Proficiency Tests
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Evaluation Report
proficiency test

DLA 08/2018

Allergens VIII:

Almond and Cashew

in Veggie Burger Powder

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

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Unteraufträge *Subcontractors*	Falls im Rahmen der Eignungsprüfung eine Prüfung der Gehalte, Homogenität und Stabilität von EP-Parametern durchgeführt wurde, hat DLA diese im Unterauftrag vergeben. In case the analysis of the content, homogeneity and stability of PT-parameters was part of the proficiency test, the determinations were subcontracted by DLA.
Vertraulichkeit *Confidentiality*	Die Teilnehmerergebnisse sind im EP-Bericht in anonymisierter Form mit Auswertenummern benannt. Daten einzelner Teilnehmer werden ausschließlich nach vorheriger Zustimmung des Teilnehmers an Dritte weitergegeben. Participant result are named anonymously with evaluation numbers in the PT report. Data of individual participants will be passed on to third parties only with prior consent of the participant.

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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 with the same food matrix were provided for the detection and quantitative determination of the allergens in the range of mg/kg as well as one spiking level sample with a simple matrix. One of the samples (spiked sample) and the spiking level sample contain the respective allergenic ingredients in a similar concentration range. The results of the spiking level 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 of the food matrix samples is a common in commerce veggie burger mixture (powder). The basic composition of samples A and B was the same (see table 1). After crushing and sieving (mesh 1,5 mm) the basic mixture was homogenized.
Afterwards the **spiked samples B** were produced as follows:
The spiking materials containing the allergenic ingredients almond and cashew were milled and homogenized with ball mills. Afterwards added to an aliquot of the basic mixture and the mixture was homogenized. Subsequently, the basic mixture was again added in up to 3 additional steps and homogenized in each case until the total quantity had been reached.

For the **spiking level sample,** the allergenic compounds above mentioned were added during a multi-stage addition of potato powder and homogenization.

The samples A and B were portioned to approximately 25 g, the spiking levels sample to approximately to 15 g in metallized PET film bags.

Table 1: Composition of DLA-Samples

Ingredients	**Sample A**	**Sample B**	**Spiking Level Sample**
Veggie burger powder Ingredients: Oat wholemeal flakes, vegetables (carrots, parsnips, celery, leek, fried onions, parsley root), breadcrumbs (wheat flour, salt, yeast), buckwheat groats, wholemeal semolina, wholemeal flour, wheat bran, sea salt, parsley, spices (onions, marjoram, celery leaves, garlic, pepper), yeast flakes Nutrients per 100 g: Fat 4,6 g, Carbohydrates 55 g, Fiber 14 g, Protein 13 g, Salt 2,9 g	100 g/100 g	99,6 g/100g	-
Potato Powder Ingredients: Potatoes, E471, E304, E223, E100	-	-	99,8 g/100 g
Almond butter, white - as Almond* - thereof 16% total protein**	-	 23,4 mg/kg 3,79 mg/kg	 24,1 mg/kg 3,91 mg/kg
Cashew: - as Cashew* - thereof 16% total protein**	-	 33,0 mg/kg 5,15 mg/kg	 32,3 mg/kg 5,05 mg/kg
further Ingredients: *Maltodextrin, sodium sulfate and silica dioxide*	-	<0,5 g/100 g	<0,5 g/100 g

* 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 with F=5,18 for almond protein and with F=5,30 for cashew protein)

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 A and the spiking level sample showed a probability of 55% and 90%. Additionally particle number results were converted into concentrations, statistically evaluated according to normal distribution and compared to the standard deviation according to Horwitz. For the assessment HorRat values between 0,3 and 1,3 are to be accepted under repeat conditions (measurements within the laboratory) [17].
This gave a HorRat value of 1,5 and 0,8 respectively. Aufgrund der ausreichenden Wahrscheinlichkeit wurde der HorRat-Wert für Probe B akzeptiert. The HorRat value of sample B was accepted because of the sufficient probability and results of homgeneity testing by ELISA (see below). 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 13528:2015 Annex B (possibly with Notes 1 and 2).

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 almond (Immunolab, AgraQuant and Veratox) and cashew (Immunolab and AgraQuant), respectively (see page 7). Recommendations for repeatability standard deviations of ELISA and PCR methods are usually ≤ 25% [18, 19, 22, 23].

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].

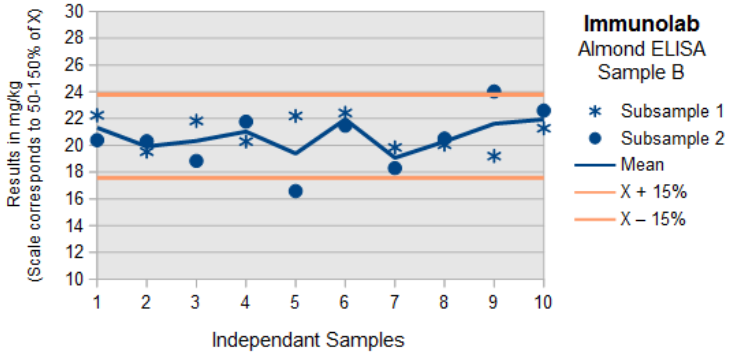
ELISA-Tests: Homogenität Mandel / Homogeneity Almond

Immunolab Almond ELISA

Sample weights: 1,0 g (0,9 – 1,1 g)
Number of replicates: 2
Overall result: Almond 20,7 ± 0,4 mg/kg

Sample B	Subsample 1 mg/kg	Subsample 2 mg/kg	Mean mg/kg
1	22,2	20,4	21,3
2	19,5	20,3	19,9
3	21,8	18,8	20,3
4	20,3	21,8	21,0
5	22,2	16,6	19,4
6	22,4	21,5	21,9
7	19,8	18,3	19,1
8	20,0	20,5	20,3
9	19,2	24,0	21,6
10	21,3	22,6	21,9

General average X	20,7	
SD of sample means Sx	1,04	5,0%
SD within-samples Sw	1,37	6,6%
SD between-samples Ss	0,37	1,8%

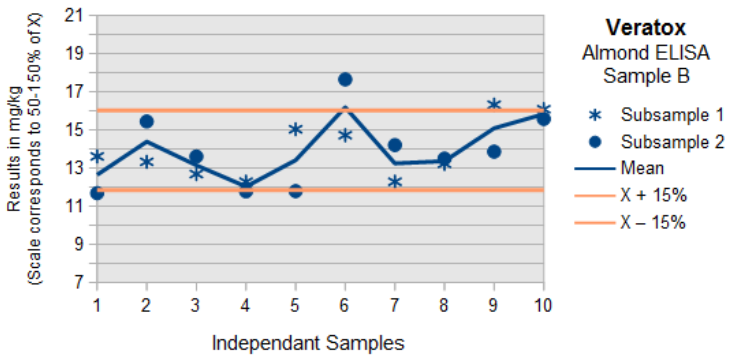


Neogen Veratox ELISA Almond

Sample weights: 5,0 g (4,5 – 5,5 g)
Number of replicates: 2
Overall result: Almond 13,9 ± 1,2 mg/kg

Sample B	Subsample 1 mg/kg	Subsample 2 mg/kg	Mean mg/kg
1	13,6	11,7	12,6
2	13,3	15,4	14,4
3	12,7	13,6	13,1
4	12,3	11,8	12,0
5	15,0	11,8	13,4
6	14,7	17,6	16,2
7	12,3	14,2	13,2
8	13,2	13,5	13,4
9	16,3	13,9	15,1
10	16,1	15,6	15,8

General average X	13,9	
SD of sample means Sx	1,38	9,9%
SD within-samples Sw	0,98	7,0%
SD between-samples Ss	1,19	8,6%

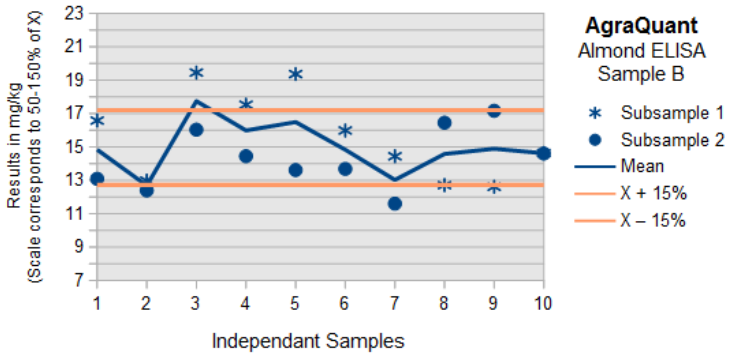


Romerlabs AgraQuant Almond

Sample weights: 1,0 g (0,9 – 1,1 g)
Number of replicates: 2
Overall result: Almond 15,0 ± 0,9 mg/kg

Sample B	Subsample 1 mg/kg	Subsample 2 mg/kg	Mean mg/kg
1	16,6	13,1	14,8
2	13,0	12,4	12,7
3	19,5	16,0	17,7
4	17,5	14,4	16,0
5	19,4	13,6	16,5
6	16,0	13,7	14,8
7	14,4	11,6	13,0
8	12,7	16,4	14,6
9	12,6	17,2	14,9
10	14,6	14,6	14,6

General average X	15,0	
SD of sample means Sx	1,50	10,1%
SD within-samples Sw	1,69	11,3%
SD between-samples Ss	0,91	6,1%



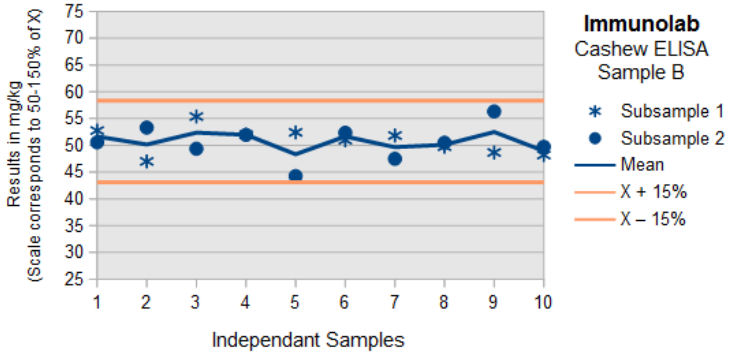
ELISA-Tests: Homogenität Cashew / Homogeneity Cashew

Immunolab Cashew ELISA

Sample weights: 1,0 g (0,9 – 1,1 g)
Number of replicates: 2
Overall result: Cashew 50,7 ± 1,5 mg/kg

Sample B	Subsample 1 mg/kg	Subsample 2 mg/kg	Mean mg/kg
1	52,8	50,6	51,7
2	47,0	53,3	50,1
3	55,4	49,4	52,4
4	52,0	51,9	52,0
5	52,4	44,3	48,3
6	51,0	52,3	51,6
7	51,8	47,5	49,6
8	49,7	50,5	50,1
9	48,7	56,3	52,5
10	48,2	49,7	48,9

General average X	50,7	
SD of sample means Sx	1,48	2,9%
SD within-samples Sw	2,40	4,7%
SD between-samples Ss	1,48	2,9%

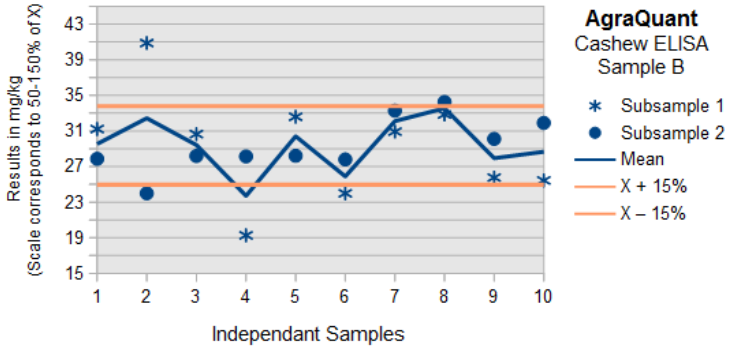


Romerlabs AgraQuant Cashew

Sample weights: 1,0 g (0,9 – 1,1 g)
Number of replicates: 2
Overall result: Cashew 29,4 ± 1,8 mg/kg

Sample B	Subsample 1 mg/kg	Subsample 2 mg/kg	Mean mg/kg
1	31,2	27,9	29,5
2	40,9	24,0	32,4
3	30,6	28,2	29,4
4	19,3	28,1	23,7
5	32,6	28,2	30,4
6	24,0	27,8	25,9
7	30,9	33,3	32,1
8	32,8	34,2	33,5
9	25,8	30,1	27,9
10	25,4	31,9	28,7

General average X	29,4	
SD of sample means Sx	3,02	10,3%
SD within-samples Sw	3,47	11,8%
SD between-samples Ss	1,76	6,0%



2.1.2 Stability

A water activity (a_W) of < 0,5 is an important factor to ensure the stability of dry or dried products during storage. Optimum conditions for storage is the a_W value range of 0,15 - 0,3. In this range the lowest possible degradation rate is to be expected [16].

