Proficiency Tests

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Evaluation Report
proficiency test

DLA 07/2016

Allergens VII:

Crustaceae and Cashew

in Instant Soup Powder

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Allgemeine Informationen zur Eignungsprüfung (EP)
General Information on the proficiency test (PT)

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Unteraufträge *Subcontractors*	Die Prüfung der Gehalte, Homogenität und Stabilität von EP-Parametern wird von DLA im Unterauftrag vergeben. The analysis of the content, homogeneity and stability of PT-parameters are subcontracted by DLA.

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1. Introduction

The participation in proficiency testing schemes is an essential element of the quality-management-system of every laboratory testing food and feed, cosmetics and food contact materials. The implementation of proficiency tests enables the participating laboratories to prove their own analytical competence under realistic conditions. At the same time they receive valuable data regarding the verification and/or validation of the particular testing method [1, 5].
The purpose of DLA is to offer proficiency tests for selected parameters in concentrations with practical relevance.
Realisation and evaluation of the present proficiency test follows the technical requirements of DIN EN ISO/IEC 17043 (2010) and DIN ISO 13528:2009 / ISO 13528:2015 [2, 3].

2. Realisation

2.1 Test material

Two PT-samples for the detection of allergens in the range of mg/kg and one spiking material sample were provided for analysis. The spiking material sample contains the respective allergenic ingredients in the range of 1-10 % and was added to the spiked PT-sample. The results of the spiking material sample should give the possibility of a comparison with the spiked sample in respect to the detectability of the allergens with and without the influence of matrix and / or food processing.

The test material was a common in commerce instant soup powder - "onion soup powder". The basic composition of both sample A and sample B was the same (see table 1). After crushing, sieving and homogenization of the basic mixture the spiked sample B was produced as following:
The spiking material containing the allergenic ingredients Crustaceae and Cashew was added to an aliquot of the basic mixture and the mixture was homogenized. Subsequently, the basic mixture was again added in 3 additional steps and mechanically homogenized in each case until the total quantity had been reached.
The raw materials of the allergen premix were sieved (mesh 400 µm) or sieved by means of a centrifugal mill (mesh 500 µm) prior to use.

The composition of the spiking material sample and the amounts of allergens in sample B is given in table 2.

After homogenization the samples were portioned to approximately 25 g into metallised PET film bags.

Table 1: Composition of DLA-Samples

Ingredients	**Sample A**	**Sample B**
Onion soup (Powder) Ingredients: Onion (45%), starch, salt, fried onion (7%), vegetable fats, yeast extract, flavor, sugar, thickening agents: guar gum, maltodextrin, garlic, spices, aroma, caramel sugar Nutrients per 100 g: Protein 11 g, Carbohydrates 43 g, Fat 8,0 g	67,2 g/100g	66,9 g/100g
Potato flour Ingredients: Potato flour Nutrients per 100 g: Protein <0,1 g, Carbohydrates 80 g, Fat <0,1 g	21,1 g/100g	21,0 g/100g
Maltodextrin Ingredients: Maltodextrin	11,8 g/100g	11,7 g/100g
Spiking material sample	-	0,480 g/100g

Table 2: Added amounts of allergenic ingredients

Ingredients	**Spiking material sample**	**Amounts in Sample B**
Potato flour	89,3 %	0,42 %
Cashew mush Ingredients: Cashew - as Cashew* - *thereof 18% total protein***	11200 mg/kg (1,12 %) 2020 mg/kg	54 mg/kg 9,7 mg/kg
Shrimps (Litopenaeus vannamei) - as Shrimp, dried* - *thereof 63% total protein***	9440 mg/kg (0,944 %) 5950 mg/kg	45 mg/kg 28 mg/kg
additional ingredients: *maltodextrin, sodium chloride, sodium sulfate, and silicon dioxide*	< 7,50 %	< 0,04 %

*Allergen contents as „total food" as described in column ingredients according to gravimetric mixture
** Protein contents according to laboratory analysis of raw material (total nitrogen according to Kjeldahl)

Note: *The metrological traceability of temperature, mass and volume during production of the PT samples is ensured by DAkkS calibrated reference materials.*

2.1.1 Homogeneity

The **mixture homogeneity before bottling** was examined 8-fold by **microtracer analysis**. It is a standardized method that is part of the international GMP certification system for feed [14].
Before mixing dye coated iron particles of µm size are added to the sample and the number of particles is determined after homogenization in taken aliquots. The evaluation of the mixture homogeneity is based on the Poisson distribution using the chi-square test. A probability of ≥ 5 % is equivalent to a good homogeneous mixture and of ≥ 25% to an excellent mixture [14, 15].
The microtracer analysis of the present PT samples showed a probability of 99% for the spiked sample B and of 80% for the spiking material sample. Additionally particle number results were converted into concentrations, statistically evaluated according to normal distribution and compared to the standard deviation according to Horwitz. This gave a Hor-Rat value of 0,7 and 1,1 respectively. The results of microtracer analysis are given in the documentation.

Homogeneity of bottled spiked sample B

Implementation of homogeneity tests
The homogeneity tests were carried out in cooperation with the laboratories of the specified test kit providers. Ten samples of the bottled spiked sample were chosen randomly by DLA, thereof 2 subsamples were weighed into previously randomly encoded sample containers, and then sent to the laboratories for analysis. The sample weights were made with a deviation of ± 10% from recommended sample weight of the test kit instructions and not communicated to the laboratories. After transmission of analysis results by the laboratories, the valid results were calculated on the basis of the exact weightings by DLA and the statistical calculation was carried out according to ISO 13528:2009 Annex B.

Valuation of homogeneity
The homogeneity is regarded as sufficient when the standard deviation between the samples Ss is ≤ 15% („heterogeneity standard deviation"). This criterion is fulfilled for sample B by all ELISA tests for cashew (Immunolab and AgraQuant) (see page 7). The criterion is not fulfilled by the ELISA tests for crustaceae (Immunolab, AgraQuant and Veratox). For crustaceae the heterogeneity standard deviations were in the range of 15-20% and >25%, respectively (see page 8).
Recommendations for repeatability standard deviations of ELISA and PCR methods are usually ≤ 25% [16, 17, 20, 21].

In case the criterion for sufficient homogeneity of the test items is not fulfilled the impact on the target standard deviation will be verified. If necessary the evaluation of results will be done considering the standard uncertainty of the assigned value by z'-scores (s. 3.6 and 3.8) [3].

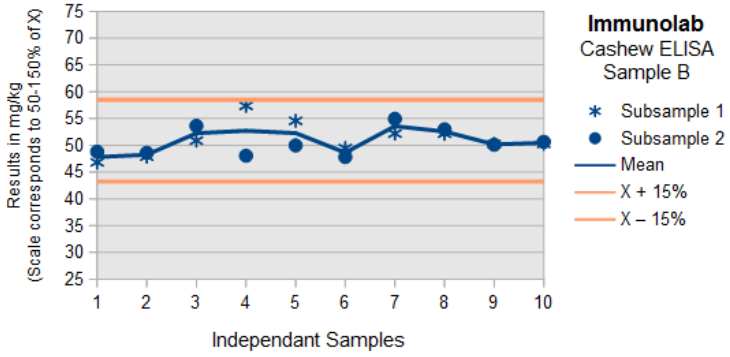
ELISA-Tests: Homogenität Cashew / Homogeneity Cashew

Immunolab Cashew ELISA

Sample weights: 1,0 g (0,9 – 1,1 g)
Number of replicates: 2
Overall result: Cashew 50,9 ± 2,09 mg/kg

Sample B	Subsample 1 mg/kg	Subsample 2 mg/kg	Mean mg/kg
1	46,8	48,8	47,8
2	47,9	48,6	48,2
3	50,9	53,6	52,2
4	57,4	48,0	52,7
5	54,6	50,0	52,3
6	49,5	47,8	48,6
7	52,2	54,9	53,6
8	52,2	53,0	52,6
9	50,2	50,1	50,2
10	50,3	50,6	50,5

General average X	50,9	
SD of sample means Sx	2,09	4,1%
SD within-samples Sw	2,56	5,0%
SD between-samples Ss	2,09	4,1%

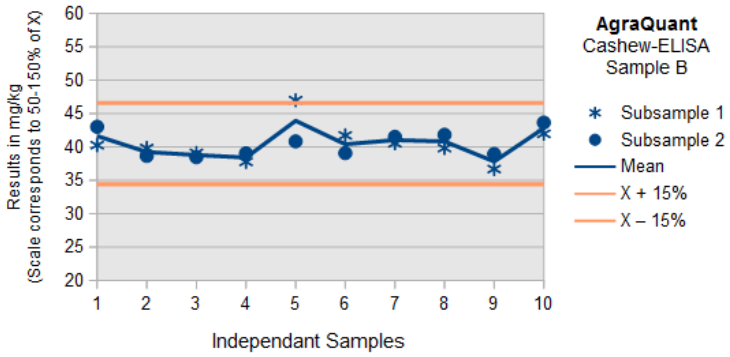


Romerlabs AgraQuant Cashew

Sample weights: 1,0 g (0,9 – 1,1 g)
Number of replicates: 2
Overall result: Cashew 40,5 ± 1,23 mg/kg

Sample B	Subsample 1 mg/kg	Subsample 2 mg/kg	Mean mg/kg
1	40,3	43,0	41,6
2	39,8	38,7	39,2
3	39,1	38,5	38,8
4	37,8	39,1	38,4
5	47,0	40,8	43,9
6	41,7	39,1	40,4
7	40,6	41,5	41,0
8	39,8	41,8	40,8
9	36,7	38,9	37,8
10	42,0	43,6	42,8

General average X	40,5	
SD of sample means Sx	1,97	4,9%
SD within-samples Sw	1,86	4,6%
SD between-samples Ss	1,23	3,0%



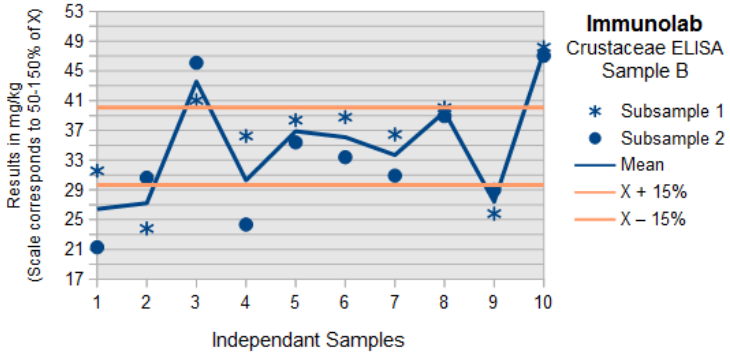
ELISA-Tests: Homogenität Crustacea / Homogeneity Crustaceae

