

Two tetraplex real-time PCR for the detection and quantification of DNA from eight allergens in food

René Köppel · Veronika Dvorak · Franziska Zimmerli ·
Alda Breitenmoser · Albert Eugster ·
Hans-Ulrich Waiblinger

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Abstract According to the EU and Swiss legislation, food has to be labelled for allergens to enable allergic consumers to avoid such food and its products. To provide efficient and reliable methods, two novel quantitative multiplex real-time polymerase chain reaction systems were developed and validated. They simultaneously determine DNA of peanut, hazelnut, celery, soy, egg, milk, almond and sesame, respectively. The tests exhibit good specificity and sensitivity in the range of 0.01%. Due to low DNA amounts, lower sensitivities for egg and milk were obtained. First comparisons of ELISA results with PCR results suggest a qualitative accordance, but a low correlation of quantitative results.

Keywords Allergens · Multiplex real-time quantitative PCR · Peanut · Hazelnut · Celery · Soy · Egg · Milk · Almond · Sesame · Validation

Introduction

As much as 2–6% of the population are estimated to exhibit allergic reactions due to the consumption of food

containing allergens. To increase the well-being and safety of these people, ingredients that cause allergic reactions must be labelled on foods [1, 2]. To control that these regulations are implemented by the producers, food samples are regularly examined for the presence of allergens and the compliance with the declaration. Currently, there are 20 allergens listed by the food law of European Union and Switzerland. It comprises cereals with gluten, crustacean, egg, fish, milk, molluscs, soy, nuts (almonds, peanuts, cashew, hazelnut, macadamia, walnut, Brazil nut, pecan, pistachio and Queensland nuts), sesame, celery, mustard, lupine and sulphites. Sulphites are analysed by other methods than protein or DNA-based methods. For most of the analytes, polymerase chain reaction (PCR) together with ELISA are well-recognized methods [3, 4], although PCR does not detect proteins that have the allergenic potential. Many qualitative and quantitative PCR systems were described in the past, most of them detecting single analytes [3, 5–11], multiplex qualitatively [12, 13] or duplex quantitatively [14]. In principle, food samples need to be analysed for all allergens. However, these analyses are very laborious and time-consuming. In addition, many allergen combinations are unlikely to be present in certain food products. If analysed separately, the amount of isolated DNA may be insufficient to analyse all possible allergens in a food sample. Therefore, multiplex PCR is a necessary and promising approach to produce results in a more economic and faster way. Here, we present two tetraplex real-time PCR systems called AllAll A and AllAll B. AllAll A simultaneously determines the contents of DNA from peanut, hazelnut, celery and soy, while AllAll B determines that from almond, sesame, egg (DNA of chicken) and milk (DNA of cow) in food.

R. Köppel (✉) · V. Dvorak · F. Zimmerli · A. Breitenmoser
Official Food Control Authority of the Canton
Zürich (KLZH), Zurich, Switzerland
e-mail: rene.koeppel@klzh.ch

A. Eugster
Official Food Control Authority of the Canton
Aargau (KLAG), Aargau, Switzerland

H.-U. Waiblinger
State Institute of Chemical and Veterinarian Analysis,
Freiburg, Germany

Materials and methods

Plant material and samples

Fresh and dried plant and animal material were obtained from local suppliers.

Spiked reference material (cookies)

Two portions of dough were mixed with the following ingredients each: rice flour (200 g), butter (90 g), sugar (100 g) and two eggs. To one portion, defatted and finely ground peanuts, hazelnuts, almonds and sesame as well as flour from soybeans were added to give the spiked material. Spiking amounts were chosen to give 400 mg of allergenic compound per kilogram. Each of the doughs were baked in an oven for 10 min at 180 °C, finely ground and mixed to give allergen concentrations of 10 and 50 mg/kg.

DNA extraction

DNA extraction was performed using a Wizard Plus Minipreps DNA purification system (Promega, Madison, USA). As much as 200 mg of ground sample material was extracted and DNA was eluted in 50 µl of elution buffer according to the producer's manual.

Some of the laboratories participating in the interlaboratory validation (see below) used CTAB extraction protocol followed by a column purification step (QiaQuick DNA purification system, QIAGEN, Germany) [15] or commercial DNA extraction kit (Sure Food, Congen, Berlin).

Primers and probes

The primers and probes were taken from published single PCR systems or were established in this work (for details see Tables 1, 2). The labelling of the probes was done according to the recommendations of the manual of the Rotorgene 6000 real-time system (Corbett, Australia) and based on the availability of existing labelling dyes for probes.

