

One-step multiplex PCR method for the determination of pecan and Brazil nut allergens in food products

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Abstract

BACKGROUND: A one-step polymerase chain reaction (PCR) method for the simultaneous detection of the major allergens of pecan and Brazil nuts was developed. Primer pairs for the amplification of partial sequences of genes encoding the allergens were designed and tested for their specificity on a range of food components.

RESULTS: The targeted amplicon size was 173 bp of *Ber e 1* gene of Brazil nuts and 72 bp of *vicilin-like seed storage protein* gene in pecan nuts. The primer pair detecting the noncoding region of the chloroplast DNA was used as the internal control of amplification. The intrinsic detection limit of the PCR method was 100 pg mL⁻¹ pecan or Brazil nuts DNA. The practical detection limit was 0.1% w/w (1 g kg⁻¹). The method was applied for the investigation of 63 samples with the declaration of pecans, Brazil nuts, other different nut species or nuts generally. In 15 food samples pecans and Brazil nuts allergens were identified in the conformity with the food declaration.

CONCLUSION: The presented multiplex PCR method is specific enough and can be used as a fast approach for the detection of major allergens of pecan or Brazil nuts in food.

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Keywords: allergy; DNA; identification; vicilin-like seed storage protein; *Ber e 1*

INTRODUCTION

Nuts are considered to be the main source of allergens originating in food. The prevention of life-threatening reactions caused by food allergens relies on a strict avoidance of allergenic food. Therefore the declaration of allergens on food products package is very important and in addition is assessed in EU legislation. The compulsory indication of the presence of all major allergens regardless of their specific amount on the packaging of products results from the Directive 2003/89/EC.¹ Since the occurrence of allergic reactions is reported even after consumption of very low amounts of allergenic proteins, the presence of hidden or undeclared allergens in food products poses a serious health risk for allergic individuals.

Food allergens are usually proteins or glycoproteins with molecular mass ranging from 10 to 70 kDa.² *Ber e 1*–2S albumin³ – and *Ber e 2* – excelsin or so-called 11S legumin-like seed storage protein⁴ – are the main allergens of *Bertholletia excelsa* (Brazil nuts). *Car a 1*–2S albumin or so-called napin and vicilin-like seed storage protein – are the main allergens of *Carya illinoensis* (pecan nuts). Following consumption of Brazil nuts or pecans several cases of allergic or anaphylactic reaction have been reported.⁵ A different symptom of an allergic reaction was observed also in a patient who experienced an acute vesicular cutaneous reaction after prolonged contact with pecans.⁶ Most patients with symptoms suggesting nut allergy have specific IgE to more than one nut.⁷ Therefore the patient should be tested for other nut allergens since a cross-reactivity effect is well known for tree nuts.^{8,9} Multiple allergies appeared progressively with age.

Facial angio-oedema and laryngeal oedema are observed as the most common symptoms.¹⁰

The allergenicity of proteins can be modified during technological, culinary or digestive processing. Thermal processing leads sometimes to an increase in allergenicity, through the formation of neoallergens. Neoallergens appear during heating or storage of foods and may be important in some anaphylactic reactions.¹¹ On the other hand, some studies demonstrate the reduction of allergenicity as the successful decrease of IgE-binding capacity in pistachio nuts using steam-roast processing.¹² Many allergens have been found to be consistently more resistant to digestion by pepsin than other proteins.^{13–16}

For the detection of pecan and Brazil nut allergens various different methods have been reported. Protein or DNA molecule can be used as the target marker. Methods based on DNA molecule detection¹⁷ are considered to be more sensitive by some authors because, in comparison with proteins, DNA is more resistant to heat and pressure technologies used in the food industry. On the other hand, the methods detect only a marker (gene encoding the allergenic protein) that indicates the presence of the harmful food; they do not detect directly the allergen (protein) itself.¹⁸ For the detection of DNA of pecan and Brazil nuts in foods real-time PCR

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has also been described.^{19,20} Protein is used as the target marker in immunoassays.^{21–26}

In this study a conventional multiplex PCR method for the simultaneous detection of *vicilin-like seed storage protein* gene of pecan nuts and for *Ber e 1 (2S albumin)* gene of Brazil nuts was developed. The method was tested by the investigation of commercial food products with pecan or Brazil nuts or other nuts or without nut declaration.

MATERIALS AND METHODS

Plant and food materials

Three cultivar samples of pecans (*Carya illinoensis*) – two imported from the USA and one from Mexico – and also three cultivars of Brazil nuts (*Bertholletia excelsa*) – two imported from Brazil and one from Bolivia – and processed food products as well as other food commodities used for primer specificity testing were obtained from different distributors located in the Czech market. In total 30, samples with nut declaration just as ‘contains nuts’, 15 samples with pecan or Brazil nut declaration and 18 samples without nut declaration or labelled ‘may contain nuts’ or ‘produced in a factory where nuts are processed’ were purchased.

