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Schaflandstraße 3/2, 70736 Fellbach, Germany
Phone: +49 711 3426 1234
Email: postfach@cvuas.bwl.de
Internet: www.cvuas.de

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Layout, graphics and image editing:
Pat Schreiter, CVUA Stuttgart

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Standard Materials for Allergen Analysis and Their Use in a Ring Trial Validation

Hans-Ulrich Waiblinger[1]*, Carina Stengg[1], Britta Boernsen[1], Daniela Bartsch[2], Matthias Besler-Scharf[3], Stefanie Dobrovolny[4], Rupert Hochegger[4], René Koeppel[5], Ingo Krujatz[6], Petra Reihl[7] and Jakob Frenzel[8]

[1] State Institute for Chemical and Veterinary Analysis Freiburg (CVUA Freiburg), Bissierstraße 5, 79114 Freiburg, Germany
[2] State Institute for Chemical and Veterinary Analysis Münsterland-Emscher-Lippe (CVUA-MEL), Joseph-Koenig-Straße 40, 48147 Muenster, Germany
[3] DLA - Proficiency Tests GmbH, Hauptstraße 80, 23845 Oering, Germany
[4] Austrian Agency for Health and Food Safety (AGES), Spargelfeldstraße 191, 1220 Vienna, Austria
[5] Official Food Control Authority of the Canton Zurich, Fehrenstraße 15, 8032 Zürich, Switzerland
[6] Chemical and Veterinary Analytical Institute, Jägerstraße 8/10, 01099 Dresden, Germany
[7] Hessian State Laboratory (LHL), Druseltalstraße 67, 34131 Kassel, Germany
[8] Federal Office of Consumer Protection and Food Safety (BVL), Gerichtstraße 49, 13347 Berlin, Germany

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Abstract

The results of the interlaboratory validation of a new multiplex real-time PCR method for the detection and semi-quantitative determination of the allergenic species brown/black mustard, white mustard, celery, and pistachio in food are presented in this article. A new approach was pursued, based on specially produced standard allergen stock mixtures. These pre-mixtures were incorporated (incurred) into five different foods on a laboratory scale and further processed (sausage, cookies, chocolate, ice cream, falafel). The results show that the allergen mixtures are well suited for the production of calibration standards for allergen analysis on the one hand and for the production of specific matrix control samples on the other. With the help of these materials, the multiplex PCR method was successfully validated in a ring trial involving 11 laboratories. Detection of the allergenic ingredients was possible in the range of 1.0 mg/kg (i.e., approx. 0.2 – 0.3 mg allergenic proteins/kg). The suitability for – at least – semi-quantitative estimation of the allergenic ingredients was confirmed by analysing the five matrix control samples spiked in the range of 10 to 50 mg/kg. The new approach allows the flexible verification of the suitability of the method for the matrix relevant to control practice. In the interlaboratory test, sufficient recovery rates and measurement uncertainties were determined for three matrices. Too low recoveries were obtained for two matrices (ice cream, falafel), which led to measurement uncertainties of more than 50%.

1. Introduction

A review of the latest publications in food allergy over the past couple of years confirmed that food allergy is a major public health concern, affecting about 8% of children and 10% of adults in developed countries. The prevalence of food allergy varies around the world and is mainly driven by environmental factors, possibly together with genetic susceptibility to environmental changes [1]. People with food allergies rely on allergen information provided with the food. Labelling provisions have been set in many countries. Within the European Union, labelling is mandatory for food ingredients containing celery, mustard and pistachio, among others [2].

***CORRESPONDING AUTHOR:** Hans-Ulrich Waiblinger, email: hans-ulrich.waiblinger@cvuafr.bwl.de, tel.: +49 761-8855-0
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The official food control in Germany uses so-called action values as internal minimal thresholds for analytical allergen control purposes. These values support laboratories in the decision-making process, whether further inspections at the production facility may be neglected or not [3]. Current action values (as of April 2024) are published on the homepage of the German Federal Office of Consumer Protection and Food Safety (BVL) [4]. The values range between 40 mg/kg (mustard) and 2100 mg/kg (crustacea), expressed as the allergenic food item. However, values even in the range of 10 to 50 mg/kg may have to be considered if food is specified or promoted for people with food allergies [5–6].

For this purpose, the working group ''Food Allergens'', hosted by the Federal Office of Consumer Protection and Food Safety, has validated a set of multiplex real-time PCR methods in the past ten years for the sensitive screening and the quantification covering most of the allergens relevant in terms of food labelling [7–10].