Immunolab Crustaceae ELISA

Sample weights: 1,0 g (0,9 – 1,1 g)
Number of replicates: 2
Overall result: Shrimps (raw, dry) 34,9 ± 6,52 mg/kg

Sample B	Subsample 1 mg/kg	Subsample 2 mg/kg	Mean mg/kg
1	31,6	21,3	26,4
2	23,8	30,6	27,2
3	41,0	46,1	43,6
4	36,3	24,3	30,3
5	38,4	35,4	36,9
6	38,8	33,4	36,1
7	36,5	30,9	33,7
8	40,1	38,9	39,5
9	25,8	29,0	27,4
10	48,2	47,0	47,6

General average X	34,9	
SD of sample means Sx	7,24	20,8%
SD within-samples Sw	4,48	12,8%
SD between-samples Ss	6,52	18,7%

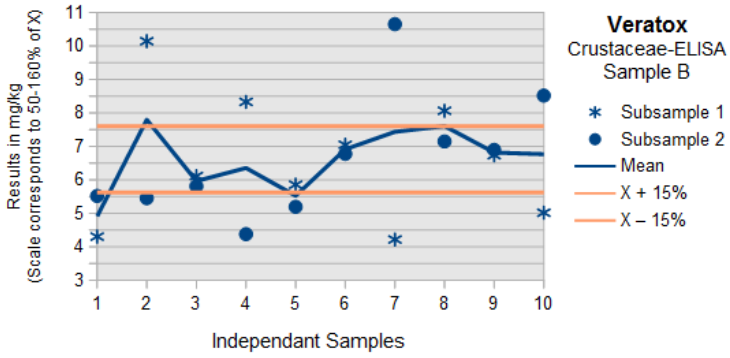


Neogen Veratox ELISA Crustaceae

Sample weights: 5,0 g (4,5 – 5,5 g)
Number of replicates: 2
Overall result: Crustaceae 6,61 ± 2,07 mg/kg

Sample B	Subsample 1 mg/kg	Subsample 2 mg/kg	Mean mg/kg
1	4,30	5,52	4,91
2	10,1	5,45	7,80
3	6,11	5,81	5,96
4	8,33	4,38	6,35
5	5,85	5,19	5,52
6	7,05	6,78	6,91
7	4,22	10,65	7,43
8	8,06	7,15	7,60
9	6,73	6,89	6,81
10	5,02	8,52	6,77

General average X	6,61	
SD of sample means Sx	0,93	14,1%
SD within-samples Sw	2,17	32,8%
SD between-samples Ss	2,07	31,3%

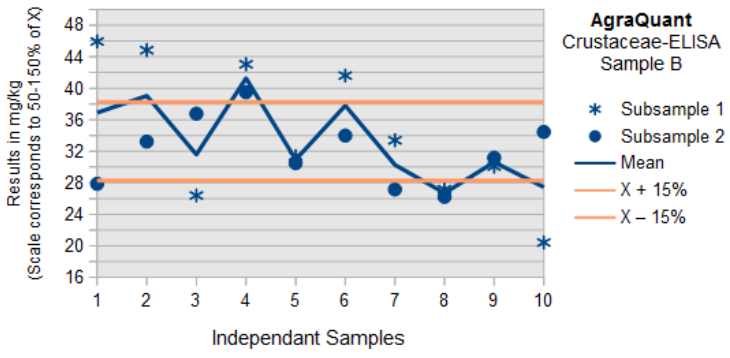


Romerlabs AgraQuant Crustaceae

Sample weights: 1,0 g (0,9 – 1,1 g)
Number of replicates: 2
Overall result: Shrimps (dry) 33,3 ± 5,57 mg/kg

Sample B	Subsample 1 mg/kg	Subsample 2 mg/kg	Mean mg/kg
1	45,9	27,9	36,9
2	44,8	33,2	39,0
3	26,4	36,8	31,6
4	43,0	39,5	41,3
5	31,4	30,5	30,9
6	41,6	34,0	37,8
7	33,4	27,2	30,3
8	27,2	26,2	26,7
9	30,1	31,2	30,6
10	20,4	34,5	27,4

General average X	33,3	
SD of sample means Sx	5,08	15,3%
SD within-samples Sw	6,63	19,9%
SD between-samples Ss	5,57	16,7%



2.2 Sample shipment and information to the test

The portions of test material (sample A and sample B as well as the spiking material sample) were sent to every participating laboratory in the 46th week of 2016. The testing method was optional. The tests should be finished at December 30th 2016 the latest.

With the cover letter along with the sample shipment the following information was given to participants:

There are two different samples, sample A and sample B, of onion soup powder possibly containing the allergenic ingredients crustaceae and/or cashew in the range of mg/kg. Additionally a "Spiking Material Sample" is provided which was used for the spiking of the positive sample (A or B). It contains 1-10% of the allergenic items in potato flour and should be analysed like a normal sample (eventually diluted).
In general we recommend to homogenize a representative sample amount before analysis according to good laboratory practice, especially in case of low sample weights. Every suitable method for detection or determination of the analytes may be applied (e.g. ELISA, PCR).

2.3 Submission of results

The participants submitted their results in standard forms, which have been sent by email or were available on our website. On one hand the results given as positive/negative and on the other hand the indicated results of the allergenic ingredients e.g. total food item or protein in mg/kg were evaluated.
During evaluation DLA eventually requests detailed information by email on the type of indicated quantitative results from participants concerned.
Queried and documented were the indicated results and details of the test methods like specifity, test kit manufacturer and hints about the procedure.
In case participants submitted several results for the same parameter obtained by different methods these results were evaluated with the same evaluation number with a letter as a suffix and indication of the related method.
All 14 participants submitted their results in time.

3. Evaluation

Different ELISA-methods for the determination of allergens in foods are eventually using different antibodies, are usually calibrated with different reference materials and may utilize differing extraction methods. Among others this can induce different results of the content of the analyte [23, 24, 25, 26]. It is for this reason that we contrast the results of the present proficiency test with several assigned values. Thereby it is possible to evaluate each single result in comparison to the mean of all results and/or in comparison to the mean of results obtained by a single method. For comparison the actually added amount is plotted in the figures of the results.

For quantitative results of the spiking material sample and the spiked sample recovery rates were calculated with respect to the known content of spiked allergens. The recovery rates were given for information only. *No* statistical evaluation was done. The recovery rates should exclusively give an estimation of the matrix- and/or processing influences.

ELISA- and PCR results were valuated qualitatively with respect to the percentages of positive and negative results, respectively. If there are ≥ 75 % positive or negative results, a consensus result is determined for each sample.

3.1 Consensus value from participants (assigned value)

The robust mean of the submitted results was used as assigned value (X_{pt}) („consensus value from participants") providing a normal distribution. The calculation was done according to algorithm A as described in annex C of ISO 13528 [3].
The condition is that the majority of the participants' results show a normal distribution or are distributed unimodal and symmetrically. To this end, an examination of the distribution is carried out, inter alia, using the kernel density estimate [3, 12].

In case there are indications for sources of higher variability such as a bimodal distribution of results, a cause analysis is performed. Frequently different analytical methods may cause an anomaly in results' distribution. If this is the case, separate evaluations with own assigned values (X_{pti}) are made whenever possible.

If possible, this is the standard procedure for the evaluation of ELISA methods for the determination of allergens:

i) ***Robust mean of all results*** - $X_{pt_{ALL}}$
ii) ***Robust mean of single methods*** - $X_{pt_{METHOD\ i}}$
with at least 5 quantitative results given.

Single results giving values outside the measuring range of the participating laboratory or given as „0" are not considered for statistical evaluation (e.g. results given as > 25 mg/kg and < 2,5 mg/kg, respectively) [3].

3.2 Robust standard deviation

For comparison to the target standard deviation σ_{pt} (standard deviation for proficiency assessment) a robust standard deviation (S^x) was calculated. The calculation was done according to algorithm A as described in annex C of ISO 13528 [3].

The following robust standard deviations were considered:

i) ***Robust standard deviation of all results*** - S^x_{ALL}
ii) ***Robust standard deviation of single methods*** - $S^x_{METHOD\ i}$
with at least 5 quantitative results given.

3.3 Exclusion of results and outliers

Before statistical evaluation obvious blunders, such as those with incorrect units, decimal point errors, and results for a another proficiency test item can be removed from the data set [2]. All results should be given at least with 2 significant digits. Specifying 3 significant digits is usually sufficient.

Results obtained by different analytical methods causing an increased variability and/or a bi- or multimodal distribution of results, are treated separately or could be excluded in case of too few numbers of results.
For this results are checked by kernel density estimation [3, 12].

Results are identified as outliers by the use of robust statistics. If a value deviates from the robust mean by more than 3 times the robust standard deviation, it is classified as an outlier [3]. Detected outliers are stated for information only, when z-score are < -2 or > 2. Due to the use of robust statistics outliers are not excluded, provided that no other reasons are present [3].

3.4 Target standard deviation (for proficiency assessment)

The target standard deviation of the assigned value σ_{pt} (= standard deviation for proficiency assessment) can be determined according to the following methods.
In the present PT the target standard deviation was determined according to 3.4.3 value by perception.

3.4.1 General model (Horwitz)

Based on statistical characteristics obtained in numerous PTs for different parameters and methods Horwitz has derived a general model for estimating the reproducibility standard deviation σ_R [6]. Later the model was modified by Thompson for certain concentration ranges [10]. The reproducibility standard deviation σ_R can be applied as the relative target standard deviation σ_{pt} in % of the assigned values and calculated according to the following equations [3]. For this the assigned value X_{pt} is used for the concentration c.

Equations	**Range of concentrations**	**corresponds to**
$\sigma_R = 0{,}22c$	$c < 1{,}2 \times 10^{-7}$	< 120 µg/kg
$\sigma_R = 0{,}02c^{0{,}8495}$	$1{,}2 \times 10^{-7} \leq c \leq 0{,}138$	≥ 120 µg/kg
$\sigma_R = 0{,}01c^{0{,}5}$	$c > 0{,}138$	> 13,8 g/100g

with c = mass content of analyte (as relative size, e.g. 1 mg/kg = 1 ppm = 10^{-6} kg/kg)

The target standard deviation according to Horwitz is currently not achievable by ELISA-methods for values in the mg/kg range and was therefore not considered for evaluation.

3.4.2 Value by precision experiment

Using the reproducibility standard deviation σ_R and the repeatability standard deviation σ_r of a precision experiment (collaborative trial or proficiency test) the target standard deviation σ_{pt} can be derived considering the number of replicate measurements m of participants in the present PT [3]:

$$\sigma_{pt} = \sqrt{\sigma_R^2 - \sigma_r^2\left(m-1/m\right)}$$

The relative repeatability standard deviations (RSD_r) and relative reproducibility standard deviations (RSD_R) given in table 3a (ELISA) and table 3b (PCR) were obtained in precision experiments by the indicated methods. The resulting target standard deviations σ_{pt} were calculated for a number of m = 2 replicate measurements. With a number of m = 1 replicate measurements the reproducibility standard deviation σ_R is identical to the target standard deviation σ_{pt}.

