Proficiency Tests
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Evaluation Report
proficiency test

DLA 06/2018

Allergens VI:

Hazelnut and Pecan

in Chocolate

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1st Correction 07/02/2019:
For evaluation of the ELISA results of hazelnut the result set of participant no. 9 was converted to hazelnut by mistake. However, the data were submitted by the participant as hazelnut in time.
Therefore the evaluation chapter of the ELISA results for hazelnut (p.22-29) were corrected accordingly including z-scores.

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

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EP-Bericht Freigabe *PT-Report Authorization*	Dr. Matthias Besler-Scharf (Technischer Leiter / Technical Manager) *- gezeichnet / signed M. Besler-Scharf* Alexandra Scharf MSc. (QM-Beauftragte / Quality Manager) *- gezeichnet / signed A. Scharf* Datum / Date: 7 February 2019
Unteraufträge *Subcontractors*	Falls im Rahmen der Eignungsprüfung eine Prüfung der Gehalte, Homogenität und Stabilität von EP-Parametern durchgeführt wurde, hat DLA diese im Unterauftrag vergeben. In case the analysis of the content, homogeneity and stability of PT-parameters was part of the proficiency test, the determinations were subcontracted by DLA.
Vertraulichkeit *Confidentiality*	Die Teilnehmerergebnisse sind im EP-Bericht in anonymisierter Form mit Auswertenummern benannt. Daten einzelner Teilnehmer werden ausschließlich nach vorheriger Zustimmung des Teilnehmers an Dritte weitergegeben. Participant result are named anonymously with evaluation numbers in the PT report. Data of individual participants will be passed on to third parties only with prior consent of the participant.

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1. Introduction

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

2. Realisation

2.1 Test material

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

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

After mixing and homogenizing of the basic matrix at 60°C with stirring the spiking material containing the allergenic ingredients hazelnut and pecan were added to an aliquot of the basic matrix in order to prepare the spiked **sample B**. Then it was homogenized again at 60°C with stirring. Subsequently, the basic mixture was again added in 3 additional steps and homogenized in each case until the total quantity had been reached.

For the **spiking level sample**, the above mentioned allergenic compounds 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 500 µm).

After homogenization the samples A and B were portioned to approx. 25 g into PE container and metallised PET film bags. The spiking level sample was portioned to approx. 15 g in metallized PET film bags.

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

Table 1: Composition of DLA-Samples

Ingredients	**Sample A**	**Sample B**	**Spiking Level Sample**
Dark Chocolate (cocoa: 70% at least) Ingredients: Cocoa mass, sugar, cocoa butter, emulsifier: soy lecithin, vanilla extract Nutrients per 100 g: Fat 43 g, Carbohydrates 34 g, Protein 79,6 g	100 g/100 g	99,8 g/100 g	-
Potato Powder Ingredients: Potatoes, E471, E304, E223, E100	-	-	99,8 g/100 g
Hazelnuts, roasted ground, mixture (5 countries / Europe) - as Hazelnut* - *thereof 14,1% total protein***	-	 43,9 mg/kg 6,19 mg/kg	 38,9 mg/kg 5,48 mg/kg
Pecan - as Pecan* - *thereof 10,3% total protein***	-	 27,2 mg/kg 2,80 mg/kg	 26,2 mg/kg 2,70 mg/kg
further Ingredients: Maltodextrin, sodium sulfate and silicon dioxide	-	<0,2 g/100 g	<0,2 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=5,3)

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

2.1.1 Homogeneity

The **mixture homogeneity before bottling** was examined 8-fold by **microtracer analysis**. It is a standardized method that is part of the international GMP certification system for feed [14].
Before mixing dye coated iron particles of µm size are added to the sample and the number of particles is determined after homogenization in taken aliquots. The evaluation of the mixture homogeneity is based on the Poisson distribution using the chi-square test. A probability of ≥ 5 % is equivalent to a good homogeneous mixture and of ≥ 25% to an excellent mixture [14, 15].
Because stuck solid samples can not be analysed by the microtracer method, only the spiking level sample was measured. The microtracer analysis of the present PT showed a probability of 93%. Additionally particle number results were converted into concentrations, statistically evaluated according to normal distribution and compared to the standard deviation according to Horwitz. For the assessment HorRat values between 0,3 and 1,3 are to be accepted under repeat conditions (measurements within the laboratory) [17]. This gave a HorRat value 0,7. The results of microtracer analysis are given in the documentation.

Homogeneity of bottled spiked sample B

Implementation of homogeneity tests
The homogeneity tests were carried out in cooperation with the laboratories of the specified test kit providers. Ten samples of the bottled spiked sample were chosen randomly by DLA, thereof 2 subsamples were weighed into previously randomly encoded sample containers, and then sent to the laboratories for analysis. The sample weights were made with a deviation of ± 10% from recommended sample weight of the test kit instructions and not communicated to the laboratories. After transmission of analysis results by the laboratories, the valid results were calculated on the basis of the exact weightings by DLA and the statistical calculation was carried out according to ISO 13528:2015 Annex B (possibly with Notes 1 and 2).

Valuation of homogeneity
The homogeneity is regarded as sufficient when the standard deviation between the samples Ss is ≤ 15% („heterogeneity standard deviation"). This criterion is fulfilled for sample B by all ELISA tests for hazelnut (Immunolab, Veratox and AgraQuant) and pecan (Immunolab and AgraQuant), respectively (see page 7-8). 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].

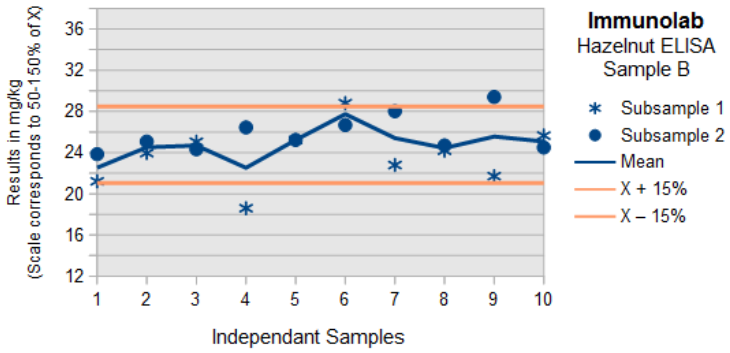
ELISA-Tests: Homogenität Haselnuss / Homogeneity Hazelnut

Immunolab Hazelnut ELISA
(with extraction additive)

Sample weights: 1,0 g (0,9 – 1,1 g)
Number of replicates: 2
Overall result: Hazelnut 24,8 ± 0,5 mg/kg

Sample B	Subsample 1 mg/kg	Subsample 2 mg/kg	Mean mg/kg
1	21,2	23,8	22,5
2	24,0	25,1	24,5
3	25,1	24,3	24,7
4	18,6	26,4	22,5
5	25,2	25,2	25,2
6	28,8	26,7	27,7
7	22,8	28,0	25,4
8	24,2	24,7	24,4
9	21,7	29,4	25,6
10	25,7	24,5	25,1

General average X	24,8	
SD of sample means Sx	1,51	6,1%
SD within-samples Sw	2,02	8,1%
SD between-samples Ss	0,48	1,9%

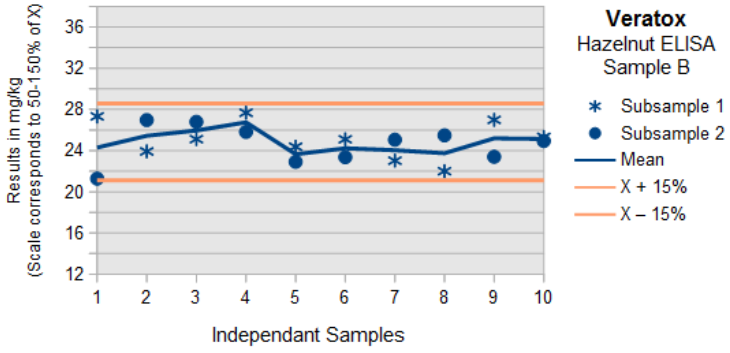


Neogen Veratox ELISA Hazelnut

Sample weights: 5,0 g (4,5 – 5,5 g)
Number of replicates: 2
Overall result: Hazelnut 24,8 ± 1,0 mg/kg

