Proficiency Tests
DLA
food
cosmetics
consumer goods
www.dla-lvu.de

Evaluation Report
proficiency test

DLA 03/2017

Allergens III:

β-Lactoglobulin, Casein and Gluten

in Infant Food

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

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

Coordinator of this PT:
Dr. Matthias Besler

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 Waldemar-Bonsels-Weg 170, 22926 Ahrensburg, Germany Tel. ++49(0)171-1954375 Fax. ++49(0)4102-9944976 eMail. proficiency-testing@dla-lvu.de
EP-Nummer *PT-Number*	DLA 03/2017
EP-Koordinator *PT-Coordinator*	Dr. Matthias Besler
Status des EP-Bericht *Status of PT-Report*	Abschlussbericht / Final report (23 June 2017) 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 (Technischer Leiter / Technical Manager) - *gezeichnet / signed M. Besler* Dr. Gerhard Wichmann (QM-Beauftragter / Quality Manager) - *gezeichnet / signed G. Wichmann* Datum / Date: 23 June 2017
Unteraufträge *Subcontractors*	Die Prüfung der Gehalte, Homogenität und Stabilität von EP-Parametern wird von DLA im Unterauftrag vergeben. The analysis of the content, homogeneity and stability of PT-parameters are 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.

Inhalt / Content

1. Introduction

The participation in proficiency testing schemes is an essential element of the quality-management-system of every laboratory testing food and feed, cosmetics and food contact materials. The implementation of proficiency tests enables the participating laboratories to prove their own analytical competence under realistic conditions. At the same time they receive valuable data regarding the 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 was a common in commerce infant food product "millet pap with rice" for children from up to 4 month (labeled as milk- and gluten-free). The basic composition of both sample A and sample B was the same (see table 1).
After crushing and sieving using an impact mill (mesh 1,5 mm) the basic mixture was homogenized. Afterwards the **spiked sample A** was produced as follows:
The spiking material containing the allergenic ingredients skimmed milk powder and wheat flour were sieved (mesh 600 µm) or sieved with a centrifugal mill (mesh 500 µm) and then added to an aliquot of the basic mixture and homogenized. Subsequently, basic mixture was again added in 4 additional steps and mechanically homogenized in each case until the total quantity had been reached.
For the **spiking level sample**, the allergenic compounds skimmed milk powder and wheat flour were added during a multi-stage addition of potato flour and homogenization. Afterwards the whole sample was sieved by means of a centrifugal mill (mesh 500 µm).

The samples A and B were portioned to approximately 25 g, the spiking level sample to approximately to 10 g in metallized PET film bags.

The composition of the PT samples and the spiking level sample is given in table 1.

Table 1: Composition of DLA-Samples

Ingredients	**Sample A**	**Sample B**	**Spiking Level Sample**
Organic-Millet-Pap with rice, infant pap after 4th month Ingredients: Millet whole flour (75%), brown rice flour (25%), vitamin B1 Nutrients per 100g: Protein 12 g, carbohydrates 77 g, fat 3,7 g	99,8 g/100 g	100 g/100g	-
Potato powder Ingredients: Potatoes, E471, E304, E223, E100	-	-	99,8 g/100 g
Milk: - as skimmed milk powder* - thereof 37% total protein** - thereof casein*** - thereof β-lactoglobulin***	 158 mg/kg 58,1 mg/kg 46,5 mg/kg 5,8 mg/kg	-	 155 mg/kg 57,0 mg/kg 45,6 mg/kg 5,7 mg/kg
Wheat: - as wheat flour* - thereof 10% total protein** - thereof gluten***	 648 mg/kg 64,8 mg/kg 56,4 mg/kg	-	 549 mg/kg 54,9 mg/kg 47,8 mg/kg
further Ingredients: Maltodextrin, sodium sulfate and silicon dioxide	<0,02 g/100 g	-	<0,02 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 materials (total nitrogen according to Kjeldahl with F=6,25 for milk protein and F=5,7 for wheat protein)
*** Protein contents according to literature values (approx. 80% casein and 10% β-latcoglobulin in total milk protein [31]; approx. 8,7% gluten in wheat flour [32, 33, 34])

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

2.1.1 Homogeneity

The **mixture homogeneity before bottling** was examined 8-fold by **microtracer analysis**. It is a standardized method that is part of the international GMP certification system for feed [14].
Before mixing dye coated iron particles of µm size are added to the sample and the number of particles is determined after homogenization in taken aliquots. The evaluation of the mixture homogeneity is based on the Poisson distribution using the chi-square test. A probability of ≥ 5 % is equivalent to a good homogeneous mixture and of ≥ 25% to an excellent mixture [14, 15].
The microtracer analysis of the present PT sample A and the spiking level sample showed a probability of 72% and 75%. Additionally particle number results were converted into concentrations, statistically evaluated according to normal distribution and compared to the standard deviation according to Horwitz. This gave a HorRat value of 0,8 and 0,7 respectively. The results of microtracer analysis are given in the documentation.

Homogeneity of bottled spiked sample A

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 13528:2009 Annex B.

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 A by the ELISA tests for β-lactoglobulin (Immunolab) and milk (Veratox as non fat dry milk) as well as for gluten (Immunolab and AgraQuant) (see pages 7-9). One additional ELISA test for gluten gave a Ss of 15,6% close to the above mentioned criterion (Veratox). And one ELISA test for casein gave a Ss below 25% (AgraQuant). Recommendations for repeatability standard deviations of ELISA and PCR methods are usually ≤ 25% [16, 17, 20, 21].

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

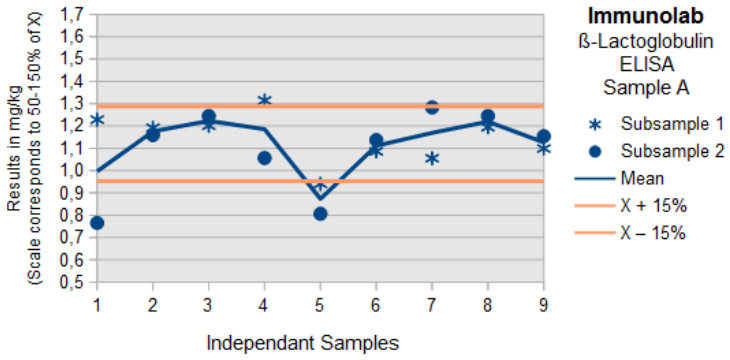
ELISA-Tests: Homogenität / Homogeneity beta-Lactoglobulin

Immunolab β-Lactoglobulin ELISA

Sample weights: 1,0 g (0,9 – 1,1 g)
Number of replicates: 2
Overall result: β-Lactoglobulin 1,12 ± 0,12 mg/kg

Sample A	Subsample 1 mg/kg	Subsample 2 mg/kg	Mean mg/kg
1	1,23	0,77	1,00
2	1,19	1,16	1,18
3	1,20	1,24	1,22
4	1,31	1,06	1,19
5	0,94	0,81	0,87
6	1,09	1,14	1,11
7	1,06	1,28	1,17
8	1,19	1,24	1,22
9	1,10	1,15	1,13

General average X	1,12	
SD of sample means Sx	0,12	10,3%
SD within-samples Sw	0,14	12,7%
SD between-samples Ss	0,12	10,4%



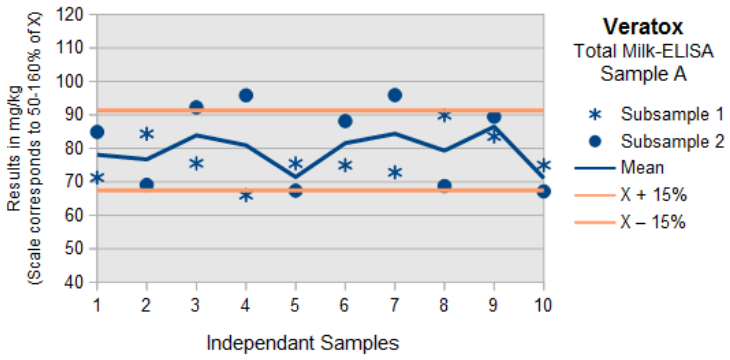
ELISA-Tests: Homogenität Milch / Homogeneity Milk (Protein / Casein)

Neogen Veratox ELISA Total Milk

Sample weights: 5,0 g (4,5 – 5,5 g)
Number of replicates: 2
Overall result: as Non Fat Dry Milk 79,4 ± 11,5 mg/kg

Sample A	Subsample 1 mg/kg	Subsample 2 mg/kg	Mean mg/kg
1	71,23	84,87	78,05
2	84,31	69,06	76,68
3	75,50	92,21	83,86
4	65,99	95,82	80,90
5	75,45	67,33	71,39
6	74,94	88,12	81,53
7	72,80	95,89	84,34
8	89,87	68,71	79,29
9	83,50	89,41	86,46
10	74,97	67,11	71,04

General average X	79,4	
SD of sample means Sx	5,21	6,6%
SD within-samples Sw	12,1	15,2%
SD between-samples Ss	11,5	14,5%

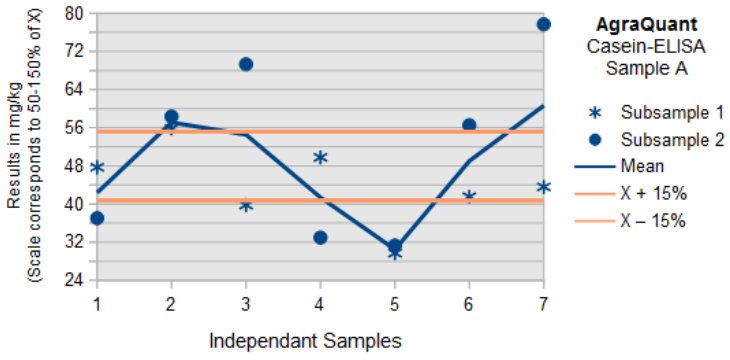


Romerlabs AgraQuant Casein

Sample weights: 1,0 g (0,9 – 1,1 g)
Number of replicates: 2
Overall result: Casein 47,9 ± 11,6 mg/kg

Sample A	Subsample 1 mg/kg	Subsample 2 mg/kg	Mean mg/kg
1	47,7	37,0	42,4
2	55,9	58,4	57,1
3	39,8	69,3	54,5
4	49,8	33,0	41,4
5	29,7	31,3	30,5
6	41,5	56,5	49,0
7	43,6	77,7	60,6

General average X	47,9	
SD of sample means Sx	10,6	22,0%
SD within-samples Sw	13,8	28,8%
SD between-samples Ss	11,6	24,2%



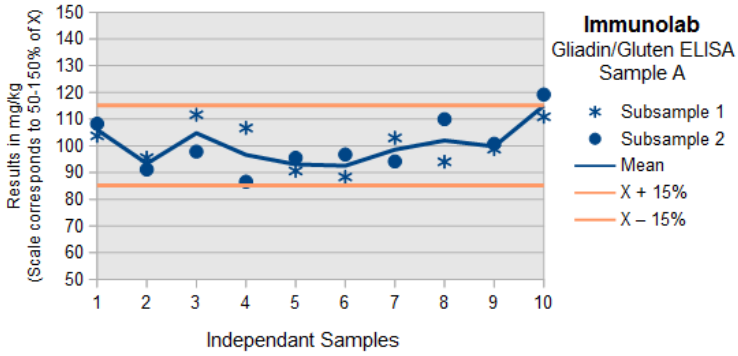
ELISA-Tests: Homogenität Gluten / Homogeneity Gluten

Immunolab Gliadin/Gluten ELISA

Sample weights: 1,0 g (0,9 – 1,1 g)
Number of replicates: 2
Overall result: Gluten 100 ± 5,6 mg/kg

Sample A	Subsample 1 mg/kg	Subsample 2 mg/kg	Mean mg/kg
1	103,7	108,3	106,0
2	95,57	91,14	93,35
3	111,6	97,84	104,7
4	106,7	86,54	96,61
5	90,59	95,51	93,05
6	88,34	96,74	92,54
7	103,0	94,15	98,56
8	94,02	110,0	102,0
9	98,76	100,8	99,76
10	110,9	119,2	115,0

General average X	100,2	
SD of sample means Sx	7,07	7,1%
SD w ithin-samples Sw	7,54	7,5%
SD betw een-samples Ss	5,64	5,6%

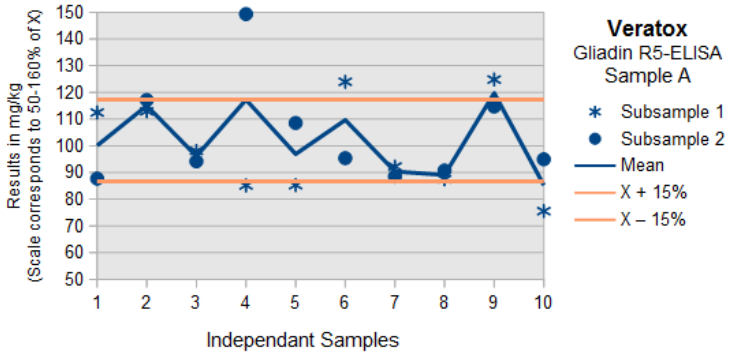


Neogen Veratox ELISA Gliadin R5

Sample weights: 1,0 g (0,9 – 1,1 g)
Number of replicates: 2
Overall result: Gluten 102 ± 16 mg/kg

Sample A	Subsample 1 mg/kg	Subsample 2 mg/kg	Mean mg/kg
1	112,5	87,72	100,1
2	112,9	117,1	115,0
3	98,10	94,19	96,14
4	85,17	149,3	117,2
5	85,26	108,5	96,90
6	124,0	95,38	109,7
7	92,23	88,51	90,37
8	87,45	90,75	89,10
9	124,8	114,7	119,8
10	75,53	94,95	85,24

General average X	102	
SD of sample means Sx	12,6	12,3%
SD w ithin-samples Sw	18,2	17,9%
SD betw een-samples Ss	15,9	15,6%

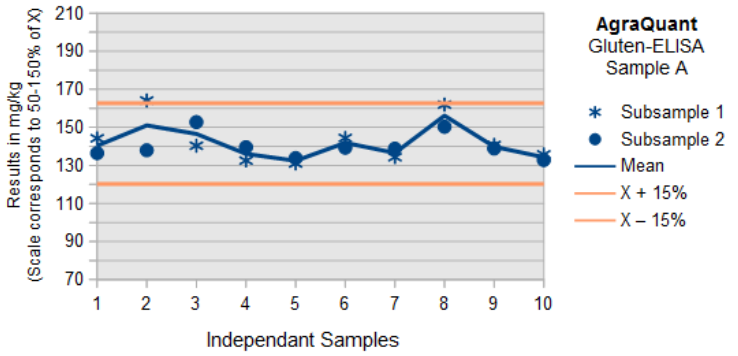


Romerlabs AgraQuant Gluten

Sample weights: 1,0 g (0,9 – 1,1 g)
Number of replicates: 2
Overall result: Gluten 142 ± 5,3 mg/kg

Sample A	Subsample 1 mg/kg	Subsample 2 mg/kg	Mean mg/kg
1	144,2	136,5	140,4
2	164,1	137,9	151,0
3	140,3	152,7	146,5
4	132,5	139,6	136,1
5	131,0	133,8	132,4
6	144,4	139,3	141,9
7	134,3	138,8	136,6
8	161,9	150,3	156,1
9	140,8	138,9	139,8
10	135,8	132,8	134,3

General average X	141,5	
SD of sample means Sx	7,62	5,4%
SD w ithin-samples Sw	7,59	5,4%
SD betw een-samples Ss	5,34	3,8%



2.1.2 Stability

The experience with various DLA reference materials showed good storage stability with respect to the durability of the sample (spoilage) and the content of the PT parameters milk proteins and gluten for comparable food matrices and water activity (a_W value <0,5). The stability of the sample material is therefore given during the investigation period under consideration of given storage conditions.

