

Ring trial validation of single and multiplex real-time PCR methods for the detection and quantification of the allergenic food ingredients mustard, celery, soy, wheat and rye

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Abstract We describe an interlaboratory ring trial validation for quantification methods of 3 food allergens using real-time PCR: a real-time PCR method for the joint simultaneous detection and quantification of brown and black mustard, a real-time PCR method for the detection and quantification of wheat and rye, and a multiplex real-time PCR method combining the detection and quantification of brown/black mustard, white mustard, celery and soybean. Mixtures with defined copy numbers of the target sequences from brown mustard, white mustard, celery, soybean and wheat were applied as calibrants for the real-time PCR-based quantification. Boiled sausages incurred with these allergenic ingredients in the range of 0–100 mg/kg were used as ring trial samples. Within the ring trial, we demonstrated that 40 mg/kg of the respective allergens (wheat/rye 80 mg/kg) can reliably be detected and quantified by all PCR systems even in boiled sausage meat. Brown/black mustard was detectable at the lowest concentration level of 10 mg/kg even after autoclaving. Results for brown/black mustard in

terms of sensitivity, trueness and precision were comparable in single- and multiplex PCR. The results of the study revealed the importance of matrix-matched control samples for allergen analysis in terms of both conversion to mg/kg and of normalization variations caused by different extraction procedures.

Keywords Allergen · Mustard · Celery · Soybean · Wheat · Real-time PCR · Interlaboratory validation · Quantification

1 Introduction

About 3–6% of the worldwide population suffer from food allergies (Sicherer 2011). To ensure the availability of information about the presence of relevant allergens in food, labelling regulations have been implemented in many countries. According to the European regulation 1169/2011, labelling is mandatory for food ingredients based on mustard, celery, soybean and gluten-containing cereals, among others (The European Parliament and the Council of the European Union 2011). For surveillance purposes, methods for the detection and quantification of allergenic ingredients are needed. In practice, protocols based on real-time polymerase chain reaction (PCR) and immunological methods are predominantly used (EFSA NDA Panel 2014).

In case of positive results, the quantification of the respective allergen or allergenic ingredient may be necessary for risk assessment, allergen management,¹ and surveillance purposes (CVUA 2016). However, the European Union has not yet set legal threshold values

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above which the labelling of food allergens would be mandatory, regardless of whether they are present due to cross-contact or due to their use as an ingredient. An important prerequisite for setting such thresholds is the availability of standardized quantification methods. In previous works, we have described the interlaboratory validation of single and multiplex real-time PCR methods for the quantification of the allergenic ingredients soybean and white mustard in sausages and sesame, almond, Brazil nut and lupine in baked products and sauce powder (Siegel et al. 2012; Waiblinger et al. 2014). Those validation studies (ring trials) were planned and organized by the working group “Food Allergens” of the Federal Office of Consumer Protection and Food Safety within the scope of the official method collection according to § 64 of the German food and feed code (LFGB). In both ring trials, the quantification approach was based upon a calibration curve generated from matrix-matched food standards incurred with defined levels of the respective allergenic food ingredients (Siegel et al. 2013).

In this work, we present another ring trial validation of published real-time PCR methods following a similar approach: 2 single real-time PCR methods for a simultaneous joint detection and quantification of brown/black mustard and of wheat/rye, respectively (Palle-Reisch et al. 2013; Zeltner et al. 2009). Additionally, a multiplex real-time PCR method to combine the detection and quantification of brown/black mustard, white mustard, celery and soybean was validated (Luber et al. 2015).

2 Materials and methods

2.1 Spiked (=incurred) food material

As ring trial samples, we used sausages of the type ‘Lyoner’ (French ‘Cervelas’) that were incurred with defined quantities of brown, black and white mustard, soybean, celery seed, wheat and rye (amongst others). Details on the material production and information about the incurred allergenic ingredients, as well as about the homogeneity testing of these materials, are described by Siegel et al. (2012; 2013). As described in these references, the material was filled into cans and then boiled for 1.5 h for conservation (material type “C”). Instead of boiling at normal pressure, one part of the material was sterilized by autoclaving for 60 min (material type “A”). All materials were stored frozen until analysis. The

composition of the materials used in this ring trial shows Table 1.

2.2 DNA extraction

For DNA extraction, a protocol for CTAB (Cetyl Trimethyl Ammonium Bromide) extraction was used: 2 g of the homogenized sample was mixed with the CTAB extraction buffer and consecutively incubated with Proteinase K solution (Siegel et al. 2012). The lysates were centrifuged and then purified by extraction with a mixture of Chloroform and Isoamyl alcohol. The DNA was precipitated using Iso-propanol. The pellet was washed with diluted ethanol (70%), centrifuged, dried and dissolved in 0.2× TE buffer. The DNA was further purified with the QIAQuick PCR Purification Kit (QIAGEN, Hilden, Germany) according to the manufacturer’s instructions and eluted using 50 µl of the elution buffer. The DNA extracts were stored at 2–8 °C until analysis.

The estimation of the DNA concentration by photometry (OD₂₆₀) was optional. 5 µl of the undiluted DNA was used for real-time PCR. For the PCR analysis of black/brown mustard (single PCR) and for the detection of PCR inhibitors, a part of the DNA was adjusted to a concentration of 40 ng/µl with 0.2× TE.

2.3 DNA calibration standards

Genomic DNA, extracted from ground seeds or kernels of brown mustard, white mustard, celery, soybeans and wheat was extracted using the same protocol as described in 2.1. The number of copies of the target DNA per volume was measured by Droplet DigitalTM PCR (QX200TM, Bio-Rad Laboratories, Cressier, CH). For this, the same primers as described in this work were used. Probes were labelled with a fluorophore, either HEX or FAM according to the recommendations of the QX200TM manual. The concentration of DNA extracts were determined and diluted to produce DNA extracts containing a mixture of genomic DNA containing 1000 copies of each target sequence per 5 µl. The DNA extracts were stored at 2–8 °C until analysis. Before analysis, the DNA mixture was diluted serially to yield the levels 1000 (undiluted), 316, 100, 31.6 and 10 copies per 5 µl.

2.4 Matrix standard DNA

DNA prepared by 2-fold extraction as described above from the 400 mg/kg sausage materials “A

Table 1 Sausage materials used in the ring trial

Sausage/type/name	Spiked allergenic ingredient (mg/kg)						
	Brown mustard	Black mustard	White mustard	Celery	Soy	Wheat	Rye
A 100	50	50	100	100	100	550	1160
A 40	20	20	40	40	40	220	464
A 10	5	5	10	10	10	55	116
C 100	0	0	100	100	100	100	100
C 40	0	0	40	40	40	40	40
C 0	0	0	0	0	0	0	0
A 400*	200	200	400	400	400	400	400
C 400*	0	0	400	400	400	400	400

* For material used to extract matrix standard DNA, see Sect. 2.3

400” and “C 400” was used for calibration (Table 1). For each material, both DNA extracts were pooled. The DNA extracts were stored at 2–8 °C until analysis.

