Simultaneous detection of peanut and hazelnut allergens in food matrices using multiplex PCR method

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Received September 12, 2014
Accepted April 1, 2015

Abstract

Multiplex PCR analysis for the detection of two targeting segments of genes coding major food protein allergens as peanut (*Arachis hypogaea) Ara h* 1 gene and hazelnut (*Corylus avellana*) *Cor a* 1 gene was developed. Two sets of primers were designed and tested to their specificity on a broad range of ingredients. The identity of amplicons (*Ara h* 1- 180 bp, *Cor a* 1 – 258 bp) by sequencing and alignment of sequences with sequences deposited in Genbank was confirmed. When testing the specificity of designed primer pairs on a spectrum of food ingredients, no cross reactions were detected. A potential inhibition of PCR reaction was eliminated using the universal plant primers of *chloroplast* gene 124 bp for the plant matrices confirmation. The intrinsic detection limit was 10 $pg \cdot ml^{-1}$ and the practical detection limit was 0.001% w/w (10 $mg \cdot kg^{-1}$) for both peanuts and hazelnuts. The method was applied to the investigation of 60 commercial food samples. The developed multiplex PCR method is cheap, specific and sensitive enough and can be used as a simple, one day procedure for the checking of undeclared peanut and hazelnut major allergens in food.

Plant allergens, DNA, Ara h 1, Cor a 1, food

People suffering from food allergies are dependent on accurate food labelling to prevent the allergic reaction. The detection of peanut (*Arachis hypogaea) Ara h* 1 gene coding the main allergen protein, and hazelnut (*Corylus avellana*) *Cor a* 1 gene is very important for retail food products labelling control. Food allergies are the fourth most important public health problem according to the World Health Organization (WHO 1999).

Allergens labelling legislation can improve the consumers' protection because the most reliable possibility of preventing an allergic reaction is strict avoidance of the consumption of the allergenic food. Several studies have shown that the allergenicity of proteins can be modifyied during technological, culinary or digestive processing. Thermal processing sometimes leads to an increase of allergenicity through the formation of neoallergens. Neoallergens appear during the heating or storing of foods and may be important in some anaphylactic reactions (Malanin et al. 1995). On the other hand, some studies have demonstrated reduction of allergenicity as the succesfull decrease of IgE-binding capacity in pistachio nuts using steam-roast processing (Noorbakhsh et al. 2010). Many plant allergens have been found to be consistently more resistant to digestion by pepsin than other proteins (Koppelmann et al. 2005).

Currently there are several different techniques for the detection of food allergens (Poms et al. 2004), such as enzyme linked immunosorbent assays – ELISA (Holzhauser et al. 2002; Doi et al. 2008), biosensor immunoassays (Yman et al. 2006; Bettazi et al. 2008), random amplified polymorphic DNA technique – RAPD (Galderisi et al. 1999), conventional polzmerase chain reaction – PCR (Hubalkova and Rencova 2011), real-time PCR methods (Arlorio et al. 2007; Mafra et al. 2008; D'Andrea et al. 2009; Köppel et al. 2010) or liquid chromatography/tandem mass spectrometry - LC/MS/MS methods (Schefcheck and Musser 2004).

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The purpose of this study was to develop a multiplex PCR method for the detection of two major food allergens (peanut and hazelnut) via targeting segments of genes as protein markers at one time in food products. Although the method is not quantitative, it can serve as a quick screening method to check the presence of allergens based on their declaration in food products. The method was verified by monitoring residues of these allergens in declared or undeclared food products on the retail market.

Materials and Methods

Samples of nuts and processed food products as well as other food commodities used for the primer specificity testing were obtained from different distributors on the Czech market. In total 9 samples with peanut declaration (two various cultivar samples from Georgia and Turkey) and 30 samples with hazelnut (three various cultivar samples from Argentina, Brazil, and India) declaration were purchased. For verification of the method, 18 samples without nut declaration or labelled "may contain nuts" or "produced in a factory, where nuts are processed" and 3 samples with declaration "contains nuts" were investigated. Furthemore, 30 samples of different food ingredients that could be a component of food products were tested for the potential cross-reactivities.

