

Detection of hazelnut DNA traces in chocolate by PCR

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Summary By use of the DneasyTM Plant Tissue kit (Qiagen Inc.) plant DNA could be extracted from chocolate and related matrices. The polymerase chain reaction (PCR) detection of mitochondrial plant DNA is directly correlated with the length of the amplified fragment indicating shearing of DNA during chocolate production. Hazelnut DNA could be specifically detected in chocolate matrices with primers derived from the intron between exon B and C of the mitochondrial gene *nad1*. Specificity was confirmed towards individual chocolate ingredients and in 20 hazelnut negative chocolates. From taxonomically closely related plant species, only *Carpinus turczaninowii*, *Ostrya carpinifolia* and *Corylus americana* showed cross reaction, this was because of the identical sequence of the *nad1* fragment. Application of extra MgCl₂ throughout the DNA extraction procedure and of a specially designed Mg²⁺ buffered PCR, increased the detection sensitivity of co-processed hazelnut in chocolate to 0.001% or 10 ppm.

Keywords Allergen, chocolate, *Corylus colurna* L., DNA alkalisolation, hazelnut, Mg²⁺ buffered PCR, Nad1, PCR inhibition.

Introduction

Hazelnut (*Corylus colurna* L.) has been identified as a source that can cause food allergy (Ortolani *et al.*, 2000). People suffering from such a food allergy are obliged to maintain strict avoidance diets. Therefore, mislabelling of food products has to be avoided and cross-contamination in food processing facilities has to be traced. Ensuring that a food product is free from allergenic residues is difficult and dependent on adequate and sensitive detection methods.

Immunochemical methods have been developed by using antibodies raised against hazelnut protein (Holzhauser & Vieths, 1999). A sandwich ELISA, constructed with immunopurified antibodies from rabbits sensitized with hazelnut protein, was evaluated to ascertain whether it reached an appropriate sensitivity and specificity for use in the food industry (Holzhauser & Vieths, 1999; Koppelman

et al., 1999). Immunochemical techniques based on polyclonal antisera have, however, some restrictions, particularly concerning availability of high quality antisera with good batch-to-batch consistency (Holzhauser *et al.*, 2000). Alternatives to these immunoassays include DNA based technology and especially application of the polymerase chain reaction (PCR). This can offer a very suitable tool to assess the presence of small traces of allergens in food products. Holzhauser *et al.* (2000) reported the application of PCR for the detection of hazelnut traces in different food matrices. The results obtained with a commercial hazelnut-specific PCR-ELISA correlated well with those obtained with a sandwich-type protein-ELISA for 41 commercial food products with and without hazelnut components (Holzhauser *et al.*, 2002).

The sensitivity of the PCR method is expected to be dependent on the shearing of DNA during processing. (Milk) chocolate can be considered as a suspension of (milk solids), cocoa solids and sugar in a matrix of (milk fat and) cocoa butter. The most important step in the manufacturing

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of chocolate is the conching process. During conching, the chocolate is heated for several hours in containers in which an intense kneading process takes place at temperatures between 55 and 105 °C. The cocoa mass, which is used in the pressing industry, is often obtained from alkalized cocoa nib. The process of alkalization is executed by adding a solution of an alkali (mostly potassium carbonate) to the cocoa and results in a darker colour and a milder taste.

In this article, a specific PCR was developed to confirm the absence of hazelnut traces in hazelnut free chocolate and the detection sensitivity was optimized as a function of the shearing of the DNA and the alkalization of some chocolate matrices. Because of the dramatic influence of the alkalization process on the PCR efficiency, the sample preparation method was adapted and a unique PCR buffer system has been designed.

Materials and methods

Extraction of DNA

The DNA of different plant species (leaves taken from the plants) and of different chocolate ingredients was extracted with the DNeasy™ Plant Tissue kit (Qiagen Inc., Valencia, CA, USA) in combination with liquid N₂. The same kit was used to extract DNA from 100 mg of chocolate and related matrices. The samples were melted in 400 µL lysis buffer with 4 µL RnaseA stock solution (100 µg mL⁻¹) as described by the manufacturers. To extract DNA from products made from alkylised cocoa mass, 100 mM MgCl₂ was added as the first step in the extraction procedure. Before elution the column was washed two times with 200 µL 0.2 M MgCl₂. Finally the DNA was eluted in a total volume of 200 µL H₂O, resulting in an average concentration of DNA of 98 µg mL⁻¹ as determined spectrophotometrically.

