

Multiplex real-time PCR using SYBR[®] GreenER[™] for the detection of DNA allergens in food

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Abstract We describe the development of a six-target real-time multiplex PCR assay with the SYBR[®] GreenER[™] fluorescent dye, targeted to genes encoding for allergenic proteins commonly present in many processed food products (patent application pending). The assay was successfully trialled on reconstructed food matrices and on a range of commercial foodstuffs, and is proposed as a ready-to-use analytical tool for food manufacturers to detect the presence or confirm the absence of sequences encoding for important allergenic proteins of plant origin.

Keywords Allergen · Multiplex real-time PCR · Traceability · Food · Melting curve

Introduction

SYBR[®] Green I, a non-specific fluorescent dye which has a high affinity for double-stranded DNA [1,2], has been widely used to determine the melting temperature (T_m) of PCR amplicons [3]. The use of this reagent has also been reported for the detection of specific viruses [3–7], for the typing of bacterial strains [8,9], and as a molecular diagnostics reagent in both humans [10,11] and plants [12]. It has been applied in the area of food analysis to detect various classes of biological contaminants, including fungi [13], pathogens [14], transgenes [15], and allergens [16,17]. A particularly attractive feature of the SYBR[®] Green assay is its compatibility with the multiplex real-time PCR platform,

which demands a set of distinct assays for each amplicon target. This can be achieved by attaching different fluorescent dyes to each assay, as in the TaqMan assay, but the emission spectra of the dyes commonly used in TaqMan-based real-time PCR overlap extensively with one another. When the intention is solely to detect the presence/absence (as opposed to the quantity) of, for example, a given set of pathogens [18], allergens [19,20], or transgenes [21], the degree of multiplexing achievable can be quite high, as in the case of the ligation-dependent probe amplification system which allowed the simultaneous detection of ten allergens [19]. This system is based on the amplification of products resulting from the ligation of bipartite hybridization probes, followed by detection through capillary electrophoresis. The use of SYBR[®] Green may result in a high multiplexing index in less time, in a single reaction and without the use of toxicant reagents. The TaqMan assay represents an effective quantification platform, but its per assay cost is higher than that of the SYBR[®] Green assay. The SYBR[®] Green assay of plant allergens was reported firstly by us [16] and by D'Andrea et al. [17] but was limited to hazelnut, although a number of DNA-based assays have been developed for this purpose [20,22–28], including the previously mentioned ligation-dependent probe amplification method [19].

Here we describe the development of a multiple-target real-time multiplex PCR assay (patent application pending) based on SYBR[®] GreenER[™], a modified SYBR[®] Green molecule. The targets chosen were genes encoding for allergenic proteins, important because of the dangerous nature of the anaphylactic shock reaction which their ingestion can induce [29]. The avoidance of allergens in the diet requires an analytical capacity to detect them in foodstuffs. Furthermore, at the regulatory level, European legislation [30–32] insists on adequate labelling with respect to the presence of allergens.

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Materials and methods

Target gene selection and primers design

The DNA sequences corresponding to the genes encoding the allergenic proteins of the six target species were retrieved from the Genbank database. Specific primers were designed using the software program Primer Express™ version 2.0 (Applied Biosystems, Foster City, CA, USA). Designed primers were compared with sequences present in databases, using the BlastN program at NCBI. Both unlabelled and Cy5 5'-labelled primers were synthesized by MWG (Ebersberg, Germany).

Food and plant materials

A detailed list of food and plant materials is given in Table 1. Nut materials of almond, hazelnut, cashew, peanut, and walnut, sesame seeds, and various commercial brands of food were purchased from local stores and stored at room temperature in the dark. Leaves of barley, maize, wheat, oats, rice, and olive were each collected from a single plant and stored at -20°C .

Reconstructed biscuits were prepared with a food processor (Braun, Germany) as follows: a dough containing 50% wheat flour, 20% sugar, 15% butter, and 15% eggs was divided in five parts and spiked with 1, 0.5, 0.25, 0.05, or 0% of ground almond. The reference biscuits with 0% almond and the spiked biscuits were baked separately at 180°C for 10 min.

DNA extraction from plant materials and foodstuffs

DNA was extracted from leaves using the cetyltrimethylammonium bromide method reported by Doyle and Doyle [33]. Seeds, reconstructed biscuits, and food materials were ground using Knifetec™1095 (Foss Tecator, Höganäs, Sweden) and DNA was extracted following the protocol of Meyer et al. [34]. DNA was then purified with a Wizard® resin kit (Promega, Madison, WI, USA). Four independent extractions were performed for each sample, together with a negative control of the extraction, in which no biological sample was present, but only water. The resulting DNA was quantified spectrophotometrically.

Genomic DNAs extracted from reconstructed biscuits were diluted 1:5 for PCR analyses.