Method validation is not possible without the availability of calibrants that enable the transformation of the analytical measurement into a result with the dimension ''mg/kg'' [11]. So far, certified reference materials for allergen analysis do not exist for several reasons [12]. One of them is the lack of officially recognized reference methods for certification of the material, another one is the complexity of allergenic foods consisting of a multitude of allergenic proteins. Siegel *et al.* [11] proposed a simple and pragmatic approach for the production of matrix standard material (e.g. rice cookies) containing all relevant allergenic ingredients at the same time. The allergens were spiked as allergenic ingredients (e.g. kernels, seeds, freeze dried shrimp), most of them provided by local importers. The materials have proven to be very useful for validation of the above-mentioned real-time PCR methods as well as for quality control using other methodologies for allergen analysis such as ELISA or mass spectrometry. However, the approach is limited to some food matrices, that may not reflect the foods to be analysed in a routine lab. In addition, due to the spiking of whole food materials, detailed information in terms of "mass of total protein of the allergenic food ingredient per mass of food" as the favored dimension of allergen quantification [13] was not provided with these materials.

Below we describe a modified practical approach for production of materials for quality control and validation of methods for allergen analysis. Additionally, the working group "Food Allergens" has used these new materials for ring trial validation of another multiplex real-time PCR method for the sensitive screening and quantification of the allergenic ingredients white mustard, black resp. brown mustard, celery and pistachio [14]. The results of the inter-laboratory study are summarized in this work.

2. Materials and Methods

2.1. Quality control test materials (Allergen mixtures, AM)

Four mixtures of allergenic foods spiked at a level of 1000 mg/kg each in potato flour were produced by the company DLA - Proficiency Tests GmbH (Oering, Germany). For selection of allergens, mixtures of untreated seeds or kernels of allergenic food items were used. For example, for the allergen almond, a mixture of 18 products originating from 5 countries were used. Premixes containing the allergenic foods were added during a multi-stage addition of potato powder, milled, sieved (mesh 500 µm) and homogenized. Afterwards, the samples were filled into metallized, recyclable PP foil bags in portions of approx. 25 g. The mixture homogeneity after bottling was examined 8-fold by microtracer analysis. Allergen contents in potato powder were given as „total food" according to gravimetric mixture with an extended combined uncertainty of gravimetric allergen-contents of ± 4.0%. Protein contents of raw materials were determined by total nitrogen according to Kjeldahl with specified factors. Mixture "Nuts I Allergens" contained the allergens almond, cashew, hazelnut, peanut and walnut; mixture "Nuts II Allergens" contained Brazil nuts, macadamia nuts, pecan nuts and pistachio; mixture "Animal Allergens" crustaceae (king prawns freeze dried), egg (whole egg powder), fish (cod freeze-dried), milk (skimmed milk powder) and molluscs (yesso scallop freeze dried) and finally mixture "Other Plant Allergens" with celery seeds, lupin flour, white mustard, brown mustard, sesame, soya flour (toasted) and wheat flour. As an example, in Table 1, the composition of the mixture "Other Plant Allergens" is compiled.

Table 1. Quality Control Material "Other Plant Allergens"

Ingredients	Content
Celery Seeds milled, mixture (8 products from Asia and Europe) – as celery seed – thereof 19.4% total protein[a)]	 1,001 mg/kg 194 mg/kg
Lupin Flour milled, mixture (4 products from Europe) – as sweet lupin flour (white lupin / no species information available for other 3 products) – thereof 38.6% total protein[a)]	 1,001 mg/kg 386 mg/kg
Mustard, white (*Sinapis alba*) milled, mixture (9 products from Asia and Europe) – as mustard seed – thereof 30.7% total protein[a)]	 1,001 mg/kg 307 mg/kg
Mustard, brown (*Brassica juncea*) milled, mixture (1 product from Canada) – as mustard seed – thereof 27.6% total protein[a)]	 1,001 mg/kg 276 mg/kg
Sesame, white (*Sesamum indicum*), raw milled, mixture (10 products from Africa, Asia and South America) – as sesame seed – thereof 24.5% total protein[a)]	 1,001 mg/kg 245 mg/kg
Soya Flour, toasted milled, mixture (6 products from Asia, Europe and USA) – as soya flour – thereof 37.8% total protein[a)]	 1,001 mg/kg 380 mg/kg
Wheat Flour milled, mixture (21 products from Europe, Asia, USA) – as wheat flour[a)] – thereof 10.3% total protein[a)] – calculated as gluten[b)]	 13,100 mg/kg 1,350 mg/kg 998 mg/kg
Potato Flour ingredients: Potato, E471, E304, E223, E100	 89 g/100 g
further Ingredients: Maltodextrin, sodium sulfate and silicon dioxide	< 11 g/100 g

