Evaluation Report
proficiency test

ptAL01 (2020)

Allergens I:

Egg and Fish

in Instand Soup Powder

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

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Unteraufträge *Subcontractors*	Im Rahmen dieser Eignungsprüfung wurden nachstehende Leistungen im Unterauftrag vergeben: Homogenitätsprüfung der EP-Parameter, Proteinbestimmung As part of the present proficency test the following services were subcontracted: Homogeneity tests of PT-parameter(s), protein determination
Vertraulichkeit *Confidentiality*	Die Teilnehmerergebnisse sind im EP-Bericht in anonymisierter Form mit Auswertenummern benannt. Daten einzelner Teilnehmer werden ausschließlich nach vorheriger Zustimmung des Teilnehmers an Dritte weitergegeben. Participant result are named anonymously with evaluation numbers in the PT report. Data of individual participants will be passed on to third parties only with prior consent of the participant.

Contents

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 of the food matrix samples is a common in commerce instant soup powder with addition of potato flour. The basic composition of both sample A and sample B was the same (see table 1).
After crushing and sieving by means of an impact mill (mesh 1,5 mm) the basic mixture was homogenized.
Afterwards the **spiked sample B** was produced as follows:
The spiking materials containing the allergenic ingredients whole egg powder and fish powder were added to an aliquot of the basic mixture and the mixture was homogenized. Subsequently, the basic mixture was again added in up to 3 additional steps and homogenized in each case until the total quantity had been reached.
The **spiking level sample** was produced with the allergenic compounds above mentioned by multi-stage addition of potato powder (mesh 500 µm) and homogenization.

The samples A and B were portioned to approximately 25 g, the spiking levels sample to approximately to 15 g in metallized PET film bags.

Table 1: Composition of DLA-Samples

Ingredients	**Sample A**	**Sample B**	**Spiking Level Sample**
Vegetable Soup Powder Ingredients: Iodized salt, sugar, flavoring, yeast extract, carrots, rapeseed oil, parsley, onions, leeks, spices, antioxidant extract from rosemary Nutrients per 100 g: Fat o g, Carbohydrates 35 g, Protein 5 g, Salt 55 g	90,8 g/100g	90,8 g/100g	-
Potato Flour Nutrients per 100 g: Protein 0g	9,2 g/100g	9,1 g/100g	-
Potato Powder Ingredients: Potatoes, E471, E304, E223, E100	-	-	99,9 g/100g
Whole Egg Powder: pasteurized, spray dried, mixture (6 products from 2 countries, Europe) - as Whole Egg Powder* - *thereof 47,6% total protein*** - *thereof 26,0% egg white protein****	-	 28,6 mg/kg 13,4 mg/kg 7,43 mg/kg	 28,7 mg/kg 13,5 mg/kg 7,46 mg/kg
Fish Powder: Codfish (Gadus morhua), frozen, freeze-dried, mixture (2 products, Northeast Atlantic) - as Fish Powder* - *thereof 78,4% total protein*** converted to: - *Cod, fresh (wet weight, muscle tissue)****	-	 73,2 mg/kg 57,4 mg/kg 366 mg/kg	 83,2 mg/kg 65,2 mg/kg 416 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=6,25 for egg protein and F=5,6 for fish protein [38, 39])
*** Egg white protein calculated according to literature [36, 37]/ test kit instructions (r-Biopharm) and cod, fresh, with a water content of 80% (Nutrient tables, Souci/Fachmann/Kraut)

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 B and the spiking level sample showed a probability of 31% and 65%. 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 1,3 or 1,1. 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 egg (Immunolab, Veratox and AgraQuant) and fish (Immunolab and AgraQuant) (see page 7). Recommendations for repeatability standard deviations of ELISA and PCR methods are usually ≤ 25% [18, 19, 22, 23].

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

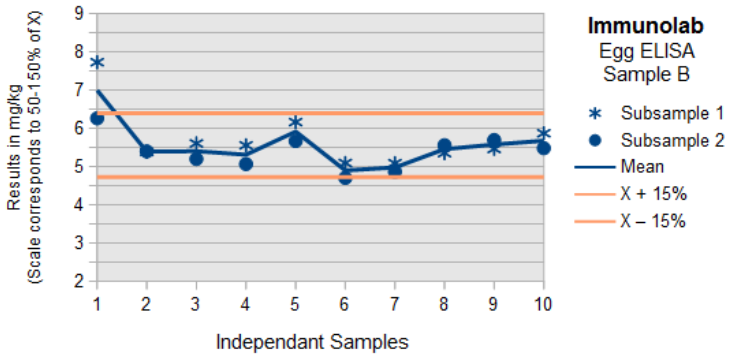
ELISA-Tests: Homogenität Ei / Homogeneity Egg

Immunolab Egg ELISA

Sample weights: 1,0 g (0,9 – 1,1 g)
Number of replicates: 2
Overall result: Egg white proteins 5,56 ± 0,55 mg/kg

Sample B	Subsample 1 mg/kg	Subsample 2 mg/kg	Mean mg/kg
1	7,72	6,26	6,99
2	5,38	5,39	5,39
3	5,60	5,20	5,40
4	5,55	5,06	5,31
5	6,16	5,66	5,91
6	5,09	4,70	4,89
7	5,09	4,86	4,97
8	5,35	5,55	5,45
9	5,46	5,69	5,57
10	5,87	5,48	5,67

General average X	5,56	
SD of sample means Sx	0,59	10,6%
SD within-samples Sw	0,29	5,1%
SD between-samples Ss	0,55	9,9%

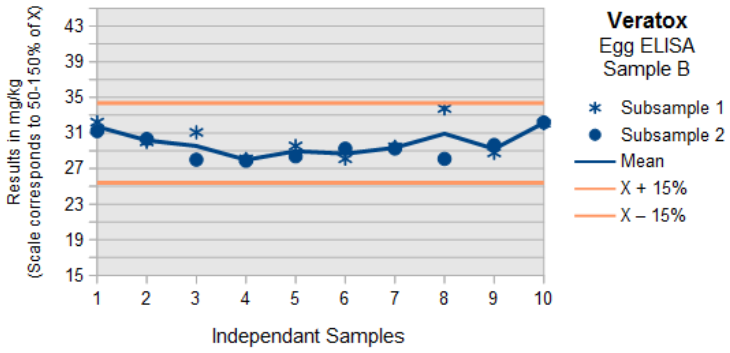


Veratox Egg ELISA

Sample weights: 5,0 g (4,5 – 5,5 g)
Number of replicates: 2
Overall result: Whole egg powder 29,9 ± 1,1 mg/kg

Sample B	Subsample 1 mg/kg	Subsample 2 mg/kg	Mean mg/kg
1	32,2	31,2	31,7
2	30,0	30,3	30,2
3	31,1	28,0	29,5
4	28,1	27,9	28,0
5	29,5	28,4	29,0
6	28,1	29,2	28,7
7	29,4	29,2	29,3
8	33,7	28,1	30,9
9	28,8	29,7	29,2
10	32,1	32,2	32,1

General average X	29,9	
SD of sample means Sx	1,35	4,5%
SD within-samples Sw	1,07	3,6%
SD between-samples Ss	1,11	3,7%

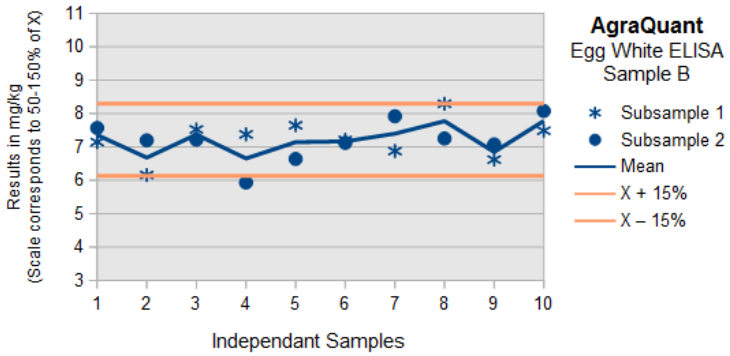


AgraQuant Egg White ELISA

Sample weights: 1,0 g (0,9 – 1,1 g)
Number of replicates: 2
Overall result: Egg white proteins 7,21 ± 0,27 mg/kg

Sample B	Subsample 1 mg/kg	Subsample 2 mg/kg	Mean mg/kg
1	7,14	7,57	7,35
2	6,16	7,20	6,68
3	7,52	7,21	7,37
4	7,37	5,93	6,65
5	7,65	6,64	7,14
6	7,21	7,12	7,16
7	6,87	7,92	7,39
8	8,29	7,25	7,77
9	6,62	7,07	6,85
10	7,48	8,07	7,78

General average X	7,21	
SD of sample means Sx	0,40	5,6%
SD within-samples Sw	0,42	5,9%
SD between-samples Ss	0,27	3,7%



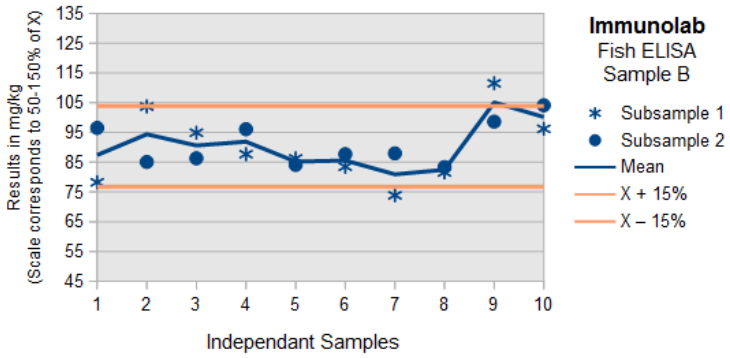
ELISA-Tests: Homogenität Fisch / Homogeneity Fish

Immunolab Fish ELISA

Sample weights: 1,0 g (0,9 – 1,1 g)
Number of replicates: 2
Overall result: Cod, fresh 90,4 ± 6,7 mg/kg

Sample B	Subsample 1 mg/kg	Subsample 2 mg/kg	Mean mg/kg
1	78,2	96,5	87,4
2	103,7	85,1	94,4
3	94,9	86,3	90,6
4	87,7	96,1	91,9
5	86,3	84,1	85,2
6	83,5	87,7	85,6
7	73,9	88,0	80,9
8	81,5	83,3	82,4
9	111,5	98,6	105,1
10	96,2	104,1	100,2

