Evaluation Report
proficiency test

DLA ptAL08 (2021)

Allergens VIII:

Buckwheat, Almond and Macadamia

in Cereal Muesli

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

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Unteraufträge *Subcontractors*	Im Rahmen dieser Eignungsprüfung wurden nachstehende Leistungen im Unterauftrag vergeben: Homogenitätsprüfung der EP-Parameter, Proteinbestimmung As part of the present proficency test the following services were subcontracted: Homogeneity tests of PT-parameter(s), protein determination
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.
Akkreditierung *Accreditation* ilac-MRA DAkkS Deutsche Akkreditierungsstelle D-EP-21534-01-00	nach / according to: ISO/IEC 17.043-2010 Konformitätsbewertung - Allgemeine Anforderungen an Eignungsprüfungen Conformity Assessment - General Requirements for Proficiency Testing Die Akkreditierung gilt für den in der Urkundenanlage genannten Umfang. The accreditation is valid for the scope of the annex to the certificate of accreditation

Contents

1. Introduction

The participation in proficiency test (PT) 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 mixture of commercially available cereal mueslis with fruits and oatmeal from European manufacturers. The basic composition was the same for both samples A and B (see Table 1).
After crushing and sieving (mesh 1,5 mm) of the raw materials, the basic mixture was homogenized.

Afterwards, the **spiked sample A** was produced as follows:
The spiking materials containing the allergenic ingredients buckwheat, almond and macadamia were sieved, then added to an aliquot of the base matrix and the mixture was homogenized. Subsequently, basic matrix was again added in portions in further steps and in each case homogenized until the total amount was reached.
For the **spiking level sample**, the allergenic compounds mentioned above were added during a multi-stage addition of potato powder (mesh 500 µm) and homogenization.

Samples A and B were filled into metallized PET foil bags in portions of approx. 25 g and the spiking level sample of approx. 15 g.

Table 1: Composition of DLA-Samples

Ingredients	**Sample A**	**Sample B**	**Spiking Level Sample**
Muesli Crunchy Fruits, organic Ingredients: Oatmeal, sugar, sunflower oil, puffed wheat, black currant juice concentrate, freeze-dried berries (raspberries, strawberries, blackberries), salt Nutrients per 100 g: Fat 14 g, Carbohydrates 63 g, Protein 10 g	33,3 g/100 g	33,3 g/100 g	-
Porridge Berry, organic Ingredients: Wholemeal oat flakes, dried dates (dates, rice flour), dried and sweetened cranberries (cranberries, apple juice concentrate, sunflower oil), freeze-dried raspberries, beetroot juice concentrate, freeze-dried raspberry powder Nutrients per 100 g: Fat 5,8 g, Carbohydrates 60 g, Protein 13 g	33,3 g/100 g	33,3 g/100 g	-
Muesli Crunchy Oats, organic Ingredients: Wholemeal oat flakes, raw cane sugar, sunflower oil, rice extrudate (rice flour, barley malt flour, sea salt), rice syrup, grated coconut Nutrients per 100 g: Fat 14 g, Carbohydrates 64 g, Protein 10 g	33,3 g/100 g	33,3 g/100 g	-
Potato Powder Ingredients: Potatoes, E471, E304, E223, E100	-	-	99,9 g/100 g
Buckwheat - as buckwheat* - *thereof 12,2% total protein***	 20,2 mg/kg 2,46 mg/kg	-	 20,8 mg/kg 2,53 mg/kg
Macadamia - as macadamia* - *thereof 8,0% total protein***	 10,2 mg/kg 0,82 mg/kg	-	 9,22 mg/kg 0,74 mg/kg
Almond, roasted milled, mixture (23 products from USA, Europe, Australia, Western Asia) - as almond* - *thereof 21,1% total protein***	 13,9 mg/kg 2,94 mg/kg	-	 15,5 mg/kg 3,27 mg/kg
further Ingredients: *Maltodextrin, sodium sulfate and silicon dioxide*	<0,3 g/100 g	-	<0,3 g/100 g

* Allergen contents as "total food" as described in the column ingredients according to the gravimetric mixture
** Protein contents according to laboratory analysis of the raw material (total nitrogen according to Kjeldahl with F=6,25 for buckwheat protein, F=5,30 for macadamia protein and F=5,18 for almond protein)

Note: *The metrological traceability of temperature, mass and volume during the 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 sample A and the spiking level sample showed a probability of 21% and 97%, respectively. 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]. HorRat values of 1,8 and 0,59 were obtained in this PT. The higher HorRat value was accepted based on the probability results and the following homogeneity tests by ELISA. The results of microtracer analysis are given in the documentation.

Homogeneity of bottled spiked sample A

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 (exception: Morinaga ELISA II performed by DLA). 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 A by all ELISA tests for buckwheat, macadamia and almond (Morinaga, Immunolab, AgraQuant, Veratox) (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].

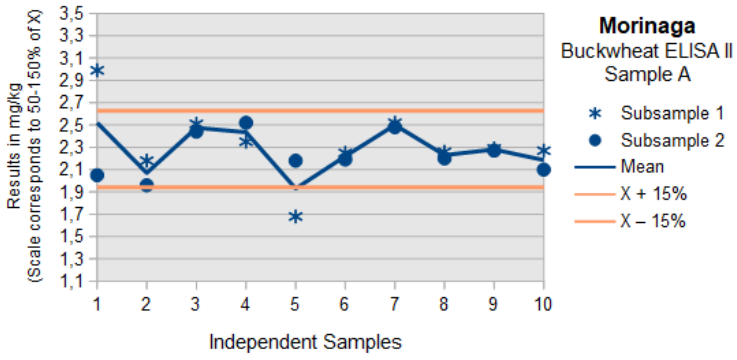
ELISA-Tests: Homogenität Buchweizen / Homogeneity Buckwheat

Morinaga Buckwheat ELISA Kit II

Sample weights: 1,0 g (0,9 – 1,1 g)
Number of replicates: 2
Overall result: Buckwheat Protein 2,28 ± 0,15 mg/kg

Sample A	Subsample 1 mg/kg	Subsample 2 mg/kg	Mean mg/kg
1	2,99	2,05	2,52
2	2,18	1,96	2,07
3	2,51	2,44	2,48
4	2,35	2,52	2,44
5	1,68	2,18	1,93
6	2,25	2,19	2,22
7	2,52	2,48	2,50
8	2,26	2,20	2,23
9	2,29	2,27	2,28
10	2,27	2,10	2,19

General average X	2,28	
SD of sample means Sx	0,197	8,6%
SD within-samples Sw	0,177	7,8%
SD between-samples Ss	0,152	6,7%



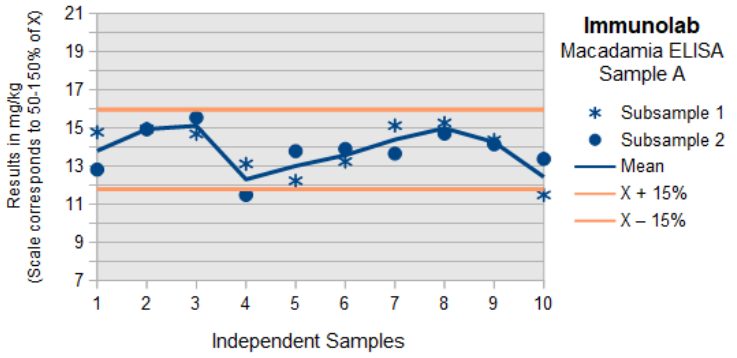
ELISA-Tests: Homogenität Macadamia / Homogeneity Macadamia

Immunolab Macadamia ELISA

Sample weights: 1,0 g (0,9 – 1,1 g)
Number of replicates: 2
Overall result: Macadamia 13,9 ± 0,94 mg/kg

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

General average X	13,9	
SD of sample means Sx	1,04	7,5%
SD within-samples Sw	0,636	4,6%
SD between-samples Ss	0,936	6,7%



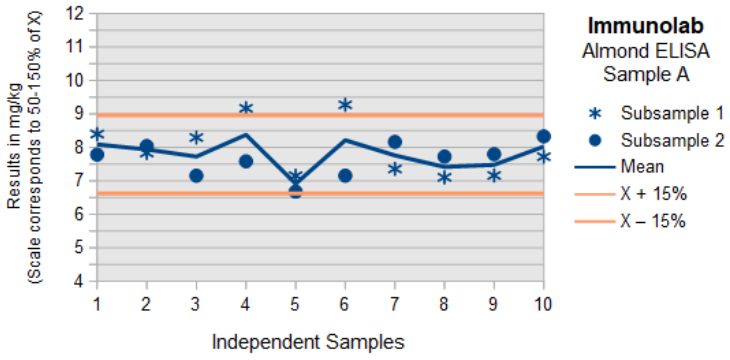
ELISA-Tests: Homogenität Mandel / Homogeneity Almond

Immunolab Almond ELISA

Sample weights: 1,0 g (0,9 – 1,1 g)
Number of replicates: 2
Overall result: Almond 7,79 ± 0,23 mg/kg

Sample A	Subsample 1 mg/kg	Subsample 2 mg/kg	Mean mg/kg
1	8,39	7,78	8,09
2	7,83	8,04	7,93
3	8,29	7,15	7,72
4	9,17	7,58	8,37
5	7,15	6,67	6,91
6	9,27	7,15	8,21
7	7,35	8,16	7,76
8	7,10	7,72	7,41
9	7,16	7,79	7,48
10	7,72	8,33	8,02

General average X	7,79	
SD of sample means Sx	0,434	5,6%
SD within-samples Sw	0,519	6,7%
SD between-samples Ss	0,232	3,0%

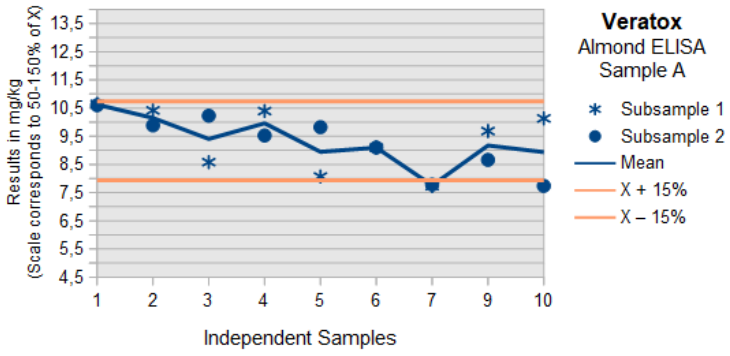


Veratox Almond ELISA

Sample weights: 5,0 g (4,5 – 5,5 g)
Number of replicates: 2
Overall result: Almond 9,34 ± 0,72 mg/kg

Sample A	Subsample 1 mg/kg	Subsample 2 mg/kg	Mean mg/kg
1	10,6	10,6	10,6
2	10,4	9,88	10,15
3	8,58	10,2	9,40
4	10,4	9,53	9,96
5	8,07	9,82	8,95
6	9,09	9,10	9,10
7	7,73	7,78	7,76
9	9,68	8,66	9,17
10	10,1	7,74	8,94

General average X	9,34	
SD of sample means Sx	0,837	9,0%
SD within-samples Sw	0,615	6,6%
SD between-samples Ss	0,715	7,7%

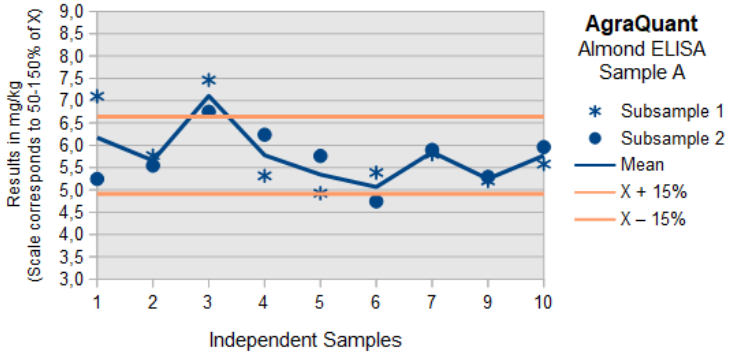


AgraQuant Almond ELISA

Sample weights: 1,0 g (0,9 – 1,1 g)
Number of replicates: 2
Overall result: Almond 5,78 ± 0,53 mg/kg

Sample A	Subsample 1 mg/kg	Subsample 2 mg/kg	Mean mg/kg
1	7,10	5,25	6,17
2	5,77	5,55	5,66
3	7,46	6,75	7,11
4	5,32	6,24	5,78
5	4,93	5,76	5,34
6	5,39	4,74	5,07
7	5,80	5,90	5,85
9	5,20	5,29	5,25
10	5,58	5,96	5,77

General average X	5,78	
SD of sample means Sx	0,604	10,5%
SD within-samples Sw	0,412	7,1%
SD between-samples Ss	0,529	9,2%



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 PT samples was approx. 0,40 (19,2°C) and 0,36 (19,3°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 2021. The testing method was optional. The tests should be finished at 04 February 2022 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 Buckwheat, Almond and Macadamia in the range of mg/kg in the matrix of Cereal Muesli. 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 and should be analysed like a normal sample.