Optimization of the primers was performed by titrating the optimal primer concentration for each analyte. The combination of the analytes was taken randomly as the purpose was always to run both systems in parallel irrespective of the sample to be analysed.

Real-time PCR procedure

DNA extracts (5 µl) were added to 20 µl of the reaction mix containing QuantiTect multiplex PCR no ROX Master Mix (QIAGEN), primers and probes (for final concentration see Tables 1, 2, all primers and probes were

Table 1 Multiplex real-time PCR system AllAll A for the simultaneous detection of hazelnut, peanut, celery and soy

Primer/probe	Final conc. (μM)	Sequence	Size	GenBank acc. no./source/labelling
Tetraplex qPCR system AllAll A				
Hazelnut				
cor 145 F	0.15	GGA GGT CCC GGA ACC ATT A	85 bp	Z72440, major allergen Cor a 1
cor 229 R	0.15	CAC AAA ACG TAC AAC TCC TTG G		This study
cor 166 TMP	0.2	AAG ATC TGT TTC GAT GAA GGT TAA TTT CTA CCA C		Cy5 BHQ2
Peanut				
Arah2 F	0.4	GCT CGA GAG GGC GAA CCT	66 bp	EU267272, Ara d 2
Arah2 R	0.4	TCC TCG TCA CGT TGG ATC TTC		This study
Arah2 TMP	0.05	AGG CCC TGC GAG CAA CAT CTC ATG		FAM-TAMRA
Celery				
man03	0.25	AAA CGC CTA TAT GTT CTT TGT C	145 bp	AF067082, <i>Apium graveolens</i> mannitol dehydrogenase (Mtd) gene
man04	0.25	TTG CCA TTC ATC AGA TAC CAT T		UniBE ^a
Cel_Joe	0.05	TAT GGC CCC TTT AGC CTA ATT AAA		JOE-BHQ1, this study
Soy				
Lectin-F	0.15	TCC ACC CCC ATC CAC ATT T	81 bp	K00821, Soybean lectin (Le1) gene
Lectin-R	0.15	GGC ATA GAA GGT GAA GTT GAA GGA		This study
Lectin-TMP	0.03	AAC CGG TAG CGT TGC CAG CTT CG		ROX-BHQ2

^a Primers were developed at the University Bern, Department of Biochemistry; the probe was developed in this study

Table 2 Multiplex qPCR System AllAll B for the simultaneous detection of sesame, milk, almonds and egg

Primer/probe	Final conc. (μM)	Sequence	Size	GenBank acc.no./source/
Tetraplex qPCR system AllAll B				
Sesame				
SesF	0.12	CCC GCT CTT CGT CAT CTT CAG	133 bp	U97700, <i>Sesamum indicum</i> 15.5 kDa oleosin mRNA
SesR	0.12	GTA GTT CAG CAA CCA CGA AAT CG		This work
Ses_Cy5	0.03	CAT CTT GGT CCC CGC CGC CCT AAC		Cy5, BHQ2
Milk (beef)				
RindAG1	0.1	AGT TAG AGA TTG AGA GCC ATA TAC TCT CC	83 bp	AY526085, <i>Bos taurus</i> mitochondrion, tRNA-Lys
RindAG2	0.1	TTG ATA AGA TCA TTG TCA GTC ATG TTG		Cantonal Laboratory Aargau
Rind TMP	0.05	TGG TGA CAT GCC GCA ACT AGA CAC G		FAM-TAMRA
Almonds				
MadlF	0.16	CCT AGC GGA GGA TCC ATC ATC	129 bp	BQ641046, Major Allergen PRU AV 1(PRU A 1)-gene
MadlR	0.16	GTA GGT CTC AAT GAG CTT GAA GAG		This work
Madl_Joe	0.05	AGA GCA CCA GCC ACT ACC ACA CCA		JOE BHQ1
Egg (chicken)				
Gallus1 F	0.2	CAG CTG GCC TGC CGG	76 bp	AY685072, <i>Gallus gallus</i> transforming growth factor beta 3 gene
Gallus1 R	0.2	CCC AGT GGA ATG TGG TAT TCA		Earlier work of the Cantonal Laboratory Zürich
Gallus1 TMP	0.06	TCT GCC ACT CCT CTG CAC CCA GT		ROX BHQ2

synthesized by Microsynth AG, Balgach, Switzerland). PCR was performed on the Rotorgene 6000 according to the following cycling protocol: Initial step of 15 min at 95 °C; followed by 45 cycles of 15 s at 95 °C and 1 min at 60 °C and 1 min at 72 °C (according to the recommendations for the QuantiTect multiplex PCR no ROX Master Mix).

