Proficiency Tests
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Evaluation Report
proficiency test

DLA 08/2016

Allergens VIII:

Lupin, Brazil Nuts and Pistachio

in Veggie Burger Powder

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

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EP-Nummer *PT-Number*	DLA 08/2016
EP-Koordinator *PT-Coordinator*	Dr. Matthias Besler
Status des EP-Bericht *Status of PT-Report*	Abschlussbericht / Final report (20 April 2017) Gültig ist die jeweils letzte Version/Korrektur des Berichts. Sie ersetzt alle vorangegangenen Versionen. Only the latest version/correction of the report is valid. It replaces all preceding versions.
EP-Bericht Freigabe *PT-Report Authorization*	Dr. Matthias Besler (Technischer Leiter / Technical Manager) - *gezeichnet / signed M. Besler* Dr. Gerhard Wichmann (QM-Beauftragter / Quality Manager) - *gezeichnet / signed G. Wichmann* Datum / Date: 20 April 2017
Unteraufträge *Subcontractors*	Die Prüfung der Gehalte, Homogenität und Stabilität von EP-Parametern wird von DLA im Unterauftrag vergeben. The analysis of the content, homogeneity and stability of PT-parameters are subcontracted by DLA.
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 for the detection of allergens in the range of mg/kg and one spiking material sample were provided for analysis. The spiking material sample contains the respective allergenic ingredients in the range of 1-10 % and was added to the spiked PT-sample. The results of the spiking material sample should give the possibility of a comparison with the spiked sample in respect to the detectability of the allergens with and without the influence of matrix and / or food processing.

The test material was a mixture of two common in commerce veggie burger powders with additional wheat flour. The basic composition of both sample A and sample B was the same (see table 1). After crushing, sieving and homogenization of the basic mixture the spiked sample A was produced as follows:
The spiking material containing the allergenic ingredients Lupin, Brazil-Nut and Pistachio was added to an aliquot of the basic mixture and the mixture was homogenized. Subsequently, the basic mixture was again added in 4 additional steps and mechanically homogenized in each case until the total quantity had been reached.
The raw materials of the allergen premix were sieved (mesh 1,5 mm) or sieved by means of a centrifugal mill (mesh 500 µm) prior to use.

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

After homogenization the samples A and B were portioned to approximately 25 g and the spiking material sample to 10 g into metallised PET film bags.

Table 1: Composition of DLA-Samples

Ingredients	**Sample A**	**Sample B**
Veggie Burger (Powder) "Soy + Vegetables" Ingredients: oat flakes, soy granules, soy flakes (soy in total approx. 21%), buckwheat flour, buck-wheats grouts, wheat flour, -semolina, -bran, salt, parsley, yeast, wholemeal rice flour, fried onion, carrots, parsnips, onion, celery leaves, leek, marjoram, celery, garlic, sun-flower oil, pepper, tomato, parsley root, macis, lovage, curcuma, natural aroma, chive, laurel Nutrients per 100 g: Protein 21 g, Carbohydrates 40 g, Fat 8,8 g	57,2 g/100 g	57,5 g/100 g
Organic-Wheat flour, Type 1050	42,3 g/100g	42,5 g/100g
Spiking material sample	0,443 g/100 g	-

Table 2: Added amounts of allergenic ingredients

Ingredients	**Spiking material sample**	**Amounts in Sample A**
Potato flour	89 %	0,39 %
Lupin - as sweet lupin flour* - *thereof 37% total protein***	 9040 mg/kg (0,90 %) 3340 mg/kg	 40 mg/kg 15 mg/kg
Brazil-Nut - as Brazil-Nut* - *thereof 15% total protein***	 7780 mg/kg (0,78 %) 1150 mg/kg	 35 mg/kg 5,1 mg/kg
Pistachio - as pistachio* - *thereof 26% total protein***	 11100 mg/kg (1,11 %) 2840 mg/kg	 49 mg/kg 13 mg/kg
additional ingredients: *maltodextrin, sodium sulfate, and silicon dioxide*	< 8,5 %	< 0,04 %

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

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

2.1.1 Homogeneity

The **mixture homogeneity before bottling** was examined 8-fold by **microtracer analysis**. It is a standardized method that is part of the international GMP certification system for feed [14].
Before mixing dye coated iron particles of µm size are added to the sample and the number of particles is determined after homogenization in taken aliquots. The evaluation of the mixture homogeneity is based on the Poisson distribution using the chi-square test. A probability of ≥ 5 % is equivalent to a good homogeneous mixture and of ≥ 25% to an excellent mixture [14, 15].
The microtracer analysis of the present PT samples showed a probability of 99% and 70% for the samples A and B, and of 100% for the spiking material sample. Additionally particle number results were converted into concentrations, statistically evaluated according to normal distribution and compared to the standard deviation according to Horwitz. This gave a HorRat value of 0,7, 1,3 and 0,5 respectively. 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. The sample weights were made with a deviation of ± 10% from recommended sample weight of the test kit instructions and not communicated to the laboratories. After transmission of analysis results by the laboratories, the valid results were calculated on the basis of the exact weightings by DLA and the statistical calculation was carried out according to ISO 13528:2009 Annex B.

Valuation of homogeneity
The homogeneity is regarded as sufficient when the standard deviation between the samples Ss is ≤ 15% („heterogeneity standard deviation"). This criterion is fulfilled for sample A by the Immunolab ELISA tests for lupin, brazil nut and pistachio, respectively (see page 7). The heterogeneity standard deviation in the AgraQuant ELISA tests was about 20% for lupin and pistachio. Recommendations for repeatability standard deviations of ELISA and PCR methods are usually ≤ 25% [16, 17, 20, 21].

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

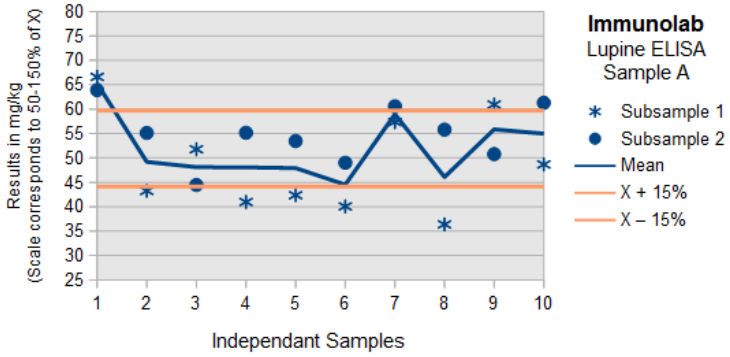
ELISA-Tests: Homogenität Lupine / Homogeneity Lupin

Immunolab Lupine ELISA

Sample weights: 1,0 g (0,9 – 1,1 g)
Number of replicates: 2
Overall result: Lupin 51,9 ± 6,4 mg/kg

Sample A	Subsample 1 mg/kg	Subsample 2 mg/kg	Mean mg/kg
1	66,6	63,9	65,2
2	43,2	55,1	49,2
3	51,8	44,5	48,1
4	41,0	55,2	48,1
5	42,4	53,5	47,9
6	40,1	49,0	44,5
7	57,3	60,6	59,0
8	36,4	55,8	46,1
9	60,9	50,8	55,9
10	48,7	61,3	55,0

General average X	51,9	
SD of sample means Sx	6,61	12,7%
SD within-samples Sw	7,93	15,3%
SD between-samples Ss	6,40	12,3%

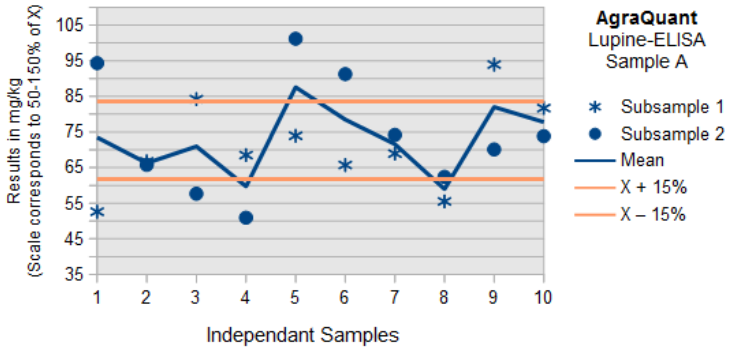


Romerlabs AgraQuant Lupin

Sample weights: 1,0 g (0,9 – 1,1 g)
Number of replicates: 2
Overall result: Lupin 72,7 ± 14,1 mg/kg

Sample A	Subsample 1 mg/kg	Subsample 2 mg/kg	Mean mg/kg
1	52,6	94,3	73,5
2	66,7	65,8	66,3
3	84,2	57,7	70,9
4	68,5	50,9	59,7
5	73,9	101,2	87,6
6	65,7	91,3	78,5
7	68,9	74,2	71,6
8	55,5	62,4	59,0
9	93,9	70,1	82,0
10	81,7	73,8	77,8

General average X	72,7	
SD of sample means Sx	9,25	12,7%
SD within-samples Sw	15,6	21,4%
SD between-samples Ss	14,1	19,5%



