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Alexandra Ehlert ^a; Anja Demmel ^a; Christine Hupfer ^b; Ulrich Busch ^b; Karl-Heinz Engel ^a

^a Department of General Food Technology, Technische Universität München, D-85350 Freising-Weißenstephan, Germany ^b Bavarian Health and Food Safety Authority, Germany

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Simultaneous detection of DNA from 10 food allergens by ligation-dependent probe amplification

Alexandra Ehlert^a, Anja Demmel^a, Christine Hupfer^b, Ulrich Busch^b and Karl-Heinz Engel^{a*}

^aDepartment of General Food Technology, Technische Universität München, D-85350 Freising-Weihenstephan, Germany;

^bBavarian Health and Food Safety Authority, Veterinärstrasse 2, D-85764 Oberschleißheim, Germany

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The simultaneous detection of DNA from different allergenic food ingredients by a ligation-dependent probe amplification (LPA) system is described. The approach allows detection of several targets in a one-tube assay. Synthetic oligonucleotides were designed to detect DNA from peanuts, cashews, pecans, pistachios, hazelnuts, sesame seeds, macadamia nuts, almonds, walnuts and brazil nuts. The specificity of the system was tested with DNA from more than 50 plant and animal species. The sensitivity of the method was suitable to detect allergenic ingredients in the low mg kg⁻¹ range. The limit of detection (LOD) for single allergens in different food matrices was 5 mg kg⁻¹. The novel analytical strategy represents a useful tool for the surveillance of established legislation on food allergens within the European Union.

Keywords: food allergen; tree nuts; peanut; sesame; detection; ligation; PCR

Introduction

In industrialized countries, 1–2% of adults and up to 8% of children and adolescents are affected by food allergies (Sicherer and Sampson 2006). The symptoms may range from skin irritations to severe anaphylactic reactions with fatal consequences. Approximately 90% of the adverse reactions observed have been associated with eight food groups: cow's milk, eggs, fish, crustaceans, peanuts, soybeans, tree nuts and wheat. In addition to these major food allergens, a broad spectrum of fruits, vegetables, seeds, spices and meats have been reported to possess allergenic potential (Breiteneder and Ebner 2000; Ellman et al. 2002; Poms et al. 2004).

Taking into account the recommendations of the Codex Alimentarius Commission (2007), the European Commission amended the European Food Labelling Directive 2000/13/EC with a list of ingredients to be labeled (EC 2000). Currently, Annex IIIa of Directive 2007/68/EC (EC 2007) comprises gluten-containing cereals, crustaceans, molluscs, fish, peanuts, soybeans, eggs, milk and dairy products (including lactose), nuts, celery, mustard, sesame seeds, lupine, sulfite and products thereof. To protect the health of consumers, the declaration of these ingredients has been made mandatory, regardless of their amounts in the final product.

Allergens are proteins whose routine food analysis is based on immunological detection using either specific IgE from human serum or antibodies raised

in animals. Major challenges are the requirement to check for the presence of food allergens at extremely low levels and to detect trace amounts of hidden allergens in composite and processed foods (Poms et al. 2004). PCR-based methods amplifying specific DNA sequences offer alternative tools for the detection of allergenic or marker proteins for the species (Goodwin 2004; Poms and Anklam 2004). In most cases, DNA presents a more stable analyte compared to proteins and is less affected by denaturation (Poms and Anklam 2004). In addition, species-specific sequences allow the discrimination of closely related organisms.

Appropriate PCR assays for the detection and identification of individual food allergens have been developed for cashew nuts (Brzezinski 2006; Ehlert et al. 2008a), celery (Dovicovicova et al. 2004; Stephan et al. 2004; Hupfer et al. 2006), cereals (wheat, barley, rye) (Dahinden et al. 2001; Sandberg et al. 2003; Hernandez et al. 2005), peanuts (Hird et al. 2003; Stephan and Vieths 2004), pistachios (Barbieri and Frigeri 2006) and tree nuts (walnut, pecan, hazelnut) (Holzhauser et al. 2000; Herman et al. 2003; Brezna et al. 2005; Germini et al. 2005; Arlorio et al. 2007; Brezna and Kuchta 2007). Conventional and real-time PCR methods for the detection of soybean, sesame, mustard, peanut, hazelnut and almond have recently been compared (Pancaldi et al. 2005). At present, only a few duplex-PCR systems are known allowing the simultaneous detection of peanut and hazelnut or wheat and barley (Rossi et al. 2005; Ronning et al. 2006).