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

General average X	24,8	
SD of sample means Sx	1,02	4,1%
SD within-samples Sw	1,47	5,9%
SD between-samples Ss	< 1,02	< 4,1%

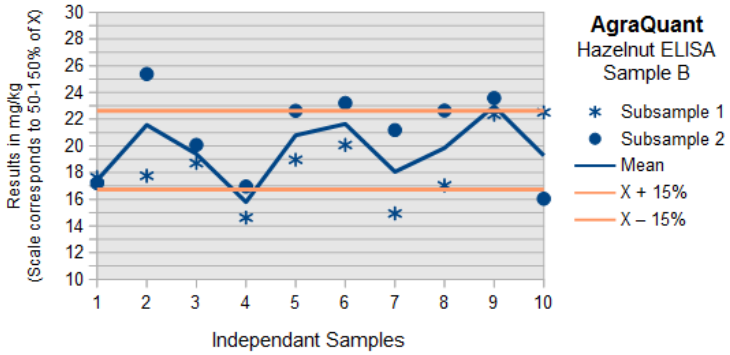


Romerlabs AgraQuant Hazelnut

Sample weights: 1,0 g (0,9 – 1,1 g)
Number of replicates: 2
Overall result: Hazelnut 19,7 ± 1,5 mg/kg

Sample B	Subsample 1 mg/kg	Subsample 2 mg/kg	Mean mg/kg
1	17,6	17,2	17,4
2	17,8	25,4	21,6
3	18,7	20,1	19,4
4	14,6	17,0	15,8
5	19,0	22,6	20,8
6	20,1	23,2	21,6
7	14,9	21,2	18,1
8	17,0	22,6	19,8
9	22,3	23,6	23,0
10	22,5	16,0	19,3

General average X	19,7	
SD of sample means Sx	2,17	11,0%
SD within-samples Sw	2,25	11,4%
SD between-samples Ss	1,48	7,5%



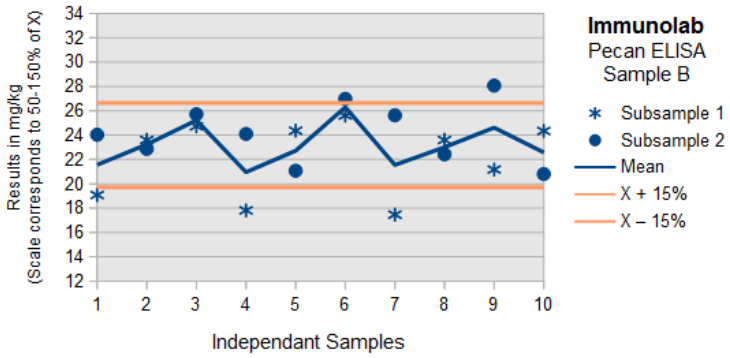
ELISA-Tests: Homogenität Pecannuss / Homogeneity Pecan

Immunolab Pecan ELISA
(with extraction additive)

Sample weights: 1,0 g (0,9 – 1,1 g)
Number of replicates: 2
Overall result: Pecan 23,2 ± 0,6 mg/kg

Sample B	Subsample 1 mg/kg	Subsample 2 mg/kg	Mean mg/kg
1	19,1	24,0	21,6
2	23,6	22,9	23,2
3	24,7	25,7	25,2
4	17,8	24,1	21,0
5	24,4	21,1	22,7
6	25,6	27,0	26,3
7	17,5	25,6	21,5
8	23,6	22,4	23,0
9	21,2	28,1	24,6
10	24,3	20,8	22,6

General average X	23,2	
SD of sample means Sx	1,72	7,4%
SD within-samples Sw	2,27	9,8%
SD between-samples Ss	0,63	2,7%

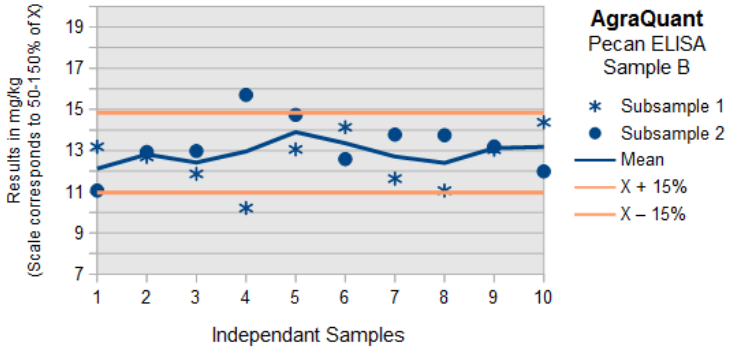


Romerlabs AgraQuant Pecan

Sample weights: 1,0 g (0,9 – 1,1 g)
Number of replicates: 2
Overall result: Pecan 12,9 ± 0,5 mg/kg

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

General average X	12,9	
SD of sample means Sx	0,52	4,1%
SD within-samples Sw	1,21	9,4%
SD between-samples Ss	< 0,52	< 4,1%



2.1.2 Stability

The food matrix of the sample material is chocolate, which is known to be stable for years because of its low water content. The storage stability and durability of the samples (microbial spoilage) was thus ensured during the investigation period under the specified storage conditions.

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

The experience with various DLA test materials showed good storage stability with respect to the durability of the sample (spoilage) and the content of the PT parameters for comparable food matrices and water activity (a_W value <0,5).
The a_W value of the PT samples was approx. 0,34 (20,6°C). The stability of the sample material was thus ensured during the investigation period under the specified storage conditions.

2.2 Sample shipment and information to the test

The portions of test materials sample A, B and the spiking level sample were sent to every participating laboratory in the 42nd week of 2018. The testing method was optional. The tests should be finished at November 30th 2018.

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 **Pecannut** in the range of mg/kg in the matrix of **Chocolate**. One of these samples and the "spiking level sample" were prepared adding the allergenic ingredients. The **"spiking level sample"** contains the allergens in a simple matrix in **similar amounts** without further processing.*

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

2.3 Submission of results

The participants submitted their results in standard forms, which have been sent by email.
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 10 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 level sample and the spiked sample recovery rates were calculated with respect to the known content of spiked allergens. The recovery rates were given for information only. *No* statistical evaluation was done. The recovery rates should exclusively give an estimation of the matrix- and/or processing influences.

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

3.1 Consensus value from participants (assigned value)

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

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

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

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

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

3.2 Robust standard deviation

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

The following robust standard deviations were considered:

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

3.3 Exclusion of results and outliers

Before statistical evaluation obvious blunders, such as those with incorrect units, decimal point errors, and results for a another proficiency test item can be removed from the data set [2]. Even if a result e.g. with a factor >10 deviates significantly from the mean and has an influence on the robust statistics, a result of the statistical evaluation can be excluded [3].
All results should be given at least with 2 significant digits. Specifying 3 significant digits is usually sufficient.

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

Results are tested for outliers by the use of robust statistics (algorithm A): If a value deviates from the robust mean by more than 3 times the robust standard deviation, it can be classified as an outlier (see above) [3]. Due to the use of robust statistics outliers are not excluded, provided that no other reasons are present [3]. Detected outliers are only mentioned in the results section, if they have been excluded from the statistical evaluation.

