DLA
Dienstleistung
Lebensmittel
Analytik GbR

Evaluation Report
proficiency test

01/2016

Allergens I:

Egg and Fish

in Sauce Powder

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Content

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 validity of the particular testing method.
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. Realisation

2.1 Test material

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

The test material is a common in commerce instant sauce powder. The basic composition of both sample A and sample B was the same (see table 1). After sieving and homogenisation of the basic mixture an aliquot of it was added stepwise during several homogenisations to the spiking material which contained the allergenic ingredients egg and fish for preparation of sample B.
Then the samples were packaged in portions to approximately 25 g.

The composition of the spiking material sample and the amounts of allergens in sample B is given in table 2.

Table 1: Composition of DLA-Samples

Ingredients	**Sample A**	**Sample B**
Gravy Powder Ingredients: Starch, palm oil, iodized salt, rice flour, maltodextrin, yeast extract, flavors, tomato, caramel sugar syrup, pepper, sugar, garlic, onions, pepper, sunflower oil Nutrients per 100g: Protein 7.8 g, carbohydrates 52 g, fat 20 g Allergen information: may contain traces of egg, gluten, milk and celery.	100 g/100 g	99,6 g/100 g
Spiking material sample	-	0,36 g/100g

Table 2: Added amounts of allergenic ingredients

Ingredients	**Spiking material sample**	**Amounts in Sample B**
Potato flour Nutrients per 100g: Protein 0 g	94,9 %	0,34 %
Whole Egg Powder Ingredients: Hen's egg (pasteurized, spray dried) Nutrients per 100g: Protein > 45 g	21900 mg/kg (= 2,19 %)	78 mg/kg
- thereof Protein, total* - thereof Egg white protein*	10500 mg/kg 5690 mg/kg	38 mg/kg 20 mg/kg
Fish Powder Ingredients: Coalfish *(Pollachius virens)* (cooked, dried, milled) Nutrients per 100g: Protein 87 g	28700 mg/kg (= 2,87 %)	103 mg/kg
- thereof Fishprotein*	25000 mg/kg	89 mg/kg
calculated to: - Coalfish, fresh ** (wet weight, muscle tissue)	144000 mg/kg	514 mg/kg

* Protein content calculated according to labeling/specification/literature
** with water content of 80% (nutrient tables, Souci/Fachmann/Kraut)

2.1.1 Homogeneity

Homogeneity of the spiking material sample and spiked sample B was checked by ELISA-test for egg white proteins (fig. 1). The resulting standard deviation between the samples of < 15% ensured sufficient homogeneity (17, 18, 20).

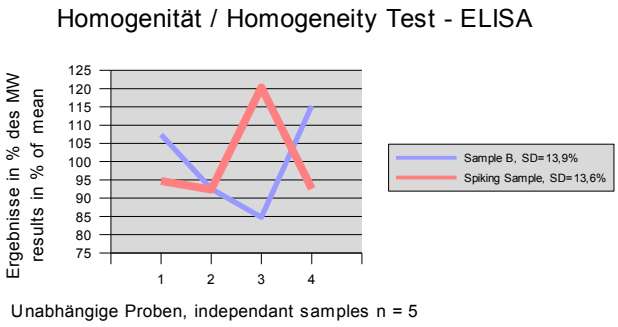

Fig. 1: Testing of homogeneity of DLA-sample B and spiking material sample. Results are given in percent of the arithmetic mean

2.2 Test

The portions of test material (sample A and sample B as well as the spiking material sample) were sent to every participating laboratory in the 2nd week of 2016. The testing method was optional. The tests should be finished at February 26th 2016 the latest.

2.3 Submission of results

The participants submitted their results in standard forms, which have been sent by email or were available on our website. On one hand the results given as positive/negative and on the other hand the indicated results of the allergenic ingredients e.g. whole egg powder and fresh fish in mg/kg were evaluated. The species of added fish was announced to the participants in the letter accompanied with the shipment of samples.
Queried and documented were the indicated results and details of the test methods like specifity, test kit manufacturer and hints about the procedure.
In case participants submitted several results for the same parameter obtained by different methods these results were evaluated with the same evaluation number with a letter as a suffix and indication of the related method.
One participant submitted no results. All other 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 (20, 21, 22, 23). It is for this reason that we contrast the results of the present proficiency test with several assigned values. Thereby it is possible to evaluate each single result in comparison to the mean of all results and/or in comparison to the mean of results obtained by a single method. For comparison the actually added amount is plotted in the figures of the results.

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

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

3.1 Consensus value from participants (assigned value)

The robust mean of the submitted results was used as assigned value (X) („consensus value from participants") providing a normal distribution. The calculation was done according to algorithm A as described in annex C of ISO 13528 (6).

In case an examination of the distribution of the submitted results, e.g. using the kernel density estimation (23), implies 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 Xi are made whenever possible.

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

i) ***Robust mean of all results*** - X_{ALL}
ii) ***Robust mean of single methods*** - $X_{METHOD\ i}$
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) (6).

3.2 Standard deviation

For comparison to the target standard deviation a robust standard deviation (S^x) was calculated. The calculation was done according to algorithm A as described in annex C of ISO 13528 (6).

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 (1, 6).
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 (6, 23).

Results are identified as outliers by the use of robust statistics. If a value deviates from the robust mean by more than 3 times the robust standard deviation, it is classified as an outlier (6). Detected outliers are stated for information only, when z-score are < -2 or > 2. Due to the use of robust statistics outliers are not excluded, provided that no other reasons are present (6).

3.4 Target standard deviation

The target standard deviation of the assigned value is determined according to the following methods.

3.4.1 General model (Horwitz)

The relative target standard deviation in % of the assigned value is derived from following equation (Horwitz)

$$\hat{\sigma}_{(\%)} = 2^{(1-0,5\log X)}$$

From the result the target standard deviation is calculated

$$\hat{\sigma} = X * \hat{\sigma}_{(\%)} / 100.$$

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

3.4.2 Value by precision experiment

Using the reproducibility standard deviation σ_R and the repeatability standard deviation σ_r of a precision experiment the between-laboratories standard deviation can be calculated σ_L :

$$\sigma_L = \sqrt{(\sigma_R^2 - \sigma_r^2)} \quad .$$

And then, using the number of replicate measurements n, each participant is to perform, the target standard deviation for proficiency assessment is calculated :

$$\hat{\sigma} = \sqrt{(\sigma_L^2 + (\sigma_r^2/n))} \quad .$$

Because in the present proficiency test the number of replicate measurements is n = 1, the reproducibility standard deviation σ_R is identical to the target standard deviation $\hat{\sigma}$.