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

For the detection of two nut allergens, two sets of primers amplifying partial sequences of encoding genes were designed. The main peanut allergen Ara h 1 is encoded by *Ara h* 1 gene (GenBank accession number AF432231), and the major hazelnut allergen Cor a 1 is encoded by *Cor a* 1 gene (GenBank accession number Z72440). The primers were designed using program Primer3 (Anonymous 2013) or the simplification multiplex PCR system was constructed. For these purposes Multiplex PCR kit (Qiagen, Hilden, Germany) was used. The reaction mix contained 25 µl of 2 × Qiagen Multiplex PCR Master Mix, 5 µl of primer mix (three primer pairs – for the amplification of *Ara h* 1, *Cor a* 1 and *chloroplast* gene), 15 µl RNase-free water and 5 µl of DNA template. The total volume of the reaction mix was 50 µl. The primer mix was prepared according to the instructions recommended in the handbook, so that each primer was of 2 µM concentration. Ten µl of each defined DNAs peanut or hazelnut (10 $ng \cdot \mu l^{-1}$ were mixed in 70 µl of AE buffer and 5 µl of this DNA mix served as the PCR positive control. Distilled water instead of DNA served as negative control. The PCR program was modified according to the experimental studies. Finally, it consisted of the initial denaturation step (95 °C, 15 min), 35 cycles composed of the denaturation step (94 °C, 30 s), annealing (65 °C, 90 s) and extension (72 °C, 90 s), and the final extension step (72 °C, 10 min).

Separation and visualization of PCR products was carried out using horizontal electrophoresis in 2.5% agarose gel on a 1 X TBE containing 0.1 $\mu l \cdot ml^{-1}$ GelGreen stain. A 50 bp ladder (Qiagen, Hilden, Germany) was used as a marker. The amplification fragments were analyzed under UV light using an ultraviolet transluminator (Spectroline, Westbury, New York).

The sensitivity (practical limit of detection) of the method was tested on a model mixtures composed of 0.0001, 0.001, 0.01, 0.1, and 1 g (%) peanut/hazelnut content in 100 g (%) of neutral matrix, wheat flour (Figs. 1a and 1b). The practical detection limit was 0.001% w/w (10 $mg \cdot kg^{-1}$) both for peanuts and and for hazelnuts. This process was repeated × 4 and the homogeneity of spikes was guaranteed using a special blender (Retch, Germany).

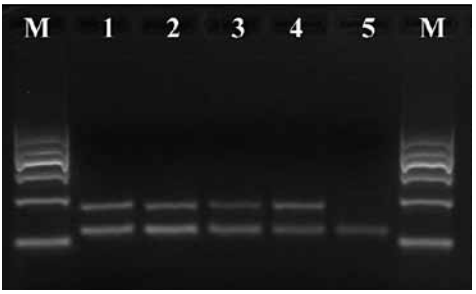

Fig. 1a. Limit of detection of multiplex PCR method for peanut allergen *Ara h 1*. Lane M, GelPilot 100 bp Ladder (Qiagen GmbH, Hiden, Germany) fragments 600, 500, 400, 300, 200, 100 base pairs); lane 1, 1%; lane 2, 0.1%; lane 3, 0.01%; lane 4, 0.001%; lane 5, 0.0001%.

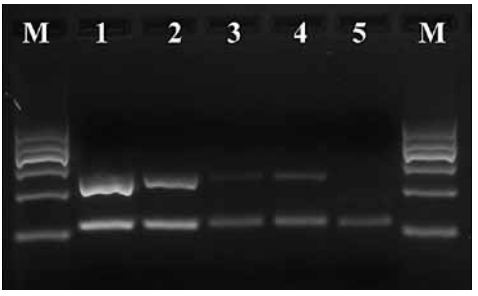

Fig. 1b. Limit of detection of multiplex PCR method for the hazelnut allergen *Cor a 1*. Lane M, GelPilot 100 bp Ladder (Qiagen GmbH, Hiden, Germany) fragments 600, 500, 400, 300, 200, 100 base pairs); lane 1, 1%; lane 2, 0.1%; lane 3, 0.01%; lane 4, 0.001%; lane 5, 0.0001%.

The intrinsic detection limit was obtained by dilution of DNA isolated of peanuts and hazelnuts and is 10 pg·ml^{-1} (data not shown).