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, limit of quantifications, test kit manufacturer and remarks 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 16 participants submitted at least one result.

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 methods for the quantitative 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*) 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^*_{ALL}***
ii) ***Robust standard deviation of single methods - $S^*_{METHOD\ i}$***
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]. Also, 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. 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]:

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

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 evaluation 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 to the square root of quadrat sum of the target standard deviation (σ_{pt}) and the standard uncertainty ($U_{(Xpt)}$) [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*/σ_{pt}

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 and traceability

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 of assigned values

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 participants' 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.
The corresponding z-scores were calculated according to 3.5 with the target standard deviation of 25% (see 3.4.3).

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 are reported then.

In the result chapter all quantitative results of the participants are displayed formatted to 3 decimal places (valid digits). 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.

The ELISA results, which were given as **almond-** and **macadamia protein,** have been converted to the **total food (almond, macadamia)** using the experimentally determined protein content of the raw materials (see page 5). The ELISA results for **buckwheat** were consistently given as **protein** and evaluated without conversion.

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.

If 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 $U_{(Xpt)}$		
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 Buckwheat

4.1.1 ELISA Results: Buckwheat (as protein)

Qualitative valuation of the 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		
14	positive	3,18	negative	<2.5	2/2 (100%)	ES	
4	positive	2,58	negative		2/2 (100%)	MI-II	
15	positive	3,00	negative	<1	2/2 (100%)	MI-II	

	Sample A		Sample B	
Number positive	3		0	
Number negative	0		3	
Percent positive	100		0	
Percent negative	0		100	
Consensus value	positive		negative	

Methods:
ES = ELISA-Systems
MI-II = Morinaga Institute ELISA Kit II

Comment:
The consensus values are in qualitative agreement with the spiking of sample A.

Quantitative evaluation of ELISA-results: Sample A

The quantitative results were not evaluated because too few single results were available.

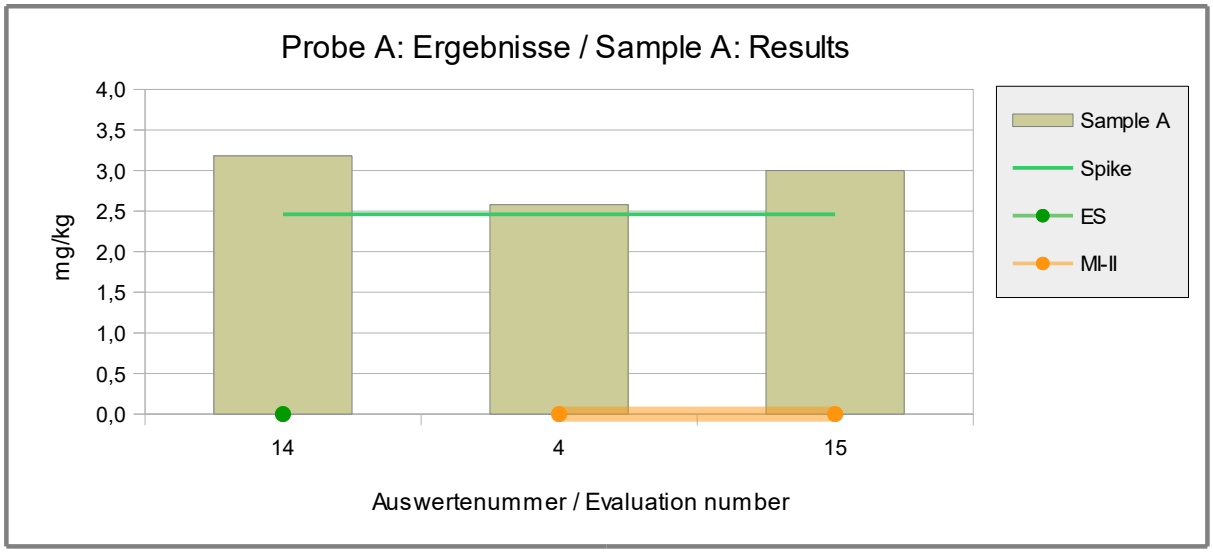


Abb./Fig. 1: ELISA Results Buckwheat (as protein)
green line = Spiking level (Spike)
round symbols = Applied methods (see legend)

Quantitative evaluation of ELISA-results: Spiking Level Sample

The quantitative results were not evaluated because too few single results were available.

Evaluation number	Buckwheat	Buckwheat	z-Score Xpt_{ALL}	Method	Remarks
	pos/neg	[mg/kg]			
14	positive	7,49		ES	
4	positive	1,89		MI-II	
15	positive	2,50		MI-II	

Number positive	3
Number negative	0
Percent positive	100
Percent negative	0
Consensus value	positive

Methods:
ES = ELISA-Systems
MI-II = Morinaga Institute ELISA Kit II

Comment:
100% positive results were obtained for the spiking level sample.

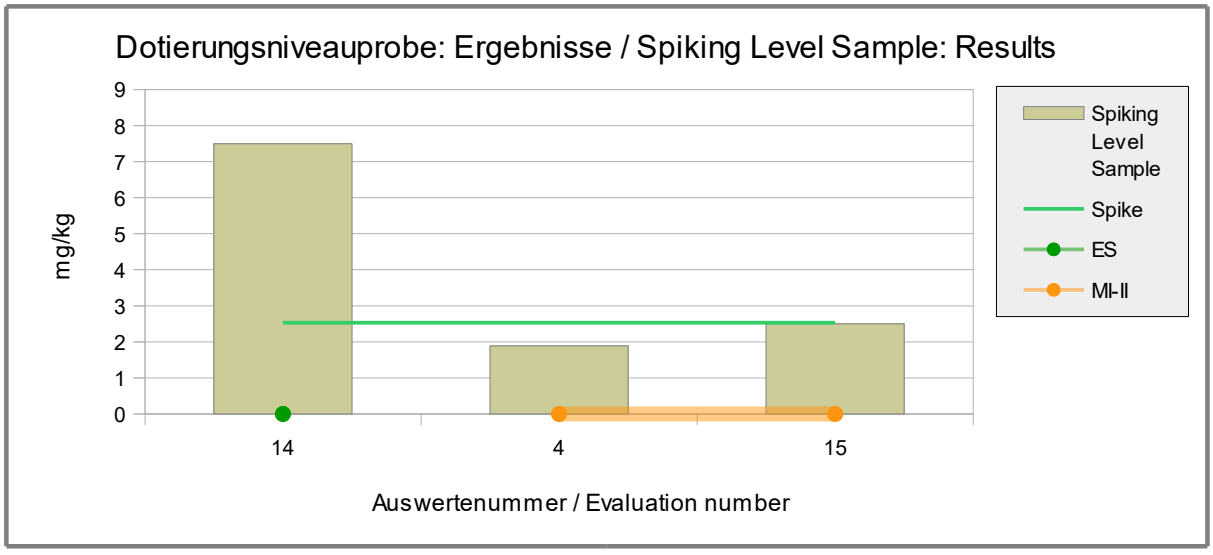


Abb./Fig. 2: ELISA Results Buckwheat (as protein)
green line = Spiking level (Spike)
round symbols = Applied methods (see legend)

Recovery Rates with z-Scores ELISA for Buckwheat (as Protein): Spiking Level Sample and Sample A

Evaluation number	Spiking Level Sample	Recovery rate*		Sample A	Recovery rate*		Method	Remarks
	[mg/kg]	[%]	[Z_{RR}]	[mg/kg]	[%]	[Z_{RR}]		
14	7,49	296	7,8	3,18	**129**	1,2	ES	
4	1,89	**74,7**	-1,0	2,58	**105**	0,20	MI-II	
15	2,50	**98,8**	-0,05	3,00	**122**	0,88	MI-II	

RA**	50-150 %	RA**	50-150 %
Number in RA	2	Number in RA	3
Percent in RA	67	Percent in RA	100

Methods:
ES = ELISA-Systems
MI-II = Morinaga Institute ELISA Kit II

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

** Range of acceptance of AOAC for allergen ELISAS

Comments:

Two out of three participants obtained a recovery rate in the range of the AOAC recommendation of 50-150% with the spiking level sample using ELISA. For the spiked food matrix sample A, all three recovery rates were within this range of acceptance.
The related z-scores are based on the target standard deviation of 25%.

4.1.2 PCR Results: Buckwheat

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	positive	14,6	negative		2/2 (100%)	SFA	
10	positive		negative		2/2 (100%)	SFA-ID	
14	positive	17,4	negative	<1	2/2 (100%)	SFA-ID	
15	positive		negative		2/2 (100%)	div	

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

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

Comment:
The consensus values are in qualitative agreement with the spiking of sample A.

Quantitative evaluation PCR: Sample A

The quantitative results were not evaluated because too few single results were available.

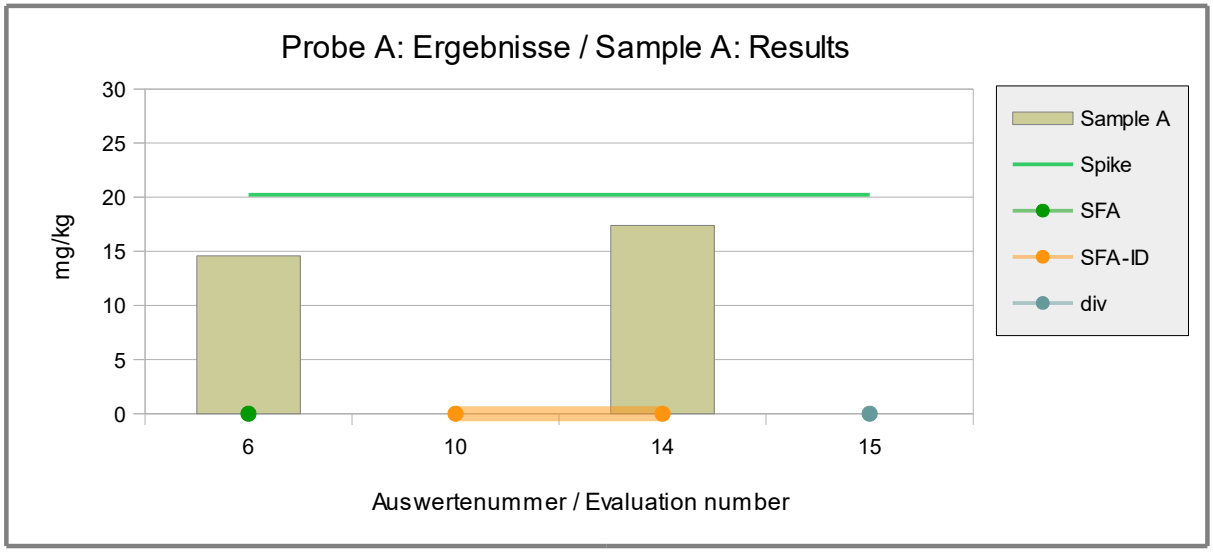


Abb./Fig. 3: PCR Results Buckwheat
green line = Spiking level
round symbols = Applied methods (see legend)

Quantitative evaluation PCR: Spiking Level Sample

The quantitative results were not evaluated because too few single results were available.

Evaluation number	Buckwheat	Buckwheat	z-Score Xpt_{ALL}	Method	Remarks
	pos/neg	[mg/kg]			
6	positive	9,00		SFA	
10	positive			SFA-ID	
14	positive	20,3		SFA-ID	
15	positive			div	

Number positive	4
Number negative	0
Percent positive	100
Percent negative	0
Consensus value	positive

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

Comment:
100% positive results were obtained for the spiking level sample.

