Proficiency Tests
DLA
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Evaluation Report
proficiency test

DLA 05/2017

Allergens V:

Hazelnut and Walnut

in Pastry (Butter Cookies)

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

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

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1. Introduction

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

2. Realisation

2.1 Test material

Two PT-samples with the same food matrix were provided for the detection and quantitative determination of the allergens in the range of mg/kg as well as one spiking level sample with a simple matrix. One of the samples (spiked sample) and the spiking level sample contain the respective allergenic ingredients in a similar concentration range. The results of the spiking level sample should give the possibility of a comparison with the spiked sample in respect to the detectability of the allergens with and without the influence of matrix and / or food processing.

The test material are a common in commerce butter cookies. The basic composition of both sample A and sample B was the same (see table 1).

After crushing and sieving using an impact mill (mesh 1,5 mm) the basic mixture was homogenized. Afterwards the **spiked sample A** was produced as follows:
After crushing and homogenization a baked cookie (170°C, 30 min) containing the allergenic ingredients hazelnut and walnut was added to an aliquot of the basic mixture and the mixture was homogenized. Subsequently, the basic mixture was again added in 3 additional steps and mechanically homogenized in each case until the total quantity had been reached.

For the **spiking level sample**, the allergenic compounds hazelnut and walnut were added during a multi-stage addition of potato powder and homogenization. Afterwards the whole sample was sieved by means of a centrifugal mill (mesh 250 µm).

The samples A and B were portioned to approximately 25 g, the spiking level sample to approximately to 10 g in metallized PET film bags.

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

Table 1: Composition of DLA-Samples

Ingredients	**Sample A**	**Sample B**	**Spiking Level Sample**
Butter Cookies Ingredients: Wheat flour, sugar, butter, barley malt extract, glucose syrup, raising agent: ammonium hydrogencarbonate, salt, emulsifier lecithin Nutrients per 100 g: Protein 7,1 g, Carbohydrates 76 g, Fat 12 g	96,0 g/100 g	100 g/100g	-
Cookies (baked 170°C, 30 min) Ingredients: Wheat flour, sugar, butter, eggs, salt and hazelnuts, walnuts and further ingredients (see below)	4,0 g/100 g	-	-
Potato Powder Ingredients: Potatoes, E471, E304, E223, E100	-	-	99,9 g/100 g
Hazelnuts, roasted ground, mixture (5 countries / Europe) - as Hazelnut* - *thereof 14,1% total protein***	 25,5 mg/kg 3,6 mg/kg	-	 33,7 mg/kg 4,8 mg/kg
Walnuts, raw ground, mixture (5 countries / North and South Amerika, Europe) - as Walnut* - *thereof 13,6% total protein***	 31,6 mg/kg 4,3 mg/kg	-	 38,0 mg/kg 5,2 mg/kg
further Ingredients: Maltodextrin, sodium sulfate and silicon dioxide	<0,15 g/100 g	-	<0,1 g/100 g

*Allergen contents as „total food" as described in column ingredients according to gravimetric mixture
** Protein contents according to laboratory analysis of raw material (total nitrogen according to Kjeldahl with F=5,30 for hazelnuts and walnuts)

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 sample A and the spiking level sample showed a probability of 95% and 100%. Additionally particle number results were converted into concentrations, statistically evaluated according to normal distribution and compared to the standard deviation according to Horwitz. For the assessment HorRat values between 0,3 and 1,3 are to be accepted under repeat conditions (measurements within the laboratory) [16, 17]. This gave HorRat values of 0,7 and 0,4, 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 all ELISA tests for hazelnut (Immunolab, Veratox, AgraQuant Plus) and walnut (Immunolab, AgraQuant), respectively (see page 8 and 9). Recommendations for repeatability standard deviations of ELISA and PCR methods are usually ≤ 25% [18, 19, 22, 23].

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

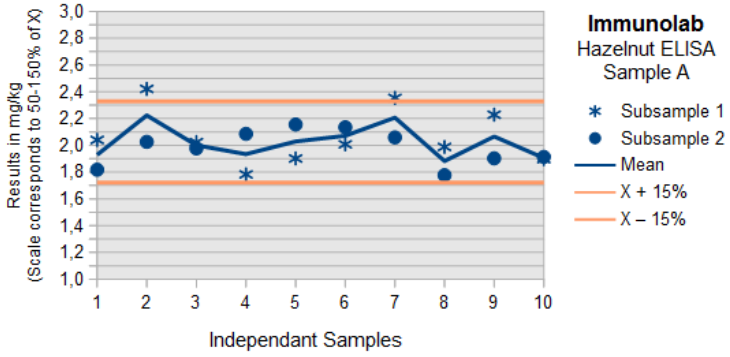
ELISA-Tests: Homogenität Haselnuss / Homogeneity Hazelnut

Immunolab Hazelnut ELISA

Sample weights: 1,0 g (0,9 – 1,1 g)
Number of replicates: 2
Overall result: Hazelnut 2,02 ± 0,15 mg/kg

Sample A	Subsample 1 mg/kg	Subsample 2 mg/kg	Mean mg/kg
1	2,04	1,82	1,93
2	2,42	2,03	2,22
3	2,02	1,98	2,00
4	1,78	2,09	1,93
5	1,90	2,16	2,03
6	2,01	2,13	2,07
7	2,35	2,06	2,21
8	1,99	1,78	1,88
9	2,23	1,90	2,07
10	1,90	1,91	1,91

General average X	2,02	
SD of sample means Sx	0,120	5,9%
SD within-samples Sw	0,176	8,7%
SD between-samples Ss	0,154	7,6%

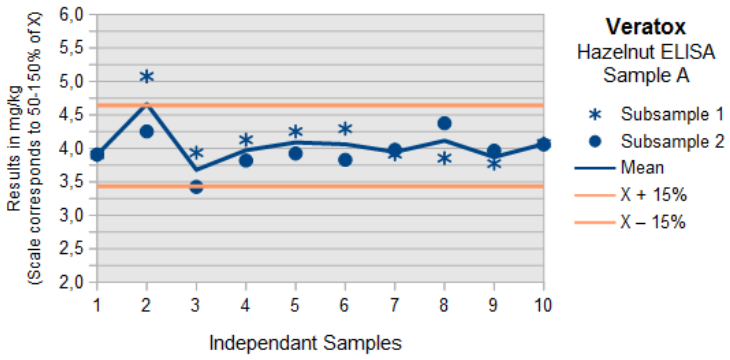


Neogen Veratox ELISA Hazelnut

Sample weights: 5,0 g (4,5 – 5,5 g)
Number of replicates: 2
Overall result: Hazelnut 4,04 ± 0,23 mg/kg

Sample A	Subsample 1 mg/kg	Subsample 2 mg/kg	Mean mg/kg
1	3,90	3,91	3,90
2	5,08	4,25	4,66
3	3,93	3,42	3,68
4	4,13	3,82	3,97
5	4,25	3,92	4,09
6	4,29	3,83	4,06
7	3,91	3,98	3,95
8	3,85	4,37	4,11
9	3,77	3,96	3,87
10	4,07	4,06	4,07

General average X	4,04	
SD of sample means Sx	0,256	6,3%
SD within-samples Sw	0,289	7,2%
SD between-samples Ss	0,225	5,6%

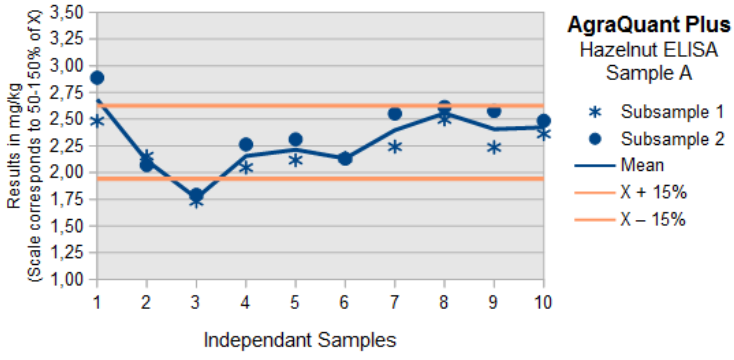


Romerlabs AgraQuant Plus Hazelnut

Sample weights: 1,0 g (0,9 – 1,1 g)
Number of replicates: 2
Overall result: Hazelnut 2,28 ± 0,25 mg/kg

Sample A	Subsample 1 mg/kg	Subsample 2 mg/kg	Mean mg/kg
1	2,48	2,89	2,68
2	2,15	2,07	2,11
3	1,73	1,79	1,76
4	2,05	2,26	2,15
5	2,12	2,31	2,21
6	2,14	2,13	2,13
7	2,24	2,55	2,40
8	2,50	2,61	2,56
9	2,24	2,58	2,41
10	2,36	2,48	2,42

General average X	2,28	
SD of sample means Sx	0,264	11,6%
SD within-samples Sw	0,158	6,9%
SD between-samples Ss	0,245	10,7%



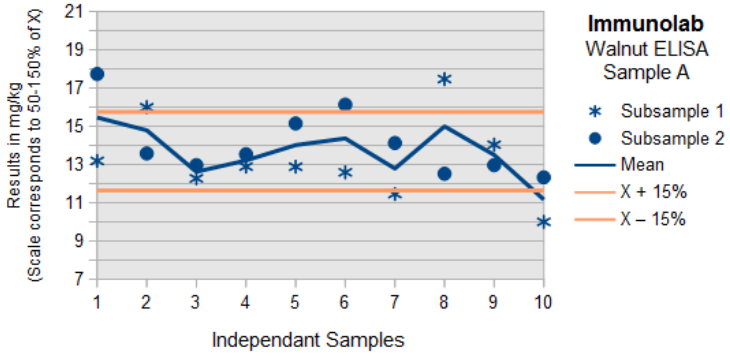
ELISA-Tests: Homogenität Walnuss / Homogeneity Walnut

Immunolab Walnut ELISA

Sample weights: 1,0 g (0,9 – 1,1 g)
Number of replicates: 2
Overall result: Walnut 13,7 ± 2,0 mg/kg

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

General average X	13,7	
SD of sample means Sx	1,30	9,5%
SD within-samples Sw	2,04	14,9%
SD between-samples Ss	1,82	13,3%

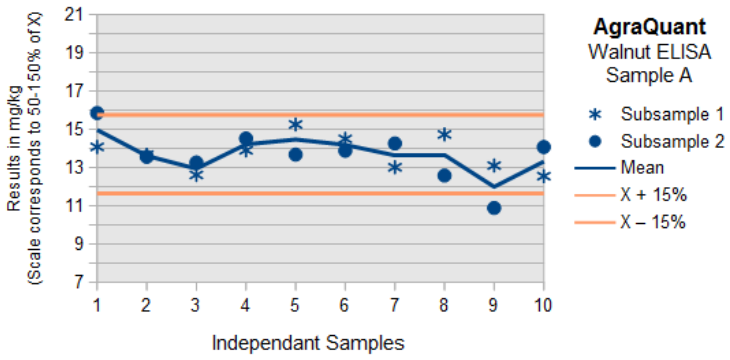


Romerlabs AgraQuant Walnut

Sample weights: 1,0 g (0,9 – 1,1 g)
Number of replicates: 2
Overall result: Walnut 13,7 ± 1,2 mg/kg

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

General average X	13,7	
SD of sample means Sx	0,84	6,1%
SD within-samples Sw	1,01	7,3%
SD between-samples Ss	1,17	8,5%



2.1.2 Stability

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

2.2 Sample shipment and information to the test

The portions of test material (sample A and sample B as well as the spiking level sample) were sent to every participating laboratory in the 37th week of 2017. The testing method was optional. The tests should be finished at October 27th 2017 the latest.

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

There are two different samples A and B possibly containing the allergenic parameters hazelnut and/or walnut in the range of mg/kg in the matrix of butter cookies. 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, while the allergens in samples A and/or B were baked. The "spiking level sample" should be analysed like a regular sample too.

Please note the attached information on the proficiency test.
(see documentation, section 5.4 Information on the PT)

2.3 Submission of results

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

All 20 participants submitted their results in time.

3. Evaluation

Different ELISA-methods for the determination of allergens in foods are eventually using different antibodies, are usually calibrated with different reference materials and may utilize differing extraction methods. Among others this can induce different results of the content of the analyte [25, 26, 27, 28]. It is for this reason that we contrast the results of the present proficiency test with several assigned values. Thereby it is possible to evaluate each single result in comparison to the mean of all results and/or in comparison to the mean of results obtained by a single method. For comparison the actually added amount is plotted in the figures of the results.

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

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

3.1 Consensus value from participants (assigned value)

The **robust mean** of the submitted results was used as assigned value (X_{pt}) („consensus value from participants") providing a normal distribution. The calculation was done according to algorithm A as described in annex C of ISO 13528 [3]. If there are < 12 quantitative results and an increased difference between robust mean and median, the **median** may be used as the assigned value (criterion: Δ median - rob. mean > 0,3 σ_{pt}) [3].
The condition is that the majority of the participants' results show a normal distribution or are distributed unimodal and symmetrically. To this end, an examination of the distribution is carried out, inter alia, using the kernel density estimate [3, 12].

