



Analytical Methods

High resolution melting analysis as a new approach to detect almond DNA encoding for Pru du 5 allergen in foods

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ABSTRACT

Almond is responsible for triggering adverse immune responses in allergic individuals, and since it is present in many processed food, it is considered as a potential hidden allergen. Here we propose a novel, simple and highly specific 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 Pru du 5 allergen in almond, using the fluorescent EvaGreen® dye combined with high resolution melting (HRM) analysis. The new approach allowed the detection of trace amounts of almond down to the level of 0.005% (w/w) and was successfully applied to processed foods. HRM analysis increased the specificity of the assay and was effective in distinguishing almonds from other plant foods, including the closely related fruits from the Rosaceae family. It was demonstrated for the first time that HRM analysis can provide a powerful tool for the identification of allergens in foods.

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1. Introduction

Food allergies are, by definition, adverse responses of the immune system to food proteins, mainly glycoproteins. These reactions are attributed to Immunoglobulin E (IgE) or non-IgE (cellular) mechanisms and they affect almost 3–4% of adult population and 6% of young children (Sicherer & Sampson, 2006). Tree nuts comprise one of the eight groups of potentially allergenic foods known to be responsible for almost 90% of human allergies caused by food ingestion. In recent years, their use in food also led to an increasing concern about the rising number of sensitised individuals to tree nuts and peanut, which has intensified in Europe and USA (Sathe, Sharma, & Roux, 2008). Presently, in order to protect allergic individuals from atypical immune reactions, the total avoidance of any allergen containing food is recommended. However, this is a hard task to achieve, since processed food products commonly contain all sorts of ingredients, including potential allergens (Poms, Klein, & Anklam, 2004). To safeguard sensitised individuals, legislation on food labelling has established clear guidelines in European Union (EU), from which the recent Directive 2007/68/EC requires 14 groups of allergenic food ingredients with mandatory labelling: cereals containing gluten, crustaceans, eggs, fish, peanuts, soybeans, milk, nuts, celery, mustard, sesame, sulphur dioxide and, more recently, lupine and molluscs.

Almond, taxonomically designated as *Prunus dulcis* or *Amygdalus communis* L., belongs to the Rosaceae family, subfamily of the

Prunoideae and is genetically related to fruits such as peach, plum, apples and cherries (Chen et al., 2008; Shahidi, Zhong, Wijeratne, & Ho, 2008). In conjunction with almond consumption, numerous mild to severe immune reactions, including potentially fatal anaphylactic shocks, have been described in sensitised and allergic individuals (Chen et al., 2008; Tawde et al., 2006). Until now, eight groups of allergens have been identified in almond, comprising the following proteins: Pru du 1 (PR-10 proteins), Pru du 2 (thaumatin-like proteins), Pru du 2S albumin (lipid transfer proteins), Pru du 3 (lipid transfer proteins), Pru du 4 (profilins), Pru du 5 (60S acidic ribosomal protein P2), Pru du 6 (amandin–legumins) and Pru du γ-conglutinin (vicilins) (Costa, Mafra, & Oliveira, 2012).

Pru du 5, also known as 60S acidic ribosomal protein P2, is encoded by the *P. dulcis* 60S acidic ribosomal protein gene (AL60SRP) (<http://www.ncbi.nlm.nih.gov/nuccore/AY081851.1>), has a sequence of 113 amino acids and a calculated molecular mass of 11.4 kDa, being recently reported as an almond allergen (Abolhassani & Roux, 2009). The presence of IgE antibodies for 60S acidic ribosomal protein P2 in 50% of almond sensitised patient's sera seems to classify Pru du 5 as a major allergen in almond, according to the allergen nomenclature guidelines specified by the International Union of Immunological Societies (I.U.I.S.) Allergen Nomenclature Sub-committee (<http://www.allergen.org>).

To ensure compliance with the food labelling, reliable methods have been proposed for the detection and quantification of allergens in foods. Most of the commercially available and published techniques for allergen detection are based on the determination of potentially allergenic proteins by immunological assays (Albillos et al., 2008; Kirsch et al., 2009; van Hengel, 2007), including some applications to almond detection (Garber & Perry, 2010; Rejeb,

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Abbott, Davies, Cl  roux, & Delahaut, 2005; Roux, Teuber, Robotham, & Sathe, 2001; Scheibe, Weiss, Ru  ff, Przybilla, & G  rg, 2001). More recently, DNA-based methods have provided reliable tools of detecting hidden allergens in a wide range of foods (Mafra, Ferreira, & Oliveira, 2008; Monaci & Visconti, 2010). However, only a few polymerase chain reaction (PCR) applications have been reported for almond allergen detection (K  ppel et al., 2010; Pafundo, Gull  , & Marmiroli, 2009; Pafundo, Gull  , & Marmiroli, 2010).