Interlaboratory validation study

Samples of finely ground rice cookies containing 0, 10 and 50 mg/kg of different allergens were sent to nine Swiss and German laboratories. Two DNA extractions were performed for each sample; each DNA extract was run in PCR in duplicate. In addition, most of the laboratories analysed the DNA extracts using their in-house validated PCR method in parallel.

Results and discussion

Design of the multiplex real-time PCR systems

Before combining with multiplex assays, the specificity of each PCR system has to be demonstrated. After designing

primers by the Beacon-designer 5.1 software (Premier Biosoft, Palo Alto, USA), they were first tested without probes in the single SYBR-green format. This approach is useful to detect amplification of unspecific products by the melting curve analysis. After this, a first check of the specificity was performed. Therefore, DNA of several related plant species was isolated and used as template for the PCR reaction. In addition, a dilution series of each target was performed to estimate the amplification efficiency reach maximal sensitivity for each target. If these first results were satisfactory, Taqman probes were designed according to the channels of the Rotorgene 6000. We chose the fluorophores FAM, JOE, ROX and Cy5 for labelling (for details see Tables 1, 2).

Specificity

During the design, all primers and probes were successfully checked for relevant homologies by BLASTNr search within GenBank databases. To test the specificity extensively, DNA from a wide range of plant species and other food ingredients was isolated. DNA of the following organisms was isolated and tested in these two multiplex PCR systems: almonds, aniseed, apricots, beans, beef, brazil nut, carrot, cashew, celery, cloves, coconut, chive,

cinnamon, chicken chickpeas, egg powder, garlic, hazelnut, goat, kidney beans, lamb, *Lupinus albus*, *Lupinus angustifolius*, mung beans, nutmeg, onion, parsley, paprika, peach, peanut, peas, pecan, pig, pistachio, plum, potato, runner beans, sesame, soy, tomato, turkey, walnut, wheat, white pepper, white lentils and whole egg powder. Cross-reactivity above 1% emerged only on using peach and apricot as template. Peach led to a 10% signal in the hazelnut system and in case of apricot to a 1.2% signal in the almond system. Usually, allergens are found either as contamination or declared ingredient. It is unlikely that food products become contaminated by peach and apricots. Therefore, this cross-reactivity has to be considered.

Sensitivity, precision and uncertainty

To evaluate the sensitivity, DNA extracts with a concentration of 20 ng/μl (corresponds to 100%) were diluted in herring sperm DNA solution (20 ng/μl) down to 0.0032% in half-logarithmic steps. These single analyte dilution series were mixed according to the Tables 3 and 4 to produce two multiplex standards, simulating samples with signals for all four analytes per multiplex PCR system. Each data point was analysed ten times ($N = 10$) over a

Table 3 Multiplex dilution series of standards for real-time PCR system AllAll A in the range of 0.0032–1%

No.	Hazelnut	Peanut	Celery	Soy
AllAll A: percentage of DNA in total DNA				
1	0.32	0.0032	0.032	1
2	0.1	1	0.01	0.32
3	0.032	0.32	0.0032	0.1
4	0.01	0.1	1	0.032
5	0.0032	0.032	0.32	0.01
6	1	0.01	0.1	0.0032

As background, herring sperm DNA was used. Total DNA concentration was for all combinations 20 ng/μl

Table 4 Multiplex dilution series of standards for real-time PCR system AllAll B in the range of 0.0032–1%. As background, herring sperm-DNA was used. Total DNA concentration was for all combinations 20 ng/μl

No.	Sesam	Milk	Almond	Egg
AllAll B: percent DNA in total DNA				
1	0.32	0.0032	0.032	1
2	0.1	1	0.01	0.32
3	0.032	0.32	0.0032	0.1
4	0.01	0.1	1	0.032
5	0.0032	0.032	0.32	0.01
6	1	0.01	0.1	0.0032

period of 1 month. In every run, two dilution rows were analysed. One of the two served as calibration row, whereas the other row served as “unknown samples” and inverse. Data in percentage according to the diluted DNA solutions were collected and the relative standard deviation (rSD) for the estimation of the precision, mean deviation from the true value for the estimation of the accuracy was calculated (see compilation in Tables 5, 6). All samples showed 100% positive signals down to 0.0032% concentrations of the analytes. None of the ten negative controls showed a positive signal.

