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Analytical Methods

SYBR®GreenER™ Real-Time PCR to detect almond in traces in processed food

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ABSTRACT

Because food ingredients are sometimes considered as causative factors in IgE mediated food allergies, DNA-based tests may prove to be very useful to establish whether allergenic species have been used in foodstuffs production. The development of two SYBR®GreenER™ Real-Time PCR assays, targeting Pru 1 and rbcL genes, based on melting curve analysis, to detect allergen species in food has been presented. Applicability of these methods was assessed with several commercial products containing processed almond.

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1. Introduction

Food ingredients are sometimes considered as causative factors in IgE mediated food allergies (Taylor, 2006; Taylor & Hefle, 2001). These are recognised as a major health concern with as much as 4% of the total population affected by food-allergic disorders. The most effective way of preserving an allergic individual from the contact with the allergen is through the avoidance of any allergen-containing food. Total avoidance may result sometimes difficult, because processed food products usually contain a plethora of ingredients, including potential allergens. For the allergic consumer, full information about the presence of any potential allergens in a given food product is often obviously of survival value (Poms & Anklam, 2004). At this purpose, in many countries, the label of a food product must indicate the presence of an allergenic ingredient or the potential contamination along the production chain with an allergenic ingredient (van Hengel, 2007).

In principle, any molecule that is specific for an allergenic ingredient can serve as a marker of its presence in food, but mostly proteins and DNA are targeted for the purpose. Since DNA is more stable than other macro-molecules to physical and chemical treatments, the DNA-based tests may prove to be very useful to establish whether allergenic species have been used in foodstuffs production (Hernández, Esteve, & Pla, 2005) and to solve controversial instances.

This paper describes the development of Real-Time PCR with SYBR®GreenER™, a modified SYBR Green molecule, for almond detection in processed food.

2. Materials and methods

2.1. Food and plant materials

Leaves of almond (*Prunus dulcis* sp.) cultivars were collected in spring 2007 in Sicily and kindly provided by Prof. A. Scialabba (University of Palermo), single plants for each cultivar were stored at −20 °C. Almonds, hazelnuts, cashews, peanuts, nuts, sesame seeds, and different types of biscuits were purchased from different retailers and stored at room temperature in the dark. The complete list of materials used for DNA extraction and for PCR analyses is contained in Table 1.

2.2. DNA extraction from leaves and food materials

DNA was extracted from leaves using the CTAB method reported by Doyle and Doyle (1988). Food materials were grinded using Knifetec™1095 (Foss Tecator AB, Höganäs, Sweden) and DNA was extracted according to Meyer, Chardonnens, Hübner, and Lüthy (1996). DNA was purified with Wizard®Resin (Promega, Madison, WI) and analysed and quantified spectrophotometrically.

2.3. Target gene selection and primers design

Nucleotidic sequences of almond target genes (Pru 1, and rbcL) were retrieved from Genebank. Specific primers were designed using the software Primer Express™ v.2.0 (Applied Biosystems division of Perkin–Elmer Corp., Foster City, CA). The information about genes and primers used in this study are shown in Table 2. Designed primers were compared with sequences present in databases, using the BlastN programme at NCBI. Primers were purchased from MWG (Ebersberg, Germany).

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Table 1
List of plant species and food matrices used for PCR and ELISA analyses. Specificity test for Pru 1 and rbcL primers was performed on genomic DNA of leaves and fruit. ELISA was carried out on proteins purified from fruits and biscuits. Legend: + positive result (corresponding to a peak of predicted T_m); − negative result (corresponding to absence of peak); ≠ different result (corresponding to a peak or two peaks with different T_m or a false positive result for ELISA).

