Proficiency Tests
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Evaluation Report
proficiency test

DLA 01/2018

Allergens I:

Egg and Fish

in Sauce Powder

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

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

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1. Introduction

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

2. Realisation

2.1 Test material

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

The test material is a common in commerce instant sauce powder. The basic composition of both sample A and sample B was the same (see table 1). After crushing and sieving by means of a impact mill (mesh 1,5 mm) the basic mixture was homogenized.

Afterwards the **spiked sample B** was produced as follows:
The spiking materials (premix) 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 4 additional steps and homogenized in each case until the total quantity had been reached. Afterwards the total sample was sieved by means of a centrifugal mill (mesh 500 µm) and homogenized again.
For the **spiking level sample**, the allergenic compounds above mentioned were added during a multi-stage addition of potato powder and homogenization. Afterwards the total sample was sieved by means of a centrifugal mill (mesh 250 µm) and homogenized again.

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

Table 1: Composition of the DLA-Samples

Ingredients	**Sample A**	**Sample B**	**Spiking Level Sample**
Onion Instant Soup Powder Ingredients: onions, starch, salt, fried onions, vegetable fats (palm, shea), yeast extract, seasoning, sugar, thickener: guar gum, maltodextrin, garlic, spices, flavoring, caramel sugar Nutrients per 100 g: Protein 4,0 g, Carbohydrates 17 g, Fat 3,4 g	76,6 g/100 g	76,5 g/100g	-
Potato Flour Nutrients per 100 g: Protein 0 g	23,4 g/100 g	23,4 g/100g	-
Potato Powder Ingredients: Potatoes, E471, E304, E223, E100	-	-	99,8 g/100 g
Volleipulver: Ingredients: Hen's egg (pasteurized, spray dried) - as Whole Egg Powder* - *thereof 47,6% total protein*** - *thereof 26,0% egg white protein****	-	 23,0 mg/kg 10,9 mg/kg 6,0 mg/kg	 22,5 mg/kg 10,6 mg/kg 5,9 mg/kg
Fish Powder: Ingredients: Codfish (Gadus morhua), dried - as Fish Powder* - *thereof 55,8% total protein*** converted to: - *Cod, fresh (wet weight, muscle tissue)****	-	 101 mg/kg 56,4 mg/kg 505 mg/kg	 93,7 mg/kg 52,3 mg/kg 469 mg/kg
further Ingredients: Maltodextrin, sodium chloride, 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 fish protein)
*** Egg white protein calculated according to literature [37, 38] / 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 sample B showed a probability of 85% and 91% for the spiking level sample, respectively. 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 HorRat values of 1,1 and 0,89, respectively. 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 pages 7-8). Recommendations for repeatability standard deviations of ELISA and PCR methods are usually ≤ 25% [18, 19, 22, 23].

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

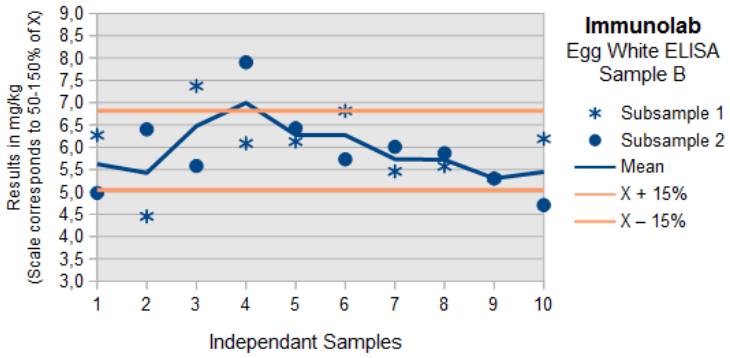
ELISA-Tests: Homogenität Ei / Homogeneity Egg

Immunolab Egg White ELISA

Sample weights: 1,0 g (0,9 – 1,1 g)
Number of replicates: 2
Overall result: Egg white protein 5,93 ± 0,32 mg/kg

Sample B	Subsample 1 mg/kg	Subsample 2 mg/kg	Mean mg/kg
1	6,27	4,98	5,62
2	4,45	6,40	5,43
3	7,37	5,58	6,48
4	6,09	7,90	7,00
5	6,13	6,43	6,28
6	6,81	5,73	6,27
7	5,46	6,01	5,74
8	5,57	5,87	5,72
9	5,31	5,30	5,30
10	6,19	4,71	5,45

General average X	5,93	
SD of sample means Sx	0,55	9,3%
SD w ithin-samples Sw	0,63	10,6%
SD betw een-samples Ss	0,32	5,5%

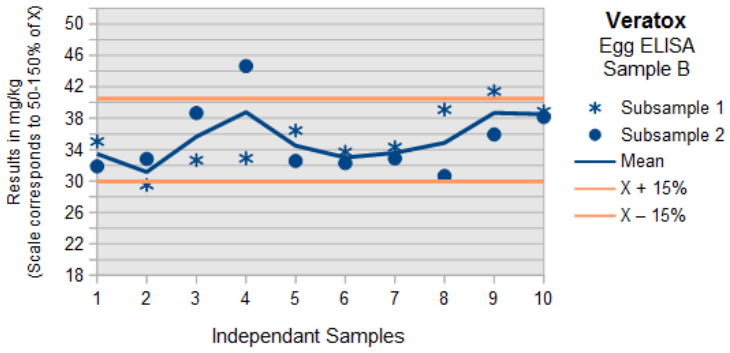


Neogen Veratox ELISA Egg

Sample weights: 5,0 g (4,5 – 5,5 g)
Number of replicates: 2
Overall result: Whole dried egg 35,2 ± 1,8 mg/kg

Sample B	Subsample 1 mg/kg	Subsample 2 mg/kg	Mean mg/kg
1	35,0	31,9	33,5
2	29,5	32,8	31,2
3	32,7	38,7	35,7
4	32,9	44,6	38,8
5	36,4	32,6	34,5
6	33,7	32,3	33,0
7	34,3	32,9	33,6
8	39,1	30,6	34,9
9	41,4	35,9	38,7
10	38,9	38,2	38,5

General average X	35,2	
SD of sample means Sx	2,66	7,5%
SD w ithin-samples Sw	2,81	8,0%
SD betw een-samples Ss	1,76	5,0%

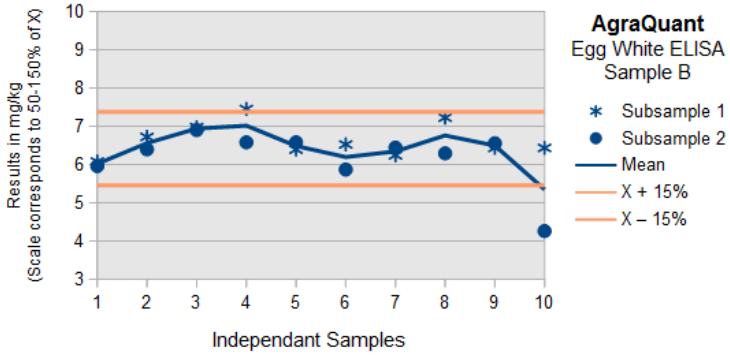


Romerlabs AgraQuant Egg White

Sample weights: 1,0 g (0,9 – 1,1 g)
Number of replicates: 2
Overall result: Egg whit protein 6,41 ± 0,39 mg/kg

Sample B	Subsample 1 mg/kg	Subsample 2 mg/kg	Mean mg/kg
1	6,08	5,96	6,02
2	6,72	6,39	6,56
3	6,98	6,90	6,94
4	7,45	6,58	7,01
5	6,39	6,58	6,48
6	6,52	5,87	6,19
7	6,24	6,44	6,34
8	7,22	6,29	6,76
9	6,43	6,55	6,49
10	6,43	4,26	5,34

General average X	6,41	
SD of sample means Sx	0,49	7,6%
SD w ithin-samples Sw	0,42	6,5%
SD betw een-samples Ss	0,39	6,0%



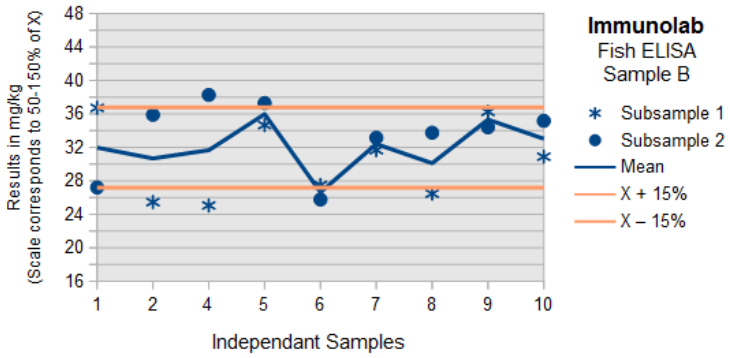
ELISA-Tests: Homogenität Fisch / Homogeneity Fish

Immunolab Fish ELISA

Sample weights: 1,0 g (0,9 – 1,1 g)
Number of replicates: 2
Overall result: Cod, fresh 32,0 ± 1,2 mg/kg

Sample B	Subsample 1 mg/kg	Subsample 2 mg/kg	Mean mg/kg
1	36,7	27,2	32,0
2	25,5	35,9	30,7
4	25,1	38,3	31,7
5	34,7	37,3	36,0
6	27,5	25,8	26,6
7	31,7	33,1	32,4
8	26,5	33,7	30,1
9	36,2	34,4	35,3
10	30,9	35,2	33,0

