

A novel real-time polymerase chain reaction method for the detection of macadamia nuts in food

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Abstract A real-time PCR-based method for the detection of macadamia nuts (fruits of *Macadamia integrifolia* or *M. tetraphylla* or their hybrids) in food products is described. The method consists of DNA isolation by chaotropic solid phase extraction and subsequent PCR with macadamia-specific primers and a TaqMan fluorescent probe. The primers and the probe were targeted to the gene encoding for vicilin precursor. The method was positive for *M. integrifolia* and *M. tetraphylla* and negative for 16 other plant species used in food industry, including peanuts, walnuts, hazelnuts, almonds, pistachio nuts, cashew nuts, Brazil nuts, and chestnuts. The DNA-based detection limit of the method was 1.45 pg. Using a series of model samples with defined macadamia nut contents, a practical detection limit of 0.02% (w/w) macadamia nuts was determined. Practical applicability of the PCR method was tested by the analysis of 14 confectionery samples. For all of the samples, results conforming to the labeling were obtained. The presented PCR method is useful for relatively fast, highly selective, and moderately sensitive detection of macadamia nuts in food samples.

Keywords Macadamia · Allergen · Polymerase chain reaction (PCR) · Food · Confectionery · DNA

Introduction

Macadamia nuts are deshelled kernels of the fruits of two species of trees native to eastern Australia, namely, *Macadamia integrifolia* and *M. tetraphylla* or their hybrids, which are currently grown in various countries including Australia and Hawaii as main producers of this crop. Because of their low production volumes, macadamia nuts belong to most expensive nuts in the market [1].

Macadamia nuts are valued in particular for their extraordinary organoleptic properties and are widely consumed either directly, roasted, or as a component of confectionery products such as filled chocolates or nougats [2]. However, macadamia nuts may have negative effects on certain consumers due to the contents of allergenic proteins [3]. The allergenic proteins are different from those contained in nuts of the Northern hemisphere (hazelnut, chestnut, walnut), which belong to rose-like plants (Rosidae). Macadamia allergies are not very prevalent, but the symptoms are severe, including life-threatening anaphylaxis [4–6]. In response to this fact, European legislation included these nuts in the list of components which are required to be declared on the label of food products [7].

Although, due to their considerable high price, it is not probable that undeclared macadamia nuts may be present in food products as a result of fraudulent substitution, adventitious contamination may take place when various nuts are used in the same food processing facility. Also, lower-quality macadamia nuts may be used for the production of inadequately specified nut pastes, flours, meals or powders and these may then lead to contamination of food products by undeclared macadamia nuts.

Methods for analysis of macadamia nuts in food products are necessary to control the proper labelling, but currently no such method is available. Here, we present a

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Table 1 Oligonucleotides designed; the predicted length of the amplicon was 73 bp

Designation	Length (bp)	T_m (°C)	P _{GC} ^a (%)	Sequence (5'–3')
macF1	20	58.88	55.00	cctcgagtgtggagacgtaa
macR1	20	59.87	50.00	ctcggtgtgtctcgttga
macP1	23	68.28	52.17	FAM-tcagaatcccagctggaaccaca-TAMRA

^a Portion of GC pairs

a probe were designed using Primer Express software (Applied Biosystems, Foster City, Calif., USA).

Polymerase chain reaction

PCR was performed in a volume of 25 μ L. Each reaction contained 5 μ L of template DNA, 1.5 U DNA polymerase HotStarTaq Plus (Qiagen), 1 $\times$ reaction buffer for the DNA polymerase, 2.5 mmol/L MgCl₂, 200 μ mol/L dNTP mixture (Applied Biosystems), 500 nmol/L of the forward primer, 500 nmol/L of the reverse primer, and 200 nmol/L of the probe. Oligonucleotides were synthesized by Qiagen Operon, Köln, Germany. PCR was performed in an Opticon 2 real-time PCR cycler (MJ Research, Waltham, Mass., USA). The PCR program consisted of the initial denaturation at 95 °C for 5 min, followed by 60 cycles of denaturation at 95 °C for 15 s, and annealing with polymerization at 60 °C for 60 s. Fluorescence was measured in the optical channel 1, which is optimal for the FAM dye. The threshold cycle c_T values were calculated for individual samples by the internal software of the cycler using a manual threshold setting at a fluorescence value of 0.035.

Results and discussion

Vicilin precursor gene was chosen as a marker for macadamia, based on of the previous positive experience with the vicilin gene in the design of the walnut-specific PCR [9]. Three alleles of this gene were found in the GenBank database (accession numbers AF161883, AF161884 and AF161885). The region was selected, where all these three alleles were identical, i.e., base pairs 993–1226 according to AF161883 sequence (Fig. 1). Primers targeting this DNA sequence were designed to be preferably 20 bp long, to have an optimal T_m of 60 °C and to produce an amplicon 50–75 bp long. The 5'-nuclease (TaqMan) probe was designed to be preferably 23 bp long and to have an optimal T_m of 70 °C. From the combinations in the program output, those were selected where primers ended with A and where a probe did not start with G. The resulting oligonucleotides are presented in Table 1 and Fig. 1.

