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► **To cite this version:**

Karl-Heinz Engel, Anja Demmel, Alexandra Ehlert, Christine Hupfer, Ulrich Busch. Simultaneous detection of DNA from ten food allergens by ligation-dependent probe amplification. *Food Additives and Contaminants*, 2009, 26 (04), pp.409-418. 10.1080/02652030802593529 . hal-00577350

**HAL Id: hal-00577350**

**<https://hal.science/hal-00577350>**

Submitted on 17 Mar 2011

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

Journal:	<i>Food Additives and Contaminants</i>
Manuscript ID:	TFAC-2008-268.R1
Manuscript Type:	Original Research Paper
Date Submitted by the Author:	29-Oct-2008
Complete List of Authors:	Engel, Karl-Heinz; Technische Universitaet Muenchen, Chair of General Food Technology Demmel, Anja; Technische Universitaet Muenchen, Chair of General Food technology Ehlert, Alexandra; Technische Universitaet Muenchen, Chair of General Food technology Hupfer, Christine; Bavarian Health and Food safety Authority Busch, Ulrich; Bavarian Health and Food Safety Authority
Methods/Techniques:	Molecular biology - PCR
Additives/Contaminants:	Allergens
Food Types:	Bakery products, Processed foods

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# 1 Simultaneous detection of DNA from ten food 2 allergens by ligation-dependent probe 3 amplification

## 4 Abstract

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

## 18 Keywords

19 *Food allergen, tree nuts, peanut, sesame, detection, ligation, PCR*

## 20 Introduction

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

30 Taking into account the recommendations of the Codex Alimentarius  
31 Commission (Codex Alimentarius Commission 2007), the European  
32 Commission amended the European Food Labelling Directive 2000/13/EC by a  
33 list of ingredients to be labeled (EC 2000). Currently, Annex IIIa of Directive  
34 2007/68/EC (EC 2007) comprises gluten-containing cereals, crustaceans,  
35 molluscs, fish, peanuts, soybeans, eggs, milk and dairy products (including  
36 lactose), nuts, celery, mustard, sesame seeds, lupine, sulfite and products  
37 thereof. To protect the health of consumers, the declaration of these ingredients  
38 has been made mandatory regardless of their amounts in the final product.

39 Allergens are proteins whose routine food analysis is based on  
40 immunological detection using either specific IgE from human serum or  
41 antibodies raised in animals. Major challenges are the needs to check for the  
42 presence of food allergens at extremely low levels and to detect trace amounts  
43 of hidden allergens in composite and processed foods (Poms et al. 2004). PCR-  
44 based methods amplifying specific DNA sequences offer alternative tools to the

1  
2  
3 45 detection of allergenic or marker proteins for the species (Goodwin 2004, Poms  
4  
5 46 and Anklam 2004). In most cases, DNA presents a more stable analyte  
6  
7  
8 47 compared to proteins and is less affected by denaturation (Poms and Anklam,  
9  
10 48 2004). In addition, species-specific sequences allow the discrimination of  
11  
12 49 closely related organisms.

13  
14  
15 50 Appropriate PCR assays for the detection and identification of individual  
16  
17 51 food allergens have been developed for cashew nuts (Brzezinski 2006, Ehlert et  
18  
19 52 al. 2008a), celery (Dovicovicova et al. 2004, Stephan et al. 2004, Hupfer et al.  
20  
21 53 2006), cereals (wheat, barley, rye) (Dahinden et al. 2001, Hernandez et al.  
22  
23 54 2005, Sandberg et al. 2003), peanuts (Hird et al. 2003, Stephan and Vieths  
24  
25 55 2004), pistachios (Barbieri and Frigeri 2006) and tree nuts (walnut, pecan,  
26  
27 56 hazelnut) (Holzhauser et al. 2000; Herman et al. 2003; Brezna et al. 2005;  
28  
29 57 Brezna and Kuchta 2007; Germini et al. 2005; Arlorio et al. 2007). Conventional  
30  
31 58 and Real-time PCR methods for the detection of soybean, sesame, mustard,  
32  
33 59 peanut, hazelnut and almond have recently been compared (Pancaldi et al.  
34  
35 60 2005). At present, only a few Duplex-PCR systems are known allowing the  
36  
37 61 simultaneous detection of peanut and hazelnut or wheat and barley (Ronning et  
38  
39 62 al. 2006; Rossi et al. 2005).