The novel approach of high-resolution melting (HRM) analysis emerged with the recent advances in high resolution instrumentation and with the specialised fluorescent DNA-binding dyes (Herrmann, Durtschi, Voelkerding, & Wittwer, 2006; Reed, Kent, & Wittwer, 2007). The method involves the gradual denaturation (melting) of PCR amplicons and detection of subsequent subtle fluorescent changes by the so-called new generation dyes present in the amplification reaction, such as LC Green PLUS (Idaho Technologies Ltd.), EvaGreen (Biotium) and SYTO9 (Invitrogen). The advantage of the new dyes over SYBR Green I is related to the possibility of using higher concentrations, generating greater fluorescent signals and increased sensitivity without causing PCR inhibition. HRM analysis offers a rapid high-throughput and

cost-effective method that has been applied to genotyping, mutation scanning (Herrmann et al., 2006; Reed et al., 2007) and RNA editing (Chateigner-Boutin & Small, 2007). Applications of HRM for the development of single nucleotide polymorphism of almond cultivars (Wu, Wirthensohn, Hunt, Gibson, & Sedgley, 2008; Wu et al., 2009), for the verification of grapevine and olive cultivars using microsatellite markers (Mackay, Wright, & Bonfiglioli, 2008) and for the authentication of berry species using DNA barcodes (Jaakola, Suokasa, & H  ggmana, 2010) have also been recently described.

In this work, we propose the application of HRM analysis as a highly specific and sensitive approach to detect trace amounts of almond in foods, targeting the *ALGOSRP* gene encoding for the Pru du 5 allergen.

2. Materials and methods

2.1. Sample preparation

Almond kernels were obtained from selected regional ("Casa Nova", "Duro Italiano", "Marcelina", "Orelha de mula", "Pegarinhos",

Table 1
Results of PCR amplification of *ALGOSRP* gene and HRM analysis applied to 12 almond cultivars and other plant species.

Name	Scientific denomination	Country of origin	End-point PCR	HRM analysis ^b
Almond "Pegarinhos" cultivar	<i>Prunus dulcis</i>	Portugal	+	Cluster 1 (99.4%)
Almond "Orelha de mula" cultivar	<i>Prunus dulcis</i>	Portugal	+	Cluster 1 (96.4%)
Almond "Verdeal" cultivar	<i>Prunus dulcis</i>	Portugal	+	Cluster 1 (98.1%)
Almond "Duro Italiano" cultivar	<i>Prunus dulcis</i>	Portugal	+	Cluster 1 (98.0%)
Almond "Ferragnes" cultivar	<i>Prunus dulcis</i>	Portugal	+	Cluster 1 (85.1%)
Almond "Ferrastar" cultivar	<i>Prunus dulcis</i>	Portugal	+	Cluster 1 (99.4%)
Almond "Ferraduel" cultivar	<i>Prunus dulcis</i>	Portugal	+	Cluster 1 (97.3%)
Almond "Casa Nova" cultivar	<i>Prunus dulcis</i>	Portugal	+	Cluster 1 (95.1%)
Almond "Gloriette" cultivar	<i>Prunus dulcis</i>	Portugal	+	Cluster 1 (97.8%)
Almond "Refego" cultivar	<i>Prunus dulcis</i>	Portugal	+	Cluster 1 (98.5%)
Almond "Marcelina" cultivar	<i>Prunus dulcis</i>	Portugal	+	Cluster 1 (98.2%)
Almond "Marcona" cultivar	<i>Prunus dulcis</i>	Portugal	+	Cluster 1 (98.3%)
Walnut kernel	<i>Juglans regia</i>	Portugal	–	NA
Walnut ^a	<i>Juglans regia</i>	France	UA	Cluster 3 (100.0%) ^c
Macadamia nut ^a	<i>Macadamia tetraphylla</i>	Austria	UA	Cluster 2 (99.8%) ^c
Hazelnut	<i>Corylus avellana</i>	Portugal	UA	Cluster 4 (99.9%) ^c
Pine nut	<i>Pinus pinea</i>	Portugal	–	NA
Brazil nut ^a	<i>Bertholletia excelsa</i>	Bolivia	–	NA
Pistachio ^a	<i>Pistacia vera</i>	USA	–	NA
Cashew ^a	<i>Anacardium occidentale</i>	India	–	NA
Chestnut	<i>Castanea sativa</i>	Portugal	–	NA
Lupine	<i>Lupinus albus</i>	Portugal	–	NA
Fava bean ^a	<i>Vicia faba</i>	Greece	UA	ND
Peanut	<i>Arachis hypogaea</i>	USA	UA	Cluster 6 (89.4%)
Soybean	<i>Glycine max</i>	USA	UA	ND
Maize	<i>Zea mays</i>	USA	–	NA
Wheat	<i>Triticum aestivum</i>	Portugal	–	NA
Rice	<i>Oryza sativa</i>	Portugal	–	NA
Oat	<i>Avena sativa</i>	Portugal	–	NA
Barley	<i>Hordeum vulgare</i>	Spain	–	NA
Pumpkins seeds ^a	<i>Cucurbita mixta</i>	Greece	–	NA
Rapeseed	<i>Brassica napus</i>	Brazil	–	NA
Sunflower	<i>Helianthus annuus</i>	Brazil	–	NA
Rye	<i>Secale cereale</i>	Portugal	–	NA
Tomato	<i>Solanum lycopersicum</i>	Spain	–	NA
Peach	<i>Prunus persica</i>	Portugal	+	Cluster 5 (97.3%)
Nectarine	<i>Prunus persica</i>	Spain	+	Cluster 4 (82.7%)
Cherry	<i>Prunus avium</i>	Chile	–	NA
Plum	<i>Prunus domestica</i>	Chile	–	NA
Prune	<i>Prunus spinosa</i>	France	–	NA
Cherry plum	<i>Prunus cerasifera</i>	South Africa	–	NA
Apricot	<i>Prunus armeniaca</i>	Turkey	+	Cluster 3 (75.5%)
Black Apricot	<i>Prunus armeniaca</i>	Turkey	+	Cluster 2 (94.6%)

UA, unspecific amplification; NA, not applied; ND, not detected; +, Positive detection; –, Not detected.