Genomic DNAs extracted from almonds, hazelnuts, cashews, peanuts, nuts, and sesame seeds were mixed to obtain a total amount of 5 ng containing different proportion of each species: (1) a mixture of DNA from the six species in the same amount (16.66% of each species) (mix A) and (2) six mixtures in which DNA of one species was present at 1% of the total DNA and in which DNA of five species was present at 99% (19.8% for each species) (mix B).

Serial dilutions (1:1, 1:10, and 1:100) were prepared for each mix B, resulting in samples with 5, 0.5, and 0.05 ng of total DNA, and therefore 50, 5, and 0.5 pg of DNA of the species present at 1%.

PCR conditions

Three replicate amplifications and a negative control were performed for each sample, and each experiment was repeated three times. For standard PCRs, both with unlabelled and Cy5-labelled primers, the amplifications were carried out in an ABI Prism® 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA). Some unlabelled amplification products were analysed by electrophoresis through 3% (w/v) agarose gels (Sigma, St. Louis, MO, USA) in $1\times$ TAE [40 mM tris(hydroxymethyl)aminomethane acetate, 1 mM EDTA] and were visualized by ethidium bromide staining. For standard PCRs using labelled primers, the reaction products were separated using a CEQ™ 2000 XL sequencer (Beckman Coulter, Fullerton, CA, USA), applying the following parameters: capillary temperature 50°C , denaturation temperature 90°C for 120 s, injection voltage 2 V for 30 s, and separation voltage 6 V for 50 min. The resulting profiles were analysed using the Fragment Analysis component of the CEQ 2000 DNA analysis system (Beckman Coulter). SYBR® GreenER™ (Invitrogen, Carlsbad, CA, USA) DNA melting curve temperature analyses were carried out using either the ABI Prism® 7000 sequence detection system or an Applied Biosystems 7900 HT fast real-time PCR system (Applied Biosystems). The resulting melting curves were converted into their negative first-order derivatives, which allows the T_m of the amplicons to be determined from the peak. With the LCGreen® melting dye using LCGreen® Plus+ (Idaho Technology, Salt Lake City, UT USA), each 25- μl reaction contained 50 ng template DNA, $1\times$ Taq buffer, optimized primer concentrations, 0.4 mM dNTP, 2 mM MgCl_2 , 1.5 U HotStarTaq DNA polymerase (QIAGEN, Milan, Italy), and $1\times$ LCGreen® melting dye, in an ABI Prism® 7000 sequence detection system (Applied Biosystems). The PCR conditions were as previously described. For the Fast SYBR® Green using Fast SYBR® Green master mix (Applied Biosystems), reactions were carried out following the manufacturer's instructions in an Applied Biosystems 7900 HT fast real-time PCR system, using optimized primer concentrations.

Cloning and sequencing of PCR amplicons

PCR products were cloned using the pGEM®-T easy vector system (Promega, Madison, WI, USA, USA). Plasmid DNA was extracted from transformed recombinant cells, with a Wizard® Plus SV minipreps DNA purification kit (Promega).

Three clones for each fragment were sequenced, with a CEQ 2000 dye terminator cycle sequencing quick start kit (Beckman Coulter), using M13 universal primers. Nucleotide BLAST searches were carried out for similarities

within fragments sequences and those present in databases, using the NCBI server (<http://www.ncbi.nlm.nih.gov/BLAST>). BLAST parameters were set to default values.

Table 1 Primer specificity was tested using six primer pairs on a template derived from a range of matrices. Food products were tested for verification of the precautionary labelling with the six-target multiplex assay

Type of material	Plant species	Results	T_m (°C) of amplicon(s)	Amplicon's length (bp)	Species detected
Nuts	Almond (<i>Prunus dulcis</i>)	+	77.8±0.2	76	Almond
	Hazelnut (<i>Corylus avellana</i>)	+	73.3±0.1	54	Hazelnut
	Cashew (<i>Anacardium occidentale</i>)	+	70.5±0.1	57	Cashew
	Peanut (<i>Arachis hypogea</i>)	+	75.9±0.1	87	Peanut
	Walnut (<i>Juglans regia</i>)	+	76.3±0.2	81	Walnut
Seeds	Sesame (<i>Sesamum indicum</i>)	+	85±0.2	100	Sesame
	Pistachio (<i>Pistacia vera</i>)	-	—	—	—
Leaves	Barley (<i>Hordeum vulgare</i>)	-	—	—	—
	Maize (<i>Zea mays</i>)	-	—	—	—
	Wheat (<i>Triticum</i> ssp.)	-	—	—	—
	Oat (<i>Avena sativa</i>)	-	—	—	—
	Rice (<i>Oryza sativa</i>)	-	—	—	—
	Olive (<i>Olea europaea</i>)	-	—	—	—

