Proficiency Tests

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Evaluation Report
proficiency test

DLA 06/2017

Allergens VI:

Almond and Pistachio

in Spread (Cocoa Cream)

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

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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 spread "nut-nougat cream". The basic composition of both sample A and sample B was the same (see table 1). The basic mixture was homogenized stirring at approx. 40°C.

Afterwards the **spiked sample B** was produced as follows:
The spiking material containing the allergenic ingredients almond and pistachio (sieved mesh 400 µm) was added to an aliquot of the basic mixture and the mixture was homogenized at approx. 40°C. Subsequently, the basic mixture was again added in 3 additional steps and homogenized at approx. 40°C 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. Afterwards the whole sample was sieved by means of a centrifugal mill (mesh 250 µm).

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

The composition of the PT samples and the spiking level sample is given in table 1.

Table 1: Composition of DLA-Samples

Ingredients	**Sample A**	**Sample B**	**Spiking Level Sample**
Spread (Nut-Nougat Cream) Ingredients: Sugar, palm oil, hazelnuts (13%), low-fat cocoa powder, skimmed milk powder (7,5%), emulsifier: lecithin, vanillin Nutrients per 100 g: Protein 6,6 g, Carbohydrates 57 g, Fat 32 g	100 g/100 g	99,5 g/100g	-
Potato Powder Ingredients: Potatoes, E471, E304, E223, E100	-	-	99,6 g/100 g
Almond Butter, white - as Almonds* - *thereof 16,2% total protein***	-	 29,3 mg/kg 4,8 mg/kg	 24,0 mg/kg 3,9 mg/kg
Pistachios, raw ground - as Pistachios* - *thereof 21,7% total protein***	-	 33,9 mg/kg 7,4 mg/kg	 28,0 mg/kg 6,1 mg/kg
further Ingredients: Maltodextrin, sodium sulfate and silicon dioxide	-	<0,5 g/100 g	<0,4 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 almonds and F=5,30 for pistachios)

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].
Because no pasty samples can be analysed by the microtracer method, only the spiking level samples was measured. The microtracer analysis of the present PT showed a probability of 59%. 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) [16, 17]. This gave a HorRat value 0,85. 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.3 (with notes 1-3).

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, Veratox, AgraQuant) and pistachio (Immunolab, AgraQuant), respectively (see page 8 and 9). 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].

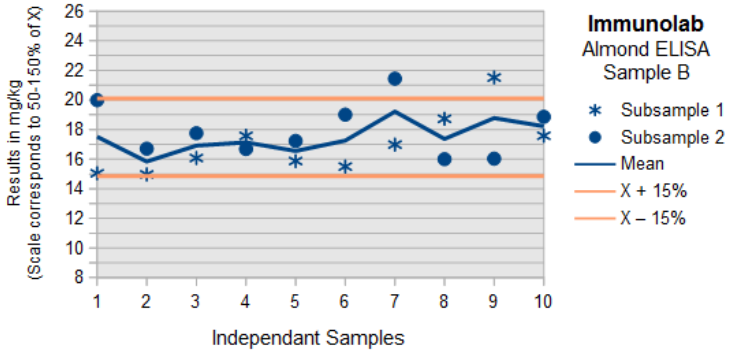
ELISA-Tests: Homogenität Mandel / Homogeneity Almond

Immunolab Almond ELISA

Sample weights: 1,0 g (0,9 – 1,1 g)
Number of replicates: 2
Overall result: Almond 17,5 ± 1,0 mg/kg

Sample B	Subsample 1 mg/kg	Subsample 2 mg/kg	Mean mg/kg
1	15,0	20,0	17,5
2	15,0	16,7	15,8
3	16,1	17,8	16,9
4	17,6	16,7	17,1
5	15,9	17,2	16,6
6	15,5	19,0	17,3
7	17,0	21,4	19,2
8	18,7	16,0	17,4
9	21,5	16,0	18,8
10	17,6	18,9	18,2

General average X	17,5	
SD of sample means Sx	1,02	5,8%
SD within-samples Sw	1,61	9,2%
SD between-samples Ss	< 1,0	< 5,8%

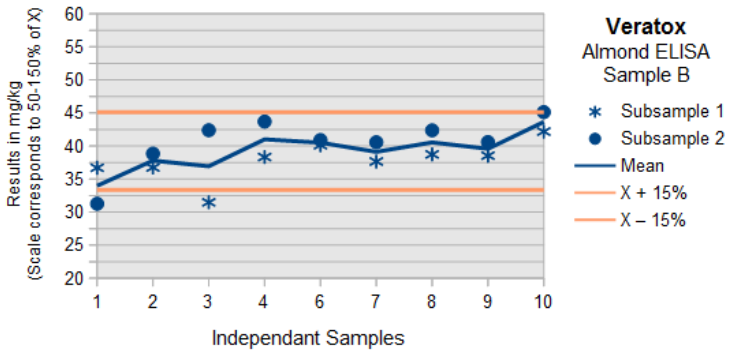


Neogen Veratox ELISA Almond

Sample weights: 5,0 g (4,5 – 5,5 g)
Number of replicates: 2
Overall result: Almond 39,2 ± 2,2 mg/kg

Sample B	Subsample 1 mg/kg	Subsample 2 mg/kg	Mean mg/kg
1	36,7	31,3	34,0
2	36,7	38,9	37,8
3	31,5	42,4	36,9
4	38,3	43,7	41,0
6	40,1	40,9	40,5
7	37,7	40,6	39,1
8	38,7	42,4	40,6
9	38,5	40,6	39,6
10	42,2	45,1	43,7

General average X	39,2	
SD of sample means Sx	2,76	7,0%
SD within-samples Sw	2,46	6,3%
SD between-samples Ss	2,15	5,5%

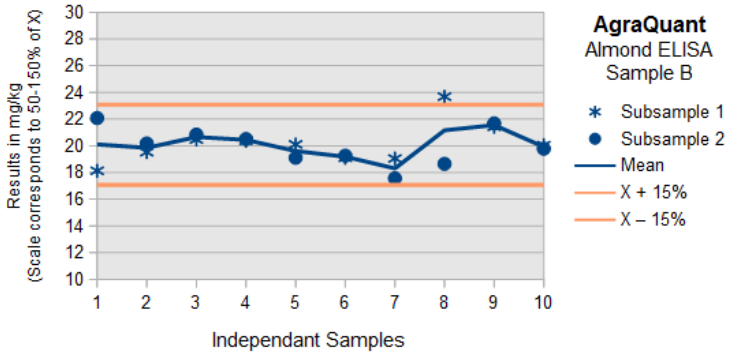


Romerlabs AgraQuant Almond

Sample weights: 1,0 g (0,9 – 1,1 g)
Number of replicates: 2
Overall result: Almond 20,1 ± 0,6 mg/kg

Sample B	Subsample 1 mg/kg	Subsample 2 mg/kg	Mean mg/kg
1	18,1	22,1	20,1
2	19,5	20,2	19,8
3	20,5	20,8	20,7
4	20,4	20,5	20,4
5	20,1	19,1	19,6
6	19,1	19,3	19,2
7	19,0	17,6	18,3
8	23,7	18,6	21,2
9	21,4	21,7	21,6
10	20,1	19,8	19,9

General average X	20,1	
SD of sample means Sx	0,95	4,7%
SD within-samples Sw	1,06	5,3%
SD between-samples Ss	0,58	2,9%



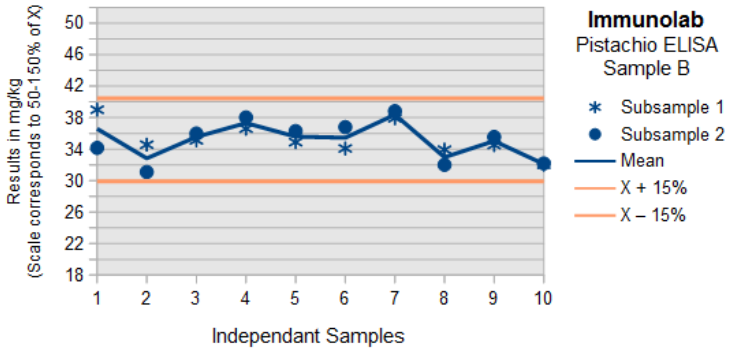
ELISA-Tests: Homogenität Pistazie / Homogeneity Pistachio

Immunolab Pistachio ELISA

Sample weights: 1,0 g (0,9 – 1,1 g)
Number of replicates: 2
Overall result: Pistachio 35,2 ± 1,9 mg/kg

Sample B	Subsample 1 mg/kg	Subsample 2 mg/kg	Mean mg/kg
1	39,0	34,2	36,6
2	34,6	31,1	32,8
3	35,1	36,0	35,5
4	36,6	38,0	37,3
5	34,9	36,3	35,6
6	34,1	36,8	35,4
7	37,9	38,8	38,4
8	33,9	32,0	33,0
9	34,5	35,5	35,0
10	32,0	32,2	32,1

General average X	35,2	
SD of sample means Sx	2,03	5,8%
SD within-samples Sw	1,16	3,3%
SD between-samples Ss	1,86	5,3%

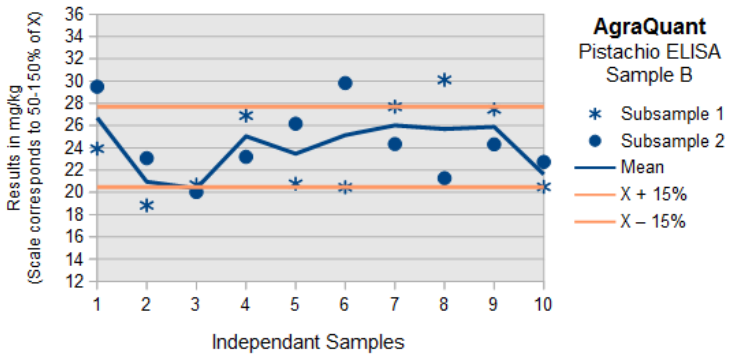


Romerlabs AgraQuant Pistachio

Sample weights: 1,0 g (0,9 – 1,1 g)
Number of replicates: 2
Overall result: Pistachio 24,1 ± 1,4 mg/kg

Sample B	Subsample 1 mg/kg	Subsample 2 mg/kg	Mean mg/kg
1	23,9	29,5	26,7
2	18,8	23,1	20,9
3	20,7	20,0	20,4
4	26,9	23,2	25,0
5	20,8	26,2	23,5
6	20,5	29,8	25,1
7	27,7	24,3	26,0
8	30,1	21,3	25,7
9	27,4	24,3	25,9
10	20,5	22,7	21,6

General average X	24,1	
SD of sample means Sx	2,33	9,7%
SD within-samples Sw	2,66	11,1%
SD between-samples Ss	1,36	5,7%



2.1.2 Stability

The experience with various DLA reference materials showed good storage stability with respect to the durability of the sample (spoilage) and the content of the PT parameters almonds and pistachios for comparable food matrices and water activity (a_W value <0,5). The stability of the sample material is therefore given during the investigation period under consideration of given storage conditions.