^a Declaration in product label "may contain traces of peanut or other nuts".

^b Percentage of confidence according the reference cluster "cluster 1".

^c Samples included in different set of real-time PCR runs, where almond standards and commercial food containing almond were defined as reference cluster 1 (Fig. 4 and Table 2).

“Refego”, and “Verdeal”) and commercial cultivars (“Ferradual”, “Ferragnes”, “Ferrastar”, “Gloriette”, and “Marcona”), collected in orchards located in Southwest region of Trás-os-Montes, Northeast of Portugal. Walnut kernels were collected from an orchard situated in Beira Alta region, Eastern of Portugal. Other tree nuts (walnut, macadamia nut, hazelnut, pine nut, Brazil nut, chestnut, cashew and pistachio), fruits from the Rosaceae family (peach, nectarine, apricot, cherry and plum) and other plant foods (peanuts, soybean, lupine, fava bean, maize, oat, barley, rice, pumpkin seeds, rapeseed, sunflower and tomato) were obtained in local markets. Processed foods (30 samples) containing almond and/or other tree nuts were also acquired at local markets, including nut snacks (3), cereal foods (3), biscuits (2), cake (1) and chocolate bars (21). All samples were ground and homogenised using a laboratory knife mill (Grindomix GM200, Retsch, Haan, Germany), separately using different material and different containers formerly treated with DNA decontamination solution.

Almonds and walnuts kernels, previously triturated, were combined using the laboratory knife mill to prepare model mixtures as binary reference standards containing 0.001%, 0.005%, 0.01%, 0.05%, 0.1%, 0.5%, 1%, 5% and 10% of almond in walnut.

All food samples and binary reference mixtures were immediately stored at -20°C after preparation until DNA extraction.

2.2. DNA extraction

DNA from all samples was extracted using Nucleospin® Food kit (Macherey–Nagel, Düren, Germany) according to the manufacturer's instructions with minor modifications. To 200 mg of each sample, 700 μL of CF lysis solution pre-heated at 65°C and 10 μL of proteinase K (20 mg/mL) were added. The mixture was incubated in a thermal block (Thermomixer Compact, Eppendorf AG,

Hamburg, Germany) at 65°C for 1 h with continuous stirring and centrifuged for 10 min (18,500g, 4°C). The supernatant was transferred (approximately 600 μL) to a new sterile reaction tube and the same volume of precipitation solution C4 and ethanol 100% were added. The mixture was homogenised by inversion and eluted through a spin column by centrifugation (1 min, 13,000g). The column was then washed three times: the first wash with 400 μL of CQW solution, the second and third washes with 700 and 200 μL of C5 solution, respectively, followed by 1 min centrifugation (13,000g) after the first two washings and a 2 min centrifugation after the final one. The DNA was eluted from the column by adding 100 μL of CE solution at 70°C , followed by a 5 min incubation period and centrifugation (1 min, 13,000g). All the extracts were kept at -20°C until further analysis. The extractions were done in duplicate assays for each sample.

Yield and purity of extracts were assessed by agarose gel electrophoresis and by UV spectrophotometry using a spectrophotometer UV1800 Shimadzu (Kyoto, Japan). The DNA concentration was determined by UV absorbance at 260 nm (1 absorbance unit corresponds to 50 $\mu\text{g}/\text{mL}$ of dsDNA). The purity of the extracted DNA was determined by the ratio of the absorbance at 260 and 280 nm.

2.3. Target gene selection and oligonucleotide primers

The DNA sequence corresponding to the gene encoding for the allergenic 60S acidic ribosomal protein of *P. dulcis* was retrieved from the Genbank database (Accession No. DQ836316). Specific primers Prd5-1F (GGT TGT TGC AGC ATA CTT GTT GGC) and Prd5-1R (GCT CCA ACA GAG CCA AGG ATG TCC) to produce a DNA fragment of 90 bp, were designed using the software Primer-BLAST designing tool (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>). The nucleotide sequence was submitted to a basic

Table 2

Results of PCR amplification of *AL60SRP* gene and HRM analysis applied to commercial food samples.

Food samples	Almond declaration	End-point PCR	HRM analysis ^c
Tree nut snack	+ ^a	++	Cluster 1 (99.9%)
Tree nut and fruit snack	+ ^a	++	Cluster 1 (99.6%)
Almond cereals	+ ^a	++	Cluster 1 (99.7%)
Cereal bars with almond and chocolate	+	++	Cluster 1 (99.9%)
Cereal bars with almond and apricot	+	++	Cluster 1 (99.7%)
Walnut cake	—	++	Cluster 1 (99.9%)
Almond biscuits	+	++	Cluster 1 (99.9%)
Nougat and honey chocolate	+	+	Cluster 1 (99.9%)
Nougat chocolate	+ ^a	++	Cluster 1 (99.3%)
Milk fruit and nut chocolate	+ ^a	+	Cluster 1 (99.8%)
Milk chocolate with almonds 1	+ ^a	+	Cluster 1 (98.9%)
Milk chocolate with almonds 2	+ ^a	+	Cluster 1 (99.8%)
Milk chocolate with almonds 3	+ ^a	+	Cluster 1 (99.7%)
Milk chocolate with almonds 4	+ ^a	++	Cluster 1 (100.0%)
Milk chocolate with almonds 5	+ ^a	++	Cluster 1 (99.5%)
Milk chocolate with caramelised almonds	+ ^a	++	Cluster 1 (100.0%)
Milk chocolate with almonds, hazelnuts and raisins	+ ^a	++	Cluster 1 (99.9%)
Chocolate with marzipan and strawberry	+ ^a	++	Cluster 1 (99.5%)
White chocolate with hazelnuts	—	UA	Cluster 2 (98.8%)
Chocolate crispy nuts (hazelnuts)	— ^a	—	NA
Milk chocolate with hazelnuts	— ^a	—	NA
Milk chocolate with hazelnut cream	— ^a	—	NA
Black chocolate with nuts 1	+ ^{a,b}	—	NA
Black chocolate with nuts 2	+ ^{a,b}	—	NA
Black chocolate with nuts 3	+ ^{a,b}	—	NA
Honey chocolate with nuts	+ ^{a,b}	—	NA
Honey chocolate with fruits and nuts	+ ^{a,b}	—	NA
Honey chocolate with nuts and crispy rice	+ ^{a,b}	—	NA
Chocolate salami	+ ^{a,b}	—	NA
Cookies with chocolate and nuts	—	—	NA