3.4 Target standard deviation (for proficiency assessment)

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

3.4.1 General model (Horwitz)

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

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

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

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

3.4.2 Value by precision experiment

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

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

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

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

Parameter	Matrix	Mean [mg/kg]	Recovery	rob RSD	RSD_r	RSD_R	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 for the allergens peanut, hazelnut, almond and brazil nut 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. An error or cause analysis can be carried out by checking the analysis process including understanding and implementation of the measurement by the staff, details of the measurement process, calibration of equipment and composition of reagents, transmission or calculation errors, trueness and precision, and use of reference material. If necessary, the problems must be addressed through appropriate corrective action [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 (xi) of the participant from the respective consensus value to the square root of quadrat sum of the target standard deviation (σ_{pt}) and the standard uncertainty (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*/σ_{pt}

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 and traceability

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

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

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

too low with respect to the standard uncertainty of the assigned value.

The traceability of the assigned value is ensured on the basis of the consensus value as a robust mean of the participant results.

3.9 Figures

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

3.10 Recovery rates: Spiking

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

4. Results

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

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

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

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

ELISA results given as **hazelnut protein** were converted by DLA to the **total food item (hazelnut)** using the analyzed protein content of the raw material (see page 5).

ELISA results given as **pecan protein** were converted by DLA to the **total food item (pecan)** using the analyzed protein content of the raw material (see page 5).

In the present PT, the quantitative PCR results e.g. for celery were sometimes given unclear or implausible as DNA, seed, tuber and/or only as celery, mustard and sesame. It was therefore not intended to standardize the PCR results.

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

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

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

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

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

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

° Target range is calculated with z-score or z'-score

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

4.1 Proficiency Test 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		
4	negative	0	positive	51,7	2/2 (100%)	BF	
7	negative	<3,5	positive	24,8	2/2 (100%)	ES	result converted °
2	negative	<1	positive	30,4	2/2 (100%)	IL	
10	negative	<0,3	positive	26,0	2/2 (100%)	IL	
1	negative		positive	38,7	2/2 (100%)	RS-F	
3	negative	<2.5	positive	50,7	2/2 (100%)	RS-F	
6	negative	<2,5	positive	>20	2/2 (100%)	RS-F	
8	negative		positive	35,0	2/2 (100%)	RS-F	
5	negative	<2.5	positive	15,2	2/2 (100%)	VT	
9	negative		positive	14,8	2/2 (100%)	VT	

° calculation p. 20

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

Methods:

BF = MonoTrace ELISA, BioFront Technologies
ES = ELISA-Systems
IL = Immunolab
RS-F= Ridascreen® Fast, R-Biopharm
VT = Veratox, Neogen

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

Quantitative valuation of ELISA-results: Sample B

Evaluation number	Hazelnut	z'-Score Xpt_{ALL}	Method	Remarks
	[mg/kg]			
4	51,7	1,9	BF	
7	24,8	-0,69	ES	result converted °
2	30,4	-0,15	IL	
10	26,0	-0,58	IL	
1	38,7	0,66	RS-F	
3	50,7	1,8	RS-F	
6	>20		RS-F	
8	35,0	0,30	RS-F	
5	15,2	-1,6	VT	
9	14,8	-1,7	VT	

° calculation p. 20

Methoden:

BF = MonoTrace ELISA, BioFront Technologies

ES = ELISA-Systems

IL = Immunolab

RS-F= Ridascreen® Fast, R-Biopharm

VT = Veratox, Neogen

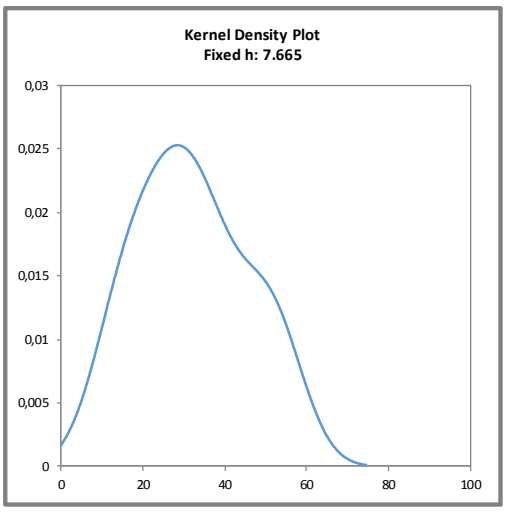


Abb. / Fig. 1:
Kerndichte-Schätzung aller ELISA-Ergebnisse (mit h = 0,75 x σ_{pt} von Xpt_{ALL})

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

Comments:
The kernel density estimation shows nearly a symmetrical distribution of results with a shoulder at approx. at 50 mg/kg (methods BF and RS-F).

Characteristics: Quantitative evaluation ELISA: Hazelnut

Sample B

Statistic Data	**All Results** *[mg/kg]*
Assigned value (X_{pt})	$\boldsymbol{X_{pt_{ALL}}}$
Number of results	9
Number of outliers	0
Mean	31,9
Median	30,4
Robust Mean (X_{pt})	**31,9**
Robust standard deviation (S*)	**15,3**
Target range:	
Target standard deviation $\sigma_{pt'}$	**10,2**
lower limit of target range	**11,5**
upper limit of target range	**52,4**
Quotient S/$\sigma_{pt'}$*	*1,5*
Standard uncertainty $U_{(Xpt)}$	*6,38*
Results in the target range	*9*
Percent in the target range	*100*

Comments to the statistical characteristics and assigned values:

The kernel density estimation showed almost a symmetrical distribution of results and no clear method-dependent differences.

Due to the relatively broad distribution and small number of results of the single methods, only an evaluation of all methods using z'-scores was done.

The evaluation of all methods showed a normal variability of results. The quotient S/$\sigma_{pt'}$ was below 2,0. The robust standard deviation is in the upper 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, because there were only a few results for some methods.*

The assigned value of the evaluation of all results was 73% of the spiking level of hazelnut to sample B and thus within the recommendations for the applied methods (s. 3.4.3 and "Recovery rates of Hazelnut" p.29).

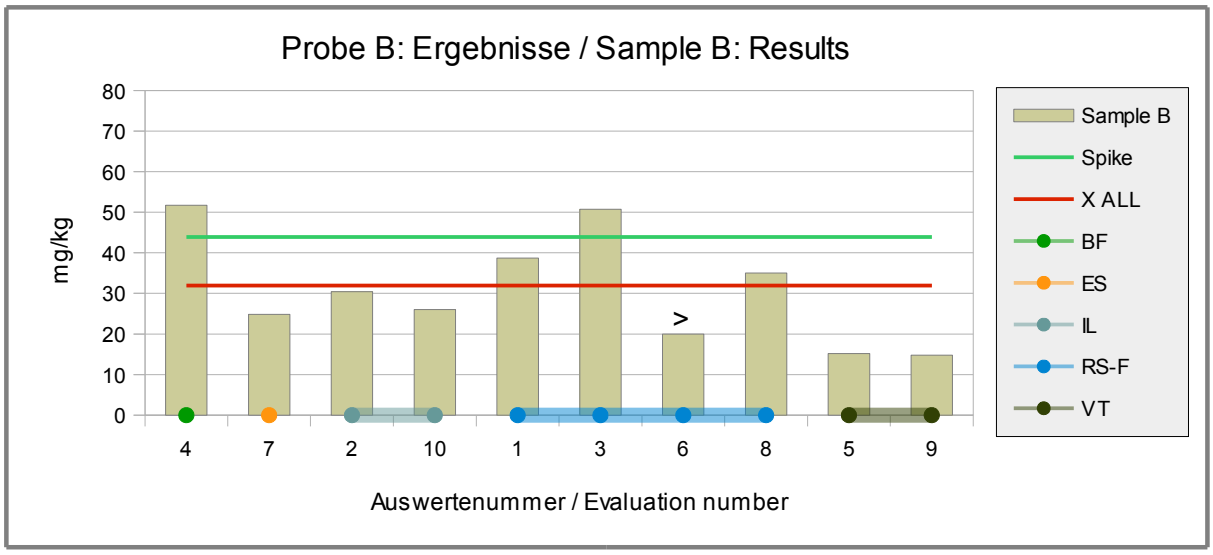


Abb./Fig. 2: ELISA Results Hazelnut
green line = Spiking level
red line = Assigned value robust mean all results
round symbols = Applied methods (see legend)