2.2 Sample shipment and information to the test

The portions of test material (sample A and sample B as well as the spiking level sample) were sent to every participating laboratory in the 10th week of 2017. The testing method was optional. The tests should be finished at November 17th 2017 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/or pistachio in the range of mg/kg in the matrix of spread (nut nougat spread with hazelnut and cocoa). 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 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.
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.

All 18 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 [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 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]. 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, 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 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]:

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

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 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 11 – 32% 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. 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 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.

ELISA results given as **almond protein** or **pistachio protein** were converted by DLA to total food items (almonds, pistachios) using the analyzed protein content of the raw materials (see page 5).

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 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	positive	0,5	positive	26,0	1/2 (50%)	AQ	
3	negative	ND	positive	41,9	2/2 (100%)	BF	outlier X_{all}
4	positive	0,53	positive	16,8	1/2 (50%)	BM	
1	negative	< 0,4	positive	21,8	2/2 (100%)	IL	
2	negative	< 0,4	positive	22,6	2/2 (100%)	IL	
12	negative	< 1	positive	25,0	2/2 (100%)	IL	
5	negative	< 1,2	positive	23,0	2/2 (100%)	RS-F	
8	negative		positive	147	2/2 (100%)	RS-F	result converted °
9	negative	< 2,5	positive	16,2	2/2 (100%)	RS-F	
10	negative	< 2,5	positive	22,4	2/2 (100%)	RS-F	
15	negative	< 2,5	positive	13,0	2/2 (100%)	RS-F	
17	negative	< 2,5	positive	>20	2/2 (100%)	RS-F	
18a	negative	< 2,5	positive	17,0	2/2 (100%)	RS-F	
6	negative	< 2,5	positive	41,0	2/2 (100%)	VT	
11	positive	3,8	positive	20,1	1/2 (50%)	VT	
14	negative	< 2,5	positive	11,0	2/2 (100%)	VT	
18b	negative	< 2,5	positive	16,0	2/2 (100%)	VT	

° calculation p. 19

	Sample A		Sample B	
Number positive	3		17	
Number negative	14		0	
Percent positive	18		100	
Percent negative	82		0	
Consensus value	negative		positive	

Methods:

AQ = AgraQuant, RomerLabs
BF = MonoTrace ELISA, BioFront Technologies
BM = AlerTox ELISA, Biomedal
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. Three positive results were obtained for sample A, all in the range of the limit of quantitation of the methods (Test kit manuals / Handbooks: AQ 0,4 mg/kg, BM 0,5 mg/kg, VT 2,5 mg/kg).

Quantitative valuation of ELISA-results: Sample B

Evaluation number	Almond	z-Score Xpt_{ALL}	z-Score Xpt_{RS-F}	Method	Remarks
	[mg/kg]				
13	26,0	1,0		AQ	
3	41,9	4,0		BF	
4	16,8	-0,78		BM	
1	21,8	0,18		IL	
2	22,6	0,34		IL	
12	25,0	0,80		IL	
5	23,0	0,41	1,0	RS-F	
8	147	24	28	RS-F	result converted ° / and excluded
9	16,2	-0,90	-0,47	RS-F	
10	22,4	0,30	0,89	RS-F	
15	13,0	-1,5	-1,2	RS-F	
17	>20			RS-F	
18a	17,0	-0,74	-0,29	RS-F	
6	41,0	3,9		VT	
11	20,1	-0,14		VT	
14	11,0	-1,9		VT	
18b	16,0	-0,93		VT	

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Methods:

AQ = AgraQuant, RomerLabs
BF = MonoTrace ELISA, BioFront Technologies
BM = AlerTox ELISA, Biomedal
IL = Immunolab
RS-F= Ridascreen® Fast, R-Biopharm
VT = Veratox, Neogen

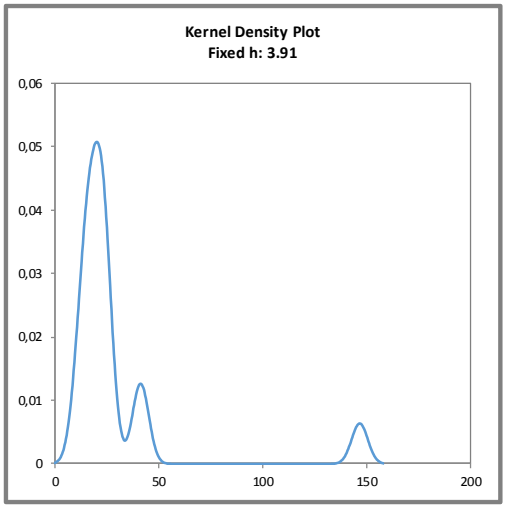

Abb. / Fig. 1:
Kerndichte-Schätzung aller ELISA-Ergebnisse (mit h = 0,75 x σ_{pt} von Xpt_{ALL})

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

Comments:
The kernel density estimation shows nearly a symmetrical distribution of results with two smaller peaks at approx. 40 mg/kg (2 single results methods BF and VT) and at approx. 150 mg/kg (method RS-F) due to the excluded result.

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 ***	15	5
Number of outliers	0	0
Mean	22,3	18,3
Median	21,8	17,0
Robust Mean (X)	**20,9**	**18,3**
Robust standard deviation (S*)	**6,75**	**4,85**
Target range:		
Target standard deviation σ_{pt}	**5,21**	**4,58**
lower limit of target range	**10,4**	**9,16**
upper limit of target range	**31,3**	**27,5**
Quotient S/σ_{pt}*	*1,3*	*1,10*
Standard uncertainty $U_{(Xpt)}$	*2,18*	*2,71*
Quotient $U_{(Xpt)}/\sigma_{pt}$	*0,42*	*0,59*
Results in the target range	*13*	*5*
Percent in the target range	*87*	*100*

*** without evaluation no. 8*

Methods:
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 all methods and the evaluation of results from method RS-F showed a normal variability of results, respectively. The quotients S/σ_{pt} were well 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 71% and 62% 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).

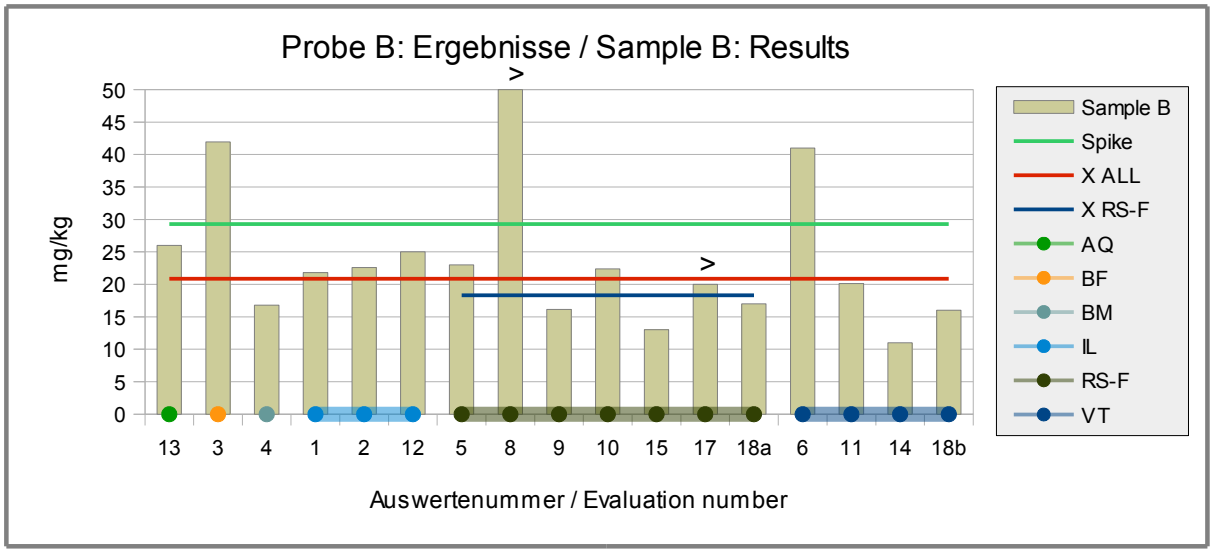

Abb./Fig. 2: ELISA Results Almond
green line = Spiking level
red line = Assigned value robust mean all results
blue line = Assigned value robust mean results method RS-F
round symbols = Applied methods (see legend)

