Collaborative trial validation of a new multiplex real-time PCR to sensitively detect allergenic nuts in food

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Abstract
In this article, we present a multiplex real-time PCR method for a simultaneous, sensitive and specific detection and semi-quantitative estimation of the allergenic species peanut, hazelnut, walnut and cashew in food. Due to the use of multicopy target sequences, a very sensitive detection of the allergenic ingredients was possible. The method was validated in-house as well as by a collaborative trial with 12 laboratories. Within the ring trial, 0.64 mg/kg (i.e. approx. 0.1–0.2 mg of peanut and tree nut-derived protein/kg) could still be detected in a processed cookie matrix, confirmed by results of incurred, processed samples spiked at very low levels between 0.9 and 50 mg/kg of the corresponding allergenic ingredient (peanut, tree nut). In addition, the method revealed good precision data. With regard to quantitative analysis though, insufficient recovery data (bias) were determined in some cases, resulting in measurement uncertainties of more than 50%.

Keywords Allergen management · Nuts · Multiplex real-time PCR · Inter-laboratory validation · Quantification

1 Introduction
There is extensive data suggesting that food allergies are already ubiquitous (up to 10% of people affected) with sharp increases mainly in the last 2–3 decades (Sicherer and Sampson 2018). The occurrence of food allergies can be observed more frequently among children than adults. However, the list of foods which account for most of the severe disease courses is rather short, namely peanut, tree nuts, fish, shellfish, egg, milk, wheat, soy, and seeds (Sicherer and Sampson 2018), with the first two being among the most frequent triggers for food allergies in Europe (Nwaru et al. 2014).

To ensure the availability of information about the presence of relevant allergens in food, labelling provisions have been set in many countries. Within the European Union, labelling is mandatory for food ingredients containing—amongst others—peanut, hazelnut, walnut and cashew (Regulation 2011/1169/EC). In the case of positive findings, a quantification of the respective allergens or allergenic ingredients may be crucial for risk assessment, allergen management, and surveillance (The Allergen Bureau; Waiblinger and Schulze 2018). The implementation of legal threshold values is currently under discussion. An important factor for setting thresholds for food allergens is the availability of standardized methods for detection and quantification with sufficient sensitivity to detect even low amounts of allergenic components, if relevant. The official food control laboratories in Germany are working with an internal action value of 1 mg protein/kg (derived from peanut or tree nut) or 5 mg/kg whole peanut or tree nut, respectively. These action values serve as internal minimal thresholds by food control authorities and support laboratories in the decision-making process, whether further inspections at the production facility may be

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neglected or not (Waiblinger and Schulze 2018). The values are taking into account the published eliciting doses (Remington et al. 2020). In routine analysis, predominantly protocols based on real-time polymerase chain reaction (PCR) and immunological methods have been used (EFSA 2014). A real-time PCR method targeting a multicopy sequence from mitochondrial DNA has proven suitable for sensitive detection and even quantification of the allergenic ingredient peanut at the level of 5 mg/kg (Ladenburger et al. 2018; Waiblinger et al. 2019). Several single and multiplex real-time PCR methods for detection of the tree nuts cashew, hazelnut and walnut have already been published (Iniesto et al. 2013; Ehlert et al. 2008; Brežná et al. 2006; Koeppel et al. 2010, 2012). The sensitivity (limit of detection) of these methods ranges between 5 and 20 mg/kg not covering such low concentrations as to protect very sensitive consumers. By the use of multicycopy instead of singlecopy target sequences the sensitivity of the allergen detection can be increased significantly (Ladenburger 2018).

In this article, the design, intra- and inter-laboratory validation of a multiplex real-time PCR method “AllNut” for the simultaneous sensitive detection and semi-quantitative estimation of the allergenic foods peanut, hazelnut, cashew and walnut is presented.

2 Material and methods

2.1 Spiked (= incurred) food material

The composition of the materials used for in-house and inter-laboratory (= ring-trial) validation is compiled in Table 1. Materials of different food matrices were artificially contaminated (incurred) with defatted flours of peanut, hazelnut, cashew and walnut, respectively (hereafter referred to as “nuts”) and then processed. Rice cookies were baked 10 min at 200 °C, sausage meat mixtures of the type “Lyoner” were filled into cans and boiled at 100 °C for 1.5 h. For sauce powder no further processing step followed. Details on the production of the materials and information about the allergenic ingredients as well as on the homogeneity testing of these materials are provided in the reference Siegel et al. (2012). Artificially contaminated vegan cookies and veggie burgers were provided by DLA Proficiency Testing (Oerling, Germany). Only these two materials were spiked with roasted nuts. All materials were stored frozen at −18 °C (or below) until analysis.

2.2 DNA extraction

A cetyltrimethylammonium bromide (CTAB) extraction protocol with a precipitation step described in detail in Siegel et al. (2013) was used for extraction of DNA from all materials. 5 µL of the DNA samples were used for real-time PCR. Within the collaborative validation study, DNA extraction was performed by a single lab (see 2.6, ring-trial validation).