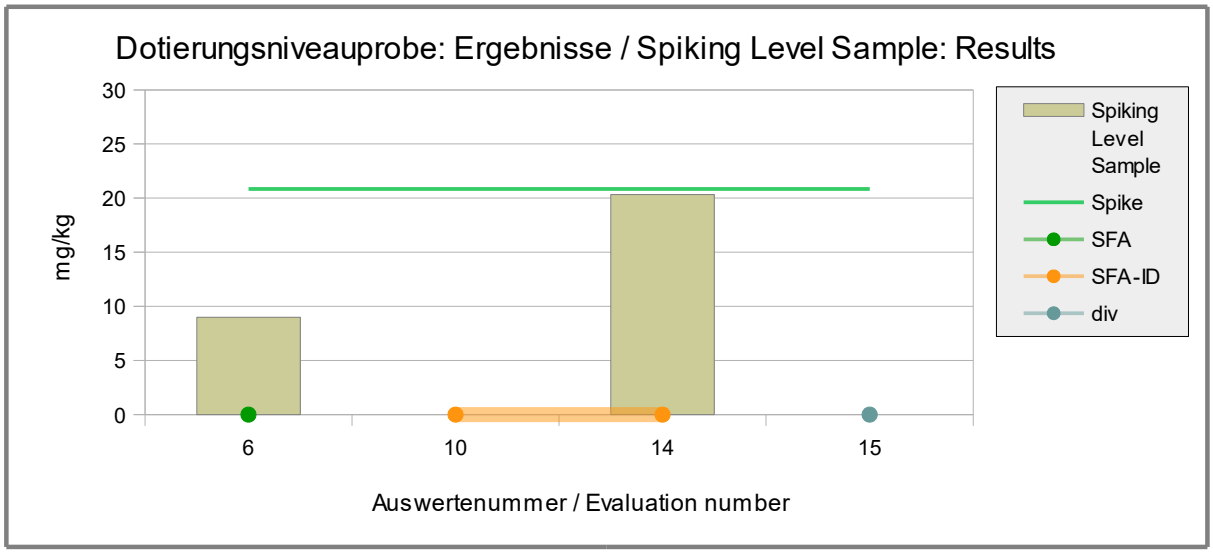


Abb./Fig. 4: PCR Results Buckwheat
green line = Spiking level
round symbols = Applied methods (see legend)

Recovery Rates with z-Scores PCR for Buckwheat: Spiking Level Sample and Sample A

Evaluation number	Spiking Level Sample	Recovery rate*		Sample A	Recovery rate*		Method	Remarks
	[mg/kg]	[%]	[Z_{RR}]	[mg/kg]	[%]	[Z_{RR}]		
6	9,00	43,2	-2,3	14,6	**72,1**	-1,1	SFA	
10							SFA-ID	
14	20,3	**97,6**	-0,10	17,4	**86,0**	-0,56	SFA-ID	results given as DNA
15							div	

RA**	50-150 %	RA**	50-150 %
Number in RA	1	Number in RA	2
Percent in RA	50	Percent in RA	100

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

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

** Range of acceptance of AOAC for allergen ELISAS

Comments:

One out of two participants obtained a recovery rate in the range of the AOAC requirement of 50-150% with the spiking level sample using PCR. For the spiked food matrix sample A, both recovery rates were within this range of acceptance.

Since the results of participant 14 were given as DNA, the calculation of the recovery rates in relation to buckwheat was purely informative with reservations.

The related z-scores are based on the target standard deviation of 25%.

4.2 Proficiency Test Macadamia

4.2.1 ELISA Results: Macadamia

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	positive	15,6	negative	<4,16	2/2 (100%)	3M	Result converted °
12a	positive	25,0	negative	0	2/2 (100%)	AQ-P	
15	positive	35,0	negative	<1	2/2 (100%)	AQ-P	
5	positive	18,6	negative	<2	2/2 (100%)	BF	
11	positive	13,0	positive	3,10	1/2 (50%)	BF	
13	positive	16,1	negative	<4.0	2/2 (100%)	BF	
1	positive	23,9	negative	<1	2/2 (100%)	IL	
14	positive	15,7	negative	<1	2/2 (100%)	IL	
2	positive	16,0	-		1/1 (100%)	SP	
16	positive	13,3	*	1,00	1/1 (100%)	SP	* Sample B: borderline
12b	positive		negative		2/2 (100%)	div	Lateral Flow

° calculation see p. 19

	Sample A		Sample B	
Number positive	11		1	
Number negative	0		8	
Percent positive	100		11	
Percent negative	0		89	
Consensus value	positive		negative	

Methods:

3M = 3M Protein ELISA Kit
AQ-P = AgraQuant Plus, RomerLabs
BF = MonoTrace ELISA, BioFront Technologies
IL = Immunolab
SP = SensiSpec ELISA Kit, Eurofins
div = not indicated / other method

Comment:
The consensus values are in qualitative agreement with the spiking of sample A.

Quantitative evaluation of ELISA-results: Sample A

Evaluation number	Macadamia	z-Score Xpt_{ALL}	Method	Remarks
	[mg/kg]			
7	15,6	-0,61	3M	Result converted °
12a	25,0	1,4	AQ-P	
15	35,0	3,6	AQ-P	
5	18,6	0,04	BF	
11	13,0	-1,2	BF	
13	16,1	-0,50	BF	
1	23,9	1,2	IL	
14	15,7	-0,59	IL	
2	16,0	-0,53	SP	
16	13,3	-1,1	SP	
12b			div	Lateral Flow

° calculation see p. 19

Methoden:

3M = 3M Protein ELISA Kit
AQ-P = AgraQuant Plus, RomerLabs
BF = MonoTrace ELISA, BioFront Technologies
IL = Immunolab
SP = SensiSpec ELISA Kit, Eurofins
div = not indicated / other method

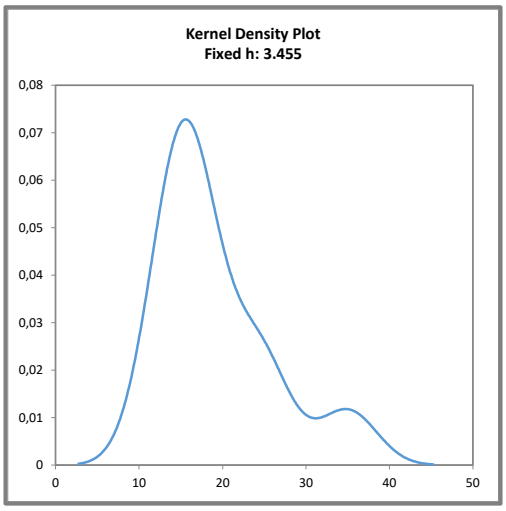


Abb. / Fig. 5:
Kerndichte-Schätzung aller ELISA-Ergebnisse (mit h = 0,75 x σpt von Xpt_{ALL})

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

Comments:
The kernel density estimation shows an approximately symmetrical distribution of the results with a slight shoulder at about > 22 mg/kg (2 higher single values, methods AQ-P and IL) and a secondary peak at about 35 mg/kg due to a participant result outside of the target range (method AQ-P).

Characteristics: Quantitative evaluation ELISA Macadamia

Sample A

Statistic Data	**All Results** *[mg/kg]*
Assigned value (X_{pt})	$\boldsymbol{X_{pt_{ALL}}}$
Number of results	10
Number of outliers	0
Mean	19,2
Median	16,1
Robust Mean (X_{pt})	**18,4**
Robust standard deviation (S*)	**5,72**
Target range:	
Target standard deviation σ_{pt}	**4,61**
lower limit of target range	**9,21**
upper limit of target range	**27,6**
Quotient S/σ_{pt}*	*1,2*
Standard uncertainty $U_{(Xpt)}$	*2,26*
Results in the target range	*9*
Percent in the target range	*90*

Comments to the statistical characteristics and assigned values:

The kernel density estimation showed an approximately symmetrical distribution and no clear method-dependent differences.

The evaluation of the results of all methods showed a normal variability of the results. The quotient S/σ_{pt} was below 2,0. The robust standard deviation is in the range of established values for the reproducibility standard deviation of the applied methods (cf. 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 was 180% of the spiking level of macadamia to sample A and thus above the range of the recommendations for the applied methods (see 3.4.3 and p.34 "Recovery rates with z-scores ELISA for Macadamia").

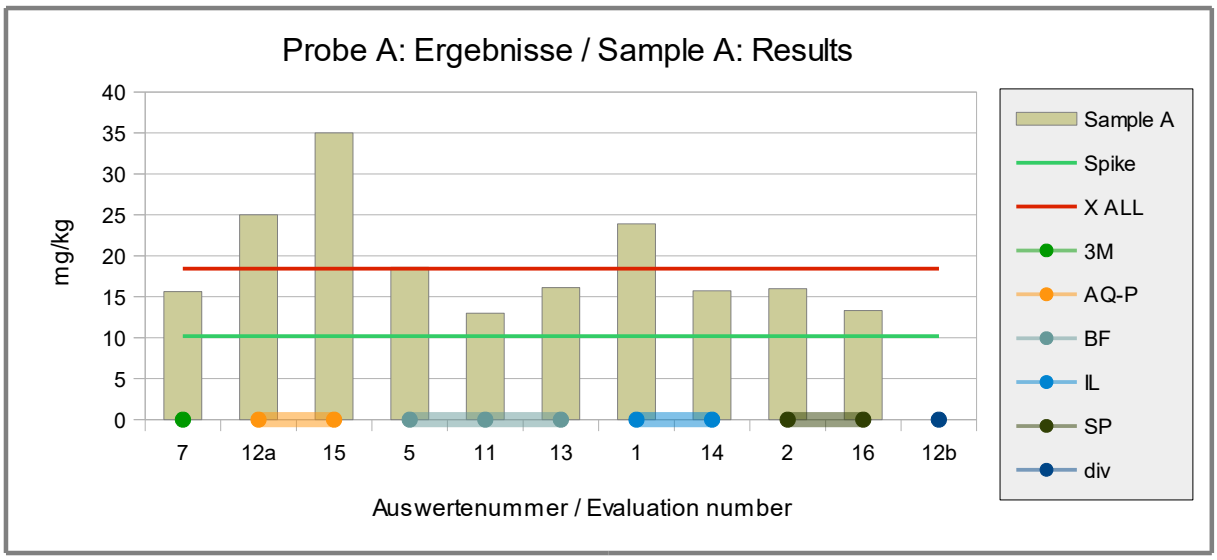


Abb./Fig. 6: ELISA Results Macadamia
green line = Spiking level (Spike)
red line = robust mean of all results
round symbols = Applied methods (see legend)

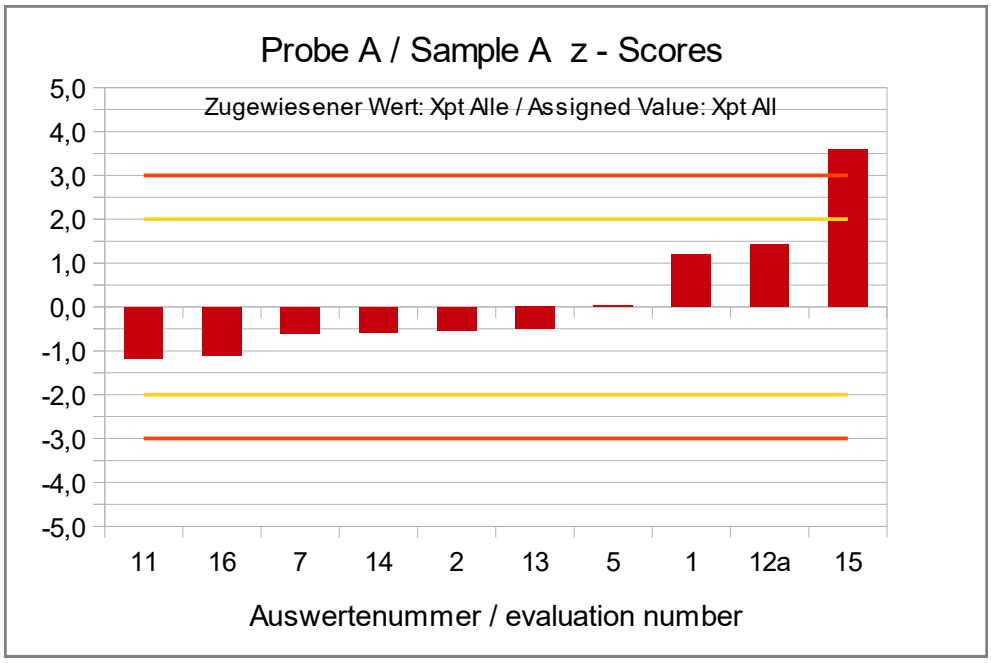


Abb./Fig. 7:
z-Scores (ELISA Results as macadamia)
Assigned value: robust mean of all results

Quantitative evaluation of ELISA-results: Spiking Level Sample

Evaluation number	Macadamia	z-Score Xpt_{ALL}	Method	Remarks
	[mg/kg]			
7	19,5	-0,19	3M	Result converted °
12a	23,0	0,49	AQ-P	
15	34,0	2,6	AQ-P	
5			BF	
11	3,10	-3,4	BF	
13			BF	
1	21,0	0,10	IL	
14	18,7	-0,35	IL	
2	23,0	0,49	SP	
16	17,7	-0,54	SP	
12b			div	Lateral Flow

° Calculation see p. 19

Methods:

3M = 3M Protein ELISA Kit
AQ-P = AgraQuant Plus, RomerLabs
BF = MonoTrace ELISA, BioFront Technologies
IL = Immunolab
SP = SensiSpec ELISA Kit, Eurofins
div = not indicated / other method

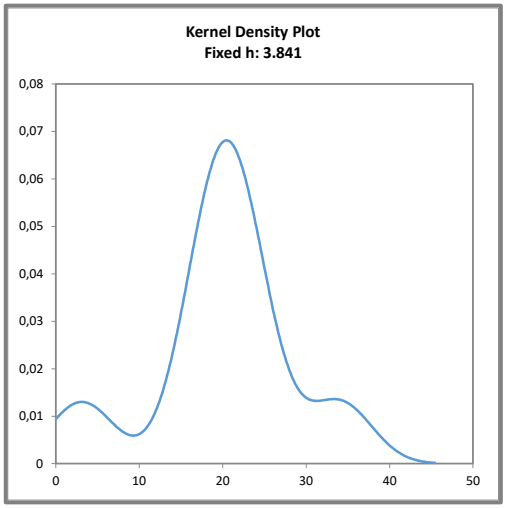


Abb. / Fig. 8:
Kerndichte-Schätzung aller ELISA-Ergebnisse (mit h = 0,75 x σpt von Xpt_{ALL})

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

Comments:
The kernel density estimation shows an approximately symmetrical distribution of the results with 2 secondary peaks outside the target range at approx. 3 mg/kg (method BF) and at 34 mg/kg (method AQ-P).