For determination of the PCR detection sensitivity of hazelnut chocolate, hazelnut free chocolate (Callebaut Gourmet Product Range, Barry Callebaut, Belgium) was mixed with decreasing amounts of pure hazelnut paste (Callebaut Gourmet Product Range). The mixtures were homogenized after co-melting at 65 °C for 10 min. DNA was extracted as described above.

PCR amplification

Standard PCR was performed in a final volume of 25 µL containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 200 µM of each dNTP, 0.75 U of AmpliTaq DNA polymerase (Perkin Elmer, Norwalk, CT, USA), 25 pmol of each primer and 8 µL of DNA. For the products made from alkalized cocoa mass, only 0.8 µL of DNA was added to the PCR. For Mg²⁺ buffered PCR 9 mM ethyleneglycoltetraacetic acid (EGTA) was added to the standard PCR mix and the MgCl₂ concentration was increased to 9 mM MgCl₂. The mixture was subjected to 30 cycles of amplification in a thermal cycler (Cetus 9600; Perkin Elmer). The application of a higher number of cycles is also shown in the result section. The first cycle was preceded by denaturation for 1 min at 95 °C. Each cycle consisted of denaturation for 15 s at 95 °C, annealing for 15 s at 62 °C or at a temperature indicated in the result section, and elongation for 30 s at 72 °C. A final elongation for 8 min at 72 °C followed the last cycle. The PCR products were analysed on a 1.5% (w/v) Seakem ME agarose gel (FMC Bioproducts, Rockland, ME, USA). For all samples a PCR inhibition control was performed by adding 0.5 ng of hazelnut DNA to the PCR mix.

To evaluate the influence of Mg²⁺ buffered PCR on the detection sensitivity, 280 and 56 ng of DNA, extracted from a hazelnut free chocolate (Barry Callebaut) was mixed with dilutions of DNA (15–0.03 ng) extracted from pure roasted chopped hazelnuts (Callebaut Gourmet Product Range).

DNA sequencing and analysis

The sequences were obtained using the ABI PRISM Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) directly on the PCR amplified DNA and using *nad1* exonB (5'GCATTACGATCTGCAGCTCA 3'), *nad1* exonC (5'GGAGCTCGATTAGTTTC-TGC3') (Demesure *et al.*, 1995), NAD1, NAD2, NAD3 and NAD4 (this work, Fig. 1) as sequencing primers. The results were analysed on a 373A automated DNA sequencer (Applied Biosystems). Sequence alignment was made with the Gene-Compar software package (version 2.0, Applied Maths, Kortrijk, Belgium).