Table 3a: ELISA-Methods - Relative repeatability standard deviations (RSD_r) and relative reproducibility standard deviations (RSD_R) from precision experiments and resulting target standard deviations σ_{pt} [28-29]

Parameter	**Matrix**	**Mean [mg/kg]**	**Recovery**	**rob RSD**	**RSD_r**	**RSD_R**	**σ_{pt}**	**Method / Literature**
Peanut	Milk chocolate	173,7 33,8 5,9	87 % 85 % 59 %	- - -	8,8% 5,2% 7,8%	31% 20% 31%	30,4% 19,7% 30,5%	ELISA Manuf. A ASU 00.00-69
Peanut	Milk chocolate	215,7 40,1 10,1	108 % 100 % 101 %	- - -	5,9% 7,2% 7,3%	32% 14% 16%	31,7% 13,0% 15,1%	ELISA Manuf. B ASU 00.00-69
Peanut	Dark chocolate	148,2 30,9 5,7	74 % 77 % 57 %	- - -	6,0% 13% 6,1%	22% 25% 33%	21,6% 23,2% 32,7%	ELISA Manuf. A ASU 00.00-69
Hazelnut	Dark chocolate	16,3 7,56 3,73 1,62	81 % 76 % 75 % 81 %	- - - -	4,7% 8,9% 13% 15%	12% 15% 24% 33%	11,5% 13,6% 22,2% 31,2%	ELISA Manuf. A ASU 44.00-7
Hazelnut	Dark chocolate	21,3 10,7 4,69 2,37	106 % 107 % 94 % 119 %	- - - -	7,1% 11% 11% 9,3%	14% 19% 17% 17%	13,1% 17,3% 15,1% 16,4%	ELISA Manuf. B ASU 44.00-7

From the precision data of the official German ASU §64 methods the calculated relative target standard deviations are in the range of 11 – 33% for the ELISA methods and 15 – 43% for the PCR methods depending on the matrix, processing and concentration level of allergens (s. Tab. 3a and 3b).

The Working Group on Prolamin Analysis and Toxicity (WGPAT) coordinated a collaborative study with two commercial ELISA test kits for the determination of gluten using the monoclonal R5 antibody [22]. 12 food samples with gliadin in the range of 0 - 168 mg/kg were analyzed by 20 laboratories. Recovery rates ranged between 65 and 110%, relative repeatability deviations ranged from 13 - 25% (method 1) and 11 - 22% (method 2) while the relative reproducibility standard deviations ranged from 23 - 47% (method 1) and 25 - 33% (method 2). According to the authors both ELISA test kits fulfilled therefore the current validation criteria for ELISA methods [22].

The IRMM (Institute for Reference Materials and Measurements) performed an interlaboratory comparison for five different ELISA test kits for the quantification of peanut [25]. The mean values for two matrices were in the concentration range of 0,3 - 16,1 mg/kg and 1,2 - 20,4 mg/kg, respectively. The lowest relative reproducibility standard deviations of the five test kits were for dark chocolate in the range of 20 - 42% and for cookies in the range of 23 – 61%.

Table 3b: PCR-Methods - Relative repeatability standard deviations (RSD_r) and relative reproducibility standard deviations (RSD_R) from precision experiments and resulting target standard deviations σ_{pt} [30-32]

Parameter	**Matrix**	**Mean [mg/kg]**	**Recov-ery**	**rob RSD**	**RSD_r**	**RSD_R**	**σ_{pt}**	**Method / Literature**
Almond	Rice cookie	105,2 18,0 10,5	105 % 90 % 105 %	-	19,3% 44,0% 32,0%	27,5% 49,1% 38,8%	23,9% 38,0% 31,5%	rt-PCR ASU 18.00-20
Almond	Wheat cookie Sauce powder	114,3 88,1	94,6 % 88,1 %	-	22,1% 43,9%	41,8% 43,1%	38,8% - %	rt-PCR ASU 18.00-20
Almond	Rice cookie	109 21,3 12,3	109 % 107 % 121 %	-	17,6% 35,8% 32,0%	32,8% 45,0% 47,8%	30,3% 37,2% 42,1%	rt-PCR ASU 18.00-22
Almond	Wheat cookie Sauce powder	120,7 112	98,2 % 94,1 %	-	15,7% 36,2%	32,5% 42,8%	30,5% 34,3%	rt-PCR ASU 18.00-22
Sesame	Rice cookie	94,6 15,7 9,8	95 % 79 % 98 %	-	22,5% 26,0% 20,9%	27,5% 39,5% 33,5%	22,4% 35,0% 30,0%	rt-PCR ASU 18.00-19
Sesame	Wheat cookie Sauce powder	96,9 59,8	79 % 60 %	-	21,8% 22,2%	33,0% 43,2%	29,2% 40,2%	rt-PCR ASU 18.00-19
Sesame	Rice cookie	88,9 17,8 9,8	89 % 89 % 98 %	-	18,2% 34,2% 26,2%	30,5% 37,8% 37,0%	27,7% 29,1% 32,0%	rt-PCR ASU 18.00-22
Sesame	Wheat cookie Sauce powder	115 58,5	93 % 59 %	-	16,7% 30,8%	41,1% 44,4%	39,4% 38,7%	rt-PCR ASU 18.00-22

3.4.3 Value by perception

The target standard deviation for proficiency assessment can be set at a value that corresponds to the level of performance that the coordinator would wish laboratories to be able to achieve [3].
Criteria for the level of performance of analytical methods for the quantitative determination of allergens in foods were recently elaborated e.g. by the Ministry of Health and Welfare (MHLW) in Japan [20], by the working group 12 „Food Allergens" of the technical committee CEN/TC 275 [17-19], by an international "Food Allergen Working Group" under the advice of the AOAC Presidential Task Force on Food Allergens [21] and by the Codex Alimentarius Committee (CAC/GL 74-2010) [16].

Some of the relevant ELISA and PCR validation criteria of the mentioned panels are listed in tables 4 and 5, respectively.

Table 4: ELISA-Validation

Literature [16-22]	**Recovery rate**	**Repeatability standard deviation**	**Reproducibility standard deviation**
MHLW 2006	50 - 150%		≤ 25%
CEN 2009		≤ 20%	
AOAC 2010	50 - 150%	6,9 - 34,4% [(a)]	19,5 - 57,2% [(a)]
CAC 2010	70 - 120%	≤ 25%	≤ 35%

(a) = Example from an hypothetical proficiency scheme in the range of 0,5 - 5 mg/kg

Table 5: PCR-Validation

Literature [16]	**Recovery rate**	**Repeatability standard deviation**	**Reproducibility standard deviation**
CAC 2010	± 25% [(a)]	≤ 25%	≤ 35%

(a) = Trueness / Richtigkeit

Based on the currently achievable level of performance of ELISA and PCR methods for the quantitative determination of allergens in foods, which could be deduced from the data of precision experiments and from validation criteria, we set a relative target standard deviation σ_{pt} of 25%.
This target standard deviation was applied for the statistical evaluation of the results by z-score and was used for all assigned values mentioned in 3.1.

3.5 z-Score

To assess the results of the participants the z-score is used. It indicates about which multiple of the target standard deviation (σ_{pt}) the result (x_i) of the participant is deviating from the assigned value (X_{pt}) [3].
Participants' z-scores are derived from:

$$z_i = \frac{\left(x_i - x_{pt}\right)}{\sigma_{pt}}$$

The requirements for the analytical performance are generally considered as fulfilled if

$$-2 \leq z \leq 2 \ .$$

For information the z-scores below are calculated with a target standard deviation of 25%:

i) ***z-Score*** - z_{ALL} *(with respect to all methods)*
ii) ***z-Score*** - $z_{METHOD\ i}$ *(with respect to single methods)*

3.5.1 Warning and action signals

In accordance with the norm ISO 13528 it is recommended that a result that gives rise to a *z*-score above 3,0 or below −3,0, shall be considered to give an "action signal" [3]. Likewise, a *z*-score above 2,0 or below −2,0 shall be considered to give a "warning signal". A single "action signal", or "warning signal" in two successive PT-rounds, shall be taken as evidence that an anomaly has occurred which requires investigation. For example a fault isolation or a root cause analysis through the examination of transmission error or an error in the calculation, in the trueness and precision must be performed and if necessary appropriate corrective measures should be applied [3].

In the figures of z-scores DLA gives the limits of warning and action signals as yellow and red lines respectively. According to ISO 13528 the signals are valid only in case of a number of ≥ 10 results [3].

3.6 z'-Score

The z'-score can be used for the valuation of the results of the participants, in cases the standard uncertainty has to be considered (s. 3.8). The z'-score represents the relation of the deviation of the result (x) of the participant from the respective consensus value (X) to the square root of quadrat sum of the target standard deviation ($\hat{\sigma}$) and the standard uncertainty (Ux_{pt}) [3].

The calculation is performed by:

$$z'_i = \frac{x_i - x_{pt}}{\sqrt{\sigma_{pt}^2 + u_{(x_{pt})}^2}}$$

If carried out an evaluation of the results by means of z 'score, we have defined below the expression in the denominator as a target standard deviation σ_{pt}'.

The requirements for the analytical performance are generally considered as fulfilled if

$$-2 \leq z' \leq 2 \ .$$

For warning and action signals see 3.5.1.

3.7 Quotient S*/σpt

Following the HorRat-value the results of a proficiency-test (PT) can be considered convincing, if the quotient of robust standard deviation S* and target standard deviation σ_{pt} does not exceed the value of 2.
A value > 2 means an insufficient precision, i.e. the analytical method is too variable, or the variation between the test participants is higher than estimated. Thus the comparability of the results is not given [3].

3.8 Standard uncertainty of the assigned value

Every assigned value has a standard uncertainty that depends on the analytical method, differences between the analytical methods used, the test material, the number of participating laboratories (P) and on other factors. The standard uncertainty ($U_{(x_{pt})}$) for this PT is calculated as follows [3]:

$$u_{(x_{pt})} = 1{,}25 \times \frac{s^*}{\sqrt{p}}$$

If $U_{(x_{pt})} \leq 0{,}3\ \sigma_{pt}$ the standard uncertainty of the assigned value needs not to be included in the interpretation of the results of the PT [3]. Values exceeding 0,3 imply, that the target standard deviation could be

too low with respect to the standard uncertainty of the assigned value. The Quotient $U(x_{pt})/\sigma_{pt}$ is reported in the characteristics of the test.