Characteristics: Quantitative evaluation ELISA Macadamia

Spiking Level Sample

Statistic Data	**All Results** *[mg/kg]*
Assigned value (X_{pt})	$\boldsymbol{X_{pt_{ALL}}}$
Number of results	8
Number of outliers	–
Mean	20,0
Median	20,3
Robust Mean (X_{pt})	**20,5**
Robust standard deviation (S*)	**5,10**
Target range:	
Target standard deviation σ_{pt}	**5,12**
lower limit of target range	**10,2**
upper limit of target range	**30,7**
Quotient S/σ_{pt}*	*1,0*
Standard uncertainty $U_{(Xpt)}$	*2,26*
Results in the target range	*6*
Percent in the target range	*75*

Comments to the statistical characteristics and assigned values:

The kernel density estimation showed an approximately symmetrical distribution and no clear method-dependent differences.

The evaluation of the results of all methods showed a low variability of the results. The quotient S/σ_{pt} was 1,0. The robust standard deviation is in the range of established values for the reproducibility standard deviation of the applied methods (cf. 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 was 222% of the spiking level of macadamia to the spiking level sample and thus above the range of the recommendations for the applied methods (see 3.4.3 and p.34 "Recovery rates with z-scores ELISA for Macadamia").

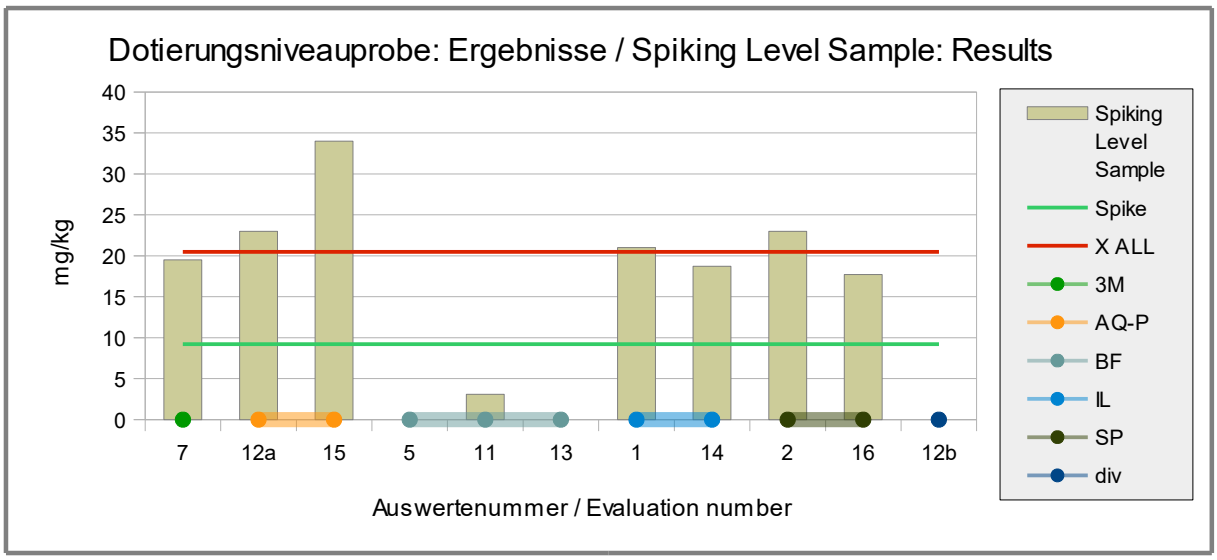


Abb./Fig. 9: ELISA Results Macadamia
green line = Spiking level (Spike)
red line = robust mean of all results
round symbols = Applied methods (see legend)

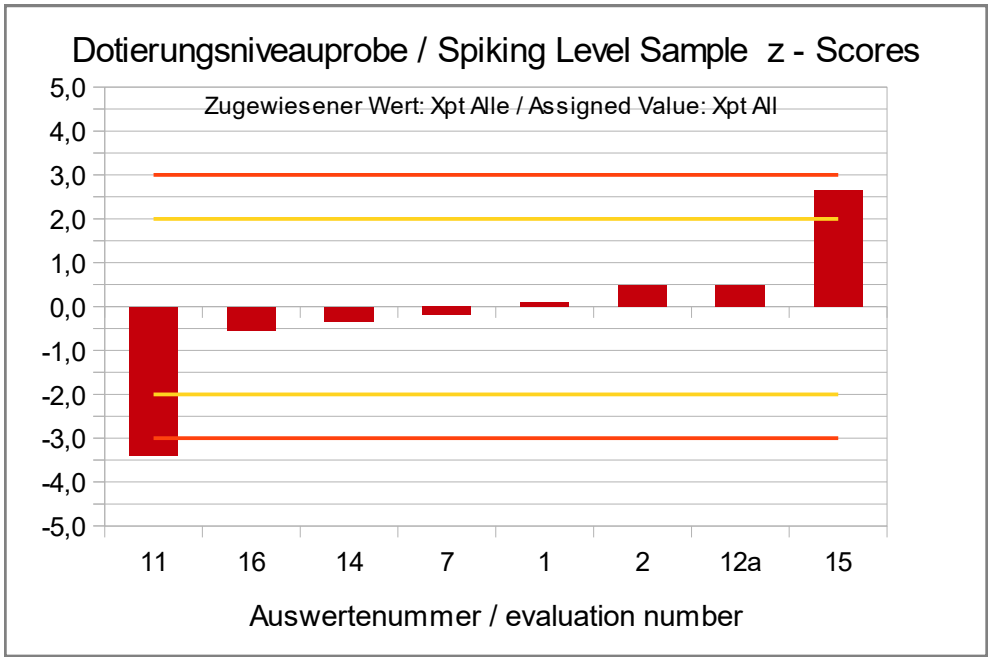


Abb./Fig. 10:
z-Scores (ELISA Results as macadamia)
Assigned value: robust mean of all results

Recovery Rates with z-Scores ELISA for Macadamia: Spiking Level Sample and Sample A

Evaluation number	Spiking Level Sample	Recovery rate*		Sample A	Recovery rate*		Method	Remarks
	[mg/kg]	[%]	[Z_{RR}]	[mg/kg]	[%]	[Z_{RR}]		
7	19,5	211	4,5	15,6	153	2,1	3M	Result converted °
12a	23,0	249	6,0	25,0	245	5,8	AQ-P	
15	34,0	369	11	35,0	343	9,7	AQ-P	
5				18,6	182	3,3	BF	
11	3,10	33,6	-2,7	13,0	**127**	1,1	BF	
13				16,1	158	2,3	BF	
1	21,0	228	5,1	23,9	234	5,4	IL	
14	18,7	203	4,1	15,7	154	2,2	IL	
2	23,0	249	6,0	16,0	157	2,3	SP	
16	17,7	192	3,7	13,3	**130**	1,2	SP	
12b							div	Lateral Flow

° calculation see p. 19

RA**	50-150 %	RA**	50-150 %
Number in RA	0	Number in RA	2
Percent in RA	0	Percent in RA	20

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

** Range of acceptance of AOAC for allergen ELISAS

Methods:

3M = 3M Protein ELISA Kit

AQ-P = AgraQuant Plus, RomerLabs

BF = MonoTrace ELISA, BioFront Technologies

IL = Immunolab

SP = SensiSpec ELISA Kit, Eurofins

div = not indicated / other method

Comments:

None of the participants obtained a recovery rate in the range of the AOAC recommendation of 50-150% with the spiking level sample by ELISA. With one exception, all recovery rates were well over 150%. *For the spiked food matrix sample A, 20% (2) of the recovery rates were within this range of acceptance.*

The related z-scores are based on the target standard deviation of 25%.

4.2.2 PCR Results: Macadamia

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	positive	4,40	negative		2/2 (100%)	SFA	
9	positive		negative		2/2 (100%)	SFA	
8	positive		negative		2/2 (100%)	SFA-4p	
3	positive		negative		2/2 (100%)	SFA-ID	
10	positive		negative		2/2 (100%)	SFA-ID	
14	positive	17,0	negative	<1	2/2 (100%)	SFA-ID	
2	positive		negative		2/2 (100%)	div	
15	positive		negative		2/2 (100%)	div	

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

Methods:
SFA = Sure Food ALLERGEN, R-Biopharm / Congen
SFA-4p = Sure Food Allergen 4plex, R-Biopharm / Congen
SFA-ID = Sure Food Allergen ID, R-Biopharm / Congen
div = not indicated / other method

Comment:
The consensus values are in qualitative agreement with the spiking of sample A.

Quantitative evaluation PCR: Sample A

The quantitative results were not evaluated because too few single results were available.

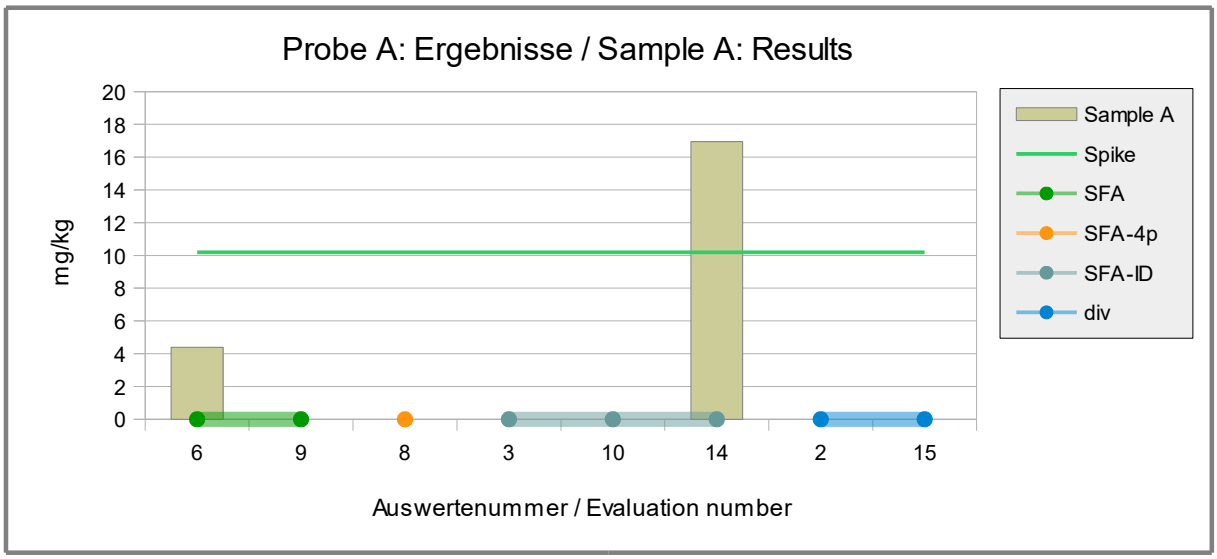


Abb./Fig. 11: PCR Results Macadamia
green line = Spiking level (Spike)
round symbols = Applied methods (see legend)

Quantitative evaluation PCR: Spiking Level Sample

The quantitative results were not evaluated because too few single results were available.

Evaluation number	Macadamia	Macadamia	z-Score Xpt_{ALL}	Method	Remarks
	pos/neg	[mg/kg]			
6	positive	1,40		SFA	
9	positive			SFA	
8	positive			SFA-4p	
3	positive			SFA-ID	
10	positive			SFA-ID	
14	positive	6,52		SFA-ID	
2	positive			div	
15	positive			div	

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

Methods:
SFA = Sure Food ALLERGEN, R-Biopharm / Congen
SFA-4p = Sure Food Allergen 4plex, R-Biopharm / Congen
SFA-ID = Sure Food Allergen ID, R-Biopharm / Congen
div = not indicated / other method

Comment:
For the spiking level sample, 100% positive results were obtained.

