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Multiplex, Quantitative, Ligation-Dependent Probe Amplification for Determination of Allergens in Food

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ABSTRACT: Legislation requires labeling of foods containing allergenic ingredients. Here, we present a robust 10-plex quantitative and sensitive ligation-dependent probe amplification method, the allergen−multiplex ligation-dependent probe amplification (MLPA) method, for specific detection of eight allergens: sesame, soy, hazelnut, peanut, lupine, gluten, mustard, and celery. Ligated probes were amplified by polymerase chain reaction (PCR), and amplicons were detected using capillary electrophoresis. Quantitative results were obtained by comparing signals with an internal positive control. The limit of detection varied from approximately 5 to 400 gene copies, depending on the allergen. The method was tested using different foods spiked with mustard, celery, soy, or lupine flour in the 1−0.001% range. Depending on the allergen, sensitivities were similar or better than those obtained with qPCR. The allergen-MLPA method is modular and can be adapted by adding probe pairs for other allergens. The DNA-based allergen-MLPA method will constitute a complementary method to the traditional protein-based methods.

KEYWORDS: Ligation, DNA amplification, allergen, capillary electrophoresis, MLPA

INTRODUCTION

Food allergies are a major health concern in industrialized countries and may affect up to 3% of the adult population and 6−8% of the children in Europe.[1−3] The number of food allergen-induced life-threatening syndromes is increasing. The consumption of allergens by patients with hypersensitivity can trigger a variety of immunological reactions ranging from hives, pruritus, atopic dermatitis, swelling of the throat or facial tissues, vomiting, diarrhea, asthmatic wheeze, difficulty in breathing, and hypotension to life-threatening anaphylaxis.[4] A large number of anaphylactic reactions to food are treated in emergency departments each year, and it is estimated that food allergy causes several deaths annually (see ref 5 and references therein). The level of exposure to provoke a reaction varies from food to food and from person to person. Most often, reactions are elicited after exposure of 1−100 ppm of an allergen, but sometimes, only minute amounts are required. Treatment of food allergy is difficult, and avoiding the allergen-containing food is often the only option. This may sometimes be difficult, especially for processed foods, which may contain allergens either added deliberately, for example, when using spices containing celery powder, or unintentionally, when foods are contaminated during shipping and storage and from food production lines, etc. In addition, labeling errors may occur before the product leaves the manufacturing facility.

Allergenic foods are used as ingredients in many food products due to their nutritional properties, or as food additives, processing aids, and edible films (e.g., wheat gluten, casein, and soy). For example, soy may be found as an ingredient or food additive in vegetarian products, ice cream, sauces, cream, desserts, chocolate, meat products, milk products, cakes, peanut butter, tuna, muesli, infant food, soups, and convenience foods. Allergic consumers rely on accurate food labels to make informed choices to be able to avoid offending allergens. To minimize or eliminate the risk of cross-contamination, the industry needs to have in place an allergen control program, which is usually integrated as part of its Hazard Analysis and Critical Control Point (HACCP) program. Analytical techniques can be used as tools to monitor potential errors before, during, and after manufacturing activities. Detection methods for allergens are used for screening, routine, and confirmatory analysis.[6]

Legislation has been implemented in many countries aiming to achieve a high level of health protection for allergic consumers. The Food Allergen Labeling & Consumer Protection Act (FALCPA) came into effect in the United States at the beginning of 2006.[7] The FALCPA addresses the most common allergenic foods in the United States and requires the identification on the food label of ingredients derived from common allergenic sources. This implies mandatory labeling of foods containing milk, eggs, fish, crustacean shell fish, peanuts, soybeans, wheat, and tree nuts (hazelnut, walnut, almond, etc.).[8] Except for the lack of labeling of sulfites, this list is in accordance with the recommendations of the Codex Alimentarius Commission. Canada, Australia, and New Zealand, in addition to the allergens listed in FALCPA, require labeling of sesame seeds and sulfites. EU Regulation No. 852/2004[9] states that the primary responsibility for food safety stays with the food business operator. In addition to this general regulation, Directives 2000/13/EC[10] and 2003/89/EC[11] require mandatory declaration of allergenic foods, namely, those listed by the FALCPA and in addition to these celery, mustard, sesame seeds, and sulfites. Commission Directive 2006/142/EC has also included lupine and molluscs in the list.[12] The EU allergen list is intended to be dynamic, and more allergens may be included over time.

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Table 1. Foods Analyzed Using Allergen-MLPA

species	ingredient	allergen-MLPA[a] sesame	soy	hazelnut	peanut	lupine	gluten	mustard	celery
sesame (*Sesamum indicum*)	white sesame seeds	+	−	−	−	−	−	−	−
	black sesame seeds	+	−	−	−	−	−	−	−
soy (*Glycine max*)	flour	−	+	−	−	−	−	−	−
hazelnut (*Corylus avellana*)	hazelnuts	−	−	+	−	−	−	−	−
peanut (*Arachis hypogaea*)	peanuts	−	−	−	+	−	−	−	−
lupine (*Lupinus* spp.)	flour	−	−	−	−	+	−	−	−
wheat (*Triticum aestivum*)	flour	−	−	−	−	−	+	−	−
barley (*Hordeum vulgare*)	flour	−	−	−	−	−	+	−	−
rye (*Secale cereale*)	flour	−	−	−	−	−	+	−	−
oat (*Avena sativa*)	flour	−	−	−	−	−	+	−	−
black mustard (*Brassica nigra*)	mustard seeds	−	−	−	−	−	−	+	−
Oriental mustard (*Brassica juncea*)	mustard seeds	−	−	−	−	−	−	+	−
yellow mustard (*Sinapis alba*)	mustard seeds	−	−	−	−	−	−	+	−
celery (*Apium graveolens*)	celery sticks	−	−	−	−	−	−	−	+
	celeriac	−	−	−	−	−	−	−	+
	celery seeds	−	−	−	−	−	−	−	+
cashew nuts (*Anacardium occidentale*)		−	−	−	−	−	−	−	−
Brazil nuts (*Bertholletia excelsa*)		−	−	−	−	−	−	−	−
almond (*Prunus dulcis*)		−	−	−	−	−	−	−	−
carrot (*Daucus carota*)		−	−	−	−	−	−	−	−
paprika (*Capsicum annuum*)		−	−	−	−	−	−	−	−
parsley (*Petroselinum crispum*)		−	−	−	−	−	−	−	−
leek (*Allium ampeloprasum*)		−	−	−	−	−	−	−	−
maize (*Zea mays*)		−	−	−	−	−	−	−	−
garlic (*Allium sativum*)		−	−	−	−	−	−	−	−
pea (*Pisum sativum*)		−	−	−	−	−	−	−	−
radish (*Raphanus sativus*)		−	−	−	−	−	−	+	−
broccoli (*Brassica oleracea*)		−	−	−	−	−	−	+	−
Swede (*Brassica napobrassica*)		−	−	−	−	−	−	+	−
Chinese cabbage (*Brassica rapa*)		−	−	−	−	−	−	+	−
cabbage (*Brassica oleracea*)		−	−	−	−	−	−	+	−

[a] Analyzed using undiluted and 10-fold diluted template DNA. For probes binding to several templates, cross-reactivity was 100% since the template DNAs were similar.