Real-time PCR employing novel primers and the probe was used to analyse DNA isolated from leaves of *M. tetraphylla*. When serial dilutions of the isolated

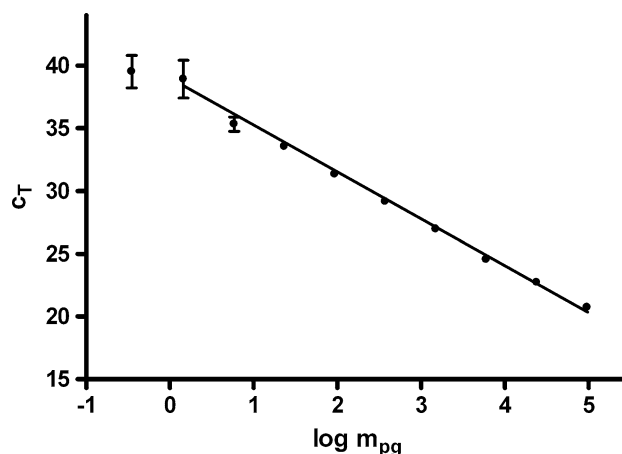


Fig. 2 Relationship ($y = -3.682 \log_{10}x + 38.72$; $R^2 = 0.9896$) between DNA amount per reaction [pg] and the threshold cycle c_T ($\pm$ standard deviation) in real-time PCR (triplicate analyses). Two out of three parallel samples of the lowest concentration were positive

DNA were analysed by real-time PCR in triplicate, a detection limit of 1.45 pg macadamia DNA was determined (Fig. 2). Thus, the method had a sensitivity comparable to other real-time PCR-based methods for nuts [9, 13, 14]. DNA concentration lower than this value, i.e., 0.35 pg could be detected only in two out of three parallel reactions.

In order to determine specificity of the elaborated real-time PCR system, DNA was isolated from various plant species, including various nuts, and analysed by the macadamia-specific real-time PCR. Samples of leaves of *M. integrifolia* and *M. tetraphylla* as well as five brands of macadamia nuts available on the market were positive, while all non-macadamia samples were negative (Table 2). These results demonstrate the appropriate specificity of the method.

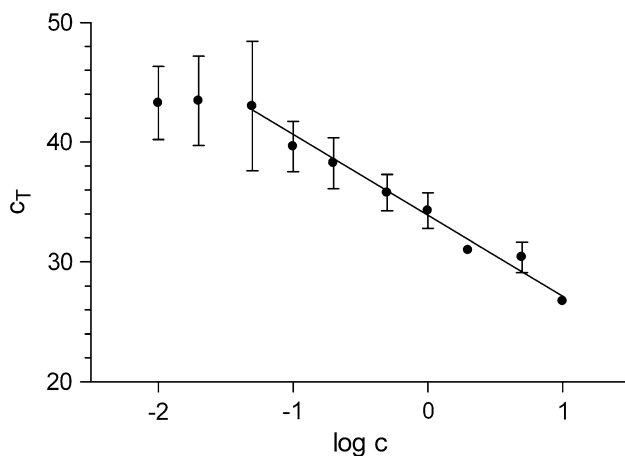
By the analysis of model samples with defined macadamia nut contents, a practical detection limit of 0.02% (w/w) was determined. The detection of lower concentration was possible but not reproducible between parallel reactions. A linear relationship between the logarithm of the macadamia nut content and the threshold cycle was observed for model samples with macadamia nut contents of 0.05% (w/w) or higher, when the template DNA amount was adjusted to 150 ng per reaction (Fig. 3). The PCR method was then applied to the analysis of 14 nut and confectionery products. PCR results were found to conform to the labelling in all samples (Table 3).

Table 2 Plant species analysed and the results of macadamia-specific PCR

Sample	PCR result
<i>Macadamia integrifolia</i>	+
<i>Macadamia tetraphylla</i>	+
Almond	–
Brazil nut	–
Cashew	–
Chestnut	–
Hazelnut	–
Peanut	–
Pistachio	–
Sunflower	–
Walnut	–
Maize	–
Oat	–
Rice	–
Soya	–
Wheat	–
Cow's milk	–
Cocoa	–

Table 3 Confectionery products analysed and results of the macadamia-specific PCR

Food sample	Country of production	Macadamia declaration	PCR result
Macadamia nuts, roasted and salted 1	Germany	+	+
Macadamia nuts, roasted and salted 2	Germany	+	+
Macadamia nuts, fat-free roasted and salted	Germany	+	+
Macadamia nuts in white chocolate	Germany	+	+
Macadamia nuts in honey cream	Germany	+	+
Milk chocolate with macadamia nuts	Belgium	+	+
Edible nuts in milk chocolate	Austria	+	+
Cashew nuts	Germany	–	–
Cashew nuts in white chocolate	Germany	–	–
Edible nuts mixture, salted	Germany	–	–
Dark chocolate	Belgium	–	–
Brazil nuts	Austria	–	–
Almonds	Austria	–	–
Filled chocolate	Austria	–	–

**Fig. 3** Relationship between the contents [% (w/w)] of macadamia nuts in model samples (six replicate analyses) and the threshold cycle c_T ($\pm$ standard deviation) in real-time PCR. The template DNA amount was adjusted to 150 ng per reaction. Two out of six parallel samples of the lowest concentration were positive

As demonstrated, the presented real-time PCR fulfills the requirements for highly specific and relatively rapid detection of macadamia nuts in food. Regarding sensitivity in food samples, the method is by one or two orders of magnitude less sensitive than required by the allergologists [15]. However, it may be useful until more sensitive, probably immunochemical methods for the detection of macadamia nuts in food are available. The method may be

also used for highly specific confirmation of the results obtained by immunochemical methods.

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