General average X	90,4	
SD of sample means Sx	7,76	8,6%
SD within-samples Sw	5,66	6,3%
SD between-samples Ss	6,65	7,4%

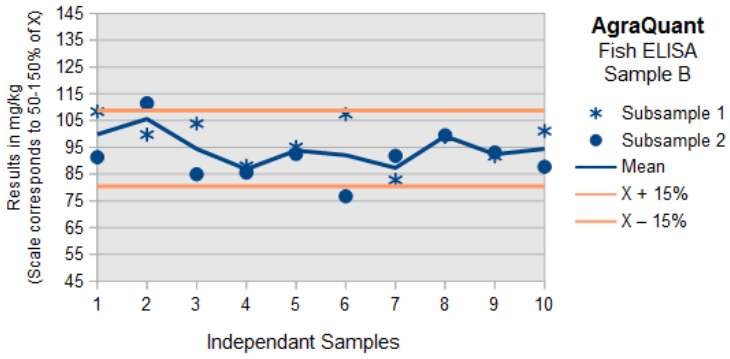


AgraQuant Fish ELISA

Sample weights: 1,0 g (0,9 – 1,1 g)
Number of replicates: 2
Overall result: Cod, fresh 94,6 ± 2,8 mg/kg

Sample B	Subsample 1 mg/kg	Subsample 2 mg/kg	Mean mg/kg
1	108,3	91,3	99,8
2	99,7	111,5	105,6
3	103,9	84,9	94,4
4	88,1	85,5	86,8
5	95,1	92,5	93,8
6	107,4	76,7	92,0
7	82,8	91,8	87,3
8	98,9	99,5	99,2
9	91,7	93,1	92,4
10	101,1	87,7	94,4

General average X	94,6	
SD of sample means Sx	5,73	6,1%
SD within-samples Sw	7,08	7,5%
SD between-samples Ss	2,78	2,9%



2.1.2 Stability

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

The experience with various DLA test materials showed good storage stability with respect to the durability of the sample (spoilage) and the content of the PT parameters for comparable food matrices and water activity (a_W value <0,5).
The a_W value of the spiking level sample was approx. 0,40 (19,0°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 4th week of 2020. The testing method was optional. The tests should be finished at 6th March 2020 the latest.

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

There are two different samples A and B possibly containing the allergenic parameters egg and fish in the range of mg/kg in the matrix of instant soup powder. One of these samples and the "spiking level sample" were prepared adding the allergenic ingredients. The "spiking level sample" contains the allergens in a simple matrix in similar amounts without further processing and should be analysed like a normal sample.

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

2.3 Submission of results

The participants submitted their results in standard forms, which have been handed out with the samples (by email).
On one hand the results given as positive/negative and on the other hand the indicated results of the allergenic ingredients e.g. total food item or protein in mg/kg were evaluated.
Queried and documented were the indicated results and details of the test methods like specificity, limit of quantifications, test kit manufacturer and 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.

12 of 13 participants submitted their results in time. One participant submitted no results.

3. Evaluation

Different ELISA-methods for the determination of allergens in foods are eventually using different antibodies, are usually calibrated with different reference materials and may utilize differing extraction methods. Among others this can induce different results of the content of the analyte [25, 26, 27, 28]. It is for this reason that we contrast the results of the present proficiency test with several assigned values.
Thereby it is possible to evaluate each single result in comparison to the mean of all results and/or in comparison to the mean of results obtained by a single method. For comparison the actually added amount is plotted in the figures of the results.
For quantitative results of the spiking level sample and the spiked sample recovery rates were calculated with respect to the known content of spiked allergens. The recovery rates were given for information only. *No* statistical evaluation was done. The recovery rates should exclusively give an estimation of the matrix- and/or processing influences.

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

3.1 Consensus value from participants (assigned value)

The **robust mean** of the submitted results was used as assigned value (X_{pt}) („consensus value from participants") providing a normal distribution. The calculation was done according to algorithm A as described in annex C of ISO 13528 [3]. If there are < 12 quantitative results and an increased difference between robust mean and median, the **median** may be used as the assigned value (criterion: Δ median - rob. mean > 0,3 σ_{pt}) [3].
The condition is that the majority of the participants' results show a normal distribution or are distributed unimodal and symmetrically. To this end, an examination of the distribution is carried out, inter alia, using the kernel density estimate [3, 12].
In case there are indications for sources of higher variability such as a bimodal distribution of results, a cause analysis is performed. Frequently different analytical methods may cause an anomaly in results' distribution. If this is the case, separate evaluations with own assigned values (X_{pti}) are made whenever possible.

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

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

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

3.2 Robust standard deviation

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

The following robust standard deviations were considered:

i) ***Robust standard deviation of all results*** - S^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, too few significant digits (valid digits) or results for 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	σpt	Method / Literature
Peanut	Milk chocolate	173,7 33,8 5,9	87 % 85 % 59 %	- - -	8,8% 5,2% 7,8%	31% 20% 31%	30,4% 19,7% 30,5%	ELISA Manuf. A ASU 00.00-69
Peanut	Milk chocolate	215,7 40,1 10,1	108 % 100 % 101 %	- - -	5,9% 7,2% 7,3%	32% 14% 16%	31,7% 13,0% 15,1%	ELISA Manuf. B ASU 00.00-69
Peanut	Dark chocolate	148,2 30,9 5,7	74 % 77 % 57 %	- - -	6,0% 13% 6,1%	22% 25% 33%	21,6% 23,2% 32,7%	ELISA Manuf. A ASU 00.00-69
Hazelnut	Dark chocolate	16,3 7,56 3,73 1,62	81 % 76 % 75 % 81 %	- - - -	4,7% 8,9% 13% 15%	12% 15% 24% 33%	11,5% 13,6% 22,2% 31,2%	ELISA Manuf. A ASU 44.00-7
Hazelnut	Dark chocolate	21,3 10,7 4,69 2,37	106 % 107 % 94 % 119 %	- - - -	7,1% 11% 11% 9,3%	14% 19% 17% 17%	13,1% 17,3% 15,1% 16,4%	ELISA Manuf. B ASU 44.00-7

From the precision data of the official German ASU §64 methods the calculated relative target standard deviations are in the range of 12 – 33% for the ELISA methods and 12 – 37% 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} [32-35]

Parameter	Matrix	Mean [mg/kg]	Recovery	rob RSD	RSD_r	RSD_R	σ_{pt}	Method / Literature
Peanut	Rice cookie	23,5 5,29	113 % 100 %	-	11,6% 14,7%	14,4% 18,1%	11,8% 14,8%	rt-PCR ASU 00.00-169
Peanut	Wheat cookie	1,97	39 %		16,0%	19,5%	15,9%	rt-PCR ASU 00.00-169
Peanut	Milk powder	3,66	73 %		12,8%	14,8%	11,7%	rt-PCR ASU 00.00-169
Peanut	Boiled Sausage	2,44	49 %		11,9%	15,9%	13,5%	rt-PCR ASU 00.00-169
Soya	Wheat flour Maize flour	107 145	107 % 145 %	63 % 34 %	- -	31 % 24 %	- -	rt-PCR ASU 16.01-9
Soya flour	Boiled sausage (100°C, 60 min)	114,1 64,4	114 % 161 %	-	14,7% 27,7%	22,2% 41,4%	19,6% 36,5%	rt-PCR ASU 08.00-65
Soya flour	Sausage, autoclaved	33,1	33 %	-	21,5%	30,8	26,8%	rt-PCR ASU 08.00-65
Soya flour	Boiled sausage (100°C, 60 min)	82,0 39,6 19,6 9,3	82 % 99 % 98 % 93 %	-	17,3% 22,9% 22,9% 31,1%	24,1% 31,8% 24,0% 30,2%	20,8% 27,4% 17,7% -	rt-PCR ASU 08.00-59

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 procedure, calibration of equipment and composition of reagents, transmission or calculation errors, trueness and precision and use of reference material. If necessary appropriate corrective measures should be applied [3].

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

3.6 z'-Score

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

The calculation is performed by:

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

If carried out an evaluation of the results by means of z'score, we have defined below the expression in the denominator as a target standard deviation σ_{pt}'.
The requirements for the analytical performance are generally considered as fulfilled if

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

For warning and action signals see 3.5.1.

3.7 Quotient S*/σ_{pt}

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

3.8 Standard uncertainty and traceability

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

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

If $U_{(Xpt)} \leq 0{,}3\ \sigma_{pt}$ the standard uncertainty of the assigned value needs not to be included in the interpretation of the results of the PT [3]. Values exceeding 0,3 imply, that the target standard deviation could be too low with respect to the standard uncertainty of the assigned value.
The traceability of the assigned value is ensured on the basis of the consensus value as a robust mean of the participant results.

3.9 Figures of assigned values

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

3.10 Recovery rates: Spiking

For the results of the spiking level sample and the spiked sample recovery rates were calculated with respect to the known content of added allergens. The related values of added allergens are given in 2.1 test material in table 1. As a range of acceptance RA for valuating participant's results the range of 50 - 150% for the recovery rates of llergen-ELISAs proposed by the AOAC was used [23]. For quantitative PCR or LC/MS determinations we use the same range of acceptance.
The corresponding z-scores were calculated according to 3.5 with the target standard deviation of 25% (see 3.4.3).

4. Results

All following tables are anonymized. With the delivering of the evaluation report the participants are informed about their individual evaluation number.
Evaluation was done separately for ELISA and PCR-techniques. The results were grouped according to the applied methods (e.g. test kits) and sorted chronologically according to the evaluation number of the participants.
The following result sections are structured equally for the allergenic components. First all results of ELISA or PCR methods for a certain parameter are reported for samples A and B (qualitative / possibly quantitative) and afterwards for the spiking level sample (quantitative). The recovery rates of results for the spiking level sample and the spiked sample A 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 **egg white protein** or **egg protein (egg white and yolk proteins)** were converted to **whole egg powder**. When possible the information supplied by the test kit manufacturer was used. A content of 26,0 % egg white protein in whole egg powder was taken. Total egg protein results (Moringa und Moringa Kit II) were converted by DLA to total food item (whole egg powder) using the analysed protein content of the raw materials (see page 5).
All ELISA results for **fish** were reported as **fresh fish**. The fish powder contained in the samples was converted into fresh fish for the evaluation of the results (see page 5).