Traditionally, in vitro allergen testing has consisted of detecting the presence of the allergenic proteins. However, the detection of allergens in food can be very difficult, as they are often present in trace amounts and are masked by the food matrix.[13] In many such cases, or when good immunological methods are not readily available, DNA-based methods constitute an alternative. Reviews on current detection methods have been published.[14−16] The choice of method is mainly dependent on the food and the allergen concerned (i.e., the availability of specific antibodies/DNA primers and the achievable detection limit) and on the history of processing during food production.[17] The benefits and disadvantages of the protein and DNA based methods have been reviewed and involve considerations in conjunction with detectability, specificity, limit of detection (LOD), variability of target, matrix effects, existence of reference materials, sample preparation, required equipment, and costs.[17,18]

We have previously reported quantitative polymerase chain reaction (qPCR) methods for the detection of celery, mustard, and sesame.[19] As the number of food allergen increases, multiplex detection may be preferable when enforcing the legislation in relation to labeling. Multiplex DNA-based methods have been developed for wheat, buckwheat, and peanut,[20] hazelnut and peanut,[21] and two major allergens of hazelnut.[22] A multiplex ligation-dependent probe amplification (MLPA) method for the detection of nine different nuts and sesame has been described.[23] Recently also, two tetraplex qPCRs were developed for the detection of peanut, hazelnut, celery, soy, egg, milk, almond, and sesame[24] and a six-plex qPCR for the detection of almond, hazelnut, cashew, peanut, walnut, and sesame.[25] In addition, a multiplex detection system for eight fish species based on xMAP technology has been devised.[26] Toward the establishment of thresholds for the major food allergens, allergen detection methods should preferably quantitative.[27,28]

Here, we present a novel quantitative, 10-plex, competitive MLPA method for simultaneous detection of sesame, soy, hazelnut, peanut, lupine, gluten, mustard, and celery together with an internal positive control (IPC).

Table 2. Detection of DNA from Allergen Material in Spiked Foods Using Allergen-MLPA

spiked food	added allergen	allergen (% w/w)	detection by allergen-MLPA[a]	C_T value from qPCR[b]
hot chili	mustard	1	9/9	39.7
spice	(powder)	0.1	12/12	ND
		0.05	6/6	ND
		0.01	15/15	ND
		0.001	7/15	ND
	celery (spice)	1	6/6	38.3
		0.1	8/12	ND
		0.05	3/9	ND
		0.01	2/3	ND
		0.001	1/3	ND
jytte flour[c]	soy flour	1	6/6	31.4
		0.1	5/9	34.4
		0.05	3/3	35.3
		0.01	1/3	37.6
	lupine flour	1	9/9	33.4
		0.1	10/10	37.4
		0.05	6/6	38.4
		0.01	14/14	ND
		0.001	15/15	ND
	soy + lupine	0.1 + 0.1	3/3 + 3/3	–
wheat flour	soy flour	0.1	6/6	–
		0.05	3/3	–
		0.01	3/3	–

[a] X/Y, X positive of Y determinations. [b] The same DNA samples analyzed by qPCR; ND, not detected (after 43 cycles); –, not determined. [c] Jytte flour (Holmen Crisp): A flour mixture substitute for wheat containing rice flour, buckwheat, psyllium shells, potato starch, tara gum (E417), and *Caesalpinia spinosa* (E410 and E415). Samples tested in jytte flour were without gluten probes because of presence of strong gluten signals from the unspiked flour.

MATERIALS AND METHODS

Materials and DNA Isolation. The materials analyzed in Tables 1 and 2 were from local retail stores. Lupine flour was a gift from L. H. Moen, Norwegian Veterinary Institute, Oslo, Norway. The samples were homogenized in a food processor and thereafter ground in a mortar. Nuts were ground with liquid nitrogen. DNA was isolated by adsorption to columns using the DNeasy plant mini kit (Qiagen, Hilden, Germany) as described by the manufacturer with the following modifications. The initial buffer volume was doubled, and lysis was carried out for 30 min at 65 °C in a shaking incubator. When eluting DNA bound to the column, 2 × 50 μL of buffer was used. Chromosomal DNA was analyzed by agarose gel electrophoresis. The isolated DNA was analyzed for quality and quantity with a Nano-Drop ND-1000 spectrophotometer (Saveen Werner, United States) and stored at −20 °C.

Allergen-MLPA Reaction. A total of 10 sets of MLPA probes specific for eight allergens were designed according to the general suggestions of MRC-Holland (Amsterdam, The Netherlands) http://www.mrc-holland.com/WebForms/WebFormMain.aspx) (Table 3). The probes were purchased from Eurofins mwg operon (Ebensburg, Germany). The MLPA method is described in ref 29. Allergen-MLPA was performed according to a standard protocol developed by MRC-Holland (Amsterdam, The Netherlands) with modifications. Routinely, 50−300 ng of DNA samples in 5 μL of ddH_2O was denatured at 98 °C for 5 min. After the samples were cooled to 25 °C, 3 μL of the probe solution consisting of 1.5 μL of SALSA MLPA buffer and 1.5 μL of probe mix was added. We used 0.6 fmol of each of the probes. Hybridization of probes was performed at 60 °C for 17−18 h. Ligation of the hybridized probes in 40 μL of reaction mixtures was performed at 54 °C for 25 min followed by inactivation of the ligase-65 at 98 °C for 5 min. The ligated probes were used as templates for the universal SALSA PCR primers (LMunivBF/LmunivBR, Table 3) in the following PCR reactions. For PCR, 10 μL of the MLPA ligation reaction was mixed with 2 μL of SALSA PCR primers and 25 μL of 2 × Qiagen Multiplex PCR kit (Qiagen, Hilden, Germany) in 50 μL of total reaction. The PCR program included a treatment of 95 °C for 15 min followed by 43 cycles of 94 °C, 30 s; 60 °C, 90 s; 72 °C, 90 s; and final extension at 72 °C for 10 min. The PCRs were carried out on a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA). Only ligated probes are amplified in this step, thus selecting for DNA from allergenic materials. In the allergen-MLPA, 7400 copies of linearized and denatured recombinant plasmid pSIP409[30] were added as an IPC. The amplicon was spanning the junction region between the plasmid vector and the insert and was thus unique. All dilutions of plasmid DNA were done in *Escherichia coli* DNA (10 ng/μL) to avoid unspecific adsorption. In some cases, the probe pairs for gluten were omitted. In these cases, no other changes in the method were done. Experiments were performed with three parallels. Dilution experiments and spiking experiments were repeated twice with three parallels for each repetition.