[a)] Protein contents according to laboratory analysis of raw materials determined by total nitrogen according to Kjeldahl with following factors: 6.25 for celery protein, lupin protein, mustard protein and sesame protein, 5.71 for soya protein and 5.83 for wheat protein
[b)] Gluten content in wheat protein 74% according to [15]

2.2. Matrix control materials

Quality control test materials containing allergen mixtures (see 2.1) were used to prepare five different matrix control materials in laboratory-scale, including different degrees of processing. The matrix samples were rice cookies, cooked sausage, ice cream, chocolate, and falafel, each of which was enriched with defined proportions of black or brown mustard, white mustard, celery, and pistachio. Details of the procedures are described in Table 2 a) – e), p. 4.

The materials were each produced on a scale of approximately 300 – 400 grams using a standard household food processor (Thermomix TM 5-1, Vorwerk, Wuppertal). Commercially available premixes were used to produce the ice cream matrix and the falafel matrix. Further preparation after adding the allergen mixtures was carried out according to the instructions on the packaging. All commercial mixtures and products used (ice cream and falafel powder, chocolate) as well as the main ingredients rice flour and minced meat were checked in advance for the absence of the allergenic components to be investigated.

The composition of the materials used for interlaboratory (=ring trial) validation is summarized in Table 3, p. 5. All materials were stored frozen at –18 °C (or below) until analysis.

Table 2. Preparation of the Matrix Control Materials

a) Rice crackers ("rice cookies")

Ingredients	Rice Cookie#2 (RC#2)	Rice Cookie blank (RC#0)
Rice flour [g]	157.2	169.88
AM#Pi [a] [g]	2.40	0
AM#CeMbMw [a] [g]	12.00	0
Sugar [g]	57	57
Eggs (homogenized) [g]	30	30.15
Water [g]	150	105.1
Knead each (2 min)		
Total [g]	408.72	362.1

- spread each dough on a baking sheet, weigh the baking sheet before and after adding the dough
- bake at 180 °C for 20 min
- Weigh the baking tray with the biscuits and calculate the baking loss. Take into account the baking loss for allergen concentration in rice biscuits
- Finely grind the biscuits
- Divide into aliquots (5 g each) and pack in airtight containers

b) Boiled sausage (based on [15])

Ingredients	Boiled Sausage #1 (BS#1)	Boiled Sausage #Blank (BS#0)
Preparation of sausage meat base 600 g		
Water [g]	168	
Gelatine [g]	20.4	
Salt [g]	3	
Sorbic acid [g]	0.6	
• mix all ingredients thoroughly and divide meat base into two portions		
Sausage meat base [g]	282	300
AM#Pi [a] [g]	15.0	0
AM#CeMbMw [a] [g]	3.00	0

- Mix each carefully
- Divide into portions of approx. 50 g each in 50 ml centrifuge tubes and heat in a water bath at 100 °C for 60 min
- Combine the boiled material each (BS#1 resp. BS#0) and mix again in a food processor
- Divide into portions (5 g) and pack in airtight containers

c) Milk ice cream

Ingredients	Ice Cream #1 (IC#1)
Water [g]	206.3
• Weigh in a food processor and add while stirring:	
Ice cream powder* [g] (e.g., powder for making ice cream in soft ice cream machines, Hagena, Henstedt (D); ingredients: sugar, skimmed milk powder, dextrose, coconut fat powder, sweet whey powder, whey protein concentrate, thickening agents: sodium alginate, sodium carboxymethylcellulose, carrageenan, flavoring, stabilizer diphosphates, coloring agent beta-carotene)	75
AM#Pi [a] [g]	15.0
AM#CeMbMw [a] [g]	3.00

- Continue stirring for 90 seconds (medium speed)
- Leave to stand for 10 minutes and stir again for 30 seconds at medium speed
- Divide into aliquots (5 g) immediately and pack in airtight containers

d) Falafel (chickpea patties)

Ingredients	Falafel#1 (Fa#1)
Commercial falafel mix, powdered* [g] (e.g., organic falafel curry coconut, gluten-free, Bauck GmbH, Rosche (D); ingredients: chickpea groats, chickpea flour, coconut flour, coconut flakes, curry powder, salt, banana granules, onion pieces, parsley, garlic powder, coriander, chili powder)	103.7
AM#Pi [a] [g]	15.0
AM#CeMbMw [a] [g]	3.00
• Weigh into a food processor and mix well	
Water, boiling hot [g]	178.2