2.5 Real-time PCR

Three real-time PCR systems were used for the amplification of sequences specific for

- black mustard (*Brassica nigra*) and brown mustard (*Brassica juncea*) (single PCR, joint detection)
- wheat (*Triticum*) and rye (*Secale cereale*) (single PCR, joint detection)
- black mustard (*Brassica nigra*) and brown mustard (*Brassica juncea*) (joint detection), white mustard (*Sinapis alba*), celery (*Apium graveolens*) and soybean (*Glycine max*) (multiplex PCR).

The respective primers and probes are shown in Table 2. The compositions of the PCR reaction mixes for the two single PCR systems and the multiplex PCR are listed in Table 3. The mastermix used for real-time PCRs was the Quantitect Multiplex Mastermix (2×) (QIAGEN; Hilden, Germany). Primers and probes were synthesized by Microsynth (Balgach, Switzerland). The following temperature–time-protocols were applied: initial denaturation for 15 min at 95 °C for 1 cycle, followed by 40 cycles of 30 s at 95 °C and 60 s at 60 °C for wheat/rye single PCR and for multiplex PCR; initial step for 15 min at 95 °C for 1 cycle, followed by 40 cycles of 15 s at 95 °C and 60 s at 55 °C for brown/black mustard single PCR. In each PCR, the fluorescence was measured during elongation.

2.6 Specificity testing and comparison between species/cultivars

For specificity testing, DNA was extracted from the respective material (kernels/seeds) of the following species: amaranth, apricot, barley, beet, white bean, broccoli, cashew, chervil, codfish, coriander, fennel, hazelnut, horseradish, kohlrabi, red lentil, linseed, lovage, lupine, pumpkin seed, maize, Brazil nut, oat, parsnip, parsley, green pea, peanut, sweet pepper, pistachio, poppy seed, potato, radish, rice, sesame, shrimps, sunflower seed, squid, tomato, walnut, and wheat.

In addition, DNA was extracted from kernels/seed derived from different cultivars of the targeted species (e.g. mustard, wheat and rye). Kernels and seeds were purchased at the local market, seeds from specified cultivars were provided by German breeders. The DNA concentration was measured photometrically, adjusted to a concentration of 10 ng/μl and analyzed using the 3 PCR systems described above.

2.7 Interlaboratory validation

The reliability of the methods was tested using an interlaboratory validation study with 13 participating laboratories (Table 4), mainly members of the working group “Food Allergens” of the Federal Office of Consumer Protection and Food Safety within the scope of the official method collection according to § 64 of the German food and feed code (LFGB).

We used material from sausage incurred with allergenic ingredients as mentioned in Sect. 2.1 and sent to the participants:

Table 2 Real-time PCR primer and probe sequences

Name	Sequence
(a) Single real-time PCR systems	
Black and brown mustard: partial RT gene for reverse transcriptase from Gypsy-like retro element 13G42-26 (multicopy-sequence), 76 bp (Palle-Reisch et al. 2013)	
11f	5'-GTT GAG CCG AGG GTC ATA ATT TC-3'
11r	5'-TCG ACT TAG GCA TCC TTA CGG-3'
11p	5'-ROX-CGA GAG TCC GAA TAC TGG GCT GGG GTC-BBQ-3'
Wheat and rye: high molecular weight (HMW) glutenin genes B1-1 (wheat) and 1-R (rye), 85 bp (Zeltner et al. 2009)	
tritprglut 3 F	5'-CCT CTT TGC GGC AGT AGT YGT-3'
tritprglut 3 R	5'-CTC GCR CTC ACA BTG TAG TTG-3'
tritglut	5'-FAM- CCT CGT GGC TCT CAC CRY CGC T- BHQ1 -3'
(b) Multiplex real-time PCR (Luber et al. 2015)	
Black and brown mustard: see single PCR	
White mustard: mRNA sequence coding for MADS-D protein of <i>Sinapis alba</i> (NCBI accession no. Y08626), 74 bp (Fuchs et al. 2010)	
MADS D F	5'-TGA AAA CTC TCT TCC CCT CTT AGG-3'
MADS D R	5'-ACA AAT GCA CAC AAG ACA GAG ATA TAG A-3'
MADS D pr	5'-HEX-TAC ATG ATG CTT ACC TCG C-BBQ-3'
Celery: sequence of mannitol dehydrogenase gene of <i>Apium graveolens</i> , 101 bp (Hupfer et al. 2007)	
Cel-MDH-iF	5'-CGA TGA GCG TGT ACT GAG TC -3'
Cel-MDH-iR	5'-AAT AGG AAC TAA CAT TAA TCA TAC CAA AC-3'
Cel-MDH-probe	5'-FAM-AAC AGA TAA CGC TGA CTC ATC ACA CCG -BBQ
Soybean: sequence of lectin gene of <i>Glycine max.</i> (Official collection of Test methods 2012)	
Lectin-F	5'-TCC ACC CCC ATC CAC ATT T-3'
Lectin-R	5'-GGC ATA GAA GGT GAA GTT GAA GGA-3'
Lectin probe	5'-CY5- AAC CGG TAG CGT TGC CAG CTT CG-BBQ-3'

FAM 6-Carboxyfluorescein, ROX 6-Carboxyl-X-Rhodamine, BBQ Black berry quencher, BHQ 1 Black hole quencher 1, HEX 6-Hexachloro-fluorescein, CY5 Cyanin-5, FAM 6-carboxyfluorescein, BBQ Black berry quencher, BHQ 1 and 2 Black hole quencher 1 and 2

- 18 encoded samples (3 samples for each of the 6 spiking levels)
- 2 samples containing the matrix standard (400 mg/kg; "A400" and "C400", see Sect. 2.3)
- Aliquots of the dried oligonucleotides and probes (Tables 2, 3)
- An aliquot of a mixture of genomic DNA from brown mustard, white mustard, celery, soybeans, and wheat, adjusted to 1000 copies of each target sequence per 5 µl each (see Sect. 2.2)

The laboratories were asked to order the mastermixes for the real-time PCR analyses individually. The suitability of the following commercially available mastermixes had been tested before the study: QuantiTect Multiplex PCR Kit (No ROX), QIAGEN, Hilden (D); Perfecta Multiplex qPCR ToughMix, Bionline, Luckenwalde (D) and SensiMix™ II Probe No-Rox Kit (Quanta/VWR, Darmstadt (D). All these mastermixes were recommended to the participants, but all laboratories used the "Quantitect Multiplex

Mastermix (2×) no ROX" (QIAGEN, Hilden). There was no restriction in terms of the type of real-time PCR system used. A summary of the layout of the inter-laboratory validation is given in Table 5.

The DNA extraction and the real-time PCRs had to be performed according to the above-mentioned protocol. Each coded sample had to be extracted once. Each DNA extract had to be analyzed in duplicates in all 3 real-time PCR methods and each level of the calibration standard DNA dilution series in triplicates.