Type of material	Matrix	Almond (%)	Pru 1 primers	rbcL primers	ELISA
Leaves	*Prunus dulcis* cv. *Cavalera muddrisa*	100	+	+	
	Prunus dulcis cv. *Rappa dura*	100	+	+	
	Prunus dulcis Franco di mandorlo *Muddrisa nostralis*	100	+	+	
	Prunus dulcis Franco di mandorlo *Dura nostralis*	100	+	+	
	Barley (*Hordeum vulgare*)	−	≠	+	
	Maize (*Zea mays*)	−	−	+	
	Wheat (*Triticum ssp*)	−	−	+	
Fruits	Almond (*Prunus dulcis*)	100	+	+	+
	Bald almond (*Prunus dulcis*)	100	+	+	+
	Hazelnut (*Corylus avellana*)	−	−	+	−
	Cashew (*Anacardium occidentalis*)	−	−	+	−
	Peanut (*Arachis hypogea*)	−	−	≠	−
	Nut (*Juglans regia*)	−	−	+	−
	Sesame (*Sesamum indicum*)	−	≠	≠	−
Biscuit	With almond	>80	+	+	+
	With almond flour	2	+	+	+
	With traces of nuts	Unknown	+	+	+
	With chocolate and traces of nuts	Unknown	−	≠	+
	With hazelnut and traces of nuts	Unknown	+	+	+
	With hazelnut cream and traces of nuts	Unknown	+	+	+
	With eggs without traces of nuts	Unknown	−	+	≠
	With butter without traces of nuts	Unknown	≠	+	−

Table 2
Target sequences selected for primer design. For each gene selected the accession number and the species from which they are derived is reported. For each primer pair sequences, length and T_m of the amplicon are reported.

Specie	Gene	Accession number	Primer for 5′–3′	Primer rev 5′–3′	Amplicon length (bp)	Theoretical T_m amplicon (°C)
Almond (*Prunus dulcus*)	Pru 1	Patent pending	Patent pending	Patent pending	76	81
Peach (*Prunus persica*)	rbcL (RUBISCO) gene	AF206813.1	AGC TCG TAA TGA GGG ACG TG	CTC CAT TTA CTA GCC TCG CG	69	79

2.4. PCR conditions

Amplifications were carried out in a final volume of 20 μl starting from 50 ng of DNA in presence of 1× *Taq* Buffer, 0.25 μM of each forward and reverse primers, 0.2 mM dNTPs, 2 mM $MgCl_2$, 2.5 U HotStartTaq DNA polymerase (Qiagen, Milan, Italy), in a Techne TC 512 thermal cycler (Techne, Duxford, Cambridge, UK). For all samples, three replicates of the amplification and a negative control were done. Standard PCR conditions were used with annealing step at 58 °C. Amplification products were analysed by electrophoresis on 2% (w/v) agarose gel. SYBR®GreenER™ DNA Melting Curve Temperature Analyses were carried out using an ABI Prism®7000 Sequence detection system and also with Applied Biosystems 7900HT Fast Real-Time PCR System (Applied Biosystems division of Perkin–Elmer Corp., Foster City, CA). For all samples, three replicates of the amplification and a negative control were done. Each experiment was repeated 10-fold. The reactions were carried out in a final volume of 25 μl starting from different amounts of DNA in the presence of 1× SYBR®GreenER™qPCR SuperMix for ABI PRISM® (Invitrogen Corporation, Carlsbad, CA, USA), 0.25 μM of each forward and reverse primers. PCR conditions were: 2 min at 50 °C, 15 min at 95 °C followed by 40 cycles of 15 s at 95 °C, 1 min at 60 °C. The PCR programme was followed by a "Dissociation" stage, during which the temperature was gradually increased from 60 to 95 °C at a rate of 0.25 °C/s. The melting curves obtained were converted into their negative first derivatives, which showed a maximum corresponding to the melting temperature (T_m) of the amplification product.

