DLA
Dienstleistung
Lebensmittel
Analytik GbR

Evaluation Report
proficiency test

03/2014

Allergens III:

β-Lactoglobulin and Gluten

in Infant Food

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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 material is an infant food mixture of common in commerce "millet-pap" and "millet-fruit-pap" powders from 4th and 6th month respectively (labeled "milk and gluten free"). The basic composition of both sample A and sample B was the same (see table 1). The spiking material sample containing milk powder and wheat flour was added to sample B. The composition of the spiking material sample and the amounts of allergens in sample B is given in table 2.

After homogenisation the samples were portioned to approximately 25 g.

Table 1: Composition of DLA-Samples

Ingredients	**Sample A**	**Sample B**
Organic-Millet-Pap, infant pap after 4th month Ingredients: Millet whole flour, Vitamin B1 Nutrients per 100 g: Protein 13 g, carbohydrates 75 g, fat 4,1 g	50,0 g/100 g	49,6 g/100 g
Organic-Millet-Fruit-Pap, infant pap after 6th month Ingredients: Millet whole flour (90%), apple-flakes with rice flour (5%), pear flakes with rice flour (5%), Vitamin B1 Nutrients per 100 g: Protein 12 g, carbohydrates 76 g, fat 4,2 g	50,0 g/100 g	49,6 g/100 g

Table 2: Added amounts of allergenic ingredients

Ingredients	**Spiking material sample**	**Sample B**
Potato flour	83 %	0,71 %
Soy flour	6,21 %	0,053 %
Whole egg powder	3,41 %	0,029 %
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	 410 mg/kg 145 mg/kg 116 mg/kg 14 mg/kg
Wheat: - as Wheat flour Typ 550 - thereof total protein - *thereof gluten***	 26500 mg/kg (2,65 %) 2809 mg/kg 2528 mg/kg	 212 mg/kg 23 mg/kg 22 mg/kg

* according to labelling
** Definition of "gluten" from the Gluten Intolerance Labelling Regulation (EU/41/2009) corresponds to 85-91% of wheat protein according to 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).

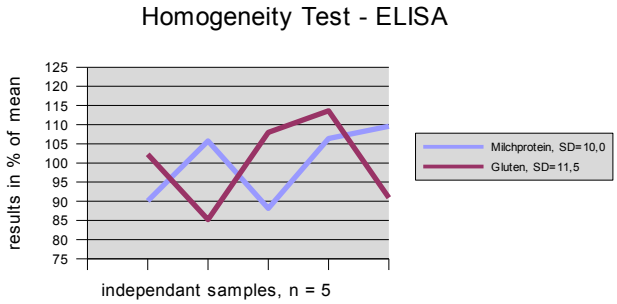

Fig. 1: Testing of homogeneity of DLA-sample B
Results are given in percent of the arithmetic mean

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 17th week of 2014. The testing method was optional. The tests should be finished at May 10th 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. beta-lactoglobulin or gluten 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.
All 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).

The Working Group on Prolamin Analysis and Toxicity (WGPAT) coordinated a collaborative study with two commercial ELISA-Test-Kits for the determination of gluten using the monoclonal R5 antibody (21). 12 food samples with gliadin in the range of 0 - 168 mg/kg were analyzed by 20 laboratories. Recovery rates ranged between 65 and 110%, relative repeatability deviations ranged from 13 - 25% (method 1) and 11 - 22% (method 2) while the relative reproducibility standard deviations ranged from 23 - 47%

(method 1) and 25 - 33% (method 2). According to the authors both ELISA-Test-Kits fulfilled therefore the current validation criteria for ELISA methods (21).

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 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 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 (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, which were given as gliadin, were converted into gluten with respect to the instructions of the test kit manufacturers. The original results are given in the documentation.

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 β-Lactoglobulin

4.1.1 ELISA-Results: β-Lactoglobulin (beta-LG)

Qualitative valuation of results: Samples A and B

Evaluation number	Sample A	Sample A	Sample B	Sample B	Qualitative Valuation	Method	Remarks
	pos/neg	[mg/kg]	pos/neg	[mg/kg]	Agreement with Consensus Value		
3	negative	<0,2	positive	13,4	2/2 (100%)	ES	
14	positive	0,005	positive	>1	1/2 (50%)	ES	
18	negative	<0,2	positive	20	2/2 (100%)	ES	
19	negative	<0,5	positive	11	2/2 (100%)	IG	
5	negative	<0,50	positive	5,42	2/2 (100%)	RS1	
7	negative	<0,2	positive	2,2	2/2 (100%)	RS1	
11	negative	<5,0	positive	16,2	2/2 (100%)	RS1	
16	negative	< BG	negative	< BG	1/2 (50%)	RS1	
6	negative	<0,5	positive	5,34	2/2 (100%)	RS2	
12	negative	<0,5	positive	4,43	2/2 (100%)	RS2	
10	negative	<5	positive	6,26	2/2 (100%)	RS?	
8	negative	< 0,1	-		2/2 (100%)	div	