*Corresponding author. Email: k.h.engel@wzw.tum.de

The aim of this study was to develop and validate a multi-target method for the simultaneous detection of allergens in different food matrices. The application of a ligation-dependent probe amplification (LPA) technique for the simultaneous detection of DNA from peanut, cashew nut, pecan nut, pistachio nut, hazelnut, sesame seeds, macadamia nut, almond, walnut and brazil nut in a single reaction is described. Ligation-dependent PCR was originally introduced to allow the detection of nucleic acid sequences (Carrino 1996; Hsuih et al. 1996; Belgrader et al. 1997). First applications in the field of medical diagnostics allowed the detection and the relative quantification of up to 40–50 target sequences in a single assay (Gille et al. 2002; Schouten et al. 2002; Eldering et al. 2003; Hogervorst et al. 2003; Taylor et al. 2003). The suitability of this method for the event-specific detection and relative quantification of DNA from two genetically modified organisms (GMO) has been demonstrated using commercially available maize and soya standards (Moreano et al. 2006). The technique does not amplify the target sequences, but is rather based on the amplification of products resulting from the ligation of bipartite hybridization probes. The use of this analytical strategy results in a flexible system that can be complemented with further hybridization probes to broaden the range of target sequences to be detected. This approach has been realized by the development of a modular system allowing the simultaneous detection of several GMO targets corresponding to different levels of specificity in a one-tube assay (Ehlert et al. 2008b).

Materials and methods

Materials and samples

Nut materials, sesame seeds, ingredients of self-prepared walnut cookies and commercial food samples were purchased from local grocery stores. DNA plant and animal materials used to test the specificity of the method and spiked samples of chocolate, cookies and pesto used to determine sensitivity were obtained from the Bavarian Health and Food Safety Authority (Oberschleißheim, Germany). Chocolate samples had been spiked with peanuts (100, 10, 5, 1 and 0.5 mg kg⁻¹) and hazelnuts (20, 10 and 5 mg kg⁻¹). Cookies spiked with peanuts contained 100, 10, 5, 1 and 0.5 mg kg⁻¹ of peanuts and pesto spiked with cashew nuts contained 100, 20, 10, 5, 2 and 1 mg kg⁻¹ of cashew nuts. In all cases samples of the unspiked material were included in the analysis.

Preparation of walnut cookies spiked with different nuts

Nuts ground with a Thermomixer (Vorwerk, Wuppertal, Germany) as well as the other cookie

ingredients were analyzed by LPA to ensure the purity of the starting materials. Two doughs containing 25% wheat flour, 25% sugar, 25% butter and 25% ground nuts (either walnuts only or walnuts spiked with 10% of peanut, hazelnut, pecan and macadamia) were prepared using a food processor (Braun, Germany). The reference cookies containing only walnuts and the cookies with all five nuts were baked separately at 180°C for 10 min and ground afterwards. The concentrations of peanut, hazelnut, pecan and macadamia were adjusted in the spiked cookies to 10,000, 1000, 100, 10 and 1 mg kg⁻¹, respectively, by mixing the corresponding amounts of ground spiked cookies and ground walnut reference cookies in a food processor. The mixtures became fluid due to the high fat contents; however, the procedure resulted in visually homogeneous dispersions.

