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A novel real-time polymerase chain reaction (PCR) method for the detection of walnuts in food

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Abstract A real-time polymerase chain reaction (PCR)-based method for the detection of the walnut (*Juglans regia*) component in food is described here. The method consists of DNA isolation by chaotropic solid phase extraction and the subsequent PCR with walnut-specific primers and a TaqMan fluorescent probe. The primers and the probe are targeted to the *jug r2*, a major allergen gene of walnut. The method was positive for 8 varieties of walnut and negative for all other tested plant materials used in food industry, including pecan nuts. The intrinsic detection limit of the method was 0.24 ng walnut DNA. Using a series of model pastry samples with defined walnut contents, a practical detection limit of 0.01% walnut content was estimated. Practical applicability of the PCR method was tested by the analysis of 13 food samples (bakery and confectionery products), out of which two cakes were found to contain walnuts although they were not adequately labelled. The presented PCR method is useful for sensitive and selective detection of walnuts in food samples and can be performed in one working day.

Keywords Walnut · Allergen · Polymerase chain reaction (PCR) · Bakery · Confectionery

Introduction

Walnuts, the fruits of the walnut tree (*Juglans regia*), belong to widely consumed tree nuts. They are used in bakery products as a basic component of nut fillings in various cakes, in a range of confectionery products like filled chocolates, in cereal müsli mixtures and in other food products. However, walnuts may be a threat to some consumers since they contain allergens and consumption of food products containing walnuts may cause severe allergy in a part of the population [1, 2]. In response to this fact, the recently introduced

European legislation requires labelling of food products in respect to the contents of walnuts [3]. To enforce this legislation, methods for analysis of walnut content in food are necessary. Currently, there is only one published method available for the detection of walnuts in food products, which is a sandwich ELISA. A drawback of this method is a cross-reactivity with hazelnuts, pecan nuts and sesame [4]. Although several food analytical laboratories have reported that PCR-based tests for walnut detection are available on request [5], the data necessary for the reproduction of these methods were neither published, nor were the genes disclosed, to which the PCR methods are targeted. Here we present a method for the specific detection of walnut DNA by real-time PCR, which is a technology becoming widely used for the detection of allergenic components in food [6].

Materials and methods

Plant and food materials

Nuts of various walnut varieties were obtained from Central Institute for Supervising and Testing, Brno, Czech Republic. Pecan nuts (*Carya illinoensis*) were provided by Dr. C. E. Cerniglia, National Center for Toxicological Research, US FDA, Jefferson, Arkansas, USA. Sprays of non-food species of *Juglans* and *Carya* genera were provided by Dr. I. Štefančík, Forestry Research Institute, Zvolen, Slovakia. Wheat, barley and rye flour as well as potato starch were obtained from Dr. F. Schwägele, Federal Research Centre for Nutrition and Food, Kulmbach, Germany. The other nuts and food samples were obtained from grocery stores in Bratislava.

Preparation of standard pastry with defined walnut concentration

The basic, nut-free dough was prepared according to the traditional recipe for “bear paws” pastry, which contains 600 g wheat flour, 500 g butter, 300 g sugar powder, 0.5 g

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hydrocarbonate-based baking powder, 12.5 g (a spoonful) cocoa and 102.7 g (2 pieces) eggs. Separately, mixtures of walnuts and almonds with 0, 0.1, 0.5, 2, 10 and 100% walnut content were weighed. Individual nut mixtures were homogenized in coffee mills. Twenty grams of each resulting nut paste was mixed with 180 g of the basic dough. The dough was filled into greased and flour-powdered baking forms and the individual batches with the same walnut concentration were wrapped in the aluminium foil. The pastry was baked in a hot air oven with forced convection HS 202A (Chirana, Piestany, Slovakia) without overpressure. The baking temperature was 180 °C and baking was continued for 30 min to simulate regular baking conditions.

DNA extraction and quantification

Prior to DNA extraction, food samples were homogenized using mortar and pestle. Wood-containing samples, i.e. nutshells and sprays, were ground to a fine powder using emery (rasp paper). Subsequently, DNA was isolated by chaotropic solid phase extraction using the GeneSpin kit (GeneScan, Freiburg, Germany), according to the instructions for use attached to the kit. DNA was quantified by UV-spectrometry. If the absorption peak at $\lambda=260$ nm was present, the DNA concentration was calculated from A_{260} and adjusted to 30 ng/ μ l for use in subsequent PCR reactions. If the absorption peak was not observed as, for instance, in some wood-containing samples, the concentration calculation was omitted and the 10^0 , 10^{-1} and 10^{-2} dilutions were used in subsequent PCR reactions. DNA concentration was considered sufficient, if the sample was positive at the amplification check.

