DLA
Dienstleistung
Lebensmittel
Analytik GbR

Evaluation Report
proficiency test

01/2014

Allergens I:

Egg and Milk

in Sausage Meat

Dienstleistung Lebensmittel Analytik GbR
Pinnberg 5
22927 Großhansdorf, Germany

proficiency-testing@dla-lvu.de www.dla-lvu.de

Coordinator of this PT:
Dr. Matthias Besler

Inhalt

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.

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 materials are sausage meat preserves produced exclusively for the present proficiency test. There are two different samples A and B. The spiking material containing the allergenic components egg and milk powder were added to sample B (see below). The basic recipe of sample A and B was the same:

Ingredients	**Sample A**	**Sample B**
Mixed Organic Minced Meat	68 %	67 %
Crushed Ice	17 %	17 %
Water	14 %	14 %
Sodium Chloride	0,2 %	0,2 %
Sodium Citrate	0,05 %	0,05 %
Spiking Material	-	2,03 %
Dye E100	0,025 %	-
Dye E120	-	0,025 %

The composition of the spiking material sample and the content of allergenic compounds in sample B is given in Table 1.

The samples were produced in a 9 L cutter. Portions of more than 50 g were filled in capped glasses. All glasses were heated in an oven to 110°C for approximately 30 min. Until shipment the samples were stored at

4-8°C.

Table 1: Added amounts of allergenic ingredients

Ingredients	Spiking Material Sample	Sample B
Potato Flour	83 %	1,68 %
Soy Flour	6,21 %	0,23 %
Hen's Egg: - as Whole Egg Powder - thereof Egg White Proteins*	 34100 mg/kg (3,41 %) 8866 mg/kg	 691 mg/kg 180 mg/kg
Milk: - as Skimmed Milk Powder - thereof Total Protein - thereof Casein* - thereof β-Lacto-globulin*	 48400 mg/kg (4,84 %) 17400 mg/kg 13900 mg/kg 1740 mg/kg	 981 mg/kg 353 mg/kg 283 mg/kg 35 mg/kg
Wheat Flour	2,65 %	0,054 %

* calculated with data from the literature

2.1.1 Homogeneity

Homogeneity of the spiked sample B was checked by 5fold ELISA-test. The resulting standard deviation between the samples of < 15% ensured sufficient homogeneity (17, 18, 20).

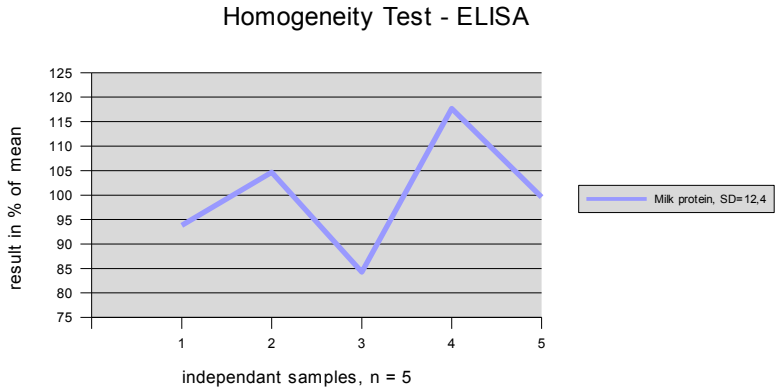

Fig. 1: Testing of homogeneity of DLA-sample B
Results are given in percent of the arithmetic mean

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 9th week of 2014. The testing method was optional. The tests should be finished at April 11th 2014 the latest.

2.3 Submission of results

The participants submitted their results in standard forms, which have been handed out along with the samples. On one hand the results given as positive/negative and on the other hand the indicated results of the allergenic ingredients e.g. egg powder or milk proteins in mg/kg were evaluated.
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. 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 actually added amount, in comparison to the mean of all results and/or in comparison to the mean of results obtained by a single method.

For ELISA-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 Assigned value

Because the analysed material was no certified reference material the robust mean of the submitted results was used as assigned value X (6). In case the submitted results show hints for bimodal distribution or other reasons for a higher variability the evaluation will be performed additionally with respect to the robust mean of single methods. 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 were considered for statistical evaluation (e.g. results given as > 25 mg/kg and < 2,5 mg/kg, respectively) when a result indicating „>" is above and a result indicating „<" is below the target range.

3.2 Standard deviation

For comparison to the target standard deviation a robust standard deviation (S^x) was calculated (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 Outliers

Statistical outliers were determined by Mandel´s-H-Statistic for 95% significance niveau (5). Detected outliers were stated for information only, when z-score was < -2 or > 2.

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 (13, 14, 15):

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 (16). 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%.

Štumr et al. conducted two interlaboratory studies for the validation of commercial ELISA-Test-Kits for the determination of β-lactoglobulin and for the determination of casein (22, 23).
20 food samples with β-lactoglobulin contents in the range of 0 - 33 mg/kg were analyzed by 6 laboratories. Recovery rates ranged between 91 - 118%. Relative repeatability standard deviations ranged from 5,8 - 13% and the relative reproducibility standard deviations ranged from 26 - 49% (22).
Casein was analyzed by 8 laboratories in 10 food samples in the range of 0 - 30 mg/kg and in 3 food samples with contents >30 mg/kg. Recovery rates ranged between 67 - 81%. Relative repeatability standard deviations ranged from 11 - 52% and was for one sample Probe 99% and the relative reproducibility standard deviations ranged from 13 - 61% and were for two samples 96% and 111%, respectively (23).
According to the authors both ELISA-Test-Kits were acceptable for routine control of food samples (22, 23).

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 quan-

titative determination of allergens in foods with ELISA were recently elaborated 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 (18, 19) and by an international "Food Allergen Working Group" under the advice of the AOAC Presidential Task Force on Food Allergens (20).

Some of the relevant ELISA validation criteria of the three panels are listed below:

Literature (17, 18, 20)	**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)

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

Based on the currently achievable level of performance of ELISA 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)*

3.6 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 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 (20).

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.

ELISA-Results given as egg white proteins were converted to whole egg powder. Results given as skimmed milk powder were converted to total milk protein. When possible the information supplied by the test kit manufacturer was used.

The results were grouped according to the applied methods and sorted chronologically according to the evaluation-number of the participants. Only ELISA-results were submitted by 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		
13	negative	0	positive	20,8	2/2 (100%)	BK	Results converted
14	negative	< 2,5	positive	3,2	2/2 (100%)	BK	
3a	negative		positive	< 19	2/2 (100%)	OX	Results converted
1	negative	< 0,5	positive	10,6	2/2 (100%)	RS	
2	negative	< 0,5	positive	1,4	2/2 (100%)	RS	
3b	negative		positive	12	2/2 (100%)	RS	Results converted
4	negative	< 0,1	positive	32	2/2 (100%)	RS	Outlier X_{all} a. X_{RS}
5	negative	< 0,5	positive	2,41	2/2 (100%)	RS	
6	negative	< 0,5	positive	3,69	2/2 (100%)	RS	
7	negative	< 0,12	positive	1,5	2/2 (100%)	RS	Results converted
8	negative	< 0,5	positive	7	2/2 (100%)	RS	
9	negative	< 0,5	positive	1,3	2/2 (100%)	RS	
10	negative	< 0,5	positive	1,7	2/2 (100%)	RS	
11	negative	< 0,5	positive	9,8	2/2 (100%)	RS	
12	negative	< 0,10	positive	8,82	2/2 (100%)	RS	
15	negative	< NWG	positive	4,35	2/2 (100%)	RS	