The following table shows the relative reproducibility standard deviations from proficiency tests of ELISA-methods from German ASU §64 methods (24, 25, 26):

Method	**Parameter**	**Matrix**	**Mean values**	**Relative σ_R**	**Literature**
ELISA	Soy protein	Sausage	0,36 - 4,07%	14 - 28%	L 06.00-56
ELISA (Manuf. A)	Peanut	Milk chocolate	5,9 - 174 mg/kg	20 - 31%	L 00.00-69
ELISA (Manuf. B)	Peanut	Milk chocolate	10,1 - 216 mg/kg	14 - 32%	L 00.00-69
ELISA (Manuf. A)	Peanut	Dark chocolate	5,7 - 148 mg/kg	22 - 33%	L 00.00-69
ELISA (Manuf. A)	Hazelnut	Dark chocolate	1,6 - 16,3 mg/kg	12 - 33%	L 44.00-7
ELISA (Manuf. A)	Hazelnut	Dark chocolate	2,4 - 21,3 mg/kg	14 - 19%	L 44.00-7

From these precision data of the ASU §64 methods the calculated relative target standard deviations are in the range of 12 - 33%.

The IRMM (Institute for Reference Materials and Measurements) performed an interlaboratory comparison for five different ELISA-test kits for the quantification of peanut (22). 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%.

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 (6).
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 (17), by the working group 12 „Food Allergens" of the technical committee CEN/TC 275 (14 - 16), by an international "Food Allergen Working Group" under the advice of the AOAC Presidential Task Force on Food Allergens (18) and by the Codex Alimentarius Committee (CAC/GL 74-2010) (13).

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 (14, 17, 18, 13)	**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 (13)	**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 $\hat{\sigma}$ of 25%.
This target standard deviation was applied for the statistical evaluation of the results by z-score and was used for all assigned values mentioned in 3.1.

3.5 z-Score

To assess the results of the participants the z-score is used. It indicates about which multiple of the target standard deviation ($\hat{\sigma}$) the result (x) of the participant is deviating from the assigned value (X) (6).
Participants' z-scores were derived as:

$$z = (x - X) / \hat{\sigma} \ ;$$

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 DIN ISO 13528 (6) 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". Likewise, a *z*-score above 2,0 or below −2,0 shall be considered to give a "warning signal". A single "action signal", or "warning signal" in two successive PT-rounds, shall be taken as evidence that an anomaly has occurred which requires investigation. For example a fault isolation or a root cause analysis through the examination of transmission error or an error in the calculation, in the trueness and precision must be performed and if necessary appropriate corrective measures should be applied (6).

In the figures of z-scores DLA gives the limits of warning and action signals as yellow and red lines respectively. According to ISO 13528:2009 the signals are valid only in case of a number of ≥ 10 results (6).

3.6 Quotient $S^x/\hat{\sigma}$

Following the Horrat-value the results of a proficiency-test (PT) can be considered convincing, if the quotient of robust standard deviation and target standard deviation 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 (11).

3.7 Standard uncertainty

The assigned value X has a standard uncertainty u_X that depends on the analytical method, differences between the analytical methods used, the test material, the number of participant laboratories and perhaps on other factors. The standard uncertainty u_X for this PT is calculated as follows (6).

$$u_X = 1{,}25 * S^x / \sqrt{(p)}$$

If $u_X \leq 0{,}3 * \hat{\sigma}$ the standard uncertainty of the assigned value needs not to be included in the interpretation of the results of the PT (6). The Quotient $u_X/\hat{\sigma}$ is reported in the characteristics of the test.

3.8 Figures

The assigned values 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.

3.9 Recovery rates: Spiking

For the results of the spiking material sample and the spiked sample recovery rates were calculated with respect to the known content of added allergens. The related values of added allergens are given in 2.1 test material in table 2. As a range of acceptance RA for valuating participant's results the range of 50 - 150% for the recovery rates of allergen-ELISAs proposed by the AOAC was used (18). For quantitative PCR determinations we use the same range of acceptance.

4. Results

All following tables are anonymized. With the delivering of the evaluation-report the participants are informed about their individual evaluation-number.
The following result sections are structured equally for the allergenic components. First all results for a certain analyte are reported together for sample A and afterwards for sample B.

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 and a content of 48,1 % egg protein was taken.

ELISA-results given as codfish (or fish in general) (test kit AgraQuant) were multiplied with a conversion factor of 3,2 for **coalfish**.
For calculating recovery rates the added amount of fish powder was converted to fresh fish (wet weight). A content of 80 % water was considered (Souci/Fachmann/Kraut nutrient tables).

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.

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 X_{ALL}	z-Score $X_{M\,i}$	Method	Remarks
	pos/neg	[mg/kg]	X All	X Method i		

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:

	All Results *[mg/kg]*	**Method i** *[mg/kg]*
Assigned value	X_{ALL}	$X_{Method\ i}$
Number of results		
Robust mean (X)		
Robust standard deviation (S^x)		
Median		
Target range:		
Target standard deviation ($\hat{\sigma}$)		
lower limit of target range (X - 2 $\hat{\sigma}$)		
upper limit of target range (X + 2 $\hat{\sigma}$)		
Quotient $S^x/\hat{\sigma}$		
Standard uncertainty u_X		
Quotient $u_X/\hat{\sigma}$		
Number of results in the target range		

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

4.1 Proficiency Test 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		
1	negative		positive	123	2/2 (100%)	AQ	Result converted *
4	negative	< 1,5	positive	102	2/2 (100%)	AQ	Result converted *
9	negative	< 0,39	positive	119	2/2 (100%)	BK	
10	negative	< 1,9	positive	105	2/2 (100%)	BK	Result converted *
16	negative		positive		2/2 (100%)	ES	
18	negative	< 1,5	positive	92	2/2 (100%)	IL	Result converted *
7	negative	< 0,7	positive	77	2/2 (100%)	MR	
8	negative	< 0,6	positive	81,3	2/2 (100%)	MR	Result converted *
11	negative	nd	positive	96	2/2 (100%)	MR	Result converted *
2	negative	< 0,5	positive	86,5	2/2 (100%)	RS	
3	negative	< 0,19	positive	>127	2/2 (100%)	RS	Result converted *
5	negative	< 0.5	positive	62,9	2/2 (100%)	RS	Mean calculated by DLA
6	negative	< 0,5	positive	<50	2/2 (100%)	RS	
12	negative	< 0,5	positive	79	2/2 (100%)	RS	
13	negative		positive	89,5	2/2 (100%)	RS	
14	negative	< 0,5	positive	120	2/2 (100%)	RS	
17	negative		positive	>3,6	2/2 (100%)	RS	

* calculation see p. 12

	Sample A		Sample B	
Number positive	0		17	
Number negative	17		0	
Percent positive	0		100	
Percent negative	100		0	
Consensus value	negative		positive	

Methods:

AQ = AgraQuant, RomerLabs
BK = BioKits, Neogen
ES = ELISA Systems
IL = Immunolab
MR = Morinaga
RS = Ridascreen®, R-Biopharm

Comments:

There were 100% negative results for sample A and 100% positive results for sample B by the ELISA-methods. The consensus values are in agreement with the spiking of sample B.