UA, unspecific amplification; NA, not applied; +, Faint to moderate bands. ++, Strong bands; —, Not detected.

^a Declaration “may contain traces of peanut or other nuts”.

^b May contain traces of almonds.

^c Percentage of confidence according the reference cluster “cluster 1”.

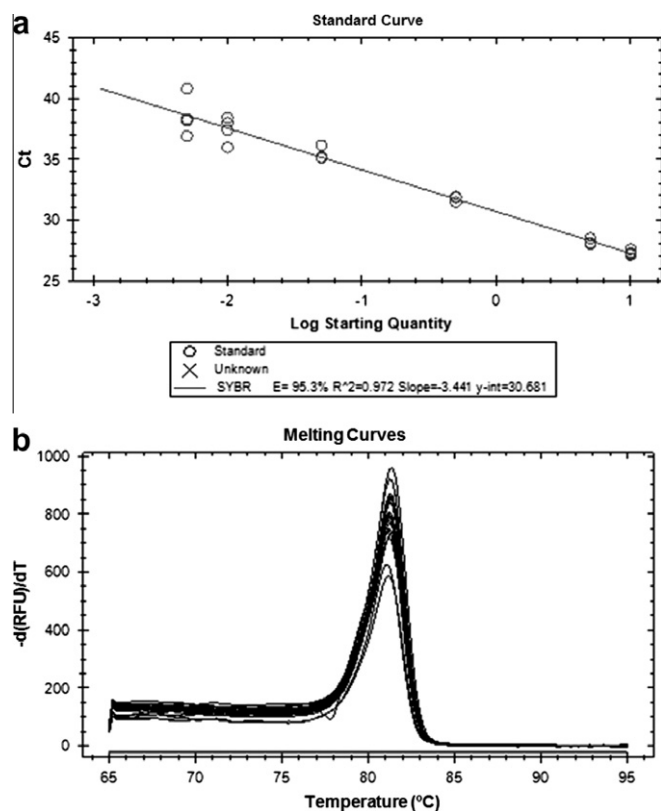


Fig. 1. Standard curve (a) and melting curve analysis (b) obtained by real-time PCR with EvaGreen[®] dye amplification targeting the Pru du 5 gene of almond and applied to reference mixtures containing 10%, 5%, 0.5%, 0.05%, 0.01% and 0.005% of almond.

local alignment search tool BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>), which identifies regions of local similarity among homologue sequences from different species and calculates the statistical significance of the matches. The programme confirmed the specificity of the designed primers for the selected *ALGOSRP* gene sequence with 100% identity. The program did not find homology with any other sequence from different plant species. The primers were synthesised by Eurofins MWG Operon (Ebersberg, Germany).

2.4. End-point PCR

Amplifications by end-point PCR were performed in 25 μ L of total reaction volume containing 2 μ L of DNA extract (100 ng),

15 mmol/L of Tris-HCl (pH 8.3), 50 mmol/L of KCl, 200 nmol/L of each primer Prd5-1F/Prd5-1R, 200 μ mol/L of each dNTP (Invitrogen, Carlsbad, CA, USA), 3.5 mmol/L of MgCl₂ and 1.5 U of DNA polymerase AmpliTaq Gold[®] (Applied Biosystems, Branchburg, NJ, USA). The reactions were performed in a thermal cycler MJ Mini (Bio-Rad Laboratories, Hercules, CA, USA) using the following program: initial denaturation at 95 °C for 5 min, with 40 cycles at 95 °C for 30 s, 64 °C for 30 s and 72 °C for 30 s, and a final extension at 72 °C for 5 min.

The amplified fragments were analysed by electrophoresis in a 2.0% agarose gel containing Gel Red 1 $\times$ (Biotium, Hayward, CA, USA) for staining and carried out in TAE buffer (40 mmol/L Tris-acetate, 1 mmol/L EDTA) for 60 min at 120 V. The agarose gel was visualised under UV light and a digital image was obtained using a Kodak Digital Science[™] DC120 (Rochester, NY, USA). Each extract was amplified at least in duplicate assays.