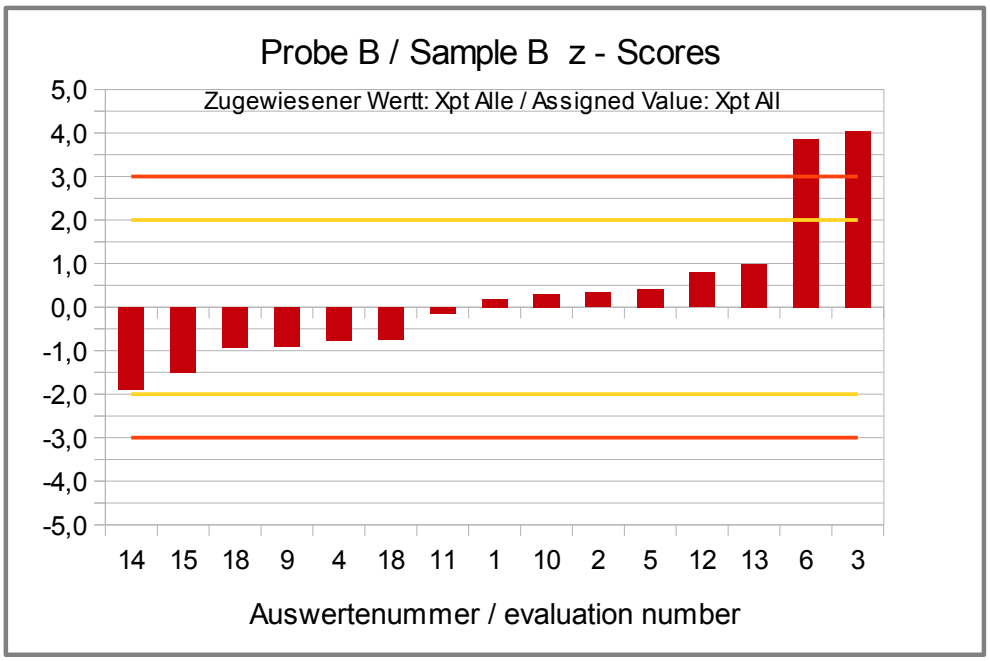

Abb./Fig. 3:
z-Scores (ELISA Results Almond)
Assigned value robust mean of all results

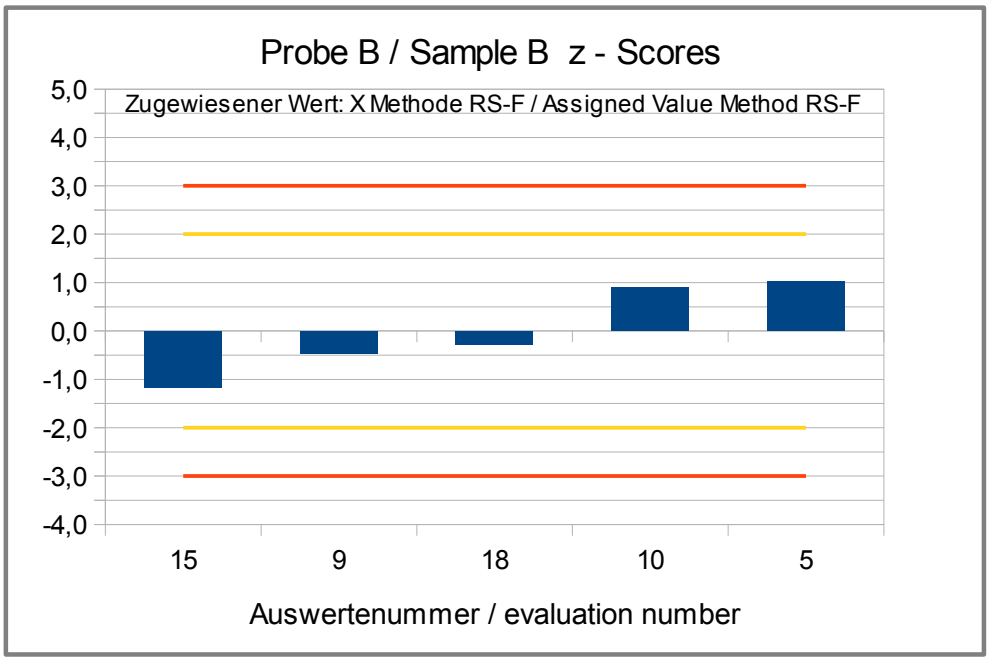

Abb./Fig. 4:
z-Scores (ELISA Results Almond)
Assigned value robust mean of method RS-F (R-Biopharm, Ridascreen® Fast)

Quantitative valuation of results: Spiking level sample

Evaluation number	Almond	z-Score Xpt_{ALL}	z-Score Xpt_{RS-F}	Method	Remarks
	[mg/kg]				
13	22,0	1,2		AQ	
3	30,6	3,2		BF	outlier X_{all}
4	13,9	-0,72		BM	
1	18,6	0,38		IL	
2	16,8	-0,04		IL	
12	15,0	-0,47		IL	
5	16,0	-0,23	-0,01	RS-F	
8	85,2	16	17,3	RS-F	result converted ° / and excluded
9	19,9	0,69	1,0	RS-F	
10	13,8	-0,75	-0,56	RS-F	
15	13,5	-0,82	-0,63	RS-F	
17	16,0	-0,23	-0,01	RS-F	
18a	17,0	0,00	0,24	RS-F	
6				VT	
11	18,8	0,43		VT	
14				VT	
18b	15,0	-0,47		VT	

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Methods:

AQ = AgraQuant, RomerLabs
BF = MonoTrace ELISA, BioFront Technologies
BM = AlerTox ELISA, Biomedal
IL = Immunolab
RS-F= Ridascreen® Fast, R-Biopharm
VT = Veratox, Neogen

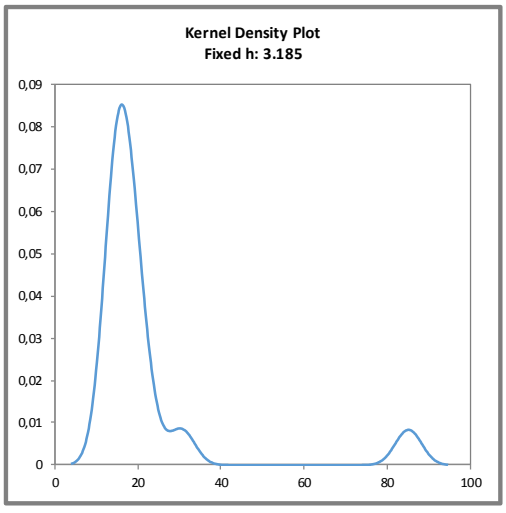

Abb. / Fig. 5:
Kerndichte-Schätzung aller ELISA-Ergebnisse (mit h = 0,75 x σpt von Xpt_{ALL})

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 two smaller peaks at approx. 30 mg/kg (method BF) and at approx. 85 mg/kg (method RS-F) due to the excluded result.

Characteristics: Quantitative evaluation Almond

Spiking level sample

Statistic Data	**All Results** *[mg/kg]*	**Method RS-F** *[mg/kg]*
Assigned value (X_{pt})	$X_{pt_{ALL}}$	$X_{pt_{METHOD\ RS\text{-}F}}$
*Number of results ***	14	6
Number of outliers	1	0
Mean	17,6	16,0
Median	16,4	16,0
Robust Mean (X)	**17,0**	**16,0**
Robust standard deviation (S*)	**3,16**	**2,65**
Target range:		
Target standard deviation σ_{pt}	**4,25**	**4,01**
lower limit of target range	**8,49**	**8,02**
upper limit of target range	**25,5**	**24,1**
Quotient S/σ_{pt}*	*0,74*	*0,66*
Standard uncertainty $U_{(Xpt)}$	*1,06*	*1,35*
Quotient $U_{(Xpt)}/\sigma_{pt}$	*0,25*	*0,34*
Results in the target range	*13*	*6*
Percent in the target range	*93*	*100*

*** without evaluation no. 8*

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 all methods and the evaluation of results from method RS-F showed a low variability of results, respectively. The quotients S/σ_{pt} were 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 71% and 67% 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).

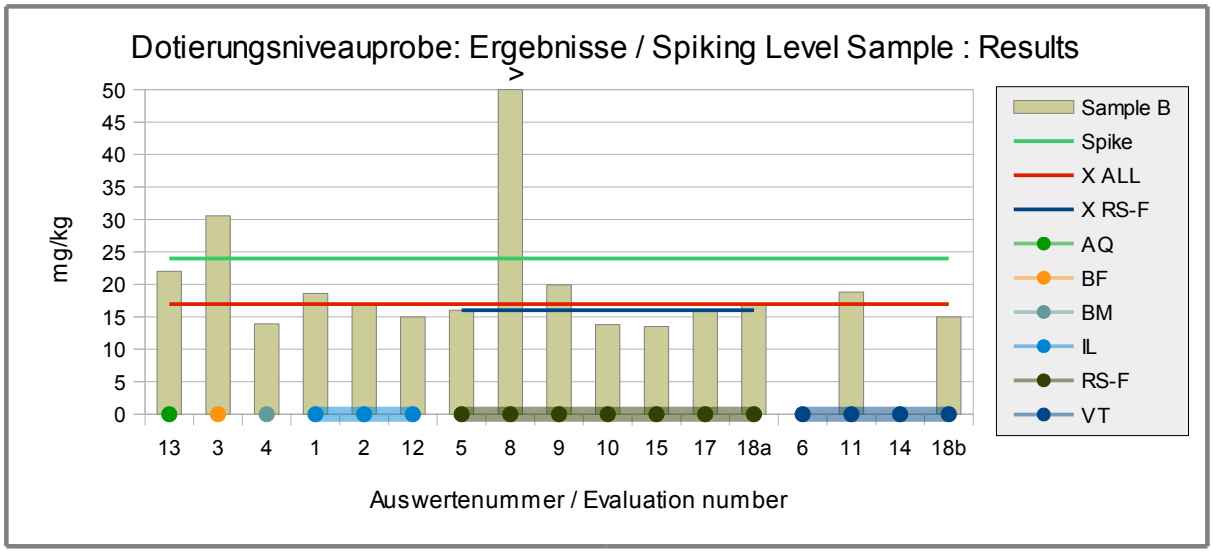

Abb./Fig. 6: ELISA Results Almond
green line = Spiking level
red line = Assigned value robust mean all results
blue line = Assigned value robust mean results method RS-F
round symbols = Applied methods (see legend)