2.3 Matrix standard DNA

For the calibration of the results in mg/kg, DNA was prepared by dual extraction (see Sect. 2.2.) from the 400 mg/kg rice cookie material (Table 1). Both DNA extracts were blended and then stored at 2–8 °C until analysis. Before the real-time PCR setup, rice cookie DNA was diluted with

| Material                  | Spiked allergenic ingredient (mg/kg) | Spiked ingredients roasted? |
|---------------------------|-------------------------------------|-----------------------------|
|                           | Peanut | Hazelnut | Cashew | Walnut | Prot. | Peanut | Hazelnut | Cashew | Walnut | Prot. |
| Sausage “Lyoner”          | 5      | 1.3      | 20     | 2.8    | 50    | 8.8    | 20      | 2.9    | No     |
| Rice cookie #1            | 5      | 1.3      | 5      | 0.7    | 5     | 0.9    | 5       | 0.7    | No     |
| Rice cookie #2            | 0.9    | 0.23     | 1.9    | 0.27   | 9.4   | 1.7    | 5       | 0.7    | No     |
| Rice cookie #3            | 0      | 0        | 0      | 0      | 0     | 0      | 0       | 0      | No     |
| Sauce powder              | 5      | 1.3      | 5      | 0.7    | 5     | 0.9    | 5       | 0.7    | No     |
| Vegan cookie              | 5      | 1.3      | 5      | 0.7    | 5     | 0.9    | 5       | 0.7    | No     |
| Veggie burger (powder)    | 10     | 2.6      | “0”    | 0      | 2     | 0.35   | “0”     | 0      | Yes    |
| Rice cookie 400f          | 400    | 104      | 400    | 56     | 400   | 70     | 400     | 58     | No     |

Except for rice cookie 400 and rice cookie #2, spiked allergenic ingredients were not identical

a Spiked (incurred) allergenic ingredient (ground “nuts”) in mg/kg
b “nut” protein (calculated from literature values according to BLS 3.02)
c “0” for hazelnut and walnut in veggie burger: both ingredients were not spiked (incurred), however the materials were not specifically produced and tested as “blank controls” for these allergens
d Material used for extraction of matrix standard DNA, see section: material and methods/matrix standard DNA.
0.2 × TE-solution to yield DNA-solutions corresponding to the following theoretical concentrations: 400 (undiluted DNA), 100, 25, 10, 4, 1.6 and 0.64 mg of each nut per kg (Siegel et al. 2012, 2013).

2.4 Primer and probes

2.4.1 Selection of target genes for real-time PCR

For the specific detection and quantification of peanut, hazelnut, walnut and cashew TaqMan™ real-time PCR methods were used targeting at multiplex sequences from mitochondrial, ribosomal RNA genes and chloroplasts, respectively. The primers and probes for the amplification of a peanut-specific sequence were taken from Ladenburger et al. (2018). Other PCR-systems were established in this work. Details of target sequences, primers and probes together with final concentrations in the PCR reaction mix are listed in Table 2. The Quantitect Multiplex Mastermix no ROX (QIAGEN, Hilden, Germany) was used for qPCR according to the manufacturer’s instructions.

The following temperature–time–protocol was applied using the QuantStudio 5 thermocycler (Thermo Fisher): initial denaturation for 15 min at 95 °C for one cycle, followed by 40 cycles: 95 °C for 15 s and 60 °C for 1 min.

Table 2 qPCR primer and probe sequences

(a) Peanut: multiplex sequence from mitochondrial DNA close to the coding region of the ATPase subunit 6 of Arachis hypogaea, 104 bp (Ladenburger 2018)

| Primer/probe | Final conc. μM | Sequence | Amplicon | Target sequence: GenBank acc.no./labelling |
|--------------|----------------|----------|----------|------------------------------------------|
| atp6-F       | 0.3            | CAG GGC ATC CTT AAC TGG AG | 104 bp   | MW448460.1                               |
| atp6-R       | 0.3            | GGA AAG ACG GGT TGG TGA TA |         |                                          |
| atp6-P       | 0.04           | AAG GCG AAG AAG GGT CAG AT |         |                                          |

(b) Hazelnut: multiplex sequence from the internal transcribed spacer 2 (ITS2) region from the 5.8S ribosomal RNA gene of Corylus spp. (this work)

| Primer/probe | Final conc. μM | Sequence | Amplicon | Target sequence: GenBank acc.no./labelling |
|--------------|----------------|----------|----------|------------------------------------------|
| H-nuss-F     | 0.6            | GCG GCT GGC CTA AAA GC | 62 bp    | MG237811.1                               |
| H-nuss-R     | 0.6            | GGT TTG TCA ACC ACC GAT TGT |        |                                          |
| H-nuss-MGB   | 0.04           | AGT CCT CGG CGA G |         | FAM-MGB-BMN-Q535                          |

(c) Walnut: multiplex sequence from a non-coding segment of the large single copy (LSC) region of the chloroplast genome of Juglans regia (this work)

| Primer/probe | Final conc. μM | Sequence | Amplicon | Target sequence: GenBank acc.no./labelling |
|--------------|----------------|----------|----------|------------------------------------------|
| WalN F       | 0.3            | TTT CCA TAT CGA TCC ATG CG | 67 bp    | MF167463.1                               |
| WalN R       | 0.3            | TCT CTT TGT CAC ATC CGT TCT CC |      |                                          |
| WalN ROX LNA | 0.01           | A-LNA TCC CAT TGC CGG A5C A |         | ROX-LNA-BMN-Q620                          |

(d) Cashew: multiplex sequence from the internal transcribed spacer 2 (ITS2) region from the 5.8S ribosomal RNA gene of Anacardium occidentale (this work)

| Primer/probe | Final conc. μM | Sequence | Amplicon | Target sequence: GenBank acc.no./labelling |
|--------------|----------------|----------|----------|------------------------------------------|
| CashITS-F    | 0.2            | GAA CGA ACC CGA TGA TCC | 69 bp    | AB071690.1                               |
| CashITS-R    | 0.2            | CCA TCG AGG GTC AAG GAG |         |                                          |
| CashITS-CY 5 | 0.04           | GGA CGC GCT CTC TCT GTG | CY5-BHQ2 |                                          |

Primer Express 3.0.1 (Thermo Fisher Scientific, Waltham, MA, USA) and Primer3Plus software (Untergasser 2007) were used for primer and probe design. The labelling of the probes for multiplexing was done according to the recommendations of manufacturers of real-time PCR instruments and availability of existing labelling dyes for probes. To increase the specificity of the walnut system, a modified nucleotide (Locked Nucleic Acid-enhancement; LNA) was incorporated at the 3' end of the reverse primer. The designed primer systems were analysed with the OligoAnalyzer™ software, version 3.1 (Integrated DNA Technologies, Inc. (IDT), Coralville, IO, USA), for the absence of interfering dimers and hairpin structures. Primers and probes were synthesized by biomers.net (Ulm, Germany).