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

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

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

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

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

Characteristics	**All Results** *[mg/kg]*	**Method i** *[mg/kg]*
Assigned value (X_{pt})	$X_{pt_{ALL}}$	$X_{pt_{METHOD\ i}}$
Number of results		
Number of outliers		
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 $U_{(X_{pt})}$		
Number of results in target range		
Percent in target range		

° Target range calculated using z-score or z'-score

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

4.1 Proficiency Test Egg

4.1.1 ELISA Results: Egg (as whole egg powder)

Qualitative valuation of results: Samples A and B

Evaluation number	Sample A	Sample A	Sample B	Sample B	Qualitative Valuation	Method	Remarks
	pos/neg	[mg/kg]	pos/neg	[mg/kg]	Agreement with consensus value		
8	negative	<1,5	positive	15,2	2/2 (100%)	AQ	Result converted °
11	negative	0	positive	24,4	2/2 (100%)	BF	
10	negative	<0,77	positive	32,7	2/2 (100%)	EF	Result converted °
5	negative	<1,7	positive	35,0	2/2 (100%)	IL	Result converted °
9a	negative	<0,66	positive	33,9	2/2 (100%)	MI	Result converted °
1a	negative		positive	19,5	2/2 (100%)	MI-II	Result converted °
4	negative	<0,66	positive	36,2	2/2 (100%)	MI-II	Result converted °
9b	negative	<0,66	positive	21,5	2/2 (100%)	MI-II	Result converted °
2	negative	<0,25	positive	33,0	2/2 (100%)	RS	
7a	negative	<0,25	positive	24,0	2/2 (100%)	RS	
1b	negative		positive	24,0	2/2 (100%)	RS-F	
6	negative		positive	20,7	2/2 (100%)	RS-F	
7b	negative	<0,5	positive	22,2	2/2 (100%)	RS-F	
12	negative		positive	29,2	2/2 (100%)	RS-F	

° calculation see p. 19

	Sample A		Sample B	
Number positive	0		14	
Number negative	14		0	
Percent positive	0		100	
Percent negative	100		0	
Consensus value	negative		positive	

Methods:

AQ = AgraQuant, RomerLabs
BF = MonoTrace ELISA, BioFront Technologies
EF = SensiSpec ELISA Kit, Eurofins
IL = Immunolab
MI = Morinaga Institute ELISA
MI-II = Morinaga Institute ELISA Kit II
RS = Ridascreen®, R-Biopharm
RS-F= Ridascreen® Fast, R-Biopharm

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

Quantitative valuation of ELISA-results: Sample B

Evaluation number	Whole egg powder	z-Score Xpt_{ALL}	Method	Remarks
	[mg/kg]			
8	15,2	-1,7	AQ	Result converted °
11	24,4	-0,32	BF	
10	32,7	0,93	EF	Result converted °
5	35,0	1,3	IL	Result converted °
9a	33,9	1,1	MI	Result converted °
1a	19,5	-1,1	MI-II	Result converted °
4	36,2	1,5	MI-II	Result converted °
9b	21,5	-0,75	MI-II	Result converted °
2	33,0	0,97	RS	
7a	24,0	-0,38	RS	
1b	24,0	-0,38	RS-F	
6	20,7	-0,88	RS-F	
7b	22,2	-0,65	RS-F	
12	29,2	0,40	RS-F	

° calculation see p. 19

Methods:

AQ = AgraQuant, RomerLabs
BF = MonoTrace ELISA, BioFront Technologies
EF = SensiSpec ELISA Kit, Eurofins
IL = Immunolab
MI = Morinaga Institute ELISA
MI-II = Morinaga Institute ELISA Kit II
RS = Ridascreen®, R-Biopharm
RS-F= Ridascreen® Fast, R-Biopharm

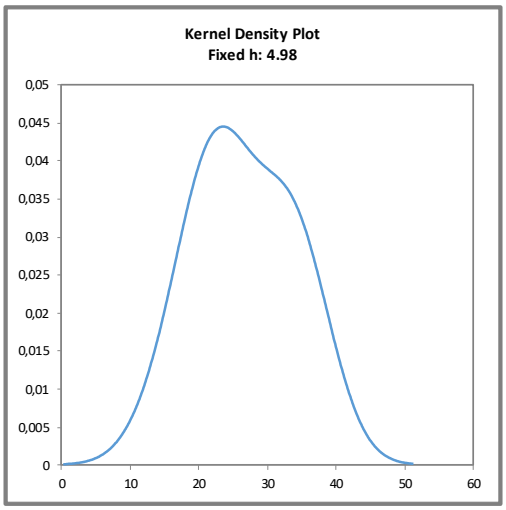


Abb. / Fig. 1:
Kerndichte-Schätzung aller ELISA-Ergebnisse (mit h = 0,75 x σpt von Xpt_{ALL})

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

Comments:
The kernel density estimation shows nearly a symmetric distribution of results with a shoulder at > 30 mg/kg (several methods).

Characteristics: Quantitative evaluation ELISA egg (as whole egg powder)

Sample B

Statistic Data	**All Results** *[mg/kg]*
Assigned value (X_{pt})	$X_{pt_{ALL}}$
Number of results	14
Number of outliers	0
Mean	26,5
Median	24,2
Robust Mean (X_{pt})	**26,5**
Robust standard deviation (S*)	**7,57**
Target range:	
Target standard deviation σ_{pt}	**6,64**
lower limit of target range	**13,3**
upper limit of target range	**39,8**
Quotient S/σ_{pt}*	*1,1*
Standard uncertainty $U_{(Xpt)}$	*2,53*
Results in the target range	*14*
Percent in the target range	*100*

Comments to the statistical characteristics and assigned values:

The kernel density estimation showed no method-dependent differences.

The evaluation of all methods showed a normal variability of results, with a quotient S/σ_{pt} well below 2,0.*
The robust standard deviation is in the range of established values for the repeatability and 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 was 93% of the spiking level of whole egg powder to sample B and was thus in the range of the recommendations for the applied methods (s. 3.4.3 and p.28 "Recovery rates ELISA for egg").

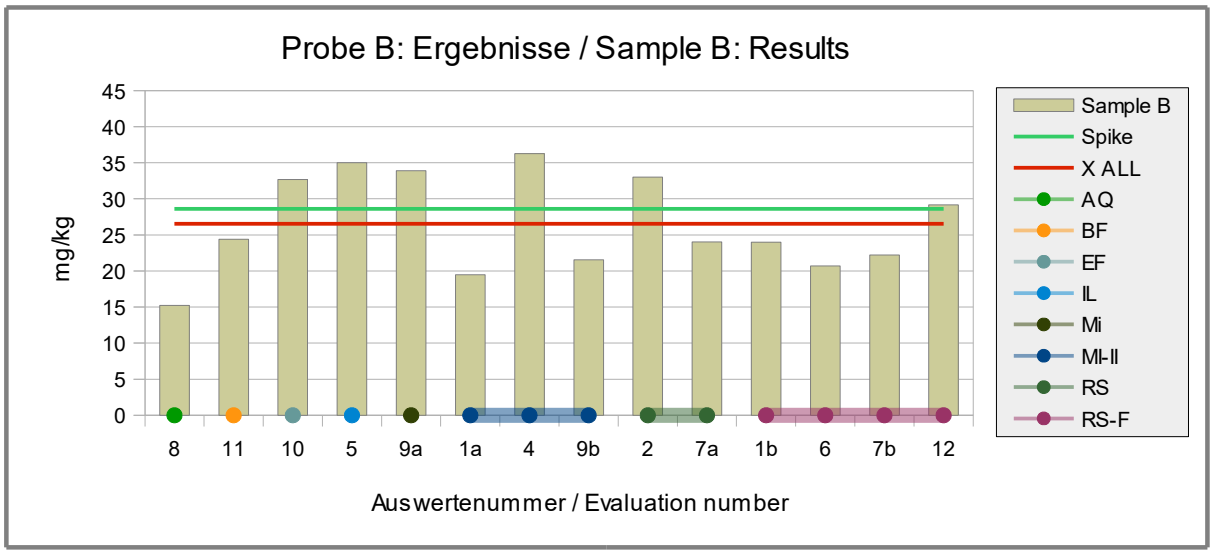


Abb./Fig. 2: ELISA Results egg (as whole egg powder)
green line = Spiking level (Spike)
red line = Assigned value robust mean all results
round symbols = Applied methods (see legend)

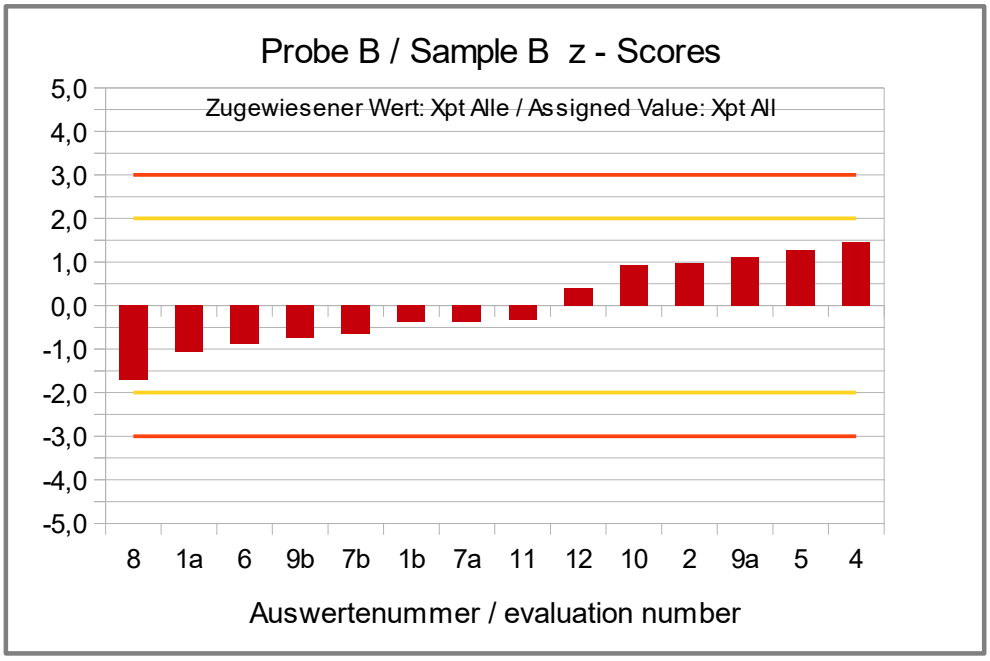


Abb./Fig. 3:
z-Scores ELISA Results egg (as whole egg powder)
Assigned value robust mean of all results