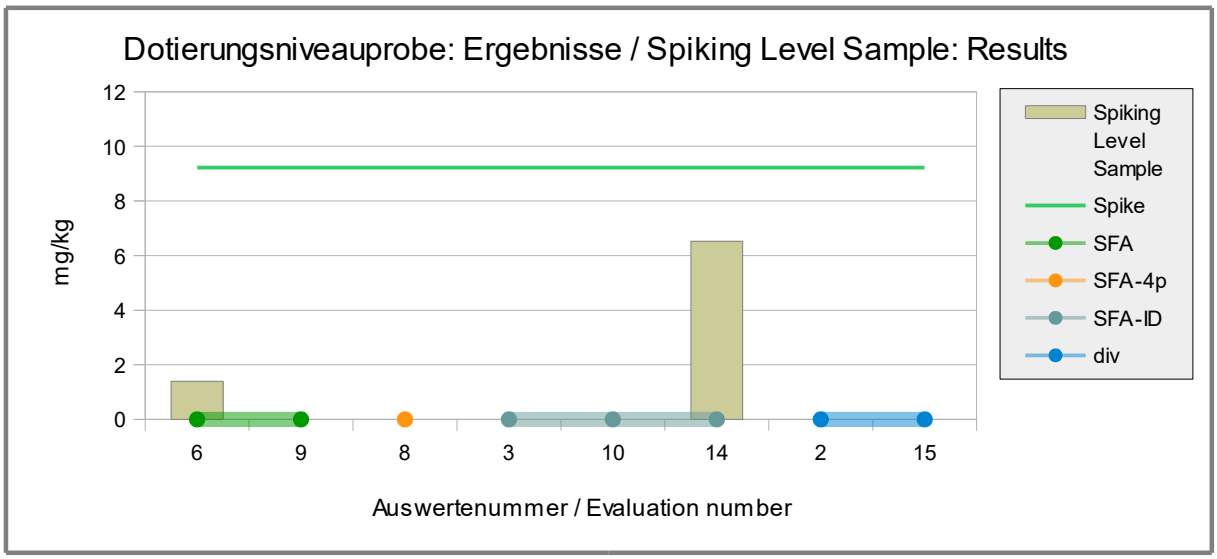


Abb./Fig. 12: PCR Results Macadamia
green line = Spiking level (Spike)
round symbols = Applied methods (see legend)

Recovery Rates with z-Scores PCR for Macadamia: Spiking Level Sample and Sample A

Evaluation number	Spiking Level Sample	Recovery rate*		Sample A	Recovery rate*		Method	Remarks
	[mg/kg]	[%]	[Z_{RR}]	[mg/kg]	[%]	[Z_{RR}]		
6	1,40	15,2	-3,4	4,40	43,1	-2,3	SFA	
9							SFA	
8							SFA-4p	
3							SFA-ID	
10							SFA-ID	
14	6,52	**70,7**	-1,2	17,0	166	2,6	SFA-ID	results given as DNA
2							div	
15							div	

RA**	50-150 %	RA**	50-150 %
Number in RA	1	Number in RA	0
Percent in RA	50	Percent in RA	0

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

** Range of acceptance of AOAC for allergen ELISAS

Methods:
SFA = Sure Food ALLERGEN, R-Biopharm / Congen
SFA-4p = Sure Food Allergen 4plex, R-Biopharm / Congen
SFA-ID = Sure Food Allergen ID, R-Biopharm / Congen
div = not indicated / other method

Comments:
One participant obtained a recovery rate in the range of the AOAC recommendation of 50-150% with the spiking level sample using PCR. For the spiked food matrix sample A, none of the recovery rates were in this range of acceptance.
Since the results of participant 14 were given as DNA, the calculation of the recovery rates in relation to macadamia was purely informative with reservations.
The related z-scores are based on the target standard deviation of 25%.

4.3 Proficiency Test Almond

4.3.1 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		
12a	positive	13,0	negative	0	2/2 (100%)	AQ-P	
14	positive	5,48	negative	<0.5	2/2 (100%)	BC	
5	positive	10,3	negative	<1	2/2 (100%)	BF	
11	positive	9,00	negative	<2,37	2/2 (100%)	ES	Result converted °
1	positive	5,90	negative	<0,4	2/2 (100%)	IL	
4	positive	9,69	negative		2/2 (100%)	RS-F	
9	positive	17,6	negative	< BG	2/2 (100%)	RS-F	
14	positive	14,4	negative	<2.5	2/2 (100%)	RS-F	
15a	positive	7,00	negative	<2.5	2/2 (100%)	RS-F	
2	positive	6,50	negative	<0,4	2/2 (100%)	SP	
16	positive	9,50	negative	< 0.2	2/2 (100%)	SP	
7	positive	6,59	negative	<2,5	2/2 (100%)	VT	
13	positive	11,4	negative	<2.5	2/2 (100%)	VT	
15b	positive	8,00	negative	<2.5	2/2 (100%)	VT	
12b	positive		negative		2/2 (100%)	div	Lateral Flow

° calculation see p. 19

	Sample A		Sample B	
Number positive	15		0	
Number negative	0		15	
Percent positive	100		0	
Percent negative	0		100	
Consensus value	positive		negative	

Methods:

AQ-P = AgraQuant Plus, RomerLabs
BC = BioCheck ELISA
BF = MonoTrace ELISA, BioFront Technologies
ES = ELISA-Systems
IL = Immunolab
RS-F= Ridascreen® Fast, R-Biopharm
SP = SensiSpec ELISA Kit, Eurofins
VT = Veratox, Neogen
div = not indicated / other method

Comment:
The consensus values are in qualitative agreement with the spiking of sample A.

Quantitative evaluation of ELISA-results: Sample A

Evaluation number	Almond	z-Score Xpt_{ALL}	Method	Remarks
	[mg/kg]			
12a	13,0	1,5	AQ-P	
14a	5,48	-1,7	BC	
5	10,3	0,39	BF	
11	9,00	-0,16	ES	Result converted °
1	5,90	-1,5	IL	
4	9,69	0,13	RS-F	
9	17,6	3,5	RS-F	
14b	14,4	2,1	RS-F	
15a	7,00	-1,0	RS-F	
2	6,50	-1,2	SP	
16	9,50	0,05	SP	
7	6,59	-1,2	VT	
13	11,4	0,86	VT	
15b	8,00	-0,59	VT	
12b			div	Lateral Flow

° calculation see p. 19

Methoden:

AQ-P = AgraQuant Plus, RomerLabs
BC = BioCheck ELISA
BF = MonoTrace ELISA, BioFront Technologies
ES = ELISA-Systems
IL = Immunolab
RS-F= Ridascreen® Fast, R-Biopharm
SP = SensiSpec ELISA Kit, Eurofins
VT = Veratox, Neogen
div = not indicated / other method

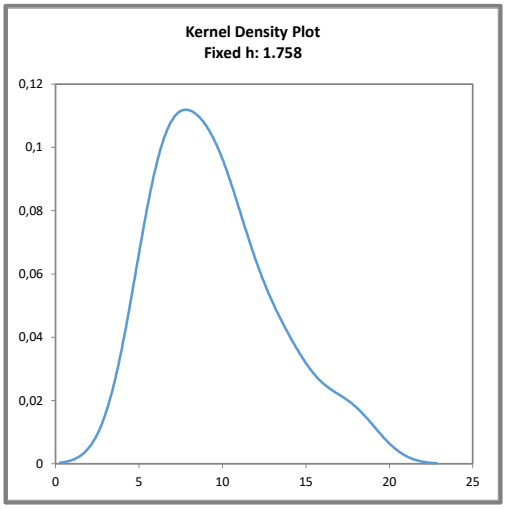


Abb. / Fig. 13:
Kerndichte-Schätzung aller ELISA-Ergebnisse (mit h = 0,75 x σpt von Xpt_{ALL})

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

Comments:
The kernel density estimation shows an approximately symmetrical distribution of results with a slight shoulder at approximately > 15 mg/kg due to 2 results outside the target range (method RS-F).

Characteristics: Quantitative evaluation ELISA Almond

Sample A

Statistic Data	**All Results** *[mg/kg]*
Assigned value (X_{pt})	$X_{pt_{ALL}}$
Number of results	14
Number of outliers	0
Mean	9,59
Median	9,25
Robust Mean (X_{pt})	**9,38**
Robust standard deviation (S*)	**3,46**
Target range:	
Target standard deviation σ_{pt}	**2,34**
lower limit of target range	**4,69**
upper limit of target range	**14,1**
Quotient S/σ_{pt}*	*1,5*
Standard uncertainty $U_{(Xpt)}$	*1,15*
Results in the target range	*12*
Percent in the target range	*86*

Comments to the statistical characteristics and assigned values:

The kernel density estimation showed an approximately symmetrical distribution and no clear method-dependent differences.

The evaluation of the results of all methods showed a normal variability of the results. The quotient S/σ_{pt} was below 2,0. The robust standard deviation is in the range of established values for the reproducibility standard deviation of the applied methods (cf. 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 was 67% of the spiking level of almond to sample A and thus within the range of the recommendations for the applied methods (see 3.4.3 and p.45 "Recovery rates with z-scores ELISA for Almond").

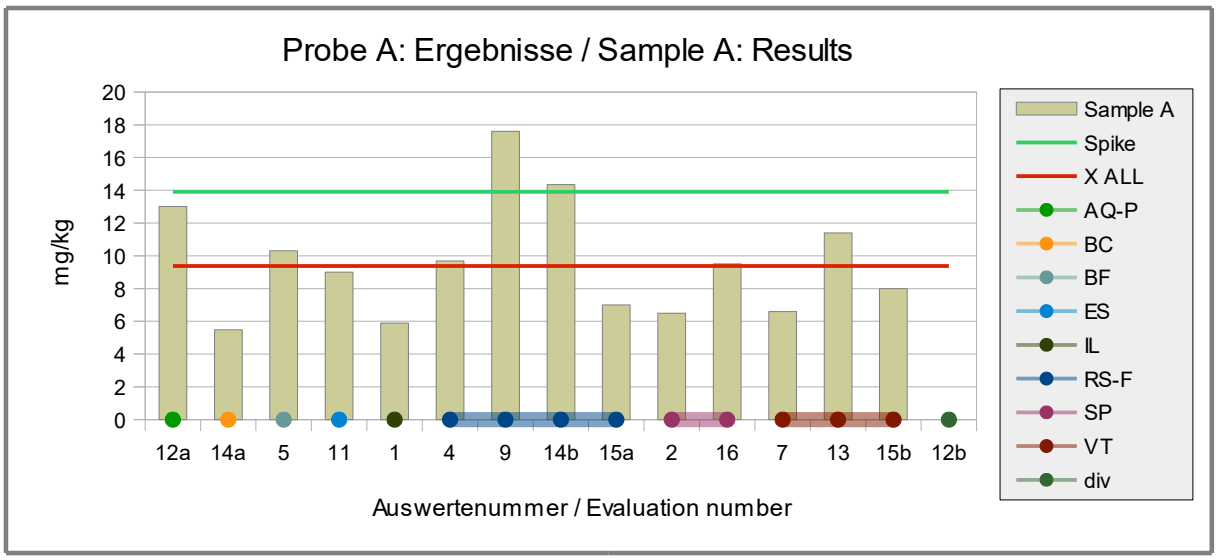


Abb./Fig. 14: ELISA Results Almond
green line = Spiking level (Spike)
red line = robust mean of all results
round symbols = Applied methods (see legend)

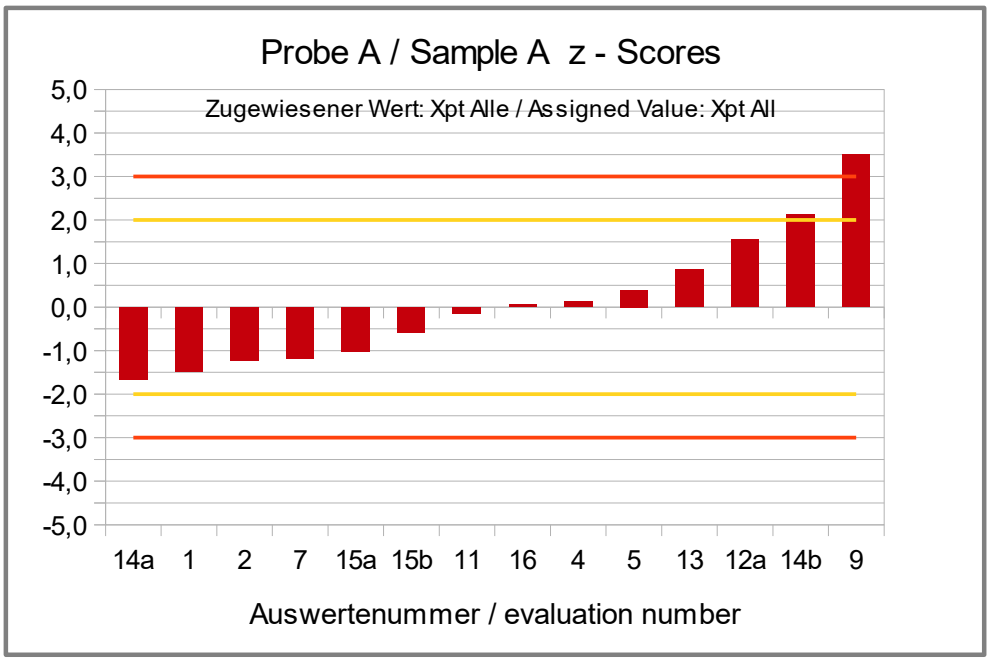


Abb./Fig. 15:
z-Scores (ELISA Results as almond)
Assigned value: robust mean of all results