Quantification of allergens

The quantification of allergens in milligram per kilogram of the allergenic food or protein by PCR and ELISA is difficult. Both methods measure different analytes, which in most cases are not identical to the allergenic protein-epitope on an allergen. The quantification performance of the DNA mixtures is shown in Tables 5 and 6. These data show the feasibility of quantitative determination of DNA derived from allergens of a product. However, the values gained applying DNA dilution series for the calibration need not to quantitatively comply with the mass amount of allergenic material in a product.

In case of PCR, this may be caused by degradation of the DNA during production (heat, acids) or storage and/or the use of plant and animal material with different contents of DNA (e.g. seeds with or without chaff, skimmed or not skimmed milk). Although these mechanisms may bias

Table 5 Performance of the AllAll A system using DNA mixtures

AllAll A	Hazelnut	Peanut	Celery	Soy
rSD (%)	42	41	35	32
Diff. true value (%)	14	11	10	10

Six independent runs with standard rows of all analytes were accomplished. The values in percentage generated during all five runs were averaged. To estimate the accuracy the highest difference of the averaged measured values to the true value was taken (diff. true value %). To estimate the precision, the highest relative standard deviation resulting from the five runs for all analytes was taken (rSD %)

Table 6 Performance of the AllAll B system using DNA mixtures

AllAll B	Sesam	Milk	Almond	Egg
rSD (%)	24	13	29	20
Diff. true value (%)	19	7	17	9

Six independent runs with standard rows of all analytes were accomplished. The values in percentage generated during all six runs were averaged. To estimate the accuracy, the highest difference of the averaged measured values to the true value was taken (diff. true value %). To estimate the precision, the highest relative standard deviation resulting from the five runs for all analytes was taken (rSD %)

quantification, it is important to realize that all these mechanisms diminish DNA contents and therefore lead to an underestimation of the content of allergen when measured by PCR.

To estimate the amount of the allergenic ingredient or contamination (in milligram per kilogram), further investigation has to be done. One approach could be the use of matrix-adapted reference materials. Spiking levels should be in the quantification range and therefore higher than those used in this work to estimate the limit of sensitivity.

Interlaboratory validation of sensitivity in food matrix (rice cookies)

Results of an interlaboratory validation of the two multiplex systems using spiked food samples (rice cookies) are shown in Table 7. Materials were spiked in the estimated range of limit of detection of the method (10 and 50 mg of allergenic ingredient per kilogram). Celery, which would be detectable by the system AllAll A, was not spiked to the rice cookies; butter (milk protein) and egg were used as ingredients in the dough (also non-spiked material). Estimation of the sensitivity of the detection systems for celery, egg and milk is thus not included in this study.

To compare the multiplex real-time PCR described in this study with their own, routinely used in-house single real-time PCR methods, laboratories analysed the DNA extracts from the rice cookie materials in parallel. For all allergens, no significant difference of sensitivities was found between multiplex PCR (MP) and the different single PCR methods (IM).

Table 7 Results of interlaboratory validation using rice cookies spiked with allergens in the range of estimated limit of detection

Spiked allergen	Spiking level and number of positive reactions					
	0 mg/kg		10 mg/kg		50 mg/kg	
	MP	IM	MP	IM	MP	IM
Peanut	0/34	0/25	17/34	12/25	34/34	20/25
Hazlenut	0/26	0/25	21/26	22/25	25/26	25/25
Almond	0/34	0/17	27/34	13/17	27/34	17/17
Soybean	6/34	1/12	22/30	16/21	26/30	21/21
Sesame	3/30	4/25	16/30	14/21	23/30	20/21
Milk ^a	34/34	–	34/34	–	34/34	–
Egg ^a	11/31	–	22/31	–	22/31	–

Spiking levels were 10 and 50 mg/kg of allergenic ingredient. Results of multiplex systems described in this study (MP) and routinely used in-house real-time PCR methods (IM) are compared. For example, 0/34 means no amplification out of 34 PCR reactions; four PCR reactions were performed per allergen

^a Milk (butter) and egg were used as ingredients for the preparation of the cookies, to obtain results for information. In case of milk, the results may serve as inhibition control

In both cases, 50 mg of the allergen per kilogram could be detected with acceptable false-negative rates (MP, 0% of all PCR reactions for peanut up to 23% of sesame; IM, 0% for hazelnut, almond and soybean up to 20% of peanut).