The potential cross reactions were tested on a broad range of ingredients, that could be components of investigated food products. The list of 30 ingredients tested was following: peanuts/hazelnuts, walnuts, Brazil nuts, cashew nuts, pistachios, wheat, barley, rye, oat, sunflower seeds, cucurbit, sesame seeds, lentils, peas, beans, maize, rice, potato starch, apples, pineapples, strawberries, kiwi, plums, apricots, peaches, cherries, sour cherries, raisins, apricots kernels, and almonds. No cross reactions occurred.

To check the efficiency of DNA isolation, universal plant primers of *chloroplast* gene (Acc. No. AF076774) for plant DNA amplification were added to the multiplex PCR reaction (Taberlet et al. 1991).

The authenticity of two plant allergens was confirmed via the sequencing of corresponding marker sequences of *Ara h* 1 and *Cor a* 1 genes and the alignment of obtained sequencing with the sequences deposited in GenBank. The PCR products were purified using the PCR Purification Kit (Qiagen, Hilden, Germany) and sent to a commercial facility for sequencing (MWG Biotech, Ebersberg, Germany).

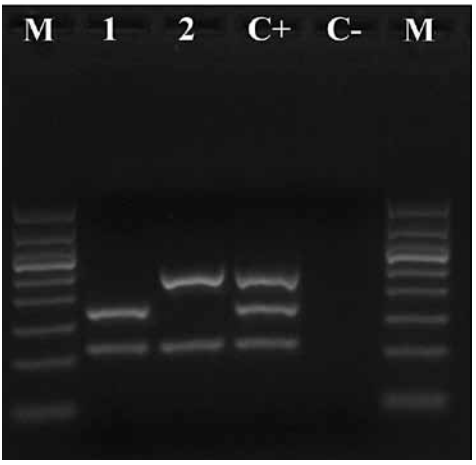

Fig. 2. The separation and visualisation of PCR products of the multiplex PCR method for the nut allergens detection. Lane M, GelPilot 50 bp Ladder (Qiagen GmbH, Hiden, Germany) fragments 500, 400, 350, 300, 250, 200, 150, 100, 50 base pairs); lane 1, primers for *Ara h* 1 gene (180 bp); lane 2, primers for *Cor a* 1 gene (258 bp); C+, positive control; C-, negative control; lanes 1, 2, and C+; primers for *Corythophora alta chloroplast* gene (124 bp).

Results

In this study polymerase chain reactions were designed for the amplification of partial sequences of *Ara h* 1 (peanuts) gene (180 bp) and *Cor a* 1 (hazelnuts) gene (258 bp) which is demonstrated in Fig. 2. The identity of amplicons was confirmed by sequencing and alignment of sequences with sequences deposited in Genbank. While testing the specificity of designed primer pairs on a spectrum of food ingredients, no cross reactions were detected. A multiplex PCR for the simultaneous detection of two main allergens was constructed with the sensitivity of 0.001% w/w (10 mg·kg^{-1}) for both peanuts and hazelnuts. A potential inhibition of PCR reaction was eliminated using the universal plant primers (Taberlet et al. 1991) (Table 1).

This multiplex PCR method was applied to the analysis of 60 commercial food products declared as "with nuts, peanuts, hazelnuts or without nuts". In all samples with peanut declaration, peanut DNA was detected. In samples with hazelnut declaration, hazelnut

Table 1. Primer pairs designed for PCR.

Plant species	Allergen	Gene	Sequences of primers (5‘ - 3‘)	Accesion Number (Gen Bank)	The PCR product (bp)
Arachis hypogaea	Ara h 1	*Ara h* 1	agagggagatatcaccaacccaatc gagttgaagtgtgggagcatcaaag	AF432231	180
Corylus avellana	Cor a 1	*Cor a* 1	aaaggccatcaagagcattg catcgccttcaatcacactg	Z72440	258
Corythophora alta	-	*Chloroplast*	cggacgagaataaagatagagt ttttggggatagagggacttg	AF076774	124

Table 2. The investigation of food products and heat-processed cookies with declared or undeclared peanut and hazelnut content.