Quantitative valuation of ELISA: Spiking Level Sample

Evaluation number	Whole egg powder	z-Score Xpt_{ALL}	Method	Remarks
	[mg/kg]			
8	30,6	-0,07	AQ	Result converted °
11	27,3	-0,49	BF	
10	34,6	0,45	EF	Result converted °
5	41,2	1,3	IL	Result converted °
9a	34,8	0,47	MI	Result converted °
1a	25,9	-0,67	MI-II	Result converted °
4	34,1	0,38	MI-II	Result converted °
9b	22,0	-1,2	MI-II	Result converted °
2	31,0	-0,02	RS	
7a	29,7	-0,19	RS	
1b	30,5	-0,08	RS-F	
6	27,8	-0,43	RS-F	
7b	28,1	-0,39	RS-F	
12	40,8	1,2	RS-F	

° calculation see p. 19

Methods:

AQ = AgraQuant, RomerLabs
BF = MonoTrace ELISA, BioFront Technologies
EF = SensiSpec ELISA Kit, Eurofins
IL = Immunolab
MI = Morinaga Institute ELISA
MI-II = Morinaga Institute ELISA Kit II
RS = Ridascreen®, R-Biopharm
RS-F= Ridascreen® Fast, R-Biopharm

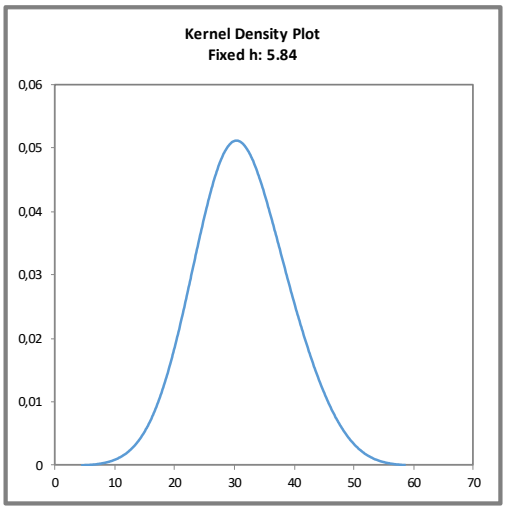


Abb. / Fig. 4:
Kerndichte-Schätzung aller ELISA-Ergebnisse (mit h = 0,75 x σ_{pt} von Xpt_{ALL})

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

Comment:
The kernel density estimation shows a symmetric distribution of results.

Characteristics: Quantitative evaluation ELISA egg (as whole egg powder)

Spiking Level Sample

Statistic Data	**All Results** *[mg/kg]*
Assigned value (X_{pt})	$\boldsymbol{X_{pt_{ALL}}}$
Number of results	14
Number of outliers	0
Mean	31,3
Median	30,5
Robust Mean (X_{pt})	**31,1**
Robust standard deviation (S*)	**5,44**
Target range:	
Target standard deviation σ_{pt}	**7,78**
lower limit of target range	**15,6**
upper limit of target range	**46,7**
Quotient S/σ_{pt}*	*0,70*
Standard uncertainty $U_{(X_{pt})}$	*1,82*
Results in the target range	*14*
Percent in the target range	*100*

Comments to the statistical characteristics and assigned values:

The kernel density estimation showed a symmetrical distribution of results.

The evaluation of all methods showed a low variability of results, with a quotient S/σ_{pt} below 1,0.*
The robust standard deviation is in the range of established values for the repeatability and 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 was 108% of the spiking level of whole egg powder to the spiking level sample and were in the range of the recommendations for the applied methods (s. 3.4.3 and p.28 "Recovery rates ELISA for egg").

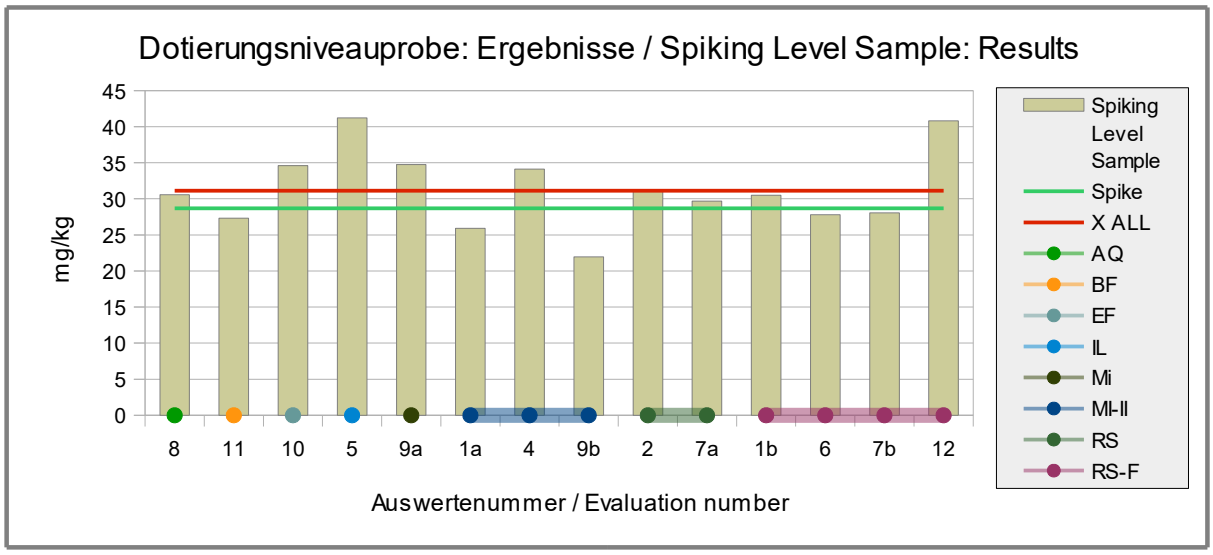


Abb./Fig. 5: ELISA Results egg (as whole egg powder)
green line = Spiking level (Spike)
red line = Assigned value robust mean all results
round symbols = Applied methods (see legend)

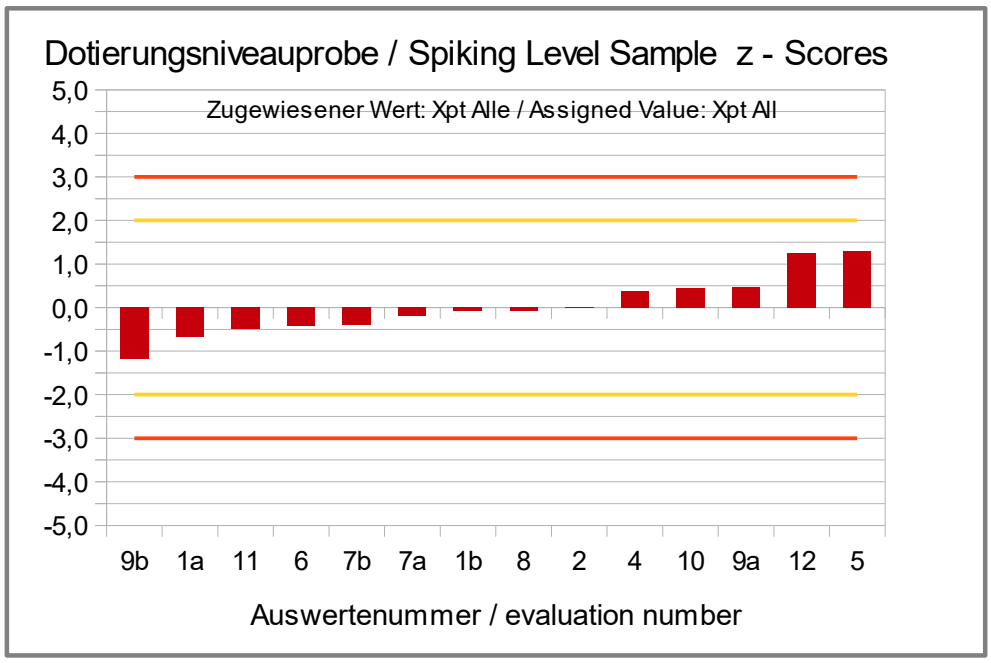


Abb./Fig. 6:
z-Scores ELISA Results egg (as whole egg powder)
Assigned value robust mean of all results

Recovery Rates with z-Scores ELISA for egg: Spiking Level Sample and Sample B

Evaluation number	Spiking Level Sample	Recovery rate*		Sample B	Recovery rate*		Methode	Remarks
	[mg/kg]	[%]	[Z_{RR}]	[mg/kg]	[%]	[Z_{RR}]		
8	30,6	**107**	0,26	15,2	**53**	-1,9	AQ	Result converted °
11	27,3	**95**	-0,20	24,4	**85**	-0,59	BF	
10	34,6	**121**	0,82	32,7	**114**	0,57	EF	Result converted °
5	41,2	**144**	1,7	35,0	**122**	0,90	IL	Result converted °
9a	34,8	**121**	0,84	33,9	**119**	0,74	MI	Result converted °
1a	25,9	**90**	-0,39	19,5	**68**	-1,3	MI-II	Result converted °
4	34,1	**119**	0,75	36,2	**127**	1,1	MI-II	Result converted °
9b	22,0	**77**	-0,94	21,5	**75**	-0,99	MI-II	Result converted °
2	31,0	**108**	0,32	33,0	**115**	0,62	RS	
7a	29,7	**103**	0,14	24,0	**84**	-0,64	RS	
1b	30,5	**106**	0,25	24,0	**84**	-0,64	RS-F	
6	27,8	**97**	-0,13	20,7	**72**	-1,1	RS-F	
7b	28,1	**98**	-0,09	22,2	**78**	-0,89	RS-F	
12	40,8	**142**	1,7	29,2	**102**	0,08	RS-F	

° calculation see p. 19

RA**	50-150 %	RA**	50-150 %
Number in RA	14	Number in RA	14
Percent in RA	100	Percent in RA	100

* Recovery rate 100% relative size: whole egg powder, s. Page 5

** Range of acceptance of AOAC for allergen ELISAS

Methods:

AQ = AgraQuant, RomerLabs
BF = MonoTrace ELISA, BioFront Technologies
EF = SensiSpec ELISA Kit, Eurofins
IL = Immunolab
MI = Morinaga Institute ELISA
MI-II = Morinaga Institute ELISA Kit II
RS = Ridascreen®, R-Biopharm
RS-F= Ridascreen® Fast, R-Biopharm

Comment:
All participants obtained for the spiking level sample and for the spiked food matrix sample B, which had a high salt content of approx. 55%, a recovery rate by ELISA methods within the range of the AOAC-recommendation of 50-150%. The related z-scores are based on the target standard deviation of 25%.