Capillary Electrophoresis (CE). For each sample, 1 μL of the PCR reaction, 9 μL of HiDi formamide and 0.5 μL of Genescan 500 LIZ size standard were mixed. The samples were denatured for 2 min at 94 °C and then cooled on ice. The plate was run on a 3130xl Genetic analyzer (Applied Biosystems), and data were collected during fragment separation. The injection time was 5−22 s, and the electrophoresis conditions were as follows: run time 1800 s at 15000 V; run current, 5 μA; and 60 °C run temperature. GeneMapper 4.0 (Applied Biosystems) was used to extract data on the height, area, and size of fluorescent PCR fragments in the obtained electropherograms. For quantitative considerations, 2-fold dilutions of template DNA were analyzed while keeping the amount of IPC constant. When the amplification efficiency of the allergen DNA is the same as that of the IPC, the relative amounts of DNA can be found by plotting log(area allergen peak/area IPC peak) vs log(dilution of allergen DNA). Ideally, this should give a straight line with slope = 1.[31] Statistical analyses were performed using Excel 2003 SP2.

qPCR. 5′-Nuclease PCR was performed on a 7900 HT Sequence Detection System (Applied Biosystems) using oligonucleotide probes labeled with 5′-FAM reporter dye and a 3′-TAMRA quencher dye (Table 4) as described previously.[32] Samples were analyzed with three parallels.

RESULTS

Development of the Allergen-MLPA Method. Specific ligation probes were designed by going through the literature on PCR detection of the various allergens and choosing regions in the vicinity of or overlapping with known specific PCR primer/probe binding sites (Table 3). The allergen-MLPA probes were equipped with a suitable number of linker bases to give amplicons that were easily separated by CE. No redesigning of probe sets was necessary with regards to hybridizing sequence. However, one celery probe was redesigned with an expanded linker region to avoid overlapping with other signals.

Preliminary experiments using single templates from known allergens in combination with single probe sets gave single peaks with the expected sizes and showed that all probe pairs amplified their specific targets (results not shown). The amount of IPC,

Table 3. Probes and Primers Used in the Allergen-MLPA Method

target	probe name	sequence[a]	probe size (bases)	amplicon size		Genbank accession/reference[b]
				calculated (bp)	observed (bp)	
sesame	SesLMF1	**F**-cAGGGCTAGGGACCTTCCTCGCAGGTGCAA	49	99	97	AF240005.1
	SesLMF2	Pho-CATGCGACCCCAGCAATGCCAATTCCg-**RE**	50			
soya	SoyLMF1	**F**-cGGTAGCGTTGCCAGCTTCGCCGCT	44	105	103	K00821
	SoyLMF2	Pho-TCCTTCAACTTCACCTTCTATGCCCCTGACACAAAAAG-**RE**	61			
hazelnut	Has1LMF1	**F**-cGAGGATCCATCTTGAAGATCACCAGCAAGTACCACA	56	109	106	AF136945
	Has1LMF2	Pho-CCAAGGGCAACGCTTCAATCAATGAGGAGG-**RE**	53			
peanut	PeaLMF1	**F**-caagtctgacaaAGGGCGAACCTXAGGCCCTGCGAG	55	112	111	AY007229
	PeaLMF2	Pho-CAACATCTCATGCAGAAXATCCAACGTGACGAGGAg-**RE**	59			
hazelnut	Has2LMF1	**F**-cGAGATTGACCAAGCAAACTTTTCATATCGCTACAGTGTGATT	62	119	117	Z72440
	Has2LMF2	Pho-GAAGGCGATGCTTTGTCCGACAAACTGGAGAAAg-**RE**	57			
lupine	LupLMF1	**F**-ccgtcgtgaaagtctgacaaCCCCTCGTGTCAGGAGGCGCCXCAXCCTXTGC	71	127	124	Z72202
	LupLMF2	Pho-GGTXTCCTCCTGGCCTAATAACAAAACCCCGGg-**RE**	56			
gluten	GluLMF1	**F**-cgcaGAGACTCAATGGAAGCTGTTCTAACGAATCGAGTTAATTA	63	134	133	36
	GluLMF2	Pho-CGTTGTGTTGTTAGTGGAATTCCTTCTAATTCTAAATTAGagaaagag-**RE**	71			
mustard	SenLMF1	**F**-cgcagcaXXTXXTXAGXCGTATCTACCAGACXGCTACXCACTTACCTAXAGTT	72	139	138	S54101
	SenLMF2	Pho-TGCAACATXXXXCAAGTXAGXXTTTGTCCCTTCXAGaagaccag-**RE**	67			
IPC[c]	SIPLMF1	**F**-cacgtcgtgaaagtctgacaaAGCATATATGTATTCTATAAAATACTATTACAAGGAGATTTTAGCCATGGTA	92	143	141	pSIP409[30]
	SIPLMF2	Pho-CGTCCTGTAGAAACCCCAACCCGTGAAg-**RE**	51			
celery	CelLMBF1	**F**-ctgccacgtcgtgaaagtctgacaaGCTGAGTCACGATGAGCGTGTACTGAGTCAGTGTTATGTT	84	148	149	AF067082
	CelLMF2	Pho-TGGATTACGGTGTGATGAGTCAGCGTTATCTGTTTTTATAg-**RE**	64			
	LmunivBF[d,e]	FAM-GGGTTCCCTAAGGGTTGGA				
	LmunivBR[d]	GTGCCAGCAAGATCCAATCTAGA				

[a] Common probe sequences are **F** = GGGTTCCCTAAGGGTTGGA, and **RE** (Right extension) = TCTAGATTGGATCTTGCTGGCAC. Hybridizing sequences are in upper case, and extension sequences (linker bases) to give appropriate sizes of amplicons (in bp) and to increase amplification efficiency are in lower case. All LMF1 primers were hypur-purified, all LMF2 were purified by HPLC, and all LMF2 probes are 5′-phosphorylated (Pho). X, inosin. [b] The genes employed for MLPA probe construction were the same as those used in qPCR (Table 4), except for gluten for which the *trnL* intron was used and hazelnut (AF136945), which employed a *Cor a1* gene with a different sequence than that reported in Z72440. [c] IPC, plasmid pSIP409. [d] MLPA SALSA primers used for PCR amplification. [e] Primer is labeled with FAM in the 5′-end.

