Evaluation Report
proficiency test

DLA 10/2019

Allergens X:

Gluten from Durum Wheat

in “gluten free” Noodles

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

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Unteraufträge *Subcontractors*	Falls im Rahmen der Eignungsprüfung eine Prüfung der Gehalte, Homogenität und Stabilität von EP-Parametern durchgeführt wurde, hat DLA diese im Unterauftrag vergeben. In case the analysis of the content, homogeneity and stability of PT-parameters was part of the proficiency test, the determinations were subcontracted by DLA.
Vertraulichkeit *Confidentiality*	Die Teilnehmerergebnisse sind im EP-Bericht in anonymisierter Form mit Auswertenummern benannt. Daten einzelner Teilnehmer werden ausschließlich nach vorheriger Zustimmung des Teilnehmers an Dritte weitergegeben. Participant result are named anonymously with evaluation numbers in the PT report. Data of individual participants will be passed on to third parties only with prior consent of the participant.

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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. One of the samples (spiked sample) contains the respective allergenic ingredients.

The test material of the food matrix samples are customary "gluten free" noodles. The basic composition of samples A and B was the same (see table 1). After crushing and sieving (mesh <1,5 mm) the basic mixture was homogenized.
Afterwards the **spiked sample B** was produced as follows:
The spiking material, noodles from durum wheat, were crushed and sieved by a centrifugal mill (mesh <250 µm), added to an aliquot of the basic mixture and the mixture was homogenized. Subsequently, the basic mixture was again added in up to two additional steps and homogenized in each case until the total quantity had been reached.

The samples A and B were portioned to approximately 25 g in metallized PET film bags.

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Table 1: Composition of DLA-Samples

Ingredients	**Sample A**	**Sample B**
"Gluten free" noodles, Ingredients: Maize flour, emulgator: mono and diglycerides of fatty acids Nutrients per 100 g: Fat 1,0 g, Carbohydrates 79 g, fiber 2,0 g Protein 6,5 g, salt 0,03 g	100 g/100 g	99,95 g/100g
Noodles, organic Ingredients: Durum wheat semolina - as durum wheat* - *thereof 12% total protein*** - *thereof gluten****	-	 544 mg/kg 65,6 mg/kg 41,9 mg/kg

* 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=5,7 for wheat protein)
*** Protein contents according to literature values: 7,7% gluten in durum wheat [36]

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

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2.1.1 Homogeneity

The **mixture homogeneity before bottling** was examined 8-fold by **microtracer analysis.** It is a standardized method that is part of the international GMP certification system for feed [14].
Before mixing dye coated iron particles of µm size are added to the sample and the number of particles is determined after homogenization in taken aliquots. The evaluation of the mixture homogeneity is based on the Poisson distribution using the chi-square test. A probability of ≥ 5 % is equivalent to a good homogeneous mixture and of ≥ 25% to an excellent mixture [14, 15].
The microtracer analysis of the present PT sample B showed a probability of 84%. Additionally particle number results were converted into concentrations, statistically evaluated according to normal distribution and compared to the standard deviation according to Horwitz. For the assessment HorRat values between 0,3 and 1,3 are to be accepted under repeat conditions (measurements within the laboratory) [17].
This gave a HorRat value of 1,3. The results of microtracer analysis are given in the documentation.

2.1.2 Stability

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

The experience with various DLA test materials showed good storage stability with respect to the durability of the sample (spoilage) and the content of the PT parameters for comparable food matrices and water activity (a_W value <0,5).
The a_W value of the EP samples was approx. 0,47 (24,0°C). The stability of the sample material was thus ensured during the investigation period under the specified storage conditions.

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2.2 Sample shipment and information to the test

The portions of test materials sample A and B were sent to every participating laboratory in the 26th week of 2019. The testing method was optional. The tests should be finished at 23rd August 2019 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 parameter Gluten in the range of mg/kg in the matrix of gluten-free Noodles. One of these samples was prepared adding the allergenic ingredient.