	Sample A		Sample B	
Number positive	0		16	
Number negative	16		0	
Percent positive	0		100	
Percent negative	100		0	
Consensus	negative		positive	

Methods:

BK = Biokits, Neogen RS = R-Biopharm, Ridascreen®

OX = Oxoid Egg Residue

Comments:

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

Quantitative evaluation of results: Sample B

Evaluation number	Whole Egg Powder	z-Score X_{ALL}	z-Score X_{RS}	Method	Remarks
	[mg/kg]	Bezug X_{ALL}	$X_{Methode\ RS}$		
13	20,8	8,8		BK	Results converted
14	3,2	-2,0		BK	
3a	< 19			OX	Results converted
1	10,6	2,5	3,0	RS	
2	1,4	-3,1	-3,1	RS	
3b	12	3,4	4,0	RS	Results converted
4	32	15,6	17,3	RS	Outlierr X_{all} a. X_{RS}
5	2,41	-2,5	-2,4	RS	
6	3,69	-1,7	-1,5	RS	
7	1,5	-3,1	-3,0	RS	Results converted
8	7	0,3	0,7	RS	
9	1,3	-3,2	-3,1	RS	
10	1,7	-3,0	-2,9	RS	
11	9,8	2,0	2,5	RS	
12	8,82	1,4	1,9	RS	
15	4,35	-1,3	-1,1	RS	

Methods:

BK = Biokits, Neogen RS = R-Biopharm, Ridascreen®

OX = Oxoid Egg Residue

Characteristics: Quantitative evaluation Egg (as Whole Egg Powder)

Sample B

	All Results *[mg/kg]*	**Method RS** *[mg/kg]*
Assigned value	X_{ALL}	$X_{Method\ RS}$
Number of results	*15*	*13*
Robust mean (X)	6,52	6,02
Robust standard deviation (S^x)	5,67	5,09
Median	4,35	4,35
Target range:		
Target standard deviation ($\hat{\sigma}$)	1,63	1,51
lower limit of target range (X - 2 $\hat{\sigma}$)	3,26	3,01
upper limit of target range (X + 2 $\hat{\sigma}$)	9,78	9,03
Quotient $S^x/\hat{\sigma}$	*3,5*	*3,4*
Standard uncertainty u_X	*1,8*	*1,8*
Quotient $u_X/\hat{\sigma}$	*1,1*	*1,2*
Number of results in the target range	*6 (40%)*	*4 (31%)*

Method:
RS = R-Biopharm, Ridascreen Fast®

Comments:
The evaluation of all methods and of method RS showed an increased variability. The quotients $S^x/\hat{\sigma}$ were clearly above. Therefore the comparability was limited.
The mean of the evaluations of all results and of method RS were about 100 times below the spiking level (s. also "Recovery rates of Whole Egg Powder" p.18).

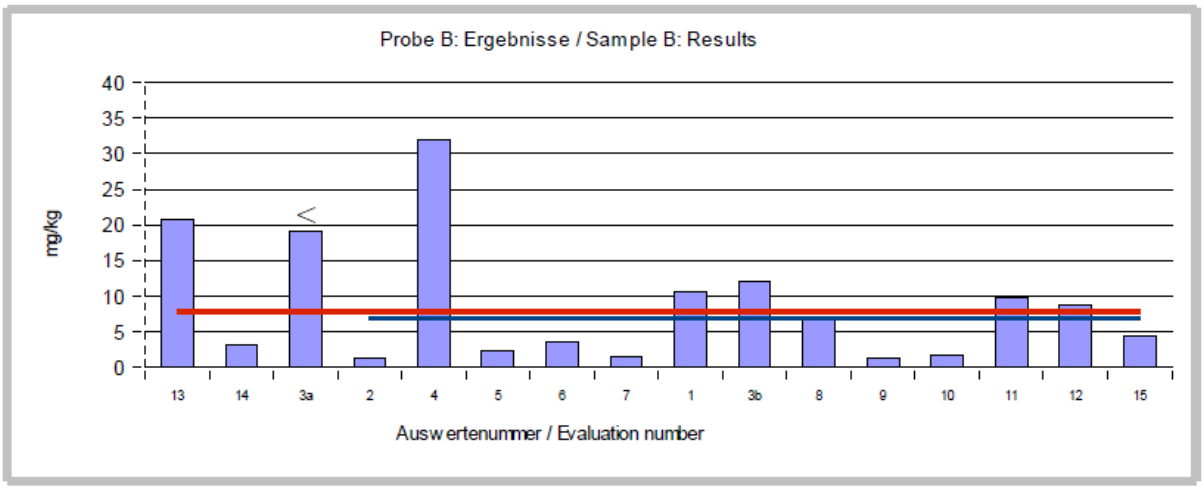

Fig. 2: ELISA-Results Egg (as Whole Egg Powder)
red line = Assigned value robust mean all results
blue line = Assigned value robust mean results method RS

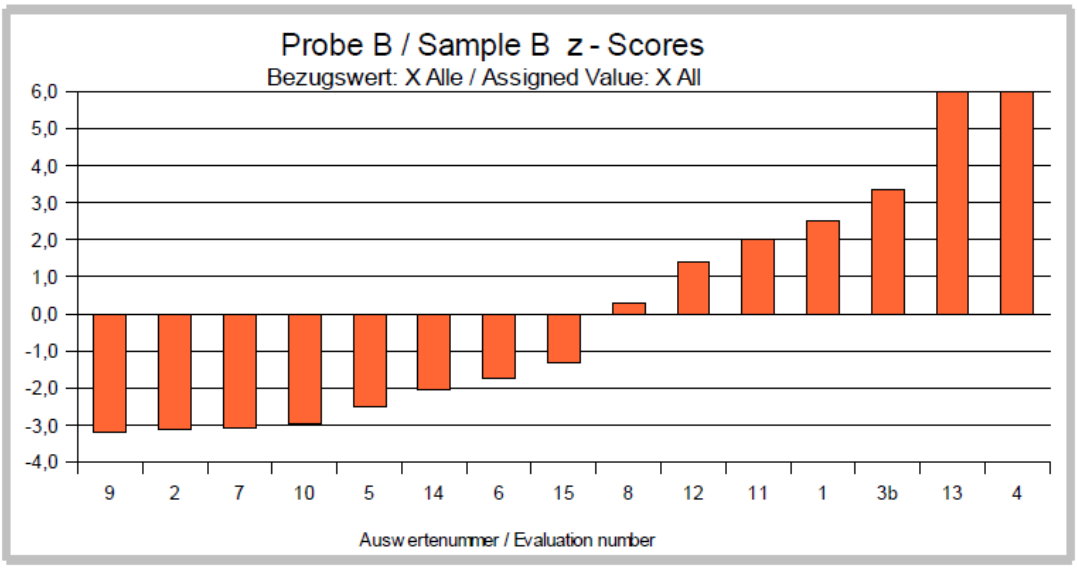

Fig. 3: z-Scores (ELISA-Results as Whole Egg Powder)
Assigned value robust mean of all results

