Proficiency Tests

DLA

food
cosmetics
consumer goods
www.dla-lvu.de

Evaluation Report

proficiency test

DLA 02/2018

Allergens II:

Soya and Rye

in "gluten-free" Pastry

Dienstleistung Lebensmittel Analytik GbR
Waldemar-Bonsels-Weg 170
22926 Ahrensburg, Germany

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

Coordinator of this PT:
Matthias Besler-Scharf, PhD

1st Correction 16/07/2018:
There was a mistake of the z-scores in the table on page 26 (Evaluation ELISA: Gluten in spiking level sample):
The z-scores were not correct, there was a wrong reference in the calculation. This has been corrected. The z-scores in the figures 7 and 8 on pages 28-29 were not affected.

Reprint, also in part, only with written permission from DLA-Ahrensburg

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

EP-Anbieter *PT-Provider*	**DLA - Dienstleistung Lebensmittel Analytik GbR** Gesellschafter: Dr. Gerhard Wichmann und Dr. Matthias Besler-Scharf Waldemar-Bonsels-Weg 170, 22926 Ahrensburg, Germany Tel. ++49-(0)4532-9183358 Mob. ++49(0)171-1954375 Fax. ++49(0)4102-9944976 eMail. proficiency-testing@dla-lvu.de
EP-Nummer *PT-Number*	DLA 02/2018
EP-Koordinator *PT-Coordinator*	Dr. Matthias Besler-Scharf
Status des EP-Bericht *Status of PT-Report*	Abschlussbericht / Final report (16 July 2018) 1. Korrektur / 1st Correction Gültig ist die jeweils letzte Version/Korrektur des Berichts. Sie ersetzt alle vorangegangenen Versionen. Only the latest version/correction of the report is valid. It replaces all preceding versions.
EP-Bericht Freigabe *PT-Report Authorization*	Dr. Matthias Besler-Scharf (Technischer Leiter / Technical Manager) *- gezeichnet / signed M. Besler-Scharf* Dr. Gerhard Wichmann (QM-Beauftragter / Quality Manager) *- gezeichnet / signed G. Wichmann* Datum / Date: 16 July 2018
Unteraufträge *Subcontractors*	Falls im Rahmen der Eignungsprüfung eine Prüfung der Gehalte, Homogenität und Stabilität von EP-Parametern durchgeführt wurde, hat DLA diese im Unterauftrag vergeben. In case the analysis of the content, homogeneity and stability of PT-parameters was part of the proficiency test, the determinations were subcontracted by DLA.
Vertraulichkeit *Confidentiality*	Die Teilnehmerergebnisse sind im EP-Bericht in anonymisierter Form mit Auswertenummern benannt. Daten einzelner Teilnehmer werden ausschließlich nach vorheriger Zustimmung des Teilnehmers an Dritte weitergegeben. Participant result are named anonymously with evaluation numbers in the PT report. Data of individual participants will be passed on to third parties only with prior consent of the participant.

Reprint, also in part, only with written permission from DLA-Ahrensburg

Inhalt / Content

1. Introduction..4
2. Realisation...4
2.1 Test material...4
2.1.1 Homogeneity...6
2.1.2 Stability...9
2.2 Sample shipment and information to the test.....................9
2.3 Submission of results...9
3. Evaluation..10
3.1 Consensus value from participants (assigned value)............10
3.2 Robust standard deviation......................................11
3.3 Exclusion of results and outliers..............................11
3.4 Target standard deviation (for proficiency assessment)........12
3.4.1 General model (Horwitz)......................................12
3.4.2 Value by precision experiment................................12
3.4.3 Value by perception..15
3.5 z-Score..16
3.6 z'-Score...17
3.7 Quotient S*/σpt..17
3.8 Standard uncertainty and traceability..........................17
3.9 Figures..18
3.10 Recovery rates: Spiking.......................................18
4. Results...19
4.1 Proficiency Test Rye ("Gluten")................................21
4.1.1 ELISA-Results: Gluten..21
4.1.2 PCR Results: Gluten-containing cereals.......................31
4.2 Proficiency Test Soya..35
4.2.1 ELISA-Results: Soya (as Soy Protein).........................35
4.2.2 PCR Results: Soya (as Soy flour, Soybean)....................45
5. Documentation...49
5.1 Details by the participants....................................49
5.1.1 ELISA: Gluten..49
5.1.2 ELISA: Soya..51
5.1.3 PCR: Gluten-containing Cereals...............................53
5.1.4 PCR: Soya..54
5.2 Homogeneity..55
5.2.1 Mixture homogeneity before bottling..........................55
5.3 Information on the Proficiency Test (PT).......................56
6. Index of participant laboratories...............................57
7. Index of references...58

Reprint, also in part, only with written permission from DLA-Ahrensburg

1. Introduction

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

2. Realisation

2.1 Test material

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

The test material is a common in commerce "gluten-free" cake baking mix. The basic composition of both sample A and sample B was the same (see table 1). The basic mixture was homogenized.

Afterwards the **spiked sample B** was produced as follows:
The spiking materials (premix) containing the allergenic ingredients rye flour and soy flour were crushed and sieved by means of a centrifugal mill (mesh 250 µm), added to an aliquot of the basic mixture and the mixture was homogenized. Subsequently, the basic mixture was again added in 3 additional steps and homogenized in each case until the total quantity had been reached.
For the **spiking level sample**, the allergenic compounds above mentioned were added during a multi-stage addition of potato powder (mesh 500 µm) and homogenization.

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

Reprint, also in part, only with written permission from DLA-Ahrensburg

Table 1: Composition of the DLA-Samples

Ingredients	**Sample A**	**Sample B**	**Spiking Level Sample**
Cake Baking Mix, organic Ingredients: Sugar, rice flour, corn-starch, tapioca starch, raising agent: sodium bicarbonate, acidifier: monopotassium tartrate, vanilla, salt Nutrients per 100 g: Fat <0,5 g, Carbohydrates 90 g, Protein 2,1 g	100 g/100 g	99,5 g/100g	-
Potato Powder Ingredients: Potatoes, E471, E304, E223, E100	-	-	99,6 g/100 g
Soya: Soy flour mixture, toasted (6 products from Asia, Europe, North America) - as soy flour * - thereof 37,8% total protein **	-	 78,0 mg/kg 29,5 mg/kg	 66,8 mg/kg 25,2 mg/kg
Rye: - as rye flour* - *thereof 7,42% total protein* ** - *thereof 3,3% "gluten"* ***	-	 496 mg/kg 36,8 mg/kg 16,4 mg/kg	 389 mg/kg 28,9 mg/kg 12,8 mg/kg
further Ingredients: Maltodextrin, sodium sulfate and silicon dioxide	-	<0,5 g/100 g	<0,4 g/100 g

*Allergen contents as „total food" as described in column ingredients according to gravimetric mixture
** Protein contents according to laboratory analysis of raw material (total nitrogen according to Kjeldahl with F=5,83 for rye protein and F=5,71 for soy protein)
*** Protein calculated according to literature (approx. 3,3% gluten in rye flours [36]).

Note: *The metrological traceability of temperature, mass and volume during production of the PT samples is ensured by DAkkS calibrated reference materials.*

Reprint, also in part, only with written permission from DLA-Ahrensburg

2.1.1 Homogeneity

The **mixture homogeneity before bottling** was examined 8-fold by **microtracer analysis**. It is a standardized method that is part of the international GMP certification system for feed [14].
Before mixing dye coated iron particles of µm size are added to the sample and the number of particles is determined after homogenization in taken aliquots. The evaluation of the mixture homogeneity is based on the Poisson distribution using the chi-square test. A probability of ≥ 5 % is equivalent to a good homogeneous mixture and of ≥ 25% to an excellent mixture [14, 15].
The microtracer analysis of the present PT sample B showed a probability of 67% and 93% for the spiking level sample, respectively. Additionally particle number results were converted into concentrations, statistically evaluated according to normal distribution and compared to the standard deviation according to Horwitz. For the assessment HorRat values between 0,3 and 1,3 are to be accepted under repeat conditions (measurements within the laboratory) [17]. This gave HorRat values of 1,2 and 0,9, respectively. The results of microtracer analysis are given in the documentation.

Homogeneity of bottled spiked sample B

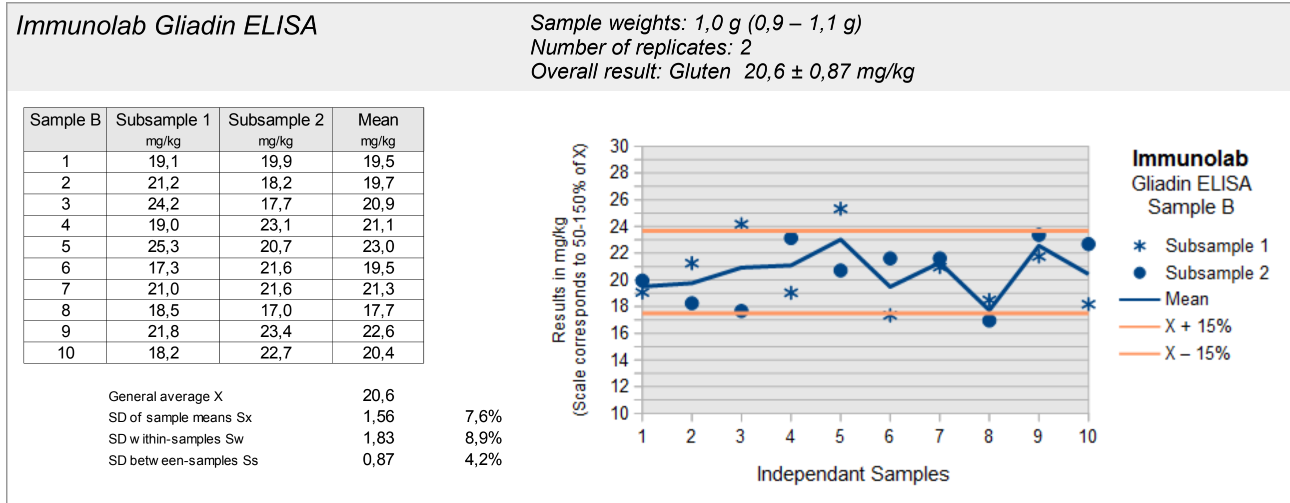
Implementation of homogeneity tests
The homogeneity tests were carried out in cooperation with the laboratories of the specified test kit providers. Ten samples of the bottled spiked sample were chosen randomly by DLA, thereof 2 subsamples were weighed into previously randomly encoded sample containers, and then sent to the laboratories for analysis. The sample weights were made with a deviation of ± 10% from recommended sample weight of the test kit instructions and not communicated to the laboratories. After transmission of analysis results by the laboratories, the valid results were calculated on the basis of the exact weightings by DLA and the statistical calculation was carried out according to ISO 13528:2015 Annex B (possibly with Notes 1 and 2).

Valuation of homogeneity
The homogeneity is regarded as sufficient when the standard deviation between the samples Ss is ≤ 15% („heterogeneity standard deviation"). This criterion is fulfilled for sample B by all ELISA tests for gluten (Immunolab, Veratox and AgraQuant G12) and soy (Immunolab, Veratox and AgraQuant) (see pages 7-8). Recommendations for repeatability standard deviations of ELISA and PCR methods are usually ≤ 25% [18, 19, 22, 23].

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

Reprint, also in part, only with written permission from DLA-Ahrensburg

ELISA-Tests: Homogenität Gluten / Homogeneity Gluten

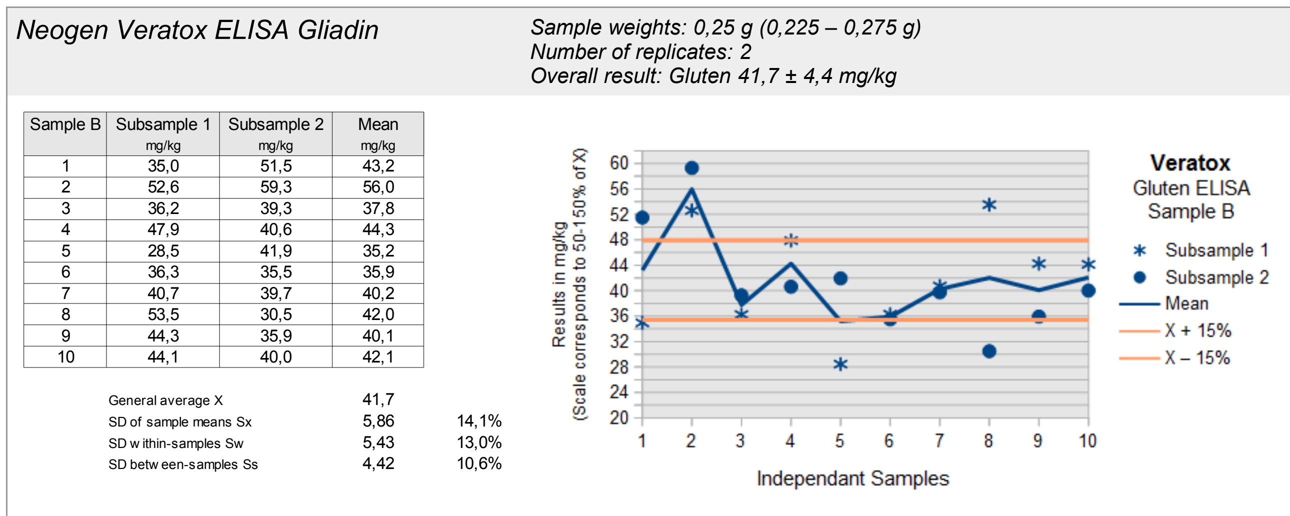


Immunolab Gliadin ELISA

Sample weights: 1,0 g (0,9 – 1,1 g)
Number of replicates: 2
Overall result: Gluten 20,6 ± 0,87 mg/kg

Sample B	Subsample 1 mg/kg	Subsample 2 mg/kg	Mean mg/kg
1	19,1	19,9	19,5
2	21,2	18,2	19,7
3	24,2	17,7	20,9
4	19,0	23,1	21,1
5	25,3	20,7	23,0
6	17,3	21,6	19,5
7	21,0	21,6	21,3
8	18,5	17,0	17,7
9	21,8	23,4	22,6
10	18,2	22,7	20,4

General average X	20,6	
SD of sample means Sx	1,56	7,6%
SD within-samples Sw	1,83	8,9%
SD between-samples Ss	0,87	4,2%

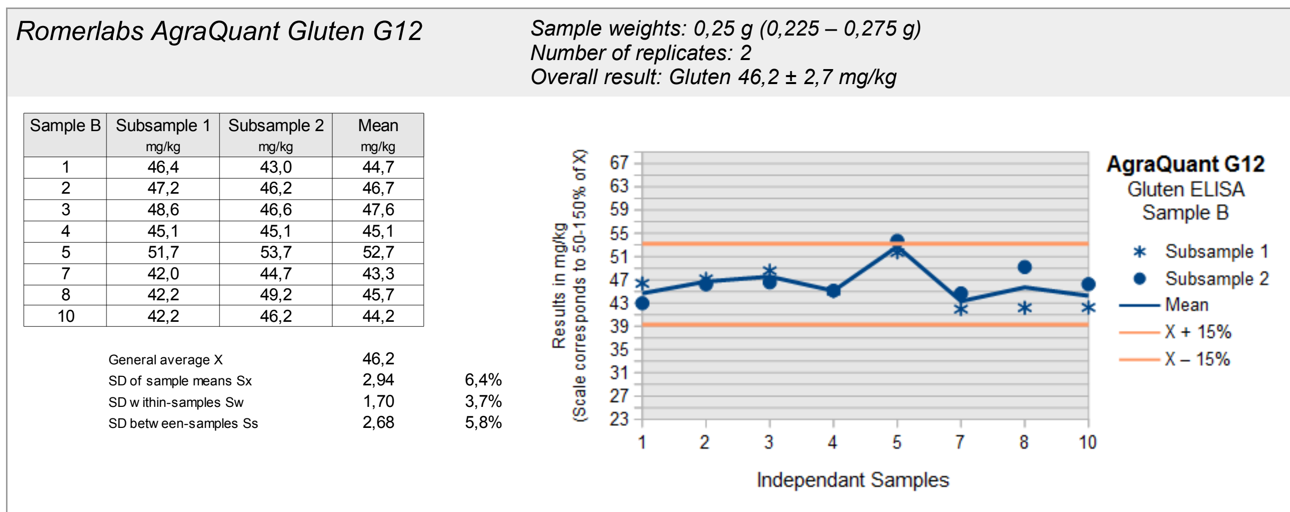


Neogen Veratox ELISA Gliadin

Sample weights: 0,25 g (0,225 – 0,275 g)
Number of replicates: 2
Overall result: Gluten 41,7 ± 4,4 mg/kg