Quantitative evaluation of ELISA-results: Spiking Level Sample

Evaluation number	Almond	z-Score Xpt_{ALL}	Method	Remarks
	[mg/kg]			
12a	13,0	0,32	AQ-P	
14a	10,9	-0,39	BC	
5	16,0	1,3	BF	
11	7,58	-1,5	ES	Result converted °
1	9,20	-0,94	IL	
4	11,2	-0,28	RS-F	
9	17,8	1,9	RS-F	
14b	17,4	1,8	RS-F	
15a	14,0	0,65	RS-F	
2	9,60	-0,81	SP	
16	11,5	-0,18	SP	
7	11,0	-0,36	VT	
13	9,20	-0,94	VT	
15b	11,0	-0,35	VT	
12b			div	Lateral Flow

° calculation see p. 19

Methods:

AQ-P = AgraQuant Plus, RomerLabs
BC = BioCheck ELISA
BF = MonoTrace ELISA, BioFront Technologies
ES = ELISA-Systems
IL = Immunolab
RS-F= Ridascreen® Fast, R-Biopharm
SP = SensiSpec ELISA Kit, Eurofins
VT = Veratox, Neogen
div = not indicated / other method

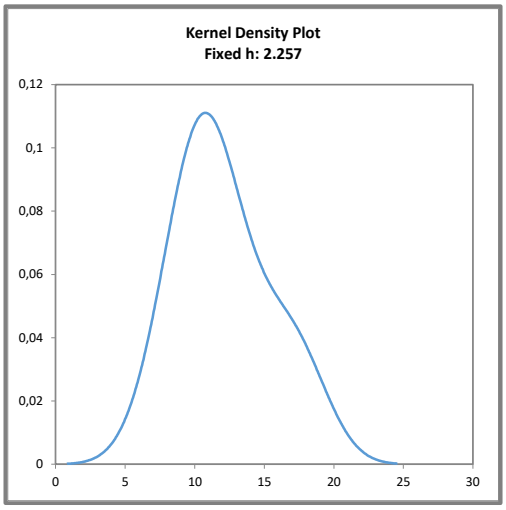


Abb. / Fig. 16:
Kerndichte-Schätzung aller ELISA-Ergebnisse (mit h = 0,75 x σpt von Xpt_{ALL})

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

Comments:
The kernel density estimation shows an approximately symmetrical distribution of the results with a slight shoulder at approx. > 15 mg/kg, which can be attributed to 2 higher single values using the method RS-F.

Characteristics: Quantitative evaluation ELISA Almond

Spiking Level Sample

Statistic Data	**All Results** *[mg/kg]*
Assigned value (X_{pt})	$\boldsymbol{X_{pt_{ALL}}}$
Number of results	14
Number of outliers	0
Mean	12,1
Median	11,1
Robust Mean (X_{pt})	**12,0**
Robust standard deviation (S*)	**3,45**
Target range:	
Target standard deviation σ_{pt}	**3,01**
lower limit of target range	**6,02**
upper limit of target range	**18,1**
Quotient S/σ_{pt}*	*1,1*
Standard uncertainty $U_{(Xpt)}$	*1,15*
Results in the target range	*14*
Percent in the target range	*100*

Comments to the statistical characteristics and assigned values:

The kernel density estimation showed an approximately symmetrical distribution and no clear method-dependent differences.

The evaluation of the results of all methods showed a normal variability of the results. The quotient S/σ_{pt} was below 2,0. The robust standard deviation is in the range of established values for the reproducibility standard deviation of the applied methods (cf. 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 was 77% of the spiking level of almond to the spiking level sample and thus within the range of the recommendations for the applied methods (see 3.4.3 and p.45 "Recovery rates with z-scores ELISA for Almond").

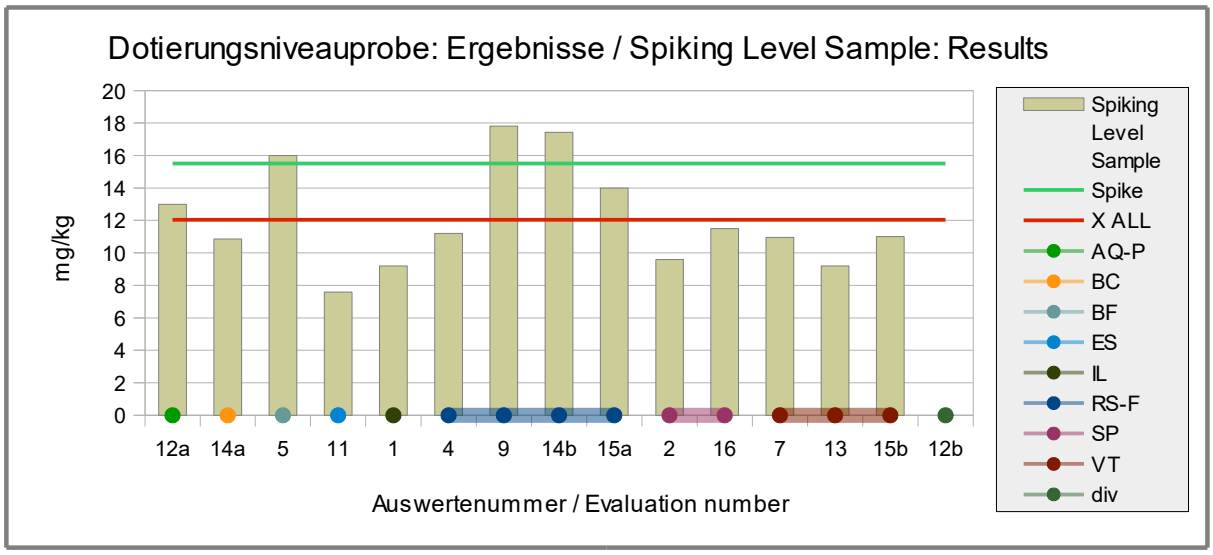


Abb./Fig. 17: ELISA Results Almond
green line = Spiking level (Spike)
red line = robust mean of all results
round symbols = Applied methods (see legend)

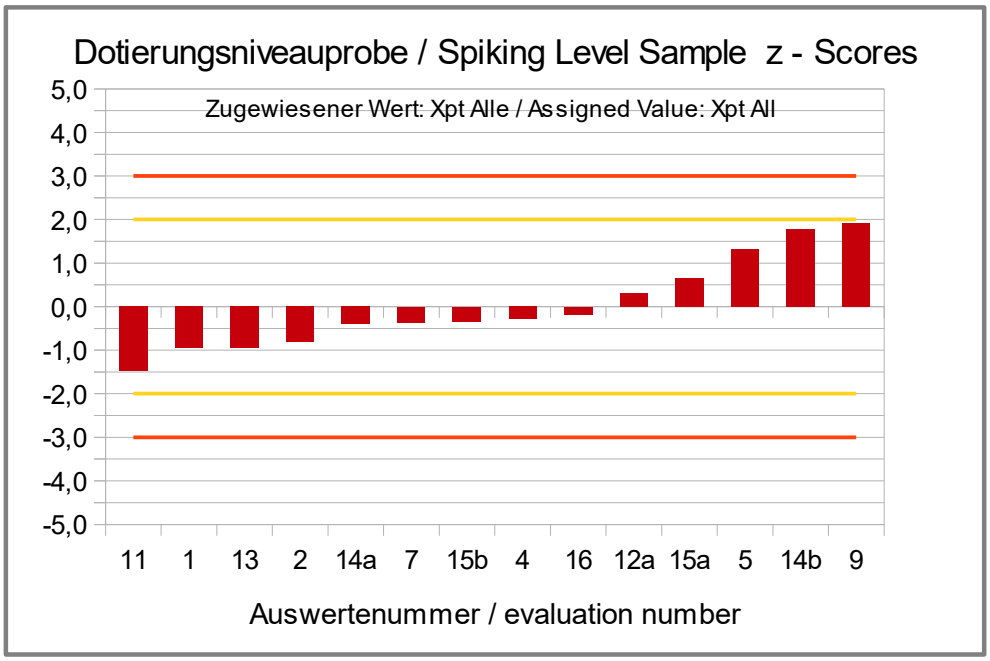


Abb./Fig. 18:
z-Scores (ELISA Results as almond)
Assigned value: robust mean of all results

Recovery Rates with z-Scores ELISA for Almond: Spiking Level Sample and Sample A

Evaluation number	Spiking Level Sample	Recovery rate*		Sample A	Recovery rate*		Method	Remarks
	[mg/kg]	[%]	[Z_{RR}]	[mg/kg]	[%]	[Z_{RR}]		
12a	13,0	**83,9**	-0,65	13,0	**93,5**	-0,26	AQ-P	
14a	10,9	**70,1**	-1,2	5,48	39,4	-2,4	BC	
5	16,0	**103**	0,13	10,3	**74,1**	-1,0	BF	
11	7,58	48,9	-2,0	9,00	**64,7**	-1,4	ES	Result converted °
1	9,20	**59,4**	-1,6	5,90	42,4	-2,3	IL	
4	11,2	**72,3**	-1,1	9,69	**69,7**	-1,2	RS-F	
9	17,8	**115**	0,59	17,6	**127**	1,1	RS-F	
14b	17,4	**112**	0,50	14,4	**103**	0,13	RS-F	
15a	14,0	**90,3**	-0,39	7,00	**50,4**	-2,0	RS-F	
2	9,60	**61,9**	-1,5	6,50	46,8	-2,1	SP	
16	11,5	**74,2**	-1,0	9,50	**68,3**	-1,3	SP	
7	11,0	**70,7**	-1,2	6,59	47,4	-2,1	VT	
13	9,20	**59,4**	-1,6	11,4	**82,0**	-0,72	VT	
15b	11,0	**71,0**	-1,2	8,00	**57,6**	-1,7	VT	
12b							div	Lateral Flow

° calculation see p. 19

RA**	50-150 %	AB**	50-150 %
Number in RA	13	Number in RA	10
Percent in RA	93	Percent in RA	71

* Recovery rate 100% relative size: almond, 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
ES = ELISA-Systems
IL = Immunolab
RS-F= Ridascreen® Fast, R-Biopharm
SP = SensiSpec ELISA Kit, Eurofins
VT = Veratox, Neogen
div = not indicated / other method

Comments:

93% (13) of the participants obtained a recovery rate in the range of the AOAC recommendation of 50-150% with the spiking level sample using ELISA. For the spiked food matrix sample A, 71% (10) of the recovery rates were within this range of acceptance.
The related z-scores are based on the target standard deviation of 25%.

4.3.2 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		
6	positive	1,00	negative		2/2 (100%)	SFA	
9	positive		negative		2/2 (100%)	SFA	
3	positive		negative		2/2 (100%)	SFA-ID	
5	positive	>0,4	negative	<0,4	2/2 (100%)	SFA-ID	
10	positive		negative		2/2 (100%)	SFA-ID	
14	positive	1,57	negative	<1	2/2 (100%)	SFA-ID	
8	negative		negative		1/2 (50%)	div	no positive sample identified
15	negative		negative		1/2 (50%)	div	no positive sample identified

	Sample A		Sample B	
Number positive	6		0	
Number negative	2		8	
Percent positive	75		0	
Percent negative	25		100	
Consensus value	positive		negative	

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

Comment:
The consensus values are in qualitative agreement with the spiking of sample A. Two negative results were obtained for sample A.

Quantitative evaluation PCR: Sample A

The quantitative results were not evaluated because too few single results were available.