2.5. Cloning and sequencing of PCR amplicons

PCR amplicons were cloned using the pGEM®-T Easy Vector System (Promega, Madison, WI, USA), following the manufacturer instructions. Three clones for each fragment were sequenced, with the CEQ 2000 Dye Terminator Cycle Sequencing Quick Start Kit (Beckman-Coulter, Fullerton, CA). Three sequences from each fragment were aligned and compared using the programme CLUSTAL-W.

2.6. Generation of a standard curve

Genomic DNA extracted from almonds was serially diluted (10-fold) to obtain samples containing 100, 10, and 1 theoretical copies of the almond haploid genome, one haploid almond genome being 0.30 ng (Arumuganathan & Earle, 1991a, 1991b).

Plasmid DNA was serially diluted (10-fold) to obtain samples containing from 5×10^5 to 5 copies of plasmid. Salmon sperm DNA was added to dilutions of genomic DNA containing 1 and 10 theoretical copies, and to dilutions of plasmid DNA containing 5 copies.

The standard curves were obtained for both series of samples. The data were analysed statistically using the parametric *t* test and the non-parametric Mann-Whitney U test and Kolmogorov-Smirnov Z test for two independent samples when the data are not normally distributed (Vickers, 2005). Probability values for significance were considered below 0.05 ($p \leqslant 0.05$).

2.7. Quantitative almond allergen test using a sandwich enzyme-linked immunosorbent assay (s-ELISA)

The analysis was carried out employing the VERATOX® quantitative almond allergen test (Neogen Corporation, Lansing, MI) and the proteins were extracted using the Allergen Extraction kit (Neogen). Experiments were carried out following the instruction of the manufacturer, starting from 5 g for each matrix reported in Table 1.

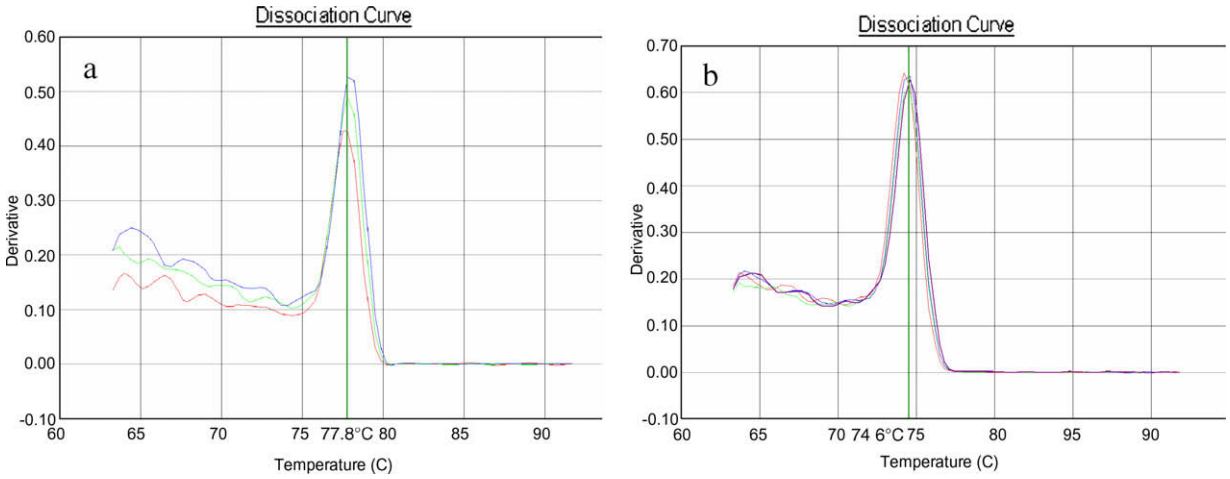

Fig. 1. *Specificity and robustness:* derivatives of melting curves of Pru 1 amplicons (a) and rbcL amplicons (b) obtained from two/three cultivars of *Prunus dulcis* and almond fruits DNA in the presence of SYBR®GreenER™ performed on ABI Prism 7000 Sequence detection system.