Food sample	Product description	Matrix declaration (%)	*Ara h* 1	*Cor a* 1	Plant DNA
1	Peanuts	Peanuts	+	-	+
2	Peanut groats	Peanuts	+	-	+
3	Chocolate with peanuts	Peanuts 12,0	+	-	+
4	Gaufre with peanuts	Peanuts 11,0	+	-	+
5	Gaufre with peanuts	Peanuts 11,5	+	-	+
6	Gaufre with peanuts	Peanuts 11,0	+	-	+
7	Peanuts	Peanuts	+	-	+
8	Peanut spread	Peanuts 31,0	+	-	+
9	Peanuts	Peanuts	+	-	+
10	Hazelnuts	Hazelnuts	-	+	+
11	Wafer with hazelnut filling	Hazelnuts 5,1[a]	-	+	+
12	Wafer with hazelnut filling	Hazelnuts 3,5	-	+	+
13	Chocolate with hazelnut	Hazelnuts 20,0[a]	-	+	+
14	Wafer with hazelnut filling	Hazelnuts 3,0[a]	-	+	+
15	Wafer with hazelnut filling	Hazelnut aroma[a]	-	-	+
16	Hazelnut cake	Hazelnuts[a]	-	+	+
17	Wafer with hazelnut filling	Hazelnut aroma[a]	-	-	+
18	Cookies with hazelnut flavour	Hazelnut cream[a]	-	-	+
19	Wafer with whole hazelnuts	Hazelnuts 17,8	-	+	+
20	Müsli with hazelnuts	Hazelnuts 9,0[a]	-	+	+
21	Cereals with chocolate and nuts	Hazelnut cream 1,8	-	-	+
22	Crunchy cookies	Hazelnut aroma[a]	-	-	+
23	Cereal biscuits with nuts and honey	Hazelnuts 3,2[a]	-	-	+
24	Cocoa-nut biscuits with cereals	Hazelnuts 3,2[a]	-	-	+
25	Müsli with apricots in yoghurt sauce	Hazelnuts[a]	+	+	+
26	Wafer with hazelnut filling in chocolate	Hazelnuts 1,6[a]	+	+	+
27	Wafer with hazelnut filling	Hazelnut aroma	-	-	+
28	Wafer with hazelnut filling	Hazelnuts[a]	-	+	+
29	Wafer with hazelnut filling	Hazelnuts 3,0	-	+	+
30	Müsli with nuts	Hazelnuts 5,0[a]	-	+	+
31	Cocoa biscuits with hazelnut flavour	Hazelnuts 1,5	-	+	+
32	Müsli with chocolate and nuts	Hazelnuts 1,0	-	+	+
33	Chocolate müsli with nuts	Hazelnuts 1,0[a]	-	+	+
34	Müsli with nuts	Hazelnuts 5,0[a]	-	+	+
35	Hazelnuts in caramel	Hazelnuts	-	+	+
36	Müsli with nuts and almonds	Hazelnuts[a]	-	+	+
37	Yoghurt with hazelnuts	Hazelnuts	-	-	+
38	Milk chocolate	Hazelnut cream[a]	-	+	+
39	Milk chocolate	Hazelnut cream[a]	-	+	+
40	Müsli	-	-	-	+
41	Oat flakes	-	-	-	+
42	Gaufre with cocoa filling	- [a]	-	-	+

[a] Declared as “may contain residues of nuts” or “produced in a nut-processing factory”.

Table 2. The investigation of food products and heat-processed cookies with declared or undeclared peanut and hazelnut content.

Food sample	Product description	Matrix declaration (%)	*Ara h* 1	*Cor a* 1	Plant DNA
43	Oatmeal with apples and cinnamon	- [a]	-	-	+
44	Shortbread cookies	-	-	-	+
45	Yoghurt with chocolate flakes	- [a]	-	-	+
46	Candy with chocolate filling	- [a]	-	-	+
47	Oatflakes with chocolate	-	-	-	+
48	Croissant	- [a]	-	-	+
49	Gingerbread with strawberries	- [a]	-	-	+
50	Müsli with yoghurt, cranberries and honey	-	-	-	+
51	Gaufre with cocoa filling	- [a]	-	-	+
52	Gaufre with cocoa filling	- [a]	-	-	+
53	Müsli	- [a]	-	-	+
54	Cereal stick with milk filling	- [a]	-	-	-
55	Gingerbread with chocolate filling	-	-	-	+
56	Gofri waffle	- [a]	-	-	+
57	Whole bread	-	-	-	+
58	Nut gaufre	- [a]	-	-	+
59	Nut croissant	-	+	-	+
60	Nut biscuit	-	-	-	+

[a] Declared as "may contain residues of nuts" or "produced in a nut-processing factory".

