Proficiency Tests

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Evaluation Report
proficiency test

DLA 06/2016

Allergens VI:

Hazelnut and Milk

in "milk-free" Chocolate

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

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Unteraufträge *Subcontractors*	Die Prüfung der Gehalte, Homogenität und Stabilität von EP-Parametern wird von DLA im Unterauftrag vergeben. The analysis of the content, homogeneity and stability of PT-parameters are subcontracted by DLA.

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1. Introduction

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

2. Realisation

2.1 Test material

Two PT-samples for the detection of allergens in the range of mg/kg and one spiking material sample were provided for analysis. The spiking material sample contains the respective allergenic ingredients in the range of 1-10 % and was added to the spiked PT-sample. The results of the spiking material sample should give the possibility of a comparison with the spiked sample in respect to the detectability of the allergens with and without the influence of matrix and / or food processing.

The test material 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 the basic matrix at 60°C with stirring the spiking material containing the allergenic ingredients hazelnut and milk were added to an aliquot of the basic matrix in order to prepare the spiked sample A. Then it was homogenized again at 60°C with stirring. Subsequently, the basic mixture was again added in 4 additional steps and mechanically homogenized in each case until the total quantity had been reached.
The composition of the spiking material sample and the amounts of allergens in sample A is given in table 2.

After homogenization the samples were portioned to approximately 25 g into PE container and metallised PET film bags.

Table 1: Composition of DLA-Samples

Ingredients	**Sample A**	**Sample B**
Dark-Chocolate **(cacao: 85% at least)** Ingredients: Cocoa mass, cocoa butter, sugar, low-fat cocoa, emulsifier: soy lecithin, vanilla extract Nutrients per 100 g: Protein 11 g, carbohydrates 20 g, fat 50 g Allergen-Information: may contain traces of peanuts, almonds, nuts and milk.	99,8 g/100 g	100 g/100 g
Spiking material sample	0,205 g/100 g	-

Table 2: Added amounts of allergenic ingredients

Ingredients	**Spiking material sample**	**Amounts in Sample A**
Potato flour	93 %	0,19%
Hazelnut mush Ingredients: Hazelnuts - as Hazelnut* - *thereof 16% total protein***	11800 mg/kg (= 1,18 %) 11800 mg/kg 1890 mg/kg	 24,1 mg/kg 3,9 mg/kg
Milk: - as Skimmed Milk Powder* - *thereof 37% total protein*** - *thereof Casein****	 19600 mg/kg (1,96 %) 7220 mg/kg 5780 mg/kg	 40,2 mg/kg 14,8 mg/kg 11,8 mg/kg
additional ingredients: *soy flour and wheat flour*	 < 3,50 %	 < 0,03 %

*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)
*** Protein contents (appr. 80% caseins in total milkprotein) calculated according to literature [31]

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

2.1.1 Homogeneity

The **mixture homogeneity before bottling** was examined 8-fold by **microtracer analysis**. It is a standardized method that is part of the international GMP certification system for feed [14].
Before mixing dye coated iron particles of µm size are added to the sample and the number of particles is determined after homogenization in taken aliquots. The evaluation of the mixture homogeneity is based on the Poisson distribution using the chi-square test. A probability of ≥ 5 % is equivalent to a good homogeneous mixture and of ≥ 25% to an excellent mixture [14, 15].
The microtracer analysis of the present PT samples showed a probability of 43% for the spiking material sample. Additionally particle number results were converted into concentrations, statistically evaluated according to normal distribution and compared to the standard deviation according to Horwitz. This gave a HorRat value of 1,0. 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 not fulfilled for sample A by all ELISA tests for hazelnut (Immunolab, AgraQuant Plus and Veratox) and milk / casein (Immunolab, AgraQuant Plus and Veratox), respectively (see page 7). For hazelnut heterogeneity standard deviations were in the range of 15-20% and >25%, respectively. For milk they were in the range of 20-30%. Recommendations for repeatability standard deviations of ELISA and PCR methods are usually ≤ 25% [16, 17, 20, 21].

In case the criterion for sufficient homogeneity of the test items is not fulfilled the impact on the target standard deviation will be verified. The evaluation of ELISA results from participants for hazelnut and milk protein was therefore done by an extended target standard deviation considering the standard uncertainty of the assigned value by z'-scores (s. 3.6 and 3.8) [3].

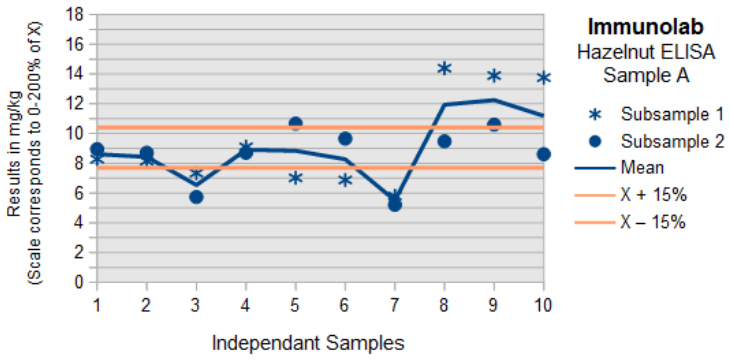
ELISA-Tests: Homogenität Haselnuss / Homogeneity Hazelnut

Immunolab Hazelnut ELISA

Sample weights: 1,0 g (0,9 – 1,1 g)
Number of replicates: 2
Overall result: Hazelnut 9,05 ± 1,39 mg/kg

Sample A	Subsample 1 mg/kg	Subsample 2 mg/kg	Mean mg/kg
1	8,28	8,95	8,62
2	8,15	8,71	8,43
3	7,34	5,73	6,54
4	9,11	8,70	8,91
5	7,02	10,7	8,84
6	6,88	9,66	8,27
7	5,80	5,21	5,51
8	14,4	9,48	11,9
9	13,9	10,6	12,2
10	13,8	8,61	11,2

General average X	9,05	
SD of sample means Sx	2,19	24,2%
SD w ithin-samples Sw	2,08	23,0%
SD betw een-samples Ss	1,39	15,3%

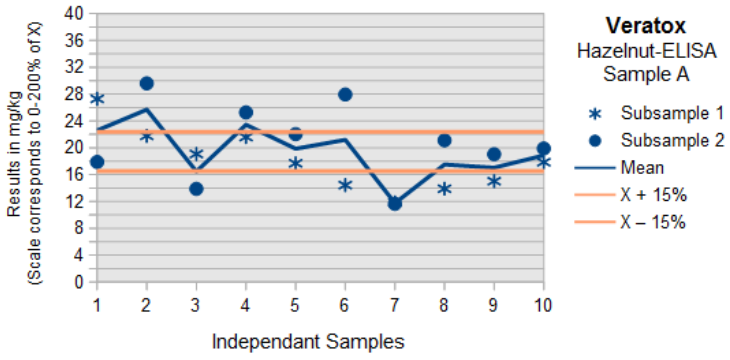


Neogen Veratox ELISA Hazelnut

Sample weights: 5,0 g (4,5 – 5,5 g)
Number of replicates: 2
Overall result: Hazelnut 19,4 ± 3,88 mg/kg

Sample A	Subsample 1 mg/kg	Subsample 2 mg/kg	Mean mg/kg
1	27,3	17,9	22,6
2	21,8	29,6	25,7
3	19,1	13,9	16,5
4	21,6	25,3	23,4
5	17,7	22,0	19,9
6	14,4	27,9	21,2
7	11,9	11,6	11,8
8	13,9	21,1	17,5
9	15,0	19,0	17,0
10	17,9	19,9	18,9

General average X	19,4	
SD of sample means Sx	4,03	20,7%
SD w ithin-samples Sw	4,82	24,8%
SD betw een-samples Ss	3,88	20,0%

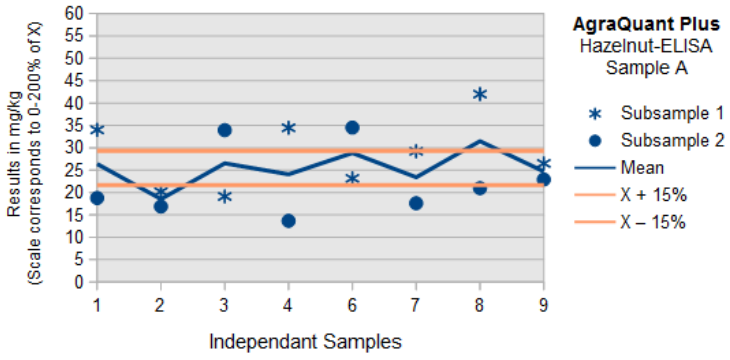


Romerlabs AgraQuant Plus Hazelnut

Sample weights: 1,0 g (0,9 – 1,1 g)
Number of replicates: 2
Overall result: Hazelnut 25,5 ± 9,66 mg/kg