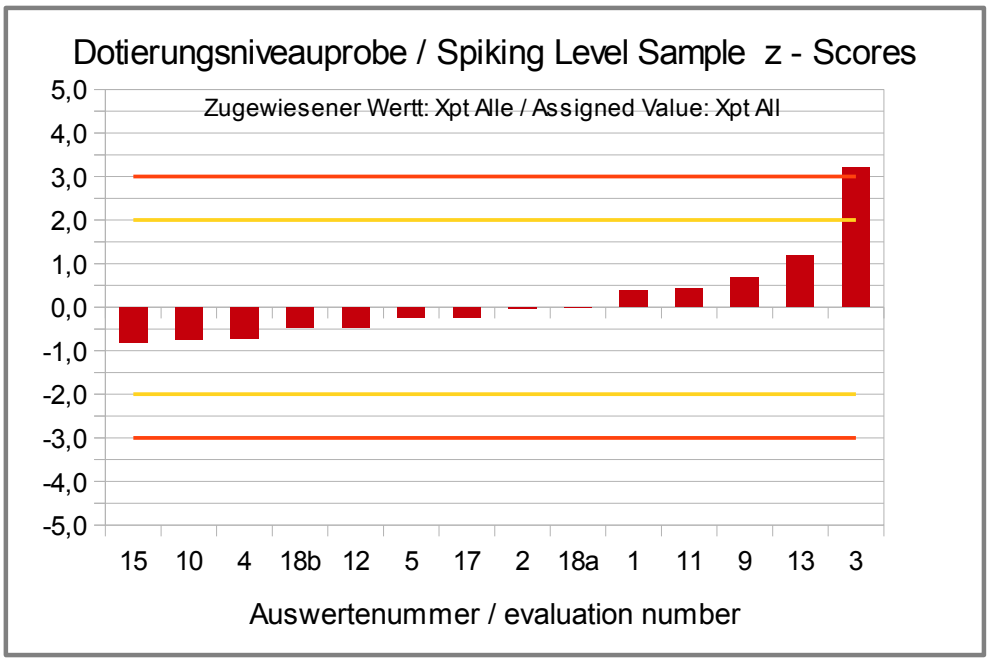

Abb./Fig. 7:
z-Scores (ELISA Results Almond)
Assigned value robust mean of all results

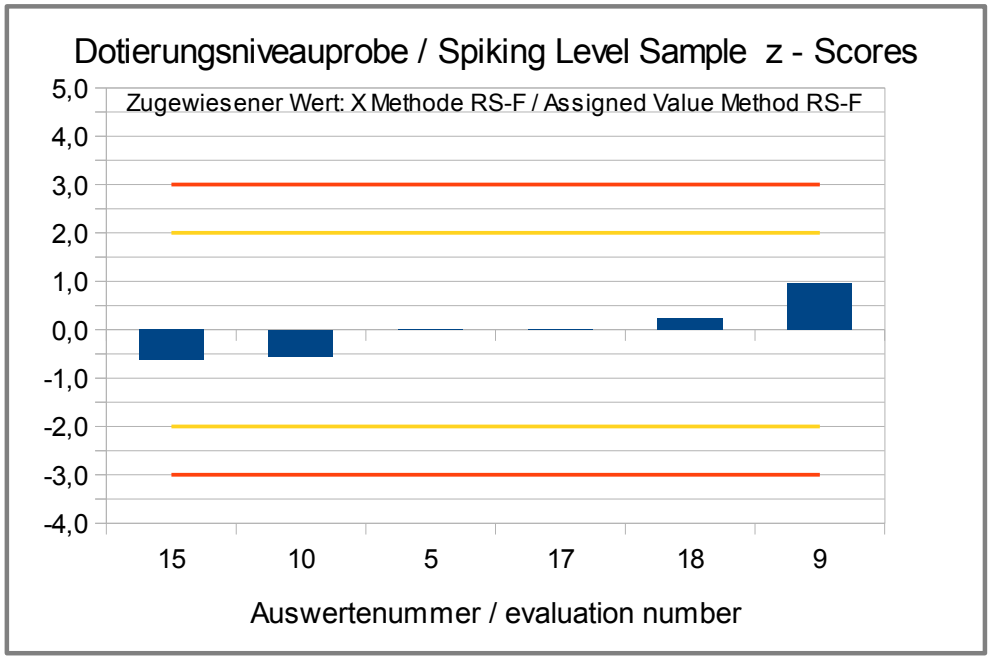

Abb./Fig. 8:
z-Scores (ELISA Results Almond)
Assigned value robust mean of method RS-F (R-Biopharm, Ridascreen® Fast)

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	22,0	**92**	26,0	**89**	AQ	
3	30,6	**127**	41,9	**143**	BF	
4	13,9	**58**	16,8	**57**	BM	
1	18,6	**78**	21,8	**74**	IL	
2	16,8	**70**	22,6	**77**	IL	
12	15,0	**63**	25,0	**85**	IL	
5	16,0	**67**	23,0	**78**	RS-F	
8	85,2	355	147	502	RS-F	result converted °
9	19,9	**83**	16,2	**55**	RS-F	
10	13,8	**58**	22,4	**76**	RS-F	
15	13,5	**56**	13,0	44	RS-F	
17	16,0	**67**	20,0	**68**	RS-F	
18a	17,0	**71**	17,0	**58**	RS-F	
6			41,0	**140**	VT	
11	18,8	**78**	20,1	**69**	VT	
14			11,0	38	VT	
18b	15,0	**63**	16,0	**55**	VT	

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RA**	50-150 %	RA**	50-150 %
Number in RA	**14**	Number in RA	**14**
Percent in RA	**93**	Percent in RA	**82**

* Recovery rate 100% relative size: Almond, s. page 5

** Range of acceptance of AOAC for allergen ELISAS

Methods:
AQ = AgraQuant, RomerLabs
BF = MonoTrace ELISA, BioFront Technologies
BM = AlerTox ELISA, Biomedal
IL = Immunolab
RS-F= Ridascreen® Fast, R-Biopharm
VT = Veratox, Neogen

Comments:
For the spiking level sample 93% 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 82% of the recovery rates were within the range of acceptance.

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		
13	negative		positive		2/2 (100%)	ASU	
16	negative		positive		2/2 (100%)	ASU	
7	negative		positive		2/2 (100%)	MS	
5	negative	< 4	positive	> 4	2/2 (100%)	SFA-ID	
8	negative		positive		2/2 (100%)	SFA-ID	
9	negative	< 1	positive	13,1	2/2 (100%)	SFA-ID	
15	negative		positive		2/2 (100%)	SFA-ID	
18	negative		positive		2/2 (100%)	div	

	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:
ASU = ASU §64 Methode/method
MS = Microsynth
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 PCR: Sample B

No quantitative evaluation was done, because there were < 5 quantitative results.

(Quantitative) Valuation PCR: Spiking Level Sample

No quantitative evaluation was done, because there were < 5 quantitative results.

Evaluation number	Almond	Almond	z-Score Xpt_{ALL}	Method	Remarks
	pos/neg	[mg/kg]			
13	positive			ASU	
16	positive			ASU	
7	positive			MS	
5	positive	> 4		SFA-ID	
8	positive			SFA-ID	
9	positive	7,22		SFA-ID	
15	positive			SFA-ID	
18	positive			div	

Number positive	8	
Number negative	0	
Percent positive	100	
Percent negative	0	
Consensus value	positive	

Methods:

ASU = ASU §64 Methode/method

MS = Microsynth

SFA-ID = Sure Food Allergen ID, 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.