Quantitative valuation of results: Sample B

Evaluation number	Whole Egg Powder	z-Score X_{ALL}	z-Score X_{RS}	Method	Remarks
	[mg/kg]	X_{ALL}	X_{RS}		
1	123	1,2		AQ	Result converted *
4	102	0,3		AQ	Result converted *
9	119	1,0		BK	
10	105	0,4		BK	Result converted *
16				ES	
18	92	-0,1		IL	Result converted *
7	77	-0,8		MR	
8	81,3	-0,6		MR	Result converted *
11	96	0,0		MR	Result converted *
2	86,5	-0,4	0,0	RS	
3	>127			RS	Result converted *
5	62,9	-1,4	-1,1	RS	Mean calculated by DLA
6	<50			RS	
12	79	-0,7	-0,4	RS	
13	89,5	-0,2	0,1	RS	
14	120	1,1	1,5	RS	
17	>3,6			RS	

* calculation see p. 12

Methods:

AQ = AgraQuant, RomerLabs
BK = BioKits, Neogen
ES = ELISA Systems
IL = Immunolab
MR = Morinaga
RS = Ridascreen®, R-Biopharm

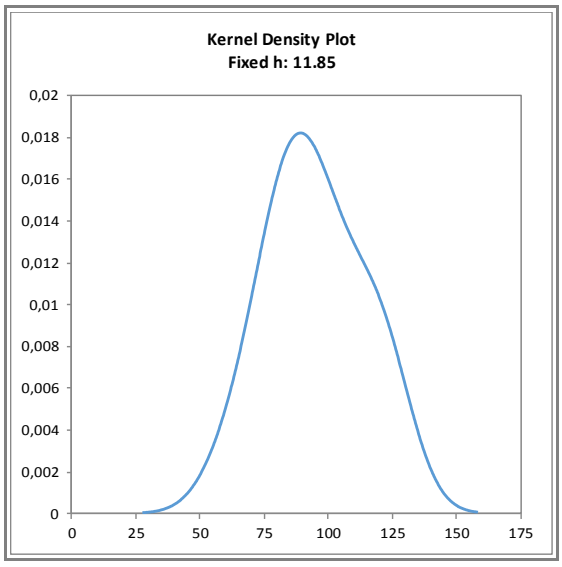

Fig. 2: Kernel Density Plot of all ELISA-results egg (with h = 0,5 x $\hat{\sigma}$ of X_{ALL})

Characteristics: Quantitative evaluation Egg (as Whole Egg Powder)

Sample B

Characteristics	**All Results** *[mg/kg]*	**Method RS** *[mg/kg]*
Assigned value	X_{ALL}	$X_{Method\ RS}$
Number of results	*13*	*5*
Robust mean (X)	94,9	87,6
Robust standard deviation (S^x)	20,6	21,9
Median	92,0	86,5
Target range:		
Target standard deviation ($\hat{\sigma}$)	23,7	21,9
lower limit of target range (X - 2 $\hat{\sigma}$)	47,5	43,8
upper limit of target range (X + 2 $\hat{\sigma}$)	142	131
Quotient $S^x/\hat{\sigma}$	*0,87*	*1,1*
Standard uncertainty u_X	*7,16*	*13,2*
Quotient $u_X/\hat{\sigma}$	*0,30*	*0,60*
Number of results in the target range	*13 (100%)*	*5 (100%)*

Method:
RS = R-Biopharm, Ridascreen Fast®

Comments to the statistical characteristics:

The evaluation of all methods and the evaluation of results from method RS showed a low variability, respectively. The quotients $S^x/\hat{\sigma}$ were 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.

The robust means of the evaluations were with 122% and 112% slightly higher than the spiking level of egg powder to sample B but within the recommendations for the applied methods (s. 3.4.3 and "Recovery rates of Egg" p.19).

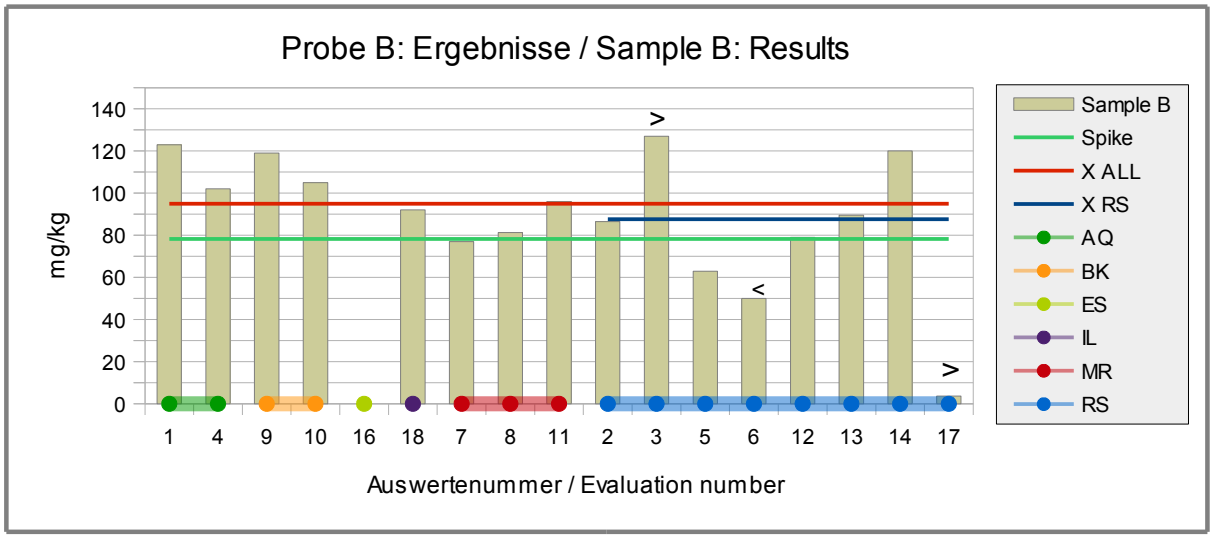

Fig. 3: ELISA-Results Egg (as Whole Egg Powder)
green line = Spiking level
red line = Assigned value robust mean all results
blue line = Assigned value robust mean results method RS
round symbols = Applied methods (see legend)