Sample B	Subsample 1 mg/kg	Subsample 2 mg/kg	Mean mg/kg
1	35,0	51,5	43,2
2	52,6	59,3	56,0
3	36,2	39,3	37,8
4	47,9	40,6	44,3
5	28,5	41,9	35,2
6	36,3	35,5	35,9
7	40,7	39,7	40,2
8	53,5	30,5	42,0
9	44,3	35,9	40,1
10	44,1	40,0	42,1

General average X	41,7	
SD of sample means Sx	5,86	14,1%
SD within-samples Sw	5,43	13,0%
SD between-samples Ss	4,42	10,6%



Romerlabs AgraQuant Gluten G12

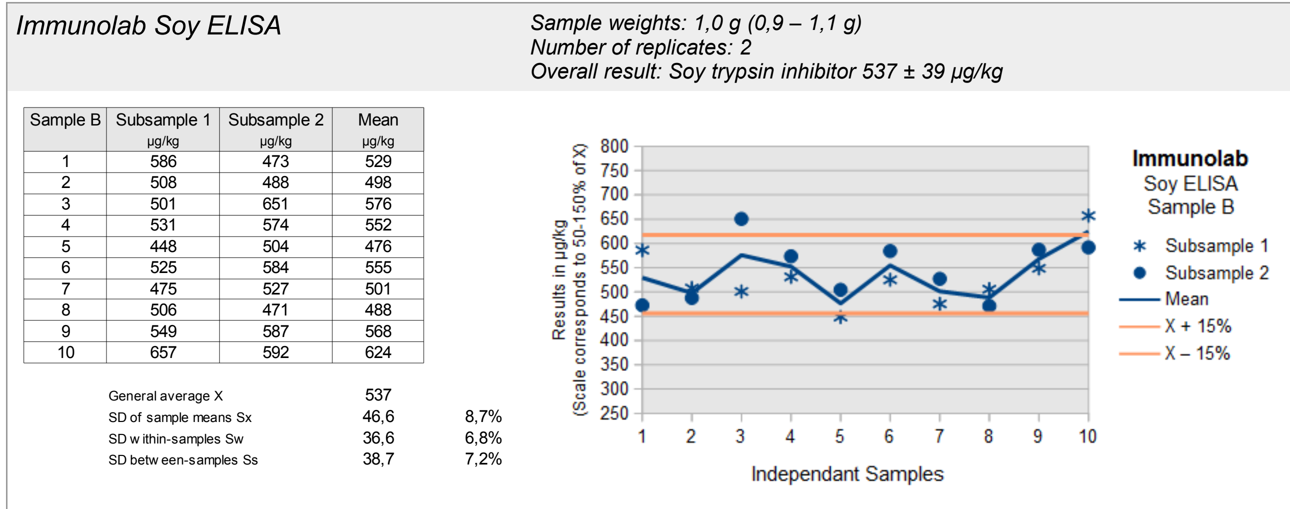
Sample weights: 0,25 g (0,225 – 0,275 g)
Number of replicates: 2
Overall result: Gluten 46,2 ± 2,7 mg/kg

Sample B	Subsample 1 mg/kg	Subsample 2 mg/kg	Mean mg/kg
1	46,4	43,0	44,7
2	47,2	46,2	46,7
3	48,6	46,6	47,6
4	45,1	45,1	45,1
5	51,7	53,7	52,7
7	42,0	44,7	43,3
8	42,2	49,2	45,7
10	42,2	46,2	44,2

General average X	46,2	
SD of sample means Sx	2,94	6,4%
SD within-samples Sw	1,70	3,7%
SD between-samples Ss	2,68	5,8%

Reprint, also in part, only with written permission from DLA-Ahrensburg

ELISA-Tests: Homogenität Soja / Homogeneity Soya

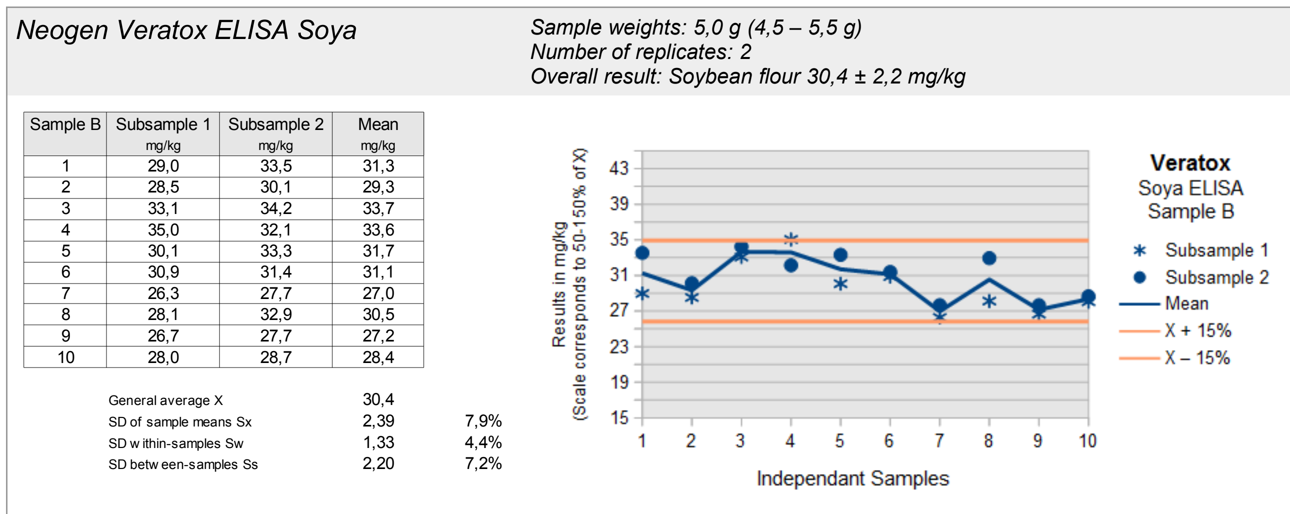


Immunolab Soy ELISA

Sample weights: 1,0 g (0,9 – 1,1 g)
Number of replicates: 2
Overall result: Soy trypsin inhibitor 537 ± 39 µg/kg

Sample B	Subsample 1 µg/kg	Subsample 2 µg/kg	Mean µg/kg
1	586	473	529
2	508	488	498
3	501	651	576
4	531	574	552
5	448	504	476
6	525	584	555
7	475	527	501
8	506	471	488
9	549	587	568
10	657	592	624

General average X	537	
SD of sample means Sx	46,6	8,7%
SD within-samples Sw	36,6	6,8%
SD between-samples Ss	38,7	7,2%

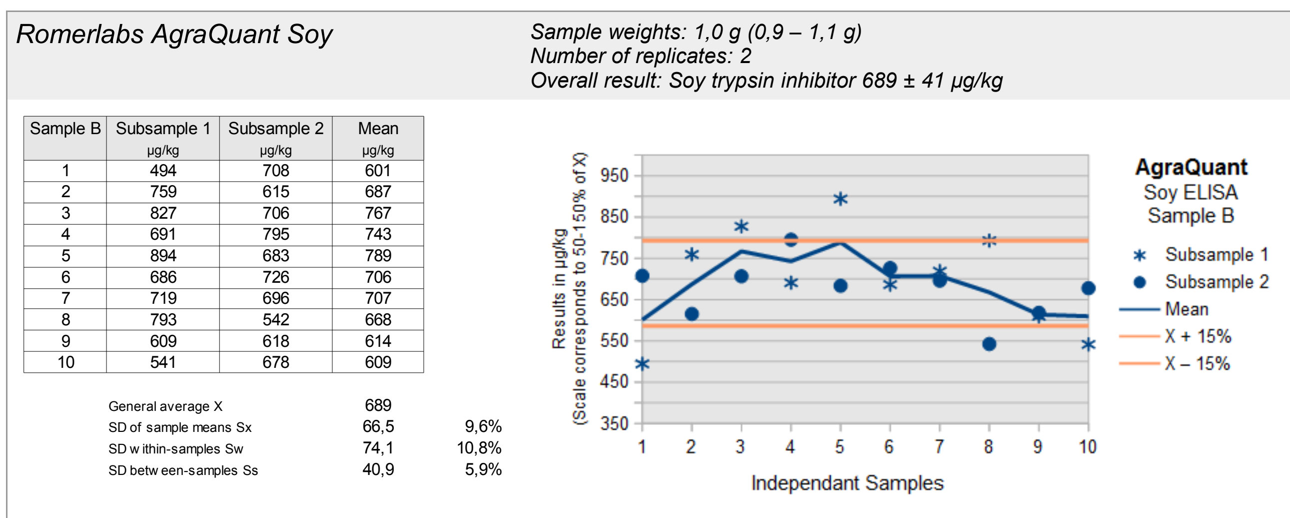


Neogen Veratox ELISA Soya

Sample weights: 5,0 g (4,5 – 5,5 g)
Number of replicates: 2
Overall result: Soybean flour 30,4 ± 2,2 mg/kg

Sample B	Subsample 1 mg/kg	Subsample 2 mg/kg	Mean mg/kg
1	29,0	33,5	31,3
2	28,5	30,1	29,3
3	33,1	34,2	33,7
4	35,0	32,1	33,6
5	30,1	33,3	31,7
6	30,9	31,4	31,1
7	26,3	27,7	27,0
8	28,1	32,9	30,5
9	26,7	27,7	27,2
10	28,0	28,7	28,4

General average X	30,4	
SD of sample means Sx	2,39	7,9%
SD within-samples Sw	1,33	4,4%
SD between-samples Ss	2,20	7,2%



Romerlabs AgraQuant Soy

Sample weights: 1,0 g (0,9 – 1,1 g)
Number of replicates: 2
Overall result: Soy trypsin inhibitor 689 ± 41 µg/kg

Sample B	Subsample 1 µg/kg	Subsample 2 µg/kg	Mean µg/kg
1	494	708	601
2	759	615	687
3	827	706	767
4	691	795	743
5	894	683	789
6	686	726	706
7	719	696	707
8	793	542	668
9	609	618	614
10	541	678	609

General average X	689	
SD of sample means Sx	66,5	9,6%
SD within-samples Sw	74,1	10,8%
SD between-samples Ss	40,9	5,9%

Reprint, also in part, only with written permission from DLA-Ahrensburg

2.1.2 Stability

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

The experience with various DLA test materials showed good storage stability with respect to the durability of the sample (spoilage) and the content of the PT parameters for comparable food matrices and water activity (a_W value <0,5).
The a_W value of the EP samples was approx. 0,35 (19,8°C). The stability of the sample material was thus ensured during the investigation period under the specified storage conditions.

2.2 Sample shipment and information to the test

The portions of test materials sample A, B and the spiking level sample were sent to every participating laboratory in the 8th week of 2018. The testing method was optional. The tests should be finished at April 6th 2018.

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

There are two different samples A and B possibly containing the allergenic parameters Soya and/or Rye in the range of mg/kg in the matrix of cake bake mix. One of these samples and the "spiking level sample" were prepared adding the allergenic ingredients. The ***"spiking level sample"*** *contains the allergens in a simple matrix in* ***similar amounts*** *without further processing.*

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

2.3 Submission of results

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

All 13 participants submitted their results in time.

Reprint, also in part, only with written permission from DLA-Ahrensburg

3. Evaluation

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

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

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

3.1 Consensus value from participants (assigned value)

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

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

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

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

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

Reprint, also in part, only with written permission from DLA-Ahrensburg

3.2 Robust standard deviation

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

The following robust standard deviations were considered:

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

3.3 Exclusion of results and outliers

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

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

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

Reprint, also in part, only with written permission from DLA-Ahrensburg

3.4 Target standard deviation (for proficiency assessment)

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

3.4.1 General model (Horwitz)

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

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

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

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

3.4.2 Value by precision experiment

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

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

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

Reprint, also in part, only with written permission from DLA-Ahrensburg

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

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

From the precision data of the official German ASU §64 methods the calculated relative target standard deviations are in the range of 11 – 32% for the ELISA methods and 18 – 38% for the PCR methods depending on the matrix, processing and concentration level of allergens (s. Tab. 2a and 2b).

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

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

Reprint, also in part, only with written permission from DLA-Ahrensburg

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

Parameter	**Matrix**	**Mean [mg/kg]**	**Recov-ery**	**rob RSD**	**RSD_r**	**RSD_R**	**σ_{pt}**	**Method / Literature**
Soya	Wheat flour Maize flour	107 145	107 % 145 %	63 % 34 %	- -	31 % 24 %	- -	rt-PCR ASU 16.01-9
Soya flour	Boiled saus-age (100°C, 60 min)	114,1 64,4	114 % 161 %	-	14,7% 27,7%	22,2% 41,4%	19,6% 36,5%	rt-PCR ASU 08.00-65
Soya flour	Sausage, autoclaved	33,1	33,1 %	-	21,5%	30,8	26,8%	rt-PCR ASU 08.00-65
Soya flour	Boiled saus-age (100°C, 60 min)	82,0 39,6 19,6 9,3	82 % 99 % 98 % 93 %	-	17,3% 22,9% 22,9% 31,1%	24,1% 31,8% 24,0% 30,2%	20,8% 27,4% 17,7% -	rt-PCR ASU 08.00-59
Wheat + Rye	Boiled saus-age (100°C, 60 min)	96,1	120 %	-	21,3%	35,4%	32,0%	rt-PCR ASU 08.00-66
Wheat + Rye	Sausage, autoclaved	74,9	11,0 %	-	24,6%	32,7%	27,7%	rt-PCR ASU 08.00-66

Reprint, also in part, only with written permission from DLA-Ahrensburg

3.4.3 Value by perception

The target standard deviation for proficiency assessment can be set at a value that corresponds to the level of performance that the coordinator would wish laboratories to be able to achieve [3].
Criteria for the level of performance of analytical methods for the quantitative determination of allergens in foods were recently elaborated e.g. by the Ministry of Health and Welfare (MHLW) in Japan [22], by the working group 12 „Food Allergens‟ of the technical committee CEN/TC 275 [19-21], by an international "Food Allergen Working Group" under the advice of the AOAC Presidential Task Force on Food Allergens [23] and by the Codex Alimentarius Committee (CAC/GL 74-2010) [18].

Some of the relevant ELISA and PCR validation criteria of the mentioned panels are listed in tables 3 and 4, respectively.

Table 3: ELISA-Validation

Literature [18-24]	**Recovery rate**	**Repeatability standard deviation**	**Reproducibility standard deviation**
MHLW 2006	50 - 150%		≤ 25%
CEN 2009		≤ 20%	
AOAC 2010	50 - 150%	6,9 - 34,4% (a)	19,5 - 57,2% (a)
CAC 2010	70 - 120%	≤ 25%	≤ 35%

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

Table 4: PCR-Validation

Literature [18]	**Recovery rate**	**Repeatability standard deviation**	**Reproducibility standard deviation**
CAC 2010	± 25% (a)	≤ 25%	≤ 35%

(a) = Trueness / Richtigkeit

Based on the currently achievable level of performance of ELISA and PCR methods for the quantitative determination of allergens in foods, which could be deduced from the data of precision experiments and from validation criteria, we set a relative target standard deviation σ_{pt} of 25%.
This target standard deviation was applied for the statistical evaluation of the results by z-score or if necessary by z´-Score and was used for all assigned values mentioned in 3.1.