40  
41  
42 63 The aim of this study was therefore to develop and to validate a multi-  
43  
44 64 target method for the simultaneous detection of allergens in different food  
45  
46 65 matrices. The application of a ligation-dependent probe amplification (LPA)  
47  
48 66 technique for the simultaneous detection of DNA from peanut, cashew nut,  
49  
50 67 pecan nut, pistachio nut, hazelnut, sesame seeds, macadamia nut, almond,  
51  
52 68 walnut and brazil nut in a single reaction is described. Ligation-dependent PCR  
53  
54 69 was originally introduced to allow the detection of nucleic acid sequences  
55  
56 70 (Belgrader et al. 1997, Carrino 1996, Hsuih et al. 1996). First applications in the

1  
2  
3 71 field of medical diagnostics allowed the detection and the relative quantification  
4  
5 72 of up to 40 – 50 target sequences in a single assay (Eldering et al. 2003, Gille  
6  
7  
8 73 et al. 2002, Hogervorst et al. 2003, Schouten et al. 2002, Taylor et al. 2003).  
9  
10 74 The suitability of this method for the event-specific detection and relative  
11  
12 75 quantification of DNA from two genetically modified organisms (GMO) has been  
13  
14 76 demonstrated using commercially available maize and soya standards  
15  
16 77 (Moreano et al. 2006). The technique does not amplify the target sequences,  
17  
18 78 but is rather based on the amplification of products resulting from the ligation of  
19  
20 79 bipartite hybridization probes. The use of this analytical strategy results in a  
21  
22 80 flexible system that can be complemented with further hybridization probes to  
23  
24 81 broaden the range of target sequences to be detected. This approach has been  
25  
26 82 realized by the development of a modular system allowing the simultaneous  
27  
28 83 detection of several GMO targets corresponding to different levels of specificity  
29  
30 84 in a one-tube assay (Ehlert et al. 2008b).  
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## 40 **Materials and Methods**

### 41 42 43 44 45 *Materials and samples*

46  
47  
48 88 Nut materials, sesame seeds, ingredients of self-prepared walnut cookies  
49  
50 89 and commercial food samples were purchased from local grocery stores. DNA  
51  
52 90 plant and animal materials used for testing the specificity of the method and  
53  
54 91 spiked samples of chocolate, cookies and pesto used to determine the  
55  
56 92 sensitivity were obtained from the Bavarian Health and Food Safety Authority  
57  
58 93 (Oberschleißheim, Germany). Chocolate samples had been spiked with  
59  
60 94 peanuts (100, 10, 5, 1 and 0.5 mg/kg) and hazelnuts (20, 10 and 5 mg/kg).

1  
2  
3 95 Cookies spiked with peanuts contained 100, 10, 5, 1 and 0.5 mg/kg of peanuts  
4  
5 96 and pesto spiked with cashew nuts contained 100, 20, 10, 5, 2 and 1 mg/kg of  
6  
7 97 cashew nuts. In all cases samples of the unspiked material were included in the  
8  
9 98 analysis.  
10  
11  
12  
13 99

### 100 *Preparation of walnut cookies spiked with different nuts*

101 Nuts ground with a Thermomixer (Vorwerk, Wuppertal, Germany) as well  
102 as the other cookie ingredients were analyzed by LPA to ensure the purities of  
103 the starting materials. Two doughs containing 25 % wheat flour, 25 % sugar,  
104 25 % butter and 25 % ground nuts (either walnuts only or walnuts spiked with  
105 10 % of peanut, hazelnut, pecan and macadamia) were prepared using a food  
106 processor (Braun, Germany). The reference cookies containing only walnuts  
107 and the cookies with all five nuts were baked separately at 180°C for 10 min  
108 and ground afterwards. The concentrations of peanut, hazelnut, pecan and  
109 macadamia were adjusted in the spiked cookies to 10000, 1000, 100, 10 and  
110 1 mg/kg, respectively, by mixing the corresponding amounts of ground spiked  
111 cookies and ground walnut reference cookies in a food processor. The mixtures  
112 became fluid due to the high fat contents; however the procedure resulted in  
113 visually homogeneous dispersions.

### 114 115 *DNA extraction*

116 Each sample (2 g) was mixed with 10 mL CTAB-extraction buffer [2 %  
117 (w/v) cetyltrimethylammoniumbromide, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-  
118 OH/HCl] and 30 µL proteinase K (20mg/mL) in a 50 mL Falcon-tube and

1  
2  
3 119 incubated at 65°C overnight. After 5 min centrifugation at 5000 g, 1000 µL  
4  
5 120 supernatant was transferred to a 1.5 mL tube and centrifuged again at 14000 g  
6  
7  
8 121 for 5 min. 500 µL chloroform/isoamylalcohol (Ready Red™, MP Biomedicals,  
9  
10 122 Heidelberg, Germany) was mixed with 700 µL supernatant and centrifuged at  
11  
12 123 16000 g for 15 min. 500 µL supernatant was added to 500 µL isopropanol  
13  
14  
15 124 (stored at -20°C) and incubated at room temperature (RT) for 30 min. After  
16  
17 125 15 min centrifugation at 16000 g the supernatant was removed, the pellet was  
18  
19 126 washed with 500 µL ethanol (70 %; stored at -20°C) and centrifuged 5 min at  
20  
21 127 16000 g. After removal of ethanol the pellet was dried 1 h at 50°C and  
22  
23 128 afterwards diluted in 100 µL TE-buffer (1x). Additionally, the DNA extracts were  
24  
25 129 purified using spin filter columns (Qiagen, Hilden, Germany).