3.9 Figures

The assigned values and spiking levels are indicated as coloured lines in the figures of results. This allows the comparison of a single result with different possible target values like the spiked level, the robust mean of all results and the robust mean of a single method.

3.10 Recovery rates: Spiking

For the results of the spiking material sample and the spiked sample recovery rates were calculated with respect to the known content of added allergens. The related values of added allergens are given in 2.1 test material in table 1. As a range of acceptance RA for valuating participant's results the range of 50 - 150% for the recovery rates of allergen-ELISAs proposed by the AOAC was used [21]. For quantitative PCR determinations we use the same range of acceptance.

4. Results

All following tables are anonymized. With the delivering of the evaluation report the participants are informed about their individual evaluation number.
Evaluation was done separately for ELISA and PCR-techniques. The results were grouped according to the applied methods (e.g. test kits) and sorted chronologically according to the evaluation number of the participants.

The following result sections are structured equally for the allergenic components. First all results of ELISA or PCR methods for a certain analyte are reported for sample A and afterwards for sample B. The results of the spiking material sample are reported together with the referring spiked sample in the recovery section.

In the result chapter all quantitative results of the participants are displayed formatted to 3 decimal places. In the documentation, all results are given as they were transmitted by the participants.

To ensure the **comparability of quantitative results** DLA harmonized participants' results giving different specifications (e.g. as protein or as allergenic food) as far as possible.

ELISA-results for crustaceae (wet), crustaceae protein and tropomyosin were converted in crustaceae/shrimp dry weight. According to different kit specifications results were converted as followed:

Immunolab (IL): Submitted results were given as crustaceae/shrimp dry weight, thus no recalculation was necessary.
AgraQuant (AQ): Results submitted as crustaceae protein were calculated in dry weight (factor 70, as indicated by test kit instructions). Results submitted as tropomyosin were first converted into crustaceae protein and afterwards into dry weight (20% tropomyosin in total protein – information from test kit).
Ridascreen Fast (RS-F): Results given as crustaceae (wet weight) were first converted into crustaceae protein (20% protein in crustaceae – information from test kit) and afterwards into dry weight (factor 1,6), considering the experimentally determined protein content of 63% (s. page 5) in shrimps (dry).

The ELISA-results given as cashew protein were converted into cashew, considering the experimentally determined protein content of 18% (s. page 5) of the raw material.

Results were valuated qualitatively with respect to the percentages of positive and negative results, respectively. If there are ≥ 75 % positive or negative results, a consensus result is determined for each sample. Each participant result is valuated qualitatively with respect to the consensus value. The valuation was given as a percentage of results in agreement with the consensus values.

When there are at least 5 quantitative results for all methods or for single methods a statistical evaluation was done.

In cases when a statistical evaluation of the quantitative values was done the result table was given as indicated below:

Evaluation number	Result	Result	z-Score Xpt_{ALL}	z-Score $Xpt_{M\,i}$	Method	Remarks
	pos/neg	[mg/kg]				

The statistical evaluation of results for each parameter was calculated in cases where at least 50% results were positive and at least 5 quantitative values were given:

Characteristics	**All Results** *[mg/kg]*	**Method i** *[mg/kg]*
Assigned value (X_{pt})	$\boldsymbol{X_{pt_{ALL}}}$	$\boldsymbol{X_{pt_{METHOD\ i}}}$
Number of results		
Number of outliers		
Median		
Robust mean (X_{pt})		
Robust standard deviation (S*)		
Target data:		
Target standard deviation σ_{pt}		
lower limit of target range (X_{pt} - 2σ_{pt})		
upper limit of target range (X_{pt} + 2σ_{pt})		
Quotient S*/σ_{pt}		
Standard uncertainty $U_{(Xpt)}$		
Quotient $U_{(Xpt)}/\sigma_{pt}$		
Number of results in target range		
Percent in target range		

After that the recovery rates of the results for the spiking sample and the spiked sample are reported. The number of results within the range of acceptance of 50-150% is given.

4.1 Proficiency Test Crustaceae

4.1.1 ELISA Results: Crustaceae (Shrimps, dry)

Qualitative valuation of results: Samples A and B

Evaluation number	Sample A	Sample A	Sample B	Sample B	Qualitative Valuation	Method	Remarks
	pos/neg	[mg/kg]	pos/neg	[mg/kg]	Agreement with consensus value		
1	negative	<1,4	positive	32,2	2/2 (100%)	AQ	Result converted °
7	negative		positive	23,8	2/2 (100%)	AQ	Result converted °
9	negative	<1,4	positive	36,4	2/2 (100%)	AQ	Result converted °
8	negative	<1,4	positive	32,9	2/2 (100%)	IL	
11	negative	< 1	positive	41,0	2/2 (100%)	IL	
2	negative	<0,64	positive	6,72	2/2 (100%)	RS-F	Mean w as determined by DLA, Result converted °
3	negative	<6,4	positive	6,95	2/2 (100%)	RS-F	Result converted °
5	negative		positive	40,0	2/2 (100%)	RS-F	Result converted °
6	negative	<6,4	positive	8,84	2/2 (100%)	RS-F	Result converted °
10	negative		positive		2/2 (100%)	RS-F	Result converted °
12	negative	< 0,64	positive	32,0	2/2 (100%)	RS-F	Result converted °
13	negative	< LOD	positive	3,78	2/2 (100%)	RS-F	Mean w as determined by DLA, Result converted °

° Conversion p. 19

	Sample A		Sample B	
Number positive	0		12	
Number negative	12		0	
Percent positive	0		100	
Percent negative	100		0	
Consensus value	negative		positive	

Methods:
AQ = AgraQuant, RomerLabs
IL = Immunolab
RS-F= Ridascreen® Fast, R-Biopharm

Comments:
The consensus values are in agreement with the spiking of sample B.

Quantitative valuation of results: Sample B

Evaluation number	Crusta-ceae	z-Score Xpt_{ALL}	Method	Remarks
	[mg/kg]			
1	32,2	-0,1	AQ	Result converted °
7	23,8	-1,1	AQ	Result converted °
9	36,4	0,4	AQ	Result converted °
8	32,9	0,0	IL	
11	41,0	0,9	IL	
2	6,72		RS-F	Mean w as determined by DLA, Result converted °
3	6,95		RS-F	Result converted °
5	40,0		RS-F	Result converted °
6	8,84		RS-F	Result converted °
10			RS-F	Result converted °
12	32,0		RS-F	Result converted °
13	3,78		RS-F	Mean w as determined by DLA, Result converted °

° Conversion p. 19

Methods:

AQ = AgraQuant, RomerLabs

IL = Immunolab

RS-F= Ridascreen® Fast, R-Biopharm

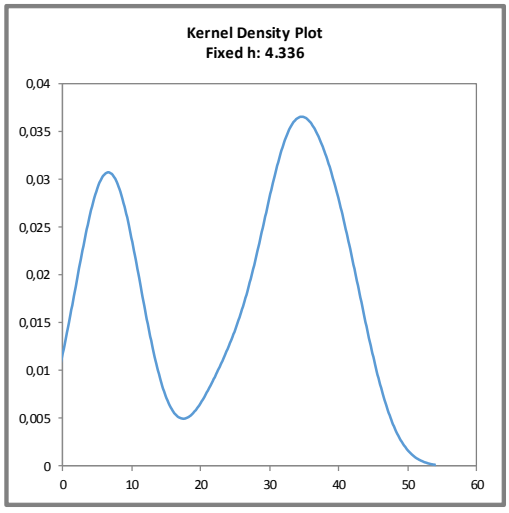


Abb. / Fig. 1:
Kerndichte-Schätzung aller ELISA-Ergebnisse (mit h = 0,5 x σpt von Xpt_{ALL})

Kernel density plot of all ELISA results (with h = 0,5 x σpt of Xpt_{ALL})

Comments:
The kernel density estimation shows two maxima: one at < 10 mg/kg (method RS-F) the other at > 30 mg/kg (methods AQ, IL, in part RS-F).

Characteristics: Quantitative evaluation Crustacea (Shrimps, dry)

Sample B

Statistic Data	**All Results*** *[mg/kg]*
Assigned value (X_{pt})	$X_{pt_{ALL}}$
Number of results	5
Number of outliers	0
Mean	33,3
Median	32,9
Robust Mean (X)	**33,3**
Robust standard deviation (S*)	**7,18**
Target range:	
Target standard deviation σ_{pt}	**8,32**
lower limit of target range	**16,6**
upper limit of target range	**49,9**
Quotient S/σ_{pt}*	*0,86*
Standard uncertainty $U_{(Xpt)}$	*4,02*
Quotient $U_{(Xpt)}/\sigma_{pt}$	*0,48*
Results in the target range	*5*
Percent in the target range	*100*

** Results without method RS-F (see comments)*

Methods:
RS-F = R-Biopharm, Ridascreen®FAST

Comments to the statistical characteristics and assigned values:

The kernel density plot shows a bimodal distribution of results (s Fig. 1). The side peak at < 10 mg/kg is due to 3 results of method RS-F. Thus the statistical evaluation was performed without the results of method RS-F, which gave in total inconsistent results.

The evaluation of all methods (without method RS-F) showed a normal variability of results. The quotient S/σ_{pt} was below 1,0. The robust standard deviation is in the range of established values for the reproducibility standard deviation of the applied methods (see 3.4.2 value by precision experiments and 3.4.3 value by perception). The comparability of results is given.*
This conclusion is limited for the evaluation across the methods, because there were only a few results for the methods.

The robust mean of the evaluation was 74% of the spiking level of Crustaceae to sample B and within the recommendations for the applied methods (s. 3.4.3).