Reprint, also in part, only with written permission from DLA-Ahrensburg

3.5 z-Score

To assess the results of the participants the z-score is used. It indicates about which multiple of the target standard deviation (σ_{pt}) the result (x_i) of the participant is deviating from the assigned value (X_{pt}) [3].
Participants’ z-scores are derived from:

$$z_i = \frac{\left(x_i - x_{pt}\right)}{\sigma_{pt}}$$

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

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

For information the z-scores below are calculated with a target standard deviation of 25%:

i) ***z-Score*** - z_{ALL} *(with respect to all methods)*
ii) ***z-Score*** - $z_{METHOD\ i}$ *(with respect to single methods)*

3.5.1 Warning and action signals

In accordance with the norm ISO 13528 it is recommended that a result that gives rise to a *z*-score above 3,0 or below −3,0, shall be considered to give an “action signal” [3]. Likewise, a *z*-score above 2,0 or below −2,0 shall be considered to give a “warning signal”. A single “action signal”, or “warning signal” in two successive PT-rounds, shall be taken as evidence that an anomaly has occurred which requires investigation. An error or cause analysis can be carried out by checking the analysis process including understanding and implementation of the measurement by the staff, details of the measurement process, calibration of equipment and composition of reagents, transmission or calculation errors, trueness and precision, and use of reference material. If necessary, the problems must be addressed through appropriate corrective action [3].

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

Reprint, also in part, only with written permission from DLA-Ahrensburg

3.6 z'-Score

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

The calculation is performed by:

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

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

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

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

For warning and action signals see 3.5.1.

3.7 Quotient S*/σ_{pt}

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

3.8 Standard uncertainty and traceability

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

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

If $U_{(Xpt)} \leq 0{,}3\ \sigma_{pt}$ the standard uncertainty of the assigned value needs not to be included in the interpretation of the results of the PT [3]. Values exceeding 0,3 imply, that the target standard deviation could be

Reprint, also in part, only with written permission from DLA-Ahrensburg

too low with respect to the standard uncertainty of the assigned value.

The traceability of the assigned value is ensured on the basis of the consensus value as a robust mean of the participant results.

3.9 Figures

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

3.10 Recovery rates: Spiking

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

Reprint, also in part, only with written permission from DLA-Ahrensburg

4. Results

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

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

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

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

ELISA-results, which were given as **soy flour** or **soybean**, were converted into total **soy protein** using the analysed protein content of the raw material (see page 5). The PCR-results were submitted as soy flour or soybean and evaluated as thus.

ELISA-results given as **gliadin** were converted into **gluten** multiplying the gliadin-content with the factor of 2.

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.

Reprint, also in part, only with written permission from DLA-Ahrensburg

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

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

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

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

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

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

Reprint, also in part, only with written permission from DLA-Ahrensburg

4.1 Proficiency Test Rye ("Gluten")

4.1.1 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		
1	negative	0	positive	126	2/2 (100%)	BF	
13	negative	<1,0	positive	21,2	2/2 (100%)	IL	
2	negative	<5,0	positive	64,0	2/2 (100%)	RS	
3	negative	0,68	positive	133	2/2 (100%)	RS	
4a	negative	< 5,0	positive	84,1	2/2 (100%)	RS	
4b	negative	< 5,0	positive	58,9	2/2 (100%)	RS	
5	negative		positive	71,0	2/2 (100%)	RS	
8	negative	<5,0	positive	110	2/2 (100%)	RS	
9	negative	<10	positive	245	2/2 (100%)	RS	results converted °
10	-	<3,0	-	83,1	2/2 (100%)	RS	
11	negative	<5,0	positive	114	2/2 (100%)	RS	
12	negative		positive	92,2	2/2 (100%)	RS	
6a	negative	<5,0	positive	110	2/2 (100%)	RS	
7	negative	<10	positive	>80	2/2 (100%)	RS-F	
6b	negative	<3,12	positive	115	2/2 (100%)	SP	* mean calculated by DLA

° calculation p.19

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

Methods:
BF = MonoTrace ELISA, BioFront Technologies
IL = Immunolab
RS = Ridascreen®, R-Biopharm
RS-F= Ridascreen® Fast, R-Biopharm
SP = SENSISpec Ingezim

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

Reprint, also in part, only with written permission from DLA-Ahrensburg

Quantitative valuation of ELISA-results: Sample B

Evaluation number	Gluten	z-Score Xpt_{ALL}	z-Score Xpt_{RS}	Method	Remarks
	[mg/kg]				
1	126	1,2		BF	
13	21,2	-3,1		IL	
2	64,0	-1,4	-1,4	RS	
3	133	1,5	1,5	RS	
4a	84,1	-0,52	-0,52	RS	
4b	58,9	-1,6	-1,6	RS	
5	71,0	-1,1	-1,1	RS	
8	110	0,55	0,55	RS	
9	245	6,1	6,1	RS	results converted °
10	83,1	-0,56	-0,56	RS	
11	114	0,71	0,72	RS	
12	92,2	-0,19	-0,19	RS	
6a	110	0,55	0,55	RS	
7	>80			RS-F	
6b	115	0,75		SP	* mean calculated by DLA

° calculation p.19

Methods:
BF = MonoTrace ELISA, BioFront Technologies
IL = Immunolab
RS = Ridascreen®, R-Biopharm
RS-F= Ridascreen® Fast, R-Biopharm
SP = SENSISpec Ingezim

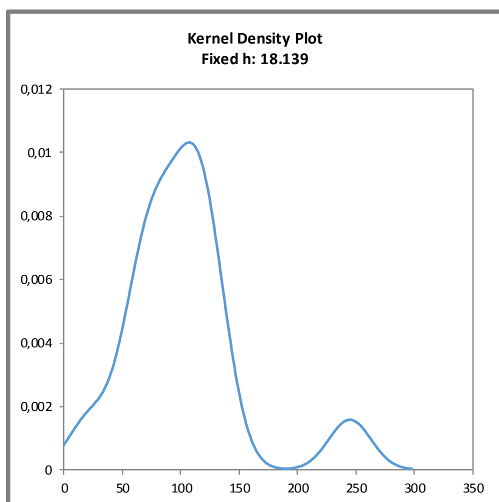


Abb. / Fig. 1:
Kerndichte-Schätzung aller ELISA-Ergebnisse (mit h = 0,75 x σ_{pt} von Xpt_{ALL})

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

Comments:
The kernel density estimation shows nearly a symmetrical distribution of results with a shoulder at approx. 20 mg/kg (method IL) and a side-peak caused at 245 mg/kg (method RS) due to a single result above the target range (eventually submitted as gliadin by mistake).

Reprint, also in part, only with written permission from DLA-Ahrensburg

Characteristics: Quantitative evaluation ELISA Gluten

Sample B

Statistic Data	**All Results** *[mg/kg]*	**Method RS** *[mg/kg]*
Assigned value (X_{pt})	$X_{pt_{ALL}}$	$X_{pt_{METHOD\ RS}}$
Number of results	14	11
Number of outliers	1	1
Mean	102	106
Median	101	92,2
Robust Mean (X)	**96,7**	**96,7**
Robust standard deviation (S*)	**34,5**	**31,5**
Target range:		
Target standard deviation σ_{pt}	**24,2**	**24,2**
lower limit of target range	**48,4**	**48,4**
upper limit of target range	**145**	**145**
Quotient S/σ_{pt}*	*1,4*	*1,3*
Standard uncertainty $U_{(Xpt)}$	*11,5*	*11,9*
Results in the target range	*12*	*10*
Percent in the target range	*86*	*91*

Methods:
RS = R-Biopharm, Ridascreen®

Comments to the statistical characteristics and assigned values:

The kernel density estimation showed almost a symmetrical distribution of results without clear method-dependent differences.

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

The assigned values X_{pt} of the evaluation of all results and method RS-F were both 590% of the spiking level of "gluten" to sample B and thus above the range of the recommendations for the applied methods (s. 3.4.3 and "Recovery rates of Gluten" p.30).

Reprint, also in part, only with written permission from DLA-Ahrensburg

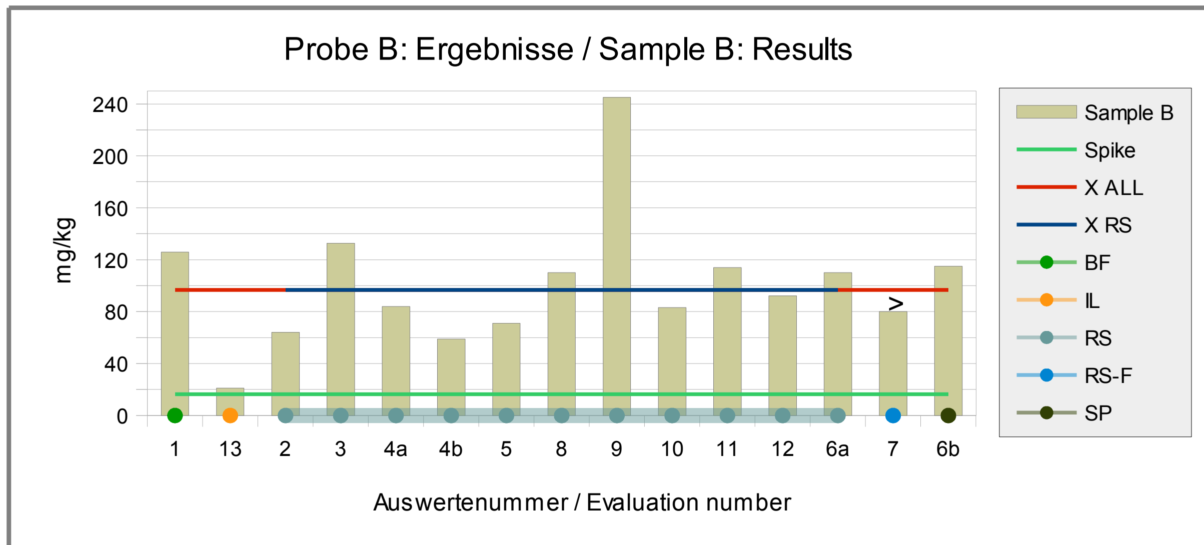


Abb./Fig. 2: ELISA Results Gluten
green line = Spiking level
red line = Assigned value robust mean all results
blue line = Assigned value robust mean results method RS
round symbols = Applied methods (see legend)

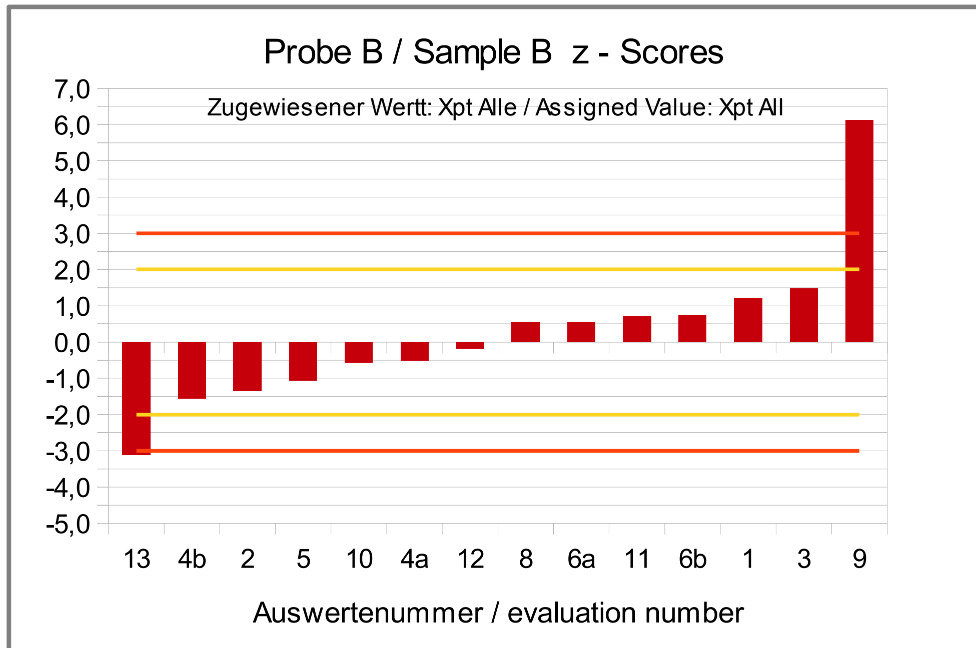


Abb./Fig. 3:
z-Scores (ELISA Results as Gluten
Assigned value robust mean (algorithm A) of all results

Reprint, also in part, only with written permission from DLA-Ahrensburg

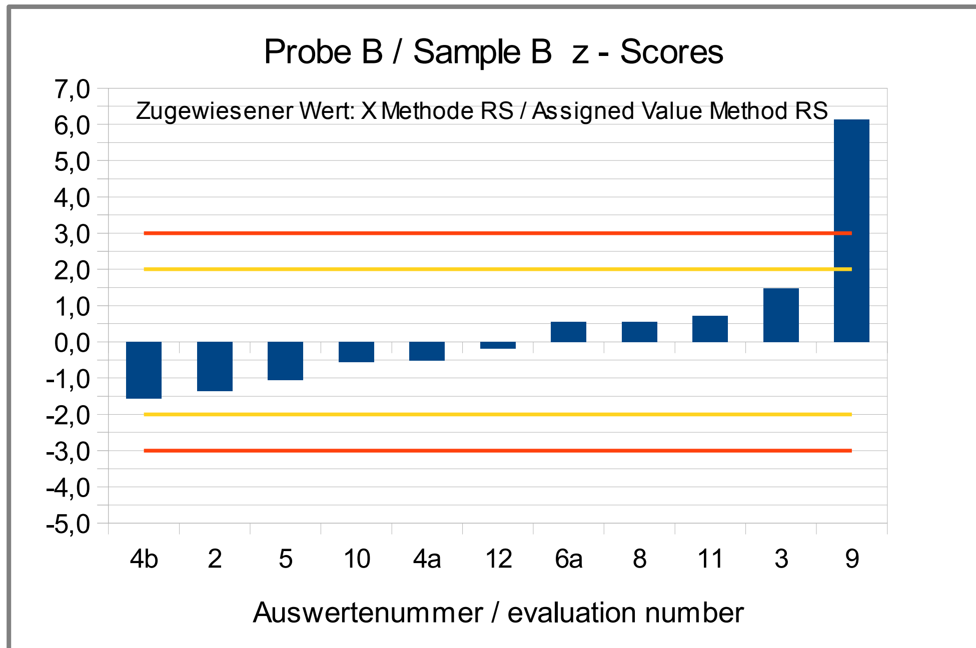


Abb./Fig. 4:
z-Scores (ELISA Results as Gluten)
Assigned value median of method RS (R-Biopharm, Ridascreen®)

Reprint, also in part, only with written permission from DLA-Ahrensburg

Quantitative evaluation of ELISA results: Spiking level sample

Evaluation number	Gluten	z-Score Xpt_{ALL}	z-Score Xpt_{RS}	Method	Remarks
	[mg/kg]				
1	90,6	1,4		BF	
13	31,6	-2,1		IL	
2	41,4	-1,5	-1,5	RS	
3	76,1	0,51	0,65	RS	
4a	50,9	-1,0	-0,89	RS	
4b	31,7	-2,1	-2,1	RS	
5	58,0	-0,56	-0,45	RS	
8	74,0	0,39	0,53	RS	
9	206	8,2	8,6	RS	result converted °
10	81,2	0,82	1,0	RS	
11	86,0	1,1	1,3	RS	
12	60,2	-0,43	-0,32	RS	
6a	60,0	-0,44	-0,33	RS	
7	>80			RS-F	
6b	95,0	1,6		SP	* mean calculated by DLA

° calculation p.19

Methods:
BF = MonoTrace ELISA, BioFront Technologies
IL = Immunolab
RS = Ridascreen®, R-Biopharm
RS-F= Ridascreen® Fast, R-Biopharm
SP = SENSISpec Ingezim

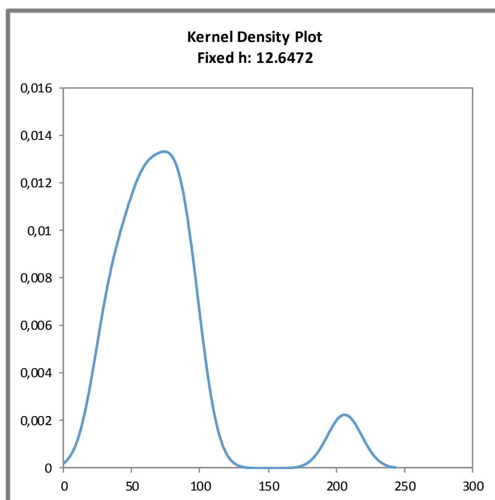


Abb. / Fig. 5:
Kerndichte-Schätzung aller ELISA-Ergebnisse (mit h = 0,75 x σ_{pt} von Xpt_{ALL})

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

Comments:
The kernel density estimation shows nearly a symmetrical distribution of results with a side-peak caused at 206 mg/kg (method RS) due to a single result above the target range (eventually submitted as gliadin by mistake).