Reconstructed matrices	Almond (%)	Results	T_m (°C) of amplicon	Allergen detected
Biscuit with ground almond	1, 0.5, 0.1, 0.05	+	77.8	Almond
	0	-	Undetermined	—

Commercial matrices	Precautionary labelling	Results	T_m (°C) of amplicon(s)	Allergen detected	
Biscuits with	Almond	—	+	77.8	Almond
	Almond flour (2%)	Traces of nuts, sesame	+	77.8	Almond
	Sugar	Traces of nuts, sesame, peanuts	-	—	—
	Chocolate	Traces of nuts,sesame	-	—	—
	Hazelnut	Traces of nuts, sesame	+	73.3	Hazelnut
	Hazelnut cream	—	+	73.3	Hazelnut
	Eggs	—	-	—	—
	Butter	Traces of nuts	+	77.8, 73.3	Almond and hazelnut
	Cashews and honey	Traces of nuts, sesame	+	70.4	Cashew
	Walnuts and chocolate	Traces of nuts, sesame	+	76.3, 73.3	Walnut, hazelnut
Chocolate	Dark chocolate	Traces of hazelnut	-	—	—
	Chocolate with peanuts and hazelnut paste	Traces of nuts	+	75.9, 73.3	Peanuts, hazelnut
Crackers with	Sesame seeds	Traces of nuts	+	84.9, 73.3, 75.9, 77.8	Sesame, hazelnut, peanuts, almond

+ positive result (detection of a peak of predicted T_m), - negative result (absence of a peak)

Generation of standard curves from plasmid-based templates

Plasmid preparations were diluted to obtain preset numbers of plasmid copies, as calculated from the known size of each plasmid. A mixture containing all six plasmids was prepared and serial dilutions were performed to obtain samples containing 10^6 , 10^5 , or 10^4 copies of each plasmid. The plasmid DNA template was analysed by real-time multiplex PCR with SYBR® GreenER™, as described elsewhere in this paper. A semilogarithmic plot was generated ($\log_{10}$ of the concentration of DNA versus C_t) by ABI Prism® 7000 SDS software (Applied Biosystems) and the standard curve was calculated as $y = -ax + b$.

The efficiency of the reaction (E) was calculated as $E = (10^{-1/a}) - 1$.

Results

Selection of target genes and primers design

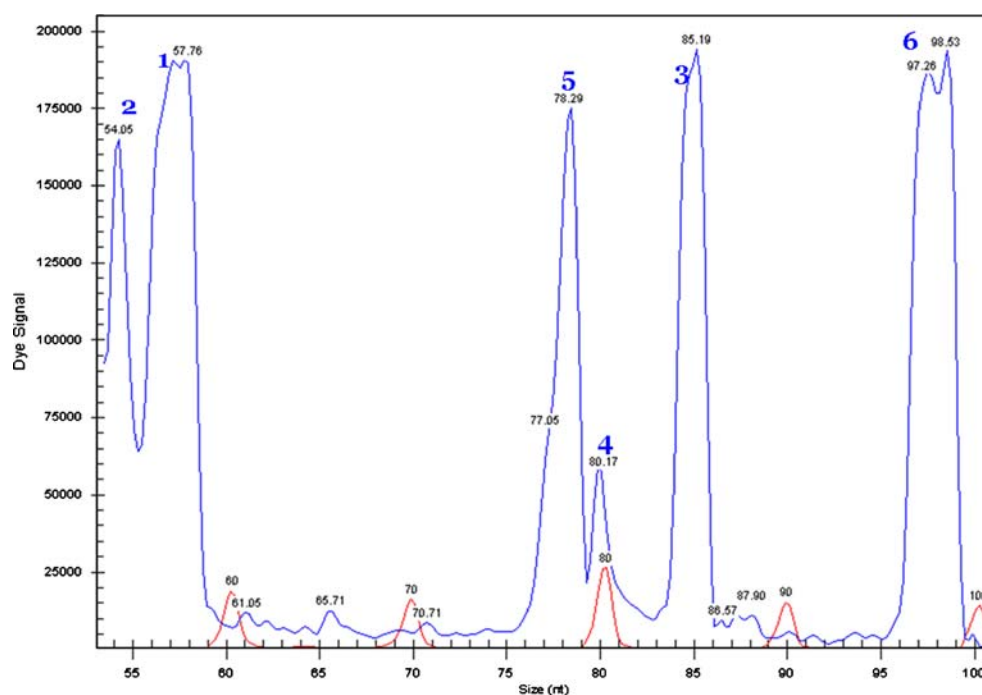
The following allergenic plant species were considered: *Prunus dulcis* (almond), *Corylus avellana* (hazelnut), *Anacardium occidentale* (cashew), *Arachis hypogaea* (peanut), *Juglans regia* (walnut), and *Sesamum indicum* (sesame) because a declaration of their presence on the labels of food that contains or may contain them is mandatory in many countries [35]. The target genes encode the major allergen present in each of these six species, since