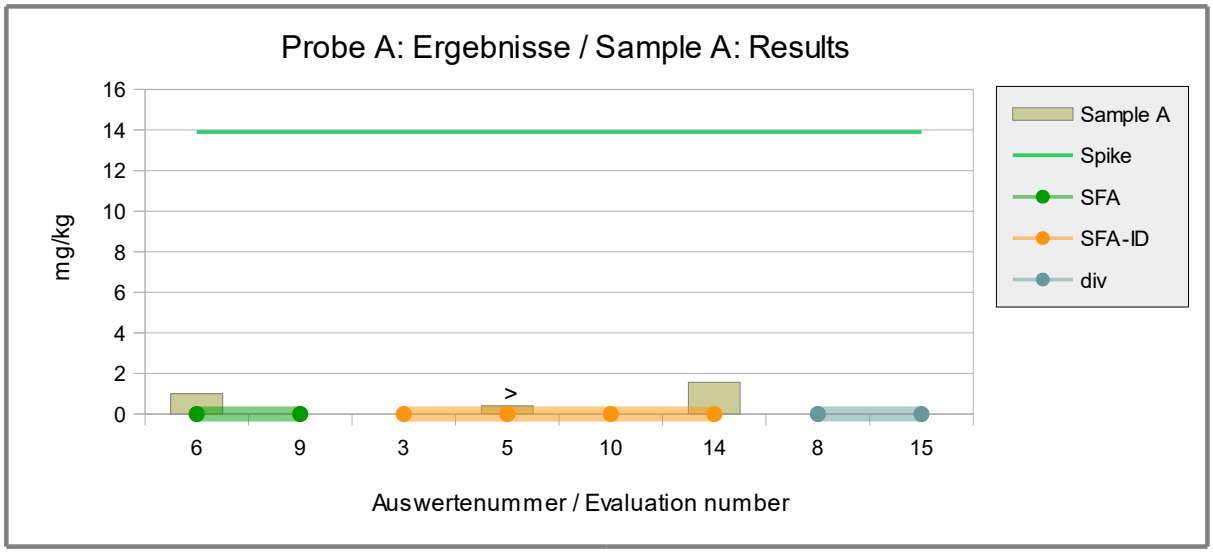


Abb./Fig. 19: PCR Results Almond
green line = Spiking level (Spike)
round symbols = Applied methods (see legend)

Quantitative evaluation PCR: Spiking Level Sample

The quantitative results were not evaluated because too few single results were available.

Evaluation number	Almond	Almond	z-Score Xpt_{ALL}	Method	Remarks
	pos/neg	[mg/kg]			
6	positive	1,00		SFA	
9	positive			SFA	
3	positive			SFA-ID	
5	positive	>0,4		SFA-ID	
10	positive			SFA-ID	
14	positive	1,31		SFA-ID	
8	negative			div	no positive sample identified
15	negative			div	no positive sample identified

Number positive	6
Number negative	2
Percent positive	75
Percent negative	25
Consensus value	positive

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

Comment:
For the spiking level sample, 75% positive results were obtained.

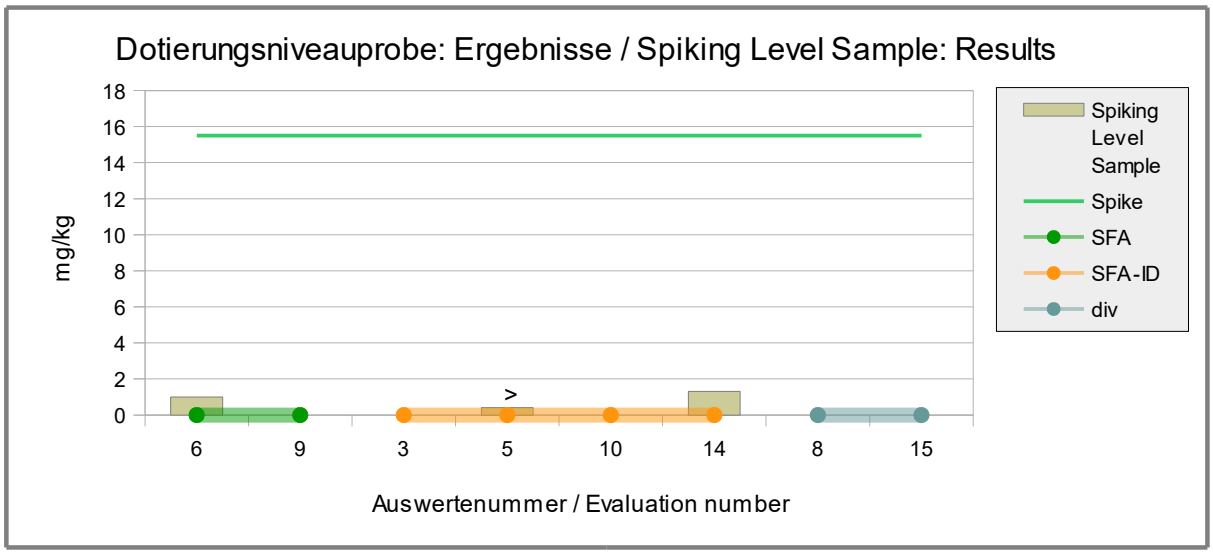


Abb./Fig. 20: PCR Results Almond
green line = Spiking level (Spike)
round symbols = Applied methods (see legend)

Recovery Rates with z-Scores PCR for Almond: Spiking Level Sample and Sample A

Evaluation number	Spiking Level Sample	Recovery rate*		Sample A	Recovery rate*		Method	Remarks
	[mg/kg]	[%]	[Z_{RR}]	[mg/kg]	[%]	[Z_{RR}]		
6	1,00	6,45	-3,7	1,00	7,19	-3,7	SFA	
9							SFA	
3							SFA-ID	
5	>0,4			>0,4			SFA-ID	
10							SFA-ID	
14	1,31	8,45	-3,7	1,57	11,3	-3,5	SFA-ID	results given as DNA
8							div	
15							div	

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

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

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

** Range of acceptance of AOAC for allergen ELISAS

Comments:
None of the participants obtained a PCR recovery rate in the range of the AOAC recommendation of 50-150% with the spiking level sample or the spiked food matrix sample A.
Since the results of participant 14 were given as DNA, the calculation of the recovery rates in relation to almond was purely informative with reservations.
The related z-scores are based on the target standard deviation of 25%.

4.4 Participant z-Scores: overview table

Z-Scores for the assigned values from participants' results (consensus values)

Evaluation number	ELISA Macadamia: **Xpt** (div. Methods)		ELISA Almond: **Xpt** (div. Methods)	
	Sample A	Spiking Level Sample	Sample A	Spiking Level Sample
1	1,2	0,10	-1,5	-0,94
2	-0,53	0,49	-1,2	-0,81
3				
4			0,13	-0,28
5	0,04		0,39	1,3
6				
7	-0,61	-0,19	-1,2	-0,36
8				
9			3,5	1,9
10				
11	-1,2	-3,4	-0,16	-1,5
12 / 12a	1,4	0,49	1,5	0,32
12b				
13	-0,50		0,86	-0,94
14 / 14a	-0,59	-0,35	-1,7	-0,39
14b			2,1	1,8
15 / 15a	3,6	2,6	-1,0	0,65
15b			-0,59	-0,35
16	-1,1	-0,54	0,05	-0,18

Bewertung des z-Scores / valuation of z-score (DIN ISO 13528:2009-01):
-2 ≤ z-score ≤ 2 erfolgreich / successful (in green)
-2 > z-score > 2 „Warnsignal“ / warning signal (in yellow)
-3 > z-score > 3 „Eingriffssignal“ / action signal (in red)

z-Scores for the assigned values from spiking level (recovery rates)

Evaluation number	ELISA Buckwheat (as Protein): Xpt (Spike)		ELISA Macadamia: Xpt (Spike)		ELISA Almond: Xpt (Spike)		PCR Buckwheat: Xpt (Spike)		PCR Macadamia: Xpt (Spike)		PCR Almond: Xpt (Spike)	
	Sample A	Spik. Lev. Sample	Sample A	Spik. Lev. Sample	Sample A	Spik. Lev. Sample	Sample A	Spik. Lev. Sample	Sample A	Spik. Lev. Sample	Sample A	Spik. Lev. Sample
1			5,4	5,1	-2,3	-1,6						
2			2,3	6,0	-2,1	-1,5						
3												
4	0,20	-1,0			-1,2	-1,1						
5			3,3		-1,0	0,13						
6							-1,1	-2,3	-2,3	-3,4	-3,7	-3,7
7			2,1	4,5	-2,1	-1,2						
8												
9					1,1	0,59						
10												
11			1,1	-2,7	-1,4	-2,0						
12 / 12a			5,8	6,0	-0,26	-0,65						
12b												
13			2,3		-0,72	-1,6						
14 / 14a	1,2	7,8	2,2	4,1	-2,4	-1,2	-0,56	-0,10	2,6	-1,2	-3,5	-3,7
14b					0,13	0,50						
15 / 15a	0,88	-0,05	9,7	11	-2,0	-0,39						
15b					-1,7	-1,2						
16			1,2	3,7	-1,3	-1,0						

Bewertung des z-Scores / valuation of z-score (DIN ISO 13528:2009-01):
-2 ≤ z-score ≤ 2 erfolgreich / successful (in green)
-2 > z-score > 2 „Warnsignal“ / warning signal (in yellow)
-3 > z-score > 3 „Eingriffssignal“ / action signal (in red)

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: Buckwheat

Meth. Abr.	Evaluation number	Date of analysis	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	%		Test-Kit + Manufacturer
ES	14	13.01.2022	positive	3,18	negative	<2.5	positive	7,49	2,5	2,5		Buckwheat protein	ELISA Systems Buckwheat ESBWPRD-48
MI-II	4	18. Jan	positive	2,58	negative		positive	1,89	0,31	0,31	32	Buckwheat protein	Buckwheat ELISA Kit-II, Morinaga
MI-II	15	12.1./3.2.22	positive	3	negative	<1	positive	2,5	1	1		Buckwheat protein	Buckwheat ELISA Kit-II, Morinaga

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

Meth. Abr.	Evaluation number	Specifity	Remarks on the method (extraction and determination)	Method accredited ISO/IEC 17025	Further Remarks
		Antibody	e.g. extraction solution / time / temperature	yes/no	
ES	14	As Per Kit Instructions	As Per Kit Instructions	Yes	
MI-II	4		extraction buffer/ 10 min./ 100 °C	yes	
MI-II	15				

5.1.2 ELISA: Macadamia

Meth. Abr.	Evaluation number	Date of analysis	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	%		Test-Kit + Manufacturer
3M	7	28.01.2022	positive	1,25	negative	<0,333	positive	1,56	NA	0,333		Macadamia protein	3M Macadamia nut Protein ELISA Kit E96MAC
AQ-P	12a	20.01.22	positive	25	negative	0	positive	23	1	1	24	Macadamia	AgraQuant Plus ELISA Macadamia CO-KAL1648F, RomerLabs
AQ-P	15	14.01.22	positive	35	negative	<1	positive	34	1	1		Macadamia	AgraQuant Plus ELISA Macadamia CO-KAL1648F, RomerLabs
BF	5		positive	18,6	negative	<2	-			2		Macadamia	MonoTrace Almond ELISA kit, BioFront Technologies
BF	11	16 Dec	positive	13	positive	3,1	positive	3,1				Macadamia	MonoTrace Macadamia ELISA kit, BioFront Technologies
BF	13	03. Feb	Detected	16,1	Not detected	<4.0	Detected			4		Food	MonoTrace -BioFront
IL	1		positive	23,9	negative	<1	positive	21				Macadamia	Immunolab Macadamia ELISA
IL	14	15.12.2022	positive	15,72	negative	<1	positive	18,72	1	1	12,4	Macadamia	Immunolab Macadamia ELISA
SP	2	27.12.21	positive	16	-		positive	23	1	1		Macadamia	Eurofins SensiSpec Macadamia nut ELISA Kit
SP	16	22.01.22	positive	13,3	borderline	1.0	positive	17,7	0,1	1		Macadamia	Eurofins SensiSpec Macadamia nut ELISA Kit
div °	12b	27.01.22	positive		negative		positive			2		Macadamia	Lateral Flow

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

Meth. Abr.	Evaluation number	Specifity	Remarks on the method (extraction and determination)	Method accredited ISO/IEC 17025	Further Remarks
		Antibody	e.g. extraction solution / time / temperature	yes/no	
3M	7		as stipulated in kit insert	yes	
AQ-P	12a	Macadamia protein, polyclonal	according to manual	Yes	
AQ-P	15				
BF	5			yes	
BF	11				This kit has cross-reactivity MN1-EK-96
BF	13			yes	
IL	1				
IL	14	As Per Kit Instructions	As Per Kit Instructions	Yes	
SP	2	detects macadamia nut protein	according to manufacturer information	Yes	Sample B: not analysable due to low cross-reactivity w ith coconut
SP	16			Sample B at LOQ level	
div °	12b				