- Add, stir for 30 seconds (medium speed) and leave to soak for at least 15 minutes
- Stir again for 30 seconds (medium speed)
- Form patties and weigh
- Add approx. 5 g vegetable oil to a pan and fry the patties over medium heat until golden brown
- Weigh the fried mixture of all patties: take into account the loss of allergen concentration in the finished product
- Divide into aliquots (5 g) and pack in airtight containers

e) Chocolate

Ingredients	chocolate#2 (Ch#2)
dark chocolate [g] (ingredients: sugar, cocoa mass, cocoa butter, emulsifier soy lecithin, vanilla extract)	282.0
AM#Pi [a] [g]	3.0
AM#CeMbMw [a] [g]	15.0

- Weigh into food processor and stir (medium speed) to 50 °C
- Stir for a total of 5 minutes at 50 °C and medium speed
- Immediately pour the liquid mixture into small molds (e.g., silicone) and allow to harden at room temperature
- Pack the "chocolates" or pieces thereof (aliquots of at least 5 g each) in airtight containers

[a] Allergen mixtures according to Section 2.1:
AM#CeMbMw with 1,001 mg/kg each of celery, white mustard and brown mustard (amongst others);
AM#Pi with 1,001 mg/kg pistachio (amongst others).

Table 3. Materials used for ring trial validation

Material	Spiked allergenic ingredient [mg/kg]							
	Mustard, brown		Mustard, white		Celery		Pistachio	
	spike[1)]	prot.[2)]	spike[1)]	prot.[2)]	spike[1)]	prot.[2)]	spike[1)]	prot.[2)]
Rice cookie#2	48.8	13.5	48.8	15.0	48.8	9.5	9.8	2.0
Rice cookie#0	0	0	0	0	0	0	0	0
Boiled sausage#2	10	2.8	10	3.1	10	1.9	50	10.2
Boiled sausage#2	0	0	0	0	0	0	0	0
Ice cream#1	10	2.8	10	3.1	10	1.9	50	10.2
Falafel#1	10.8	3.0	10.8	3.3	10.8	2.1	53.8	10.9
Chocolate#2	48.8	13.5	48.8	15.0	48.8	9.5	9.8	2.0
AM#CeMbMw[3)]	1001	307	1001	386	1001	194	–	–
AM#Pi[3)]	–	–	–	–	–	–	1001	203

[1)] spiked (incurred) allergenic ingredient in mg/kg
[2)] total protein determined by total nitrogen according to Kjeldahl (see Section 2.1 and Table 1)
[3)] AM#CeMbMw and AM#Pi were used for extraction of matrix standard DNA, see Section 2.1 „Material and Methods/Matrix standard DNA“

2.3. Analytical method

All samples were examined using the multiplex real-time PCR method described by Koeppel *et al.* [14]. A CTAB (cetyltrimethylammonium bromide) extraction protocol with a precipitation step described in detail by Siegel *et al.* [16] was used for extraction of DNA from all materials. 5 µL of the DNA samples were used for real-time PCR.

Allergen Standard DNA:

For calibration of results in mg/kg, DNA was prepared by extraction from allergen mixtures “Nuts II Allergens” and “Other Plants Allergens” according to 2.1 and then mixed in a ratio of 1+1. This mixture should correspond to a respective proportion of the four allergenic components of 500 mg/kg. Before the setup of the real-time PCR, this DNA mixture was diluted with 0.2 × TE-solution to yield DNA-solutions corresponding to the following theoretical concentrations: 125, 31.3, 7.81, 1.95 and 0.66 mg of each allergenic ingredient per kg [11, 17].

2.4. Ring trial

The reliability of the method was tested in a collaborative trial with 11 participating labs (Suppl. 1). Participants of the collaborative trial received DNA previously extracted by the organising lab from the samples listed in Table 3. 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 3.
- One sample containing the allergen standard DNA (500 mg/kg), see Section 2.3.
- Aliquots of oligonucleotides and probes and mastermixes for the qPCR according to Koeppel et al (2025); the PCR mastermix provided was the Quantitect Multiplex Mastermix no ROX (QIAGEN; Hilden, Germany).

There were no restrictions regarding the real-time PCR system used. The qPCR had to be performed according to the detailed protocol based on the method described by Koeppel *et al.* [14]. Each DNA extract had to be analysed in duplicate and each level of the matrix standard DNA dilution series in triplicates. The lowest level of the allergen standard DNA dilution series (0.66 mg/kg) was used as sensitivity control and was therefore analysed in ten replicates.

The qualitative and quantitative evaluation of the ring trial was carried out in accordance with the procedure described by Waiblinger [10]:

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 (0.66 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” [17].