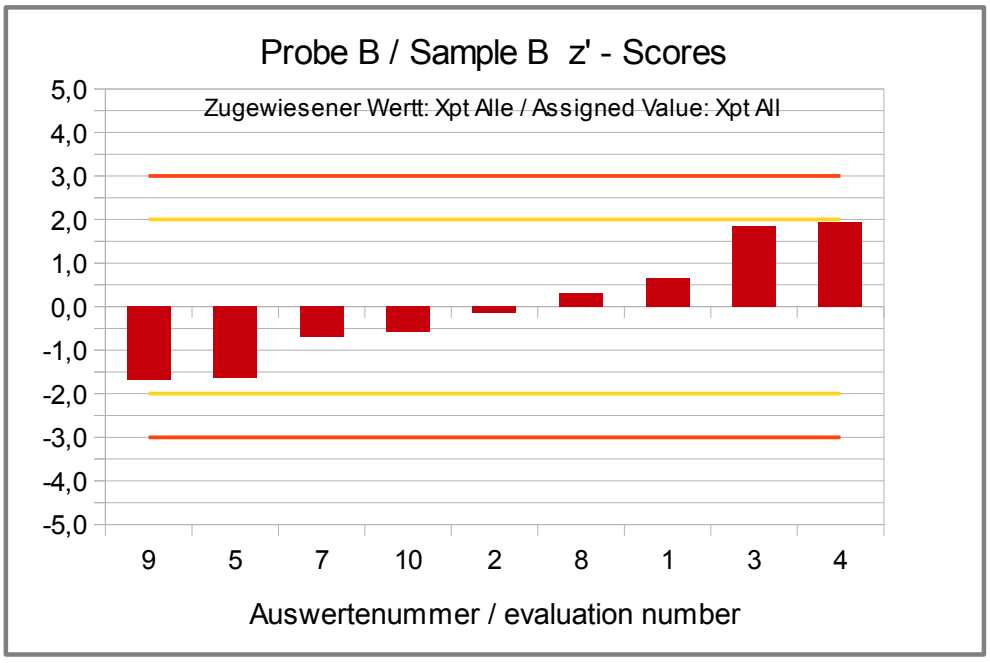


Abb./Fig. 3:
z'-Scores (ELISA Results Hazelnut)
Assigned value robust mean (algorithm A) of all results

Quantitative valuation ELISA: Spiking level sample

Evaluation number	Hazelnut	z'-Score Xpt_{ALL}	Method	Remarks
	[mg/kg]			
4	52,3	1,8	BF	
7	19,2	-1,4	ES	result converted °
2	36,2	0,27	IL	
10	34,0	0,06	IL	
1	36,0	0,25	RS-F	
3	51,8	1,8	RS-F	
6	>20		RS-F	
8	30,0	-0,33	RS-F	
5	17,8	-1,5	VT	
9	23,2	-1,0	VT	

° calculation p. 20

Methods:

BF = MonoTrace ELISA, BioFront Technologies

ES = ELISA-Systems

IL = Immunolab

RS-F= Ridascreen® Fast, R-Biopharm

VT = Veratox, Neogen

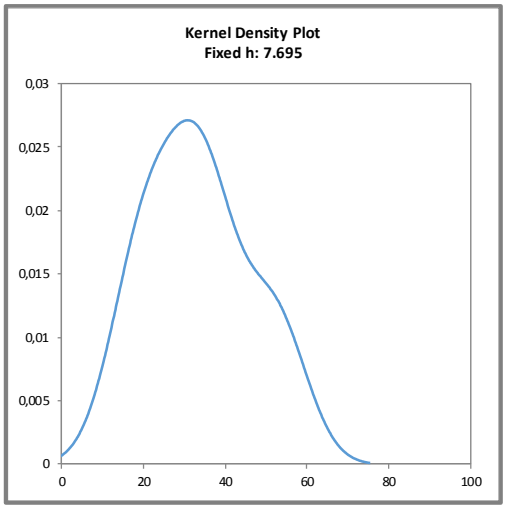


Abb. / Fig. 4:
Kerndichte-Schätzung aller ELISA-Ergebnisse (mit h = 0,75 x σ_{pt} von Xpt_{ALL})

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

Comments:
The kernel density estimation shows nearly a symmetrical distribution of results with a shoulder at approx. at 60 mg/kg (methods BF and RS-F).

Characteristics: Quantitative evaluation Hazelnut

Spiking level sample

Statistic Data	**All Results** *[mg/kg]*
Assigned value (X_{pt})	$X_{pt_{ALL}}$
Number of results	9
Number of outliers	0
Mean	33,4
Median	34,0
Robust Mean (X_{pt})	**33,4**
Robust standard deviation (S*)	**14,3**
Target range:	
Target standard deviation $\sigma_{pt'}$	**10,26**
lower limit of target range	**12,9**
upper limit of target range	**53,9**
Quotient S/$\sigma_{pt'}$*	*1,4*
Standard uncertainty $U_{(Xpt)}$	*5,96*
Results in the target range	*9*
Percent in the target range	*100*

Comments to the statistical characteristics and assigned values:

The kernel density estimation showed almost a symmetrical distribution of results and no clear method-dependent differences.

Due to the relatively broad distribution and small number of results of the single methods, only an evaluation of all methods using z'-scores was done.

The evaluation of all methods showed a normal variability of results. The quotient S/$\sigma_{pt'}$ was below 2,0. The robust standard deviation is in the upper 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, because there were only a few results for some methods.*

The assigned value of the evaluation of all results was 86% 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.29).

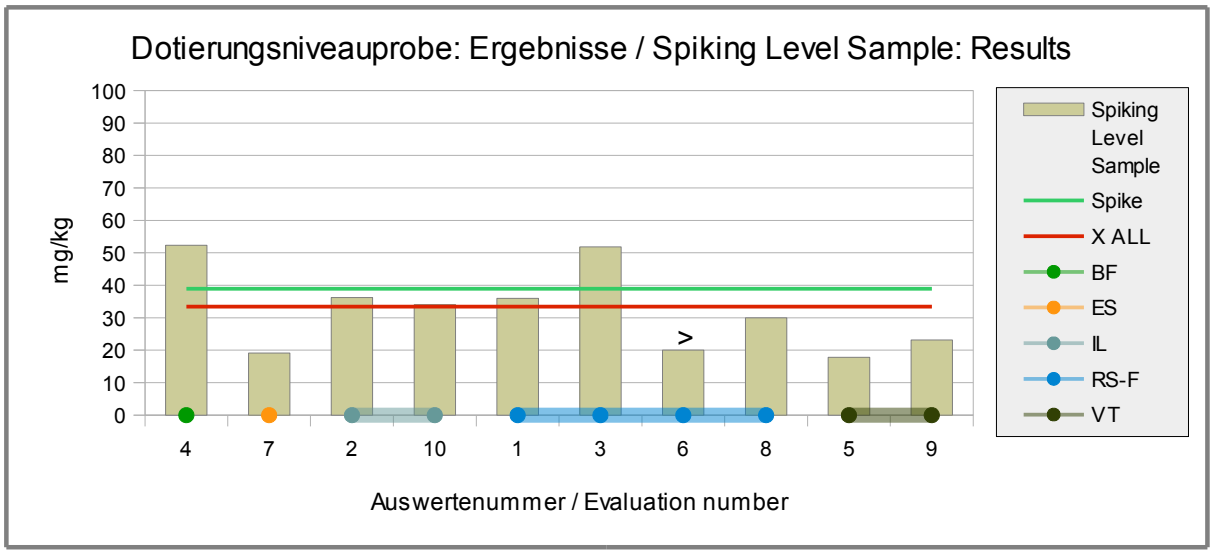


Abb./Fig. 5: ELISA Results Hazelnut
green line = Spiking level
red line = Assigned value robust mean all results
round symbols = Applied methods (see legend)