26  
27  
28  
29 130 DNA concentrations were determined fluorimetrically using PicoGreen®  
30  
31 131 dsDNA quantification reagent (Invitrogen, Karlsruhe, Germany) on a Tecan  
32  
33 132 GENios™ plus reader (Männedorf, Switzerland).

34  
35  
36 133

#### 37 38 39 134 *Probes and primers*

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42  
43 135 Probes used for the detection of peanut, cashew nut, pecan nut,  
44  
45 136 pistachio, hazelnut, sesame, macadamia nut, almond, walnut and brazil nut  
46  
47 137 were designed using the Beacon Designer 4.0 software (Premier Biosoft Int.,  
48  
49 138 USA) and FastPCR software (University of Helsinki, Finland). The synthesis of  
50  
51 139 the probes was done by Biolegio B.V. (Nijmegen, The Netherlands). Primers  
52  
53 140 are included in the MLPA reagents kit (MRC-Holland, Amsterdam, The  
54  
55 141 Netherlands). Sequences of LPA probes and primers as well as GenBank  
56  
57 142 accession numbers of the selected targets are listed in Table 1.

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59  
60 143



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3 144 [Insert Table 1 about here]  
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8

9 146 *Ligation-dependent probe amplification*  
10

11  
12 147 The LPA reaction was essentially carried out as described by Schouten  
13 et al. (Schouten et al. 2002). Hybridization was performed overnight in 0.5 mL  
14  
15 148 et al. (Schouten et al. 2002). Hybridization was performed overnight in 0.5 mL  
16  
17 149 reaction vessels using a thermocycler (Mastercycler Gradient, Eppendorf,  
18 Hamburg, Germany) and 100 ng DNA sample. After 5 min of DNA denaturation  
19  
20 150 Hamburg, Germany) and 100 ng DNA sample. After 5 min of DNA denaturation  
21 at 98°C, 1.5 µL of MLPA buffer and 1.5 µL of a mixture of the synthesized  
22  
23 151 at 98°C, 1.5 µL of MLPA buffer and 1.5 µL of a mixture of the synthesized  
24 probes containing 1 fmol of each LPA probe for the detection of peanut,  
25  
26 152 probes containing 1 fmol of each LPA probe for the detection of peanut,  
27 cashew, pecan nut, pistachio, hazelnut, sesame, macadamia nut, almond,  
28  
29 153 cashew, pecan nut, pistachio, hazelnut, sesame, macadamia nut, almond,  
30 walnut and brazil nut, respectively, were added and held at 60°C for 16 h.  
31  
32 154 walnut and brazil nut, respectively, were added and held at 60°C for 16 h.  
33 Ligation reaction was performed at 54°C for 15 min after adding 3 µL Ligase-65  
34  
35 155 Ligation reaction was performed at 54°C for 15 min after adding 3 µL Ligase-65  
36 buffer A, 3 µL Ligase-65 buffer B, 25 µL H<sub>2</sub>O and 1 µL Ligase-65 (MRC-  
37  
38 156 buffer A, 3 µL Ligase-65 buffer B, 25 µL H<sub>2</sub>O and 1 µL Ligase-65 (MRC-  
39 Holland, Amsterdam, The Netherlands). After ligation, reaction mixes were  
40  
41 157 Holland, Amsterdam, The Netherlands). After ligation, reaction mixes were  
42 heated for 5 min at 98°C to inactivate the enzyme.  
43  
44 158 heated for 5 min at 98°C to inactivate the enzyme.

45  
46 159 For amplification of the ligation products 10 µL Polymerase mix with  
47 primers, dNTPs, buffer and Polymerase enzyme of the MLPA kit (MRC-Holland,  
48  
49 160 primers, dNTPs, buffer and Polymerase enzyme of the MLPA kit (MRC-Holland,  
50 Amsterdam, The Netherlands) were added to 40 µL ligation reaction mixture at  
51  
52 161 Amsterdam, The Netherlands) were added to 40 µL ligation reaction mixture at  
53 60°C. 35 amplification cycles at 95°C for 30 sec, 60°C for 30 sec and 72°C for  
54  
55 162 60°C. 35 amplification cycles at 95°C for 30 sec, 60°C for 30 sec and 72°C for  
56 60 sec were followed by a final step of 20 min at 72°C and cooled down to 4°C.  
57  
58 163 60 sec were followed by a final step of 20 min at 72°C and cooled down to 4°C.