Sample preparation and DNA extraction

Before DNA extraction food matrices were homogenized using a blender, avoiding any cross-contamination. Subsequently, DNA was isolated with a commercial Plant Mini Kit (Qiagen, Hilden, Germany), in agreement with the manufacturer's instructions.

Polymerase chain reaction

For the detection of the main allergen *Ber e 1 (2S albumin)* of Brazil nuts a specific primer pair amplifying the partial sequence of coding gene *Ber e 1* (GenBank accession number AB044391) was designed. Similarly, for the identification of major allergen vicilin-like seed storage protein of pecans a specific set of primers for the amplification of partial sequence of *vicilin-like seed storage protein* gene (GenBank accession number DQ156215) was produced. The primers were designed using the program Primer3. Additionally, universal plant primers were added to the PCR reaction for the confirmation of plant DNA in the sample.²⁷ A multiplex PCR system for the simultaneous detection of these two allergens was constructed. The Multiplex PCR Kit (Qiagen) was chosen for these purposes. The reaction mix contained 25 µL of 2× Qiagen Multiplex PCR Master Mix, 5 µL primer mix (three primer pairs – for the amplification of *Ber e 1*, *vicilin-like seed storage protein* and plant DNA genes), 15 µL RNase-free water and 5 µL DNA template. The total volume of the reaction mix was 50 µL. The primer mix was prepared according to the instructions recommended in the manufacturer's handbook, so that each primer was of 2 µmol L⁻¹ concentration. The negative control was composed of 5 µL RNase-free water instead of DNA. Ten microlitres of each defined DNA (10 ng µL⁻¹) of pecans and Brazil nuts mixed together in AE buffer were used as the positive control. The PCR program was modified according to the experimental studies, and consisted of an initial denaturation step at 95 °C for 15 min, followed by 35 cycles composed of a denaturation step at 94 °C for 30 s, annealing at 65 °C for 90 s and extension at 72 °C for 90 s, and a final extension step at 72 °C for 10 min.

Separation and visualization of PCR products

Separation and visualization of the PCR products was carried out using horizontal electrophoresis in 2.5% agarose gel on a 1 × TRIS-Boric acid-EDTA-Ethylene diaminetetraacetic acid (TBE) containing 0.1 µL mL⁻¹ GelGreen stain. A 50 bp ladder (Qiagen) was used as a marker. The amplification fragments were analyzed under UV light using an ultraviolet transilluminator (Spectroline, Westbury, NY, USA).

Sensitivity of designed primer pairs

To investigate the detection limit of multiplex PCR, samples containing decreasing amounts of mixed DNA of Brazil and pecan nuts diluted in elution buffer (1 ng to 1 fg mL⁻¹) were prepared. The practical limit of detection of the method for pecans and for Brazil nuts was tested on model samples composed of 0.001, 0.01, 0.1, 1 and 10 g (%) pecan/Brazil nut tissue in 100 g (%) of a mixture of all tested neutral matrices as wheat flour, barley flour, oats flour and chocolate. Also, four model samples of the matrices as wheat flour, barley flour, oats flour and chocolate were mixed with the pecans and Brazil nuts separately and its content was determined to be 0.1% w/w (1 g kg⁻¹).

Specificity of designed primers

Various food ingredients that could be components of food products were tested for potential cross-reactivities. The list of ingredients tested was as follows: peanuts, hazelnuts, almonds, walnuts, Brazil nuts, cashew nuts, pistachios, wheat, barley, rye, oat, sunflower seeds, cucurbit, sesame seeds, lentils, peas, beans, maize, rice, potato starch and dried fruit – apples, pineapples, strawberries, kiwi, plums, apricots and raisins.

Amplification control

To check the efficiency of the DNA isolation a primer pair for universal detection of the DNA derived from plants was used.²⁷ The primer pair detects the noncoding region of the chloroplast DNA that was reported as highly conserved over a wide taxonomic range.²⁷ According to the sequence information, the primer pair generates a 124 bp amplified fragment in *Corythophora alba*, so the primer pair would generate amplified fragments of almost the same size (123 bp and 129 bp) from a broad range of plant species.²⁸ This setting of primer pair enables detection of the plant tissue in the investigated samples, and controls the correct DNA isolation.