Sample A	Subsample 1 mg/kg	Subsample 2 mg/kg	Mean mg/kg
1	34,0	18,8	26,4
2	20,2	16,9	18,5
3	19,1	33,9	26,5
4	34,5	13,7	24,1
6	23,2	34,5	28,8
7	29,2	17,6	23,4
8	42,0	21,0	31,5
9	26,5	22,9	24,7

General average X	25,5	
SD of sample means Sx	3,86	15,1%
SD w ithin-samples Sw	10,0	39,4%
SD betw een-samples Ss	9,66	37,9%



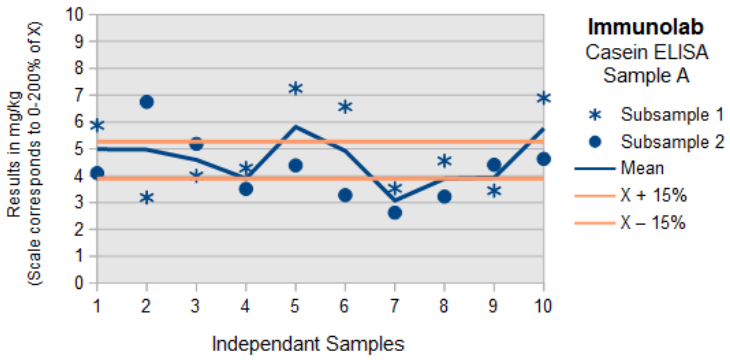
ELISA-Tests: Homogenität Milch / Homogeneity Milk

Immunolab Casein ELISA

Sample weights: 0,50 g (0,45 – 0,55 g)
Number of replicates: 2
Overall result: Casein 4,58 ± 1,38 mg/kg

Sample A	Subsample 1 mg/kg	Subsample 2 mg/kg	Mean mg/kg
1	5,87	4,10	4,99
2	3,19	6,75	4,97
3	3,99	5,19	4,59
4	4,28	3,51	3,90
5	7,25	4,38	5,82
6	6,57	3,28	4,92
7	3,53	2,62	3,08
8	4,55	3,22	3,89
9	3,44	4,41	3,92
10	6,89	4,63	5,76

General average X	4,58	
SD of sample means Sx	0,88	19,2%
SD within-samples Sw	1,51	33,0%
SD between-samples Ss	1,38	30,0%

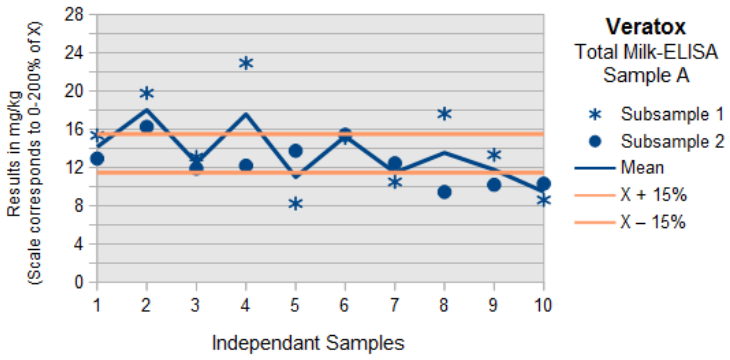


Neogen Veratox ELISA Total Milk

Sample weights: 5,0 g (4,5 – 5,5 g)
Number of replicates: 2
Overall result: Non Fat Dry Milk 13,5 ± 2,92 mg/kg

Sample A	Subsample 1 mg/kg	Subsample 2 mg/kg	Mean mg/kg
1	15,4	12,9	14,1
2	19,8	16,3	18,0
3	13,1	11,9	12,5
4	22,9	12,2	17,6
5	8,24	13,7	11,0
6	15,1	15,4	15,3
7	10,5	12,4	11,5
8	17,6	9,44	13,5
9	13,3	10,2	11,8
10	8,61	10,3	9,46

General average X	13,5	
SD of sample means Sx	2,81	20,9%
SD within-samples Sw	3,53	26,2%
SD between-samples Ss	2,92	21,6%

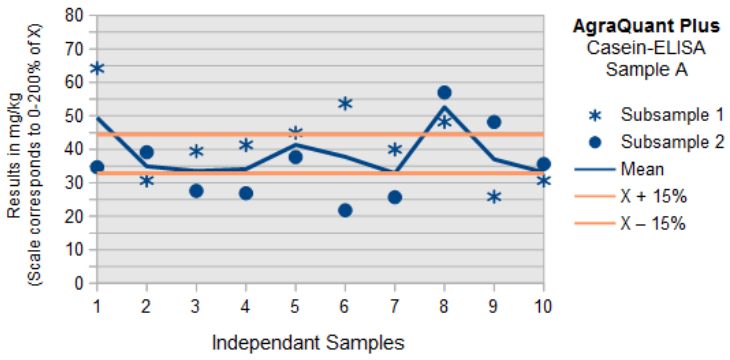


Romerlabs AgraQuant Plus Casein

Sample weights: 1,0 g (0,9 – 1,1 g)
Number of replicates: 2
Overall result: Casein 38,7 ± 11,6 mg/kg

Sample A	Subsample 1 mg/kg	Subsample 2 mg/kg	Mean mg/kg
1	64,2	34,7	49,4
2	30,7	39,1	34,9
3	39,4	27,6	33,5
4	41,3	26,9	34,1
5	45,0	37,7	41,3
6	53,7	21,8	37,7
7	40,0	25,7	32,8
8	48,2	57,0	52,6
9	25,9	48,2	37,0
10	30,7	35,6	33,2

General average X	38,7	
SD of sample means Sx	7,04	18,2%
SD within-samples Sw	12,6	32,5%
SD between-samples Ss	11,6	29,9%



2.2 Sample shipment and information to the test

The portions of test material (sample A and sample B as well as the spiking material sample) were sent to every participating laboratory in the 42nd week of 2016. The testing method was optional. The tests should be finished at December 2nd 2016 the latest.

With the cover letter along with the sample shipment the following information was given to participants:
There are two different test samples Sample A and Sample B. Both are chocolates possibly containing the allergenic foods hazelnut and/or milk in the range of mg/kg. Additionally a "Spiking Material Sample" is provided. It contains 1-10% of the allergenic items in potato flour.
The homogeneity of the material was tested. In general we recommend to homogenize a representative sample amount before analysis according to good laboratory practice, especially in case of low sample weights. Every suitable method for detection or determination of the analytes may be applied (e.g. ELISA, PCR).

2.3 Submission of results

The participants submitted their results in standard forms, which have been sent by email or were available on our website. On one hand the results given as positive/negative and on the other hand the indicated results of the allergenic ingredients e.g. total food item or protein in mg/kg were evaluated.
During evaluation DLA eventually requests detailed information by email on the type of indicated quantitative results from participants concerned.
Queried and documented were the indicated results and details of the test methods like specifity, test kit manufacturer and hints about the procedure.
In case participants submitted several results for the same parameter obtained by different methods these results were evaluated with the same evaluation number with a letter as a suffix and indication of the related method.
All 17 participants submitted their results in time.

3. Evaluation

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

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

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

3.1 Consensus value from participants (assigned value)

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

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

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

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

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

3.2 Robust standard deviation

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

The following robust standard deviations were considered:

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

3.3 Exclusion of results and outliers

Before statistical evaluation obvious blunders, such as those with incorrect units, decimal point errors, and results for a another proficiency test item can be removed from the data set [2]. Even if a result clearly deviates from the robust mean (e.g. factor >10) and has an influence on the robust statistics, a result can be excluded from statistical evaluation [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 identified as outliers by the use of robust statistics. If a value deviates from the robust mean by more than 3 times the robust standard deviation, it is classified as an outlier [3]. Detected outliers are stated for information only, when z-score are < -2 or > 2. Due to the use of robust statistics outliers are not excluded, provided that no other reasons are present [3].

3.4 Target standard deviation (for proficiency assessment)

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

3.4.1 General model (Horwitz)

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

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

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

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

3.4.2 Value by precision experiment

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

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

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

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

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

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

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

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

Table 3b: PCR-Methods - Relative repeatability standard deviations (RSD_r) and relative reproducibility standard deviations (RSD_R) from precision experiments and resulting target standard deviations σ_{pt} [29-30]

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 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 ASU 18.00-22

3.4.3 Value by perception

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

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

Table 4: ELISA-Validation

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

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

Table 5: PCR-Validation

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

(a) = Trueness / Richtigkeit

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

3.5 z-Score

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

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

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

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

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

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

3.5.1 Warning and action signals

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

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

3.6 z'-Score

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

The calculation is performed by:

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

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

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

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

For warning and action signals see 3.6.1.

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 ($U_{(Xpt)}$) 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 Quotient $U(x_{pt})/\sigma_{pt}$ is reported in the characteristics of the test.

3.9 Figures

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

3.10 Recovery rates: Spiking

For the results of the spiking material sample and the spiked sample recovery rates were calculated with respect to the known content of added allergens. The related values of added allergens are given in 2.1 test material in table 2. As a range of acceptance RA for valuating participant's results the range of 50 - 150% for the recovery rates of allergen-ELISAs proposed by the AOAC was used [21]. For quantitative PCR determinations we use the same range of acceptance.