4.1.2 PCR Results: Egg

Comment:
No PCR determinations were carried out by the participants.

4.2 Proficiency Test Fish

4.2.1 ELISA Results: Fish (as fresh cod)

Qualitative valuation of results: Samples A and B

Evaluation number	Sample A	Sample A	Sample B	Sample B	Qualitative Valuation	Method	Remarks
	pos/neg	[mg/kg]	pos/neg	[mg/kg]	Agreement with consensus value		
8	negative	<4	positive	60,6	2/2 (100%)	AQ	
9	negative	<4	positive	81,3	2/2 (100%)	AQ	
7	negative	<5	positive	300	2/2 (100%)	BC	
11	negative	0	positive	11,7	2/2 (100%)	BF	
10	negative	< 5	positive	95,0	2/2 (100%)	EF	
5	negative	<4	positive	132	2/2 (100%)	IL	

	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:
AQ = AgraQuant, RomerLabs
BC = BioCheck ELISA
BF = MonoTrace ELISA, BioFront Technologies
EF = SensiSpec ELISA Kit, Eurofins
IL = Immunolab

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

Quantitative valuation of ELISA-results: Sample B

Evaluation number	fresh fish	z-Score Xpt_{ALL}	Method	Remarks
	[mg/kg]			
8	60,6	-1,2	AQ	
9	81,3	-0,31	AQ	
7	300	9,6	BC	
11	11,7	-3,5	BF	
10	95,0	0,31	EF	
5	132	2,0	IL	

Methods:
AQ = AgraQuant, RomerLabs
BC = BioCheck ELISA
BF = MonoTrace ELISA, BioFront Technologies
EF = SensiSpec ELISA Kit, Eurofins
IL = Immunolab

Comment:
A kernel density estimation was not made due to the number of <8 results.

Characteristics: Quantitative evaluation ELISA fish (as fresh fish)

Sample B

Statistic Data	**All Results** *[mg/kg]*
Assigned value (X_{pt})	$X_{pt_{ALL}}$
Number of results	6
Number of outliers	0
Mean	114
Robust Mean	101
Median (X_{pt})	**88,2**
Robust standard deviation (S*)	**81,6**
Target range:	
Target standard deviation σ_{pt}	**22,0**
lower limit of target range	**44,1**
upper limit of target range	**132**
Quotient S/σ_{pt}*	*3,7*
Standard uncertainty $U_{(Xpt)}$	*41,7*
Results in the target range	*4*
Percent in the target range	*67*

Comments to the statistical characteristics and assigned values:

The evaluation of all methods showed an increased variability of results, with a quotient S/σ_{pt} of 3,7. The standard uncertainty was not considered by using the z'-score evaluation, because the target range would otherwise be rendered unsuitably great for an evaluation. The evaluation was done just for information.*

The median was used as the assigned value (see 3.1).
The robust standard deviation is well above the range of established values for the repeatability and 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 limited because only a few results were available.

The median of the evaluation was 24% of the spiking level of fish (as fresh cod) to sample B below the range of the recommendations for the applied methods (s. 3.4.3 and p.36 "Recovery rates ELISA for fish").

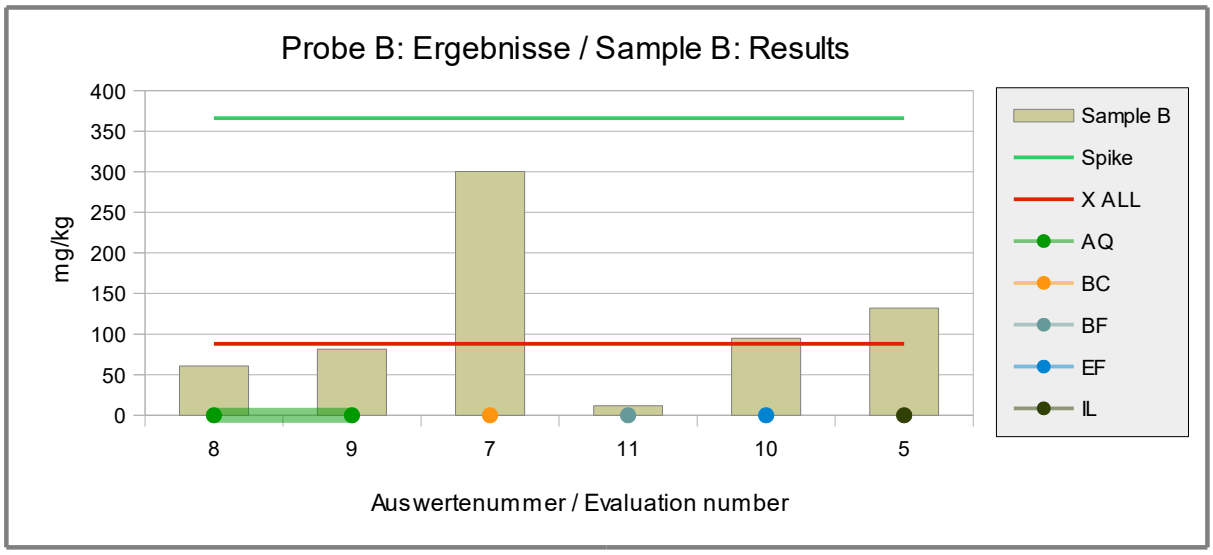


Abb./Fig. 7: ELISA Results Fish (as fresh cod)
green line = Spiking level (Spike)
red line = Assigned value robust mean all results
round symbols = Applied methods (see legend)

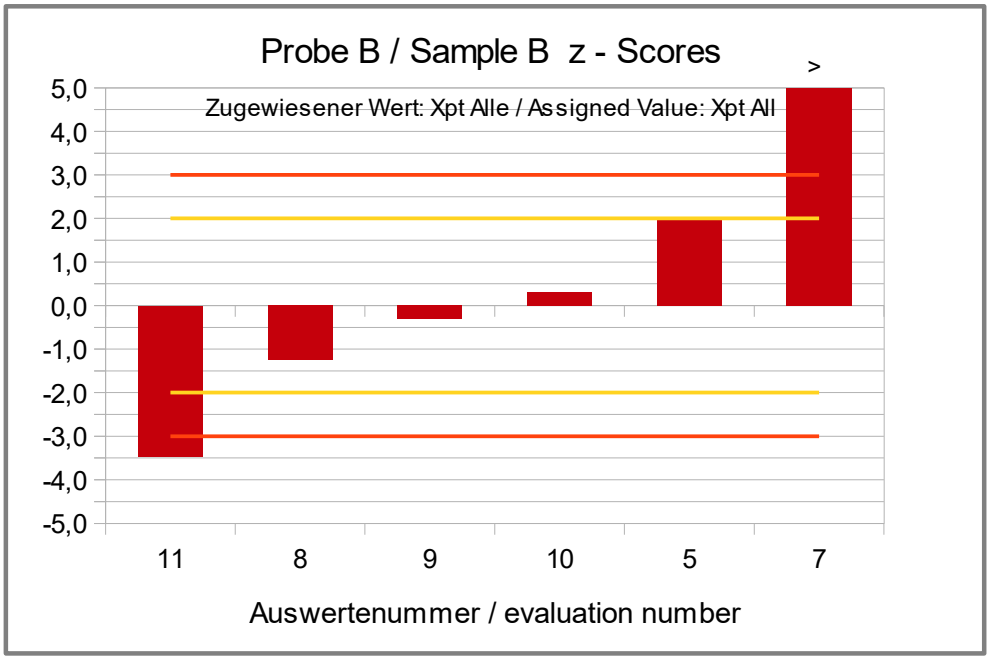


Abb./Fig. 8:
z'-Scores ELISA Results Fish (as fresh cod)
Assigned value median of all results

Quantitative valuation of ELISA: Spiking Level Sample

Evaluation number	fresh fish	z-Score Xpt_{ALL}	Method	Remarks
	[mg/kg]			
8	207	0,75	AQ	
9	109	-1,5	AQ	
7	187	0,28	BC	
11	163	-0,28	BF	
10	130	-1,0	EF	
5	253	1,8	IL	

Methods:
AQ = AgraQuant, RomerLabs
BC = BioCheck ELISA
BF = MonoTrace ELISA, BioFront Technologies
EF = SensiSpec ELISA Kit, Eurofins
IL = Immunolab

Comment:
A kernel density estimation was not made due to the number of <8 results.

Characteristics: Quantitative evaluation ELISA fish (as fresh fish)

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	175
Median	175
Robust Mean (X_{pt})	**175**
Robust standard deviation (S*)	**59,6**
Target range:	
Target standard deviation σ_{pt}	**43,7**
lower limit of target range	**87,4**
upper limit of target range	**262**
Quotient S/σ_{pt}*	*1,4*
Standard uncertainty $U_{(Xpt)}$	*30,4*
Results in the target range	*6*
Percent in the target range	*100*

Comments to the statistical characteristics and assigned values:

The evaluation of all methods showed a normal variability of results, with a quotient S/σ_{pt} below 2,0.*
The robust standard deviation is in the range of established values for the repeatability and 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 was 42% of the spiking level of fish to the spiking level sample and were just below the range of the recommendations for the applied methods (s. 3.4.3 and p.36 "Recovery rates ELISA for fish").