Confirmation of results of the developed PCR method

The authenticity of two nut allergens was confirmed by sequencing of corresponding marker sequences of *Ber e 1* and *vicilin-like seed storage protein* genes, and alignment of obtained sequences with those deposited in the GenBank database. The PCR products were purified using PCR Purification Kit (Qiagen) and sent for sequencing to a commercial facility (MWG Biotech, Ebersberg, Germany).

RESULTS AND DISCUSSION

Ber e 1 (2S albumin) has been reported as the major allergen in Brazil nuts, while vicilin-like seed storage protein has been described, with *Car a 1 (2S albumin)*, as the main allergens of pecan nut. The sequences of genes encoding these two proteins (*Ber e 1/2S albumin* and *vicilin-like seed storage protein*) are deposited in GenBank under unique accession numbers (Table 1).

Table 1. Primer pairs designed for PCR

Plant species	Allergen	Gene	Sequence of primers (5'–3')	Accession No. (GenBank)	PCR product (bp)
<i>Bertholletia excelsa</i>	Ber e 1 (2S albumin)	Ber e 1	Atggcgaagatttcagttgc Ctcattgtacatccggcagtg	AB044391	173
<i>Carya illinoensis</i>	Vicilin-like seed storage protein	<i>vicilin-like seed storage protein</i>	Agagaatggggaccagatca gtgcctctgtgtcctctt	DQ156215	72
<i>Corythophora alta</i>	–	<i>chloroplast</i>	Cggacgagaataaagatagagt ttttggggatagagggacttg	AF076774 ²⁷	124

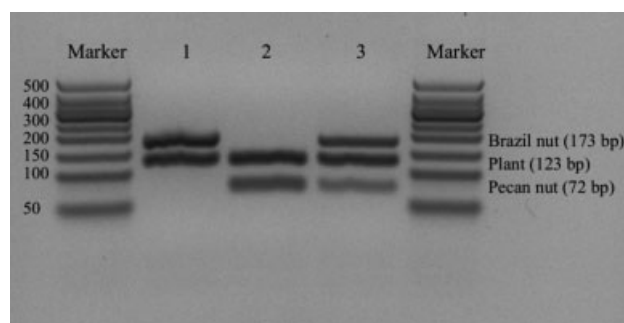


Figure 1. Size of amplification products for particular genes encoding the allergens – multiplex PCR. Line M, marker (50 bp); line 1, PCR product for *Ber e 1* (2S albumin) gene (173 bp) and PCR product for plant DNA (123 bp); line 2, PCR product for plant DNA (123 bp) and for *vicilin-like seed storage protein* gene (72 bp); line 3, PCR product for *Ber e 1* (2S albumin) gene (173 bp), PCR product for plant DNA (123 bp) and PCR product for *vicilin-like seed storage protein* gene (72 bp).

The partial sequences of these two genes were used as the targeted sequences for primer pair production. The predicted amplicon size was 173 bp in *Ber e 1* gene of Brazil nuts and 72 bp in *vicilin-like seed storage protein* gene in pecan nuts (Fig. 1). The short length of amplicons facilitates the detection of allergens in food products processed under different technological approaches that can cause fragmentation of DNA molecule. The identity of amplicons was confirmed by sequencing and alignment of sequences with those deposited in GenBank. The universal primers for plant DNA detection generated an amplicon of 123 bp, which confirms that the sequence amplified with the selected primers (Table 1) was highly conserved, as described by Watanabe *et al.*²⁸ The fragment sizes consisting of approximately 50 bp differences from each other enabled construction of a multiplex PCR method for the simultaneous identification of two allergens and plant DNA, which was used for the confirmation of plant tissue in the sample. The specificity of designed primer pairs for Brazil nut and pecan DNA detection was evaluated with the plant material that could be a component of food products with nut content. No cross-reactivity was observed.

The sensitivity of the multiplex PCR method was tested on a decreasing range of diluted DNA of Brazil nuts and pecan nuts and was assessed to be 100 pg μL^{-1} (Fig. 2). The practical detection limit of the multiplex PCR method was analysed using model samples composed of 0.001, 0.01, 0.1, 1 and 10 g (%) pecan/Brazil nut tissue in 100 g (%) and of the mixture of all tested matrices as wheat flour, barley flour, oats flour and chocolate and was determined in both as 0.1% w/w (Fig. 3). The detection limit was assessed as the least concentration for which results were consistently positive. Many of the allergen assays are targeted to