HEX hexachloro-fluorescein, FAM 6-carboxyfluorescein, ROX carboxy-X-rhodamine, CY5 cyanin 5, BHQ 1/2 black hole quencher 1/2, BMN-Q535 and BMN-Q620 Quencher of the manufacturer biomers.net (Ulm; Germany), equivalent to BHQ1 and BHQ2 resp., LNA locked nucleic acid, MGB minor groove binder
by 38 cycles each with three steps of 10 s at 95 °C, 60 s at 60 °C and 60 s at 72 °C. The fluorescence signal was measured during elongation.

2.5 Validation of the method

Validation of the method was performed in two steps:

- in-house validation by the organizing lab (Sect. 2.5.1),
- collaborative trial (ring-trial) validation with 12 participating labs (Sect. 2.6).

2.5.1 In‑house validation

In a first in-house validation, the following parameters were checked: specificity, crosstalk, sensitivity [limit of detection (LOD) including asymmetric LOD], precision and trueness (ENGL 2021).

a. Specificity testing and comparison between species/cultivars

Specificity testing was done with non-target organisms/taxa and response testing was carried out with target-organisms or cultivars. DNA was extracted from test material (kernels and seeds) using a commercial kit (Qiagen, Hilden, Germany) and following a CTAB-based protocol (Siegel et al. 2013).

- Exclusivity tests with non-target species/taxa: almond, anis, apricot, baker’s yeast, boletus, white bean, Brazil nut, cabbage, (cashew), celery, chestnut, coconut, garlic, (hazelnut), lentil, linseed, lupin, macadamia, maize, mango, millet, mustard, oat, paprika, parsley, pea, (peanut), pecan nut, pig, pistachio, potato, rice, sesame, soy, tomato, thym, (walnut), wheat (in brackets: specificity test only relevant if species is not the target).
- Inclusivity (response) tests with target species/cultivars: kernels of peanuts (n = 11), hazelnuts (n = 11), walnuts (n = 15), and cashew (n = 5) from different cultivars/origins and/or different market samples (n = whole number of tested materials). Available material varied in the degree of traceability: for cashew, only different market samples (brands) without specification of origin could be purchased, whereas for peanut, different cultivars from Argentina, Brazil, China, South Africa and USA were available. Market samples with declared origin USA, Chile and France (walnut), Turkey, France, Italy (hazelnut) as well as self-harvested walnuts from different regions in Germany were included.

The response of obtained amplifications was determined using external standard curves prepared from market samples of peeled, unroasted peanuts, hazelnuts, walnuts and cashews as reference (= 100%). The response values were each calculated as the ratio between the concentration of DNA of the analysed species/cultivar and the reference.

Before actual testing, using the PCR systems described above, the concentration of extracted DNA was determined by a Qubit fluorometer (Thermo Fisher Scientific, Waltham, USA) and adjusted to a concentration of 10 ng/µl (+ for hazelnut and walnut: 1 ng/µl). Additionally, for response testing of walnut and hazelnut samples, the DNA-dilutions were analysed in parallel by real-time PCR amplifying a species-specific single-copy target sequence (Koeppel et al. 2010).

b. Crosstalk

The eventual bleed-through of fluorescence signals between detection channels during multiplex PCR was tested in accordance to ENGL (2021). Test samples containing no target DNA of the PCR module were tested in the presence of 100 ng of the target species DNA for the other PCR modules (n = 3 per test).

iii. Sensitivity

The sensitivity of the method was estimated by the organising laboratory using serial dilution of matrix standard DNA (see Sect. 2.3). Dilutions corresponding to the concentrations 10, 5, 2 and 1 mg of each nut per kg were analysed in six replicates each. Additionally, the LOD under asymmetric conditions was tested to check for competitive effects in the presence of high amounts of the other targets (ENGL 2021). The serial dilution of the matrix standard DNA corresponding to 5 mg/kg of each “nut” was analysed in the presence of 250 ng DNA per PCR reaction of the three other target species in six replicates each.

iv. Incurred food material: Sensitivity, precision and trueness

Incurred food material according to 2.1 was analysed in-house by duplicate extraction and PCR analysis of each extract in four replicates yielding eight results for each material.

e. Proficiency test hazelnut

The suitability of the hazelnut system was additionally tested by the analysis of materials from a proficiency test (PT) (DLA 2018). For this PT, hazelnut at different degrees of processing [unroasted, nut butter (roasted), nut spread with cocoa, nut nougat and nut crocant] was spiked at levels of 50 mg/kg to a potato powder matrix.

f. Peanut: comparison unroasted vs. roasted
The influence of roasting on the recovery was estimated exemplarily for peanut. 1000 mg/kg of a ground mixture of unroasted or roasted kernels, respectively, of different peanut cultivars was spiked to rice flour and analysed according to Sects. 2.2–2.4 (two extracts, PCR duplicates each).

2.6 Ring-trial

The reliability of the method was tested in a collaborative trial with 12 participating labs (Table S1, Supplementary Material) within the § 64 working group “Food Allergens”, hosted by the Federal Office of Consumer Protection and Food Safety. Participants of the collaborative trial received DNA samples previously extracted (Table 1) by the organising lab. Thus, the term “samples” mentioned below refers to DNA extracts. The following material was sent to the participating laboratories:

- 21 encoded samples: Three aliquots of each of the seven materials according to Table 1.
- One sample containing the matrix standard, see section “matrix standard DNA”.
- Aliquots of oligonucleotides and probes and mastermixes for the qPCR [see 2.4, Quantitect Multiplex Mastermix no ROX (QIAGEN, Hilden, Germany)].

