et al., 2009), thus evidencing their adequacy for the detection of almond Pru du 6 allergen.

Comparing the performances of the two real-time PCR methods, the single-tube nested real-time PCR proved to be a specific and sensitive technique for the detection of almond in foods. Some caution should be taken when analysing samples susceptible of containing apricot or peach since the system can also amplify these fruits, but only if the quantity of initial DNA is high. The homology between almond and apricot regarding DNA encoding the Pru du 6 allergen is high. Conversely, the existence of a single difference in the DNA sequences of almond and apricot is enough to produce a shift in the Ct value of approximately 3 for the same DNA concentration. In identical conditions, almond and peach presented a shift of 8 Ct, which is a consequence of the 3 nucleotide mismatches encountered in respective sequences. Almond and other fruits from the Rosaceae family have been extensively described as genetically related with high homology, still the molecular methods available so far present very few (Köppel et al., 2010) or no information at all (Pafundo et al., 2009, 2010) about possible cross-reactivity among them.

In summary, the developed single-tube nested real-time PCR method presented in this work constitutes an alternative approach to detect almond at trace levels in foods. The effective application of the single-tube nested real-time PCR to different DNA targets like almond, hazelnut (Costa et al., 2012a) or peanut (Bergerová et al., 2011) highlights the usefulness of this new tool and its potentiality for the identification of diverse allergens in foods, for which further research work is still required.

Acknowledgements

This work has been supported by the Fundação para a Ciência e a Tecnologia (FCT) through grant no. PEst-C/EQB/LA0006/2011, by the

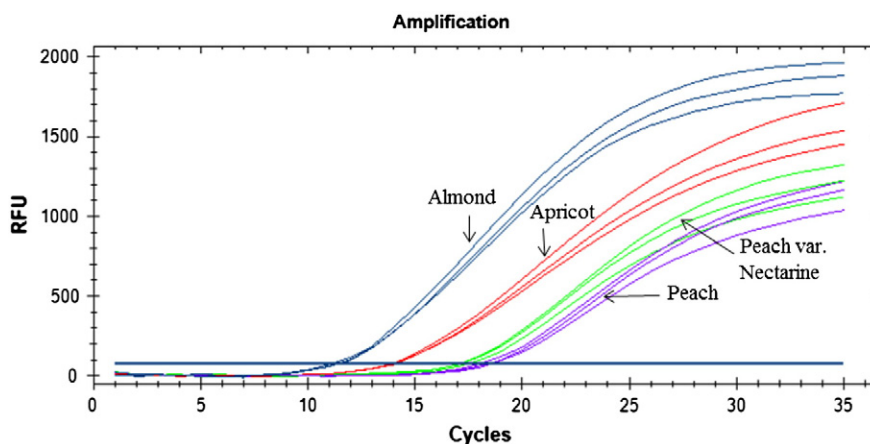


Fig. 5. Amplification curves obtained by nested real-time PCR of DNA from almond and other genetically related fruits from the Rosaceae family (apricot, peach and peach var. nectarine).

project “Improvement of molecular biology methods for authenticity assessment and detection of allergens in foods” (FCT/2489/3/6/2011/S) of Technological and Scientific Cooperation FCT/Slovakia and by the University of Porto “Projectos Pluridisciplinares” IJUP. Joana Costa is grateful to FCT PhD grant (SFRH/BD/64523/2009) financed by POPH-QREN (subsidised by FSE and MCTES).

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