The experience with various DLA test materials showed good storage stability with respect to the durability of the sample (spoilage) and the content of the PT parameters for comparable food matrices and water activity (a_W value <0,5).
The a_W value of the EP samples was approx. 0,37 (21,3°C) The stability of the sample material was thus ensured during the investigation period under the specified storage conditions.

2.2 Sample shipment and information to the test

The portions of test materials sample A, B and the spiking level sample were sent to every participating laboratory in the 49th week of 2018. The testing method was optional. The tests should be finished at 28th November 2018 the latest.

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

There are two different samples A and B possibly containing the allergenic parameters Almond and Cashew in the range of mg/kg in the matrix of Veggie Burger Powder. One of these samples and the "spiking level sample" were prepared adding the allergenic ingredients. The ***"spiking level sample"*** *contains the allergens in a simple matrix in* ***similar amounts*** *without further processing.*

Please note the attached information on the proficiency test.
(see documentation, section 5.3 Information on the PT)

2.3 Submission of results

The participants submitted their results in standard forms, which have been handed out with the samples (by email).
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.
Queried and documented were the indicated results and details of the test methods like specificity, 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.

Out of 14 participants, 13 participants submitted their results in time. One participant submitted results delayed.

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 [25, 26, 27, 28]. 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 level 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]. If there are < 12 quantitative results and an increased difference between robust mean and median, the **median** may be used as the assigned value (criterion: Δ median - rob. mean > 0,3 σ_{pt}) [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) ***Assigned value of all results - $X_{pt_{ALL}}$***
ii) ***Assigned value 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, too few significant digits (valid digits) or results for another proficiency test item can be removed from the data set [2]. Even if a result e.g. with a factor >10 deviates significantly from the mean and has an influence on the robust statistics, a result of the statistical evaluation can be excluded [3].
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 tested for outliers by the use of robust statistics (algorithm A): If a value deviates from the robust mean by more than 3 times the robust standard deviation, it can be classified as an outlier (see above) [3]. Due to the use of robust statistics outliers are not excluded, provided that no other reasons are present [3]. Detected outliers are only mentioned in the results section, if they have been excluded from the statistical evaluation.

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 or PCR-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]:

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

The relative repeatability standard deviations (RSD_r) and relative reproducibility standard deviations (RSD_R) given in table 2a (ELISA) and table 2b (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 2a: 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} [30-31]

Parameter	**Matrix**	**Mean [mg/kg]**	**Recovery**	**rob RSD**	**RSD_r**	**RSD_R**	**σpt**	**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 12 – 33% for the ELISA methods and 24 – 42% for the PCR methods depending on the matrix, processing and concentration level of allergens (s. Tab. 2a and 2b).

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 [24]. 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 [24].

The IRMM (Institute for Reference Materials and Measurements) performed an interlaboratory comparison for five different ELISA test kits for the quantification of peanut [27]. 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 2b: 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} [32-34]

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 multiplex 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 multiplex ASU 18.00-22
Brazil Nut	Rice cookie	89,1 17,3 9,8	89,1 % 86,5 % 98 %	-	34,1% 36,2% 40,2%	34,4% 38,2% 41,8%	24,5% 28,4% 30,6%	rt-PCR ASU 18.00-21
Brazil Nut	Wheat cookie Sauce powder	80,8 42,6	65,7 % 42,6 %	-	25,6% 27,5%	36,4% 39,7%	31,6% 34,6%	rt-PCR ASU 18.00-21
Brazil Nut	Rice cookie	96,6 14,2	96,6 % 71 %	-	16,8% 54,2%	31,8% 56,5%	29,5% 41,5%	rt-PCR multiplex ASU 18.00-22
Brazil Nut	Wheat cookie Sauce powder	76,5 48,4	62,2 % 48,4 %	-	15,6% 34,4%	35,8% 37,5%	34,1% 28,5%	rt-PCR multiplex 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 [22], by the working group 12 „Food Allergens" of the technical committee CEN/TC 275 [19-21], by an international "Food Allergen Working Group" under the advice of the AOAC Presidential Task Force on Food Allergens [23] and by the Codex Alimentarius Committee (CAC/GL 74-2010) [18].

Some of the relevant ELISA and PCR validation criteria of the mentioned panels are listed in tables 3 and 4, respectively.

Table 3: ELISA-Validation

Literature [18-24]	**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 4: PCR-Validation

Literature [18]	**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 or if necessary 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.

An error or cause analysis can be carried out by checking the analysis process including understanding and implementation of the measurement by the staff, details of the measurement procedure, calibration of equipment and composition of reagents, transmission or calculation errors, trueness and precision and use of reference material. 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_i) of the participant from the respective consensus value (X) to the square root of quadrat sum of the target standard deviation (σ_{pt}) 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 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 ($U_{(Xpt)}$) for this PT is calculated as follows [3]:

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

If $U_{(Xpt)} \leq 0{,}3\ \sigma_{pt}$ 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 traceability of the assigned value is ensured on the basis of the consensus value as a robust mean of the participant results.

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 level 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 [23]. For quantitative PCR or LC/MS 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 parameter are reported for samples A and B (qualitative / possibly quantitative) and afterwards for the spiking level sample (quantitative). The recovery rates of results for the spiking level sample and the spiked sample A or B are reported then.
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.

One ELISA result, expressed as cashew protein, has been converted to total food item (cashew) using the analysed protein content of the raw material (see page 5).
In the present PT all other results were expressed as cashew and almond, so no further conversion was necessary.

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})	$X_{pt_{ALL}}$	$X_{pt_{METHOD\ i}}$
Number of results		
Number of outliers		
Mean		
Median		
Robust mean (X_{pt})		
Robust standard deviation (S*)		
Target data°:		
Target standard deviation σ_{pt} *or* σ_{pt}'		
lower limit of target range ($X_{pt} - 2\sigma_{pt}$) or ($X_{pt} - 2\sigma_{pt}'$)°		
upper limit of target range ($X_{pt} + 2\sigma_{pt}$) or ($X_{pt} + 2\sigma_{pt}'$)°		
Quotient S*/σ_{pt} *or* S*/σ_{pt}'		
Standard uncertainty $U_{(X_{pt})}$		
Number of results in target range		
Percent in target range		

° Target range calculated using z-score or z'-score

After that the recovery rates of the results for the spiking level 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 Almond

4.1.1 ELISA Results: Almond

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		
13	negative	0	positive	20,0	2/2 (100%)	BF	
10a	negative	< 0.2	positive	21,0	2/2 (100%)	EF	
12	negative	<0,2	positive	25,2	2/2 (100%)	IL	
1	negative	<2,5	positive	23,3	2/2 (100%)	RS-F	
2	negative	<2,5	positive	20,0	2/2 (100%)	RS-F	
4	negative		positive	22,0	2/2 (100%)	RS-F	
5	negative	<2,5	positive	16,0	2/2 (100%)	RS-F	
8	negative	< BG	positive	24,0	2/2 (100%)	RS-F	
9	negative	< 2.5	positive	24,0	2/2 (100%)	RS-F	
10b	negative	< 1.5	positive	17,0	2/2 (100%)	RS-F	
14	negative	<2.5	positive	28,0	2/2 (100%)	RS-F	
3	negative	<2.5	positive	17,3	2/2 (100%)	VT	
6	negative		positive	12,3	2/2 (100%)	VT	

	Sample A		Sample B	
Number positive	0		13	
Number negative	13		0	
Percent positive	0		100	
Percent negative	100		0	
Consensus value	negative		positive	

Methods:

BF = MonoTrace ELISA, BioFront Technologies
EF = SensiSpec ELISA Kit, Eurofins
IL = Immunolab
RS-F= Ridascreen® Fast, R-Biopharm
VT = Veratox, Neogen

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

Quantitative valuation of ELISA-results: Sample B

Evaluation number	Almond	z-Score Xpt_{ALL}	z-Score $Xpt_{RS\text{-}F}$	Method	Remarks
	[mg/kg]				
13	20,0	-0,2		BF	
10a	21,0	0,0		EF	
12	25,2	0,8		IL	
1	23,3	0,5	0,3	RS-F	
2	20,0	-0,2	-0,3	RS-F	
4	22,0	0,2	0,0	RS-F	
5	16,0	-0,9	-1,1	RS-F	
8	24,0	0,6	0,4	RS-F	
9	24,0	0,6	0,4	RS-F	
10b	17,0	-0,7	-0,9	RS-F	
14	28,0	1,4	1,1	RS-F	
3	17,3	-0,7		VT	
6	12,3	-1,6		VT	

Methods:

BF = MonoTrace ELISA, BioFront Technologies
EF = SensiSpec ELISA Kit, Eurofins
IL = Immunolab
RS-F= Ridascreen® Fast, R-Biopharm
VT = Veratox, Neogen

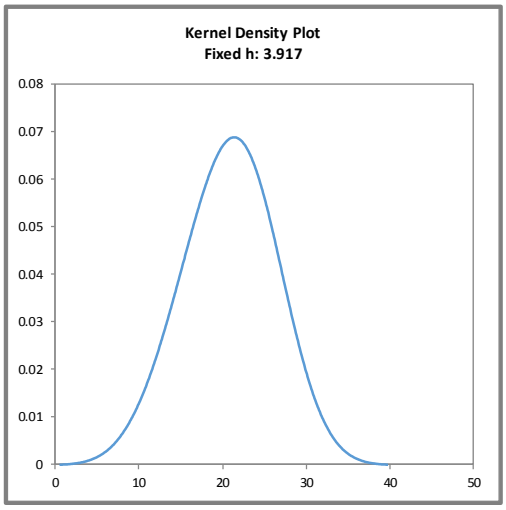


Abb. / Fig. 1:
Kerndichte-Schätzung aller ELISA-Ergebnisse (mit h = 0,75 x σpt von Xpt_{ALL})

Kernel density plot of all ELISA results (with h = 0,75 x σpt of Xpt_{ALL})

Comments:
The kernel density estimation shows nearly a symmetrical distribution of results.