4. Results

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

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

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

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

ELISA results given as **hazelnut protein** were converted by DLA to hazelnut. The conversion based on 10% protein in hazelnuts as indicated by the referring test kit provider (ELISA-Systems).

ELISA results given as **skimmed milk powder** were converted by DLA to milk protein. The conversion based on 35% protein in skimmed milk powder as indicated by the referring test kit provider (Veratox, Neogen).

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

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

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

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

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

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

After that the recovery rates of the results for the spiking 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		
10	positive	20,8	negative	< 5	2/2 (100%)	ES	
13	positive	27,0	negative	< 5	2/2 (100%)	ES	result converted °
17	positive	9,10	negative	< 0,3	2/2 (100%)	IL	
1	positive	45,2	negative	<2,5	2/2 (100%)	RS-F	
4	positive	19,1	negative	< 2,5	2/2 (100%)	RS-F	
5	negative	< 2,5	positive	18,6	0/2 (0%)	RS-F	result excluded
8	positive	16,5	negative	<2,5	2/2 (100%)	RS-F	
9	positive	10,5	negative		2/2 (100%)	RS-F	mean calculated by DLA
12	positive	13,0	negative	<2.5	2/2 (100%)	RS-F	
14	positive	35,9	negative	<2,5	2/2 (100%)	RS-F	mean calculated by DLA
15	positive	14,7	negative	< 1,5	2/2 (100%)	RS-F	
16	-		-		-	div	

° calculation p. 19

	Sample A		Sample B	
Number positive	10		1	
Number negative	1		10	
Percent positive	91		9	
Percent negative	9		91	
Consensus value	positive		negative	

Methods:

ES = ELISA-Systems

IL = Immunolab

RS-F= Ridascreen® Fast, R-Biopharm

div = not indicated / other method

Comments:

The consensus values are in agreement with the spiking of sample A. The single negative and positive results (both evaluation no. 5) could be due to a confusion of sample A and sample B.

Quantitative valuation of results: Sample A

Evaluation number	Hazelnut	z'-Score Xpt_{ALL}	z'-Score Xpt_{RS-F}	Method	Remarks
	[mg/kg]				
10	20,8	0,1		ES	
13	27,0	1,0		ES	result converted °
17	9,10	-1,7		IL	
1	45,2	3,7	2,8	RS-F	
4	19,1	-0,2	-0,3	RS-F	
5	< 2,5			RS-F	result excluded
8	16,5	-0,6	-0,6	RS-F	
9	10,5	-1,5	-1,3	RS-F	mean calculated by DLA
12	13,0	-1,1	-1,0	RS-F	
14	35,9	2,3	1,7	RS-F	mean calculated by DLA
15	14,7	-0,8	-0,8	RS-F	
16				div	

° calculation p. 19

Methods:

ES = ELISA-Systems

IL = Immunolab

RS-F= Ridascreen® Fast, R-Biopharm

div = not indicated / other method

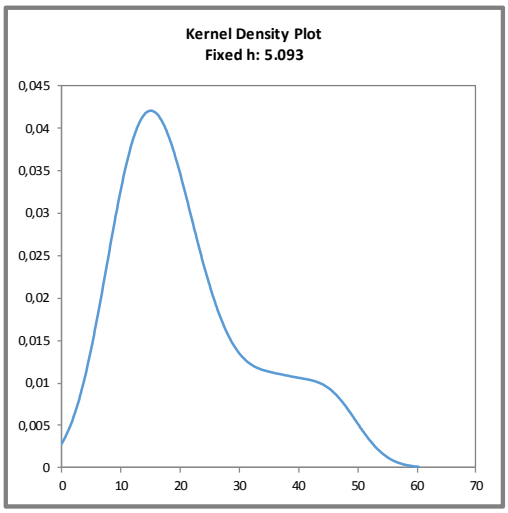


Abb. / Fig. 1:
Kerndichte-Schätzung aller ELISA-Ergebnisse (mit h = 0,75 x σpt von Xpt_{ALL})

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 of results with a slight shoulder at > 35 mg/kg (method RS-F).

Characteristics: Quantitative evaluation Hazelnut

Sample A

Statistic Data	**All Results** *[mg/kg]*	**Method RS** *[mg/kg]*
Assigned value (X_{pt})	$X_{pt_{ALL}}$	$X_{pt_{METHOD\ RS\text{-}F}}$
Number of results	10	7
Number of outliers	0	0
Mean	21,2	22,1
Median	17,8	16,5
Robust Mean (X)	**20,4**	**21,6**
Robust standard deviation (S*)	**11,4**	**13,8**
Target range:		
Target standard deviation $\sigma_{pt'}$	**6,79**	**8,48**
lower limit of target range	**6,81**	**4,68**
upper limit of target range	**34,0**	**38,6**
Quotient S/$\sigma_{pt'}$*	*1,70*	*1,60*
Standard uncertainty $U_{(Xpt)}$	*4,49*	*6,53*
Quotient $U_{(Xpt)}/\sigma_{pt'}$	*0,66*	*0,80*
Results in the target range	*8*	*6*
Percent in the target range	*80*	*86*

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

Comments to the statistical characteristics and assigned values:

The evaluation of all methods and the evaluation of results from method RS-F showed a slightly increased variability of results, respectively. The quotients S/σ_{pt} were 2,2 and 2,6. Therefore evaluation was done by z'-score considering the standard uncertainty. The quotients S*/$\sigma_{pt'}$ were below 2,0 then.*
The robust standard deviations are 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 across the methods, because there were only a few results for some methods.

The robust means of the evaluation of all results and method RS-F were 85% and 90% of the spiking level of hazelnut to sample A and within the recommendations for the applied methods (s. 3.4.3 and "Recovery rates of Hazelnut" p.26).

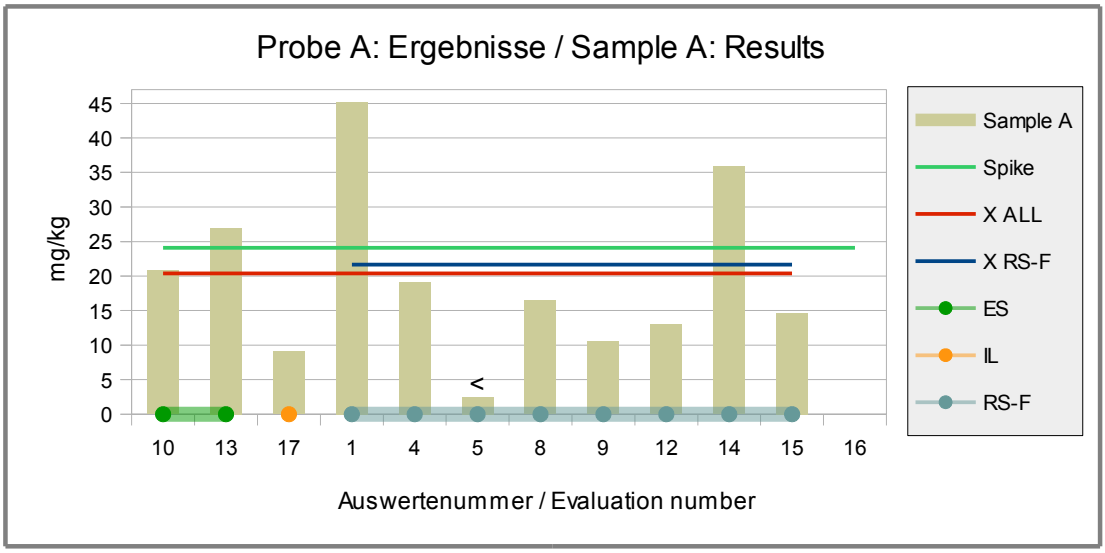


Abb./Fig. 2: ELISA Results Hazelnut
green line = Spiking level
red line = Assigned value robust mean all results
blue line = Assigned value robust mean results method RS-F
round symbols = Applied methods (see legend)