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Table 4. Primers and Probes for qPCR[a]

allergen	gene[1]	comments
sesame	*Sesamum indicum* 2S albumin AF240005	19
soy	lectin (*Le1*) K00821	Eurofins analysis note 050403
hazel	*Cor a1.04* Z72440	37
peanut	*AraH2* AY007229	38
lupine	*ITS1* internal spacer	39
gluten	*Glud1* U86029.1	40
mustard	*Sinapis alba* SinA1 S54101	19
IPC	plasmid pSIP409[b]	SIPF: CTATTACAAGGA-GATTTTAGCCATGGT SIPR: TTTCGCGAT-CCAGACTGAA SIPPR: fam-CGTCCTGT-AGAAACCCCAA-CCCGTG-tamra
celery	*Apium graveolens* mannitol dehydrogenase AF067082	19

[a] The code denotes the entry number in Genbank, NCBI. Primers and probes were from MWG Biotech (Germany). [b] Gift from Dr. L. Axelsson, Nofima Mat AS.

the recombinant plasmid pSIP409, was adjusted to give appropriate signal strength relative to the allergen signals (not shown). In a previously developed GMO-MLPA, we achieved higher sensitivity of the MLPA system by lowering the probe concentrations.[33] In the allergen-MLPA system, lowering of the probe concentrations, however, no similar increase in sensitivity was observed, and consequently, the probe concentrations were left unchanged. The signals for lupine and mustard were weaker when the probe concentrations were lowered (not shown).

Specificity. The fully developed system was tested for specificity using purified DNA from a number of different foods (Table 1). Results for selected samples are presented in Figure 1. The allergens all gave specific single signals close to their calculated sizes (Table 3). As was evident from Figure 1A,B, all eight allergens could be detected concomitantly when present in a mixture. The system gave very little unspecific background signals as can be seen from Figure 1C, where IPC gives a strong signal. When the system was overloaded, each allergen in addition showed an extra peak with an apparent size approximately 6−8 bases smaller than expected, as can be seen in Figure 1D,F,G. Both the hazelnut and the peanut probe pairs tested negative for cashew nuts, brazil nuts, and almonds. The mustard probe set also gave signals for radish, broccoli, Swede, Chinese cabbage, and cabbage as expected since the probe was specific for *Brassica* sp. rather than mustard. The gluten-specific probe gave positive signals for wheat, barley, and rye as expected. It also gave a strong positive signal for oat. For all other foods tested, no allergen signals were obtained. In these cases, only the IPC was detected, showing that the ligation and PCR amplification had worked properly.

Quantitative Considerations and Sensitivity. Because all ligated template DNA and the IPC were amplified using the same universal primer pair, the allergen-MLPA comprised a quantitative, multiplex, competitive PCR system where the amount of a specific

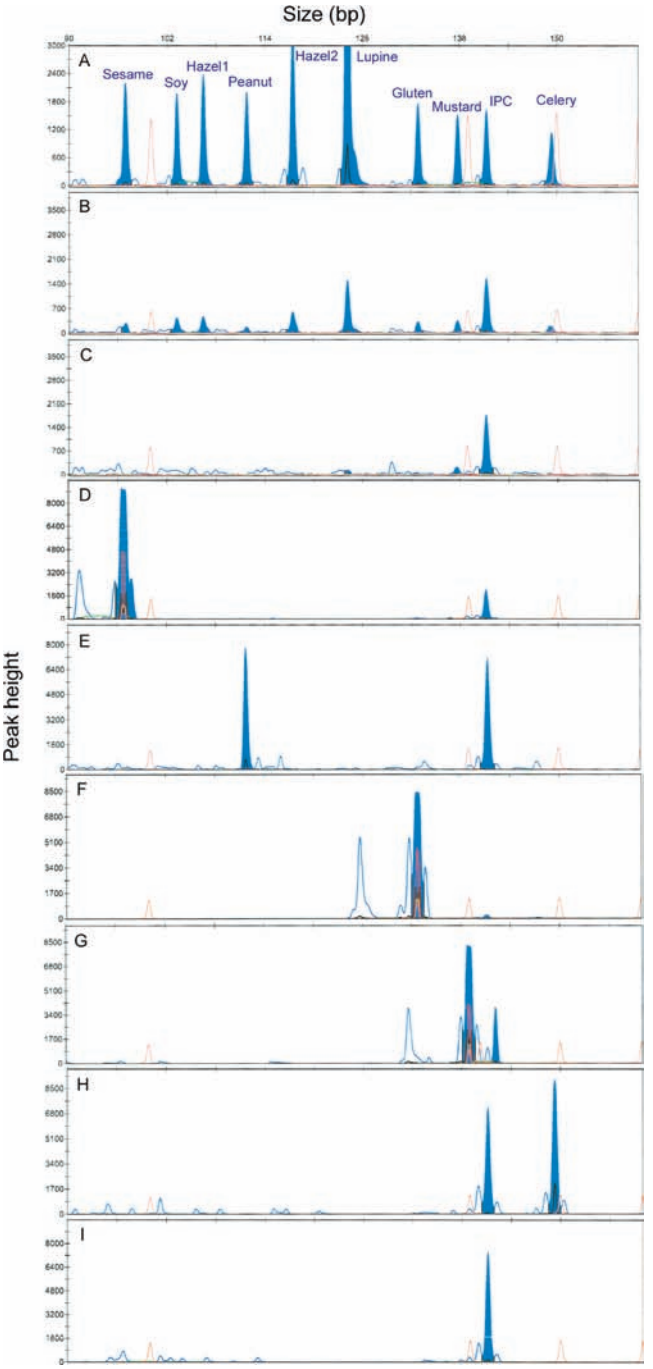

Figure 1. CE after allergen-MLPA of DNA from different allergenic materials. (A) Mix of DNA from sesame (3.8 ng), soy (13.6 ng), hazelnut (1.4 ng), peanut (18.4 ng), lupine (0.09 ng), gluten (2.6 ng), mustard (1.2 ng), and celery (1.2 ng). (B) Same mix as in panel A but with DNA diluted 8-fold. (C) No template control (NTC) with IPC. (D) Sesame. (E) Peanut. (F) Wheat. (G) Mustard. (H) Celery. (I) Brazil nut. IPC (5×10^{-5} ng) was added in all samples. Orange peaks are Genescan 500 Liz molecular weight markers. The extra peaks in panels D, F, and G are due to overload of the system (see the text).