	Sample A		Sample B	
Number positive	1		10	
Number negative	11		1	
Percent positive	8		91	
Percent negative	92		9	
Consensus	negative		positive	

Method:

ES = ELISA Systems
IG = Ingenzim, Ingenasa
RS1 = Ridascreen® R4901, R-Biopharm
RS2 = Ridascreen® R4902, R-Biopharm
RS? = Ridascreen®, R-Biopharm
div = not indicated / other method

Comments:

There were 92% negative results for sample A and 91% positive results for sample B for detection of beta-Lactoglobulin by the ELISA-methods. The results are in qualitative agreement with the spiking of sample B.

Quantitative evaluation of results: Sample B

Evaluation number	beta-LG	z-Score X_{ALL}	Method	Remarks
	[mg/kg]			
3	13,4	1,8	ES	
14	>1		ES	
18	20	4,6	ES	
19	11	0,7	IG	
5	5,42	-1,7	RS1	
7	2,2	-3,1	RS1	
11	16,2	3,0	RS1	
16	< BG		RS1	
6	5,34	-1,7	RS2	
12	4,43	-2,1	RS2	
10	6,26	-1,3	RS?	
8			div	

Methods:

ES = ELISA Systems
IG = Ingenzim, Ingenasa
RS1 = Ridascreen® R4901, R-Biopharm
RS2 = Ridascreen® R4902, R-Biopharm
RS? = Ridascreen®, R-Biopharm
div = not indicated / other method

Characteristics: Quantitative evaluation β-Lactoglobulin

Sample B

	All Results *[mg/kg]*
Assigned value	X_{ALL}
Number of results	*9*
Robust mean (X)	9,30
Robust standard deviation (S^x)	6,76
Median	6,26
Target range:	
Target standard deviation ($\hat{\sigma}$)	2,33
lower limit of target range (X - 2 $\hat{\sigma}$)	4,65
upper limit of target range (X + 2 $\hat{\sigma}$)	14,0
Quotient $S^x/\hat{\sigma}$	*2,9*
Standard uncertainty u_X	*2,82*
Quotient $u_X/\hat{\sigma}$	*1,2*
Number of results in the target range	*5 (56%)*

Comments:
The evaluation of results from all methods showed a slightly increased variability. The quotient $S^x/\hat{\sigma}$ was above 2,0.
The mean of the evaluation was about 62% of the spiking level (s. "Recovery rates of β-Lactoglobulin" p.17).

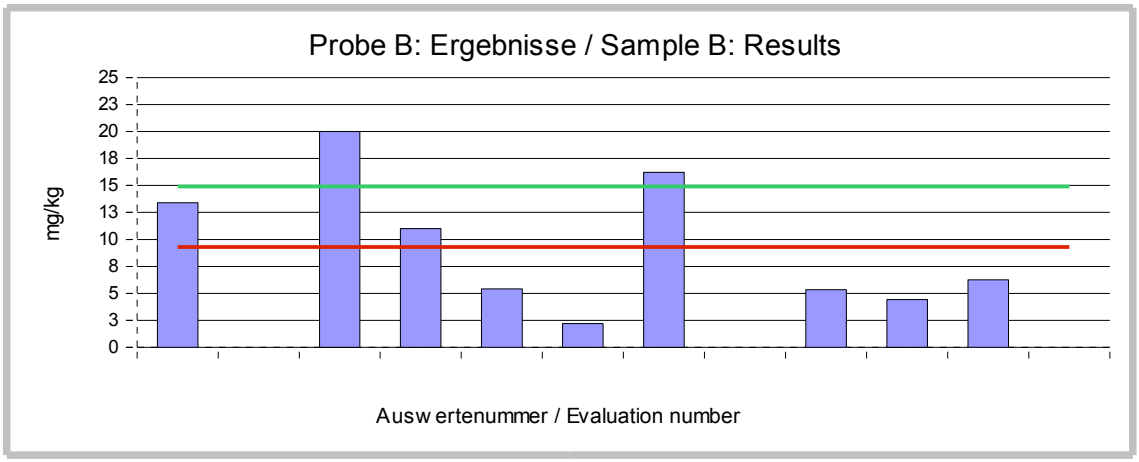

Fig. 2: ELISA-Results β-Lactoglobulin
green line = Spiking level
red line = Assigned value robust mean all results