2.8 Qualitative and quantitative evaluation

For the qualitative evaluation, Ct values <40 were considered as amplification (=positive result). Additionally, the sample Ct values were compared with the average Ct value of the lowest standard (10 copies per 5 µl). If the Ct value of the sample is at least 1 higher than the Ct of the lowest standard (10 cp/

Table 3 Reaction mixes for real-time PCR

Reagent	Working concentration	Final concentration	Volume per reaction (μl)
Single PCR: Black and brown mustard			
Quantitect Multiplex Mastermix, QIAGEN, Hilden	2×	1×	12.5
Primer 11f	3 μM	0.3 μmol/L	2.5
Primer 11r	3 μM	0.3 μmol/L	2.5
Probe 11p	0.5 μM	0.05 μmol/L	2.5
DNA-extract	40 ng/μl		5.0
Water			–
Total reaction volume each PCR			25
Single PCR: wheat and rye			
Quantitect Multiplex Mastermix, QIAGEN, Hilden	2×	1×	12.5
Primer tritprglut 3 F	6 μM	0.6 μmol/L	2.5
Primer tritprglut 3 R	6 μM	0.6 μmol/L	2.5
Probe tritprglut	1.5 μM	0.15 μmol/L	2.5
DNA-extract	Undiluted		5.0
Water			–
Total reaction volume each PCR			25
Multiplex PCR: black and brown mustard, white mustard, celery and soybean			
Quantitect Multiplex Mastermix, QIAGEN, Hilden.	2×	1×	12.5
Primer 11f	^a	0.3 μM	^a
Primer 11r		0.3 μM	
Probe 11p		0.05 μM	
Primer MADS D F		0.1 μM	
Primer MADS D R		0.1 μM	
Probe MADS D pr		0.12 μM	
Primer Cel-MDH-iF		0.3 μM	
Primer Cel-MDH-iR		0.3 μM	
Probe Cel-MDH-probe		0.2 μM	
Primer Lectin-F		0.9 μM	
Primer Lectin-R		0.9 μM	
Probe Lectin		0.2 μM	
DNA-extract	Undiluted		5.0
Total reaction volume each PCR			25

^a The volumes of solutions of primers and probes and added water depend on the concentrations of the working solutions. Within the ring trial, the participants were provided with dried aliquots of prime/probe mixtures to be stored in the dark until analysis. Aliquots were dissolved in a defined amount of water

5 μl), the result was considered as “negative” (Waiblinger et al. 2011).

In order to evaluate the results of the samples quantitatively, the Ct values were first assigned to concentrations in copy numbers of the respective target sequence per PCR using the DNA calibration standard curve by linear regression of the data of the calibration series. Average Ct values obtained by real-time PCR amplification of the DNA standard dilution levels were plotted against the logarithm of the corresponding DNA concentration (1000, 316, 100, 31.6

and 10 copies/5 μl). For each DNA extraction of the samples including the matrix standards, mean values of the concentrations obtained for the PCR duplicates were calculated. The DNA concentrations given in copy number of the target sequence per PCR were assigned to concentrations in mg/kg using the averaged results of the matrix standard DNA.

The participants were asked to report results of each PCR as Ct values as well as concentrations. Median, mean and standard deviation were calculated from these average values (n = 3 per

Table 4 Interlaboratory validation—list of participants

Laboratory	City	Country
AGES Österreichische Agentur für Gesundheit und Ernährungssicherheit GmbH	Vienna	Austria
Bayerisches Landesamt für Gesundheit und Lebensmittelsicherheit	Oberschleißheim	Germany
Bundesforschungsinstitut für Ernährung und Lebensmittel (MRI)	Kulmbach	Germany
Chemisches und Veterinäruntersuchungsamt (CVUA) Freiburg	Freiburg	Germany
CVUA Karlsruhe	Karlsruhe	Germany
CVUA Sigmaringen	Sigmaringen	Germany
Congen Biotechnologie GmbH	Berlin	Germany
Institut für Hygiene und Umwelt	Hamburg	Germany
Kantonales Laboratorium Zürich	Zuerich	Switzerland
Landesbetrieb Hessisches Landeslabor (LHL)	Kassel	Germany
Landeslabor Schleswig-Holstein	Neumünster	Germany
LAVES Niedersachsen, Lebensmittel- und Veterinärinstitut Braunschweig/Hannover	Braunschweig	Germany
Landesuntersuchungsanstalt für das Gesundheits- und Veterinärwesen Sachsen	Dresden	Germany

Table 5 Layout of the ring-trial

Sausage/type/name (see Table 1)	Samples per lab	Extraction per sample	PCRs per extraction	Number of PCR reactions per level
A 400	1	Standard material		
C 400	1	Standard material		
A 100	3	1	2	6
A 40	3	1	2	6
A 10	3	1	2	6
C100	3	1	2	6
C 40	3	1	2	6
C 0	3	1	2	6

concentration level). For comparison of the trueness of the results of the individual laboratories, the difference between the individual mean and the median result of all laboratories was calculated. If the result was below 5 copies per μl DNA extract (mean of PCR duplicates), it was not considered for quantitative evaluation.

Besides, if the slopes of the standard curves per material and allergen were below -4.1 (corresponding to a PCR efficiency below 80%), the data were not considered, also if the mean of the 3 extracts deviated from the median value was more than 50% (in copy number/5 μl and mg/kg). After exclusion of these data, robust mean and robust standard deviation in copy number/5 μl and mg/kg were calculated with an algorithm according to ISO 5725-5 (1998).

Before the calculation of the mean and median values, the repeatability standard deviation (RSD_r), and reproducibility standard deviation (RSD_R),

outliers were identified using the Grubbs test (5% significance level) and removed from the dataset. All calculations were performed by using the software² Valoo 2.4.

3 Results and discussion

3.1 Specificity tests

Prior to the ring trial, the specificity of previously reported methods was randomly confirmed using DNA extracted from material as mentioned in Sect. 2 (Palle-Reisch et al. 2013; Zeltner et al. 2009; Lubner et al. 2015; Fuchs et al. 2010; Hupfer et al. 2007; Official collection of test methods 2012). No cross reactivity was observed for the black and brown

² <http://www.analytik-software.de/>.

mustard single PCR. The other PCRs showed weak cross-reactivity between 0.01 and 0.1%:

- Wheat (*Triticum*) and rye, single PCR: cross reaction with amaranth and lupine ($\leq 0.02\%$ each)
- Multiplex-PCR:
 - Brown/black mustard: Celery (ca. 0.01%)
 - White mustard: Alfalfa (ca. 0.1%)
 - Celery: Coriander, Lovage (ca. 0.01% each)
 - Soy: Potato (ca. 0.01%).

3.2 Between cultivar/species variation

Furthermore, complementing the data published previously, the response of the methods depending on the variation of cultivars or species originally targeted by the methods shows Table 6. According to these results, brown and black mustard are detected with a similar response in both the single and the multiplex PCR. This is also the case for species and subspecies within the genus *Triticum* as well as for rye in the “wheat and rye” PCR. Barley is also detected, however with a response of only about 0.1% compared to *Triticum* and rye. The method can therefore not be used for the detection of the gluten containing cereals barley and oat.

3.3 In-house testing for sensitivity

Complementing the ring trial, the method sensitivity was estimated in our laboratory using a dilution series of the DNA calibration standard. Following the procedure described by the German Federal Office of Consumer Protection and Food Safety (BVL 2016), the DNA mixture was serially diluted to yield the levels 20, 10, 5, 2, 1 and 0.1 copies per 5 μ l (BVL 2016). The LOD_{95%} was estimated using an online tool³:

- Black/brown mustard single PCR: 7.5 copies (brown mustard DNA)
- Multiplex PCR:
- Black/brown mustard: 9.7 copies (brown mustard DNA)
- White mustard: 8.5 copies
- Celery: <10 copies
- Soy: 3 copies

In the course of the in-house validation and further application of the method within routine testing, the detection of celery in the multiplex PCR

yielded weak false positive signals for the non-template control (NTC) (Ct values >38, corresponding to concentrations far below 5 copies and 5 mg/kg, resp.). Therefore, a more precise determination of the LOD was not possible for celery in the multiplex PCR.