2.2 Sample shipment and information to the test

The portions of test material (sample A and sample B as well as the spiking level sample) were sent to every participating laboratory in the 9th week of 2017. The testing method was optional. The tests should be finished at April 13th 2017 the latest.

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 β-Lactoglobulin, Casein and/or Gluten in the range of mg/kg in the matrix of Infant Food ("gluten-free" cereal pap). 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 or were available on our website. On one hand the results given as positive/negative and on the other hand the indicated results of the allergenic ingredients e.g. total food item or protein in mg/kg were evaluated.
During evaluation DLA eventually requests detailed information by email on the type of indicated quantitative results from participants concerned.
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 18 participants submitted their results in time.

3. Evaluation

Different ELISA-methods for the determination of allergens in foods are eventually using different antibodies, are usually calibrated with different reference materials and may utilize differing extraction methods. Among others this can induce different results of the content of the analyte [23, 24, 25, 26]. 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].
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) ***Robust mean of all results*** - $X_{pt_{ALL}}$
ii) ***Robust mean of single methods*** - $X_{pt_{METHOD\ i}}$
with at least 5 quantitative results given.

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

3.2 Robust standard deviation

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

The following robust standard deviations were considered:

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

3.3 Exclusion of results and outliers

Before statistical evaluation obvious blunders, such as those with incorrect units, decimal point errors, and results for a another proficiency test item can be removed from the data set [2]. 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 identified as outliers by the use of robust statistics. If a value deviates from the robust mean by more than 3 times the robust standard deviation, it is classified as an outlier [3]. Detected outliers are stated for information only, when z-score are < -2 or > 2. Due to the use of robust statistics outliers are not excluded, provided that no other reasons are present [3].

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 3a (ELISA) and table 3b (PCR) were obtained in precision experiments by the indicated methods. The resulting target standard deviations σ_{pt} were calculated for a number of m = 2 replicate measurements. With a number of m = 1 replicate measurements the reproducibility standard deviation σ_R is identical to the target standard deviation σ_{pt}.

Table 2a: ELISA-Methods - Relative repeatability standard deviations (RSD_r) and relative reproducibility standard deviations (RSD_R) from precision experiments and resulting target standard deviations σ_{pt} [28-29]

Parameter	**Matrix**	**Mean [mg/kg]**	**Recovery**	**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 – 33% for the ELISA methods and 15 – 43% 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 [22]. 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 [22].

The IRMM (Institute for Reference Materials and Measurements) performed an interlaboratory comparison for five different ELISA test kits for the quantification of peanut [25]. The mean values for two matrices were in the concentration range of 0,3 - 16,1 mg/kg and 1,2 - 20,4 mg/kg, respectively. The lowest relative reproducibility standard deviations of the five test kits were for dark chocolate in the range of 20 - 42% and for cookies in the range of 23 – 61%.

Table 2b: PCR-Methods - Relative repeatability standard deviations (RSD_r) and relative reproducibility standard deviations (RSD_R) from precision experiments and resulting target standard deviations σ_{pt} [30-32]

Parameter	**Matrix**	**Mean [mg/kg]**	**Recov-ery**	**rob RSD**	**RSD_r**	**RSD_R**	**σ_{pt}**	**Method / Literature**
Soya flour	Boiled sausage (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 sausage (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 sausage (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

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 [20], by the working group 12 „Food Allergens" of the technical committee CEN/TC 275 [17-19], by an international "Food Allergen Working Group" under the advice of the AOAC Presidential Task Force on Food Allergens [21] and by the Codex Alimentarius Committee (CAC/GL 74-2010) [16].

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

Table 4: ELISA-Validation

Literature [16-22]	**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 5: PCR-Validation

Literature [16]	**Recovery rate**	**Repeatability standard deviation**	**Reproducibility standard deviation**
CAC 2010	± 25% [(a)]	≤ 25%	≤ 35%

(a) = Trueness / Richtigkeit

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

3.5 z-Score

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

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

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

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

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

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

3.5.1 Warning and action signals

In accordance with the norm ISO 13528 it is recommended that a result that gives rise to a *z*-score above 3,0 or below −3,0, shall be considered to give an "action signal" [3]. Likewise, a *z*-score above 2,0 or below −2,0 shall be considered to give a "warning signal". A single "action signal", or "warning signal" in two successive PT-rounds, shall be taken as evidence that an anomaly has occurred which requires investigation. For example a fault isolation or a root cause analysis through the examination of transmission error or an error in the calculation, in the trueness and precision must be performed and if necessary appropriate corrective measures should be applied [3].

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

3.6 z'-Score

The z'-score can be used for the valuation of the results of the participants, in cases the standard uncertainty has to be considered (s. 3.8). The z'-score represents the relation of the deviation of the result (x) of the participant from the respective consensus value (X) to the square root of quadrat sum of the target standard deviation ($\hat{\sigma}$) 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*/σpt

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 of the assigned value

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 ($U_{(x_{pt})}$) for this PT is calculated as follows [3]:

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

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

too low with respect to the standard uncertainty of the assigned value. The Quotient $U(x_{pt})/\sigma_{pt}$ is reported in the characteristics of the test.

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 [21]. For quantitative PCR determinations we use the same range of acceptance.

4. Results

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

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

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

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

ELISA-results, given as skimmed milk powder, were converted to casein considering literature data (approx. 80% casein in 37% total milk protein, see p. 5) (ELISA-Systems).

For β-lactoglobulin and gluten all present results were submitted as β-lactoglobulin or gluten, thus no recalculation was necessary.

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

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

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

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

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

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

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

4.1 Proficiency Test β-Lactoglobulin

4.1.1 ELISA Results: β-Lactoglobulin

Qualitative valuation of results: Samples A and B

Evaluation number	Sample A	Sample A	Sample B	Sample B	Qualitative Valuation	Method	Remarks
	pos/neg	[mg/kg]	pos/neg	[mg/kg]	Agreement with consensus value		
3	positive	7,80	negative	< 2	2/2 (100%)	BK	
17	positive	13,0	negative	<5	2/2 (100%)	BK	
2	positive	>1	negative	<0,1	2/2 (100%)	ES	
18	positive	6,70	negative	< 1	2/2 (100%)	ES	
1	positive	2,30	negative	<0,5	2/2 (100%)	IG	
14	positive	1,60	negative	< 0,01	2/2 (100%)	IL	
8	positive	3,40	negative	<0,031	2/2 (100%)	MI	
4	positive	0,608	negative	negative	2/2 (100%)	RS	
11	positive	3,01	negative	<1,00	2/2 (100%)	RS	
13	negative	<5	negative	<5	1/2 (50%)	RS	
16	positive	1,40	negative		2/2 (100%)	RS	
7	positive	2,10	negative	< 0,5	2/2 (100%)	RS-F	
12	positive	2,37	negative	<0,5	2/2 (100%)	RS-F	
15	positive	2,37	negative	<0.5	2/2 (100%)	RS-F	

	Probe A		Probe B	
Number positive	13		0	
Number negative	1		14	
Percent positive	93		0	
Percent negative	7		100	
Consensus value	positive		negative	

Methoden:

BK = BioKits, Neogen
ES = ELISA-Systems
IG = Ingezim ELISA, Ingenasa
IL = Immunolab
MI = Morinaga Institute ELISA
RS = Ridascreen®, R-Biopharm
RS-F= Ridascreen® Fast, R-Biopharm

Comments:
The consensus values are in agreement with the spiking of sample A. One negative result for sample A was obtained with method RS (Ridascreen).

Quantitative valuation of ELISA-results: Sample A

Evaluation number	β-Lacto-globulin	z-Score Xpt_{ALL}	Method	Remarks
	[mg/kg]			
3	7,80	9,4	BK	Result excluded
17	13,0	18,0	BK	Result excluded
2	>1		ES	
18	6,70	7,6	ES	Outlier X_{all}
1	2,30	0,0	IG	
14	1,60	-1,2	IL	
8	3,40	1,9	MI	
4	0,608	-3,0	RS	
11	3,01	1,2	RS	
13	<5		RS	
16	1,40	-1,6	RS	
7	2,10	-0,4	RS-F	
12	2,37	0,1	RS-F	
15	2,37	0,1	RS-F	

Methods:

BK = BioKits, Neogen
ES = ELISA-Systems
IG = Ingezim ELISA, Ingenasa
IL = Immunolab
MI = Morinaga Institute ELISA
RS = Ridascreen®, R-Biopharm
RS-F= Ridascreen® Fast, R-Biopharm

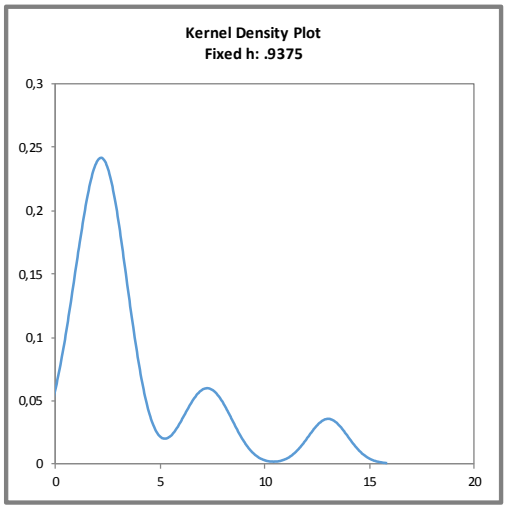


Abb. / Fig. 1:
Kerndichte-Schätzung aller ELISA-Ergebnisse (mit h = 0,75 x σpt von Xpt_{ALL})

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

Comments:
The kernel density estimation shows nearly a normal distribution with two side-peaks at approx. 7 mg/kg (method BK and ES) and 13 mg/kg (method BK).

Characteristics: Quantitative evaluation ELISA: β-Lactoglobulin

Sample A

Statistic Data	**All Results** *[mg/kg]*
Assigned value (X_{pt})	$\boldsymbol{X_{pt_{ALL}}}$
Number of results	10
Number of outliers	1
Mean	2,59
Median	2,34
Robust Mean (X)	**2,32**
Robust standard deviation (S*)	**1,11**
Target range:	
Target standard deviation σ_{pt}	**0,580**
lower limit of target range	**1,16**
upper limit of target range	**3,48**
Quotient S/σ_{pt}*	*1,9*
Standard uncertainty $U_{(Xpt)}$	*0,437*
Quotient $U_{(Xpt)}/\sigma_{pt}$	*0,75*
Results in the target range	*8*
Percent in the target range	*80*

Comments to the statistical characteristics and assigned values:

The kernel density plot showed two divergent high results from method BK, which were excluded prior to statistic evaluation. The also increased result from method ES was not excluded, as it was a single result only. All three results were evaluated by z-Score.

The evaluation of results of all methods showed a normal variability of results, after exclusion of the two above mentioned results. The quotient S/σ_{pt} was 1,9. The robust standard deviation is in the range of established values for the repeatability and 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 the methods.*

The robust mean of the evaluation was 40% of the spiking level of β-lactoglobulin to sample A and thus below the recommendations for the applied methods (s. 3.4.3 and "recovery rates for β-Lactoglobulin").

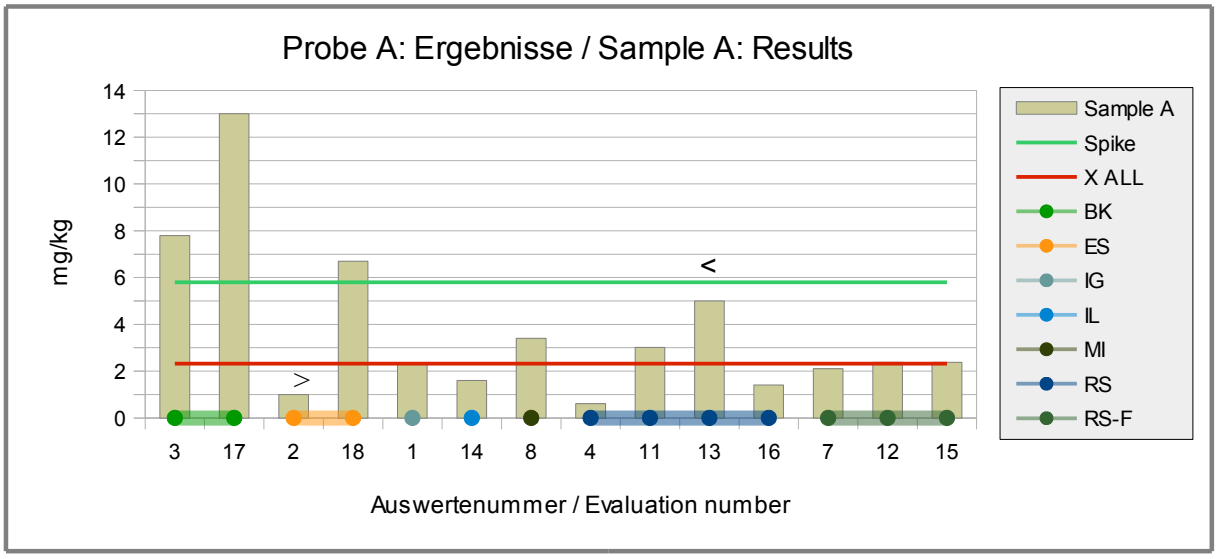


Abb./Fig. 2: ELISA Results β-Lactoglobulin
green line = Spiking level
red line = Assigned value robust mean all results
round symbols = Applied methods (see legend)

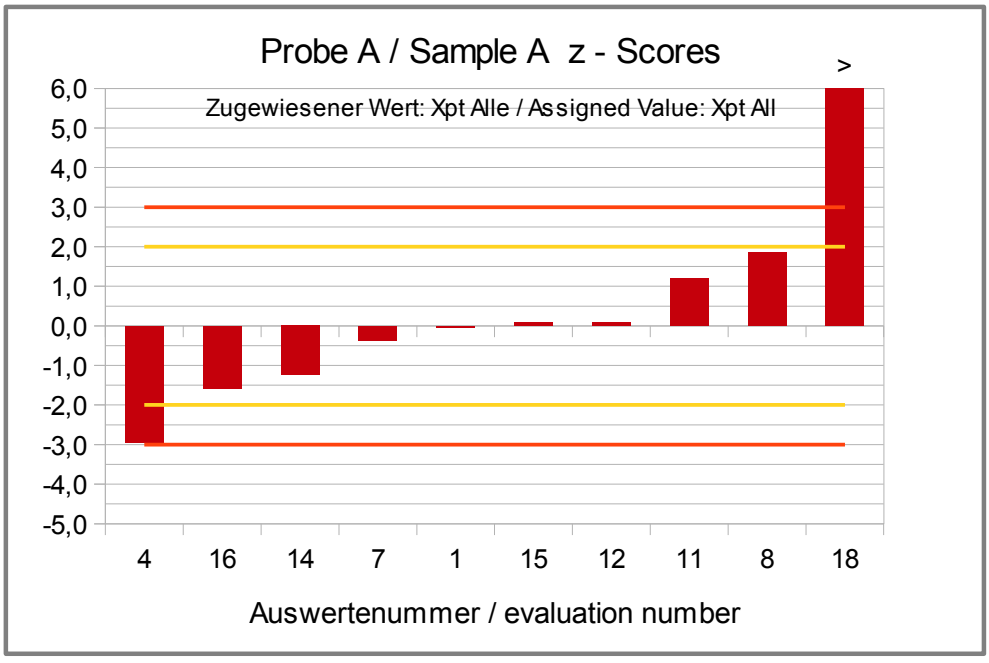


Abb./Fig. 3:
z-Scores (ELISA Results β-Lactoglobulin) Assigned value robust mean of all results