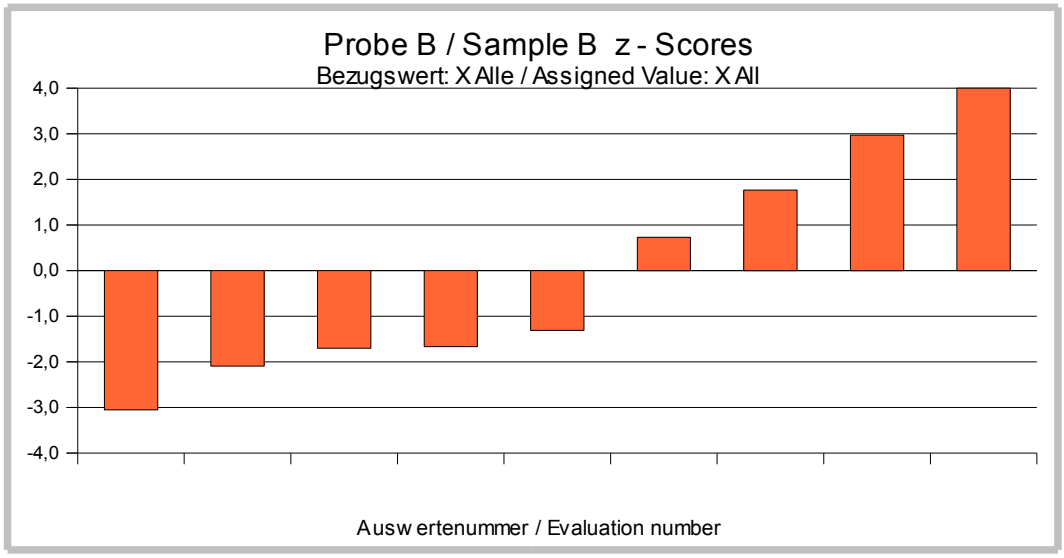

Fig. 3: z-Scores (ELISA-Results as β-Lactoglobulin)
Assigned value robust mean of all methods

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

Evaluation number	Spiking material	Recovery rate	Sample B	Recovery rate	Method	Remarks
	[mg/kg]	[%]	[mg/kg]	[%]		
3	1390	**80**	13,4	**90**	ES	
14	>1		>1		ES	
18	1500	**86**	20	**134**	ES	
19	/		11	**74**	IG	
5	594,17	34	5,42	36	RS1	
7	72	4	2,2	15	RS1	
11	731,8	42	16,2	**109**	RS1	
16	165	9	< LOD		RS1	
6	751,07	43	5,34	36	RS2	
12	1518,34	**87**	4,43	30	RS2	
10	182,17	10	6,26	42	RS?	
8					div	

RA*	50-150 %	RA*	50-150 %
Number in RA	3	Number in RA	4
Percent in RA	33	Percent in RA	44

* Range of acceptance of AOAC for allergen ELISAs

Methods:

ES = ELISA Systems
IG = Ingenzim, Ingenasa
RS1 = Ridascreen® R4901, R-Biopharm
RS2 = Ridascreen® R4902, R-Biopharm
RS? = Ridascreen®, R-Biopharm
div = not indicated / other method

Comments:

For the spiking material sample 3 participants obtained recovery rates within the range of the AOAC-recommendation of 50-150%. For the infant pap mixture-sample B produced with the spiking material sample 4 recovery rates were in the range of acceptance.

4.1.2 PCR-Results: β-Lactoglobulin (bovine DNA)

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		
7	positive	+	positive	+	-	div	

Methods:
div = not indicated / other method

Comments:
For the detection of bovine DNA by PCR only one participant submitted a result. Therefore no evaluation was performed.

4.2 Proficiency Test Wheat

4.2.1 ELISA-Results: Gluten

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		
19	negative	< 5	positive	31	2/2 (100%)	IG	
1	negative	< 5,0	positive	46,7	2/2 (100%)	RS1	
4	negative	< 5	positive	33	2/2 (100%)	RS1	
5	negative	< 5,00	positive	33,23	2/2 (100%)	RS1	
6	negative	< 5	positive	24,04	2/2 (100%)	RS1	
7	positive	6-10	positive	60,6	1/2 (50%)	RS1	result converted
9	negative	< 5	positive	20,52	2/2 (100%)	RS1	
12	positive	5	positive	33,86	1/2 (50%)	RS1	
14	negative	<5	positive	35,14	2/2 (100%)	RS1	
16	negative	< LOD	positive	28,7	2/2 (100%)	RS1	
17	negative	< 5	positive	26,7	2/2 (100%)	RS1	
18	negative	< 5	positive	32	2/2 (100%)	RS1	
2	positive	8,7	positive	20,3	1/2 (50%)	RS2	
11	negative	< 10	positive	19,2	2/2 (100%)	RS2	result converted
10	negative	< 5	positive	17,6	2/2 (100%)	RS?	
3	positive	6,1	positive	44,4	1/2 (50%)	VT	
8	negative	< 10	positive	38,4	2/2 (100%)	div	
13	negative	< 10	positive	28	2/2 (100%)	div	result converted
15	negative	< 3	positive	17	2/2 (100%)	div	
20	negative	3	positive	15	2/2 (100%)	div	