Characteristics: Quantitative evaluation ELISA Almond

Sample B

Statistic Data	**All Results** *[mg/kg]*	**Method RS-F** *[mg/kg]*
Assigned value (X_{pt})	$X_{pt_{ALL}}$	$X_{pt_{METHOD\ RS\text{-}F}}$
Number of results	13	8
Number of outliers	0	0
Mean	20,8	21,8
Median	21,0	22,7
Robust Mean (X)	**20,9**	**21,8**
Robust standard deviation (S*)	**4,51**	**4,50**
Target range:		
Target standard deviation σ_{pt}	**5,22**	**5,45**
lower limit of target range	**10,4**	**10,9**
upper limit of target range	**31,3**	**32,7**
Quotient S/σ_{pt}*	*0,86*	*0,83*
Standard uncertainty $U_{(Xpt)}$	*1,57*	*1,99*
Results in the target range	*13*	*8*
Percent in the target range	*100*	*100*

Method:
RS-F = R-Biopharm, Ridascreen® Fast

Comments to the statistical characteristics and assigned values:

The kernel density estimation showed almost a symmetrical distribution of results.

The evaluation of the results of all methods and the evaluation of results from method RS-F showed a low variability of results, respectively. The quotients S/σ_{pt} were well below 1,0. The robust standard deviations are 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 some methods.*

The robust means of the evaluation of all results and method RS-F were 89% and 93% of the spiking level of almond to sample B and within the range of the recommendations for the applied methods (s. 3.4.3 and "Recovery rates of Almond" p.30).

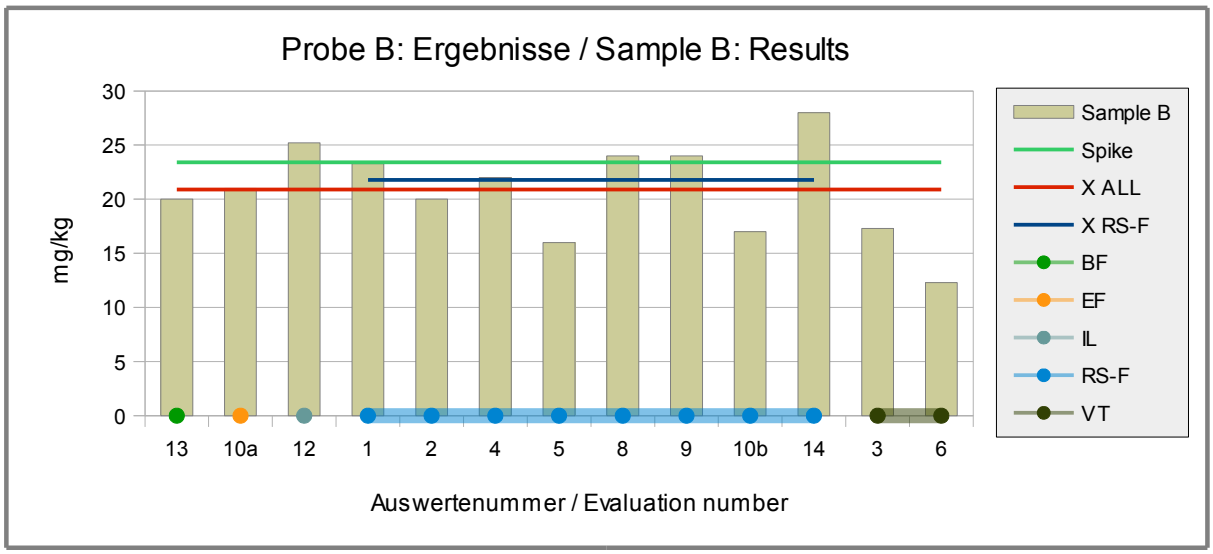


Abb./Fig. 2: ELISA Results Almond
green line = Spiking level
red line = Assigned value robust mean all results
blue line = Assigned value robust mean results method RS-F
round symbols = Applied methods (see legend)

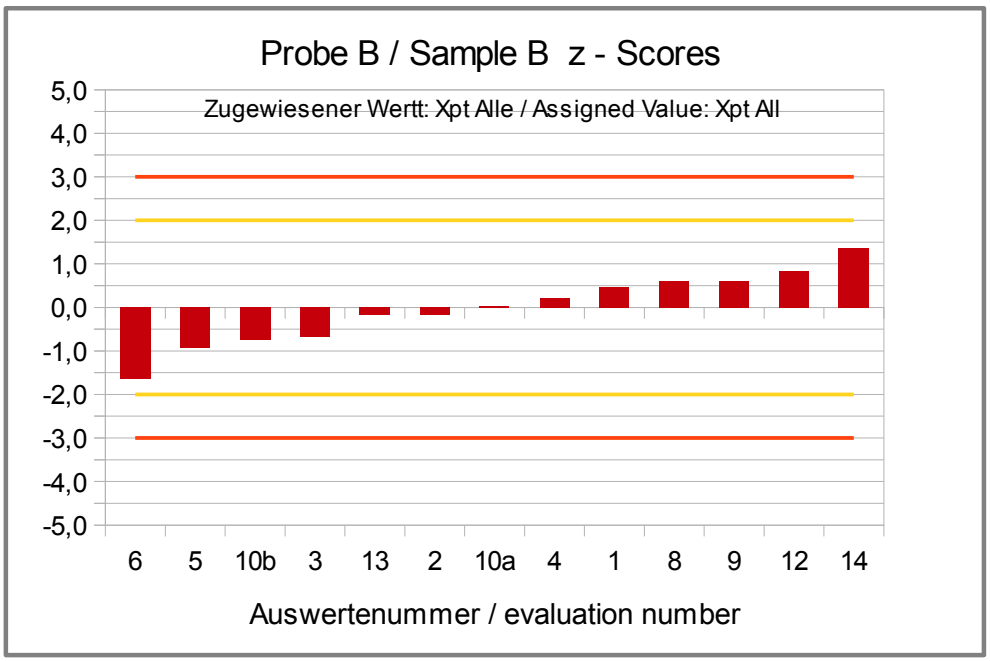


Abb./Fig. 3:
z-Scores (ELISA Results Almond)
Assigned value robust mean of all results

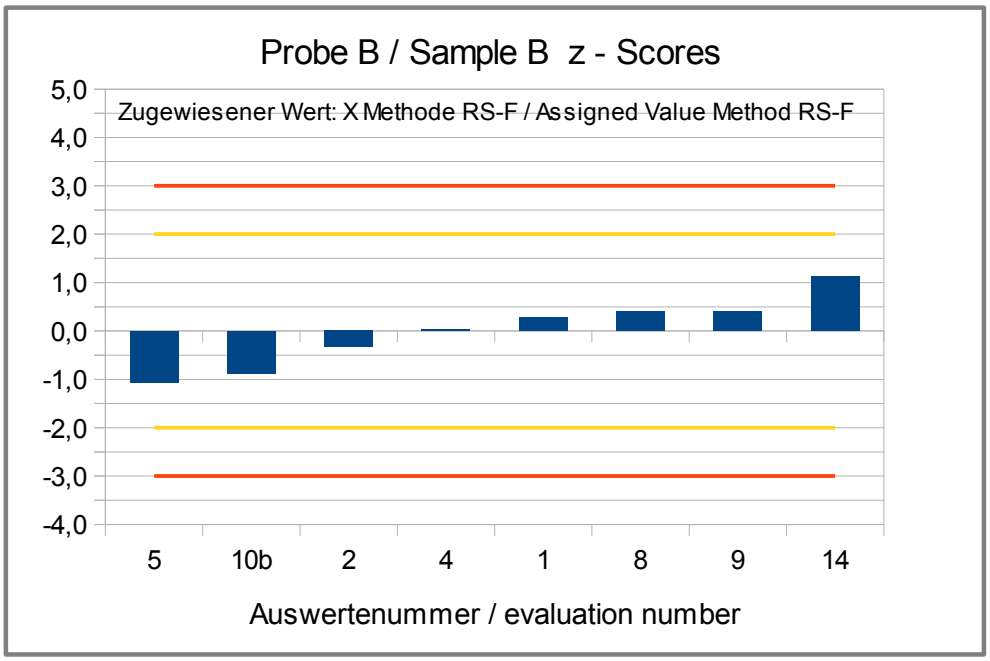


Abb./Fig. 4:
z-Scores (ELISA Results Almond)
Assigned value robust mean of method RS-F (R-Biopharm, Ridascreen® Fast)

Quantitative valuation of results: Spiking level sample

Evaluation number	Almond	z-Score Xpt_{ALL}	z-Score Xpt_{RS-F}	Method	Remarks
	[mg/kg]				
13	22,1	-0,5		BF	
10a	28,0	0,5		EF	
12	25,3	0,0		IL	
1	93,5	10,9	10,6	RS-F	
2	20,0	-0,8	-0,9	RS-F	
4	23,0	-0,3	-0,4	RS-F	
5	16,0	-1,5	-1,5	RS-F	
8	27,0	0,3	0,2	RS-F	
9	27,0	0,3	0,2	RS-F	
10b	22,0	-0,5	-0,6	RS-F	
14	32,9	1,2	1,1	RS-F	
3	25,2	0,0		VT	
6	24,0	-0,2		VT	

Methods:

BF = MonoTrace ELISA, BioFront Technologies
EF = SensiSpec ELISA Kit, Eurofins
IL = Immunolab
RS-F= Ridascreen® Fast, R-Biopharm
VT = Veratox, Neogen

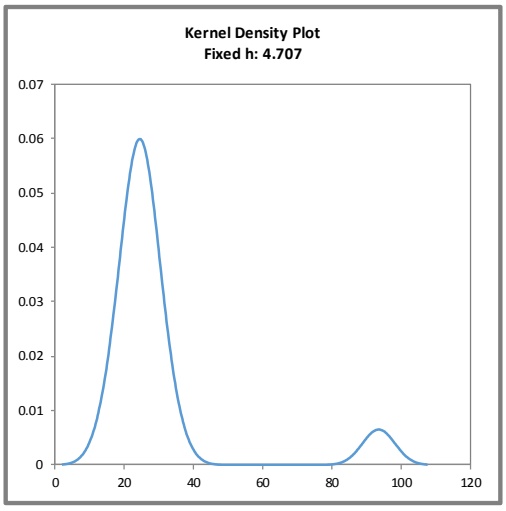


Abb. / Fig. 5:
Kerndichte-Schätzung aller ELISA-Ergebnisse (mit h = 0,75 x σpt von Xpt_{ALL})

Kernel density plot of all ELISA results (with h = 0,75 x σpt of Xpt_{ALL})

Comments:
The kernel density estimation shows nearly a symmetrical distribution of results with a smaller peak at approx. 95 mg/kg due to a single result (method RS-F).

Characteristics: Quantitative evaluation ELISA Almond

Spiking level sample

Statistic Data	**All Results** *[mg/kg]*	**Method RS-F** *[mg/kg]*
Assigned value (X_{pt})	$X_{pt_{ALL}}$	$X_{pt_{METHOD\ RS-F}}$
Number of results	13	8
Number of outliers	-	-
Mean	29,7	32,7
Median	25,2	25,0
Robust Mean (X)	**25,1**	**25,7**
Robust standard deviation (S*)	**5,00**	**7,95**
Target range:		
Target standard deviation σ_{pt}	**6,28**	**6,42**
lower limit of target range	**12,6**	**12,8**
upper limit of target range	**37,7**	**38,5**
Quotient S/σ_{pt}*	*0,80*	*1,2*
Standard uncertainty $U_{(Xpt)}$	*1,73*	*3,51*
Results in the target range	*12*	*7*
Percent in the target range	*92*	*88*

Method:
RS-F = R-Biopharm, Ridascreen® Fast

Comments to the statistical characteristics and assigned values:

The kernel density estimation showed no clear method-dependent differences (one high single value).