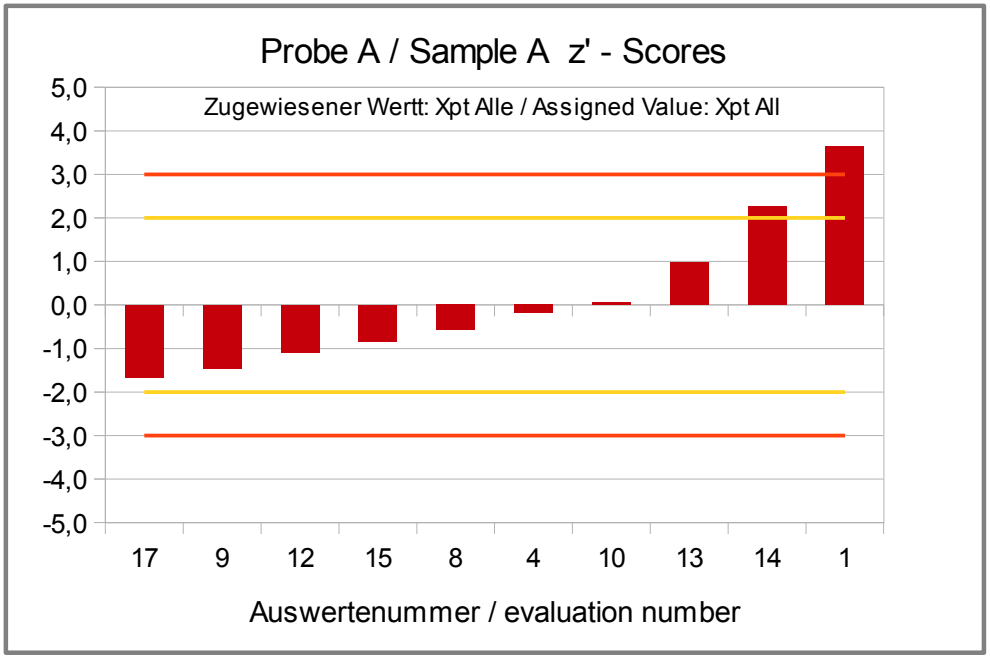


Abb./Fig. 3:
z'-Scores (ELISA Results Hazelnut) Assigned value robust mean of all results

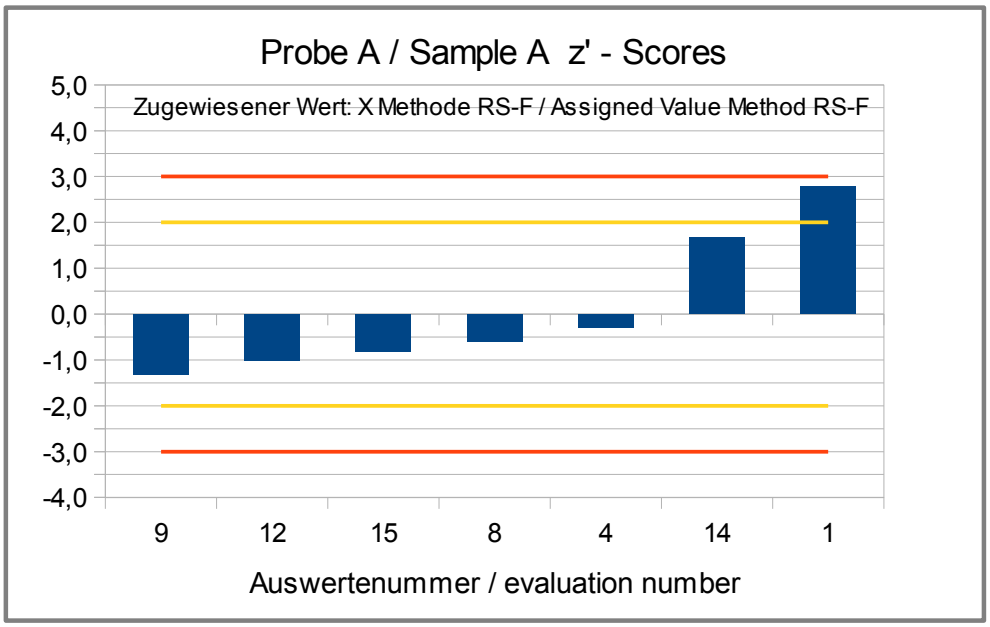


Abb./Fig. 4:
z'-Scores (ELISA Results Hazelnut)
Assigned value robust mean of method RS-F (R-Biopharm, Ridascreen FAST)

Recovery Rates for Hazelnut:
Spiking Material Sample and Sample A

Evaluation number	Spiking material	Recovery rate*	Sample A	Recovery rate*	Method	Remarks
	[mg/kg]	[%]	[mg/kg]	[%]		
10	11200	**95**	20,8	**86**	ES	
13	18000	153	27,0	**112**	ES	result converted °
17	8000	**68**	9,10	38	IL	
1	11700	**100**	45,2	188	RS-F	
4	12300	**105**	19,1	**79**	RS-F	
5	1420	12	< 2,5		RS-F	result excluded
8	10000	**85**	16,5	**68**	RS-F	
9	8630	**73**	10,5	44	RS-F	mean calculated by DLA
12	9800	**83**	13,0	**54**	RS-F	
14	11190	**95**	35,9	**149**	RS-F	mean calculated by DLA
15	12500	**106**	14,7	**61**	RS-F	
16	negative				div	

° calculation p. 19

RA**	50-150 %	RA**	50-150 %
Number in RA	9	Number in RA	7
Percent in RA	82	Percent in RA	70

Methods:
ES = ELISA-Systems
IL = Immunolab
RS-F= Ridascreen® Fast, R-Biopharm
div = not indicated / other method

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

** Range of acceptance of AOAC for allergen ELISAS

Comments:
For the spiking material sample 82% (9) of the participants obtained a recovery rate within the range of the AOAC-recommendation of 50-150%. For the food matrix sample A produced with the spiking material sample 70% (7) of the recovery rates were in 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		
10	positive		negative		1/1 (100%)	ASU	
3	negative		negative		1/1 (100%)	MS	traces in sample A
11	positive	34,5	positive	2	0/1 (0%)	SFA-Q	see comments
2	negative		negative		1/1 (100%)	div	
6	negative		negative		1/1 (100%)	div	
16	positive	24000	negative		1/1 (100%)	div	

	Sample A		Sample B	
Number positive	3		1	
Number negative	3		5	
Percent positive	50		17	
Percent negative	50		83	
Consensus value	none		negative	

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

Comments:
The negative consensus value of sample B is in qualitative agreement with the spiking of sample A. For the spiked sample A there were 50% positive and negative results each. Therefore no consensus value of ≥ 75% could be established.
The results of participant no. 11 are in qualitative agreement with the ELISA results for sample A (positive) and are not contrary for sample B (10 of 11 ELISA results < 5 mg/kg).

Quantitative valuation of results: Sample A

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

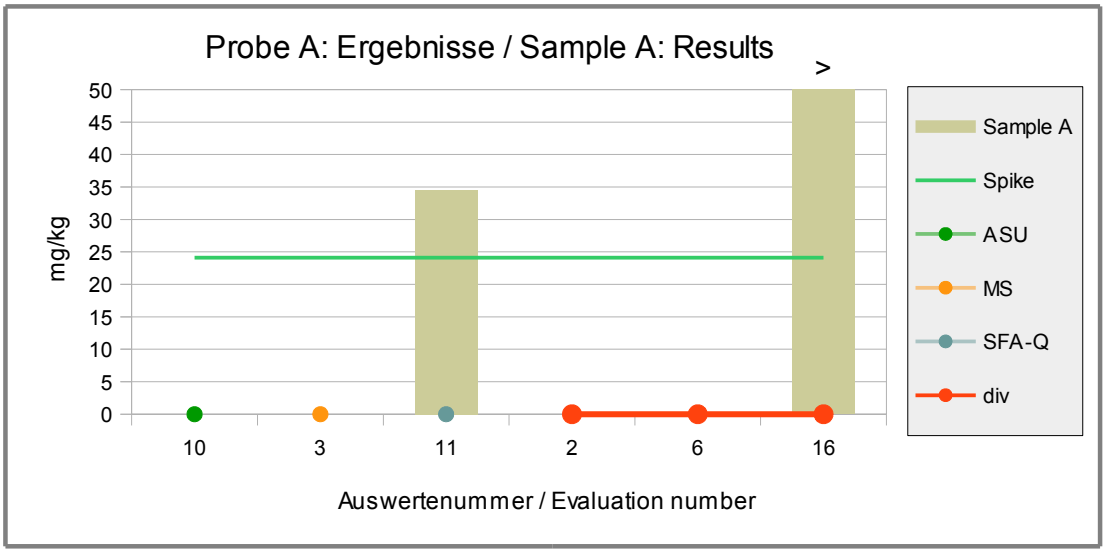


Abb./Fig. 5: PCR Results Hazelnut
green line = Spiking level
round symbols = Applied methods (see legend)

Recovery Rates for Hazelnut:
Spiking Material Sample and Sample A

	Spiking material	Recovery rate*	Sample A	Recovery rate*	Method	Remarks
	[mg/kg]	[%]	[mg/kg]	[%]		
10					ASU	
3					MS	
11	2220	19	34,5	**143**	SFA-Q	
2					div	
6					div	
16			24000	99585	div	confusion of samples?

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

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

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

** Range of acceptance of AOAC for allergen ELISAS

Comments:
The recovery rate of the submitted PCR-result was below the range of the AOAC-recommendation of 50-150% for the spiking material sample, while the participant obtained a recovery rate within the range of the AOAC-recommendation for the food matrix sample A produced with the spiking material sample.
The result of participant no. 16 is not plausible, maybe the samples were mixed up.