	Sample A		Sample B	
Number positive	4		20	
Number negative	16		0	
Percent positive	20		100	
Percent negative	80		0	
Consensus	negative		positive	

Methods:

IG = Ingenzim, Ingenasa
RS1 = R-Biopharm, R7001 Ridascreen®
RS2 = R-Biopharm, R7002 Ridascreen®
VT = Veratox, Neogen
div = not indicated / other method

Comments:

There were 80% negative results for sample A (with 4 positive results up to 10 mg/kg) and 100% positive results for sample B by the ELISA-methods. The results are in qualitative agreement with the spiking of sample B.

Quantitative valuation of results: Sample B

Evaluation number	Gluten	z-Score X_{ALL}	z-Score X_{RS}	Method	Remarks
	[mg/kg]	X_{ALL}	$X_{Method\ RS}$		
19	31	0,2		IG	
1	46,7	2,3	1,7	RS1	
4	33	0,5	0,0	RS1	
5	33,23	0,5	0,1	RS1	
6	24,04	-0,7	-1,1	RS1	
7	60,6	4,2	3,4	RS1	result converted, outlier X_{All} a. X_{RS}
9	20,52	-1,2	-1,5	RS1	
12	33,86	0,6	0,1	RS1	
14	35,14	0,8	0,3	RS1	
16	28,7	-0,1	-0,5	RS1	
17	26,7	-0,4	-0,7	RS1	
18	32	0,3	-0,1	RS1	
2	20,3	-1,2		RS2	
11	19,2	-1,4		RS2	result converted
10	17,6	-1,6		RS?	
3	44,4	2,0		VT	
8	38,4	1,2		div	
13	28	-0,2		div	result converted
15	17	-1,7		div	
20	15	-2,0		div	

Methods:

IG = Ingenzim, Ingenasa
RS1 = R-Biopharm, R7001 Ridascreen®
RS2 = R-Biopharm, R7002 Ridascreen®
VT = Veratox, Neogen
div = not indicated / other method

Characteristics: Quantitative evaluation Gluten

Sample B

	All Results *[mg/kg]*	**Method RS** *[mg/kg]*
Assigned value	X_{ALL}	$X_{Method\ RS}$
Number of results	*20*	*11*
Robust mean (X)	29,5	32,8
Robust standard deviation (S^x)	10,8	9,34
Median	29,9	33,0
Target range:		
Target standard deviation ($\hat{\sigma}$)	7,38	8,20
lower limit of target range (X - 2 $\hat{\sigma}$)	14,6	16,4
upper limit of target range (X + 2 $\hat{\sigma}$)	44,3	49,2
Quotient $S^x/\hat{\sigma}$	*1,5*	*1,1*
Standard uncertainty u_X	*3,02*	*3,52*
Quotient $u_X/\hat{\sigma}$	*0,41*	*0,43*
Number of results in the target range	*18 (90%)*	*10 (91%)*

Method:
RS1 = R-Biopharm, R7001 Ridascreen®

Comments:
The evaluation of all methods and of method RS showed low variabilities. 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 one third higher than the spiking level (s. also "Recovery rates of Gluten" p.24). It should be noted, that a gluten-content of the basic matrix "infant pap mixture" could not be excluded (sample A: 80% of results < 10 mg/kg).