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

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

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

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

respectively) [3].

3.2 Robust standard deviation

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

The following robust standard deviations were considered:

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

3.3 Exclusion of results and outliers

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

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

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

3.4 Target standard deviation (for proficiency assessment)

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

3.4.1 General model (Horwitz)

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

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

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

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

3.4.2 Value by precision experiment

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

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

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

Table 2a: ELISA-Methods - Relative repeatability standard deviations (RSD_r) and relative reproducibility standard deviations (RSD_R) from precision experiments and resulting target standard deviations σ_{pt} [30-31]

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

From the precision data of the official German ASU §64 methods the calculated relative target standard deviations are in the range of 11 – 32% for the ELISA methods and 24 – 42% for the PCR methods depending on the matrix, processing and concentration level of allergens (s. Tab. 2a and 2b).

The Working Group on Prolamin Analysis and Toxicity (WGPAT) coordinated a collaborative study with two commercial ELISA test kits for the determination of gluten using the monoclonal R5 antibody [24]. 12 food samples with gliadin in the range of 0 - 168 mg/kg were analyzed by 20 laboratories. Recovery rates ranged between 65 and 110%, relative repeatability deviations ranged from 13 - 25% (method 1) and 11 - 22% (method 2) while the relative reproducibility standard deviations ranged from 23 - 47% (method 1) and 25 - 33% (method 2). According to the authors both ELISA test kits fulfilled therefore the current validation criteria for ELISA methods [24].

The IRMM (Institute for Reference Materials and Measurements) performed an interlaboratory comparison for five different ELISA test kits for the quantification of peanut [27]. The mean values for two matrices were in the concentration range of 0,3 - 16,1 mg/kg and 1,2 - 20,4 mg/kg, respectively. The lowest relative reproducibility standard deviations of the five test kits were for dark chocolate in the range of 20 - 42% and for cookies in the range of 23 – 61%.

Table 2b: PCR-Methods - Relative repeatability standard deviations (RSD_r) and relative reproducibility standard deviations (RSD_R) from precision experiments and resulting target standard deviations σ_{pt} [32-34]

Parameter	**Matrix**	**Mean [mg/kg]**	**Recov-ery**	**rob RSD**	**RSD_r**	**RSD_R**	**σ_{pt}**	**Method / Literature**
Almond	Rice cookie	105,2 18,0 10,5	105 % 90 % 105 %	-	19,3% 44,0% 32,0%	27,5% 49,1% 38,8%	23,9% 38,0% 31,5%	rt-PCR ASU 18.00-20
Almond	Wheat cookie Sauce powder	114,3 88,1	94,6 % 88,1 %	-	22,1% 43,9%	41,8% 43,1%	38,8% - %	rt-PCR ASU 18.00-20
Almond	Rice cookie	109 21,3 12,3	109 % 107 % 121 %	-	17,6% 35,8% 32,0%	32,8% 45,0% 47,8%	30,3% 37,2% 42,1%	rt-PCR multiplex ASU 18.00-22
Almond	Wheat cookie Sauce powder	120,7 112	98,2 % 94,1 %	-	15,7% 36,2%	32,5% 42,8%	30,5% 34,3%	rt-PCR multiplex ASU 18.00-22
Brazil Nut	Rice cookie	89,1 17,3 9,8	89,1 % 86,5 % 98 %	-	34,1% 36,2% 40,2%	34,4% 38,2% 41,8%	24,5% 28,4% 30,6%	rt-PCR ASU 18.00-21
Brazil Nut	Wheat cookie Sauce powder	80,8 42,6	65,7 % 42,6 %	-	25,6% 27,5%	36,4% 39,7%	31,6% 34,6%	rt-PCR ASU 18.00-21
Brazil Nut	Rice cookie	96,6 14,2	96,6 % 71 %	-	16,8% 54,2%	31,8% 56,5%	29,5% 41,5%	rt-PCR multiplex ASU 18.00-22
Brazil Nut	Wheat cookie Sauce powder	76,5 48,4	62,2 % 48,4 %	-	15,6% 34,4%	35,8% 37,5%	34,1% 28,5%	rt-PCR multiplex ASU 18.00-22

3.4.3 Value by perception

The target standard deviation for proficiency assessment can be set at a value that corresponds to the level of performance that the coordinator would wish laboratories to be able to achieve [3].
Criteria for the level of performance of analytical methods for the quantitative determination of allergens in foods were recently elaborated e.g. by the Ministry of Health and Welfare (MHLW) in Japan [22], by the working group 12 „Food Allergens" of the technical committee CEN/TC 275 [19-21], by an international "Food Allergen Working Group" under the advice of the AOAC Presidential Task Force on Food Allergens [23] and by the Codex Alimentarius Committee (CAC/GL 74-2010) [18].

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

Table 3: ELISA-Validation

Literature [18-24]	**Recovery rate**	**Repeatability standard deviation**	**Reproducibility standard deviation**
MHLW 2006	50 - 150%		≤ 25%
CEN 2009		≤ 20%	
AOAC 2010	50 - 150%	6,9 - 34,4% (a)	19,5 - 57,2% (a)
CAC 2010	70 - 120%	≤ 25%	≤ 35%

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

Table 4: PCR-Validation

Literature [18]	**Recovery rate**	**Repeatability standard deviation**	**Reproducibility standard deviation**
CAC 2010	± 25% (a)	≤ 25%	≤ 35%

(a) = Trueness / Richtigkeit

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

3.5 z-Score

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

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

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

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

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

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

3.5.1 Warning and action signals

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

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

3.6 z'-Score

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

The calculation is performed by:

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

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

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

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

For warning and action signals see 3.5.1.

3.7 Quotient S*/σpt

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

3.8 Standard uncertainty of the assigned value

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

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

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

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

3.9 Figures

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

3.10 Recovery rates: Spiking

For the results of the spiking level sample and the spiked sample recovery rates were calculated with respect to the known content of added allergens. The related values of added allergens are given in 2.1 test material in table 1. As a range of acceptance RA for valuating participant's results the range of 50 - 150% for the recovery rates of allergen-ELISAs proposed by the AOAC was used [23]. For quantitative PCR or LC/MS determinations we use the same range of acceptance.

4. Results

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

The following result sections are structured equally for the allergenic components. First all results of ELISA, PCR or LC/MS methods for a certain parameter are reported for samples A and B (qualitative / possibly quantitative) and afterwards for the spiking level sample (quantitative). The recovery rates of results for the spiking level sample and the spiked sample A or B are reported then.

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

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

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

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

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

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

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

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

Characteristics	**All Results** *[mg/kg]*	**Method i** *[mg/kg]*
Assigned value (X_{pt})	$X_{pt_{ALL}}$	$X_{pt_{METHOD\ i}}$
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 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 Hazelnut

4.1.1 ELISA Results: Hazelnut

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		
3	positive	8,33	negative	< 5	2/2 (100%)	ES	
13	positive	6,24	negative	< 0,35	2/2 (100%)	ES	result converted °
7	positive	1,80	negative	< 1	2/2 (100%)	IL	
8	positive	2,30	negative	< 0,5	2/2 (100%)	IL	
17	positive	1,48	negative	< 0,3	2/2 (100%)	IL	
1	positive	3,50	negative	< 2,5	2/2 (100%)	RS-F	
2	positive	3,90	negative	< 1,5	2/2 (100%)	RS-F	
4	positive	3,00	negative	< 2,5	2/2 (100%)	RS-F	
12	positive	3,60	negative	< 2,5	2/2 (100%)	RS-F	
15	positive	3,90	negative	< 1,5	2/2 (100%)	RS-F	
16	positive	3,50	negative	< 2,5	2/2 (100%)	RS-F	
18	positive	3,01	negative	< 2,5	2/2 (100%)	RS-F	
5	positive	16,2	positive	0,51	1/2 (50%)	VT	
9	positive	4,30	negative	< 2,5	2/2 (100%)	VT	
11	positive	3,70	negative	< 2,5	2/2 (100%)	VT	

° calculation p. 19

	Sample A		Sample B	
Number positive	15		1	
Number negative	0		14	
Percent positive	100		7	
Percent negative	0		93	
Consensus value	positive		negative	

Methods:

ES = ELISA-Systems

IL = Immunolab

RS-F= Ridascreen® Fast, R-Biopharm

VT = Veratox, Neogen

Comments:

The consensus values are in agreement with the spiking of sample A.
One positive result was obtained for sample B by method VT (Veratox).
The value was below the range of determination given by the test kit manufacturer (Food Allergen Handbook, 9th Ed., Neogen & FARRP).

Quantitative valuation of ELISA-results: Sample A

Evaluation number	Hazelnut	z-Score Xpt_{ALL}	z-Score $Xpt_{RS\text{-}F}$	Method	Remarks
	[mg/kg]				
3	8,33	4,8		ES	
13	6,24	2,6		ES	result converted °
7	1,80	-2,1		IL	
8	2,30	-1,6		IL	
17	1,48	-2,4		IL	
1	3,50	-0,31	0,01	RS-F	
2	3,90	0,12	0,47	RS-F	
4	3,00	-0,83	-0,56	RS-F	
12	3,60	-0,20	0,13	RS-F	
15	3,90	0,12	0,47	RS-F	
16	3,50	-0,31	0,01	RS-F	
18	3,01	-0,82	-0,55	RS-F	
5	16,2	13,1		VT	
9	4,30	0,54		VT	
11	3,70	-0,09		VT	

° calculation S. 19

Methodes:

ES = ELISA-Systems

IL = Immunolab

RS-F= Ridascreen® Fast, R-Biopharm

VT = Veratox, Neogen

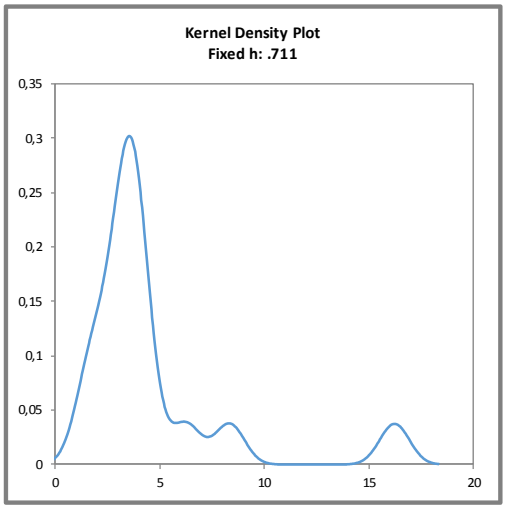


Abb. / Fig. 1:
Kerndichte-Schätzung aller ELISA-Ergebnisse (mit h = 0,75 x σpt von Xpt_{ALL})

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

Comments:
The kernel density estimation shows nearly a normal distribution with a slight shoulder at < 3 mg/kg (method IL), two side-peaks at > 5 mg/kg (method ES) and a side-peak at 16 mg/kg (method VT) due to an outlier.

Characteristics: Quantitative evaluation ELISA: Hazelnut

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	15	7
Number of outliers	1	0
Mean	4,58	3,49
Median	3,60	3,50
Robust Mean (X)	**3,79**	**3,49**
Robust standard deviation (S*)	**1,71**	**0,419**
Target range:		
Target standard deviation σ_{pt}	**0,948**	**0,872**
lower limit of target range	**1,90**	**1,74**
upper limit of target range	**5,69**	**5,23**
Quotient S/σ_{pt}*	*1,8*	*0,48*
Standard uncertainty $U_{(Xpt)}$	*0,553*	*0,198*
Quotient $U_{(Xpt)}/\sigma_{pt}$	*0,58*	*0,23*
Results in the target range	*10*	*7*
Percent in the target range	*67*	*100*

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

Comments to the statistical characteristics and assigned values:

Although the kernel density estimation showed indications for method-dependent difference a joint evaluation was carried out, because of the fair statistical characteristics (relatively small difference between median and robust mean, quotient S/σ_{pt} < 2,0).*

The evaluation of all methods and the evaluation of results from method RS-F showed a normal and low variability of results, respectively. The quotients S/σ_{pt} were below 2,0 and 1,0. The robust standard deviations are in the range of established values for the reproducibility standard deviation of the applied methods (see 3.4.2 value by precision experiments and 3.4.3 value by perception). The comparability of results is given. 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 evaluation of all results and method RS-F were 15% and 14% of the spiking level of hazelnut to sample A and thus below the recommendations for the applied methods (s. 3.4.3 and "Recovery rates of Hazelnut" p.31).