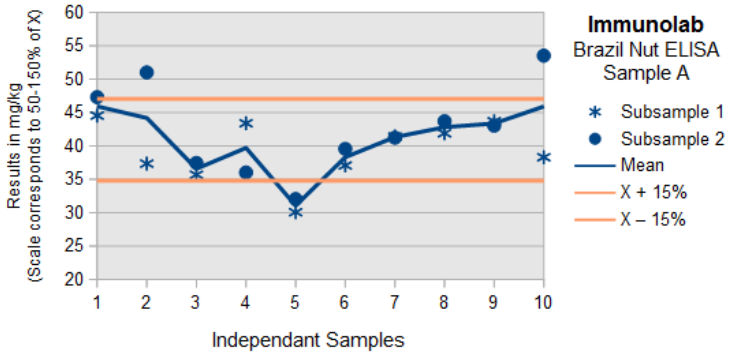
ELISA-Tests: Homogenität Paranuss / Homogeneity Brazil Nut

Immunolab Brazil Nut ELISA

Sample weights: 1,0 g (0,9 – 1,1 g)
Number of replicates: 2
Overall result: Brazil Nut 40,9 ± 3,8 mg/kg

Sample A	Subsample 1 mg/kg	Subsample 2 mg/kg	Mean mg/kg
1	44,5	47,3	45,9
2	37,3	51,0	44,2
3	35,7	37,4	36,6
4	43,4	36,0	39,7
5	30,1	32,0	31,0
6	37,1	39,5	38,3
7	41,4	41,2	41,3
8	41,9	43,7	42,8
9	43,7	43,0	43,3
10	38,3	53,5	45,9

General average X	40,9	
SD of sample means Sx	4,65	11,4%
SD within-samples Sw	4,98	12,2%
SD between-samples Ss	3,75	9,16%



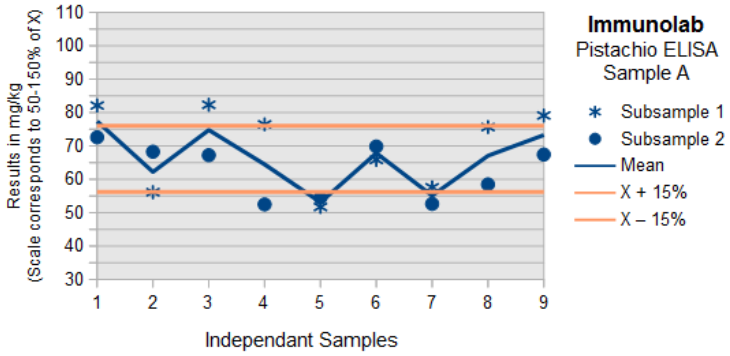
ELISA-Tests: Homogenität Pistazie / Homogeneity Pistachio

Immunolab Pistachio ELISA

Sample weights: 1,0 g (0,9 – 1,1 g)
Number of replicates: 2
Overall result: Pistachio 66,1 ± 7,0 mg/kg

Sample A	Subsample 1 mg/kg	Subsample 2 mg/kg	Mean mg/kg
1	82,1	72,5	77,3
2	56,1	68,2	62,2
3	82,3	67,2	74,7
4	76,4	52,5	64,4
5	51,7	54,5	53,1
6	65,8	69,8	67,8
7	57,6	52,6	55,1
8	75,6	58,4	67,0
9	79,1	67,4	73,2

General average X	66,1	
SD of sample means Sx	8,39	12,7%
SD w ithin-samples Sw	9,19	13,9%
SD betw een-samples Ss	7,02	10,6%

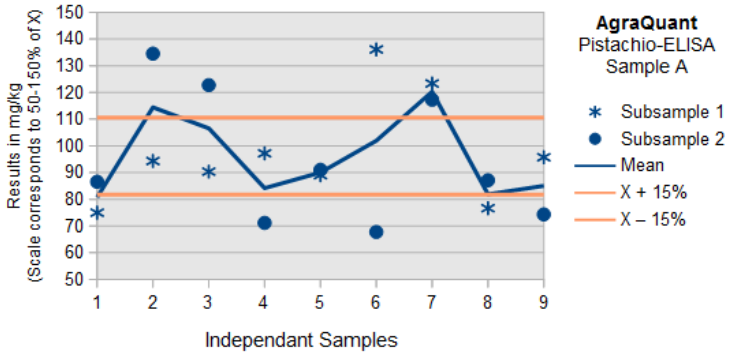


Romerlabs AgraQuant Pistachio

Sample weights: 1,0 g (0,9 – 1,1 g)
Number of replicates: 2
Overall result: Pistachio 96,1 ± 19,3 mg/kg

Sample A	Subsample 1 mg/kg	Subsample 2 mg/kg	Mean mg/kg
1	75,0	86,6	80,8
2	94,3	134,5	114,4
3	90,4	122,7	106,6
4	97,2	71,2	84,2
5	89,0	91,0	90,0
6	136,1	67,8	101,9
7	123,4	117,2	120,3
8	76,7	87,1	81,9
9	95,7	74,3	85,0

General average X	96,1	
SD of sample means Sx	15,0	15,6%
SD w ithin-samples Sw	22,1	22,9%
SD betw een-samples Ss	19,3	20,1%



2.2 Sample shipment and information to the test

The portions of test material (sample A and sample B as well as the spiking material sample) were sent to every participating laboratory in the 49th week of 2016. The testing method was optional. The tests should be finished at January 20th 2017 the latest.

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

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

2.3 Submission of results

The participants submitted their results in standard forms, which have been sent by email or were available on our website. On one hand the results given as positive/negative and on the other hand the indicated results of the allergenic ingredients e.g. total food item or protein in mg/kg were evaluated.
During evaluation DLA eventually requests detailed information by email on the type of indicated quantitative results from participants concerned.
Queried and documented were the indicated results and details of the test methods like specifity, test kit manufacturer and hints about the procedure.
In case participants submitted several results for the same parameter obtained by different methods these results were evaluated with the same evaluation number with a letter as a suffix and indication of the related method.
All 15 participants submitted their results in time.

3. Evaluation

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

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

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

3.1 Consensus value from participants (assigned value)

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

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

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

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

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

3.2 Robust standard deviation

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

The following robust standard deviations were considered:

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

3.3 Exclusion of results and outliers

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

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

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

3.4 Target standard deviation (for proficiency assessment)

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

3.4.1 General model (Horwitz)

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

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

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

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

3.4.2 Value by precision experiment

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

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

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

Table 3a: ELISA-Methods - Relative repeatability standard deviations (RSD_r) and relative reproducibility standard deviations (RSD_R) from precision experiments and resulting target standard deviations σ_{pt} [27, 28]

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

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

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

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

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

Parameter	**Matrix**	**Mean [mg/kg]**	**Recov-ery**	**rob RSD**	**RSD_r**	**RSD_R**	**σ_{pt}**	**Method / Literature**
Brazil Nut	Rice cookie	100,1 18,5 9,2	89,1% 86,5% 98%	-	34,1% 36,2% 40,2%	34,4% 38,2% 41,8%	24,5% 28,4% 30,7%	rt-PCR ASU 18.00-21
Brazil Nut	Wheat cookie	82,5	65,7%	-	25,6%	36,4%	31,2%	rt-PCR ASU 18.00-21
Brazil Nut	Sauce powder	46,8	42,6%	-	27,5%	39,7%	34,6%	rt-PCR ASU 18.00-21
Brazil Nut	Rice cookie	99,3 12,5	96,6% 71%	-	16,8% 54,2%	31,8% 56,5%	29,5% 41,5%	rt-PCR ASU 18.00-22
Brazil Nut	Wheat cookie	67,1	62,2%	-	15,6%	35,8%	34,1%	rt-PCR ASU 18.00-22
Brazil Nut	Sauce powder	45,1	48,4%	-	34,4%	37,5%	28,5%	rt-PCR ASU 18.00-22
Lupin	Rice cookie	99,1 17,5 9,3	102% 87% 95%	-	14,6% 26,5% 39,1%	23,0% 33,1% 42,6%	20,0% 27,3% 32,4%	rt-PCR ASU 18.00-22
Lupin	Wheat cookie	64,8	64,1%	-	10,5%	29,5%	28,6%	rt-PCR ASU 18.00-22
Lupin	Sauce powder	48,9	53,6%	-	23,9%	48,0%	44,9%	rt-PCR ASU 18.00-22

3.4.3 Value by perception

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

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

Table 4: ELISA-Validation

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

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

Table 5: PCR-Validation

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

(a) = Trueness / Richtigkeit

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

3.5 z-Score

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

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

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

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

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

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

3.5.1 Warning and action signals

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

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

3.6 z'-Score

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

The calculation is performed by:

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

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

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

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

For warning and action signals see 3.5.1.

3.7 Quotient S*/σpt

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

3.8 Standard uncertainty of the assigned value

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

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

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

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

3.9 Figures

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

3.10 Recovery rates: Spiking

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

4. Results

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

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

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

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

ELISA-results for lupin were converted into lupin protein, considering the literature and test kit values (approx. 40% protein; AgraQuant and Immunolab).

ELISA-results given as brazil-nut protein (Elution technologies) were converted into brazil-nut considering the experimentally determined protein content of 15%.

For pistachio all present results were submitted as pistachio, thus no recalculation was necessary.