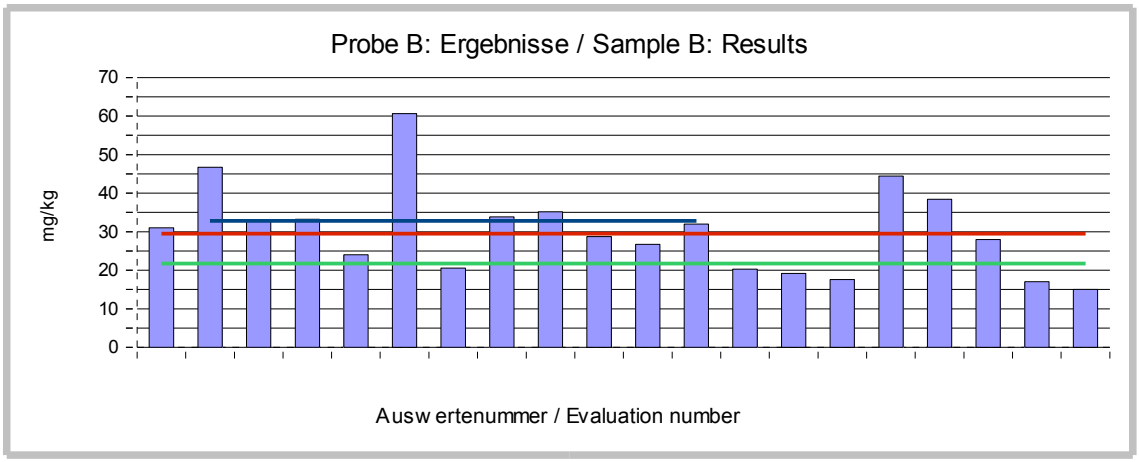

Fig. 5: ELISA-Results Gluten
green line = Spiking level
red line = Assigned value robust mean all results
blue line = Assigned value robust mean results method RS1

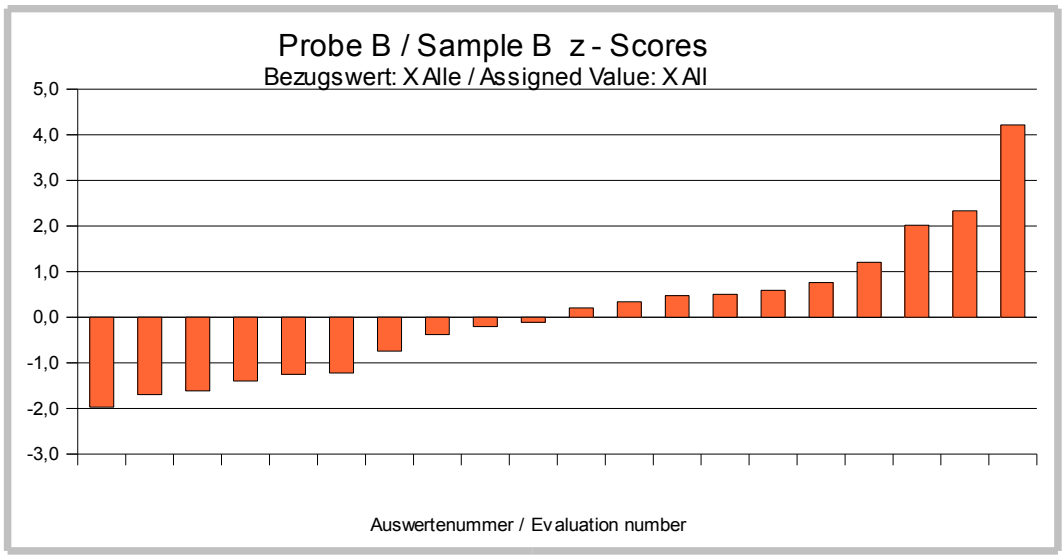

Fig. 6: z-Scores (ELISA-Results as Gluten)
Assigned value robust mean of all results

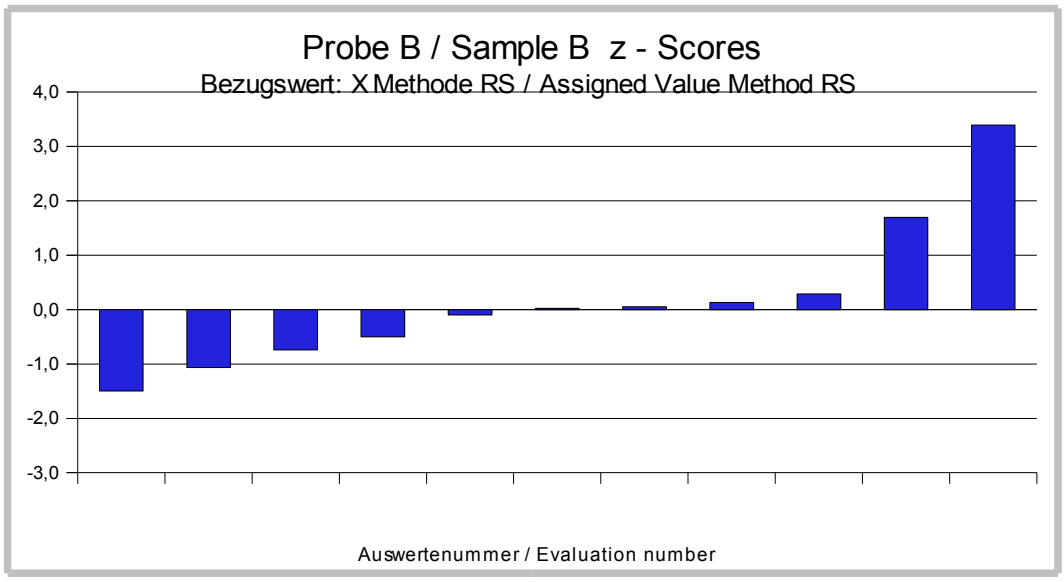

Fig. 7: z-Scores (ELISA-Results as Gluten) Assigned value robust mean of method RS (R-Biopharm, R7001 Ridascreen)