Nucleotide sequence sources and alignment

The partial genomic DNA sequences of the pecan and walnut *jug r2* gene were obtained in our laboratory as follows: partial PCR amplicons of *jug r2* were prepared using the sequencing primers JuglseqF and JuglseqR (Table 1), which were designed according to the walnut mRNA sequence in the GenBank database, accession number AF066055. DNA extracts of walnut (unspecified market sample), pecan 1 and pecan 2, respectively, were used as a DNA template. PCR conditions were the same as described below, however, no FAM-labelled probe was involved. Amplified DNA fragments were purified by GFX PCR DNA and gel band purification kit (Amersham Pharmacia Biotech, Uppsala, Sweden), and sequenced with the original primers by BigDye Terminator 3.1 Cycle Sequencing Kit and Prism 3100 Avant (Applied Biosystems, Foster City, Calif., USA) at the Department of Molecular Biology, Faculty of Natural Sciences, Bratislava, Slovakia. The resultant sequences were aligned with the following sequences from the GenBank database coding for vicilin-like proteins: macadamia nut AF161883, cocoa X62625, sesame AF240006, hazelnut AF441864. The alignment was performed using the software Megalign (DNASTar, Madison, Wisconsin, USA).

Table 1 Primers used in PCR

Designation	Sequence (5'–3')	Target	Reference
JuglF	gcgcagagaagcagag	Walnut	This article
JuglR	ctcatgtctcgacctaatgct	Walnut	This article
JuglP	FAM-attgtgcctctgtt-gctcctcttcccg-TAMRA	Walnut	This article
TR03	tctgccctatcaactttcgaaggta	Eukaryotic	7
TR04	aatttgcgcgcctgctgccttcctt	Eukaryotic	7
TRPb	FAM-ccgtttctcaggctc-cctctccggaatcgaacc-TAMRA	Eukaryotic	8
JuglseqF	ggcagagaccgacaagatcc	Walnut	This article
JuglseqR	gaaggagctcggttctct	Walnut	This article
M13F-40	gttttccagtcacgac	pDrive vector	Qiagen ^a
M13R	aacagctatgacctatg	pDrive vector	Qiagen

^aSequences of these primers were obtained from the Qiagen PCR cloning handbook (Qiagen, Hilden, Germany)

Design of the walnut DNA-targeted PCR primers and a probe

Primers and a FAM-labelled, TAMRA-quenched oligonucleotide probe for walnut detection were designed manually to target the *jug r2* gene coding for vicilin-like seed storage allergenic protein of walnut. The primers were designed to produce an amplicon 50–100 bp long, to have a melting temperature (T_m) between 58 and 60 °C, to be between 17 and 24 bases long. Additionally, the reverse primer was designed in a way to match the DNA region divergent from pecan (Fig. 1). The probe was designed to have a T_m by 10 °C higher than that of the primers, to be between 20 and 24 bases long, to have a T, C or A at the 5' end and to contain more cytosines than guanines. The T_m values were calculated using the Davis, Botstein, Roth Melting Temperature option of the Editseq software (DNASTar).

Assessment of DNA amplifiability

To check the amplifiability of the isolated DNA, real-time PCR with universal eukaryotic primers and a FAM-labelled, TAMRA-quenched oligonucleotide probe targeted to 18S rRNA gene ([7, 8], Table 1) was performed. Only those DNA samples, which demonstrated PCR amplifiability in this test, were included in subsequent PCR experiments with primers and the probe targetting *jug r2* gene.

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 (Qiagen, Hilden, Germany), 1 $\times$ reaction buffer for the DNA polymerase, 2.5 mmol/l $MgCl_2$, 200 μ mol/l dNTP mixture (Invitrogen, Carlsbad, Calif.,

Fig. 1 Region of the *jug r2* gene of walnut, to which the PCR primers and the probe were designed, and the comparison to vicilin-like seed storage protein genes of selected agricultural plants. GenBank accession numbers: walnut AF066055; pecan DQ156215; macadamia AF161883; cocoa X62625; sesame AF240006; hazelnut AF441864. Symbols: (°) mRNA sequence; identity with the genomic DNA was confirmed by sequencing; (*) mRNA sequence; (†) genomic DNA sequence