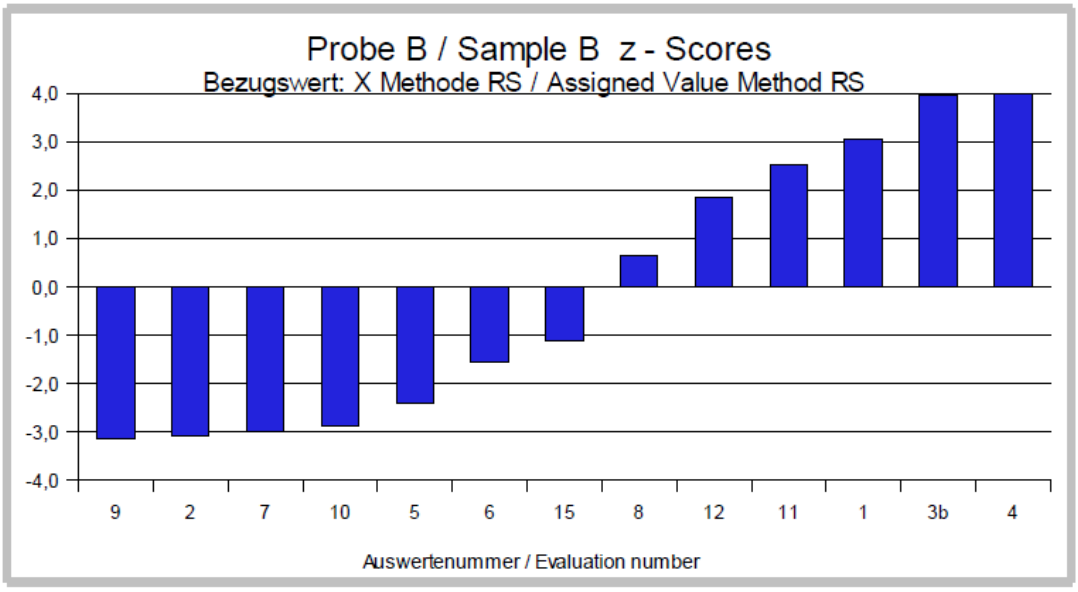

Fig. 4: z-Scores (ELISA-Results as Whole Egg Powder) Assigned value robust mean of method RS (R-Biopharm, Ridascreen Fast)

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

Evaluation number	Spiking material	Recovery rate	Sample B	Recovery rate	Method
	[mg/kg]	[%]	[mg/kg]	[%]	
13	28719	**84**	20,8	3,0	BK
14	33700	**99**	3,2	0,5	BK
3a			< 19		OX
1	42244	**124**	10,6	1,5	RS
2	-		1,4	0,2	RS
3b			12	1,7	RS
4			32	4,6	RS
5	39066,86	**115**	2,41	0,3	RS
6	30570	**90**	3,69	0,5	RS
7	> 15		1,5	0,2	RS
8	>13,5		7	1,0	RS
9	42600	**125**	1,3	0,2	RS
10	77000	226	1,7	0,2	RS
11	36378	**107**	9,8	1,4	RS
12	>13,5		8,82	1,3	RS
15	101446	297	4,35	0,6	RS

RA*	50-150 %	RA*	50-150 %
Number in RA	**7**	Number in RA	0
Percent in RA	**78**	Percent in RA	0

* Range of Acceptance of AOAC for Allergen-ELISAs

Methods:
BK = Biokits, Neogen RS = R-Biopharm, Ridascreen®
OX = Oxoid Egg Residue

Comments:
For the spiking material sample 78% of participants obtained recovery rates within the range of the AOAC-recommendation of 50-150%. For the sausage-sample B produced with the spiking material sample all recovery rates were below 5%.

4.2 Proficiency Test Milk

4.2.1 ELISA-Results: Total Milk Protein

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	<2,5	positive	101,4	2/2 (100%)	RS	
5	negative	<2,5	positive	80,33	2/2 (100%)	RS	
6	negative	<2.5	positive	52,5	2/2 (100%)	RS	
8	negative	<2,5	positive	92	2/2 (100%)	RS	
9	negative	<2,5	positive	100	2/2 (100%)	RS	
11	negative	<2.5	positive	49	2/2 (100%)	RS	
15	negative	< LOD	positive	44,5	2/2 (100%)	RS	
14	negative	<0,9	positive	57,6	2/2 (100%)	VT	Result converted

	Probe A		Probe B	
Number positive	0		8	
Number negative	8		0	
Percent positive	0		100	
Percent negative	100		0	
Consensus	negative		positive	

Method:
RS = R-Biopharm, Ridascreen® VT = Veratox, Neogen

Comments:
There were 100% negative results for sample A and 100% positive results for sample B by the ELISA-methods for total milk protein. The results are in qualitative agreement with the spiking of sample B.

Quantitative valuation of results: Sample B

Evaluation number	Milk protein	z-Score X_{ALL}	z-Score X_{RS}	Method	Remarks
	[mg/kg]	Bezug X_{ALL}	$X_{Methode\ RS}$		
1	101,4	1,6	1,5	RS	
5	80,33	0,5	0,3	RS	
6	52,5	-1,1	-1,2	RS	
8	92	1,1	1,0	RS	
9	100	1,5	1,4	RS	
11	49	-1,3	-1,4	RS	
15	44,5	-1,5	-1,6	RS	
14	57,6	-0,8		VT	Results converted

Method:
RS = R-Biopharm, Ridascreen® VT = Veratox, Neogen

Characteristics: Quantitative evaluation Milk Protein

Sample B

	All Results *[mg/kg]*	**Method RS** *[mg/kg]*
Assigned value	X_{ALL}	$X_{Method\ RS}$
Number of results	*8*	*7*
Robust mean (X)	72,2	74,2
Robust standard deviation (S^x)	27,1	28,3
Median	69,0	80,3
Target range:		
Target standard deviation ($\hat{\sigma}$)	18,1	18,6
lower limit of target range (X - 2 $\hat{\sigma}$)	36,1	37,1
upper limit of target range (X + 2 $\hat{\sigma}$)	108	111
Quotient $S^x/\hat{\sigma}$	*1,5*	*1,5*
Standard uncertainty u_X	*12,0*	*13,4*
Quotient $u_X/\hat{\sigma}$	*0,66*	*0,72*
Number of results in the target range	*8 (100%)*	*7 (100%)*

Method:
RS = R-Biopharm, Ridascreen Fast®

Comments:
The evaluation of all methods and of method RS showed an acceptable variability. The quotients $S^x/\hat{\sigma}$ were clearly below 2,0. Therefore the comparability was fair.
The mean of the evaluations of all results and of method RS were about 5 times lower than the spiking level (s. also "Recovery rates of Total Milk Protein" p.24).

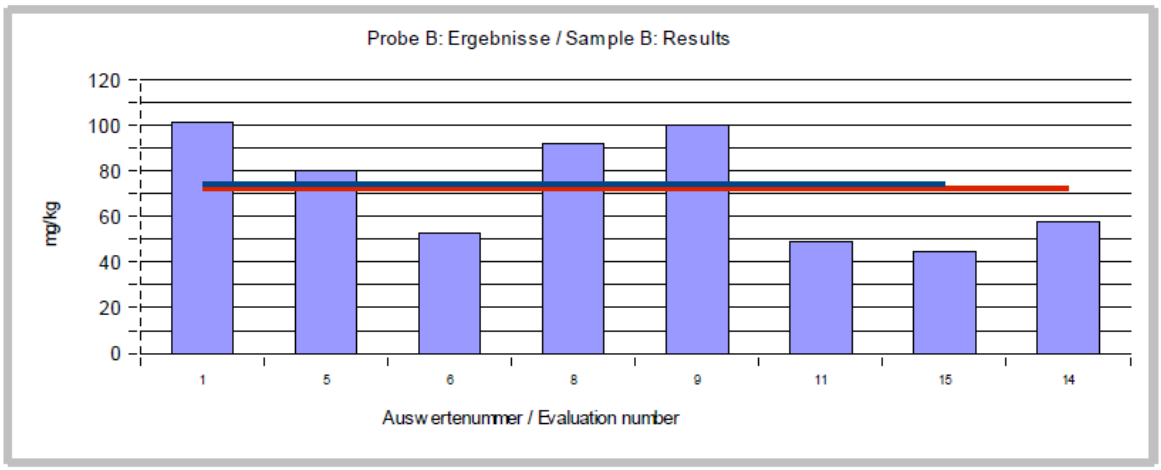

Fig. 5: ELISA-Results Milk Protein
red line = Assigned value robust mean all results
blue line = Assigned value robust mean results method RS