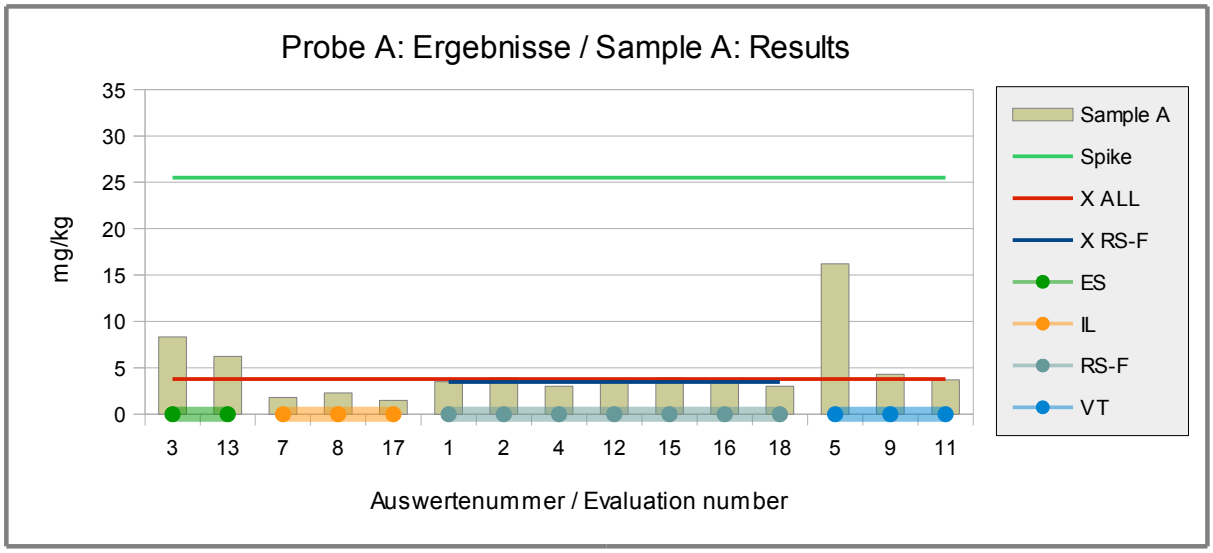


Abb./Fig. 2: ELISA Results Hazelnut
green line = Spiking level
red line = Assigned value robust mean all results
blue line = Assigned value robust mean results method RS-F
round symbols = Applied methods (see legend)

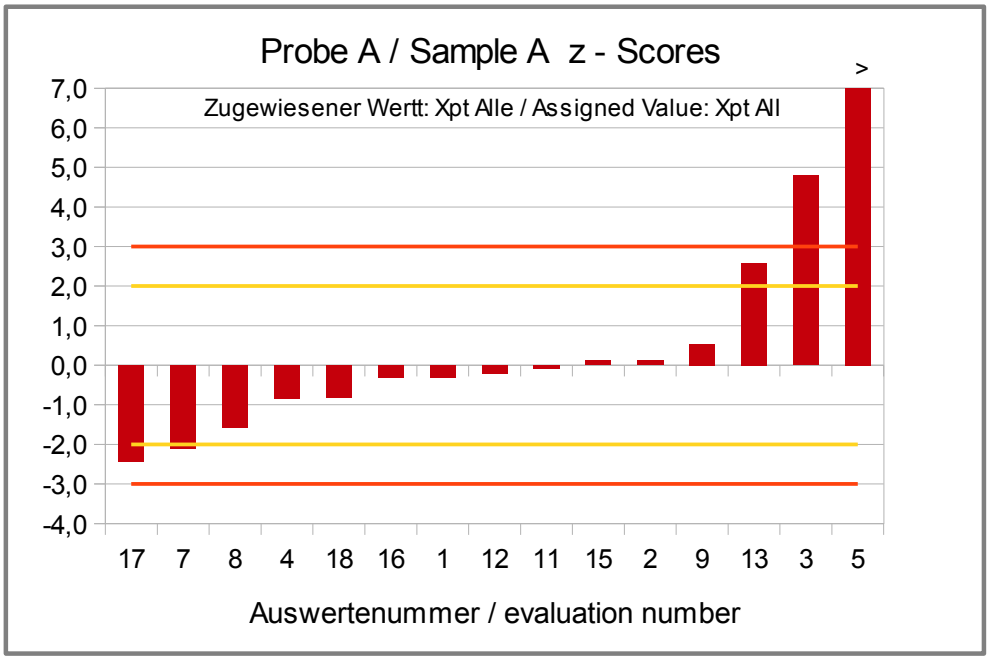


Abb./Fig. 3:
z-Scores (ELISA Results Hazelnut)
Assigned value robust mean of all results

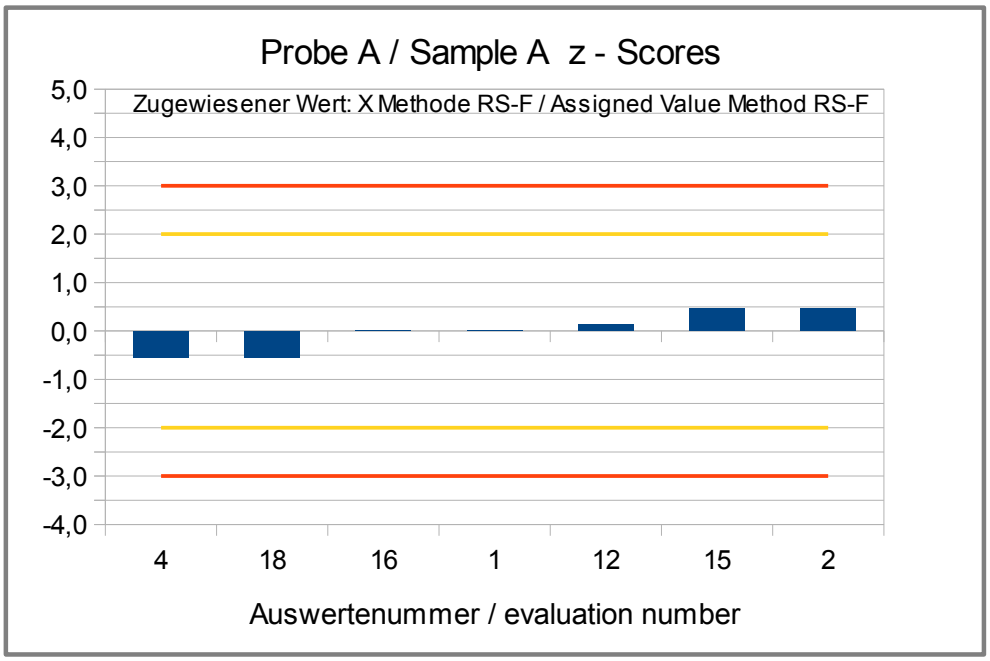


Abb./Fig. 4:
z-Scores (ELISA Results Hazelnut)
Assigned value robust mean of method RS-F (R-Biopharm, Ridascreen® Fast)

Quantitative valuation of results: Spiking level sample

Evaluation number	Hazelnut	z-Score Xpt_{ALL}	z-Score $Xpt_{RS\text{-}F}$	Method	Remarks
	[mg/kg]				
3	8,13	-2,7		ES	
13	18,4	-1,0		ES	result converted °
7	31,6	1,2		IL	
8	30,1	0,9		IL	
17	23,5	-0,2		IL	
1	36,0	1,89	1,15	RS-F	
2	-			RS-F	
4	25,0	0,09	-0,42	RS-F	
12	26,8	0,39	-0,16	RS-F	
15	33,0	1,40	0,72	RS-F	
16	19,0	-0,89	-1,28	RS-F	
18	27,82	0,56	-0,02	RS-F	
5	23,62	-0,1		VT	
9	16,0	-1,38		VT	
11	18,5	-0,97		VT	

° calculation p. Umrechnung S. 19

Methods:

ES = ELISA-Systems

IL = Immunolab

RS-F= Ridascreen® Fast, R-Biopharm

VT = Veratox, Neogen

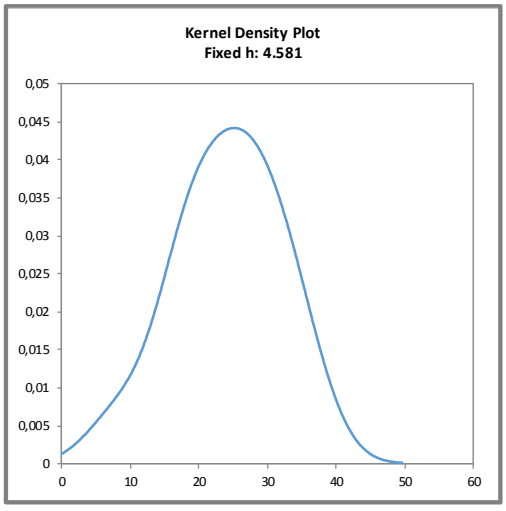


Abb. / Fig. 5:
Kerndichte-Schätzung aller ELISA-Ergebnisse (mit h = 0,75 x σpt von Xpt_{ALL})

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

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

Characteristics: Quantitative evaluation Hazelnut

Spiking level sample

Statistic Data	**All Results** *[mg/kg]*	**Method RS-F** *[mg/kg]*
Assigned value (X_{pt})	$X_{pt_{ALL}}$	$X_{pt_{METHOD\ RS\text{-}F}}$
Number of results	14	6
Number of outliers	0	0
Mean	24,1	27,9
Median	24,3	27,3
Robust Mean (X)	**24,4**	**27,9**
Robust standard deviation (S*)	**7,83**	**6,81**
Target range:		
Target standard deviation σ_{pt}	**6,11**	**6,98**
lower limit of target range	**12,2**	**14,0**
upper limit of target range	**36,6**	**41,9**
Quotient S/σ_{pt}*	*1,3*	*0,98*
Standard uncertainty $U_{(Xpt)}$	*2,62*	*3,48*
Quotient $U_{(Xpt)}/\sigma_{pt}$	*0,43*	*0,50*
Results in the target range	*13*	*6*
Percent in the target range	*93*	*100*

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

Comments to the statistical characteristics and assigned values:

The kernel density plot showed nearly a normal distribution.

The evaluation of all methods and the evaluation of results from method RS-F showed a normal to low variability of results. The quotients S/σ_{pt} were below 2,0. The robust standard deviations are in the range of established values for the reproducibility standard deviation of the applied methods (see 3.4.2 value by precision experiments and 3.4.3 value by perception). The comparability of results is given. 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 evaluation of all results and method RS-F were 72% and 83% of the spiking level of hazelnut to the spiking level sample and thus within the recommendations for the applied methods (s. 3.4.3 and "Recovery rates of Hazelnut" p.31).

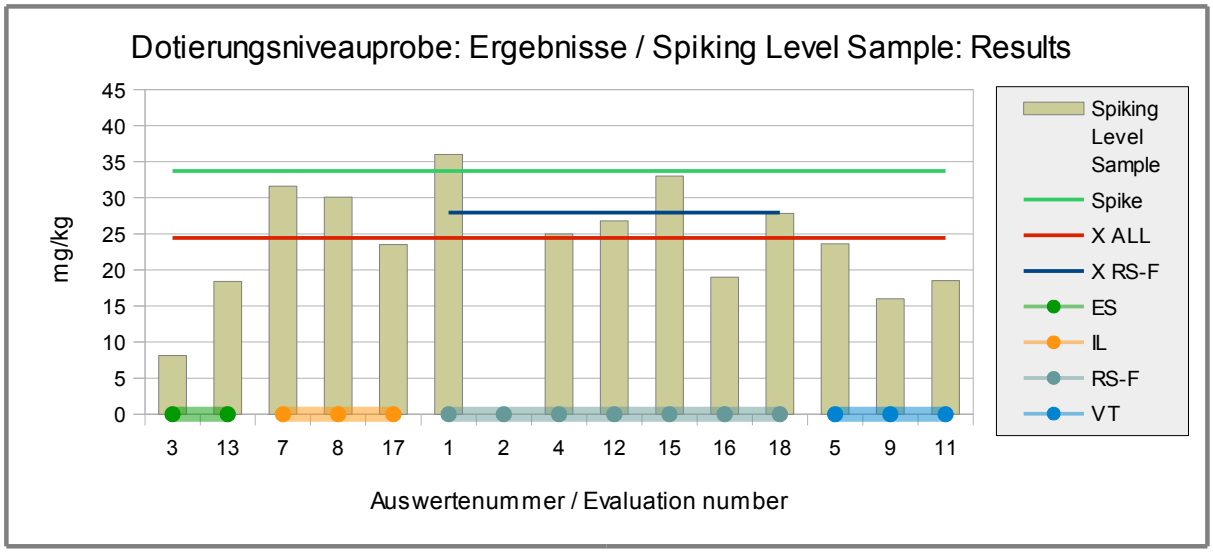


Abb./Fig. 6: ELISA Results Hazelnut
green line = Spiking level
red line = Assigned value robust mean all results
blue line = Assigned value robust mean results method RS-F
round symbols = Applied methods (see legend)