DNA extraction

Each sample (2 g) was mixed with 10 ml CTAB-extraction buffer [2% (w/v) cetyltrimethylammoniumbromide, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-OH/HCl] and 30 µl proteinase K (20 mg ml⁻¹) in a 50 ml Falcon tube and incubated at 65°C overnight. After 5 min centrifugation at 5000 g, 1000 µl supernatant was transferred to a 1.5-ml tube and centrifuged again at 14,000 g for 5 min. Then, 500 µl chloroform/isoamylalcohol (Ready Red™; MP Biomedicals, Heidelberg, Germany) was mixed with 700 µl supernatant and centrifuged at 16,000 g for 15 min. A 500 µl aliquot of supernatant was added to 500 µl isopropanol (stored at -20°C) and incubated at room temperature (RT) for 30 min. After 15 min centrifugation at 16,000 g, the supernatant was removed, the pellet washed with 500 µl ethanol (70%; stored at -20°C) and centrifuged for 5 min at 16,000 g. After removal of ethanol, the pellet was dried for 1 h at 50°C and afterwards diluted in 100 µl TE-buffer (1×). Additionally, the DNA extracts were purified using spin filter columns (Qiagen, Hilden, Germany).

DNA concentrations were determined fluorometrically using PicoGreen® dsDNA quantification reagent (Invitrogen, Karlsruhe, Germany) on a Tecan GENios™ plus reader (Männedorf, Switzerland).

Probes and primers

Probes used for the detection of peanut, cashew nut, pecan nut, pistachio, hazelnut, sesame, macadamia nut, almond, walnut and brazil nut were designed using Beacon Designer 4.0 software (Premier Biosoft Int., Palo Alto, CA, USA) and FastPCR software (University of Helsinki, Finland). The synthesis of the probes was done by Biolegio B.V. (Nijmegen, The Netherlands). Primers are included in the

MLPA reagents kit (MRC-Holland, Amsterdam, The Netherlands). Sequences of LPA probes and primers, as well as GenBank accession numbers of the selected targets, are listed in Table 1.

Ligation-dependent probe amplification

The LPA reaction was essentially carried out as described by Schouten et al. (2002). Hybridization was performed overnight in 0.5 ml reaction vessels using a thermocycler (Mastercycler Gradient; Eppendorf, Hamburg, Germany) and 100 ng DNA sample. After 5 min of DNA denaturation at 98°C, 1.5 µl of MLPA buffer and 1.5 µl of a mixture of the synthesized probes containing 1 fmol of each LPA probe for the detection of peanut, cashew, pecan nut, pistachio, hazelnut, sesame, macadamia nut, almond, walnut and brazil nut, respectively, were added and held at 60°C for 16 h. Ligation reaction was performed at 54°C for 15 min after adding 3 µl Ligase-65 buffer A, 3 µl Ligase-65 buffer B, 25 µl H₂O and 1 µl Ligase-65 (MRC-Holland). After ligation, reaction mixes were heated for 5 min at 98°C to inactivate the enzyme.

For amplification of the ligation products, 10 µl polymerase mixed with primers, dNTPs, buffer and polymerase enzyme of the MLPA kit (MRC-Holland) were added to 40 µl ligation reaction mixture at 60°C. Thirty-five amplification cycles at 95°C for 30 s, 60°C for 30 s and 72°C for 60 s were followed by a final step of 20 min at 72°C and cooled down to 4°C.

Fragment length analysis

Fragment length analysis was performed on an ABI PRISM® 310 Genetic Analyzer using capillaries (47 cm) and polymer (POP-6TM performance optimized polymer); reagents were obtained from Applied Biosystems (Forster City, CA, USA). One µl of the PCR product (pure or diluted) was mixed with 0.3 µl size standard (GeneScan®-500 [TAMRA]TM) and 14.7 µl Hi-DiTM formamide. Prior to analysis, DNA was denatured at 94°C for 3 min and cooled on ice. Electrokinetic injections were performed at 15 kV for 5 s. Electrophoretic separations were run at 60°C and 15 kV.

Sequencing

Ligation products were generated and amplified separately prior to sequencing. Primers used for sequencing were identical to those listed in Table 1. FAM-labeling did not interfere with cycle sequencing. Amplified products were cleaned up using a PCR purification kit (QIAquick, Qiagen GmbH, Hilden, Germany) and used as template for the sequencing PCR. This reaction was performed using a BigDye

Terminator v1.1 cycle sequencing kit (Applied Biosystems). One reaction mix (20 µl) contained 2 µl 5× buffer, 4 µl RR-mix, 2 µl primer (10 pmol), 8 µl H₂O and 4 µl template. Reaction conditions were as follows: initial denaturation (1 min at 96°C), 30 cycle denaturation steps (10 s at 96°C) and primer annealing (5 s at 56°C), and a final step (4 min at 60°C).