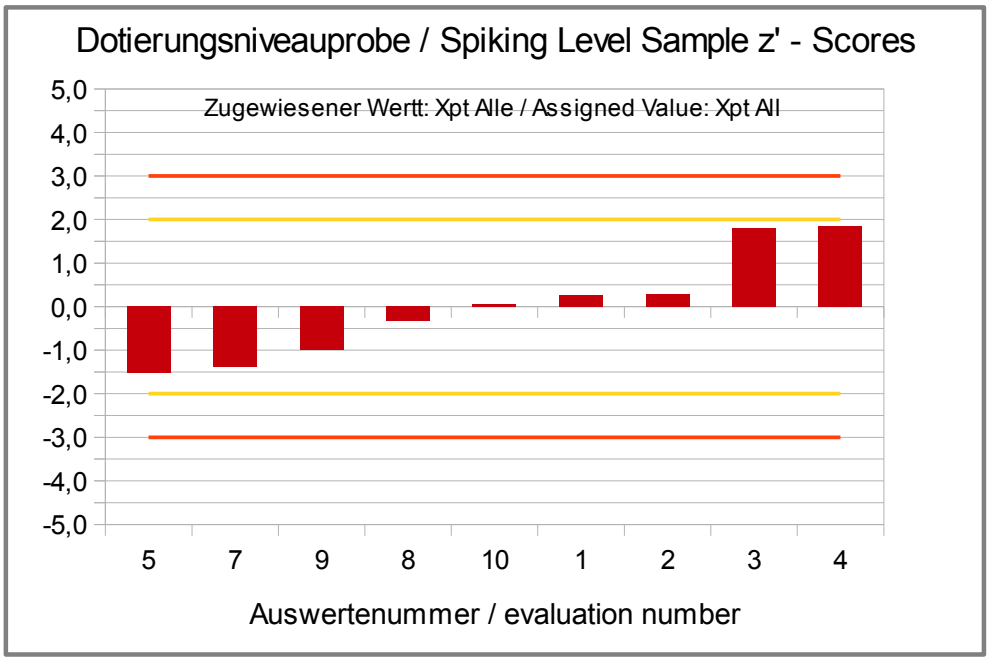


Abb./Fig. 6:
z'-Scores (ELISA Results Hazelnut)
Assigned value: robust mean (algorithm A) of all results

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

Evaluation number	Spiking Level Sample	Recovery rate*	Sample B	Recovery rate*	Method	Remarks
	[mg/kg]	[%]	[mg/kg]	[%]		
4	52,3	**134**	51,7	**118**	BF	
7	19,2	49	24,8	**57**	ES	result converted °
2	36,2	**93**	30,4	**69**	IL	
10	34,0	**87**	26,0	**59**	IL	
1	36,0	**93**	38,7	**88**	RS-F	
3	51,8	**133**	50,7	**116**	RS-F	
6	>20		>20		RS-F	
8	30,0	**77**	35,0	**80**	RS-F	
5	17,8	46	15,2	35	VT	
9	23,2	**60**	14,8	34	VT	

° calculation p. 20

RA**	50-150 %	RA**	50-150 %
Number in RA	**7**	Number in RA	**7**
Percent in RA	**78**	Percent in RA	**78**

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

** Range of acceptance of AOAC for allergen ELISAS

Methods:

BF = MonoTrace ELISA, BioFront Technologies

ES = ELISA-Systems

IL = Immunolab

RS-F= Ridascreen® Fast, R-Biopharm

VT = Veratox, Neogen

Comments:

For the spiking level sample 78% (7) of the participants obtained a recovery rate by ELISA methods within the range of the AOAC-recommendation of 50-150%. For the spiked food matrix sample B also 78% (7) of the recovery rates were within the range of acceptance.

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		
7	negative		positive		2/2 (100%)	div	
8	negative		positive	150	2/2 (100%)	div	indicated as Hazelnut-DNA

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

Methods:
div = not indicated / other method

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

Quantitative Valuation PCR: Sample B

A quantitative evaluation was not done, because there were not enough results.

(Quantitative) Valuation PCR: Spiking Level Sample

A quantitative evaluation was not done, because there were not enough results.

Evaluation number	Hazelnut	Spiking Level Sample	z-Score Xpt_{ALL}	Method	Remarks
	pos/neg	[mg/kg]			
7	positive			div	
8	positive	100		div	given as Hazelnut-DNA

Number positive	2	
Number negative	0	
Percent positive	100	
Percent negative	0	
Consensus value	positive	

Methods:
div = not indicated / other method

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

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

Since no quantitative results were available given as hazelnut, but as hazelnut DNA only, no recovery rates were calculated.

4.2 Proficiency Test Pecan

4.2.1 ELISA Results: Pecan

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		positive	13,6	2/2 (100%)	3M	result converted °
4	negative	0	positive	42,1	2/2 (100%)	BF	
5	negative	<2,0	positive	42,5	2/2 (100%)	BF	
6	negative	<2	positive	17,8	2/2 (100%)	BF	
3	negative	<2	positive	10,7	2/2 (100%)	IL	
10	negative	<0,2	positive	26,0	2/2 (100%)	IL	

° calculation p. 20

	Sample A		Sample B	
Number positive	0		6	
Number negative	6		0	
Percent positive	0		100	
Percent negative	100		0	
Consensus value	negative		positive	

Methods:

3M = 3M Protein ELISA Kit

BF = MonoTrace ELISA, BioFront Technologies

IL = Immunolab

Comments:

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

Quantitative valuation of ELISA-results: Sample B

Due to the heterogeneous distribution of data and the low number, no evaluation of the results by z-scores was performed (see next page).

Evaluation number	Pecan	z-Score Xpt_{ALL}	Method	Remarks
	[mg/kg]			
1	13,6		3M	result converted °
4	42,1		BF	
5	42,5		BF	
6	17,8		BF	
3	10,7		IL	
10	26,0		IL	

° calculation p. 20

Methods:

3M = 3M Protein ELISA Kit

BF = MonoTrace ELISA, BioFront Technologies

IL = Immunolab

Comments:

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

Characteristics: Quantitative evaluation ELISA: Almond

Sample B

Statistic Data	**All Results** *[mg/kg]*
Assigned value (X_{pt})	$\boldsymbol{X_{pt_{ALL}}}$
Number of results	6
Number of outliers	0
Mean	25,5
Median (X_{pt})	**25,5**
Robust Mean	21,9
Robust standard deviation (S*)	**15,9**
Target range:	
Target standard deviation σ_{pt}	
lower limit of target range	
upper limit of target range	
Quotient S/σ_{pt}*	
Standard uncertainty $U_{(Xpt)}$	
Results in the target range	
Percent in the target range	

Comments to the statistical characteristics and assigned values:

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

The distribution of the results of all methods showed an increased variability with a quotient S/σ_{pt} of 2,9. Due to the heterogeneous data and the low number, no evaluation of the results by z-scores was performed.*

The median of the participant results was 94% of the spiking level of pecan to sample B and thus within the recommendations for the applied methods (s. 3.4.3 and "Recovery rates of Pecan" p.39).