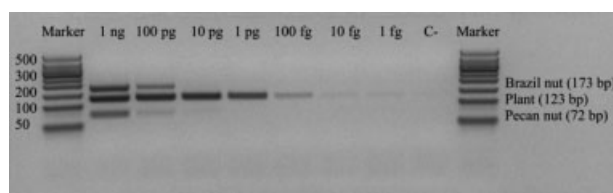


Figure 2. Intrinsic detection limit of multiplex PCR method for the detection of DNA of Brazil and pecan nut mixed together. Marker (50 bp); C–, negative control (without DNA).

the detection of protein and the limit of detection (LOD) is about 1 μg allergen protein g^{-1} of sample with various matrices.^{21,24,25} The detection limit of 0.1 μg g^{-1} was reached using enzyme-linked immunosorbent assay (ELISA) for the simultaneous detection of peanuts, hazelnuts and Brazil nuts.²³ The detection limit of 10–90 ng mL^{-1} was assessed by competitive ELISA for Brazil nut detection.²⁶ The sensitivity of the methods based on DNA detection are relative to the copies of DNA, the concentration of DNA or the detection of DNA in spiked samples. The detection limit of pecan nuts using real-time PCR was determined as 1 pg of pecan DNA or 0.01% (w/w) of pecans.¹⁹ The ligation-dependent probe amplification for 10 food allergens including pecans and Brazil nuts achieved an LOD of 5 mg kg^{-1} .¹⁷ Using real-time PCR for the detection of Brazil nuts a detection limit of 10 pg of Brazil nut DNA or a practical detection limit of 0.1% (w/w), similar to our results, was achieved.²⁰ Real-time PCR is a quantitative method and the detection limit is relatively lower. The equipment for real-time PCR, in comparison with conventional PCR, is more sophisticated and also more expensive. This multiplex PCR is specific and enables the detection of both major allergens of pecans and Brazil nuts simultaneously in one-step PCR.

The developed method was applied to the investigation of 30 food products with nuts declared as 'contains nuts', 18 products declared as 'without nuts' or 'may contain nuts' and 15 samples with pecan and Brazil nut declarations. Four samples of this total were model samples with defined pecan and Brazil nut contents consisting of four various matrices as wheat, barley and oats flours and chocolate determined to 0.1% (w/w); and the remaining 11 samples with a declaration of pecan and Brazil nuts were obtained in the market. The products included nuts, gaufres, muesli, chocolates, wafers, cookies or biscuits. For the DNA isolation the whole size package sample, or 100 g in the case of muesli sample, were processed. 'Neither pecan nor Brazil nut major allergen DNA' was detected in samples declared as 'contains nuts' or 'without nuts'. Eleven samples (muesli, cookies, biscuits and chocolate) with the declaration of pecans and Brazil nuts were investigated in conformity with the declared composition. Also the pecan and Brazil nut DNA in four model samples adjusted to a

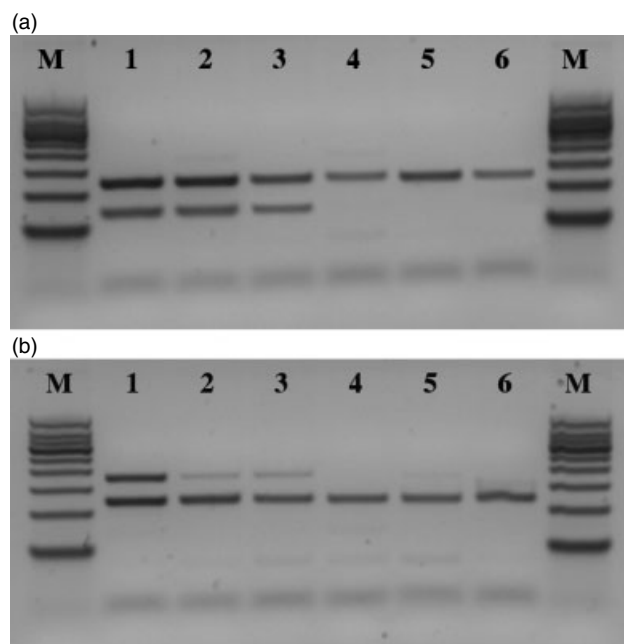


Figure 3. (a) Practical detection limit of multiplex PCR method for the detection of pecan nut major allergen vicilin-like seed storage protein content in model sample mixtures composed of wheat, barley and oats flours and chocolate. Line M, marker (50 bp); line 1, PCR product for 10% concentration; line 2, PCR product for 1% concentration; line 3, PCR product for 0.1% concentration; line 4, PCR 0.01% concentration; line 5, 0.001% concentration; line 6, 0% concentration; lines 1–6 always contain PCR product for plant chloroplast DNA (123 bp); line M, marker (50 bp). (b) Practical detection limit of multiplex PCR method for the detection of Brazil nut major allergen Ber e 1 (2S albumin) content in model sample mixtures composed of wheat, barley and oats flours and chocolate. Line M, marker (50 bp); line 1, PCR product for 10% concentration; line 2, PCR product for 1% concentration; line 3, PCR product for 0.1% concentration; line 4, 0.01% concentration; line 5, 0.001% concentration; line 6, 0% concentration; lines 1–6 always contain PCR product for plant chloroplast DNA (123 bp); line M, marker (50 bp).