Purification of the PCR products was carried out following amplification. PCR products (10 µl) were mixed with 16 µl H₂O, 4 µl Na-acetate (3 M) and 50 µl EtOH (100%) in a 1.5 ml reaction vessel and centrifuged at 15,000 rpm for 15 min. EtOH was removed carefully without damaging the precipitated DNA pellet. The pellet was vortexed with 50 µl EtOH and centrifuged at 15,000 rpm for 5 min. After carefully removing EtOH, the pellet was allowed to dry at 50°C for 1 h. Finally, DNA was dissolved in 20 µl H₂O.

Sequencing of the diluted PCR products (10 µl H₂O + 6 µl purified DNA) was carried out on an ABI PRISM® 310 genetic analyzer (Applied Biosystems) using 47 cm capillaries and POP-6TM performance optimized polymer. Electrokinetic injections were performed at 2 kV for 30 s. Runs were carried out at 50°C and 15 kV.

Real-time PCR and ELISA assays

SureFood® allergen kits for the qualitative detection of DNA from hazelnut and peanut from Congen Biotechnology GmbH (Berlin, Germany) were used for real-time PCR analysis. The limits of detection for both kits were 10 copies of genomic DNA (Congen 2006a,b). For the detection of cashew nut, a recently described real-time PCR system was used (Ehlert et al. 2008a). Ridascreen® enzyme immunoassays for the quantitative analysis of hazelnut and peanut were obtained from R-Biopharm AG (Darmstadt, Germany). The limit of detection as indicated by the manufacturer were 2.5 mg kg⁻¹ for both hazelnut and peanut (R-Biopharm 2006; 2007).

Results and discussion

Design of LPA probes and choice of target sequences

The principle of ligation-dependent probe amplification reaction, as well as suggestions and rules for the design of LPA probes, have been specified in detail previously (Schouten et al. 2002; Moreano et al. 2006, MRC Holland). The detection of each target sequence requires the design of two probes containing the respective target-specific hybridization sites as well as identical primer binding sites (PBS) at their 5'- or 3'-ends. The LPA system designed in this study uses the advantages of synthetic oligonucleotides for hybridization, as described by Moreano et al. (2006).

Table 1. Probes and primers.