3. Results and discussion

3.1. Target gene selection and primer design

The gene for allergenic protein Pru 1, a 11S globulin (prunin) was chosen as allergen-specific target. The ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene of *P. persica* was chosen, since it is a multicopy chloroplastic sequence. Primers to amplify genomic DNA of almond were designed to obtain amplicons shorter than 100 bp, to increase the possibility of amplifying the target sequence, although starting material was a complex and processed matrix (Pafundo, Agrimonti, Maestri, & Marmiroli 2007). The designed primers were checked for their specificity using BLAST. All primers were preliminary tested on almond genomic DNA samples by standard PCR. All the amplicons were sequenced and they showed the expected length and a complete

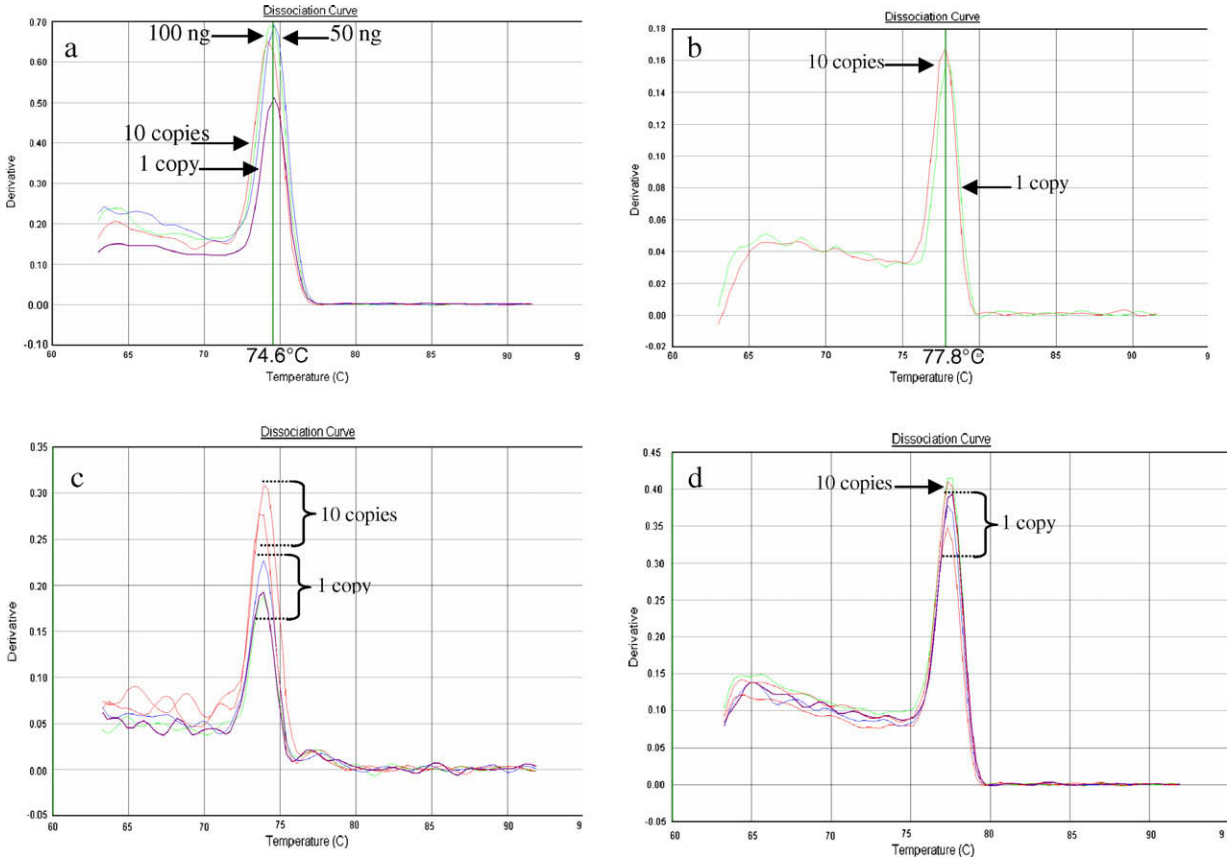

Fig. 2. *Sensitivity:* amplifications were performed on ABI Prism 7000 Sequence detection system in the presence of SYBR®GreenER™. Derivatives of melting curves of the amplicons obtained from different samples are shown. Samples containing different amounts (100 ng, 50 ng) or different number of copies of almond genome (10 copies, 1 copy), were amplified with rbcL primers (a) or with Pru 1 primers (b). Samples containing different amount of recombinant plasmids DNA (10 copies, 1 copy) were amplified with rbcL primers (c) or with Pru 1 primers (d).

homology with the sequences on which they were designed (data not shown).

3.2. Analyses of specificity, robustness, sensitivity and efficiency

Real-Time PCRs were performed using the ABI Prism 7000 Sequence detection system with both the Pru 1 and rbcL primers on almond DNA. The maximum of the derivative of the melting curve was reached at 77.8 °C ± 0.1 for Pru 1 primers (Fig. 1a) and at 74.6 °C ± 0.1 for rbcL primers (Fig. 1b). Both curves were shaped and clear with no trace of aspecific products or of primer–dimers.

Specificity of the two primer pairs was analysed by testing the primers on DNA extracted from cereals species and other nuts (Table 1). Considering the obtained results, the conclusion was that Pru 1 primers were specific for almond DNA and, if an aspecific amplification was observed on matrix different from almond, the amplicon obtained had a different T_m, whilst rbcL primers could amplify DNA from several species, since it is a highly conserved sequence in plants.

The robustness of the two reactions was checked performing the same experiments on a different instrument (Applied Biosystems 7900HT Fast Real-Time PCR System), obtaining the same results.