		forward primer	probe (antisense)	
walnut°	493	GCCCGCGCAGAGAAAGCAGAGGCCGGGAAGAGGAGCA---	ACAGAGGCACAATCCCTACT	550
pecan†	158	GCCCGCGAAGAGAAAGCAGAGGCCGGGAAGAGGAGCA---	ACAGAGGCACAATCCCTACT	215
macadamia*	712	GGGGAGGCGAGCGGCAGATACGAGGAGGGAGAAGAGGA---	GCAAGCGACAACCCCTACT	769
cocoa†	986	ATCACAAAAAATAGGAGCGAAGAAGAAGAGGCA---	ACAAGAAACAATCCCTACT	1043
sesame*	540	ATGAAGGGAGAGAAAGAGAGGAGCAAGAAGAACAGGGACGAGGGCGGATTCCGTACG		600
hazelnut*	131	AGGAGGGCAATAGTAGCGAGGAA-AGTTATGGGAA--	GGAGCAGGAGGAAAATCCTTATG	188
		reverse primer		
walnut°	551	ACTTTCCTCCAGAGCATTAGGTCTGA---	GACATGAGTCCGAGGAAGGTGAAGTTAAAT	606
pecan†	216	ACTTTCCTCCAGGSACTCAGGTCTGA---	GACATGAGTCCGAGGAAGGTGAAGTTAAAT	271
macadamia*	770	ACTTCGAGCAACGAAGCTTAAGTACAA---	GGTTCAGGACCGAGGAAGGCCACATCTCAG	825
cocoa†	1044	ATTTTCCTAAAAGAAGATCATTCCAACTCGATTGAGGGATGAAGAGGGCAACTTCAAGA		1102
sesame*	601	TGTTTGAGGACCAAGCATTTCTTA---	CTGGTTTCGGACGACGACGGCCGGATGCGGG	656
hazelnut*	189	TTTTTCAAGATGAGCATTTTGAAT---	CCAGAGTTAAACAGAGGAAGGCAGAGTGCAGG	244

USA), 500 nmol/l of the forward primer, 500 nmol/l of the reverse primer and 200 nmol/l of the probe. PCR was performed in a PTC-200 PCR cycler with an optical unit Chromo 4 (MJ Research, Waltham, Mass., USA). The PCR programme consisted of the initial denaturation at 94 °C for 15 min followed by 60 cycles of denaturation at 94 °C for 15 s and annealing with polymerization at 60 °C for 60 s. The fluorescence was measured in channel 1 (FAM). The threshold cycle C_T was calculated for individual samples by an internal software of the cycler using the manual threshold setting at a fluorescence value of $10^{-1.8}$. For the interpretation of the results in case of the TaqMan system *juglF*, *juglR* and *juglP* (Table 1), all C_T values below 35 were considered as positive whereas all C_T values above 35 were treated as negative. For the interpretation of the DNA amplifiability test using TR03, TR04 and TRPb, a similar positivity threshold was set at $C_T=24$, which corresponds to the minimal DNA amount that allows amplification of *jug r2* in the model pastry containing 0.01% walnut.

Cloning of the PCR products

To confirm the identity of the PCR products obtained with *JuglF* and *JuglR* primers, PCR products were cloned into pDrive cloning vector using the Qiagen PCR Cloning Kit (Qiagen) with DH5 α competent cells used instead of Qiagen EZ competent cells. From the correct clones, PCR amplicons were prepared using M13F-40 and M13R primers, purified by GFX PCR DNA and gel band purification kit (Amersham Pharmacia Biotech) and sequenced with the original primers as described above.

Results and discussion

In our attempt to design PCR primers and a TaqMan fluorescent probe specific for walnut (*Juglans regia*), we decided to use a gene encoding the walnut allergenic protein as a DNA target, because analogous approach had been reported to lead to the design of a highly specific assay for the detection of peanut [9]. We intended to avoid cross-reactivity with pecan nut (*Carya illinoensis*), which is closely re-

Table 2 Species analysed and the results of walnut-specific PCR

Plant species	PCR result	Plant species	PCR result
<i>Juglans regia</i> Apollo	+	Peanut	—
<i>Juglans regia</i> Buchlov	+	Peach	—
<i>Juglans regia</i> Jupiter	+	Cashew	—
<i>Juglans regia</i> Lake	+	Hazelnut	—
<i>Juglans regia</i> Magdon	+	Almond	—
<i>Juglans regia</i> Mars	+	Apricot	—
<i>Juglans regia</i> Saturn	+	Brazil nut	—
<i>Juglans regia</i> Seiferdorfský	+	Pistachio	—
<i>Juglans regia</i> (unspecified market sample)	+	Sesame	—
<i>Juglans cinerea</i>	+	Sunflower	—
<i>Juglans cordiformis</i>	+	Chestnut	—
<i>Juglans mandshurica</i>	+	Macadamia nut	—
<i>Juglans nigra</i>	+	Cocoa	—
<i>Carya cordiformis</i>	—	Potato	—
<i>Carya illinoensis</i> "Pecan 1"	—	Maize	—
<i>Carya illinoensis</i> "Pecan 2"	—	Wheat	—
<i>Carya lacciniosa</i>	—	Barley	—
<i>Carya ovata</i>	—	Rye	—
<i>Carya tomentosa</i>	—		