Target/GenBank Accession No.	Left probe	Right probe	Ligation products (retention time) [nt]
Peanut/L77197	5'-GGGTTCCTAAGGGTTGGAGCGAG GCAGCAGTGGGAATC-3'	P-5'-CAAGGAGACAGAAAGATGCCAGAG CCTCTAGATTGGATCTTGCTGGCAC-3'	88 (87.10 ± 0.13)
Cashew/AY081853	5'-GGGTTCCTAAGGGTTGGACTTATTA GATTAATTCACTGGACTGC-3'	P-5'-CATGAAGTGAAGCAGTAGTAGAAGTCT AGATTGGATCTTGCTGGCAC-3'	92 (91.16 ± 0.17)
Pecan nut/DQ156215	5'-GGGTTCCTAAGGGTTGGACACAATC CCTACTACTTTCACCTCCAGGGA-3'	P-5'-CTCAGGTCGAGACATGAGTCCG GGTCTAGATTGGATCTTGCTGGCAC-3'	96 (94.67 ± 0.17)
Pistachio nut/Y07600	5'-GGGTTCCTAAGGGTTGGACCTGAA CACGGCGAGCACAAAG-3'	P-5'-AGGACTGGTGAAGATCAAGAC AAgtgtgtgtTCTAGATTGGATCTTGCTGGCAC-3'	100 (99.37 ± 0.18)
Hazelnut/AF136945	5'-GGGTTCCTAAGGGTTGGAGATCACC AGCAAGTACCACACCAAGG-3'	P-5'-GCAA CGCTTCAATCAATGAGGAGGA GAgtgtgtgtTCTAGATTGGATCTTGCTGGCAC-3'	104 (102.75 ± 0.12)
Sesame seeds/ AF240006	5'-GGGTTCCTAAGGGTTGGAggtgtttGA AGGGAGAGAAAGAGAGGAGGAGCAA-3'	P-5'-GAAACAAGGACGAGGCGGATtg tgtgtgtTCTAGATTGGATCTTGCTGGCAC-3'	108 (108.11 ± 0.13)
Macadamia nut/ AF161883	5'-GGGTTCCTAAGGGTTGGACTTA ATCAACCGAGACAAACGAGAGG-3'	P-5'-CTCCACATAGCCAAAGTTCTTACAGACCA TtgtgtgtgtgtTCTAGATTGGATCTTGCTGGCAC-3'	112 (111.07 ± 0.14)
Almond/X65718	5'-GGGTTCCTAAGGGTTGGAggtgtgtgt CCATTACAAGTCTCCACCAACCAAC-3'	P-5'-CTTCTCTCTCTCCAGTCTACTCACC ACCgtgtgtgtTCTAGATTGGATCTTGCTGGCAC-3'	116 (114.84 ± 0.12)
Walnut/AF066055	5'-GGGTTCCTAAGGGTTGGAggtgtgtgtGG CACAAATCCCTACTACTTTCACCTCCAGAG-3'	P-5'-CATTAGTTCGAGACATGATCCGAGGA AGGgtgtgtgtTCTAGATTGGATCTTGCTGGCAC-3'	120 (119.80 ± 0.11)
Brazil nut/M17146	5'-GGGTTCCTAAGGGTTGGAggtgtgtgtgtgt gtgGAGGAGGAGAACCAAGGAGGTGTC-3'	P-5'-GCGAGCAGATGCAGAGACAGCAGgtgtgt gtgtgtgtgtTCTAGATTGGATCTTGCTGGCAC-3'	124 (124.84 ± 0.09)
Primer R – unlabeled	5'-GTGCCAGCAAGATCCAACTAGA-3'		
Primer F – labeled	FAM-5'-GGGTTCCCTAAGGGTTGGA-3'		

Capitals: plant DNA.

Bold capitals: primer binding site.

Lowercase fonts: spacer DNA.

Different tree nuts (macadamia, cashew, pecan, walnut, brazil nut, pistachio, almond and hazelnut), as well as peanut and sesame, were chosen as examples for the detection of allergenic components in foods by LPA. They are often present as hidden allergens in pastries, candies or chocolate, and pose potential health risks for allergic individuals.

Details of the selected target sequences and the GenBank accession numbers of the LPA primers and probes are listed in Table 1. The probes mostly detect genes encoding for plant food proteins of the cupin and prolamin superfamily, which are known to cause IgE-mediated allergic reactions. The simultaneous detection of all targets is shown in Figure 1.

The primer sequences described by Schouten et al. (2002) for MLPA analysis were also used in this study. They were tested regarding their suitability for analyzing foods by database enquiry via NCBI GenBank and PCR using DNA of different plant species. The use of spacer sequences between PBS and hybridization sites rendered ligation products with lengths characteristic for each target DNA. Differences of four nucleotides (nt) in length are sufficient for unequivocal

determination of the amplification products using POP-6TM polymer (Ehlert et al. 2008b). Simple repeats of GT bases were used for the spacer sequences to avoid intra- and intermolecular hybridizations.

Using this approach, all target signals were sufficiently separated. The signal obtained for pistachio nut overlapped one of the standard signals (100 nt); however, owing to the use of different fluorescent dyes for standard and target probes, a discrimination of the two signals is possible.

