

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

L. Piknová · D. Pangallo · T. Kuchta

Received: 12 December 2006 / Revised: 23 March 2007 / Accepted: 6 April 2007 / Published online: 3 May 2007
© Springer-Verlag 2007

Abstract A real-time PCR (polymerase chain reaction)-based method for the detection of hazelnuts (nuts of *Corylus avellana* or *C. maxima*) in confectionery and bakery products is described. The method consists of DNA isolation by chaotropic solid phase extraction and the subsequent PCR with hazelnut-specific primers and a TaqMan fluorescent probe. The primers and the probe are targeted to the *hsp1* gene encoding for a low molecular weight heat-shock protein. The method was positive for five hazelnut varieties approved in Slovakia and negative for all other tested plant materials used in food industry including peanuts, walnuts, almonds, pistachio nuts, cashews and chestnuts. The intrinsic detection limit of the method was 13 pg hazelnut DNA, which corresponds to approximately 27 genome equivalents (1C). Using a series of model pastry samples with defined hazelnut contents, a practical detection limit of 0.01% (w/w) hazelnut was determined. Practical applicability of the PCR method was tested by the analysis of 20 food samples (confectionery and bakery products) along with ELISA. For all of the food samples, identical results were obtained by both methods, which conformed to the labelling. The presented PCR method is useful for sensitive and selective detection of hazelnuts in food samples and can be performed in one working day.

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

Introduction

Hazelnuts, the fruits of the hazel bush (*Corylus avellana* or *C. maxima*), belong to widely consumed tree nuts. They are used in a range of confectionery products like filled chocolates or wafers, in cereal müsli mixtures, in bakery products as a basic component of nut fillings in various cakes, and in other food products. However, hazelnuts may be a threat to some consumers since they contain allergens and consumption of food products containing hazelnuts may cause severe allergy in a part of population [1, 2]. In response to this fact, European legislation requires labelling of food products in respect to the contents of hazelnuts [3]. Methods for analysis of the hazelnut content in food are necessary to support this legislation. Several sandwich ELISA-based methods are available for the detection of hazelnuts in food [4–6]. These immunochemical methods are highly sensitive; however, they may suffer of cross-reactivity with other tree nuts. An alternative method based on polymerase chain reaction (PCR) has been developed and tested for 100% specificity [7]. Although PCR-based methods for the detection of tree nuts are highly specific, they are usually less sensitive than the ELISA-based methods because the content of DNA as their target is much lower than the content of proteins. However, the importance of PCR-based methods is that they may facilitate independent confirmation of the results of the immunochemical methods [8]. Here we present a method for the specific detection of hazelnut DNA by real-time PCR, which is a technology, becoming widely used for the detection of allergenic components in food [9].

L. Piknová · T. Kuchta (✉)
Department of Microbiology and Molecular Biology,
Food Research Institute, Priemyselná 4, P. O. Box 25,
842 75 Bratislava 26, Slovakia
e-mail: kuchta@vup.sk

D. Pangallo
Institute of Molecular Biology, Slovak Academy of Sciences,
Dúbravská cesta 21, 845 51 Bratislava, Slovakia

Materials and methods

Plant and food materials

Nuts and leaves of five hazelnut cultivars approved in Slovakia were obtained from Botanical Garden, Slovak Agricultural University, Nitra, Slovakia. These were *C. avellana* cv. Hallská obrovská, *C. avellana* cv. Lombardská biela, *C. avellana* cv. Webbowa, *C. avellana* cv. Contorta and *C. maxima* cv. Purpurea. Wheat, barley, rye and soya flours were obtained from Federal Research Centre for Nutrition and Food, Kulmbach, Germany. Other nuts and food samples were obtained from grocery stores in Bratislava.

Preparation of model pastry with defined hazelnut contents

Hazelnuts and walnuts were milled in separate coffee mills and mixtures containing 0; 0.01; 0.05; 0.1; 0.2; 0.5; 1; 2; 5; 10 and 100% (w/w) hazelnuts were prepared. These were added to pieces of a commercial nut-free Blätterteig dough in portions of 5 g per each 15 g cake and baked for 30 min at 180 °C.

DNA extraction, quantification and amplifiability determination

DNA was extracted from leaves by chaotropic solid phase extraction (SPE) using DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the instructions for use included in the kit. Prior to DNA extraction from food samples, these were homogenised using mortar and pestle, and DNA was isolated by chaotropic SPE using the NucleoSpin kit (Macherey-Nagel, Düren, Germany) according to the instructions manual included in the kit. DNA was quantified by fluorimetry using Quant-iT PicoGreen kit (Invitrogen Molecular Probes, Eugene, OR, USA) according to the instruction manual included in the kit. Amplifiability of the isolated DNA was determined using universal eukaryotic real-time PCR [10].