During the inter-laboratory validation, false positive reactions for the NTC were not reported. The sensitivity of the wheat (*Triticum*) and rye single PCR was estimated using a 6-fold dilution of wheat DNA. The lowest level yielding positive reactions in all replicates was 10 copies per PCR. In-house routine analysis using spiked (= incurred) control samples of boiled sausage meat and rice cookies demonstrated that 20 to 40 mg/kg of wheat or rye and 5 to 10 mg/kg of the other allergenic ingredients can be reliably detected with these methods (data not shown).

3.4 Interlaboratory validation

The reliability of these methods was tested in an interlaboratory validation study with 13 participating laboratories.

3.4.1 Deviations from the ring trial protocol

Some deviations from the ring trial protocol were reported by participating laboratories: In one laboratory, the purification of the DNA was performed using the mericon Food Kit (QIAGEN, Hilden) instead of the recommended QIAQuick PCR Purification Kit. The reported DNA concentration (assessed photometrically) was about half of the average value of the other participants. Another laboratory purified the DNA from 2 samples using the QIAQuick purification kit. Incubation with extraction buffer was done at 50 °C overnight instead of for 90 min at 65 °C in a third laboratory. Finally, one laboratory used fluorimetric instead of photometric estimation of the DNA concentration. However, initially the data of all 13 laboratories were considered for evaluation. The criteria for the exclusion of results are described in Sect. 2. Moreover, results were not included in the evaluation if the data of the calibration curves were far out of the expected range (see below).

3.4.2 Calibration standard DNA and matrix standard DNA

The results of the calibration series are summarized in Table 7. In addition, it shows the results for the matrix standard DNA using the calibration standard DNA. Performance criteria for standard curves are described e.g. in guidelines for GMO analysis (ENGL

³ <https://quodata.de/content/validation-qualitative-pcr-methods-single-laboratory>.

Table 6 Between species/cultivar variation of Ct values (10 ng/μl of DNA analyzed in PCR)

Species (number of tested cultivars)	Ct values single PCR		Ct values multiplex PCR	
	Mean	Standard deviation	Mean	Standard deviation
(a) Brown and black mustard in single-/multiplex PCR				
Brown mustard (<i>B. juncea</i>) (3)	19.2	0.85	18.8	0.95
Black mustard (<i>B. nigra</i>) (1)	19.6		18.9	
Species (number of tested cultivars)	Ct values			
	Mean		Standard deviation	
(b) <i>Triticum</i> (wheat) and <i>secale</i> (rye) PCR				
Wheat (<i>T. aestivum</i>) (13)	27.21		0.27	
Spelt (<i>T. spelta</i>) (5)	27.31		0.30	
Durum wheat (<i>T. durum</i>) (2)	26.9			
Emmer (<i>Triticum dicoccum</i>) (1)	27.3			
Khorasan wheat (<i>Triticum turgidum</i> × <i>polonicum</i>) (1)	26.5			
Barley (<i>Hordeum vulgare</i>) (8)	37.30		0.87	
Rye (<i>Secale cereale</i>) (5)	27.10		0.13	
Oat (<i>Avena sativa</i>) (5)	No Ct			

2015). According to these guidelines, the slope of the standard curve should be between -3.1 and -3.6 and R^2 should be >0.98 . For multiplex PCR used for qualitative analysis, a slope between -2.9 and -3.9 deemed acceptable (Broeders et al. 2014).

The number of laboratories fulfilling these criteria was higher when the lowest standard of 10 copies/5 μl was not taken into account for the standard curves (standard dilution from 1000 to 31.6 cp/ 5 μl instead of 1000 to 10 cp/ 5 μl). This was the case for both the single PCR systems (*Triticum/Secale* and brown/black mustard) and the brown/black mustard PCR within the multiplex PCR (Table 7a–c). For example, in the black/brown mustard single PCR, the reported slopes and R^2 were out of the recommended range for 6 and 7 laboratories, respectively. Without considering the lowest standard of 10 copies/5 μl, slope and R^2 were not acceptable for 2 or 3 laboratories, respectively. Some of the slopes of the calibrations curves obtained in laboratory 5 and 9 were far outside the expected range. Therefore, the respective results of these laboratories were not taken into account for quantitative evaluation. The lowest standard of the dilution series (10 cp/PCR) was simultaneously used to estimate the sensitivity of the respective PCR. For 38 of 39 reactions ($=97\%$), amplifications were obtained in the brown/black mustard single PCR and in the *Triticum/Secale* single PCR. In the multiplex PCR, for all 4

systems 95% of reactions (37 out of 39) were positive. Without considering the results of laboratory 5 (see above), the rate of positive results was 100% each ($=0\%$ false negatives).

3.4.3 Results of the encoded samples

The samples were decoded and assigned to the 6 sausage meat materials. Per material and lab, 6 PCR results (3 DNA extracts analyzed in duplicates each) were obtained.

3.4.3.1 Qualitative evaluation The results are shown in Table 8a/b. At the levels of 100 mg/kg (autoclaved and boiled sausages) and 40 mg/kg (boiled sausage meat), for all PCR systems (2 single, 4 included in multiplex PCR) the expected positive results were obtained. Unexpected positive results for brown/black mustard in both single and multiplex PCR were reported in the boiled sausage materials C 100 and C 40. Additional analyses showed that the white mustard material also contained in the sausages C 100 and C 40 was contaminated with brown/black mustard. The higher level of positive results detected by multiplex PCR can be explained by the use of undiluted DNA for the PCR instead of DNA diluted to 40 ng/μl in the single PCR.

At 40 and 10 mg/kg, none of the incurred allergenic ingredients were detected reliably in the autoclaved material, except for brown/black mustard.

Table 7 Interlaboratory validation: real-time PCR systems and results of the dilution series of the calibration standard DNA (1000–10 copies/PCR)