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

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

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

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

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

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

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

4.1 Proficiency Test Lupin

4.1.1 ELISA Results: Lupin (as Lupin-protein)

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		
8	positive	33,6	positive	1,32	2/2 (100%)	AQ	Result converted °
15	positive	460	positive	420	2/2 (100%)	ES	Result excluded
14	positive	20,0	positive	0,92	2/2 (100%)	IL	Result converted °
3	positive	21,1	positive	3,40	2/2 (100%)	RS-F	
5	positive	34,3	positive	4,07	2/2 (100%)	RS-F	
6	positive	17,0	positive	2,50	2/2 (100%)	RS-F	
7	positive	30,9	positive	4,72	2/2 (100%)	RS-F	
10	positive	22,9	positive	2,80	2/2 (100%)	RS-F	
11	positive	13,1	positive	1,53	2/2 (100%)	RS-F	

° Conversion p. 19

	Sample A		Sample B	
Number positive	9		9	
Number negative	0		0	
Percent positive	100		100	
Percent negative	0		0	
Consensus value	positive		positive	

Methods:

AQ = AgraQuant, RomerLabs

ES = ELISA-Systems

IL = Immunolab

RS-F= Ridascreen® Fast, R-Biopharm

Comments:

The consensus values were positive for both, the spiked sample A and the none-spiked sample B. The result is in agreement with the obtained PCR results. Sample B (not spiked) contains small contents of lupin protein in the range of 0,9 to 5 mg/kg. The results of participant 15 are probably based on a high cross-reactivity of used method between lupin and soy, as soy is included (approx. 12%) in the basic matrix.

Quantitative valuation of results: Sample A

Evaluation number	Lupin	z-Score Xpt_{ALL}	z-Score Xpt_{RS}	Method	Remarks
	[mg/kg]				
8	33,6	1,6		AQ	Result converted °
15	460			ES	Result excluded
14	20,0	-0,7	3,4	IL	Result converted °
3	21,1	-0,5	-0,4	RS-F	
5	34,3	1,7	1,9	RS-F	
6	17,0	-1,2	-1,1	RS-F	
7	30,9	1,1	1,3	RS-F	
10	22,9	-0,2	-0,1	RS-F	
11	13,1	-1,8	-1,7	RS-F	

° Conversion p. 19

Methods:

AQ = AgraQuant, RomerLabs

ES = ELISA-Systems

IL = Immunolab

RS-F= Ridascreen® Fast, R-Biopharm

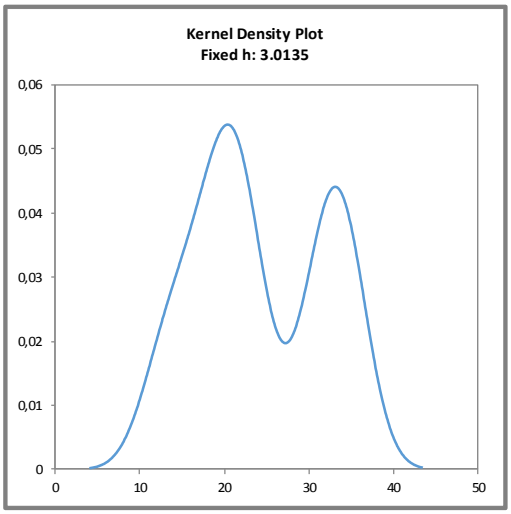


Abb. / Fig. 1:
Kerndichte-Schätzung aller ELISA-Ergebnisse (mit h = 0,5 x σpt von Xpt_{ALL})

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

Comments:
The kernel density estimation shows a main maximum at 20 mg/kg and a side peak at >30 mg/kg. The results of the side peak can not be assigned exclusively to a single method.

Characteristics: Quantitative evaluation Lupin (as lupin-protein)

Sample A

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	8	6
Number of outliers	0	0
Mean	24,1	23,2
Median	22,0	22,0
Robust Mean (X)	**24,1**	**23,2**
Robust standard deviation (S*)	**9,00**	**9,19**
Target range:		
Target standard deviation σ_{pt}	**6,03**	**5,80**
lower limit of target range	**12,1**	**11,6**
upper limit of target range	**36,2**	**34,8**
Quotient S/σ_{pt}*	*1,5*	*1,6*
Standard uncertainty $U_{(Xpt)}$	*3,98*	*4,69*
Quotient $U_{(Xpt)}/\sigma_{pt}$	*0,66*	*0,81*
Results in the target range	*8*	*6*
Percent in the target range	*100*	*100*

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

Comments to the statistical characteristics and assigned values:

The kernel density plot shows no clear method dependent differences. The evaluation of results showed a normal variability of results. The quotient S/σ_{pt} was below 2,0.*
The robust standard deviations of 37,3% and 39,6% 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 the methods.

The robust means of the evaluations were 165% and 159% of the spiking level of lupin to sample A and slightly over the recommendations for the applied methods (s. 3.4.3). It should be considered that an additional small content of lupin is present in the basic matrix (see sample B).

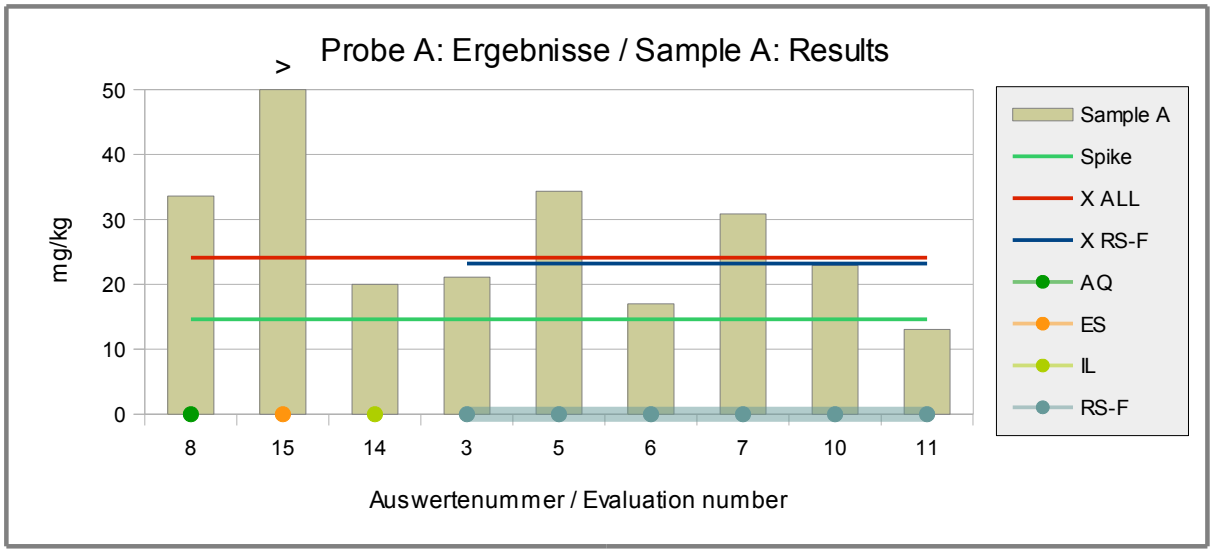


Abb./Fig. 2: ELISA Results Lupin-protein
green line = Spiking level
red line = Assigned value robust mean all results
blue line = Assigned value robust mean method RS-F
round symbols = Applied methods (see legend)

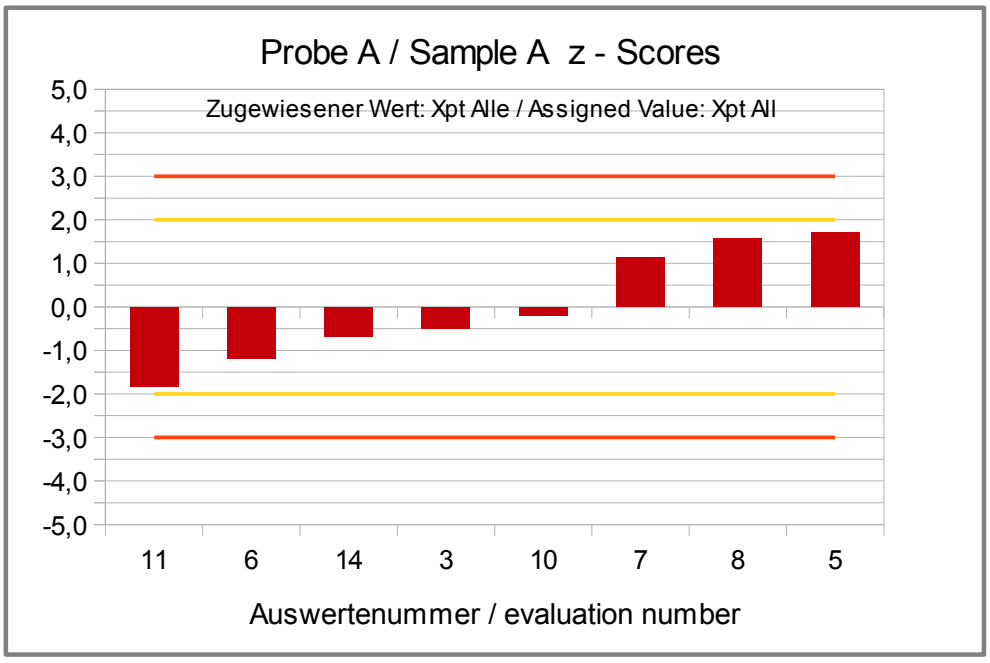


Abb./Fig. 3:
z-Scores (ELISA Results Lupin-protein) Assigned value robust mean of all results