Reprint, also in part, only with written permission from DLA-Ahrensburg

Characteristics: Quantitative evaluation ELISA: Gluten

Spiking Level Sample

Statistic Data	**All Results** [mg/kg]	**Method RS** [mg/kg]
Assigned value (X_{pt})	$X_{pt_{ALL}}$	$X_{pt_{METHOD\ RS}}$
Number of results	14	11
Number of outliers	1	1
Mean	74,5	75,1
Median	67,1	60,2
Robust Mean (X_{pt})	**67,5**	**65,4**
Robust standard deviation (S*)	**26,8**	**22,9**
Target range:		
Target standard deviation σ_{pt}	**16,9**	**16,3**
lower limit of target range	**33,7**	**32,7**
upper limit of target range	**101**	**98,1**
Quotient S/σ_{pt}*	*1,6*	*1,4*
Standard uncertainty $U_{(X_{pt})}$	*8,97*	*8,63*
Results in the target range	*11*	*9*
Percent in the target range	*79*	*82*

Comments to the statistical characteristics and assigned values:

The kernel density estimation showed almost a symmetrical distribution of results without clear method-dependent differences.

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

The assigned values X_{pt} of the evaluation of all results and method RS were 525% and 509% of the spiking level of "gluten" to the spiking level sample and thus above the range of the recommendations for the applied methods (s. 3.4.3 and "Recovery rates of Gluten" p.30).

Reprint, also in part, only with written permission from DLA-Ahrensburg

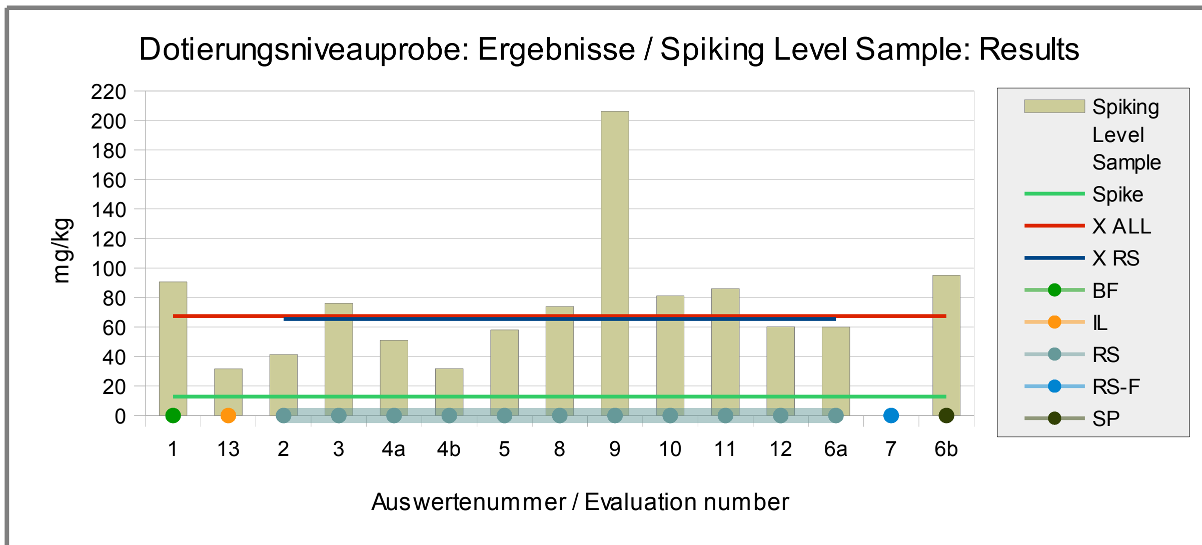


Abb./Fig. 6: ELISA Results Gluten
green line = Spiking level
red line = Assigned value robust mean all results
blue line = Assigned value robust mean results method RS
round symbols = Applied methods (see legend)

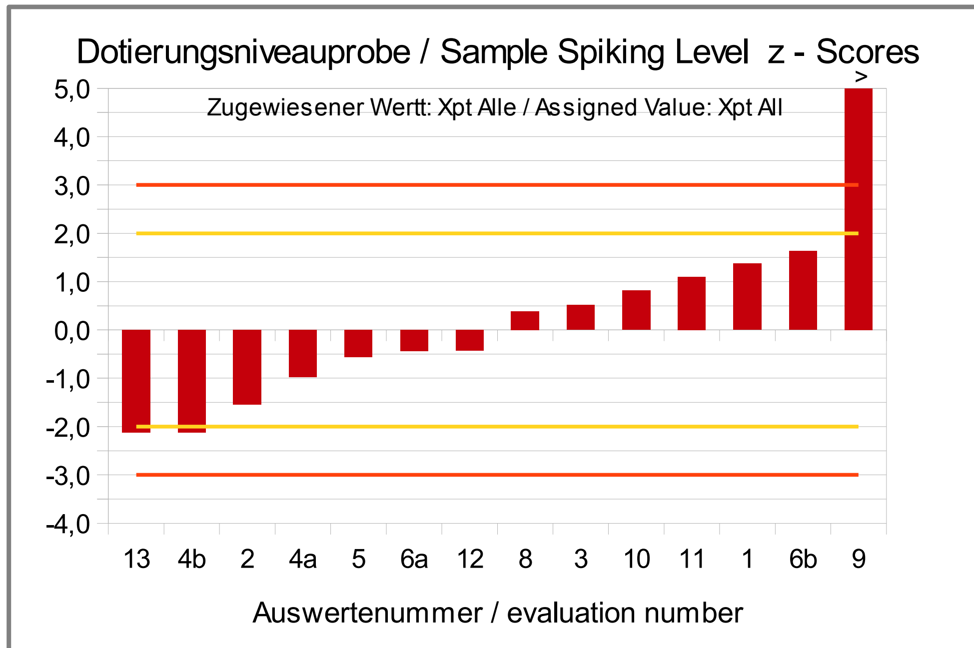


Abb./Fig. 7:
z-Scores (ELISA Results as Gluten)
Assigned value robust mean (algorithm A) of all results

Reprint, also in part, only with written permission from DLA-Ahrensburg

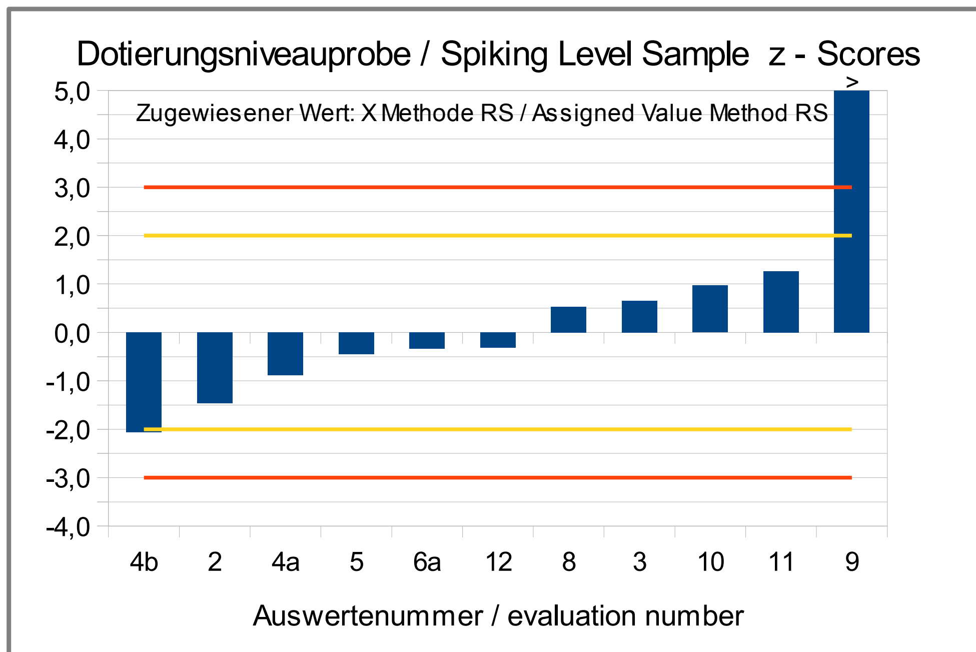


Abb./Fig. 8:
z-Scores (ELISA Results as Gluten)
Assigned value median of method RS (R-Biopharm, Ridascreen®)

Recovery Rates ELISA for Gluten: Spiking Level Sample and Sample B

Evaluation number	Spiking Level Sample	Recovery rate*	Sample B	Recovery rate*	Method	Remarks
	[mg/kg]	[%]	[mg/kg]	[%]		
1	90,6	706	126	770	BF	
13	31,6	246	21,2	**130**	IL	
2	41,4	322	64,0	391	RS	
3	76,1	593	133	810	RS	
4a	50,9	397	84,1	514	RS	
4b	31,7	247	58,9	360	RS	
5	58,0	452	71,0	434	RS	
8	74,0	576	110	672	RS	
9	206	1610	245	1497	RS	result converted °
10	81,2	633	83,1	508	RS	
11	86,0	670	114	696	RS	
12	60,2	469	92,2	563	RS	
6a	60,0	467	110	672	RS	
7	>80		>80		RS-F	
6b	95,0	740	115	703	SP	* mean calculated by DLA

° calculation p.19

RA**	50-150 %	RA**	50-150 %
Number in RA	**0**	Number in RA	**1**
Percent in RA	**0**	Percent in RA	**7**

* Recovery rate 100% relative size: Gluten, see p. 5

** Range of acceptance of AOAC for allergen ELISAS

Methods:

BF = MonoTrace ELISA, BioFront Technologies

IL = Immunolab

RS = Ridascreen®, R-Biopharm

RS-F= Ridascreen® Fast, R-Biopharm

SP = SENSISpec Ingezim

Comments:

When valuating the recovery rates, it should be noted that the samples contained rye flour, whereas the ELISA methods are commonly using wheat gluten as a standard reference.

One participant obtained a recovery rate for the spiked food matrix sample B within the range of the AOAC-recommendation of 50-150%. For the spiking level sample none of the recovery rates were in the range of acceptance.
The other results are with one exception up to about 7-fold above the spiked level.

Reprint, also in part, only with written permission from DLA-Ahrensburg

4.1.2 PCR Results: Gluten-containing cereals

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		
12a	negativ		positiv		2/2 (100%)	ASU	
6a	negativ		positiv		2/2 (100%)	div	
12b	negativ		negativ		1/2 (50%)	div	
2	negativ	< 10	positiv	58,9	2/2 (100%)	div	as rye
6b	negativ		positiv		2/2 (100%)	div	

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

Methods:
ASU = ASU §64 Methode/method
div = not indicated / other method

Comments:
The consensus values are in qualitative agreement with the spiking of sample B.
There was one negative result for sample B obtained by a PCR method specific for wheat (as specified by the participant).

Quantitative Valuation PCR: Samples B

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

Reprint, also in part, only with written permission from DLA-Ahrensburg

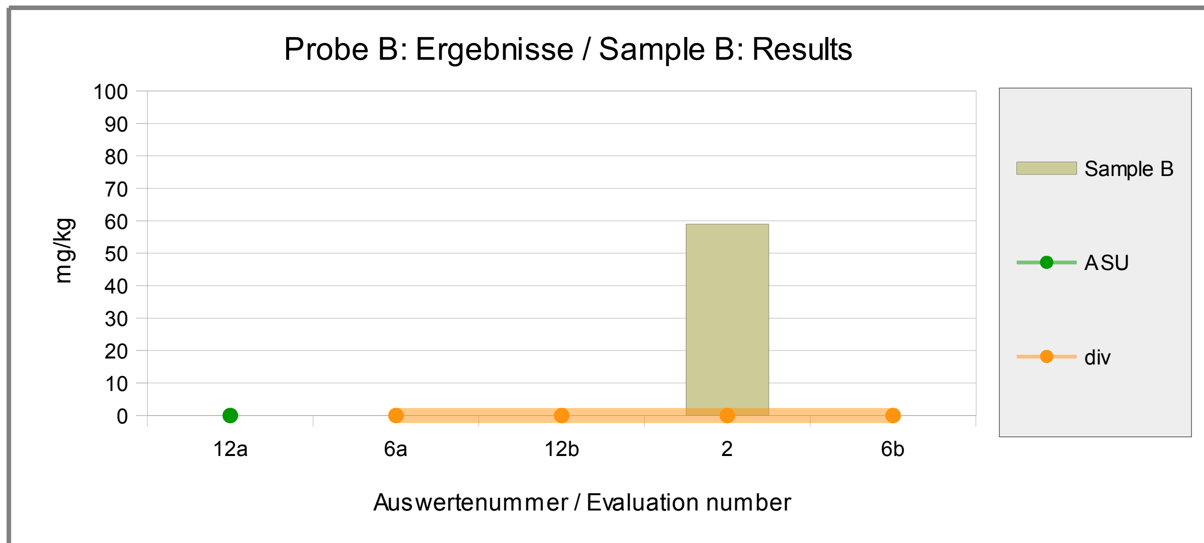


Abb./Fig. 9: PCR Results Gluten-containing Cereals Sample B
(result no. 2 as rye)
round symbols = Applied methods (see legend)

Reprint, also in part, only with written permission from DLA-Ahrensburg

(Quantitative) valuation of PCR results: Spiking Level Samples

No quantitative evaluation was done, because there was no quantitative result.

Evaluation number	Gluten-containing Cereals	Gluten-containing Cereals	Method	Remarks
	pos/neg	[mg/kg]		
12a	positive		ASU	as rye DNA
6a	positive		div	as gluten-containing cereals DNA
12b	negative		div	as wheat flour
2	positive		div	as rye
6b	positive		div	as rye DNA

Number positive	4
Number negative	1
Percent positive	80
Percent negative	20
Consensus value	positive

Methods:

ASU = ASU §64 Methode/method

div = not indicated / other method

Comments:

There was one negative result for the spiking level sample obtained by a PCR method specific for wheat (as specified by the participant).

Reprint, also in part, only with written permission from DLA-Ahrensburg

Recovery Rates PCR for Gluten-containing Cereals: Spiking level Sample and Sample B

Evaluation number	Spiking Level Sample	Recovery rate*	Sample B	Recovery rate*	Method	Remarks
	[mg/kg]	[%]	[mg/kg]	[%]		
12a					ASU	
6a					div	
12b					div	
2			58,9	11,9	div	as rye
6b					div	

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

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

** Range of acceptance of AOAC for allergen ELISAS

Methods:
ASU = ASU §64 Methode/method
div = not indicated / other method

Comments:
One participant submitted a quantitative result for the spiked food matrix sample B and obtained a recovery rate below the range of the AOAC recommendation of 50-150%.