Evaluation of target specificity

To avoid cross homologies, the specificities of the probe target sequences were first evaluated by a BLAST search within the NCBI GenBank using Beacon Designer software. Additionally, each probe was checked with DNA extracted from the other target species. The performance of the LPA system was examined using DNA extracts of the 10 targets adjusted to 20 ng μL^{-1} and different mixtures prepared to simulate composite food products with different nut proportions. No unspecific signals were observed for almond, peanut, pecan, pistachio, hazelnut, sesame, macadamia, walnut or brazil nut probes. Furthermore, differentiation between the phylogenetically closely related tree nuts, pecan and walnut could be achieved. Published sequencing data were used to design the ligation probes with only four different DNA bases (Brezna et al. 2005; Brezna and Kuchta 2007). Cross-reactivity of the cashew probes with other members of the Anacardiaceae family, specifically pistachio and mango, could be eliminated by detailed characterization of the target and a new design of the probes. Different pairs of primer were designed to amplify the target region of the 2s albumin gene (ana o3 allergen). PCR analysis using one of the primer pairs and DNA of cashew, mango and pistachio resulted in specific amplification of a 103 bp DNA section of cashew DNA (Ehlert et al. 2008a). No signals were observed when DNA of pistachio or mango was used in the PCR reaction (data not shown). The region that was also used for specific detection of cashew DNA by real-time PCR analysis (Ehlert et al. 2008a) was, therefore, chosen as hybridization site for the cashew LPA probes.

Subsequently, the specificity of the method was tested by analyzing DNA from organisms related to the selected targets and from other organisms that are expected to be ingredients of composed foods. Non-coding regions of chloroplast DNA were amplified for verification (Taberlet et al. 1991). The species tested with all LPA probes are listed in Table 2. No false positive signals were observed for peanut, cashew, pecan, pistachio, hazelnut, sesame, macadamia, walnut or brazil nut. The probes developed for the detection of

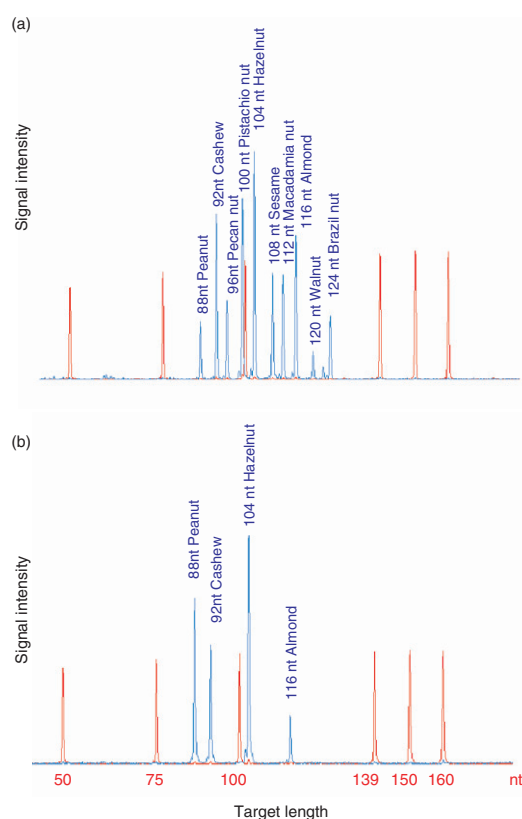


Figure 1. (a) Electropherogram of a sample containing a DNA mixture of peanut, cashew, pecan nut, pistachio nut, hazelnut, sesame, macadamia nut, almond, walnut and brazil nut. (b) Analysis of the commercial sample "organic mixed nut butter". Electropherogram showing the detection of four tree nuts contained in the sample. Non-assigned signals correspond to the used length standards.

Table 2. Species used for determining the target specificity of the LPA method.

Plants		Animals	
Anise	Garlic	Plum (leaf)	Beef
Apple	Ginger	Pumkin seed	Chicken
Apricot (leaf)	Lemon grass	Raspberry (leaf and fruit)	Duck
Banana (leaf)	Linseed	Rice	Turkey
Basil	Maize	Rye	Pork
Blackberry (leaf)	Mango (pulp and paring)	Sour cherry	
Cardamom seeds	Nectarine (leaf)	Soya	Microorganism
Carrot	Nutmeg	Strawberry (leaf and fruit)	Yeast
Cinnamon	Oregano	Sunflower seed	
Coconut	Parsley	Sultana	
Coriander	Peach	Sweet cherry	
Cumin	Pear	Wheat	
Curcuma	Pepper (black)		
Currant	Pimento		
Fennel	Pine nut		

almond DNA also yielded positive results in the presence of DNA from apricot, nectarine, peach and plum due to their phylogenetical relationship. Further characterization of the target by sequencing will be necessary to increase the specificity of the almond detection.