Note: The sample matrix was crushed by means of an impact mill (mesh <1,5 mm). Accordingly, it has a relatively broad particle size distribution. Therefore, before analysis, the whole sample quantities should be homogenized.

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 handed out with the samples (by email).
On one hand the results given as positive/negative and on the other hand the indicated results of the allergenic ingredients e.g. total food item or protein in mg/kg were evaluated.
Queried and documented were the indicated results and details of the test methods like specificity, limit of quantifications, 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 16 participants submitted results.

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3. Evaluation

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

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

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

3.1 Consensus value from participants (assigned value)

The **robust mean** of the submitted results was used as assigned value (X_{pt}) („consensus value from participants") providing a normal distribution. The calculation was done according to algorithm A as described in annex C of ISO 13528 [3]. If there are < 12 quantitative results and an increased difference between robust mean and median, the **median** may be used as the assigned value (criterion: Δ median - rob. mean > 0,3 σ_{pt}) [3].
The condition is that the majority of the participants' results show a normal distribution or are distributed unimodal and symmetrically. To this end, an examination of the distribution is carried out, inter alia, using the kernel density estimate [3, 12].
In case there are indications for sources of higher variability such as a bimodal distribution of results, a cause analysis is performed. Frequently different analytical methods may cause an anomaly in results' distribution. If this is the case, separate evaluations with own assigned values (X_{pti}) are made whenever possible.

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

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

Single results giving values outside the measuring range of the participating laboratory or given as „0" are not considered for statistical evaluation (e.g. results given as > 25 mg/kg and

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< 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*) 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^*_{ALL}***
ii) ***Robust standard deviation of single methods - $S^*_{METHOD\ i}$***
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, too few significant digits (valid digits) or results for another proficiency test item can be removed from the data set [2]. Even if a result e.g. with a factor >10 deviates significantly from the mean and has an influence on the robust statistics, a result of the statistical evaluation can be excluded [3].
All results should be given at least with 2 significant digits. Specifying 3 significant digits is usually sufficient.

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

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

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3.4 Target standard deviation (for proficiency assessment)

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

3.4.1 General model (Horwitz)

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

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

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

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

3.4.2 Value by precision experiment

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

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

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

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

Parameter	Matrix	Mean [mg/kg]	Recovery	rob RSD	RSD_r	RSD_R	σ_{pt}	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 12 – 33% for the ELISA methods and 18 – 37% for the PCR methods depending on the matrix, processing and concentration level of allergens (s. Tab. 2a and 2b).

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

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

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Table 2b: PCR-Methods - Relative repeatability standard deviations (RSD_r) and relative reproducibility standard deviations (RSD_R) from precision experiments and resulting target standard deviations σ_{pt} [32-35]

Parameter	**Matrix**	**Mean [mg/kg]**	**Recovery**	**rob RSD**	**RSD_r**	**RSD_R**	**σ_{pt}**	**Method / Literature**
Soya	Wheat flour Maize flour	107 145	107 % 145 %	63 % 34 %	- -	31 % 24 %	- -	rt-PCR ASU 16.01-9
Soya flour	Boiled 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

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3.4.3 Value by perception

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

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

Table 3: ELISA-Validation

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

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

Table 4: PCR-Validation

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

(a) = Trueness / Richtigkeit

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

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3.5 z-Score

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

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

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

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

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

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

3.5.1 Warning and action signals

In accordance with the norm ISO 13528 it is recommended that a result that gives rise to a *z*-score above 3,0 or below −3,0, shall be considered to give an "action signal" [3]. Likewise, a *z*-score above 2,0 or below −2,0 shall be considered to give a "warning signal". A single "action signal", or "warning signal" in two successive PT-rounds, shall be taken as evidence that an anomaly has occurred which requires investigation.

An error or cause analysis can be carried out by checking the analysis process including understanding and implementation of the measurement by the staff, details of the measurement procedure, calibration of equipment and composition of reagents, transmission or calculation errors, trueness and precision and use of reference material. 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].