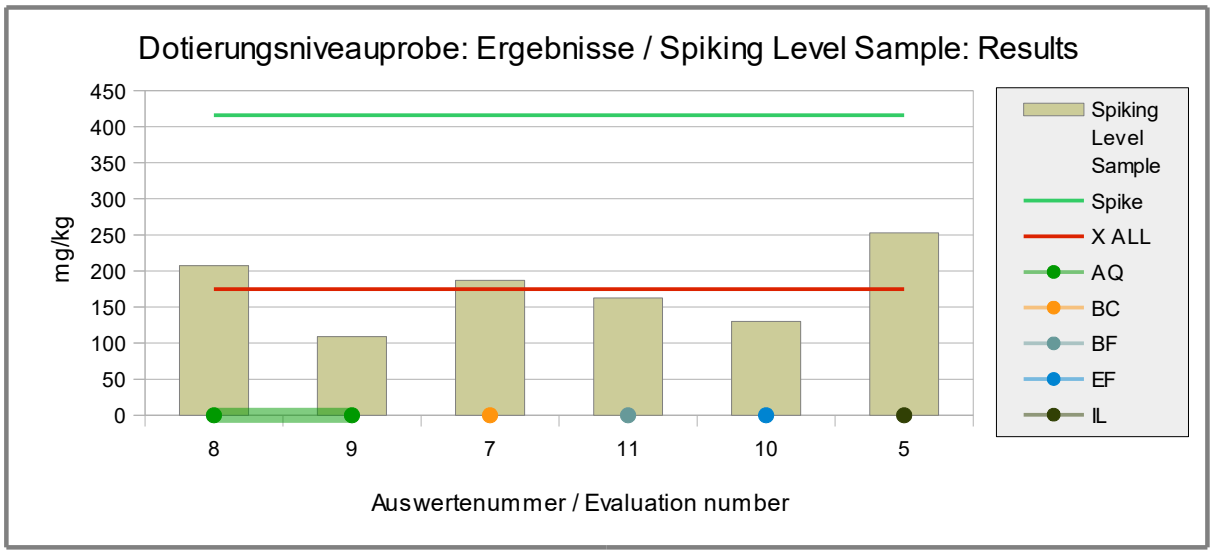


Abb./Fig. 9: ELISA Results Fish (as fresh cod)
green line = Spiking level (Spike)
red line = Assigned value robust mean all results
round symbols = Applied methods (see legend)

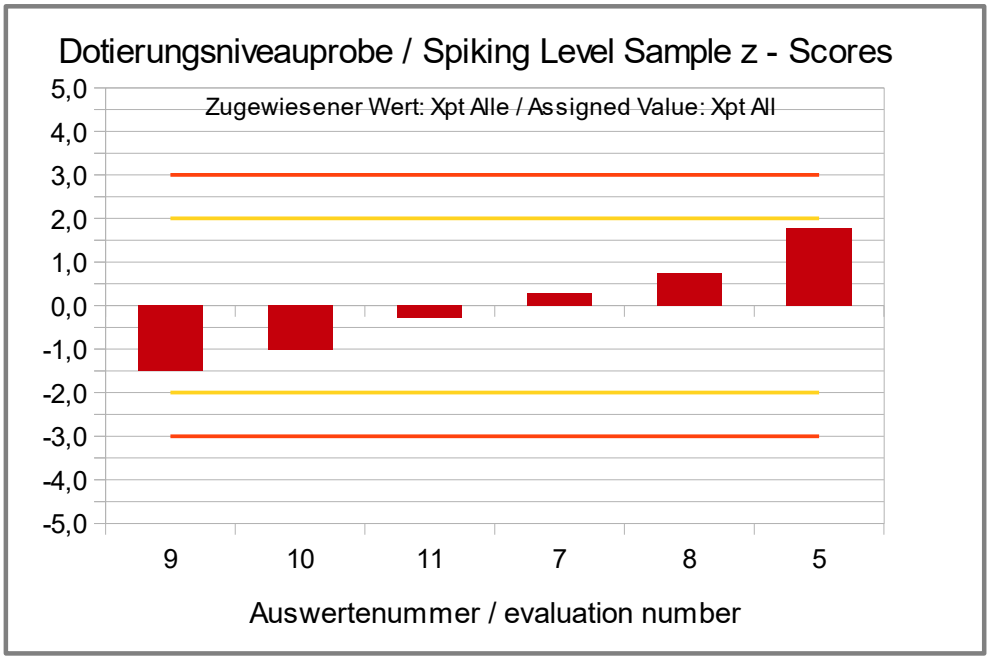


Abb./Fig. 10:
z-Scores ELISA Results fish (as fresh cod)
Assigned value robust mean of all results

Recovery Rates with z-Scores ELISA for fish: Spiking Level Sample and Sample B

Evaluation number	Spiking Level Sample	Recovery rate*		Sample B	Recovery rate*		Method	Remarks
	[mg/kg]	[%]	[Z_{RR}]	[mg/kg]	[%]	[Z_{RR}]		
8	207	**50**	-2,0	60,6	17	-3,3	AQ	
9	109	26	-3,0	81,3	22	-3,1	AQ	
7	187	45	-2,2	300	**82**	-0,72	BC	
11	163	39	-2,4	11,7	3,2	-3,9	BF	
10	130	31	-2,8	95,0	26	-3,0	EF	
5	253	**61**	-1,6	132	36	-2,6	IL	

RA**	50-150 %	RA**	50-150 %
Number in RA	**2**	Number in RA	**1**
Percent in RA	**33**	Percent in RA	**17**

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

** Range of acceptance of AOAC for allergen ELISAS

Methods:

AQ = AgraQuant, RomerLabs

BC = BioCheck ELISA

BF = MonoTrace ELISA, BioFront Technologies

EF = SensiSpec ELISA Kit, Eurofins

IL = Immunolab

Comments:

Two (33%) participants obtained for the spiking level sample a recovery rate by ELISA methods within the range of the AOAC-recommendation of 50-150%. For the spiked food matrix sample B, which had a high salt content of approx. 55%, one recovery rate (17%) was in this range of acceptance. All other recovery rates were below 50%. The related z-scores are based on the target standard deviation of 25%.

4.2.2 PCR Results: fish (as fresh cod)

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		positive		2/2 (100%)	SFA	
7	negative	<1	positive	17,0	2/2 (100%)	SFA	
12	negative		positive		2/2 (100%)	SFA	
1	negative		positive		2/2 (100%)	div	
2	negative	< 1,0	positive		2/2 (100%)	div	
4	negative		positive		2/2 (100%)	div	
6	negative		positive		2/2 (100%)	div	

	Sample A		Sample B	
Number positive	0		7	
Number negative	7		0	
Percent positive	0		100	
Percent negative	100		0	
Consensus value	negative		positive	

Methods:
SFA = Sure Food ALLERGEN, R-Biopharm / Congen
div = keine genaue Angabe / andere Methode
div = not indicated / other method

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

Quantitative valuation of PCR-results: Sample B

No quantitative valuation was done, because there were too few results available.

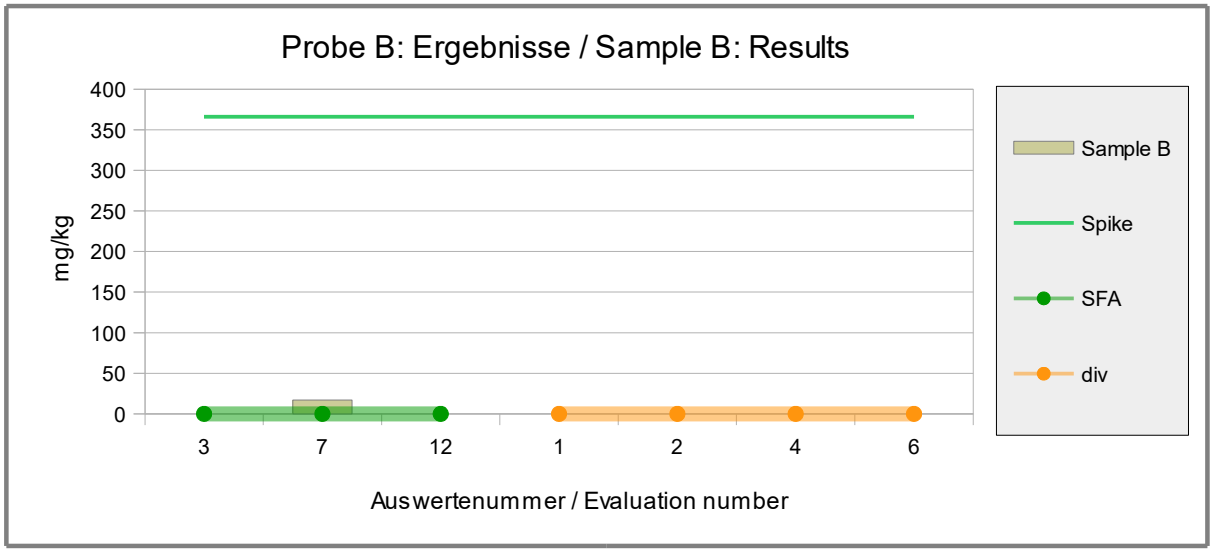


Abb./Fig. 11: PCR Results Fish (as fresh cod)
green line = Spiking level
round symbols = Applied methods (see legend)

Quantitative Valuation of PCR: Spiking level sample

No quantitative valuation was done, because there were too few results available.

Evaluation number	fresh fish	Spiking Level Sample	Method	Remarks
	pos/neg	[mg/kg]		
3	-		SFA	
7	positive	189	SFA	
12	positive		SFA	
1	positive		div	
2	positive		div	
4	positive		div	
6	positive		div	

Number positive	6
Number negative	0
Percent positive	100
Percent negative	0
Consensus value	positive

Methods:
SFA = Sure Food ALLERGEN, R-Biopharm / Congen
div = keine genaue Angabe / andere Methode
div = not indicated / other method

Comment:
For the spiking level sample only positive results were obtained.