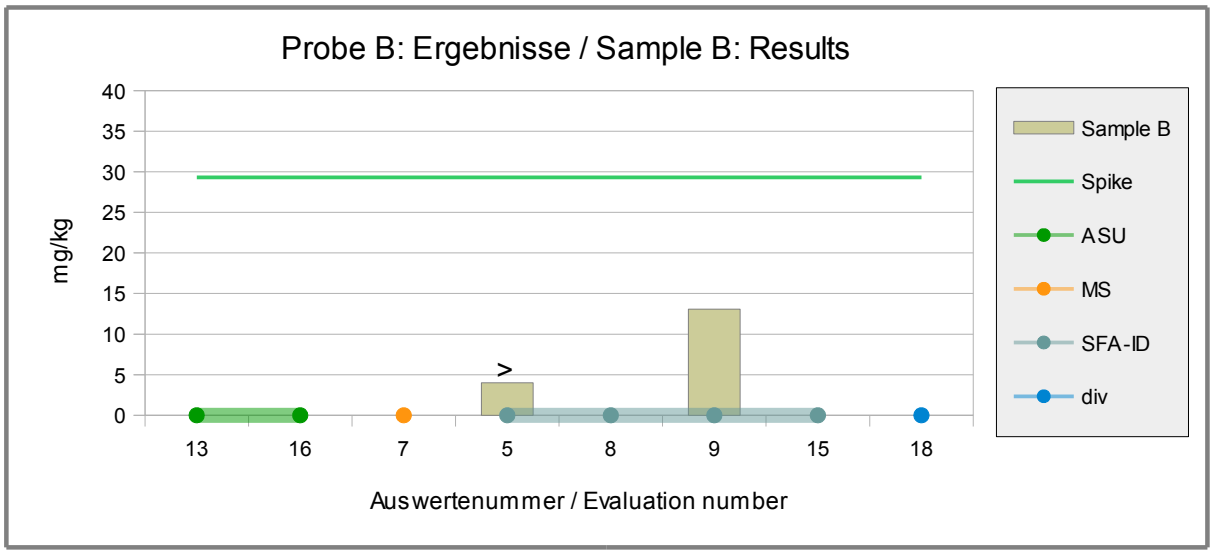

Abb./Fig. 9: PCR Results Almond Sample B
green line = Spiking level
round symbols = Applied methods (see legend)

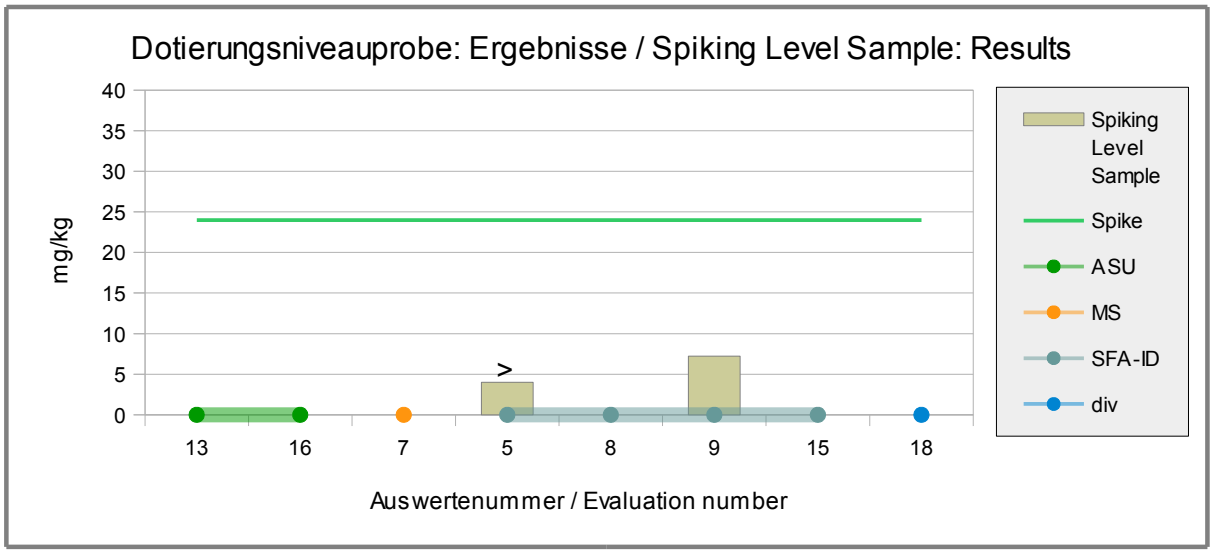

Abb./Fig. 10: PCR Results Almond Spiking Level Sample
green line = Spiking level
round symbols = Applied methods (see legend)

Recovery Rates PCR for Almond:
Spiking Material Sample and Sample B

Evaluation number	Spiking Level Sample	Recovery rate*	Sample B	Recovery rate*	Method	Remarks
	[mg/kg]	[%]	[mg/kg]	[%]		
13					ASU	
16					ASU	
7					MS	
5	> 4		> 4		SFA-ID	
8					SFA-ID	
9	7,22	30	13,1	45	SFA-ID	
15					SFA-ID	
18					div	

RA**	50-150 %	RA**	50-150 %
Number in RA	0	Number in RA	0
Percent in RA	0	Percent in RA	0

* Recovery rate 100% relative size: Almond, s. page 5

** Range of acceptance of AOAC for allergen ELISAS

Methods:
ASU = ASU §64 Methode/method
MS = Microsynth
SFA-ID = Sure Food Allergen ID, R-Biopharm / Congen
div = not indicated / other method

Comments:
One participant submitted quantitative results by PCR. The recovery rates were for the spiking material sample and for the spiked food matrix sample B slightly below the range of the AOAC-recommendation of 50-150% for.

4.2 Proficiency Test Pistachio

4.2.1 ELISA Results: Pistachio

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		
18	negative	< 10	positive	50,0	1/2 (100%)	AQ-P	
9	positive	6,88	positive	111	1/2 (100%)	BC	
3	negative	ND	positive	42,4	1/2 (100%)	BF	
11	negative	< 2,0	positive	55,6	1/2 (100%)	BF	
17	negative	< 2	positive	47,0	1/2 (100%)	BF	
6	negative	< 5	positive	18,0	1/2 (100%)	ET	result converted °
2	positive	9,20	positive	126	1/2 (100%)	IL	
12	positive	5,30**	positive	34,0**	1/2 (100%)	IL	** w eak cross-reactivity to hazelnut (s. documentation)

° calculation p. 19

	Sample A		Sample B	
Number positive	3		8	
Number negative	5		0	
Percent positive	38		100	
Percent negative	63		0	
Consensus value	none		positive	

Methods:

AQ-P = AgraQuant Plus, RomerLabs
BC = BioCheck ELISA
BF = MonoTrace ELISA, BioFront Technologies
ET = Elution Technologies ELISA Kit
IL = Immunolab

Comments:

For the spiked sample a consensus value of 100% positive results were obtained. For sample A 3 positive and 5 negative (< 75%) results were obtained. A weak cross-reactivity of 0,17% against hazelnut is described in the test kit instructions of method IL. Hazelnut is contained in the food matrix of samples A and B.

Quantitative valuation of ELISA-results: Sample B

Evaluation number	Pistachio	z-Score Xpt_{ALL}	Method	Remarks
	[mg/kg]			
18	50,0	0,5	AQ-P	
9	111	5,9	BC	result excluded
3	42,4	-0,2	BF	
11	55,6	1,0	BF	
17	47,0	0,2	BF	
6	18,0	-2,4	ET	result converted °
2	126	7,3	IL	result excluded
12	34,0**	-1,0	IL	** w eak cross-reactivity to hazelnut (s. documentation)

° calculation p. 19

Methodes:

AQ-P = AgraQuant Plus, RomerLabs

BC = BioCheck ELISA

BF = MonoTrace ELISA, BioFront Technologies

ET = Elution Technologies ELISA Kit

IL = Immunolab

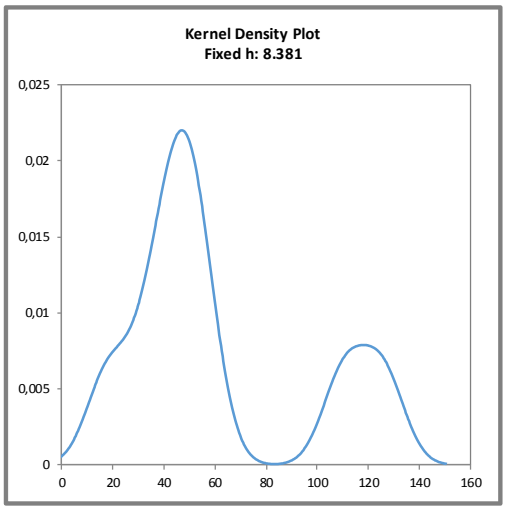

Abb. / Fig. 11:
Kerndichte-Schätzung aller ELISA-Ergebnisse (mit h = 0,75 x σpt von Xpt_{ALL})

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

Comments:
The kernel density estimation shows a main peak with almost symmetrical distribution and a shoulder at approx. 18 mg/kg (method ET) and a side-peak at approx. 100 mg/kg > 100 mg/kg (methods BC and IL) due to two excluded results.

Characteristics: Quantitative evaluation ELISA: Pistachio

Sample B

Statistic Data	**All Results** *[mg/kg]*
Assigned value (X_{pt})	$X_{pt_{ALL}}$
*Number of results ***	6
Number of outliers	0
Mean	41,2
Robust Mean	41,3
Median (X)	**44,7**
Robust standard deviation (S*)	**14,9**
Target range:	
Target standard deviation σ_{pt}	**11,2**
lower limit of target range	**22,35**
upper limit of target range	**67,1**
Quotient S/σ_{pt}*	*1,3*
Standard uncertainty $U_{(Xpt)}$	*7,6*
Quotient $U_{(Xpt)}/\sigma_{pt}$	*0,68*
Results in the target range	*5*
Percent in the target range	*83*

*** without evaluation no. 2 and 9*

Comments to the statistical characteristics and assigned values:

The kernel density estimation showed a main peak with almost symmetrical distribution of results. The results of a side peak at > 100 mg/kg were excluded for the calculation of the statistical data above.

The median was applied as the assigned value (see 3.1). The evaluation of all methods showed a normal variability of results. The quotient S*/σ_{pt} *was well below 2,0. The robust standard deviation was 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. 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 median of the evaluation of all results was 132% of the spiking level of pistachio to sample B within the recommendations for the applied methods (s. 3.4.3 and "Recovery rates of Pistachio" p.43).