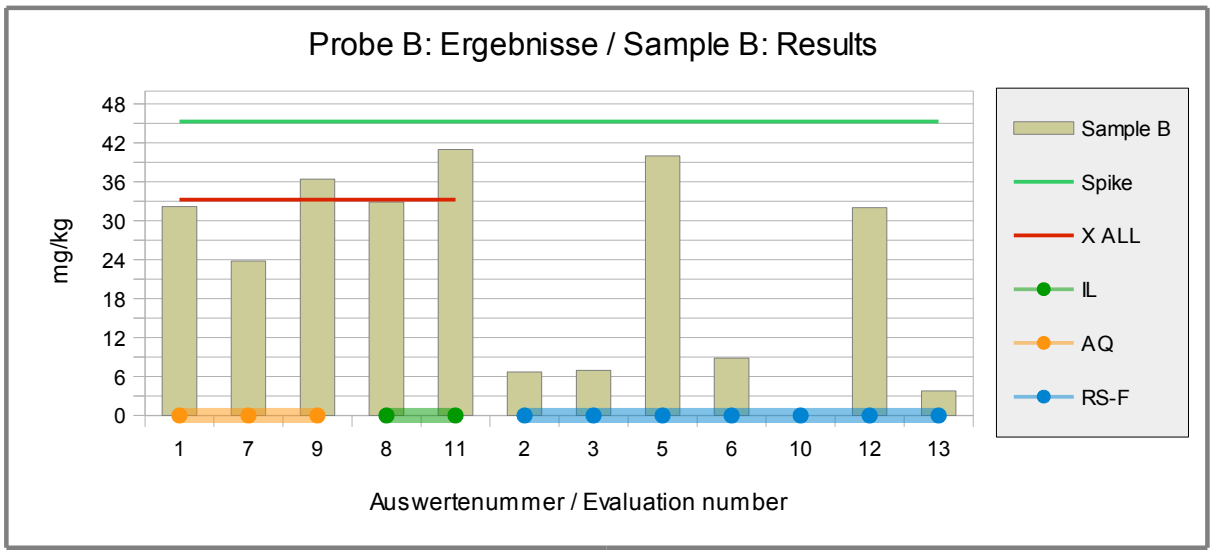


Abb./Fig. 2: ELISA Results Crustaceae
green line = Spiking level
red line = Assigned value robust mean all results (without RS-F)
round symbols = Applied methods (see legend)

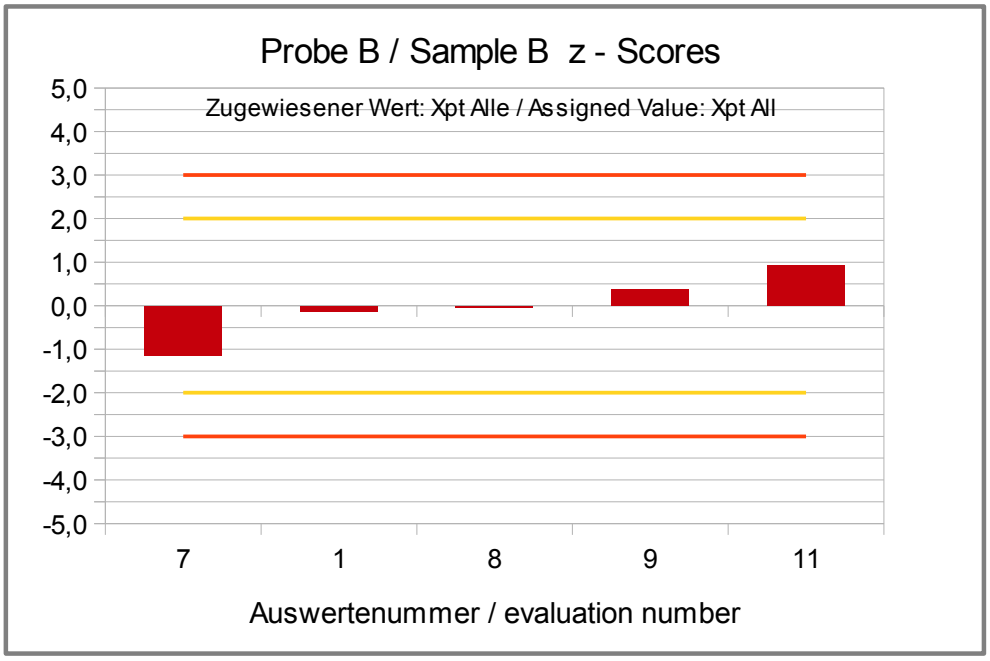


Abb./Fig. 3:
z-Scores (ELISA Results Crustaceae) Assigned value robust mean of all results (without method RS-F)

Recovery Rates for Crustaceae (Shrimps, dry): Spiking Material Sample and Sample B

Evaluation number	Spiking material	Recovery rate*	Sample B	Recovery rate*	Method	Remarks
	[mg/kg]	[%]	[mg/kg]	[%]		
1	7980	**85**	32,20	**71**	AQ	Result converted °
7	9840	**104**	23,80	**53**	AQ	Result converted °
9			36,40	**80**	AQ	Result converted °
8	10500	**111**	32,9	**73**	IL	
11	9660	**102**	41	**91**	IL	
2	7520	**80**	6,72	15	RS-F	Mean was determined by DLA, Result converted °
3	5690	**60**	6,95	15	RS-F	Result converted °
5	49900	528	40	**88**	RS-F	Result converted °
6			8,84	20	RS-F	Result converted °
10					RS-F	Result converted °
12	80,0	1	32,0	**71**	RS-F	Result converted °
13	1560	17	3,78	8	RS-F	Mean was determined by DLA, Result converted °

° Conversion p. 19

RA*	50-150 %	RA*	50-150 %
Number in RA	**6**	Number in RA	**7**
Percent in RA	**67**	Percent in RA	**64**

Methods:
AQ = AgraQuant, RomerLabs
IL = Immunolab
RS-F= Ridascreen® Fast, R-Biopharm

* Recovery rate 100% relative size: Crustaceae, s. Page 5

** Range of acceptance of AOAC for allergen ELISAS

Comments:
For the spiking material sample 6 (67%) participants obtained a recovery rate within the range of the AOAC-recommendation of 50-150% by PCR. For the food matrix sample B produced with the spiking material sample 64% (64%) participants obtained a recovery rate of in the range of acceptance.

4.1.2 PCR Results: Crustaceae (Shrimps, dry)

Qualitative valuation of results: Samples A and B

Evaluation number	Sample A	Sample A	Sample B	Sample B	Qualitative Valuation	Method	Remarks
	pos/neg	[mg/kg]	pos/neg	[mg/kg]	Agreement with consensus value		
2	negative	<0,4	positive	>0,4	2/2 (100%)	SFA-ID	
3	negative	<10	positive	51,1	2/2 (100%)	SFA-ID	
4	negative		positive		2/2 (100%)	SFA-ID	
14	positive	> 0,4	positive	> 0,4	1/2 (50%)	SFA-ID	

	Sample A		Sample B	
Number positive	1		4	
Number negative	3		0	
Percent positive	25		100	
Percent negative	75		0	
Consensus value	negative		positive	

Methods:
SFA-ID = Sure Food Allergen ID, R-Biopharm / Congen

Comments:
The consensus values are in qualitative agreement with the spiking of sample B. One positive result was obtained by method SFA-ID.

Quantitative valuation of results: Sample B

There were < 5 quantitative results, therefore no statistical evaluation was done.

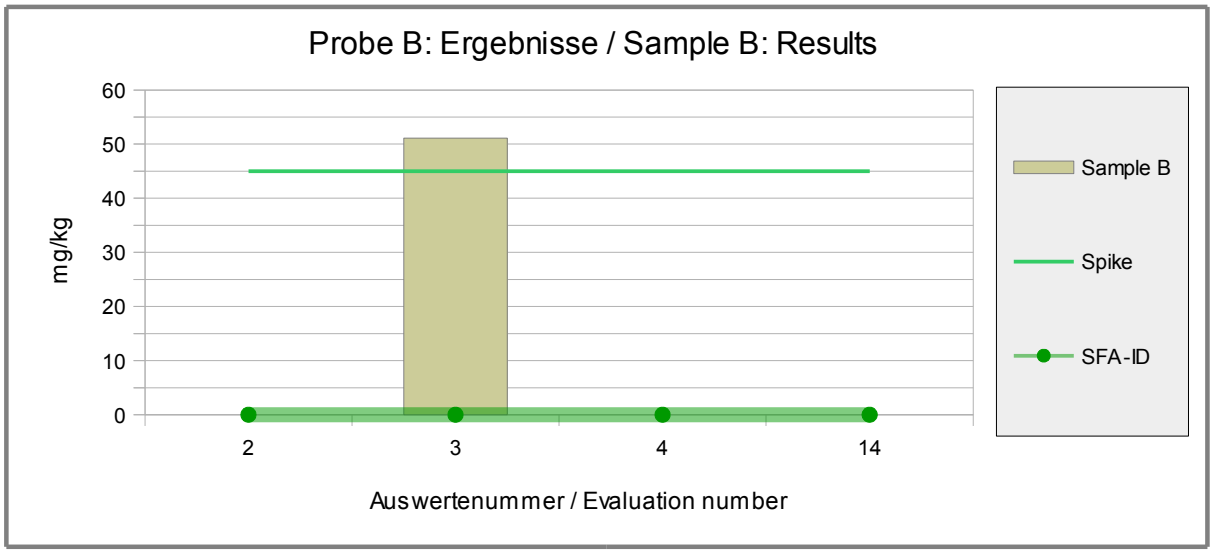


Abb./Fig. 4: PCR Results Crustaceae
green line = Spiking level
round symbols = Applied methods (see legend)

Recovery Rates for Crustaceae (Shrimps, dry): Spiking Material Sample and Sample B

Evaluation number	Spiking material	Recovery rate*	Sample B	Recovery rate*	Method	Remarks
	[mg/kg]	[%]	[mg/kg]	[%]		
2	>0,4		>0,4		SFA-ID	
3	3860	41	51,1	**114**	SFA-ID	
4					SFA-ID	
14	> 0,4		> 0,4		SFA-ID	

RA**	50-150 %	RA**	50-150 %
Number in RA	0	Anzahl im AB	1
Percent in RA	0	Prozent im AB	100

Methods:
SFA-ID = Sure Food Allergen ID, R-Biopharm / Congen

* Recovery rate 100% relative size: Crustaceae, s. Page 5

** Range of acceptance of AOAC for allergen ELISAS

Comments:
One participant quantified crustaceae by PCR. For the food matrix sample B produced with the spiking material sample the recovery rate was in the range of the AOAC-recommendation of 50-150%.

4.2 Proficiency Test Cashew

4.2.1 ELISA Results: Cashew

Qualitative valuation of results: Samples A and B

Evaluation number	Sample A	Sample A	Sample B	Sample B	Qualitative Valuation	Method	Remarks
	pos/neg	[mg/kg]	pos/neg	[mg/kg]	Agreement with consensus value		
7	negative		positive	83,6	2/2 (100%)	AQ	
8	negative	< 2	positive	90,0	2/2 (100%)	AQ	
3	negative	< 2	positive	55,4	2/2 (100%)	BC	
1	negative	< 5	positive	150	2/2 (100%)	ET	Result converted °
9	negative	< 5	positive	126	2/2 (100%)	ET	Result converted °
2	negative	< 0,2	positive	87,0	2/2 (100%)	IL	Mean w as determined by DLA
11	negative	< 1	positive	53,0	2/2 (100%)	IL	
13	negative	< LOD	positive	86,1	2/2 (100%)	RS-F	Mean w as determined by DLA

° Conversion p. 19

	Sample A		Sample B	
Number positive	0		8	
Number negative	8		0	
Percent positive	0		100	
Percent negative	100		0	
Consensus value	negative		positive	

Methods:

AQ = AgraQuant, RomerLabs
BC = BioCheck ELISA
ET = Elution Technologies ELISA Kit
IL = Immunolab
RS-F= Ridascreen® Fast, R-Biopharm

Comments:
The consensus values are in qualitative agreement with the spiking of sample B.