Reprint, also in part, only with written permission from DLA-Ahrensburg

4.2 Proficiency Test Soya

4.2.1 ELISA-Results: Soya (as Soy 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		
11	negative	<0,27	positive	2,72	2/2 (100%)	AQ	
9	negative	<0.9	positive	11,7	2/2 (100%)	BC	
1	negative	0	positive	11,5	2/2 (100%)	BF	result converted °
13	negative	< 0,38	positive	28,4	2/2 (100%)	IL	result converted °
6a	negative	<0,31	positive	22,0	2/2 (100%)	MI-II	
2	negative	<2.5	positive	21,2	2/2 (100%)	RS-F	
3	negative	0,03	positive	13,8	2/2 (100%)	RS-F	
4	negative	< 2,5	positive	23,1	2/2 (100%)	RS-F	
5	negative		positive	7,56	2/2 (100%)	RS-F	result converted °
8	negative	<2.5	positive	20,0	2/2 (100%)	RS-F	
12	negative	nd	positive	21,3	2/2 (100%)	RS-F	
2	negative	<0,95	positive	10,2	2/2 (100%)	VT	result converted °
6b	negative	<0,95	positive	10,2	2/2 (100%)	VT	result converted °

° calculation p.19

	Sample A		Sample B	
Number positive	0		13	
Number negative	13		0	
Percent positive	0		100	
Percent negative	100		0	
Consensus value	negative		positive	

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

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

Reprint, also in part, only with written permission from DLA-Ahrensburg

Quantitative valuation of ELISA-results: Sample B

Evaluation number	Soy protein	z-Score $Xpt_{ALL\ 10}$	z-Score $Xpt_{ALL\ 20}$	z-Score Xpt_{RS-F}	Method	Remarks
	[mg/kg]					
11	2,72	-2,9			AQ	
9	11,7	0,65			BC	
1	11,5	0,57			BF	result converted °
13	28,4		1,6		IL	result converted °
6a	22,0		0,38		MI-II	
2	21,2		0,22	0,70	RS-F	
3	13,8		-1,3	-0,94	RS-F	
4	23,1		0,59	1,1	RS-F	
5	7,6		-2,5	-2,3	RS-F	result converted °
8	20,0		-0,02	0,43	RS-F	
12	21,3		0,24	0,71	RS-F	
2	10,2	0,06			VT	result converted °
6b	10,2	0,06			VT	result converted °

° calculation p.19

Methods:

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

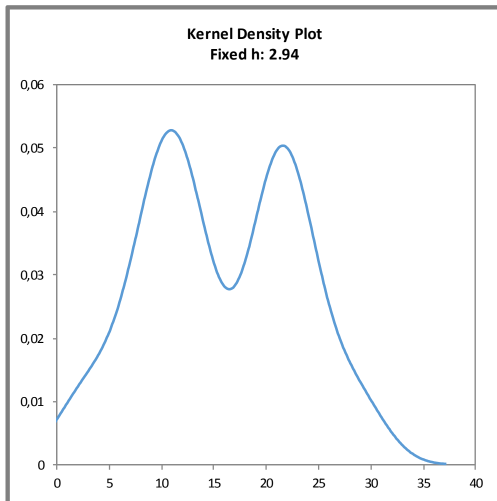


Abb. / Fig. 10:
Kerndichte-Schätzung aller ELISA-Ergebnisse (mit h = 0,75 x σ_{pt} von Xpt_{ALL})

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

Comments:
The kernel density estimation shows a distribution of results on two peaks at approx. 10 mg/kg and approx. 20 mg/kg, referred to below as peak 10 and peak 20.

Reprint, also in part, only with written permission from DLA-Ahrensburg

Characteristics: Quantitative evaluation ELISA: Soya (as Soy Protein)

Sample B

Statistic Data	Meth.Peak 10 [mg/kg]	Meth.Peak 20 [mg/kg]	Meth. RS-F [mg/kg]
Assigned value (X_{pt})	$X_{pt\,ALL\,10}$	$X_{pt\,ALL\,20}$	$X_{pt\,METHOD\,RS\text{-}F}$
Number of results	5	8	6
Number of outliers	0	0	0
Mean	9,26	19,7	17,8
Median	10,2	21,3	20,6
Robust Mean (X_{pt})	**10,1**	**20,1**	**18,1**
Robust standard deviation (S*)	**2,27**	**6,13**	**6,21**
Target range:			
Target standard deviation σ_{pt}	**2,52**	**5,03**	**4,52**
lower limit of target range	**5,03**	**10,1**	**9,04**
upper limit of target range	**15,1**	**30,2**	**27,1**
Quotient S/σ_{pt}*	*0,90*	*1,2*	*1,4*
Standard uncertainty $U_{(Xpt)}$	*1,27*	*2,71*	*3,17*
Results in the target range	*4*	*7*	*5*
Percent in the target range	*80*	*88*	*83*

Methods:
Peak 10 = AgraQuant, BioCheck, Biofront, Veratox
Peak 20 = Immunolab, Morinaga, Ridascreen Fast®
RS-F = R-Biopharm, Ridascreen Fast®

Comments to the statistical characteristics and assigned values:

The kernel density estimation showed almost a bimodal distribution of results. Therefore, no common evaluation of all methods was made, but two evaluations separated by methods that can be assigned to the first peak (peak 10) and the second peak (peak 20) (assignment see above below the table).
The evaluations of peak 10 and peak 20 results as well as the evaluation of results from method RS-F showed a normal variability of results. The quotients S/σ_{pt} were well below 2,0. The robust standard deviations are in the range of established values for the reproducibility standard deviation of the applied methods (see 3.4.2 value by precision experiments and 3.4.3 value by perception). The comparability of results is given in the respective set of results. This conclusion is limited for the evaluation across the methods, because there were only a few results for some methods.*
The assigned value X_{pt} of the evaluation of peak 10 results was 34% of the spiking level of "soy protein" to sample B and thus below the recommendations for the applied methods while the results of peak 20 with 68% and method RS-F with 61% were within the recommendations (s. 3.4.3 and "Recovery rates of Soya" p.44).

Reprint, also in part, only with written permission from DLA-Ahrensburg

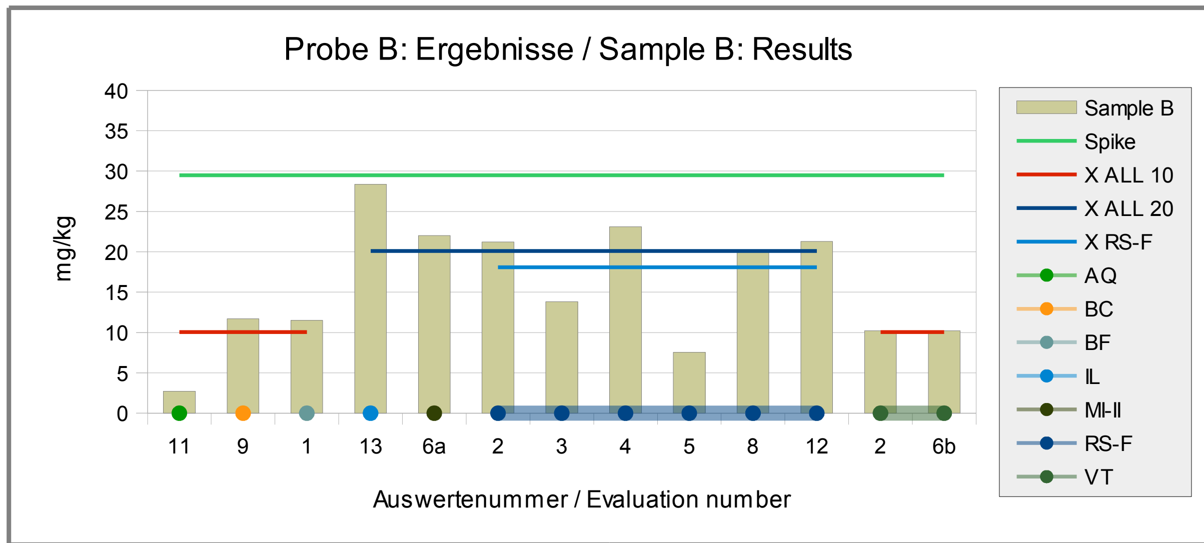


Abb./Fig. 11: ELISA Results Soy Protein
green line = Spiking level
red line = Assigned value robust mean all results of peak 10
blue line = Assigned value robust mean all results of peak 10
light blue line = Assigned value robust mean results method RS-F
round symbols = Applied methods (see legend)

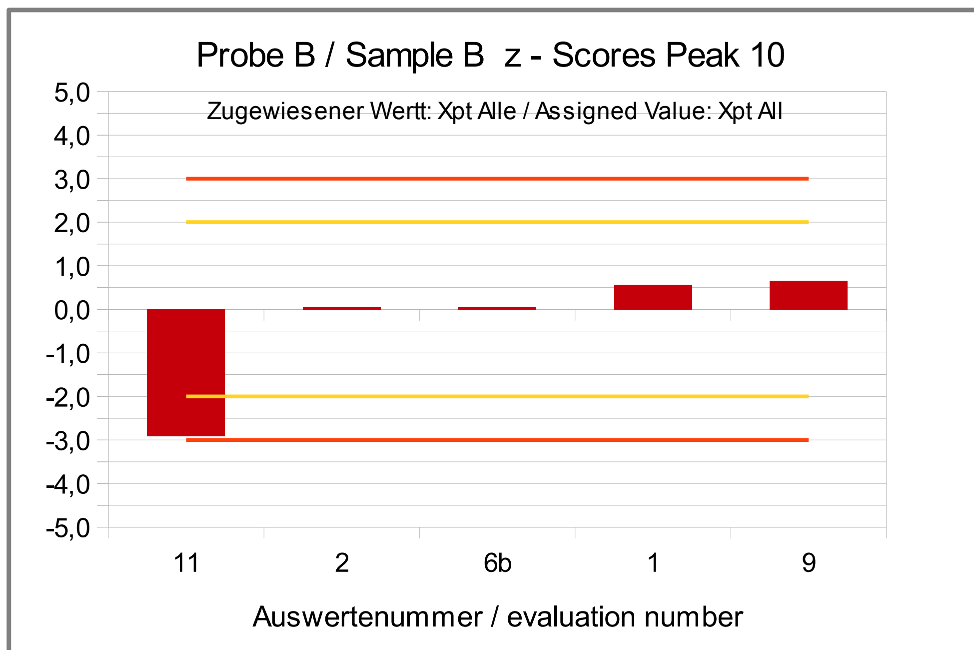


Abb./Fig. 12:
z-Scores (ELISA Results as Soy Protein
Assigned value robust mean (algorithm A) of all results for Peak 10

Reprint, also in part, only with written permission from DLA-Ahrensburg

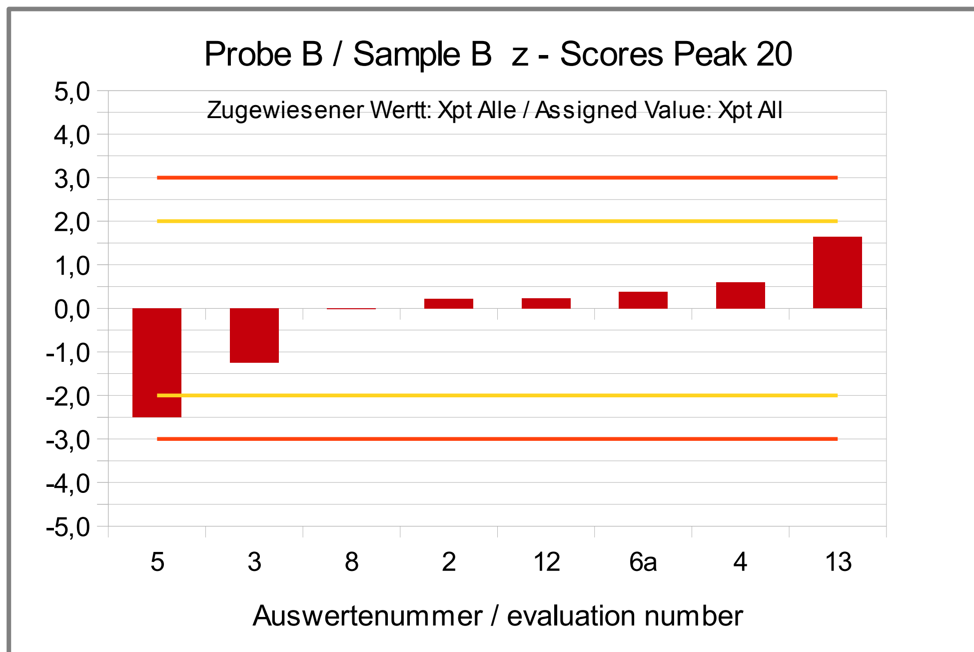


Abb./Fig. 13:
z-Scores (ELISA Results as Soy Protein
Assigned value robust mean (algorithm A) of all results for Peak 20

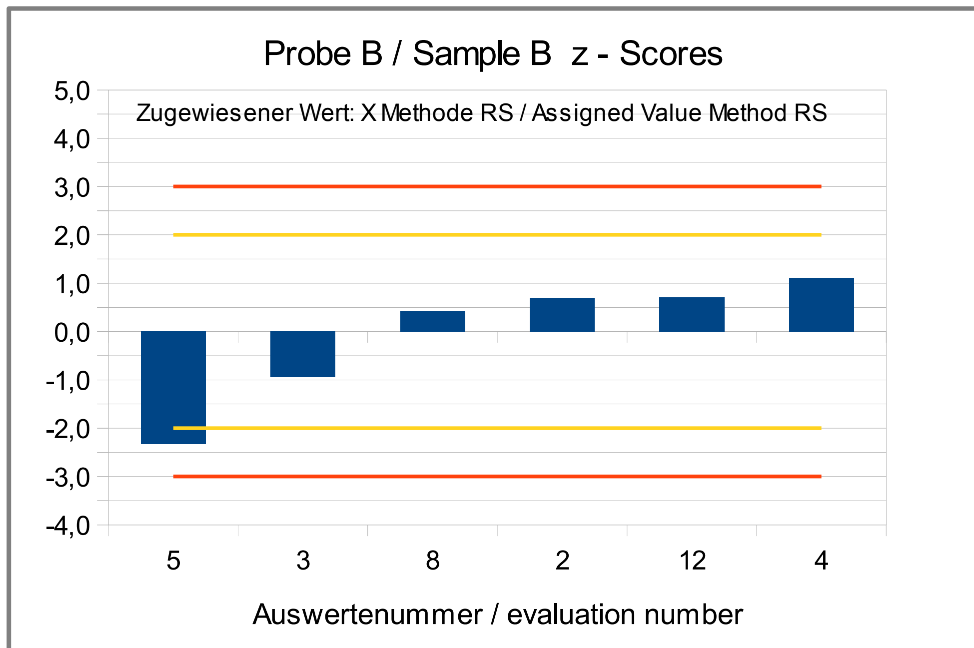


Abb./Fig. 14:
z-Scores (ELISA Results as Soy Protein)
Assigned value median of method RS (R-Biopharm, Ridascreen® Fast)

Reprint, also in part, only with written permission from DLA-Ahrensburg

Quantitative evaluation of ELISA results: Spiking level sample

Evaluation number	Soy protein	z-Score $Xpt_{ALL\ 10}$	z-Score $Xpt_{ALL\ 20}$	z-Score Xpt_{RS-F}	Method	Remarks
	[mg/kg]					
11	3,20	-2,6			AQ	
9	12,4	1,25			BC	
1	11,8	0,98			BF	result converted °
13	29,9		1,7		IL	result converted °
6a	18,0		-0,58		MI-II	
2	25,1		0,76	0,97	RS-F	
3	17,2		-0,73	-0,59	RS-F	
4	20,7		-0,07	0,10	RS-F	
5	7,2		-2,6	-2,6	RS-F	result converted °
8	23,0		0,37	0,56	RS-F	
12	22,9		0,35	0,54	RS-F	
2	9,45	0,00			VT	result converted °
6b	9,83	0,16			VT	result converted °

° calculation p.19

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

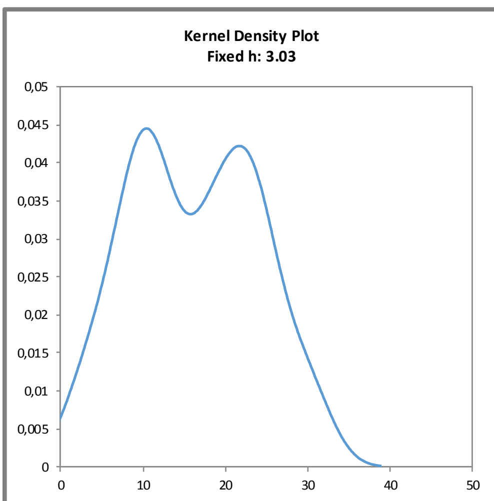


Abb. / Fig. 15:
Kerndichte-Schätzung aller ELISA-Ergebnisse (mit h = 0,75 x σpt von Xpt_{ALL})

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

Comments:
The kernel density estimation shows a distribution of results on two peaks at approx. 10 mg/kg and approx. 20-25 mg/kg, referred to below as peak 10 and peak 20.