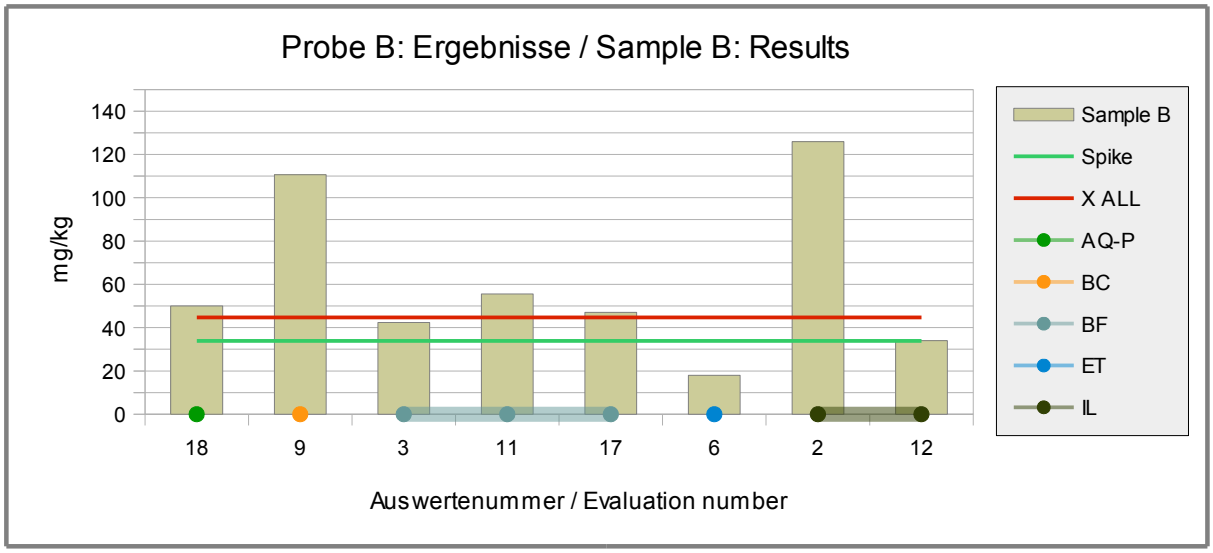

Abb./Fig. 12: ELISA Results Pistachio
green line = Spiking level
red line = Assigned value median of all results
round symbols = Applied methods (see legend)

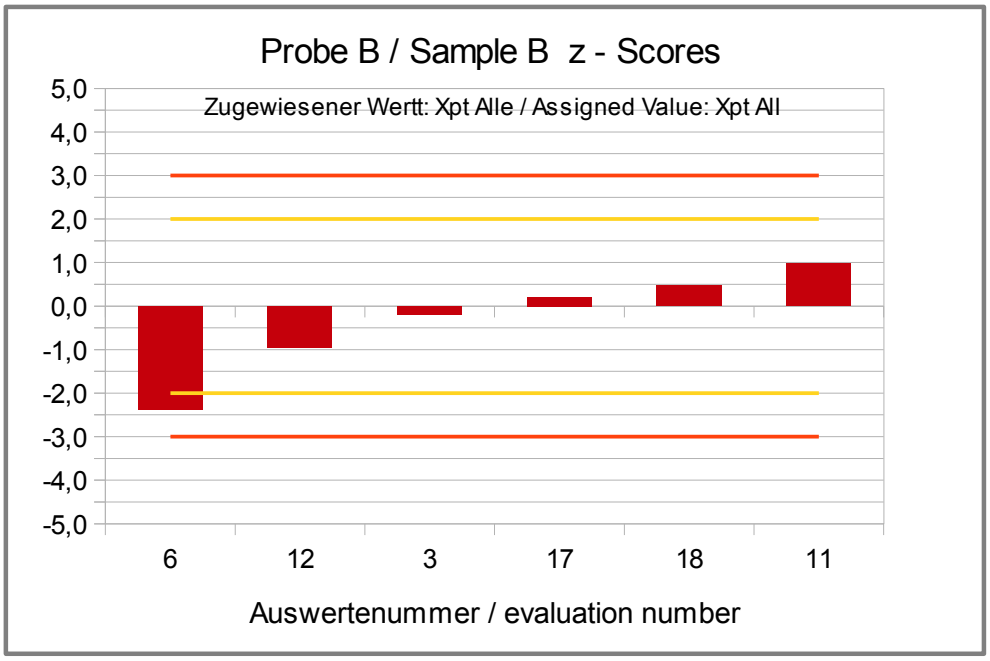

Abb./Fig. 13:
z-Scores (ELISA Results Pistachio)
Assigned value median of all results

Quantitative valuation of results: Spiking level sample

Evaluation number	Pistachio	z-Score Xpt_{ALL}	Method	Remarks
	[mg/kg]			
18	37,0	-1,1	AQ-P	
9	83,4	2,4	BC	
3	44,4	-0,6	BF	
11	52,8	0,1	BF	
17	> 80		BF	
6			ET	
2	88,2	2,8	IL	
12	51,0	-0,1	IL	** weak cross-reactivity to hazelnut (s. documentation)

° calculation p. 19

Methods:

AQ-P = AgraQuant Plus, RomerLabs

BC = BioCheck ELISA

BF = MonoTrace ELISA, BioFront Technologies

ET = Elution Technologies ELISA Kit

IL = Immunolab

Comments:

A kernel density estimation was not done, because there were < 8 results.

Characteristics: Quantitative evaluation Pistachio

Spiking level sample

Statistic Data	**All Results** *[mg/kg]*
Assigned value (X_{pt})	$X_{pt_{ALL}}$
Number of results	6
Number of outliers	0
Mean	59,5
Robust Mean	59,5
Median (X)	**51,9**
Robust standard deviation (S*)	**24,0**
Target range:	
Target standard deviation σ_{pt}	**13,0**
lower limit of target range	**26,0**
upper limit of target range	**77,9**
Quotient S/σ_{pt}*	*1,9*
Standard uncertainty $U_{(Xpt)}$	*12,3*
Quotient $U_{(Xpt)}/\sigma_{pt}$	*0,95*
Results in the target range	*4*
Percent in the target range	*67*

Comments to the statistical characteristics and assigned values:

A kernel density estimation was not done due to the low number of results.

The median was applied as the assigned value (see 3.1). The evaluation of all methods showed a normal variability of results. The quotient S/σ_{pt} was well below 2,0. The robust standard deviation was 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. 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 median of the evaluation of all results was 179% of the spiking level of pistachio to the spiking level sample and thus above the recommendations for the applied methods (s. 3.4.3 and "Recovery rates of Pistachio" p.43).

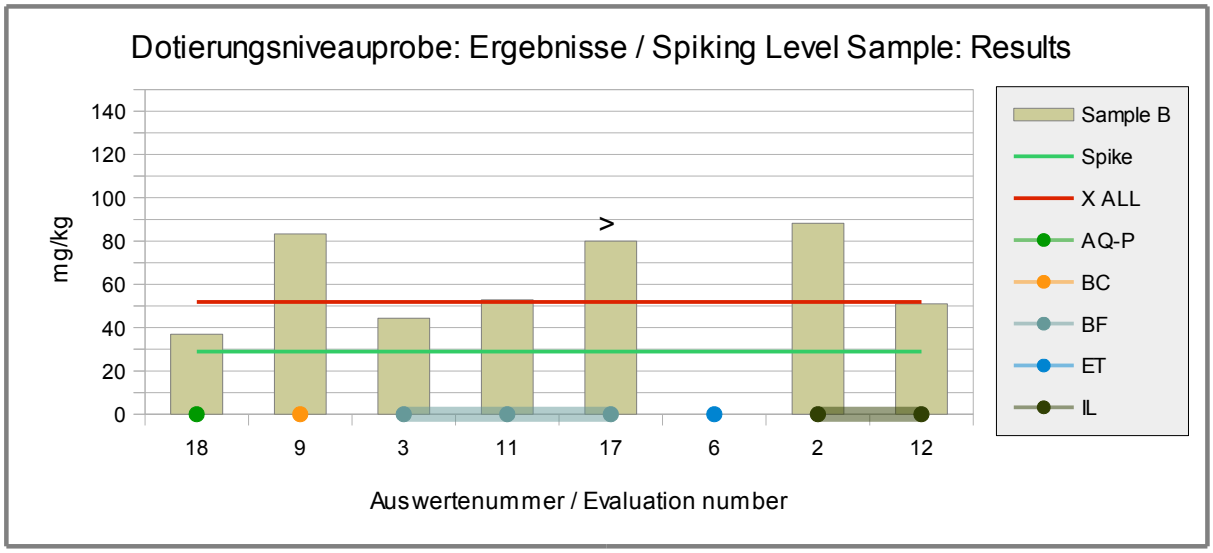

Abb./Fig. 14: ELISA Results Pistachio
green line = Spiking level
red line = Assigned value robust mean all results
round symbols = Applied methods (see legend)

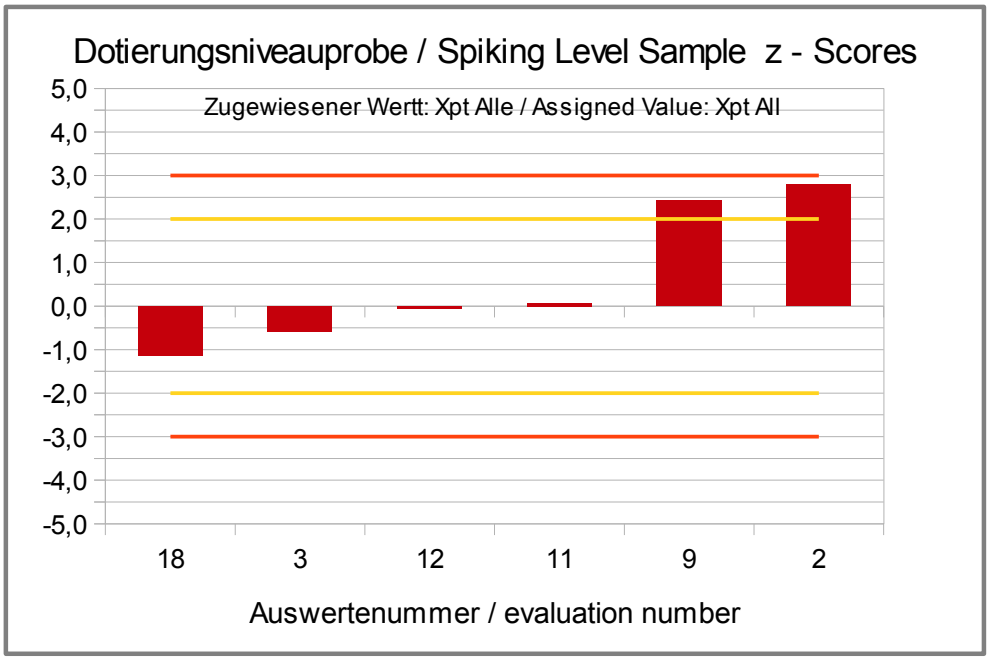

Abb./Fig. 15:
z-Scores (ELISA Results Pistachio)
Assigned value robust mean of all results