There was no restriction in terms of the real-time PCR system used. The qPCR had to be performed according to the procedure described in 2.4. Each DNA extract had to be analysed in duplicates and each level of the matrix standard DNA dilution series in triplicates. The lowest level of the matrix standard DNA dilution series (0.64 mg/kg) was used as sensitivity control and therefore analysed in ten replicates.

2.7 Qualitative and quantitative evaluation of the inter-laboratory validation

As for the qualitative evaluation, Cq values < 38 were considered as amplification (= positive result). Additionally, the sample Cq values were compared with the average Cq value of the lowest standard (0.64 mg/kg). If the Cq value of the sample was at least 1 higher than the Cq of the lowest standard, the result would be considered “negative” (Waiblinger et al. 2011).

In order to evaluate the results of the samples quantitatively, the Cq values were first assigned to concentrations in mg/kg using the DNA matrix standard curve by linear regression of the data of the calibration series. Average Cq values obtained by qPCR amplification of the DNA standard dilution levels were plotted against the logarithm of the corresponding DNA concentration (400, 100, 25, 10, 4, 1.6 mg/kg). For each DNA extraction of the samples including the matrix standards, mean values of the concentrations obtained for the PCR duplicates were calculated. The participating laboratories were asked to report results of each PCR as Cq values as well as concentrations. Median, mean and standard deviation were calculated from the average values of concentrations in mg/kg (n = 3 per concentration level). Prior to the calculation of the mean and median values, the repeatability standard deviation (S_r), and reproducibility standard deviation (S_p), outliers were identified using the Grubbs test (1% significance level) and removed from the dataset. All calculations were performed using the software Valoo 2.4. The full method protocol has been published in the Official Collection of Methods of Analysis (BVL 2022).

3 Results and discussion

3.1 In-house testing prior to the collaborative trial

3.1.1 Specificity

The specificity of the multiplex PCR method was first evaluated by theoretical in silico tests in order to predict potential false positive results (ENGL 2021). Unexpected similarities to other target sequences could not be identified. No negative PCR effects were observed during the primer check with the software tool OligoAnalyzer for possible formation of secondary structures (dimers and hairpins). The deltaG value for the dimer analysis was below − 10 kcal/mol, indicating a suitable primer design. The melting temperature of the formed hairpins showed in each case the recommended minimum temperature of 50 °C below the annealing temperature of the PCR.

Prior to the inter-laboratory validation, the specificity of the method was tested in-house. No amplification was obtained when testing DNA from non-target species according to Sect. 2.5.1a). For the species birch, an amplification signal was detected in the hazelnut system. However, to our knowledge birch has no relevance in terms of food. Furthermore, the variation of the response was tested when analysing materials of the target species from different origins and—if available—of different cultivars.

A sufficiently low variation of the response was observed for all detection systems ranging from 17 to 37% (data not shown). In table S2 (Supplementary Material), the results for walnut and hazelnut are shown as an example. Relative standard deviations of all values were 16.8% (walnut) and 36.9% (hazelnut), compared to 11.0% (walnut) and 36.4% (hazelnut) when using the singlecopy target sequence according Köppel (2010). Additionally, it has to be mentioned, that for hazelnut the detection method is only genus specific (Corylus spp.). The test of the quantitative response was carried out with commercially available references predominantly derived from Corylus maxima. Equally,
nuts from Corylus avellana are detected by the method. The results of the tests with the available references hint to a response of C. avellana being about factor two to four stronger than the response of C. maxima.

3.1.2 Crosstalk

In none of the four detection channels, crosstalk was observed. Thus, the acceptance criteria example of ENGL (2021) could be fulfilled for the parameter crosstalk.

3.1.3 Sensitivity

The levels of the serial dilutions 10, 5, 2 and 1 mg/kg of each “nut” were detectable in six of six PCR replicates each. Additionally, the level of 5 mg/kg was still detectable in co-presence of high amounts of DNA of the other target species. Therefore, an asymmetric LOD of at least 5 mg/kg of each “nut” was estimated by in-house validation. The lowest spiked levels of incurred materials were detectable in eight of eight PCR reactions each: hazelnut and peanut: 0.9 mg/kg (rice cookie); walnut, cashew: 5 mg/kg (sauce powder).

3.1.4 Precision and trueness

Good precision data with relative standard deviations > 25% were obtained for all tested materials and spiked levels (detailed data not shown). However, bias, i.e. deviation of measured concentration in mg/kg from incurred level, was more than 50% for some materials and levels. This was the case, e.g., for the quantification of peanut, hazelnut and walnut in some sausage and sauce powder materials. The overall measurement uncertainty (see also data from collaborative trial) was estimated to be in the range of ± 50% (detailed data not shown).

3.1.5 PT samples hazelnut

All six samples were correctly classified as positive or negative. The recoveries of the five spiked potato powder materials spiked with different preparations of hazelnut were 59% (nut nougat and nut crocant), 64% (nut spread with cocoa), 68% (nut crocant), 108% (hazelnut roasted) and 223% (hazelnut unroasted).

3.1.6 Peanut: comparison roasted and unroasted

The influence of the roasting process on recovery was exemplarily tested for the peanut quantification. The results of quantification of 1000 mg peanut/kg in rice flour was 100 mg/kg for roasted peanut mix and 960 mg/kg for the unroasted peanut material corresponding to recovery rates of 10.0% and 96.0%, respectively.

3.2 Collaborative trial validation

No deviations from the protocol were reported by none of the 12 participating laboratories. Three labs asked to repeat the analysis with a new set of samples for different reasons (use of unsuitable plastic ware, deviation from the protocol by mistake, suspicious results and amplification curves of the sensitivity control and two samples respectively).