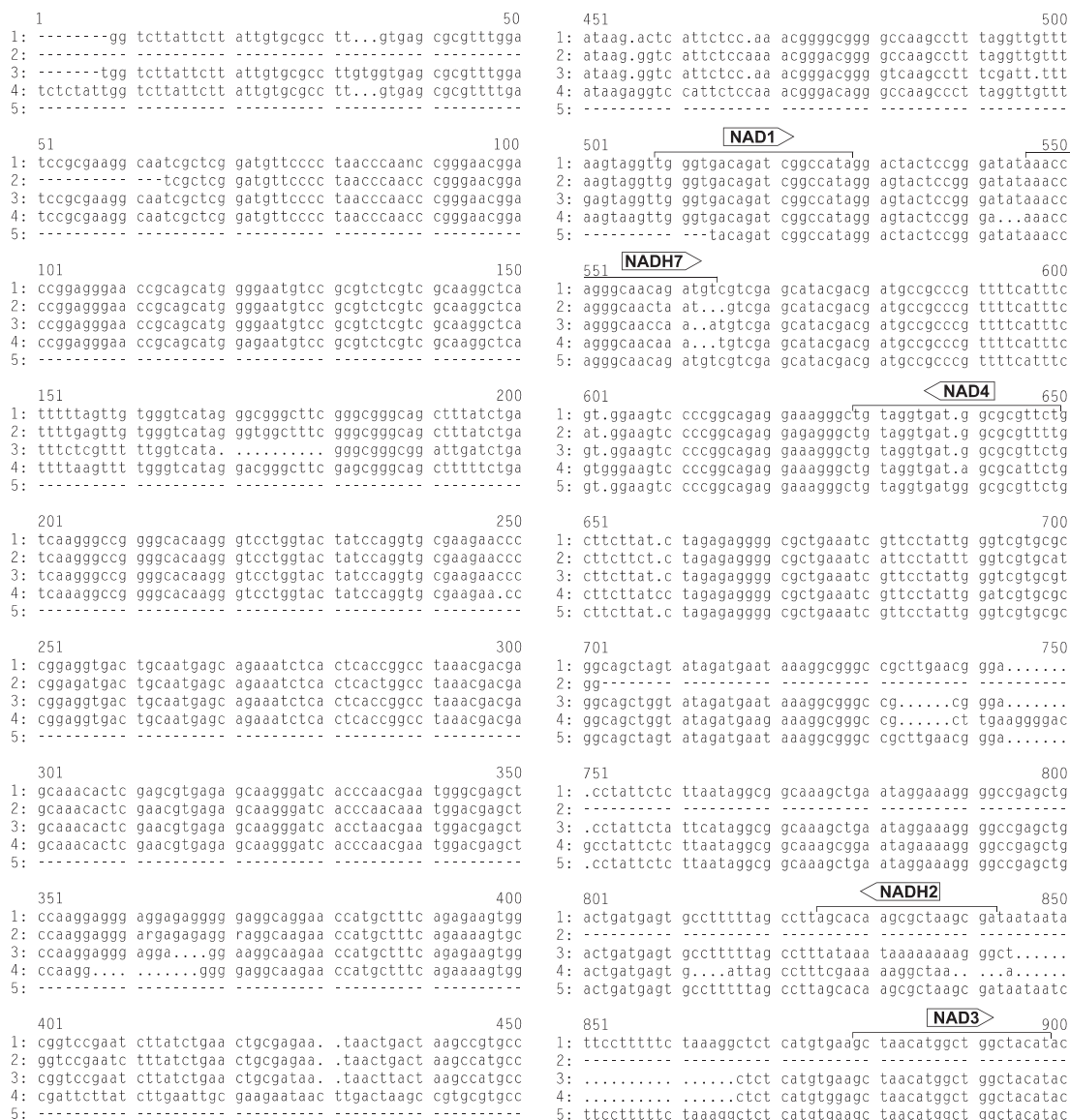


Figure 1 Sequence alignment of the *nad1* mitochondrial gene fragment amplified with primers *nad1* exonB and *nad1* exonC from *Corylus colurna* (1), *Theobroma cacao* (2), *Glycine max* (3), *Beta vulgaris* (4) and *Ostrya carpinifolia* (5). The sequences are available at the EMBL database: hazelnut, *Corylus colurna* L.: AJ428871; *Theobroma cacao* L.: AJ428874; *Glycine max*: AJ428875; *Beta vulgaris*: AJ428873; *Ostrya carpinifolia* Scop.: AJ428872. The different primers sequences used are indicated.

Results and discussion

PCR detection of plant DNA in chocolate ingredients and in different chocolate matrices

By application of the DNeasy™ Plant Tissue kit (Qiagen Inc.), plant DNA could be extracted from

chocolate and from chocolate matrices obtained during the production process. The presence of mitochondrial plant DNA was detected by different primer sets amplifying DNA fragments of different sizes (Table 1). The primers *nad1* exonB and *nad1* exonC are derived from the plant mitochondrial *nad1* gene and amplify an intron



Figure 1 (Contd.)

between exon B and C (Demesure *et al.*, 1995). The other primers are internally located within the *nad1* exonB and *nad1* exonC amplified fragment (Fig. 1). NAD1 and NAD3 show total homology for hazelnut, soy, cocoa, sugar beet and *Ostrya* sp., while minor sequence differences are present in NAD2 and NAD4. In spite of this, the PCR detection of plant DNA was shown to be directly correlated with the length of the amplified fragment indicating a considerable shearing of the

DNA during chocolate production. In crunchy pieces of roasted hazelnuts, coated with caramelized sugar (Callebaut Gourmet Product Range) and in praline paste (Callebaut Gourmet Product Range) a maximum length of about 1500, 1000 and 300 bp is amplified, respectively. In 1 soy lecithin sample, no plant DNA or plant DNA of decreasing size could be detected. In cocoa powder only a plant DNA fragment of 138 bp was detected.