Reprint, also in part, only with written permission from DLA-Ahrensburg

Characteristics: Quantitative evaluation ELISA: Soya (as Soy Protein)

Spiking Level Sample

Statistic Data	**Meth.Peak 10** [mg/kg]	**Meth.Peak 20** [mg/kg]	**Meth. RS-F** [mg/kg]
Assigned value (X_{pt})	$X_{pt\ ALL\ 10}$	$X_{pt\ ALL\ 20}$	$X_{pt\ METHOD\ RS\text{-}F}$
Number of results	5	8	6
Number of outliers	0	0	0
Mean	9,33	20,5	19,3
Median	9,83	21,8	21,8
Robust Mean (X_{pt})	**9,45**	**21,1**	**20,2**
Robust standard deviation (S*)	**3,84**	**6,26**	**5,34**
Target range:			
Target standard deviation σ_{pt}	**2,36**	**5,26**	**5,05**
lower limit of target range	**4,73**	**10,5**	**10,1**
upper limit of target range	**14,2**	**31,6**	**30,3**
Quotient S/σ_{pt}*	*1,6*	*1,2*	*1,1*
Standard uncertainty $U_{(Xpt)}$	*2,15*	*2,77*	*2,72*
Results in the target range	*4*	*7*	*5*
Percent in the target range	*80*	*88*	*83*

Methods:
Peak 10 = AgraQuant, BioCheck, Biofront, Veratox
Peak 20 = Immunolab, Morinaga, Ridascreen Fast®
RS-F = R-Biopharm, Ridascreen Fast®

Comments to the statistical characteristics and assigned values:

The kernel density estimation showed almost a bimodal distribution of results. Therefore, no common evaluation of all methods was made, but two evaluations separated by methods that can be assigned to the first peak (peak 10) and the second peak (peak 20) (assignment see above below the table).

The evaluations of peak 10 and peak 20 results as well as the evaluation of results from method RS-F showed a normal variability of results. The quotients S/σ_{pt} were well below 2,0. The robust standard deviations are in the range of established values for the reproducibility standard deviation of the applied methods (see 3.4.2 value by precision experiments and 3.4.3 value by perception). The comparability of results is given in the respective set of results. This conclusion is limited for the evaluation across the methods, because there were only a few results for some methods.*

The assigned value X_{pt} of the evaluation of peak 10 results was 38% of the spiking level of "soy protein" to sample B and thus below the recommendations for the applied methods while the results of peak 20 with 84% and method RS-F with 80% were within the recommendations (s. 3.4.3 and "Recovery rates of Soya" p.44).

Reprint, also in part, only with written permission from DLA-Ahrensburg

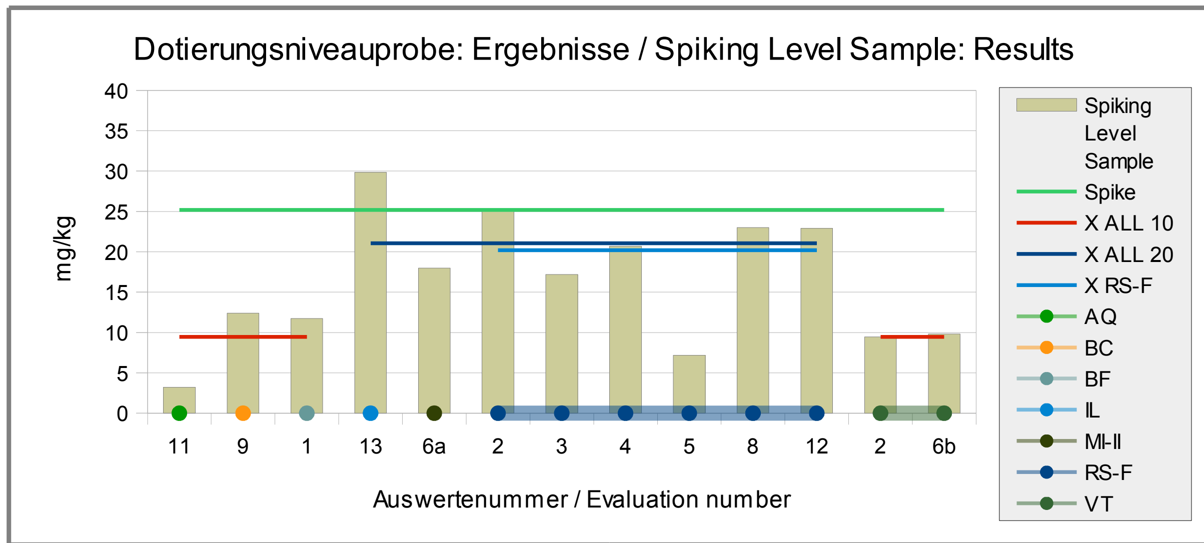


Abb./Fig. 16: ELISA Results Soy Protein
green line = Spiking level
red line = Assigned value robust mean all results of peak 10
blue line = Assigned value robust mean all results of peak 10
light blue line = Assigned value robust mean results method RS-F
round symbols = Applied methods (see legend)

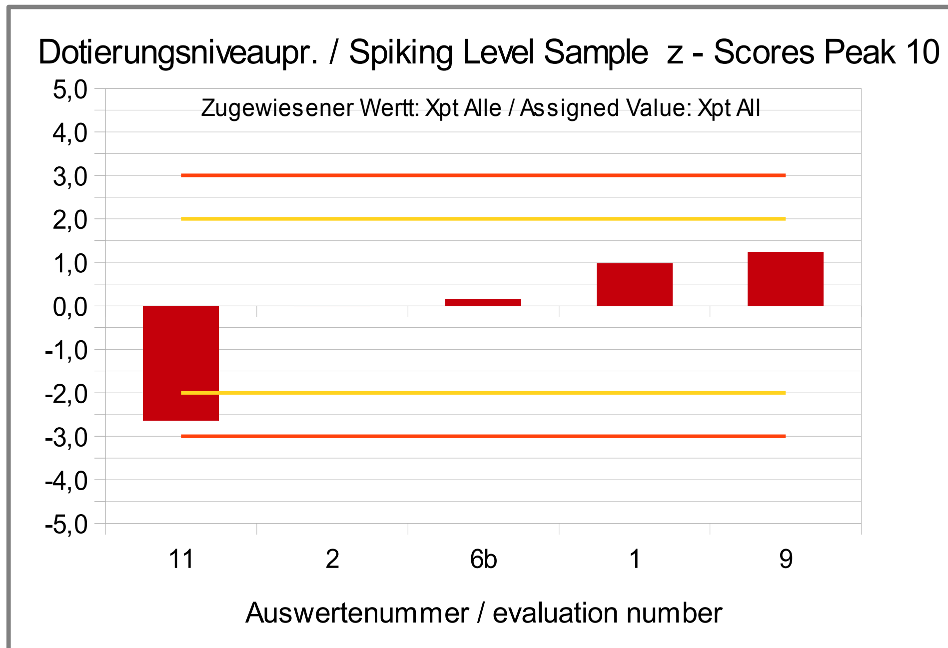


Abb./Fig. 17:
z-Scores (ELISA Results as Soy Protein
Assigned value robust mean (algorithm A) of all results for Peak 10

Reprint, also in part, only with written permission from DLA-Ahrensburg

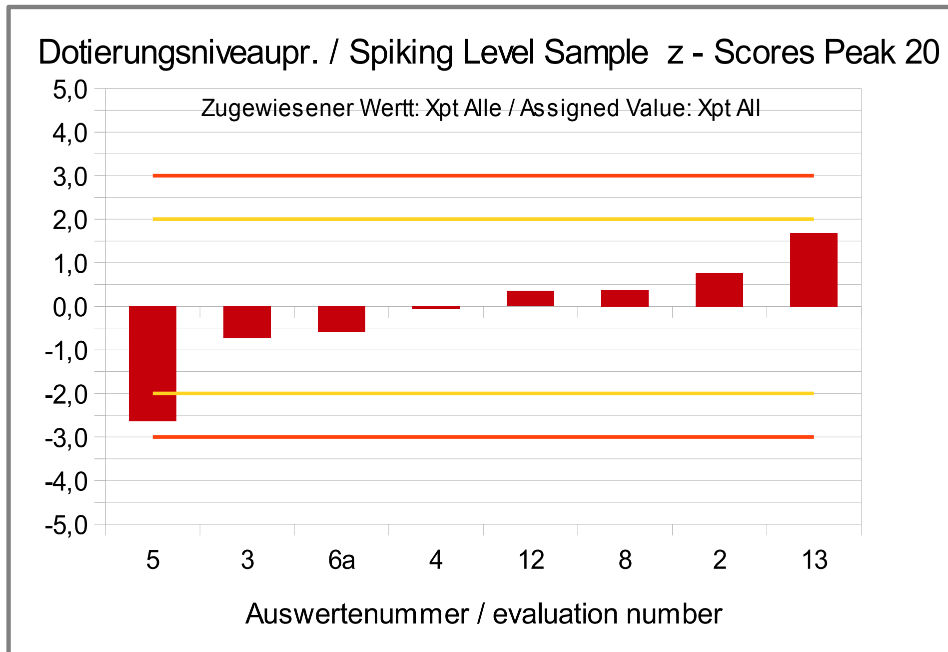


Abb./Fig. 18:
z-Scores (ELISA Results as Soy Protein
Assigned value robust mean (algorithm A) of all results for Peak 20

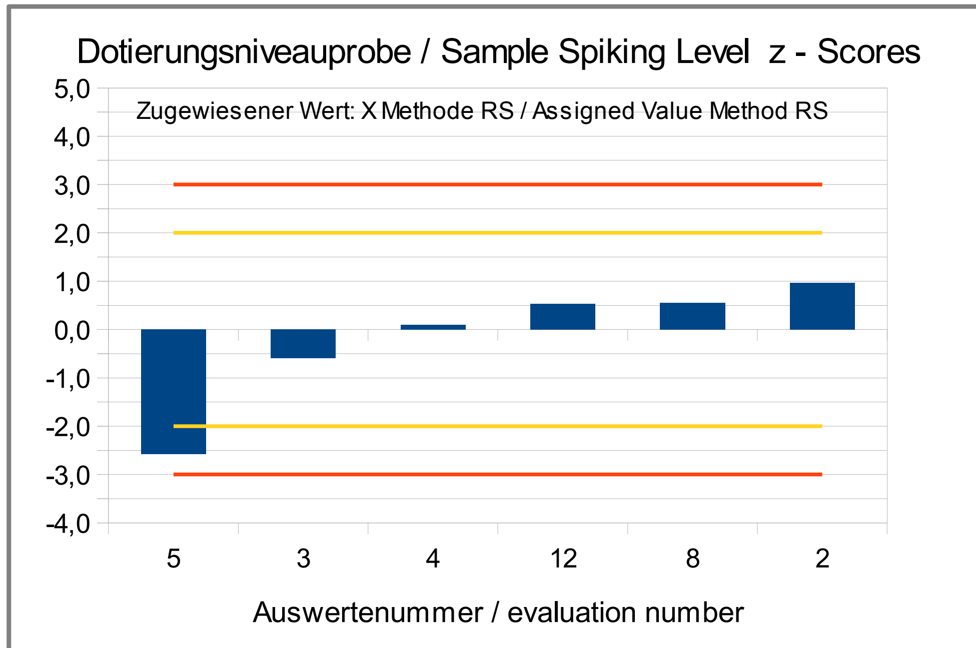


Abb./Fig. 19:
z-Scores (ELISA Results as Soy Protein)
Assigned value median of method RS (R-Biopharm, Ridascreen® Fast)

Reprint, also in part, only with written permission from DLA-Ahrensburg

Recovery Rates ELISA for Soya (as Soy Protein):
Spiking level Sample and Sample B

Evaluation number	Spiking Le-vel Sample	Recovery rate*	Sample B	Recovery rate*	Method	Remarks
	[mg/kg]	[%]	[mg/kg]	[%]		
11	3,20	13	2,72	9,2	AQ	
9	12,4	49	11,7	40	BC	
1	11,8	47	11,5	39	BF	result converted °
13	29,9	**119**	28,4	**96**	IL	result converted °
6a	18,0	**71**	22,0	**75**	MI-II	
2	25,1	**99**	21,2	**72**	RS-F	
3	17,2	**68**	13,8	47	RS-F	
4	20,7	**82**	23,1	**78**	RS-F	
5	7,18	29	7,6	26	RS-F	result converted °
8	23,0	**91**	20,0	**68**	RS-F	
12	22,9	**91**	21,3	**72**	RS-F	
2	9,45	38	10,2	35	VT	result converted °
6b	9,83	39	10,2	35	VT	result converted °

° calculation p.19

RA**	50-150 %	RA**	50-150 %
Number in RA	7	Anzahl im AB	6
Percent in RA	54	Prozent im AB	46

* Recovery rate 100% relative size: Soy Protein, see p. 5

** Range of acceptance of AOAC for allergen ELISAS

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

Comments:
For the spiking material sample 54% (7) of the participants obtained a recovery rate within the range of the AOAC-recommendation of 50-150%. For the spiked food matrix sample B 46% (6) of the recovery rates were in the range of acceptance.

It should be noted that some test kit manufacturers have different conversion factors for e.g. toasted and non-toasted soy flours, factors can differ about a factor of 10 (test kit instructions: methods AQ, IL).
The present soy flour of the PT samples is a mixture of toasted soybean flours, some of which still have measurable trypsin inhibitor activities. Thus the predetermined conversion factors of the methods may not be exactly applicable.

Reprint, also in part, only with written permission from DLA-Ahrensburg

4.2.2 PCR Results: Soya (as Soy flour, Soybean)

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		
12a	negative		positive		2/2 (100%)	ASU	1-plex
12b	negative		positive		2/2 (100%)	ASU	4-plex
2b	negative	<1	positive	7,68	2/2 (100%)	SFA	
2a	negative	<1	positive	10,2	2/2 (100%)	SFA-ID	
4	negative	< 0,4	positive	> 0,4	2/2 (100%)	SFA-ID	
6	negative		positive		2/2 (100%)	div	
10	negative	< 10	positive	131	2/2 (100%)	div	

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

Methods:
ASU = ASU §64 Methode/method
SFA = Sure Food ALLERGEN, R-Biopharm / Congen
SFA-ID = Sure Food Allergen ID, R-Biopharm / Congen
div = not indicated / other method

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

Quantitative Valuation PCR: Samples B

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

Reprint, also in part, only with written permission from DLA-Ahrensburg

(Quantitative) valuation of PCR results: Spiking Level Samples

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

Evaluation number	Soy flour	Soy flour	Method	Remarks
	pos/neg	[mg/kg]		
12a	positive		ASU	1-plex
12b	positive		ASU	4-plex
2b	positive	2,76	SFA	
2a	positive	3,96	SFA-ID	
4	positive	> 0,4	SFA-ID	
6	positive		div	
10	positive	60	div	

Number positive	7
Number negative	0
Percent positive	100
Percent negative	0
Consensus value	positive

Methods:

ASU = ASU §64 Methode/method

SFA = Sure Food ALLERGEN, R-Biopharm / Congen

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

div = not indicated / other method

Comments:

For the spiking level sample there were 100% positive results.