allergen DNA could be determined by plotting the Log(area allergen-specific peak/area IPC peak) vs Log(amount allergen-specific DNA) from dilution series of template DNA. The amount of IPC was kept constant in all samples. Two-fold dilution series of mixtures of DNA from all eight allergenic foods were analyzed in

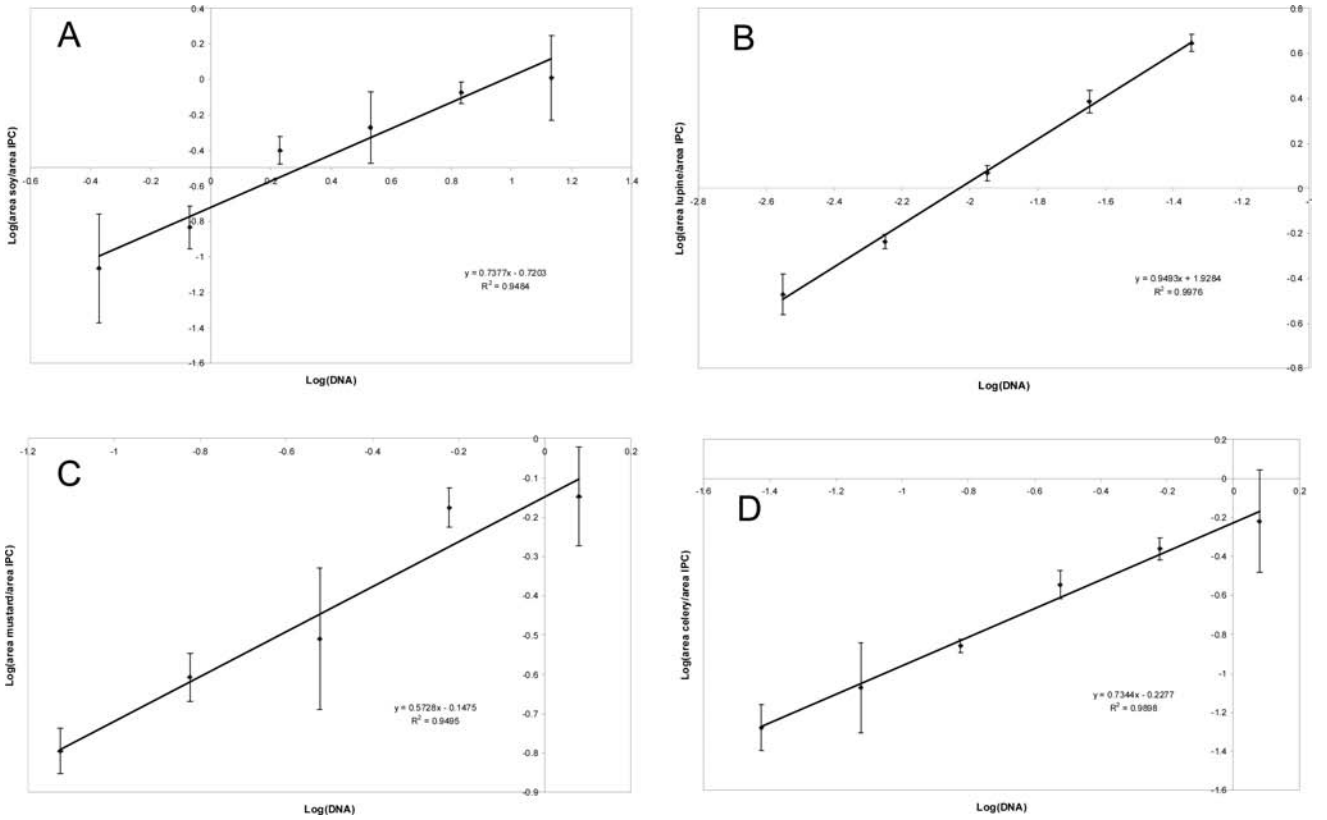

Figure 2. Two-fold dilution curves for (A) soy, (B) lupine, (C) mustard, and (D) celery. Log (area allergen-specific peak/area IPC peak) was plotted against Log(amount DNA) from the dilution samples. Data are from samples containing mixtures of DNA from eight different allergenic foods (examples are shown in Figure 1A,B). Each dilution was analyzed with three parallels.

this way. An example of an undiluted and an 8-fold dilution is presented in Figure 1A,B, respectively. Examples of 2-fold dilution plots for soy, lupine, mustard, and celery are presented in Figure 2. The curves confirmed the quantitative nature of the assay with DNA from all allergenic foods present concomitantly. Similar curves were obtained when different allergen-specific DNA preparations were tested separately (not shown).

To determine the sensitivity of the allergen-MLPA, the end points of the 2-fold dilution series of mixtures of DNA from all eight allergenic foods were determined. From the amount of template DNA for each allergen used in the analysis, the known haploid genome sizes and the highest dilutions giving specific signals, it was possible to calculate an approximate LOD (Table 5). The LOD was calculated as if the genes were single copy genes/haploid genome. This may not always be the case. Multicopy genes would increase the sensitivity of the assay but would influence quantification only if the copy number was different from that of the cultivar used in the reference material. Also, the LOD for allergen-MLPA does not take into consideration that only 10 μL of the ligation mix of 40 μL is used for PCR, so the numbers of template molecules detected are in fact 4-fold lower than those reported in the table. The sensitivity varied with the allergen in question. For some allergens like lupine, wheat, mustard, and celery, sensitivity was very high, whereas sensitivity was somewhat lower for soy and peanut. The sensitivity remained essentially unchanged when a dilution series of allergen DNA were tested separately (not shown). Ten-fold dilution series of the same eight allergen DNA preparations were also analyzed by qPCR. All dilution series gave linear curves over five 10-fold dilutions with slopes in the range −2.4 to −4.4 and with regression coefficients (R^2) in the range 0.98−1.00 (not shown). From these curves and qPCR of the template DNA used, it was calculated that DNA in the allergen-MLPA assay at LOD would correspond approximately to the C_T values listed in Table 5.

Table 5. Sensitivity of the Allergen-MLPA System

allergen	DNA (ng)[a]	haploid genome size[b] (bp, $\times 10^9$)	LOD (gene copy number)[c]	corresponding C_T by qPCR at LOD for allergen-MLPA[d]
sesame	3.8	0.95	173 ± 93	33
soy	13.6	1.09	431 ± 451	34
hazel	1.4	0.46	105 ± 103	31
peanut	18.4	2.77	242 ± 225	33
lupine	0.09	0.59	5 ± 6	44
wheat (gluten)	2.6	9.99	16 ± 0	40
mustard (brown)	1.2	1.47	35 ± 19	39
celery	1.2	2.90	27 ± 3	34

[a] Amounts of DNA used in undiluted sample (Figure 1A). [b] Haploid genome sizes: sesame,[41] soy and hazel,[42] peanut,[43] lupine,[44] wheat and mustard (The RBG Kew DNA Bank, http://data.kew.org/cvalues/), and celery.[45] [c] Average LOD from two separate experiments with three parallels each, with standard deviations. Calculated LOD if genes are single copy genes/haploid genome. [d] Estimated C_T values from qPCR, using DNA amounts at LOD for the allergen-MLPA assay.