Table 1 Polymerase chain reaction (PCR) detection of plant DNA in plant material, chocolate ingredients and chocolate matrices by different primer combinations

	<i>Nad1</i> exonB-exonC	<i>nad1</i> exonB-NAD2	NAD1- <i>nad1</i> exonC	NAD3- <i>nad1</i> exonC	NAD1-NAD2	NAD3-NAD2	NAD1-NAD4
	1664 bp AT: 62 °C	1156 bp AT: 62 °C	1150 bp AT: 57 °C	893 bp AT: 57 °C	641 bp AT: 57 °C	282 bp AT: 57 °C	138 bp AT: 57 °C
Hazelnut raw	+	+	+	+	+	+	+
Soy beans	+	+	+	+	+	+	+
Lecithin 1*	–	–	–	+	+	+	+
Lecithin 2*	–	–	–	–	–	–	+
Lecithin 3*	–	–	–	–	–	–	–
Cocoa powder	–	–	–	–	–	–	+
Hazelnut 1*	–	+	+	+	+	+	+
Hazelnut 2*	+	+	+	+	+	+	+
Hazelnut 3*	–	–	–	–	–	+	+
Praline paste*	–	–	+	+	+	+	+

In this analysis a PCR of 30 cycles is applied. The length of the fragment amplified by each primer pair is indicated.

*Lecithin 1, 2, 3: different samples of lecithin; Hazelnut 1 and 2 : different samples of pure roasted chopped hazelnuts; Hazelnut 3: crunchy pieces of roasted hazelnuts, coated with caramelized sugar; Praline paste (Callebaut Gourmet Product Range).

AT, annealing temperature.

Hazelnut specific PCR

The sequence of the *nad1* intron between exon B and C was determined for the most abundant plant components of chocolate: sugar beet, soy, cocoa and hazelnut. Sequence alignment (Fig. 1) revealed a lot of sequence deviations between these plant species. One primer pair (NADH2-NADH7) (Fig. 1), amplifying a fragment of 294 bp, was tested for specific amplification of hazelnut DNA from plant components related with chocolate and on taxonomically related plant species (Table 2). Because of their evolutionary significance, taxonomic data are forming a strong basis for validating PCR specificity of plant mitochondrial targets. No cross amplification was obtained with any plant species related to chocolate. The best results were obtained with an annealing temperature of 62 °C. Cross-reaction still occurred with *Carpinus turezaninovi* Hance, *Ostrya carpinifolia* Scop., *Corylus colurna* L. and *Corylus americana* Walter.

Sequence analysis of the *nad1* intron between exon B and C of *C. turezaninovi*, *O. carpinifolia* and *C. colurna* revealed a total homology with the fragment from hazelnut. This proves the impossibility of obtaining complete specificity, even under extreme stringent PCR annealing conditions, towards these species based on the intron between

exon B and exon C of the mitochondrial *nad1* gene.

The NADH2-NADH7 PCR, however, showed sufficient specificity to detect hazelnut traces in chocolate. A total of 20 hazelnut negative milk chocolates (Callebaut Gourmet Product Range) were all confirmed as negative in a PCR of 45 cycles. All individual chocolate ingredients were also negative (Table 3). The positive controls, executed by added 0.5 ng of hazelnut DNA to the PCR mixes, reacted positively for all samples.

Positive results were thus only obtained within a group of very closely related species. *Carpinus*, *Ostrya*, and *Corylus*, belonging to the Betulaceae subfamily Coryloideae, which is sometimes considered a distinct family Corylaceae, especially in Europe (Tutin & Walters, 1993). Although this distinction is confirmed by the present and other molecular studies this view is not widely accepted, and most modern authors maintain the family as a single group composed of two subfamilies, Betuloideae (*Alnus* and *Betula*) and Coryloideae (*Carpinus*, *Corylus*, *Ostrya*, and *Ostryopsis*). The family is held together on the basis of many features, e.g. general habit, leaf and wood anatomy, trichome and inflorescence morphology, ovary and ovule structure, pollen morphology, embryo structure, and fertilization