The evaluation of all methods and the evaluation of results from method RS-F showed a low to normal variability of results, respectively. The quotients S/σ_{pt} were below 2,0. The robust standard deviations are 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 some methods.*

The robust means of the evaluation of all results and method RS-F were 104% and 107% of the spiking level of almond to the spiking level sample and within the range of the recommendations for the applied methods (s. 3.4.3 and "Recovery rates of Almond" p.30).

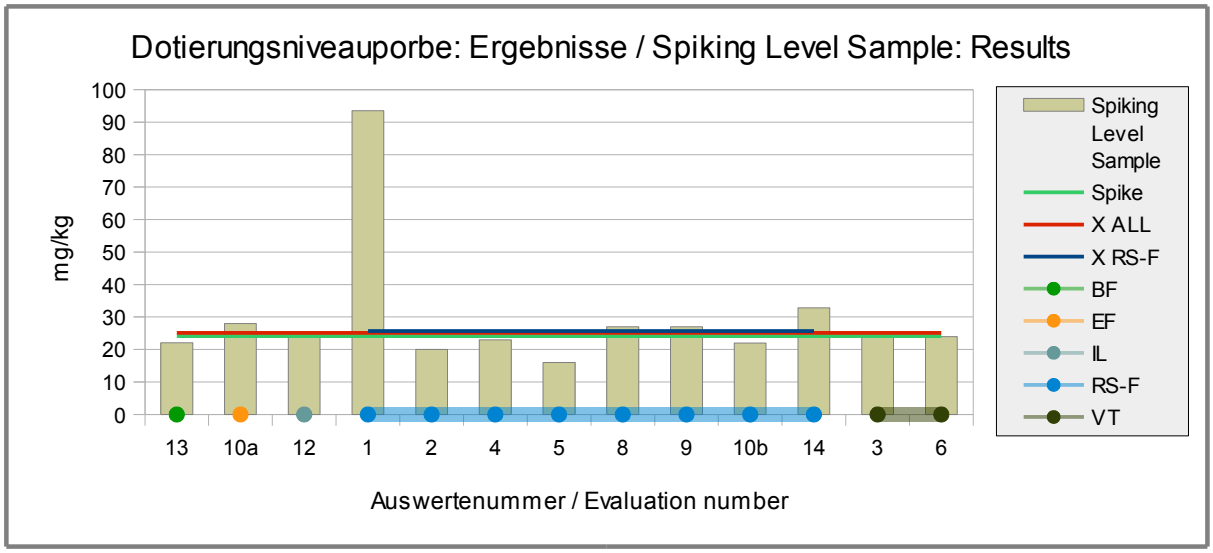


Abb./Fig. 6: ELISA Results Almond
green line = Spiking level
red line = Assigned value robust mean all results
blue line = Assigned value robust mean results method RS-F
round symbols = Applied methods (see legend)

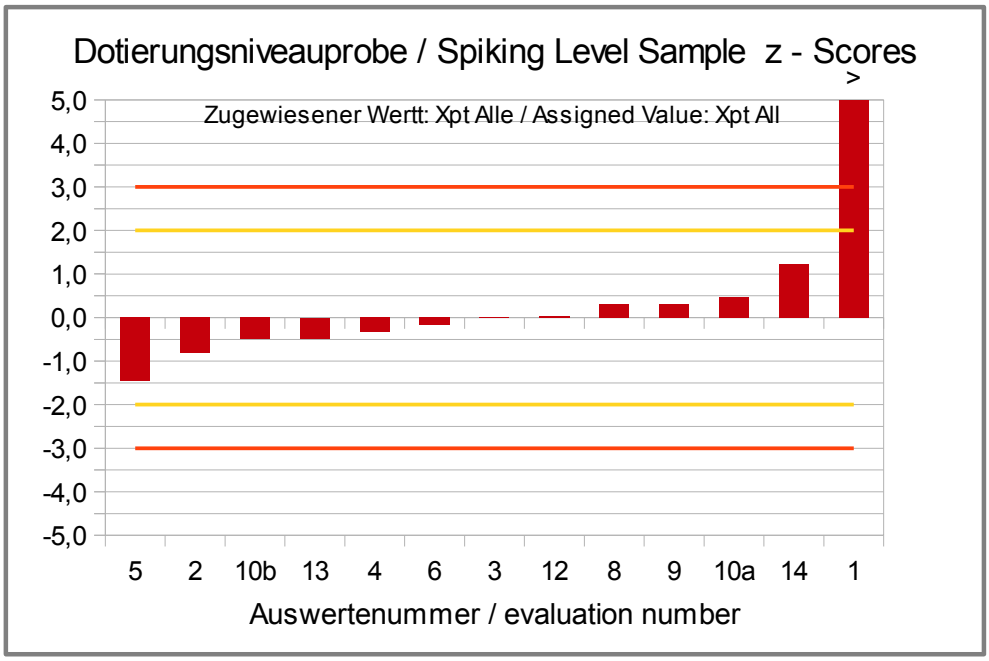


Abb./Fig. 7:
z-Scores (ELISA Results Almond)
Assigned value robust mean of all results

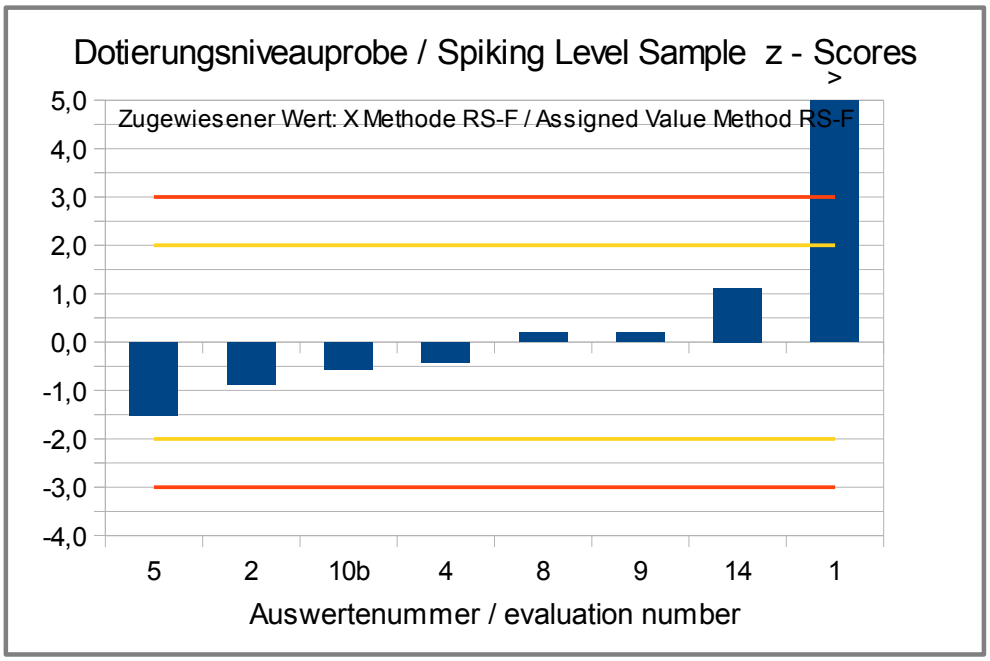


Abb./Fig. 8:
z-Scores (ELISA Results Almond)
Assigned value robust mean of method RS-F (R-Biopharm, Ridascreen® Fast)

Recovery Rates ELISA for Almond:
Spiking Level Sample and Sample B

Evaluation number	Spiking Level Sample	Recovery rate*	Sample B	Recovery rate*	Method	Remarks
	[mg/kg]	[%]	[mg/kg]	[%]		
13	22,1	**92**	20,0	**85**	BF	
10a	28,0	**116**	21,0	**90**	EF	
12	25,3	**105**	25,2	**108**	IL	
1	93,5	388	23,3	**100**	RS-F	
2	20,0	**83**	20,0	**85**	RS-F	
4	23,0	**95**	22,0	**94**	RS-F	
5	16,0	**66**	16,0	**68**	RS-F	
8	27,0	**112**	24,0	**103**	RS-F	
9	27,0	**112**	24,0	**103**	RS-F	
10b	22,0	**91**	17,0	**73**	RS-F	
14	32,9	**136**	28,0	**120**	RS-F	
3	25,2	**105**	17,3	**74**	VT	
6	24,0	**100**	12,3	**53**	VT	

RA**	50-150 %	RA**	50-150 %
Number in RA	12	Number in RA	13
Percent in RA	92	Percent in RA	100

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

** Range of acceptance of AOAC for allergen ELISAS

Methods:
BF = MonoTrace ELISA, BioFront Technologies
EF = SensiSpec ELISA Kit, Eurofins
IL = Immunolab
RS-F= Ridascreen® Fast, R-Biopharm
VT = Veratox, Neogen

Comments:
With one exception all participants obtained for the spiking level sample (92%, 12) and for the spiked processed food matrix sample B (100%, 13) recovery rates within the range of the AOAC-recommendation of 50-150%.

4.1.2 PCR Results: Almond

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	18	1/1 (100%)	ASU	
8	negative		positive		1/1 (100%)	SFA	
10	negative		positive		1/1 (100%)	SFA	
9	negative	< 0.01%	negative	< 0.01%	1/1 (100%)*	div	* no positive sample identified
11	negative		negative		1/1 (100%)*	div	* no positive sample identified

	Probe A		Probe B	
Number positive	0		3	
Number negative	5		2	
Percent positive	0		60	
Percent negative	100		40	
Consensus value	negative		none	

Methoden:
ASU = ASU §64 Methode/method
SFA = Sure Food ALLERGEN, R-Biopharm / Congen
div = keine genaue Angabe / andere Methode
div = not indicated / other method

Comments:
For Sample A, a consensus value of 100% negative results was obtained. For the spiked sample B no consensus value of ≥ 75% could be determined. In qualitative agreement with the spiking 3 positive results for sample B were obtained, as well as 2 negative results with unspecified PCR-methods.

Quantitative Valuation PCR: Sample B

No quantitative evaluation was done, because there were too few individual results.