Determination of Allergens in Spiked Samples. Selected allergens were chosen for spiking of different foods. Hot chili

spice was spiked with brown mustard powder or celery spice at concentrations from 1 to 0.001%. Likewise, "jytte flour", a flour that contains a large amount of buckwheat and can substitute for wheat in bread making, was spiked with soy flour, lupine flour, or a mixture of soy and lupine flour. In addition, wheat was spiked with soy flour. DNA was purified from the spiked foods and subjected to allergen-MLPA (Table 2). When spiked wheat was used as a template, the gluten probes were omitted to avoid overloading the system with strong gluten signals. For comparison, the same DNA was subjected to qPCR. The sensitivity varied with the allergen in question and was very high for lupine, which could be detected at 50-fold lower concentration as compared to qPCR. For both mustard and celery, the sensitivity was higher than with qPCR. This may to some degree be explained by part inhibition in the qPCR. A 10-fold dilution of DNA from the 1% samples spiked with mustard and celery still gave C_T values of 39.2 and 38.0, respectively. The same undiluted samples appeared not to give inhibition in the allergen-MLPA, indicating that this assay is relatively insensitive to contaminants in the template DNA. For samples spiked with soy, the sensitivity was approximately that of qPCR.

DISCUSSION

Multiplexing PCR is fraught with problems. The number of potential side reactions, yielding unspecific primer−dimer amplification products, increases with the number of primers in a reaction. In addition, small differences in amplification efficiencies for the different primer pairs might result in preferential amplification of some of the PCR products, leaving other PCR products at subdetectable levels. Ligation-mediated amplification offers many advantages over traditional multiplex PCR. Little interaction between the ligation probes is observed. The method shows high specificity and reproducibility. In addition, possibilities for higher levels of multiplexing exist. Schouten et al. reported a 40-plex system for detection of human mutations.[29] By using bipartite ligation probes, all PCR fragments are amplified using the same primer set. This creates a competitive PCR where all fragments are amplified with the same efficiency, and thus, the initial ratios of amplicons are conserved throughout the PCR. Competitive PCR is considered an accurate method for quantification. By comparing a specific allergen signal with an IPC reference signal, quantitative results can be obtained. In addition, the IPC acts as a control showing that annealing, ligation, and PCR have functioned properly. A relatively low copy number of IPC was used to not reduce sensitivity of the allergen signals. All dilutions of the IPC were done in water containing 10 ng/μL *E. coli* DNA. This was important to avoid unspecific adsorption of IPC to plastic ware.

The PCR step was performed using the Qiagen Multiplex PCR kit. In a previously developed MLPA method for detection of eight different GMO maize events, this gave increased signal strength and increased reproducibility.[32] Even though the PCR step constitutes a quantitative, competitive reaction, deviations may occur due to differences in annealing and/or ligation of probes. This might explain the results, for example, for soy, which showed somewhat lower sensitivity as compared to the other allergens. One must keep in mind the possibilites of false negative results when sensitivity is low. To ensure performance of the assay, reference samples of known concentrations should always be analyzed together with unknown samples. The reasons for the consistent differences between the observed and the calculated amplicon sizes (Table 3) are unknown but may in part be caused by nontemplate addition of an adenine nucleotide by DNA polymerase.[34] Generally, background signals are low and pose no problems unless they have the same molecular weight as a specific signal. Some small peaks usually appear adjacent to the specific peaks. The reason for the presence of these peaks is not known but may be caused by slippage of DNA polymerase or minor impurities from the oligo nucleotide synthesis.[34] The reason for the occurrence of peaks 6−8 bases in front of the allergen-specific peaks during overload of the system is also unknown. The system was designed so that these peaks would not overlap with other specific signals.

Specificity of ligation-mediated PCR is considered to be very high due to the requirement of two different probes annealing directly adjacent to each other for ligation to occur. For mustard, the probes detected a group of closely related *Brassica* sp. genes. This highlights the importance of knowing the history of the food so that the results are interpreted correctly. For instance, if a food containing broccoli is analyzed for mustard, false positive results may occur. In other cases, it may be beneficial to detect the allergen even though mustard is not present, since allergic persons might cross react with homologous allergens from other sources. Gluten probes (Table 3) tested positive for wheat, rye, barley, and oat. Again, care must be exercised when interpreting the results. Negative signals would show that gluten is absent, whereas positive gluten signals could stem from oats. During development of DNA-based detection methods for hazelnut, most researchers have used the gene encoding the major allergen Cor a1. Two different genes for this major allergen are known (Genbank accession no. AF136945 and Z72440). Because the prevalence of these genes in hazel was unknown, the allergen-MLPA was designed using probe pairs from both of these genes.

Sensitivity of the allergen-MLPA method was high both when assayed using dilutions of purified DNA and when using DNA from spiked foods (Tables 2 and 5). Some variations in sensitivity between the different allergens were observed. For the spiked foods, the sensitivity was of the same order or more sensitive than that obtained by qPCR. Sensitivity may differ between templates of pure DNA and DNA from naturally contaminated or spiked foods due to partial degradation of DNA or the presence of inhibiting compounds in the latter.[16] This could partly explain the lower sensitivity of qPCR in hot chili spice spiked with mustard or celery. This also indicates a lower sensitivity of the allergen-MLPA to contaminations. The detection limit for DNA-based methods is often reported to be in the range of 1−50 pg of DNA or 0.01−0.1% of the allergen-causing ingredient in a food,[16] that is, similar to those obtained with the allergen-MLPA.

It is still an open question as to how sensitive a detection method needs to be. Threshold levels for specific allergic reactions determined by double blind placebo-controlled food challenges range between less than 1 mg and more than 1 g of allergenic protein depending on the food in question and the sensitivity of the allergic individual.[35] The sensitivity of the assay, however, is highly dependent on the sample preparation and the food product. Also, results from DNA-based analyses must be interpreted with caution. It is important to obtain knowledge about the food that is analyzed and the processing to which it has been subjected. Depending on the food and the processing, DNA may be partly degraded. Sometimes, DNA can be totally degraded, leading to no PCR product and consequently giving false negative results, while the food still may contain the allergen. In addition, food matrix contaminants may interfere with the

ligation and PCR and lower the amplification efficiency. This problem is usually circumvented by diluting the template DNA and thereby concomitantly diluting the inhibitors. The DNeasy plant mini kit DNA purification method used in this work was suitable for a large number of food matrices. For specific food samples, however, other special DNA purification methods may be required.