Recovery Rates ELISA for Pistachio:
Spiking level Sample and Sample B

Evaluation number	Spiking Level Sample	Recovery rate*	Sample B	Recovery rate*	Method	Remarks
	[mg/kg]	[%]	[mg/kg]	[%]		
18	37,0	**128**	50,0	**148**	AQ-P	
9	83,4	288	111	327	BC	
3	44,4	153	42,4	**125**	BF	
11	52,8	182	55,6	164	BF	
17	>80		47,0	**139**	BF	
6			18,0	**53**	ET	result converted °
2	88,2	304	126	372	IL	
12	51,0	176	34	**100**	IL	** weak cross-reactivity to hazelnut (s. documentation)

° calculation p. 19

RA**	50-150 %	RA**	50-150 %
Number in RA	**1**	Number in RA	**5**
Percent in RA	**17**	Percent in RA	**63**

* Recovery rate 100% relative size: Pistachio, s. page 5

** Range of acceptance of AOAC for allergen ELISAS

Methods:
AQ-P = AgraQuant Plus, RomerLabs
BC = BioCheck ELISA
BF = MonoTrace ELISA, BioFront Technologies
ET = Elution Technologies ELISA Kit
IL = Immunolab

Comments:
For the spiking level sample one of the participants obtained a recovery rate by ELISA methods within the range of the AOAC-recommendation of 50-150%. All other recovery rates were above 150%.
For the spiked food matrix sample B 63% of the recovery rates were within the range of acceptance, and 3 other were above 150%.
It should be noted for evaluation numbers 9, 2 and 12, that small amounts were determined in sample A (not spiked) which may cause higher recovery rates for sample B.

4.2.2 PCR Results: Pistachio

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		
5	negative	< 0,4	positive	> 0,4	2/2 (100%)	SFA-ID	
10	negative		positive		2/2 (100%)	SFA-ID	
8	negative		positive	9,6	2/2 (100%)	SFA-Q	
13	negative		positive		2/2 (100%)	div	
16	negative		positive		2/2 (100%)	div	
18	negative		positive		2/2 (100%)	div	

	Sample A		Sample B	
Number positive	0		6	
Number negative	6		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 PCR: Sample B

No quantitative evaluation was done, because there were < 5 quantitative results.

(Quantitative) Valuation PCR: Spiking Level Sample

No quantitative evaluation was done, because there were < 5 quantitative results.

Evaluation number	Pistachio	Pistachio	Method	Remarks
	pos/neg	[mg/kg]		
5	positive	> 0,4	SFA-ID	
10	positive		SFA-ID	
8	positive	5,7	SFA-Q	
13	positive		div	
16	positive		div	
18	positive		div	

Number positive	6
Number negative	0
Percent positive	100
Percent negative	0
Consensus value	positive

Methods:

SFA-ID = Sure Food Allergen ID, 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.

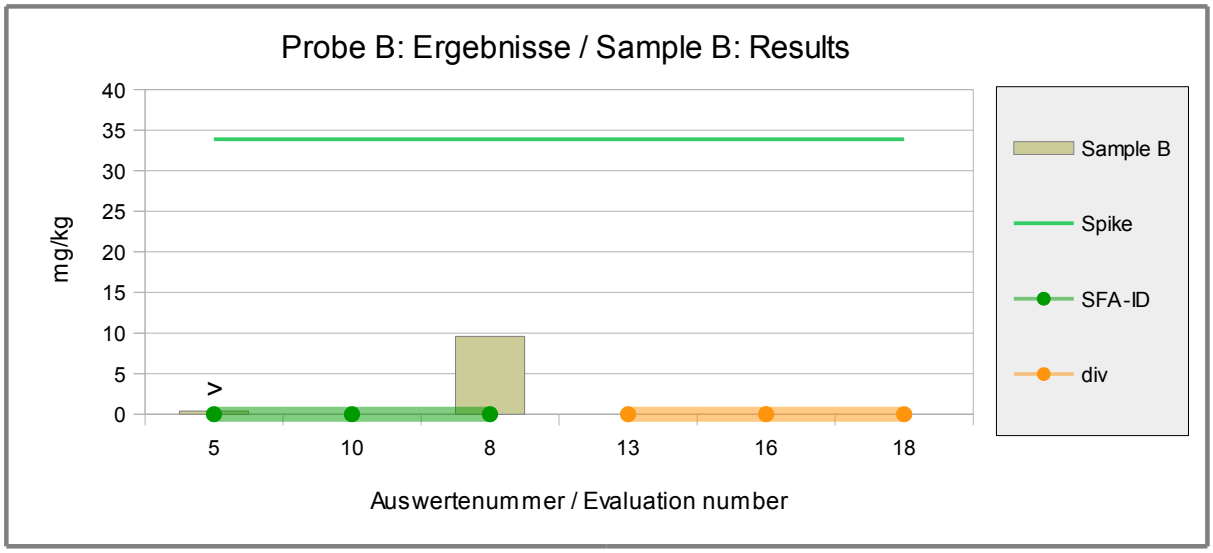

Abb./Fig. 16: PCR Results Pistachio Sample B
green line = Spiking level
round symbols = Applied methods (see legend)

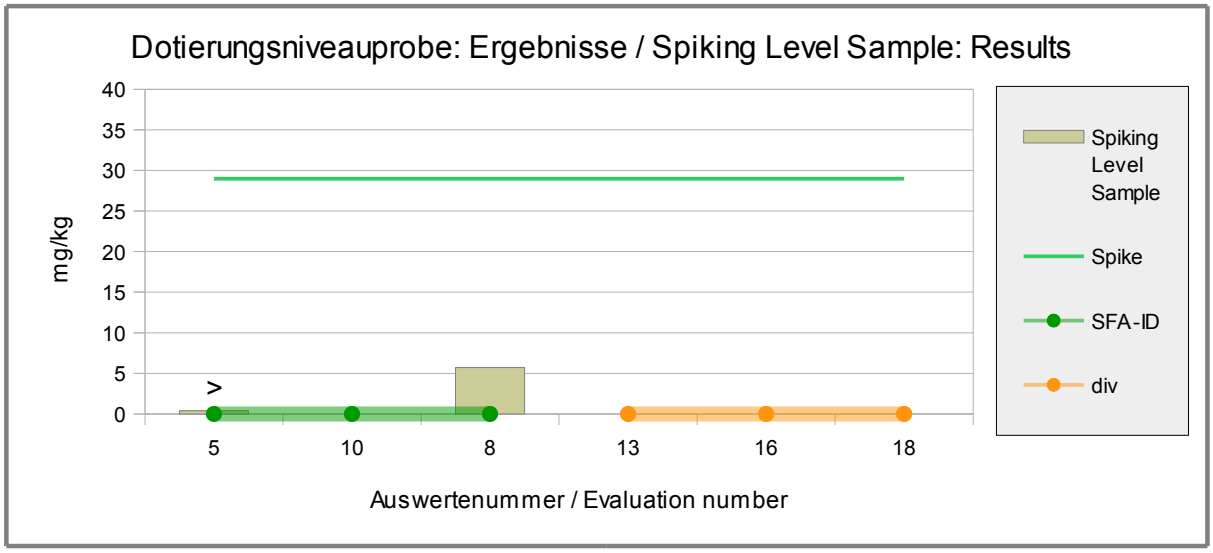

Abb./Fig. 17: PCR Results Pistachio Spiking Level Sample
green line = Spiking level
round symbols = Applied methods (see legend)

Recovery Rates PCR for Pistachio:
Spiking Material Sample and Sample B

Evaluation number	Spiking Level Sample	Recovery rate*	Sample B	Recovery rate*	Method	Remarks
	[mg/kg]	[%]	[mg/kg]	[%]		
5	> 0,4		> 0,4		SFA-ID	
10					SFA-ID	
8	5,7	20	9,6	28	SFA-Q	
13					div	
16					div	
18					div	

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

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

* Recovery rate 100% relative size: Pistachio, s. page 5

** Range of acceptance of AOAC for allergen ELISAS

Comments:
One participant submitted quantitative results by PCR. The recovery rates were for the spiking material sample and for the spiked food matrix sample B below the range of the AOAC-recommendation of 50-150% for.