Quantitative valuation of results: Spiking level sample

Evaluation number	β-Lactoglo-bulin	z-Score Xpt_{ALL}	Method	Remarks
	[mg/kg]			
3	9,50	11,9	BK	Result excluded
17	15,0	21,1	BK	Result excluded
2	>1		ES	
18	5,80	5,7	ES	Outlier X_{all}
1			IG	
14	1,70	-1,2	IL	
8	3,40	1,7	MI	
4	1,75	-1,1	RS	
11	2,84	0,8	RS	
13	<5		RS	
16	1,40	-1,7	RS	
7	1,50	-1,5	RS-F	
12	2,23	-0,3	RS-F	
15	2,78	0,7	RS-F	

Methods:

BK = BioKits, Neogen
ES = ELISA-Systems
IG = Ingezim ELISA, Ingenasa
IL = Immunolab
MI = Morinaga Institute ELISA
RS = Ridascreen®, R-Biopharm
RS-F= Ridascreen® Fast, R-Biopharm

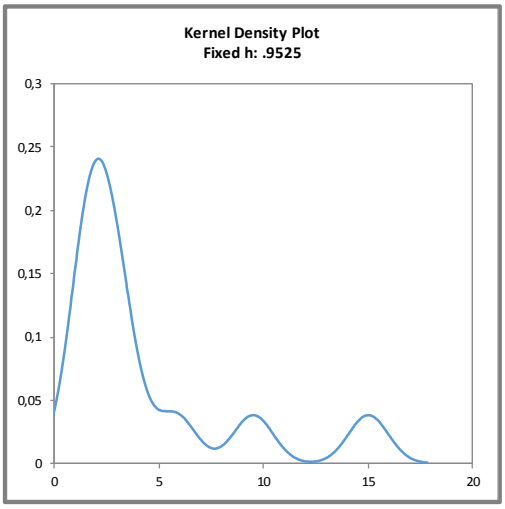


Abb. / Fig. 4:
Kerndichte-Schätzung aller ELISA-Ergebnisse (mit h = 0,75 x σpt von Xpt_{ALL})

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

Comments:
The kernel density estimation shows nearly a normal distribution of results with a shoulder (method ES) and two side peaks at approx. 7 mg/kg, and 13 mg/kg (method BK).

Characteristics: Quantitative evaluation β-Lactoglobulin

Spiking level sample

Statistic Data	**All Results** *[mg/kg]*
Assigned value (X_{pt})	$X_{pt_{ALL}}$
Number of results	9
Number of outliers	1
Mean	2,60
Median	2,23
Robust Mean (X)	**2,39**
Robust standard deviation (S*)	**1,01**
Target range:	
Target standard deviation σ_{pt}	**0,597**
lower limit of target range	**1,19**
upper limit of target range	**3,58**
Quotient S/σ_{pt}*	*1,7*
Standard uncertainty $U_{(Xpt)}$	*0,421*
Quotient $U_{(Xpt)}/\sigma_{pt}$	*0,70*
Results in the target range	*8*
Percent in the target range	*89*

Comments to the statistical characteristics and assigned values:

The kernel density plot showed two divergent high results from method BK, which were excluded prior to statistic evaluation. The also increased result from method ES was not excluded, as it was a single result only. All three results were evaluated via z-Score.

The evaluation of results of all methods showed an normal variability of results, after exclusion of the two above mentioned results. The quotient S/σ_{pt} was 1,7. The robust standard deviation is in the range of established values for the repeatability and 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 the methods.*

The robust mean of the evaluation was 42% of the spiking level of β-lactoglobulin to the spiking level sample and thus below the recommendations for the applied methods (s. 3.4.3 and "recovery rates for β-Lactoglobulin").

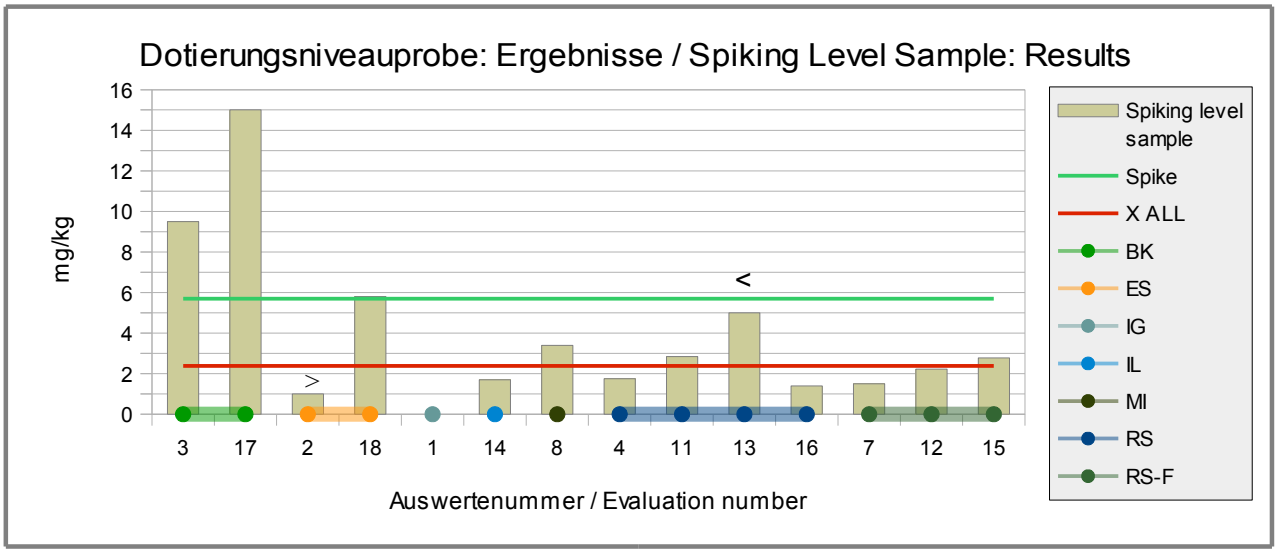


Abb./Fig. 5: ELISA Results β-Lactoglobulin
green line = Spiking level (Spike)
red line = Assigned value robust mean all results
round symbols = Applied methods (see legend)

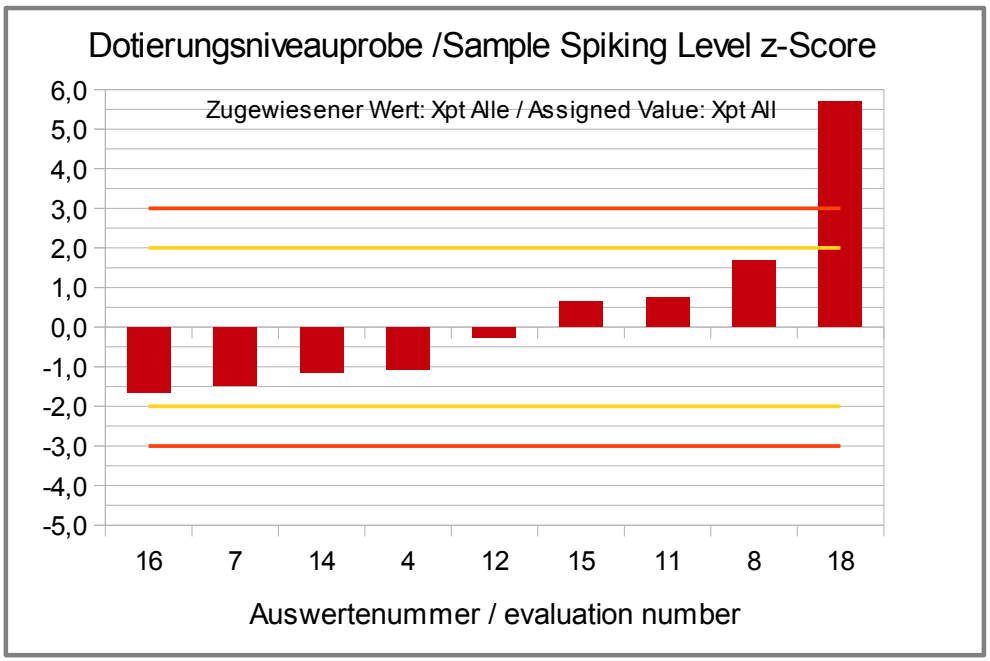


Abb./Fig. 6:
z-Scores (ELISA Results β-Lactoglobulin) Assigned value robust mean of all results

Recovery Rates ELISA for β-Lactoglobulin:
Spiking level Sample and Sample A

Evaluation number	Spiking Level Sample	Recovery rate*	Sample A	Recovery rate*	Method	Remarks
	[mg/kg]	[%]	[mg/kg]	[%]		
3	9,50	167	7,8	**134**	BK	
17	15,0	263	13	224	BK	
2	>1		>1		ES	
18	5,80	**102**	6,7	**116**	ES	
1			2,3	40	IG	
14	1,70	30	1,6	28	IL	
8	3,40	**60**	3,4	**59**	MI	
4	1,75	31	0,61	10	RS	
11	2,84	**50**	3,01	**52**	RS	
13	<5		<5		RS	
16	1,40	25	1,4	24	RS	
7	1,50	26	2,1	36	RS-F	
12	2,23	39	2,37	41	RS-F	
15	2,78	49	2,37	41	RS-F	

RA**	50-150 %	RA**	50-150 %
Number in RA	**3**	Number in RA	**4**
Percent in RA	**27**	Percent in RA	**33**

* Recovery rate 100% relative size: β-Lactoglobulin, s. page 5

** Range of acceptance of AOAC for allergen ELISAS

Methods:
BK = BioKits, Neogen
ES = ELISA-Systems
IG = Ingezim ELISA, Ingenasa
IL = Immunolab
MI = Morinaga Institute ELISA
RS = Ridascreen®, R-Biopharm
RS-F= Ridascreen® Fast, R-Biopharm

Comments:
For the spiking level sample 27% (3) of the participants obtained a recovery rate by ELISA methods within the range of the AOAC-recommendation of 50-150%. For the spiked food matrix sample A 33% (4) of the participants obtained a recovery rate within the range of acceptance.

4.2 Proficiency test: Casein / Milk protein

4.2.1 ELISA Results: Casein

Qualitative valuation of results: Samples A and B

Evaluation number	Sample A	Sample A	Sample B	Sample B	Qualitative Valuation	Method	Remarks
	pos/neg	[mg/kg]	pos/neg	[mg/kg]	Agreement with consensus value		
3	positive	102	negative		2/2 (100%)	AQ	
10	positive	45,4	negative	<1	2/2 (100%)	AQ	
17	positive	31,5	negative	<1,5	2/2 (100%)	AQ	
2	positive	>3	negative	<0,3	2/2 (100%)	ES	Result converted °
1	positive	32,0	negative	<1	2/2 (100%)	IG	
9a	positive	136	negative	< 0,2	2/2 (100%)	IL	
14	positive	36,0	negative	< 0,2	2/2 (100%)	IL	
8	positive	46,0	negative	<0,25	2/2 (100%)	MI	
4	positive	10,0	negative	negative	2/2 (100%)	RS-F	
7	positive	12,9	negative	<0,5	2/2 (100%)	RS-F	
9b	positive	42,9	negative	<0,5	2/2 (100%)	RS-F	
12	positive	30,9	negative	<2,5	2/2 (100%)	RS-F	
15	positive	49,7	negative	<2,5	2/2 (100%)	RS-F	
16a	positive	44,0	negative		2/2 (100%)	RS-F	
18	positive	38,0	negative		2/2 (100%)	RS-F	
16b	positive	46,0	negative		2/2 (100%)	VT	
5	positive	31,3	negative	<2,0	2/2 (100%)	div.	

° Conversion p. 19

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

Methods:

AQ = AgraQuant, RomerLabs
ES = ELISA-Systems
IG = Ingezim ELISA, Ingenasa
IL = Immunolab
MI = Morinaga Institute ELISA
RS-F= Ridascreen® Fast, R-Biopharm
VT = Veratox, Neogen
div = not indicated / other method

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

Quantitative valuation of results: Sample A

Evaluation number	Casein	z-Score Xpt_{ALL}	z-Score Xpt_{RS-F}	Method	Remarks
	[mg/kg]				
3	102	6,3		AQ	Outlier X_{all}
10	45,4	0,6		AQ	
17	31,5	-0,8		AQ	
2	>3			ES	Result converted °
1	32,0	-0,8		IG	
9a	136	9,8		IL	Outlier X_{all}
14	36,0	-0,4		IL	
8	46,0	0,7		MI	
4	10,0	-3,0	-1,9	RS-F	
7	12,9	-2,7	-1,7	RS-F	
9b	42,9	0,4	0,9	RS-F	
12	30,9	-0,9	-0,1	RS-F	
15	49,7	1,0	1,5	RS-F	
16a	44,0	0,5	1,0	RS-F	
18	38,0	-0,1	0,5	RS-F	
16b	46,0	0,7		VT	
5	31,3	-0,8		div.	

° Conversion p. 19

Methods:

AQ = AgraQuant, RomerLabs
ES = ELISA-Systems
IG = Ingezim ELISA, Ingenasa
IL = Immunolab
MI = Morinaga Institute ELISA
RS-F= Ridascreen® Fast, R-Biopharm
VT = Veratox, Neogen
div = not indicated / other method

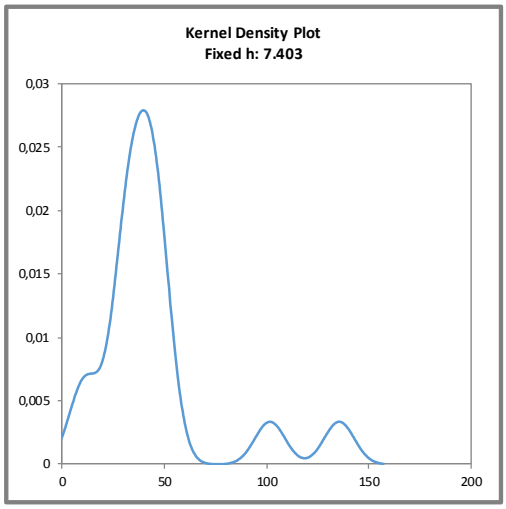


Abb. / Fig. 7:
Kerndichte-Schätzung aller ELISA-Ergebnisse (mit h = 0,75 x σpt von Xpt_{ALL})

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

Comments:
The kernel density estimation shows nearly a normal distribution of results with a shoulder at <15 mg/kg (method RS-F) and two side peaks at > 100 mg/kg (method AQ and IL).

Characteristics: Quantitative evaluation ELISA Casein

Sample A

Statistic Data	**All Results** *[mg/kg]*	**Method RS-F** *[mg/kg]*
Assigned value (X_{pt})	$X_{pt_{ALL}}$	$X_{pt_{METHOD\ RS-F}}$
Number of results	16	7
Number of outliers	2	0
Mean	45,9	32,6
Median	40,5	38,0
Robust Mean (X)	**39,5**	**32,6**
Robust standard deviation (S*)	**14,0**	**17,7**
Target range:		
Target standard deviation σ_{pt} and $\sigma_{pt'}$	**9,87**	**11,7**
lower limit of target range	**19,7**	**9,3**
upper limit of target range	**59,2**	**56,0**
Quotient S/σ_{pt}*	*1,4*	*1,5*
Standard uncertainty $U_{(Xpt)}$	*4,37*	*8,3*
Quotient $U_{(Xpt)}/\sigma_{pt}$	*0,44*	*0,72*
Results in the target range	*12*	*7*
Percent in the target range	*75*	*100*

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

Comments to the statistical characteristics and assigned values:

The kernel density plot showed no clear method dependent differences.