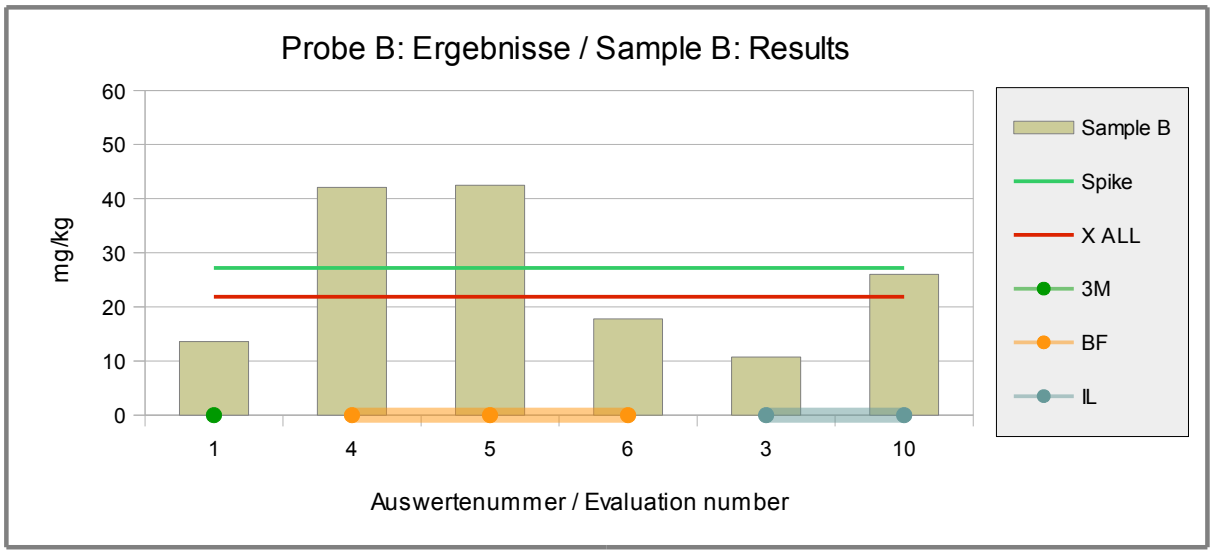


Abb./Fig. 7: ELISA Results Pecan
green line = Spiking level
red line = robust mean all results
round symbols = Applied methods (see legend)

Quantitative valuation of results: Spiking level sample

Evaluation number	Pecan	z-Score Xpt_{ALL}	Method	Remarks
	[mg/kg]			
1	23,3	-2,3	3M	result converted °
4	69,8	1,0	BF	
5	61,1	0,40	BF	
6	64,4	0,64	BF	
3	52,6	-0,21	IL	
10	51,0	-0,33	IL	

° calculation p. 20

Methods:

3M = 3M Protein ELISA Kit

BF = MonoTrace ELISA, BioFront Technologies

IL = Immunolab

Comments:

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

Characteristics: Quantitative evaluation Pecan

Spiking level sample

Statistic Data	**All Results** *[mg/kg]*
Assigned value (X_{pt})	$X_{pt_{ALL}}$
Number of results	6
Number of outliers	0
Mean	53,7
Median	56,9
Robust Mean (X_{pt})	**55,5**
Robust standard deviation (S*)	**14,3**
Target range:	
Target standard deviation σ_{pt}	**13,9**
lower limit of target range	**27,8**
upper limit of target range	**83,3**
Quotient S/σ_{pt}*	*1,0*
Standard uncertainty $U_{(Xpt)}$	*7,30*
Results in the target range	*5*
Percent in the target range	*83*

Comments to the statistical characteristics and assigned values:

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

The evaluation of all methods showed a normal to low variability of results. The quotient S/σ_{pt} was well 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 some methods.*

The assigned value of the evaluation of all results was 212% of the spiking level of pecan to the spiking level sample and above the recommendations for the applied methods (s. 3.4.3 and "Recovery rates of Pecan" p.39).

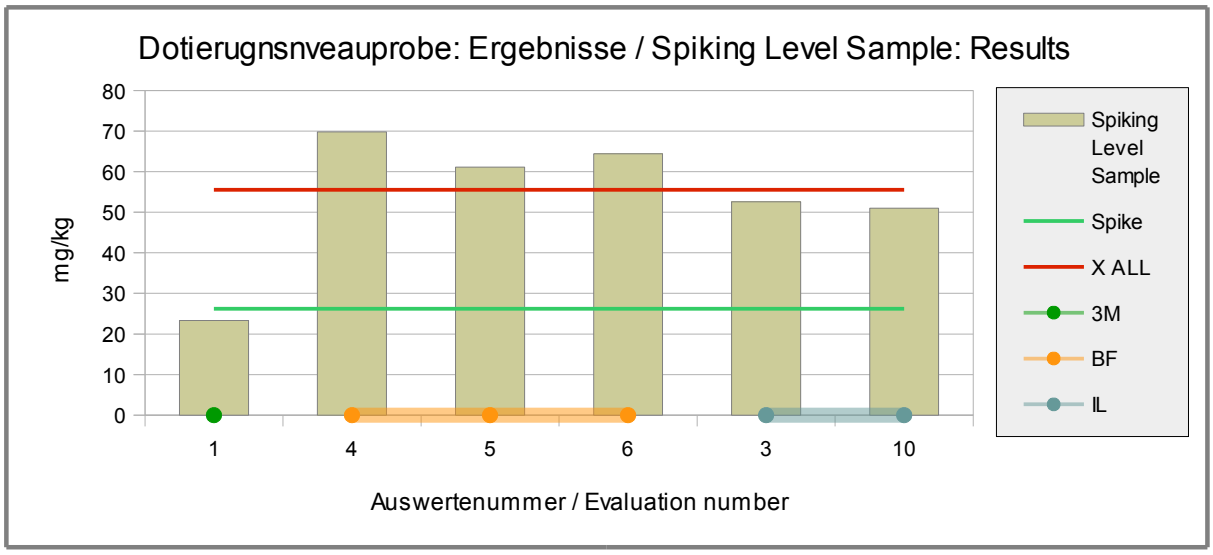


Abb./Fig. 8: ELISA Results Pecan
green line = Spiking level
red line = Assigned value robust mean all results
round symbols = Applied methods (see legend)

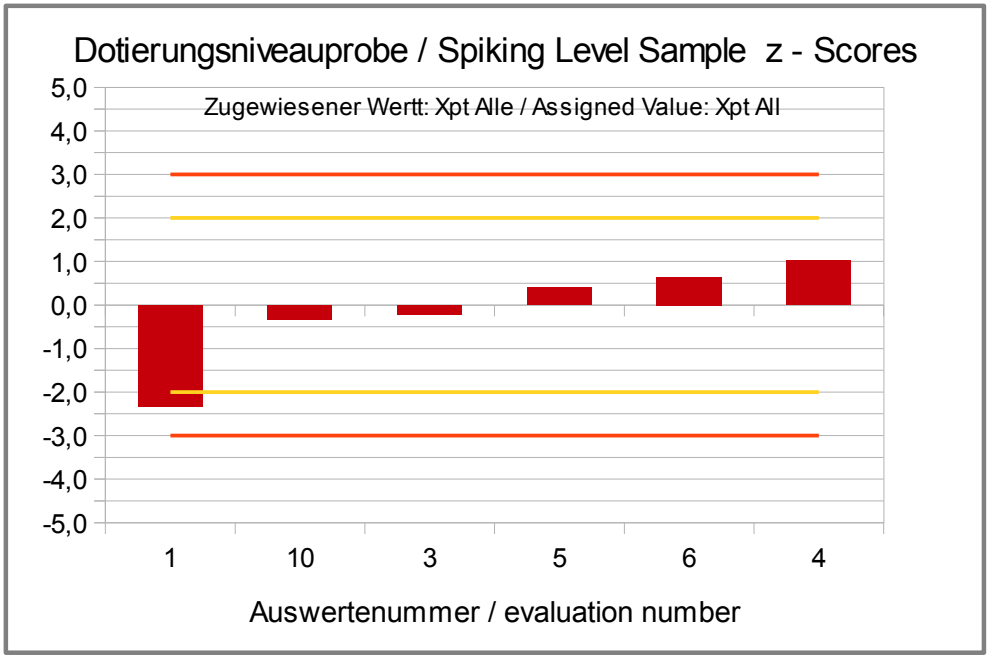


Abb./Fig. 9:
z-Scores (ELISA Results Pecan)
Assigned value: robust mean (algorithm A) of all results