Design of PCR primers and a probe

DNA sequences were obtained from GenBank (National Center for Biotechnology Information, Bethesda, MD, USA). Primers with a theoretical melting temperature of 60 °C and the corresponding 5'-nuclease (TaqMan) probe were designed using the Primer Express software (Applied Biosystems, Foster City, CA, USA).

Polymerase chain reaction

PCR was performed in a volume of 25 µl. Each reaction contained 5 µl of template DNA, 1.5 U DNA polymerase

HotStarTaq (Qiagen), 1× reaction buffer for the DNA polymerase, 2.5 mmol/l MgCl₂, 200 µmol/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 synthesised by Qiagen Operon, Köln, Germany. PCR was performed in a PTC-200 PCR cycler with an optical unit Chromo 4 (MJ Research, Waltham, MA, 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 the internal software of the cycler using the manual threshold setting at a fluorescence value of 0.03. The c_T values lower than 35 were considered as positive, whereas all c_T values greater than 35 were considered as negative.

ELISA

Protein isolation and immunochemical detection of hazelnuts was carried out using RidaScreen FAST Hazelnut kit (R-Biopharm, Darmstadt, Germany) according to the instruction manual included in the kit. The protein isolate was diluted 100× before applying to ELISA.

Results and discussion

Based on the search in the GenBank database, the gene *hsp1* encoding for a low molecular weight heat-shock protein (access number AF021807) was selected as a target for hazelnut-specific PCR. No sequences encoding for a homologue in related plant species were available in the database. Primers as well as a TaqMan probe were designed (Table 1).

Real-time PCR employing novel primers and the probe was used to analyse DNA isolated from leaves of five hazelnut varieties approved in Slovakia. Positive results were obtained in all cases. When decimal dilutions of the isolated DNA were analysed by real-time PCR in six parallels, a detection limit of 13 pg was determined. This corresponds to 27 genome equivalents, given the genome size of *C. avellana* is 0.48 pg [11]. Regarding variability of the threshold cycle values for the concentrations tested, relative

Table 1 Oligonucleotides used in PCR specific for hazelnuts

Designation	Sequence (5'–3')
Nocc2F	ggcaagttcgtgagcaggttc
Nocc1Rbis	cttcggaatagtcacagtgg
Nocc1P	FAM-cctgacgatgcgatgctcgaccag-BHQ1

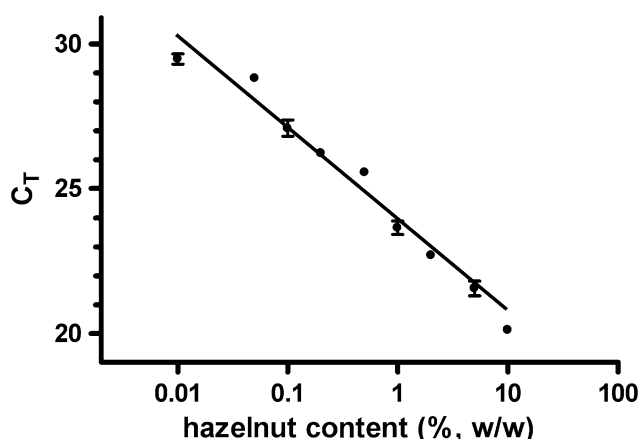


Fig. 1 Calibration line of the hazelnut contents versus threshold cycle (C_T) in real-time PCR, $y = -3.267 \log_{10}x + 23.82$, $r^2 = 0.9744$. The template DNA amount was adjusted to 150 ng per reaction. DNA isolated from individual samples was analysed in duplicate and the standard deviations are depicted as bars

standard deviations ranged from 1 to 3% (data not shown). These parameters mean that the method had a sensitivity comparable to other real-time PCR-based methods for nuts [10, 12, 13].

DNA was isolated from various plant, nut and food samples, and analysed by the hazelnut-specific real-time PCR. All hazelnut samples were positive (seven samples), while all non-hazelnut samples comprising walnuts, pecan nuts, peanuts, cashew nuts, almonds, pistachios, sunflower seeds,

sesame, chestnuts, soya, wheat, barley, rye, oat, rice, fig, cocoa, alfalfa and dried milk were negative (data not shown). These results mean that the method had an appropriate specificity.