The evaluation of results of all methods showed a normal variability of results. The quotient S/σ_{pt} was below 2,0. The evaluation of the results from method RS-F showed an increased variability of results. Thus the evaluation was performed by z´-scores considering the standard uncertainty. The quotient S*/$\sigma_{pt`}$ was then below 2,0. The robust standard deviation is in the range of established values for the repeatability and 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 robust means of the evaluations were 85% and 70% of the spiking level of casein to sample A and thus within the recommendations for the applied methods (s. 3.4.3 and "recovery rates for casein").

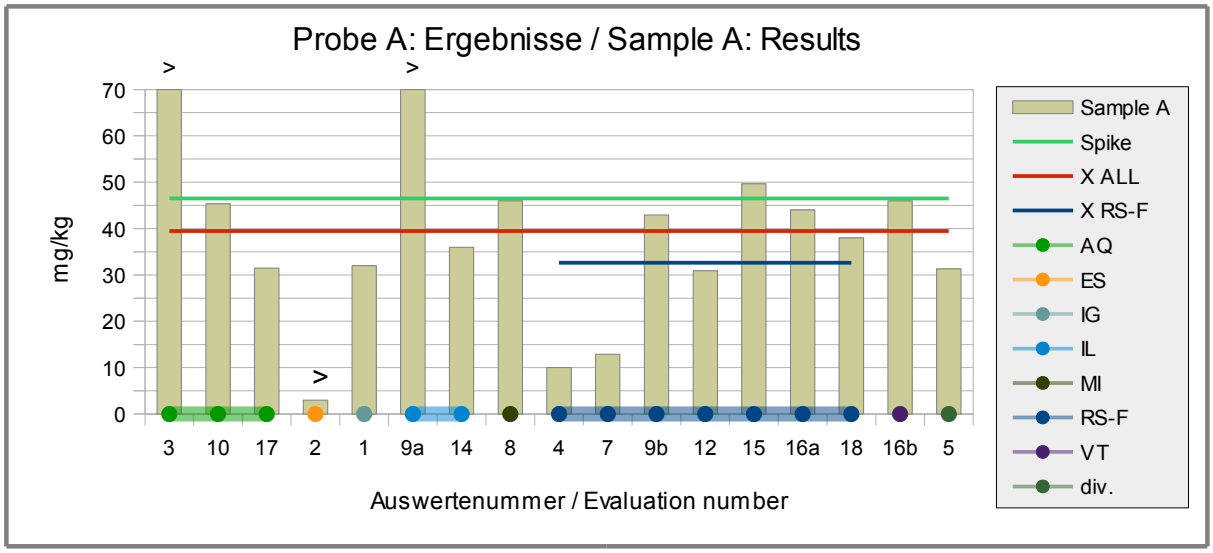


Abb./Fig. 8: ELISA Results Casein
green line = Spiking level (Spike)
red line = Assigned value robust mean all results
blue line = Assigned value robust mean method RS-F
round symbols = Applied methods (see legend)

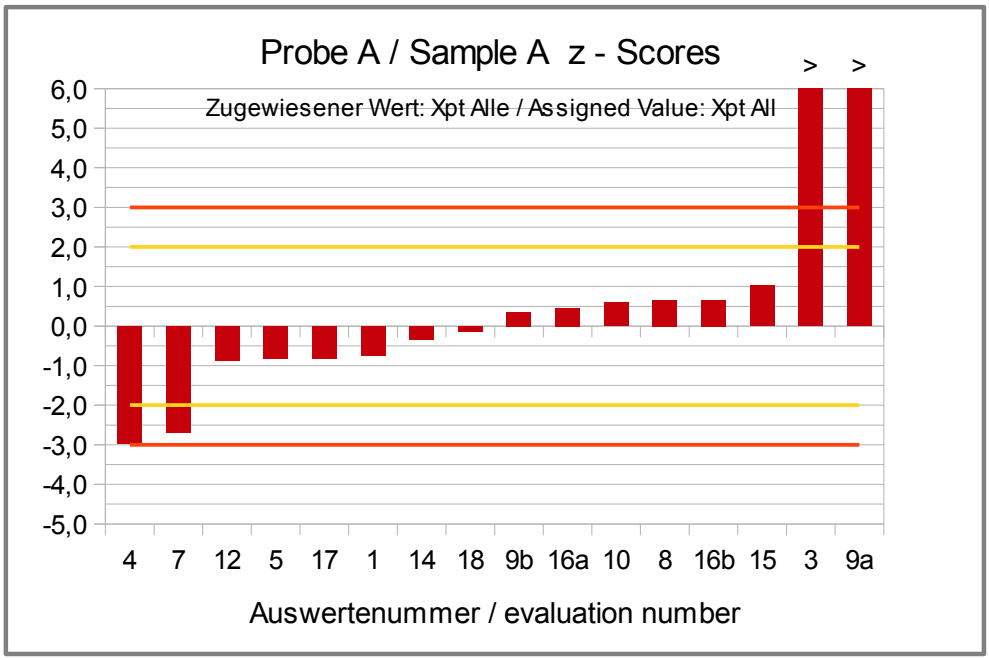


Abb./Fig. 9:
z´-Scores (ELISA Results Casein) Assigned value robust mean of all results

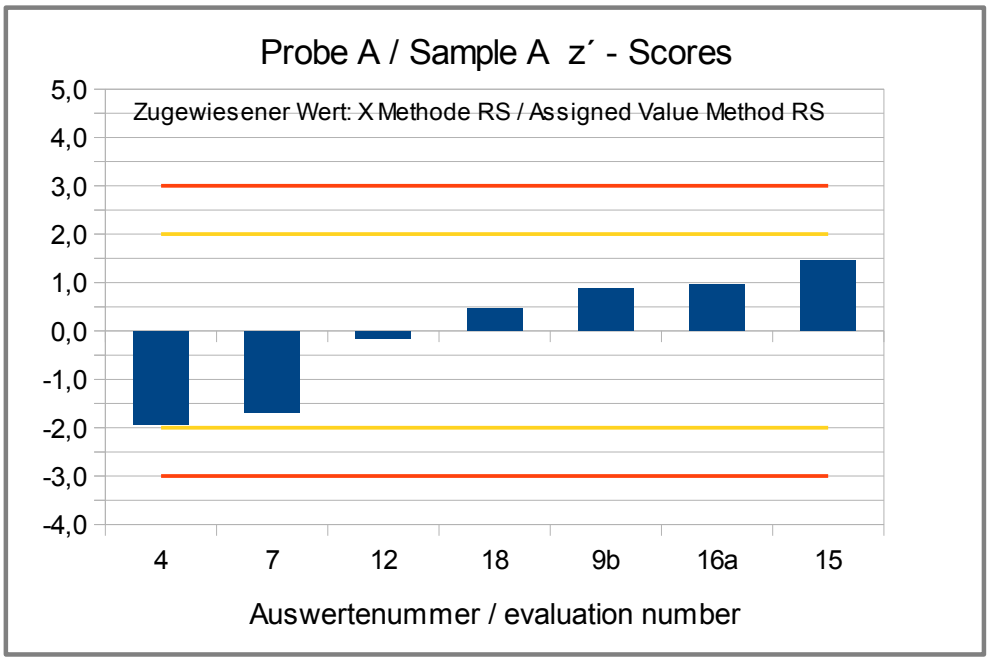


Abb./Fig. 10:
z´-Scores (ELISA Results Casein) Assigned value robust mean of results method RS-F (R-Biopharm, Ridascreen Fast)

Quantitative valuation of ELISA results: Spiking level sample

Evaluation number	Casein	z-Score Xpt_{ALL}	z-Score Xpt_{RS}	Method	Remarks
	[mg/kg]				
3	77,5	3,1		AQ	Outlier X_{all}
10	43,4	0,0		AQ	
17	28,8	-1,4		AQ	
2	>3			ES	Result converted °
1		-4,0		IG	
9a	98,9	5,1		IL	Outlier X_{all}
14	44,0	0,0		IL	
8	43,0	0,0		MI	
4	49,6	0,6	1,2	RS-F	
7	7,00	-3,4	-3,3	RS-F	
9b	40,1	-0,3	0,2	RS-F	
12	22,8	-1,9	-1,6	RS-F	
15	42,0	-0,1	0,4	RS-F	
16a	56,0	1,1	1,9	RS-F	
18	45,0	0,1	0,7	RS-F	
16b	48,0	0,4		VT	
5	38,6	-0,5		div.	

° Conversion p. 19

Methods:

AQ = AgraQuant, RomerLabs
ES = ELISA-Systems
IG = Ingezim ELISA, Ingenasa
IL = Immunolab
MI = Morinaga Institute ELISA
RS-F= Ridascreen® Fast, R-Biopharm
VT = Veratox, Neogen
div = not indicated / other method

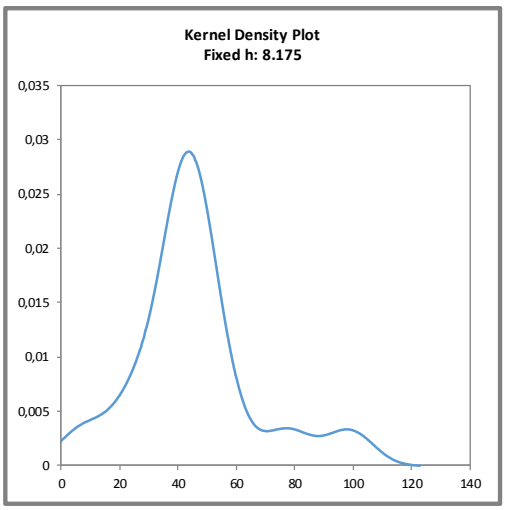


Abb. / Fig. 11:
Kerndichte-Schätzung aller ELISA-Ergebnisse (mit h = 0,75 x σ_{pt} von Xpt_{ALL})

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

Comments:
The kernel density plot shows nearly a normal distribution of results with a shoulder at <10 mg/kg (method RS-F) and two side peaks at > 70 mg/kg (method AQ and IL).

Characteristics: Quantitative evaluation ELISA Casein

Spiking level sample

Statistic Data	**All Results** *[mg/kg]*	**Method RS-F** *[mg/kg]*
Assigned value (X_{pt})	$X_{pt_{ALL}}$	$X_{pt_{Method\ RS\text{-}F}}$
Number of results	15	7
Number of outliers	1	0
Mean	45,6	37,5
Median	43,4	42,0
Robust Mean (X)	**43,5**	**38,2**
Robust standard deviation (S*)	**14,4**	**17,5**
Target range:		
Target standard deviation σ_{pt}	**10,9**	**9,55**
lower limit of target range	**21,8**	**19,1**
upper limit of target range	**65,3**	**57,3**
Quotient S/σ_{pt}*	*1,3*	*1,8*
Standard uncertainty $U_{(Xpt)}$	*4,65*	*8,28*
Quotient $U_{(Xpt)}/\sigma_{pt}$	*0,43*	*0,87*
Results in the target range	*12*	*6*
Percent in the target range	*80*	*86*

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

Comments to the statistical characteristics and assigned values:

The kernel density plot showed no clear method dependent differences.

The evaluation of results of all methods as well as the results of method RS-F showed a normal variability of results. The quotients S/σ_{pt} were below 2,0. The robust standard deviation is in the range of established values for the repeatability and 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 robust means of the evaluations were 95% and 84% of the spiking level of casein to the spiking level sample and thus within the recommendations for the applied methods (s. 3.4.3 and "recovery rates for casein").

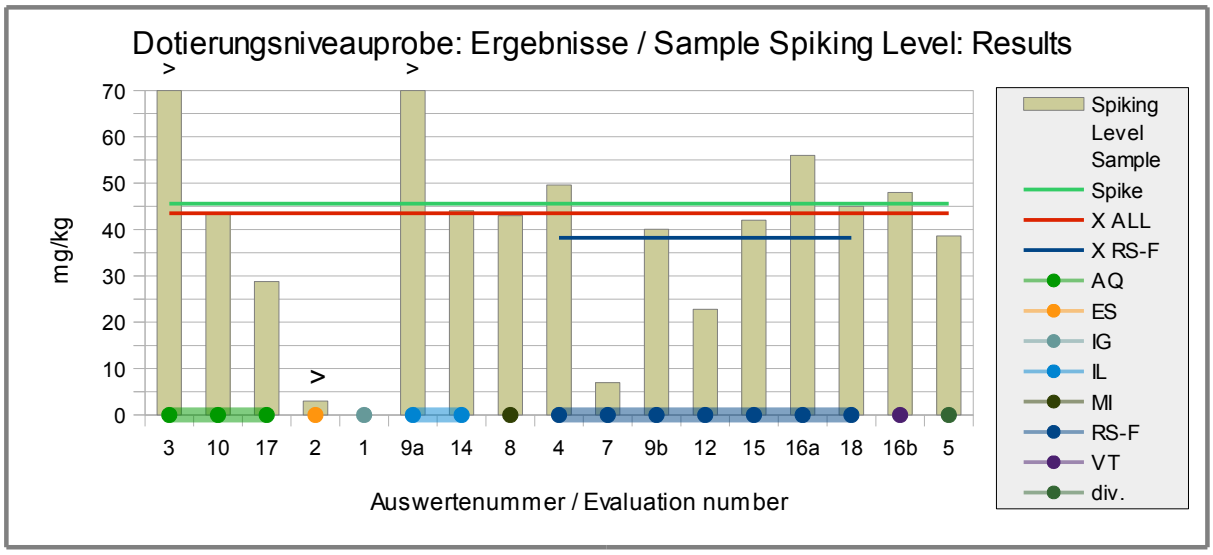


Abb./Fig. 12: ELISA Results Casein
green line = Spiking level (Spike)
red line = Assigned value robust mean all results
blue line = Assigned value robust mean method RS-F
round symbols = Applied methods (see legend)

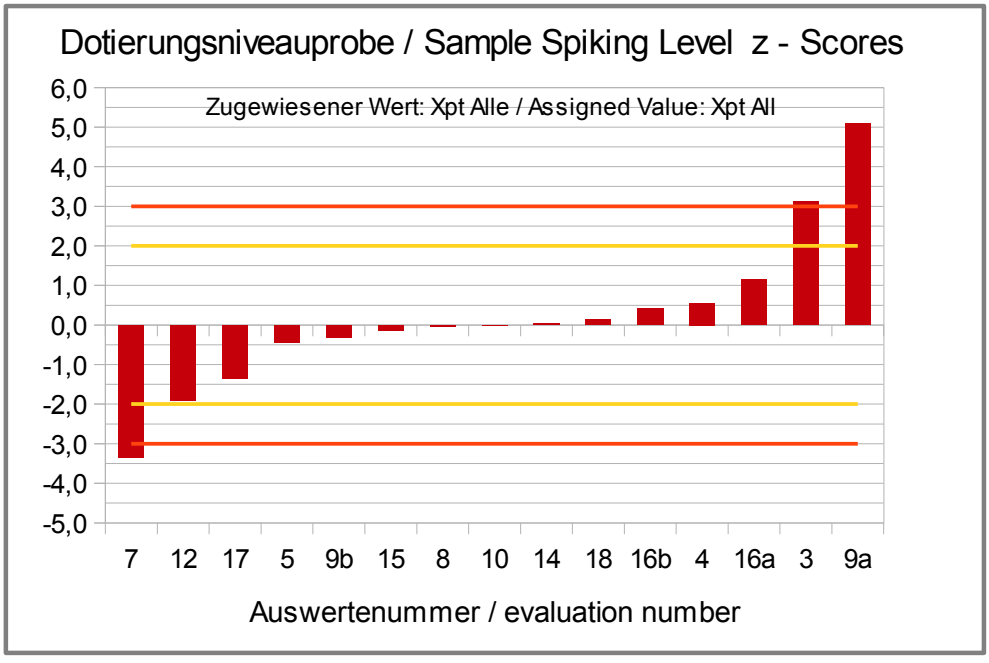


Abb./Fig. 13:
z-Scores (ELISA Results Casein) Assigned value robust mean of all results