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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_i) of the participant from the respective consensus value (X) to the square root of quadrat sum of the target standard deviation (σ_{pt}) and the standard uncertainty (Ux_{pt}) [3].

The calculation is performed by:

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

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

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

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

For warning and action signals see 3.5.1.

3.7 Quotient S*/σ_{pt}

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

3.8 Standard uncertainty and traceability

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

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

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

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

3.9 Figures

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

3.10 Recovery rates: Spiking

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

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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). The recovery rates of results for 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.

In the present PT all gluten ELISA results were submitted as gluten, therefore no conversion 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.

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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})	$\boldsymbol{X_{pt_{ALL}}}$	$\boldsymbol{X_{pt_{METHOD\ i}}}$
Number of results		
Number of outliers		
Mean		
Median		
Robust mean (X_{pt})		
Robust standard deviation (S*)		
Target data°:		
Target standard deviation σ_{pt} *or* σ_{pt}'		
lower limit of target range ($X_{pt} - 2\sigma_{pt}$) or ($X_{pt} - 2\sigma_{pt}'$)°		
upper limit of target range ($X_{pt} + 2\sigma_{pt}$) or ($X_{pt} + 2\sigma_{pt}'$)°		
Quotient S*/σ_{pt} *or* S*/σ_{pt}'		
Standard uncertainty $U_{(Xpt)}$		
Number of results in target range		
Percent in target range		

° Target range calculated using z-score or z'-score

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

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4.1 Proficiency Test Durum Wheat (Gluten)

4.1.1 ELISA-Results: Gluten

Qualitative valuation of results: Samples A and B

Evaluation number	Sample A	Sample A	Sample B	Sample B	Qualitative Valuation	Method	Remarks
	pos/neg	[mg/kg]	pos/neg	[mg/kg]	Agreement with consensus value		
5a	negative	<3,12	positive	57,0	2/2 (100%)	EF	
8	negative	<0,8	positive	148	2/2 (100%)	IL	
10	positive	63,3	positive	415	1/2 (50%)	IL	
2	negative	<3	positive	19,3	2/2 (100%)	RS	
4	negative		positive	22,2	2/2 (100%)	RS	
5b	negative	<5	positive	30,0	2/2 (100%)	RS	
7	negative	<3	positive	25,0	2/2 (100%)	RS	
9	negative		positive	25,2	2/2 (100%)	RS	
11	negative		positive	36,5	2/2 (100%)	RS	
13	negative	<5,00	positive	43,4	2/2 (100%)	RS	
14a	negative	< 5,0	positive	21,8	2/2 (100%)	RS	
14b	negative	< 5,0	positive	22,4	2/2 (100%)	RS	
15	negative		positive	45,0	2/2 (100%)	RS	
16	negative	< BG	positive	31,0	2/2 (100%)	RS	
12	negative	< 10	positive	94,0	2/2 (100%)	RS-C	
6	negative	<10	positive	21,1	2/2 (100%)	RS-F	
1a	negative	<5	positive	40,0	2/2 (100%)	VT	
1b	negative	<5	positive	50,0	2/2 (100%)	VT	
3	positive	12,4	positive	31,4	1/2 (50%)	VT	Mean calculated by DLA

	Sample A		Sample B	
Number positive	2		19	
Number negative	17		0	
Percent positive	11		100	
Percent negative	89		0	
Consensus value	negative		positive	

Methods:

EF-R5 = SensiSpec Ingezim Gluten R5, Eurofins
IL = Immunolab
RS = Ridascreen®, R-Biopharm
RS-C = Ridascreen® competitive, R-Biopharm
RS-F= Ridascreen® Fast, R-Biopharm
VT-R5 = Veratox, Neogen

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

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Quantitative valuation of ELISA-results: Sample B