4.2 Proficiency Test Milk

4.2.1 ELISA Results: Milkprotein

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	6,50	negative	<2,5	2/2 (100%)	RS-F	
3	positive	15,6	negative		2/2 (100%)	RS-F	
4	positive	16,0	negative	< 2,5	2/2 (100%)	RS-F	
5	positive	9,20	negative	< 2,5	2/2 (100%)	RS-F	
7	positive	8,66	negative	<2,5	2/2 (100%)	RS-F	
12	positive	6,65	negative	<2.5	2/2 (100%)	RS-F	
14	positive	17,0	negative	<2,5	2/2 (100%)	RS-F	mean calculated by DLA
15	positive	22,7	negative	<0,70	2/2 (100%)	RS-F	
1	positive	2,29	negative	<0,88	2/2 (100%)	VT	result converted °
6	positive	13,4	negative	0,62	2/2 (100%)	VT	result converted °
8	positive		negative		2/2 (100%)	VT	
10	positive	5,09	negative	<1	2/2 (100%)	VT	
11	positive	1,93	negative	<0,35	2/2 (100%)	VT	result converted °

° calculation p. 19

	Sample A		Sample B	
Number positive	13		0	
Number negative	0		13	
Percent positive	100		0	
Percent negative	0		100	
Consensus value	positive		negative	

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

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

Quantitative valuation of results: Sample A

Evaluation number	Milk -protein	z'-Score Xpt_{ALL}	z'-Score Xpt_{RS}	Method	Remarks
	[mg/kg]				
2	6,50	-1,0	-1,4	RS-F	
3	15,6	1,5	0,6	RS-F	
4	16,0	1,6	0,7	RS-F	
5	9,20	-0,3	-0,8	RS-F	
7	8,66	-0,4	-0,9	RS-F	
12	6,65	-1,0	-1,4	RS-F	
14	17,0	1,9	1,0	RS-F	mean calculated by DLA
15	22,7	3,5	2,3	RS-F	
1	2,29	-2,2		VT	result converted °
6	13,4	0,9		VT	result converted °
8				VT	
10	5,09	-1,4		VT	
11	1,93	-2,3		VT	result converted °

° calculation p. 19

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

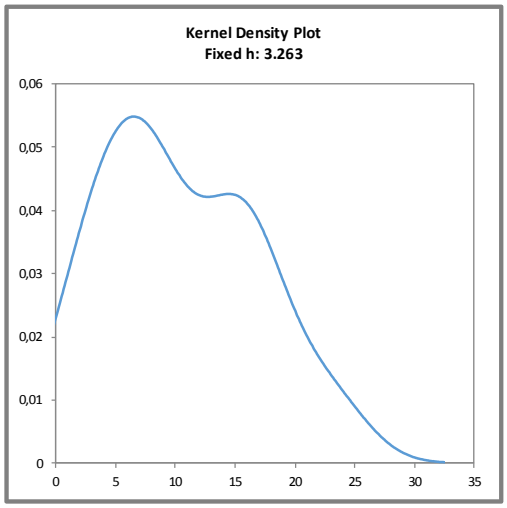


Abb. / Fig. 6:
Kerndichte-Schätzung aller ELISA-Ergebnisse (mit h = 0,75 x σpt von Xpt_{ALL})

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

Comments:
The kernel density estimation shows a broad distribution of results with a smaller second peak at approximately 15 - 20 mg/kg. However, the information provided by the participants on the methods gave no obvious indications of such an array of results.

Characteristics: Quantitative evaluation Milkprotein

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	12	8
Number of outliers	0	0
Mean	10,4	12,8
Median	8,93	12,4
Robust Mean (X)	**10,3**	**12,8**
Robust standard deviation (S*)	**6,98**	**6,66**
Target range:		
Target standard deviation σ_{pt}	**3,59**	**4,35**
lower limit of target range	**3,07**	**4,10**
upper limit of target range	**17,4**	**21,5**
Quotient S/σ_{pt}*	*1,90*	*1,50*
Standard uncertainty $U_{(Xpt)}$	*2,52*	*2,94*
Quotient $U_{(Xpt)}/\sigma_{pt}$	*0,70*	*0,68*
Results in the target range	*9*	*7*
Percent in the target range	*75*	*88*

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

Comments to the statistical characteristics and assigned values:

The evaluation of all methods and the evaluation of results from method RS-F showed a slightly increased variability of results, respectively. The quotients S/σ_{pt} were 2,7 and 2,1. Therefore evaluation was done by z'-score considering the standard uncertainty. The quotients S*/σ_{pt}' were below 2,0 then.*
The robust standard deviations are 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 across the methods, because there were only a few results for method VT.

The robust means of the evaluation of all results and method RS-F were 70% and 86% of the spiking level of milkprotein to sample A and within the recommendations for the applied methods (s. 3.4.3 and "Recovery rates of Milkprotein" p.36).

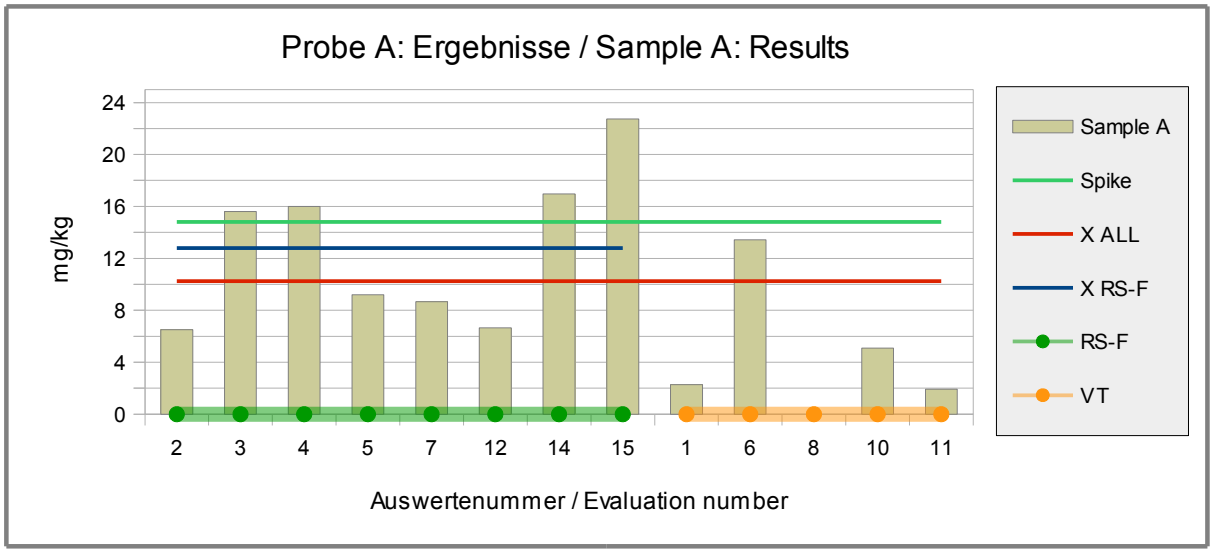


Abb./Fig. 7: ELISA Results Milkprotein
green line = Spiking level
red line = Assigned value robust mean all results
blue line = Assigned value robust mean results method RS-F
round symbols = Applied methods (see legend)

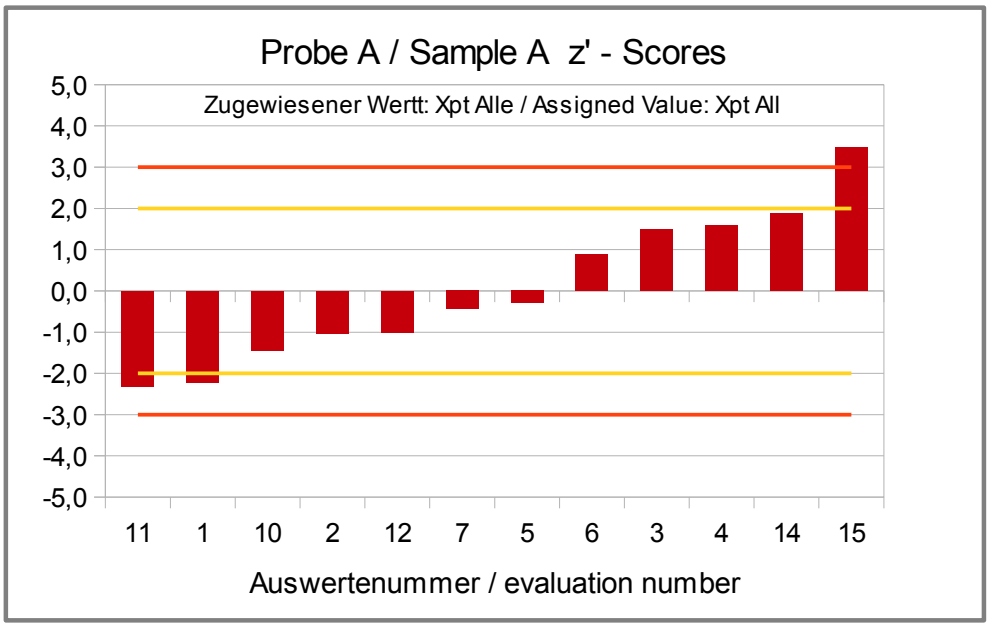


Abb./Fig. 8:
z'-Scores (ELISA Results Milkprotein) Assigned value robust mean of all results