Plant species	PCR*	Plant family and subfamily
Industrial hazelnut, <i>Corylus</i> sp.	+	Betulaceae – Coryloideae
Industrial sugar beat, <i>Beta vulgaris</i> L. subsp. <i>vulgaris</i> cv. Altissima	–	Chenopodiaceae
Industrial cocoa powder, <i>Theobroma cacao</i> L.	–	Sterculiaceae
Industrial soy shoot, <i>Glycine max</i> (L.) Merr.	–	Fabaceae
<i>Aesculus carnea</i> Hayne	–	Hippocastanaceae
<i>Aesculus octandra</i> Marshall	–	Hippocastanaceae
<i>Alnus cordata</i> (Lois.) Duby	–	Betulaceae – Betuloideae
<i>Alnus crispa</i> (Ait.) Pursh	–	Betulaceae – Betuloideae
<i>Alnus incana</i> (L.) Moench	–	Betulaceae – Betuloideae
<i>Alnus pendula</i> Matsum.	–	Betulaceae – Betuloideae
<i>Alnus rubra</i> Bong.	–	Betulaceae – Betuloideae
<i>Alnus viridis</i> (Chaix) DC.	–	Betulaceae – Betuloideae
<i>Betula apoiensis</i> Nak.	–	Betulaceae – Betuloideae
<i>Betula platyphylla</i> Sukachev.	–	Betulaceae – Betuloideae
<i>Betula schmidtii</i> Reg.	–	Betulaceae – Betuloideae
<i>Betula pendula</i> Roth.	–	Betulaceae – Betuloideae
<i>Betula papyrifera</i> Marshall	–	Betulaceae – Betuloideae
<i>Carpinus turczaninowii</i> Hance	+	Betulaceae – Coryloideae
<i>Corylus americana</i> Walter	+	Betulaceae – Coryloideae
<i>Corylus colurna</i> L.	+	Betulaceae – Coryloideae
<i>Ostrya carpinifolia</i> Scop.	+	Betulaceae – Coryloideae
<i>Castanea sativa</i> Mill.	–	Fagaceae
<i>Fagus sylvatica</i> L. cv. Atropunicea	–	Fagaceae
<i>Fagus sylvatica</i> L. cv. Pendula	–	Fagaceae
<i>Quercus acutissima</i> Carruth.	–	Fagaceae
<i>Quercus cerris</i> L.	–	Fagaceae
<i>Quercus frainetto</i> Ten.	–	Fagaceae
<i>Quercus macrocarpa</i> Michx.	–	Fagaceae
<i>Quercus marilandica</i> Münchh.	–	Fagaceae
<i>Quercus petraea</i> Liebl.	–	Fagaceae
<i>Juglans regia</i> L.	–	Juglandaceae
<i>Nothofagus antarctica</i> Oerst.	–	Nothofagaceae
<i>Ginkgo biloba</i> L.	–	Ginkgoaceae

*Polymerase chain reaction with primers NADH2–NADH7 at an annealing temperature of 62 °C.

Table 2 Polymerase chain reaction (PCR) specificity of NADH2–NADH7

and germination patterns. Members of Betulaceae are economically important as timber or as ornamental trees and shrubs, or as the source of hazelnuts (*Corylus*), and also as aids in soil nitrification and stabilization (*Alnus*). Some cause pollen allergies in regions where they grow abundantly.

Other material showing negative results for the test included the related Fagaceae (*Fagus*, *Quercus*), Nothofagaceae (*Nothofagus*) and Juglandaceae (*Juglans*); the even more distantly related family Hippocastanaceae (*Aesculus*), and to the totally unrelated gymnosperms (*Ginkgo*).

Sensitivity of hazelnut PCR detection in chocolate matrices

In different chocolate preparations, alkalization of the cocoa nib was performed. We hypothesized that carbonates could be co-extracted with the DNA and, because of Mg^{2+} capturing, could negatively influence the PCR sensitivity. Therefore, extra $MgCl_2$ was applied throughout the DNA extraction procedure (see Materials and methods). The sensitivity of the NADH2–NADH7 PCR for detection of hazelnut sequences in chocolate could be clearly improved by using a