2.5. Real-time PCR and HRM analysis

The amplifications by real-time PCR were carried out in 20 μ L of total reaction volume containing 2 μ L of DNA extract of almond mixtures (50 ng) or commercial samples (100 ng), 1 $\times$ of SsoFast[™] Evagreen[®] Supermix (Bio-Rad Laboratories, Hercules, CA, USA) and 200 nmol/L of each primer Prd5-1F/Prd5-1R. The real-time PCR assays were performed on a fluorometric thermal cycler CFX96 Real-time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) using the following conditions: 95 °C for 5 min; 50 cycles at 95 °C for 10 s and 66.6 °C for 30 s, with collection of fluorescence signal at the end of each cycle. Data were collected and processed using the software Bio-Rad CFX Manager 2.0 (Bio-Rad Laboratories, Hercules, CA, USA).

For HRM analysis, PCR products were denatured at 95 °C for 1 min and then annealed at 65 °C for 5 min in order to allow the correct annealing of the DNA duplexes. These two steps were followed by melting curve ranging from 65 to 95 °C with temperature increments of 0.2 °C every 10 s. The fluorescence data were acquired by the end of each melting temperature.

The collected fluorescence data were processed using the Precision Melt Analysis Software 1.0 (Bio-Rad Laboratories, Hercules, CA, USA) to generate melting curves as a function of temperature and difference curves for easier visual identification of clusters. Cluster detection settings were defined targeting high sensitivity and threshold yields for more heterozygote clusters. Melting curve shape sensitivity determines the stringency used to classify melting curves into different clusters, thus high percentage value for this parameter allows increasing stringency and present results

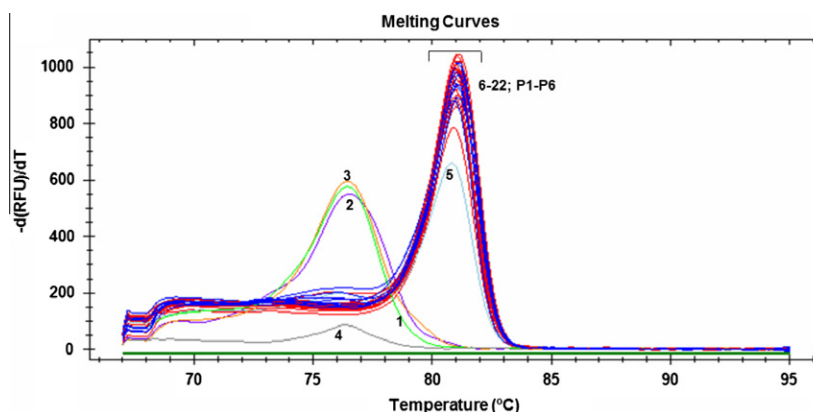


Fig. 2. Melting curves obtained by real-time PCR amplification with EvaGreen[®] dye targeting the Pru du 5 gene of almond and applied to food samples. Legend: 1 – macadamia nut, 2 – hazelnut chocolate, 3 – hazelnut snack, 4 – walnut, 5 – walnut cake, 6–22 – processed foods (chocolates, cereals foods, nut mixes, biscuit), P1–P6 – 100% almond extracts serially diluted (4-fold).

in more heterozygote clusters. Temperature of melting (T_m) difference threshold is a parameter that determines the lowest amount of T_m difference between samples and enables the inclusion of samples into different clusters. Therefore, melting curve shape sensitivity parameter was adjusted to percentage value >65% and T_m difference threshold parameter was set as default value of 0.15. Reference mixtures and samples were analysed in replicates ($n = 4$) in two independent assays.

2.6. Sequencing of PCR products

To confirm the identity of the PCR products obtained with the Prd5-1F/Prd5-1R primers for almond and related species, the DNA fragments were sequenced. PCR products of peach, nectarine, apricot and two varieties of almond were purified with Cut&Spin

DNA gel extraction columns (GRISP Research Solutions, Porto, Portugal) to remove interfering components. The purified products were 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 good quality.

3. Results and discussion

3.1. End-point PCR amplification

To specifically detect almond, the gene *AL60SRP* coding for Pru du 5 allergen was chosen from the available *P. dulcis* DNA/mRNA