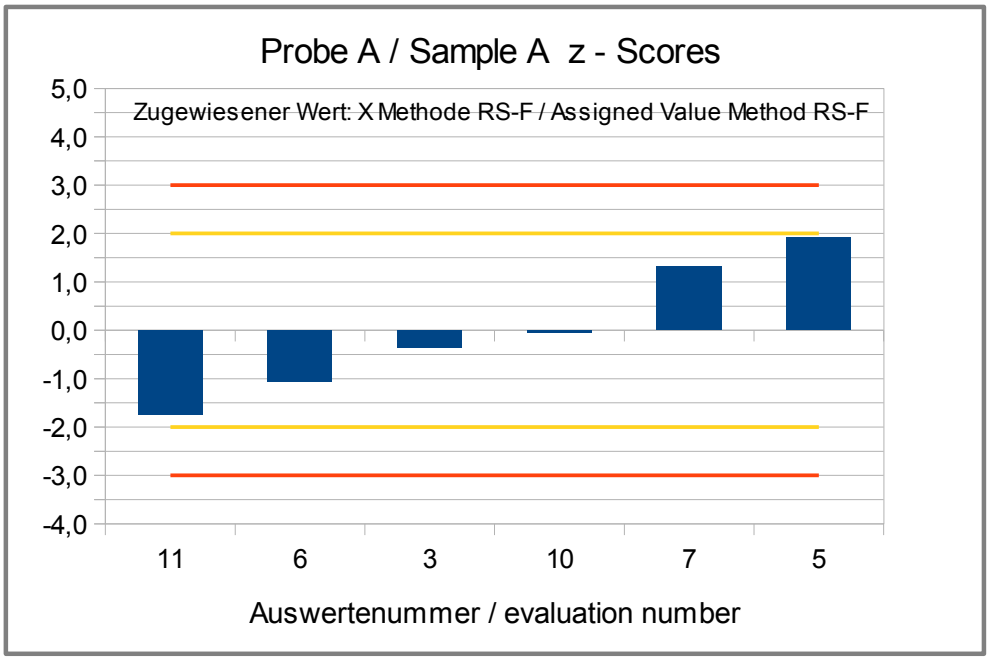
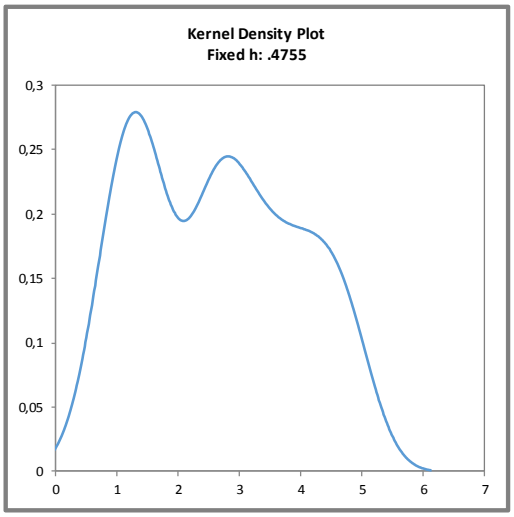


Abb./Fig. 4:
z-Scores (ELISA Results Lupin-protein) Assigned value robust mean of results method RS-F (R-Biopharm, Ridascreen Fast)

Quantitative valuation of results: Sample B

Evaluation number	Lupin	z-Score Xpt_{ALL}	z-Score Xpt_{RS}	Method	Remarks
	[mg/kg]				
8	1,32	-1,4		AQ	Result converted °
15	420			ES	Result excluded
14	0,92	-1,8	1,2	IL	Result converted °
3	3,40	0,8	0,3	RS-F	
5	4,07	1,5	1,1	RS-F	
6	2,50	-0,2	-0,8	RS-F	
7	4,72	2,2	2,0	RS-F	
10	2,80	0,1	-0,5	RS-F	
11	1,53	-1,2	-2,1	RS-F	

° Conversion p. 19



Methods:

AQ = AgraQuant, RomerLabs

ES = ELISA-Systems

IL = Immunolab

RS-F= Ridascreen® Fast, R-Biopharm

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

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

Comments:
The kernel density estimation exhibits a mean maximum at approx. 1 mg/kg and a wide side peak with a shoulder at >3 mg/kg. None of the maxima can be assigned exclusively to a single method.

Characteristics: Quantitative evaluation Lupin (as Lupin-protein)

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	8	6
Number of outliers	0	0
Mean	2,66	3,17
Median	2,65	3,10
Robust Mean (X)	**2,66**	**3,17**
Robust standard deviation (S*)	**1,54**	**1,30**
Target range:		
Target standard deviation σ_{pt} and $\sigma_{pt'}$	**0,951**	**0,793**
lower limit of target range	**0,755**	**1,59**
upper limit of target range	**4,56**	**4,76**
Quotient S/σ_{pt}*	*1,6*	*1,6*
Standard uncertainty $U_{(Xpt)}$	*0,681*	*0,662*
Quotient $U_{(Xpt)}/\sigma_{pt}$	*0,72*	*0,84*
Results in the target range	*7*	*5*
Percent in the target range	*88*	*83*

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

Comments to the statistical characteristics and assigned values:

The kernel density plot shows no clear method dependent differences. The evaluation of results of all methods showed a slightly increased variability with a quotient S/σ_{pt} from 2,3. Thus the evaluation was performed using the z'-score considering the standard uncertainty. The resulting quotient S*/$\sigma_{pt'}$ was below 2,0.*
The evaluation of the results of method RS-F showed a normal variability of results. The quotient S/σ_{pt} was below 2,0. The robust standard deviation is in the range of established values for the reproducibility standard deviation of the applied methods (see 3.4.2 value by precision experiments and 3.4.3 value by perception). The 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.

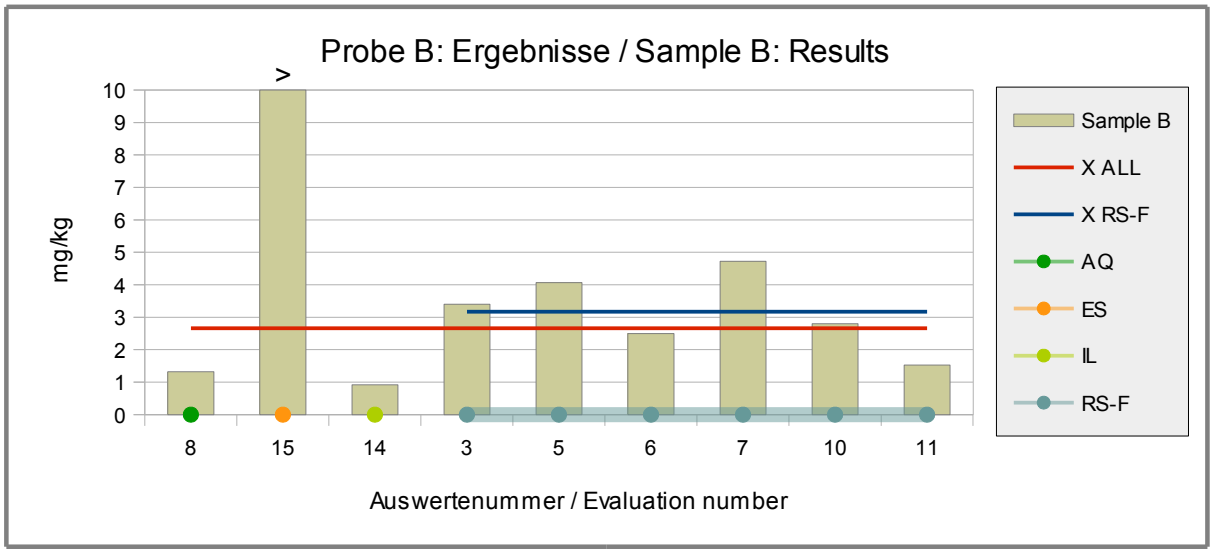


Abb./Fig. 6: ELISA Results Lupin-protein
red line = Assigned value robust mean all results
blue line = Assigned value robust mean method RS-F
round symbols = Applied methods (see legend)

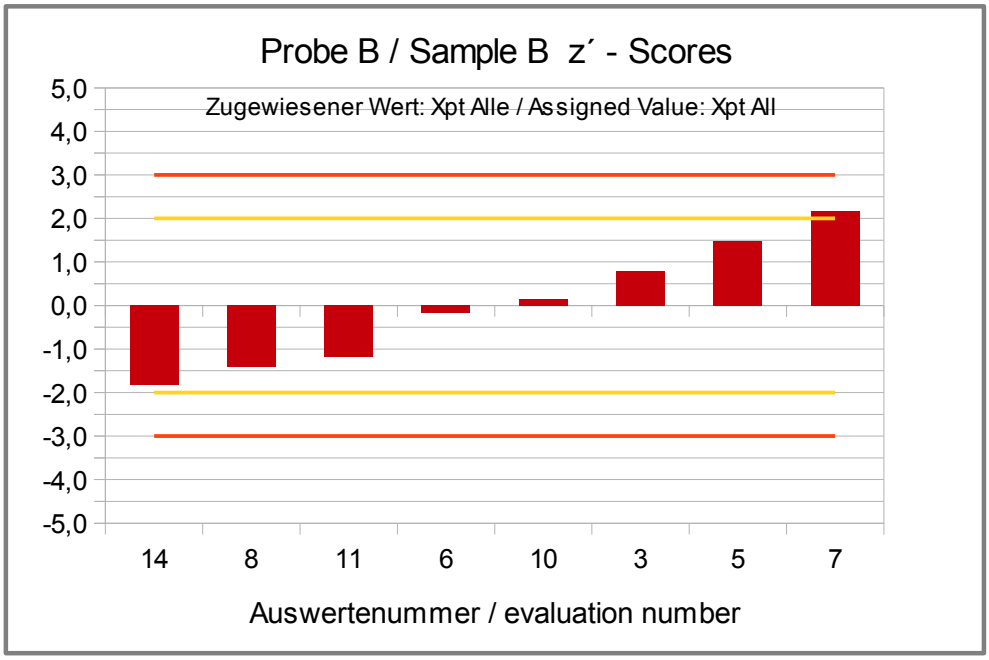


Abb./Fig. 7:
z´-Scores (ELISA Results Lupin-protein) Assigned value robust mean of all results