3.2.1 Calibration standard DNA and matrix standard DNA

Table 3 summarises the results of the calibration series. Performance criteria for standard curves are described, e.g., in guidelines for GMO analysis (BVL 2016; ENGL 2015, 2021; Broeders et al. 2014). According to these guidelines, the slope of the standard curve should be between − 3.1 and − 3.6 and R2 should be > 0.98. For multiplex PCR used for qualitative analysis, a slope between − 2.9 and − 3.9 is considered to be acceptable (Broeders et al. 2014).

The results of lab No. 12 were considerably outside of these requirements for all PCR systems and therefore could not be taken into account, neither for qualitative nor quantitative evaluation. Furthermore, the results of two laboratories were excluded from the quantitative evaluation of the walnut PCR due to clearly deviating values from the standard curve for walnut (Table 3). In Table 3, for each PCR system the number of results (labs) fulfilling the standard curve criteria are given. In total, range of labs with satisfactory results was from 9 of 12 (walnut, slope and R2) to 11 of 12 (cashew, slope).

Sensitivity control of 0.64 mg “nut”/kg could be amplified in 110 out of 110 reactions for each PCR module (0% false negative rate; lab 12 was not considered, see above).

3.2.2 Qualitative evaluation of samples

The samples were decoded and assigned to the seven different materials. Per material and lab, six PCR results (three DNA extracts analysed in duplicates each) were obtained.

Table 4a–d show the results of the qualitative evaluation of the inter-laboratory validation. For each of the four spiked allergenic ingredients, the expected results were obtained, with some exceptions: for the rice cookie not incurred with hazelnut, in 15 of total 66 reactions, amplifications with Cq values from 34.4 to 38, mainly between 37 and 38 were obtained. No false-positive reaction resulted when comparing the Cq with the lowest standard (see Sect. 2.7) though. Similar results were reported for the walnut “blank” material. Unavoidable contaminations of the rice cookie material

Previous studies indicate that the considerable decrease of recovery is caused by the degradation of DNA during the process of roasting (see e.g., DLA 2019; Rossmanith 2011).
Table 3  Inter-laboratory validation: real-time PCR systems and results of the dilution series of the matrix calibration DNA (400–1.6 mg/kg)

| Lab No | qPCR system | Peanut | | Hazelnut | | Walnut | | Cashew |
|--------|-------------|--------|---|---------|---|---------|---|---------|
|        |             | Slope  | R² | Std 0.64 mg/kg | Slope  | R² | Std 0.64 mg/kg | Slope  | R² | Std 0.64 mg/kg | Slope  | R² | Std 0.64 mg/kg |
| 1      | a           | − 3.36 | 0.998 | 10 | − 3.54 | 0.998 | 10 | − 3.41 | 0.996 | 10 | − 3.42 | 0.997 | 10 |
| 2      | a           | − 3.56 | 0.997 | 10 | − 3.40 | 0.997 | 10 | − 3.51 | 0.994 | 10 | − 3.46 | 0.995 | 10 |
| 3      | b           | − 3.46 | 0.997 | 10 | − 3.22 | 0.996 | 10 | − 3.28 | 0.996 | 10 | − 3.28 | 0.997 | 10 |
| 4      | b           | − 3.35 | 0.991 | 10 | − 3.40 | 0.985 | 10 | − 3.35 | 0.985 | 10 | − 3.41 | 0.989 | 10 |
| 5      | c           | − 3.45 | 0.987 | 10 | − 3.27 | 0.992 | 10 | − 3.32 | 0.986 | 10 | − 3.31 | 0.981 | 10 |
| 6      | a           | − 3.37 | 0.997 | 10 | − 3.37 | 0.998 | 10 | − 3.33 | 0.994 | 10 | − 3.45 | 0.998 | 10 |
| 7      | b           | − 3.39 | 0.997 | 10 | − 3.39 | 0.998 | 10 | − 3.44 | 0.999 | 10 | − 3.39 | 0.998 | 10 |
| 8      | d           | − 3.03 | 0.994 | 10 | − 3.07 | 0.993 | 10 | − 2.74 | 0.953 | 10 | − 3.11 | 0.993 | 10 |
| 9      | a           | − 3.33 | 0.997 | 10 | − 3.42 | 0.995 | 10 | − 3.44 | 0.996 | 10 | − 3.41 | 0.992 | 10 |
| 10     | e           | − 3.18 | 0.974 | 10 | − 3.26 | 0.976 | 10 | − 3.91 | 0.865 | 10 | − 3.18 | 0.971 | 10 |
| 11     | f           | − 3.16 | 0.998 | 10 | − 3.37 | 0.989 | 10 | − 3.24 | 0.994 | 10 | − 3.26 | 0.996 | 10 |
| 12     | g           | − 3.88 | 0.862 | 8  | − 4.26 | 0.850 | 9  | − 3.93 | 0.897 | 9  | − 5.13 | 0.765 | 10 |

Slope: − 3.1 to − 3.6
R² > 0.98?
(ENGL 2015, 2021)

No. labs
Mean:

| Peanut | Hazelnut | Walnut | Cashew |
|--------|---------|--------|--------|
| Slope  | R²      | Std 0.64 mg/kg | Slope  | R² | Std 0.64 mg/kg | Slope  | R² | Std 0.64 mg/kg | Slope  | R² | Std 0.64 mg/kg |
| − 3.35 | 0.993 | 110/110 (= 100%) | − 3.34 | 0.995 | 110/110 (= 100%) | − 3.39 | 0.993 | 110/110 (= 100%) | − 3.34 | 0.991 | 110/110 (= 100%) |

The results out of specification for slope (between − 3.1 and − 3.6) and R² ( > 0.98) are in italic (ENGL 2015, 2021)

a Real-time PCR systems used in the collaborative trial:
a CFX 96 (Biorad)
b QuantStudio 5 (Thermo Fisher)
c ABI 7500 bzw. ABI 7500 Fast (Applied Biosystems/Life Technologies)
d Rotor Gene (Qiagen)
e Light Cycler 480 (Roche)
f Aria Mx (Agilent)
g Rotorgene RS 6000 (Corbett)