Lab no.	Real-time PCR-system ¹	Slope	Slope (without 10 cp)	R ²	R ² (without 10 cp)	Standard 10 cp ²	MS-DNA ³
(a) PCR system <i>Triticum</i> and <i>Secale</i>							
1	a	-3.88	-3.84	0.963	0.994	3	184
2	b	-4.00	-3.51	0.940	0.990	3	251
3	c	-3.14	-3.04	0.963	0.972	3	134
4	d	-3.54	-3.59	0.989	0.985	3	281
5	a	-3.69	-3.77	0.947	0.980	3	378
6	c	-3.26	-3.21	0.990	0.993	3	420
7	e	-3.92	-3.84	0.984	0.983	3	292
8	a	-3.50	-3.42	0.994	0.993	2	205
9	f	-4.58	-4.34	0.972	0.984	3	329
10	b	-3.31	-3.28	0.970	0.970	3	345
11	b	-3.28	-3.12	0.991	0.993	3	254
12	g	-3.36	-3.36	0.992	0.992	3	177
13	c	-3.28	-3.57	0.985	0.990	3	314
Mean		-3.60	-3.55	0.975	0.986	38 of 39 (=97%)	274 (StDev: 31%)
(b) Single PCR system "brown/black mustard"							
1	a	-3.98	-3.49	0.95	0.99	3	1080
2	b	-3.64	-3.74	0.96	0.98	3	643
3	c	-3.59	-3.70	0.98	0.986	3	1497
4	d	-3.99	-3.71	0.97	0.99	3	1312
5	a	-3.69	-3.76	0.95	0.98	3	2309
6	c	-3.67	-3.67	0.99	0.995	3	1810
7	e	-3.28	-3.46	0.96	0.986	3	1977
8	a	-3.24	-3.03	0.77	0.96	2	1093
9	f	-4.20	-4.04	0.97	0.99	3	–
10	b	-3.53	-3.28	0.98	0.97	3	2079
11	b	-3.15	-3.36	0.99	0.99	3	1852
12	g	-3.17	-3.56	0.98	0.995	3	1825
13	c	-3.52	-3.56	0.99	0.984	3	1630
Mean		-3.59	-3.57	0.96	0.984	38 of 39 (=97%)	1592 (StDev: 30%)
(c) Multiplex PCR							
1. System "brown/black mustard"							
1	a	-3.62	0.97	-3.57	0.98	3	1352
2	b	-3.73	0.97	-3.9	0.99	3	2927
3	c	-3.29	0.98	-3.57	0.98	3	1813
4	d	-3.51	0.99	-3.69	0.99	3	4639
5	a	-4.58	0.99	-4.43	0.99	1	2773
6	c	-3.31	0.98	-3.63	0.99	3	5349
7	e	-3.79	0.96	-3.48	0.99	3	2466
8	a	-2.9	0.96	-3.34	0.99	3	3129
9	f	-4.43	0.94	-4.15	0.95	3	2630
10	b	-3.74	0.99	-3.73	0.99	3	4044
11	b	-3.48	0.97	-3.15	0.99	3	3691
12	g	-3.35	0.98	-3.29	0.99	3	2389
13	c	-3.36	0.99	-3.45	0.99	3	3448
Mean		-3.62	0.97	-3.64	0.98	37 of 39 (=95%)	3127 (StDev: 35%)

Table 7 continued

Lab no.	Real-time PCR-system ¹	Slope	R ²	Standard 10 cp ²	MS-DNA ³
2. System "White mustard"					
1	a	-3.75	0.99	3	814
2	b	-4.72	0.98	3	–
3	c	-3.88	0.99	3	923
4	d	-3.75	0.99	3	1522
5	a	-5.60	0.98	1	–
6	c	-3.55	0.99	3	1594
7	e	-3.88	0.99	3	1158
8	a	-3.65	1.00	3	1215
9	f	-4.00	0.99	3	1361
10	b	-3.80	0.99	3	1577
11	b	-3.76	0.99	3	1208
12	g	-3.62	1.00	3	916
13	c	-3.48	0.98	3	1852
<i>Mean</i>		<i>-3.74 (without L 2 and 5)</i>	<i>0.99 (without L 2 and 5)</i>	<i>37 of 39 (=95%)</i>	<i>1285 (StDev: 26%)</i>
3. System "Celery"					
1	a	-3.62	0.99	3	684
2	b	-3.89	1.00	3	1166
3	c	-3.52	0.98	3	732
4	d	-3.76	1.00	3	935
5	a	-4.49	0.98	1	–
6	c	-3.54	0.97	3	1311
7	e	-3.33	0.99	3	996
8	a	-3.46	0.98	3	1147
9	f	-4.46	0.98	3	–
10	b	-3.58	0.99	3	1292
11	b	-3.43	0.99	3	924
12	g	-3.38	0.98	3	854
13	c	-3.40	0.99	3	1498
<i>Mean</i>		<i>-3.54 (without L 5 and 9)</i>	<i>0.99 (without L 2 and 5)</i>	<i>37 of 39 (=100%)</i>	<i>1049 (StDev: 24%)</i>
4. System "Soybean"					
1	a	-4.00	0.98	3	322
2	b	-4.72	0.98	3	575
3	c	-3.69	0.99	3	330
4	d	-3.54	0.99	3	579
5	a	-4.7	0.98	1	814
6	c	-3.51	0.99	3	581
7	e	-3.77	0.99	3	483
8	a	-3.5	0.99	3	339
9	f	-4.2	0.99	3	607
10	b	-3.59	0.99	3	655
11	b	-3.61	0.99	3	426
12	g	-3.69	0.98	3	525

Table 7 continued

Lab no.	Real-time PCR-system ¹	Slope	R ²	Standard 10 cp ²	MS-DNA ³
13	c	-3.33	0.98	3	729
Mean		-3.83	0.986	37 of 39 (=100%)	536 (StDev: 29%)

Data with and without inclusion of the lowest dilution level of 10 copies/5 µl in the standard curve. In bold: results out of specification for slope (between -3.1 and -3.6) and R² (>0.98), specification for multiplex PCR: slope between -2.9 and -4.1, R² > 0.98 (Broeders et al. 2014)

a ABI 7500 and ABI 7500 Fast resp. (Applied Biosystems/Life Technologies)

b MX3005P (Agilent)

c Rotorgene RS 6000 (Corbett)

d CFX 96 (Biorad)

e Rotorgene Q (QIAGEN, Hilden)

f Aria Mx (Agilent)

g Light Cycler 480 (Roche)

¹ Real-time PCR systems used in the ring-trial

² Std 10 cp = number of positive reactions for standard dilution 10 cp/5 µl (n = 3)

³ MS-DNA = results of matrix standard DNA (cp/5 µl; mean of 2 reactions)

Obviously, the sensitivity of detection is reduced by autoclaving the materials after spiking with the allergenic ingredients. However, due to the higher sensitivity of the brown/black mustard PCR, which targets a multi-copy sequence, 40 and 10 mg/kg of brown and black mustard can still be detected after autoclaving.

For “blank” sausage meat material (not containing the relevant allergic ingredients), sufficient ratios (<5%) of false positive reactions were obtained. In the *Triticum/Secale* PCR, 9 positive reactions close to Ct values of 40 were reported, corresponding to a false positive ratio of 11.5%. This ratio is reduced to 1.3%, if a different mode of evaluation is used (Table 8, legend remark 2).

In summary, 40 mg/kg of the allergenic ingredients in boiled sausage meat could reliably be detected in the interlaboratory validation study for all PCR systems. For wheat and rye, a limit of detection of 80 mg wheat or rye per kg can be assumed in this matrix, since both wheat and rye were spiked with 40 mg/kg each in this material and a similar response of amplification was observed for both species.

3.4.3.2 Quantitative evaluation Table 9 shows a summary of the statistical evaluation of all interlaboratory validation results. Due to the lack of appropriate guidelines and performance criteria related to allergen analysis, guidelines for real-time PCR-based GMO analysis were used for the evaluation of the results (ENGL 2015; Broeders et al. 2014).

3.4.3.3 Trueness/recoveries According to guidelines, the deviation of the mean value from the true value or an accepted reference value should not exceed 25% (ENGL 2015; Broeders et al. 2014). Except for the quantification of brown/black mustard, the non-autoclaved sausage standard material was used for the calculation of the recoveries. The recoveries thus express the percentage deviations of the results in relation to this matrix standard (=100%), (Siegel et al. 2013).