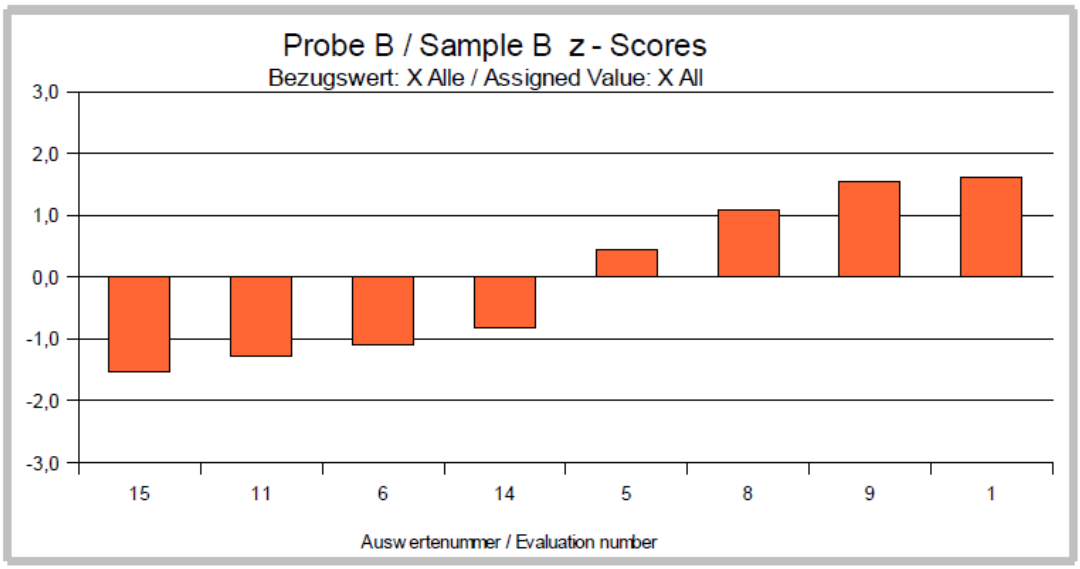

Fig. 6: z-Scores (ELISA-Results as Milk Protein)
Assigned value robust mean of all results

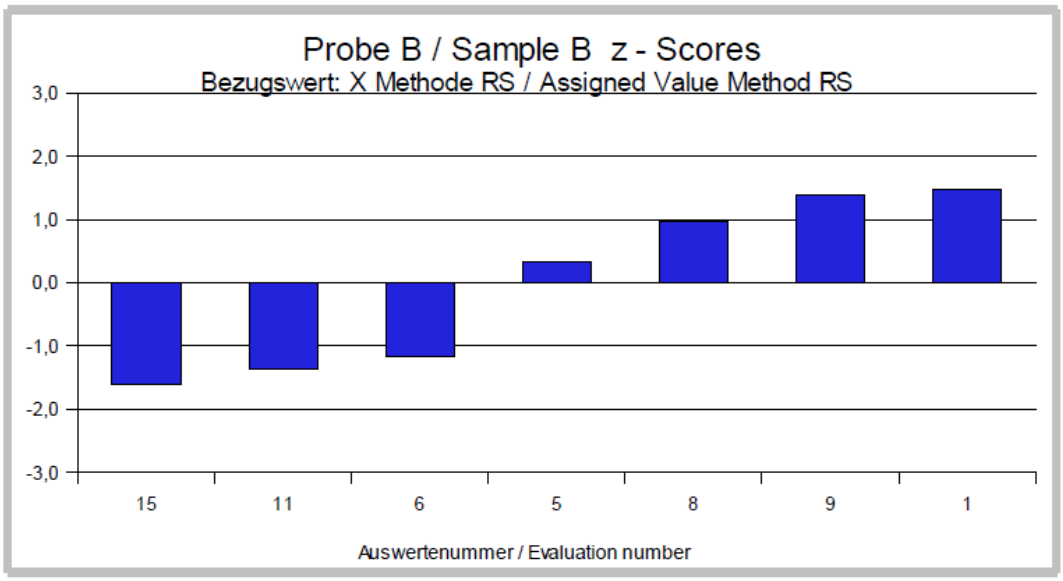

Fig. 7: z-Scores (ELISA-Results as Milk Protein) Assigned value robust mean of method RS (R-Biopharm, Ridascreen Fast)

Recovery Rates for Total Milk Protein:
Spiking Material Sample and Sample B

Evaluation number	Spiking material	Recovery rate	Sample B	Recovery rate	Method	Remarks
	[mg/kg]	[%]	[mg/kg]	[%]		
1	19398	**111**	101,4	29	RS	
5	17449,28	**100**	80,33	23	RS	
6	5518,77	32	52,5		RS	
8	>67,5		92	26	RS	
9	17148	**98**	100	28	RS	
11	15379	**88**	49	14	RS	
15	12930	**74**	44,5	13	RS	
14	12312	**71**	57,6	16	VT	Result converted

RA*	50-150 %	RA*	50-150 %
Number in RA	**6**	Number in RA	0
Percent in RA	**86**	Percent in RA	0

* Range of Acceptance of AOAC for Allergen-ELISAs

Method:
RS = R-Biopharm, Ridascreen® VT = Veratox, Neogen

Comments:
For the spiking material sample 86% of participants obtained recovery rates within the range of the AOAC-recommendation of 50-150%. For the sausage-sample B produced with the spiking material sample recovery rates were in the range of 14-29%.

4.2.2 ELISA-Results: Casein

Qualitative valuation of results: Samples A and B

Evaluation number	Sample A	Sample A	Sample B	Sample B	Qualitative Valuation	Method	Remarks
	pos/neg	[mg/kg]	pos/neg	[mg/kg]	Agreement with consensus value		
13	negative	0	positive	31,6	2/2 (100%)	AQ	
1	negative	< 0,5	positive	2,8	2/2 (100%)	RS	
3a	negative		positive	16	2/2 (100%)	RS	
4	negative	< 1,36	positive	3,4	2/2 (100%)	RS	
6	negative	< 0,5	positive	7,86	2/2 (100%)	RS	
7	negative	< 1,36	positive	14,9	2/2 (100%)	RS	
10	negative	< 0,5	positive	25	2/2 (100%)	RS	
12	negative	< 1,36	positive	14,77	2/2 (100%)	RS	
15	negative	< NWG	positive	2,96	2/2 (100%)	RS	
3b	negative		positive	> 7	2/2 (100%)	VT	

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

Methods:

AQ = AgraQuant, RomerLabs VT = Veratox, Neogen
RS = R-Biopharm, Ridascreen®

Comments:

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

Quantitative valuation of results: Sample B

Evaluation number	Casein	z-Score X_{ALL}	z-Score X_{RS}	Method	Remarks
	[mg/kg]	X_{ALL}	$X_{Method\ RS}$		
13	31,6	5,7		AQ	
1	2,8	-3,1	-3,0	RS	
3a	16	0,9	1,9	RS	
4	3,4	-3,0	-2,8	RS	
6	7,86	-1,6	-1,1	RS	
7	14,9	0,6	1,5	RS	
10	25	3,7	5,2	RS	
12	14,77	0,5	1,4	RS	
15	2,96	-3,1	-2,9	RS	
3b	> 7			VT	

Method:
AQ = AgraQuant, RomerLabs VT = Veratox, Neogen
RS = R-Biopharm, Ridascreen®

Characteristics: Quantitative evaluation Casein

Sample B

	All Results *[mg/kg]*	**Method RS** *[mg/kg]*
Assigned value	X_{ALL}	$X_{Method\ RS}$
Number of results	*9*	*8*
Robust mean (X)	13,0	10,9
Robust standard deviation (S^x)	11,0	8,84
Median	14,8	11,3
Target range:		
Target standard deviation ($\hat{\sigma}$)	3,25	2,73
lower limit of target range (X - 2 $\hat{\sigma}$)	6,50	5,45
upper limit of target range (X + 2 $\hat{\sigma}$)	19,5	16,4
Quotient $S^x/\hat{\sigma}$	*3,4*	*3,2*
Standard uncertainty u_X	*4,58*	*3,91*
Quotient $u_X/\hat{\sigma}$	*1,4*	*1,4*
Number of results in the target range	*4 (44%)*	*4 (50%)*

Method:
RS = R-Biopharm, Ridascreen Fast®

Comments:
The evaluation of all methods and of method RS showed an increased variability. The quotients $S^x/\hat{\sigma}$ were clearly above 2,0. Therefore the comparability was limited.
The mean of the evaluations of all results and of method RS were about 20 times lower than the spiking level (s. also "Recovery rates of Casein" p.30).

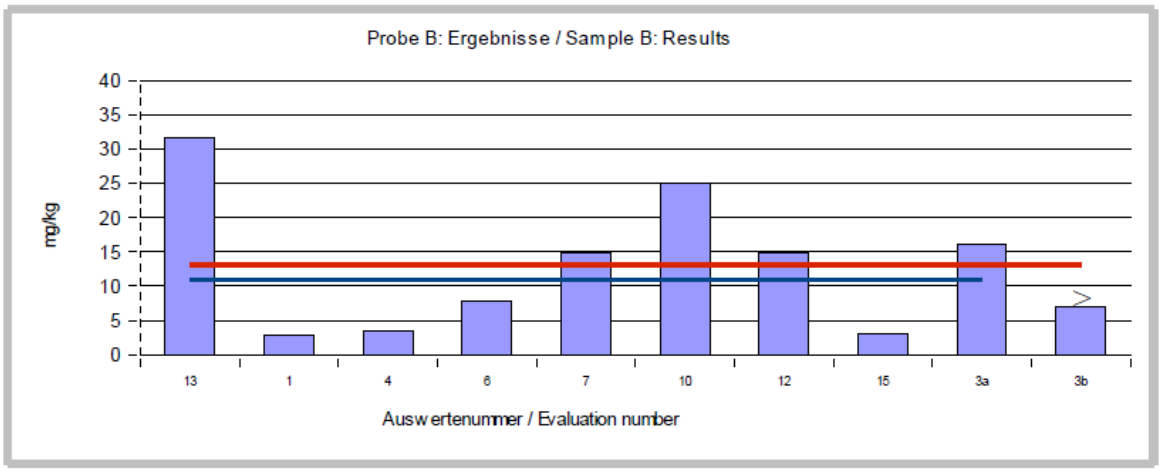

Fig. 8: ELISA-Results Casein
red line = Assigned value robust mean all results
blue line = Assigned value robust mean results method RS

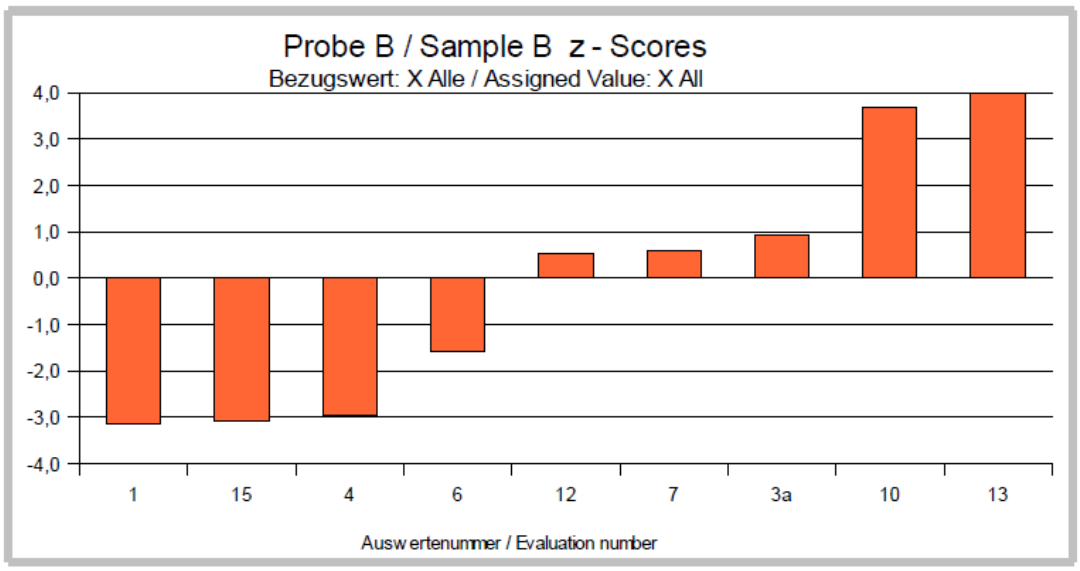

Fig. 9: z-Scores (ELISA-Results as Casein)
Assigned value robust mean of all results

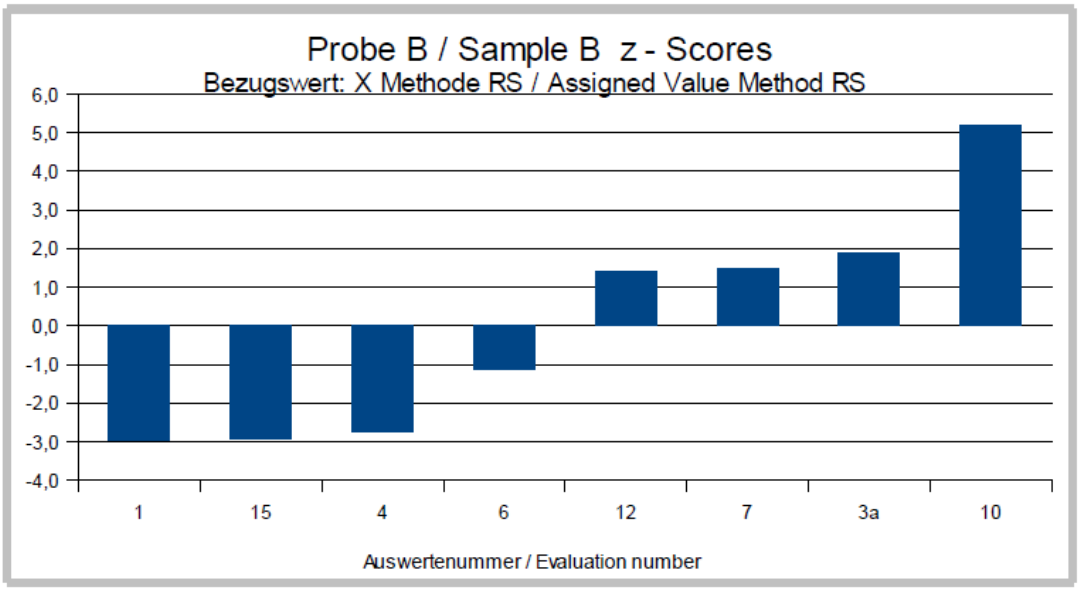

Fig. 10: z-Scores (ELISA-Results as Casein) Assigned value robust mean of method RS (R-Biopharm, Ridascreen Fast)