in previous work [16] it was demonstrated that the use of allergen-specific primers allows for a specific and highly sensitive detection of the DNA of the target species with a PCR assay. The primer sequences were designed to ensure that each amplicon was of a recognizably different length, and that the T_m s of all six amplicons differed from one another by at least 0.25°C (patent application pending). The six primer pairs are hereafter referred to as PRU1 (almond allergen), CORA1 (hazelnut allergen), JUGR1 (walnut allergen), ANAO1 (cashew allergen), ARAH1 (peanut allergen), and SESI (sesame allergen). A BLAST search (<http://www.ncbi.nlm.nih.gov/BLAST>) of the primer sequences confirmed that they were all species-specific.

Simplex PCR

A standard PCR was performed with each primer pair in turn, and the resulting amplicons were separated by agarose gel electrophoresis. Each reaction produced the predicted single amplicon of expected length, with no primer-dimer products being generated (data not shown). Each of the fragments was eluted from the gel, cloned, and sequenced, following the method of Pafundo et al. [16]. In all six cases, the resulting nucleotide sequence matched the sequence used for primer design. To determine whether the T_m of each amplicon was as predicted, and that the set of T_m s would allow discrimination between the species in a multiplex format, a set of SYBR® GreenER™ based real-time PCRs was performed. Each primer pair amplified a specific DNA fragment with a T_m close to that expected

Fig. 1 Capillary electrophoresis with an automatic sequencer of a multiplex mix. The electropherogram shows the results obtained after a standard PCR of a mixture of six DNAs, in equal parts (mix A), diluted 1:50. Six primer pairs were used together, and each forward primer was labelled with Cy5. The standard deviation of the length of each fragment was ± 2 bp. The height of the peaks is not correlated with the amount of DNA in the sample. 1 cashew, 2 hazelnut, 3 peanut, 4 walnut, 5 almond, 6 sesame



exclusively when DNA from its target species was provided as a template (Table 1). The T_m s of the amplicon derived from a PCR where the template provided was from the respective target species are documented in Table 1. No amplification was obtained when the template consisted of DNA extracted from the leaves of the set of non-target plant species likely to be present in biscuits and other bakery foods.

Multiplex PCR and real-time PCR

A preliminary standard PCR was performed including all six primer pairs in the same reaction, providing as a template either the DNA of each of the target species on its own or an equimolar mixture of all six DNAs (mix A). The former reactions produced the expected single amplicon

Fig. 2 Real-time multiplex PCR. The six primer pairs were used together on the DNA of a single-target species. **a** Derivatives of melting curves obtained with SYBR® GreenER™ in an ABI Prism® 7000 sequence detection system. **b** Derivatives of melting curves obtained with Fast SYBR® Green in an Applied Biosystems 7900 HT fast real-time PCR system. **c** Derivatives of melting curves obtained with SYBR® GreenER™ in an ABI Prism® 7000 sequence detection system, using all six primers together on the DNA of the single-target species and on a mixture of all six target DNAs. 1 cashew, 2 hazelnut, 3 peanut, 4 walnut, 5 almond, 6 sesame, 7 mixture