59  
60 164 *Fragment length analysis*

165  
166  
167 165 Fragment length analysis was performed on an ABI PRISM<sup>®</sup> 310 Genetic  
Analyzer using capillaries (47 cm), polymer (POP-6<sup>™</sup> Performance Optimized  
Polymer); reagents were obtained from Applied Biosystems (Forster City, USA).

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2  
3 168 One microliter of the PCR product (pure or diluted) was mixed with 0.3  $\mu\text{L}$  size  
4  
5 169 standard (GeneScan<sup>®</sup>-500 [TAMRA]<sup>™</sup>) and 14.7  $\mu\text{L}$  Hi-Di<sup>™</sup> formamide. Prior to  
6  
7  
8 170 analysis, DNA was denatured at 94°C for 3 min and cooled down on ice.  
9  
10 171 Electrokinetic injections were performed at 15 kV for 5 sec. Electrophoretic  
11  
12 172 separations were run at 60°C and 15 kV.  
13  
14  
15 173

### 174 *Sequencing*

175 Ligation products were generated and amplified separately prior to  
176 sequencing. Primers used for sequencing were identical to those listed in Table  
177 1. FAM-labeling did not interfere with cycle sequencing. Amplified products  
178 were cleaned up using a PCR purification kit (QIAquick, Quiagen GmbH, Hilden,  
179 Germany) and used as template for the sequencing PCR. This reaction was  
180 performed using a BigDye, Terminator v1.1 Cycle Sequencing Kit (Applied  
181 Biosystems, Foster City, USA). One reaction mix (20  $\mu\text{L}$ ) contained 2  $\mu\text{L}$  5x  
182 buffer, 4  $\mu\text{L}$  RR-mix, 2  $\mu\text{L}$  primer (10 pmol), 8  $\mu\text{L}$  H<sub>2</sub>O and 4  $\mu\text{L}$  template.  
183 Reaction conditions were as follows: initial denaturation (1 min at 96°C), 30  
184 cycle denaturation steps (10 sec at 96°C) and primer annealing (5 sec at 56°C),  
185 and a final step (4 min at 60°C).

186 Purification of the PCR products was carried out following amplification.  
187 PCR products (10  $\mu\text{L}$ ) were mixed with 16  $\mu\text{L}$  H<sub>2</sub>O, 4  $\mu\text{L}$  Na-acetate (3M) and  
188 50  $\mu\text{L}$  EtOH (100%) in a 1.5 mL reaction vessel and centrifuged at 15.000 rpm  
189 for 15 min. EtOH was removed carefully without damaging the precipitated DNA  
190 pellet. The pellet was vortexed with 50  $\mu\text{L}$  EtOH and centrifuged at 15.000 rpm  
191 for 5 min. After carefully removing EtOH, the pellet was allowed to dry at 50°C  
192 for 1 hr. Finally, DNA was dissolved in 20  $\mu\text{L}$  H<sub>2</sub>O.

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3 193 Sequencing of the diluted PCR products (10  $\mu$ L H<sub>2</sub>O + 6  $\mu$ L purified DNA)  
4  
5 194 was carried out on an ABI PRISM<sup>®</sup> 310 Genetic Analyzer (Applied Biosystems,  
6  
7 195 Forster City, USA) using 47 cm capillaries and POP-6<sup>™</sup> Performance  
8  
9 196 Optimized Polymer. Electrokinetic injections were performed at 2 kV for 30 sec.  
10  
11 197 Runs were carried out at 50 °C and 15 kV.  
12  
13  
14

### 16 198 *Real-time PCR and ELISA assays*

18  
19 199 SureFood<sup>®</sup> Allergen Kits for the qualitative detection of DNA from  
20  
21 200 hazelnut and peanut from Congen Biotechnology GmbH (Berlin, Germany)  
22  
23 201 were used for Real-time PCR analysis. The limits of detection for both kits were  
24  
25 202 indicated to be 10 copies of genomic DNA. For the detection of cashew nut a  
26  
27 203 recently described Real-time PCR system was used (Ehlert et al. 2008a).  
28  
29 204 Ridascreen<sup>®</sup> enzyme immunoassays for the quantitative analysis of hazelnut  
30  
31 205 and peanut were obtained from r-biopharm AG (Darmstadt, Germany). The limit  
32  
33 206 of detection as indicated by the manufacturer were 2.5 mg/kg hazelnut and  
34  
35 207 peanut, respectively (r-biopharm; 2006, 200x).  
36  
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43