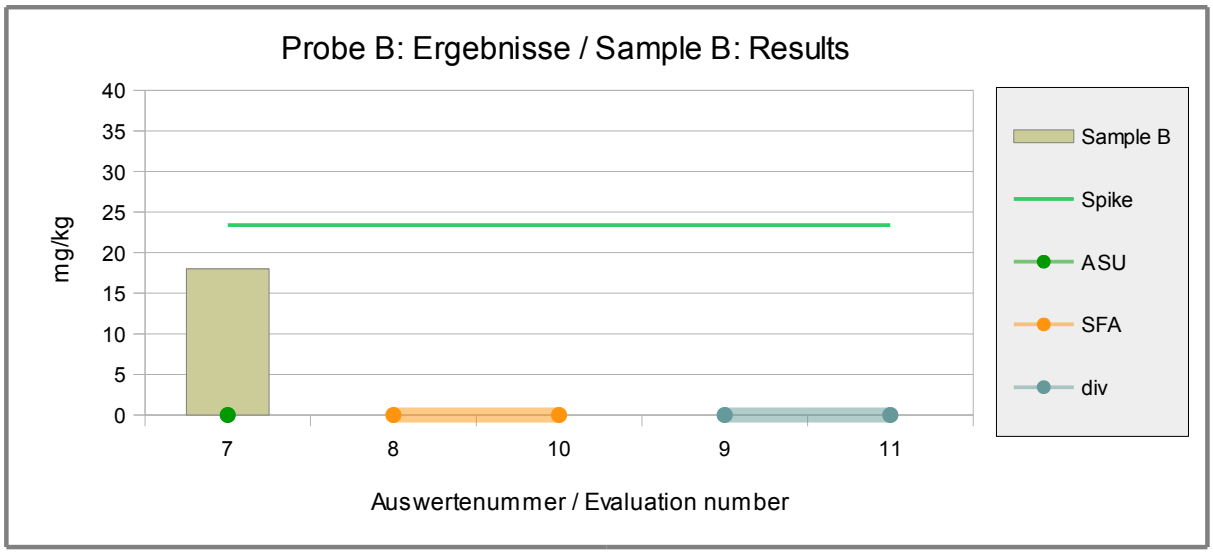


Abb./Fig. 9: PCR Results Almond
green line = Spiking level
round symbols = Applied methods (see legend)

(Quantitative) Valuation PCR: Spiking Level Sample

No quantitative evaluation was done, because there were to few quantitative results.

Evaluation number	Almond	Almond	z-Score Xpt_{ALL}	Method	Remarks
	pos/neg	[mg/kg]			
7	positive	26		ASU	
8	positive			SFA	
10	positive			SFA	
9	positive			div	
11	positive			div	

Number positive	5	
Number negative	0	
Percent positive	100	
Percent negative	0	
Consensus value	positive	

Methods:
ASU = ASU §64 Methode/method
SFA = Sure Food ALLERGEN, R-Biopharm / Congen
div = keine genaue Angabe / andere Methode
div = not indicated / other method

Comments:
For the spiking level sample there were 100% positive results.

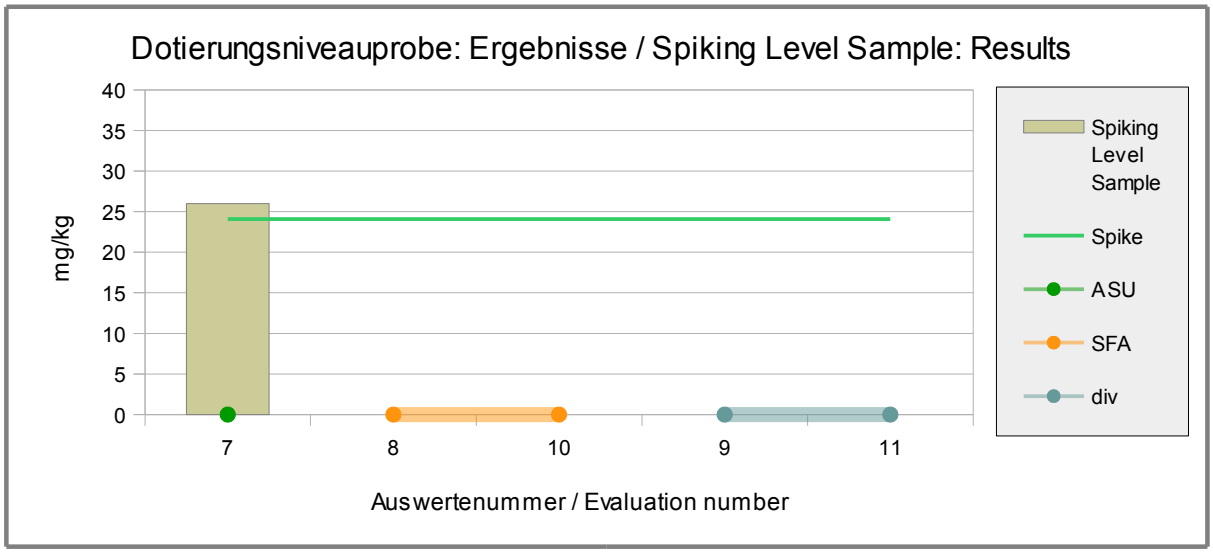


Abb./Fig. 10: PCR Results Almond
green line = Spiking level
round symbols = Applied methods (see legend)

Recovery Rates PCR for almond:
Spiking Level Sample and Sample B

Evaluation number	Spiking Level Sample	Recovery rate*	Sample B	Recovery rate*	Method	Remarks
	[mg/kg]	[%]	[mg/kg]	[%]		
7	26	**108**	18	**77**	ASU	
8					SFA	
10					SFA	
9			< 0.01%		div	
11					div	

RA**	50-150 %	RA**	50-150 %
Number in RA	**1**	Number in RA	**1**
Percent in RA	**100**	Percent in RA	**100**

Methods:
ASU = ASU §64 Methode/method
SFA = Sure Food ALLERGEN, R-Biopharm / Congen
div = keine genaue Angabe / andere Methode
div = not indicated / other method

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

** Range of acceptance of AOAC for allergen ELISAS

Comments:
One participant submitted quantitative results by PCR and obtained for the spiking level sample and the spiked food matrix sample B recovery rates within 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		
6	negative		positive	101	2/2 (100%)	3M	Result converted °
14	negative	<2	positive	46,4	2/2 (100%)	BC	
3	negative	<2.0	positive	32,7	2/2 (100%)	BF	
5	negative	<2	positive	32,0	2/2 (100%)	BF	
13	negative	0	positive	52,6	2/2 (100%)	BF	
2	negative	<2	positive	80,0	2/2 (100%)	EF	
10	negative	< 0.2	positive	50,0	2/2 (100%)	EF	
12	negative	<0,2	positive	49,3	2/2 (100%)	IL	
7	negative		positive	47,0	2/2 (100%)	RS-F	
8	negative	< BG	positive	41,0	2/2 (100%)	RS-F	
9	negative	< 2.5	positive	57,0	2/2 (100%)	RS-F	

° calculation see p. 19

	Sample A		Sample B	
Number positive	0		11	
Number negative	11		0	
Percent positive	0		100	
Percent negative	100		0	
Consensus value	negative		positive	

Methods:

3M = 3M Protein ELISA Kit
BF = MonoTrace ELISA, BioFront Technologies
EF = SensiSpec ELISA Kit, Eurofins
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 ELISA-results: Sample B

Evaluation number	Cashew	z-Score Xpt_{ALL}	Method	Remarks
	[mg/kg]			
6	101	4,0	3M	Result converted °
14	46,4	-0,3	BC	
3	32,7	-1,4	BF	
5	32,0	-1,5	BF	
13	52,6	0,2	BF	
2	80,0	2,3	EF	
10	50,0	0,0	EF	
12	49,3	-0,1	IL	
7	47,0	-0,3	RS-F	
8	41,0	-0,8	RS-F	
9	57,0	0,5	RS-F	

° calculation see p. 19

Methods:

3M = 3M Protein ELISA Kit

BF = MonoTrace ELISA, BioFront Technologies

EF = SensiSpec ELISA Kit, Eurofins

IL = Immunolab

RS-F= Ridascreen® Fast, R-Biopharm

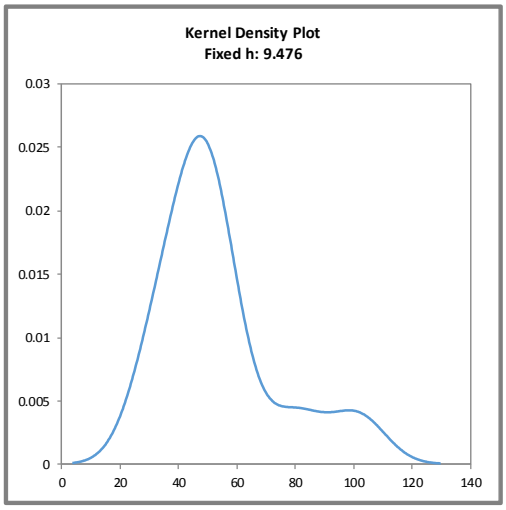


Abb. / Fig. 11:
Kerndichte-Schätzung aller ELISA-Ergebnisse (mit h = 0,75 x σpt von Xpt_{ALL})

Kernel density plot of all ELISA results (with h = 0,75 x σpt of Xpt_{ALL})

Comments:
The kernel density estimation shows nearly a symmetrical distribution of results with a second broader peak at approx. >80 mg/kg due to two single results of method 3M and EF (Fig. 11).

Characteristics: Quantitative evaluation ELISA Cashew

Sample B

Statistic Data	**All Results** *[mg/kg]*
Assigned value (X_{pt})	$X_{pt_{ALL}}$
Number of results	11
Number of outliers	-
Mean	53,6
Median	49,3
Robust Mean (X)	50,5
Robust standard deviation (S*)	**15,7**
Target range:	
Target standard deviation σ_{pt}	**12,6**
lower limit of target range	**25,3**
upper limit of target range	**75,8**
Quotient S/σ_{pt}*	*1,2*
Standard uncertainty $U_{(Xpt)}$	*5,93*
Results in the target range	*9*
Percent in the target range	*82*

Comments to the statistical characteristics and assigned values:

The kernel density estimation showed no clear method-dependent differences.

The evaluation of all methods showed a normal variability of results, respectively. The quotient S/σ_{pt} was below 2,0. The robust standard deviations are 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 some methods.*

The robust mean of the evaluation of all results was 153% of the spiking level of cashew to the spiking level sample and just above the range of the recommendations for the applied methods (s. 3.4.3 and "Recovery rates of Cashew" p.42).

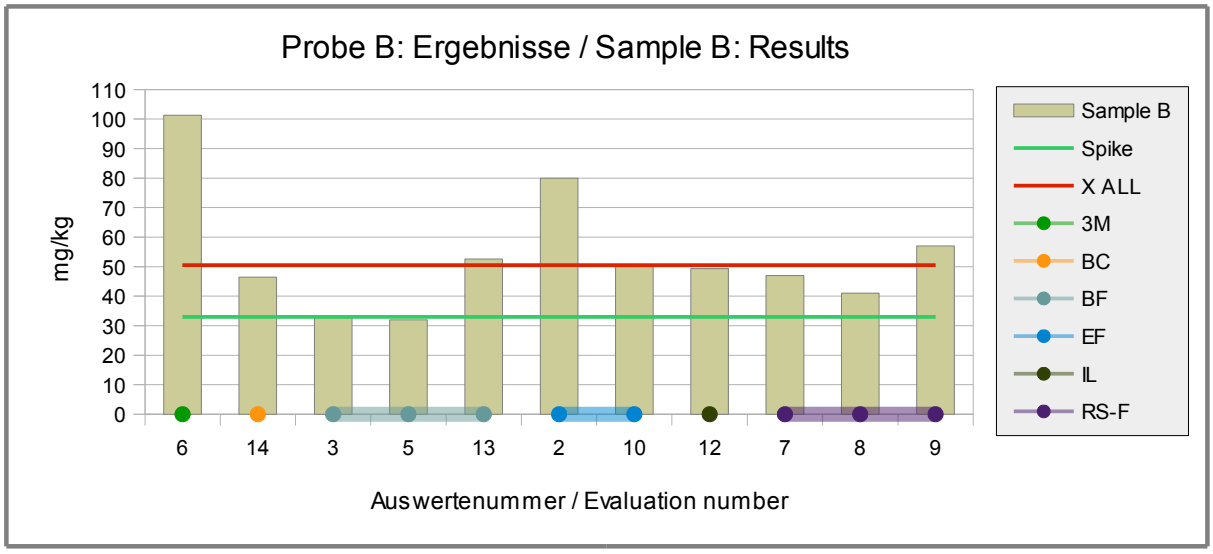


Abb./Fig. 12: ELISA Results Cashew
green line = Spiking level
red line = Assigned value median of all results
round symbols = Applied methods (see legend)