Std 0.64 mg/kg = number of positive reactions for standard dilution 0.64 mg “nut”/kg (n = 10 per lab)

After removal of outliers: all data of lab 12 and data for walnut, lab 8 and 10
### Table 4  a–d Inter-laboratory validation, qualitative evaluation

| Material and spike level (mg/kg)<sup>a</sup> | RC 1 | RC 2 | RC 3 | SL | SP | VC | VB |
|--------------------------------------------|------|------|------|----|----|----|----|
|                                           | 5    | 0.9  | 0    | 5  | 5  | 5  | 10 |

| Number of samples per lab                  | 3    | 3    | 3    | 3  | 3  | 3  | 3  |
| Number of samples total                    | 33   | 33   | 33   | 33 | 33 | 33 | 33 |
| Number of PCR results total                | 66   | 65   | 66   | 66 | 66 | 66 | 66 |
| Number of positive PCR results             | 66   | 65   | 1<sup>b</sup>, 0<sup>c</sup> | 66 | 66 | 66 | 66 |
| False positive results                     | –    | –    | 1<sup>b</sup>, 0<sup>c</sup> | –  | –  | –  | –  |
| False positive results (%)                 | –    | –    | 1<sup>b</sup>, 0<sup>c</sup> | –  | –  | –  | –  |
| False negative results                     | 0<sup>b</sup>, 1<sup>c</sup> | 0    | –    | 0  | 0  | 0<sup>b</sup>, 29<sup>c</sup> | 0  |
| False negative results (%)                 | 0<sup>b</sup>, 1.5<sup>c</sup> | 0    | –    | 0  | 0  | 0<sup>b</sup>, 44<sup>c</sup> | 0  |

#### (b) Hazelnut (11 labs considered)

| Material and spike level (mg/kg)<sup>a</sup> | RC 1 | RC 2 | RC 3 | SL | SP | VC | VB |
|--------------------------------------------|------|------|------|----|----|----|----|
|                                           | 5    | 1.9  | 0    | 20 | 5  | 5  | “0”|

| Number of samples per lab                  | 3    | 3    | 3    | 3  | 3  | 3  | 3  |
| Number of samples total                    | 33   | 33   | 33   | 33 | 33 | 33 | 33 |
| Number of PCR results total                | 66   | 65   | 66   | 66 | 66 | 66 | 66 |
| Number of positive PCR results             | 66   | 65   | 1<sup>b</sup>, 0<sup>c</sup> | 0  | 66 | 66 | 66 |
| False positive results                     | –    | –    | 1<sup>b</sup>, 0<sup>c</sup> | –  | –  | –  | –  |
| False positive results (%)                 | –    | –    | 1<sup>b</sup>, 0<sup>c</sup> | –  | –  | –  | –  |
| False negative results                     | 0    | 0    | –    | 0  | 0  | 0<sup>b</sup>, 29<sup>c</sup> | –  |
| False negative results (%)                 | 0    | 0    | –    | 0  | 0  | 0<sup>b</sup>, 44<sup>c</sup> | –  |

#### (c) Walnut (11 labs considered)

| Material and spike level (mg/kg)<sup>a</sup> | RC 1 | RC 2 | RC 3 | SL | SP | VC | VB |
|--------------------------------------------|------|------|------|----|----|----|----|
|                                           | 5    | 0    | 0    | 20 | 5  | 5  | “0”|

| Number of samples per lab                  | 3    | 3    | 3    | 3  | 3  | 3  | 3  |
| Number of samples total                    | 33   | 33   | 33   | 33 | 33 | 33 | 33 |
| Number of PCR results total                | 66   | 65   | 66   | 66 | 66 | 66 | 66 |
| Number of positive PCR results             | 66   | 1<sup>b</sup>, 0<sup>c</sup> | 0    | 66 | 66 | 66 | 38<sup>b</sup>, 0<sup>c</sup> |
| False positive results                     | –    | –    | 1<sup>b</sup>, 0<sup>c</sup> | 0   | –  | –  | –  |
| False positive results (%)                 | –    | –    | 1<sup>b</sup>, 0<sup>c</sup> | 0   | –  | –  | –  |
| False negative results                     | 0    | –    | –    | 0  | 0  | 0<sup>b</sup>, 1<sup>c</sup> | 0  |
| False negative results (%)                 | 0    | –    | –    | 0  | 0  | 0<sup>b</sup>, 1.5<sup>c</sup> | 0  |

#### (d) Cashew (11 labs considered)

| Material and spike level (mg/kg)<sup>a</sup> | RC 1 | RC 2 | RC 3 | SL | SP | VC | VB |
|--------------------------------------------|------|------|------|----|----|----|----|
|                                           | 5    | 9.4  | 0    | 50 | 5  | 5  | 2  |

| Number of samples per lab                  | 3    | 3    | 3    | 3  | 3  | 3  | 3  |
| Number of samples total                    | 33   | 33   | 33   | 33 | 33 | 33 | 33 |
| Number of PCR results total                | 66   | 65   | 66   | 66 | 66 | 66 | 66 |
| Number of positive PCR results             | 66   | 65   | 0    | 66 | 66 | 66 | 66 |
| False positive results                     | –    | –    | 0    | –  | –  | –  | –  |
| False positive results (%)                 | –    | –    | 0    | –  | –  | –  | –  |
| False negative results                     | 0<sup>b</sup>, 1<sup>c</sup> | 0    | –    | 0  | 0<sup>b</sup>, 1<sup>c</sup> | 0  |
| False negative results (%)                 | 0<sup>b</sup>, 1.5<sup>c</sup> | 0    | –    | 0  | 0<sup>b</sup>, 1.5<sup>c</sup> | 0  |

<sup>a</sup>Abbreviations of the used materials (numbers indicate the amount of spiked allergenic ingredient in mg/kg; “0” = material not specifically prepared as blank): RC rice cookie, SL sausage “Lyoner”, SP sauce powder, VC vegan cookie, VB veggie burger

<sup>b</sup>Amplifications considered with Ct < 38

<sup>c</sup>Evaluation by comparison of the sample Cq value with the average Cq value of the lowest standard (10.2 mg/kg). If Cq value of the sample is at least 1 higher than the Cq (10.2 mg/kg), the result is evaluated as “negative” (Waiblinger et al. 2011)
used as “blank standard”, are most probably the cause for these observations. Due to a calculated level of less than 0.1 mg/kg, the impact of the contamination on the qualitative evaluation is negligible.