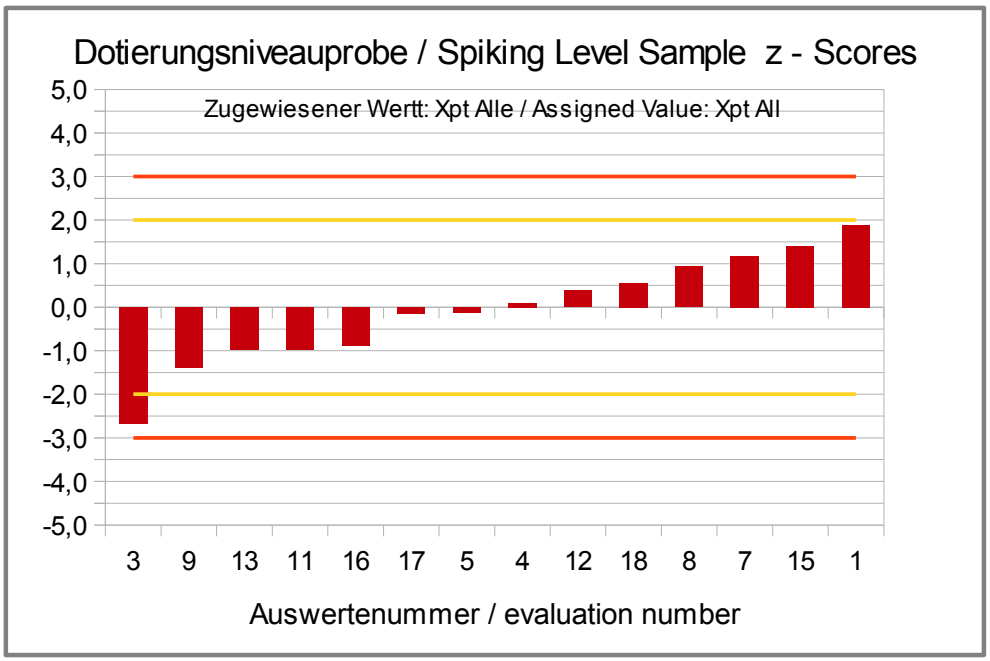


Abb./Fig. 7:
z-Scores (ELISA Results Hazelnut)
Assigned value robust mean of all results

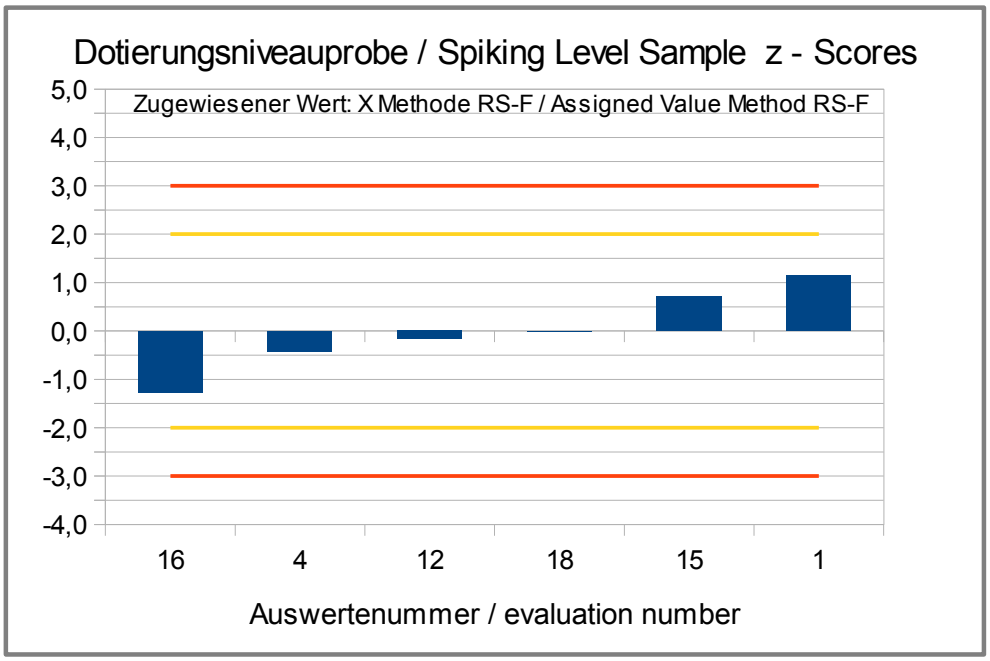


Abb./Fig. 8:
z-Scores (ELISA Results Hazelnut)
Assigned value robust mean of method RS-F (R-Biopharm, Ridascreen® Fast)

Recovery Rates ELISA for Hazelnut:
Spiking level Sample and Sample A

Evaluation number	Spiking Level Sample	Recovery rate*	Sample A	Recovery rate*	Method	Remarks
	[mg/kg]	[%]	[mg/kg]	[%]		
3	8,13	24	8,33	33	ES	
13	18,4	**55**	6,24	24	ES	result converted °
7	31,6	**94**	1,80	7,1	IL	
8	30,1	**89**	2,30	9,0	IL	
17	23,5	**70**	1,48	5,8	IL	
1	36,0	**107**	3,50	14	RS-F	
2	-		3,90	15	RS-F	
4	25,0	**74**	3,00	12	RS-F	
12	26,8	**80**	3,60	14	RS-F	
15	33,0	**98**	3,90	15	RS-F	
16	19,0	**56**	3,50	14	RS-F	
18	27,8	**83**	3,01	12	RS-F	
5	23,6	**70**	16,2	**64**	VT	
9	16,0	47	4,30	17	VT	
11	18,5	**55**	3,70	15	VT	

° calculation S. 19

RA**	50-150 %	RA**	50-150 %
Number in RA	**12**	Number in RA	**1**
Percent in RA	**86**	Percent in RA	**7**

Methods:
ES = ELISA-Systems
IL = Immunolab
RS-F= Ridascreen® Fast, R-Biopharm
VT = Veratox, Neogen

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

** Range of acceptance of AOAC for allergen ELISAS

Comments:
For the spiking level sample 86% of the participants obtained a recovery rate by ELISA methods within the range of the AOAC-recommendation of 50-150%. For the spiked processed food matrix sample A only one recovery rates was within the range of acceptance, while all others were clearly below 50%.

4.1.2 PCR Results: Hazelnut

Qualitative valuation of results: Samples A and B

Evaluation number	Sample A	Sample A	Sample B	Sample B	Qualitative Valuation	Method	Remarks
	pos/neg	[mg/kg]	pos/neg	[mg/kg]	Agreement with consensus value		
1	negative		negative		1/1 (100%)*	ASU	*no positive sample detected
3	negative		negative		1/1 (100%)*	ASU	*no positive sample detected
20	negative		negative		1/1 (100%)*	MS	*no positive sample detected
14a	negative		negative		1/1 (100%)*	SFA-4p	*no positive sample detected
14b	positive	< 5	negative		1/1 (100%)	SFA-ID	
15	positive	> 0,4	negative	<0.4	1/1 (100%)	SFA-ID	
6	positive		negative		1/1 (100%)	SFA-Q	
10	negative	< 5	negative	< 5	1/1 (100%)*	div	*no positive sample detected
19	negative		negative		1/1 (100%)*	div	*no positive sample detected

	Sample A		Sample B	
Number positive	3		0	
Number negative	6		9	
Percent positive	33		0	
Percent negative	67		100	
Consensus value	none		negative	

Methods:
ASU = ASU §64 Methode/method
MS = Microsynth
SFA-4p = Sure Food Allergen 4plex, R-Biopharm / Congen
SFA-ID = Sure Food Allergen ID, R-Biopharm / Congen
SFA-Q = Sure Food Allergen Quant, R-Biopharm / Congen
div = not indicated / other method

Comments:
For sample B a consensus value of 100% negative results was obtained. For the spiked sample A no consensus of ≥ 75% was obtained. There were only 3 positive results for sample A by PCR methods.

Quantitative Valuation PCR: Sample A

No quantitative evaluation was done, because there were no quantitative results.

(Quantitative) Valuation PCR: Spiking Level Sample

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

Evaluation number	Hazelnut	Hazelnut	z-Score Xpt_{ALL}	Method	Remarks
	pos/neg	[mg/kg]			
1	negative			ASU	
3	positive			ASU	
20	negative			MS	
14a	positive	30		SFA-4p	
14b	positive	27,5		SFA-ID	
15	positive	>0.4		SFA-ID	
6	positive			SFA-Q	
10	positive	15		div	
19	positive			div	

Number positive	7	
Number negative	2	
Percent positive	78	
Percent negative	22	
Consensus value	positive	

Methods:

ASU = ASU §64 Methode/method

MS = Microsynth

SFA-4p = Sure Food Allergen 4plex, R-Biopharm / Congen

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

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

div = not indicated / other method

Comments:

For the spiking level sample there were 78% positive results and 2 negative results.

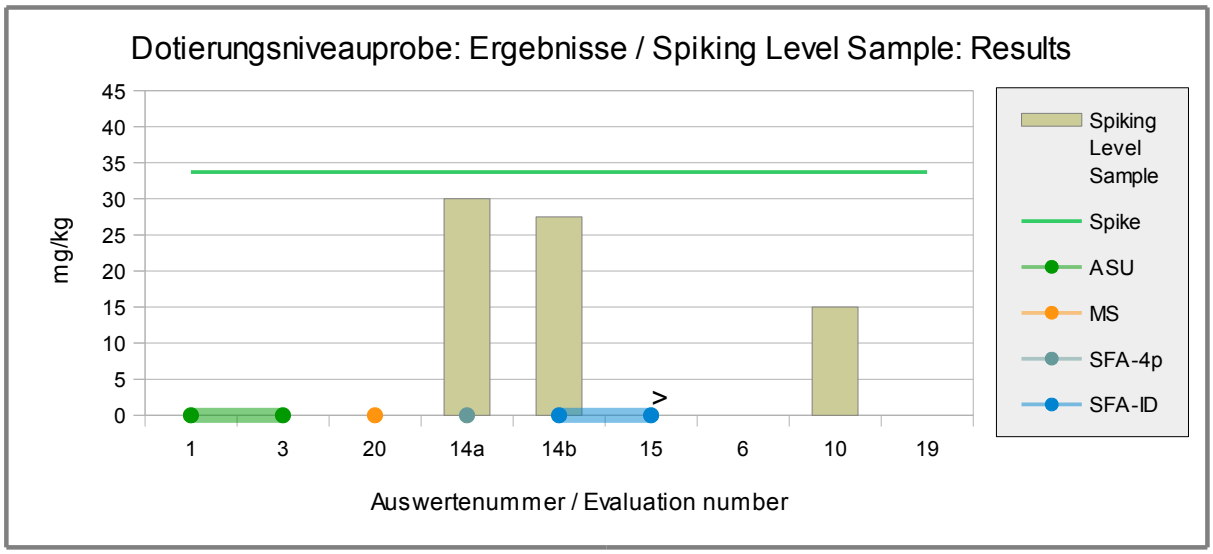


Abb./Fig. 9: PCR Results Hazelnut
green line = Spiking level
round symbols = Applied methods (see legend)

Recovery Rates PCR for Hazelnut:
Spiking Material Sample and Sample A

Evaluation number	Spiking Level Sample	Recovery rate*	Sample A	Recovery rate*	Method	Remarks
	[mg/kg]	[%]	[mg/kg]	[%]		
1					ASU	
3					ASU	
20					MS	
14a	30	**89**			SFA-4p	
14b	27,5	**82**	< 5	-	SFA-ID	
15	>0.4		> 0,4	-	SFA-ID	
6					SFA-Q	
10	15	45	< 5	-	div	
19					div	

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

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

** Range of acceptance of AOAC for allergen ELISAS

Methods:
ASU = ASU §64 Methode/method
MS = Microsynth
SFA-4p = Sure Food Allergen 4plex, R-Biopharm / Congen
SFA-ID = Sure Food Allergen ID, R-Biopharm / Congen
SFA-Q = Sure Food Allergen Quant, R-Biopharm / Congen
div = not indicated / other method

Comments:
2 out of 3 participants obtained a recovery rate within the range of the AOAC-recommendation of 50-150% for the spiking material sample. One result was slightly below this range. For the processed spiked food matrix sample A there were no quantitative results.

4.1.3 LC/MS Results: Hazelnut

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 spiking		
6	positive	17,0	negative	< 10	2/2 (100%)	LC-MS	

Methods:
LC-MS = Liquid Chromatography / Mass Spectrometry

Comments:
One set of results of LC/MS methods was submitted. The results are in qualitative agreement with the spiking of sample A.

Quantitative Valuation LC/MS: Sample A

No quantitative evaluation was done, because there were only one quantitative results.