The above-named criterion for trueness was fulfilled for the analysis of the materials C 40 and C 100 for the single PCR wheat/rye and for the PCR systems celery and white mustard in the multiplex PCR. The soybean quantification in the multiplex PCR yielded acceptable recoveries for the material C 100.

Considerably lower recoveries (range 11–33%) were obtained for the autoclaved material.

In contrast, recoveries of more than 100% were obtained for brown/black mustard. As mentioned, this allergen was quantified using the autoclaved sausage material and the obtained values in mg/kg thus refer to this material. The autoclaved material contained significantly lower quantities of black/brown mustard DNA compared to non-autoclaved material of the same spike level. Despite that, the recoveries for black/brown mustard ranged from 125 to 176% (single and multiplex PCR) and thus did not fulfil the criterion of ±25%.

Moreover, in the quantitative range, the repeatability standard deviation should not exceed 25% and the reproducibility standard deviation should be

Table 8 Inter-laboratory validation, qualitative evaluation

Material and spike level (mg/kg)	A 100	A 40	A 10	C 100	C 40	C 0
(a) Single PCR systems (13 labs considered)						
<i>Triticum</i> and <i>Secale</i>						
Number of samples per lab	3	3	3	3	3	3
Number of samples total	39	39	39	39	39	39
Number of PCR results total	78	78	78	78	78	78
Number of positive PCR results	78	78 ^a , 77 ^b	73 ^a , 62 ^b	78	78 ^a , 77 ^b	9 ^a , 1 ^b
False positive results	–	–	–	–	–	9 ^a , 1 ^b
False positive results (%)	–	–	–	–	–	11.5 ^a , 1.3 ^b
False negative results	0	0 ^a , 1 ^b	5 ^a , 16 ^b	0	0 ^a , 1 ^b	–
False negative results (%)	0	0 ^a , 1.3 ^b	6.4 ^a , 20.5 ^b	0	0 ^a , 1.3 ^b	–
Brown/black mustard						
Number of samples per lab	3	3	3	3	3	3
Number of samples total	39	39	39	39	39	39
Number of PCR results total	78	78	78	78	78	78
Number of positive PCR results	78	78 ^a , 77 ^b	76 ^{a,b}	26 ^a , 9 ^b	2 ^a , 0 ^b	0 ^{a,b}
False positive results	–	–	–	26 ^a , 9 ^b	2 ^a , 0 ^b	0 ^{a,b}
False positive results (%)	–	–	–	36 ^a , 11.5 ^b	2.6 ^a , 0 ^b	0 ^{a,b}
False negative results	0	0 ^a , 1 ^b	2 ^{a,b}	–	–	–
False negative results (%)	0	0 ^a , 1.3 ^b	2.6 ^{a,b}	–	–	–
(b) Multiplex PCR system (13 labs considered)						
Brown/black mustard						
Number of samples per lab	3	3	3	3	3	3
Number of samples total	39	39	39	39	39	39
Number of PCR results total	78	78	78	78	78	78
Number of positive PCR results	78	76 ^{a,b}	78	76 ^a , 43 ^b	60 ^a , 30 ^b	2 ^a , 0 ^b
False positive results	–	–	–	76 ^a , 43 ^b	60 ^a , 30 ^b	2 ^a , 0 ^b
False positive results (%)	–	–	–	97 ^a , 55 ^b	77 ^a , 39 ^b	2.6 ^a , 0 ^b
False negative results	0	2 ^{a,b}	0	–	–	–
False negative results (%)	0	2.6 ^{a,b}	0	–	–	–
White mustard						
Number of samples per lab	3	3	3	3	3	3
Number of samples total	39	39	39	39	39	39
Number of PCR results total	78	78	78	78	78	78
Number of positive PCR results	78	75 ^a , 62 ^b	60 ^a , 29 ^b	78	78	4 ^a , 1 ^b
False positive results	–	–	–	–	–	4 ^a , 1 ^b
False positive results (%)	–	–	–	–	–	5.1 ^a , 1.3 ^b
False negative results	0	3 ^a , 16 ^b	18 ^a , 49 ^b	0	0	–
False negative results (%)	0	3.8 ^a , 20.5 ^b	23 ^a , 63 ^b	0	0	–
Celery						
Number of samples per lab	3	3	3	3	3	3
Number of samples total	39	39	39	39	39	39
Number of PCR results total	78	78	78	78	78	78
Number of positive PCR results	76 ^a , 75 ^b	76 ^a , 40 ^b	70 ^a , 20 ^b	78	78	4 ^a , 1 ^b
False positive results	–	–	–	–	–	4 ^a , 1 ^b
False positive results (%)	–	–	–	–	–	5.1 ^a , 1.3 ^b
False negative results	2 ^a , 3 ^b	2 ^a , 38 ^b	8 ^a , 58 ^b	0	0	–
False negative results (%)	2.6 ^a , 3.8 ^b	2.6 ^a , 48.7 ^b	10 ^a , 74 ^b	0	0	–

Table 8 continued

Material and spike level (mg/kg)	A 100	A 40	A 10	C 100	C 40	C 0
Soy						
Number of samples per lab	3	3	3	3	3	3
Number of samples total	39	39	39	39	39	39
Number of PCR results total	78	78	78	78	78	78
Number of positive PCR results	78 ^{a,b}	74 ^a , 68 ^b	63 ^a , 41 ^b	78	77 ^{a,b}	5 ^{a,c} , 0 ^{b,c}
False positive results	–	–	–	–	–	5 ^{a,c} , 0 ^{b,c}
False positive results (%)	–	–	–	–	–	6.4 ^{a,c} , 0 ^{b,c}
False negative results	0 ^{a,b}	4 ^a , 10 ^b	15 ^a , 37 ^b	0	1 ^{a,b}	–
False negative results (%)	0 ^{a,b}	5.1 ^a , 12.8 ^b	19 ^a , 47.4 ^b	0	1.3 ^{a,b}	–

^a All amplifications considered with Ct < 40

^b Evaluation by comparison of the sample Ct value with the average Ct value of the lowest standard (10.2 mg/kg). If Ct value of the sample is at least 1 higher than the Ct (10.2 mg/kg), the result is evaluated as “negative” (Waiblinger et al. 2011)

^c Results of one lab not considered (6 of 6 false-positive reactions)

below 35%. At the level of the quantification limit, a value below 50% is accepted (ENGL 2015; Broeders et al. 2014).

In this interlaboratory validation study; the repeatability standard deviation was below 25% or slightly above for all PCR systems in the analysis of the materials which could be evaluated quantitatively.

The criterion of <35% for reproducibility standard deviation (RSD_R) was met for both single PCR methods. In the multiplex PCR, at least the requirement related to the RSD_R at the quantification limit (<50%) could be fulfilled for all 4 PCR systems.

In general, the precision data obtained from the evaluation of the results in mg/kg were better than those obtained from the evaluation as copy numbers. A possible explanation for this observation could be the normalization of diverging DNA extraction efficiencies by parallel extraction of the matrix standard sample (Table 7). For example, if generally low DNA quantities were extracted within one laboratory, low copy numbers of target DNA were measured. When converting copy numbers to concentrations in mg/kg, this result of low copy number in the sample is relativized if the matrix standard and the sample were both extracted with similar poor extraction efficiencies.