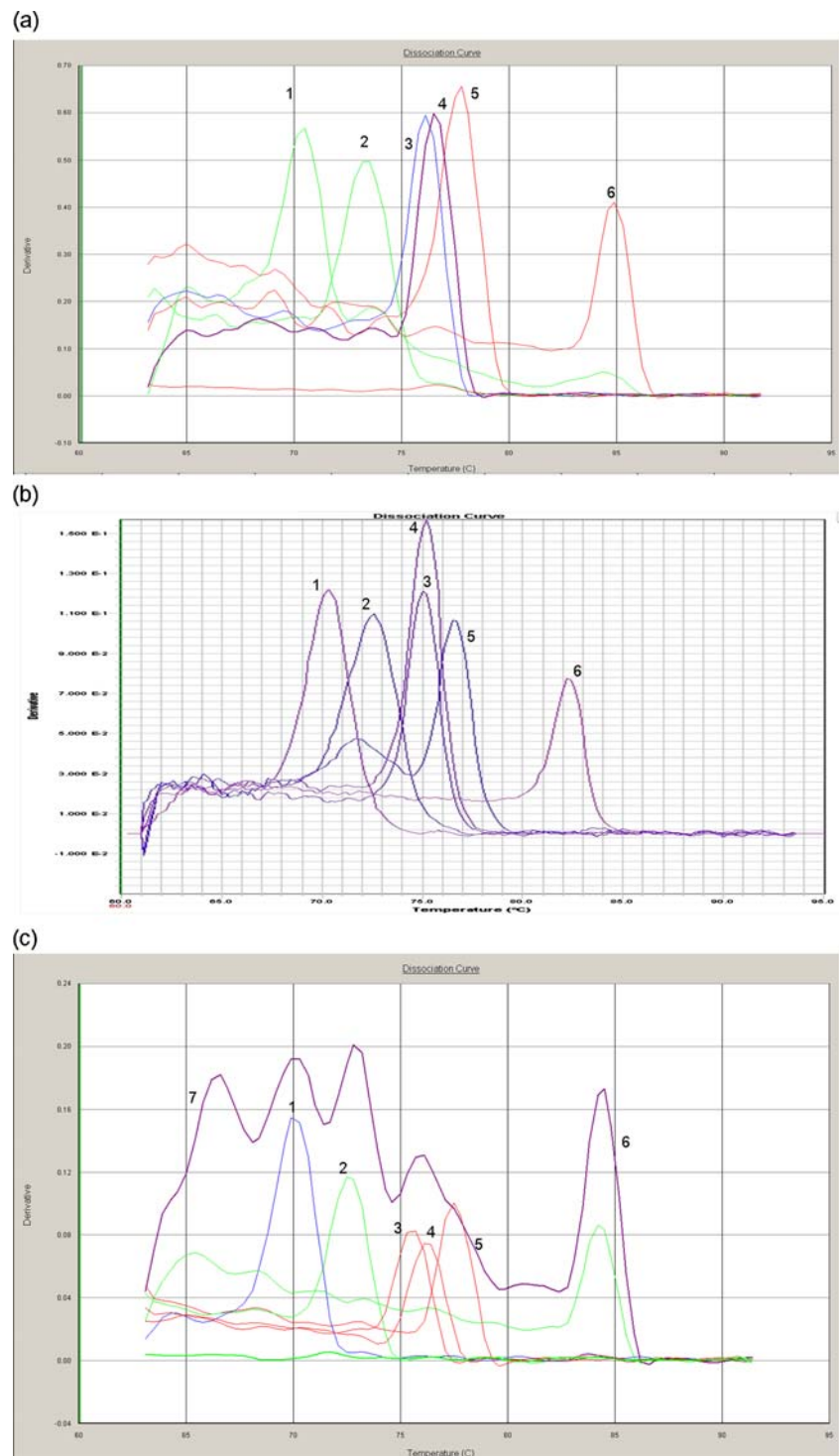


Table 2 Sensitivity of the multiplex PCR. The real-time multiplex PCR was tested using mixtures of the target DNAs (mix B) diluted at 1:10 and 1:100. One species at a time was present at 5 or 0.5 pg (as indicated in rows). The T_m s of each amplicon measured by the instrument are reported

		Amplicon T_m					
		Cashew 70.5°C±0.1	Hazelnut 73.3°C±0.1	Walnut 75.9°C±0.1	Peanut 76.3°C±0.2	Almond 77.8°C±0.2	Sesame 85°C±0.2
0.5 ng of total DNA with 1% (5 pg) of	Almond	+	+	+	+	+	+
	Peanut	+	+	+	+	+	+
	Cashew	+	+	+	+	+	+
	Walnut	+	+	+	+	+	+
	Sesame	+	+	+	+	+	+
	Hazelnut	+	+	+	+	+	+
0.05 ng of total DNA with 1% (0.5 pg) of	Almond	+	+	+	+	-	+
	Peanut	+	+	+	-	+	+
	Cashew	+	+	+	+	+	+
	Walnut	+	+	+	+	+	+
	Sesame	+	+	+	+	+	+
	Hazelnut	+	-	+	+	+	+

+ presence of a peak of predicted T_m , - absence of a peak

(data not shown), whereas the latter ones produced all six expected fragments (recognized by their different lengths) (Fig. 1). The multiplex PCR was then transferred to a real-time PCR/SYBR® GreenER™ platform on the ABI Prism® 7000 sequence detection system. Some re-optimization of primers concentrations was needed to avoid preferential amplification of some of the targets (patent application pending). The monitoring of the reaction started at the 18th cycle. The output of the optimization process was the amplification of each expected fragment with its expected T_m (Fig. 2a). The optimized assay was tested using the LCGreen® melting dye stained with LCGreen® Plus+, but with less than satisfactory results. It was also tested using both SYBR® GreenER™ and the Fast SYBR® Green dyes on the Applied Biosystems 7900 HT fast real-time PCR system. In the latter case, the reaction time for the experiment was under 30 min. As shown in Fig. 2b, a single peak with its characteristic T_m was amplified from DNA extracted from each respective species, but the peanut and walnut melting curves overlapped with one another, preventing discrimination between these two templates. As a result, we concluded that full discrimination required the use of the ABI Prism® 7000 sequence detection system.