General average X	32,0	
SD of sample means Sx	2,79	8,7%
SD within-samples Sw	3,58	11,2%
SD between-samples Ss	1,18	3,7%

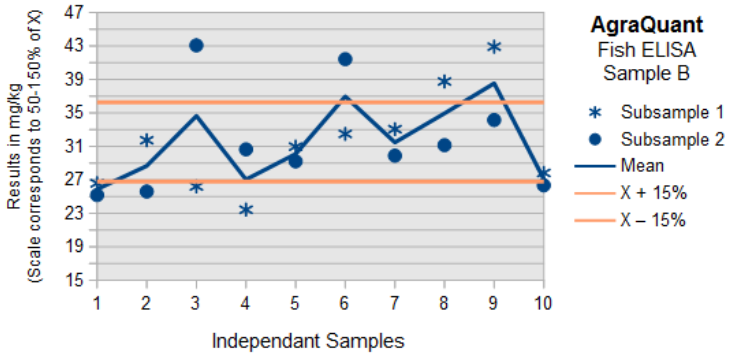


Romerlabs AgraQuant Fish

Sample weights: 1,0 g (0,9 – 1,1 g)
Number of replicates: 2
Overall result: Cod, fresh 31,5 ± 3,6 mg/kg

Sample B	Subsample 1 mg/kg	Subsample 2 mg/kg	Mean mg/kg
1	26,6	25,2	25,9
2	31,7	25,6	28,7
3	26,2	43,1	34,6
4	23,4	30,6	27,0
5	31,0	29,2	30,1
6	32,5	41,4	36,9
7	33,0	29,9	31,5
8	38,7	31,1	34,9
9	42,9	34,1	38,5
10	27,8	26,3	27,1

General average X	31,5	
SD of sample means Sx	4,50	14,3%
SD within-samples Sw	3,89	12,3%
SD between-samples Ss	3,55	11,3%



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 EP samples was approx. 0,44 (23,4°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 5th week of 2018. The testing method was optional. The tests should be finished at February 16th March 2018.

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

*There are two different samples A and B possibly containing the allergenic parameters egg (whole egg powder) and/or fish (cod, dried) in the range of mg/kg in the matrix of sauce 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.*

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

2.3 Submission of results

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

All 13 participants submitted their results in time.

3. Evaluation

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

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

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

3.1 Consensus value from participants (assigned value)

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

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

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

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

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

3.2 Robust standard deviation

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

The following robust standard deviations were considered:

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

3.3 Exclusion of results and outliers

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

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

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

3.4 Target standard deviation (for proficiency assessment)

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

3.4.1 General model (Horwitz)

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

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

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

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

3.4.2 Value by precision experiment

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

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

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

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

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

From the precision data of the official German ASU §64 methods the calculated relative target standard deviations are in the range of 11 – 32% for the ELISA methods and 18 – 42% for the PCR methods depending on the matrix, processing and concentration level of allergens (s. Tab. 2a and 2b).

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

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

Table 2b: PCR-Methods - Relative repeatability standard deviations (RSD_r) and relative reproducibility standard deviations (RSD_R) from precision experiments and resulting target standard deviations σ_{pt} [32-36]

Parameter	**Matrix**	**Mean [mg/kg]**	**Recov-ery**	**rob RSD**	**RSD_r**	**RSD_R**	**σ_{pt}**	**Method / Literature**
Almond	Rice cookie	105,2 18,0 10,5	105 % 90 % 105 %	-	19,3% 44,0% 32,0%	27,5% 49,1% 38,8%	23,9% 38,0% 31,5%	rt-PCR ASU 18.00-20
Almond	Wheat cookie Sauce powder	114,3 88,1	94,6 % 88,1 %	-	22,1% 43,9%	41,8% 43,1%	38,8% - %	rt-PCR ASU 18.00-20
Almond	Rice cookie	109 21,3 12,3	109 % 107 % 121 %	-	17,6% 35,8% 32,0%	32,8% 45,0% 47,8%	30,3% 37,2% 42,1%	rt-PCR multiplex ASU 18.00-22
Almond	Wheat cookie Sauce powder	120,7 112	98,2 % 94,1 %	-	15,7% 36,2%	32,5% 42,8%	30,5% 34,3%	rt-PCR multiplex ASU 18.00-22
Soya	Wheat flour Maize flour	107 145	107 % 145 %	63 % 34 %	- -	31 % 24 %	- -	rt-PCR ASU 16.01-9
Soya flour	Boiled saus-age (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	33,1 %	-	21,5%	30,8	26,8%	rt-PCR ASU 08.00-65
Soya flour	Boiled saus-age (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 process, calibration of equipment and composition of reagents, transmission or calculation errors, trueness and precision, and use of reference material. If necessary, the problems must be addressed through appropriate corrective action [3].

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

3.6 z'-Score

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

The calculation is performed by:

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

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

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

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

For warning and action signals see 3.5.1.

3.7 Quotient S*/σ_{pt}

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

3.8 Standard uncertainty and metrological 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 metrological traceability of the assigned value is ensured on the basis of the consensus value as a robust mean of the participant results.

3.9 Figures

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

3.10 Recovery rates: Spiking

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

4. Results

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

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

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

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

ELISA-Results given as **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 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).
One ELISA result was given as ovalbumin (Moringa Kit I). The result was first converted to egg white protein using a content of 54% ovalbumin in egg white protein [37] and afterwards to whole egg powder (see above).

ELISA and PCR results given as **fish protein** or **fish powder** were converted to **fresh fish** (wet weight) using the analysed protein content of the raw materials (see page 5) and a content of 80 % water in fresh fish, respectively (Souci/Fachmann/Kraut nutrient tables).

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

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

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

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

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

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

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

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

4.1 Proficiency Test 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		
4	negative	< LOD	positive	12,2	1/1 (100%)	AQ	result converted°
11	negative	< 1,5	positive	18,1	1/1 (100%)	BC	result converted°
6	negative	0	positive	44,3	1/1 (100%)	BF	
13	negative	< 1,5	positive	20,7	1/1 (100%)	IL	result converted°
1	positive	3,6	positive	89	1/1 (100%)	MI	result converted°
10	positive	<10	positive	12,0	1/1 (100%)	MI	
8	positive	1,30	positive	23,0	1/1 (100%)	MI-II	
12	positive	1,49	positive	20,8	1/1 (100%)	MI-II	result converted°
2	positive	1,20	positive	18,2	1/1 (100%)	RS-F	
3	positive	1,07	positive	24,2	1/1 (100%)	RS-F	
5	positive	1,32	positive	21,2	1/1 (100%)	RS-F	
7	positive	1,30	positive	19,0	1/1 (100%)	RS-F	
9	positive	1,29	positive	20,3	1/1 (100%)	RS-F	

° calculation p. 19

	Sample A		Sample B	
Number positive	9		13	
Number negative	4		0	
Percent positive	69		100	
Percent negative	31		0	
Consensus value	none		positive	

Methods:

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

Comments:
The consensus value for sample B is in qualitative agreement with the spiking of sample B. For sample A no consensus value was obtained for qualitative valuation, because there were less than 75% negative and positive results, respectively. The positive results were only slightly higher than the stated limits of quantification of the methods.

Quantitative valuation of ELISA-results: Sample A

Evaluation number	Whole Egg Powder	z-Score Xpt_{ALL}	z-Score Xpt_{RS}	Method	Remarks
	[mg/kg]				
4	< LOD	-		AQ	result converted°
11	< 1,5	-		BC	result converted°
6	0	-		BF	
13	< 1,5	-		IL	result converted°
1	3,6	7,2		MI	result converted ° / and excluded
10	<10	-		MI	
8	1,30	0,06		MI-II	
12	1,49	0,66		MI-II	result converted°
2	1,20	-0,25	-0,12	RS-F	
3	1,07	-0,66	-0,54	RS-F	
5	1,32	0,12	0,27	RS-F	
7	1,30	0,06	0,21	RS-F	
9	1,29	0,03	0,17	RS-F	

° calculation p. 19

Methods:

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

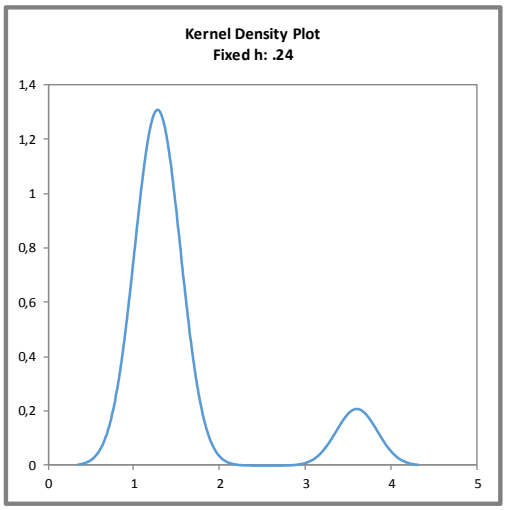


Abb. / Fig. 1:
Kerndichte-Schätzung aller ELISA-Ergebnisse (mit h = 0,75 x σ_{pt} von Xpt_{ALL})

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

Comments:
The kernel density estimation shows nearly a symmetrical distribution of results with a side-peak caused by one result at 3,6 mg/kg (method MI), which was the excluded outlier.