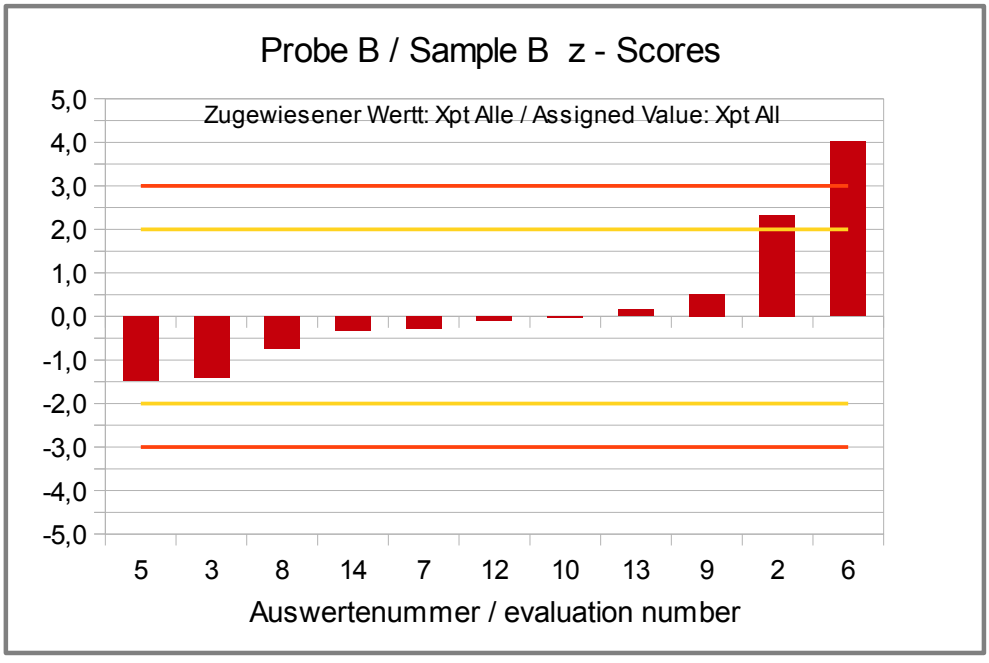


Abb./Fig. 13:
z-Scores (ELISA Results Cashew)
Assigned value robust mean of all results

Quantitative Valuation of results: Spiking level sample

Evaluation number	Cashew	z-Score Xpt_{ALL}	Method	Remarks
	[mg/kg]			
6	83,0	1,8	3M	Result converted °
14	69,7	0,9	BC	
3	48,7	-0,6	BF	
5	30,0	-1,9	BF	
13	60,7	0,3	BF	
2	95,0	2,7	EF	
10	44,5	-0,9	EF	
12	62,1	0,3	IL	
7	57,0	0,0	RS-F	
8	47,0	-0,7	RS-F	
9	38,0	-1,3	RS-F	

° calculation see p. 19

Methods:

3M = 3M Protein ELISA Kit

BC = BioCheck ELISA

BF = MonoTrace ELISA, BioFront Technologies

EF = SensiSpec ELISA Kit, Eurofins

IL = Immunolab

RS-F= Ridascreen® Fast, R-Biopharm

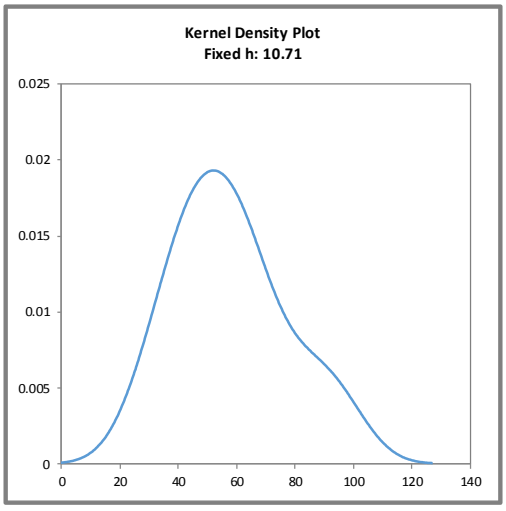


Abb. / Fig. 14:
Kerndichte-Schätzung aller ELISA-Ergebnisse (mit h = 0,75 x σpt von Xpt_{ALL})

Kernel density plot of all ELISA results (with h = 0,75 x σpt of Xpt_{ALL})

Comments:
The kernel density estimation shows nearly a symmetrical distribution of results with a soulder peak at approx. 90 mg/kg due to two single results of method 3M and EF.

Characteristics: Quantitative evaluation ELISA Cashew

Spiking Level Sample

Statistic Data	**All Results** *[mg/kg]*
Assigned value (X_{pt})	$X_{pt_{ALL}}$
Number of results	11
Number of outliers	-
Mean	57,8
Median	57,0
Robust Mean (X)	**57,1**
Robust standard deviation (S*)	**20,4**
Target range:	
Target standard deviation σ_{pt}	**14,3**
lower limit of target range	**28,6**
upper limit of target range	**85,7**
Quotient S/σ_{pt}*	*1,4*
Standard uncertainty $U_{(Xpt)}$	*7,69*
Results in the target range	*10*
Percent in the target range	*91*

Comments to the statistical characteristics and assigned values:

The kernel density estimation showed no clear method-dependent differences (two high single values).

The evaluation of all methods showed a normal variability of results, respectively. The quotient S/σ_{pt} was below 2,0. The robust standard deviations are 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 some methods.*

The robust mean of the evaluation of all results was 177% of the spiking level of cashew to the spiking level sample and thus above the range of the recommendations for the applied methods (s. 3.4.3 and "Recovery rates of cashew" p.42).

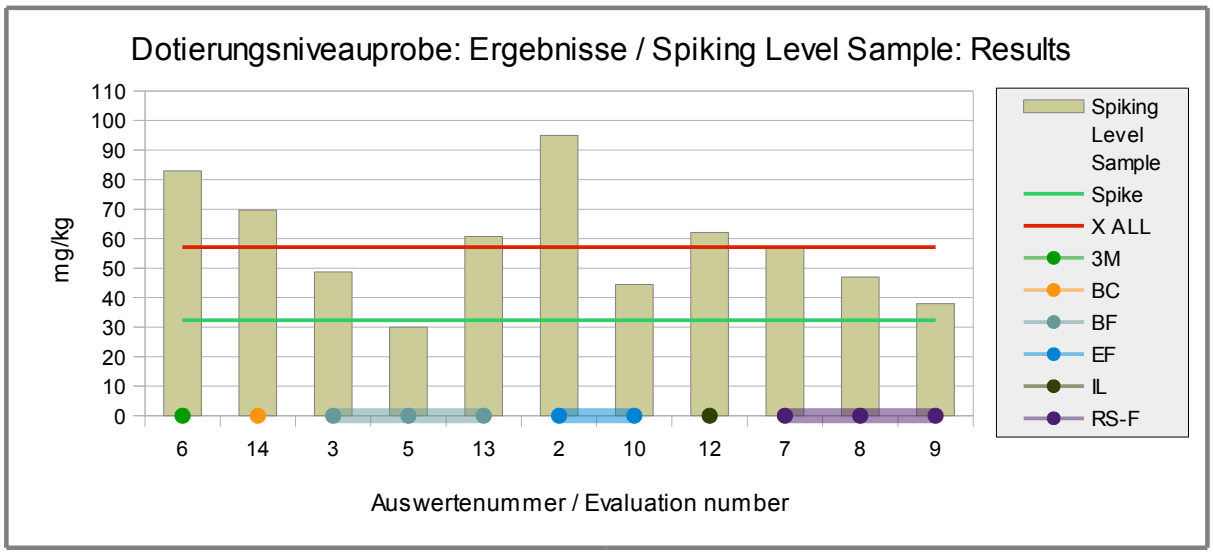


Abb./Fig. 15: ELISA Results Cashew
green line = Spiking level
red line = Assigned value median of all results
round symbols = Applied methods (see legend)

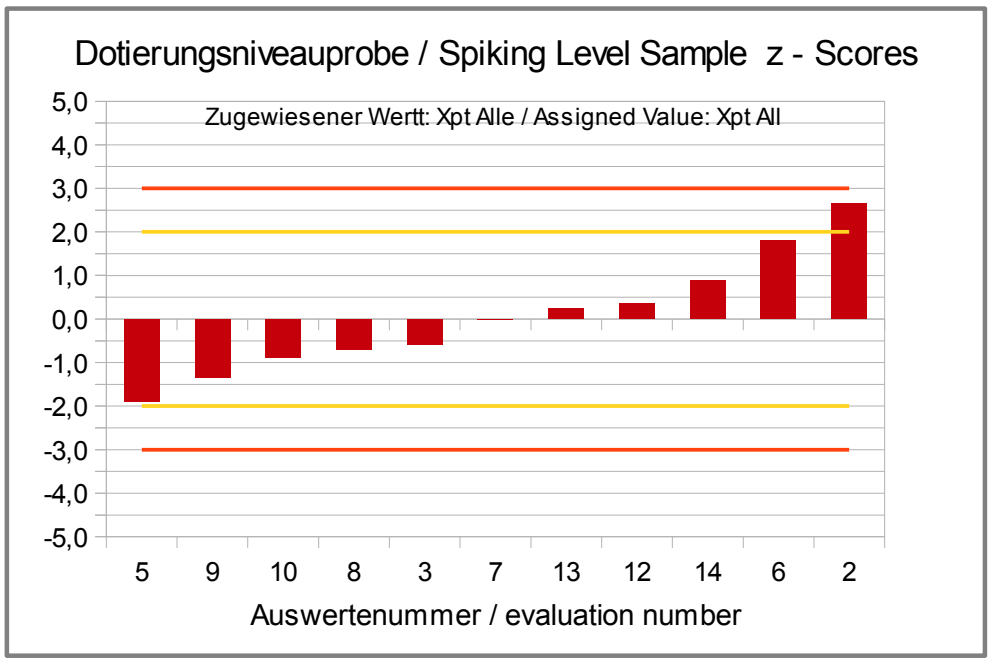


Abb./Fig. 16:
z-Scores (ELISA Results Cashew)
Assigned value robust mean of all results

Recovery Rates ELISA for Cashew:
Spiking Level Sample and Sample B

Evaluation number	Spiking Level Sample	Recovery rate*	Sample B	Recovery rate*	Method	Remarks
	[mg/kg]	[%]	[mg/kg]	[%]		
6	83,0	257	101	307	3M	Result converted °
14	69,7	216	46,4	**141**	BC	
3	48,7	151	32,7	**99**	BF	
5	30,0	**93**	32,0	**97**	BF	
13	60,7	188	52,6	159	BF	
2	95,0	294	80,0	242	EF	
10	44,5	**138**	50,0	152	EF	
12	62,1	192	49,3	**149**	IL	
7	57,0	176	47,0	**142**	RS-F	
8	47,0	**146**	41,0	**124**	RS-F	
9	38,0	**118**	57,0	173	RS-F	

° calculation see p. 19

RA**	50-150 %	RA**	50-150 %
Number in RA	4	Number in RA	6
Percent in RA	36	Percent in RA	55

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

** Range of acceptance of AOAC for allergen ELISAS

Methods:

3M = 3M Protein ELISA Kit
BC = BioCheck ELISA
BF = MonoTrace ELISA, BioFront Technologies
EF = SensiSpec ELISA Kit, Eurofins
IL = Immunolab
RS-F= Ridascreen® Fast, R-Biopharm

Comments:
36% (4) of the participants obtained a recovery rate by ELISA methods within the range of the AOAC-recommendation of 50-150%. For the spiked food matrix sample B 55% (6) of the recovery rates were within the range of acceptance.