Recovery Rates for Gluten:
Spiking Material Sample and Sample B

Evaluation number	Spiking material	Recovery rate	Sample B	Recovery rate	Method	Remarks
	[mg/kg]	[%]	[mg/kg]	[%]		
19	/		31	**143**	IG	
1	1904	**75**	46,7	215	RS1	
4	5200	206	33	152	RS1	
5	1449,38	**57**	33,23	153	RS1	
6	1797	**71**	24,04	**111**	RS1	
7	1550	**61**	60,6	279	RS1	Result converted
9	> 80		20,52	**95**	RS1	
12	1963,86	**78**	33,86	156	RS1	
14	> 80		35,14	162	RS1	
16	2105	**83**	28,7	**132**	RS1	
17	> 400		26,7	**123**	RS1	
18	2100	**83**	32	**147**	RS1	
2			20,3	**94**	RS2	
11	1638,4	**65**	19,2	**88**	RS2	Result converted
10	1653,78	**65**	17,6	**81**	RS?	
3	1180	47	44,4	205	VT	
8			38,4	177	div	
13	>2000		28	**129**	div	Result converted
15	2600	**103**	17	**78**	div	
20	< 50		15	**69**	div	

RA*	50-150 %	RA*	50-150 %
Number in RA	**10**	Number in RA	**12**
Percent in RA	**83**	Percent in RA	**60**

* Range of acceptance of AOAC for allergen ELISAs

Methods:

IG = Ingenzim, Ingenasa
RS1 = R-Biopharm, R7001 Ridascreen®
RS2 = R-Biopharm, R7002 Ridascreen®
VT = Veratox, Neogen
div = not indicated / other method

Comments:
For the spiking material sample 83% of participants obtained recovery rates within the range of the AOAC-recommendation of 50-150%. For the infant pap mixture-sample B produced with the spiking material sample 60% of the recovery rates were in the range of acceptance.
Because a minimum gluten-content in the basic matrix "infant pap mixture" could not be excluded (sample A: 80% of < 10 mg/kg), it could be estimated for sample B, that the range of acceptance could be extended for the recovery rates to approximately 183%.

4.2.2 PCR-Results: Wheat

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		
1	positive	4,3	positive	15,2	1/2 (50%)	SFA	
7	negative	-	positive	+	2/2 (100%)	div	
13	negative	<100	positive	200	2/2 (100%)	div	
18a	negative	-	positive	-	2/2 (100%)	div	
18b	negative	-	positive	-	2/2 (100%)	div	

	Sample A		Sample B	
Number positive	1		5	
Number negative	4		0	
Percent positive	20		100	
Percent negative	80		0	
Consensus	negative		positive	

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

Comments:
There were 80% negative results for sample A (with one positive result) and 100% positive results for sample B by the PCR-methods for wheat. The results are in qualitative agreement with the spiking of sample B.

Quantitative valuation of results: Sample B

There were too less numbers of results for evaluation.

Recovery Rates for Wheat:
Spiking Material Sample and Sample B

Evaluation number	Spiking material	Recovery rate	Sample B	Recovery rate	Method	Remarks
	[mg/kg]	[%]	[mg/kg]	[%]		
1	37361	47	15,2	**70**	SFA	
13	>1000	-	200		div	Indicated as Gluten or Wheat?

RA*	50-150 %	RA*	50-150 %
Number in RA	**0**	Number in RA	**1**
Percent in RA	**0**	Percent in RA	**100**

* Range of acceptance of AOAC for allergen ELISAs

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

Comments:
One participant submitted results for the analyte gluten by PCR. For the spiking material sample the recovery rate was a little below the range of the AOAC-recommendation of 50-150%. For the infant pap mixture-sample B produced with the spiking material sample recovery rate was in the range acceptance.