Table 3 NADH2-NADH7 polymerase chain reaction (PCR) on chocolate ingredients

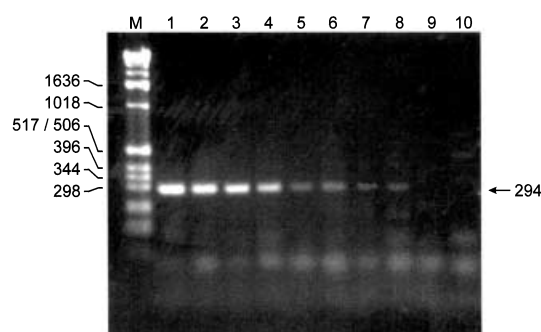
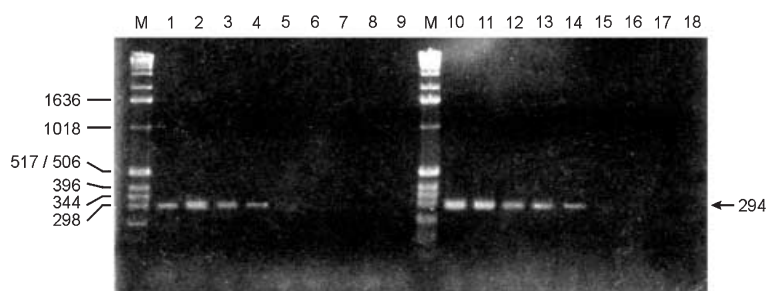
Chocolate ingredient	PCR NADH2-NADH7 AT: 62 °C; PCR 45 cycles
Skimmed milkpowder, spray dried	–
Cocoa powder	–
Spice	–
Soluble coffee	–
Cane-sugar	–
Coriander	–
Nuts aroma	–
Powder from forest fruits	–
Vanilline	–
Cinnamon	–
Almond	–
Sun-flower oil	–
Lecithin	–
Vegetable fat	–
Natural vanilla powder	–

AT, annealing temperature.

special designed Mg^{2+} buffered PCR. With a PCR of 30 cycles 3–0.3 ng of hazelnut DNA could be detected by the standard PCR mix while this detection sensitivity was increased 10 times to 0.3–0.03 ng using the Mg^{2+} buffered PCR (Fig. 2). The principle of using a modified Mg^{2+} buffer for this type of PCR is, to our knowledge, described for the first time in this study. Mg^{2+} buffers can be calculated according to Caldwell (Caldwell, 1970). A given ratio of defined quantities of added $MgCl_2$ and EGTA at a given pH results in a well-defined concentration of free Mg^{2+} ions in the solution.

The sensitivity of hazelnut PCR detection was tested on a hazelnut free chocolate with addition of different concentrations of hazelnut paste. This approach was preferred to mixing with pure hazelnut DNA because this corresponds more closely to the practical situation where the detection of co-processed hazelnuts has to be performed. With a Mg^{2+} buffered PCR of 45 cycles 0.001 % of hazelnut could be detected in hazelnut free chocolate (Fig. 3).

In dark coloured chocolate matrices with cocoa powder > 7% alkalized (Callebaut Gourmet Product Range) no hazelnut DNA could be detected

**Figure 3** Detection sensitivity of hazelnut chocolate by polymerase chain reaction (PCR) NADH2-NADH7 PCR was performed on DNA (1.5 µg) extracted from chocolates with decreasing concentrations of hazelnut paste: Lane 1, 5%; Lane 2, 1%; Lane 3, 0.5%; Lane 4, 0.1%; Lane 5, 0.05%; Lane 6, 0.01%; Lane 7, 0.005%; Lane 8, 0.001%; Lane 9, 0.0005%; Lane 10, 0%; M, molecular size marker, 1-kb DNA ladder (Gibco BRL Life Technologies, Paisley, UK).**Figure 2** Influence of the application of Mg^{2+} buffered polymerase chain reaction (PCR) on the detection sensitivity of hazelnut DNA in chocolate NADH2-NADH7 PCR was performed by mixing 0.75 µg DNA from hazelnut free chocolate with decreasing concentrations of hazelnut DNA: Lanes 1 and 10, 0.5 µg pure hazelnut DNA; Lanes 2 and 11, 15 ng; Lanes 3 and 12, 3 ng; Lanes 4 and 13, 1.5 ng; Lanes 5 and 14, 0.3 ng; Lanes 6 and 15, 0.15; Lanes 7 and 16, 0.03 ng; Lanes 8 and 17, 0 ng; Lanes 9 and 18, no DNA; Lanes 1–8, standard PCR mix; Lanes 9–16, Mg^{2+} buffered PCR mix; M, molecular size marker, 1-kb DNA ladder (Gibco BRL Life Technologies, Paisley, UK).

when a standard PCR mix was used. We hypothesize that, because of alkalyzation, carbonates are abundantly present in the matrix and expected to be tightly adhered to the DNA molecules.