Quantitative valuation of results: Sample B

Evaluation number	Cashew	z-Score Xpt_{ALL}	Method	Remarks
	[mg/kg]			
7	83,6	-0,3	AQ	
8	90,0	0,0	AQ	
3	55,4	-1,6	BC	
1	150	2,6	ET	Result converted °
9	126	1,6	ET	Result converted °
2	87,0	-0,2	IL	Mean w as determined by DLA
11	53,0	-1,7	IL	
13	86,1	-0,2	RS-F	Mean w as determined by DLA

° Conversion p. 19

Methods:

AQ = AgraQuant, RomerLabs

BC = BioCheck ELISA

ET = Elution Technologies ELISA Kit

IL = Immunolab

RS-F= Ridascreen® Fast, R-Biopharm

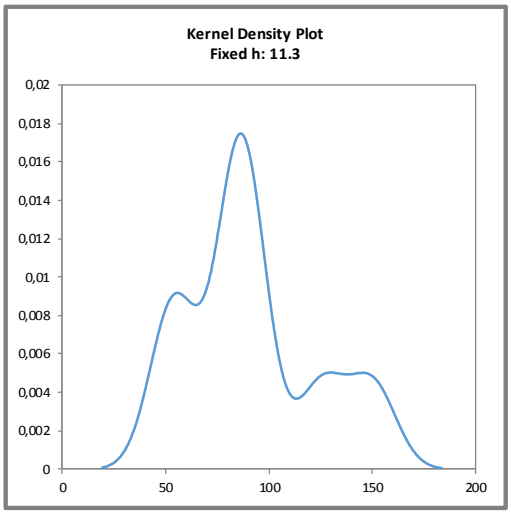


Abb. / Fig. 5:
Kerndichte-Schätzung aller ELISA-Ergebnisse (mit h = 0,5 x σpt von Xpt_{ALL})

Kernel density plot of all ELISA results (with h = 0,5 x σpt of Xpt_{ALL})

Comments:
The kernel density estimation shows in the middle nearly a normal distribution of results with a shoulder at <60 mg/kg and a side peak at >120 mg/kg (method ET).

Characteristics: Quantitative evaluation Cashew

Sample B

Statistic Data	**All Results** *[mg/kg]*
Assigned value (X_{pt})	$X_{pt_{ALL}}$
Number of results	8
Number of outliers	0
Mean	91,3
Median	86,5
Robust Mean (X)	**90,5**
Robust standard deviation (S*)	**35,1**
Target range:	
Target standard deviation σ_{pt}	**22,6**
lower limit of target range	**45,2**
upper limit of target range	**136**
Quotient S/σ_{pt}*	*1,6*
Standard uncertainty $U_{(Xpt)}$	*15,5*
Quotient $U_{(Xpt)}/\sigma_{pt}$	*0,69*
Results in the target range	*7*
Percent in the target range	*88*

Comments to the statistical characteristics and assigned values:

The comparability of results is formally given. This conclusion is limited for the evaluation across the methods, because there were only a few results for some methods and the kernel density estimation indicates method dependant differences (e.g. higher results method ET).

In total the evaluation of all methods shows a normal variability of results. The quotient S/σ_{pt} was below 2,0. The robust standard deviation is in the range of established values for the reproducibility standard deviation of the applied methods (see 3.4.2 value by precision experiments and 3.4.3 value by perception).*

The robust mean of the evaluation of all results was 180% of the spiking level of cashew to sample B and above the recommendations for the applied methods (s. 3.4.3 and "Recovery rates" p.35).

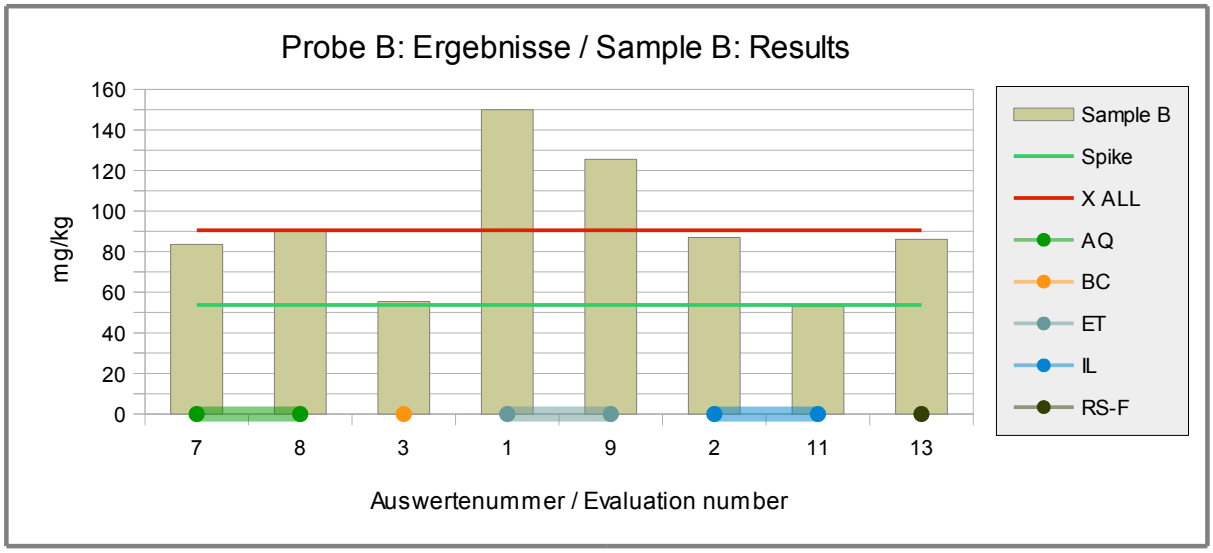


Abb./Fig. 6: ELISA Results Cashew
green line = Spiking level
red line = Assigned value robust mean all results
round symbols = Applied methods (see legend)

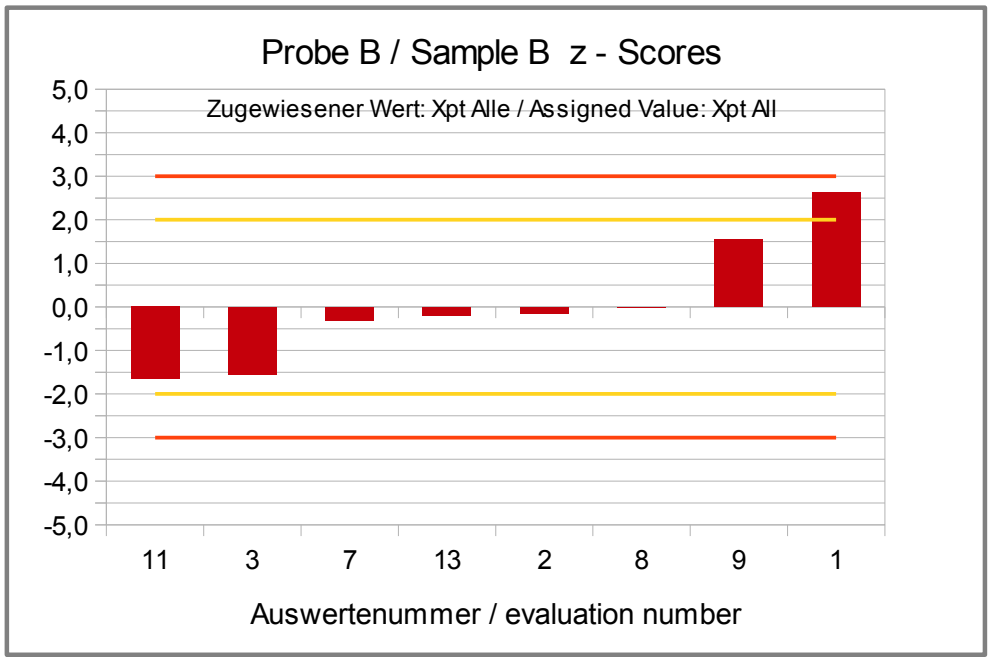


Abb./Fig. 7:
z-Scores (ELISA Results Cashew) Assigned value robust mean of all results

Recovery Rates for Cashew:
Spiking Material Sample and Sample B

Evaluation number	Spiking material	Recovery rate*	Sample B	Recovery rate*	Method	Remarks
	[mg/kg]	[%]	[mg/kg]	[%]		
7	18700	167	83,6	155	AQ	
8	17000	152	90,0	167	AQ	
3	16900	151	55,4	**103**	BC	
1	30600	273	150	279	ET	Result converted °
9			126	233	ET	Result converted °
2	28000	250	87,0	162	IL	Mean was determined by DLA
11	11200	**100**	53,0	**99**	IL	
13	21100	188	86,1	160	RS-F	Mean was determined by DLA

° Conversion p. 19

RA**	50-150 %	RA**	50-150 %
Number in RA	**1**	Number in RA	**2**
Percent in RA	**14**	Percent in RA	**25**

* Recovery rate 100% relative size: Cashew, s. Page 5

** Range of acceptance of AOAC for allergen ELISAS

Methods:
AQ = AgraQuant, RomerLabs
BC = BioCheck ELISA
ET = Elution Technologies ELISA Kit
IL = Immunolab
RS-F= Ridascreen® Fast, R-Biopharm

Comments:
For the spiking material sample one of the participants obtained a recovery rate within the range of the AOAC-recommendation of 50-150%. For the food matrix sample B produced with the spiking material sample two (25%) of the recovery rates were in the range of acceptance. All other results were between 151-279% and above 150%.

4.2.2 PCR Results: Cashew

Qualitative valuation of results: Samples A and B

Evaluation number	Sample A	Sample A	Sample B	Sample B	Qualitative Valuation	Method	Remarks
	pos/neg	[mg/kg]	pos/neg	[mg/kg]	Agreement with consensus value		
2	negative	<0,4	positive	>0,4	2/2 (100%)	SFA-ID	
14	negative	< 0,4	positive	> 0,4	2/2 (100%)	SFA-ID	
4	negative		positive		2/2 (100%)	div	
8	negative		positive		2/2 (100%)	div	

	Sample A		Sample B	
Number positive	0		4	
Number negative	4		0	
Percent positive	0		100	
Percent negative	100		0	
Consensus value	negative		positive	

Methods:
SFA-ID = Sure Food Allergen ID, R-Biopharm / Congen
div = not indicated / other method

Comments:
The consensus values are in qualitative agreement with the spiking of sample B.

Quantitative valuation of results: Sample B

There were no quantitative results, therefore no statistical evaluation was done.

Recovery Rates for Cashew: Spiking Material Sample and Sample B

Recovery rates could not be determined as no quantitative results were submitted.