Slight peaks at the $n-1$ and $n-2$ nucleotides are caused by impurities of the synthesized oligonucleotides, which could be reduced by a new synthesis but could not be eliminated completely (data not shown).

The fragment lengths determined for each target were highly reproducible ($SD \pm 0.2$ nt). The observed slight shifts (≤ 1 nt) of the absolute length values (Table 1) are well-known effects arising from differences in the mobilities of the length standards and the analyzed fragments caused by different labeling dyes and by differences in the structures and base compositions of the DNA (Magnuson et al. 1996; Wenz et al. 1998).

Sensitivity of the LPA system

Due to the lack of appropriate reference materials for the detection of allergens, different food matrices, in which the selected plants typically occur, were chosen to evaluate the sensitivity of the LPA system. Chocolate was spiked with peanuts and hazelnuts, pesto with cashew nuts, and cookies with peanuts. In addition, walnut cookies spiked with peanuts, hazelnuts, pecan and macadamia nuts were self-prepared. These examples were selected to simulate fraudulent labeling or the unintended contamination of foods with different nuts (Table 3). Starting materials and ingredients were checked for the absence of any target of the LPA system.

Two independent DNA extractions of the spiked samples were analyzed in duplicate by LPA to determine the limits of detection (LOD).

Fragment-length analysis was performed with the pure and a 1:20-diluted PCR product. The LOD was assessed as the least concentration for which all results were consistently positive. For peanut, hazelnut and cashew nut, limits of detection of 5 mg kg^{-1} were determined in the chocolate, cookie and pesto matrices.

To study the influence of unequal proportions of allergenic components in a food, i.e. high excess of one of the LPA targets in the sample, on the sensitivity of the method, cookies containing 25% ($250\,000 \text{ mg kg}^{-1}$) walnuts were spiked with defined amounts ($1-10\,000 \text{ mg kg}^{-1}$) of four other nuts. Due to competitive amplification of the probes during PCR, under these conditions the added reagents are mainly used to amplify the excess of walnut ligation products. Consequently, the sensitivity for the nuts present as trace amounts is reduced, resulting in LODs of 1000 mg kg^{-1} for peanut, hazelnut, pecan, macadamia and 100 mg kg^{-1} for hazelnut (Table 3).

This inherent feature of the multi-target LPA method has to be taken into account when traces of targets are to be simultaneously detected in the presence of a high amount of one of the other LPA targets.

The samples used to determine the limits of detection of the LPA method were also analyzed by real-time PCR and ELISA (Table 3). For the samples analyzed by the commercially available ELISA kits, the LODs determined for hazelnut and peanut were slightly higher than those given by the manufacturer (R-Biopharm 2006, 2007). For the LODs determined for these targets by real-time PCR methods, such a comparison was not possible because the information given by the manufacturer refers to copies of genomic DNA (Congen, 2006a, b). The limits of detection observed for peanuts, hazelnuts and cashew nuts in chocolate and pesto by LPA

Table 3. Comparison of limits of detection.

Sample	Detection limit [mg kg ⁻¹]		
	LPA	Real-time PCR	ELISA
Peanut chocolate	5	5	5
Hazelnut chocolate	5	10	10
Peanut-spiked cookie	5	0.5	5
Pesto cashew	5	2	*
Walnut cookies with peanut, hazelnut, pecan, macadamia	1000 (peanut, pecan, macadamia) 100 (hazelnut)	1 (peanut) 10 (hazelnut)	100 (peanut) 1 (hazelnut)

*No method available.

Table 4. Analysis of commercially available samples by LPA.