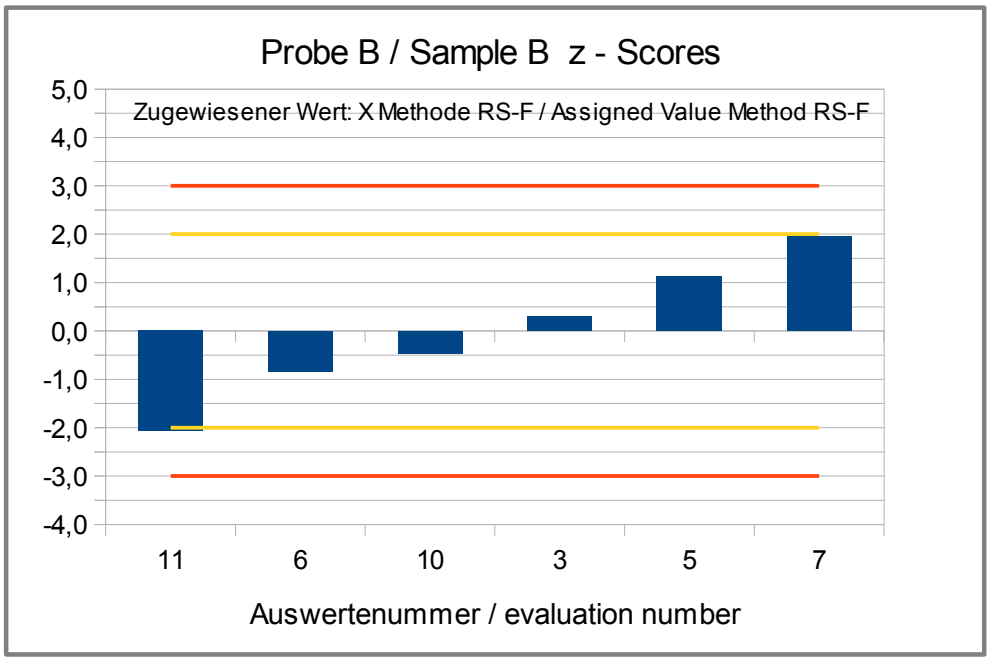


Abb./Fig. 8:
z-Scores (ELISA Results Lupin-protein) Assigned value robust mean of results method RS-F (R-Biopharm, Ridascreen Fast)

Recovery Rates for Lupin (as Lupin-protein):
Spiking Material Sample and Sample A

Evaluation number	Spiking material	Recovery rate*	Sample A	Recovery rate*	Method	Remarks
	[mg/kg]	[%]	[mg/kg]	[%]		
8	7270	220	33,6	230	AQ	Result converted °
15	7300	221	460	3151	ES	Result excluded
14	4400	**133**	20,0	**137**	IL	Result converted °
3	4550		21,1	**145**	RS-F	
5	4353	**132**	34,3	235	RS-F	
6	3500		17,0	**116**	RS-F	
7	3489	**106**	30,9	211	RS-F	
10	4091	**124**	22,9	157	RS-F	
11	2834	**86**	13,1	**89**	RS-F	

°Conversion p. 19

RA**	50-150 %	RA**	50-150 %
Number in RA	5	Number in RA	4
Percent in RA	71	Percent in RA	44

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

* Recovery rate 100% relative size: Lupin-protein, s. Page 5

** Range of acceptance of AOAC for allergen ELISAS

Comments:
For the spiking material sample 71% (5) of the participants obtained a recovery rate by ELISA methods within the range of the AOAC-recommendation of 50-150%. For the food matrix sample A produced with the spiking material sample 44% (4) of the participants obtained a recovery rate within the range of acceptance.
It should be considered that the basic matrix (see sample B) contains a small content of lupin. Considering an additional content of approx. 3 mg/kg of the spiking level participant no. 10 would have obtained a recovery rate of 127% within the range of acceptance.

4.1.2 PCR Results: Lupin

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		
8	positive		positive		2/2 (100%)	ASU	
6	positive	>0,4	positive	>0,4	2/2 (100%)	SFA-ID	
9	positive		negative		1/2 (50%)	SFA-ID	
13a	positive		positive		2/2 (100%)	SFA-ID	
5	positive	16,5	positive	2,12	2/2 (100%)	SFA-Q	
12	positive	9,10	positive	< 2,6	2/2 (100%)	SFA-Q	
13b	positive	6,87	positive	1,56	2/2 (100%)	SFA-Q	
1	positive		positive		2/2 (100%)	div.	
4	positive		positive		2/2 (100%)	div.	
15	positive		positive		2/2 (100%)	div.	

	Sample A		Sample B	
Number positive	10		9	
Number negative	0		1	
Percent positive	100		90	
Percent negative	0		10	
Consensus value	positive		positive	

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

Comments:
The consensus values were positive for both, the spiked sample A and none-spiked sample B. The result is in agreement with the obtained ELISA-results. A negative result was obtained for sample B by method SFA-ID.

Quantitative valuation of results: Samples A and B

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

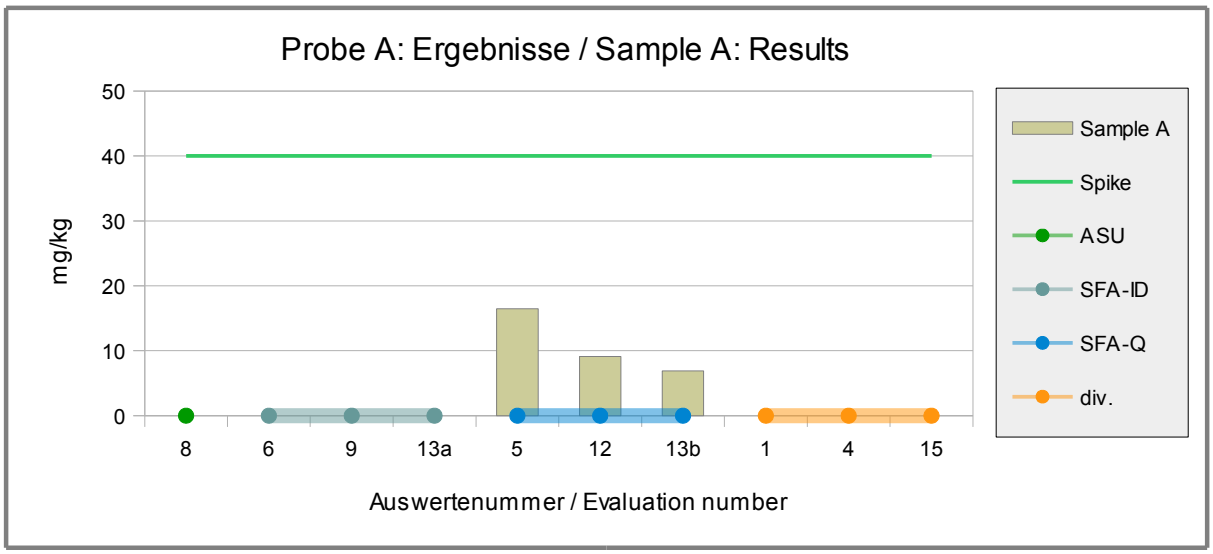


Abb./Fig. 9: PCR Results Lupin
green line = Spiking level
round symbols = Applied methods (see legend)

Recovery Rates for Lupin:
Spiking Material Sample and Sample A

Evaluation number	Spiking material	Recovery rate*	Sample A	Recovery rate*	Method	Remarks
	[mg/kg]	[%]	[mg/kg]	[%]		
8					ASU	
6	>0,4		>0,4		SFA-ID	
9					SFA-ID	
13a					SFA-ID	
5	858	9	16,5	41	SFA-Q	
12	693	8	9,10	23	SFA-Q	
13b	782	9	6,87	17	SFA-Q	
1					div.	
4					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:
ASU = ASU §64 Methode/method
SFA-ID = Sure Food Allergen ID, R-Biopharm / Congen
SFA-Q = Sure Food Allergen Quant, R-Biopharm / Congen
div = not indicated / other method

* Recovery rate 100% relative size: Lupin-protein, s. Page 5

** Range of acceptance of AOAC for allergen ELISAS

Comments:
None of the participants obtained a recovery rate in the spiking material sample nor the spiked sample A within the range of the AOAC-recommendation of 50-150%.