Recovery Rates for Casein:
Spiking Material Sample and Sample B

Evaluation number	Spiking material	Recovery rate	Sample B	Recovery rate	Method	Remarks
	[mg/kg]	[%]	[mg/kg]	[%]		
13	30775	221	31,6	11	AQ	
1	22296	160	2,8	1,0	RS	
3a			16	5,7	RS	
4			3,4	1,2	RS	
6	18759,77	**135**	7,86	2,8	RS	
7	>67,5		14,9	5,3	RS	
10	19000	**136**	25	8,8	RS	
12	> 13,5		14,77	5,2	RS	
15	1819	13	2,96	1,0	RS	
3b			> 7		VT	

RA*	50-150 %	RA*	50-150 %
Number in RA	**2**	Number in RA	0
Percent in RA	**40**	Percent in RA	0

* Range of Acceptance of AOAC for Allergen-ELISAs

Methods:
AQ = AgraQuant, RomerLabs VT = Veratox, Neogen
RS = R-Biopharm, Ridascreen®

Comments:
For the spiking material sample 40% (2 of 5) of participants obtained recovery rates within the range of the AOAC-recommendation of 50-150%. For the sausage-sample B produced with the spiking material sample recovery rates were in the range of 1-11%.

4.2.3 ELISA-Results: beta-Lactoglobulin

Qualitative valuation of results: Samples A and B

Evaluation number	Sample A	Sample A	Sample B	Sample B	Qualitative Valuation	Method	Remarks
	pos/neg	[mg/kg]	pos/neg	[mg/kg]	Agreement with consensus value		
13	negative	0	positive	2,7	2/2 (100%)	BK	
10	negative	< 0,1	negative	< 0,1	1/2 (50%)	ES	
2	negative	< 0,5	positive	6,2	2/2 (100%)	RS	
4	negative	< 0,19	positive	7,5	2/2 (100%)	RS	
6	negative	< 0,5	positive	4,84	2/2 (100%)	RS	
15	negative	< NWG	positive	4,44	2/2 (100%)	RS	

	Sample A		Sample B	
Number positive	0		5	
Number negative	6		1	
Percent positive	0		83	
Percent negative	100		17	
Consensus	negative		positive	

Methods:

BK = BioKits, Neogen RS = R-Biopharm, Ridascreen®

ES = ELISA-Systems

Comments:

There were 100% negative results for sample A and 83% positive results for sample B by the ELISA-methods for beta-Lactoglobulin. The results are in qualitative agreement with the spiking of sample B.

Quantitative valuation of results: Sample B

Evaluation number	beta-LG	z-Score X_{ALL}	Method	Remarks
	[mg/kg]	X_{ALL}		
13	2,7	-1,5	BK	
10	< 0,1	< -3,9	ES	
2	6,2	1,8	RS	
4	7,5	3,0	RS	
6	4,84	0,5	RS	
15	4,44	0,1	RS	

Methods:

BK = BioKits, Neogen
ES = ELISA-Systems
RS = R-Biopharm, Ridascreen®

Characteristics: Quantitative evaluation beta-Lactoglobulin

Sample B

	All Results *[mg/kg]*
Assigned value	X_{ALL}
Number of results	*6*
Robust mean (X)	4,3
Robust standard deviation (S^x)	3,0
Median	4,6
Target range:	
Target standard deviation ($\hat{\sigma}$)	1,1
lower limit of target range (X - 2 $\hat{\sigma}$)	2,15
upper limit of target range (X + 2 $\hat{\sigma}$)	6,45
Quotient $S^x/\hat{\sigma}$	*2,8*
Standard uncertainty u_X	*1,5*
Quotient $u_X/\hat{\sigma}$	*1,4*
Number of results in the target range	*4 (66%)*

Comments:
The evaluation of all methods showed an increased variability. The quotients $S^x/\hat{\sigma}$ were clearly above 2,0. Therefore the comparability was limited.
The mean of the evaluation of all results were about 9 times lower than the spiking level (s. also "Recovery rates of beta-Lactoglobulin" p.35).

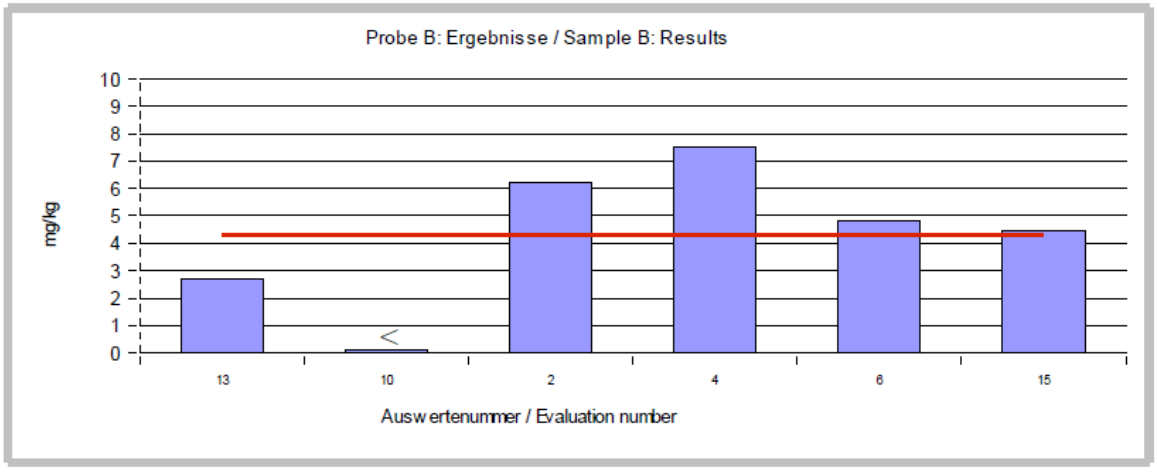

Fig. 11: ELISA-Results beta-Lactoglobulin
red line = Assigned value robust mean all results