4 Conclusion and further work

Following previous studies on interlaboratory validation to detect and quantify allergenic ingredients (Siegel et al. 2012; Waiblinger et al. 2014), the present work shows the results of another interlaboratory (ring trial) validation on PCR methods for the joint detection and quantification of brown/black mustard and wheat/rye, respectively; as well as for a multiplex

PCR method combining the detection and quantification of brown/black mustard, white mustard, celery and soybean. According to in-house method testing with incurred materials, the methods are able to detect levels down to 20 to 40 mg/kg for wheat and rye and down to 5 to 10 mg/kg of the other allergenic ingredients. Within the ring trial, it was demonstrated that the spike level of 40 mg/kg (wheat/rye 80 mg/kg) can reliably be detected and quantified with all PCR systems even after boiling of the sausage meat. However, after an extremely strong processing step using autoclaving, the allergenic ingredients incurred at the levels 40 and 10 mg/kg could not anymore be detected sufficiently. The sensitivity of the detection was reduced 5- to 10-fold by this procedure compared to the sensitivity in boiled sausage meat. Nevertheless, due to the higher sensitivity of the brown/black mustard PCR targeting a multi-copy sequence, levels of 40 and 10 mg/kg of brown and black mustard can still be detected after autoclaving. The results for black/brown mustard in terms of sensitivity, trueness, and precision were comparable in the single- and the multiplex PCR.

In general, the validated results obtained in this study are comparable to those generated in the previous ring trial validating PCR methods for the detection and quantification of allergenic ingredients in food matrices (Siegel et al. 2012; Waiblinger et al. 2014). Accordingly, the precision data obtained in this study suggest that the allergen levels under investigation (10 to 100 mg/kg of the allergenic food ingredient) are very close to the limit of quantification.

Compared to the previous ring trial studies, a new approach was used for the quantification: the participants were provided with a mixture of genomic DNA for calibration. Copy numbers of this standard

Table 9 Interlaboratory validation, quantitative evaluation

Material and spike level	A 100	A 40	A 10	C 100	C 40
(a) Single PCR systems					
Triticum and Secale					
Spike level wheat (mg/kg)	550	220	55	100	40
Spike level rye (mg/kg)	1160	464	116	100	40
Number of samples per lab	3	3	3	3	3
Number of samples/PCR results total	39/78	39/78	39/78	39/78	39/78
Number of samples considered ^a	33/66	30/60	0/0	39/78	36/72
Results in copy numbers (cp) per 5 µl					
Mean value (cp/5 µl) ^a	97.4	24.0	n.a.	72.3	32.2
Median (cp/5 µl) ^a	97.4	25.8	n.a.	71.8	29.3
Robust mean (algorithm A) (cp/5 µl)	96.7	23.7	n.a.	73.1	34.5
Standard deviation (cp/5 µl) ^a	26.9	8.0	n.a.	26.1	15.2
Robust StdDev (algorithm A) (cp/5 µl)	24.9	9.9	n.a.	20.8	16.7
Repeatability standard deviation RSD _r (cp/5 µl) ^a	14.0	7.0	n.a.	12.8	7.6
RSD _r (%)	14.4	29.2	n.a.	17.7	23.6
Reproducibility standard deviation RSD _R (cp/5 µl) ^a	27.5	14.1	n.a.	26.6	15.6
RSD _R (%) ^a	28.2	58.8	n.a.	36.8	48.4
Results in mg/kg (calculation using matrix control standard DNA)					
Mean value (mg/kg) ^a	282.6	74.9	n.a.	219.9	96.1
Recovery (%) ^b	16.5	11.0	n.a.	109.9	120.1
Median (mg/kg) ^a	279.6	67.5	n.a.	224.9	92.1
Robust mean (algorithm A) (mg/kg)	282.6	86.4	n.a.	220.1	100.3
Repeatability standard deviation RSD _r (mg/kg) ^a	21.3	4.08	n.a.	21.13	13.26
RSD _r (%) ^a	14.4	24.6	n.a.	19.8	21.3
Reproducibility standard deviation RSD _R (mg/kg) ^a	62.7	24.5	n.a.	67.1	34.0
RSD _R (%) ^a	22.2	32.7	n.a.	67.1	35.4
Material and spike level	A 100		A 40		A 10
Brown/black mustard					
Spike level brown mustard (mg/kg)	50		20		5
Spike level black mustard (mg/kg)	50		20		5
Number of samples per lab	3		3		3
Number of samples/PCR results total	39/78		39/78		39/78
Number of samples considered ^a	36/72		36/72		36/72
Results in copy numbers (cp) per 5 µl					
Mean value (cp/5 µl) ^a	566.4		188.1		61.8
Median (cp/5 µl) ^a	576.4		182.5		62.0
Robust mean (algorithm A) (cp/5 µl)	570.4		187.0		62.4
Standard deviation (cp/5 µl) ^a	144.7		54.7		15.5
Robust StdDev (algorithm A) (cp/5 µl)	119.4		57.6		12.7
Repeatability standard deviation RSD _r (cp/5 µl) ^a	65.1		26.9		13.2
RSD _r (%)	11.5		14.3		21.3
Reproducibility standard deviation RSD _R (cp/5 µl) ^a	148.2		55.9		15.7
RSD _R (%) ^a	26.2		29.7		25.4
Results in mg/kg (calculation using matrix control standard DNA)					
Mean value (mg/kg) ^a	146.7		50.0		15.8
Recovery (%) ^b	146.7		125.1		158.4
Median (mg/kg) ^a	144.3		49.9		14.7
Robust mean (algorithm A) (mg/kg)	145.8		49.6		62.4