The evaluation of the peanut results for the vegan cookie (5 mg/kg), compared with the mean Cq value of the lowest standard dilution, yielded 29 (of 66) false-negative results (= 44%). As previously mentioned, the use of roasted peanuts for spiking leads to significantly lower recoveries (see Sect. 3.1). Anyhow, for the veggie burger spiked with roasted peanuts at the level 10 mg/kg, the results were positive consistently, i.e. applying both methods for evaluation. Finally, for the rice cookie incurred with 5 mg/kg one false-negative reaction was obtained (= 1.5% of all reactions), when evaluated by comparison of Cq values.

Overall, the results demonstrate that a detectability of 5 mg peanut, hazelnut, walnut and cashew per kg [equivalent to approx. 1.3 mg (peanut), 0.7 (hazelnut), 0.7 mg (walnut) and 0.9 (cashew) protein per kg (BLS 3.02)] is still feasible in processed foods. If, as a “worst case”, in addition to being processed, the incurred food contains roasted instead of unroasted “nuts”, a LOD of 10 mg/kg can still be achieved.

3.2.3 Quantitative evaluation of samples

Table 5a–d summarise the statistical evaluation of all quantitative inter-laboratory 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, 2021). Due to the high proportion of false-negative results, data received for the vegan cookie spiked with roasted peanuts at the level 5 mg/kg were not taken into consideration for quantitative evaluation.

3.2.4 Trueness/recoveries

According to the guidelines for quantitative GMO analysis (ENGL 2015), the deviation of the mean value from the true value or an accepted reference value should not exceed 25%.

According to the results of the inter-laboratory validation study, this requirement could not be fulfilled in most cases for the different materials [peanut: criterion fulfilled in one of six materials, hazelnut (3/5), walnut (2/4) and cashew (2/6)].

The deviations from the true value (incurred level) ranged from − 4.7% for cashew in sausage to − 84.7% for walnut in vegan cookie (see Table 5a–d, “recovery”). It has to be noted that for the calibration of all results, a dilution series prepared from rice cookie material was used and the calculated recoveries are referring to %-deviations of the results in relation to the rice cookie material (= 100%) (Siegel et al. 2013). For none of the materials, the spiked

3.2.5 Precision

Moreover, in the quantitative range, the relative repeatability standard deviation (S_r, rel) should not exceed 25% and the relative reproducibility standard deviation (S_r, rel) should be below 35%. At the level of the quantification limit, a value below 50% is still acceptable (ENGL 2015, 2021). Except for S_r, rel of 1.9 mg/kg hazelnut in rice cookies (25.5%) and of 5 mg/kg hazelnut in sauce powder (28.1%), the data of these two precision criteria were in the acceptable range for all four “nut” species, all spiked levels and all materials. This is even the case for the rice cookie material at the very low spiked level of 0.9 mg peanut/kg (see Table 5a–d, “S_r, rel” and “S_r, rel”). However, it has to be pointed out, that the collaboration trial was designed in such way, that the extraction was performed by one laboratory and the analysis of unknown samples was limited to already prepared DNA solutions. A certain increase of S_r, rel and S_r, rel could be expected, if the DNA extraction step was performed individually by each lab. In previous validation studies, DNA extraction had been included (Siegel et al. 2013; Waiblinger et al. 2014, 2017), thus experience to what extent the individual DNA preparation leads to additional uncertainty is already available. Within the collaboration trial, the performance of the PCR method had to be evaluated.

3.2.6 Measurement uncertainty

Finally, the relative measurement uncertainty was estimated by pythagorean addition of the relative deviation of the true value and the relative reproducibility standard deviation. Measurement uncertainties of less than 50% were calculated for peanut in two of six materials, for hazelnut in three of five, for walnut in three of four and cashew in four of six materials.
Table 5  Inter-laboratory validation, quantitative evaluation

(a) Peanut (11 labs considered)

| Material and spike levela (mg/kg) | RC | RC | SL | SP | VB |
|---------------------------------|----|----|----|----|----|
| 5 | 0.9 | 5 | 5 | 10 |
| Number of samples per lab | 3 | 3 | 3 | 3 | 3 |
| Number of samples/PCR results total | 33/66 | 33/66 | 33/66 | 33/66 | 33/66 |
| Number of samples/PCR results considered | 32/64 | 32/64 | 33/66 | 32/64 | 30/60 |
| Results in mg/kg (calculation using matrix standard DNA) | | | | | |
| Mean value (mg/kg) and confidence interval U | 2.33 ± 0.23 | 0.72 ± 0.08 | 2.99 ± 0.27 | 0.81 ± 0.09 | 2.88 ± 0.16 |
| Minimum value (mg/kg) | 0.84 | 0.39 | 1.16 | 0.37 | 1.94 |
| Maximum value (mg/kg) | 4.01 | 1.30 | 4.95 | 1.32 | 3.82 |
| Recovery (%) | 46.5 | 80.0 | 59.8 | 16.2 | 28.8 |
| Repeatability standard deviation $S_r$ (mg/kg) | 2.74 | 0.78 | 0.31 | 0.06 | 0.29 |
| $S_r$ relative (%)b | 11.6 | 14.7 | 16.0 | 20.2 | 11.9 |
| Reproducibility standard deviation $S_R$ (mg/kg) | 3.38 | 0.96 | 0.38 | 0.07 | 0.39 |
| $S_R$ relative (%) | 14.4 | 18.1 | 19.5 | 24.3 | 15.9 |
| Measurement uncertainty (%)b | 59.9 | 36.6 | 47.6 | 88.9 | 72.8 |