DNA was detected, although in some samples with the labelling of hazelnut aroma content (samples 15, 17, 18, 21, 22, and 27) the DNA was not detected. The aroma is probably added as an artificial nut flavour during food processing. Plant DNA was amplified in all analyzed samples (124 bp amplicon), thereby the potential inhibition of PCR or possible errors in pippeting or in DNA isolation could be excluded. In samples 25 and 26 with hazelnut declaration only, the presence of peanut was detected (Table 2). Indeed, these food products present a notice on their package that they were produced in nut-processing factories. In samples with undeclared nut content, no peanuts or hazelnuts were identified. In three samples with "contains nuts" labelling, peanuts were detected in sample 59 only.

Discussion

Köppel et al. (2010) present two tetraplex real-time PCR for the detection and quantification of DNA from eight allergens in food where peanuts, hazelnuts are also included with the sensitivity of 0.01%. The sensitivity of reported SYBR-Green I real-time PCR to detect hazelnut (*Corylus avellana*) in foods through calibration via plasmid standard was 0.001% using model samples based on *Cor a* 8 gene as a marker (D'Andrea et al. 2009). Shefcheck and Musser (2004) used LC/MS/MS for the determination of specific *Ara h* 1 peptides at a level of 0.001%. Wang et al. (2011) report a novel method for the rapid identification of eight food allergens through the use of a silicon-based optical thin-film bisensor chips with the practical detection limit of 0.001%. Bergerova et al. (2011) and Costa et al. (2012) described a novel single-tube nested PCR for the detection of peanuts and hazelnuts traces with high sensitivity of 0.375 pg DNA isolated from leaves of *Arachis*

hypogaea and 50 mg·kg^{-1} of hazelnut in wheat material with the intrinsic LOD of 0.5 pg of hazelnut DNA. López-Calleja et al. (2013) developed a real-time PCR for detection of trace amounts of peanut in processed food with detection levels of 10 and 0.1 ppm. And again Costa et al. (2014) assessed hazelnut allergens detection using three approches as ELISA, LC-MS/MS and real-time PCR with similar sensitivity levels of approximately 1 mg·kg $^{-1}$ and limits of quantification of 50–100 mg·kg^{-1} in all performed methods.

Regarding the sensitivity which is very important in case of food allergens detection where 5% of the allergic population is likely to respond with objective reaction to the minimal allergenic protein dose of 1.6 mg for peanut and 0.9 mg for hazelnut but thresholds for any symptom can be × 2–6 lower (Blom et al. 2013), our method is in conformity with the majority of discussed studies, and can serve as a helpful tool for the confirmation of the presence of peanut and hazelnut allergens in a food product.

The primer pair generating a 124 bp amplified fragment in *Corythophora alta*, prevents the detection of false negative results caused by PCR inhibitors.

The advantage of the method is the detection of two plant allergens in one step and at one-time reaction. This multiplex PCR method is inexpensive, specific, and sensitive enough and can be used as a simple, one day procedure for the checking of undeclared peanut and hazelnut major allergens in retail food products with satisfactory sensitivity, and expensive laboratory equipment is not required for routine every-day screening control.

Acknowledgements

The results of the project LO1218 were obtained with the financial support from the the Ministry of Education, Youth and Sports of the Czech Republic under the NPU I program and grant of the Ministry of Agriculture of the Czech Republic (QG 60090/2005).

References

Anonymous 2013: Program Primer 3. http://bioinfo.ut.ee/primer3/

Arlorio M, Cereti E, Coisson JD, Travaglia F, Martelli A 2007: Detection of hazelnut (*Corylus* spp.) in processed foods using real-time PCR. Food Control **18**: 140-148

Bettazi F, Lucarelli F, Palchetti I, Berti F, Marrazza G, Mascini M 2008: Disposable electrochemical DNA-array for PCR amplified detection of hazelnut allergens in foodstuffs. Anal Chem Acta **614**: 93-102

Bergerova E, Brezna B, Kuchta T 2011: A novel method with improved sensitivity for the detection of peanuts based upon single-tube nested real time polymerase chain reaction. Eur Food Res Technol **232**: 1087-1091