The minimal dose of hazelnut protein that can cause an allergic reaction is unknown. For peanut, it was demonstrated that 100 µg peanut protein could cause subjective symptoms in an allergic individual (Hourihane *et al.*, 1997). This corresponds with the consumption of 100 g chocolate containing 0.0001% hazelnut protein. The detection limits for the immunochemical methods for hazelnut were reported to be 0.001% (10 ppm) (Holzhauser & Vieths, 1999) and 0.0001% (1 ppm) (Koppelman *et al.*, 1999; Holzhauser *et al.*, 2002). These detection sensitivities were determined by spiking food matrices or food extracts with pure, unprocessed hazelnut extracts or with roasted hazelnuts. In our study detection sensitivities were determined on chocolate containing processed hazelnut matrices. By PCR it was possible to detect 0.001% of hazelnut traces by a PCR of 45 cycles. Holzhauser *et al.* (2000) obtained, in different food products, the same detection sensitivity with a 55 cycles two-step PCR. Their DNA extraction protocol took 7 h to avoid PCR inhibition compared to our 2-h protocol, which resembles the protocol time applied by Holzhauser *et al.* (2002). Based on the analysis of 41 commercial food samples by both a hazelnut-specific protein-ELISA and PCR-ELISA, both methods showed about the same detection sensitivity for hazelnut traces at the level of ≤10 ppm (Holzhauser *et al.*, 2002). Only for some food products was a difference in detection sensitivity between both methods observed. This can be because of a difference in stability of DNA and proteins in the products (e.g. a higher stability of DNA in some dairy products) (Holzhauser *et al.*, 2002) or to a lower sensitivity obtained by PCR inhibition. Application of the Mg²⁺ buffered PCR overcomes inhibition due to alkalization of cocoa and enhances the efficiency of the PCR detection protocol. This newly designed buffer can be applied for detection of other target DNA's (e.g. the specific detection of genetically modified

organisms) in chocolate matrices, including those, which underwent an alkalization process.

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References

- Caldwell, P.C. (1970). Calcium chelation and buffers. In: *Calcium and Cellular Function* (edited by A.W. Cuthbert). Pp. 10–16. New York: St Martin's Press.
- Demesure, B., Sodji, N. & Petit, R.J. (1995). A set of universal primers for amplification of polymorphic non-coding regions of mitochondrial and chloroplast DNA in plants. *Molecular Ecology*, **4**, 129–131.
- Holzhauser, T. & Vieths, S. (1999). Quantitative sandwich ELISA for determination of traces of hazelnut (*Corylus avellana*) protein in complex food matrices. *Journal of Agrichemistry and Food Chemistry*, **47**, 4209–4218.
- Holzhauser, T., Wangorsch, A. & Vieths, S. (2000). Polymerase chain reaction (PCR) for detection of potentially allergenic hazelnut residues in complex food matrices. *European Food Research and Technology*, **211**, 360–365.
- Holzhauser, T., Stephan, O. & Vieths, S. (2002). Detection of potentially allergenic hazelnut (*Corylus avellana*) residues in food: a comparative study with DNA PCR-ELISA and protein sandwich-ELISA. *Journal of Agrichemistry and Food Chemistry*, **50**, 5808–5815.
- Hourihane, J.O., Kilburn, S.A., Nordlee, J.A., Hefle, S.L., Taylor, S.L. & Warner, J.O. (1997). An evaluation of the sensitivity of subjects with peanut allergy to very low doses of peanut protein: a randomized, double-blind, placebo-controlled food challenge study. *Journal of Allergy and Clinical Immunology*, **100**, 596–600.
- Koppelman, S.J., Knulst, A.C., Koers, W.J. *et al.* (1999). Comparison of different immunochemical methods for the detection and quantification of hazelnut proteins in food products. *Journal of Immunological Methods*, **29**, 107–120.
- Ortolani, C., Ballmer-Weber, B.K., Hansen, K.S. *et al.* (2000). Hazelnut allergy: a double-blind, placebo-controlled food challenge multicenter study. *Journal of Allergy and Clinical Immunology*, **105**, 577–581.
- Tutin, T.G. & Walters, S.M. (1993). Corylaceae. In: *Flora Europaea I*, 2nd edn (edited by T.G. Tutin, N.A. Burges, A.O. Chater, J.R. Edmondson, V.H. Heywood, D.M. Moore, D.H.V. Valentine, S.M. Walters & D.A. Webb). Pp. 70–71. Cambridge: Cambridge University Press.