lated to walnut and is used in food industry as well. As a target for the primer design, we chose *jug r2* gene coding for the vicilin-like seed storage protein of walnut, because this gene had no homologue in pecan nut published at the time in GenBank database. However, our first attempt at specific primer design (not shown) indicated that such homologue does exist and we obtained a partial sequence of the *jug r2* gene homologue of pecan. This sequence was identical in two independent pecan nut samples and was submitted to GenBank database under accession number DQ156215. The homologous part of the walnut DNA was sequenced as well and was identical to GenBank walnut mRNA sequence AF066055. To reflect this new fact, the reverse primer *JuglR* of the walnut-specific TaqMan assay (Table 1) was designed in a way to match the walnut *jug r2* sequence region maximally divergent from pecan (Fig. 1). Vicilin-like protein genes from other agricultural plants

Fig. 2 A record of the real-time PCR analysis of the model pastry samples containing 10% (curve 1), 1% (curve 2), 0.05% (curve 3), 0.01% (curve 4) and 0% (curve 5) of the walnut component. Curve 6 is the negative control with no template DNA. The template DNA amount was adjusted to 150 ng per reaction

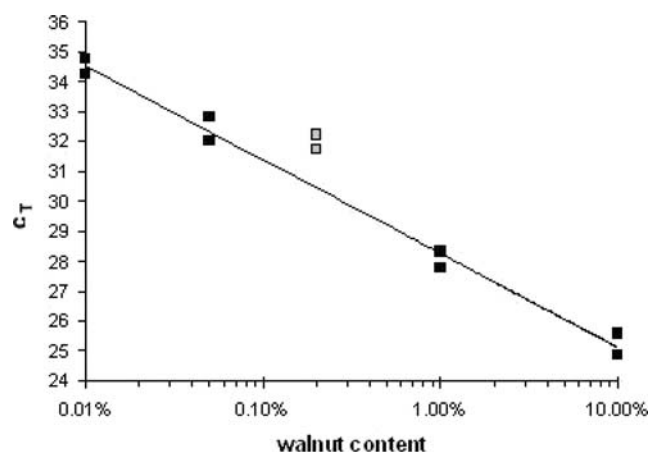
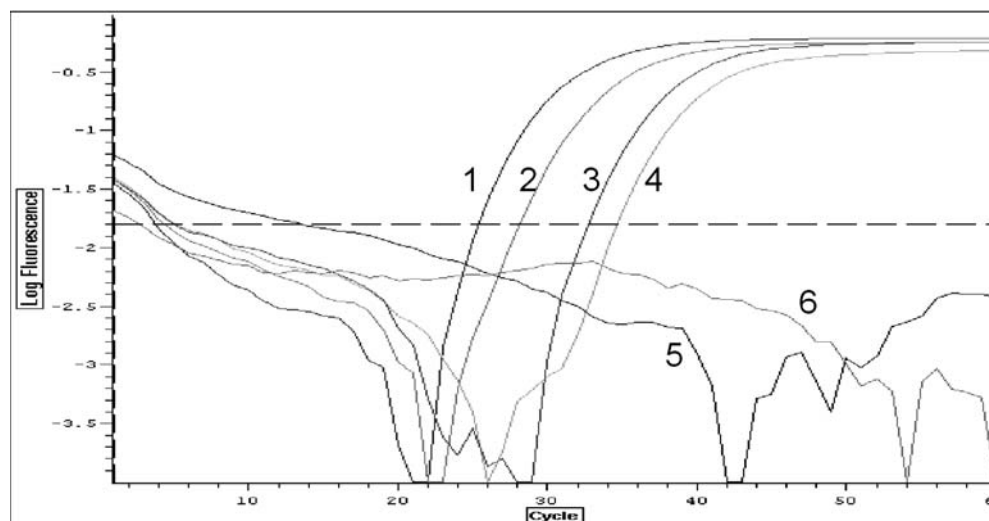


Fig. 3 Relationship ($y = -3.1329 \log_{10}x + 21.989$; $R^2 = 0.9911$) between the walnut contents in the model pastry (duplicate samples) and the threshold cycle C_T in real-time PCR. The template DNA amount was adjusted to 150 ng per reaction

have a considerably low homology to *jug r2* and, based on the sequence alignment, cross-reactivity with the walnut-targeted TaqMan system was not probable (Fig. 1).