With allergen-MLPA, accurate quantitative results can be obtained. Although this DNA-based method does not detect the allergens as such, an advantage is that DNA is efficiently extracted under harsh conditions and is less affected by extraction from food matrices as compared to proteins. The DNA-based methods will not replace the protein-based methods but exist as additional and complementary methods. Laboratories may choose different analysis formats depending on the food matrix, the availability of specific tests, the time required to obtain analysis results, and the performance characteristics of the tests.

The recognition that food allergens constitute a food hazard has led to a considerable expansion of precautionary labeling. Risk management approaches should lead to consistent and well understood management action levels across the food industry.[28] This could involve a semiquantitative approach where foods were classified as "free from", "suitable for", or "may contain" with regards to allergens. Foods in the "suitable for" class would give no reactions in the vast majority of allergic individuals. A summary on approaches to establish thresholds for major food allergens and for gluten in food has been published.[27] Several efforts regarding standardization exist. MoniQA, an EU network of excellence under Frame Work Program 6, is a collaboration between authorities, method providers, the food industry, and consumers. MoniQA is compiling information about the most important allergens, prioritizing the gaps in information, identifying needs, and developing harmonized guidelines. To this end, quantitative detection methods for allergens should be developed. The allergen-MLPA method shows many advantages in this respect in being multiplex, quantitative, specific, and sensitive. Moreover, it is modular and can thus easily be adapted by removing or adding probe pairs for special purposes.

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REFERENCES

(1) Sicherer, S. H.; Munoz-Furlong, A.; Murphy, R.; Wood, R. A.; Sampson, H. A. Symposium: Pediatric food allergy. *Pediatrics* **2003**, *111*, 1591–1594.

(2) Wuthrich, B. Lethal or life-threatening allergic reactions to food. *J. Invest. Allergol. Clin. Immunol.* **2000**, *10*, 59–65.

(3) Rona, R. J.; Keil, T.; Summers, C.; Gislason, D.; Zuidmeer, L.; Sodergren, E.; Sigurdardottir, S. T.; Lindner, T.; Goldhahn, K.; Dahlstrom, J.; McBride, D.; Madsen, C. The prevalence of food allergy: A meta-analysis. *J. Allergy Clin. Immunol.* **2007**, *120*, 638–646.

(4) Taylor, S. L.; Hefle, S. L. Food allergen labeling in the USA and Europe. *Curr. Opin. Allergy Clin. Immunol.* **2006**, *6*, 186–190.

(5) Sicherer, S. H. Epidemiology of food allergy. *J. Allergy Clin. Immunol.* **2011**, *127*, 594–602.

(6) Hernando, A.; Mujico, J. R.; Mena, M. C.; Lombardia, M.; Mendez, E. Measurement of wheat gluten and barley hordeins in contaminated oats from Europe, the United States and Canada by Sandwich R5 ELISA. *J. Gastroenterol. Hepatol.* **2008**, *20*, 545–554.

(7) Anonymous. Food Allergen Labeling and Consumer Protection Act of 2004. Public Law 108-282 August 2, 2004, 2004.

(8) Taylor, S. L.; Hefle, S. L.; Farnum, K.; Rizk, S. W.; Yeung, J.; Barnett, M. E.; Busta, F.; Shank, F. R.; Newsome, R.; Davis, S.; Bryant, C. M. Analysis and evaluation of food manufacturing practices used to address allergen concerns. *Compr. Rev. Food Sci. Food Saf.* **2006**, *5*, 138–157.

(9) Anonymous. Regulation (EC) No. 852/2004 of the European Parliament and of the Council of 29 April 2004 on the hygiene of foodstuffs. *Off. J. Eur. Union* **2004**, *L139/1*.

(10) Anonymous. European Parliament and Council. *Off. J. Eur. Commun.* **2000**, *L109*, 29−42.

(11) Anonymous. Directive 2003/89/EC of the European Parliament and of the Council of 10 November 2003 amending Directive 2000/13/EC as regards indication of the ingredients present in foodstuffs. *Off. J. Eur. Union* **2003**, *L308*, 15−18.

(12) Anonymous. Commission of the European Communities. *Off. J. Eur. Commun.* **2006**, *L368*, 15−18.

(13) Keck-Gassenmeier, B.; Benet, S.; Rosa, C.; Hischenhuber, C. Determination of peanut traces in food by a commercially-available ELISA test. *Food Agric. Immunol.* **1999**, *11*, 243–250.

(14) Diaz-Amigo, C.; Popping, B. Detection of food allergens. In *Molecular Biological and Immunological Techniques and Applications for Food Chemists*; Popping, B., Diaz-Amigo, C., Hoenicke, K., Eds.; Wiley: Hoboken, NJ, 2010; pp 175−198.

(15) van Hengel, A. J. Food allergen detection methods and the challenge to protect food-allergic consumers. *Anal. Bioanal. Chem.* **2007**, *389*, 111–118.

(16) Holck, A. L.; Diaz-Amigo, C.; Kerbach, S.; Popping, B.; Mustorp, S.; Engdahl Axelsson, C. Detection of allergens in food. In *Current Topics on Food Authentication*; Oliveira, M. B. P. P., Mafra, I., Amaral, J. S., Eds.; Transworld Research Network: Kerala, India, 2011; pp 173−210.

(17) Poms, R. E.; Anklam, E.; Kuhn, M. Polymerase chain reaction techniques for food allergen detection. *J. AOAC Int.* **2004**, *87*, 1391–1397.

(18) Poms, R. E.; Klein, C. L.; Anklam, E. Methods for allergen analysis in food: A review. *Food Addit. Contam.* **2004**, *21*, 1–31.

(19) Mustorp, S.; Engdahl Axelsson, C.; Svensson, U.; Holck, A. Detection of celery (*Apium graveolens*), mustard (*Sinapis alba*, *Brassica juncea*, *Brassica nigra*), and sesame (*Sesamum indicum*) in food by real-time PCR. *Eur. Food Res. Technol.* **2008**, *226*, 771–778.

(20) Hashimoto, H.; Makabe, Y.; Hasegawa, Y.; Sajiki, J.; Miyamoto, F. Detection of allergenic substances in foods by a multiplex PCR method. *J. Food Hyg. Soc. Jpn.* **2007**, *48*, 132–138.