Characteristics: Quantitative evaluation ELISA: Egg (as Whole Egg Powder)

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	7°	5
Number of outliers	1	0
Mean	1,28	1,24
Median	1,30	1,29
Robust Mean (X)	**1,28**	**1,24**
Robust standard deviation (S*)	**0,145**	**0,117**
Target range:		
Target standard deviation σ_{pt}	**0,320**	**0,309**
lower limit of target range	**0,641**	**0,618**
upper limit of target range	**1,92**	**1,85**
Quotient S/σ_{pt}*	*0,45*	*0,38*
Standard uncertainty $U_{(Xpt)}$	*0,068*	*0,066*
Results in the target range	7	5
Percent in the target range	*100*	*100*

° *without result no. 1 (outlier excluded)*

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

Comments to the statistical characteristics and assigned values:

Results stated quantitatively above the limits of quantification were considered.

The kernel density estimation showed almost a symmetrical distribution of results with one side-peak. An outlier was excluded from statistical calculations.

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

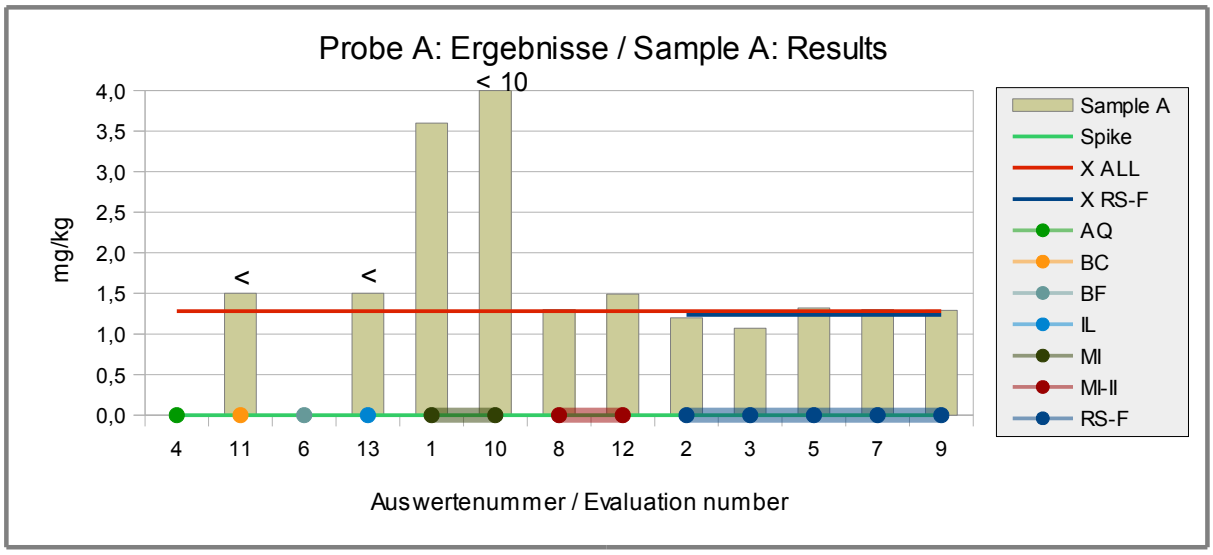


Abb./Fig. 2: ELISA Results Egg (as Whole Egg Powder)
green line = Spiking level
red line = Assigned value robust mean all results
blue line = Assigned value robust mean results method RS-F
round symbols = Applied methods (see legend)

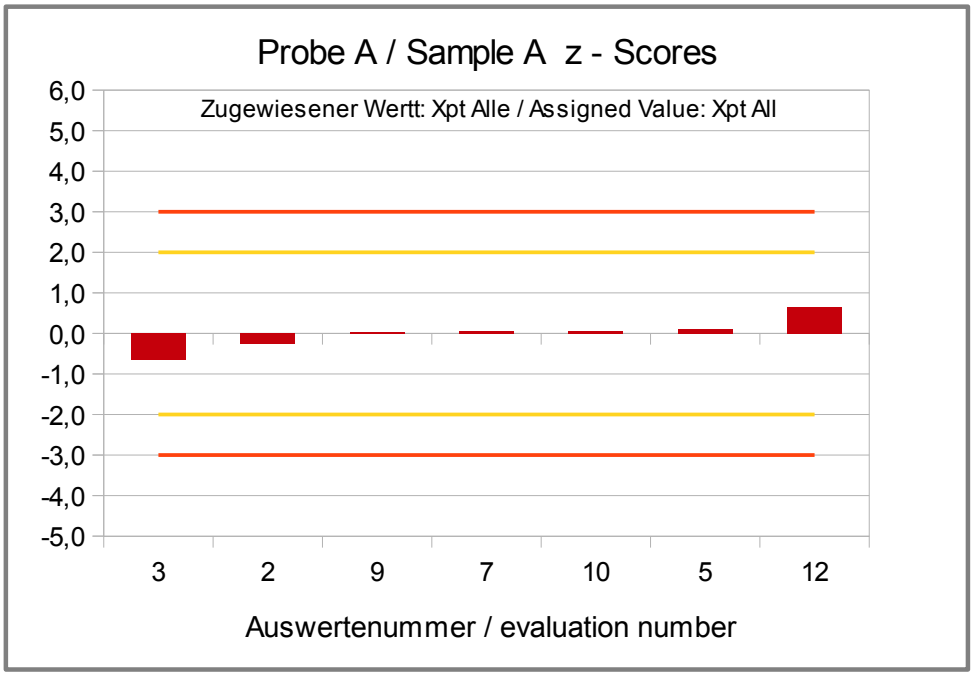


Abb./Fig. 3:
z-Scores (ELISA Results as Whole Egg Powder)
Assigned value robust mean (algorithm A) of all results

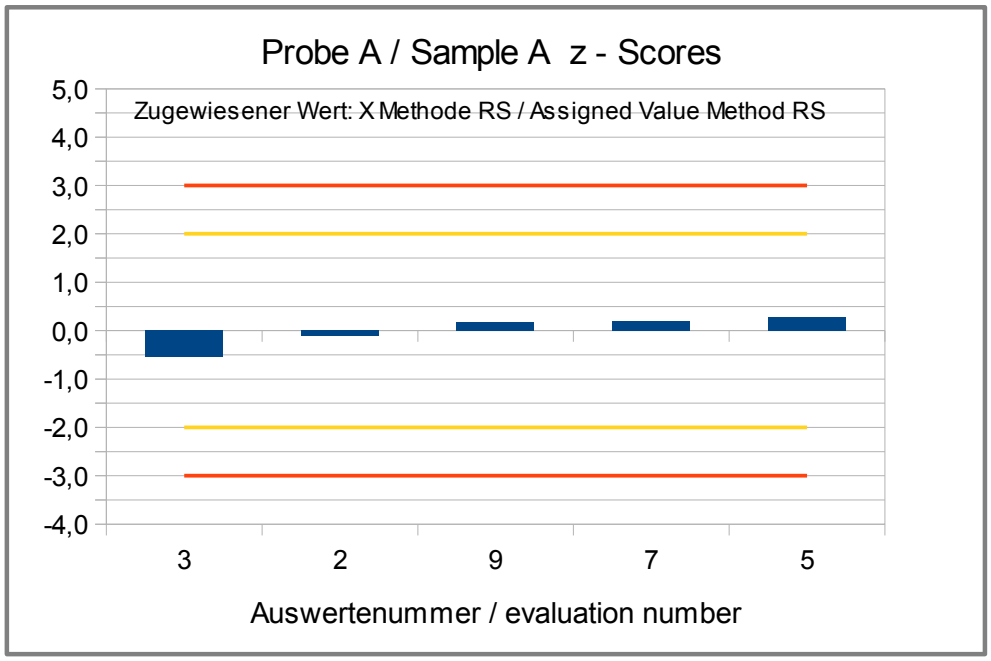


Abb./Fig. 4:
z-Scores (ELISA Results as Whole Egg Powder)
Assigned value median of method RS-F (R-Biopharm, Ridascreen® Fast)

Quantitative valuation of ELISA-results: Sample B

Evaluation number	Whole Egg Powder	z-Score Xpt_{ALL}	z-Score Xpt_{RS}	Method	Remarks
	[mg/kg]				
4	12,2	-1,5		AQ	result converted°
11	18,1	-0,36		BC	result converted°
6	44,3	4,9		BF	
13	20,7	0,17		IL	result converted°
1	89,0	14		MI	result converted ° / and excluded
10	12,0	-1,6		MI	
8	23,0	0,63		MI-II	
12	20,8	0,19		MI-II	result converted°
2	18,2	-0,34	-0,46	RS-F	
3	24,2	0,87	0,70	RS-F	
5	21,2	0,27	0,12	RS-F	
7	19,0	-0,18	-0,31	RS-F	
9	20,3	0,08	-0,06	RS-F	

° calculation p. 19

Methods:

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

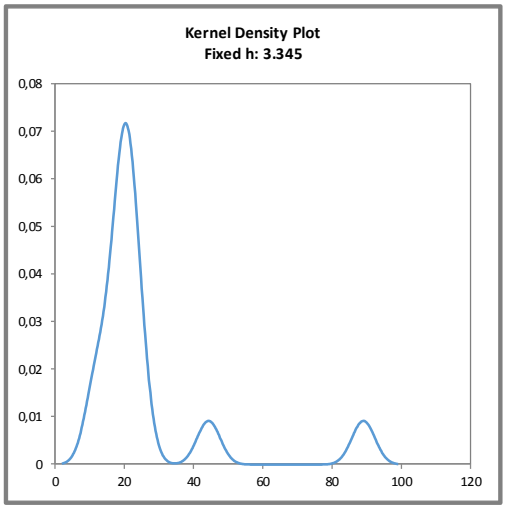


Abb. / Fig. 5:
Kerndichte-Schätzung aller ELISA-Ergebnisse (mit h = 0,75 x σ_{pt} von Xpt_{ALL})

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

Comments:
The kernel density estimation shows nearly a symmetrical distribution of results with two side-peaks at 44 mg/kg (method BF) and at approx. 90 mg/kg, which was the excluded outlier.