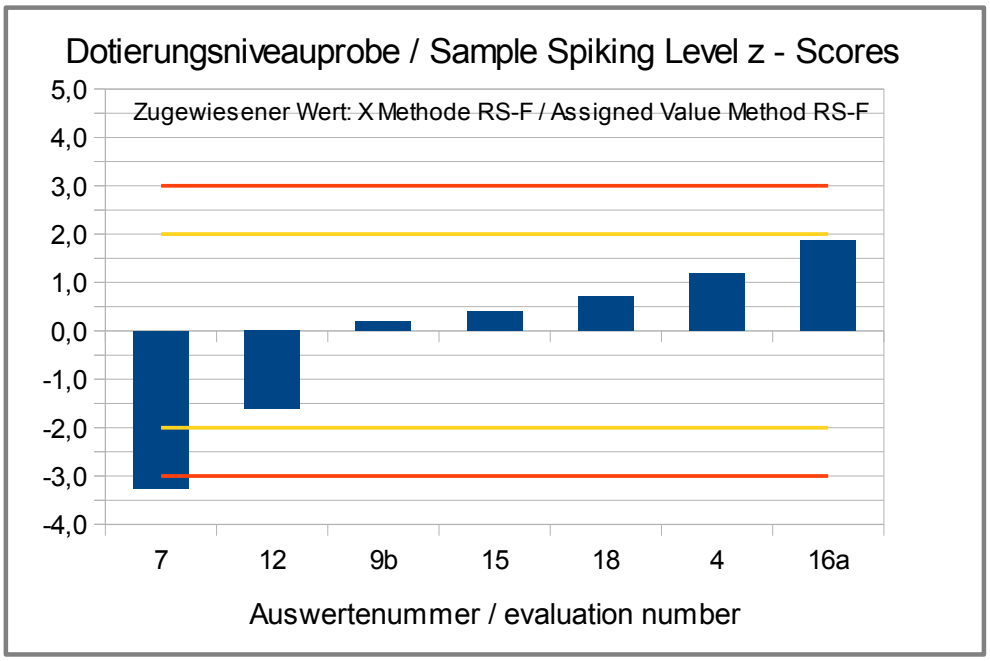


Abb./Fig. 14:
z-Scores (ELISA Results Casein) Assigned value robust mean of results method RS-F (R-Biopharm, Ridascreen Fast)

Recovery Rates for Casein:
Spiking level sample and Sample A

Evaluation number	Spiking Level Sample	Recovery rate*	Sample A	Recovery rate*	Method	Remarks
	[mg/kg]	[%]	[mg/kg]	[%]		
3	77,5	170	102	219	AQ	
10	43,4	**95**	45,4	**98**	AQ	
17	28,8	**63**	31,5	**68**	AQ	
2	>8		>10		ES	Result converted °
1			32,0	**69**	IG	
9a	98,9	217	136	292	IL	
14	44,0	**96**	36,0	**77**	IL	
8	43,0	**94**	46,0	**99**	MI	
4	49,6	**109**	10,0	22	RS-F	
7	7,00	15	12,9	28	RS-F	
9b	40,1	**88**	42,9	**92**	RS-F	
12	22,8	**50**	30,9	**66**	RS-F	
15	42,0	**92**	49,7	**107**	RS-F	
16a	56,0	**123**	44,0	**95**	RS-F	
18	45,0	**99**	38,0	**82**	RS-F	
16b	48,0	**105**	46,0	**99**	VT	
5	38,6	**85**	31,3	**67**	div.	

° Conversion p. 19

RA**	50-150 %	RA**	50-150 %
Number in RA	**12**	Number in RA	**12**
Percent in RA	**80**	Percent in RA	**75**

* Recovery rate 100% relative size: casein, s. page 5

** Range of acceptance of AOAC for allergen ELISAS

Methods:

AQ = AgraQuant, RomerLabs
ES = ELISA-Systems
IG = Ingezim ELISA, Ingenasa
IL = Immunolab
MI = Morinaga Institute ELISA
RS-F= Ridascreen® Fast, R-Biopharm
VT = Veratox, Neogen
div = not indicated / other method

Comments:
For the spiking level sample 80% (12) of the participants obtained a recovery rate within the range of the AOAC-recommendation of 50-150%. For the spiked food matrix sample A 75% (12) of the obtained recovery rates were within the recommended range.

4.2.2 ELISA Results: Milk (as milk protein)

Qualitative valuation of results: Samples A and B

Evaluation number	Sample A	Sample A	Sample B	Sample B	Qualitative Valuation	Method	Remarks
	pos/neg	[mg/kg]	pos/neg	[mg/kg]	Agreement with consensus value		
4	positive	10,9	negative	negative	2/2 (100%)	RS-F	
12	positive	49,9	negative	<2,5	2/2 (100%)	RS-F	
15	positive	76,2	negative	<2.5	2/2 (100%)	RS-F	
5	positive	39,1	negative	<2,5	2/2 (100%)	div.	

	Sample A		Sample B	
Number positive	4		0	
Number negative	0		4	
Percent positive	100		0	
Percent negative	0		100	
Consensus value	positive		negative	

Methods:
RS-F= Ridascreen® Fast, R-Biopharm
div = not indicated / other method

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

Quantitative valuation of results: Sample A

No statistical evaluation was performed, due to the low number of results.

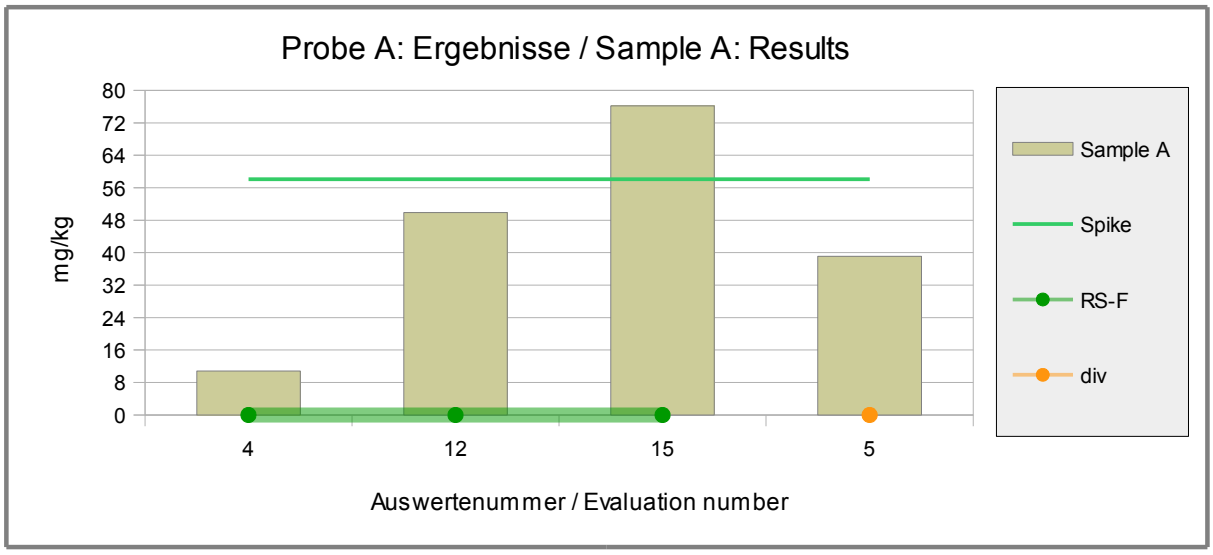


Abb./Fig. 15: ELISA Results Milk (as milk protein)
green line = Spiking level (Spike)
round symbols = Applied methods (see legend)

Quantitative valuation of results: Spiking level sample

No statistical evaluation was performed, due to the low number of results.

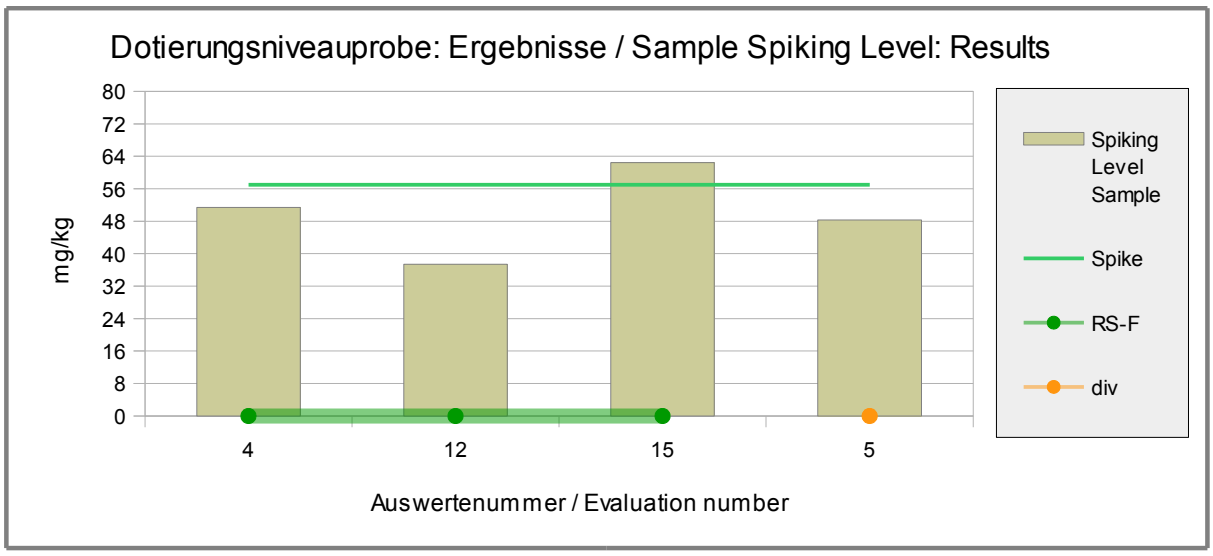


Abb./Fig. 16: ELISA Results Milk (as milk protein)
green line = Spiking level (Spike)
round symbols = Applied methods (see legend)

Recovery Rates for Milk (as milk protein):
Spiking level sample and Sample A

Evaluation number	Spiking Level Sample	Recovery rate*	Sample A	Recovery rate*	Method	Remarks
	[mg/kg]	[%]	[mg/kg]	[%]		
4	51,4	**90**	10,9	19	RS-F	
12	37,4	**66**	49,9	**86**	RS-F	
15	62,4	**110**	76,2	**131**	RS-F	
5	48,3	**85**	39,1	**67**	div.	

RA**	50-150 %	RA**	50-150 %
Number in RA	**4**	Number in RA	**3**
Percent in RA	**100**	Percent in RA	**75**

Methods:
RS-F= Ridascreen® Fast, R-Biopharm
div = not indicated / other method

* Recovery rate 100% relative size: milk protein, s. page 5

** Range of acceptance of AOAC for allergen ELISAS

Comments:
For the spiking level sample 100% (4) of the participants obtained a recovery rate by ELISA within the range of the AOAC-recommendation of 50-150%. For the spiked food matrix sample A 75% (3) of the obtained recovery rates were within the recommended range.

4.3 Proficiency test Wheat (Gluten/Wheat)

4.3.1 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		
14	positive	102	negative	< 4	2/2 (100%)	IL	
1	positive	81,0	negative	<5	2/2 (100%)	RS	
2	positive	>80	negative	<5	2/2 (100%)	RS	
3	positive	94,5	negative		2/2 (100%)	RS	
4	positive	78,3	negative	negative	2/2 (100%)	RS	
5	positive	152	negative	<4,0	2/2 (100%)	RS	
6	positive	86,0	negative	< 5,0	2/2 (100%)	RS	
7	positive	130	positive	< 10	1/2 (50%)	RS	
8	positive	89,0	negative	<5	2/2 (100%)	RS	
9	positive	107	negative	< 5,0	2/2 (100%)	RS	
10	positive	52,6	positive	<5	1/2 (50%)	RS	
12	positive	86,4	negative	<5,0	2/2 (100%)	RS	
13	positive	72,4	negative	<5	2/2 (100%)	RS	
15	positive	75,9	negative	<5	2/2 (100%)	RS	
16	positive	65,0	negative		2/2 (100%)	RS	
17	positive	55,0	negative	<5	2/2 (100%)	RS	
18	positive	71,0	negative		2/2 (100%)	RS	

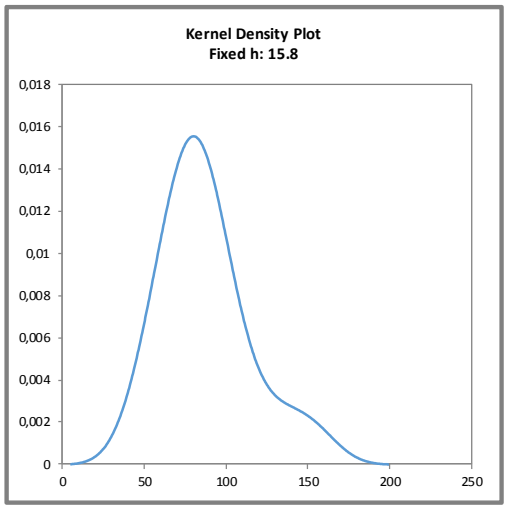
	Sample A		Sample B	
Number positive	17		2	
Number negative	0		15	
Percent positive	100		12	
Percent negative	0		88	
Consensus value	positive		negative	

Methods:
IL = Immunolab
RS = Ridascreen®, R-Biopharm

Comments:
The consensus values are in qualitative agreement with the spiking of sample A. Two positive results each below the limit of quantification were obtained for sample B with method RS.

Quantitative valuation of results: Sample A

Evaluation number	Gluten	z-Score Xpt_{ALL}	z-Score Xpt_{RS}	Method	Remarks
	[mg/kg]				
14	102	0,8		IL	
1	81,0	-0,2	-0,1	RS	
2	>80			RS	
3	94,5	0,5	0,6	RS	
4	78,3	-0,3	-0,2	RS	
5	152	3,2	3,3	RS	Outlier X_{RS}
6	86,0	0,1	0,1	RS	
7	130	2,2	2,3	RS	
8	89,0	0,2	0,3	RS	
9	107	1,0	1,1	RS	
10	52,6	-1,5	-1,5	RS	
12	86,4	0,1	0,2	RS	
13	72,4	-0,6	-0,5	RS	
15	75,9	-0,4	-0,3	RS	
16	65,0	-0,9	-0,9	RS	
17	55,0	-1,4	-1,4	RS	
18	71,0	-0,6	-0,6	RS	



Methods:
IL = Immunolab
RS = Ridascreen®, R-Biopharm

Abb. / Fig. 17:
Kerndichte-Schätzung aller ELISA-Ergebnisse (mit h = 0,75 x σpt von Xpt_{ALL})

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

Comments:
The kernel density estimation shows nearly a normal distribution of results with a shoulder at approx. 150 mg/kg, due to an outlier.