Table 9 continued

Material and spike level	A 100	A 40	A 10
Repeatability standard deviation RSD_r (mg/kg) ^a	18.1	8.6	2.4
RSD_r (%) ^a	12.3	17.2	15.4
Reproducibility standard deviation RSD_R (mg/kg) ^a	32.3	15.8	4.3
RSD_R (%) ^a	22.0	31.6	27.1
(b) Multiplex system			
Brown/black mustard			
Spike level brown mustard (mg/kg)	50	20	5
Spike level black mustard (mg/kg)	50	20	5
Number of samples per lab	3	3	3
Number of samples/PCR results total	39/78	39/78	39/78
Number of samples considered ^a	36/72	38/76	39/78
Results in copy numbers (cp) per 5 µl			
Mean value (cp/5 µl) ^a	1236.2	396.8	129.6
Median (cp/5 µl) ^a	1205.3	402.9	136.0
Robust mean (algorithm A) (cp/5 µl)	1232.6	399.0	129.3
Standard deviation (cp/5 µl) ^a	331.4	118.9	52.8
Robust StdDev (algorithm A) (cp/5 µl)	332.3	130.2	56.1
Repeatability standard deviation RSD_r (cp/5 µl) ^a	9.4	33.6	22.2
RSD_r (%)	24.5	10.3	15.2
Reproducibility standard deviation RSD_R (cp/5 µl) ^a	17.5	78.0	37.6
RSD_R (%) ^a	45.9	23.8	25.8
Results in mg/kg (calculation using matrix control standard DNA)			
Mean value (mg/kg) ^a	146.7	50.0	15.8
Recovery (%) ^b	146.7	125.1	158.4
Median (mg/kg) ^a	144.3	49.9	14.7
Robust mean (algorithm A) (mg/kg)	145.8	49.6	62.4
Repeatability standard deviation RSD_r (mg/kg) ^a	18.1	8.6	2.4
RSD_r (%) ^a	12.3	17.2	15.4
Reproducibility standard deviation RSD_R (mg/kg) ^a	32.3	15.8	4.3
RSD_R (%) ^a	22.0	31.6	27.1
White mustard			
Material and spike level	A 100	C100	C40
Spike level white mustard (mg/kg)	100	100	40
Number of samples per lab	3	3	3
Number of samples/PCR results total	39/78	39/78	39/78
Number of samples considered ^a	33/66	33/66	33/66
Results in copy numbers (cp) per 5 µl			
Mean value (cp/5 µl) ^a	38.1	396.8	129.6
Median (cp/5 µl) ^a	36.0	402.9	136.0
Robust mean (algorithm A) (cp/5 µl)	37.2	399.0	129.3
Standard deviation (cp/5 µl) ^a	17.0	118.9	52.8
Robust StdDev (algorithm A) (cp/5 µl)	17.2	130.2	56.1
Repeatability standard deviation RSD_r (cp/5 µl) ^a	9.4	33.6	22.2
RSD_r (%)	24.5	10.3	15.2
Reproducibility standard deviation RSD_R (cp/5 µl) ^a	17.5	78.0	37.6
RSD_R (%) ^a	45.9	23.8	25.8

Table 9 continued

Material and spike level	A 100	A 40	A 10
Results in mg/kg (calculation using matrix control standard DNA)			
Mean value (mg/kg) ^a	11.7	103.3	45.9
Recovery (%) ^b	11.7	103.3	114.8
Median (mg/kg) ^a	11.3	105.0	45.0
Robust mean (algorithm A) (mg/kg)	11.6	103.3	45.9
Repeatability standard deviation RSD _r (mg/kg) ^a	2.82	12.2	6.77
RSD _r (%) ^a	24.1	11.8	14.7
Reproducibility standard deviation RSD _R (mg/kg) ^a	4.01	17.7	10
RSD _R (%) ^a	34.3	17.1	21.8
Celery			
Spike level celery (mg/kg)	100	100	40
Number of samples per lab	3	3	3
Number of samples/PCR results total	39/78	39/78	39/78
Number of samples considered ^a	30/60	38/76	38/76
Results in copy numbers (cp) per 5 µl			
Mean value (cp/5 µl) ^a	26.1	259.6	118.5
Median (cp/5 µl) ^a	24.1	262.9	121.4
Robust mean (algorithm A) (cp/5 µl)	25.1	63.4	118.8
Standard deviation (cp/5 µl) ^a	9.6	59.2	41.3
Robust StdDev (algorithm A) (cp/5 µl)	7.6	255.6	36.2
Repeatability standard deviation RSD _r (cp/5 µl) ^a	6.9	54.0	19.3
RSD _r (%)	26.6	20.8	16.3
Reproducibility standard deviation RSD _R (cp/5 µl) ^a	9.8	67.7	42.2
RSD _R (%) ^a	37.5	26.1	35.6
Results in mg/kg (calculation using matrix control standard DNA)			
Mean value (mg/kg) ^a	10.5	98.1	45.5
Recovery (%) ^b	10.5	98.1	113.7
Median (mg/kg) ^a	9.8	99.4	43.2
Robust mean (algorithm A) (mg/kg)	10.1	97	43.3
Repeatability standard deviation RSD _r (mg/kg) ^a	2.7	12.4	12.7
RSD _r (%) ^a	25.8	12.6	27.9
Reproducibility standard deviation RSD _R (mg/kg) ^a	4.13	20.3	15.8
RSD _R (%) ^a	39.4	20.7	34.7
Soybean			
Spike level black mustard (mg/kg)	100	100	40
Number of samples per lab	3	3	3
Number of samples/PCR results total	39/78	39/78	39/78
Number of samples considered ^a	33/66	34/68	38/76
Results in copy numbers (cp) per 5 µl			
Mean value (cp/5 µl) ^a	45.0	148.7	72.8
Median (cp/5 µl) ^a	44.1	147.3	68.9
Robust mean (algorithm A) (cp/5 µl)	44.7	146.8	74.3
Standard deviation (cp/5 µl) ^a	16.0	50.4	28.4
Robust StdDev (algorithm A) (cp/5 µl)	19.0	38.7	26.8
Repeatability standard deviation RSD _r (cp/5 µl) ^a	7.4	20.8	19.3
RSD _r (%)	17.6	14.0	26.5
Reproducibility standard deviation RSD _R (cp/5 µl) ^a	16.7	40.2	28.8
RSD _R (%) ^a	39.7	27.0	39.6

Table 9 continued

Material and spike level	A 100	A 40	A 10
Results in mg/kg (calculation using matrix control standard DNA)			
Mean value (mg/kg) ^a	33.1	114.1	64.4
Recovery (%) ^b	33.1	114.1	161.0
Median (mg/kg) ^a	34.2	114.7	58.0
Robust mean (algorithm A) (mg/kg)	38.1	120.7	61.6
Repeatability standard deviation RSD _r (mg/kg) ^a	7.1	16.7	17.9
RSD _r (%) ^a	21.5	14.7	27.7
Reproducibility standard deviation RSD _R (mg/kg) ^a	10.2	25.4	26.7
RSD _R (%) ^a	30.8	22.2	41.4

The robust standard deviation was calculated according to algorithm A without removal of outliers. The recovery was calculated by division of the mean value by the incurred (target) level. RSD_r (%) and RSD_R (%) are referring to the mean values of each level

Values of RSD_r (%), RSD_R (%) and recovery (%) are commented in Sect. 3.4.3.2 and therefore highlighted in bold

^a All data of a laboratory were removed, if the slope of standard curve was significantly below -4.1 , or if the mean values of the 3 extractions per lab and sample showed more than 50% deviation from the median of all labs for both the evaluation of copy numbers and of the concentrations in mg/kg

^b Assumption: similar response of wheat and rye DNA (and brown and black mustard, resp.) in PCR. Recovery refers to the sum of spiked wheat and rye (black and brown mustard, resp.) material

DNA had been determined using digital PCR. This approach was selected in order to test the use of a matrix independent quantification standard. However, the variation of the results (RSD_R) in terms of copy numbers showed that an absolute quantification without further normalization is challenging, especially due to the variability of the extraction efficiency. Results of validation studies focusing on the DNA extraction confirm this observation (Waiblinger et al. 2012a, b).

The participants were provided with one exemplary matrix sample (sausage meat with a defined quantity of the allergenic ingredient in mg/kg) to enable the conversion of the results from copy numbers to mg/kg. This control sample was extracted in parallel with the unknown samples. The results of the study revealed the importance of matrix control samples for allergen analysis in terms of both conversion to mg/kg and of normalization of variations in the extraction procedure between laboratories. For the purpose of routine analysis, the combination of a uniform DNA calibration standard and an individual set of matrix control samples matching the analyzed samples might be profitable.

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