Reprint, also in part, only with written permission from DLA-Ahrensburg

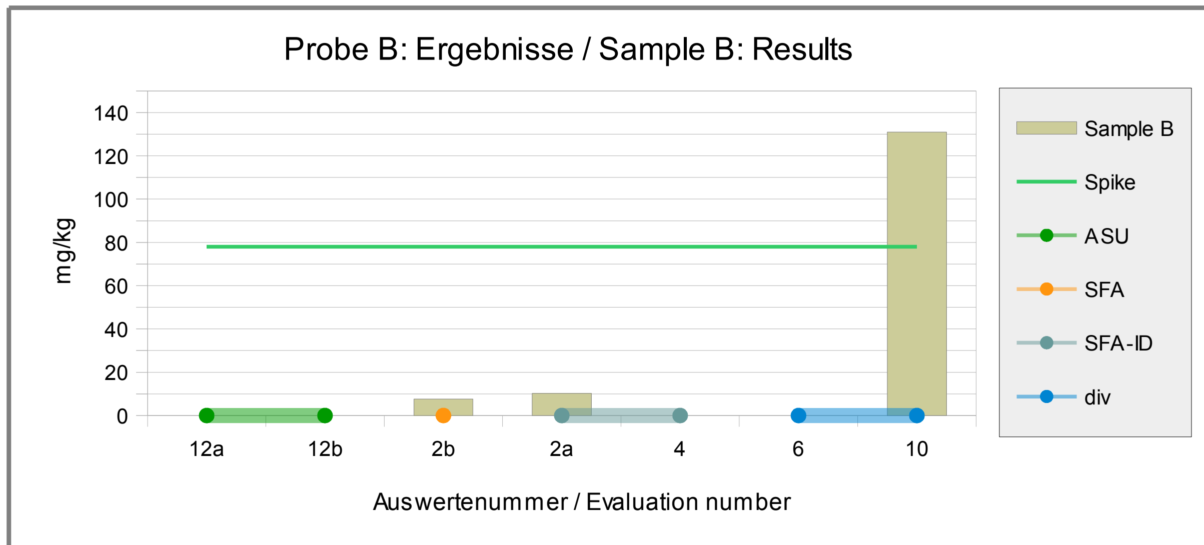


Abb./Fig. 20: PCR Results Soya (as Soy flour) Sample B
green line = Spiking level
round symbols = Applied methods (see legend)

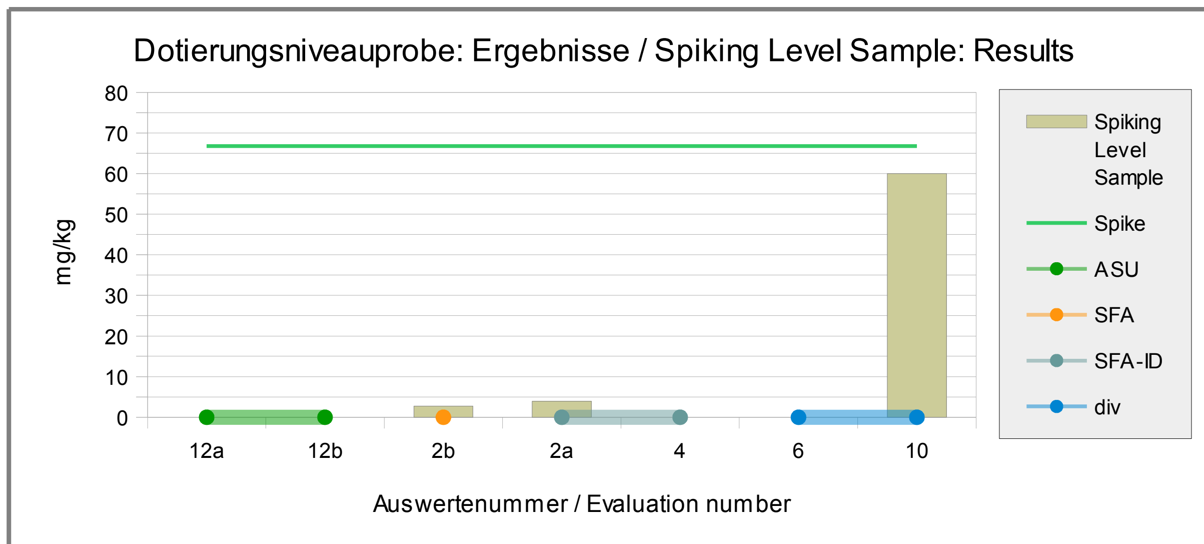


Abb./Fig. 21: PCR Results Soya (as Soy flour) Spiking Level Sample
green line = Spiking level
round symbols = Applied methods (see legend)

Reprint, also in part, only with written permission from DLA-Ahrensburg

Recovery Rates PCR for Soya (as Soy flour / Soybean): Spiking level Sample and Sample B

Evaluation number	Spiking Level Sample	Recovery rate*	Sample B	Recovery rate*	Method	Remarks
	[mg/kg]	[%]	[mg/kg]	[%]		
12a					ASU	
12b					ASU	
2b	2,76	4,1	7,68	10	SFA	
2a	3,96	5,9	10,2	13	SFA-ID	
4	> 0,4		> 0,4		SFA-ID	
6					div	
10	60	**90**	131	168	div	

RA**	50-150 %	RA**	50-150 %
Number in RA	1	Number in RA	0
Percent in RA	33	Percent in RA	0

* Recovery rate 100% relative size: Soy flour, s. Page 5

** Range of acceptance of AOAC for allergen ELISAS

Methods:
ASU = ASU §64 Methode/method
SFA = Sure Food ALLERGEN, R-Biopharm / Congen
SFA-ID = Sure Food Allergen ID, R-Biopharm / Congen
div = keine genaue Angabe / andere Methode
div = not indicated / other method

Comments:

One participant obtained for the spiking level sample a recovery rate within the range of the AOAC recommendation of 50-150% by a PCR method. For the spiked food matrix sample B the recovery rates were below the AOAC recommendation.

Reprint, also in part, only with written permission from DLA-Ahrensburg

5. Documentation

5.1 Details by the participants

Note: Information given in German were translated by DLA to the best of our knowledge (without guarantee of correctness).

5.1.1 ELISA: Gluten

Meth. Abr.	Evaluation number	Date of Analysis	Result Sample A		Result Sample B		Result Spiking Sample		NWG / LOD *	BG / LOQ *	MU*	quantitative Result given as	Method
		day/month	qualitative	mg/kg	qualitative	mg/kg	qualitative	mg/kg	mg/kg	mg/kg	mg/kg	e.g. food /protein	Test-Kit + Manufacturer
BF	1	04.04.18	negative	0	positive	126	positive	90,6	0,36	2		Gluten	MonoTrace Gluten ELISA kit, BioFront Technologies
IL	13	01.03.18	negative	< 1	positive	21,2	positive	31,6	0,6	4		Gluten	Immunolab Gliadin/Gluten ELISA
RS	2	23.03.18	negative	<5	positive	63,98	positive	41,35	5	5		Gluten	Ridascreen® Gliadin R7001, R-Biopharm
RS	3	04.04.18	negative	0,682	positive	132,64	positive	76,067	1	5		Gluten	Ridascreen® Gliadin R7001, R-Biopharm
RS	4a	01.03.	negative	< 5,0	positive	84,1	positive	50,9	1	5		Gluten	Ridascreen® Gliadin R7001, R-Biopharm
RS	4b	20.03.	negative	< 5,0	positive	58,9	positive	31,7	1	5		Gluten	Ridascreen® Gliadin R7001, R-Biopharm
RS	5	20.03.18	negative		positive	71	positive	58		5		Gluten	Ridascreen® Gliadin R7001, R-Biopharm
RS	8	23.03.18	negative	<5.0	positive	110	positive	74	5	5	0,346	Gluten	Ridascreen® Gliadin R7001, R-Biopharm
RS	9	27.02.18	negative	<5	positive	122,5	positive	103,1	5	5	0,5	Gliadin	Ridascreen® Gliadin R7001, R-Biopharm
RS	10	16.03.18	-	< 3	-	83,1	-	81,2				Gluten	Ridascreen® Gliadin R7001, R-Biopharm
RS	11		negative	<5,00	positive	114	positive	86		5		Gluten	Ridascreen® Gliadin R7001, R-Biopharm
RS	12	26.2+2.3.	negative	nb	positive	92,2	positive	60,2	1	5	14	Gluten	Ridascreen® Gliadin R7001, R-Biopharm
RS	6a	5.3.	negative	<5	positive	110	positive	60	3	5		Gluten	Ridascreen® Gliadin R7001, R-Biopharm
RS-F	7	03.04.18	negative	<10	positive	>80	positive	>80				Gluten	Ridascreen® FAST Gliadin R7002, R-Biopharm
SP	6b	27.3.	negative	<3,12	positive	110	positive	80	3,12	3,12		Gluten	SENSISpec Ingezim Gluten R5 30.GLU.K2
SP	6b	27.3.	negative	<3,12	positive	120	positive	110	3,12	3,12		Gluten	SENSISpec Ingezim Gluten R5 Quick 30.GL2.K2

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

Reprint, also in part, only with written permission from DLA-Ahrensburg

Continuation ELISA Gluten:

Meth. Abr.	Evaluation number	Specifity	Remarks to the Method (Extraction and Determination)	Method accredited ISO/IEC 17025	Further Remarks
		Antibody	e.g. Extraction Solution / Time / Temperature	yes/no	
BF	1	Monoclonal anti-gliadin	1:40 ratio, 1 hour in 1X BioFront MGEB at 60C	no	
IL	13				
RS	2	As Per Kit Instructions	As Per Kit Instructions	Yes	
RS	3	The monoclonal antibody R5 reacts with the gliadin fractions from wheat and corresponding prolamins from rye and barley.	final dilution of each sample of 500, all samples were shaking up 2h after adding 80% ethanol instead of 1h, extracts were centrifuged with high speed for 10 minutes using a microcentrifuge	AOAC method	extraction done with cocktail, the spiking level sample was not viscous or a liquid after adding the cocktail
RS	4a		As Per Kit Instructions	yes	
RS	4b		according to kit manual, extraction with 0,25g skimmed milk powder	yes	
RS	5			yes	
RS	8	R-5 (against gliadin; monoclonal Ab.)	As per kit instructions	yes	
RS	9	Gliadin	Cocktail Sol, 80% Ethanol/ 40mins @ 50c	Yes	
RS	10			yes	
RS	11			yes	
RS	12	R5	As Per Kit Instructions	yes	--
RS	6a	R5, detects prolamins from wheat, rye, barley	As Per Kit Instructions	yes	
RS-F	7		Mean of 3 determinations	validated in Codex Alimentarius Method Type 1	
SP	6b	R5, detects prolamins from wheat, rye, barley	As Per Kit Instructions	yes	
SP	6b	R5, detects prolamins from wheat, rye, barley	As Per Kit Instructions	yes	

Reprint, also in part, only with written permission from DLA-Ahrensburg

5.1.2 ELISA: Soya

Meth. Abr.	Evaluation number	Date of Analysis	Result Sample A		Result Sample B		Result Spiking Sample		NWG / LOD *	BG / LOQ *	MU*	quantitative Result given as	Method
		day/month	qualitative	mg/kg	qualitative	mg/kg	qualitative	mg/kg	mg/kg	mg/kg	mg/kg	e.g. food /protein	Test-Kit + Manufacturer
AQ	11		negative	<0,27	positive	2,72	positive	3,2		0,27		Soyprotein	AgraQuant ELISA Soy COKAL0448, RomerLabs
BC	9	28.02.18	negative	<0.9	positive	11,7	positive	12,4	0,9	0,9	0,5	Soyprotein	BioCheck ELISA Soya-Check
BF	1	04.04.18	negative	0	positive	30,4	positive	31,1	0,16	1		Soyflour	MonoTrace Soy ELISA kit, BioFront Technologies
IL	13	01.03.18	negative	< 1	positive	75*	positive	79*	1,6*	4*		Soyflour	Immunolab Soy ELISA
MI-II	6a	6.3.	negative	<0,31	positive	22	positive	18	0,31	0,31		Soyprotein	Morinaga Soja ELISA Kit II M2117
RS-F	2	29.03.18	negative	<2.5	positive	21,23	positive	25,07	2,5	2,5		Soyprotein	Ridascreen® FAST Soya R7102, R-Biopharm
RS-F	3	28.02.18	negative	0,03	positive	13,82	positive	17,21	0,24	2,5		soya protein	Ridascreen® FAST Soya R7102, R-Biopharm
RS-F	4	26.02.	negative	< 2,5	positive	23,1	positive	20,7	0,24	2,5		Soyprotein	Ridascreen® FAST Soya R7102, R-Biopharm
RS-F	5	20.03.18	negative		positive	20	positive	19		2,5		Soybean	Ridascreen® FAST Soya R7102, R-Biopharm
RS-F	8	16.03.18	negative	<2.5	positive	20	positive	23	2,5	2,5	0,32	Soyprotein	Ridascreen® FAST Soya R7102, R-Biopharm
RS-F	12	27.2.+13.3.	negative	nd	positive	21,3	positive	22,9	0,31	2,5	5,7	Soyprotein	Ridascreen® FAST Soya R7102, R-Biopharm
VT	2	29.03.18	negative	<2.5	positive	27	positive	25	2,5	2,5		Soyflour	Veratox Soy Allergen, Neogen
VT	6b	2.3.	negative	<2,5	positive	27	positive	26	0,96	2,5		Soyflour	Neogen Veratox Soja ELISA 8410

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

Reprint, also in part, only with written permission from DLA-Ahrensburg

Continuation ELISA Soya:

Meth. Abr.	Evaluation number	Specifity	Remarks to the Method (Extraction and Determination)	Method accredited ISO/IEC 17025	Further Remarks
		Antibody	e.g. Extraction Solution / Time / Temperature	yes/no	
AQ	11			yes	
BC	9	STI	Extr Buffer, Shaking for 15 mins at 60c	Yes	
BF	1	Monoclonal anti-glycinin	1:20 ratio, 10 minutes in 1X ExB while boiling	no	
IL	13	STI			* Adjustment of the conversion factor for toasted soybean flour from 470 to 100 for soybean flour with residue trypsin inhibitor activity (empirical value DLA 14/2017 Response PT Soya), the (measured) corresponding STI concentration is lower by a factor of 100
MI-II	6a	detects soyprotein beta-conglycinin	As Per Kit Instructions	yes	
RS-F	2	As Per Kit Instructions	As Per Kit Instructions	Yes	
RS-F	3	The antibodies specifically detect heated soya proteins. There is cross-reactivity to legumes of the tribe Phaseolea, the genus Vicia, to dried peas/pea flour to peanut.	extraction done by following protocol "solid samples" without caseïne, extracts were centrifuged at high speed for 10 minutes using a microcentrifuge	no	
RS-F	4		As Per Kit Instructions	yes	
RS-F	5			yes	
RS-F	8	Against Heat processed soya proteins. (Glycinin (408%, beta-conglycinin 7.3%, tripsin inhibitor 0.46%)	As per kit instructions	no	
RS-F	12	Antibodies detect specifically heated soyproteins	As Per Kit Instructions	yes	--
VT	2	As Per Kit Instructions	As Per Kit Instructions	Yes	
VT	6b	detects a specific marker in soybeans, which is extremely heat resistant	As Per Kit Instructions	yes	

Reprint, also in part, only with written permission from DLA-Ahrensburg

5.1.3 PCR: Gluten-containing Cereals

Meth. Abr.	Evaluation number	Date of Analysis	Result Sample A		Result Sample B		Result Spiking Sample		NWG / LOD *	BG / LOQ *	MU*	quantitative Result given as	Method
		day/month	qualitative	mg/kg	qualitative	mg/kg	qualitative	mg/kg	mg/kg	mg/kg	mg/kg	e.g. food /protein	Test-Kit + Manufacturer
ASU	12a	1.3.	negative		positive		positive					Roggen-DNA	ASU §64 Methode/method
div	6a	5.3.	negative		positive		positive		4			gluten-containing cereals DNA	Eur F Res Tech 212 (2001) 228ff, mod.
div	12b	1.3.	negative		negative		negative					wheat flour	M. Allmann, J. Lüthy 1993: Detection of w heat contamination in non-Wheat food products
div	2	29.03.18	negative	<10	positive	58,88	positive		10	10		Rye	other: please fill in!
div	6b	5.3.	negative		positive		positive		20			Rye DNA	internal method