Characteristics: Quantitative evaluation ELISA Gluten

Sample A

Statistic Data	**All Results** *[mg/kg]*	**Method RS** *[mg/kg]*
Assigned value (X_{pt})	$X_{pt_{ALL}}$	$X_{pt_{METHOD\ RS}}$
Number of results	16	15
Number of outliers	1	1
Mean	87,4	86,4
Median	83,5	81,0
Robust Mean (X)	**84,5**	**83,1**
Robust standard deviation (S*)	**22,6**	**22,1**
Target range:		
Target standard deviation σ_{pt} and $\sigma_{pt'}$	**21,1**	**20,8**
lower limit of target range	**42,3**	**41,5**
upper limit of target range	**127**	**125**
Quotient S/σ_{pt}*	*1,1*	*1,06*
Standard uncertainty $U_{(Xpt)}$	*7,05*	*7,1*
Quotient $U_{(Xpt)}/\sigma_{pt}$	*0,33*	*0,34*
Results in the target range	*14*	*13*
Percent in the target range	*88*	*87*

Methods:
RS = R-Biopharm, Ridascreen®

Comments to the statistical characteristics and assigned values:

The kernel density plot showed no clear method dependent differences.

The evaluation of results of all methods as well as the results of method RS showed a normal to low variability of results. The quotients S/σ_{pt} were below 2,0. The robust standard deviation is in the range of established values for the repeatability and 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 robust means of the evaluations were 150% and 147% of the spiking level of gluten to sample A and thus within the recommendations for the applied methods (s. 3.4.3 and "recovery rates for gluten").

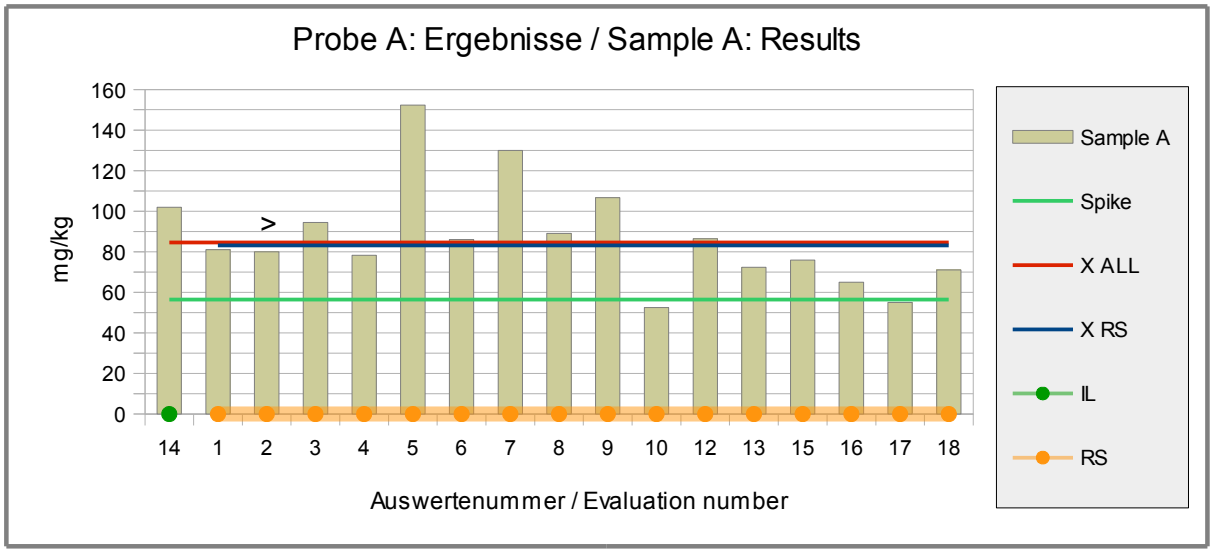


Abb./Fig. 18: ELISA Results Gluten
green line = Spiking level (Spike)
red line = Assigned value robust mean all results
blue line = Assigned value robust mean method RS
round symbols = Applied methods (see legend)

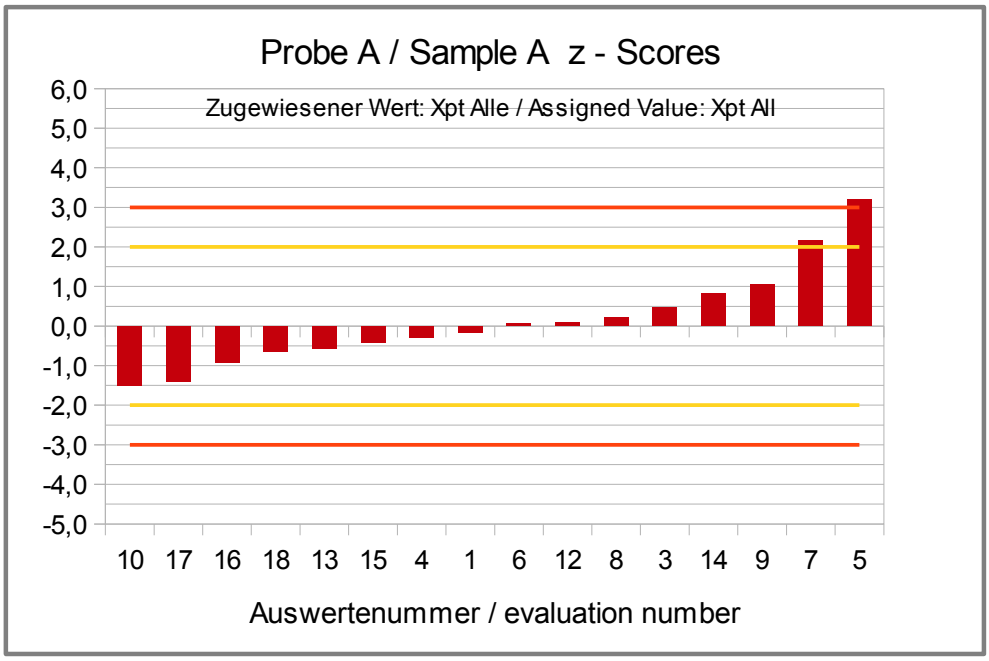


Abb./Fig. 19:
z´-Scores (ELISA Results Gluten) Assigned value robust mean of all results

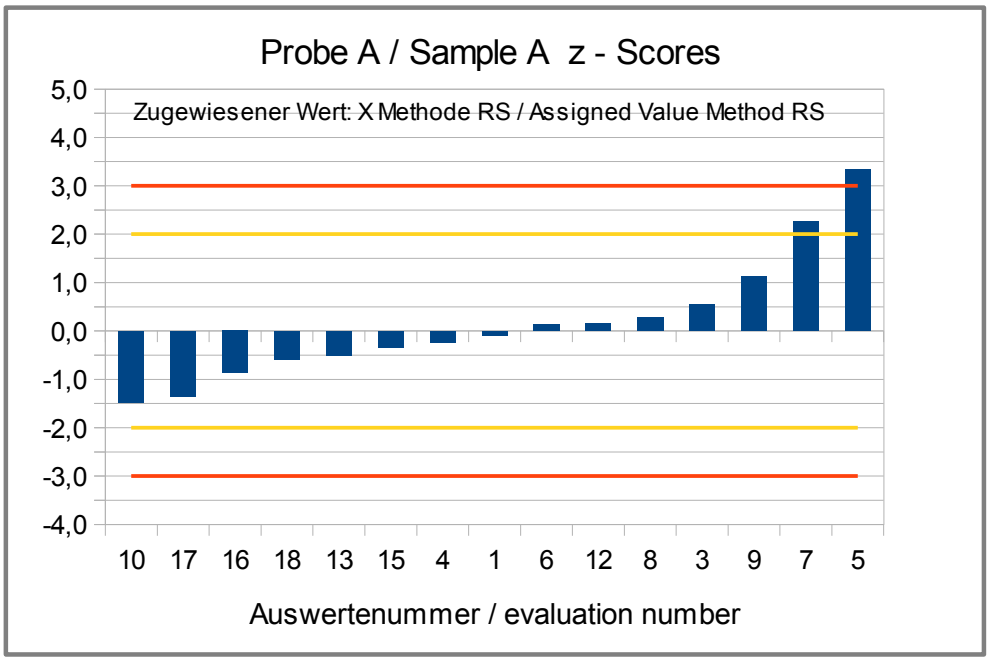
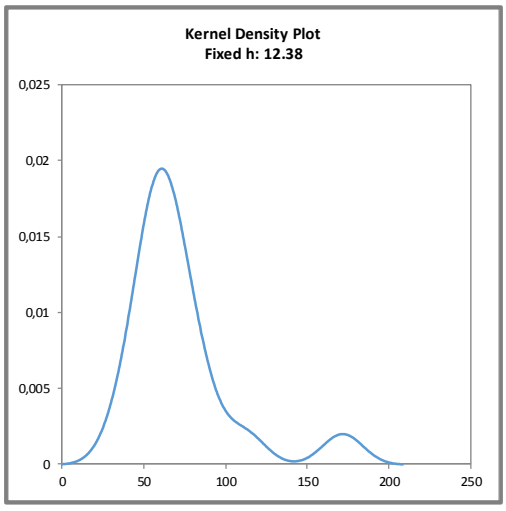


Abb./Fig. 20:
z-Scores (ELISA Results Gluten) Assigned value robust mean of results method RS (R-Biopharm, Ridascreen)

Quantitative valuation of ELISA results: Spiking level sample

Evaluation number	Gluten	z-Score Xpt_{ALL}	z-Score Xpt_{RS}	Method	Remarks
	[mg/kg]				
14	112	2,8		IL	
1				RS	
2	57,0	-0,6	-0,4	RS	
3	58,7	-0,5	-0,3	RS	
4	76,2	0,6	0,8	RS	
5	172	6,4	6,7	RS	Outlier X_{RS}
6	67,0	0,0	0,2	RS	
7	80,0	0,8	1,0	RS	
8	62,0	-0,3	-0,1	RS	
9	87,5	1,3	1,5	RS	
10	47,2	-1,1	-1,1	RS	
12	54,9	-0,7	-0,6	RS	
13	64,3	-0,1	0,0	RS	
15	49,1	-1,0	-0,9	RS	
16	69,0	0,2	0,3	RS	
17	36,0	-1,8	-1,8	RS	
18	59,5	-0,4	-0,3	RS	



Methods:
IL = Immunolab
RS = Ridascreen®, R-Biopharm

Abb. / Fig. 21:
Kerndichte-Schätzung aller ELISA-Ergebnisse (mit h = 0,75 x σpt von Xpt_{ALL})

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

Comments:
The kernel density estimation shows nearly a normal distribution of results with a shoulder and a side-peak at >100 mg/kg, due to two results above the target range.

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	16	15
Number of outliers	1	1
Mean	72,0	69,3
Median	63,2	62,0
Robust Mean (X)	**66,2**	**64,0**
Robust standard deviation (S*)	**18,8**	**16,2**
Target range:		
Target standard deviation σ_{pt}	**16,5**	**16,0**
lower limit of target range	**33,1**	**32,0**
upper limit of target range	**99,3**	**96,0**
Quotient S/σ_{pt}*	*1,1*	*1,0*
Standard uncertainty $U_{(Xpt)}$	*5,86*	*5,22*
Quotient $U_{(Xpt)}/\sigma_{pt}$	*0,35*	*0,33*
Results in the target range	*14*	*14*
Percent in the target range	*88*	*93*

Methods:
RS = R-Biopharm, Ridascreen®

Comments to the statistical characteristics and assigned values:

The kernel density plot showed no clear method dependent differences.

The evaluation of results of all methods as well as the results of method RS showed a normal variability of results. The quotients S/σ_{pt} were below 2,0. The robust standard deviation is in the range of established values for the repeatability and 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 robust means of the evaluations were 138% and 134% of the spiking level of gluten to the spiking level sample and thus within the recommendations for the applied methods (s. 3.4.3 and "recovery rates for gluten").

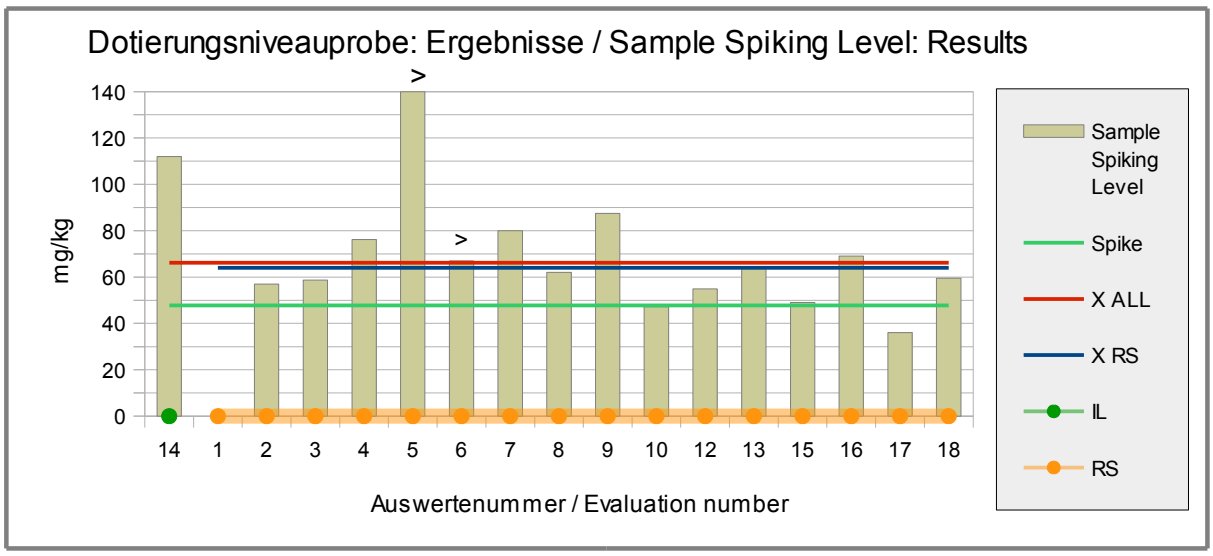


Abb./Fig. 22: ELISA Results Gluten
green line = Spiking level (Spike)
red line = Assigned value robust mean all results
blue line = Assigned value robust mean method RS
round symbols = Applied methods (see legend)