4.2.3 Other Methods: Cashew

Qualitative valuation of results: Samples A and B

Evaluation number	Sample A	Sample A	Sample B	Sample B	Qualitative Valuation	Method	Remarks
	pos/neg	[mg/kg]	pos/neg	[mg/kg]	Agreement with consensus value		
12	negative		positive		2/2 (100%)	div	Lateral Flow

Methods:
div = not indicated / other method

Comments:
The results are in qualitative agreement with the spiking of sample B.

5. Documentation

5.1 Details by the participants

Note: Information given in German/French were translated by DLA to the best of our knowledge (without guarantee of correctness).

5.1.1 ELISA: Crustaceae

Meth. Abr.	Evaluation number	Date of analysis	Result Sample A		Result Sample B		Result Spiking Sample		quantitative Result given as	Method
		Day/Month	qualitative	mg/kg	qualitative	mg/kg	qualitative	mg/kg	e.g. food / food protein	Test-Kit + Manufacturer
AQ	1	29.12.16	negative	<0.1	positive	2,3	positive	570	Crustacea-Protein	AgraQuant ELISA Crustaceae (COKAL2248), RomerLabs
AQ	7	19.12.16	negative		positive	0,34	positive	140,55	Tropomyosin	AgraQuant ELISA Crustaceae (COKAL2248), RomerLabs
AQ	9	06.12.16	negative	<0.1	positive	2,6	na	na	Crustacea-Protein	AgraQuant ELISA Crustaceae (COKAL2248), RomerLabs
IL	8	24.11.	negative	<0,02 (<1,4)	positive	0,47 (32,9)	positive	150 (10500)	Tropomyosin from Crustaceae	Immunolab Crustaceans ELISA (CRU-E01)
IL	11	22.11.16	negative	< 1	positive	41	positive	9660	Crustaceae (Shrimp)	Immunolab Crustaceans ELISA (CRU-E01)
RS-F	2	29.12.16	negative	<2	positive	>20	positive	21000	Crustaceae	Ridascreen Fast Crustacean (R7302), r-Biopharm
RS-F	2	29.12.16	negative	<2	positive	21	positive	26000	Crustaceae	Ridascreen Fast Crustacean (R7302), r-Biopharm
RS-F	3	02.12.16	negative	<20	positive	21,72	positive	17769	Crustaceae	Ridascreen Fast Crustacean (R7302), r-Biopharm
RS-F	5	22.12.16	negative		positive	25	positive	31170	Crustacea-Protein	Ridascreen Fast Crustacean (R7312), r-Biopharm
RS-F	6	22.11.16	negative	<20	positive	27,61			Crustaceae	ridascreenfast crustacean R7312
RS-F	10	01.12.16	negative		positive		positive		Crustacea-Protein	Ridascreen Fast Crustacean (R7302), r-Biopharm
RS-F	12	23.11.16	negative	< 2	positive	100	positive	250	Crustaceae	Ridascreen Fast Crustacean (R7302), r-Biopharm
RS-F	13	21.11.16	-	< LOD	-	2,71	-	998,69	Protein	r-Biopharm AG Fast Crustacean R7312
RS-F	13	21.11.16	-	< LOD	-	2,04	-	1200,79	Protein	r-Biopharm AG Fast Crustacean R7312

continued ELISA Crustaceae:

Meth. Abr.	Evaluation number	Specifity	Remarks to the Method (Extraction and Determination)	Further Remarks
		Antibody	e.g. Extraction Solution / Time / Temperature	
AQ	1		Method carried out according to test kit insert	
AQ	7			The Crustacean test measures the level of Tropomyosin, a protein found in all common crustacean species. Tropomyosin constitutes approximately 20% of the total protein in cooked crustacean samples.
AQ	9	tropomyosin		
IL	8	Crustacean-Tropomyosin	as indicated by manufacturer (result is given as Crustaceae = Tropomyosin x 70 for Shrimps w et)	
IL	11	Tropomyosin	Results Tropomyosin: < 14 ppb; 580 ppb; 138748 ppb	
RS-F	2			
RS-F	2			
RS-F	3	As Per Kit Instructions	As Per Kit Instructions	
RS-F	5			
RS-F	6	Tropomyosin	1g Sample w as extracted for 10 min at 60°C in 20 mL Extractionbuffer	
RS-F	10			
RS-F	12	Crustacea-Protein	Allergenic Extractionbuffer r-biopharm/10 min/60°C	
RS-F	13	specific tow ards Crustaceanprotein (Tropomyosin)	Allergenic extractionbuffer 10 min 60°C	
RS-F	13	spezific tow ards Crustaceanprotein (Tropomyosin)	Allergenic extractionbuffer 10 min 60°C	

5.1.2 ELISA: Cashew

Meth. Abr.	Evaluation number	Date of analysis	Result Sample A		Result Sample B		Result Spiking Sample		quantitative Result given as	Method
		Day/Month	qualitative	mg/kg	qualitative	mg/kg	qualitative	mg/kg	e.g. food / food protein	Test-Kit + Manufacturer
AQ	7	22.12.16	negative		positive	83,6	positive	18686	Cashew	AgraQuant ELISA Cashew (COKAL3148), RomerLabs
AQ	8	1.12.	negative	<2	positive	90,0	positive	17000	Cashew	AgraQuant ELISA Cashew (COKAL3148), RomerLabs
BC	3	12.12.16	negative	<2	positive	55,4	positive	16898	Cashew	Biocheck Cashew
ET	1	23.12.16	negative	<0.9	positive	27,0	positive	5500	Cashew-Protein	Elution Technologies Cashew Protein Kit (E-75CSH)
ET	9	06.12.16	negative	<0.9	positive	22,6	na	na	Cashew-Protein	Elution Technologies Cashew Protein Kit (E-75CSH)
IL	2	29.12.16	negative	<0,2	positive	81,0	positive	28000	Cashew	Immunolab Cashew ELISA (CAW-E01)
IL	2	29.12.16	negative	<0,2	positive	93,0	positive	19000	Cashew	Immunolab Cashew ELISA (CAW-E01)
IL	11	22.11.16	negative	< 1	positive	53,0	positive	11200	Cashew	Immunolab Cashew ELISA (CAW-E01)
RS-F	13	21.11.16	-	< LOD	-	87,5	-	21107	Cashew	r-Biopharm AG Fast Cashew R6872
RS-F	13	21.11.16	-	< LOD	-	84,6	-	20705	Cashew	r-Biopharm AG Fast Cashew R6872

Meth. Abr.	Evaluation number	Specifity	Remarks to the Method (Extraction and Determination)	Further Remarks
		Antibody	e.g. Extraction Solution / Time / Temperature	
AQ	7			
AQ	8	Cashew -Protein	as indicated by manufacturer	
BC	3	As Per Kit Instructions	As Per Kit Instructions	Biocheck Cashew
ET	1		Method carried out according to test kit insert	
ET	9			
IL	2			
IL	2			
IL	11			
RS-F	13	spezific tow ards Cashew protein	Allergenic extractionbuffer 10 min 60°C	
RS-F	13	spezific tow ards Cashew protein	Allergenic extractionbuffer 10 min 60°C	

5.1.3 PCR: Crustaceae

Meth. Abr.	Evaluation number	Date of analysis	Result Sample A		Result Sample B		Result Spiking Sample		quantitative Result given as	Method
		Day/Month	qualitative	mg/kg	qualitative	mg/kg	qualitative	mg/kg	e.g. food / food protein	Test-Kit + Manufacturer
SFA-ID	2	29.12.16	negative	<0,4	positive	>0,4	positive	>0,4	Crustaceae-DNA	Sure Food Allergen ID, Congen / r-Biopharm
SFA-ID	2	29.12.16	negative	<0,4	positive	>0,4	positive	>0,4	Crustaceae-DNA	Sure Food Allergen ID, Congen / r-Biopharm
SFA-ID	3	08.12.16	negative	<10	positive	51,14	positive	3863	Crustaceae	Sure Food Allergen ID, Congen / r-Biopharm
SFA-ID	4	23.11.16	negative		positive		positive		Crustaceae-DNA	Sure Food Allergen ID, Congen / r-Biopharm
SFA-ID	4	05/Dec	negative		positive		positive		Crustaceae-DNA	Sure Food Allergen ID, Congen / r-Biopharm
SFA-ID	14	17.11.16	positive	> 0,4	positive	> 0,4	positive	> 0,4	Crustaceae	Sure Food Allergen ID, Congen / r-Biopharm

Meth. Abr.	Evaluation number	Specifity	Remarks to the Method (Extraction and Determination)	Further Remarks
		Target Sequence / DNA	e.g. Extraction / Enzymes / Clean-Up / Real Time PCR / Gel electrophoresis / Cycles	
SFA-ID	2			
SFA-ID	2			
SFA-ID	3	As Per Kit Instructions	As Per Kit Instructions	
SFA-ID	4	unknown	NucleoSpin Food (Macherey Nagel)/ Real Time PCR/ 35 cycles	
SFA-ID	4	unknown	NucleoSpin Food (Macherey Nagel)/ Real Time PCR/ 35 cycles	
SFA-ID	14	-	S3112 SureFood® ALLERGEN ID Crustaceans Detection limit 0,4 mg/kg Extraction w ith S1053 SureFood® PREP Advanced, Protocol 1	-

5.1.4 PCR: Cashew

Meth. Abr.	Evaluation number	Date of analysis	Result Sample A		Result Sample B		Result Spiking Sample		quantitative Result given as	Method
		Day/Month	qualitative	mg/kg	qualitative	mg/kg	qualitative	mg/kg	e.g. food / food protein	Test-Kit + Manufacturer
SFA-ID	2	29.12.16	negative	<0,4	positive	>0,4	positive	>0,4	Cashew-DNA	Sure Food Allergen ID, Congen / r-Biopharm
SFA-ID	2	29.12.16	negative	<0,4	positive	>0,4	positive	>0,4	Cashew-DNA	Sure Food Allergen ID, Congen / r-Biopharm
SFA-ID	14	17.11.16	negative	< 0,4	positive	> 0,4	positive	> 0,4	Cashew	Sure Food Allergen ID, Congen / r-Biopharm
div.	4	09/Dec	negative		positive		positive		Cashew-DNA	in-house method
div.	4	12/Dec	negative		positive		positive		Cashew-DNA	in-house method
div.	8	22.11.	negative		positive		positive		Cashew-DNA	Selection PCR-Methoden