Characteristics: Quantitative evaluation ELISA: Egg (as Whole Egg Powder)

Sample B

Statistic Data	**All Results** *[mg/kg]*	**Method RS-F** *[mg/kg]*
Assigned value (X_{pt})	$X_{pt_{ALL}}$	$X_{pt_{METHOD\ RS\text{-}F}}$
Number of results	12	5
Number of outliers	1	0
Mean	21,2	20,6
Median	20,5	20,3
Robust Mean (X)	**19,9**	**20,6**
Robust standard deviation (S*)	**4,46**	**2,64**
Target range:		
Target standard deviation σ_{pt}	**4,97**	**5,14**
lower limit of target range	**9,94**	**10,3**
upper limit of target range	**29,8**	**30,9**
Quotient S/σ_{pt}*	*0,90*	*0,51*
Standard uncertainty $U_{(Xpt)}$	*1,61*	*1,48*
Results in the target range	*11*	*5*
Percent in the target range	*92*	*100*

° *without result no. 1 (outlier excluded)*

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

Comments to the statistical characteristics and assigned values:

The kernel density estimation showed almost a symmetrical distribution of results with two side-peaks caused by two single results. An outlier was excluded from statistical calculations.

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

The assigned values X_{pt} of the evaluation of all results and method RS-F were 87% and 90% of the spiking level of egg to sample B and thus within the range of the recommendations for the applied methods (s. 3.4.3 and "Recovery rates of Egg" p.33).

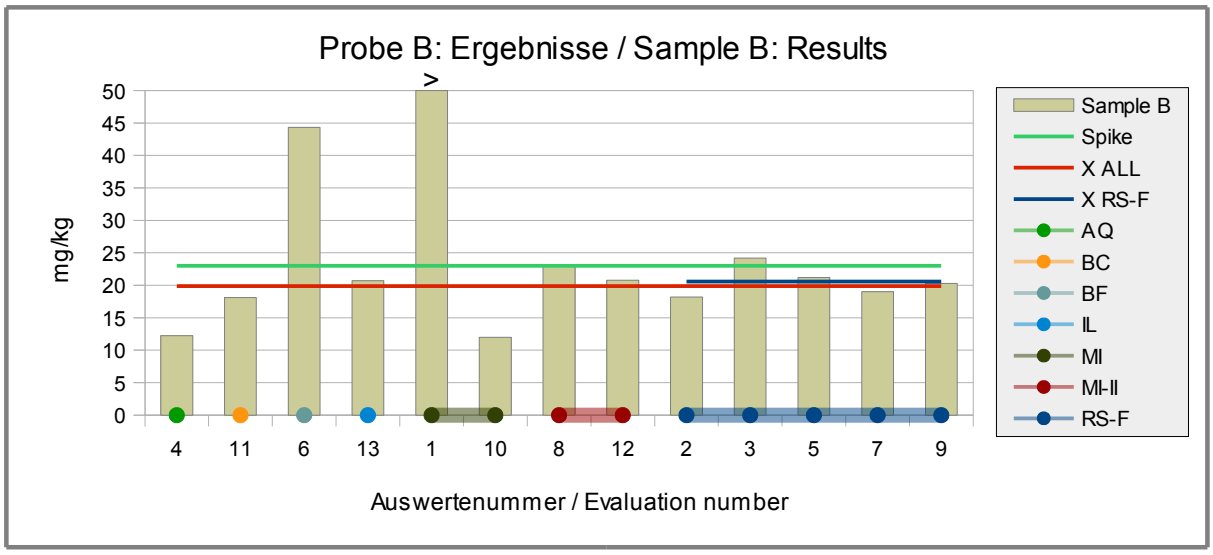


Abb./Fig. 6: ELISA Results Egg (as Whole Egg Powder)
green line = Spiking level
red line = Assigned value robust mean all results
blue line = Assigned value robust mean results method RS-F
round symbols = Applied methods (see legend)

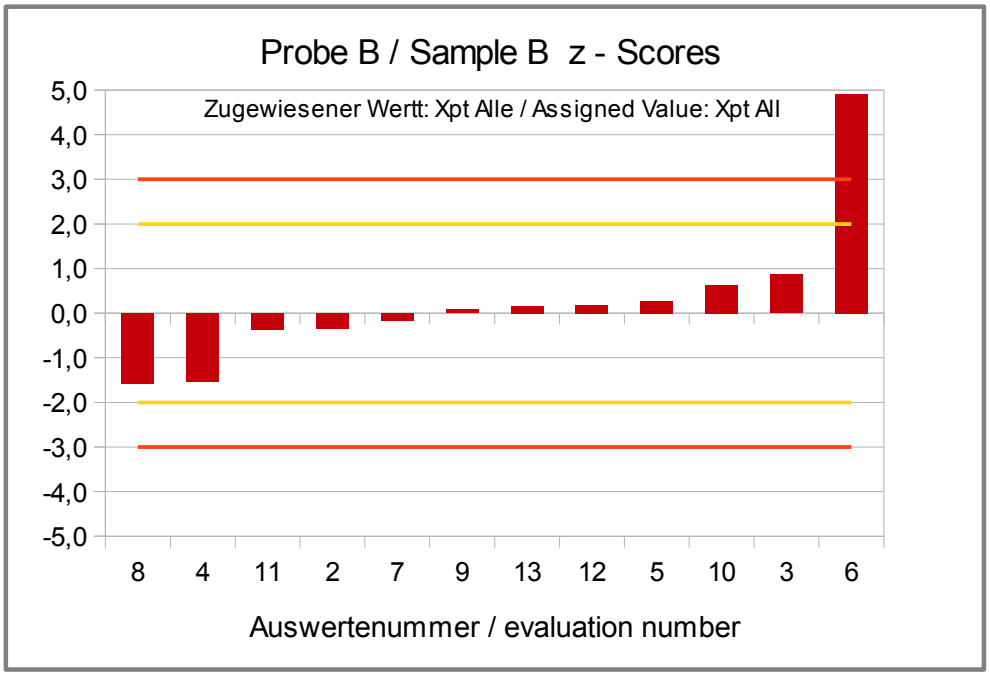


Abb./Fig. 7:
z-Scores (ELISA Results as Whole Egg Powder)
Assigned value robust mean (algorithm A) of all results

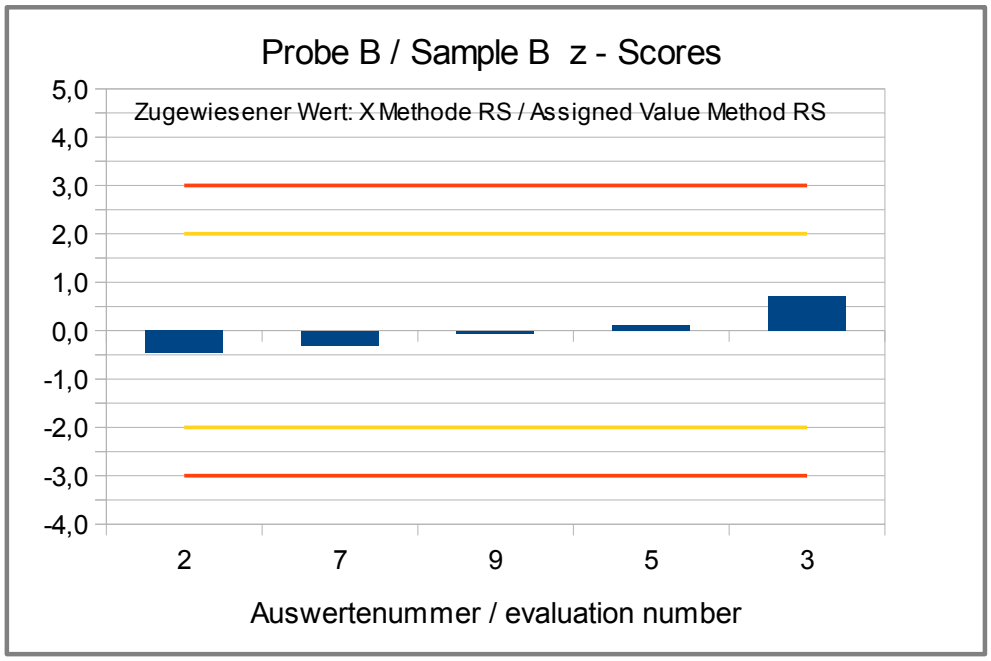


Abb./Fig. 8:
z-Scores (ELISA Results as Whole Egg Powder)
Assigned value median of method RS-F (R-Biopharm, Ridascreen® Fast)