Blom WM, Vlieg-Boestra BJ, Kruizinga AG, van der Heide S, Houben GF, Dubois AEJ 2013: Threshold dose distributions for 5 major allergenic foods in children. J Allergy Clin Immunol **131**: 172-179

Costa J, Mafra I, Kuchta T, Oliveira MBPP 2012: Single-tube nested real-time PCR as new highly sensitive approach to trace hazelnuts. J Agric Food Chem **60**: 103-8110

Costa J, Ansari P, Mafra I, Beatriz M, Oliveira PP, Baumgartner S 2014: Assessing hazelnuts allergens by protein - and DNA – based approaches: LC-MS/MS, ELISA and real-time PCR. Anal Bioanal Chem **406**: 2581-2590

D'Andrea M, Coisson JD, Travaglia F, Garino C, Arlorio M 2009: Development and validation of a SYBR-Green I real time PCR protocol to detect hazelnut (*Corylus avellana*) in foods through calibration via plasmid reference standard. J Agric Food Chem **57**: 11201-11208

Doi H, Touhata Y, Shibata H, Sakai S, Urisu A, Akiyama H, Teshima R 2008: Reliable enzyme-linked immunosorbent assay for the determination of walnut proteins in processed foods. J Agric Food Chem **56**: 7621-7630

Galderisi U, Cipollaro G, Bernardo L, De Masi G, Cascino A 1999: Identification of hazelnut (*Corylus avellana*) cultivars by RAPD Analysis. Plant Cell Rep **18**: 652-655

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. J Agric Food Chem **50**: 5808-5815

Hubalkova Z, Rencova E 2011: One step multiplex PCR method for the determination of pecan and Brazil nut allergens in food products. J Sci Food Agric **13**: 2407-2411

Köppel R, Dvorak V, Zimmerli F, Breitenmoser A, Euhster NA, Waiblinger HU 2010: Two tetraplex real-time PCR for the detection and quantification of DN from eight allergens in food. Eur Food Res Technol **230**: 367-374

Koppelman SJ, Nieuwenhuizen WF, Gaspari M, Knippels LMJ, Penninks AH, Knol EF, Hefle SL,

De Jongh HHJ 2005: Reversible denaturation of Brazil nut 2S albumin (Ber e 1) and implication of structural destabilization on digestion by pepsin. J Agric Food Chem **53**: 123-131

Lopez-Calleja IM, de la Cruz S, Pegels N, Gonzales I, Martin R 2013: Development of real-time PCR assay for detection of allergenic trase amounts of peanuts (*Arachis hypogaea*) in processed foods. Food Control **30**: 480-490

Mafra I, Ferreira I M P LVO, Oliveira MBPP 2008: Food authentication by PCR-based methods. Eur Food Res Technol **227**: 649-665

Malanin K, Lundberg M, Johansson SGO 1995: Anaphylactic reaction caused by neoallergens in heated pecan nut. Allergy **50**: 988-991

Noorbakhsh R, Mortazavi SA, Shahidi F, Maleki SJ, Nasirah LR, Falak R, Sima HR, Varasteh AR 2010: Influence of processing on the allergenic properties of pistachio nut assessed *in vitro*. J Agric Food Chem **58**: 10231-10235

Poms RE, Klein CL, Anklam E 2004: Methods for allergen analysis in food: a review. Food Add Contam Part A **21**: 1-31

Schefcheck KJ, Musser SM 2004: Confirmation of the allergenic peanut protein, *Ara h* 1, in model food matrix using liquid chromatography/tandem mass spectrometry (LC/MS/MS). J Agric Food Chem **52**: 2785-2790

Taberlet P, Gielly L, Pautou G, Bouvet J 1991: Universal primers for amplification of three non-coding regions of chloroplast DNA. Plant Mol Biol **17**: 1105–1109

Wang W, Han, JX, Wu YJ, Yuan F, Chen Y, Ge YQ 2011: Simultaneous detection of eight food allergens using optical thin-film bisensor chips. J Agric Food Chem **59**: 6889-6894

World Health Organization (WHO) 1999: International Programme on Chemical Safety Principles and Methods for Assessing Allergic Hypersensitization Associated with exposure to Chemicals. Environmental Health Criteria No. 212. World Health Organization, Geneva Switzerland

Yman IM, Eriksson A, Johansson MA, Hellenas KE 2006: Food allergen detection with biosensor immunoassays. J AOAC Int **89**: 856-861