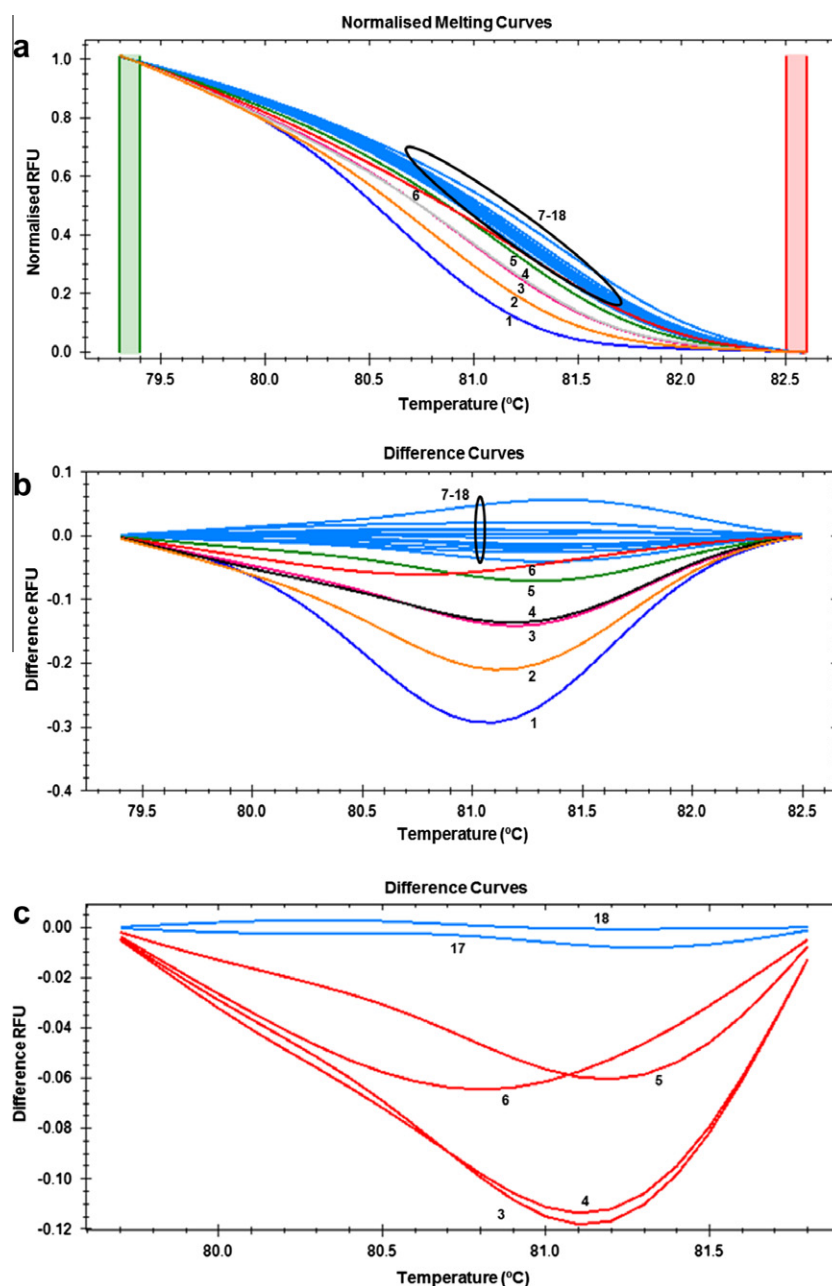


Fig. 3. HRM analysis of real-time PCR products with EvaGreen® dye targeting the Pru du 5 gene of almond and applied to different almond cultivars and other plant species. (a) Normalised melting curves and (b and c) difference curves. Legend: 1 – Hazelnut, 2 – Peanut, 3 – Apricot, 4 – Nectarine, 5 – Black Apricot, 6 – Peach, 7–18 – almond cultivars produced in Portugal, namely “Orelha de mula”, “Pegarinhos”, “Ferrestar”, “Ferragnes”, “Ferraduel”, “Gloriette”, “Casa Nova”, “Refego”, “Marcelina”, “Marcona”, “Duro Italiano” and “Verdeal”, respectively.

sequences in the Genbank database of NCBI. The choice was made because this protein was recently considered a potential major allergen for almond (Abolhassani & Roux, 2009) and based on the absence of homologue sequences in other plant sources. By using the primer-BLAST tool when designing the primers, it was possible to verify their 100% specificity towards the *AL60sRP* gene and lack of sequence similarity with other food species.

The results of estimating DNA concentration show that the extracts had, generally, high yields for all reference mixtures (403–596 ng/μL) and for the majority of the food samples (48–1253 ng/μL) under study. Complex food matrices such as chocolates also presented DNA extracts with adequate concentration range (15–130 ng/μL). The purity of extracts was generally close to 1.8 for all the reference mixtures and most foods, except for chocolates that ranged from 1.0 to 2.0.

Prior to the specific amplification of almond, all food samples were evaluated for their amplifiability with universal eukaryotic primers 18SEUDIR/18SEUINV (Fajardo et al., 2008). All samples tested positively with the universal primers, confirming the absence of false negative results that might occur due to PCR inhibition.

To test and optimise the PCR conditions with the new primers Prd5-1F/Prd5-1R, DNA extracts from binary reference mixtures containing known amounts of almond in walnut were used. The amplification results enabled detecting the addition of almond down to the level of 0.005% (w/w), with no amplification observed for the 100% walnut kernels used to prepare the binary mixtures (data not shown).

To extensively test specificity, DNA from a wide range of plant species used for food was isolated. Table 1 presents the results of

almond cultivars and several plant species tested for reactivity with primers Prd5-1F/Prd5-1R. The 12 almond cultivars tested positively for the target *AL60SRP* sequence confirming the specificity for almond species. Regarding the other eight commercial nuts analysed, walnut, hazelnut and macadamia nut produced some unspecific bands of different lengths, but none with 90 bp of the expected fragment. Peanut, soybean and fava bean also presented similar unspecific bands when tested by end-point PCR (Table 1). All the remaining species tested negatively for PCR amplification, except the four samples of *Prunus persica* and *Prunus armeniaca*, which produced PCR fragments with the same size. This can be explained due to the genetic proximity of almond and other species that belong to the same genus. Genetic relation between almond and other *Prunus* fruits, like peach (Chen et al., 2008) or cherry (Dall'Antonia, Pavkov, Fuchs, Breiteneder, & Kellera, 2005), suspected to cause cross-reactivity, has already been demonstrated.

Since a new allergen from tomato was recently identified and included in the same protein family of Pru du 5 (López-Matas et al., 2011), this species was also tested in the present work. Tomato ARP60S exhibited high identity (80.7%) and homology (93.8%) with almond 60S acidic ribosomal protein (Pru du 5), which might contribute to possible cross-reactivity between these two fruits (López-Matas et al., 2011). However, in the present study, no PCR fragment was observed for tomato fruit, confirming the absence of cross-reactivity for this species (Table 1).