Quantitative evaluation of ELISA results: Spiking level sample

Evaluation number	Whole Egg Powder	z-Score Xpt_{ALL}	Method	Remarks
	[mg/kg]			
4	8,96	-2,3	AQ	result converted°
11	15,2	-1,0	BC	result converted°
6	24,9	0,86	BF	
13	23,7	0,62	IL	result converted°
1	49,9	5,7	MI	result converted °
10	7,00	-2,6	MI	
8	21,0	0,10	MI-II	
12	21,0	0,10	MI-II	result converted°
2		-4,0	RS-F	
3	20,3	-0,04	RS-F	
5	21,1	0,12	RS-F	
7	18,0	-0,49	RS-F	
9	30,9	2,0	RS-F	

° calculation p. 19

Methods:

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

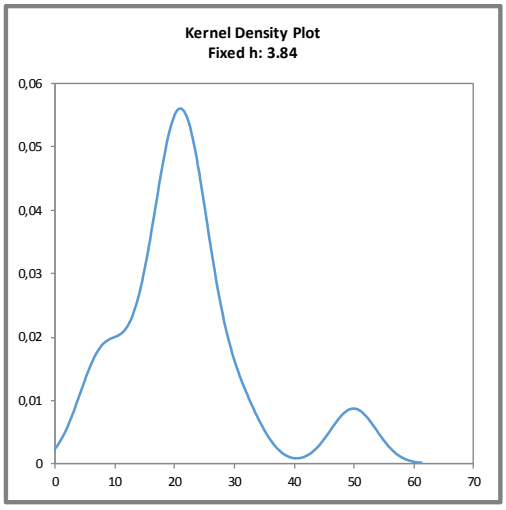


Abb. / Fig. 9:
Kerndichte-Schätzung aller ELISA-Ergebnisse (mit h = 0,75 x σpt von Xpt_{ALL})

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

Comments:
The kernel density estimation shows nearly a symmetrical distribution of results with a shoulder at approx. 10 mg/kg and a side-peak at approx. 50 mg/kg, which was out of the target range.

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	12
Number of outliers	0
Mean	21,8
Median	21,0
Robust Mean (X_{pt})	**20,5**
Robust standard deviation (S*)	**8,69**
Target range:	
Target standard deviation σ_{pt}	**5,12**
lower limit of target range	**10,2**
upper limit of target range	**30,7**
Quotient S/σ_{pt}*	*1,7*
Standard uncertainty $U_{(Xpt)}$	*3,14*
Results in the target range	*9*
Percent in the target range	*75*

Comments to the statistical characteristics and assigned values:

The kernel density estimation showed almost a symmetrical distribution of results.

The evaluation of all methods showed a normal variability of results. The quotient S/σ_{pt} was below 2,0. The robust standard deviation is in the range of established values for the reproducibility standard deviation of the applied methods (see 3.4.2 value by precision experiments and 3.4.3 value by perception). The comparability of results is given. This conclusion is limited for the evaluation across the methods, because there were only a few results for some methods.*

The assigned value X_{pt} of the evaluation of all results was 91% of the spiking level of egg to sample B and thus within the range of the recommendations for the applied methods (s. 3.4.3 and "Recovery rates of Egg" p.33).

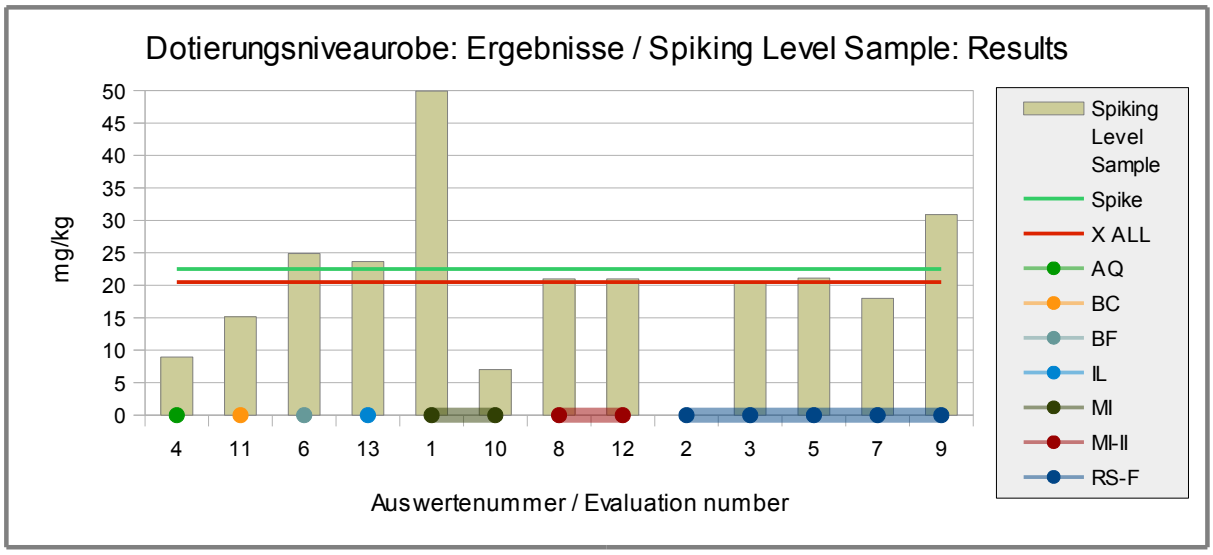


Abb./Fig. 10: ELISA Results Egg (as Whole Egg Powder)
green line = Spiking level
red line = Assigned value robust mean all results
blue line = Assigned value robust mean results method RS-F
round symbols = Applied methods (see legend)

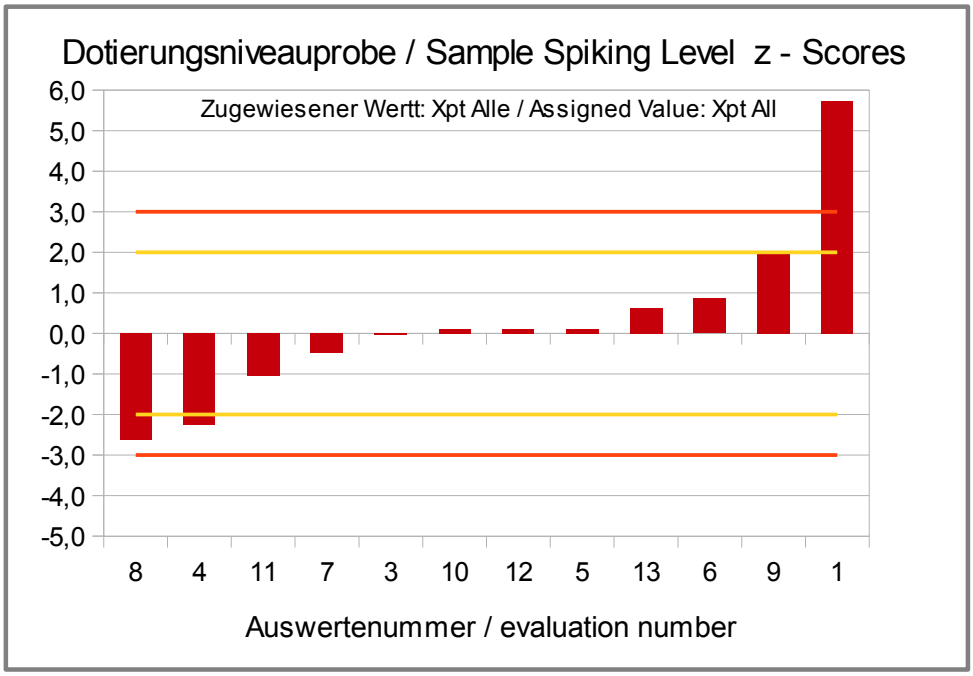


Abb./Fig. 11:
z-Scores (ELISA Results as Whole Egg Powder)
Assigned value robust mean (algorithm A) of all results

Recovery Rates ELISA for Egg (as Whole Egg Powder): Spiking level Sample and Sample B

Evaluation number	Spiking Level Sample	Recovery rate*	Sample B	Recovery rate*	Method	Remarks
	[mg/kg]	[%]	[mg/kg]	[%]		
4	8,96	40	12,2	**53**	AQ	result converted°
11	15,2	**67**	18,1	**79**	BC	result converted°
6	24,9	**111**	44,3	193	BF	
13	23,7	**105**	20,7	**90**	IL	result converted°
1	49,9	222	89,0	387	MI	result converted °
10	7,00	31	12,0	**52**	MI	
8	21,0	**93**	23,0	**100**	MI-II	
12	21,0	**93**	20,8	**90**	MI-II	result converted°
2			18,2	**79**	RS-F	
3	20,3	**90**	24,2	**105**	RS-F	
5	21,1	**94**	21,2	**92**	RS-F	
7	18,0	**80**	19,0	**83**	RS-F	
9	30,9	**137**	20,3	**88**	RS-F	

° calculation p. 19

RA**	50-150 %	RA**	50-150 %
Number in RA	**9**	Number in RA	**11**
Percent in RA	**75**	Percent in RA	**85**

* Recovery rate 100% relative size: Whole egg powder, see p. 5

** Range of acceptance of AOAC for allergen ELISAS

Methods:

AQ = AgraQuant, RomerLabs

BC = BioCheck ELISA

BF = MonoTrace ELISA, BioFront Technologies

IL = Immunolab

MI = Morinaga Institute ELISA

MI-II = Morinaga Institute ELISA Kit II

RS-F= Ridascreen® Fast, R-Biopharm

Comments:

For the spiking level sample 75% (9) of the participants obtained a recovery rate within the range of the AOAC-recommendation of 50-150%. For the spiked food matrix sample sample B 85% (11) of the recovery rates were in the range of acceptance.