Real-time multiplex PCR: sensitivity and efficiency

The sensitivity of the multiplex assay was tested by challenging it with an equimolar mixture of DNA extracted from the six nut species (mix A). This generated a curve with five rather than six peaks (Fig. 2c), because of the overlap between the peanut and walnut peaks. To define the absolute limit of detection of the method, as the lowest

amount of template that could be reliably detected [26], the primer pairs, used individually or all together, were applied to six different mixtures of DNA containing the DNA from a single species at 1% and the remaining 99% was composed of the other five species in equal amounts (19.8% each) (mix B). For each mixture three samples were tested containing 5, 0.5, or 0.05 ng of total DNA and therefore 50, 5, and 0.5 pg of DNA of the species present at 1%.

In Table 2 the results obtained using all six primer pairs together on the mixtures containing 0.5 and 0.05 ng as total DNA are reported; comparable results were obtained when mixtures containing 5 or 0.5 ng were used.

The mixtures containing 0.5 ng of total DNA gave the expected amplicons in all cases. The mixtures containing 0.05 ng of total DNA gave all the expected amplicons when the species present at 1% were cashew, walnut, or sesame, whereas when the species present at 1% were almond, peanut, or hazelnut the corresponding amplicons were missing. Therefore, the absolute sensitivity of the method was evaluated at 5 pg for almond, hazelnut, and peanut DNA, and at 0.5 pg for cashew, walnut, and sesame DNA. These results are in agreement with those obtained with the primer pairs used individually (data not shown).

The DNAs extracted from the reconstructed biscuits containing different amounts of almond were analysed with the multiplex assay: in all cases, except for the reference biscuits with 0% almond, only the amplicon for almond was detected. Dilution of the sample with the lowest quantity of almond, 0.05%, gave a limit of detection of about 100 mg kg⁻¹.

The efficiency of the multiplex assay was tested by providing serial dilutions of template plasmid DNA con-

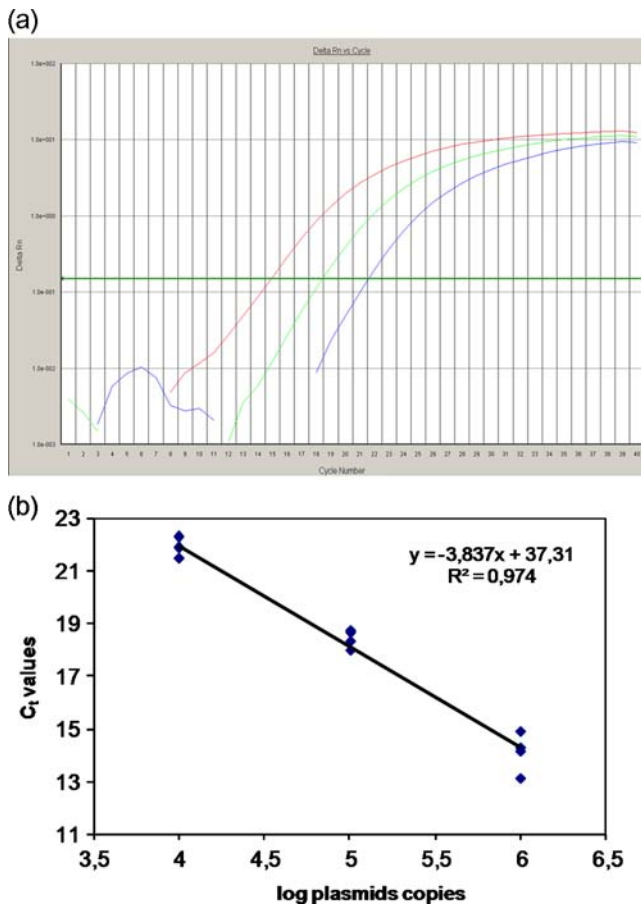


Fig. 3 Real-time multiplex PCR using SYBR® GreenER™ on plasmid DNA, containing the target amplicon of SESI primers. **a** Amplification plot of a single replicate for each dilution of a plasmid with a cloned sesame amplicon. **b** standard curve of serial dilutions of plasmid DNA, containing the sesame amplicon. Each point represents the amplification obtained using the multiplex PCR on different amounts of the plasmids. Four replicates were made for each dilution

taining either each of the six amplicons in turn or a mixture of all six amplicons. This experiment generated a set of standard curves (e.g. the sesame amplicon curve shown in Fig. 3). All the single-insert plasmids gave a result similar to that for sesame. For the multi-plasmid template, the standard curve regression was $y = -2.96x + 35.913$ ($R^2 = 0.9998$) and the efficiency was 117%.