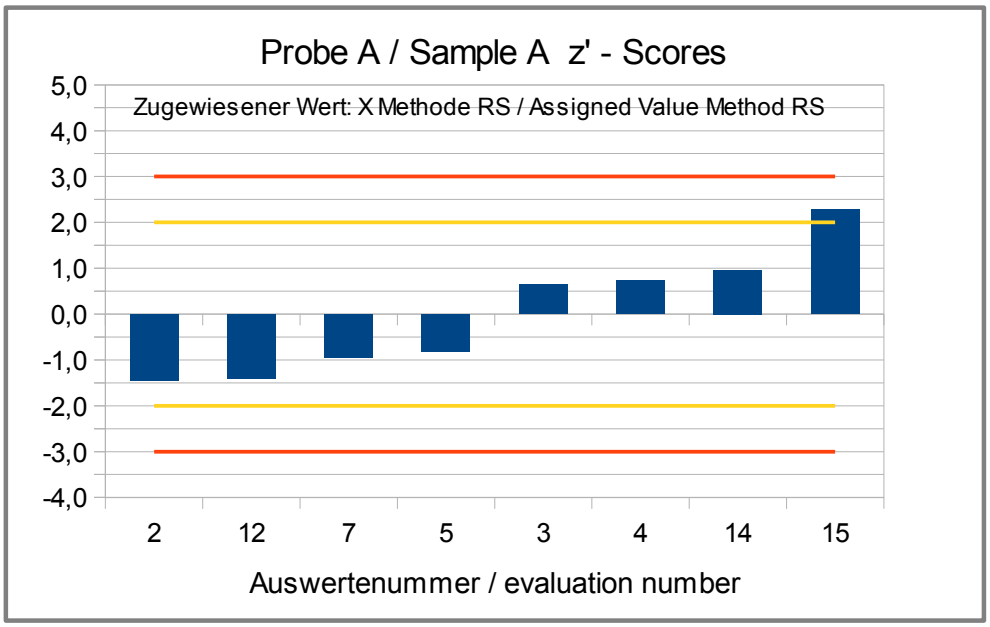


Abb./Fig. 9:
z'-Scores (ELISA Results Milkprotein)
Assigned value robust mean of method RS-F (R-Biopharm, Ridascreen FAST)

Recovery Rates for Milkprotein:
Spiking Material Sample and Sample A

Evaluation number	Spiking material	Recovery rate*	Sample A	Recovery rate*	Method	Remarks
	[mg/kg]	[%]	[mg/kg]	[%]		
2	>67,5		6,50	44	RS-F	
3	5150	**71**	15,6	**105**	RS-F	
4	6100	**84**	16,0	**108**	RS-F	
5	6260	**87**	9,20	**62**	RS-F	
7	3030	42	8,66	**59**	RS-F	
12	3970	**55**	6,65	45	RS-F	
14	3840	**53**	17,0	**115**	RS-F	mean calculated by DLA
15	4270	**59**	22,7	154	RS-F	
1	>8,75		2,29	15	VT	result converted °
6	218	3	13,4	**91**	VT	result converted °
8					VT	
10	-		5,09	34	VT	
11	>5250		1,93	13	VT	result converted °

° calculation p. 19

RA**	50-150 %	RA**	50-150 %
Number in RA	**6**	Number in RA	**6**
Percent in RA	**75**	Percent in RA	**50**

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

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

** Range of acceptance of AOAC for allergen ELISAS

Comments:
For the spiking material sample 75% (6) of the participants obtained a recovery rate within the range of the AOAC-recommendation of 50-150%. For the food matrix sample A produced with the spiking material sample 50% (6) of the recovery rates were in the range of acceptance.

4.2.2 ELISA Results: Casein

Qualitative valuation of results: Samples A and B

Evaluation number	Sample A	Sample A	Sample B	Sample B	Qualitative Valuation	Method	Remarks
	pos/neg	[mg/kg]	pos/neg	[mg/kg]	Agreement with consensus value		
13	positive	4,40	negative	<0,2	2/2 (100%)	AQ	
4a	positive	2,69	negative	< 0,2	2/2 (100%)	IL	
17	positive	6,00	negative	< 0,04	2/2 (100%)	IL	
4b	positive	6,06	negative	< 0,5	2/2 (100%)	RS-F	4b and 4c different extractions
4c	positive	15,4	negative	< 2,5	2/2 (100%)	RS-F	result excluded
9	positive	5,90	negative		2/2 (100%)	RS-F	mean calculated by DLA

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

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

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

Quantitative valuation of results: Sample A

Evaluation number	Casein	z-Score Xpt_{ALL}	Method	Remarks
	[mg/kg]			
13	4,40	-0,5	AQ	
4a	2,69	-1,9	IL	
17	6,00	0,7	IL	
4b	6,06	0,8	RS-F	4b and 4c different extractions
4c	15,4	8,1	RS-F	result excluded
9	5,90	0,6	RS-F	mean calculated by DLA

Methoden:

AQ = AgraQuant, RomerLabs

IL = Immunolab

RS-F= Ridascreen® Fast, R-Biopharm

Comments:

A kernel density estimation was not done due to < 8 results.

Characteristics: Quantitative evaluation Casein

Sample A

Statistic Data	**All Results** *[mg/kg]*
Assigned value (X_{pt})	$X_{pt_{ALL}}$
Number of results	5
Number of outliers	0
Mean	5,01
Median	5,90
Robust Mean (X)	**5,08**
Robust standard deviation (S*)	**1,51**
Target range:	
Target standard deviation σ_{pt}	**1,27**
lower limit of target range	**2,54**
upper limit of target range	**7,62**
Quotient S/σ_{pt}*	*1,20*
Standard uncertainty $U_{(Xpt)}$	*0,85*
Quotient $U_{(Xpt)}/\sigma_{pt}$	*0,67*
Results in the target range	*5*
Percent in the target range	*100*

Comments to the statistical characteristics and assigned values:

The evaluation of all methods showed a low to normal 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. This conclusion is limited for the evaluation across the methods, because there were only a few results for some methods.*

The robust mean of the evaluation of all results was 43% of the spiking level of casein to sample A and slightly below the recommendations for the applied methods (s. 3.4.3 and "Recovery rates of Casein" p.41).

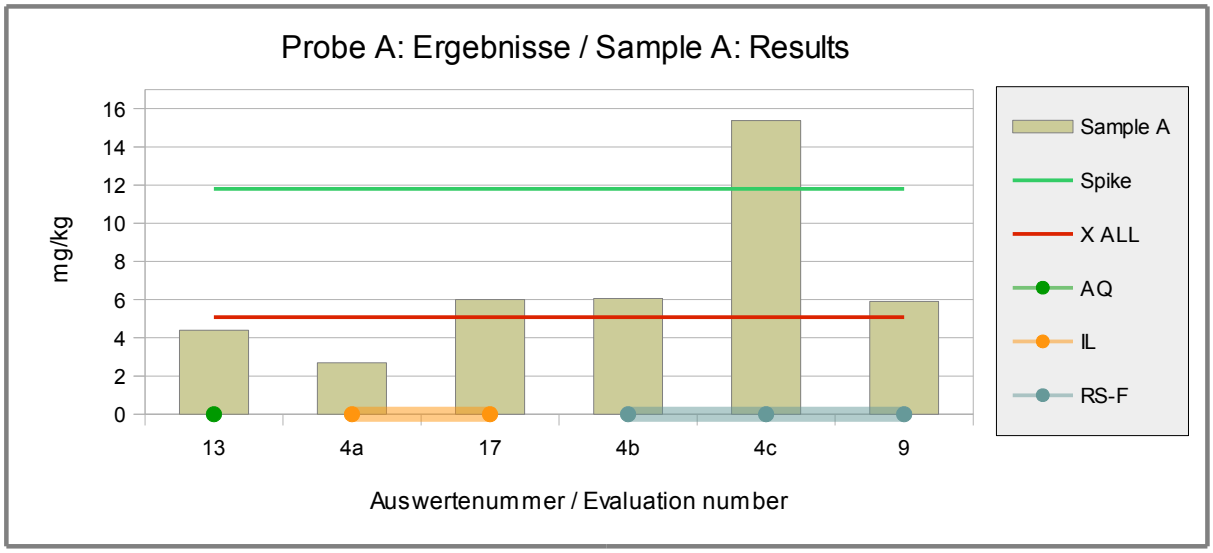


Abb./Fig. 10: ELISA Results Casein
green line = Spiking level
red line = Assigned value robust mean all results
round symbols = Applied methods (see legend)