° Lateral Flow

5.1.3 ELISA: Almond

Meth. Abr.	Evaluation number	Date of analysis	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	%		Test-Kit + Manufacturer
AQ-P	12a	14.12.21	positive	13	negative	0	positive	13	1	1	21	Almond	AgraQuant Plus ELISA Almond COKAL0748F, RomerLabs
BC	14a	15.12.2022	positive	5,48	negative	<0.5	positive	10,86	0,5	0,5		Almond	BioCheck ELISA Almond-Check
BF	5		positive	10,3	negative	<1	positive	16		1		Almond	MonoTrace Almond ELISA kit, BioFront Technologies
ES	11	22 Dec	positive	1,9	negative	<0,5	positive	1,6				Almond protein	ELISA Systems Almonds ESARD-48
IL	1		positive	5,9	negative	<0,4	positive	9,2				Almond	Immunolab Almond ELISA
RS-F	4	12. Jan	positive	9,69	negative		positive	11,21	0,1	2,5	30	Almond	Ridascreen® FAST Almond R6901, R-Biopharm
RS-F	9	31/01	-	17,6	-	< LOQ	-	17,8		2,5		Almond	Ridascreen® FAST Almond R6901, R-Biopharm
RS-F	14b	15.12.2022	positive	14,35	negative	<2.5	positive	17,43	2,5	2,5	29,32	Almond	Ridascreen® FAST Almond R6901, R-Biopharm
RS-F	15a	05.01.22	positive	7	negative	<2.5	positive	14	2,5	2,5		Please select !	Ridascreen® FAST Almond R6901, R-Biopharm
SP	2	22.12.21	positive	6,5	negative	<0,4	positive	9,6	0,4	0,4		Almond	Eurofins SensiSpec Almond ELISA Kit
SP	16	22.01.22	positive	9,5	negative	< 0.2	positive	11,5	0.06	0,4		Almond	Eurofins SensiSpec Almond ELISA Kit
VT	7	11.01.2022	positive	6,59	negative	<2,5	positive	10,96	NA	2,5		Almond	Veratox Almond, Neogen
VT	13	18/1	Detected	11,4	Not detected	<2.5	Detected	9,2		2,5		Food	Veratox 8440 - Neogen
VT	15b	04.01.22	positive	8	negative	<2.5	positive	11	2,5	2,5		Almond	Veratox Almond, Neogen
div °	12b	27.01.22	positive		negative		positive			2		Almond	Lateral Flow

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

Meth. Abr.	Evaluation number	Specifity	Remarks on the method (extraction and determination)	Method accredited ISO/IEC 17025	Further Remarks
		Antibody	e.g. extraction solution / time / temperature	yes/no	
AQ-P	12a	Almond protein, polyclonal	according to manual	Yes	
BC	14a	As Per Kit Instructions	As Per Kit Instructions	Yes	
BF	5			yes	
ES	11				
IL	1				
RS-F	4		extraction buffer/ 10 min./ 60 °C	no	
RS-F	9	Ab react specifically with almond proteins	according to test instructions	Yes	
RS-F	14b	As Per Kit Instructions	As Per Kit Instructions	Yes	
RS-F	15a				
SP	2	detects almond proteins	according to manufacturer information	Yes	
SP	16				
VT	7		as stipulated in kit insert	yes	
VT	13			yes	
VT	15b				
div °	12b				

° Lateral Flow

5.1.4 PCR: Buckwheat

Meth. Abr.	Evaluation number	Date of analysis	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	%		Test-Kit + Manufacturer
SFA	6	19.01.22	positive	14,6	negative		positive	9	0,4	1	30	Buckwheat	Sure Food ALLERGEN, R-Biopharm / Congen
SFA-ID	10		positive		negative		positive		0,4			Buckwheat-DNA	Sure Food Allergen ID, R-Biopharm / Congen
SFA-ID	14	01.02.2022	positive	17,4	negative	<1	positive	20,33	1	1		Buckwheat-DNA	Sure Food Allergen ID, R-Biopharm / Congen
div	15	31.12.21	positive		negative		positive		1			Buckwheat-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 on the method (extraction and determination)	Method accredited ISO/IEC 17025	Further Remarks
		Target-Sequence / -DNA	e.g. Extraction / enzymes / clean-up / real time PCR / gel electrophoresis / cycles	yes/no	
SFA	6	Fagopyrum esculentum	Sure Food Prep Advanced Protocol 1	No	Article no. S3620, K03
SFA-ID	10		real time PCR	no	
SFA-ID	14	As Per Kit Instructions	As Per Kit Instructions	No	
div	15	ITS	Macherey-Nagel NucleoSpin Food Mini Kit		

5.1.5 PCR: Macadamia

Meth. Abr.	Evaluation number	Date of analysis	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	%		Test-Kit + Manufacturer
SFA	6	28.12.22	positive	4,4	negative		positive	1,4	0,4	1	30	Macadamia	Sure Food ALLERGEN, R-Biopharm / Congen
SFA	9	28.12.21	positive		negative		positive					Macadamia-DNA	Sure Food ALLERGEN, R-Biopharm / Congen
SFA-4p	8	12.01.22	positive		negative		positive		0,4			Please select!	Sure Food Allergen 4plex, R-Biopharm / Congen
SFA-ID	3		positive		negative		positive		0,4			Please select!	Sure Food Allergen ID, R-Biopharm / Congen
SFA-ID	10		positive		negative		positive		0,4			Macadamia-DNA	Sure Food Allergen ID, R-Biopharm / Congen
SFA-ID	14	03.01.2022	positive	16,95	negative	<1	positive	6,52	1	1		Macadamia-DNA	Sure Food Allergen ID, R-Biopharm / Congen
div	2	27.12.21	positive		negative		positive		4			Macadamia-DNA	internal method
div	15	07.01.22	positive		negative		positive		20			Macadamia-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 on the method (extraction and determination)	Method accredited ISO/IEC 17025	Further Remarks
		Target-Sequence / -DNA	e.g. Extraction / enzymes / clean-up / real time PCR / gel electrophoresis / cycles	yes/no	
SFA	6	Macadamia ternifolia	Sure Food Prep Advanced Protocol 1	No	Article no. S3616, K02
SFA	9	characteristic sequence section of macadamia DNA	Spiking Level Sample: SureFood Prep Advanced r-biopharm/ Proteinase K/ Real Time PCR/ 45 cycles Samples A+B: Dneasy Mericon Food-Kit Qiagen/ Proteinase K/ Real Time PCR/ 45 cycles	yes	
SFA-4p	8				
SFA-ID	3				
SFA-ID	10		real time PCR	no	
SFA-ID	14	As Per Kit Instructions	As Per Kit Instructions	No	
div	2		CTAB / Proteinase K / Rnase A / Promega Maxwell / realtime PCR / 45 cycles	yes	
div	15	integrifolia vicilin precursor (AMP2)integrifolia vicilin precursor (AMP2)	Wizard Genomic DNA isolation		

5.1.6 PCR: Almond

Meth. Abr.	Evaluation number	Date of analysis	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	%		Test-Kit + Manufacturer
SFA	6	28.12.22	positive	1	negative		positive	1	0,4	1	30	Almond	Sure Food ALLERGEN, R-Biopharm / Congen
SFA	9	27.12.21	positive		negative		positive					Almond-DNA	Sure Food ALLERGEN, R-Biopharm / Congen
SFA-ID	3		positive		negative		positive		0,4			Please select!	Sure Food Allergen ID, R-Biopharm / Congen
SFA-ID	5		positive	> 0,4	negative	<0,4	positive	> 0,4	0,4			Almond-DNA	Sure Food Allergen ID, R-Biopharm / Congen
SFA-ID	10		positive		negative		positive		0,4			Almond-DNA	Sure Food Allergen ID, R-Biopharm / Congen
SFA-ID	14	03.01.2022	positive	1,57	negative	<1	positive	1,31	1	1		Almond-DNA	Sure Food Allergen ID, R-Biopharm / Congen
div	8	12.01.22	negative		negative		negative		10			Please select!	
div	15	07.01.22	negative		negative		negative		50			Almond-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 on the method (extraction and determination)	Method accredited ISO/IEC 17025	Further Remarks
		Target-Sequence / -DNA	e.g. Extraction / enzymes / clean-up / real time PCR / gel electrophoresis / cycles	yes/no	
SFA	6	Prunus dulcis	Sure Food Prep Advanced Protocol 1	No	article no. S3604, K02
SFA	9	characteristic sequence section of almond DNA	Spiking Level Sample: SureFood Prep Advanced r-biopharm/ Proteinase K/ Real Time PCR/ 45 cycles Samples A+B: Dneasy Mericon Food-Kit Qiagen/ Proteinase K/ Real Time PCR/ 45 cycles	yes	
SFA-ID	3				
SFA-ID	5			yes	
SFA-ID	10		real time PCR	no	
SFA-ID	14	As Per Kit Instructions	As Per Kit Instructions	No	
div	8				
div	15	nsLTP	Wizard Genomic DNA isolation		

5.2 Homogeneity

5.2.1 Mixture homogeneity before bottling

Microtracer Homogeneity Test
DLA ptAL08 (2021) Sample A

Weight whole sample	2,50	kg
Microtracer	FSS-red lake	
Particle size	75 – 300	µm
Weight per particle	2,0	µg
Addition of tracer	20,7	mg/kg

Result of analysis

Sample	Weight [g]	Particle number	Particles [mg/kg]
1	5,05	29	11,5
2	4,98	30	12,0
3	5,01	31	12,4
4	5,02	37	14,7
5	4,96	44	17,7
6	4,99	45	18,0
7	5,05	45	17,8
8	5,00	32	12,8

Poisson distribution		
Number of samples	8	
Degree of freedom	7	
Mean	36,6	Particles
Standard deviation	7,11	Particles
χ^2 (CHI-Quadrat)	9,66	
Probability	**21**	%
Recovery rate	71	%

Normal distribution		
Number of samples	8	
Mean	14,6	mg/kg
Standard deviation	2,84	mg/kg
rel. Standard deviaton	19,4	%
Horwitz standard deviation	10,7	%
HorRat-value	**1,8**	
Recovery rate	71	%

Microtracer Homogeneity Test
DLA ptAL08 (2021) Spiking Level Sample

Weight whole sample	1,20	kg
Microtracer	FSS-red lake	
Particle size	75 – 300	µm
Weight per particle	2,0	µg
Addition of tracer	31,8	mg/kg

Result of analysis

Sample	Weight [g]	Particle number	Particles [mg/kg]
1	4,97	77	31,0
2	5,00	87	34,8
3	5,01	88	35,1
4	4,97	82	33,0
5	5,05	80	31,7
6	4,96	86	34,7
7	5,02	81	32,3
8	4,98	90	36,1

Poisson distribution		
Number of samples	8	
Degree of freedom	7	
Mean	83,9	Particles
Standard deviation	4,63	Particles
χ^2 (CHI-Quadrat)	1,79	
Probability	**97**	%
Recovery rate	106	%

Normal distribution		
Number of samples	8	
Mean	33,6	mg/kg
Standard deviation	1,85	mg/kg
rel. Standard deviaton	5,5	%
Horwitz standard deviation	9,4	%
HorRat-value	**0,59**	
Recovery rate	106	%

5.3 Information on the Proficiency Test (PT)

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

PT number	***ptAL08 - 2021***
PT name	***Allergens VIII: Buckwheat, Almond and Macadamia in Cereal Muesli with "Spiking Level Sample"***
Sample matrix (processing)	***Samples A + B:*** *Muesli with fruits / Ingredients: Whole oat flakes, sugar, sunflower oil, rice extrudate (rice flour, barley malt flour), puffed wheat, black currant juice concentrate, freeze-dried berries (raspberries, strawberries, blackberries), dates, cranberries, rice syrup, coconut concentrate, salt, apple juice , Beetroot juice concentrate, other additives and allergenic foods (one of the two samples) , 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 + Spiking Level Sample:* *room temperature (PT period), cooled 2 - 10°C (long term)*
Intentional use	*Laboratory use only (quality control samples)*
Parameter	*qualitative + quantitative: Buckwheat (Buckwheat protein, DNA), Almond (Almond protein, DNA) and Macadamia (Macadamia 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***
Last Deadline	***the latest February 04th 2022***
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
		SWITZERLAND
		CANADA
		ITALY
		Germany
		Germany
		FRANCE
		Germany
		Germany
		Germany
		Germany
		CANADA
		GREAT BRITAIN
		SPAIN
		CZECH REPUBLIC
		ITALY

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

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

7. Index of references

1. DIN EN ISO/IEC 17025:2005; Allgemeine Anforderungen an die Kompetenz von Prüf- und Kalibrierlaboratorien / General requirements for the competence of testing and calibration laboratories
2. DIN EN ISO/IEC 17043:2010; Konformitätsbewertung – Allgemeine Anforderungen an Eignungsprüfungen / Conformity assessment – General requirements for proficiency testing
3. ISO 13528:2015 & DIN ISO 13528:2009; Statistische Verfahren für Eignungsprüfungen durch Ringversuche / Statistical methods for use in proficiency testing by inter-laboratory comparisons
4. ASU §64 LFGB: Planung und statistische Auswertung von Ringversuchen zur Methoden-validierung / DIN ISO 5725 series part 1, 2 and 6 Accuracy (trueness and preci-sion) of measurement methods and results
5. Verordnung / Regulation 882/2004/EU; Verordnung über über amtliche Kontrollen zur Überprüfung der Einhaltung des Lebensmittel- und Futtermittelrechts sowie der Bestimmungen über Tiergesundheit und Tierschutz / Regulation on official controls performed to ensure the verification of compliance with feed and food law, animal health and animal welfare rules
6. Evaluation of analytical methods used for regulation of food and drugs; W. Hor-witz; Analytical Chemistry, 54, 67-76 (1982)
7. The International Harmonised Protocol for the Proficiency Testing of Ananlytical Laboratories ; J.AOAC Int., 76(4), 926 – 940 (1993)
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