The sensitivity of the two PCR systems was assessed using as template either almond genomic DNA or recombinant plasmid DNA containing the Pru 1 or the rbcL sequences. Almond genomic (Fig. 2a and b) and plasmid DNAs (Fig. 2c and d) could be detected down to 1 copy with both PCR systems.

To assess the efficiency of these Real-Time PCR assays, standard curves were generated for the two systems (Table 3). The slopes in both cases were close to the value – 3, whilst the R^2 was higher than 0.95 (95%) in all standard curves, in particular it reached values of 99.7% using rbcL primers either on genomic and plasmidic DNA. Although SYBR®GreenER™ is a not sequence-specific binding DNA molecule, the developed systems showed an acceptable sensitivity and efficiency. Using both parametric and non-parametric statistical tests, no significant differences were found (Table 3) in agreement with Taveniers et al. (2005). The assays developed can offer a good alternative to the more commonly used Real-Time PCR with TaqMan probes (Brežná, Hudecová, & Kuchta, 2006; Brzezinski, 2007) to trace hidden allergen.

Finally, as for GMO traceability (Taveniers et al., 2005), recombinant plasmids containing target gene sequences could be used as reference material, in particular the plasmids developed in this work could be used as internal standard to quantify almond DNA in food.

Quantitation of the almond DNA is possible whenever a proper internal standard is available for Real-Time PCR.

3.3. Tests on processed foods

Pru 1 and rbcL primers were used to detect traces of almond in different types of biscuits. The assayed food matrices and the obtained results are reported in Table 1. All the matrices gave a positive result using rbcL primers, except the biscuits with chocolate and traces of nuts, which gave a peak with a different T_m. This particular result was confirmed by the analyses made with Pru 1 primers on the same matrix, which showed a negative result. In this case it was possible to exclude the presence of almond. Pru 1 primers gave a negative result also in the case of biscuits with eggs and without traces of nuts, whilst in the case of biscuits with butter and without traces of nuts they gave a different results (Fig. 3). Therefore, Pru 1 primers were able to detect almond when it was the principal ingredient and also when it was present only as traces; no false positive results were obtained in our experiments.