4.2 Proficiency Test Brazil-Nut

4.2.1 ELISA Results: Brazil-Nut

Qualitative valuation of results: Samples A and B

Evaluation number	Sample A	Sample A	Sample B	Sample B	Qualitative Valuation	Method	Remarks
	pos/neg	[mg/kg]	pos/neg	[mg/kg]	Agreement with consensus value		
2	positive	20,3	negative	<6,7	2/2 (100%)	ET	Result converted °
11	positive	22,6	negative	<6,7	2/2 (100%)	ET	Result converted °
14	positive	50,0	negative	< 1	2/2 (100%)	IL	

° Conversion p. 19

	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:
ET = Elution Technologies ELISA Kit
IL = Immunolab

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

Quantitative valuation of results: Sample A

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

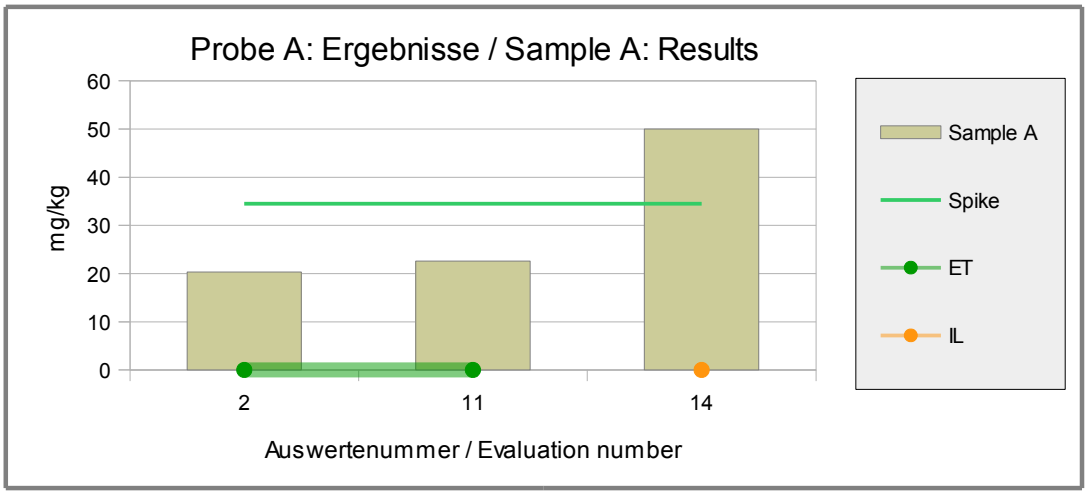


Abb./Fig. 10: ELISA Results Brazil-Nut
green line = Spiking level
round symbols = Applied methods (see legend)

Recovery Rates for Brazil-Nut:
Spiking Material Sample and Sample A

Evaluation number	Spiking material	Recovery rate*	Sample A	Recovery rate*	Method	Remarks
	[mg/kg]	[%]	[mg/kg]	[%]		
2	-		20,3	**59**	ET	Result converted °
11	6360	**82**	22,6	**66**	ET	Result converted °
14	1200	15	50,0	**145**	IL	

° Conversion p. 19

RA**	50-150 %	RA**	50-150 %
Number in RA	**1**	Number in RA	**3**
Percent in RA	**50**	Percent in RA	**100**

Methods:
ET = Elution Technologies ELISA Kit
IL = Immunolab

* Recovery rate 100% relative size: Brazil-Nut, s. Page 5

** Range of acceptance of AOAC for allergen ELISAS

Comments:
For the spiking material sample one of the participants obtained a recovery rate within the range of the AOAC-recommendation of 50-150%. For the food matrix sample A all (100%) obtained recovery rates were within the recommended range.

4.2.2 PCR Results: Brazil-Nut

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		
8	negative		negative		1/1 (100%)	ASU	no positive sample identified
15	negative		negative		1/1 (100%)	ASU	no positive sample identified
15	negative		negative		1/1 (100%)	ASU	no positive sample identified
5	positive		negative		1/1 (100%)	SFA-ID	
12	positive	> 0,4	negative	-	1/1 (100%)	SFA-ID	
1	negative		negative		1/1 (100%)	div.	no positive sample identified

	Sample A		Sample B	
Number positive	2		0	
Number negative	4		6	
Percent positive	33		0	
Percent negative	67		100	
Consensus value	none		negative	

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

Comments:
For sample A no consensus value (≥75% positive or negative) was obtained.
The consensus value for sample B is in qualitative agreement with the none-spiked sample B. The results from participant 5 and 12 are in agreement with the spiking of sample A.

Quantitative valuation of results: Sample A

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

Recovery Rates for Brazil-Nut:
Spiking Material Sample and Sample A

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

4.3 Proficiency Test Pistachio

4.3.1 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		
11	positive	129	negative	<1	2/2 (100%)	BC	
14	positive	78,0	negative	< 1	2/2 (100%)	IL	

	Sample A		Sample B	
Number positive	2		0	
Number negative	0		2	
Percent positive	100		0	
Percent negative	0		100	
Consensus value	positive		negative	

Methods:
BC = BioCheck ELISA
IL = Immunolab

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

Quantitative valuation of results: Sample A

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

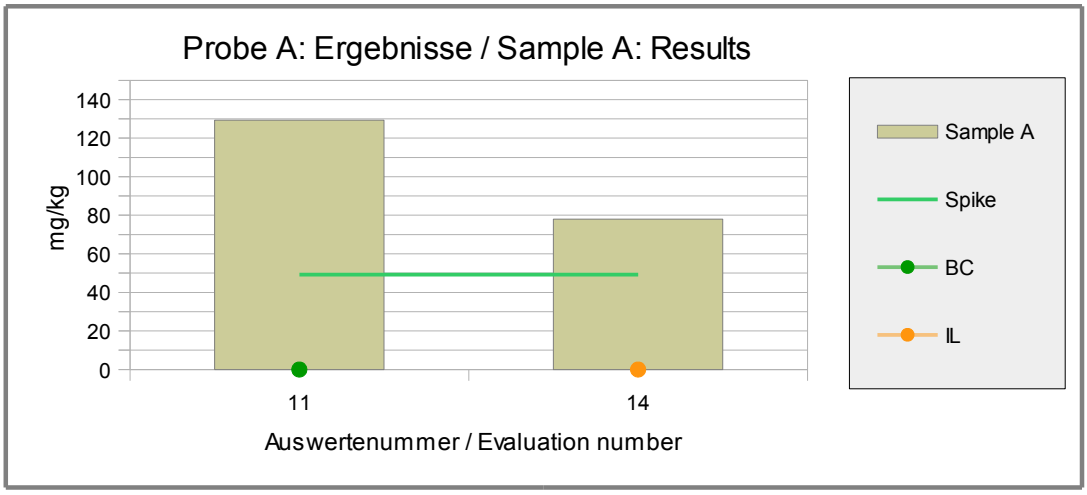


Abb./Fig. 11: ELISA Results Pistachio
green line = Spiking level
round symbols = Applied methods (see legend)

Recovery Rates for Pistachio:
Spiking Material Sample and Sample A

Evaluation number	Spiking material	Recovery rate*	Sample A	Recovery rate*	Method	Remarks
	[mg/kg]	[%]	[mg/kg]	[%]		
11	21419	193	129	263	BC	
14	17100	154	78,0	159	IL	

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

Methods:
BC = BioCheck ELISA
IL = Immunolab

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

** Range of acceptance of AOAC for allergen ELISAS

Comments:
None of the participants obtained a recovery rate in the spiking material sample or the spiked sample A via ELISA within the range of the AOAC-recommendation of 50-150%.
Increased recovery rates may be caused by a higher protein content of the raw material of the pistachios (26%) compared to the test kit specifications (approx. 20% protein in pistachio).

4.3.2 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	positive		negative		2/2 (100%)	SFA-ID	
6	positive	>0,4	negative	<0,4	2/2 (100%)	SFA-ID	
9	positive		negative		2/2 (100%)	SFA-ID	
12	positive	5,40	negative	-	2/2 (100%)	SFA-Q	
1	positive		positive		1/2 (50%)	div.	
4	positive		negative		2/2 (100%)	div.	
15	negative		negative		1/2 (50%)	div.	
15	negative		negative		1/2 (50%)	div.	

	Sample A		Sample B	
Number positive	6		1	
Number negative	2		7	
Percent positive	75		13	
Percent negative	25		88	
Consensus value	positive		negative	

Methods:

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

SFA-Q = Sure Food Allergen Quant, R-Biopharm / Congen

div = not indicated / other method

Comments:

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

Quantitative valuation of results: Sample A

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

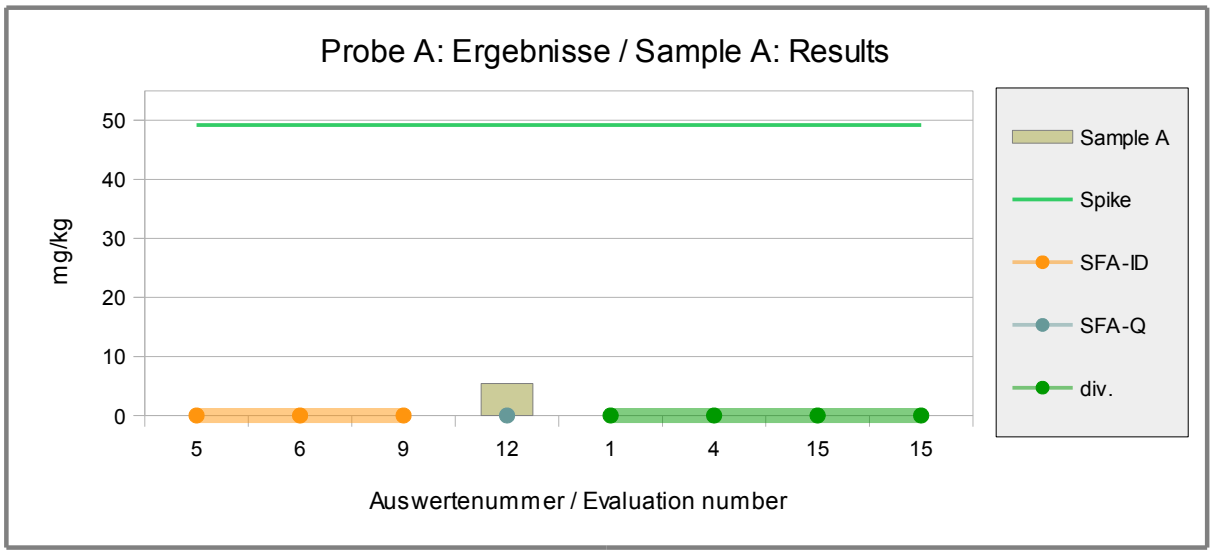


Abb./Fig. 12: PCR Results Pistachio
green line = Spiking level
round symbols = Applied methods (see legend)