(21) Rossi, S.; Searavelli, E.; Germini, A.; Corradini, R.; Fogher, C.; Marchelli, R. A PNA-array platform for the detection of hidden allergens in foodstuffs. *Eur. Food Res. Technol.* **2006**, *223*, 1–6.

(22) Bettazzi, F.; Lucarelli, F.; Palchetti, I.; Berti, F.; Marrazza, G.; Mascini, M. Disposable electrochemical DNA-array for PCR amplified detection of hazelnut allergens in foodstuffs. *Anal. Chim. Acta* **2008**, *614*, 93–102.

(23) Ehlert, A.; Demmel, A.; Hupfer, C.; Busch, U.; Engel, K. H. Simultaneous detection of DNA from 10 food allergens by ligation-dependent probe amplification. *Food Addit. Contam.* **2009**, *26*, 409–418.

(24) Koppel, R.; Dvorak, V.; Zimmerli, F.; Breitenmoser, A.; Eugster, A.; Waiblinger, H. U. Two tetraplex real-time PCR for the detection and quantification of DNA from eight allergens in food. *Eur. Food Res. Technol.* **2010**, *230*, 367–374.

(25) Pafundo, S.; Gulli, M.; Marmiroli, N. Multiplex real-time PCR using SYBR (R) GreenER (TM) for the detection of DNA allergens in food. *Anal. Bioanal. Chem.* **2010**, *396*, 1831–1839.

(26) Hildebrandt, S. Multiplexed identification of different fish species by detection of parvalbumin, a common fish allergen gene: a DNA application of multi-analyte profiling (xMAP(TM)). *Anal. Bioanal. Chem.* **2010**, *397*, 1787–1796.

(27) Buchanan, R.; Dennis, S.; Gendel, S.; Acheson, D.; Assimon, S. A.; Beru, N.; Bolger, P.; Carlson, D.; Carvajal, R.; Copp, C.; Falci, K.; Garber, E.; Harden, E.; Kane, R.; Kvenberg, J.; Luccioli, S.; Park, D.; Raybourne, R.; Troxell, T.; Vierk, K.; Threshold Working, G. Approaches to establish thresholds for major food allergens and for gluten in food. *J. Food Prot.* **2008**, *71*, 1043–1088.

(28) Ward, R.; Crevel, R.; Bell, I.; Khandke, N.; Ramsay, C.; Paine, S. A vision for allergen management best practice in the food industry. *Trends Food Sci. Technol.* **2010**, *21*, 619–625.

(29) Schouten, J. P.; McElgunn, C. J.; Waaijer, R.; Zwijnenburg, D.; Diepvens, F.; Pals, G. Relative quantification of 40 nucleic acid sequences by multiplex ligation-dependent probe amplification. *Nucleic Acids Res.* **2002**, *30*, e57.

(30) Sorvig, E.; Mathiesen, G.; Naterstad, K.; Eijsink, V. G. H.; Axelsson, L. High-level, inducible gene expression in Lactobacillus sakei and Lactobacillus plantarum using versatile expression vectors. *Microbiology-Sgm* **2005**, *151*, 2439–2449.

(31) Raeymaekers, L. A commentary on the practical applications of competitive PCR. *Genome Res.* **1995**, *5*, 91–94.

(32) Heide, B. R.; Dromtorp, S. M.; Rudi, K.; Heir, E.; Holck, A. Determination of eight genetically modified maize events by quantitative, multiplex PCR and fluoresence capillary electrophoresis. *Eur. Food Res. Technol.* **2008**, *227*, 1125–1137.

(33) Holck, A. L.; Dromtorp, S. M.; Heir, E. Quantitative, multiplex ligation-dependent probe amplification for the determination of eight genetically modified maize events. *Eur. Food Res. Technol.* **2009**, *230*, 185–194.

(34) Ehlert, A.; Moreano, F.; Busch, U.; Engel, K. H. Development of a modular system for detection of genetically modified organisms in food based on ligation-dependent probe amplification. *Eur. Food Res. Technol.* **2008**, *227*, 805–812.

(35) Taylor, S. L.; Hefle, S. L.; Bindslev-Jensen, C.; Bock, S. A.; Burks, A. W.; Christie, L.; Hill, D. J.; Host, A.; Hourihane, J. O.; Lack, G.; Metcalfe, D. D.; Moneret-Vautrin, D. A.; Vadas, P. A.; Rance, F.; Skrypec, D. J.; Trautman, T. A.; Yman, I. M.; Zeiger, R. S. Factors affecting the determination of threshold doses for allergenic foods: How much is too much? *J. Allergy Clin. Immunol.* **2002**, *109*, 24–30.

(36) Dahinden, I.; von Buren, M.; Luthy, J. A quantitative competitive PCR system to detect contamination of wheat, barley or rye in gluten-free food for coeliac patients. *Eur. Food Res. Technol.* **2001**, *212*, 228–233.

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

(38) Hird, H.; Lloyd, J.; Goodier, R.; Brown, J.; Reece, P. Detection of peanut using real-time polymerase chain reaction. *Eur. Food Res. Technol.* **2003**, *217*, 265–268.

(39) Demmel, A.; Hupfer, C.; Hampe, E. I.; Busch, U.; Engel, K. H. Development of a real-time PCR for the detection of lupine DNA (Lupinus species) in foods. *J. Agric. Food Chem.* **2008**, *56*, 4328–4332.

(40) Terzi, V.; Malnati, M.; Barbanera, M.; Stanca, A. M.; Faccioli, P. Development of analytical systems based on real-time PCR for Triticum species-specific detection and quantitation of bread wheat contamination in semolina and pasta. *J. Cereal Sci.* **2003**, *38*, 87–94.

(41) Bennett, M. D.; Leitch, I. J. Nuclear DNA amounts in angiosperms: Progress, problems and prospects. *Ann. Bot.* **2005**, *95*, 45–90.

(42) Bennett, M. D.; Smith, J. B. Nuclear-DNA amounts in angiosperms. *Philos. Trans. R. Soc., B* **1991**, *334*, 309–345.

(43) Temsch, E. M.; Greilhuber, J. Genome size variation in Arachis hypogaea and A-monticola re-evaluated. *Genome* **2000**, *43*, 449–451.

(44) Naganowska, B.; Wolko, B.; Sliwinska, E.; Kaczmarek, Z.; Schifino-Wittmann, M. 2C DNA variation and relationships among New World species of the genus Lupinus (Fabaceae). *Plant Syst. Evol.* **2005**, *256*, 147–157.

(45) Yang, X. F.; Quiros, C. F. Characterizing the celery genome with DNA-based genetic-markers. *J. Am. Soc. Hortic. Sci.* **1995**, *120*, 747–751.