The spiking level of 10 mg/kg gave false-negative rates ranging from 12% for hazelnut (IM) up to 52% for peanut (IM), and 19% for hazelnut (MP) to 50% for peanut (MP). Thus, it can be estimated that the limit of detection is for all methods in the range of 10–50 mg/kg.

DNA extraction with Wizard method, CTAB/QIAQuick and SureFood Extraction kit showed comparable results (data not shown).

False-positive results in the unspiked material (negative control) for soybean and sesame were only found in two results of one laboratory. Therefore, it is assumed that these results are rather caused by contamination within this laboratory than by the lack of specificity of the real-time PCR methods.

Analysis of commercial samples

Fifty commercial available products were analysed by AllAll A and AllAll B (see Table 8 for the results). Results should correspond qualitatively with the declarations made on the package (see D in Table 8). However, egg, which was declared in many cases, was not detected by PCR. It is well known that egg and also milk contain few DNA.

In bakery products, the amount of DNA may additionally be degraded during the baking process. The total amount of DNA that can be given in a PCR is limited (100 ng) and the target DNA may be only a small proportion of the major component such as in wheat. Hence, PCR does not seem to be an appropriate method to detect egg and/or milk in processed food. Surprisingly milk was detected in many cases.

Comparable problems are encountered using ELISA. Antibodies often do not detect degraded proteins.

Comparison of ELISA and PCR results in samples containing almonds

Of 40 samples, 5 showed positive signals for almonds using ELISA and PCR (see Tables 8, 9). Comparing the quantitative results of both methods, a correlation between the amount of amplifiable almond DNA and almond protein resp. amount of almonds can be suspected.

Comparison of ELISA and PCR results in samples containing milk

In total, 24 samples of dark chocolate were analysed for milk using ELISA and PCR. Eight samples gave positive

Table 8 Results of DNA extracts from bakery products analysed by multiplex real-time systems AllAll A and AllAll B

Product	Hazelnut (%)	Pea nut (%)	Celery (%)	Soy (%)	Sesame (%)	Milk (%)	Almond (%)	Egg (%)
Cherry cake	n.d.	n.d.	n.d.	n.d.	n.d.	D n.d.	n.d.	D n.d.
Nut cake 1	D 1.1	n.d.	n.d.	n.d.	n.d.	D n.d.	n.d.	n.d.
Almond pastry	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	D 4.0	D n.d.
Apricot pie 2	n.d.	n.d.	n.d.	D Lecithin n.d.	n.d.	n.d.	n.d.	D n.d.
Cinnamon cake	D 0.2	n.d.	n.d.	D Lecithin n.d.	n.d.	D 0.02	n.d.	D n.d.
Filled gingerbread 1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	D 1.8	n.d.
Filled gingerbread 2	D n.d.	n.d.	n.d.	0.5	n.d.	D n.d.	D 7.3	n.d.
Snack	n.d.	n.d.	n.d.	n.d.	n.d.	D 0.03	n.d.	n.d.
Flakes special red fruit	n.d.	n.d.	n.d.	n.d.	n.d.	0.02	0.1	n.d.
Mixed cereals	n.d.	n.d.	n.d.	D 1.3	n.d.	D .03	n.d.	n.d.
Milk bar	D 1.5	n.d.	n.d.	D Lecithin n.d.	n.d.	D 0.4	n.d.	n.d.
Cookie with marmalade	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.01	D n.d.
Lemon cake	n.d.	n.d.	n.d.	n.d.	n.d.	D 0.03	n.d.	D n.d.
Nut cake 3	D 0.2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	D n.d.
Nut cake with marmalade	D 3.3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	D n.d.
Chocolate chips	n.d.	n.d.	n.d.	n.d.	n.d.	D n.d.	n.d.	D n.d.
Coffee cake	n.d.	n.d.	n.d.	D Lecithin n.d.	n.d.	n.d.	n.d.	D n.d.
Amaretti	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	D 0.7	n.d.
Cereal bar	n.d.	n.d.	n.d.	n.d.	n.d.	D 1.5	n.d.	n.d.
Cereal bar with chocolate	n.d.	D 71.5	n.d.	D Lecithin n.d.	n.d.	D 0.8	n.d.	D n.d.
Filled waffle	D 26	n.d.	n.d.	D 0.17	n.d.	D 0.5	n.d.	D n.d.
Linzer torte	D 9.5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Pumpkin cake	n.d.	n.d.	n.d.	D Lecithin 0.03	D 6.2	D n.d.	D 1.3	n.d.
Müesli	D 3.7	n.d.	n.d.	D 0.9	D 0.05	n.d.	n.d.	n.d.
Butter biscuit	n.d.	n.d.	n.d.	D Lecithin n.d.	n.d.	D 0.03	n.d.	D n.d.
Butter cookie	n.d.	n.d.	n.d.	n.d.	n.d.	D 0.1	n.d.	n.d.
Chocolate cake 2	n.d.	n.d.	n.d.	D Lecithin n.d.	n.d.	D 0.5	n.d.	n.d.
Sesame snack	n.d.	n.d.	n.d.	n.d.	D 0.1	n.d.	n.d.	n.d.
Three products with declared milk only: crackers, apple poppies, wheat dips	n.d.	n.d.	n.d.	n.d.	n.d.	D n.d.	n.d.	n.d.
Seven products with declared egg only: apricot pie 1, biscuit, coco cookie, shortbread diet, biscuit, ring cookies, mini-coco cookie	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	D n.d.
Ten products without declared allergens: chocolate cake 1, nut cake 2, soft cookie, cookie of Willisau, bio-energy snack, corn dips, flaky pastry, 5 cereal bar, honey coco cookie, chocolate flakes	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Declared	9	1	0	11	3	19	5	20
Detected	8	1	0	4	3	11	5	0
Not declared but detected				1		1	2	