In order to evaluate the results of the samples quantitatively, the Cq values were first assigned to concentrations in mg/kg using the allergen DNA 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 concentration of the allergenic ingredient (500, 125, 31.3, 7.81, 1.95 mg/kg).

For each DNA extraction of the samples, 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).

After identification of outliers using the Grubbs test (5% significance level) and removing them from the dataset mean, median values, repeatability standard deviation (Sr) and reproducibility standard deviation (SR) were calculated using the software Valoo 2.4 [18].

The full method protocol will be published in the Official Collection of Methods of Analysis [19].

3. Results and Discussion

3.1. Homogeneity test of the matrix control materials

The five materials were examined in accordance with the IUPAC protocol, Appendix 1 [20], using 12 randomly selected samples in duplicate.

Multiplex real-time PCR described by Koeppel [14] showed very good precision results in the in-house and initial interlaboratory validations. This is why we decided to use this method to evaluate the homogeneity of the matrix control material samples produced. For this purpose, the DNA extraction of the samples was – starting from CTAB lysates – performed in duplicates. All extracts were then examined using the method described by Koeppel [14].

When testing for white mustard in the ice cream matrix, the homogeneity requirements were narrowly missed. In all other 19 cases (a total of 4 allergens in 5 matrices), homogeneity was achieved. No contamination of the four allergenic components was detectable in a total of six samples of the two non-spiked materials (rice cracker #blank and boiled sausage #blank), which were examined in duplicate as described above.

3.2. Collaborative trial validation

3.2.1. Deviations from the protocol and real-time PCR systems

No deviations from the interlaboratory test protocol were reported and all results were taken into account for the interlaboratory test. The participants used the following real-time PCR devices: CFX 96 (Bio-Rad) (2×), QuantStudio 5 (Thermo Fisher) (5×), ABI 7500 (Applied Biosystems/Life Technologies) (1×), Rotor Gene (Corbett/Qiagen) (1×), Light Cycler 480 (Roche) (2×).

3.2.2. Allergen standard DNA mixture: calibration series and sensitivity

Table 4 summarises the results of the calibration series. Performance criteria for standard curves are described e.g. in guidelines for GMO analysis [22–25]. 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.

These requirements for slope and R^2 were consistently met for all four PCR target sequences by all 11 laboratories. The lowest dilution of the matrix standard DNA, corresponding to 0.66 mg of the respective allergenic component (seed or kernel) per kg, was examined in ten replicates and also served as verification of sensitivity. Overall, positive reactions were obtained for all four target sequences in 110 out of 110 reactions (= 100%). A very low variation of Cq values measured in the individual labs even at this level confirmed the very good sensitivity. The median standard deviation of the Cq values obtained for the ten replicates did not exceed 0.2, correlating to a variation of less than around 20%.

3.2.3. 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. Since all results could be taken into account, 66 PCR results per sample and allergen could be used for the evaluation.

Two types of evaluation were applied. First, all amplifications with Cq values < 38 were considered as positive. Secondly, the sample Cq value was compared with the average Cq value of the lowest standard dilution (0.66 mg/kg). If Cq value of the sample is at least 1 higher than the Cq (0.66 mg/kg), the result was evaluated as “negative” [17].

Table 4. Inter-laboratory validation of results of 11 labs. Real-time PCR systems and results of the dilution series of the allergen standard DNA mixture: Calibration curve (500 – 1.95 mg/kg) and sensitivity control (0.66 mg/kg).

	Mustard, brown	Mustard, white	Celery	Pistachio
Slope ***[–3.1 – –3.6]?*** [24, 22] No. labs/total	11/11	11/11	11/11	11/11
Slope median	–3.37	–3.36	–3.32	–3.39
Slope Range	–3.24 – –3.46	3.24 – –3.50	–3.25 – –3.47	–3.28 – –3.47
$R^2 > 0.98$? [24, 22] No. labs/total	11/11	11/11	11/11	11/11
R^2 median	0.999	0.999	0.997	0.999
R^2 Range	0.996 – 1.000	0.994 – 1.000	0.994 – 1.000	0.991 – 1.000
Std 0.66 mg/kg No. positives/total [a]	110 / 110 (= 100%)	110 / 110 (= 100%)	110 / 110 (= 100%)	110 / 110 (= 100%)
Cq values median of StdDev [b]	0.20	0.08	0.16	0.11

[a] Std 0.66 mg/kg = number of positive reactions for standard dilution 0.66 mg "allergen"/kg (n=10 per lab)
[b] median of standard deviations of cq values (n= 10) per lab, obtained for the standard dilution 0.66 mg/kg

Blank materials:
For the two unspiked materials, amplifications with Cq values < 38 were obtained in three out of four PCR systems. This was particularly common for the detection of white mustard (71% and 77%, respectively).