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

Evaluation number	Spiking Level Sample	Recovery rate*	Sample B	Recovery rate*	Method	Remarks
	[mg/kg]	[%]	[mg/kg]	[%]		
1	23,3	**89**	13,6	**50**	3M	result converted °
4	69,8	266	42,1	155	BF	
5	61,1	233	42,5	156	BF	
6	64,4	246	17,8	**65**	BF	
3	52,6	201	10,7	39	IL	
10	51	195	26,0	**96**	IL	

° calculation p. 20

RA**	50-150 %	RA**	50-150 %
Number in RA	**1**	Number in RA	**3**
Percent in RA	**17**	Percent in RA	**50**

Methods:
3M = 3M Protein ELISA Kit
BF = MonoTrace ELISA, BioFront Technologies
IL = Immunolab

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

** Range of acceptance of AOAC for allergen ELISAS

Comments:
For the spiking level sample one participant (17%) obtained a recovery rate by ELISA method within the range of the AOAC-recommendation of 50-150%, while the other recovery rates were at approx. 200% or higher. For the spiked food matrix sample B 50% (3) of the recovery rates were within the range of acceptance.

4.2.2 PCR Results: Pecan

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	negative	<1	positive	11,7	2/2 (100%)	SFA	
7	negative		positive		2/2 (100%)	div	
8	negative		positive	300	2/2 (100%)	div	given as Pecan-DNA

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

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

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

Quantitative Valuation PCR: Sample B

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

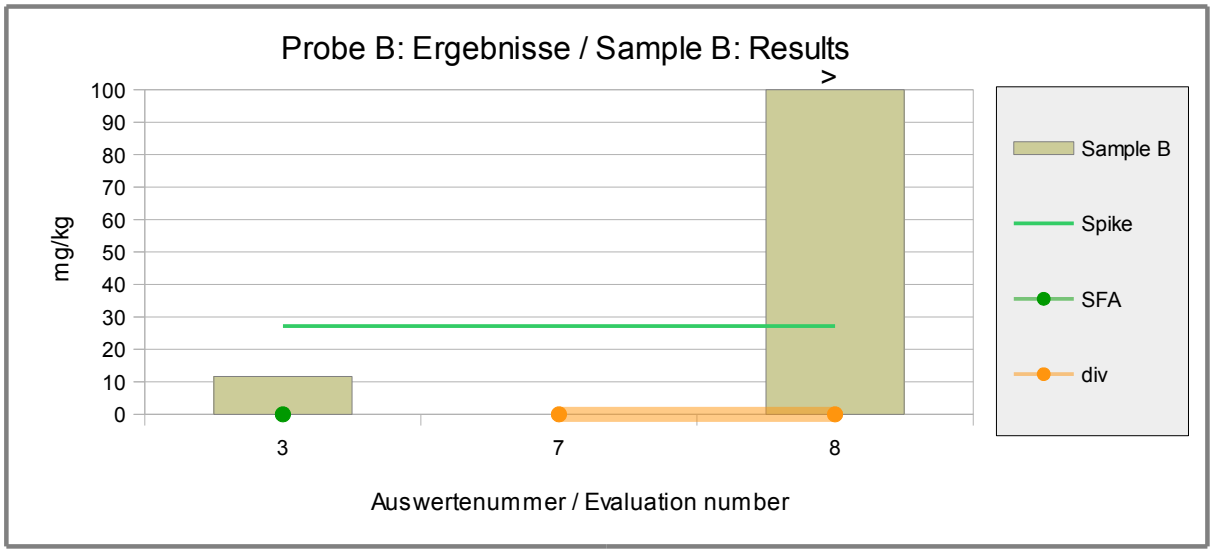


Abb./Fig. 10: PCR-Results Pecan Sample B
green line = Spiking level
round symbols = Applied methods (see legend)

(Quantitative) Valuation PCR: Spiking Level Sample

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

Evaluation number	Pecan	Pecan	Method	Remarks
	pos/neg	[mg/kg]		
3	positive		SFA	
7	positive		div	
8	positive	300	div	given as Pecan-DNA

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

Methods:
SFA = Sure Food Allergen, R-Biopharm / Congen

div = not indicated / other method

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

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

Evaluation number	Spiking Level Sample	Recovery rate*	Sample B	Recovery rate*	Method	Remarks
	[mg/kg]	[%]	[mg/kg]	[%]		
3			11,7	43	SFA	
7					div	
8	300		300		div	given as Pecan-DNA

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

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

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

** Range of acceptance of AOAC for allergen ELISAS

Comments:
One participant submitted a quantitative PCR result given as pecan. The obtained recovery rate for the spiked food matrix sample B was slightly below the range of the AOAC-recommendation of 50-150%. Results given as DNA were not considered.

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		NWG / LOD *	BG / LOQ *	MU*	quantitative Result given as	Method
		day/month	qualitative	mg/kg	qualitative	mg/kg	qualitative	mg/kg	mg/kg	mg/kg	%	e.g. food /protein	Test-Kit + Manufacturer
BF	4	25/11	negative	0	positive	51,7	positive	52,3	0,04	1		Hazelnut	MonoTrace Hazelnut ELISA kit, BioFront Technologies
ES	7	01.11.18	negative	<0,5	positive	3,5	positive	2,7	0,25	0,5		Hazelnut Protein	ELISA Systems Hazelnut ESHRD-48
IL	2	30.11.18	-	<1	-	30,4	-	36,2		1		Hazelnut	Immunolab Hazelnut ELISA
IL	10	27.10.18	negative	< 0.3	positive	26	positive	34	0.3	1		Hazelnut	Immunolab Hazelnut ELISA
RS-F	1	27.11.18	negative		positive	38,7	positive	36		2,5		Hazelnut	Ridascreen® FAST Hazelnut R6802, R-Biopharm
RS-F	3	22.10.18	negative	<2.5	positive	50,71	positive	51,82	2,5	2,5	33,3	Hazelnut	Ridascreen® FAST Hazelnut R6802, R-Biopharm
RS-F	6		negative	<2,5	positive	>20	positive	>20		2,5		Hazelnut	Ridascreen® FAST Hazelnut R6802, R-Biopharm
RS-F	8	13.11.18	negative		positive	35	positive	30	1,5	2,5			Ridascreen® FAST Hazelnut R6802, R-Biopharm
VT	5	22/11	negative	<2.5	positive	15,2	positive	17,8		2,5		Hazelnut	Veratox Hazelnut, Neogen
VT	9	17.10.18	negative		positive	14,8	positive	23,2	3	10	30	Hazelnut	Veratox Hazelnut, Neogen

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

Meth. Abr.	Evaluation number	Specifity	Remarks to the Method (Extraction and Determination)	Method accredited ISO/IEC 17025	Further Remarks
		Antibody	e.g. Extraction Solution / Time / Temperature	yes/no	
BF	4	Monoclonal antibody	1:20 extraction ratio/10 min/60 C	no	5% non-fat dry milk added to 1X extraction buffer
ES	7	detects hazelnut proteins	as per kit instructions	yes	
IL	2			yes	
IL	10		w ith Immunolab extraction additive		
RS-F	1				Test kit result - expressed as total hazelnut
RS-F	3	As Per Kit Instructions	As Per Kit Instructions	Yes	
RS-F	6			yes	
RS-F	8		As Per Kit Instructions	yes	
VT	5				
VT	9		PBS/15min/60°C	yes	