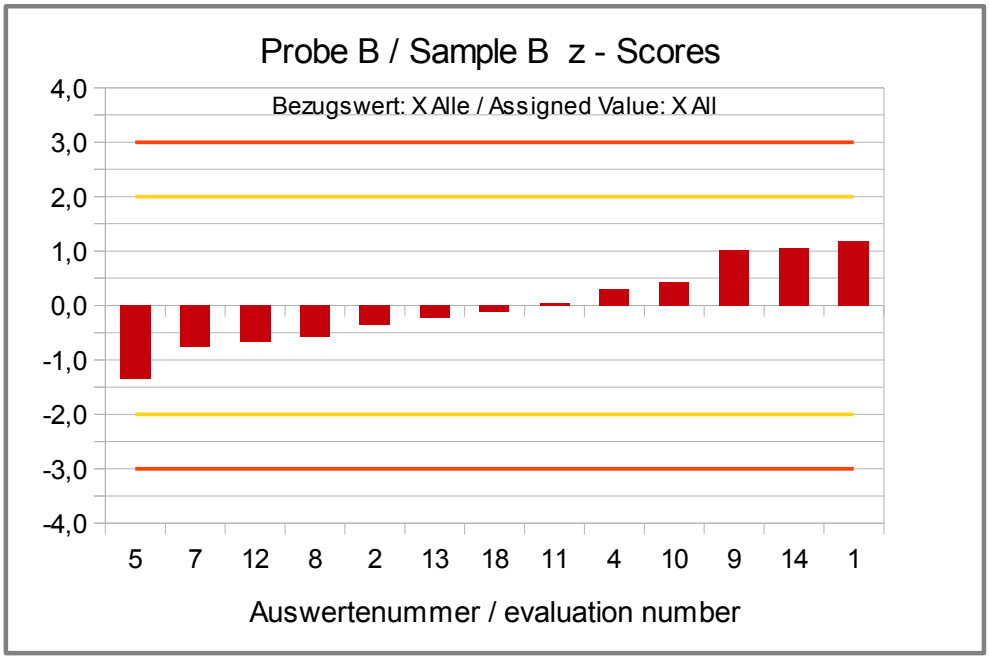

Fig. 4: z-Scores (ELISA-Results as Whole Egg Powder)
Assigned value robust mean of all results

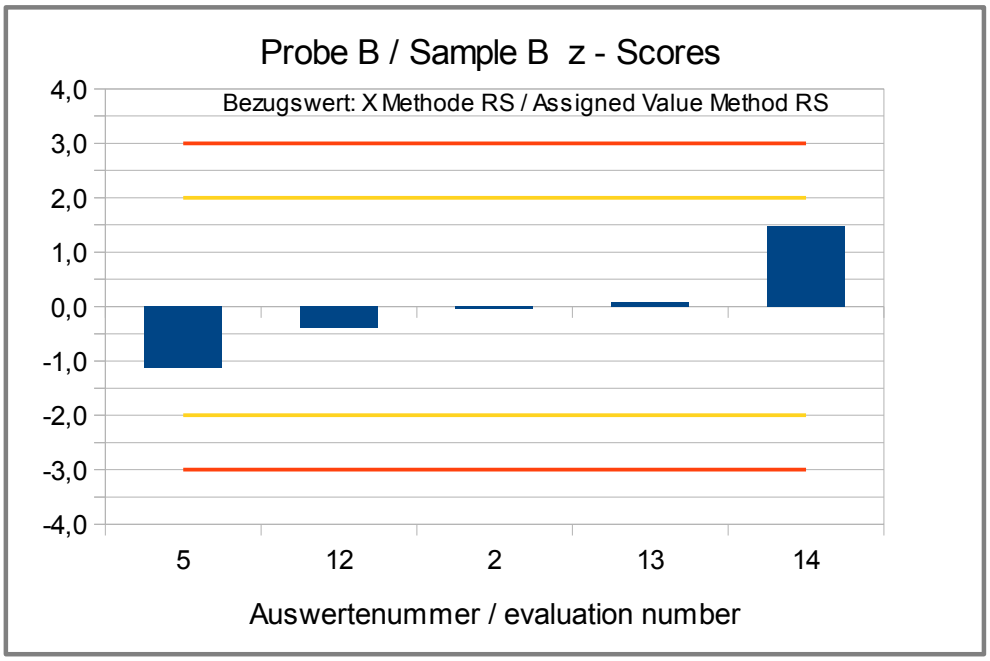

Fig. 5: z-Scores (ELISA-Results as Whole Egg Powder)
Assigned value robust mean of method RS (R-Biopharm, Ridascreen)

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

Evaluation number	Spiking material	Recovery rate	Sample B	Recovery rate	Method	Remarks
	[mg/kg]	[%]	[mg/kg]	[%]		
1	27800	**127**	123	157	AQ	Result converted *
4	28000	**128**	102	**130**	AQ	Result converted *
9	12510	**57**	119	152	BK	
10	31700	**145**	105	**134**	BK	Result converted *
16					ES	
18	29200	**133**	92	**117**	IL	Result converted *
7			77	**98**	MR	
8	na		81,3	**104**	MR	Result converted *
11	27200	**124**	96	**123**	MR	Result converted *
2	24447	**112**	86,5	**110**	RS	
3	>127		> 127		RS	Result converted *
5	14627	**67**	62,9	**80**	RS	Mean calculated by DLA
6	2567,5	12	< 50		RS	
12	21500	**98**	79	**101**	RS	
13	27535	**126**	89,5	**114**	RS	
14	38000	174	120	153	RS	
17			> 3,6		RS	

* calculation see p. 12

RA*	50-150 %	RA*	50-150 %
Number in RA	**10**	Number in RA	**10**
Percent in RA	**83**	Percent in RA	**77**

* Range of acceptance of AOAC for allergen ELISAS

Recovery rate
100% relative size:
Whole Egg Powder, s. page 4

Methods:

AQ = AgraQuant, RomerLabs
BK = BioKits, Neogen
ES = ELISA Systems
IL = Immunolab
MR = Morinaga
RS = Ridascreen®, R-Biopharm

Comments:

For the spiking material sample 83% of the participants obtained a recovery rate within the range of the AOAC-recommendation of 50-150%. For the sauce powder-sample B produced with the spiking material sample 77% of the recovery rates were in the range of acceptance.

4.1.2 PCR-Results: Egg

Evaluation number	Result Sample A	Result Sample A	Result Sample B	Result Sample B	Qualitative Valuation	Method	Remarks
	pos / neg	mg/kg	pos / neg	mg/kg	Agreement with Consensus Value		
15	negative		negative			div	Limit of detection 0,1%

Method:
div = not indicated / other method

Comments:
For sample A and sample B negative results were obtained for the detection of chicken-DNA. For the spiking material sample a positive result was obtained (see documentation).

Quantitative valuation of results: Sample B

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

Recovery Rates for Egg:
Spiking Material Sample and Sample B

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

4.2 Proficiency Test Fish

4.2.1 ELISA-Results: Fish (fresh Coalfish)

Qualitative valuation of results: Samples A and B

Evaluation number	Sample A	Sample A	Sample B	Sample B	Qualitative Valuation	Method	Remarks
	pos/neg	[mg/kg]	pos/neg	[mg/kg]	Agreement with consensus value		
2	negative	< 12,8	positive	85,1	2/2 (100%)	AQ	
4	negative	< 12,8	positive	70,7	2/2 (100%)	AQ	Result converted *
5a	negative	< 12.8	positive	84,43	2/2 (100%)	AQ	
7			positive	79,7	1/2 (50%)	AQ	Result converted *
8	negative	< 12,8	positive	65,6	2/2 (100%)	AQ	Result converted *
11	negative		positive	70,4	2/2 (100%)	AQ	Result converted *
5b	negative	< 13,55	positive	15,11	2/2 (100%)	BC	
10	negative	< 5	positive	8,7	2/2 (100%)	BC	fish species not given
18	negative	< 6,4	positive	64	2/2 (100%)	IL	

* calculation see p. 12

	Sample A		Sample B	
Number positive	0		9	
Number negative	8		0	
Percent positive	0		100	
Percent negative	100		0	
Consensus value	negative		positive	

Methods:

AQ = AgraQuant, RomerLabs IL = Immunolab
BC = Bio-check, imutest ELISA

Comments:

There were 100% negative results for sample A and 100% positive results for sample B by the ELISA-methods. The consensus values are in agreement with the spiking of sample B.