The Cq values of the observed amplifications were 34.8 and higher (mostly Cq 36 – 38). Furthermore, the evaluation by Cq comparison with the lowest standard did not result in any false-positive results. The difference in Cq values of the lowest standard (0.66 mg/kg) was at least 8, thus in this way it was possible to clearly distinguish between positive and negative samples in every case.

The causes of the amplifications observed may be minimal contaminations of the materials used as "zero standards" (calculated as below 0.1 mg/kg). However, such amplifications are not unusual in multicopy target sequences and can also be caused by minimal, negligible cross-reactivities.

Matrix samples (= matrix control materials acc. Section 2.1)
For the spiked materials, positive reactions were obtained consistently for both types of evaluation for all four detection systems (target sequences) and all five materials. The results of the spiked samples therefore confirm the results of the sensitivity assessment based on the dilutions of the DNA standard (previous section).

The four allergenic foods could be detected without doubt at concentrations of 10 mg/kg, even after heating (baking or frying). Accordingly, the results show that the detection of 3 mg/kg of mustard protein (in white or brown mustard) or 2 mg/kg of celery or pistachio protein is reliably possible in processed foods when converted to the protein content of the allergenic foods.

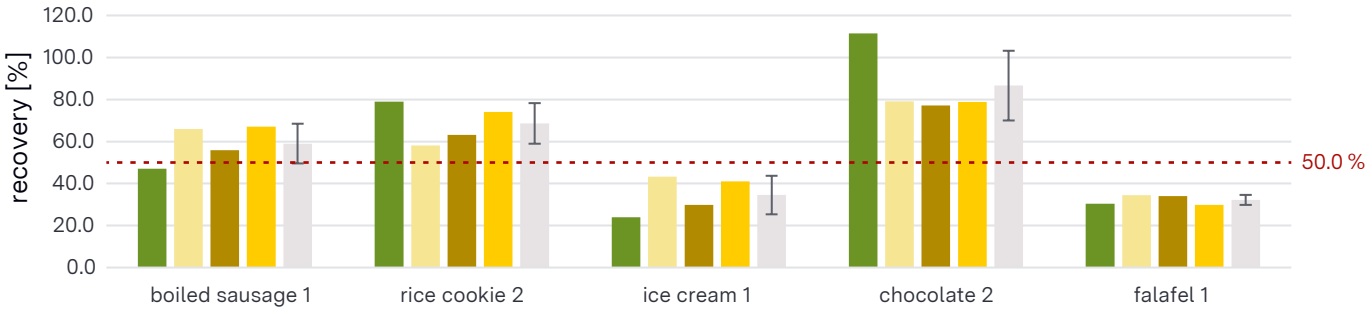
3.2.4. Quantitative evaluation of samples

Figure 1 a) – 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 using PCR, guidelines for real-time PCR-based GMO analysis were used for the evaluation of the results [24, 22]. Furthermore, recovery rates of 50% to 150% are considered acceptable for allergen analysis [26 – 27]. All 66 PCR results per sample and allergen were considered for the quantitative evaluation.

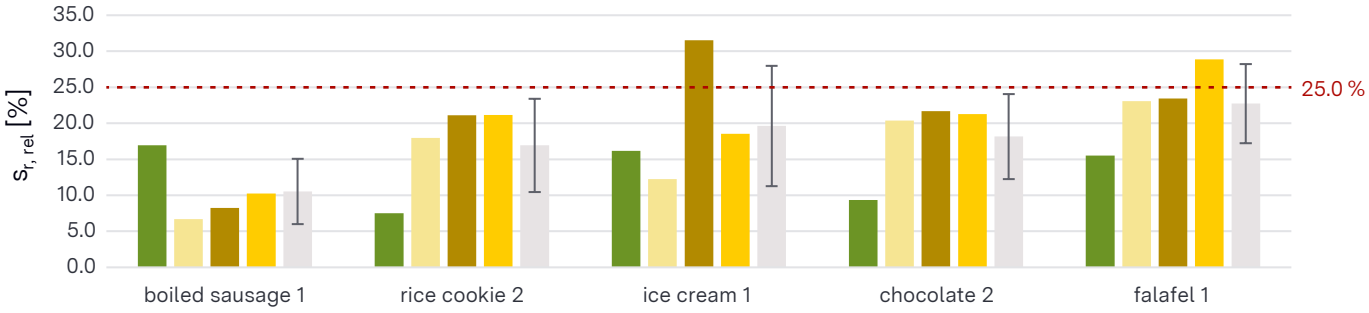
Trueness/recoveries
According to the guidelines for quantitative GMO analysis [23], the deviation of the mean value from the true value or an accepted reference value should not exceed 25%. This criterion was achieved for all four allergenic components in the chocolate matrix (11 to 22.9%). During the preparation of the samples, no further intense heating/processing took place after the