## 44 209 **Results and Discussion**

### 49 210 *Design of LPA probes and Choice of target sequences*

51  
52 211 The principle of ligation-dependent probe amplification reaction as well  
53  
54 212 as hints and rules for the design of LPA probes have been specified in detail  
55  
56 213 (Schouten et al. 2002, Moreano et al. 2006, MRC Holland). The detection of  
57  
58 214 each target sequence requires the design of two probes containing the  
59  
60 215 respective target-specific hybridization sites as well as identical primer binding

1  
2  
3 216 sites (PBS) at their 5'- or 3'- ends. The LPA system designed in this study uses  
4  
5 217 the advantages of synthetic oligonucleotides for hybridization as described by  
6  
7  
8 218 Moreano et al. (Moreano et al. 2006).  
9

10 219 Different tree nuts (macadamia, cashew, pecan, walnut, brazil nut,  
11  
12 220 pistachio, almond and hazelnut) as well as peanut and sesame were chosen as  
13  
14  
15 221 examples for the detection of allergenic components in foods by LPA. They are  
16  
17 222 often contained as hidden allergens in pastries, candies or chocolate and  
18  
19 223 present potential health risks for allergic persons.  
20  
21

22 224 Details of the selected target sequences and the GenBank accession  
23  
24 225 numbers of the LPA primers and probes are listed in Table 1. The probes  
25  
26 226 mostly detect genes encoding for plant food proteins of the cupin and prolamin  
27  
28 227 superfamily, respectively, which are known to cause IgE-mediated allergic  
29  
30 228 reactions. The simultaneous detection of all targets is shown in Figure 1.  
31  
32  
33

34 229 The primer sequences described by Schouten et al. (Schouten et al.  
35  
36 230 2002) for MLPA analysis were also used in this study. They were tested  
37  
38 231 regarding their suitability for analyzing foods by database enquiry via NCBI  
39  
40 232 GenBank and PCR using DNA of different plant species. The use of spacer  
41  
42 233 sequences between PBS and hybridization sites rendered ligation products with  
43  
44 234 lengths characteristic for each of the target DNA. Differences of 4 nucleotides  
45  
46 235 (nt) in length have been shown sufficient for unequivocal determination of the  
47  
48 236 amplification products using POP-6™ polymer (Ehlert et al. 2008b). Simple  
49  
50 237 repeats of GT bases were used for the spacer sequences to avoid intra- and  
51  
52 238 intermolecular hybridizations.  
53  
54  
55  
56

57 239 Using this approach, all target signals were sufficiently separated. The  
58  
59 240 signal obtained for pistachio nut overlapped with one of the standard signals  
60

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3 241 (100 nt); however, owing to the use of different fluorescent dyes for standard  
4  
5 242 and target probes a discrimination of the two signals is possible.  
6  
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8 243

9  
10 244 [Insert Figure 1 about here]  
11

12 245  
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14

### 15 16 246 *Evaluation of target specificity* 17

18  
19 247 To avoid cross homologies the specificities of the probe target  
20  
21 248 sequences were first evaluated by BLAST search within NCBI GenBank by  
22  
23 249 Beacon Designer software. Additionally, each probe was checked with DNA  
24  
25 250 extracted from the other target species. The performance of the LPA system  
26  
27 251 was examined using DNA extracts of the ten targets adjusted to 20 ng/ $\mu$ L and  
28  
29 252 different mixtures prepared to simulate composed food products with different  
30  
31 253 nut proportions. No unspecific signals were observed for almond, peanut,  
32  
33 254 pecan, pistachio, hazelnut, sesame, macadamia, walnut and brazil nut probes.  
34  
35 255 Furthermore, the differentiation between the phylogenetically closely related  
36  
37 256 tree nuts pecan and walnut could be achieved. Published sequencing data were  
38  
39 257 used to design the ligation probes with only four different DNA bases (Brezna  
40  
41 258 2005, Brezna and Kuchta 2007). Cross-reactivity of the cashew probes with  
42  
43 259 other members of the Anacardiaceae family, specifically pistachio and mango,  
44  
45 260 could be eliminated by detailed characterization of the target and a new design  
46  
47 261 of the probes. Different pairs of primer were designed to amplify the target  
48  
49 262 region of the 2s albumin gene (ana o3 allergen). PCR analysis using one of the  
50  
51 263 primer pairs and DNA of cashew, mango and pistachio resulted in specific  
52  
53 264 amplification of a 103 bp DNA section of cashew DNA (Ehlert et al. 2008a). No  
54  
55 265 signals were observed when DNA of pistachio or mango was used in the PCR  
56  
57  
58  
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1  
2  
3 266 reaction (data not shown). The region that was also used for specific detection  
4  
5 267 of cashew DNA by Real-time PCR analysis (Ehlert et al. 2008a) was therefore  
6  
7  
8 268 chosen as hybridization site for the cashew LPA probes.  
9