Quantitative valuation of results: Sample B

Evaluation number	Coalfish, fresh	z-Score X_{ALL}	z-Score X_{AQ}	Method	Remarks
	[mg/kg]	X_{ALL}	$X_{Method\ AQ}$		
2	85,1	0,6	0,5	AQ	
4	70,7	-0,2	-0,3	AQ	Result converted *
5a	84,43	0,5	0,4	AQ	
7	79,7	0,3	0,2	AQ	Result converted *
8	65,6	-0,5	-0,5	AQ	Result converted *
11	70,4	-0,2	-0,3	AQ	Result converted *
5b	15,11			BC	* *
10	8,7			BC	* *, fish species not given
18	64	-0,6		IL	

* calculation see p. 12

* * BC results excluded

Methods:

AQ = AgraQuant, RomerLabs
BC = Bio-check, imutest ELISA
IL = Immunolab

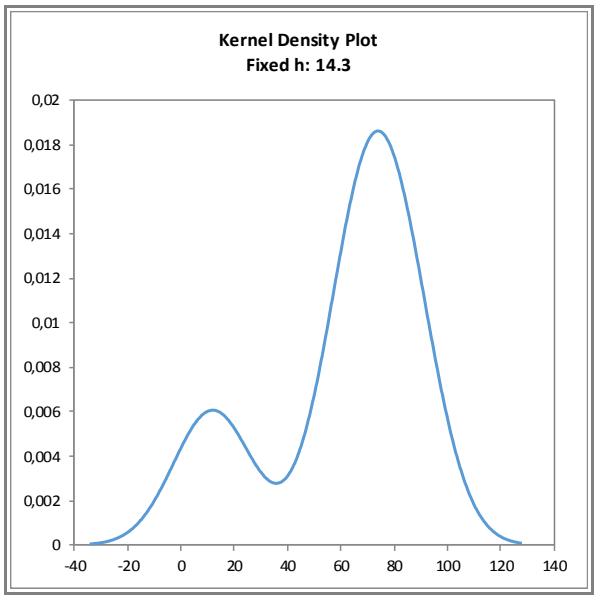

Fig. 6: Kernel Density Plot of all ELISA-results fish
(with h = 0,5 x $\hat{\sigma}$ of X_{ALL})

Comments:

For statistical evaluation the results of method BC were excluded, because they caused a bimodal distribution of results (s. fig. 6).

Characteristics: Quantitative evaluation Fish (as fresh Coalfish)

Sample B

Characteristics	**All Results** *[mg/kg]*	**Method AQ** *[mg/kg]*
Assigned value	X_{ALL}	$X_{Method\ AQ}$
Number of results	*7 **	*6*
Robust mean (X)	74,3	76,0
Robust standard deviation (S^x)	9,91	9,29
Median	70,7	75,2
Target range:		
Target standard deviation ($\hat{\sigma}$)	18,6	19,0
lower limit of target range (X - 2 $\hat{\sigma}$)	37,1	38,0
upper limit of target range (X + 2 $\hat{\sigma}$)	111	114
Quotient $S^x/\hat{\sigma}$	*0,53*	*0,49*
Standard uncertainty u_X	*4,68*	*4,74*
Quotient $u_X/\hat{\sigma}$	*0,25*	*0,25*
Number of results in the target range	*7 (100%)*	*6 (100%)*

* results of method BC were not considered

Method:
AQ = AgraQuant, RomerLabs

Comments to the statistical characteristics:

The evaluation of all methods and the evaluation of results from method AQ showed a low variability, respectively. The quotients $S^x/\hat{\sigma}$ were clearly 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.

The robust means of the evaluations were with approximately 15% clearly below the spiking level of fish to sample B not fulfilling the recommendations for the applied methods.
It should be noted, that the ELISA results were calculated as fresh fish (coalfish). The samples were spiked with fish powder, which is processed and therefore could be detected to a lower percentage. Suitable conversion factors as indicated by some test kit manufacturers could give improved quantitative results (s. 3.4.3 and "Recovery rates of Fish" p.26).

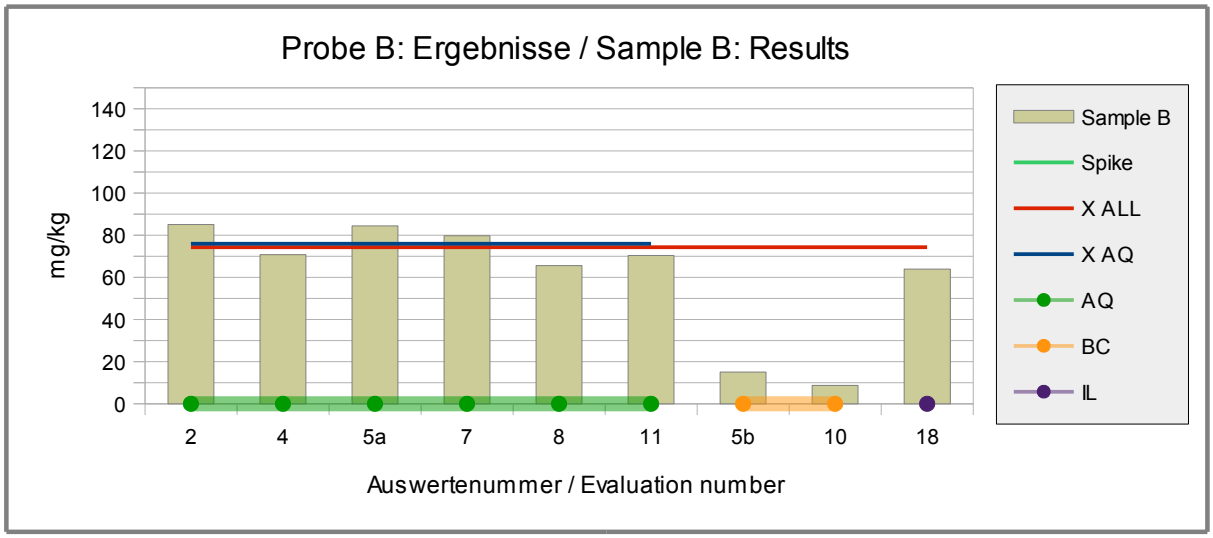

Fig. 7: ELISA-Results Fish (as fresh Coalfish / Fish)
green line = Spiking level (514 mg/kg, not indicated)
red line = Assigned value robust mean all results
blue line = Assigned value robust mean results method AQ
round symbols = Applied methods (see legend)