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		
10	negative		positive		1/1 (100%)	SFA	
7	negative		positive	17	1/1 (100%)	div	
8	negative		positive		1/1 (100%)	div	
9	negative	< 0.01%	negative	< 0.01%	1/1 (100%)*	div	* no positive sample identified
11	negative		negative		1/1 (100%)*	div	* no positive sample identified

	Sample A		Sample B	
Number positive	0		3	
Number negative	5		2	
Percent positive	0		60	
Percent negative	100		40	
Consensus value	positive		none	

Methods:
SFA = Sure Food ALLERGEN, R-Biopharm / Congen
div = keine genaue Angabe / andere Methode
div = not indicated / other method

Comments:
For sample A a consensus value of 100% negative results was obtained. For the spiked sample B no consensus value of ≥ 75% could be determined. In qualitative agreement with the spiking 3 positive results for sample B were obtained, as well as 2 negative results with unspecified PCR-methods.

Quantitative Valuation PCR: Sample B

No quantitative evaluation was done, because there were too few individual results.

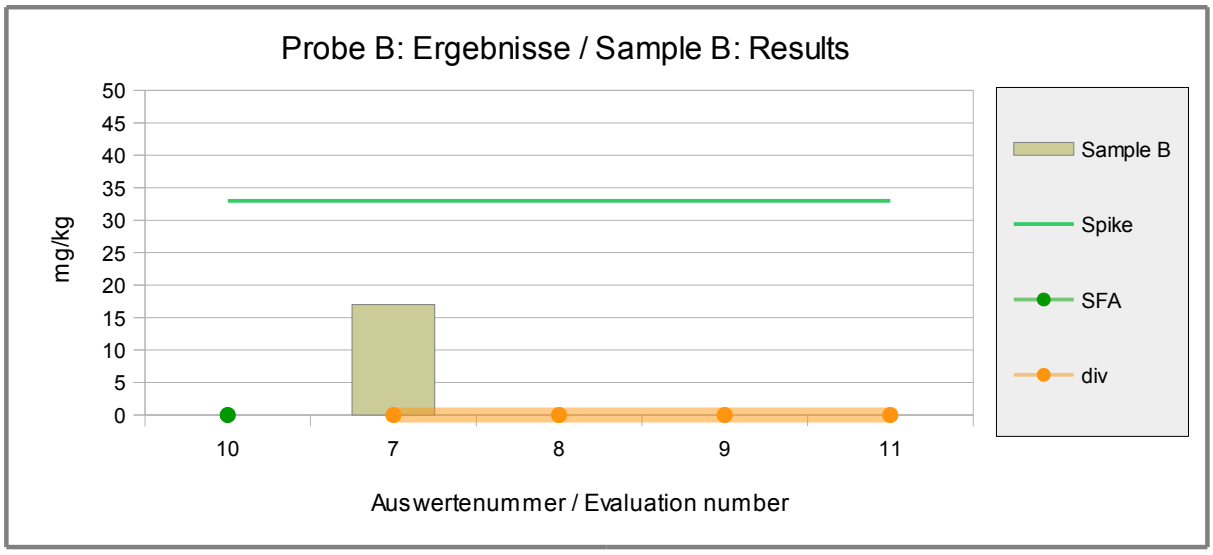


Abb./Fig. 17: PCR Results cashew
green line = Spiking level
round symbols = Applied methods (see legend)

(Quantitative) Valuation PCR: Spiking Level Sample

No quantitative evaluation was done, because there were to few quantitative results.

Evaluation number	Cashew	Cashew	z-Score Xpt_{ALL}	Method	Remarks
	pos/neg	[mg/kg]			
10	positive			SFA	
7	positive	20		div	
8	positive			div	
9	positive			div	
11	negative			div	

Number positive	4	
Number negative	1	
Percent positive	80	
Percent negative	20	
Consensus value	positive	

Methoden:
SFA = Sure Food ALLERGEN, R-Biopharm / Congen
div = keine genaue Angabe / andere Methode
div = not indicated / other method

Comments:
For the spiking level sample there were 80% positive results. One participant obtained a negative result.

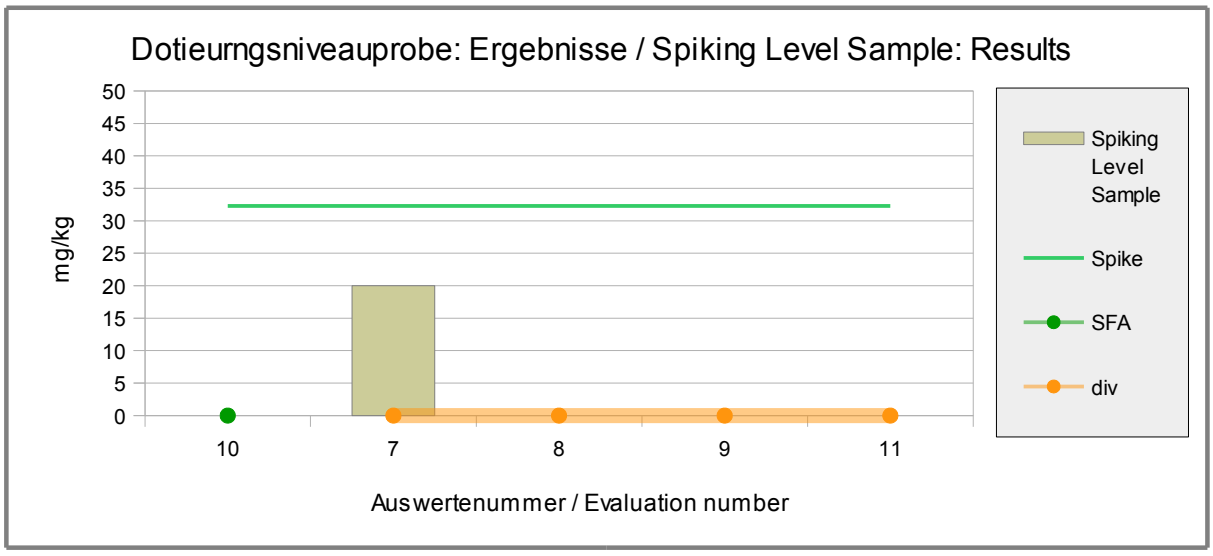


Abb./Fig. 18: PCR Results cashew
green line = Spiking level
round symbols = Applied methods (see legend)

Recovery Rates PCR for Cashew:
Spiking Level Sample and Sample B

Evaluation number	Spiking Level Sample	Recovery rate*	Sample B	Recovery rate*	Method	Remarks
	[mg/kg]	[%]	[mg/kg]	[%]		
10					SFA	
7	20	**62**	17	**52**	div	
8					div	
9			< 0.01%		div	
11					div	

RA**	50-150 %	RA**	50-150 %
Number in RA	1	Number in RA	1
Percent in RA	100	Percent in RA	100

Methods:
SFA = Sure Food ALLERGEN, R-Biopharm / Congen
div = keine genaue Angabe / andere Methode
div = not indicated / other method

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

** Range of acceptance of AOAC for allergen ELISAS

Comments:
One participant submitted quantitative results by PCR methods and obtained for the spiking level sample and the spiked food matrix sample B recovery rates within the range of the AOAC-recommendation of 50-150%.

5. Documentation

5.1 Details by the participants

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

5.1.1 ELISA: Almond

Meth. Abr.	Evaluation number	Date of analyses	Result Sample A		Result Sample B		Result Spiking Sample		NWG / LOD *	BG / LOQ *	MU*	quantitative Result given as	Method
		Day/Month	qualitative	mg/kg	qualitative	mg/kg	qualitative	mg/kg	mg/kg	mg/kg	%	e.g. food / food protein	Test-Kit + Manufacturer
BF	13	18/1	negative	0	positive	20	positive	22,1	0,15	1		Almond	MonoTrace Almond ELISA kit, BioFront Technologies
EF	10a		-	< 0.2	-	21	-	28	0,2	0,4		Almond	Eurofins SensiSpec Almond ELISA Kit
IL	12	13.12.18	negative	<0,2	positive	25,2	positive	25,3	0,2	0,4		Almond	Immunolab Almond ELISA
RS-F	1		negative	<2,5	positive	23,3	positive	93,5		2,5		Almond	Ridascreen® FAST Almond R6901, R-Biopharm
RS-F	2	17.12.18	negative	<2,5	positive	20	positive	20	1,7	2,5		Almond	Ridascreen® FAST Almond R6901, R-Biopharm
RS-F	4	14.01.19	negative		positive	22	positive	23	1	2,5	40	Almond	Ridascreen® FAST Almond R6901, R-Biopharm
RS-F	5		negative	<2,5	positive	16	positive	16		2,5		Almond	Ridascreen® FAST Almond R6901, R-Biopharm
RS-F	8	07.01.19	-	< BG	-	24	-	27		2,5		Almond	Ridascreen® FAST Almond R6901, R-Biopharm
RS-F	9	20.12.18	negative	< 2.5	positive	24	positive	27	1,7	2,5		Almond	Ridascreen® FAST Almond R6901, R-Biopharm
RS-F	10b		-	< 1.5	-	17	-	22	1,5			Almond	Ridascreen® FAST Almond R6901, R-Biopharm
RS-F	14	03.01.2019	negative	<2.5	positive	27,99	positive	32,87	2,5	2,5	21,76	Almond	Ridascreen® FAST Almond R6901, R-Biopharm
VT	3	15/1	-	<2.5	-	17,3	-	25,2		2,5		Almond	Veratox Almond, Neogen
VT	6	09.01.19	negative		positive	12,3	positive	24		2,5		Almond	Veratox Almond, Neogen

* NWG Nachweisgrenze / BG Bestimmungsgrenze
* LOD limit of detection / LOQ limit of quantitation
* MU Messunsicherheit / MU measurement uncertainty

Meth. Abr.	Evaluation number	Specifity	Remarks to the Method (Extraction and Determination)	Method accredited ISO/IEC 17025	Further Remarks
		Antibody	e.g. Extraction Solution / Time / Temperature	yes/no	
BF	13	Monoclonal antibody-based assay	1:20 extraction ratio, 10 minutes at 60C	no	
EF	10a				
IL	12				
RS-F	1			yes	
RS-F	2		according to manufacturer's instructions	yes	
RS-F	4			yes	
RS-F	5			yes	
RS-F	8	Ab reacts specifically with almond protein	As Per Kit Instructions	yes	
RS-F	9		As Per Kit Instructions	yes	Cross-reactivity with apricot kernels
RS-F	10b				
RS-F	14	As Per Kit Instructions	As Per Kit Instructions	Yes	
VT	3			yes	
VT	6				