Since traces of whole egg powder close to the LOQ in the food matrix sample A were determined by the ELISA methods, the recovery rates stated for sample B could be overestimated by about 6-7%.

4.1.2 PCR Results: Egg

Comments:
None of the participants used a PCR method for determination of egg.

4.2 Proficiency Test Fish

4.2.1 ELISA-Results: Fish (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		
1	negative	-	positive	323	2/2 (100%)	AQ	result converted °
9	negative	<5	positive	23,0	2/2 (100%)	BC	
13	negative	<0,5	positive	32,0	2/2 (100%)	IL	

° calculation p.19

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

Methods:
AQ = AgraQuant, RomerLabs
BC = BioCheck ELISA
IL = Immunolab

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

Quantitative Valuation ELISA: Samples B

No quantitative evaluation was done, because there were to few quantitative results.

(Quantitative) valuation of ELISA results: Spiking Level Sample

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

Evaluation number	Cod, fresh	Cod, fresh	Method	Remarks
	pos/neg	[mg/kg]		
1	positive	530	AQ	result converted °
9	positive	40,6	BC	
13	positive	60,0	IL	

° calculation p.19

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

Methods:

AQ = AgraQuant, RomerLabs

BC = BioCheck ELISA

IL = Immunolab

Comments:

A kernel density estimation was not made due to the number of results less than 8.

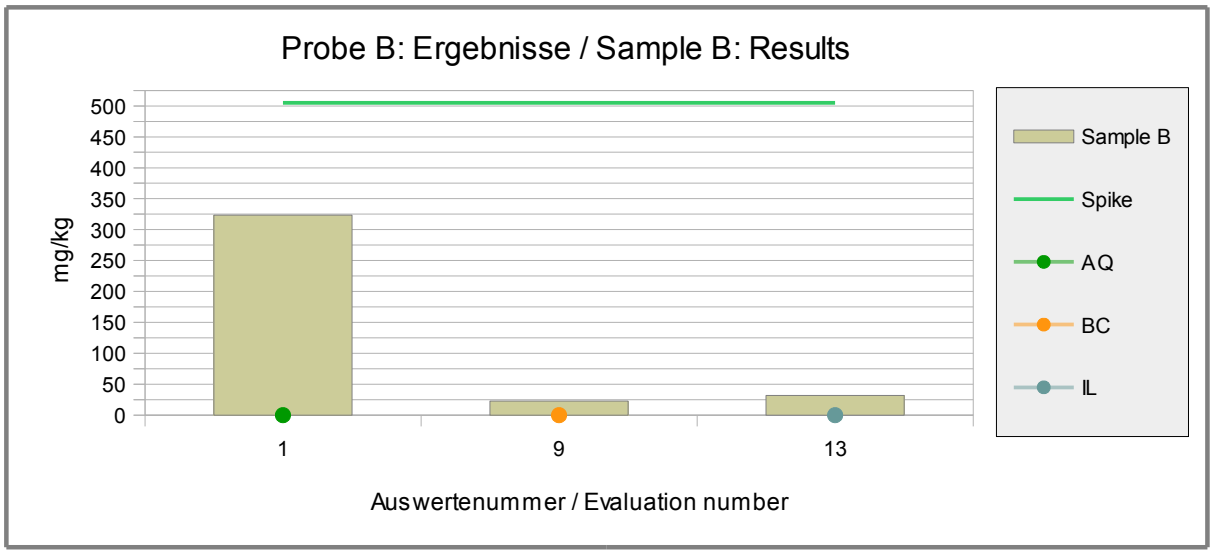


Abb./Fig. 12: ELISA Results Fish (as fresh Cod) Sample B
green line = Spiking level
round symbols = Applied methods (see legend)

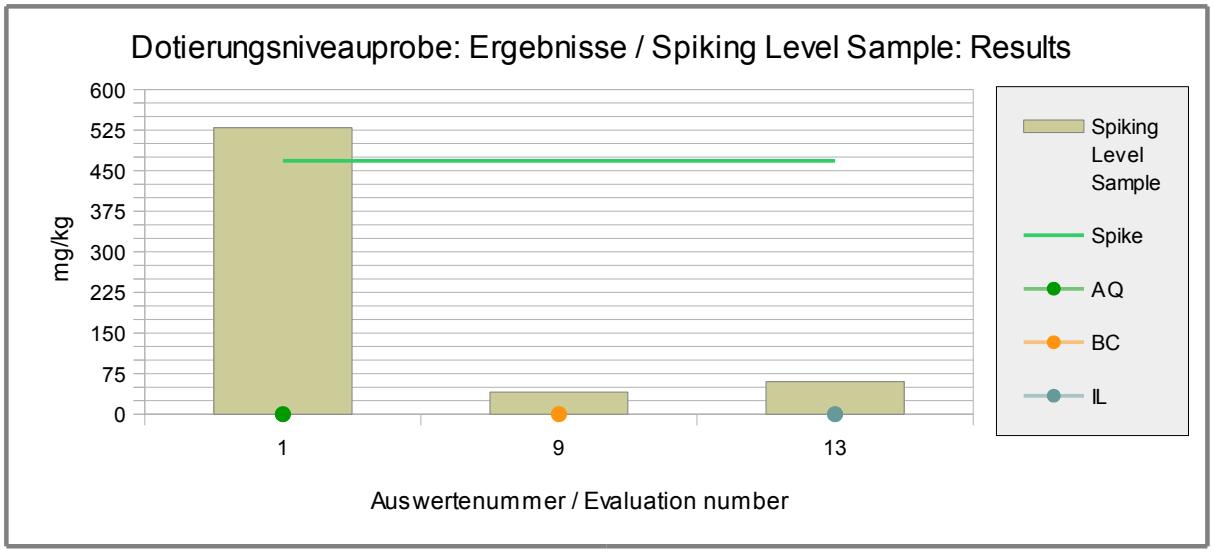


Abb./Fig. 13: ELISA Results Fish (as fresh Cod) Spiking Level Sample
green line = Spiking level
round symbols = Applied methods (see legend)

Recovery Rates ELISA for Fish (as fresh Cod): Spiking level Sample and Sample B

Evaluation number	Spiking Level Sample	Recovery rate*	Sample B	Recovery rate*	Method	Remarks
	[mg/kg]	[%]	[mg/kg]	[%]		
1	530	**113**	323	**64**	AQ	result converted °
9	40,6	8,7	23,0	4,6	BC	
13	60,0	13	32,0	6,3	IL	

° calculation p.19

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

Methods:
AQ = AgraQuant, RomerLabs
BC = BioCheck ELISA
IL = Immunolab

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

** Range of acceptance of AOAC for allergen ELISAS

Comments:
One participant obtained recoveries by ELISA for both the spiking level sample and the spiked food matrix sample B within the range of the AOAC recommendation of 50–150%.

4.2.2 PCR Results: Fish (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	negativ		positiv	224	2/2 (100%)	ASU	result converted °
10	negativ		negativ		1/2 (50%)	ASU	
9a	negativ	<10	negativ	<10	1/2 (50%)	IM	tuna specific
2	negativ		positiv		2/2 (100%)	SFA-ID	
5	negativ		positiv		2/2 (100%)	SFA-ID	
7	negativ	<1	positiv	>1	2/2 (100%)	SFA-ID	
9b	negativ	<1	positiv	27,7	2/2 (100%)	SFA-ID	
12	negativ		positiv		2/2 (100%)	div	

° calculation p.19

	Sample A		Sample B	
Number positive	0		6	
Number negative	8		2	
Percent positive	0		75	
Percent negative	100		25	
Consensus value	negativ		positiv	

Methods:

ASU = ASU §64 Methode/method
IM = Imegen Tuna ID Kit
SFA-ID = Sure Food Allergen ID, 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. There were two negative results for sample B, one result was obtained by a PCR method specific for tuna.

Quantitative Valuation PCR: Samples B

No quantitative evaluation was done, because there were to few quantitative results.

(Quantitative) valuation of PCR results: Spiking Level Samples

No quantitative evaluation was done, because there was only one quantitative result.

Evaluation number	Fish, fresh	Fish, fresh	Method	Remarks
	pos/neg	[mg/kg]		
8	positive	358	ASU	result converted °
10	negative		ASU	
9a	negative	<10	IM	tuna specific
2	positive		SFA-ID	
5	positive		SFA-ID	
7	positive	>1	SFA-ID	
9b	positive	44,4	SFA-ID	
12	positive		div	

° calculation p.19

Number positive	6
Number negative	2
Percent positive	75
Percent negative	25
Consensus value	positive

Methods:

ASU = ASU §64 Methode/method
IM = Imegen Tuna ID Kit
SFA-ID = Sure Food Allergen ID, R-Biopharm / Congen
div = keine genaue Angabe / andere Methode
div = not indicated / other method

Comments:
For the spiking level sample there were 75% (6) positive results. There were two negative results for sample B, one result was obtained by a PCR method specific for tuna.