concentration of 0.1% (w/w) in four matrices (wheat, barley, oats, chocolate) was detected and all imported and tested pecan and Brazil nut cultivars (in total, three of each species) gave positive results (Fig. 4). Cultivar No. 1 (USA) of pecans and cultivar No. 1 of Brazil nuts were used for the development of this multiplex PCR method. Two other cultivars – No. 2 and No. 3 of each species – were additionally imported and tested (Fig. 4). In all tested samples plant DNA was found. The use of plant primers prevents the detection of false negative results caused by PCR inhibitors. This implies that the amplification band produced by plant primers should be demonstrated in all samples with plant tissue content.

CONCLUSIONS

This study demonstrates the applicability of the newly developed multiplex PCR method for the simultaneous detection of pecan and Brazil nut major allergens in a one-step approach. In addition, the correct running of the PCR reaction is guaranteed by the identification of plant DNA in every sample through the gene for chloroplast amplification. Regarding sensitivity, the method is less sensitive in comparison with other studies, but it can serve as a helpful tool for the confirmation of the tangible results obtained by ELISA methods in accurate detection of allergenic proteins. The

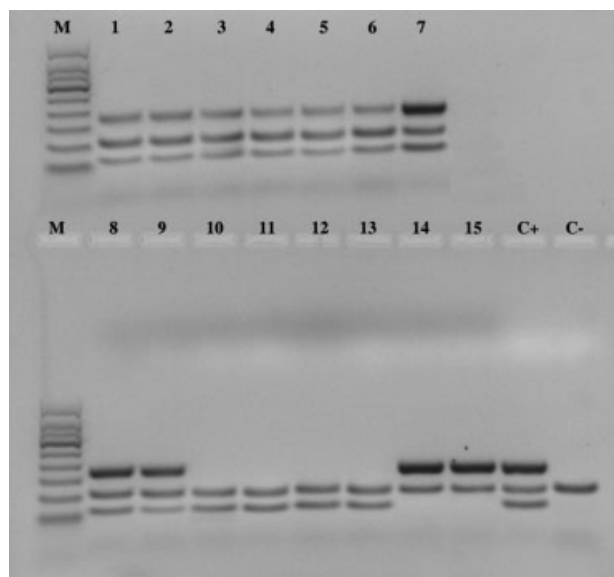


Figure 4. Investigation of food samples containing pecans and Brazil nuts. Line M, marker (50 bp); line 1, PCR product for 0.1% of pecans and Brazil nuts in wheat flour; line 2, PCR product for 0.1% of pecans and Brazil nuts in barley flour; line 3, PCR product for 0.1% of pecans and Brazil nuts in oats flour; line 4, PCR product for 0.1% of pecans and Brazil nuts in chocolate; line 5, PCR product for Jordans country crisp with pecans and Brazil nuts; line 6, PCR product for Jordans country crisp with pecans (1%) and Brazil nuts (2.5%); line 7, PCR product for Jordans country crisp with pecans (10%) and Brazil nuts (20%); line 8, PCR product for Jordans muesli with pecans (5%) and Brazil nuts (10%); line 9, PCR product for muesli with pecans and Brazil nuts; line 10, PCR product for Jordans cookies with pecans (5%); line 11, PCR product for Jordans country crisp with pecans; line 12, PCR product for pecan cultivar No. 2 from USA; line 13, PCR product for pecan cultivar No. 3 from Mexico; line 14, PCR product for Brazil nut cultivar No. 2 from Brazil; line 15, PCR product for Brazil nut cultivar No. 3 from Bolivia; C+, positive control – PCR product for *Ber e 1* (2S albumin) gene (173 bp), PCR product for plant DNA (123 bp) and PCR product for *vicilin-like seed storage protein* gene (72 bp); C–, negative control – PCR product for plant DNA (123 bp) – mixture of all matrices without the addition of pecans and Brazil nuts; lines 1–15 and C+ and C– always contain PCR product for plant chloroplast DNA (123 bp).

advantage of the method is the detection of both allergens in one step and in one time reaction. The presented PCR method is specific enough and can be used as a fast, simple, one-day procedure for the detection of pecan and Brazil nut major allergens in food products.

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