5.1.2 ELISA: Cashew

Meth. Abr.	Evaluation number	Date of analyses	Result Sample A		Result Sample B		Result Spiking Sample		NWG / LOD *	BG / LOQ *	MU*	quantitative Result given as	Method
		Day/Month	qualitative	mg/kg	qualitative	mg/kg	qualitative	mg/kg	mg/kg	mg/kg	%	e.g. food / food protein	Test-Kit + Manufacturer
3M	6	14.01.19	negative		positive	15,4	positive	18,8		0,9		Cashew protein	3M Cashew Protein Elisa Kit
BC	14	21.12.2018	negative	<2	positive	46,43	positive	69,72	2	2	29,38	Cashew	BioCheck ELISA Cashew-Check
BF	3	14/1	-	<2.0	-	32,7	-	48,7		2		Cashew	MonoTrace Cashew ELISA kit, BioFront Technologies
BF	5		negative	<2	positive	32	positive	30		2		Cashew	MonoTrace Cashew ELISA kit, BioFront Technologies
BF	13	18/1	negative	0	positive	52,6	positive	60,7	0,12	1		Cashew	MonoTrace Cashew ELISA kit, BioFront Technologies
EF	2	27.12.18	negative	<2	positive	80	positive	95	1,5	2		Cashew	Eurofins SensiSpec Cashew ELISA Kit
EF	10		-	< 0.2	-	50	-	44,5	0,2	2		Cashew	Eurofins SensiSpec Cashew ELISA Kit
IL	12	13.12.18	negative	<0,2	positive	49,3	positive	62,1	0,2	2		Cashew	Immunolab Cashew ELISA
RS-F	7	14.01.19	-		positive	47	positive	57	2,5	5		Cashew	Ridascreen® FAST Cashew R7862, R-Biopharm
RS-F	8	11.12.18	-	< BG	-	41	-	47		2,5		Cashew	Ridascreen® FAST Cashew R6872, R-Biopharm
RS-F	9	19.12.18	negative	< 2.5	positive	57	positive	38	0,09	2,5		Cashew	Ridascreen® FAST Cashew R7862, R-Biopharm

* NWG Nachweisgrenze / BG Bestimmungsgrenze

* LOD limit of detection / LOQ limit of quantitation

Meth. Abr.	Evaluation number	Specifity	Remarks to the Method (Extraction and Determination)	Method accredidet ISO/IEC 17025	Further Remarks
		Antibody	e.g. Extraction Solution / Time / Temperature	yes/no	
3M	6				
BC	14	As Per Kit Instructions	As Per Kit Instructions	Yes	
BF	3			yes	
BF	5			no	
BF	13	Monoclonal antibody-based assay	1:20 extraction ratio, 10 minutes at 60C	no	
EF	2		according to manufacturer's instructions	yes	
EF	10				
IL	12				
RS-F	7				
RS-F	8	Ab reacts specifically with cashew protein	As Per Kit Instructions	yes	
RS-F	9		As Per Kit Instructions	yes	Cross-reactivity to pistachio can not be excluded

5.1.3 PCR: Almond

Meth. Abr.	Evaluation number	Date of analyses	Result Sample A		Result Sample B		Result Spiking Sample		NWG / LOD *	BG / LOQ *	MU*	quantitative Result given as	Method
		Day/Month	qualitative	mg/kg	qualitative	mg/kg	qualitative	mg/kg	mg/kg	mg/kg	%	e.g. food / food protein	Test-Kit + Manufacturer
ASU	7	19.12.18	negative		positive	18	positive	26	10	18	50	Almond	ASU §64 Methode/method
SFA	8	13.12.18	negative		positive		positive					Almond	SureFood®Allergen Almond Congen(S3604)
SFA	10		negative		positive		positive		0,4			Almond	Sure Food ALLERGEN, R-Biopharm / Congen
div	9	12.12.18	-	< 0.01%	-	< 0.01%	positive					Almond DNA	Selection PCR-Methods
div	11		negative		negative		positive		20			Almond DNA	Selection PCR-Methods

* NWG Nachweisgrenze / BG Bestimmungsgrenze
* LOD limit of detection / LOQ limit of quantitation
* MU Messunsicherheit / MU measurement uncertainty

Meth. Abr.	Evaluation number	Specifity	Remarks to the Method (Extraction and Determination)	Method accredidet ISO/IEC 17025	Further Remarks
		Target-Sequence / -DNA	e.g. Extraction / enzymes / clean-up / real time PCR / gel electrophoresis / cycles	yes/no	
ASU	7	PRU AV1-Gene; 129bp	CTAB-precipitation method, s. e.g. ASU L 18.00-22	yes	Calibration / quantification with matrix standards, spiked material: almond, degreased.
SFA	8		Dneasy Rmericon Food Kit/ Proteinase K/ Real Time PCR/ 45 cycles	yes	
SFA	10				
div	9	PRU AV1	Wizard	yes	DNA-%, Cross-reactivity with apricot-DNA
div	11				

5.1.4 PCR: Cashew

Meth. Abr.	Evaluation number	Date of analyses	Result Sample A		Result Sample B		Result Spiking Sample		NWG / LOD *	BG / LOQ *	MU*	quantitative Result given as	Method
		Day/Month	qualitative	mg/kg	qualitative	mg/kg	qualitative	mg/kg	mg/kg	mg/kg	%	e.g. food / food protein	Test-Kit + Manufacturer
SFA	10		negative		positive		positive		0,4			Cashew	Sure Food ALLERGEN, R-Biopharm / Congen
div	7	20.12.18	negative		positive	17	positive	20	10	17	50	Cashew	other: Köppel et al. Multiplex real-time PCR for the detection and quantification of DNA from eight allergens in food.
div	8	19.12.18	negative		positive		positive					Cashew	5xQuantiFast® Pathogen PCR Fa.Qiagen Primer/Sonde: eurofins/mwg/ operon. Methode nach Ehlert et al 2008
div	9	13.12.18	-	< 0.01%	-	< 0.01%	positive					Cashew-DNA	Selection PCR-Methods
div	11		negative		negative		negative		2			Cashew DNA	Selection PCR-Methods

* NWG Nachw eisgrenze / BG Bestimmungsgrenze
* LOD limit of detection / LOQ limit of quantitation
* MU Messunsicherheit / MU measurement uncertainty

Meth. Abr.	Evaluation number	Specifity	Remarks to the Method (Extraction and Determination)	Method accredidet ISO/IEC 17025	Further Remarks
		Target-Sequence / -DNA	e.g. Extraction / enzymes / clean-up / real time PCR / gel electrophoresis / cycles	yes/no	
SFA	10				
div	7	2s albumin-Gene; 67bp	CTAB-precipitation method, s. e.g. ASU L 18.00-22	yes	Calibration / quantification w ith matrix standards, spiked material: cashew , degreased.
div	8	Cashew Gene Ana 03	Dneasy Rmericon Food Kit/ Proteinase K/ Real Time PCR/ 45 cycles	yes	
div	9	ANA O3	Wizard	yes	DNA-%
div	11				

5.2 Homogeneity

5.2.1 Mixture homogeneity before botteling

Microtracer Homogeneity Test
DLA 08-2018 Sample B

Weight whole sample	2,81	kg
Microtracer	FSS-rot lake	
Particle size	75 – 300	µm
Weight per particle	2,0	µg
Addition of tracer	14,3	mg/kg

Result of analysis

Sample	Weight [g]	Particle number	Particles [mg/kg]
1	4,97	37	14,9
2	5,05	28	11,1
3	5,05	37	14,7
4	5,05	27	10,7
5	5,01	27	10,8
6	5,07	41	16,2
7	5,02	34	13,5
8	5,02	35	13,9

Poisson distribution		
Number of samples	8	
Degree of freedom	7	
Mean	33,3	Particles
Standard deviation	5,30	Particles
χ^2 (CHI-Quadrat)	5,91	
Probability	**55**	%
Recovery rate	92	%

Normal distribution		
Number of samples	8	
Mean	13,2	mg/kg
Standard deviation	2,11	mg/kg
rel. Standard deviaton	15,9	%
Horwitz standard deviation	10,8	%
HorRat-value	**1,5**	
Recovery rate	92	%

Microtracer Homogeneity Test
DLA 08-2018 Spiking Level Sample

Weight whole sample	1,00	kg
Microtracer	FSS-rot lake	
Particle size	75 – 300	µm
Weight per particle	2,0	µg
Addition of tracer	25,1	mg/kg

Result of analysis

Sample	Weight [g]	Particle number	Particles [mg/kg]
1	5,05	82	32,5
2	4,97	65	26,2
3	5,02	80	31,9
4	5,07	73	28,8
5	5,04	74	29,4
6	4,98	68	27,3
7	5,00	70	28,0
8	5,01	75	29,9

Poisson distribution		
Number of samples	8	
Degree of freedom	7	
Mean	73,4	Particles
Standard deviation	5,44	Particles
χ^2 (CHI-Quadrat)	2,82	
Probability	**90**	%
Recovery rate	116	%

Normal distribution		
Number of samples	8	
Mean	29,2	mg/kg
Standard deviation	2,17	mg/kg
rel. Standard deviaton	7,4	%
Horwitz standard deviation	9,6	%
HorRat-value	**0,8**	
Recovery rate	116	%

5.3 Information on the Proficiency Test (PT)

Before the PT the participants received the following information in the sample cover letter:

PT number	***DLA 08/2018***
PT name	***Allergens VIII: Almond and Cashew in Veggie Burger Powder with "Spiking Level Sample"***
Sample matrix (processing)	***Samples A + B:*** *Vegetable Burger Powder / ingredients: Oat wholemeal flakes, vegetables (carrots, parsnips, celery, leeks, fried onions, parsley root), breadcrumbs (wheat flour, salt, yeast), buckwheat groats, whole wheat semolina, whole wheat flour, wheat bran, sea salt, parsley, spices (onions, marjoram, celery leaves, garlic, pepper), yeast flakes, other food additives and allergenic foods (one of both samples)* ***Spiking Level Sample:*** *potato powder, other food additives and allergenic foods*
Number of samples and sample amount	*2 different Samples A + B: 25 g each + 1 Spiking Level Sample: 15 g*
Storage	*Samples A + B: room temperature (long term cooled 2 - 10°C) Spiking Level Sample: room temperature*
Intentional use	*Laboratory use only (quality control samples)*
Parameter	*qualitative + quantitative: Almond, Cashew (as food item, protein, DNA) Samples A + B: < 500 mg/kg Spiking Level Sample: < 500 mg/kg*
Methods of analysis	*Analytical methods are optional*
Notes to analysis	*The analysis of PT samples should be performed like a routine laboratory analysis. In general we recommend to homogenize a representative sample amount before analysis according to good laboratory practice, especially in case of low sample weights. Preferably, the total sample amount is homogenized.*
Result sheet	*One result each should be determined for Samples A and B and the Spiking Level Sample. The results should be filled in the result submission file.*
Units	*mg/kg*
Number of digits	*at least 2*
Result submission	*The result submission file should be sent by e-mail to:* ***pt@dla-lvu.de***
Deadline	***the latest January 18th 2019.***
Evaluation report	*The evaluation report is expected to be completed 6 weeks after deadline of result submission and sent as PDF file by e-mail.*
Coordinator and contact person of PT	*Matthias Besler-Scharf, PhD*

* Control of mixture homogeneity and qualitative testings are carried out by DLA. Any testing of the content, homogeneity and stability of PT parameters is subcontracted by DLA.

6. Index of participant laboratories in alphabetical order

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

[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.]

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