(b) Hazelnut (11 labs considered)

| Material and spike levela (mg/kg) | RC | RC | SL | SP | VC |
|---------------------------------|----|----|----|----|----|
| 5 | 1.9 | 20 | 5 | 5 |
| Number of samples per lab | 3 | 3 | 3 | 3 | 3 |
| Number of samples/PCR results total | 33/66 | 33/66 | 33/66 | 33/66 | 33/66 |
| Number of samples/PCR results considered | 32/64 | 32/64 | 33/66 | 33/66 | 33/66 |
| Results in mg/kg (calculation using matrix standard DNA) | | | | | |
| Mean value (mg/kg) and confidence interval U | 4.42 ± 0.41 | 2.24 ± 0.21 | 31.7 ± 1.73 | 3.78 ± 0.38 | 1.33 ± 0.10 |
| Minimum value (mg/kg) | 0.84 | 1.18 | 19.0 | 6.30 | 1.89 |
| Maximum value (mg/kg) | 4.01 | 3.50 | 40.4 | 15.86 | 26.5 |
| Recovery (%) | 88.3 | 117.8 | 158.6 | 75.5 | 26.5 |
| Repeatability standard deviation $S_r$ (mg/kg) | 0.71 | 0.47 | 3.69 | 0.57 | 0.14 |
| $S_r$ relative (%)b | 16.1 | 21.2 | 11.6 | 15.1 | 10.6 |
| Reproducibility standard deviation $S_R$ (mg/kg) | 0.96 | 0.57 | 4.88 | 1.06 | 0.27 |
| $S_R$ relative (%) | 21.7 | 25.5 | 15.4 | 28.1 | 20.6 |
| Measurement uncertainty (%)b | 24.7 | 31.1 | 60.6 | 37.3 | 76.3 |

(c) Walnut (9 labs considered)

| Material and spike levela (mg/kg) | RC | SL | SP | VC |
|---------------------------------|----|----|----|----|
| 5 | 20 | 5 | 5 |
| Number of samples per lab | 3 | 3 | 3 | 3 |
| Number of samples/PCR results total | 27/54 | 27/54 | 27/54 | 27/54 |
| Number of samples/PCR results considered | 26/52 | 26/52 | 27/54 | 27/54 |
| Results in mg/kg (calculation using matrix standard DNA) | | | | |
| Mean value (mg/kg) and confidence interval U | 6.43 ± 0.46 | 15.7 ± 1.23 | 5.48 ± 0.51 | 0.77 ± 0.07 |
| Minimum value (mg/kg) | 4.43 | 7.75 | 2.79 | 0.39 |
| Maximum value (mg/kg) | 8.73 | 20.90 | 7.65 | 1.11 |
| Recovery (%) | 128.5 | 78.7 | 109.5 | 15.3 |
| Repeatability standard deviation $S_r$ (mg/kg) | 0.84 | 2.39 | 1.01 | 0.08 |
| $S_r$ relative (%)b | 13.1 | 15.2 | 18.4 | 10.8 |
| Reproducibility standard deviation $S_R$ (mg/kg) | 1.13 | 3.05 | 1.28 | 0.18 |
| $S_R$ relative (%) | 17.6 | 19.4 | 23.4 | 23.5 |
| Measurement uncertainty (%)b | 33.5 | 28.8 | 25.2 | 87.9 |
Collaborative trial validation of a new multiplex real-time PCR to sensitively detect allergenic...

Conclusion and further work

A multiplex real-time PCR for the simultaneous detection and quantification of the allergenic species peanut, hazelnut, walnut, and cashew was developed and validated as well in-house as in an inter-laboratory trial. Due to the use of multicopy target sequences, a very sensitive detection of the allergenic ingredients is possible. Within the collaborative trial, a concentration of 0.64 mg/kg (i.e. approx. 0.1–0.2 mg “nut” protein/kg) could be reliably detected in a processed cookie matrix. These excellent sensitivity data could be confirmed by inter-laboratory results of incurred, processed samples with mainly very low spiked levels in the range from 0.9 to 50 mg/kg. In addition, this method revealed good precision data.

With regards to quantitative analysis, we obtained insufficient recovery data (bias) resulting in measurement uncertainties of more than 50%. The results of in-house tests suggest that roasting of nuts is the main factor inducing deviant (low) recoveries.

We therefore propose the application of the presented method for a sensitive and simultaneous allergen screening for the presence of peanut, hazelnut, walnut and cashew in food. Moreover, a good estimation and even quantification is possible, especially if the source of allergenic ingredient (e.g., roasted peanuts) is known and can be used for the calibration of the method.

The method has recently been included into the German Official Collection of Methods of Analysis (BVL 2022, Official Collection) as a national standard method. However, even if a standardized method is applied, the use of reference material as a standard for quantification remains a prerequisite for uniform and reproducible quantification results. The availability of such materials remains a big challenge in allergen analysis.

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Declarations

Conflict of interest Each of the authors confirms that this manuscript has not been previously published and is not currently under consideration by any other journal. Additionally, all authors have approved the contents of this paper and have agreed to the submission policies. Each named author has substantially contributed to conducting the underlying research and drafting this manuscript. Additionally, to the best of our knowledge, the named authors have no conflict of interest, financial or otherwise.

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