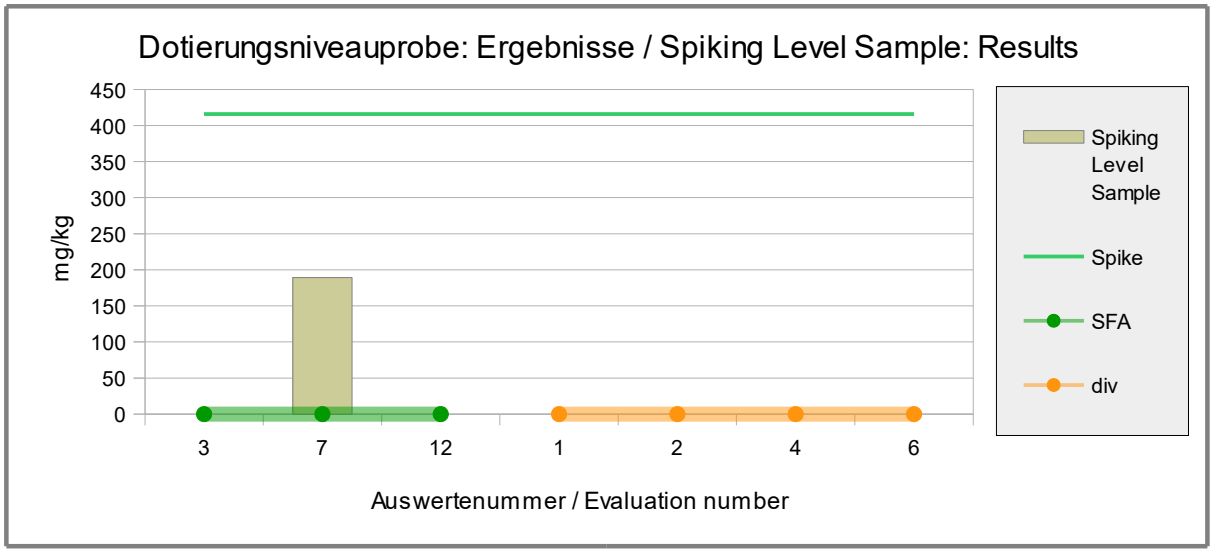


Abb./Fig. 12: PCR-Results Fish (as fresh cod)
green line = Spiking level
round symbols = Applied methods (see legend)

Recovery Rates with z-Scores PCR for fish: Spiking Level Sample and Sample B

Evaluation number	Spiking Level Sample	Recovery rate*		Sample B	Recovery rate*		Method	Remarks
	[mg/kg]	[%]	[Z_{RR}]	[mg/kg]	[%]	[Z_{RR}]		
3							SFA	
7	189	45	-2,2	17,0	4,7	-3,8	SFA	
12							SFA	
1							div	
2							div	
4							div	
6							div	

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

Methods:
SFA = Sure Food ALLERGEN, R-Biopharm / Congen
div = keine genaue Angabe / andere Methode
div = not indicated / other method

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

** Range of acceptance of AOAC for allergen ELISAS

Comments:

One participant obtained for the spiking level sample a recovery rate by PCR methods just below the range of the AOAC-recommendation of 50-150%. For the spiked food matrix sample B, which had a high salt content of approx. 55%, the recovery rate was well below this range of acceptance. The related z-scores are based on the target standard deviation of 25%.

4.3 Participant z-Scores: overview table

Z-Scores for the assigned values from participants results

Evaluation number	ELISA Egg: **Xpt** (div. methods)		ELISA Fish: **Xpt** (div. methods)	
	Sample B	**Sp. Level Sample**	**Sample B**	**Sp. Level Sample**
1a	-1,1	-0,67	-	-
1b	-0,38	-0,08	-	-
2	0,97	-0,02	-	-
3	-	-	-	-
4	1,5	0,38	-	-
5	1,3	1,3	2,0	1,8
6	-0,88	-0,43	-	-
7a / 7	-0,38	-0,19	9,6	0,28
7b	-0,65	-0,39	-	-
8	-1,7	-0,07	-1,2	0,75
9a / 9	1,1	0,47	-0,31	-1,5
9b	-0,75	-1,2	-	-
10	0,93	0,45	0,31	-1,0
11	-0,32	-0,49	-3,5	-0,28
12	0,40	1,2	-	-

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

Evaluation number	ELISA Egg: **Xpt** (div. methods)		ELISA Fish: **Xpt** (div. methods)	
	Sample B	**Sp. Level Sample**	**Sample B**	**Sp. Level Sample**
1a	-1,3	-0,39	-	-
1b	-0,64	0,25	-	-
2	0,62	0,32	-	-
3	-	-	-	-
4	1,1	0,75	-	-
5	0,90	1,7	-2,6	-1,6
6	-1,1	-0,13	-	-
7a / 7	-0,64	0,14	-0,72	-2,2
7b	-0,89	-0,09	-	-
8	-1,9	0,26	-3,3	-2,0
9a / 9	0,74	0,84	-3,1	-3,0
9b	-0,99	-0,9	-	-
10	0,57	0,82	-3,0	-2,8
11	-0,59	-0,20	-3,9	-2,4
12	0,08	1,7	-	-

Evaluation number	PCR Fish: **Xpt** (div. methods)	
	Sample B	**Sp. Level Sample**
7	-3,8	-2,2

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: Egg

Meth. Abbr.	Evaluation no.	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	ELISA Test-Kit+Manufacturer
AQ	8	03.03.20	Negative	<0.4	Positive	3,961	Positive	7,95		<0.4	25,8	Egg White Protein	AQ = AgraQuant, RomerLabs
BF	11	03.06.20	negative	0	positive	24,4	positive	27,3				Whole egg powder	MonoTrace Egg ELISA kit, BioFront Technologies
EF	10	12.02.20	negative	< 0,2	positive	"8,5"	positive	"9,0"	0.05	0.4		Egg White Protein, total	SensiSpec ELISA Egg white, Eurofins
IL	5	26.02.20	negative	<0,4	positive	8,05	positive	9,48				Egg White Protein, total	Immunolab Egg white ELISA
MI	9a	05.02.20	negative	<0,31	positive	15,9	positive	16,3		0,31		Egg proteins, total	Morinaga Egg (Ovalbumin) ELISA Kit (M2101)
MI-II	1a		negative		positive	9,14	positive	12,16	1,25	2,5		Egg proteins, total	Morinaga Egg (Ovalbumin) ELISA Kit II (M2111)
MI-II	4	03.02.20	negative	<0,31	positive	17	positive	16	0,31	0,31		Whole egg protein	Morinaga Egg (Ovalbumin) ELISA Kit II (M2111)
MI-II	9b	25.02.20	negative	<0,31	positive	10,1	positive	10,3		0,31		Egg proteins, total	Morinaga Egg (Ovalbumin) ELISA Kit II (M2111)
RS	2	30.01.	negative	<0,25	positive	33	positive	31	0,13	0,25	30	Whole egg powder	Ridascreen® Egg R6411, R-Biopharm
RS	7a	29.01.20	negative	<0.25	positive	24,04	positive	29,67	0,25	0,25		Whole egg powder	Ridascreen® Egg R6411, R-Biopharm
RS-F	1b		negative		positive	24	positive	30,5	0,5	0,5		Whole egg powder	Ridascreen® FAST Egg Protein R6402, R-Biopharm
RS-F	6		negative		positive	20,7	positive	27,8	0,1	0,5	16	Whole egg powder	Ridascreen® FAST Egg Protein R6402, R-Biopharm
RS-F	7b	29.01.20	negative	<0.5	positive	22,21	positive	28,07	0,5	0,5		Whole egg powder	Ridascreen® FAST Egg Protein R6402, R-Biopharm
RS-F	12		negative		positive	29,17	positive	40,83	0,9	3		Whole egg powder	Ridascreen® FAST Egg Protein R6402, R-Biopharm

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

Meth. Abbr.	Evaluation no.	Specifity	Remarks to the Method (Extraction and Determination)	Method Accredited ISO/IEC 17025	Further Remarks
		Antibody	e.g. Extraction Solution / Time / Temperature	yes/no	
AQ	8			yes	
BF	11	Monoclonal antibody based assay	1:20 extraction for 10 minutes @ 60C	NO	
EF	10				
IL	5				In order to calculate the whole egg powder content (NIST 4665) from the egg white protein content determined, the result must be multiplied by a factor of 4.35.
MI	9a		as per kit insert : short extraction	yes	
MI-II	1a		according to the manufacturer's instructions	yes	
MI-II	4	recognizes egg white protein ovalbumin	according to the manufacturer's instructions	yes	
MI-II	9b		as per kit insert : overnight extraction	no	
RS	2	Ovalbumin / Ovomucoid	Egg-Extractor/A-AEP; 10 min / 60°C	yes	
RS	7a	As Per Kit Instructions	As Per Kit Instructions	No	
RS-F	1b		according to the manufacturer's instructions	yes	
RS-F	6			yes	
RS-F	7b	As Per Kit Instructions	As Per Kit Instructions	Yes	
RS-F	12			yes	LFOD-TST-SOP-8966

5.1.2 ELISA: Fish

Meth. Abbr.	Evaluation no.	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	ELISA Test-Kit+Manufacturer
AQ	8	02.03.20	Negative	<4	Positive	60,622	Positive	207,45		4	53,1	Protein, total	AQ = AgraQuant, RomerLabs
AQ	9	25.02.20	negative	<4	positive	81,3	positive	109,1		4		fish, fresh	AgraQuant ELISA Fish COKAL2548, RomerLabs
BC	7	29.01.20	negative	<5	positive	300,31	positive	187,21	5	5		fish, fresh	BioCheck ELISA Fish-Check
BF	11	03.06.20	negative	0	positive	11,7	positive	162,5				fish, fresh	BioFront Technologies
EF	10	12.02.20	negative	< 5	positive	95	positive	130	1,4	10		cod, fresh	SensiSpec ELISA Fish (Parvalbumin), Eurofins
IL	5	26.02.20	negative	<4	positive	132,2	positive	253				fish, fresh	Immunolab Fish (parvalbumin) ELISA