Meth. Abr.	Evaluation number	Specifity	Remarks to the Method (Extraction and Determination)	Further Remarks
		Target Sequence / DNA	e.g. Extraction / Enzymes / Clean-Up / Real Time PCR / Gel electrophoresis / Cycles	
SFA-ID	2			
SFA-ID	2			
SFA-ID	14	-	S3115 SureFood® ALLERGEN ID Cashew Detection limit 0,4 mg/kg Extraction w ith S1053 SureFood® PREP Advanced, Protocol 1	-
div.	4	2s albumin	NucleoSpin Food (Macherey Nagel)/Real Time PCR/45 cycles	
div.	4	2s albumin	NucleoSpin Food (Macherey Nagel)/Real Time PCR/45 cycles	
div.	8		CTAB/Proteinase K/Promega Wizard DNA CleanUp/RealTimePCR/45	

5.1.5 Other Methods: Cashew

Meth. Abr.	Evaluation number	Date of analysis	Result Sample A		Result Sample B		Result Spiking Sample		quantitative Result gi-ven as	Method
		Day/Month	qualitative	mg/kg	qualitative	mg/kg	qualitative	mg/kg	e.g. food / food protein	Test-Kit + Manufacturer
div.	12	23.11.16	negative		positive		positive		Cashew	Lateral Flow Cashew Kern

Meth. Abr.	Evaluation number	Specifity	Remarks to the Method (Extraction and Determination)	Further Remarks
		Antibody	e.g. Extraction / Enzymes / Clean-Up / Real Time PCR / Gel electrophoresis / Cycles	
div.	12	Cashew-Protein	Allergen Extraktionspuffer r-biopharm/10 min/60°C	

5.2 Homogeneity

5.2.1 Mixture homogeneity before bottling

Microtracer Homogeneity Test
DLA 07-2016 Spiking material sample

Weight whole sample	1,01	kg
Microtracer	FSS-rot lake	
Particle size	75 – 300	µm
Weight per particle	2,0	µg
Addition of tracer	13,6	mg/kg

Result of analysis

Sample	Weight [g]	Particle number	Particles [mg/kg]
1	5,14	45	17,5
2	5,10	49	19,2
3	5,10	43	16,9
4	5,08	37	14,6
5	5,05	45	17,8
6	5,22	38	14,6
7	5,12	36	14,1
8	5,07	44	17,4

Poisson distribution		
Number of samples	8	
Degree of freedom	7	
Mean	42,1	Particles
Standard deviation	4,78	Particles
χ^2 (CHI-Quadrat)	3,79	
Probability	**80**	%
Recovery rate	121	%

Normal distribution		
Number of samples	8	
Mean	16,5	mg/kg
Standard deviation	1,87	mg/kg
rel. Standard deviaton	11,3	%
Horwitz standard deviation	10,5	%
HorRat-value	**1,1**	
Recovery rate	121	%

Microtracer Homogeneity Test
DLA 07-2016 Sample B

Weight whole sample	3,17	kg
Microtracer	FSS-rot lake	
Particle size	75 – 300	µm
Weight per particle	2,0	µg
Addition of tracer	11,6	mg/kg

Result of analysis

Sample	Weight [g]	Particle number	Particles [mg/kg]
1	5,02	24	9,6
2	5,06	26	10,3
3	5,16	29	11,2
4	5,00	26	10,4
5	5,15	29	11,3
6	5,03	28	11,1
7	5,15	24	9,3
8	5,24	25	9,5

Poisson distribution		
Number of samples	8	
Degree of freedom	7	
Mean	26,4	Particles
Standard deviation	2,06	Particles
χ^2 (CHI-Quadrat)	1,13	
Probability	**99**	%
Recovery rate	89	%

Normal distribution		
Number of samples	8	
Mean	10,3	mg/kg
Standard deviation	0,81	mg/kg
rel. Standard deviaton	7,82	%
Horwitz standard deviation	11,3	%
HorRat-value	**0,69**	
Recovery rate	89	%

6. Index of participant laboratories

Teilnehmer / Participant	Ort / Town	Land / Country
		SPAIN
		SPAIN
		CANADA
		CANADA
		Germany
		Germany
		ITALY
		Germany
		Germany
		ITALY
		GREAT BRITAIN
		FRANCE
		Germany
		GREAT BRITAIN

[Die Adressdaten der Teilnehmer wurden für die allgemeine Veröffentlichung des Auswerte-Berichts nicht angegeben.]

[The address data of the participants were deleted for publication of the evaluation report.]

7. Index of references

1. DIN EN ISO/IEC 17025:2005; Allgemeine Anforderungen an die Kompetenz von Prüf- und Kalibrierlaboratorien / General requirements for the competence of testing and calibration laboratories
2. DIN EN ISO/IEC 17043:2010; Konformitätsbewertung – Allgemeine Anforderungen an Eignungsprüfungen / Conformity assessment – General requirements for proficiency testing
3. ISO 13528:2015 & DIN ISO 13528:2009; Statistische Verfahren für Eignungsprüfungen durch Ringversuche / Statistical methods for use in proficiency testing by interlaboratory comparisons
4. ASU §64 LFGB: Planung und statistische Auswertung von Ringversuchen zur Methodenvalidierung / DIN ISO 5725 series part 1, 2 and 6 Accuracy (trueness and precision) of measurement methods and results
5. Verordnung / Regulation 882/2004/EU; Verordnung über über amtliche Kontrollen zur Überprüfung der Einhaltung des Lebensmittel- und Futtermittelrechts sowie der Bestimmungen über Tiergesundheit und Tierschutz / Regulation on official controls performed to ensure the verification of compliance with feed and food law, animal health and animal welfare rules
6. Evaluation of analytical methods used for regulation of food and drugs; W. Horwitz; Analytical Chemistry, 54, 67-76 (1982)
7. The International Harmonised Protocol for the Proficiency Testing of Ananlytical Laboratories ; J.AOAC Int., 76(4), 926 – 940 (1993)
8. A Horwitz-like funktion describes precision in proficiency test; M. Thompson, P.J. Lowthian; Analyst, 120, 271-272 (1995)
9. Protocol for the design, conduct and interpretation of method performance studies; W. Horwitz; Pure & Applied Chemistry, 67, 331-343 (1995)
10. Recent trends in inter-laboratory precision at ppb and sub-ppb concentrations in relation to fitness for purpose criteria in proficiency testing; M. Thompson; Analyst, 125, 385-386 (2000)
11. The International Harmonised Protocol for the Proficiency Testing of Analytical Chemistry Laboratories; Pure Appl Chem, 78, 145 – 196 (2006)
12. AMC Kernel Density - Representing data distributions with kernel density estimates, amc technical brief, Editor M Thompson, Analytical Methods Committee, AMCTB No 4, Revised March 2006 and Excel Add-in Kernel.xla 1.0e by Royal Society of Chemistry
13. EURACHEM/CITAC Leitfaden, Ermittlung der Messunsicherheit bei analytischen Messungen (2003); Quantifying Uncertainty in Analytical Measurement (1999)
14. GMP+ Feed Certification scheme, Module: Feed Safety Assurance, chapter 5.7 Checking procedure for the process accuracy of compound feed with micro tracers in GMP+ BA2 Control of residues, Version: 1st of January 2015 GMP+ International B.V.
15. MTSE SOP No. 010.01 (2014): Quantitative measurement of mixing uniformity and carry-over in powder mixtures with the rotary detector technique, MTSE Micro Tracers Services Europe GmbH
16. Codex Alimentarius Commission (2010) - Guidelines on performance criteria and validation of methods for detection, identification and quantification of specific DNA sequences and specific protiens in foods, CAC/GL 74-2010
17. DIN EN ISO 15633-1:2009; Nachweis von Lebensmittelallergenen mit immunologischen Verfahren - Teil 1: Allgemeine Betrachtungen / Foodstuffs - Detection of food allergens by immunological methods - Part 1: General considerations
18. DIN EN ISO 15634-1:2009; Nachweis von Lebensmittelallergenen mit molekularbiologischen Verfahren - Teil 1: Allgemeine Betrachtungen / Foodstuffs - Detection of food allergens by molecular biological methods - Part 1: General considerations
19. DIN EN ISO 15842:2010 Lebensmittel – Nachweis von Lebensmittelallergenen – Allgemeine Betrachtungen und Validierung von Verfahren / Foodstuffs - Detection of food allergens - General considerations and validation of

methods
20. Ministry of Health and Welfare, JSM, Japan 2006
21. Working Group Food Allergens, Abbott et al., Validation Procedures for Quantitative Food Allergen ELISA Methods: Community Guidance and Best Practices JAOAC Int. 93:442-50 (2010)
22. Working Group on Prolamin Analysis and Toxicity (WGPAT): Méndez et al. Report of a collaborative trial to investigate the performance of the R5 enzyme linked immunoassay to determine gliadin in gluten-free food. Eur J Gastroenterol Hepatol. 17:1053-63 (2005)
23. DLA Publikation: Performance of ELISA and PCR methods for the determination of allergens in food: an evaluation of six years of proficiency testing for soy (Glycine max L.) and wheat gluten (Triticum aestivum L.); Scharf et al.; J Agric Food Chem. 61(43):10261-72 (2013)
24. EFSA (2014) Scientific Opinion on the evaluation of allergenic foods and food ingredients for labelling purposes1, EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA), European Food Safety Authority (EFSA), Parma, Italy, EFSA Journal 2014;12(11):3894
25. IRMM, Poms et al.; Inter-laboratory validation study of five different commercial ELISA test kits for determination of peanut residues in cookie and dark chocolate; European Commission, Joint Research Centre, Belgium; GE/R/FSQ/D08/05/2004
26. Jayasena et al. (2015) Comparison of six commercial ELISA kits for their specificity and sensitivity in detecting different major peanut allergens. J Agric Food Chem. 2015 Feb 18;63(6):1849-55
27. ASU §64 LFGB L 06.00-56 Bestimmung von Sojaprotein in Fleisch und Fleischerzeugnissen Enzymimmunologisches Verfahren (2007)
28. ASU §64 LFGB L 00.00-69 Bestimmung von Erdnuss-Kontaminationen in Lebensmitteln mittels ELISA im Mikrotiterplattensystem (2003)
29. ASU §64 LFGB L 44.00-7 Bestimmung von Haselnuss-Kontaminationen in Schokolade und Schokoladenwaren mittels ELISA im Mikrotiterplattensystem (2006)
30. ASU §64 LFGB L 18.00-19 Untersuchung von Lebenmitteln - Nachweis und Bestimmung von Sesam (Sesamum indicum) in Reis- und Weizenkeksen sowie in Soßenpulver mittels real-time PCR (2014)
31. ASU §64 LFGB L 18.00-20 Untersuchung von Lebenmitteln - Nachweis und Bestimmung von Mandel (Prunus dulcis) in Reis- und Weizenkeksen sowie in Soßenpulver mittels real-time PCR (2014)
32. ASU §64 LFGB L 18.00-22 Untersuchung von Lebenmitteln - Simultaner Nachweis und Bestimmung von Lupine, Mandel, Paranuss und Sesam in Reis- und Weizenkeksen sowie Soßenpulver mittels real-time PCR (2014)