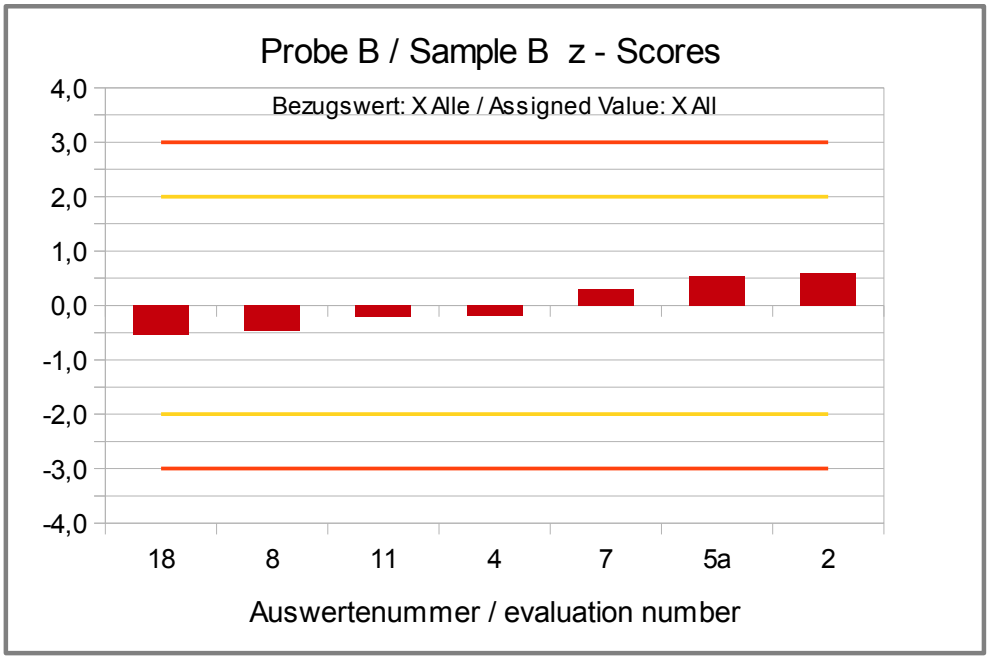

Fig. 8: z-Scores (ELISA-Results as fresh Coalfish)
Assigned value robust mean of all results (without method BC)

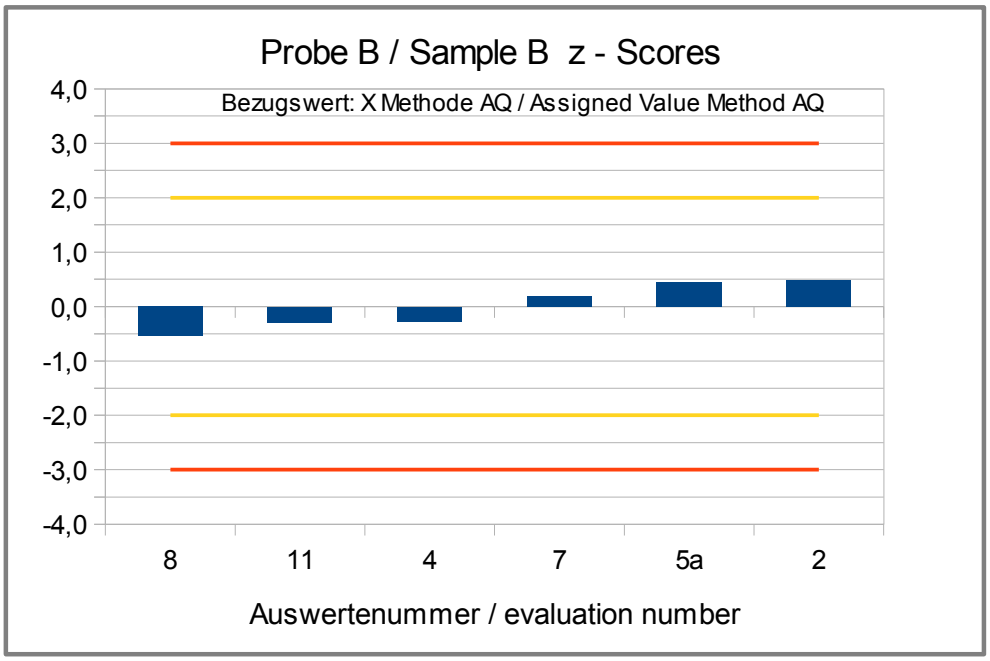

Fig. 9: z-Scores (ELISA-Results as fresh Coalfish)
Assigned value robust mean of method AQ (AgraQuant, RomerLabs)

Recovery Rates for Fish (as fresh Coalfish):
Spiking Material Sample and Sample B

Evaluation number	Spiking material	Recovery rate	Sample B	Recovery rate	Method	Remarks
	[mg/kg]	[%]	[mg/kg]	[%]		
2	29650	21	85,1	17	AQ	
4	15600	11	70,7	14	AQ	Result converted *
5a	17954	12	84,43	16	AQ	
7	18600	13	79,7	16	AQ	Result converted *
8	na		65,6	13	AQ	Result converted *
11	29100	20	70,4	14	AQ	Result converted *
5b	5116	4	15,11	3	BC	* *
10	13900	10	8,7	2	BC	* *, fish species not given
18	22400	16	64	12	IL	

* calculation see p. 12

* * BC results excluded

RA*	50-150 %	AB*	50-150 %
Number in RA	0	Anzahl im AB	0
Percent in RA	0	Prozent im AB	0

* Range of acceptance of AOAC for allergen ELISAS

Recovery rate
100% relative size:
Fresh Coalfish, s. page 4

Methods:
AQ = AgraQuant, RomerLabs
BC = Bio-check, imutest ELISA
IL = Immunolab

Comments:
For both the spiking material sample and the sauce powder-sample B produced with the spiking material sample none of the participants obtained a recovery rate within the range of the AOAC-recommendation of 50-150%.
It should be noted, that the ELISA results were calculated as fresh fish (coalfish). The samples were spiked with fish powder, which is processed and therefore could be detected to a lower percentage according to test kit manufacturers. Conversion factors may be available from the kit instructions.

4.2.2 PCR-Results: Fish

Evaluation number	Result Sample A	Result Sample A	Result Sample B	Result Sample B	Qualitative Valuation	Method	Remarks
	pos / neg	mg/kg	pos / neg	mg/kg	Agreement with Consensus Value		
2	negative		positive		2/2 (100%)	SFA-ID	
3	negative	< 0,4	positive	> 0,4	2/2 (100%)	SFA-ID	
6	negative		positive		2/2 (100%)	SFA-ID	
7a	negative	< 5	positive	44	2/2 (100%)	SFA-ID	as codfish powder
9	negative		positive		2/2 (100%)	SFA-ID	
15	negative		positive		2/2 (100%)	SFA-ID	
17	negative		positive		2/2 (100%)	SFA-ID	
1	negative		positive		2/2 (100%)	div	
7b	negative	< 20	positive	33	2/2 (100%)	div	as codfish powder
12	negative		positive		2/2 (100%)	div	
13	negative		positive		2/2 (100%)	div	
14	negative	-	positive	-	2/2 (100%)	div	

	Sample A		Sample B	
Number positive	0		12	
Number negative	12		0	
Percent positive	0		100	
Percent negative	100		0	
Consensus value	negative		positive	

Methods:

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

div = not indicated / other method

Comments:

There were 100% negative results for sample A and 100% positive results for sample B for fish by the PCR-methods. The consensus values are therefore in agreement with the spiking of sample B.