Recovery Rates for Pistachio:
Spiking Material Sample and Sample A

Evaluation number	Spiking material	Recovery rate*	Sample A	Recovery rate*	Method	Remarks
	[mg/kg]	[%]	[mg/kg]	[%]		
5					SFA-ID	
6	>0,4		>0,4		SFA-ID	
9					SFA-ID	
12	1161	10	5,40	11	SFA-Q	
1					div.	
4					div.	
15					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-ID = Sure Food Allergen ID, R-Biopharm / Congen
SFA-Q = Sure Food Allergen Quant, 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:
None of the participants obtained a recovery rate for the spiking material sample or the spiked sample A by PCR within the range of the AOAC-recommendation of 50-150%.

4.3.3 Other Methods – Lateral Flow: 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		
2	positive	>15	negative	<15	2/2 (50%)	BA	

Methods:
BA = Bioavid, R-Biopharm

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

Quantitative valuation of results: Sample A

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

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: Lupin

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	8	27.12.16	positive	84	positive	3,3	positive	18176,3	Lupin	AgraQuant ELISA Lupin (COKAL1548), RomerLabs
IL	14	08.12.16	positive	50	positive	2,3	positive	11000	Lupin	Immunolab Lupine ELISA (LUP-E01)
ES	15	28.12.	positive	460	positive	420	positive	7300	Lupin flour protein	ELISA-Systems, Lupin Residue Assay (ESLFP-48)
ES	15	27.01.17	positive	350	positive	240			Lupin flour protein	ELISA-Systems, Lupin Residue Assay (ESLFP-48)
RS-F	3	03.01.17	positive	21,1	positive	3,4	positive	4550	Lupin-Protein	Ridascreen Fast Lupine/Lupin (R6102), r-Biopharm
RS-F	5		positive	34,34	positive	4,07	positive	4353	Lupin-Protein	Ridascreen Fast Lupine/Lupin (R6102), r-Biopharm
RS-F	6	16.01.17	positive	17	positive	2,5	positive	3500	Lupin-Protein	Ridascreen Fast Lupine/Lupin (R6102), r-Biopharm
RS-F	7	13.01.17	positive	30,87	positive	4,72	positive	3489,23	Lupin-Protein	Ridascreen Fast Lupine/Lupin (R6102), r-Biopharm
RS-F	10	Januar	-	22,9	-	2,8	-	4091	Lupin-Protein	Ridascreen Fast Lupine/Lupin (R6102), r-Biopharm
RS-F	11	05.01.17	positive	13,06	positive	1,53	positive	2834	Lupin-Protein	Ridascreen Fast Lupine/Lupin (R6102), r-Biopharm

Meth. Abr.	Evaluation number	Specifity	Remarks to the Method (Extraction and Determination)	Further Remarks
		Antibody	e.g. Extraction Solution / Time / Temperature	
AQ	8		As per kit instructions	
IL	14	polyclonal		Result for sample B slightly above the quatitation limit of 2ppm, maybe due to slightly cross reactivity tow ards soy (CR Soy flour = 0.0009%)
ES	15	Lupin flour protein	As per kit instructions	
ES	15	lupin flour protein	As per kit instructions	in second sample
RS-F	3	Lupin-Protein	As per kit instructions	
RS-F	5			
RS-F	6			
RS-F	7	Lupin-Protein	As per kit instructions	Multiple Determinations: A: 30,90;27,20;30,89;34,50 B: 5,98;4,0;5,01;3,8 C: 3737,97;3392,95;3336,80
RS-F	10	Lupinprotein	Sample diluted 1 : 5, spiking material sample diluted 1: 500	
RS-F	11	As per kit instructions	As per kit instructions	

5.1.2 ELISA: Brazil-Nut

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
ET	2	23.12.17	positive	3,05	negative	<1	-		Brazil-Nut Protein	Elution Technologies Brazil Nut Protein ELISA Kit
ET	11	06.01.17	positive	3,39	negative	<1	positive	954	Brazil-Nut Protein	Elution Technologies Brazil Nut Protein ELISA Kit
IL	14	08.12.16	positive	50	negative	< 1	positive	12000	Brazil-Nut	Immunolab Brazil Nut ELISA (PAR-E01)

Meth. Abr.	Evaluation number	Specifity	Remarks to the Method (Extraction and Determination)	Further Remarks
		Antibody	e.g. Extraction Solution / Time / Temperature	
ET	2	Brazil Nut		
ET	11	As per kit instructions	As per kit instructions	
IL	14	As per kit instructions		

5.1.3 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
BC	11	19.12.16	positive	129,31	negative	<1	positive	21419	Pidtachio	Bio-Ceck Pistachio ELISA Kit
IL	14	08.12.16	positive	78	negative	< 1	positive	17100	Pistachio	Immunolab Pistachio ELISA

Meth. Abr.	Evaluation number	Specifity	Remarks to the Method (Extraction and Determination)	Further Remarks
		Target Sequence / DNA	e.g. Extraction / Enzymes / Clean-Up / Real Time PCR / Gel electrophoresis / Cycles	
BC	11	As per kit instructions	As per kit instructions	
IL	14	polyclonal		

5.1.4 PCR: Lupin

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	8	03.01.17	positive		positive		positive		Lupin-DNA	ASU §64 Method
SFA-ID	6	16.01.17	positive	>0.4	positive	>0.4	positive	>0.4	Lupin-DNA	Sure Food Allergen ID, Congen / r-Biopharm
SFA-ID	9	11.01.17	positive		negative		positive		Lupin-DNA	r-biopharm SureFood Allergen Lupine (S3111)
SFA-ID	13a	12.12.17	positive		positive		positive		Lupin	Sure Food Allergen ID, Congen / r-Biopharm
SFA-Q	5		positive	16,46	positive	2,12	positive	858,25	Lupin-DNA	Sure Food Allergen QUANT, Congen / r-Biopharm
SFA-Q	12	09.12.	positive	9,1	positive	< 2,6	positive	693	Lupin	Sure Food Allergen QUANT, Congen / r-Biopharm
SFA-Q	13b	15.12.17	positive	6,87	positive	1,56	positive	781,61	Lupin	Sure Food Allergen QUANT, Congen / r-Biopharm
div.	1	09.01.17	positive		positive		positive		Lupin-DNA	in-house method
div.	4	17.01.17	positive		positive		positive		Lupin-DNA	in-house method
div.	15	14.12.	positive		positive		positive		Lupin-DNA	in-house method
div.	15	26.01.17	positive		positive				Lupin-DNA	in-house method

Meth. Abr.	Evaluation number	Specifity	Remarks to the Method (Extraction and Determination)	Further Remarks
		Target Sequence / DNA	e.g. Extraction / Enzymes / Clean-Up / Real Time PCR / Gel electrophoresis / Cycles	
ASU	8		2 g Einw aage, Machery & Nagel NucleoSpin Food Kit	
SFA-ID	6			
SFA-ID	9		r-biopharm SureFood Prep Advanced (S1053)	
SFA-ID	13a		SureFood PREP Advanced, Congen / r-Biopharm, according to kit instructions protocol 2	
SFA-Q	5			
SFA-Q	12	-	S3211 SureFood® ALLERGEN QUANT Lupin; Detection limit 0,4 mg/kg, determination limit 2,6 mg/kg; Extraction w ith S1053 SureFood® PREP Advanced, protocol 1	-
SFA-Q	13b		SureFood PREP Advanced, Congen / r-Biopharm, according to kit instructions protocol 1, sample w eight reduced from 100mg to 50mg	
div.	1	ITS	NucleoSpin Food (Macherey Nagel)/Real Time PCR/45 cycles	
div.	4		CTAB; Magnetic Beads; Taqmann real time PCR	
div.	15		CTAB/Proteinase K/Amylase/Promega Wizard DNA CleanUp/Real Time PCR/45 Cycles	
div.	15		CTAB/Proteinase K/Amylase/Promega Wizard DNA CleanUp/Real Time PCR/45 Cycles	in second sample