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

Meth. Abbr.	Evaluation no.	Specifity	Remarks to the Method (Extraction and Determination)	Method Accredited ISO/IEC 17025	Further Remarks
		Antibody	e.g. Extraction Solution / Time / Temperature	yes/no	
AQ	8			No	
AQ	9		as per kit insert	yes	
BC	7	As Per Kit Instructions	As Per Kit Instructions	Yes	
BF	11	Monoclonal antibody based assay	1:20 extraction ratio for 1 hour while boiling	NO	MonoTrace Fish ELISA kit, BioFront Technologies
EF	10				
IL	5				The concentrations determined relate directly to the cod-equivalent fish content of the sample

5.1.3 PCR: Fish

Meth. Abbr.	Evaluation no.	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	PCR Test-Kit+Manufacturer
SFA	3		negative		positive		-					Please select!	Sure Food ALLERGEN, R-Biopharm / Congen
SFA	7	27.01.20	negative	<1	positive	17,03	positive	189,16	1	1		fish, fresh	Sure Food ALLERGEN, R-Biopharm / Congen
SFA	12		negative		positive		positive		1			DNA-Fish	Sure Food ALLERGEN, R-Biopharm / Congen
div	1		negative		positive		positive					DNA-Fish	Final report research project Technical University of Graz No. 1245: see further remarks
div	2	31.01.	negative	< 1,0	positive		positive		0,5	1		fish powder	Literatur: Sun et al. 2009: J. AOAC Int. Vol 92(1)
div	4	06.02.20	negative		positive		positive		10			DNA-Fish	internal method
div	6		negative		positive		positive		25			DNA-Fish	other: in-house method

* NWG Nachw eisgrenze / BG Bestimmungsgrenze

* LOD limit of detection / LOQ limit of quantitation

* MU Messunsicherheit / MU measurement uncertainty

Meth. Abbr.	Evaluation no.	Specifity	Remarks to the Method (Extraction and Determination)	Method Accredited ISO/IEC 17025	Further Remarks
		Target-Sequence / -DNA	e.g. Extraction / enzymes / clean-up / real time PCR / gel electrophoresis / cycles	yes/no	
SFA	3				
SFA	7	As Per Kit Instructions	As Per Kit Instructions	Yes	
SFA	12		SureFood ALLERGEN Fish - S3610		LFOD-TST-SOP-8852
div	1	18S	M&N Food Kit, conventional PCR, 35 cycles	yes	Entwicklung von Methoden zum qualitativen und quantitativen Nachweis und zur Unterscheidung von Tier- und Fischmehl in Futtermitteln durch Nachweis von DNA mit PCR; Dr. Peter Remler, Dr. Ursula Mülleder Institut für Lebensmittelchemie und –technologie, Dr. Werner Ruppitsch Bundesamt und Forschungszentrum für Landwirtschaft, Mag. Edith Rassi Lebensmitteluntersuchungsanstalt Kärnten [Development of methods for the qualitative and quantitative detection and differentiation of animal and fish meal in feed by detection of DNA by PCR; Dr. Peter Remler, Dr. Ursula Mülleder Institute of Food Chemistry and Technology, Dr. Werner Ruppitsch Federal Office and Research Center for Agriculture, Edith Rassi Food Research Institute Carinthia]
div	2	Parvalbumin Kerngen	CTAB+ Prot. K / Chloroform / Clean up: DNeasy Kit Qiagen	yes	
div	4		CTAB / Proteinase K / Promega Wizard DNA-CleanUp / Real-time PCR / 45 cycles	yes	
div	6		Limit of detection based on DNA molecules	yes	

5.2 Homogeneity

5.2.1 Mixture homogeneity before bottling

Microtracer Homogeneity Test
DLA ptA01 2020 Sample B

Weight whole sample	2,80	kg
Microtracer	FSS-rot lake	
Particle size	75 – 300	µm
Weight per particle	2,0	µg
Addition of tracer	20,7	mg/kg

Result of analysis

Sample	Weight [g]	Particle number	Particles [mg/kg]
1	5,15	73	28,3
2	4,97	76	30,6
3	5,08	62	24,4
4	5,00	77	30,8
5	5,05	56	22,2
6	5,01	79	31,5
7	4,94	65	26,3
8	4,95	59	23,8

Poisson distribution		
Number of samples	8	
Degree of freedom	7	
Mean	68,4	Particles
Standard deviation	8,98	Particles
χ^2 (CHI-Quadrat)	8,25	
Probability	**31**	%
Recovery rate	132	%

Normal distribution		
Number of samples	8	
Mean	27,3	mg/kg
Standard deviation	3,58	mg/kg
rel. Standard deviaton	13,1	%
Horwitz standard deviation	9,7	%
HorRat-value	**1,3**	
Recovery rate	132	%

Microtracer Homogeneity Test
DLA ptA01 2020 Spiking Level Sample

Weight whole sample	1,50	kg
Microtracer	FSS-rot lake	
Particle size	75 – 300	µm
Weight per particle	2,0	µg
Addition of tracer	19,6	mg/kg

Result of analysis

Sample	Weight [g]	Particle number	Particles [mg/kg]
1	5,04	59	23,4
2	4,99	54	21,6
3	4,72	48	20,3
4	4,81	61	25,4
5	4,98	64	25,7
6	5,01	50	20,0
7	4,98	47	18,9
8	5,07	53	20,9

Poisson distribution		
Number of samples	8	
Degree of freedom	7	
Mean	54,5	Particles
Standard deviation	6,28	Particles
χ^2 (CHI-Quadrat)	5,06	
Probability	**65**	%
Recovery rate	112	%

Normal distribution		
Number of samples	8	
Mean	22,0	mg/kg
Standard deviation	2,54	mg/kg
rel. Standard deviaton	11,5	%
Horwitz standard deviation	10,0	%
HorRat-value	**1,1**	
Recovery rate	112	%

5.3 Information on the Proficiency Test (PT)

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

PT number	***ptAL01 - 2020***
PT name	***Allergens I: Egg and Fish in Instant Soup Powder***
Sample matrix (processing)	***Samples A + B:*** *Vegetable soup (powder) / Ingredients: iodized salt, sugar, potato flour, flavoring, yeast extract, carrots, rapeseed oil, parsley, onions, leeks, spices, antioxidant extract from rosemary, other additives and allergenic foods (whole egg powder, freeze-dried cod) (one of both samples)* ***Spiking Level Sample:*** *potato powder, other food additives and allergenic foods*
Number of samples and sample amount	*2 different Samples A + B: 25 g each + 1 Spiking Level Sample: 15 g*
Storage	*Samples A, B + Spiking Level Sample: room temperature (PT period), cooled 2 - 10°C (long term)*
Intentional use	*Laboratory use only (quality control samples)*
Parameter	*qualitative + quantitative: Egg (egg protein, DNA), fish (fish protein, DNA) Samples A + B: < 500 mg/kg (as whole egg powder) Spiking Level Sample: < 500 mg/kg (as cod, freeze dried)*
Methods of analysis	*Analytical methods are optional*
Notes to analysis	*The analysis of PT samples should be performed like a routine laboratory analysis. In general we recommend to homogenize a representative sample amount before analysis according to good laboratory practice, especially in case of low sample weights. Preferably, the total sample amount is homogenized.*
Result sheet	*One result each should be determined for Samples A and B and the Spiking Level Sample. The results should be filled in the result submission file.*
Units	*mg/kg*
Number of digits	*at least 2*
Result submission	*The result submission file should be sent by e-mail to:* ***pt@dla-lvu.de***
Last Deadline	***the latest March 06th 2020***
Evaluation report	*The evaluation report is expected to be completed 6 weeks after deadline of result submission and sent as PDF file by e-mail.*
Coordinator and contact person of PT	*Matthias Besler-Scharf PhD*

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

6. Index of participant laboratories in alphabetical order

Teilnehmer / Participant	Ort / Town	Land / Country
		USA
		CANADA
		Germany
		Germany
		FRANCE
		Germany
		Germany
		Germany
		GREAT BRITAIN
		GREAT BRITAIN
		USA
		VIETNAM
		Germany

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

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

7. Index of references

1. DIN EN ISO/IEC 17025:2005; Allgemeine Anforderungen an die Kompetenz von Prüf- und Kalibrierlaboratorien / General requirements for the competence of testing and calibration laboratories
2. DIN EN ISO/IEC 17043:2010; Konformitätsbewertung – Allgemeine Anforderungen an Eignungsprüfungen / Conformity assessment – General requirements for proficiency testing
3. ISO 13528:2015 & DIN ISO 13528:2009; Statistische Verfahren für Eignungsprüfungen durch Ringversuche / Statistical methods for use in proficiency testing by interlaboratory comparisons
4. ASU §64 LFGB: Planung und statistische Auswertung von Ringversuchen zur Methodenvalidierung / DIN ISO 5725 series part 1, 2 and 6 Accuracy (trueness and precision) of measurement methods and results
5. Verordnung / Regulation 882/2004/EU; Verordnung über über amtliche Kontrollen zur Überprüfung der Einhaltung des Lebensmittel- und Futtermittelrechts sowie der Bestimmungen über Tiergesundheit und Tierschutz / Regulation on official controls performed to ensure the verification of compliance with feed and food law, animal health and animal welfare rules
6. Evaluation of analytical methods used for regulation of food and drugs; W. Horwitz; Analytical Chemistry, 54, 67-76 (1982)
7. The International Harmonised Protocol for the Proficiency Testing of Ananlytical Laboratories ; J.AOAC Int., 76(4), 926 – 940 (1993)
8. A Horwitz-like funktion describes precision in proficiency test; M. Thompson, P.J. Lowthian; Analyst, 120, 271-272 (1995)
9. Protocol for the design, conduct and interpretation of method performance studies; W. Horwitz; Pure & Applied Chemistry, 67, 331-343 (1995)
10. Recent trends in inter-laboratory precision at ppb and sub-ppb concentrations in relation to fitness for purpose criteria in proficiency testing; M. Thompson; Analyst, 125, 385-386 (2000)
11. The International Harmonised Protocol for the Proficiency Testing of Analytical Chemistry Laboratories; Pure Appl Chem, 78, 145 – 196 (2006)
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