Quantitative valuation of results: Sample B

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

Recovery Rates for Fish (as Fish Powder): Spiking Material Sample and Sample B

Evaluation number	Spiking material	Recovery rate	Sample B	Recovery rate	Method	Remarks
	[mg/kg]	[%]	[mg/kg]	[%]		
2					SFA-ID	
3	> 0,4		> 0,4		SFA-ID	
6					SFA-ID	
7a	130000	453	44	43	SFA-ID	as codfish powder
9					SFA-ID	
15					SFA-ID	
17					SFA-ID	
1					div	
7b	65000	226	33	32	div	as codfish powder
12					div	
13					div	
14			-		div	

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

* Range of acceptance of AOAC for allergen ELISAS

Recovery rate
100% relative size:
Coalfish as powder, s. page 4

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

Comments:
One participant submitted quantitative results obtained by two different PCR methods. The recovery rates for the spiking material sample were above and for the sauce powder-sample B produced with the spiking material sample below the range of acceptance of 50-150%.
It should be noted, that the participants' results were given as codfish and the fish species contained in the samples was coalfish.

5. Documentation

Details by the participants

5.1 ELISA: Egg

Primary data

Evaluation number	Result Sample A		Result Sample B		Result Spiking Sample		quantitative Result given as	Meth. Abr.	Method
	qualitative	mg/kg	qualitative	mg/kg	qualitative	mg/kg	e.g. food / food protein		Test-Kit + Manufacturer
1	negative		positive	32	positive	7232	Egg white proteins, total	AQ	AgraQuant Egg (COKAL0848), RomerLabs
4	negative	<0.4	positive	26,4	positive	7277	Egg White Protein	AQ	Romer Labs AgraQuant Egg White
9	-	< LOD (< 0,1 mg/kg Egg w hite prote-in)	-	119	-	12510	Whole egg powder	BK	BioKits Egg Assay Kit, Neogen
10	-	< 0,5	-	27,3	-	8250	Egg white proteins, total	BK	BioKits Egg Assay Kit, Neogen
16	negative		positive		positive		Egg white proteins, total	ES	ELISA-Systems Egg Residue Detec-tion ELISA
18	negative	< 0.4	positive	24	positive	7600	Egg white proteins, total	IL	Immunolab Eiklar ELISA
7	negative	< 0,7	positive	77	-		Whole egg powder	MR	andere: Egg (Ovalbumin) ELISA Kit. Morinaga Institute of Biolo-gical Science (MIoBS), Yoko-hama, Japan.
8	-	<0,3	-	39,1	-	na	Egg white proteins, total	MR	Morinaga Egg protein Elisa Kit 141OA
11	negative	nd	positive	46	positive	13100	egg protein	MR	Morinaga Egg
2	negative	< 0,5	positive	86,5	positive	24447	Whole egg powder	RS	Ridascreen Fast Ei (R6402), r-Biopharm
3	negative	<0.049	positive	>33	positive	>33	Egg white proteins, total	RS	Ridascreen Fast Ei (R6402), r-Biopharm
5	negative	<0.5	positive	62,68	positive	14554	Whole egg powder	RS	Ridascreen Fast Ei (R6402), r-Biopharm
5	negative	<0.13	positive	16,4	positive	3821	Egg white proteins, total	RS	Ridascreen Fast Ei (R6402), r-Biopharm
6	negative	<0,5	positive	<50	positive	2567,5	Egg	RS	r-biopharm, RIDASCREEN®FAST Egg (R6402)
12	negative	<0,5	positive	79	positive	21500	Whole egg powder	RS	Ridascreen Fast Ei (R6402), r-Bio-pharm
13	negative		positive	89,5	positive	27535	Whole egg powder	RS	Ridascreen Fast Ei (R6402), r-Bio-pharm
14	negative	<0,5	positv	120	positive	38000	Whole egg powder	RS	Ridascreen Fast Ei (R6402), r-Bio-pharm
17	negative		positive	>3,6	-		Whole egg powder	RS	Ridascreen Fast Ei (R6402), r-Biopharm
17	negative		positive	>3,6	-		Whole egg powder	RS	Ridascreen Fast Ei (R6402), r-Biopharm

Methods:

AQ = AgraQuant, RomerLabs
BK = BioKits, Neogen
ES = ELISA Systems
IL = Immunolab
MR = Morinaga
RS = Ridascreen®, R-Biopharm

Other details to the Methods

Evaluation number	Meth. Abr.	Specifity	Remarks to the Method (Extraction and Determination)	Further Remarks
		Antibody	e.g. Extraction Solution / Time / Temperature	
1	AQ			
4	AQ			
9	BK	Ovomucoid (Gal d 1)	As per Kit Instructions	
10	BK	Ovomucoid	1 g sample + 10 ml preheated extraction solution (Tris w ith gelatine). Incubation 15 minutes r.t. during sha-king.	
16	ES			
18	IL			
7	MR			
8	MR			
11	MR		1g sample/19mL kit extraction solution; extracted for 10 min at 100°C	single results
2	RS		nach Herstelleranleitung	
3	RS			
5	RS	ovalbumin and ovomucoid	As per Kit Instructions	
5	RS	ovalbumin and ovomucoid	As per Kit Instructions	Results calculated from Whole Egg Pow der results according to R-Bio-pharm Kit Instructions
6	RS		As per Kit Instructions	
12	RS			
13	RS	Ovalbumin and Ovomucoid	as per kit instructions, dilution sample B 1:50, sample C 1:50.000	
14	RS		As per Kit Instructions	
17	RS			
17	RS			

5.2 ELISA: Fish

Primary data

Evaluation number	Result Sample A		Result Sample B		Result Spiking Sample		quantitative Result given as	Meth. Abr.	Method
	qualitative	mg/kg	qualitative	mg/kg	qualitative	mg/kg	e.g. food / food protein		Test-Kit + Manufacturer
2a	negative	< 4,0	positive	26,6	positive	9266	fresh codfish	AQ	AgraQuant ELISA Fish (COKAL2548), RomerLabs
2b	negative	< 12,8	positive	85,1	positive	29.650	fresh codfish, converted to fresh coalfish	AQ	AgraQuant ELISA Fish (COKAL2548), RomerLabs
4	negative	<4	positive	22,1	positive	4877	Fish	AQ	Romer Labs AgraQuant Fish
5a	negative	<12.8	positive	84,43	positive	17954	Fish, fresh	AQ	AgraQuant ELISA Fish (COKAL2548), RomerLabs
7	-		positive	24,9	positive	5800	codfish, fresh	AQ	AgraQuant ELISA Fish (COKAL2548), RomerLabs
8	-	<4	-	20,5	-	na	Fish, fresh	AQ	AgraQuant ELISA Fish (COKAL2548), RomerLabs
11	negative	not detected	positive	22	positive	9100	Fish, fresh	AQ	AgraQuant ELISA Fish (COKAL2548), RomerLabs
5b	negative	<13.55	positive	15,11	positive	5116	Fish, fresh	BC	Bio-check / imutest Fish-check ELISA
10	-	< 5	-	8.7	-	13900	Fish, fresh	BC	Bio-check / imutest Fish-check ELISA
18	negative	< 2	positive	20	positive	7000	Fish, fresh, codfish	IL	Immunolab Fish ELISA (FIS-E01)