PCR employing novel primers and the probe targeting the *Jug r2* gene was used to analyse DNA isolated from the plant material of walnuts (*Juglans regia*), other *Juglandales* and from selected taxonomically unrelated agricultural plants which are widely present in nut-containing food products. All the eight walnut varieties registered in Slovakia, i.e. Apollo, Buchlov, Jupiter, Lake, Magdon, Mars, Saturn and Seiferdorfský, produced positive results. Cloning and sequencing of the PCR products of the unspecified market sample of walnut as well as varieties Apollo and Jupiter confirmed that the DNA region was amplified as depicted in Fig. 1. Other *Juglans* spp. (*J. cinerea*, *J. cordiformis*, *J. mandshurica* and *J. nigra*) were also amplified in this TaqMan assay. This means that the method was 100% inclusive for the genus *Juglans*. The positive detection of *Juglans* species other than *J. regia* is of a little significance for the application of the method to food analysis, since these species are not regularly used in food industry. However, broader specificity of the method to *Juglans* spp. other than *J. regia* could be an advantage, because *in vitro* studies [10] suggest that some other *Juglans* spp. might also cause food allergy to sensitive individuals if they reach the food chain.

Table 3 Bakery and confectionery products analysed and results of walnut-specific PCR

Food sample	Country of origin	Walnut declaration	PCR result
Biscuits, cocoa flavoured	Czech Republic	—	—
Biscuits with toffee and pecan	Scotland	—	—
Wafers with hazelnut filling	Czech Republic	—	—
Wafers with nut filling 1	Poland	—	—
Wafers with nut filling 2	Poland	—	—
Wafers with peanut filling	Slovakia	—	—
Wafers with vanilla filling	Bulgaria	—	—
Müsli bar with dried apricots	Poland	—	—
Plain pastry ^a	Slovakia	—	—
Roll cake with poppy seed filling	Slovakia	—	+
Roll cake with nut filling 1	Slovakia	+	+
Roll cake with nut filling 2	Slovakia	? ^b	+
Cake "Osie hniezdo"	Slovakia	— ^c	+

^aProduced in a bakery where nut-containing cakes are produced

^bAmbiguous declaration of "nuts"

^cDeclaration of hazelnuts

Cloning and sequencing of the PCR products of *J. nigra* and *J. cordiformis* showed that the amplified regions were identical to those of *J. regia* with only one mismatch (G→A) five bases downstream from the forward primer. In case of *J. mandshurica* and *J. cinerea*, the amplified regions were identical to those of *J. regia* with only one mismatch (G→A) four bases downstream from the forward primer. These mismatches apparently did not interfere with the performance of the TaqMan probe.

The method produced negative results with all other tested plant materials (Table 2). Although amplification was observed in certain samples (*Carya illinoensis* "Pecan 2"), this occurred only at threshold cycle values higher than $C_T=35$. Establishing a way of interpretation, which reported $C_T>35$ as negative, lead to exclusivity of the method be 100%. Using dilutions of the walnut DNA, the intrinsic detection limit of the method was determined as 0.24 ng, which was the minimal amount of walnut DNA amplified in all repeats and replicates.

By the analysis of model pastry samples with defined walnut contents, a practical detection limit of 0.01% was determined (Fig. 2). A linear relationship between the logarithm of the walnut concentration and the threshold cycle, C_T was observed, when the template DNA amount was adjusted to 150 ng per reaction (Fig. 3). This observation suggests that the method has a potential to be quantitative when the DNA amount is standardized.

The proposed PCR method was applied to the analysis of a collection of bakery and confectionery products (Table 3). The PCR results conformed to the labelling in all but two samples which contained undeclared walnuts. Although the relationship between the walnut contents and the threshold cycle C_T which was observed in the model pastry (Fig. 3) cannot be, in principle, correctly extrapolated to food samples of a different type, we estimated that one of the two samples with an undeclared walnut content contained less than 0.1% walnut, whereas the other contained approximately 10% walnut. The first sample was a poppy-seed

cake produced in a bakery producing also nut-containing cakes and the positivity for walnuts was probably caused by contamination during the production. The second sample was declared to contain hazelnuts and the high walnut content could be attributed to fraud. Both of these samples did not comply with Directive 2003/89/EC [3].

As demonstrated, the presented PCR method is useful for sensitive and selective detection of walnuts in food samples. The method is relatively straightforward and fast, the analysis can be performed in one working day.

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