Determined values (in nanogram DNA) are listed in bold, and negative signals assigned as not detected (n.d.) Ingredients declared are assigned as D. In contrast to the declaration, egg was not detected at all. DNA of soy from lecithin was also detected in rare cases only. For the other analytes, a reasonable correlation between declaration and detection was achieved

Table 9 Forty market samples were analysed for almonds

Positive sample no.	PCR results (ng almond DNA)	ELISA results (almond%)
1	1.8	0.32
2	7.3	0.3
3	0.007	0.02
4	0.67	0.24
5	0.15	0.24

Five samples gave positive signals in ELISA and in AllAllB for almond. Here a compilation of the quantitative results is presented

Table 10 Comparison of PCR and ELISA results of milk determination in eight dark chocolate samples

Positive sample no.	PCR results (pg milk DNA)	ELISA results (milk%)
1	11	1.80
2	1	0.70
3	1	0.80
4	3	5
5	4.5	0.70
6	1.5	2
7	2	1
8	1	0.50

Often chocolate and its products from roasting inhibits PCR. In this experiment, milk was detected indicating that no inhibition of PCR took place

results and served therefore to compare these results gained with both methods. Again, a correlation between PCR and ELISA results cannot be excluded (Table 10).

As this correlation is only suspected, it has to be investigated in the future with more samples. If a correlation can be shown, we can speculate that PCR results are linked to the amount of allergenic proteins and the results generated by PCR could possibly reflect the allergenic potential. As a consequence, PCR results could be used in a first screening step to select samples for further confirmation by ELISA. For screening of cross-contamination during production processes, PCR results may be useful.

Conclusion

The subject “Allergens in Food” can be divided into two issues. One is the impact on the consumers, estimation of risk for allergic persons and mechanisms of allergy. This is not the subject of this work. The second issue deals with methods of control of food products and production to reduce contamination and to minimize the risk to consumers. Control must enclose probable and not foreseen

contaminations. The risk is best controlled and reduced when, as much as possible, allergenic ingredients and contaminations are assessed. For broad application these assays must be cost efficient and as simple as possible. Multiplex quantitative PCR may fulfil these requirements. The quantitative multiplex real-time PCR systems that are presented allow the investigation of food products for allergenic compounds in a very efficient manner, producing results for four allergens at once. These exhibit a good sensitivity and, as these were applied by several other laboratories, a high reproducibility and robustness. In some cases, the cross-reactivity between almonds and apricots had to be considered in the context of a specific sample. Inhibitory matrices such as dark chocolate did not disturb the amplification of DNA for the detection of milk (content determined by ELISA, 0.5–2%). An interlaboratory test with spiked reference material showed sensitivities comparable to those of routinely used in-house single real-time PCR methods in the range of 10–50 mg peanut, hazelnut, almond, soybean or sesame per kilogram. For celery, this has to be determined in the future.

The comparison of ELISA and PCR results showed that a correlation between the results cannot be excluded. We assume that these two multiplex PCR systems are robust and can be applied in routine testing successfully.

Further work has to be done for quantification of allergens in food. Often, reference material covers only one or two allergens, which is insufficient for routine analysis. Therefore, one approach to overcome this could be the production of reference material spiked with a series of different allergens into matrices simulating real products.

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