The applicability of the proposed sequence-specific primers to detect *AL60SRP* gene was further tested in a wide range of processed foods commercially available (Table 2). From a total of 30 food samples, eighteen out of the 25 samples labelled as containing almond tested positively for *AL60SRP* gene, suggesting

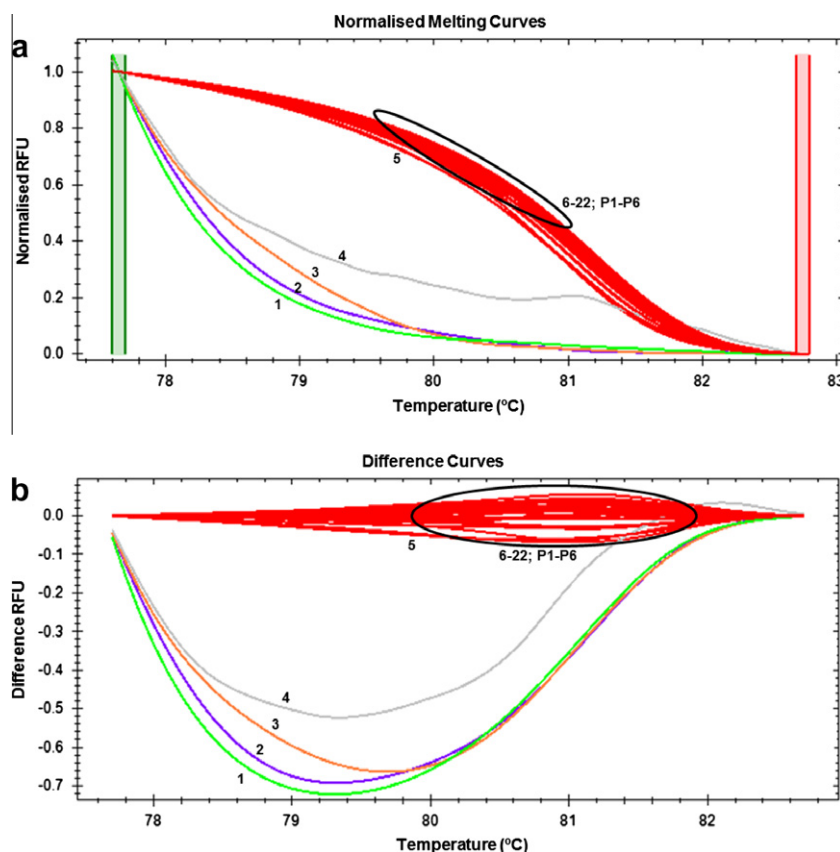


Fig. 4. HRM analysis of real-time PCR products with EvaGreen® dye targeting the Pru du 5 gene of almond and applied to different processed food samples and almond DNA extracts. (a) Normalised melting curves and (b) difference curves. Legend: 1 – Macadamia nut, 2 – hazelnut chocolate, 3 – roasted hazelnut, 4 – commercial walnut, 5 – walnut cake, 6–22 – commercial samples containing almond (chocolates, salami, cereals, cereal bars, nuts mixes), P1–P6 – Reference standards containing 100% of almond serially diluted (4-fold).

the effectiveness of the technique in processed foods. The seven samples with negative amplification declared traces of almond or nut mixtures (containing almond), probably as a precautionary labelling to safeguard any possible cross-contamination at the production facility, and not because they really contained almond. Thus, their negative results might indicate the absence of almond contamination during the production of those food samples. From the five food samples with no allegation for almond, a sample of walnut cake gave a strong positive band of the expected PCR fragment of 90 bp (Table 2), suggesting the presence of unlabelled almond in this product.

3.2. Real-time PCR amplification

To verify the specificity of the proposed primers (Prd5-1F/Prd5-1R) and their applicability to real-time PCR amplification using the fluorescent dye EvaGreen®, the DNA extracts of binary mixtures containing known amounts of almond (10%, 5%, 0.5%, 0.05%, 0.01% and 0.005%) were also used. A relative limit of detection and quantification of 0.005%, with high PCR efficiency (95.3%) and correlation ($R^2 = 0.972$) was obtained with real-time PCR (Fig. 1a), confirming the previous results of end-point PCR. The respective melting curve analysis revealed the amplification of similar products since they exhibited the same melting temperature of 81.20 °C (Fig. 1b).

The amplification of processed food samples by real-time PCR with EvaGreen® dye was in good agreement with the former end-point PCR amplifications results (Table 2). The melting curve analysis confirmed the specificity of the resultant products since all samples previously positive to almond produced fragments with the same melting temperature (Fig. 2). The unspecific amplifications for commercial walnut, macadamia nut and hazelnut (Table 1) were confirmed as such because they produced fragments with considerably lower melting temperatures than 81.20 °C (Fig. 2). The suspected presence of almond in the sample of walnut cake was reinforced by the result of melting curve analysis.

3.3. HRM analysis

HRM analysis takes advantage of the enhanced fluorescent dye and the possibility of gradually denature the PCR amplicons. Using an instrument with precise temperature control and homogeneity among the samples, slight sequence variations can be detected by denaturation of DNA fragments as a result of decreasing fluorescence due to dissociation of the double-stranded DNA-specific dye. Taking advantage of EvaGreen® dye that is able to be used at higher concentrations than SYBR Green I, better sensitivity to sequence variation during melting can be achieved, whose changes are reflected in the differing shapes of the melting curves (Mackay et al., 2008). Small differences in the nucleotide sequence of real-time PCR fragments with similar size are sufficient to alter the form of the melting curve and the melting temperature (Jaakola et al., 2010). Different base substitutions can create minor variations in the melting behaviour and the resolution of those melting alterations requires an appropriate intercalating dye such as EvaGreen® (Krypuy, Newnham, Thomas, Conron, & Dobrovic, 2006), which was chosen for the present study.