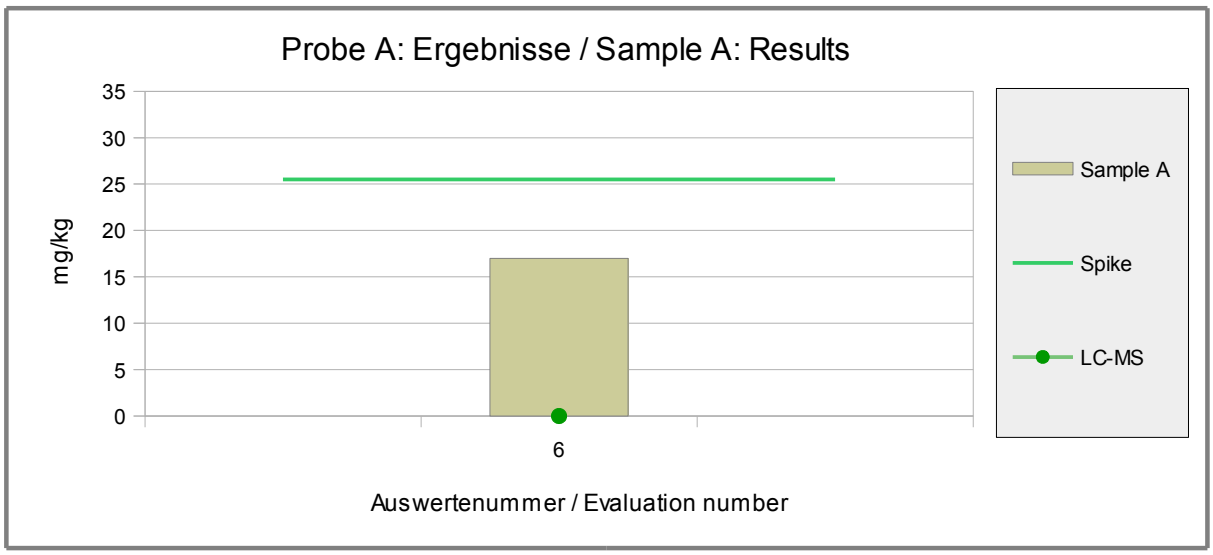


Abb./Fig. 10: LC/MS-Results Hazelnut
green line = Spiking level
round symbols = Applied methods (see legend)

(Quantitative) Valuation LC/MS: Spiking Level Sample

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

Evaluation number	Hazelnut	z-Score Xpt_{ALL}	Method	Remarks
	[mg/kg]			
6	27,6		LC-MS	

Methods:
LC-MS = Liquid Chromatography / Mass Spectrometry

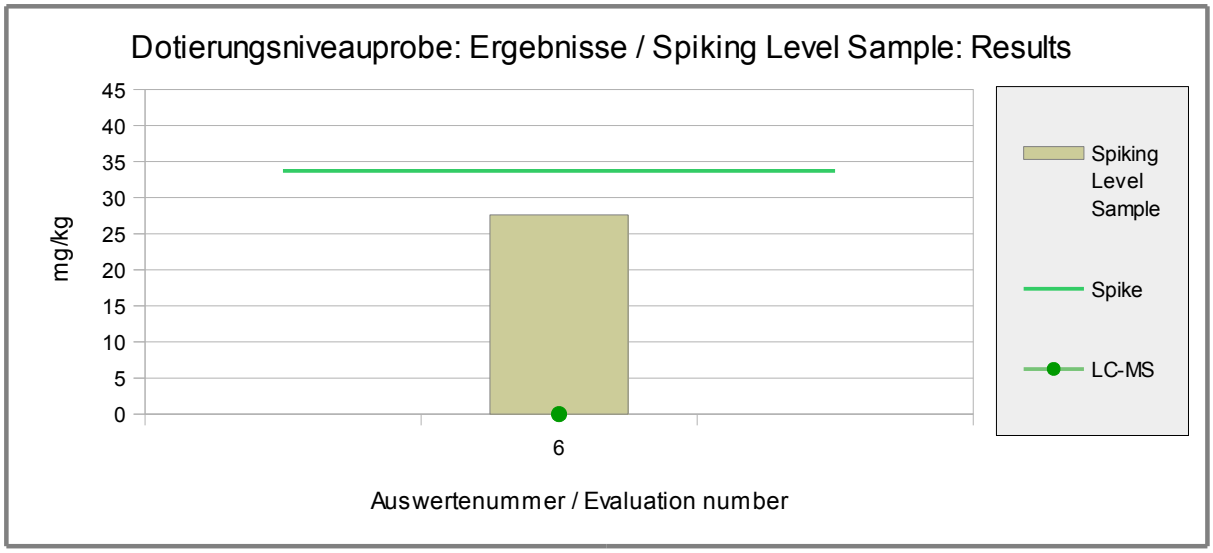


Abb./Fig. 11: LC/MS Results Hazelnut
green line = Spiking level
round symbols = Applied methods (see legend)

Recovery Rates LC/MS for Hazelnut:
Spiking Material Sample and Sample A

Evaluation number	Spiking Level Sample	Recovery rate*	Sample A	Recovery rate*	Method	Remarks
	[mg/kg]	[%]	[mg/kg]	[%]		
6	27,6	**82**	17,0	**67**	LC-MS	

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

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

** Range of acceptance of AOAC for allergen ELISAS

Methods:
LC-MS = Liquid Chromatography / Mass Spectrometry

Comments:
The participant has obtained a recovery rate within the range of the AOAC-recommendation of 50-150% for both the spiking material sample and the processed spiked food matrix sample A by a LC/MS method.

4.2 Proficiency Test Walnut

4.2.1 ELISA Results: Walnut

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	14,0	negative	< 2,0	2/2 (100%)	AQ	
20	positive	102	negative		2/2 (100%)	AQ	result converted °
18	positive	10,6	negative	< 2	2/2 (100%)	BC	
11	positive	2,70	negative	< 2,0	2/2 (100%)	BF	
16	positive	3,40	negative	< 2	2/2 (100%)	BF	
13	positive	13,0	negative	3	2/2 (100%)	BK	
15	positive	13,0	negative	< 0,25	2/2 (100%)	BK	
5	positive	20,1	negative	< 0,5	2/2 (100%)	BM	
7	positive	12,8	negative	< 2	2/2 (100%)	IL	
8	positive	14,6	negative	< 1	2/2 (100%)	IL	
17	positive	19,6	negative	< 1	2/2 (100%)	NL	result converted °

° calculation p. 19

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

Methods:

AQ = AgraQuant, RomerLabs
BC = BioCheck ELISA
BF = MonoTrace ELISA, BioFront Technologies
BK = BioKits, Neogen
BM = AlerTox ELISA, Biomedal
IL = Immunolab
NL = nutriLinia® Allergen-ELISA

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

Quantitative valuation of ELISA-results: Sample A

Evaluation number	Walnut	z-Score Xpt_{ALL}	Method	Remarks
	[mg/kg]			
2	14,0	0,15	AQ	
20	102	26,2	AQ	result converted ° / and excluded
18	10,6	-0,8	BC	
11	2,70	-3,2	BF	result excluded
16	3,40	-3,0	BF	result excluded
13	13,0	-0,15	BK	
15	13,0	-0,15	BK	
5	20,1	2,0	BM	
7	12,8	-0,21	IL	
8	14,6	0,33	IL	
17	19,6	1,8	NL	result converted °

° calculation p. 19

Methods:

AQ = AgraQuant, RomerLabs
BC = BioCheck ELISA
BF = MonoTrace ELISA, BioFront Technologies
BK = BioKits, Neogen
BM = AlerTox ELISA, Biomedal
IL = Immunolab
NL = nutriLinia® Allergen-ELISA

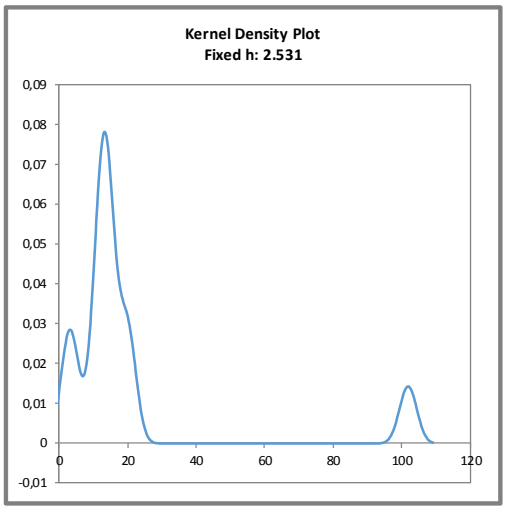


Abb. / Fig. 12:
Kerndichte-Schätzung aller ELISA-Ergebnisse (mit h = 0,75 x σpt von Xpt_{ALL})

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

Comments:
The kernel density estimation shows a main peak with nearly a normal distribution and side peak at approx. 3 mg/kg (method BF) and a side-peak at approx. 100 mg/kg (method AQ) due to an outlier.

Characteristics: Quantitative evaluation ELISA: Walnut

Sample A

Statistic Data	**All Results** *[mg/kg]*
Assigned value (X_{pt})	$X_{pt_{ALL}}$
*Number of results ***	8
Number of outliers	0
Mean	14,7
Robust Mean	14,7
Median (X_{pt})	**13,5**
Robust standard deviation (S*)	**3,82**
Target range:	
Target standard deviation σ_{pt}	**3,38**
lower limit of target range	**6,75**
upper limit of target range	**20,3**
Quotient S/σ_{pt}*	*1,1*
Standard uncertainty $U_{(Xpt)}$	*1,69*
Quotient $U_{(Xpt)}/\sigma_{pt}$	*0,50*
Results in the target range	*8*
Percent in the target range	*100*

*** without evaluation no. 11, 16 and 20*

Comments to the statistical characteristics and assigned values:

The kernel density estimation showed method-dependent differences, therefore the results of method BF (no. 11, 16) were excluded before a joint evaluation was carried out. The outlier no. 20 was also excluded.

The median was applied as the assigned value (see 3.1). The evaluation of all methods (without method BF) showed a normal variability of results. The quotient S/σ_{pt} was clearly below 2,0. The robust standard deviation was in the range of established values for the reproducibility standard deviation of the applied methods (see 3.4.2 value by precision experiments and 3.4.3 value by perception). The comparability of results is given. The comparability of results is given. This conclusion is limited for the evaluation across the methods, because there were only a few results for the methods.*

The median of the evaluation of all results was 43% of the spiking level of Walnut to sample A and thus below the recommendations for the applied methods (s. 3.4.3 and "Recovery rates of Walnut" p.46).

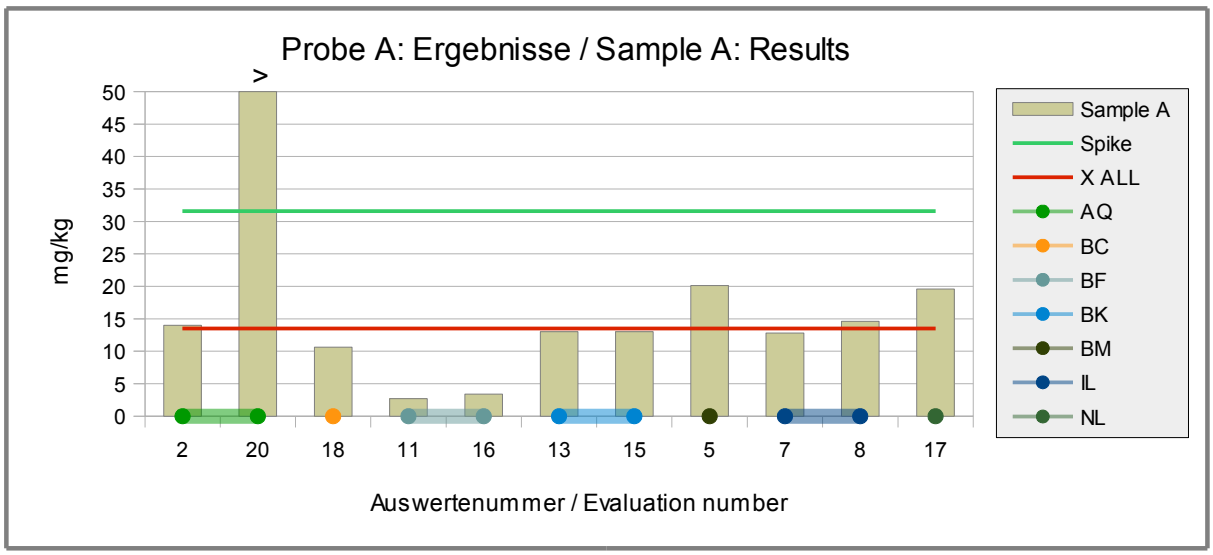


Abb./Fig. 13: ELISA Results Walnut
green line = Spiking level
red line = Assigned value median of all results
round symbols = Applied methods (see legend)

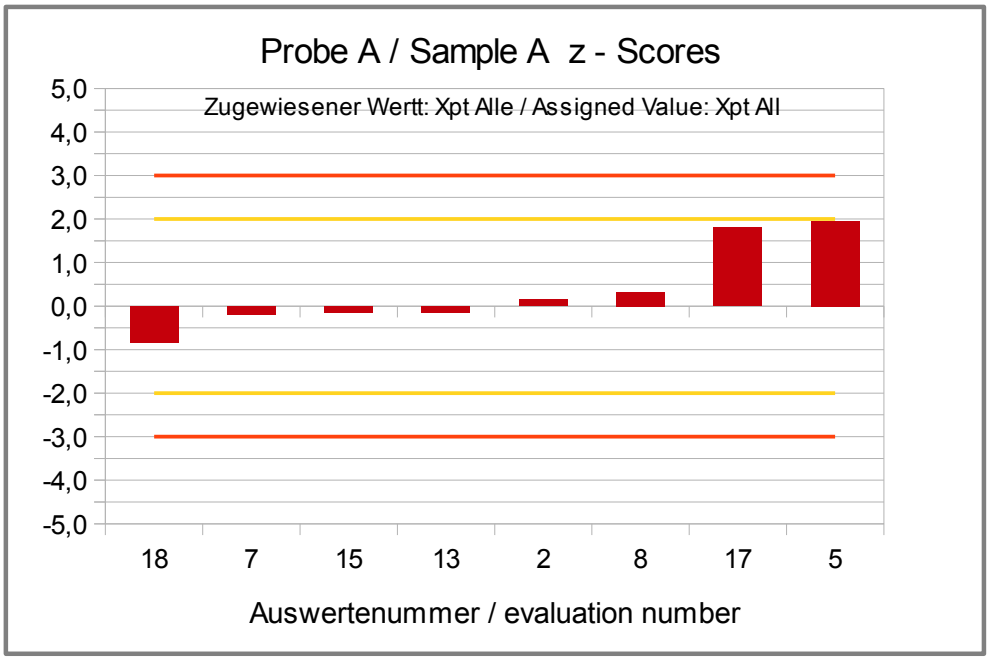


Abb./Fig. 14:
z-Scores (ELISA Results Walnut)
Assigned value median of all results