Allergens: pistachio; mustard white; mustard brown; celery; "Mean allergen" = overall mean of all allergens per matrix

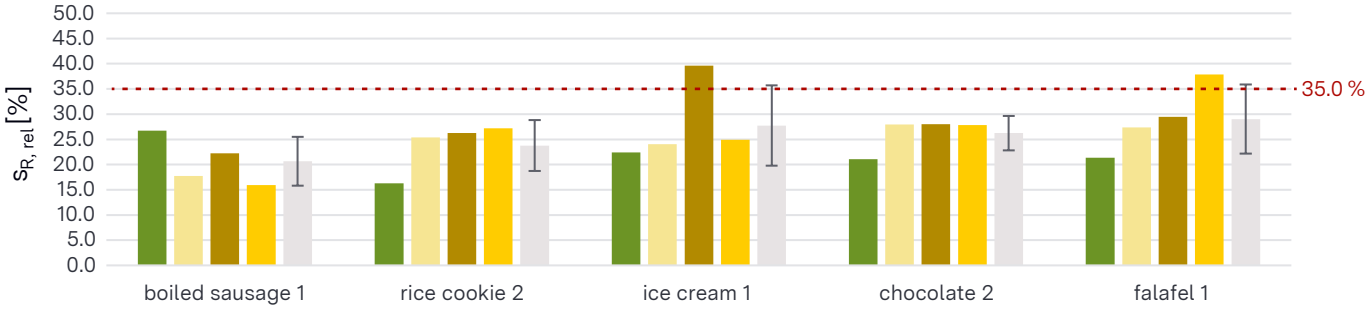
a) Recovery. The recovery was calculated by division of the mean value by the incurred (target) level.



b) Repeatability standard deviation Sr, rel (%), referring to the mean value of each level.



c) Reproducibility standard deviation S_R, rel (%), referring to the mean value of each level.



d) Measurement uncertainty (Bias/$S_{R,\,rel}$) (%). Calculated by pythagorean addidion of the bias (relative deviation of the mean value from the incurred value) (%) and the reproducibility standard deviation (%): $\sqrt{\text{bias}(\%)^2 + S_R(\%)^2}$ [27].

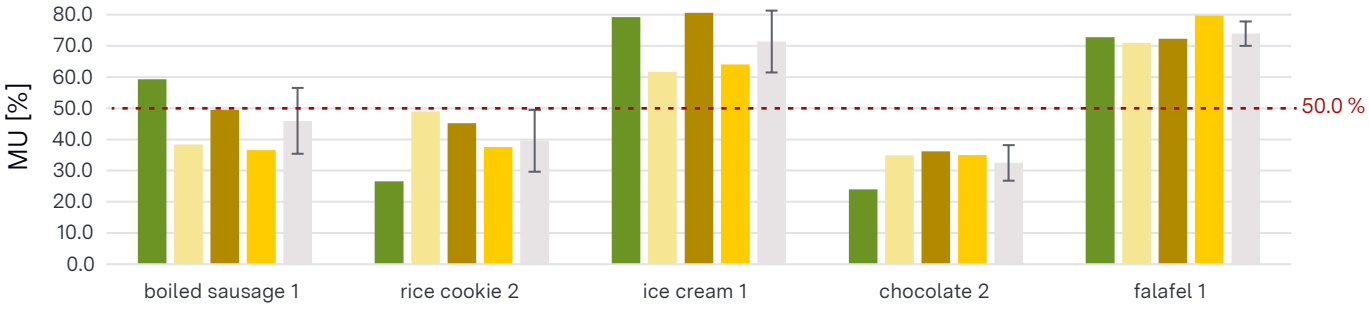


Figure 1. Interlaboratory validation: Quantitative evaluation by matrix. Error indicators show the standard deviation of all results, dotted lines in red = target values according to Section 3.2.4.

addition of the allergens (only melting of the chocolate at 50 °C).

For the cooked sausage and rice cracker matrices, this criterion was not quite met for all four allergens, but with one exception (pistachio in sausage, 47%), the recoveries were more than 50% (see also Figure 1 a)). During the production of these two matrices, even more intense and prolonged heating steps (baking, cooking) took place after the addition of the allergens.