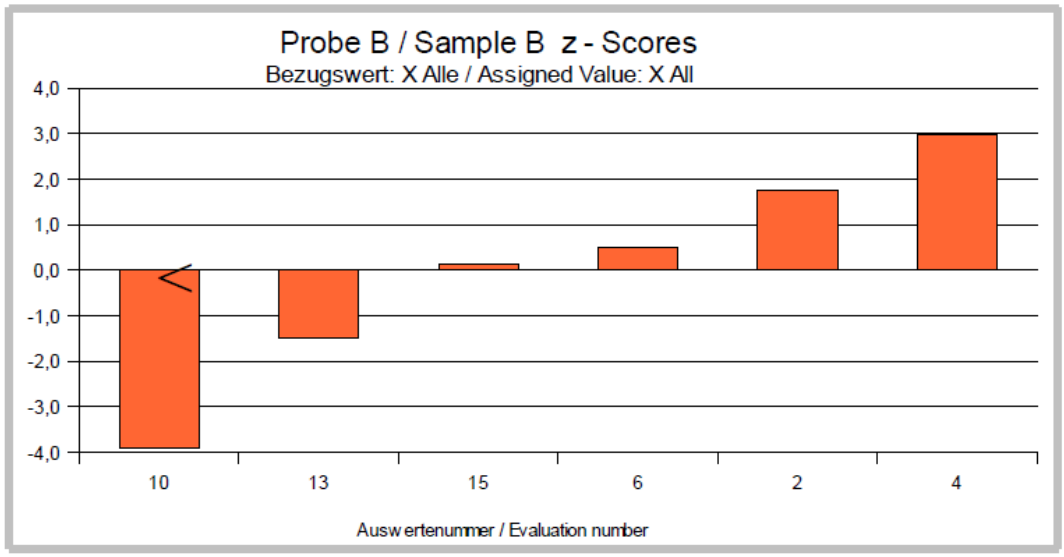

Fig. 12: z-Scores (ELISA-Results as beta-Lactoglobulin)
Assigned value robust mean of all results

Recovery Rates for beta-Lactoglobulin:
Spiking Material Sample and Sample B

Evaluation number	Spiking material	Recovery rate	Sample B	Recovery rate	Method	Remarks
	[mg/kg]	[%]	[mg/kg]	[%]		
13	3208	184	2,7	8	BK	
10	1700	**98**	< 0,1		ES	
15	1003	**58**	4,44	13	RS	
6	581,57	33	4,84	14	RS	
2	-		6,2	18	RS	
4			7,5	21	RS	

RA*	50-150 %	RA*	50-150 %
Number in RA	**2**	Number in RA	0
Percent in RA	**50**	Percent in RA	0

* Range of Acceptance of AOAC for Allergen-ELISAs

Methods:
AQ = AgraQuant, RomerLabs VT = Veratox, Neogen
RS = R-Biopharm, Ridascreen®

Comments:
For the spiking material sample 50% (2 of 4) of participants obtained recovery rates within the range of the AOAC-recommendation of 50-150%. For the sausage-sample B produced with the spiking material sample recovery rates were in the range of 8-21%.

5. Documentation

Details by the participants

5.1 ELISA: Egg

Primary data

Evaluation number	Result Sample A		Result Sample B		Result Spiking Sample		quantitatives Result given as	Meth. Abr.	Method
	qualitativ	mg/kg	qualitativ	mg/kg	qualitativ	mg/kg	z.B. Lebensmittel / Protein		Test-Kit + Manufacturer
13	-	0	-	5,4	-	7467	Egg white proteins, total	BK	BioKits Egg Assay Kit, Neogen
14	negative	<2.5	positive	3,2	positive	33700	Whole egg powder	BK	BioKits Egg Assay Kit, Neogen
3a	negative		positive	< 5			Egg white proteins, total	OX	Oxoid Egg Residue
2	negative	< 0,5	positive	1,4	positive	-	Whole egg powder	RS	Ridascreen Fast Egg (R4602), r-Biopharm
4	negative	< 0,1	positive	32	-		Whole egg powder	RS	Ridascreen Fast Egg (R4602), r-Biopharm
5	-	<0,5	-	2,41	-	39066,86	whole egg powder	RS	RIDASCREEN FAST Ei/Egg protein, r-biopharm R6402
6	negative	<0.5	positive	3,69	positive	30570	Whole egg powder	RS	Ridascreen Fast Egg (R4602), r-Biopharm
7	negative	<0,03	positive	0,38	positive	>4	Egg white proteins, total	RS	Ridascreen Fast Egg (R4602), r-Biopharm
1	negative	<0,5	positive	10,6	positive	42.244	Whole egg powder	RS	R6402, r-biopharm
3b	negative		positive	3			Egg white proteins, total	RS	Ridascreen Fast Ei
8	negative	<0,5	positive	7	positive	>13,5		RS	Ridascreen Fast Ei (R4602), r-Biopharm
9	negative	<0,5	positive	1,3	positive	42600	Whole egg powder	RS	Ridascreen Fast Ei (R4602), r-Biopharm
10	negative	<0,5	positive	1,7	positive	77000	Whole egg powder	RS	Ridascreen Fast Ei (R4602), r-Biopharm
11	negative	<0.5	positive	9,8	positive	36378	Whole egg powder	RS	Ridascreen Fast Ei (R6402), r-Biopharm
12	-	<0,10	-	8,82	-	>13,5	Whole egg powder-conzentration	RS	Ridascreen Fast Ei Art-No R6402 von r-biopharm
15	-	< LOD	-	4,35	-	101446	Whole egg powder	RS	Ridascreen Fast Ei (R4602), r-Biopharm

Methods:

BK = Biokits, Neogen RS = R-Biopharm, Ridascreen®

OX = Oxoid Egg Residue

Other details to the Methods

Evaluation number	Meth. Abr.	Specifity	Remarks to the Method (Extraction and Determination)	Further Remarks
		Antibody	e.g. Extraction Solution / Timet / Temperature	
13	BK	anti-Ovomucoid *(Gal d1)*	according to manual	
14	BK			
3a	OX		according to test kit manual	
2	RS	Antibodies against egg white proteins ovalbumin and ovomucoid	The procedure given in the instructions supplied with the kit was followed without any change	The quantitative data for the Spiking Sample is missing because, due to the limited tests we could perform, we were unable to find the correct dilution to obtain an absorbance value within the calibration curve
4	RS			
5	RS		according to kit	
6	RS	egg white proteins ovalbumin & ovomucoid	As per Kit Instructions	Spiked sample needed to be diluted to produce a result in the range of the kit. Therefore the result is only an estimate
7	RS			
1	RS		according to test kit manual	
3b	RS		according to test kit manual	13 mg/kg whole egg powder
8	RS			
9	RS	Ovalbumin/ Ovomucoid	Sample preparation according to test kit manual in shaking water bath for 10min at 60°C.	Sample A difficult to homogenize, yellowish fat-like layer
10	RS		according to test kit manual	
11	RS		according to test kit manual with addition of Casein to minimize disturbing effects	
12	RS	specific Antibodies to egg white proteins Ovalbumin and Ovomucoid. No cross-reactivities to raw and cooked hen's, turkey, pork meat or beef	Sample preparation according to point 9. of test kit manual	
15	RS		according to manual	

5.2 ELISA: Milk Protein

Primary data

Evaluation number	Result Sample A		Result Sample B		Result Spiking Sample		quantitatives Result given as	Meth. Abr.	Method
	qualitativ	mg/kg	qualitativ	mg/kg	qualitativ	mg/kg	z.B. Lebensmittel / Protein		Test-Kit + Manufacturer
1	negtive	<2,5	positive	101,4	positive	19.398	milk protein	RS	R4652, r-biopharm
5	-	<2,5	-	80,334	-	17449,28	milk protein	RS	RIDASCREEN FAST Milk, r-biopharm R4652
6	negative	<2.5	positive	52,5	positive	5518,77	Milk proteins, total	RS	Ridascreen Fast Milk (R4652), r-Biopharm
8	negtive	<2,5	positive	92	positive	>67,5		RS	Ridascreen Fast Milk (R4652), r-Biopharm
9	negtive	<2,5	positive	100	positive	17148	Milk proteins, total	RS	Ridascreen Fast Milk (R4652), r-Biopharm
11	negative	<2.5	positive	49	positive	15379	Milk proteins, total	RS	Ridascreen Fast Milk (R4652), r-Biopharm
15	-	< LOD	-	44,5	-	12930	Milk proteins, total	RS	Ridascreen Fast Milk (R4652), r-Biopharm
14	negative	<2.5	-	160	positive	34200	skimmed milk powder	VT	Veratox Total Milk Allergen, Neogen