Quantitative valuation of results: Spiking level sample

Evaluation number	Walnut	z-Score Xpt_{ALL}	Method	Remarks
	[mg/kg]			
2	-		AQ	
20	499	14,5	AQ	result converted ° / and excluded
18	113	0,21	BC	
11	80,2	-1,0	BF	
16	> 80		BF	
13	140	1,2	BK	
15	120	0,46	BK	
5	100	-0,27	BM	
7	120	0,46	IL	
8	104	-0,14	IL	
17	83,8	-0,89	NL	result converted °

° calculation p. 19

Methods:

AQ = AgraQuant, RomerLabs
BC = BioCheck ELISA
BF = MonoTrace ELISA, BioFront Technologies
BK = BioKits, Neogen
BM = AlerTox ELISA, Biomedal
IL = Immunolab
NL = nutriLinia® Allergen-ELISA

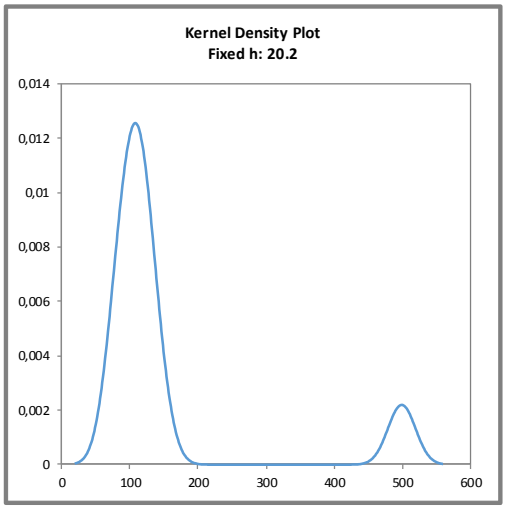


Abb. / Fig. 15:
Kerndichte-Schätzung aller ELISA-Ergebnisse (mit h = 0,75 x σpt von Xpt_{ALL})

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

Comments:
The kernel density estimation shows nearly a normal distribution of results with a side-peak due to an outlier.

Characteristics: Quantitative evaluation Walnut

Spiking level sample

Statistic Data	**All Results** *[mg/kg]*
Assigned value (X_{pt})	$X_{pt_{ALL}}$
*Number of results ***	8
Number of outliers	0
Mean	108
Median	109
Robust Mean (X_{pt})	**108**
Robust standard deviation (S*)	**22,6**
Target range:	
Target standard deviation σ_{pt}	**26,9**
lower limit of target range	**53,9**
upper limit of target range	**162**
Quotient S/σ_{pt}*	*0,84*
Standard uncertainty $U_{(Xpt)}$	*9,97*
Quotient $U_{(Xpt)}/\sigma_{pt}$	*0,37*
Results in the target range	*8*
Percent in the target range	*100*

*** without evaluation no. 16 and 20*

Comments to the statistical characteristics and assigned values:

The kernel density plot showed nearly a normal distribution.

The evaluation of all methods showed a low variability of results. The quotient S/σ_{pt} was below 1,0. The robust standard deviation is in the range of established values for the reproducibility standard deviation of the applied methods (see 3.4.2 value by precision experiments and 3.4.3 value by perception). The comparability of results is given. The comparability of results is given. This conclusion is limited for the evaluation across the methods, because there were only a few results for the methods.*

The robust mean of the evaluation of all results was 284% of the spiking level of Walnut to the spiking level sample and thus about two times above the recommendations for the applied methods (s. 3.4.3 and "Recovery rates of Walnut" p.46).

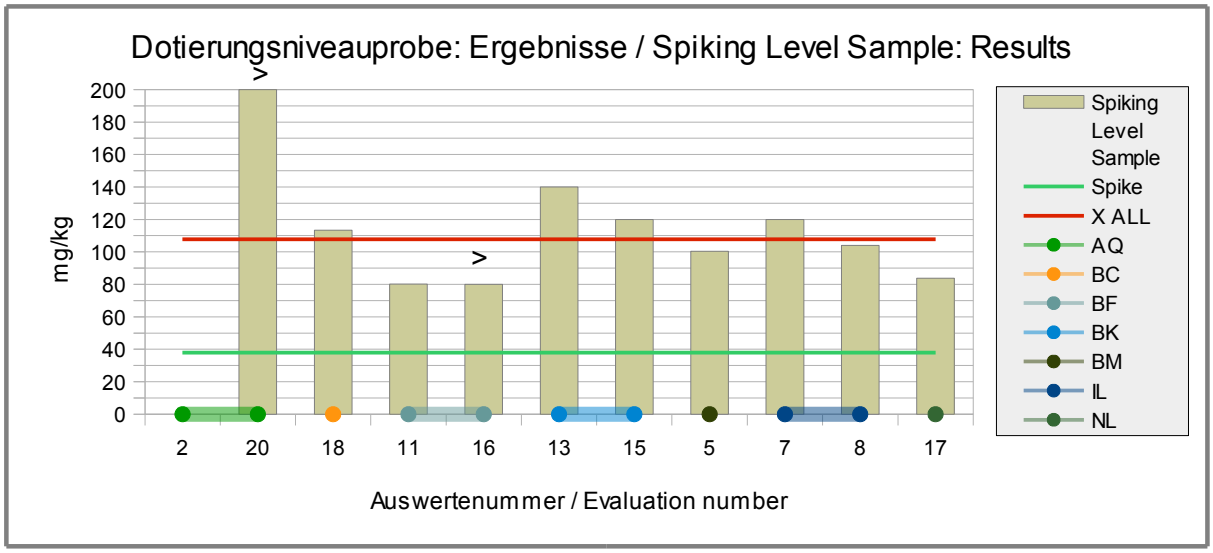


Abb./Fig. 16: ELISA Results Walnut
green line = Spiking level
red line = Assigned value robust mean all results
round symbols = Applied methods (see legend)

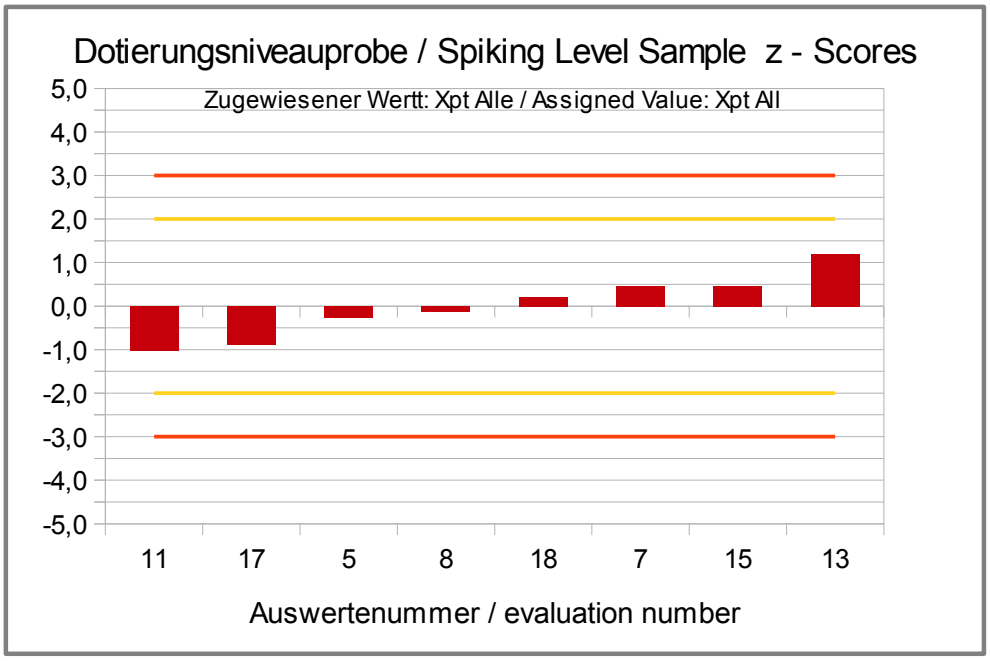


Abb./Fig. 17:
z-Scores (ELISA Results Walnut)
Assigned value robust mean of all results

Recovery Rates ELISA for Walnut:
Spiking level Sample and Sample A

Evaluation number	Spiking Level Sample	Recovery rate*	Sample A	Recovery rate*	Method	Remarks
	[mg/kg]	[%]	[mg/kg]	[%]		
2	-		14,0	44	AQ	
20	499	1313	102	323	AQ	result converted °
18	113	298	10,6	34	BC	
11	80,2	211	2,70	9	BF	
16	> 80		3,40	11	BF	
13	140	368	13,0	41	BK	
15	120	316	13,0	41	BK	
5	100	264	20,1	**64**	BM	
7	120	316	12,8	41	IL	
8	104	274	14,6	46	IL	
17	83,8	221	19,6	**62**	NL	result converted °

° calculation p. 19

RA**	50-150 %	RA**	50-150 %
Number in RA	**0**	Number in RA	**2**
Percent in RA	**0**	Percent in RA	**18**

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

** Range of acceptance of AOAC for allergen ELISAS

Methods:

AQ = AgraQuant, RomerLabs
BC = BioCheck ELISA
BF = MonoTrace ELISA, BioFront Technologies
BK = BioKits, Neogen
BM = AlerTox ELISA, Biomedal
IL = Immunolab
NL = nutriLinia® Allergen-ELISA

Comments:
For the spiking level sample none of the participants obtained a recovery rate by ELISA methods within the range of the AOAC-recommendation of 50-150%. It should be noted, that the spiked material were raw walnuts.
For the spiked processed food matrix sample A two recovery rates were within the range of acceptance, while all others with one exception were below 50%.

4.2.2 PCR Results: Walnut

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		
14	positive	< 5	negative		2/2 (100%)	SFA-4p	
4	positive		negative		2/2 (100%)	SFA-ID	
15	positive	> 0,4	negative	< 0,4	2/2 (100%)	SFA-ID	
18	positive	5,70	negative	< 1	2/2 (100%)	SFA-ID	
1	negative		negative		1/2 (50%)	div	
10	negative	< 10	negative	< 10	1/2 (50%)	div	
12	positive	1,90	negative	< 2,5	2/2 (100%)	div	
19	positive		negative		2/2 (100%)	div	

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

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

Quantitative Valuation PCR: Sample A

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

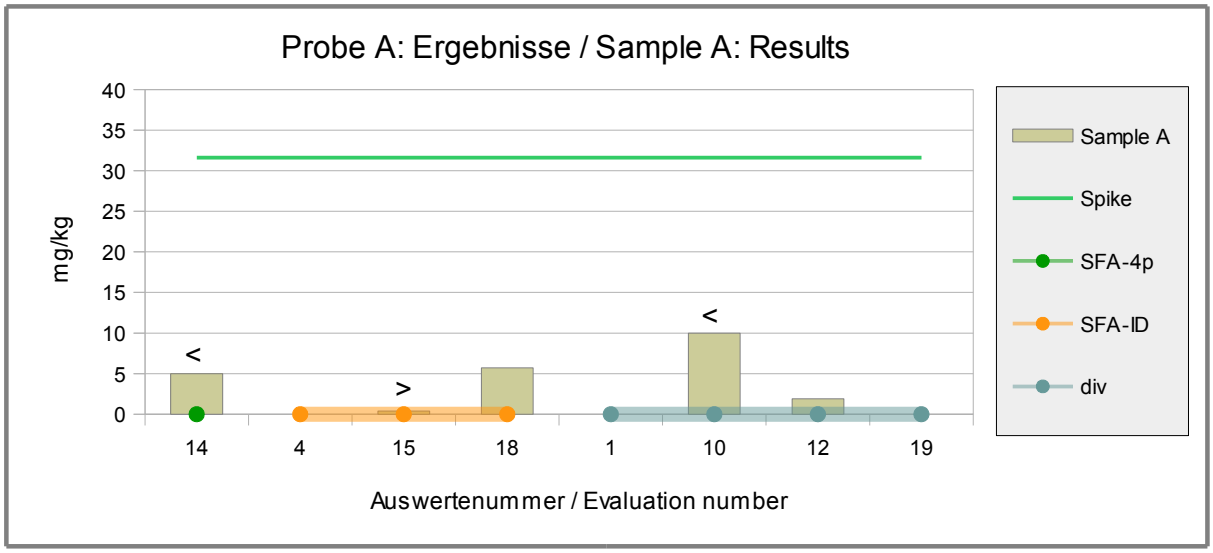


Abb./Fig. 18: PCR Results Walnut
green line = Spiking level
round symbols = Applied methods (see legend)