10 269 Subsequently, the specificity of the method was tested by analyzing DNA  
11  
12 270 from organisms related to the selected targets and from other organisms that  
13  
14  
15 271 are to be expected as ingredients of composed foods. Non-coding regions of  
16  
17 272 chloroplast DNA were amplified for verification (Taberlet et al. 1991). The  
18  
19 273 species tested with all LPA probes are listed in Table 2. No false positive  
20  
21 274 signals were observed for peanut, cashew, pecan, pistachio, hazelnut, sesame,  
22  
23 275 macadamia, walnut and brazil nut. The probes developed for the detection of  
24  
25 276 almond DNA also yielded positive results in the presence of DNA from apricot,  
26  
27 277 nectarine, peach and plum due to their phylogenetical relation. Further  
28  
29 278 characterization of the target by sequencing will be necessary to increase the  
30  
31 279 specificity of the almond detection.  
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43 281 [Insert Table 2 about here]  
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283 Slight peaks at n-1 and n-2 nucleotides are caused by impurities of the  
284 synthesized oligonucleotides that could be reduced by a new synthesis but  
285 could not be eliminated completely (data not shown).

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

292

293 *Sensitivity of the LPA system*

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

303

304 [Insert Table 3 about here]

305

306 Two independent DNA extractions of the spiked samples were analyzed  
307 in duplicate by LPA to determine the limits of detection (LOD). Fragment length  
308 analysis was performed with the pure and 1:20 diluted PCR product. The LOD  
309 was assessed as the least concentration for which all results were consistently  
310 positive. For peanut, hazelnut and cashew nut, limits of detection of 5 mg/kg  
311 were determined in the matrices chocolate, cookie and pesto, respectively.

312 To study the influence of unequal proportions of allergenic components in  
313 a food, i.e. high excess of one of the LPA targets in the sample, on the  
314 sensitivity of the method, cookies containing 25% (250,000 mg/kg) walnuts  
315 were spiked with defined amounts (1 – 10,000 mg/kg) of four other nuts. Due to  
316 competitive amplification of the probes during PCR, under these conditions the

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3 317 added reagents are mainly used to amplify the excess of walnut ligation  
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5 318 products. Consequently, the sensitivity for the nuts contained as trace amounts  
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8 319 is reduced, resulting in LODs of 1000 mg/kg for peanut, hazelnut, pecan,  
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10 320 macadamia and 100 mg/kg for hazelnut, respectively (Table 3).  
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12 321 This inherent feature of the multi-target LPA method has to be taken into  
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14 322 account, when traces of targets are to be simultaneously detected in the  
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16  
17 323 presence of a high amount of one of the other LPA targets.  
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19 324 The samples used to determine the limits of detection of the LPA method  
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21 325 were also analyzed by Real-time PCR and ELISA (Table 3). For the samples  
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23 326 analyzed by the commercially available ELISA kits, the LODs determined for  
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25 327 hazelnut and peanut were slightly higher than those given by the manufacturer  
26  
27 328 (r-biopharm 2006, 2007). For the LODs determined for these targets by the  
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29 329 Real-time PCR methods, such a comparison was not possible because the  
30  
31 330 information given by the manufacturer refers to copies of genomic DNA  
32  
33 331 (Congen, 2006a, 2006b). The limits of detection observed for peanuts,  
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35 332 hazelnuts and cashew nuts in chocolate and pesto by LPA were similar to those  
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37 333 determined for the tested ELISA and Real-time PCR approaches. The Real-  
38  
39 334 time PCR method applied for the detection of peanuts showed higher  
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41 335 sensitivity; however, the application of this kit also resulted in positive signals for  
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43 336 the non-spiked walnut reference cookie. Therefore, the reliability of these  
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45 337 results remains questionable. The hazelnut ELISA test also showed cross  
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47 338 reactivity to walnut (0.001 – 0.036 %); owing to the false-positive results a  
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49 339 detection of hazelnut traces in the presence of walnut is not possible with this kit  
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51 340 (r-Biopharm 2006, Kniel and Moser 2006).  
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3 342 *Analysis of retail samples*  
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7 343 A variety of commercial foods were tested for the presence of the LPA  
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9 344 targets. A total of 39 samples from different food categories were analyzed. All  
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11 345 DNA extracts were checked for PCR inhibition and amplifiability of DNA prior to  
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13 346 LPA analysis by amplification of non-coding regions of chloroplast DNA  
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16 347 (Taberlet et al. 1991). Results obtained for spreads, sausages, dressings, dairy  
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18 348 products and sweets are shown in Table 4.  
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23 350 [Insert Table 4 about here]  
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28 352 The results obtained for the samples “organic mixed nut butter” and  
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30 353 “gingerbread” demonstrate the advantages of the developed multi-target LPA  
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32 354 method. In both cases the simultaneous detection of the allergenic ingredients  
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34 355 declared on the label could be achieved. Figure 1B shows the results obtained  
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36 356 for the sample “organic mixed nut butter”.  
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40 357 Except for walnuts in a Thuringian sausage, the presence of ingredients  
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42 358 that had been declared on the label could be confirmed by LPA analysis in all  
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44 359 samples. In addition, information given as part of a precautionary labeling in  
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46 360 some of the retail samples could be confirmed or specified. Considering the  
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48 361 described reduction in sensitivity for targets contained as trace amounts in the  
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50 362 presence of a high amount of one of the other LPA targets, the detection of  
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52 363 undeclared allergenic ingredients may be even further improved by analyzing  
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54 364 samples with probe mixes that exclude the probes that would amplify the  
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56 365 quantitatively dominating and declared allergenic ingredients.  
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3 366 A still existing limitation of the method is the missing differentiation of  
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5 367 almond from apricot, nectarine, peach and plum. This became obvious for the  
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7 368 sample “gingerbread”: The signal detected for almond could not be assigned  
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9 369 unequivocally to almond, because apricot kernels had also been declared in the  
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11 370 list of ingredients.  
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15 371 Quantitative conclusions on the contaminations found in the retail samples are  
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17 372 difficult, but concentrations at the lower ppm range are likely.  
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## 21 22 23 374 **Conclusions**