To further confirm the results obtained by end-point PCR and real-time PCR with EvaGreen® dye, the novel approach of HRM analysis was investigated with the designated new primers. After real-time PCR amplification and melting curve analysis, all data were further analysed using the Precision Melt Analysis Software. Twelve almond cultivars were analysed together with six other species that amplified positively with real-time PCR (Fig. 3), while almond reference mixtures were tested with all the processed food samples (Fig. 4). In both cases, almond curves were included in the

same cluster (Cluster 1) and defined as the reference cluster, with percentages of confidence over 85% (Tables 1 and 2). The small differences among all melting curves presented in normalised plot (Fig. 3a) can be best visualised in the difference curve charts (Fig. 3b and c), while the great differences (Fig. 4a) are reproduced in the respective difference plot (Fig. 4b).

Fig. 3 shows the application of HRM analysis to individual genotypes of the same species (12 almond cultivars) and to six other species that amplified positively with real-time PCR. In Fig. 3b and c, it is possible to observe that the almond curves with similar shape were included in the same group, whereas the other *Prunus* fruits, namely black apricot, apricot, nectarine and peach, that formerly produced fragments with similar melting picks to almond (81.0–81.2 °C) (data not shown), were distinguished and included in clusters from 2 to 5 (Table 1). Although these species produced fragments with similar size and melting temperature due to their genetic proximity, with the application of HRM analysis that is able to distinguish small variations among closely related species, they could be discriminated from almond. This finding agrees with results of application of HRM analysis to identify berry species (Jaakola et al., 2010), grapevine and olive cultivars (Mackay et al., 2008).

The application of HRM analysis to the second set of real-time PCR data of processed foods, almond reference mixtures and other nuts (Fig. 2) confirmed and reinforced their discrimination by both normalised melting curves (Fig. 4a) and difference curves (Fig. 4b). Almond reference mixtures and all the processed samples with previous positive amplification for almond were grouped in cluster 1 with high percentage of confidence, 98.9–100.0% (Table 2). Macadamia nut, walnut and hazelnut were included in different clusters: 2–4 with percentage of confidence of 99.8%, 100.0% and 99.9%, respectively (Fig. 4a, b and Table 1). Peanut was included in a different cluster (6) with 89.4% of confidence (Fig. 3b and Table 1).

The sample of walnut cake was included in the cluster 1 with 99.9% of confidence (Fig. 4), which was in good agreement with previous PCR results (Table 2) and melting curve analysis (Fig. 2), confirming the presence of unlabelled almond.

3.4. Sequencing results

The results presented by HRM analysis were investigated and confirmed by sequencing the PCR products from almond and other fruits from the Rosaceae family. The sequencing results revealed identical nucleotide sequences of 91 bp (100% identity) for both almond varieties. In comparison with the available GenBank sequence, the obtained sequences exhibited 1 A base deletion at 58 position and insertions of 2 A bases in position 89 and 91 from 5' end of the strand.

Sequenced fragments of nectarine, peach and apricot, that revealed similar sizes as expected (90 bp for the former and 91 bp for the others), were compared with the achieved almond sequence. Nectarine nucleotide sequence presented a mismatch (G → T) in position 4, deletion of T and A bases from positions 7 and 90, respectively, and an insertion of N base in position 9 from the 5' end. Peach sequence evidenced a mismatch (T → G) in position 1 and an insertion of G base in position 11 from the 5' end. Apricot sequence revealed insertion of a T base in position 2 and a deletion of a A base from position 89. Although the differences between almond and the other *Prunus* sequences are small, they constitute the reason for HRM analysis to exclude peach, nectarine and apricot from the same cluster of almond. Since HRM analysis allow discriminating DNA sequences based on small variations such as a single nucleotide mismatch, sequencing of *Prunus* fruits enabled to confirm and reinforce the results from the HRM analysis.

4. Conclusions

The present work has demonstrated the usefulness of the designed new primers to detect a specific DNA region encoding the allergenic protein Pru du 5 in almond. The optimised conditions for both end-point PCR and real-time PCR were successfully achieved with the designated primers down to the level of 0.005% of almond addition, obtaining adequate PCR efficiency and correlation with real-time PCR. The proposed assays were also effective when applied to real processed food samples and to other non-related and related plant species. Regarding related species of *Prunus* genus such as peach, nectarine and apricot, similar fragments were identified by both end-point PCR and real-time PCR with similar melting temperatures. However, with HRM analysis it was possible to discriminate almond from other *Prunus* fruits. HRM analysis was able to demonstrate that the unspecific PCR products obtained for commercial walnut, macadamia nut and hazelnut were distinguished from almond amplification. HRM analysis also enabled the unequivocal identification of the *AL60SRP* gene present in commercial foods containing almond and the non-compliance with the labelling statement in one sample of walnut cake, which can represent a risk for allergic individuals.

In summary, we have proposed a novel and simple approach to detect almond allergens in processed foods by the use of end-point PCR and real-time PCR with EvaGreen® dye. In addition to the simple and sensitive real-time PCR assay, we apply for the first time HRM analysis as a cost-effective and powerful tool for high-throughput identification of allergens in foods.

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