In contrast, the falafel and ice cream matrices showed more significant deviations from the target, with recoveries varying from 24.0% (pistachio in ice cream) to 43.2% (mustard (white) in ice cream). The mean recovery (across all four allergens) was 32.2% for falafel and 34.5% for ice cream.

Further processing steps were carried out in the production of falafel, including frying, after the allergens were added. These steps are supposed to have reduced the amount of amplifiable DNA.

The relatively low recovery of allergenic components in the ice cream matrix can be explained by DNA loss during preparation of the allergenic components to be detected (due to a low DNA content, the presence of additives and shearing by ice crystals) or during the freezing process involved in producing the sample materials.

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 [24, 22].

As shown in Figure 1 b), the criterion for $S_{r,\,rel}$ was only narrowly missed for brown mustard in ice cream (31.5%) and celery in falafel (28.9%). For all other 18 matrix-allergen combinations, $S_{r,\,rel}$ was less than 25% and thus within the acceptable range for quantitative determinations.

With regard to $S_{R,\,rel}$, 18 of 20 matrix allergen combinations also met the requirement of $S_{R,\,rel} < 35\%$ (Figure 1 c)). Here, as well, brown mustard in ice cream (39.6%) and celery in falafel (37.9%) did not quite meet this requirement.

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 [27].

The results for the measurement uncertainty of the quantification of the four allergenic components are shown in Figure 1 d). In line with the results for trueness/recovery (see above), sufficient measurement uncertainties of less than 50% were obtained for the rice cracker, chocolate, and, for the most part, boiled sausage matrices. Such measurement uncertainties are considered sufficient for quantitative methods in allergen analysis.

In contrast, measurement uncertainties of over 50% (61.6% to 80.6%) were consistently determined for the ice cream and falafel matrices. This is due to the deviations of the mean values from the target value (= spiked content) described above. Individual DNA extraction by each laboratory would contribute to measurement uncertainty additionally.

4. Conclusion and further work

There is still a lack of official reference materials for allergen analysis and thus uniform standards that enable a quantitative assessment of allergenic contaminants in food across laboratories and methods [12]. The availability of such materials remains a big challenge in allergen analysis.

In this paper, we aim to present a new, practical approach to the production of control standards for allergen analysis. In our opinion, such an approach could also represent a step towards officially certified reference materials.

As a first step, an experienced provider of proficiency tests produced four allergen stock mixtures, each in a simple potato flour matrix. The allergens were added in the form of representative mixtures of seeds from the respective plants or animal species, characterized as well as possible. The use of heated (e.g., roasted) materials was avoided in the production of these mixtures. This is to ensure, that the material can be used for basic method calibration of the measurement result in terms of allergen content in mg/kg. A major advantage of such a material is that it can be used to calibrate further allergen analysis methods such as ELISA or LC-MS as well.

In a next step, this material might be used to produce incurred and processed foods on a laboratory scale. It has been demonstrated that these materials are sufficiently homogeneous. This allows testing laboratories to consider the analytical requirements in a flexible and matrix-specific manner.

In addition, such materials are also suitable for the validation of new allergen analysis methods, as shown here by using the multiplex real-time PCR method for the detection and quantification of white mustard, brown or black mustard, celery and pistachio. By targeting multicopy-sequences, a very sensitive detection of allergenic ingredients is possible.

The results of the interlaboratory test confirm the results of the in-house and initial interlaboratory validation of the method according to [14].

The method also proved to be highly sensitive in the interlaboratory test. The precision data also meet the requirements for quantitative analysis. By including different food matrices, it was also possible to show that certain matrices with deviating (lower) recoveries are to be expected. This must be taken into account in the quantification in individual cases. The method can be regarded as another element in the available collection of multiplex real-time PCR for sensitive, simultaneous, and at least semi-quantitative allergen analysis.

The method has been included into the German Official Collection of Methods of Analysis as a national standard method [28].

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Conflict of Interest Statement
To the best of our knowledge, the named authors have no conflict of interest, financial or otherwise.

Compliance with Ethical Standards
The work was done in line with ethical policies. All of the authors have approved the contents of this paper and have agreed to the submission policies of Aspects of Food Control and Animal Health. No research involving human participants and/or animals was done within this project. Each of the authors confirms that this manuscript has not been previously published and is not currently under consideration by any other journal.

Author contribution
Each named author has substantially contributed to conducting the underlying research and drafting this manuscript.

Data Availability
Further data will be made available upon request.

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