Functionality of the assay on commercial foodstuffs

The multiplex assay was then extended to DNA extracted from a set of commercial food products, in particular biscuits, chocolate, and crackers (Table 1). The profile of products whose ingredients included a specific allergenic species always comprised a peak at the expected T_m . When the labelling contained an indication of possible traces of nuts in general or of specific allergens, the assay could confirm if they were present or not present.

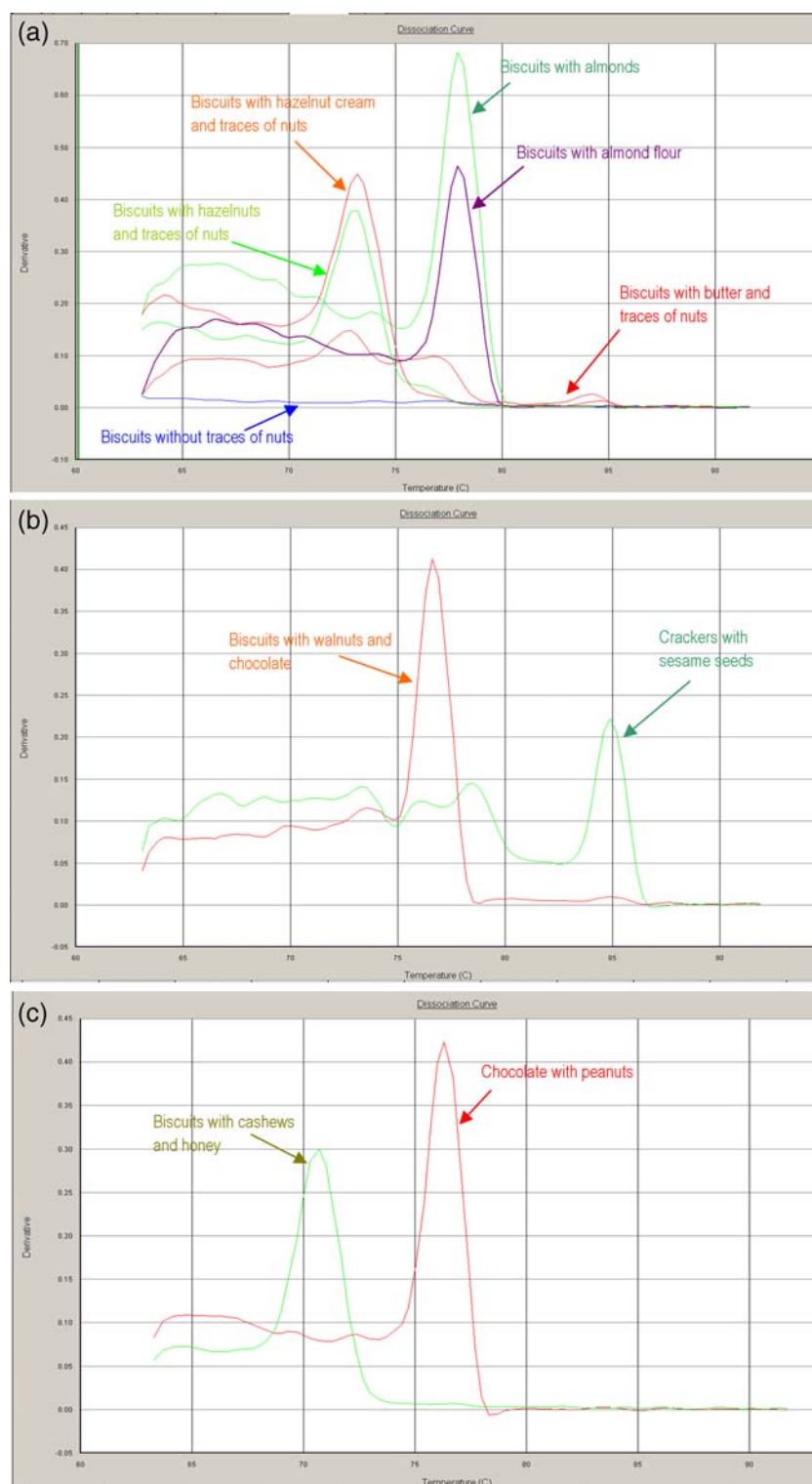
Some examples of the results obtained with the multiplex assay, targeting six species in a single-tube reaction, are shown as melting profiles in Fig. 4. In the case of biscuits with hazelnut cream, biscuits with hazelnuts, biscuits with almonds, and biscuits with almond flour (Fig. 4a) the main ingredient was detected as expected. In the case of biscuits with butter two peaks with T_m corresponding to traces of almond and hazelnut were detected, whereas no amplification was obtained from biscuits with eggs (Fig. 4a). Figure 4b shows the profiles obtained from extracts of biscuits with walnuts and chocolate, and of crackers with sesame seeds. These profiles confirmed the expected presence of, respectively, walnut and sesame DNA but, in the biscuits, a low peak with T_m corresponding to that expected for hazelnut was present, and in crackers, whose label reported the presence of traces of nuts, peaks with the T_m expected for hazelnut, peanut, and almond were detected. Finally, the profiles of both chocolate with peanuts and biscuits with cashews confirmed the presence of the DNA of the respective allergenic species (Fig. 4c); the label of chocolate with peanuts reported the presence of hazelnut paste and a low peak with the expected T_m for hazelnut was observed.