By the analysis of model pastry samples with defined hazelnut contents, a practical detection limit of 0.01% (w/w) was determined. A linear relationship between the logarithm of the hazelnut content and the threshold cycle, C_T was observed when the template DNA amount was adjusted to 150 ng per reaction (Fig. 1). This observation suggests that the method has a potential to be quantitative when the DNA amount is standardised. However, there are further prerequisites for true quantitative application of real-time PCR in food samples, in particular, the contents of amplifiable DNA in the sample should be known as well as the recovery rate of DNA isolation. On the other hand, the quantitative potential of real-time PCR is beneficial because it should facilitate at least the discrimination between the presence of traces of the analyte (detection of hidden allergens) from the presence of several percent of the analyte (detection of adulteration) in defined food sample types.

The proposed PCR method was applied to the analysis of a collection of 20 confectionery and bakery products and PCR results were found to conform to the labelling in all samples. These food samples were analysed also by ELISA and, in all cases, identical results were obtained by both methods (Table 2). This demonstrates the potential of the

Table 2 Confectionery and bakery products analysed and results of the hazelnut-specific PCR and ELISA

Food sample	Country of origin	Hazelnut declaration	PCR result	ELISA result
Wafers with hazelnut filling 1	Slovakia	+	+	+
Wafers with hazelnut filling 2	Czech Republic	+	+	+
Chocolate with nougat 1	Slovakia	+	+	+
Chocolate with nougat 2	Austria	+	+	+
Müsli bar with hazelnuts 1	Slovakia	+	+	+
Müsli bar with hazelnuts 2	Czech Republic	+	+	+
Biscuits with hazelnuts 1	Slovakia	+	+	+
Biscuits with hazelnuts 2	Spain	+	+	+
Müsli 1	Slovakia	+	+	+
Müsli 2	Spain	+	+	+
Corn flakes with hazelnuts	Spain	+	+	+
Wafers with peanut filling 1	Slovakia	T ^a	—	—
Wafers with peanut filling 2	Slovakia	T ^b	—	—
Chocolate with coconut 1	Slovakia	T ^a	+	+
Chocolate with coconut 2	Slovakia	T ^b	—	—
Biscuits with chocolate 1	Slovakia	T ^a	—	—
Biscuits with chocolate 2	Spain	—	—	—
Biscuits 1	Poland	—	—	—
Biscuits 2	Spain	—	—	—
Corn flakes	Spain	—	—	—

^a Declaration “may contain traces of nuts”

^b Declaration “may contain traces of hazelnuts”

real-time PCR-based method to produce accurate results at the analysis of confectionery and bakery products.

As demonstrated, the presented real-time PCR is useful for sensitive and selective detection of hazelnuts in food. It can be used as an alternative or complementary method to immunochemical methods. The method is relatively straightforward and fast, the analysis can be performed in one working day.

Acknowledgments This research was done in frames of the project “Development of progressive methods and procedures to support quality and safety of food production and control” of the Slovakian Department of Agriculture. The authors wish to thank Ing. J. Kuba and Ing. A. Haver, Nitra, for samples of hazelnut varieties.

References

1. Sicherer SH, Sampson HA (2000) *Curr Opin Pediatr* 12:567–573
2. Roux KH, Teuber SS, Sathe SK (2003) *Int Arch Allergy Immunol* 131:234–244
3. Directive 2003/89/EC of the European Parliament and of the Council of 10 November 2003 amending Directive 2000/13/EC as regards indication of the ingredients present in foodstuffs. *Off J Eur Union L* 308, 15–18
4. Holzhauser T, Vieths S (1999) *J Agric Food Chem* 47:4209–4218
5. Ben Rejeb S, Abbott M, Davies D, Querry J, Cleroux C, Streng C, Delahaut P, Yeung JM (2003) *J AOAC Int* 86:557–563
6. Kiening M, Niessner R, Drs E, Baumgartner S, Krška R, Bremer M, Tomkies V, Reece P, Danks C, Immer U, Weller MG (2005) *J Agric Food Chem* 53:3321–3327
7. Herman L, De Block J, Viane R (2003) *Int J Food Sci Technol* 38:633–640
8. Poms RE, Anklam E, Kuhn M (2004) *J AOAC Int* 87:1391–1397
9. Pöpping B, Holzhauser T (2004) *Dtsch Lebensm-Rundsch* 100:285–293
10. Brežná B, Hudecová L, Kuchta T (2005) *Archiv Lebensmittelhyg* 56:112–113
11. Bennett MD, Smith JB (1991) *Philos Trans R Soc London B* 334:309–345
12. Brzezinski JL (2006) *J AOAC Int* 89:1035–1038
13. Brežná B, Hudecová L, Kuchta T (2006) *Eur Food Res Technol* 223: 373–377