Methods:

AQ = AgraQuant, RomerLabs
BC = Bio-check, imutest ELISA
IL = Immunolab

Other details to the methods

Evaluation number	Meth. Abr.	Specifity	Remarks to the Method (Extraction and Determination)	Further Remarks
		Antibody	e.g. Extraction Solution / Time / Temperature	
2a	AQ		As per Kit Instructions	
2b	AQ		As per Kit Instructions	Factor 3,2
4	AQ			Conversion factors may apply for different Fish species. The species identity was not provided.
5a	AQ		As per Kit Instructions	Calculated as Fresh Coalfish
7	AQ	Fishprotein		
8	AQ			
11	AQ		1g sample/20mL kit's extraction solution; extracted for 15min in 60°C shaking waterbatch; 3 x 20min incubations prior to reading @ 450nm.	single results
5b	BC		As per Kit Instructions	Calculated as Fresh Coalfish
10	BC	Parvalbumins (Gad c1)	0,4 g sample + 3,5 ml extraction solution (Tris-glycine). Incubation 19 minutes r.t. of which 4 minutes are during shaking.	
18	IL			see below *

* for sample B 20 ppm codfish (fresh) were measured corresponding to 64 ppm coalfish, and to 4 ppm codfish (dried), and 13 ppm coalfish (dried)

for the spiking material sample 7000 ppm codfish (fresh) were measured, corresponding to 22400 ppm as coalfish, and 1400 ppm codfish (dried),and 4500 ppm coalfish (dried)

the protein content of fresh fish is for both species approximately 18% and about 90% in dry matter.

5.3 PCR: Egg (Chicken DNA)

Primary data

Evaluation number	Result Sample A		Result Sample B		Result Spiking Sample		quantitative Result given as	Meth. Abr.	Method
	qualitative	mg/kg	qualitative	mg/kg	qualitative	mg/kg	e.g. food / food protein		Test-Kit + Manufacturer
15	negative		negative		positive			div	House method

Method:
div = not indicated / other method

Other Remarks to the Methods

Evaluation number	Meth. Abr.	Specifity	Remarks to the Method (Extraction and Determination)	Further Remarks
		Antibody	e.g. Extraction / Enzymes / Clean-Up / Real Time PCR / Gel electrophoresis / Cycles	
15	div	cytochrome b/ ovalbumin/ Vitellogenin	Extraction: NucleoSpin Food (Macherey Nagel)/ Real Time PCR/ 45 cycles	LD (0,1%)

5.4 PCR: Fish

Primary data

Evaluation number	Result Sample A		Result Sample B		Result Spiking Sample		quantitative Result given as	Meth. Abr.	Method
	qualitative	mg/kg	qualitative	mg/kg	qualitative	mg/kg	e.g. food / food protein		Test-Kit + Manufacturer
2	negative		positive		positive		DNA-Fish	SFA-ID	Sure Food Allergen ID Fish, Congen / r-Biopharm
3	negative	<0.4ppm	positive	>0.4ppm	positive	>0.4ppm	DNA-Fish	SFA-ID	Sure Food Allergen ID, Congen / r-Biopharm
6	negative		positive		positive		Fish-DNA	SFA-ID	r-biopharm, SureFood® ALLERGEN ID Fish (S3110)
7a	negative	< 5	positive	44	positive	130000	codfish powder, freeze dried	SFA-ID	Sure Food Allergen ID, Congen / r-Biopharm
9	negative		positive		positive		DNA-Fish	SFA-ID	SureFood ALLERGEN ID Fish, Congen
15	negative		positive		positive			SFA-ID	Sure Food Allergen ID, Congen / r-Biopharm
17	negative		positive		-		DNA-Fish	SFA-ID	Sure Food Allergen ID, Congen / r-Biopharm
1	negative		positive		positive		DNA-Fish	div	in-house method
7b	negative	< 20	positive	33	positive	65000	codfish powder, freeze dried	div	Benedetto MC., Abete S., Squadrone S. (2011) Towards a quantitative application of real-time PCR technique for fish DNA detection in feedstuff. Food Chemistry 126, 1436-1442.
12	negative		-		positive		DNA-Fish	div	other: please fill in!
13	negative		positive		positive		DNA-Fish	div	
14	negative	-	positive	-	positive		DNA-Fish	div	internal method

Methods:

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

Other Remarks to the Methods

Evaluation number	Meth. Abr.	Specifity	Remarks to the Method (Extraction and Determination)	Further Remarks
		Antibody	e.g. Extraction / Enzymes / Clean-Up / Real Time PCR / Gel electrophoresis / Cycles	
2	SFA-ID		SureFood PREP ALLERGEN, R-Biopharm, S1053	
3	SFA-ID			
6	SFA-ID		As per Kit instructions	
7a	SFA-ID		CTAB-precipitation method	Quantification by matrix-control standards (rice cookies)
9	SFA-ID	not known, according to kit manufacturer characteristic part of fish-DNA	DNA-Extraction with 2 g-Protocol of Dneasy mericon Food Kits from Qiagen and SureFood PREP Advanced, Kit, Protocol 1, Congen/r-biopharm	
15	SFA-ID	unknown	Extraction: NucleoSpin Food (Macherey Nagel)/ Real Time PCR/ 35 cycles	
17	SFA-ID			
1	div			
7b	div	other: 12S ribosomal RNA (12S rRNA)	CTAB-precipitation method	Quantification by matrix-control standards (rice cookies)
12	div	in house	CTAB-extraction	
13	div	18S-rRNA	2 g sample weight, lysate prepared twice with Machery & Nagel Food Kit; conventional PCR	Remmler et al.; research project Technical University Graz No. 1245
14	div	Fish Myostatin	CTAB / Protease K / Chloroform + Promega Wizard/ Real-time PCR/ - / 45 cycles	

6. Index of participant laboratories

Teilnehmer / Participant	Ort / Town	Land / Country
		Germany
		SPAIN
		Germany
		FRANCE
		CANADA
		CANADA
		ITALY
		Germany
		Germany
		Germany
		Germany
		Germany
		Germany
		ITALY
		SWEDEN
		UNITED KINGDOM
		UNITED KINGDOM
		SWEDEN
		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 17043:2010; Konformitätsbewertung – Allgemeine Anforderungen an Eignungsprüfungen / Conformity assessment – General requirements for proficiency testing
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