(Quantitative) Valuation PCR: Spiking Level Sample

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

Evaluation number	Walnut	Walnut	z-Score Xpt_{ALL}	Method	Remarks
	pos/neg	[mg/kg]			
14	positive	27		SFA-4p	
4	positive			SFA-ID	
15	positive	> 0,4		SFA-ID	
18	positive	100		SFA-ID	
1	positive			div	
10	positive	59		div	
12	positive	63		div	
19	positive			div	

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

Methods:

SFA-4p = Sure Food Allergen 4plex, R-Biopharm / Congen

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

div = not indicated / other method

Comments:

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

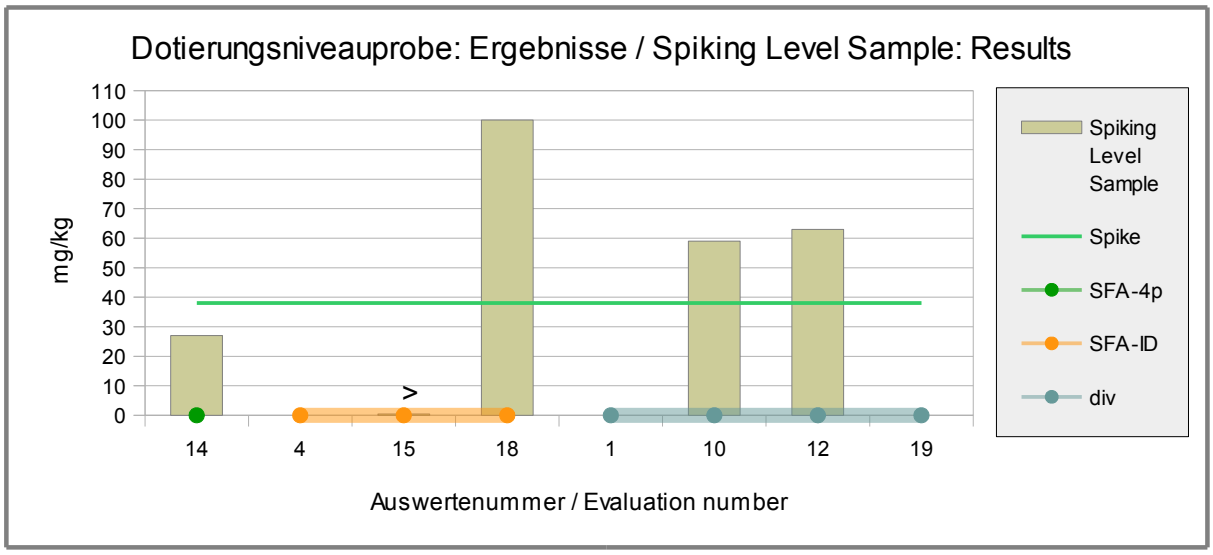


Abb./Fig. 19: PCR Results Walnut
green line = Spiking level
round symbols = Applied methods (see legend)

Recovery Rates PCR for Walnut:
Spiking Material Sample and Sample A

Evaluation number	Spiking Level Sample	Recovery rate*	Sample A	Recovery rate*	Method	Remarks
	[mg/kg]	[%]	[mg/kg]	[%]		
14	27	**71**	< 5	-	SFA-4p	
4					SFA-ID	
15	> 0,4	**-**	> 0,4	-	SFA-ID	
18	100	263	5,70	18	SFA-ID	
1					div	
10	59	155	< 10	-	div	
12	63	166	1,90	6	div	
19					div	

RA**	50-150 %	RA**	50-150 %
Number in RA	**1**	Number in RA	**0**
Percent in RA	**25**	Percent in RA	**0**

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

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

** Range of acceptance of AOAC for allergen ELISAS

Comments:
One out of 4 participants obtained a recovery rate within the range of the AOAC-recommendation of 50-150% for the spiking material sample. One result was slightly below this range. Two results were slightly above this range. For the processed spiked food matrix sample A there were two results below the range of acceptance.

4.2.3 LC/MS Results: Walnut

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 spiking		
6	positive		negative		2/2 (100%)	LC-MS	

Methods:
LC-MS = Liquid Chromatography / Mass Spectrometry

Comments:
One set of results of LC/MS methods was submitted. The results are in qualitative agreement with the spiking of sample A.

Quantitative Valuation LC/MS: Sample A

No quantitative evaluation was done, because there were no quantitative results.

Quantitative Valuation LC/MS: Spiking Level Sample

No quantitative evaluation was done, because there were no quantitative results (qualitative results see documentation).

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: Hazelnut

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
ES	3		positive	8,33	negative	< 5	positive	8,13	Food item hazelnut	ELISA Systems Hazelnut ESHRD-48
ES	13	26.9.	positive	0,88	negative	<0,5	positive	2,6	Hazelnut protein	ELISA Systems Hazelnut ESHRD-48
IL	7	24.10.17	-	1,8	-	<1	-	31,6	Hazelnut	Immunolab Hazelnut ELISA
IL	8	18.09.17	positive	2,3	negative	< 0,5 ppm	positive	30,1	Hazelnut	Immunolab Hazelnut ELISA
IL	17	09.10.17	positive	1,48	negative	< 0,3	positive	23,5	Hazelnut	Immunolab Hazelnut ELISA
RS-F	1	29.09.17	positive	3,5	negative	<2,5	positive	36	Hazelnut	Ridascreen® FAST Hazelnut R6802, R-Biopharm
RS-F	2	17.10.17	positive	3,9	negative	<1.5	-	-	Hazelnut	Ridascreen® FAST Hazelnut R6802, R-Biopharm
RS-F	4	06.10.17	positive	3	negative	< 2,5	positive	25	Hazelnut	Ridascreen® FAST Hazelnut R6802, R-Biopharm
RS-F	12	19.10.	positive	3,6	-	<2.5	-	26,8	Hazelnut	Ridascreen® FAST Hazelnut R6802, R-Biopharm
RS-F	15	10.10.17	positive	3,9	negative	<1.5	positive	33	Hazelnut	Ridascreen® FAST Hazelnut R6802, R-Biopharm
RS-F	16	15.09.17	positive	3,5	negative	<2,5	positive	19	Hazelnut	Ridascreen® FAST Hazelnut R6802, R-Biopharm
RS-F	18	04.10.17	positive	3,01	negative	<2.5	positive	27,82	Hazelnut	Ridascreen® FAST Hazelnut R6802, R-Biopharm
VT	5	20.10.17	-	16,21	-	0,51	-	23,62	Hazelnut	Veratox Hazelnut, Neogen
VT	9	20.09.17	-	4,3	-	<2.5	-	16	Hazelnut	Veratox Hazelnut, Neogen
VT	11	18/10	positive	3,7	negative	<2.5	positive	18,5	Hazelnut	Veratox Hazelnut, Neogen

***Continuation ELISA Hazelnut*:**

Meth. Abr.	Evaluation number	Specifity	Remarks to the Method (Extraction and Determination)	Method accredidet ISO/IEC 17025	Further Remarks
		Antibody	e.g. Extraction Solution / Time / Temperature	yes/no	
ES	3	anti-Hazelnut	As Per Kit Instructions	yes	
ES	13	Hazelnut proteins	As Per Kit Instructions	yes	
IL	7			no	
IL	8				
IL	17			yes	
RS-F	1		Extraction of 1 g Sample Weight with Milk Powder according to Manual	yes	
RS-F	2		20mL/10minutes at 60°C	yes	
RS-F	4		As Per Kit Instructions	yes	
RS-F	12				
RS-F	15			yes	
RS-F	16			yes	
RS-F	18	As Per Kit Instructions	As Per Kit Instructions	yes	
VT	5		ext solution TBS	no	Elisa Rombonik
VT	9	Hazelnut protein	Extraction solution phosphate butter (10 mM PBS) / 15 Min. / 60°C	no	Sample A) Tester 1=5.1 ppm, Tester 2=3.5 ppm. Sample B) Tester 1=13.4 ppm, Tester 2=18.5 ppm. Dot.-Sample) Tester 1 and 2 je <2.5 ppm. Test 1 on 20.09.17, Test 2 on 24.10.17
VT	11			yes	

5.1.2 ELISA: Walnut

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	2	18.10.17	positive	14	negative	<2.0	-	-	Walnut	AgraQuant ELISA Walnut COKAL0948, RomerLabs
AQ	20	27.09.	positive	13,9	negative		positive	67,9	Walnut protein	AgraQuant ELISA Walnut COKAL0948, RomerLabs
BC	18	04.10.17	positive	10,64	negative	<2	positive	113,4	Walnut	BioCheck ELISA Walnut-Check
BF	11	17/10	positive	2,7	negative	<2.0	positive	80,2	Walnut	BioFront Technologies
BF	16	21.09.17	positive	3,4	negative	<2	positive	>80	Walnut	MonoTrace Walnut ELISA kit, BioFront Technologies
BK	13	28.9.	positive	13	negative	3	positive	140	Walnut	BioKits Walnut Assay Kit, Neogen
BK	15	10.10.17	positive	13	negative	<0.25	positive	120	Walnut	BioKits Walnut Assay Kit, Neogen
BM	5	27/10/17	-	20,1	-	<0.5	-	100,4	Walnut	BIOMEDAL Alertox Walnut
IL	7	24.10.17	-	12,8	-	<2	-	120	Walnut	Immunolab Walnut ELISA
IL	8	18.09.17	positive	14,6	negative	< 1 ppm	positive	104	Walnut	Immunolab Walnut ELISA
NL	17	09.10.17	positive	2,66	negative	< 0,09	positive	11,4	Walnut protein	nutriLinia® Walnut-ELISA

Meth. Abr.	Evaluation number	Specifity	Remarks to the Method (Extraction and Determination)	Method accredidet ISO/IEC 17025	Further Remarks
		Antibody	e.g. Extraction Solution / Time / Temperature	yes/no	
AQ			20mL / 15min / 60°C	yes	
AQ	20		Extraction according to manual	yes	
BC	18	As Per Kit Instructions	As Per Kit Instructions	yes	
BF	11			no	
BF	16			yes	
BK	13	Walnut proteins	As Per Kit Instructions	yes	
BK	15			no	
BM	5		ext solution TBS	no	Elisa Rombonik
IL	7			no	
IL	8				
NL	17			yes	

5.1.3 PCR: Hazelnut

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	1	11.10.17	negative		negative		negative		Haselnut-DNA	
ASU	3		negative		negative		positive		Haselnut-DNA	ASU §64 L 44.00-8 (PCR Haselnut)
MS	20		negative		negative		negative		Haselnut-DNA	Microsynth
SFA-4p	14a	22.09.	negative		negative		positive	30	Haselnut	Sure Food Allergen 4plex, R-Biopharm / Congen
SFA-ID	14b	22.09.	positive	< 5	negative		positive	27,5	Haselnut	Sure Food Allergen ID, R-Biopharm / Congen
SFA-ID	15	10.10.17	positive	>0.4	negative	<0.4	positive	>0.4	Haselnut DNA	Sure Food Allergen ID, R-Biopharm / Congen
SFA-Q	6		positive		negative		positive		Haselnut	SureFood® ALLERGEN QUANT Hazelnut
div	10		negative	< 5	negative	< 5	positive	15	Haselnut	Köppel et al (2010) Eur. Food Res. Technol. 230: 367-374.
div	19	27.10.17	negative		negative		positive			in-house method

Meth. Abr.	Evaluation number	Specifity	Remarks to the Method (Extraction and Determination)	Method accredidet ISO/IEC 17025	Further Remarks
		Target-Sequence / -DNA	e.g. Extraction / enzymes / clean-up / real time PCR / gel electrophoresis / cycles	yes/no	
ASU	1	Hazelnut	Extraction of 2 g sample weight with Machery & Nagel NucleoSpin Food Kit, PCR Multiplex according to ASU §64 44.00-8	yes	weak signals in spiking level sample
ASU	3	152 bp Gen corA 1	Dneasy ®mericon Food Kit/ Proteinase K/ Real Time PCR/ 45 Cycles	yes	
MS	20		Macherey Nagel Nucleo Spin Food with optimizations: increased sample weight, buffer changing (Washing with Lysis Buffer) RNase-Step, Chloroform-Step, 2xCQW; RealTime PCR with 45 Cycles, Decontamination step with UNG; individual Thermoprofile; Inhibition control	yes	LOD 0,005% DNA; Sample A with low traces
SFA-4p	14a		CTAB + Qiaquick clean-up		
SFA-ID	14b		CTAB + Qiaquick clean-up		
SFA-ID	15			yes	
SFA-Q	6	-	Sure Food Prep Advanced, according to Prep Allergen	yes	
div	10	Cor A1 (85 bp)	Extraction: CTAB-precipitation method (s. ASU)	yes	calibration/quantitation by matrix-standards, spiked matrial: Haselnut defatted
div	19		CTAB and Nano Particles, Real-Time PCR	yes	