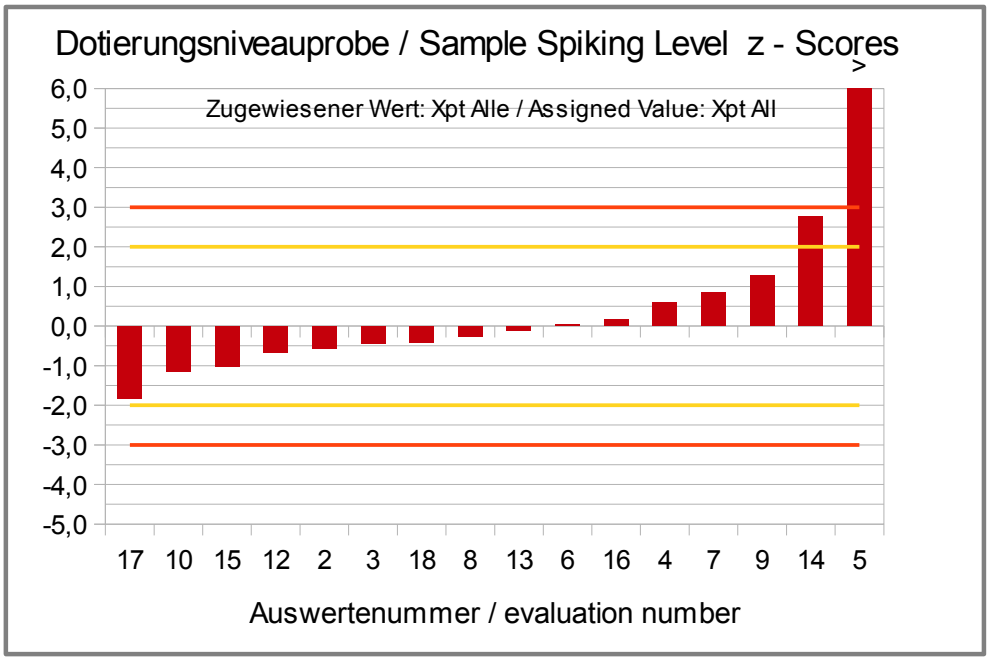


Abb./Fig. 23:
z-Scores (ELISA Results Gluten) Assigned value robust mean of all results

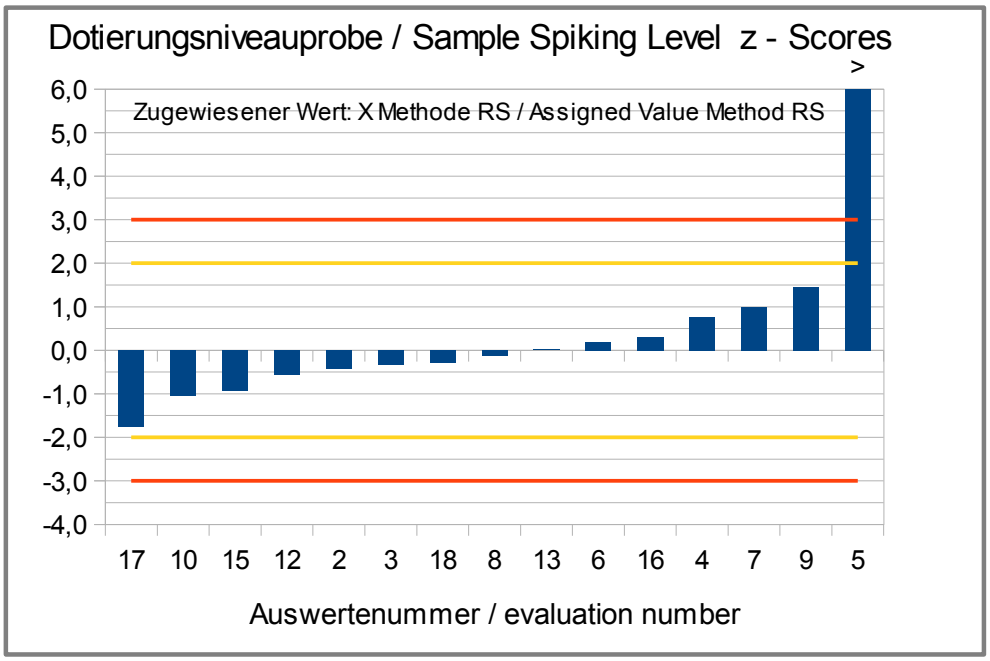


Abb./Fig. 24:
z-Scores (ELISA Results Gluten) Assigned value robust mean of results method RS (R-Biopharm, Ridascreen)

Recovery Rates for Gluten:
Spiking level sample and Sample A

Evaluation number	Spiking Level Sample	Recovery rate*	Sample A	Recovery rate*	Method	Remarks
	[mg/kg]	[%]	[mg/kg]	[%]		
14	112	234	102	181	IL	
1			81,0	**144**	RS	
2	57,0	**119**	>80		RS	
3	58,7	**123**	94,5	168	RS	
4	76,2	159	78,3	**139**	RS	
5	172	359	152	270	RS	
6	67,0	**140**	86,0	152	RS	
7	80,0	167	130	230	RS	
8	62,0	**130**	89,0	158	RS	
9	87,5	183	107	189	RS	
10	47,2	**99**	52,6	**93**	RS	
12	54,9	**115**	86,4	153	RS	
13	64,3	**135**	72,4	**128**	RS	
15	49,1	**103**	75,9	**135**	RS	
16	69,0	**144**	65,0	**115**	RS	
17	36,0	**75**	55,0	**98**	RS	
18	59,5	**124**	71,0	**126**	RS	

RA**	50-150 %	RA**	50-150 %
Number in RA	**11**	Number in RA	**8**
Percent in RA	**69**	Percent in RA	**50**

Methods:
IL = Immunolab
RS = Ridascreen®, R-Biopharm

* Recovery rate 100% relative size: gluten, s. page 5

** Range of acceptance of AOAC for allergen ELISAS

Comments:
For the spiking level sample 69% (11) of the participants obtained a recovery rate within the range of the AOAC-recommendation of 50-150%. For the processed spiked food matrix sample A 50% (8) of the obtained recovery rates were within the recommended range.

4.3.2 PCR Results: Wheat

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		
9	positive	107	positive	< 1,0	2/2 (100%)	SFA-Q	
7	positive		negative		2/2 (100%)	div.	

	Sample A		Sample B	
Number positive	2		1	
Number negative	0		1	
Percent positive	100		50	
Percent negative	0		50	
Consensus value	positive		none	

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

Comments:
The consensus value for sample A is in agreement with the spiking of sample A. For sample B one positive result was obtained with method SFA-Q, thus no consensus value for sample B was obtained.

Quantitative valuation of ELISA results: Sample A

No statistical evaluation was performed, due to the low number of results.

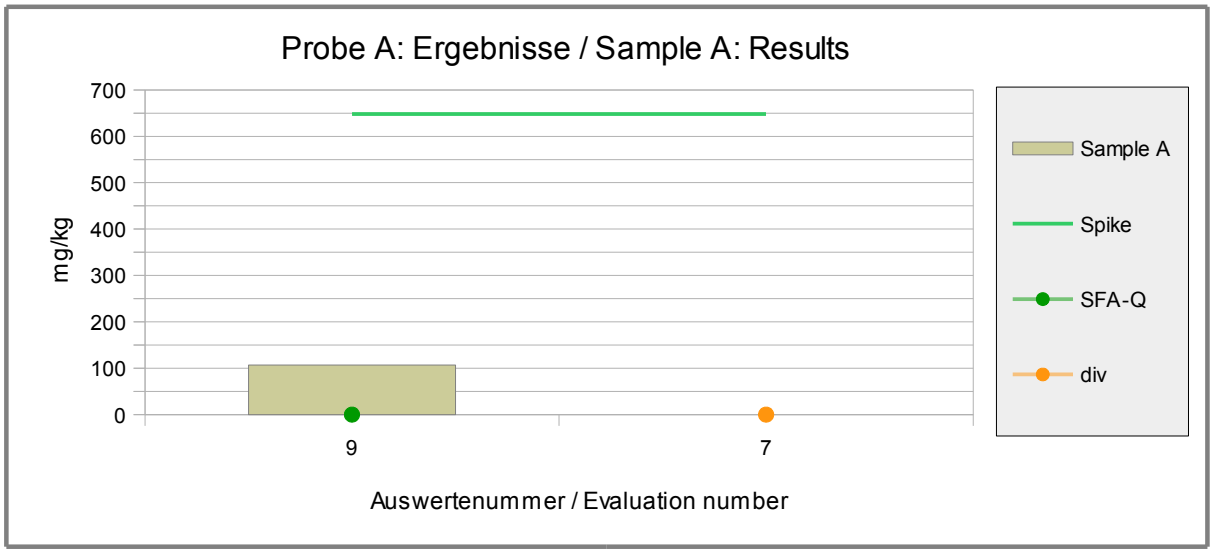


Abb./Fig. 25: PCR-Results Wheat
green line = Spiking level (Spike)
round symbols = Applied methods (see legend)

Quantitative valuation of results: Spiking level sample

No statistical evaluation was performed, due to the low number of results.

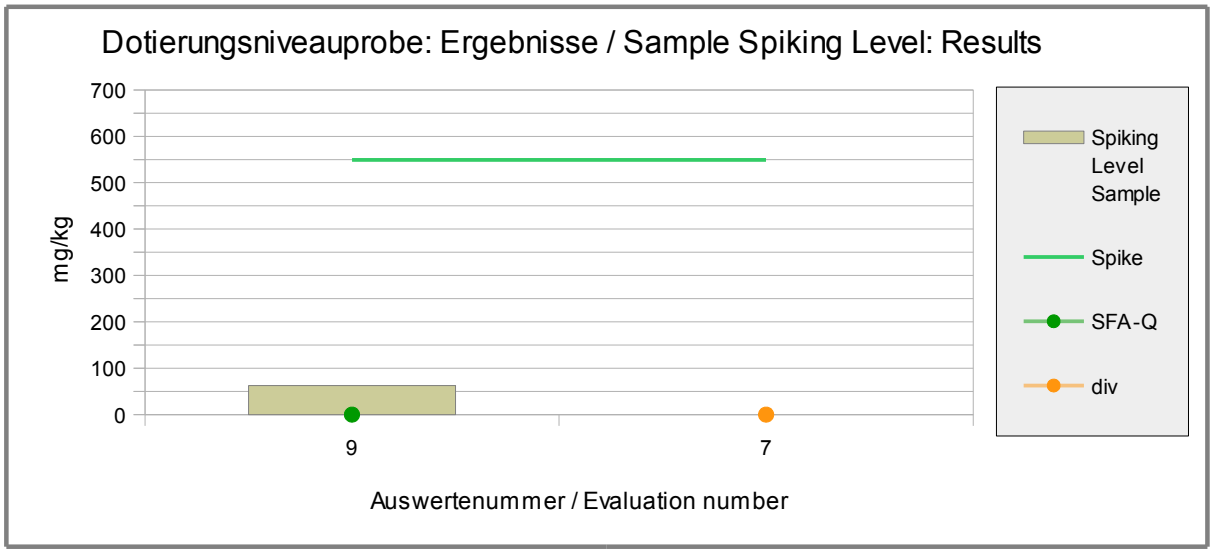


Abb./Fig. 26: PCR-Results Wheat
green line = Spiking level (Spike)
round symbols = Applied methods (see legend)

Recovery Rates for Wheat (as Wheat flour):
Spiking Level Sample and Sample A

Evaluation number	Spiking Level Sample	Recovery rate*	Sample A	Recovery rate*	Method	Remarks
	[mg/kg]	[%]	[mg/kg]	[%]		
9	62,9	10	107	17	SFA-Q	
7					div.	

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

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

* Recovery rate 100% relative size: wheat, s. page 5

** Range of acceptance of AOAC for allergen ELISAS

Comments:
One participant obtained a recovery rate for the spiking level sample and sample A below the range of the AOAC-recommendation of 50-150%.

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: β-Lactoglobulin

Meth. Abr.	Evaluation number	Date of analysis	Result Sample A		Result Sample B		Result Spiking Sample		quantitative Result given as	Method
		Day/Month	qualitative	mg/kg	qualitative	mg/kg	qualitative	mg/kg	e.g. food / food protein	Test-Kit + Manufacturer
BK	3	31.03.	positive	7,8	negative		positive	9,5	beta-Lactoglobulin	BioKits BLG Assay Kit, Neogen
BK	17	09.03.17	positive	13	negative	<5	positive	15	beta-Lactoglobulin	BLG assay kit - Biokits
ES	2	07.03.17	positive	>1	negative	<0,1	positive	>1	beta-Lactoglobulin	ELISA Systems Beta-Lactoglobulin ESMRDBLG-48
ES	18	12.04.17	positive	6,7	negative		positive	5,8	beta-Lactoglobulin	ELISA Systems Beta-Lactoglobulin ESMRDBLG-48
IG	1		positive	2,3	negative	<0,5	-		beta-Lactoglobulin	Ingenzim Blactoglobulin(ingenasa)
IL	14		positive	1,6	negative	< 0,01	positive	1,7	beta-Lactoglobulin	Immunolab Beta-Lactoglobulin ELISA
MI	8	29.03.	positive	3,4	negative	<0,031	positive	3,4	beta-Lactoglobulin	Morinaga ß Lac ELISA Kit II
RS	4	15.03.17	-	0,608	-	negative	-	1,752	beta-Lactoglobulin	Ridascreen® β-Lactoglobulin R4901, R-Biopharm
RS	11	15.03.17	positive	3,01	negative	<1,00	positive	2,84	Please select!	Ridascreen® β-Lactoglobulin R4901, R-Biopharm
RS	13		-	<5	-	<5	-	<5	beta-Lactoglobulin	Ridascreen® β-Lactoglobulin R4901, R-Biopharm
RS	16	13.04.17	Detected	1,4	Not Detected		Detected	1,4	Protein	Ridascreen Betalactoglobulin R4901
RS-F	7	30.04.17	positive	2,1	negative	< 0,5	positive	1,5	beta-Lactoglobulin	Ridascreen® FAST β-Lactoglobulin R4902, R-Biopharm
RS-F	12	08.03.17	positive	2,37	negative	<0,5	positive	2,23	beta-Lactoglobulin	Ridascreen® FAST β-Lactoglobulin R4902, R-Biopharm
RS-F	15	09.03.17	positive	2,37	negative	<0.5	positive	2,78	beta-Lactoglobulin	Ridascreen® FAST β-Lactoglobulin R4902, R-Biopharm

Continuation ELISA: β-Lactoglobulin

Meth. Abr.	Evaluation number	Specifity	Remarks to the Method (Extraction and Determination)	Method accredidet ISO/IEC 17025	Further Remarks
		Antibody	e.g. Extraction Solution / Time / Temperature	yes/no	
BK	3	beta-Lactoglobulin		no	
BK	17			yes	
ES	2			no	
ES	18			yes	
IG	1	anti betalactoglobulin antibody	kit extraction buffer	yes	operator: Poletti Alessia method MI 22 rev04 2015
IL	14	polyclonal			conversion factor to skimmed milk pow der 66,7 (correspond sample A 107 mg/kg, spiking sample 113 mg/kg)*
MI	8	ß Lactoglobulin	according to kit instruction, Overnight extraction method	yes	
RS	4	spezific for ß-Lactoglobulin cow, sheep, goat, buffalo	Extractionsolution 100 °C, Extractor 2 60 °C	yes	
RS	11	Anti-BLG	w ashing buffer, 10 minutes, 50°C	no	-
RS	13		<5		<5;<5;<5 mg/kg A
RS	16	betalactoglobulin	according to kit instruction	yes	
RS-F	7			no	
RS-F	12		according to kit instruction	no	
RS-F	15	As Per Kit Instructions	As Per Kit Instructions	Yes	

5.1.2 ELISA: Casein

Meth. Abr.	Evaluation number	Date of analysis	Result Sample A		Result Sample B		Result Spiking Sample		quantitative Result given as	Method
		Day/Month	qualitative	mg/kg	qualitative	mg/kg	qualitative	mg/kg	e.g. food / food protein	Test-Kit + Manufacturer
AQ	3	30.03.	positive	102	negative		positive	77,5	Casein	AgraQuant Casein COKAL 1200, RomerLabs
AQ	10		positive	45,35	negative	<1	positive	43,42	Casein	AgraQuant Casein COKAL 1200, RomerLabs
AQ	17	05.04.17	positive	31,48	negative	<1.5	positive	28,8	Casein	AgraQuant Casein COKAL 1200, RomerLabs
ES	2	07.03.17	positive	>10	negative	<1	positive	>10	Skimmed milk powder	ELISA Systems Casein ESCASPRD-48
IG	1		positive	32	negative	<1	-		Casein	Ingenzim casein(ingenasa)
IL	9	24.03.	positive	135,87	negative	< 0,2	positive	98,87	Casein	Immunolab Casein ELISA
IL	14		positive	36	negative	< 0,2	positive	44	Casein	Immunolab Casein ELISA
MI	8	07.04.	positive	46	negative	<0,25	positive	43	Casein	Morinaga Casein ELISA Kit II
RS-F	4	15.03.17	-	10,04	-	negative	-	49,6	Casein	Ridascreen® FAST Casein R4612, R-Biopharm
RS-F	7	28.04.17	positive	12,9	negative	< 0,5	positive	7	Casein	Ridascreen® FAST Casein R4612, R-Biopharm
RS-F	9	28.03.	positive	42,94	negative	< 0,5	positive	40,06	Casein	Ridascreen® FAST Casein R4612, R-Biopharm
RS-F	12	02.03.17	positive	30,91	negative	<2,5	positive	22,78	Casein	Ridascreen® FAST Casein R4612, R-Biopharm
RS-F	15	11.03.17	positive	49,65	negative	<2.5	positive	41,99	Casein	Ridascreen® FAST Casein R4612, R-Biopharm
RS-F	16a	13.04.17	Detected	44	Not Detected		Detected	56	protein	Ridascreen FAST Casein R4612
RS-F	18	12.04.17	positive	38	negative		positive	45	Casein	Ridascreen® FAST Casein R4612, R-Biopharm
VT	16b	13.04.17	Detected	46	Not Detected		Detected	48	protein	Neogen Veratox Casein
div.	5	06.03.17	-	31,3	-	<2,0	-	38,6	Casein	other: please fill in!