Product	Declared allergenic ingredients	Precautionary labeling	Detection
Spreads			
Organic mixed nut butter	Peanuts, hazelnuts, Cashews, almonds	–	Peanut, cashew, hazel- nut, almond
Nutella	Hazelnut 13%	–	hazelnut
Sausages			
Original Thuringian sausage	Walnuts	–	n.d.
Mortadella with pistachios	Pistachios 1%	–	Pistachio
Convenience food dressings			
Pesto alla Genovese	Cashew nuts	–	Cashew
Sat� dressing	Peanuts 21%, peanut flavor	–	Peanut
Pesto goutweed (<i>Aegopodium podagraria</i>)	Macadamia nut	–	Macadamia nut
Dairy products			
Yoghurt with almonds	Almonds 2%	–	Almond
Milkshake pistachio-cocos	Pistachio pulp	–	Pistachio
Sweets/Cookies			
Chocolate bar	Hazelnut 5%	Traces of almond, peanut and other nuts	Hazelnut
Hazelnut bar	Hazelnut mark 3.4%	Traces of nuts and other seeds	Hazelnut
Hazelnut bar with honey	Hazelnut 66%	Traces of peanut, sesame or other nuts	Hazelnut, peanut
Peanut bar with honey	Peanut 68%	Traces of sesame or nuts	Peanut, hazelnut
Sesame seed bar with honey	Sesame 68%	Traces of peanut or other nuts	Sesame
Praline hazelnut	Hazelnut 30.5%, almonds	Traces pistachio	Hazelnut, almond
Praline pistachio	Almonds 12.5%, pistachio 6.5%	Traces hazelnut	Almond, pistachio
Praline coconut	Almonds 5%	Traces egg, hazelnut, pistachio	Almond, pistachio
Gingerbread	Hazelnuts, walnuts, almonds, cashew nuts, apricot kernels	Traces of other nuts and kernels	Cashew, hazelnut, almond, walnut

were similar to those determined for the tested ELISA and real-time PCR approaches. The real-time PCR method applied for the detection of peanuts showed higher sensitivity; however, the application of this kit also resulted in positive signals for the non-spiked walnut reference cookie.

Therefore, the reliability of these results remains questionable. The hazelnut ELISA test also showed cross-reactivity to walnut (0.001–0.036%); owing to the false-positive results, a detection of hazelnut traces in the presence of walnut is not possible with this kit (Kniel and Moser 2006; R-Biopharm 2006).

Analysis of retail samples

A variety of commercial foods were tested for the presence of the LPA targets. A total of 39 samples from different food categories were analyzed. All DNA extracts were checked for PCR inhibition and amplifiability of DNA prior to LPA analysis by amplification of non-coding regions of chloroplast DNA (Taberlet et al. 1991). Results obtained for spreads, sausages, dressings, dairy products and sweets are shown in Table 4.

The results obtained for the samples "organic mixed nut butter" and "gingerbread" demonstrate the advantages of the developed multi-target LPA method. In both cases, simultaneous detection of the allergenic ingredients declared on the label could be achieved. Figure 1b shows the results obtained for the sample "organic mixed nut butter".

Except for walnuts in a Thuringian sausage, the presence of ingredients declared on the label could be confirmed by LPA analysis in all samples. In addition, information given as part of a precautionary labeling in some of the retail samples could be confirmed or specified. Considering the described reduction in sensitivity for targets contained as trace amounts in the presence of a high amount of one of the other LPA targets, the detection of undeclared allergenic ingredients may be even further improved by analyzing samples with probe mixes that exclude the probes that would amplify the quantitatively dominating and declared allergenic ingredients.

A limitation of the method is the inability to differentiate almond from apricot, nectarine, peach and plum. This became obvious for the "gingerbread" sample: the signal detected for almond could not be assigned unequivocally to almond because apricot kernels had also been declared in the list of ingredients.

Quantitative conclusions on the contamination found in the retail samples are difficult, but concentrations at the lower ppm range are likely.

Conclusions

The LPA system for the detection of food allergens was shown to be a specific and sensitive method suitable for the simultaneous detection of peanut, cashew, pecan nut, pistachio nut, hazelnut, sesame seeds, macadamia nut, walnut and brazil nut in the lower mg kg^{-1} range. The modular system allows extension to further target sequences of interest. LPA analysis of different food matrices resulted in LODs in the lower ppm range, thus confirming the suitability of the method for allergen detection (Goodwin 2004; Poms and Anklam 2004; Poms et al. 2004). The specificities of the probes targeting almond DNA have to be improved to be capable of differentiating almond from phylogenetically related species.

The lack of certified reference materials in the field of allergen detection makes development and validation of appropriate methods more difficult. Further reference materials with certified concentrations of analytes are required to gain more information on individual limits of detection of the LPA method in actual food products.

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