5.1.2 ELISA: Pecan

Meth. Abr.	Evaluation number	Date of Analysis	Result Sample A		Result Sample B		Result Spiking Sample		NWG / LOD *	BG / LOQ *	MU*	quantitative Result given as	Method
		day/month	qualitative	mg/kg	qualitative	mg/kg	qualitative	mg/kg	mg/kg	mg/kg	%	e.g. food /protein	Test-Kit + Manufacturer
3M	1	21.11.18	Negative		positive	1,4	positive	2,4		0,7		Pecan Protein	other: 3M E96PEC
BF	4	25/11	negative	0	positive	42,1	positive	69,8	0,17	1		Pecan	MonoTrace Pecan ELISA kit, BioFront Technologies
BF	5	20/11	negative	<2.0	positive	42,5	positive	61,1		2		Pecan	MonoTrace Pecan ELISA kit, BioFront Technologies
BF	6		negative	<2	positive	17,8	positive	64,4		2		Pecan	BioFront
IL	3	22.10.18	negative	<2	positive	10,74	positive	52,6	2	2	30	Pecan	Immunolab Pecan Nut ELISA
IL	10	27.10.18	negative	< 0.2	positive	26	positive	51	0.2	2		Pecan	Immunolab Pecan Nut ELISA

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

Meth. Abr.	Evaluation number	Specifity	Remarks to the Method (Extraction and Determination)	Method accredited ISO/IEC 17025	Further Remarks
		Antibody	e.g. Extraction Solution / Time / Temperature	yes/no	
3M	1				Test kit result - expressed as Pecan protein
BF	4	Monoclonal antibody	1:10 extraction ratio/10 min/60 C	no	5% non-fat dry milk added to 1X extraction buffer
BF	5				
BF	6			no	
IL	3	As Per Kit Instructions	As Per Kit Instructions	Yes	
IL	10		w ith Immunolab extraction additive		

5.1.3 PCR: Hazelnut

Meth. Abr.	Evaluation number	Date of Analysis	Result Sample A		Result Sample B		Result Spiking Sample		NWG / LOD *	BG / LOQ *	MU*	quantitative Result given as	Method
		day/month	qualitative	mg/kg	qualitative	mg/kg	qualitativee	mg/kg	mg/kg	mg/kg	%		Test-Kit + Manufacturer
div	7	01.11.18	negative		positive		positive		10			Hazelnut-DNA	ASU §64 Methode/method
div	8	31.10.18	negative		positive	150	positive	100		100		Hazelnut-DNA	

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

Meth. Abr.	Evaluation number	Specifity	Remarks to the Method (Extraction and Determination)	Method accredidet ISO/IEC 17025	Further Remarks
		Target-Sequence / -DNA	e.g. Extraction / enzymes / clean-up / real time PCR / gel electrophoresis / cycles	yes/no	
div	7	Hazelnut-DNAp	CTAB / Proteinase K / Promega Wizard DNA CleanUp / Real-time PCR / 45 Cycles	yes	
div	8		Piknova et al, 2008		related to total DNA, rough estimation, no reference samples

5.1.4 PCR: Pecan

Meth. Abr.	Evaluation number	Date of Analysis	Result Sample A		Result Sample B		Result Spiking Sample		NWG / LOD *	BG / LOQ *	MU*	quantitative Result given as	Method
		day/month	qualitative	mg/kg	qualitative	mg/kg	qualitative	mg/kg	mg/kg	mg/kg	%		Test-Kit + Manufacturer
SFA	3	27.11.18	negative	<1	positive	11,67	positive		1	1	30	Pecan	Sure Food Allergen, R-Biopharm / Congen
div	7	01.11.18	negative		positive		positive		4			Pecan-DNA	internal method
div	8	30.10.18	negative		positive	300	positive	300		100		Pecan-DNA	

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

Meth. Abr.	Evaluation number	Specifity	Remarks to the Method (Extraction and Determination)	Method accredidet ISO/IEC 17025	Further Remarks
		Target-Sequence / -DNA	e.g. Extraction / enzymes / clean-up / real time PCR / gel electrophoresis / cycles	yes/no	
SFA	3	As Per Kit Instructions	As Per Kit Instructions	No	
div	7	Pecan- and Walnut-DNAp	CTAB / Proteinase K / Promega Wizard DNA CleanUp / PCR Gel electrophoresis / 45 cycles	ja	sample B and spiking level sample tested with Walnut ELISA, result <3mg/kg Walnut
div	8		Brezna et al. 2008		related to total DNA, rough estimation, no reference samples

5.2 Homogeneity

5.2.1 Mixture homogeneity before bottling

Microtracer Homogeneity Test
DLA 06-2018 Spiking Level Sample

Weight whole sample	1,55	kg
Microtracer	FSS-rot lake	
Particle size	75 – 300	µm
Weight per particle	2,0	µg
Addition of tracer	20,4	mg/kg

Result of analysis

Sample	Weight [g]	Particle number	Particles [mg/kg]
1	4,97	92	37,0
2	5,03	89	35,4
3	5,03	91	36,2
4	5,06	80	31,6
5	5,02	88	35,1
6	4,99	79	31,7
7	5,02	81	32,3
8	5,00	82	32,8

Poisson distribution		
Number of samples	8	
Degree of freedom	7	
Mean	85,3	Particles
Standard deviation	5,40	Particles
χ^2 (CHI-Quadrat)	2,40	
Probability	**93**	%
Recovery rate	167	%

Normal distribution		
Number of samples	8	
Mean	34,0	mg/kg
Standard deviation	2,15	mg/kg
rel. Standard deviaton	6,34	%
Horwitz standard deviation	9,41	%
HorRat-value	**0,67**	
Recovery rate	167	%

5.3 Information on the Proficiency Test (PT)

Before the PT the participants received the following information in the sample cover letter (1st letter):

PT number	***DLA 06-2018***
PT name	***Allergens VI: Hazelnut and Pecannut in Chocolate***
Sample matrix (processing)	***Samples A + B:*** *Chocolate 70%/ ingredients: Cocoa mass, sugar, cocoa butter, emulsifier: lecithins, vanilla extract other food additives and allergenic foods (one of both samples)* ***Spiking Level Sample:*** *potato powder, other food additives and allergenic foods*
Number of samples and sample amount	*2 different Samples A + B: 25 g each* *+ 1 Spiking Level Sample: 15 g*
Storage	*Samples A + B: room temperature (long term cooled 2 - 10°C)* *Spiking Level Sample: room temperature*
Intentional use	*Laboratory use only (quality control samples)*
Parameter	*qualitative + quantitative:* *Hazelnut (Hazelnut protein, DNA), Pecannut (Pecannut protein, DNA)* *Samples A + B: < 500 mg/kg* *Spiking Level Sample: < 500 mg/kg*
Methods of analysis	*Analytical methods are optional*
Notes to analysis	*The analysis of PT samples should be performed like a routine laboratory analysis.* *In general we recommend to homogenize a representative sample amount before analysis according to good laboratory practice, especially in case of low sample weights. It is the best to homogenize the whole sample.*
Result sheet	*One result each should be determined for Samples A and B and the Spiking Level Sample.* *The results should be filled in the result submission file.*
Units	*mg/kg*
Number of digits	*at least 2*
Result submission	*The result submission file should be sent by e-mail to:* ***pt@dla-lvu.de***
Deadline	***the latest November 30th 2018***
Evaluation report	*The evaluation report is expected to be completed 6 weeks after deadline of result submission and sent as PDF file by e-mail.*
Coordinator and contact person of PT	*Matthias Besler-Scharf, PhD*

* Control of mixture homogeneity and qualitative testings are carried out by DLA. 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
		SPAIN
		USA
		CANADA
		Germany
		GREECE
		Germany
		SWITZERLAND
		CANADA
		GREAT BRITAIN
		SPAIN

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

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

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. Homogeneity and stability of reference materials; Linsinger et al.; Accred Qual Assur, 6, 20-25 (2001)
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 proteins 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]