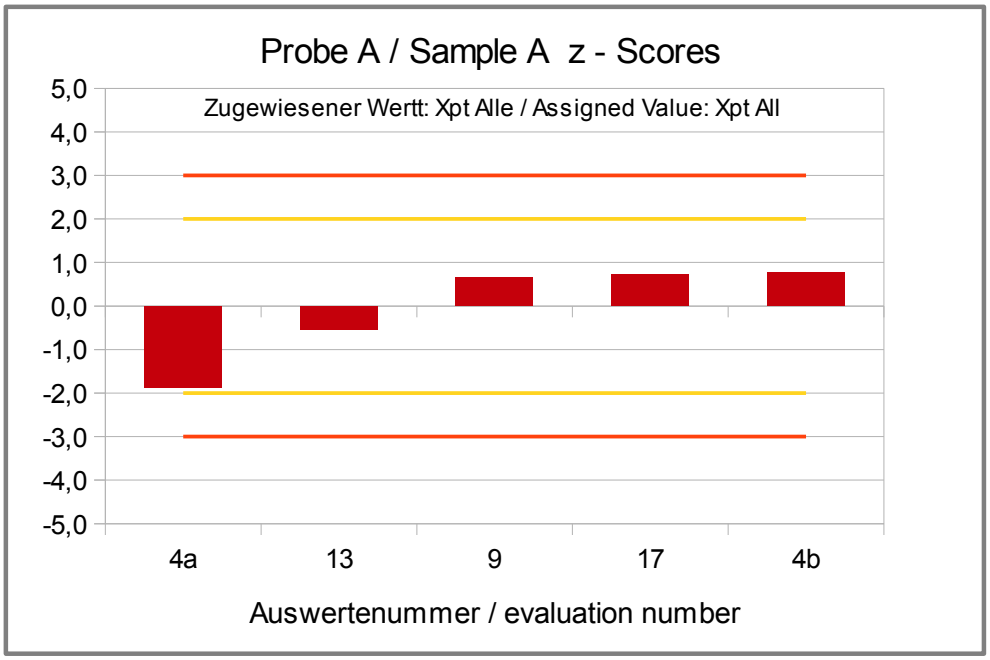


Abb./Fig. 11:
z-Scores (ELISA Results Casein) Assigned value robust mean of all results

Recovery Rates for Casein:
Spiking Material Sample and Sample A

Evaluation number	Spiking material	Recovery rate	Sample A	Recovery rate	Method	Remarks
	[mg/kg]	[%]	[mg/kg]	[%]		
13	5700	**99**	4,40	37	AQ	
4a	13200	228	2,69	23	IL	
17	5000	**87**	6,00	**51**	IL	
4b	53800	931	6,06	**51**	RS-F	4b and 4c different extractionsn
4c	7890	**137**	15,4	**130**	RS-F	
9	4490	**78**	5,90	**50**	RS-F	mean calculated by DLA

RA**	50-150 %	RA**	50-150 %
Number in RA	4	Number in RA	4
Percent in RA	67	Percent in RA	67

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

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

** Range of acceptance of AOAC for allergen ELISAS

Comments:
For the spiking material sample 67% (4) of the participants obtained a recovery rate within the range of the AOAC-recommendation of 50-150%. For the food matrix sample A produced with the spiking material sample 67% (4) of the recovery rates were in the range of acceptance.

4.2.3 ELISA Results: β-Lactoglobulin

Qualitative valuation of results: Samples A and B

Evaluation number	Sample A	Sample A	Sample B	Sample B	Qualitative Valuation	Method	Remarks
	pos/neg	[mg/kg]	pos/neg	[mg/kg]	Agreement with consensus value		
13	positive	0,39	negative	<0,1	-	ES	
16	negative		negative		-	div	

	Sample A		Sample B	
Number positive	1		0	
Number negative	1		2	
Percent positive	50		0	
Percent negative	50		100	
Consensus value	-		-	

Methods:
ES = ELISA-Systems
div = not indicated / other method

Comments:
The result of participant no. 13 is in qualitative agreement with the spiking of sample A.

Results of the spiking material sample are given in the documentation.

Quantitative valuation of results: Sample A

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

4.2.4 PCR Results: Milk (bovine DNA)

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		
16	negative		negative		-	div	

	Sample A		Sample B	
Number positive	0		0	
Number negative	1		1	
Percent positive	0		0	
Percent negative	100		100	
Consensus value	-		-	

Methods:
div = not indicated / other method

Comments:
The result sample A is not in qualitative agreement with the spiking of sample A.

Quantitative valuation of results: Sample A

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

Recovery Rates for Milk: Spiking Material Sample and Sample A

Recovery rates could not be calculated, because there were no quantitative results.

5. Documentation

5.1 Details by the participants

Note: Information given in German was 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	10		-	20,80	-	< 5	-	11200	food (hazelnut)	ELISA-Systems, Hazelnut Residue Assay (ESHRD-48)
ES	13		positive	2,7	negative	<0,5	positive	1800	hazelnut protein	ELISA-Systems, Hazelnut Residue Assay (ESHRD-48)
IL	17		positive	9,1	negative	< 0,3	positive	8000	hazelnut	Immunolab Hazelnut ELISA (HAZ-E01)
RS-F	1		positive	45,2	negative	<2,5	positive	11724,8	hazelnut	Ridascreen Fast Hazelnut (R6802), r-Biopharm
RS-F	4		positive	19,14	negative	< 2,5	positive	12303,54	hazelnut	RIDASCREEN Fast Hazelnut (R6802), r-Biopharm
RS-F	5		negative	< 2,5	positive	18,6	positive	1418,6	hazelnut	Ridascreen Fast Hazelnut (R6802), r-Biopharm
RS-F	8		-	16,5	-	<2,5	-	10000	hazelnut	Ridascreen Fast Hazelnut (R6802), r-Biopharm
RS-F	9		-	9	negative		-	8630	hazelnut	Ridascreen Fast Hazelnut (R6802), r-Biopharm
RS-F	9		-	12	negative		-	8640	hazelnut	Ridascreen Fast Hazelnut (R6802), r-Biopharm
RS-F	12	15.11.16	positive	12,96	negative	<2.5	positive	9795,15	hazelnut	Ridascreen Fast Hazelnut (R6802), r-Biopharm
RS-F	14		positive	35,4	negative	<2,5	positive	11190	hazelnut	Ridascreen Fast Hazelnut (R6802), r-Biopharm
RS-F	14		positive	36,3	negative	<2,5	-		hazelnut	Ridascreen Fast Hazelnut (R6802), r-Biopharm
RS-F	15		-	14,66	negative	< 1,50	-	12489	hazelnut	Ridascreen Fast Hazelnut (R6802), r-Biopharm
div	16		-		-		negative			
div	16		-		-		negative			

Meth. Abr.	Evaluation number	Specifity	Remarks to the Method (Extraction and Determination)	Further Remarks
		Antibody	e.g. Extraction Solution / Time / Temperature	
ES	10	anti- hazelnut	As Per Kit Instructions	
ES	13	hazelnutprotein	As Per Kit Instructions	
IL	17		using Immunolab extraction additive for polyphenl-containing samples	
RS-F	1			
RS-F	4		As Per Kit Instructions	
RS-F	5		As Per Kit Instructions	
RS-F	8	hazelnutprotein	As Per Kit Instructions	samples seems to be not homogeneous, because of strongly differing results; spiking sample lumpy; range of concentration of spiking sample not routinely measured, because of high dilutions
RS-F	9			
RS-F	9			
RS-F	12	As per Kit Instructions	As Per Kit Instructions	
RS-F	14	hazelnutprotein		
RS-F	14	hazelnutprotein		
RS-F	15	hazelnut		
div	16			
div	16			

5.1.2 ELISA: Milkprotein

Meth. Abr.	Evaluation number	Date of analysis	Result Sample A		Result Sample B		Result Spiking Sample		quantitative Result gi-ven as	Method
		Day/Month	qualitative	mg/kg	qualitative	mg/kg	qualitative	mg/kg	e.g. food / food protein	Test-Kit + Manufacturer
RS-F	2	27.10.16	positive	6,5	negative	<2,5	positive	>67,5	Milk proteins, total	Ridascreen Fast Milk (R4652), r-Biopharm
RS-F	3		positive	15,6	negative		positive	5147	Milk proteins, total	Ridascreen Fast Milk (R4652), r-Biopharm
RS-F	4		positive	15,99	negative	< 2,5	positive	6100,32	Milkprotein	RIDASCREEN Fast Milk (R4652), r-Biopharm
RS-F	5		positive	9,2	negative	< 2,5	positive	6255,3	Milkprotein	RIDASCREEN FAST Milk (R4652), r-biopharm
RS-F	7		positive	8,66	negative	<2,5	positive	3031,17	Milk proteins, total	Ridascreen Fast Milk (R4652), r-Biopharm
RS-F	12	10.11.16	positive	6,65	negative	<2.5	positive	3973,58	Milk proteins, total	Ridascreen Fast Milk (R4652), r-Biopharm
RS-F	14		positive	16,31	negative	<2,5	positive	3840	Milk proteins, total	Ridascreen Fast Milk (R4652), r-Biopharm
RS-F	14		positive	17,63	negative	<2,5	-		Milk proteins, total	Ridascreen Fast Milk (R4652), r-Biopharm
RS-F	15		-	22,73	negative	< 0,70	-	4269	Milk proteins, total	Ridascreen Fast Milk (R4652), r-Biopharm
VT	1		positive	6,53	negative	<2,5	positive	>25	Skimmed milk powder	Veratox Total Milk Allergen, Neogen
VT	6		positive	38,35	negative	1,77	positive	622,6	Skimmed milk powder	Veratox Total Milk Allergen, Neogen
VT	8		positive		negative		positive			Veratox Total Milk Allergen, Neogen
VT	10		-	5,09	-	< 1	positive	-	Milk proteins, total	Veratox Total Milk Allergen, Neogen
VT	11		positive	5,5	negative	< 1	positive	> 15000	Skimmed milk powder	Veratox Total Milk Allergen, Neogen

continued ELISA Milkprotein:

Meth. Abr.	Evaluation number	Specifity	Remarks to the Method (Extraction and Determination)	Further Remarks
		Antibody	e.g. Extraction Solution / Time / Temperature	
RS-F	2		Kits manufacture	Lot: 13386
RS-F	3		according to kit instruction; Extraction also with alternative protocol for gelling matrices (R-Biopharm)	value of spiking sample from 3 1:100 dilutions calculated; sample A: mean of 3 determinations
RS-F	4		according to kit instruction: with Extractor 2+Extraction buffer+Additive, 10min at 100°C and 10min at 60°C	
RS-F	5		As Per Kit Instructions	
RS-F	7	Milkproteins, total	As Per Kit Instructions 15-07-09	
RS-F	12	As per Kit Instructions	As Per Kit Instructions	
RS-F	14	Milkproteins, total		
RS-F	14	Milkproteins, total		
RS-F	15	Milkproteins, total		
VT	1	Milkproteins, total		
VT	6	Skimmed milk powder		
VT	8	Skimmed milk powder	As Per Kit Instructions	samples seems to be not homogeneous, because of strongly differing results; spiking sample lumpy; range of concentration of spiking sample not routinely measured, because of high dilutions
VT	10	polyclonal (rabbit) catcher antibody	As Per Kit Instructions	
VT	11		-	limit of detection 1 mg/kg

5.1.3 ELISA: Casein

Meth. Abr.	Evaluation number	Date of analysis	Result Sample A		Result Sample B		Result Spiking Sample		quantitative Result given as	Method
		Day/Month	qualitative	mg/kg	qualitative	mg/kg	qualitative	mg/kg	e.g. food / food protein	Test-Kit + Manufacturer
AQ	13		positive	4,4	negative	<0,2	positive	5700	Casein	AgraQuant Casein (COKAL1200), RomerLabs
IL	4a		positive	2,69	negative	< 0,2	positive	13159,59	Casein	Immunolab Casein ELISA
IL	17		positive	6	negative	< 0,04	positive	5000	Casein	Immunolab Casein ELISA
RS-F	4b		positive	6,06	negative	< 0,5	positive	5375,85	Casein	RIDASCREEN Fast Casein (R4652), r-Biopharm
RS-F	4c		positive	15,38	negative	< 2,5	positive	7887,56	Casein	RIDASCREEN Fast Casein (R4652), r-Biopharm
RS-F	9		-	5,4	negative		-	4300	Casein	Ridascreen Fast Casein (R4612), r-Biopharm
RS-F	9		-	6,4	negative		-	4675	Casein	Ridascreen Fast Casein (R4612), r-Biopharm

Meth. Abr.	Evaluation number	Specifity	Remarks to the Method (Extraction and Determination)	Further Remarks
		Antibody	e.g. Extraction Solution / Time / Temperature	
AQ	13	Casein	As Per Kit Instructions	
IL	4a		As Per Kit Instructions	
IL	17		using Immunolab extraction additive for polyphenl-containing samples	
RS-F	4b		according to kit instructions: with extraction buffer (without extractor 2)	
RS-F	4c		according to kit instruction: with Extractor 2+Extraction buffer+Additive, 10min at 100°C and 10min at 60°C	
RS-F	9			
RS-F	9			

5.1.4 ELISA: β-Lactoglobulin

Meth. Abr.	Evaluation number	Date of analysis	Result Sample A		Result Sample B		Result Spiking Sample		quantitative Result gi-ven as	Method
		Day/Month	qualitative	mg/kg	qualitative	mg/kg	qualitative	mg/kg	e.g. food / food protein	Test-Kit + Manufacturer
ES	13	ß Lactoglobulin	positive	0,39	negative	<0,1	positive	260	beta-Lactoglobulin	ELISA-Systems β-Lactoglobulin Residue Detection ELISA
div	16		neg		neg		positive		in house method	

Meth. Abr.	Evaluation number	Specifity	Remarks to the Method (Extraction and Determination)	Further Remarks
		Antibody	e.g. Extraction Solution / Time / Temperature	
ES	13	beta-Lactoglobulin	As Per Kit Instructions	
div	16			

5.1.5 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	10		positive		negative		positive		hazelnut-DNA	ASU §64 L 44.00-8 (PCR hazelnut)
MS	3		negative		negative		positive		hazelnut-DNA	Microsynth
SFA-Q	11		positive	34,5	positive	2	positive	2221	hazelnut	Sure Food Allergen QUANT, Congen / r-Biopharm
div	2	28.11.16	negative		negative		positive		Hazelnut-DNA	Koppel ycol., 2010
div	2	29.11.16	negative		negative		positive		Hazelnut-DNA	Koppel ycol., 2010
div	6		-		-		positive			in house method
div	16		positive	24000	neg		neg		in house method	in house method

Meth. Abr.	Evaluation number	Specifity	Remarks to the Method (Extraction and Determination)	Further Remarks
		Target Sequence / DNA	e.g. Extraction / Enzymes / Clean-Up / Real Time PCR / Gel electrophoresis / Cycles	
ASU	10	152 bp Gen corA 1	Dneasy Rmericon Food Kit/ Proteinase K/ Real Time PCR/ 45 Cycles	
MS	3		Macherey Nagel Nucleo Spin Food optimized: increased sample w eight, buffer change (w ash step w ith Lysis Buffer) RNase-step, Chloroform-step, 2xCQW; RealTime PCR w ith 45 Cycles, Decontamination step w ith UNG; ow n Thermoprofile; Inhibition control	Hazelnut traces in sample A, but far below LOD
SFA-Q	11	-	S3202 SureFood® ALLERGEN QUANT Hazelnut LOD 0,4 mg/kg, LOQ 1 mg/kg Extraction w ith S1053 SureFood® PREP Advanced	-
div	2	Cor	Macherey-Nagel/Real Time PCR/45 cycles	
div	2	Cor	Macherey-Nagel/Real Time PCR/45 cycles	
div	6		Real Time PCR / 45 Cyclen	
div	16		Wizard Real time PCR	

5.1.6 PCR: Milk

Meth. Abr.	Evaluation number	Date of analysis	Result Sample A		Result Sample B		Result Spiking Sample		quantitative Result gi-ven as	Method
		Day/Month	qualitative	mg/kg	qualitative	mg/kg	qualitative	mg/kg	e.g. food / food protein	Test-Kit + Manufacturer
div	16		neg		neg		positiv		in house method	in house method

Meth. Abr.	Evaluation number	Specifity	Remarks to the Method (Extraction and Determination)	Further Remarks
		Target Sequence / DNA	e.g. Extraction / Enzymes / Clean-Up / Real Time PCR / Gel electrophoresis / Cycles	
div	16		Wizard Real time PCR	

5.2 Homogeneity

5.2.1 Mixture homogeneity before bottling

Microtracer Homogeneity Test
Dotierungsmaterialprobe

Weight whole sample	1,21	kg
Microtracer	FSS-rot lake	
Particle size	75 – 300	µm
Weight per particle	2,0	µg
Addition of tracer	35,8	mg/kg

Result of analysis

Sample	Weight [g]	Particle number	Particles [mg/kg]
1	6,41	123	38,4
2	5,33	101	37,9
3	5,99	92	30,7
4	6,16	97	31,5
5	5,96	92	30,9
6	5,55	106	38,2
7	5,77	102	35,4
8	5,80	108	37,2

Poisson distribution		
Number of samples	8	
Degree of freedom	7	
Mean	102,8	Partikel
Standard deviation	10,1	Partikel
χ^2 (CHI-Quadrat)	6,95	
Probability	**43**	%
Recovery rate	98	%

Normal distribution		
Number of samples	8	
Mean	35,0	mg/kg
Standard deviation	3,44	mg/kg
rel. Standard deviaton	9,82	%
Horwitz standard deviation	9,37	%
HorRat-value	**1,0**	
Recovery rate	98	%

6. Index of participant laboratories

Teilnehmer / Participant	Ort / Town	Land / Country
		Germany
		SPAIN
		Germany
		Germany
		Germany
		Germany
		Germany
		Germany
		Germany
		Germany
		Germany
		Germany
		Germany
		SWITZERLAND
		AUSTRIA
		GREAT BRITAIN
		Germany

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

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

7. Index of references

1. DIN EN ISO/IEC 17025:2005; Allgemeine Anforderungen an die Kompetenz von Prüf- und Kalibrierlaboratorien / General requirements for the competence of testing and calibration laboratories
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