Continuation ELISA: Casein

Meth. Abr.	Evaluation number	Specifity	Remarks to the Method (Extraction and Determination)	Method accredidet ISO/IEC 17025	Further Remarks
		Antibody	e.g. Extraction Solution / Time / Temperature	yes/no	
AQ	3	Casein		no	
AQ	10		w eight: 0,5 g, Extraktion: Extraction buffer (1+4 diluted), 15 min at 60°C (each 2 min shaked by hand), dilution: 1:25, ELISA-Reader: Multiskan FC	no	dk
AQ	17			yes	
ES	2			no	
IG	1	anti casein antibody	kit extraction buffer	yes	operator: Poletti Alessia Method: MI 23 rev04 2015
IL	9		As Per Kit Instructions		
IL	14	polyclonal			conversion factor to skimmed milk 3,6 (corresponds sample A 130 mg/kg, spiked sample 158 mg/kg)*
MI	8	Casein	according to kit instruction, Overnight extraction method	yes	
RS-F	4	spezific for casein and ß-Lactoglobulin cow, sheep, goat, buffalo	Extraction solution 100 °C, Extractor 2 60 °C	yes	
RS-F	7			no	
RS-F	9		As Per Kit Instructions w ith extraction bufferE		
RS-F	12		As Per Kit Instructions	no	
RS-F	15	As Per Kit Instructions	As Per Kit Instructions	Yes	General Extraction Method
RS-F	16a	casein	extracted w ith extraction solution R7098 and extrcation buffer.	yes	
RS-F	18			yes	
VT	16b	Casein	according to kit instruction	yes	
div.	5				

5.1.3 ELISA: Milk

Meth. Abr.	Evaluation number	Date of analysis	Result Sample A		Result Sample B		Result Spiking Sample		quantitative Result given as	Method
		Day/Month	qualitative	mg/kg	qualitative	mg/kg	qualitative	mg/kg	e.g. food / food protein	Test-Kit + Manufacturer
RS-F	4	04.04.17	-	10,854	-	negative	-	51,41	food	Ridascreen Milk R4652
RS-F	12	08.03.17	positive	49,88	negative	<2,5	positive	37,41	Milk proteins, total	Ridascreen® FAST Milk R4652, R-Biopharm
RS-F	15	09.03.17	positive	76,15	negative	<2.5	positive	62,42	Milk proteins, total	Ridascreen® FAST Milk R4652, R-Biopharm
div.	5	06.03.17	-	39,1	-	<2,5	-	48,3	Milk proteins, total	Please select!

Meth. Abr.	Evaluation number	Specifity	Remarks to the Method (Extraction and Determination)	Method accredidet ISO/IEC 17025	Further Remarks
		Antibody	e.g. Extraction Solution / Time / Temperature	yes/no	
RS-F	4	spezific for casein cow, sheep, goat, buffalo	Extraction solution 100 °C, Extractor 2 60 °C	yes	
RS-F	12		As per test kit instructions	yes	
RS-F	15	As per test kit instructions	As per test kit instructions	yes	
div.	5				

5.1.4 ELISA: Gluten

Meth. Abr.	Evaluation number	Date of analysis	Result Sample A		Result Sample B		Result Spiking Sample		quantitative Result given as	Method
		Day/Month	qualitative	mg/kg	qualitative	mg/kg	qualitative	mg/kg	e.g. food / food protein	Test-Kit + Manufacturer
IL	14		positive	102	negative	< 4	positive	112	Gluten	Immunolab Gliadin/Gluten ELISA
RS	1		positive	81	negative	<5	-		Gluten	Ridascreen® Gliadin R7001, R-Biopharm
RS	2	15.03.17	positive	>80	negative	<5	positive	57	Gluten	Ridascreen® Gliadin R7001, R-Biopharm
RS	3	30.03.	positive	94,5	negative		positive	58,7	Gluten	Ridascreen® Gliadin R7001, R-Biopharm
RS	4	15.03.17	-	78,31	-	negative	-	76,17	Gluten	Ridascreen® Gliadin R7001, R-Biopharm
RS	5	06.03.17	-	152,3	-	<4,0	-	171,5	Gluten	andere bitte angeben!
RS	6	23.03.17	positive	86	negative	< 5,0	positive	67	Gluten	Ridascreen® Gliadin R7001, R-Biopharm
RS	7	06.04.17	positive	130	positive	< 10	positive	80	Gluten	Ridascreen® Gliadin R7001, R-Biopharm
RS	8	13.03.	positive	89	negative	<5	positive	62	Gluten	Ridascreen® Gliadin R7001, R-Biopharm
RS	9	05.04.	positive	106,7	negative	< 5,0	positive	87,45	Gluten	Ridascreen® Gliadin R7001, R-Biopharm
RS	10		positive	52,58	positive	<5	positive	47,21	Gluten	Ridascreen® Gliadin R7001, R-Biopharm
RS	12	13.03.17	positive	86,37	negative	<5,0	positive	54,92	Gluten	Ridascreen® Gliadin R7001, R-Biopharm
RS	13		-	72,4	-	<5	-	64,3	Please choose!	Ridascreen® Gliadin R7001, R-Biopharm
RS	15	06.03.17	positive	75,86	negative	<5	positive	49,11	Gluten	Ridascreen® Gliadin R7001, R-Biopharm
RS	16	13.04.17	Detected	65	Not Detected		Detected	69	Protein	Ridascreen Gliadin R7001
RS	17	17.03.17	positive	55	negative	<5	positive	36	Gluten	Ridascreen® Gliadin R7001, R-Biopharm
RS	18	12.04.17	positive	71	negative		positive	59,5	Gluten	Ridascreen® Gliadin R7001, R-Biopharm

Continuation ELISA: Gluten

Meth. Abr.	Evaluation number	Specifity	Remarks to the Method (Extraction and Determination)	Method accredidet ISO/IEC 17025	Further Remarks
		Antibody	e.g. Extraction Solution / Time / Temperature	yes/no	
IL	14	polyclonal			
RS	1	R-5 antibody	coktail solution	yes	Operator: Pletti Alessia Method: AOAC 2012.01
RS	2	R5		yes	
RS	3	Gluten		AOAC	
RS	4	spezif. F. Gliadinfraction from wheat and prolamine from rye and barley	Cocktail solution at 50°C, 80% Ethanol at 20-25 °C	yes	
RS	5				
RS	6	monoclonal R5	80% ethanol / 1h / room temperature/	yes	LAB_AR result
RS	7			no	
RS	8	R5, Prolamine from wheat, rye and barley	according to kit instruction with Cocktaill solution	ses	
RS	9		according to kit instruction		
RS	10		weight: 0,25 g, Extraction: 2,5 ml Cocktail-solution + 7,5 ml Ethanol, 40 min at 50°C, 60 min shaking, Dilution 1:500, ELISA-Reader Multiskan FC	no	ak
RS	12		As Per Kit Instructions	yes	
RS	13				57,1;>80;>80mg /kg A
RS	15	As Per Kit Instructions	As Per Kit Instructions	Yes	
RS	16	R5	according to kit instruction	yes	
RS	17			yes	
RS	18			yes	

5.1.5 PCR: Wheat

Meth. Abr.	Evaluation number	Date of analysis	Result Sample A		Result Sample B		Result Spiking Sample		quantitative Result given as	Method
		Day/Month	qualitative	mg/kg	qualitative	mg/kg	qualitative	mg/kg	e.g. food / food protein	Test-Kit + Manufacturer
SFA-Q	9	23.03.	positive	107,04	positive	< 1,0	positive	62,87	gluten cereal	Sure Food Allergen Quant, R-Biopharm / Congen
div.	7		positive		negative		positive		Please choose!	Choice PCR-Methods

Meth. Abr.	Evaluation number	Specifity	Remarks to the Method (Extraction and Determination)	Method accredidet ISO/IEC 17025	Further Remarks
		Antibody	e.g. Extraction Solution / Time / Temperature	yes/no	
SFA-Q	9		as per test kit instructions, DNA-Extraction with SureFood®PREP Advanced, Protocol 1, Fa. R-Biopharm/Congen		
div.	7				

5.2 Homogeneity

5.2.1 Mixture homogeneity before bottling

Microtracer Homogeneity Test
DLA 03-2017 Sample A

Weight whole sample	2,59	kg
Microtracer	FSS-rot lake	
Particle size	75 – 300	µm
Weight per particle	2,0	µg
Addition of tracer	52,7	mg/kg

Result of analysis

Sample	Weight [g]	Particle number	Particles [mg/kg]
1	5,00	101	40,4
2	5,00	108	43,2
3	5,00	106	42,4
4	5,00	108	43,2
5	5,00	115	46,0
6	5,00	116	46,4
7	5,00	128	51,2
8	5,00	118	47,2

Poisson distribution		
Number of samples	8	
Degree of freedom	7	
Mean	112,5	Partikel
Standard deviation	8,49	Partikel
χ^2 (CHI-Quadrat)	4,48	
Probability	**72**	%
Recovery rate	85	%

Normal distribution		
Number of samples	8	
Mean	45,0	mg/kg
Standard deviation	3,39	mg/kg
rel. Standard deviaton	7,5	%
Horwitz standard deviation	9,0	%
HorRat-value	**0,84**	
Recovery rate	85	%

Microtracer Homogeneity Test
DLA 03-2017 Spiking Level Sample

Weight whole sample	1,01	kg
Microtracer	FSS-rot lake	
Particle size	75 – 300	µm
Weight per particle	2,0	µg
Addition of tracer	62,3	mg/kg

Result of analysis

Sample	Weight [g]	Particle number	Particles [mg/kg]
1	5,00	160	64,0
2	5,00	148	59,2
3	5,00	138	55,2
4	5,00	156	62,4
5	5,00	147	58,8
6	5,00	132	52,8
7	5,00	148	59,2
8	5,00	156	62,4

Poisson distribution		
Number of samples	8	
Degree of freedom	7	
Mean	148,1	Partikel
Standard deviation	9,48	Partikel
χ^2 (CHI-Quadrat)	4,25	
Probability	**75**	%
Recovery rate	95	%

Normal distribution		
Number of samples	8	
Mean	59,3	mg/kg
Standard deviation	3,79	mg/kg
rel. Standard deviaton	6,4	%
Horwitz standard deviation	8,7	%
HorRat-value	**0,7**	
Recovery rate	95	%

5.3 Information on the Proficiency Test (PT)

Before the PT the participants received the following information in the sample cover letter:

PT number	***DLA 03-2017***
PT name	***DLA 03/2017 - Allergens III: β-Lactoglobulin, Casein and Gluten in Infant Food***
Sample matrix	***Samples A + B:*** *"Gluten-free" cereal pap (powder)/ ingredients: sorghum whole flour, natural rice flour, thiamine and other food additives and allergenic foods (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 2 - 10°C)* *Spiking Level Sample: room temperature*
Intentional use	*Laboratory use only (quality control samples)*
Parameter	*qualitative + quantitative: Milk (β-Lactoglobulin, Casein, DNA), Gluten (Wheat protein, Wheat-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.*
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. In case of several determinations the mean.*
Units	*mg/kg*
Number of digits	*at least 2 significant digits*
Result submission	*The result submission file should be sent by e-mail to:* ***pt@dla-lvu.de***
Deadline	***the latest April 13th 2017***
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, PhD*

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

6. Index of participant laboratories in alphabetical order

Teilnehmer / Participant	Ort / Town	Land / Country
		Germany
		ITALY
		Germany
		SPAIN
		Germany
		SWEDEN
		AUSTRIA
		Germany
		ITALY
		BELGIUM
		Germany
		GREAT BRITAN
		Germany
		NETHERLAND
		Germany
		SWEDEN
		SPAIN
		Germany

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

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

7. Index of references

1. DIN EN ISO/IEC 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 Anan-lytical 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. 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
17. 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
18. 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
19. DIN EN ISO 15842:2010 Lebensmittel – Nachweis von Lebensmittelallergenen – Allgemeine Betrachtungen und Validierung von Verfahren / Foodstuffs - Detection of food allergens - General considerations and validation of methods

20. Ministry of Health and Welfare, JSM, Japan 2006
21. 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)
22. 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)
23. 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)
24. 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
25. 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
26. 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
27. ASU §64 LFGB L 06.00-56 Bestimmung von Sojaprotein in Fleisch und Fleischerzeugnissen Enzymimmunologisches Verfahren (2007) [Determination of soyprotein in meat and meat products by enzyme immunoassay]
28. ASU §64 LFGB L 00.00-69 Bestimmung von Erdnuss-Kontaminationen in Lebensmitteln mittels ELISA im Mikrotiterplattensystem (2003) [Foodstuffs, determination of peanut contamintions in foodstuffs by ELISA in microtiterplates]
29. ASU §64 LFGB L 44.00-7 Bestimmung von Haselnuss-Kontaminationen in Schokolade und Schokoladenwaren mittels ELISA im Mikrotiterplattensystem (2006) [Foodstuffs, determination of hazelnut contamintions in chocolate and chocolate products by ELISA in microtiterplates]
30. 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) [Foodstuffs, detection and determination of mustard (Sinapis alba) and soya (Glycine max) in boiled sausages by real-time PCR]
31. 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) [Foodstuffs, simultaneous detection and determination of black mustard (Brassica nigra L.), brown mustard (Brassica juncea L.), white mustard (Sinapis alba), celery (Apium graveolens) and soya (Glycine max) in boiled sausages by real-time PCR]
32. 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) [Foodstuffs, detection and determination of wheat (Triticum L.) and rye (Secale cereale) in boiled sausages by real-time PCR]