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27 375 The LPA system for the detection of food allergens was shown to be a  
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29 376 specific and sensitive detection method suitable for the simultaneous detection  
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31 377 of peanut, cashew, pecan nut, pistachio nut, hazelnut, sesame seeds,  
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33 378 macadamia nut, walnut and brazil nut in the lower mg/kg range. The modular  
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35 379 system allows the extension to further target sequences of interest. LPA  
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37 380 analysis of different food matrices resulted in LODs in the lower ppm range,  
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39 381 thus confirming the suitability of the method for allergen detection (Poms et al.  
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41 382 2004, Goodwin 2004, Poms and Anklam 2004). The specificities of the probes  
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43 383 targeting almond DNA have to be improved in order to be able to differentiate  
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45 384 almond from phylogenetically related species.  
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51 385 The lack of certified reference materials in the field of allergen detection  
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53 386 makes development and validation of appropriate methods more difficult.  
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55 387 Further reference materials with certified concentrations of analytes are  
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57 388 required to gain more information on individual limits of detection of the LPA  
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59 389 method in real food products.  
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5 542 **Figure legends**  
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8 543 Figure 1. (A) Electropherogram of a sample containing a DNA mixture of peanut, cashew, pecan  
9 544 nut, pistachio nut, hazelnut, sesame, macadamia nut, almond, walnut and brazil nut. (B)  
10 545 Analysis of the commercial sample "organic mixed nut butter". Electropherogram showing the  
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12 546 detection of four tree nuts contained in the sample. Non- assigned signals correspond to the  
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14 547 used length standards.  
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Table 1 Probes and primer