Discussion

Multiplex PCR assays, based on SYBR® Green I fluorescent dye and differential melting temperatures, have been generated for the detection of microbes and viruses [6,10,12–14] and transgenes [15] in foodstuffs. Here we developed an assay able to simultaneously distinguish between six common allergenic targets. The success of the assay relied on a judicious choice of primers to generate a set of amplicons differentiated by their T_m .

Comparisons with cognate platforms (including replacing SYBR® GreenER™ with LCGreen® Plus+, and the ABI Prism® 7000 sequence detection system with the Applied Biosystems 7900 HT fast real-time PCR system) showed that the combination of staining with SYBR® GreenER™ and detection by the ABI Prism® 7000 sequence detection system represented the most effective platform for the assay. It has been claimed [36] that, in genotyping homozygotes using the T_m difference criterion and SYBR® Green I, the ABI Prism® 7000 sequence detection system suffers from a lower error rate than the ABI 7900 HT fast real-time PCR system, and the present results would agree with this assessment. The possibility of distinguishing between peanut and walnut amplicons was tested with two machines: the ABI Prism® 7000 sequence detection system and the Applied Biosystems 7900 HT fast real-time PCR system. The difference between their T_m s (0.4°C) was sufficient for their resolution with the ABI

Fig. 4 Real-time multiplex PCR using SYBR® GreenER™ on commercial products DNA. Each curve corresponds to the amplification carried out on DNA extracted from a single matrix. *Arrows* point to the curves obtained for each product. **a** DNA extracted from different types of biscuits. **b** DNA extracted from crackers with sesame and biscuits with walnut. **c** DNA extracted from chocolate with peanuts and biscuits with cashews and honey



Prism® 7000 sequence detection system, especially when they were present in real food matrices (Fig. 4).

Giglio et al. [37] suggested that it is not possible to use a system based on SYBR® Green I to quantify the amount of a target in a mixed sample, because of the preferential binding of dye to particular amplicons during the PCR,

especially when it is present in a limiting amount. The multiplex assay developed has been optimized to avoid this problem, as confirmed by the analysis of food matrices (Fig. 4). However, it is possible to estimate the sensitivity of the reaction and its efficiency. The absolute limit of detection of the method, defined as the minimum quantity

of template which can be reliably detected [26], was 5 pg for almond, hazelnut, and peanut, and 0.5 pg for cashew, walnut, and sesame. These values are similar to those reported for other simplex PCR assays developed to detect the presence of nuts, peanut, and sesame [28,29]. The limit of detection on matrices evaluated for the biscuits spiked with almond is within the range (1–100 ppm) suggested for an analytical method suitable for the detection of allergenic foods [29].

Most importantly, the application of the optimized multiplex assay for commercial foodstuff analysis confirmed that the method is sensitive enough to detect both the main ingredient and traces of any target also in commercial matrices. It is interesting the case of “crackers with sesame seeds” whose label indicated “it may contain traces of nuts” and the results of the multiplex analysis confirmed the presence of sesame as an ingredient but also traces of hazelnut, walnut, and almond (Fig. 4b). Similarly, in the case of “biscuit with walnut” the main ingredient (walnut) was detected together with traces of hazelnut (Fig. 4b).

The authors have applied for a European patent for this method (“Method for allergens detection”) and so far there has been no claim of a patent pending for a similar method.

Finally, the average estimated efficiency of the real-time multiplex PCR developed was about 107% when the method was applied on recombinant plasmid DNAs, a condition in which any possible inhibitory effect on the reaction is absent. It was demonstrated [16,38] that the efficiency of the assay based on a template of plasmid DNA is comparable to that based on DNA purified from food matrices. The use of SYBR® GreenER™ did not compromise assay efficiency.

Starting from a precautionary principle [35], the presence of an allergen should firstly be evaluated using a DNA-based method, followed, in the case of a positive signal, by some protein methods to detect the presence of the allergenic protein. We suggest therefore that the real-time multiplex PCR platform described here provides an accurate and effective method for detecting traces of common allergenic species in food, and so can be utilized as a screening test of their presence/absence in foodstuffs.

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