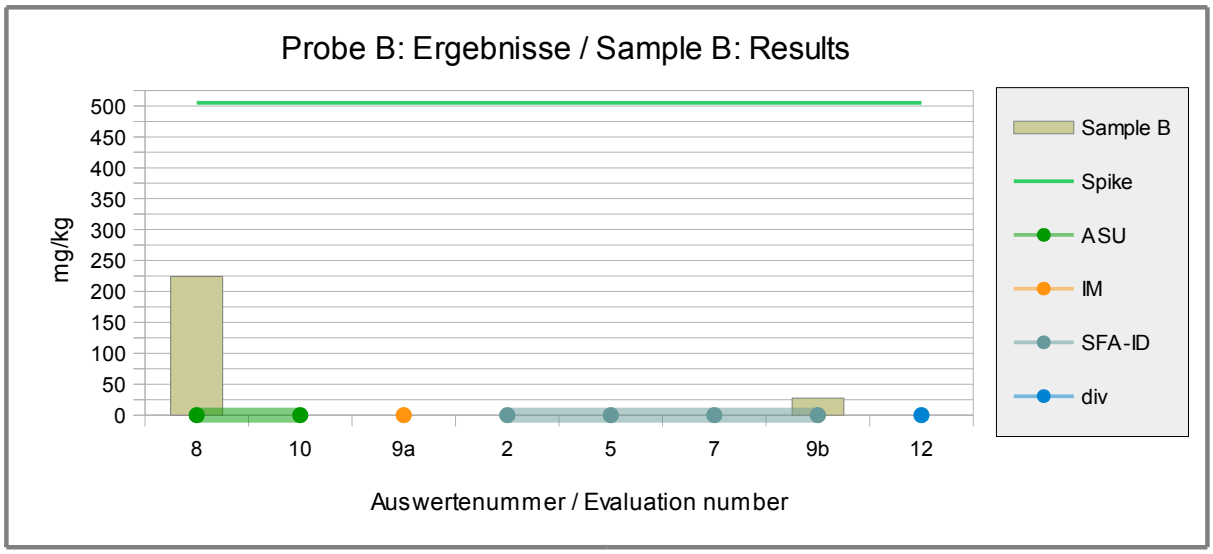


Abb./Fig. 14: PCR Results Fish Sample B
green line = Spiking level
round symbols = Applied methods (see legend)

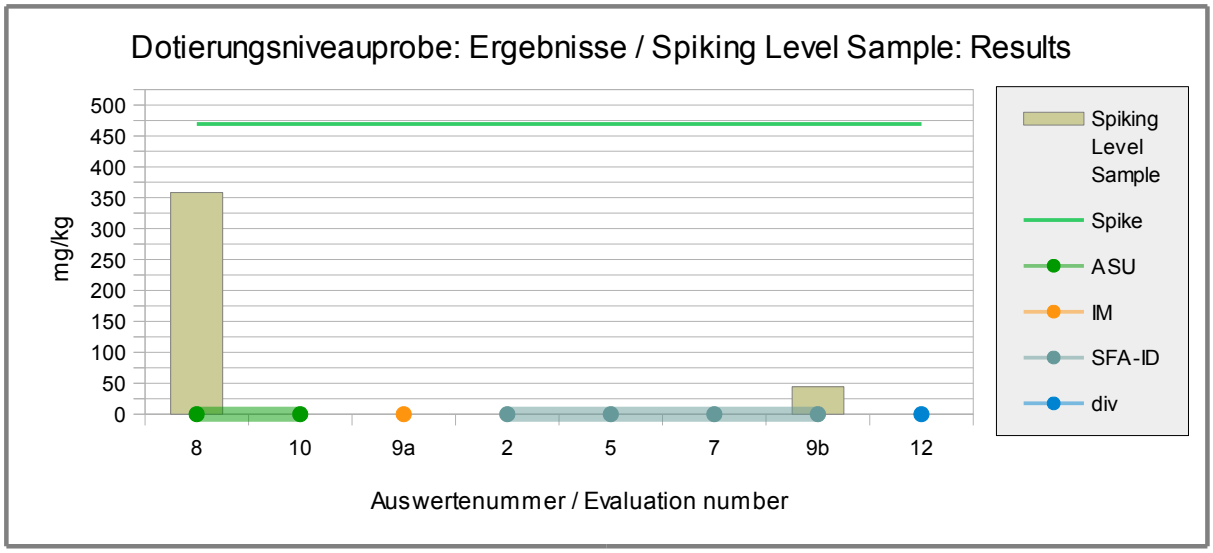


Abb./Fig. 15: PCR Results Fish Spiking Level Sample
green line = Spiking level
round symbols = Applied methods (see legend)

Recovery Rates PCR for Fish (fresh Cod): Spiking level Sample and Sample B

Evaluation number	Spiking Level Sample	Recovery rate*	Sample B	Recovery rate*	Method	Remarks
	[mg/kg]	[%]	[mg/kg]	[%]		
8	358	**76**	224	44	ASU	result converted °
10					ASU	
9a	<10		<10		IM	tuna specific
2					SFA-ID	
5					SFA-ID	
7	>1		>1		SFA-ID	
9b	44,4	9,5	27,7	5,5	SFA-ID	
12					div	

° calculation p.19

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

Methods:

ASU = ASU §64 Methode/method

IM = Imegen Tuna ID Kit

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

div = not indicated / other method

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

** Range of acceptance of AOAC for allergen ELISAS

Comments:

One participant obtained for the spiking level sample a recovery rate within the range of the AOAC recommendation of 50-150%. For the spiked food matrix sample B none of the recovery rates were within the AOAC recommendation.

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. Abr.	Evaluation number	Date of Analysis	Result Sample A		Result Sample B		Result Spiking Sample		quantitative Result given as	Method
		Tag/Monat	qualitative	mg/kg	qualitative	mg/kg	qualitative	mg/kg	z.B. Lebensmittel / Protein	Test-Kit + Anbieter
AQ	4	07.03.18	negative	< LOD	positive	3,18	positive	2,33	Egg white proteins, total	AQ = AgraQuant ELISA Egg White COKAL0848, RomerLabs
BC	11	01.02.18	negative	<0.4	positive	4,7	positive	3,94	Egg white proteins, total	BC = BioCheck ELISA Egg-Check
BF	6	16/3	negative	0	positive	44,3	positive	24,9	Whole egg powder	BF = MonoTrace Egg ELISA kit, BioFront Technologies
IL	13	05.02.18	negative	< 0,4	positive	5,38	positive	6,15	Egg white proteins, total	IL = Immunolab Egg white ELISA
MI	1	06.02.18	positive	0,5	positive	12,5	positive	13,1	ovalbumin	MI = Morinaga Egg (Ovalbumin) ELISA Kit (M2101)
MI	10	28.02.18	positive	<10	positive	12	positive	7	Whole egg powder	MI = Morinaga Egg (Ovalbumin) ELISA Kit (M2101)
MI-II	8	15.02.18	positive	1,3	positive	23	positive	21	Whole egg powder	MI = Morinaga Egg (Ovalbumin) ELISA Kit II (M2111)
MI-II	12		positive	0,71	positive	9,9	positive	10	Whole egg protein	MI = Morinaga Egg (Ovalbumin) ELISA Kit II (M2111)
RS-F	2	22.02.18	positive	1,2	positive	18,2	positive		Whole egg powder	RS-F = Ridascreen® FAST Egg Protein R6402, R-Biopharm
RS-F	3		positive	1,07	positive	24,2	positive	20,3	Whole egg powder	RS-F = Ridascreen® FAST Egg Protein R6402, R-Biopharm
RS-F	5	22.02.	positive	1,32	positive	21,2	positive	21,1	Whole egg powder	RS-F = Ridascreen® FAST Egg Protein R6402, R-Biopharm
RS-F	7	16.03.18	positive	1,3	positive	19	positive	18	Egg	RS-F = R6402 RIDASCREEN FAST Egg Protein R-Biopharm
RS-F	9	31.01.18	positive	1,29	positive	20,27	positive	30,89	Whole egg powder	RS-F = Ridascreen® FAST Egg Protein R6402, R-Biopharm

***Continuation ELISA Egg*:**

Meth. Abr.	Evaluation number	Specifity	Remarks to the Method (Extraction and Determination)	Method accredited ISO/IEC 17025	Further Remarks
		Antibody	e.g. Extraction Solution / Time / Temperature	yes/no	Meth. Abk.
AQ	4		As per kit instructions	yes	
BC	11		0.5g of sample, 10ml Extract Buffer, 15min Shaking incubator at 50°C.	yes	n/a
BF	6	Monoclonal anti-Egg OM	Extracted for 10 minutes at 60C	No	
IL	13	Ovomucoid			Conversion factor to w hole egg pow der = 4,
MI	1		as per method	yes	Sample A w as run once, and then in triplicate as follow up to confirm the low positive. Single results for Sample B and Spiking Level Sample
MI	10			no	
MI-II	8			yes	
MI-II	12	Hen's egg protein	As per kit instructions	yes	
RS-F	2		As per kit instructions	yes	
RS-F	3			no	
RS-F	5		As per kit instructions		
RS-F	7			no	
RS-F	9	As per kit instructions	As per kit instructions	Yes	