5. Documentation

Details by the participants

5.1 ELISA: β-Lactoglobulin

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
3	-	<0,2	-	13,4	-	1390	beta-Lactoglobulin	ES	ELISA systems beta-lactoglobulin residues
14	-	0,005	-	>1	-	>1	beta-Lactoglobulin	ES	ELISA-Systems β-Lactoglobulin Residue Detection ELISA
18	negative	<0,2	positive	20	positive	1500	beta-Lactoglobulin	ES	ELISA-Systems β-Lactoglobulin Residue Detection ELISA
19	-	<0,5	-	11	-	/	beta-Lactoglobulin	IG	Ingenzim beta lactoglobulin (INGENASA)
5	-	<0,50	-	5,42	-	594,17	Given as	RS1	Ridascreen β-Lactoglobulin (R4901, r-Biopharm
7	negative	<0.2ppm	positive	2.2ppm	positive	72ppm	beta-Lactoglobulin	RS1	Ridascreen β-Lactoglobulin (R4901), r-Biopharm
11	-	<5,0	-	16,2	-	731,8	beta-Lactoglobulin	RS1	Ridascreen β-Lactoglobulin (R4901), r-Biopharm
16	-	< LOD	-	< LOD	-	165	ß-Lactoglobulin	RS1	RIDASCREEN ß-Lactoglobulin R4901
6	negative	<0.5	positive	5,34	positive	751,07	beta-Lactoglobulin	RS2	Ridascreen Fast β-lactoglobulin (R4902), R-Biopharm
12	-	<0,5	-	4,433	-	1518,338	ß-Lactoglobulin	RS2	RIDASCREEN FAST ß-Lactoglobulin, r-biopharm R4902
10	-	<5	-	6,26	-	182,17	Probe B:55,2, Dotierungsprobe:2690,88	RS?	r-Biopharm RIDASCREEN ß-Lact.
8	negative	< 0,1	-		-		Given as	div	Selection beta-Lactoglobulin-Kits:

Methods:

ES = ELISA Systems
IG = Ingenzim, Ingenasa
RS1 = Ridascreen® R4901, R-Biopharm
RS2 = Ridascreen® R4902, R-Biopharm
RS? = Ridascreen®, R-Biopharm
div = not indicated / other method

Other details to the Methods

Meth. Abr.	Specifity	Remarks to the Method (Extraction and Determination)	Further Remarks
	Antibody	e.g. Extraction Solution / Timet / Temperature	
ES			
ES	anti-Beta-Lactoglobulin antibody	Extraction solution, 15min., 60°C	
ES			according to kit instructions
IG	Anti betaglobulin antibody	kit extraction buffer	The results are the mean of the tw o abilitated operators. For B test: Poletti: 12 mg/Kg; Righelli 10 mg/Kg
RS1		as kit instructions	
RS1			
RS1		according to manual	
RS1		2g sample+ 50 ml w ater, 10 min shaking, centrifugation, Extract 1:20 diluted w ith sample buffer	
RS2	β-lactoglobulin from Cow's, Sheep, Goat & Buffalo Milk	As per Kit Instructions	The spike sample result is only an estimate due to a large dilution being performed
RS2		according to kit	
RS?		Extraction w ith bidest. w ater	sample B: 5,443/5,195/8,38/6,02/<5mg/kg, Spiking sample: 196,14/ 160,91/ 205,89/ 165,75 mg/kg
div			

5.2 ELISA: Gluten

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
19	-	<5	-	31	-	/	Gluten	IG	Ingenzim gluten (Ingenasa)
1	negative	<5,0	positive	46,7	positive	1904	Gluten	RS1	Ridascreen Gluten (R7001), r-Biopharm
4	negative	< 5	positive	33	positive	5200	Gluten	RS1	Ridascreen Gluten (R7001), r-Biopharm
5	-	<5,00	-	33,23	-	1449,38	Given as	RS1	Ridascreen Gluten (R7001), r-Biopharm
6	negative	<5	positive	24,04	positive	1797	Gluten	RS1	Ridascreen Gluten (R7001), R-Biopharm
7	positive	[3-5ppm]	positive	30.3ppm	positive	775ppm	Gliadin	RS1	Ridascreen Gluten (R7001), r-Biopharm
9	negative	<5	positive	20,52	positive	>80	Gluten	RS1	Ridascreen Gluten (R7001), r-Biopharm
12	-	5	-	33,86	-	1963,86	gluten	RS1	RIDASCREEN Gliadin, r-biopharm R7001
14	-	<5	-	35,14	-	>80	Gluten	RS1	Ridascreen Gluten (R7001), r-Biopharm
16	-	< LOD	-	28,7	-	2105	Gluten	RS1	RIDASCREEN Gliadin R 7001
17	negative	<5	positive	26,7	positive	>400	Gluten	RS1	Ridascreen Gluten (R7001), r-Biopharm
18	negative	<5	positive	32	positive	2100	Gluten	RS1	Ridascreen Gluten (R7001), r-Biopharm
2	positive	8,7	positive	20,3	positive		Gluten	RS2	Ridascreen Fast Gluten (R7002), r-Biopharm
11	-	<5,0	-	9,6	-	819,2	Gliadin	RS2	Ridascreen Fast Gliadin (R7002), r-Biopharm
10	-	<5	-	17,6	-	1653,78		RS?	r-Biofarm RIDASCREEN Gliadin
3	-	6,1	-	44,4	-	1180	Gluten	VT	Veratox for Gliadin R5 (Neogen)
8	negative	< 10	positive	38,4	-		Given as	div	Selection Gluten / Gliadin-Kits:
13	negative	<5	positive	14	positive	>1000	Gliadin	div	in house
15	negative	<3	positive	17	positive	2600	Given as	div	Auswahl Gluten / Gliadin-Kits:
20	negative	3	positive	15	positive	<50	Given as	div	Auswahl Gluten / Gliadin-Kits:

Methods:

IG = Ingenzim, Ingenasa
RS1 = R-Biopharm, R7001 Ridascreen®
RS2 = R-Biopharm, R7002 Ridascreen®
VT = Veratox, Neogen
div = not indicated / other method

Other details to the methods

Meth. Abr.	Specifity	Remarks to the Method (Extraction and Determination)	Further Remarks
	Antibody	e.g. Extraction Solution / Timet / Temperature	
IG	r 5 antibody	ethanol 80%; kit extraction solution	The results are the mean of the two abilitated operators. For B test: Poletti: 30 mg/Kg; Righelli 32 mg/Kg
RS1			for sample w height of 25mg instead of 250mg, result 4128mg/kg
RS1			
RS1		as kit instructions	
RS1	R5	As per Kit Instructions - Mendez Cocktail Extraction	The spike sample result is only an estimate due to a large dilution being performed
RS1	R5		
RS1			
RS1		according to kit	
RS1	Monoklonale antibody R5	Cocktail solution (R7006), 40min., 50°C	
RS1	monoclonal R5	according to test kit instructions Cocktail sample preparation	
RS1		Extraction with Cocktail solution (AOAC Method)	
RS1	R5		according to test kit instructions
RS2	antibody R5	Extraction w ith Extraction solution Art. R7098 (colourless), as per test kit instructions Extraction w ith R7099 (coloured) . The composition of R7098 is the same as R7099, but R7098 is colourless	
RS2		Extraction w ith RIDA Extraction solution (Art. Nr. R7099)	
RS?		w ith RIDA-Extraction solution, Incubation 2 times at 60°C	sample B: 16,85/ 16,27/ 19,74/ 17,84 mg/kg; Spiking sample: 1576,8/ 1832,4/ 1540,8/ 1665,1 mg/kg
VT			
div			
div		70% MeOH	
div	see manufactuererr	aquaeous Extraction , ELISA from r-biopharm	
div		Biokit,. Limit of detection = 1 ppm. Limit of Quantification = 3 ppm	

5.3 PCR: Milk

Primary data

Evaluation number	Result Sample A		Result Sample B		Result Spiking Sample		Meth. Abr.	Method
	qualitative	mg/kg	qualitative	mg/kg	qualitative	mg/kg		Test-Kit + Manufacturer
7	positive	+	positive	+	positive	+	div	internal method

Method:
div = not idicated / other method

Other Remarks to the Methods

Evaluation number	Meth. Abr.	Methode	Specifity	Remarks to the Method (Extraction and Determination)	Further Remarks
		Test-Kit + Anbieter	Antibody	e.g. Extraction Solution / Timet / Temperature	
7	div	internal method	DNA-Cow	internal method	

5.4 PCR: Wheat

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	positive	4,3	positive	15,2	positive	37.361	Gluten	SFA	SureFood Allergen Quant Gluten, r-biopharm (S3206)
7	negative	-	positive	+	positive	+	DNA-Wheat	div	internal method
13	negative	<100	positive	200	positive	>1000	Angabe als	div	in house
18a	negative	-	positive	-	positive	-	Weizen	div	internal method
18b	negative	-	positive	-	positive	-	Weizen / Roggen / Gerste DNA - indirekt Gluten	div	internal method

Methods:
SFA = Sure Food Allergen, R-Biopharm / Congen
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 Solution / Timet / Temperature	
1	SFA		SureFood Prep Allergen, r-biopharm (S1012)	
7	div		in house method	
13	div		Wizard	
18a	div	-	DNA Extraction: CTAB+ProtK, Chloroform, Wizard, End point PCR 45 Cycles + Agarose gel	
18b	div	-	DNA Extraktion: CTAB+ProtK, Chloroform, Wizard, Endpunkt PCR 45 Cyclen + Agarosegel	

6. Index of participant laboratories

Teilnehmer / Participant	Ort / Town	Land / Country
		GERMANY
		FRANCE
		GERMANY
		GERMANY
		GERMANY
		GERMANY
		GERMANY
		GERMANY
		NEW ZEALAND
		AUSTRIA
		SWITZERLAND
		ITALY
		ITALY
		GERMANY
		GREAT BRITAIN
		GERMANY
		SPAIN
		GERMANY
		NETHERLANDS
		GERMANY

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

7. Index of references

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