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

Meth. Abr.	Evaluation number	Specifity	Remarks to the Method (Extraction and Determination)	Method accredidet ISO/IEC 17025	Further Remarks
		Target-Sequence / -DNA	e.g. Extraction / enzymes / clean-up / real time PCR / gel electrophoresis / cycles	yes/no	
ASU	12a	Glutenin system from w heat and rye	Maxw ell RSC Pure Food GMO and Authentication Kit	yes	1-plex
div	6a		CTAB / Proteinase K / Promega Wizard DNA CleanUp / PCR / Gel electrophoresis / 45 cycles	yes	
div	12b	intergenic region betw een 25S + 18S w heat ribosomal RNA Gene	Maxw ell RSC Pure Food GMO and Authentication Kit	yes	conv. PCR
div	2	As Per Kit Instructions	As Per Kit Instructions	No	Generon SPECIALfinder Rye PAV10A - Please note inhibition of spike sample meant that no level could be given
div	6b		CTAB / Proteinase K / Promega Wizard DNA CleanUp / PCR / Gel electrophoresis / 45 cycles	yes	

Reprint, also in part, only with written permission from DLA-Ahrensburg

5.1.4 PCR: Soya

Meth. Abr.	Evaluation number	Date of Analysis	Result Sample A		Result Sample B		Result Spiking Sample		NWG / LOD *	BG / LOQ *	MU*	quantitative Result given as	Method
		day/month	qualitative	mg/kg	qualitative	mg/kg	qualitative	mg/kg	mg/kg	mg/kg	mg/kg	e.g. food /protein	Test-Kit + Manufacturer
ASU	12a	1.3.	negative		positive		positive					Soyflour	ASU §64 Methode/method
ASU	12b	1.3.	negative		positive		positive					Soyflour	ASU §64 Methode/method
SFA	2b	02.03.18	negative	<1	positive	7,68	positive	2,76	1	1		Soybean	Sure Food Allergen ID, R-Biopharm / Congen
SFA-ID	2a	02.03.18	negative	<1	positive	10,19	positive	3,96	1	1		Soybean	Sure Food Allergen ID, R-Biopharm / Congen
SFA-ID	4	06.03.	negative	< 0,4	positive	> 0,4	positive	> 0,4	< 0,4			Soy-DNA	Sure Food Allergen ID, R-Biopharm / Congen
div	6	5.3.	negative		positive		positive		40			Soy-DNA	Eur F Res Tech 216 (2003) 412ff, mod.
div	10	16.03.18	-	< 10	-	131	-	60				Soyflour	in house

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

Meth. Abr.	Evaluation number	Specifity	Remarks to the Method (Extraction and Determination)	Method accredidet ISO/IEC 17025	Further Remarks
		Target-Sequence / -DNA	e.g. Extraction / enzymes / clean-up / real time PCR / gel electrophoresis / cycles	yes/no	
ASU	12a	Lectin gene 74 Bp	Maxwell RSC Pure Food GMO and Authentication Kit	yes	1-plex
ASU	12b	Lectin gene 81 Bp	Maxwell RSC Pure Food GMO and Authentication Kit	yes	4-plex
SFA	2b	As Per Kit Instructions	As Per Kit Instructions	No	New Kit Code S3601
SFA-ID	2a	As Per Kit Instructions	As Per Kit Instructions	No	Old Kit Code S3101
SFA-ID	4		Extraction: SureFood PREP Advanced, Protocol 2; Extraction and Measurement according to kit instructions	yes	
div	6		CTAB / Proteinase K / Promega Wizard DNA CleanUp / realtime PCR / 45 cycles	yes	
div	10			yes	

Reprint, also in part, only with written permission from DLA-Ahrensburg

5.2 Homogeneity

5.2.1 Mixture homogeneity before bottling

Microtracer Homogeneity Test
DLA 02-2018 Sample B

Weight whole sample	2,81	kg
Microtracer	FSS-rot lake	
Particle size	75 – 300	µm
Weight per particle	2,0	µg
Addition of tracer	15,3	mg/kg

Result of analysis

Sample	Weight [g]	Particle number	Particles [mg/kg]
1	4,98	44	17,7
2	5,10	48	18,8
3	5,01	44	17,6
4	5,05	49	19,4
5	5,06	37	14,6
6	4,99	45	18,0
7	5,08	55	21,7
8	5,08	39	15,4

Poisson distribution		
Number of samples	8	
Degree of freedom	7	
Mean	45,1	Particles
Standard deviation	5,61	Particles
χ^2 (CHI-Quadrat)	4,88	
Probability	**67**	%
Recovery rate	117	%

Normal distribution		
Number of samples	8	
Mean	17,9	mg/kg
Standard deviation	2,22	mg/kg
rel. Standard deviaton	12,4	%
Horwitz standard deviation	10,4	%
HorRat-value	**1,2**	
Recovery rate	117	%

Microtracer Homogenitätstest
DLA 02-2018 Spiking Level Sample

Weight whole sample	1,50	kg
Microtracer	FSS-rot lake	
Particle size	75 – 300	µm
Weight per particle	2,0	µg
Addition of tracer	16,5	mg/kg

Result of analysis

Sample	Weight [g]	Particle number	Particles [mg/kg]
1	5,09	49	19,3
2	5,00	49	19,6
3	4,97	41	16,5
4	5,01	46	18,4
5	5,03	43	17,1
6	4,97	51	20,5
7	5,03	40	15,9
8	4,97	47	18,9

Poisson distribution		
Number of samples	8	
Degree of freedom	7	
Mean	45,8	Particles
Standard deviation	4,05	Particles
χ^2 (CHI-Quadrat)	2,51	
Probability	**93**	%
Recovery rate	111	%

Normal distribution		
Number of samples	8	
Mean	18,3	mg/kg
Standard deviation	1,62	mg/kg
rel. Standard deviaton	8,86	%
Horwitz standard deviation	10,3	%
HorRat-value	**0,86**	
Recovery rate	111	%

Reprint, also in part, only with written permission from DLA-Ahrensburg

5.3 Information on the Proficiency Test (PT)

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

PT number	**DLA 02-2018**
PT name	***Allergens II: Soya and Rye in „gluten-free“ Pastry***
Sample matrix (processing)	***Samples A + B:*** *Cake Baking Mix, gluten free/ ingredients: Sugar, rice flour, cornstarch, raising agent: sodium bicarbonate, acidifier: monopotassium tartrate, vanilla, and other food additives and allergenic foods soyflour, toasted, and rye flour (one of both samples)* ***Spiking Level Sample:*** *potato powder, other food additives and allergenic foods*
Number of samples and sample amount	*2 different Samples A + B: 25 g each* *+ 1 Spiking Level Sample: 15 g*
Storage	*Samples A + B: room temperature (long term cooled 2 - 10°C)* *Spiking Level Sample: room temperature*
Intentional use	*Laboratory use only (quality control samples)*
Parameter	*qualitative + quantitative:* *Soya (Soya protein, DNA), "Gluten" / Rye (Rye protein, DNA)* *Samples A + B: < 500 mg/kg* *Spiking Level Sample: < 500 mg/kg*
Methods of analysis	*Analytical methods are optional*
Notes to analysis	*The analysis of PT samples should be performed like a routine laboratory analysis.* *In general we recommend to homogenize a representative sample amount before analysis according to good laboratory practice, especially in case of low sample weights. It is the best to homogenize the whole sample.*
Result sheet	*One result each should be determined for Samples A and B and the Spiking Level Sample.* *The results should be filled in the result submission file.*
Units	*mg/kg*
Number of digits	*at least 2*
Result submission	*The result submission file should be sent by e-mail to:* ***pt@dla-lvu.de***
Deadline	***the latest April 06th 2018***
Evaluation report	*The evaluation report is expected to be completed 6 weeks after deadline of result submission and sent as PDF file by e-mail.*
Coordinator and contact person of PT	*Matthias Besler-Scharf, PhD*

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

Reprint, also in part, only with written permission from DLA-Ahrensburg

6. Index of participant laboratories in alphabetical order

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

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

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

Reprint, also in part, only with written permission from DLA-Ahrensburg

7. Index of references

1. DIN EN ISO/IEC 17025:2005; Allgemeine Anforderungen an die Kompetenz von Prüf- und Kalibrierlaboratorien / General requirements for the competence of testing and calibration laboratories
2. DIN EN ISO/IEC 17043:2010; Konformitätsbewertung – Allgemeine Anforderungen an Eignungsprüfungen / Conformity assessment – General requirements for proficiency testing
3. ISO 13528:2015 & DIN ISO 13528:2009; Statistische Verfahren für Eignungsprüfungen durch Ringversuche / Statistical methods for use in proficiency testing by interlaboratory comparisons
4. ASU §64 LFGB: Planung und statistische Auswertung von Ringversuchen zur Methodenvalidierung / DIN ISO 5725 series part 1, 2 and 6 Accuracy (trueness and precision) of measurement methods and results
5. Verordnung / Regulation 882/2004/EU; Verordnung über über amtliche Kontrollen zur Überprüfung der Einhaltung des Lebensmittel- und Futtermittelrechts sowie der Bestimmungen über Tiergesundheit und Tierschutz / Regulation on official controls performed to ensure the verification of compliance with feed and food law, animal health and animal welfare rules
6. Evaluation of analytical methods used for regulation of food and drugs; W. Horwitz; Analytical Chemistry, 54, 67-76 (1982)
7. The International Harmonised Protocol for the Proficiency Testing of Ananlytical Laboratories ; J.AOAC Int., 76(4), 926 – 940 (1993)
8. A Horwitz-like funktion describes precision in proficiency test; M. Thompson, P.J. Lowthian; Analyst, 120, 271-272 (1995)
9. Protocol for the design, conduct and interpretation of method performance studies; W. Horwitz; Pure & Applied Chemistry, 67, 331-343 (1995)
10. Recent trends in inter-laboratory precision at ppb and sub-ppb concentrations in relation to fitness for purpose criteria in proficiency testing; M. Thompson; Analyst, 125, 385-386 (2000)
11. The International Harmonised Protocol for the Proficiency Testing of Analytical Chemistry Laboratories; Pure Appl Chem, 78, 145 – 196 (2006)
12. AMC Kernel Density - Representing data distributions with kernel density estimates, amc technical brief, Editor M Thompson, Analytical Methods Committee, AMCTB No 4, Revised March 2006 and Excel Add-in Kernel.xla 1.0e by Royal Society of Chemistry
13. EURACHEM/CITAC Leitfaden, Ermittlung der Messunsicherheit bei analytischen Messungen (2003); Quantifying Uncertainty in Analytical Measurement (1999)
14. GMP+ Feed Certification scheme, Module: Feed Safety Assurance, chapter 5.7 Checking procedure for the process accuracy of compound feed with micro tracers in GMP+ BA2 Control of residues, Version: 1st of January 2015 GMP+ International B.V.
15. MTSE SOP No. 010.01 (2014): Quantitative measurement of mixing uniformity and carry-over in powder mixtures with the rotary detector technique, MTSE Micro Tracers Services Europe GmbH
16. Homogeneity and stability of reference materials; Linsinger et al.; Accred Qual Assur, 6, 20-25 (2001)
17. AOAC Official Methods of Analysis: Guidelines for Standard Method Performance Requirements, Appendix F, p. 2, AOAC Int (2016)

18. Codex Alimentarius Commission (2010) - Guidelines on performance criteria and validation of methods for detection, identification and quantification of specific DNA sequences and specific protiens in foods, CAC/GL 74-2010
19. DIN EN ISO 15633-1:2009; Nachweis von Lebensmittelallergenen mit immunologischen Verfahren - Teil 1: Allgemeine Betrachtungen / Foodstuffs - Detection of food allergens by immunological methods - Part 1: General considerations
20. DIN EN ISO 15634-1:2009; Nachweis von Lebensmittelallergenen mit molekularbiologischen Verfahren - Teil 1: Allgemeine Betrachtungen / Foodstuffs - Detection of food allergens by molecular biological methods - Part 1: General considerations
21. DIN EN ISO 15842:2010 Lebensmittel – Nachweis von Lebensmittelallergenen –

Reprint, also in part, only with written permission from DLA-Ahrensburg

Allgemeine Betrachtungen und Validierung von Verfahren / Foodstuffs - Detection of food allergens - General considerations and validation of methods
22. Ministry of Health and Welfare, JSM, Japan 2006
23. Working Group Food Allergens, Abbott et al., Validation Procedures for Quantitative Food Allergen ELISA Methods: Community Guidance and Best Practices JAOAC Int. 93:442-50 (2010)
24. Working Group on Prolamin Analysis and Toxicity (WGPAT): Méndez et al. Report of a collaborative trial to investigate the performance of the R5 enzyme linked immunoassay to determine gliadin in gluten-free food. Eur J Gastroenterol Hepatol. 17:1053-63 (2005)
25. DLA Publikation: Performance of ELISA and PCR methods for the determination of allergens in food: an evaluation of six years of proficiency testing for soy (Glycine max L.) and wheat gluten (Triticum aestivum L.); Scharf et al.; J Agric Food Chem. 61(43):10261-72 (2013)
26. EFSA (2014) Scientific Opinion on the evaluation of allergenic foods and food ingredients for labelling purposes1, EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA), European Food Safety Authority (EFSA), Parma, Italy, EFSA Journal 2014;12(11):3894
27. IRMM, Poms et al.; Inter-laboratory validation study of five different commercial ELISA test kits for determination of peanut residues in cookie and dark chocolate; European Commission, Joint Research Centre, Belgium; GE/R/FSQ/D08/05/2004
28. Jayasena et al. (2015) Comparison of six commercial ELISA kits for their specificity and sensitivity in detecting different major peanut allergens. J Agric Food Chem. 2015 Feb 18;63(6):1849-55
29. ASU §64 LFGB L 06.00-56 Bestimmung von Sojaprotein in Fleisch und Fleischerzeugnissen Enzymimmunologisches Verfahren (2007)
30. ASU §64 LFGB L 00.00-69 Bestimmung von Erdnuss-Kontaminationen in Lebensmitteln mittels ELISA im Mikrotiterplattensystem (2003)
31. ASU §64 LFGB L 44.00-7 Bestimmung von Haselnuss-Kontaminationen in Schokolade und Schokoladenwaren mittels ELISA im Mikrotiterplattensystem (2006)
32. ASU §64 LFGB L 16.01-9 Untersuchung von Lebenmitteln - Bestimmung von Soja (Glycine max) in Getreidemehl mittels real-time PCR (2016)
33. ASU §64 LFGB L 08.00-59 Untersuchung von Lebenmitteln - Nachweis und Bestimmung von Senf (Sinapis alba) sowie Soja (Glycine max) in Brühwürsten mittels real-time PCR (2013)
34. ASU §64 LFGB L 08.00-65 Untersuchung von Lebenmitteln - Simultaner Nachweis und Bestimmung von schwarzem Senf (Brassica nigra L.), braunem Senf (Brassica juncea L.), weißem Senf (Sinapis alba). Sellerie (Apium graveolens) und Soja (Glycine max) in Brühwurst mittels real-time PCR (2016)
35. ASU §64 LFGB L 08.00-66 Untersuchung von Lebenmitteln - Nachweis und Bestimmung von Weizen (Triticum L.) und Roggen (Secale cereale) in Brühwurst mittels real-time PCR (2016)
36. Köhler & Andersen (2014) Analyse von Glutengehalten in Getreide und getreidehaltigen Produkten, Tabellenwerk zum Nährstoffgehalt von Lebensmitteln 3.1.5.1, Deutsche Forschungsanstalt für Lebensmittelchemie Leibniz Institut Jahresbericht 2014 [Analysis of gluten contents in cereals and cereal products, nutrient tables of foods]

Reprint, also in part, only with written permission from DLA-Ahrensburg