5.1.5 PCR: Brazil-Nut

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	8	03.01.17	negative		negative		positive		Brazil-Nut-DNA	ASU §64 Method
ASU	15	14.12.	negative		negative		positive		Brazil-Nut-DNA	ASU §64 Method
ASU	15	26.01.17	negative		negative				Brazil-Nut-DNA	ASU §64 Method
SFA-ID	5		positive		negative		positive		Brazil-Nut-DNA	Sure Food Allergen ID, Congen / r-Biopharm
SFA-ID	12	09.12.	positive	> 0,4	negative	-	positive	> 0,4	Brazil-Nut	Sure Food Allergen ID, Congen / r-Biopharm
div.	1	09.01.17	negative		negative		negative		Brazil-Nut-DNA	in-house method

Meth. Abr.	Evaluation number	Specifity	Remarks to the Method (Extraction and Determination)	Further Remarks
		Antibody	e.g. Extraction / Enzymes / Clean-Up / Real Time PCR / Gel electrophoresis / Cycles	
ASU	8		2 g sample weight, Machery & Nagel NucleoSpin Food Kit	
ASU	15		CTAB/Proteinase K/Amylase/Promega Wizard DNA CleanUp/Real Time PCR/45 cycles	
ASU	15		CTAB/Proteinase K/Amylase/Promega Wizard DNA CleanUp/Real Time PCR/45 cycles	in second sample
SFA-ID	5			
SFA-ID	12	-	S3117 SureFood® ALLERGEN ID Brazil Nut; Detection limit 0,4 mg/kg; Extraction with S1053 SureFood® PREP Advanced, Protocol 1	-
div.	1	Sulfur-rich water soluble	NucleoSpin Food (Macherey Nagel)/Real Time PCR/45 cycles	

5.1.6 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		positive		negative		positive		Pistachion-DNA	Sure Food Allergen ID, Congen / r-Biopharm
SFA-ID	6	16.01.17	positive	>0.4	negative	<0.4	positive	>0.4	Pistachio-DNA	Sure Food Allergen ID, Congen / r-Biopharm
SFA-ID	9	11.01.17	positive		negative		positive		Pistachio-DNA	r-biopharm SureFood Allergen Pistachio (S3114)
SFA-Q	12	09.12.	positive	5,4	negative	-	positive	1161	Pistachio	Sure Food Allergen QUANT, Congen / r-Biopharm
div.	1	09.01.17	positive		positive		positive		Pistachion-DNA	in-house method
div.	4	17.01.17	positive		negative		positive		Pistachion-DNA	in-house method
div.	15	14.12.	negative		negative		positive		Pistachion-DNA	in-house method
div.	15	26.01.17	negative		negative				Pistachion-DNA	in-house method

Meth. Abr.	Evaluation number	Specifity	Remarks to the Method (Extraction and Determination)	Further Remarks
		Antibody	e.g. Extraction / Enzymes / Clean-Up / Real Time PCR / Gel electrophoresis / Cycles	
div.	1	Dehidrin (cor)	NucleoSpin Food (Macherey Nagel)/Real Time PCR/45 cycles	
div.	4		CTAB; Magnetic Beads; Taqmann real time PCR	
div.	15		CTAB/Proteinase K/Amylase/Promega Wizard DNA CleanUp/PCR/Gelelectrophorese/45 cycles	
div.	15		CTAB/Proteinase K/Amylase/Promega Wizard DNA CleanUp/PCR/Gelelectrophorese/45 cycles	in second sample
SFA-ID	5			
SFA-ID	6			
SFA-ID	9		r-biopharm SureFood Prep Advanced (S1053)	
SFA-Q	12	-	S3214 SureFood® ALLERGEN QUANT Pistachio; Detection limit 0,4 mg/kg, determination limit 1 mg/kg; Extraction w ith S1053 SureFood® PREP Advanced, Protocol 1	-

5.1.7 Other Methods - Lateral Flow: 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
BA	2	23.12.17	positive	>15	negative	<15	-		Pistachio	R-Biopharm Lateral Flow Device Pistachio

Meth. Abr.	Evaluation number	Specifity	Remarks to the Method (Extraction and Determination)	Further Remarks
		Antibody	e.g. Extraction / Enzymes / Clean-Up / Real Time PCR / Gel electrophoresis / Cycles	
BA	2		NaCl solution / 5 min / room temperature	

5.2 Homogeneity

5.2.1 Mixture homogeneity before bottling

Microtracer Homogeneity Test
DLA 08-2016 Sample A

Weight whole sample	3,12	kg
Microtracer	FSS-rot lake	
Particle size	75 – 300	µm
Weight per particle	2,0	µg
Addition of tracer	12,3	mg/kg

Result of analysis

Sample	Weight [g]	Particle number	Particles [mg/kg]
1	5,13	31	12,1
2	5,27	36	13,7
3	5,00	33	13,2
4	5,04	39	15,5
5	5,12	37	14,5
6	5,02	34	13,5
7	5,10	33	12,9
8	5,05	35	13,9

Poisson distribution		
Number of samples	8	
Degree of freedom	7	
Mean	34,8	Partikel
Standard deviation	2,58	Partikel
χ^2 (CHI-Quadrat)	1,34	
Probability	**99**	%
Recovery rate	111	%

Normal distribution		
Number of samples	8	
Mean	13,7	mg/kg
Standard deviation	1,01	mg/kg
rel. Standard deviaton	7,42	%
Horwitz standard deviation	10,8	%
HorRat-value	**0,69**	
Recovery rate	111	%

Microtracer Homogeneity Test
DLA 08-2016 Sample B

Weight whole sample	3,00	kg
Microtracer	FSS-rot lake	
Particle size	75 – 300	µm
Weight per particle	2,0	µg
Addition of tracer	12,7	mg/kg

Result of analysis

Sample	Weight [g]	Particle number	Particles [mg/kg]
1	5,13	31	12,1
2	5,17	42	16,2
3	5,08	33	13,0
4	5,10	26	10,2
5	5,04	36	14,3
6	5,12	31	12,1
7	5,03	37	14,7
8	5,02	35	13,9

Poisson distribution		
Number of samples	8	
Degree of freedom	7	
Mean	33,9	Partikel
Standard deviation	4,78	Partikel
χ^2 (CHI-Quadrat)	4,71	
Probability	**70**	%
Recovery rate	105	%

Normal distribution		
Number of samples	8	
Mean	13,3	mg/kg
Standard deviation	1,88	mg/kg
rel. Standard deviaton	14,1	%
Horwitz standard deviation	10,8	%
HorRat-value	**1,3**	
Recovery rate	105	%

Microtracer Homogeneity Test
DLA 08-2016 Spiking Material Sample

Weight whole sample	0,725	kg
Microtracer	FSS-rot lake	
Particle size	75 – 300	µm
Weight per particle	2,0	µg
Addition of tracer	12,8	mg/kg

Result of analysis

Sample	Weight [g]	Particle number	Particles [mg/kg]
1	5,09	26	10,2
2	5,06	28	11,1
3	5,00	30	12,0
4	5,02	28	11,2
5	5,24	28	10,7
6	5,15	27	10,5
7	5,05	29	11,5
8	5,19	29	11,2

Poisson distribution		
Number of samples	8	
Degree of freedom	7	
Mean	28,1	Partikel
Standard deviation	1,45	Partikel
χ^2 (CHI-Quadrat)	0,52	
Probability	**100**	%
Recovery rate	86	%

Normal distribution		
Number of samples	8	
Mean	11,0	mg/kg
Standard deviation	0,57	mg/kg
rel. Standard deviaton	5,15	%
Horwitz standard deviation	11,1	%
HorRat-value	**0,46**	
Recovery rate	86	%

6. Index of participant laboratories in alphabetical order

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

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

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

7. Index of references

1. DIN EN ISO/IEC 17025:2005; Allgemeine Anforderungen an die Kompetenz von Prüf- und Kalibrierlaboratorien / General requirements for the competence of testing and calibration laboratories
2. DIN EN ISO/IEC 17043:2010; Konformitätsbewertung – Allgemeine Anforderungen an Eignungsprüfungen / Conformity assessment – General requirements for proficiency testing
3. ISO 13528:2015 & DIN ISO 13528:2009; Statistische Verfahren für Eignungsprüfungen durch Ringversuche / Statistical methods for use in proficiency testing by interlaboratory comparisons
4. ASU §64 LFGB: Planung und statistische Auswertung von Ringversuchen zur Methodenvalidierung / DIN ISO 5725 series part 1, 2 and 6 Accuracy (trueness and precision) of measurement methods and results
5. Verordnung / Regulation 882/2004/EU; Verordnung über über amtliche Kontrollen zur Überprüfung der Einhaltung des Lebensmittel- und Futtermittelrechts sowie der Bestimmungen über Tiergesundheit und Tierschutz / Regulation on official controls performed to ensure the verification of compliance with feed and food law, animal health and animal welfare rules
6. Evaluation of analytical methods used for regulation of food and drugs; W. Horwitz; Analytical Chemistry, 54, 67-76 (1982)
7. The International Harmonised Protocol for the Proficiency Testing of Ananlytical Laboratories ; J.AOAC Int., 76(4), 926 – 940 (1993)
8. A Horwitz-like funktion describes precision in proficiency test; M. Thompson, P.J. Lowthian; Analyst, 120, 271-272 (1995)
9. Protocol for the design, conduct and interpretation of method performance studies; W. Horwitz; Pure & Applied Chemistry, 67, 331-343 (1995)
10. Recent trends in inter-laboratory precision at ppb and sub-ppb concentrations in relation to fitness for purpose criteria in proficiency testing; M. Thompson; Analyst, 125, 385-386 (2000)
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