5.1.2 ELISA: Fish

Meth. Abr.	Evaluation number	Date of Analysis	Result Sample A		Result Sample B		Result Spiking Sample		quantitative Result given as	Method
		Tag/Monat	qualitative	mg/kg	qualitative	mg/kg	qualitative	mg/kg	z.B. Lebensmittel / Protein	Test-Kit + Anbieter
AQ	1	02.03.18	negative	-	positive	36,1	positive	59,1	fish protein (cod)	AQ = AgraQuant ELISA Fish COKAL2548, RomerLabs
BC	9	31.01.18	negative	<5	positive	23	positive	40,62	fresh cod	BC = BioCheck ELISA Fish-Check
IL	13	05.02.18	negative	< 0,5	positive	32	positive	60	fresh cod	IL = Immunolab Fish (parvalbumin) ELISA

Meth. Abr.	Evaluation number	Specifity	Remarks to the Method (Extraction and Determination)	Method accredited ISO/IEC 17025	Further Remarks
		Antibody	e.g. Extraction Solution / Time / Temperature	yes/no	
AQ	1		as per method	yes	Single results
BC	9	As per kit instructions	As per kit instructions	Yes	Reported as Fresh Cod
IL	13	Parvalbumin	AuExcerpt from IFU: "It has to be taken into account that the standardization as w ell as the conversion factors refer to fresh fish.The degree of processing of the respective foodstuff has to be considered in the interpretation of the results.Validation tests show ed that cooked cod (20 min) has a reactivity of 25% compared to fresh cod. "		

5.1.3 PCR: Fish

Meth. Abr.	Evaluation number	Date of Analysis	Result Sample A		Result Sample B		Result Spiking Sample		quantitative Result given as	Method
			qualitative	mg/kg	qualitative	mg/kg	qualitative	mg/kg	mg/kg	
ASU	8	28.02.18	negative		positive	25	positive	40	Cod, dried	ASU §64 Methode/method
ASU	10	28.02.18	negative		negative		negative		DNA Fish	ASU §64 Methode/method
IM	9a	10.03.18	negative	<10	negative	<10	negative	<10	other: please fill in!	
SFA-ID	2	28.02.18	negative		positive		positive		DNA Fish	SFA-ID = Sure Food Allergen ID, R-Biopharm / Congen
SFA-ID	5	27.02.18	negative		positive		positive		DNA Fish	SFA-ID = Sure Food Allergen ID, R-Biopharm / Congen
SFA-ID	7	16.03.18	negative	<1	positive	>1	positive	>1	DNA Fish	SFA-ID = SureFood® ALLERGEN Fish Art.-No. S3110 Congen
SFA-ID	9b	14.02.18	negative	<1	positive	27,7	positive	44,44	Fish, fresh	SFA-ID = Sure Food Allergen ID, R-Biopharm / Congen
div	12		negative		positive		positive		DNA Fish	

Meth. Abr.	Evaluation number	Specifity	Remarks to the Method (Extraction and Determination)	Method accredidet ISO/IEC 17025	Further Remarks
		Target-Sequence / -DNA	e.g. Extraction / enzymes / clean-up / real time PCR / gel electrophoresis / cycles	yes/no	
ASU	8		Extraction: CTAB-precipitation method. PCR: Hoxc 13 Gen. Tetzlaff, C. & Mäde, D. (2017) Development of a real-time PCR system for the detection of the potential allergen fish in food. Eur Food Res Technol 243: 849. https://doi.org/10.1007/s00217-016-2799-5.	yes	Method from § 64 WG, not published yet
ASU	10		Wizard cleanup	no	
IM	9a	As per kit instructions	As per kit instructions	No	Only Detects Tuna - Using Imegen Tuna ID Kit
SFA-ID	2		As per kit instructions	yes	
SFA-ID	5		As per kit instructions, extraction w ith SureFood Prep Advanced, Protocl 2		
SFA-ID	7			no	
SFA-ID	9b	As per kit instructions	As per kit instructions	No	
div	12		CTAB, Proteinase K, Promega Wizard DNA CleanUp, Real Time PCR, 45 Cycles	yes	internal method

5.2 Homogeneity

5.2.1 Mixture homogeneity before bottling

Microtracer Homogeneity Test
DLA 01-2018 Sample B

Weight whole sample	2,69	kg
Microtracer	FSS-rot lake	
Particle size	75 – 300	µm
Weight per particle	2,0	µg
Addition of tracer	13,4	mg/kg

Result of analysis

Sample	Weight [g]	Particle number	Particles [mg/kg]
1	5,04	36	14,3
2	5,05	26	10,3
3	4,97	33	13,3
4	5,03	30	11,9
5	5,12	35	13,7
6	4,97	30	12,1
7	5,13	27	10,5
8	4,99	27	10,8

Poisson distribution		
Number of samples	8	
Degree of freedom	7	
Mean	30,5	Particles
Standard deviation	3,81	Particles
χ^2 (CHI-Quadrat)	3,33	
Probability	**85**	%
Recovery rate	90	%

Normal distribution		
Number of samples	8	
Mean	12,1	mg/kg
Standard deviation	1,51	mg/kg
rel. Standard deviaton	12,5	%
Horwitz standard deviation	11,0	%
HorRat-value	**1,1**	
Recovery rate	90	%

Microtracer Homogeneity Test
DLA 01-2018 Spiking Level Sample

Weight whole sample	1,51	kg
Microtracer	FSS-rot lake	
Particle size	75 – 300	µm
Weight per particle	2,0	µg
Addition of tracer	15,5	mg/kg

Result of analysis

Sample	Weight [g]	Particle number	Particles [mg/kg]
1	4,99	42	16,8
2	5,06	41	16,2
3	5,03	41	16,3
4	4,99	47	18,8
5	5,03	49	19,5
6	4,97	44	17,7
7	5,08	52	20,5
8	5,03	41	16,3

Poisson distribution		
Number of samples	8	
Degree of freedom	7	
Mean	44,6	Particles
Standard deviation	4,14	Particles
χ^2 (CHI-Quadrat)	2,69	
Probability	**91**	%
Recovery rate	115	%

Normal distribution		
Number of samples	8	
Mean	17,8	mg/kg
Standard deviation	1,65	mg/kg
rel. Standard deviaton	9,3	%
Horwitz standard deviation	10,4	%
HorRat-value	**0,89**	
Recovery rate	115	%

5.3 Information on the Proficiency Test (PT)

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

PT number	***DLA 01-2018***
PT name	***Allergens I: Fish and Egg in Sauce Powder***
Sample matrix (processing)	***Samples A + B:*** *Sauce powder/ ingredients: onions, starch, salt, fried onions, vegetable fats (palm, shea), yeast extract, seasoning, sugar, thickener: guar gum, maltodextrin, garlic, spices, flavoring, caramel sugar and potato powder, other food additives and allergenic foods whole egg powder and fish powder (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: room temperature (long term cooled 2 - 10°C) Spiking Level Sample: room temperature*
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 Spiking Level Sample: < 500 mg/kg*
Methods of analysis	*Analytical methods are optional*
Notes to analysis	*The analysis of PT samples should be performed like a routine laboratory analysis. In general we recommend to homogenize a representative sample amount before analysis according to good laboratory practice, especially in case of low sample weights. It is the best to homogenize the whole sample.*
Result sheet	*One result each should be determined for Samples A and B and the Spiking Level Sample. The results should be filled in the result submission file.*
Units	*mg/kg*
Number of digits	*at least 2*
Result submission	*The result submission file should be sent by e-mail to:* ***pt@dla-lvu.de***
Deadline	***the latest March 16th 2018***
Evaluation report	*The evaluation report is expected to be completed 6 weeks after deadline of result submission and sent as PDF file by e-mail.*
Coordinator and contact person of PT	*Matthias Besler-Scharf, PhD*

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

6. Index of participant laboratories in alphabetical order

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

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

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

7. Index of references

1. DIN EN ISO/IEC 17025:2005; Allgemeine Anforderungen an die Kompetenz von Prüf- und Kalibrierlaboratorien / General requirements for the competence of testing and calibration laboratories
2. DIN EN ISO/IEC 17043:2010; Konformitätsbewertung – Allgemeine Anforderungen an Eignungsprüfungen / Conformity assessment – General requirements for proficiency testing
3. ISO 13528:2015 & DIN ISO 13528:2009; Statistische Verfahren für Eignungsprüfungen durch Ringversuche / Statistical methods for use in proficiency testing by 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
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15. MTSE SOP No. 010.01 (2014): Quantitative measurement of mixing uniformity and carry-over in powder mixtures with the rotary detector technique, MTSE Micro Tracers Services Europe GmbH
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19. DIN EN ISO 15633-1:2009; Nachweis von Lebensmittelallergenen mit immunologischen Verfahren - Teil 1: Allgemeine Betrachtungen / Foodstuffs - Detection of food allergens by immunological methods - Part 1: General considerations
20. DIN EN ISO 15634-1:2009; Nachweis von Lebensmittelallergenen mit molekularbiologischen Verfahren - Teil 1: Allgemeine Betrachtungen / Foodstuffs - Detection of food allergens by molecular biological methods - Part 1: General considerations
21. DIN EN ISO 15842:2010 Lebensmittel – Nachweis von Lebensmittelallergenen –

Allgemeine Betrachtungen und Validierung von Verfahren / Foodstuffs - Detection of food allergens - General considerations and validation of methods
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