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 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	13	06.10.17	positive	0,5	positive	26	positive	22	Almond	AgraQuant ELISA Almond COKAL0748, RomerLabs
BF	3	07.10.17	negative	ND	positive	41,93	positive	30,57	Almond	MonoTrace Almond ELISA kit, BioFront Technologies
BM	4	26.10.17	positive	0,53	positive	16,79	positive	13,91	Almond	ALERTOX ELISA ALMOND / KT-5910 / BIOMEDAL DIAGNOSTICS
IL	1	24.10.17	-	<0,4	-	21,8	-	18,6	Almond	Immunolab Almond ELISA
IL	2	17.10.17	negative	< 0,4	positive	22,6	positive	16,8	Almond	Immunolab Almond ELISA
IL	12	09.10.17	negative	< 1	positive	25	positive	15	Almond	Immunolab Almond ELISA
RS-F	5		negative	<1.2	positive	23	positive	16	Almond	R6901 RIDASCREEN FAST Almond R-Biopharm
RS-F	8	19.10.17	negative		positive	23,8	positive	13,8	Almond protein	Ridascreen® FAST Almond R6901, R-Biopharm
RS-F	9	17.10.17	negative	<2.5	positive	16,16	positive	19,9	Almond	Ridascreen® FAST Almond R6901, R-Biopharm
RS-F	10	27.10.	negative	< 2,5	positive	22,4	positive	13,8	Almond	RIDASCREEN® FAST Almond, R6901, R-Biopharm
RS-F	15		negative	< 2,5	positive	13	positive	13,5	Food item Almond	Ridascreen® FAST Almond R6901, R-Biopharm
RS-F	17	17.10.17	negative	<2,5	positive	>20	positive	16	Almond	Ridascreen® FAST Almond R6901, R-Biopharm
RS-F	18a	25.10./14.11.17	negative	<2.5	positive	17	positive	17	Almond	Ridascreen® FAST Almond R6901, R-Biopharm
VT	6	2017-10-13 et 20	negative	<2,5	positive	41	-		Almond	Veratox Almond, Neogen
VT	11	30/10	positive	3,8	positive	20,1	positive	18,8	Almond	Veratox Almond, Neogen
VT	14	17.10.17	negative	<2.5	positive	11	-		Almond	Veratox Almond, Neogen
VT	18b	09.11./13.11.17	negative	<2.5	positive	16	positive	15	Almond	Veratox Almond, Neogen

***Continuation ELISA Almond*:**

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	
AQ	13		As Per Kit Instructions	yes	
BF	3	Monoclonal Antibody	1:20 extraction ratio, 10 minutes @ 60C	no	ND = not detected
BM	4	CROSS REACTION Almond 100% Peach k. 15.95%, Plum k. 9.82%, Sweet cherry 1.74%, Mahaleb k. 1.42% Chili p. 0.00018%CROSS REACTION Almond 100%, Peach k. 15.95% Plum k. 9.82%, Sweet cherry 1.74% Mahaleb k. 1.42%, Chili p. 0.00018%	SAMPLE SIZE: 0,5G/ EXTRACTION SOLUTION SIZE: 10ml / INCUBATION TEMPERATURE 60oC FOR 15min / ELISA FILTER: 450nm /	YES	
IL	1			no	
IL	2			yes	
IL	12				
RS-F	5				
RS-F	8			yes	
RS-F	9	As Per Kit Instructions	As Per Kit Instructions	Yes	
RS-F	10		As Per Kit Instructions	yes	
RS-F	15	Almond protein	As Per Kit Instructions	yes	
RS-F	17			yes	
RS-F	18a				limit of detection: 2.5 mg/kg
VT	6			yes	
VT	11			yes	
VT	14		125mL PBS / 15min / 60°C	yes	
VT	18b				limit of detection: 2.5 mg/kg

5.1.2 ELISA: Pistachio

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-P	18	23.11./30.11.17	negative	<10	positive	50	positive	37	Pistachio	AgraQuant Plus ELISA Pistachio COKAL2748F, RomerLabs
BC	9	17.10.17	positive	6,88	positive	110,66	positive	83,36	Pistachio	BioCheck ELISA Pistachio-Check
BF	3	14.11.17	negative	ND	positive	42,4	positive	44,35	Pistachio	MonoTrace Pistachio ELISA kit, BioFront Technologies
BF	11	30/10	negative	<2.0	positive	55,6	positive	52,8	Pistachio	MonoTrace Pistachio ELISA kit, BioFront Technologies
BF	17	13.10.17	negative	<2	positive	47	positive	>80	Pistachio	BioFront
ET	6	07.11.17	negative	<1	positive	3,9	-		Pistachio protein	Elution Technologies ELISA Kit Pistachio Protein E-75PST
IL	2	16.11.17	positive	9,2	positive	125,9	positive	88,2	Pistachio	Immunolab Pistachio ELISA
IL	12	09.10.17	positive**	5,3	positive	34**	positive	51	Pistachio	Immunolab Pistachio ELISA

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	
AQ-P	18				limit of detection: 10 mg/kg
BC	9	As Per Kit Instructions	As Per Kit Instructions	Yes	
BF	3	Monoclonal Antibody	1:10 extraction ratio, 10 minutes @ 60C	no	ND = not detected
BF	11			no	
BF	17			no	
ET	6			yes	
IL	2			yes	
IL	12				** Test has weak cross-reactivity against hazelnut, sample A is eventually negative, therefore sample B is not linear in dilution

5.1.3 PCR: Almond

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
ASU	13	24.10.17	negative		positive		positive		Almond-DNA	§64 ASU L 18.00-22
ASU	16		negative		positive		positive			ASU §64 Methode/method
MS	7	25.10.17	negative		positive		positive		Almond-DNA	Microsynth
SFA-ID	5		negative	<4	positive	> 4	positive	> 4	DNA Almond	SureFood® ALLERGEN Almond Art.-No. S3104 Congen
SFA-ID	8	19.10.17	negative		positive		positive		Almond	Sure Food Allergen ID, R-Biopharm / Congen
SFA-ID	9	07.10.17	negative	<1	positive	13,08	positive	7,22	Almond	Sure Food Allergen ID, R-Biopharm / Congen
SFA-ID	15		negative		positive		positive		Almond	SureFood®Allergen ID AlmondFa. r-biopharm (S3104)
div	18	14./17./21.11.17	negative		positive		positive			realtime PCR

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	13		Extraction with Macherey & Nagel NucleoSpin Food Kit, 2 g sample weight, as Singleplex applied	yes	
ASU	16	non specific lipid transfer protein	Silica-Säulchen	no	
MS	7	Almond	Macherey Nagel Nucleo Spin Food optimized: increased sample weight, buffer change (washing step with Lysis Buffer) Rnase step, Chloroform step, 2xCQW; RealTime PCR with 45 cycles, Decontamination step with UNG; Inhibition control	yes	LOD: 0,005% DNA
SFA-ID	5				
SFA-ID	8			yes	LOD 4 ppm
SFA-ID	9	As Per Kit Instructions	As Per Kit Instructions	No	
SFA-ID	15		Dneasy Mericon Food Kit/ Proteinase K/ Real Time PCR/ 45 cycles	yes	
div	18	nsLTP			limit of detection: 10-20 DNA copies

5.1.4 PCR: Pistachio

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	5		negative	<0.4	positive	> 0.4	positive	> 0.4	DNA Pistachio	SureFood® ALLERGEN Pistachio Art.-No. S3114 Congen
SFA-ID	10	13.10.	negative		positive		positive		Pistachio	SureFood® Allergen ID Pistachio, R3114, R-Biopharm/Congen
SFA-Q	8	19.10.17	negative		positive	9,6	positive	5,7	Pistachio	Sure Food Allergen Quant, R-Biopharm / Congen
div	13	16.10.17	negative		positive		positive		Pistachio-DNA	Köppel et al 2012
div	16		negative		positive		positive			Hausmethode
div	18	17.11.17	negative		positive		positive			realtime PCR

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-ID	5				
SFA-ID	10		SureFood® PREP Allergen, Protocol 2, S1053, R-Biopharm/Congen	yes	
SFA-Q	8			no	LOD 0,4 ppm, LOQ 1 ppm
div	13	Dehydrin Y07600	Extraction with Macherey & Nagel NucleoSpin Food Kit, 2 g sample weight	yes	
div	16	Pistachio	Silica columns	no	
div	18	COR			limit of detection: 10-20 DNA copies

5.2 Homogeneity

5.2.1 Mixture homogeneity before bottling

Microtracer Homogenitätstest
DLA 06-2017 Spiking Level Sample

Weight whole sample	1,56	kg
Microtracer	FSS-rot lake	
Particle size	75 – 300	µm
Weight per particle	2,0	µg
Addition of tracer	34,6	mg/kg

Result of analysis

Sample	Weight [g]	Particle number	Particles [mg/kg]
1	5,02	152	60,6
2	5,01	162	64,7
3	5,03	157	62,4
4	4,97	139	55,9
5	5,03	149	59,2
6	4,98	127	51,0
7	5,12	154	60,2
8	5,06	159	62,8

Poisson distribution		
Number of samples	8	
Degree of freedom	7	
Mean	150	Particles
Standard deviation	11,0	Particles
χ^2 (CHI-Quadrat)	5,60	
Probability	**59**	%
Recovery rate	172	%

Normal distribution		
Number of samples	8	
Mean	59,6	mg/kg
Standard deviation	4,36	mg/kg
rel. Standard deviaton	7,31	%
Horwitz standard deviation	8,65	%
HorRat-value	**0,85**	
Recovery rate	172	%

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 06-2017***
PT name	***Allergens VI: Almond and Pistachio in Spread (Cocoa Cream)***
Sample matrix (processing)	***Samples A + B:*** *Nut-Nougat Spread / ingredients: sugar, vegetable oil, hazelnuts, low fat cocoa, skimmed milk powder, emulsifier: lecithins (soya), vanillin, 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: cooled 2 - 10°C (long term < -18°C)* *Spiking Level Sample: room temperature*
Intentional use	*Laboratory use only (quality control samples)*
Parameter	*qualitative + quantitative:* *Almond, Pistachio (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. It is the best to homogenize the whole sample.*
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 November 17th 2017***
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, PhD*

* Control of mixture homogeneity and qualitative testings are carried out by DLA. 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

[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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