5.1.4 PCR: Walnut

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-4p	14	22.09.	positive	<5	negative		positive	27	Walnut	Sure Food Allergen 4plex, R-Biopharm / Congen
SFA-ID	4	13.10.17	positive		negative		positive		Walnut-DNA	Sure Food Allergen ID, R-Biopharm / Congen
SFA-ID	15	10.10.17	positive	>0.4	negative	<0.4	positive	>0.4	Walnut DNA	Sure Food Allergen ID, R-Biopharm / Congen
SFA-ID	18	07.10.17	positive	5,7	negative	<1	positive	100	Walnut	Sure Food Allergen ID, R-Biopharm / Congen
div	1	11.10.17	negative		negative		positive		Walnut-DNA	andere: bitte eingeben!
div	10		negative	< 10	negative	< 10	positive	59	Walnut	Brezna et al (2006) Eur Food Res. Techol 223:373-377
div	12	22.09.	positive	1,9	-	<2.5	-	63	Walnut	López-Calleja I et all. 2015
div	19	25.10.17	positive		negative		positive			in-house method

Meth. Abr.	Evaluation number	Specifity	Remarks to the Method (Extraction and Determination)	Method accredidet ISO/IEC 17025	Further Remarks
		Target-Sequence / -DNA	e.g. Extraction / enzymes / clean-up / real time PCR / gel electrophoresis / cycles	yes/no	
SFA-4p	14		CTAB + Qiaquick clean-up		
SFA-ID	4		according to kit instructions, sample preparation with SureFast PREP Advanced, R-Biopharm/Congen	no	
SFA-ID	15			yes	
SFA-ID	18	As Per Kit Instructions	As Per Kit Instructions	no	
div	1	Walnut	Extraction of 2 g sample weight with Machery & Nagel NucleoSpin Food Kit ; Brezna et al, Eur Food Res Technol, 2006	yes	
div	10	Jug R2 (88bp)	Extraction: CTAB-precipitation method (s. ASU)	yes	calibration/quantitation with matrix-standards, spiked material: Walnut defatted
div	12				
div	19		CTAB, Real-Time PCR	no	

5.1.5 LC/MS: Hazelnut

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
LC-MS	6		positive	17	negative	< 10	positive	27,6	Hazelnut	LC-MS/MS

Meth. Abr.	Evaluation number	Specifity	Remarks to the Method (Extraction and Determination)	Method accredidet ISO/IEC 17025	Further Remarks
				yes/no	
LC-MS	6	Marker peptides	aqueous extraction with urea after hexane defatting, afterwards tryptic digestion and solid phase extraction	yes	

5.1.6 LC/MS: Walnut

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
LC-MS	6		positive		negative		positive		Walnut	LC-MS/MS

Meth. Abr.	Evaluation number	Specifity	Remarks to the Method (Extraction and Determination)	Method accredidet ISO/IEC 17025	Further Remarks
				yes/no	
LC-MS	6	Marker peptides	aqueous extraction with urea after hexane defatting, afterwards tryptic digestion and solid phase extraction		

5.2 Homogeneity

5.2.1 Mixture homogeneity before bottling

Microtracer Homogeneity Test
DLA 05-2017 Sample A

Weight whole sample	2,64	kg
Microtracer	FSS-rot lake	
Particle size	75 – 300	µm
Weight per particle	2,0	µg
Addition of tracer	29,8	mg/kg

Result of analysis

Sample	Weight [g]	Particle number	Particles [mg/kg]
1	5,02	72	28,7
2	5,00	61	24,4
3	5,12	74	28,9
4	5,10	74	29,0
5	5,06	72	28,5
6	5,07	72	28,4
7	4,98	68	27,3
8	5,08	63	24,8

Poisson distribution		
Number of samples	8	
Degree of freedom	7	
Mean	69,5	Particles
Standard deviation	4,71	Particles
χ^2 (CHI-Quadrat)	2,24	
Probability	**95**	%
Recovery rate	92	%

Normal distribution		
Number of samples	8	
Mean	27,5	mg/kg
Standard deviation	1,86	mg/kg
rel. Standard deviaton	6,8	%
Horwitz standard deviation	9,72	%
HorRat-value	**0,70**	
Recovery rate	92	%

Microtracer Homogeneity Test
DLA 05-2017 Spiking Level Samples

Weight whole sample	1,52	kg
Microtracer	FSS-rot lake	
Particle size	75 – 300	µm
Weight per particle	2,0	µg
Addition of tracer	23,7	mg/kg

Result of analysis

Sample	Weight [g]	Particle number	Particles [mg/kg]
1	5,06	94	37,2
2	4,99	89	35,7
3	5,03	88	35,0
4	4,99	90	36,1
5	5,02	87	34,7
6	4,99	86	34,5
7	5,04	89	35,3
8	5,07	84	33,1

Poisson distribution		
Number of samples	8	
Degree of freedom	7	
Mean	88,4	Particles
Standard deviation	2,99	Particles
χ^2 (CHI-Quadrat)	0,71	
Probability	**100**	%
Recovery rate	148	%

Normal distribution		
Number of samples	8	
Mean	35,2	mg/kg
Standard deviation	1,19	mg/kg
rel. Standard deviaton	3,39	%
Horwitz standard deviation	9,36	%
HorRat-value	**0,36**	
Recovery rate	148	%

5.3 Information on the Proficiency Test (PT)

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

PT number	***DLA 05-2017***
PT name	***Allergens V: Hazelnut and Walnut in Pastry***
Sample matrix (processing)	***Samples A + B:*** *Butter Cookies (baked at appr. 170°C)/ ingredients: Wheat flour, sugar, butter, barley malt extract, glucose syrup, baking agent ammonium carbonate, salt, emulsifier lecithins, other food additives, egg and allergenic foods (one of both samples)* ***Spiking Level Sample:*** *potato powder, other food additives and allergenic foods*
Number of samples and sample amount	*2 different Samples A + B: 25 g each + 1 Spiking Level Sample: 15 g*
Storage	*Samples A + B: cooled 2 - 10°C (long term < -18°C) Spiking Level Sample: room temperature*
Intentional use	*Laboratory use only (quality control samples)*
Parameter	*qualitative + quantitative: Hazelnut, Walnut (as food item, protein, DNA) Samples A + B: < 500 mg/kg Spiking Level Sample: < 500 mg/kg*
Methods of analysis	*Analytical methods are optional*
Notes to analysis	*The analysis of PT samples should be performed like a routine laboratory analysis. In general we recommend to homogenize a representative sample amount before analysis according to good laboratory practice, especially in case of low sample weights. It is the best to homogenize the whole sample.*
Result sheet	*One result each should be determined for Samples A and B and the Spiking Level Sample. The results should be filled in the result submission file.*
Units	*mg/kg*
Number of digits	*at least 2*
Result submission	*The result submission file should be sent by e-mail to:* ***pt@dla-lvu.de***
Deadline	***the latest October 27th 2017***
Evaluation report	*The evaluation report is expected to be completed 6 weeks after deadline of result submission and sent as PDF file by e-mail.*
Coordinator and contact person of PT	*Matthias Besler, PhD*

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

6. Index of participant laboratories in alphabetical order

Teilnehmer / Participant	Ort / Town	Land / Country

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

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

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)
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; Ana-lyst, 125, 385-386 (2000)
11. The International Harmonised Protocol for the Proficiency Testing of Analytical Chemistry Laboratories; Pure Appl Chem, 78, 145 – 196 (2006)
12. AMC Kernel Density - Representing data distributions with kernel density estim-ates, amc technical brief, Editor M Thompson, Analytical Methods Committee, AMCTB No 4, Revised March 2006 and Excel Add-in Kernel.xla 1.0e by Royal Society of Chemistry
13. EURACHEM/CITAC Leitfaden, Ermittlung der Messunsicherheit bei analytischen Messun-gen (2003); Quantifying Uncertainty in Analytical Measurement (1999)
14. GMP+ Feed Certification scheme, Module: Feed Safety Assurance, chapter 5.7 Check-ing procedure for the process accuracy of compound feed with micro tracers in GMP+ BA2 Control of residues, Version: 1st of January 2015 GMP+ International B.V.
15. MTSE SOP No. 010.01 (2014): Quantitative measurement of mixing uniformity and carry-over in powder mixtures with the rotary detector technique, MTSE Micro Tracers Services Europe GmbH
16. Horwitz Equation as Quality Benchmark in ISO/IEC 17025 Testing Laboratory, Rivera & Rodriguez, Bufete de ingenieros industriales, S.C. (Corrigendum 2014)
17. AOAC Official Methods of Analysis: Guidelines for Standard Method Performance Re-quirements, Appendix F, p. 2, AOAC Int (2016)
18. Codex Alimentarius Commission (2010) - Guidelines on performance criteria and validation of methods for detection, identification and quantification of specific DNA sequences and specific protiens in foods, CAC/GL 74-2010
19. DIN EN ISO 15633-1:2009; Nachweis von Lebensmittelallergenen mit immunologischen Verfahren - Teil 1: Allgemeine Betrachtungen / Foodstuffs - Detection of food allergens by immunological methods - Part 1: General considerations
20. DIN EN ISO 15634-1:2009; Nachweis von Lebensmittelallergenen mit molekularbiologischen Verfahren - Teil 1: Allgemeine Betrachtungen / Foodstuffs - Detection of food allergens by molecular biological methods - Part 1: General considerations
21. DIN EN ISO 15842:2010 Lebensmittel – Nachweis von Lebensmittelallergenen – Allgemeine Betrachtungen und Validierung von Verfahren / Foodstuffs - Detection of food allergens - General considerations and validation of methods
22. Ministry of Health and Welfare, JSM, Japan 2006
23. Working Group Food Allergens, Abbott et al., Validation Procedures for Quantitative Food Allergen ELISA Methods: Community Guidance and Best Practices JAOAC Int. 93:442-50 (2010)
24. Working Group on Prolamin Analysis and Toxicity (WGPAT): Méndez et al. Report of a

collaborative trial to investigate the performance of the R5 enzyme linked immunoassay to determine gliadin in gluten-free food. Eur J Gastroenterol Hepatol. 17:1053-63 (2005)
25. DLA Publikation: Performance of ELISA and PCR methods for the determination of allergens in food: an evaluation of six years of proficiency testing for soy (Glycine max L.) and wheat gluten (Triticum aestivum L.); Scharf et al.; J Agric Food Chem. 61(43):10261-72 (2013)
26. EFSA (2014) Scientific Opinion on the evaluation of allergenic foods and food ingredients for labelling purposes1, EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA), European Food Safety Authority (EFSA), Parma, Italy, EFSA Journal 2014;12(11):3894
27. IRMM, Poms et al.; Inter-laboratory validation study of five different commercial ELISA test kits for determination of peanut residues in cookie and dark chocolate; European Commission, Joint Research Centre, Belgium; GE/R/FSQ/D08/05/2004
28. Jayasena et al. (2015) Comparison of six commercial ELISA kits for their specificity and sensitivity in detecting different major peanut allergens. J Agric Food Chem. 2015 Feb 18;63(6):1849-55
29. ASU §64 LFGB L 06.00-56 Bestimmung von Sojaprotein in Fleisch und Fleischerzeugnissen Enzymimmunologisches Verfahren (2007) [Determination of soyprotein in meat and meat products by enzyme immunoassay]
30. ASU §64 LFGB L 00.00-69 Bestimmung von Erdnuss-Kontaminationen in Lebensmitteln mittels ELISA im Mikrotiterplattensystem (2003) [Foodstuffs, determination of peanut contamintions in foodstuffs by ELISA in microtiterplates]
31. ASU §64 LFGB L 44.00-7 Bestimmung von Haselnuss-Kontaminationen in Schokolade und Schokoladenwaren mittels ELISA im Mikrotiterplattensystem (2006) [Foodstuffs, determination of hazelnut contamintions in chocolate and chocolate products by ELISA in microtiterplates]
32. ASU §64 LFGB L 18.00-20 Untersuchung von Lebenmitteln - Nachweis und Bestimmung von Mandel (Prunus dulcis) in Reis- und Weizenkeksen sowie in Soßenpulver mittels real-time PCR (2014) [Foodstuffs, detection and determination of almond (Prunus dulcis) in rice and wheat cookies and sauce powders by PCR]
33. ASU §64 LFGB L 18.00-21 Untersuchung von Lebenmitteln - Nachweis und Bestimmung von Paranuss (Bertholletia exceisa) in Reis- und Weizenkeksen sowe in Soßenpulver mittels real-time PCR (2014) [Foodstuffs, detection and determination of brazil nut (Bertholletia exceisa) in rice and wheat cookies and sauce powders by PCR]
34. ASU §64 LFGB L 18.00-22 Untersuchung von Lebenmitteln - Simultaner Nachweis und Bestimmung von Lupine, Mandel, Paranuss und Sesam in Reis- und Weizenkeksen sowie Soßenpulver mittels real-time PCR (2014) [Foodstuffs, simultaneous detection and determination of lupin, almond, brazil nut and sesame in rice and wheat cookies and sauce powders by PCR]