Target / GenBank Accession Nr	Left probe	Right probe	Ligation products (retention time) [nt]
<b>Peanut / L77197</b>	5' - <b>GGGTTCCCTAAGGGTTGG</b> AGCGAG GCAGCAGTGGGA <del>ACTC</del> - 3'	P - 5' - CAAGGAGACAGAAGATGCCAGAG <b>CCTCTAGATTGGATCTTGCTGGCAC</b> - 3'	88 (87,10 ± 0,13)
<b>Cashew / AY081853</b>	5' - <b>GGGTTCCCTAAGGGTTGG</b> ACTTATTA GATTAATTCAGTGGACTGC - 3'	P - 5' - CATGAAGTGAAGCAGTAGTAGAAGTCT <b>AGATTGGATCTTGCTGGCAC</b> - 3'	92 (91,16 ± 0,17)
<b>Pecan nut / DQ156215</b>	5' - <b>GGGTTCCCTAAGGGTTGG</b> ACACAATC CCTACTACTTTCACTCCCAGGGA - 3'	P - 5' - CTCAGGTCGAGACATGAGTCCG <b>GGTCTAGATTGGATCTTGCTGGCAC</b> - 3'	96 (94,67 ± 0,17)
<b>Pistachio nut / Y07600</b>	5' - <b>GGGTTCCCTAAGGGTTGG</b> ACCTGAA CACGGCGAGCACAAG - 3'	P - 5' - AGGGACTGGTGGAGAAGATCAAAGAC AAgtgtgtgt <b>TCTAGATTGGATCTTGCTGGCAC</b> - 3'	100 (99,37 ± 0,18)
<b>Hazelnut / AF136945</b>	5' - <b>GGGTTCCCTAAGGGTTGG</b> AGATCACC AGCAAGTACCACACCAAGG - 3'	P - 5' - GCAACGCTTCAATCAATGAGGAGGA GAtgtgtgt <b>TCTAGATTGGATCTTGCTGGCAC</b> - 3'	104 (102,75 ± 0,12)
<b>Sesame seeds / AF240006</b>	5' - <b>GGGTTCCCTAAGGGTTGG</b> AgtgtgtTGA AGGGAGAGAAAGAGAGAGGAGGAGCAA - 3'	P - 5' - GAAGAACAGGGACGAGGGCGGATtg tgtgtgt <b>TCTAGATTGGATCTTGCTGGCAC</b> - 3'	108 (108,11 ± 0,13)
<b>Macadamia nut / AF161883</b>	5' - <b>GGGTTCCCTAAGGGTTGG</b> ACTTA ATCAACCGAGACAACAACGAGAGG - 3'	P - 5' - CTCCACATAGCCAAGTTCTTACAGACCA Ttgtgtgtgt <b>TCTAGATTGGATCTTGCTGGCAC</b> - 3'	112 (111,07 ± 0,14)
<b>Almond / X65718</b>	5' - <b>GGGTTCCCTAAGGGTTGG</b> Agtgtgtgtgt CCATTACAAGTCTCCACCACCACCAC - 3'	P - 5' - CTTCTCCTACTCCTCCAGTCTACTCACC ACCgtgtgt <b>TCTAGATTGGATCTTGCTGGCAC</b> - 3'	116 (114,84 ± 0,12)
<b>Walnut / AF066055</b>	5' - <b>GGGTTCCCTAAGGGTTGG</b> AgtgtgtgtgtGG CACAATCCCTACTACTTTCACTCCCAGAG - 3'	P - 5' - CATTAGGTCGAGACATGAGTCCGAGGA AGGtgtgtgt <b>TCTAGATTGGATCTTGCTGGCAC</b> - 3'	120 (119,80 ± 0,11)
<b>Brazil nut / M17146</b>	5' - <b>GGGTTCCCTAAGGGTTGG</b> Agtgtgtgtgtgtgt gtgGAGGAGGAGAACCAGGAGGAGTGTC - 3'	P - 5' - GCGAGCAGATGCAGAGACAGCAGgtgtgt gtgtgtgtgt <b>TCTAGATTGGATCTTGCTGGCAC</b> - 3'	124 (124,84 ± 0,09)
<b>Primer R - unlabeled</b>	5' - <b>GTGCCAGCAAGATCCAATCTAGA</b> - 3'		
<b>Primer F – labeled</b>	<b>FAM</b> – 5' - <b>GGGTTCCCTAAGGGTTGGA</b> - 3'		

Capitals : plant DNA  
**Bold capitals:** primer binding site  
 Lowercase fonts: spacer DNA

Table 2 Species used for the determination of the target specificity of the LPA method

plants		animals	
anise	garlic	plum (leaf)	beef
apple	ginger	pumkin seed	chicken
apricot (leaf)	lemon grass	raspberry	duck
banana (leaf)	linseed	(leaf and fruit)	turkey
basil	maize	rice	pork
blackberry (leaf)	mango	rye	
cardamom	(pulp and paring)	sour cherry	
seeds	nectarine (leaf)	soya	<b>microorganism</b>
carrot	nutmeg	strawberry	
cinnamon	oregano	(leaf and fruit)	yeast
coconut	parsley	sunflower seed	
coriander	peach	sultana	
cumin	pear	sweet cherry	
curcuma	pepper (black)	wheat	
currant	pimento		
fennel	pine nut		

Table 3 Comparison of limits of detection

Sample	Detection limit [mg/kg]		
	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
<b>Spreads</b>			
organic mixed nut butter	peanuts, hazelnuts, cashews, almonds	-	peanut, cashew, hazelnut, almond
nutella	hazelnut 13 %	-	hazelnut
<b>Sausages</b>			
original Thuringian sausage	walnuts	-	n.d.
mortadella with pistachios	pistachios 1%	-	pistachio
<b>Convenience food dressings</b>			
pesto alla Genovese	cashew nuts	-	cashew
saté dressing	peanuts 21%, peanut flavor	-	peanut
pesto goutweed ( <i>Aegopodium podagraria</i> )	macadamia nut	-	macadamia nut
<b>Dairy products</b>			
yoghurt with almonds	almonds 2%	-	almond
milkshake pistachio-cocos	pistachio pulp	-	pistachio
<b>Sweets/ Cookies</b>			
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

Figure 1