3.4. Comparison with almond specific ELISA

An almond specific ELISA was performed on all the tested matrices. The results are reported in Table 1. Most of the results obtained using SYBR®GreenER™ Real-Time PCR were confirmed. In the case of biscuits with eggs and without traces of nuts, the presence of a precipitate at the end of the ELISA could be considered as a false positive result, according to manufacturer instructions. In the case of biscuits with butter and without traces of nuts, ELISA analysis showed that no allergenic almond proteins were present. In this sample, a particular melting curve with Pru 1 primers was obtained (Fig. 3). Therefore, Pru 1 assay and ELISA test for almond could be combined to obtain a precise indication about the presence of almond allergens.

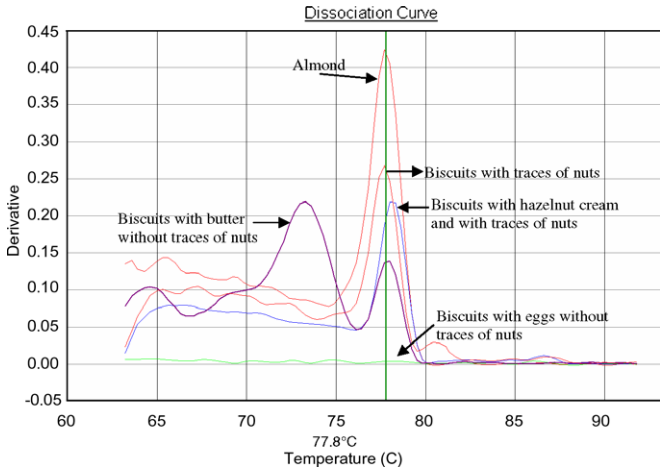

Fig. 3. *Tests on food products:* derivatives of melting curves of Pru 1 amplicons on almond DNA and four different types of biscuits DNA, in the presence of SYBR®GreenER™ performed on ABI Prism 7000 Sequence detection system.

Table 3
Comparison of the sensitivity of Pru 1- and rbcL-based systems, assessed by the standard curve obtained from the amplifications of serial dilutions of almond genomic DNA and of plasmid containing the fragment. Statistical analysis was carried out using parametric *t* test firstly, and then confirmed/belied by non-parametric analyses for two independent samples.

System	Plasmid copy number standard curve		Genome copy number standard curve	
Pru 1 assay	$y = -3.0443x + 34{,}88$	$R^2 = 0.9564$	$y = -2.9796x + 37.761$	$R^2 = 0.9897$
rbcL Assay	$y = -3.1701x + 37{,}669$	$R^2 = 0.9977$	$y = -2.7303x + 30.964$	$R^2 = 0.9978$
Pru 1 vs. rbcL		*t Test = 0.603* *U test of Mann-Whitney = 0.744* *Test of Kolmogorov-Smirnov = 0.894*		Not significant
Plasmid vs. genome		*t Test = 0.321* *U test of Mann-Whitney = 0.336* *Test of Kolmogorov-Smirnov = 0.205*		Not significant

4. Conclusion

Starting from a precautionary principle (van Hengel, 2007), the presence of an allergen should firstly be evaluated using a DNA-based method, followed, in case of a positive signal, by some protein detection methods to verify the effective presence of the allergenic protein.

The development of two SYBR®GreenER™ Real-Time PCR assays, based on melting curve analysis, has been presented. Applicability of the methods was assessed with several commercial products containing processed almond. It can be concluded that the use of SYBR®GreenER™ can offer a less expensive test respect to TaqMan assay, with a comparable efficiency and high level of sensitivity. Only Pru 1 primers allowed to trace almond in those products which have undergone a marked processing or to solve doubtful cases and it could be used to help the manufacturer and the retailer in food labelling matter.

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