Method:
RS = R-Biopharm, Ridascreen® VT = Veratox, Neogen

Other details to the methods

Meth. Abr.	Specifity	Remarks to the Method (Extraction and Determination)	Further Remarks
	Antibody	e.g. Extraction Solution / Timet / Temperature	
RS		according to test kit manual	
RS		according to kit	
RS	α- β- & κ-caseins and β-lactoglobulin from cow's milk & of sheep, goat and buffalo milk	As per Kit Instructions	Spiked sample needed to be diluted to produce a result in the range of the kit. Therefore the result is only an estimate
RS			
RS	Caseins/ ß-Lactoglobulin	Sample preparation according to test kit manual in shaking w ater bath for 10 min at 100°C and then 10-min at 60°C.	Spiking sample clotting after cooking
RS			
RS		according to test kit manual	
VT			

5.3 ELISA: Casein

Primary data

Evaluation number	Result Sample A		Result Sample B		Result Spiking Sample		quantitatives Result given as	Meth. Abr.	Method
	qualitativ	mg/kg	qualitativ	mg/kg	qualitativ	mg/kg	z.B. Lebensmittel / Protein		Test-Kit + Manufacturer
13	-	0	-	31,6	-	30775	Casein	AQ	AgraQuant Casein (COKAL1200), RomerLabs
1	negative	<0,5	positive	2,8	positive	22.296	Casein	RS	R4612, r-bipharm
4	negative	< 1,36	positive	3,4	-		Casein	RS	Ridascreen Fast Casein (R4612), r-Biopharm
6	negative	<0.5	positive	7,86	positive	18759,77	Casein	RS	Ridascreen Fast Casein (R4612), r-Biopharm
7	negative	< 1,36	positive	14,9	positive	>67,5	Casein	RS	Ridascreen Fast Casein (R4612), r-Biopharm
10	negative	<0,5	positive	25	positive	19000	Casein	RS	Ridascreen Fast Casein (R4612), r-Biopharm
12	-	<1,36	-	14,77	-	> 13,5	Casein-concentration	RS	Ridascreen Fast Casein Art-No R4612 von r-biopharm
15	-	< LOD	-	2,96	-	1819	Casein	RS	Ridascreen Fast Casein (R4612), r-Biopharm
3a	negative		positive	16			Casein	RS	Ridascreen Fast Casein
3b	negative		positive	> 7			Casein	VT	Neogen Total Milk

Methods:

AQ = AgraQuant, RomerLabs VT = Veratox, Neogen
RS = R-Biopharm, Ridascreen®

Other Remarks to the Methods

Evaluation number	Meth. Abr.	Specifity	Remarks to the Method (Extraction and Determination)	Further Remarks
		Antibody	e.g. Extraction Solution / Timet / Temperature	
13	AQ	anti-Casein	according to test kit manual	
1	RS		according to test kit manual, sample preparation without RIDA Extraction solution	
4	RS			
6	RS	α- β- & κ-caseins from cow's milk & caseins of sheep, goat and buffalo milk	As per Kit Instructions	Spiked sample needed to be diluted to produce a result in the range of the kit. Therefore the result is only an estimate
7	RS			
10	RS		according to test kit manual	Attention: Extract of sample B was measured undiluted and diluted 1:10. I submitted the result of the 1:10 dilution (25 mg/kg). Undiluted we observed inhibition, giving underestimated results. The undiluted extract gave the result 4,6 mg/kg!
12	RS	The antibodies detect specifically α-,β- and κ-Caseine from cow's milk and sheep, goat and buffalo milk	Sample preparation as described in pointt 9.3. sausage and baking mixtures of test kit manual	
15	RS		according to test kit manual for sausages	
3a	RS		according to test kit manual	
3b	VT		according to test kit manual	> 9 mg/kg total milk proteins

5.3 ELISA: beta-Lactoglobulin

Primary data

Evaluation number	Result Sample A		Result Sample B		Result Spiking Sample		quantitatives Result given as	Meth. Abr.	Method
	qualitativ	mg/kg	qualitativ	mg/kg	qualitativ	mg/kg	z.B. Lebensmittel / Protein		Test-Kit + Manufacturer
13	-	0	-	2,7	-	3208	beta-Lactoglobulin	BK	BioKits β-Lactoglobulin Assay Kit, Neogen
10	negative	<0,1	negative	<0,1	positive	1700	beta-Lactoglobulin	ES	ELISA-Systems β-Lactoglobulin Residue Detection ELISA
2	negative	< 0,5	positive	6,2	positive	-	beta-Lactoglobulin	RS	Ridascreen Fast β-Lactoglobulin (R4902), r-Biopharm
4	negative	< 0,19	positive	7,5	-		beta-Lactoglobulin	RS	Ridascreen Fast β-Lactoglobulin (R4902), r-Biopharm
6	negative	<0.5	positive	4,84	positive	581,57	beta-Lactoglobulin	RS	Ridascreen Fast β-Lactoglobulin (R4902), r-Biopharm

Methods:

BK = BioKits, Neogen RS = R-Biopharm, Ridascreen®

ES = ELISA-Systems

Other Remarks to the Methods

Evaluation number	Meth. Abr.	Specifity	Remarks to the Method (Extraction and Determination)	Further Remarks
		Antibody	e.g. Extraction Solution / Timet / Temperature	
13	AQ	anti-beta Lactoglobulin	according to test kit manual	
10	RS		according to test kit manual	
2	RS	Antibodies against beta-lactoglobulin	The procedure given in the instructions supplied with the kit was followed without any change	The quantitative data for the Spiking Sample is missing because, due to the limited tests we could perform, we were unable to find the correct dilution to obtain an absorbance value within the calibration curve
4	RS			
6	RS	β-lactoglobulin of cow, sheep, goat and buffalo milk	As per Kit Instructions	Spiked sample needed to be diluted to produce a result in the range of the kit. Therefore the result is only an estimate
15	RS		according to test kit manual	

6. Index of participant laboratories

Teilnehmer / Participant	Ort / Town	Land / Country
		SWITZERLAND
		ITALY
		GERMANY
		GERMANY
		GERMANY
		ITALY
		GERMANY
		ITALY
		SWITZERLAND
		SWITZERLAND
		GERMANY
		GERMANY
		GERMANY
		UNITED KINGDOM
		GERMANY
		NETHERLANDS

[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
2. Verordnung / Regulation 882/2004/EU; Verordnung über amtliche Kontrollen / Regulation on official controls
3. 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
4. Richtlinie / Directive 1993/99/EU; über zusätzliche Maßnahmen im Bereich der amtlichen Lebensmittelüberwachung / on additional measures concerning the official control of foodstuffs
5. ASU §64 LFGB : Planung und statistische Auswertung von Ringversuchen zur Methodenvalidierung
6. DIN ISO 13528:2009; Statistische Verfahren für Eignungsprüfungen durch Ringversuche / Statistical methods for use in proficiency testing by interlaboratory comparisons
7. The International Harmonised Protocol for the Proficiency Testing of Ananlytical Laboratories ; J.AOAC Int., 76(4), 926 – 940 (1993)
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