Evaluation number	Gluten	z-Score Xpt_{ALL}	z-Score Xpt_{RS}	Method	Remarks
	[mg/kg]				
5a	57,0	2,0		EF	
8	148	12		IL	
10	415	40		IL	
2	19,3	-2,0	-1,4	RS	
4	22,2	-1,6	-0,95	RS	
5b	30,0	-0,82	0,12	RS	
7	25,0	-1,3	-0,57	RS	
9	25,2	-1,3	-0,55	RS	
11	36,5	-0,13	1,0	RS	
13	43,4	0,60	2,0	RS	
14a	21,8	-1,7	-1,0	RS	
14b	22,4	-1,6	-0,93	RS	
15	45,0	0,77	2,2	RS	
16	31,0	-0,71	0,25	RS	
12	94,0	6,0		RS-C	
6	21,1	-1,8		RS-F	
1a	40,0	0,24		VT	
1b	50,0	1,3		VT	
3	31,4	-0,67		VT	

Methods:

EF-R5 = SensiSpec Ingezim Gluten R5, Eurofins
IL = Immunolab
RS = Ridascreen®, R-Biopharm
RS-C = Ridascreen® competitive, R-Biopharm
RS-F= Ridascreen® Fast, R-Biopharm
VT-R5 = Veratox, Neogen

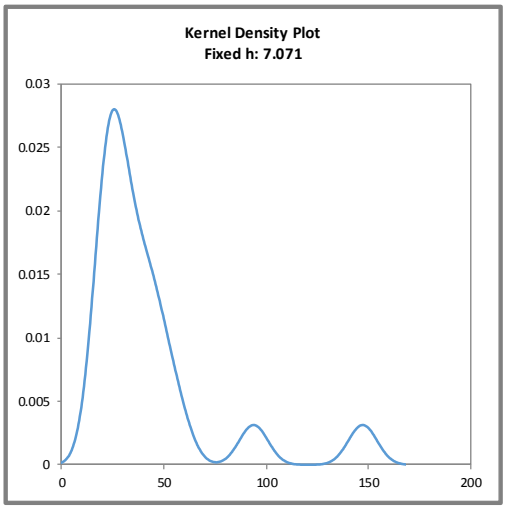

Abb. / Fig. 1:
Kerndichte-Schätzung aller ELISA-Ergebnisse (mit h = 0,75 x σ_{pt} von Xpt_{ALL})

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

Comments:
The kernel density estimation shows nearly a symmetric distribution of results with three side peak, due to single values above the target range (side peak at approx. 400 mg/kg not shown).

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Characteristics: Quantitative evaluation ELISA Gluten

Sample B

Statistic Data	**All Results** *[mg/kg]*	**Method RS** *[mg/kg]*
Assigned value (X_{pt})	$X_{pt_{ALL}}$	$X_{pt_{METHOD\ RS}}$
Number of results	19	11
Number of outliers	-	0
Mean	62,0	29,2
Median	31,4	25,2
Robust Mean (X_{pt})	**37,7**	**29,2**
Robust standard deviation (S*)	**18,3**	**9,88**
Target range:		
Target standard deviation σ_{pt}	**9,43**	**7,29**
lower limit of target range	**18,9**	**14,6**
upper limit of target range	**56,6**	**43,7**
Quotient S/σ_{pt}*	*1,9*	*1,4*
Standard uncertainty $U_{(Xpt)}$	*5,24*	*3,73*
Results in the target range	*16*	*10*
Percent in the target range	*84*	*91*

Method:
RS = R-Biopharm, Ridascreen®

Comments to the statistical characteristics and assigned values:

The kernel density estimation showed nearly a symmetrical distribution of results with single values above the target range.

The evaluations of all methods and method RS showed a normal variability of results, with quotients S/σ_{pt} below 2,0.*
The robust standard deviation is for the results of all methods above and for method RS in the upper range of established values for the reproducibility standard deviation of the applied methods (see 3.4.2 value by precision experiments and 3.4.3 value by perception). The comparability of results is limited for the evaluation across the methods, because there were only a few results for some methods.
The robust means of the evaluations were 90% and 70% of the spiking level of gluten to sample B within the range of the recommendations for the applied methods (s. 3.4.3 and "Recovery rates ELISA for Gluten" p.24).

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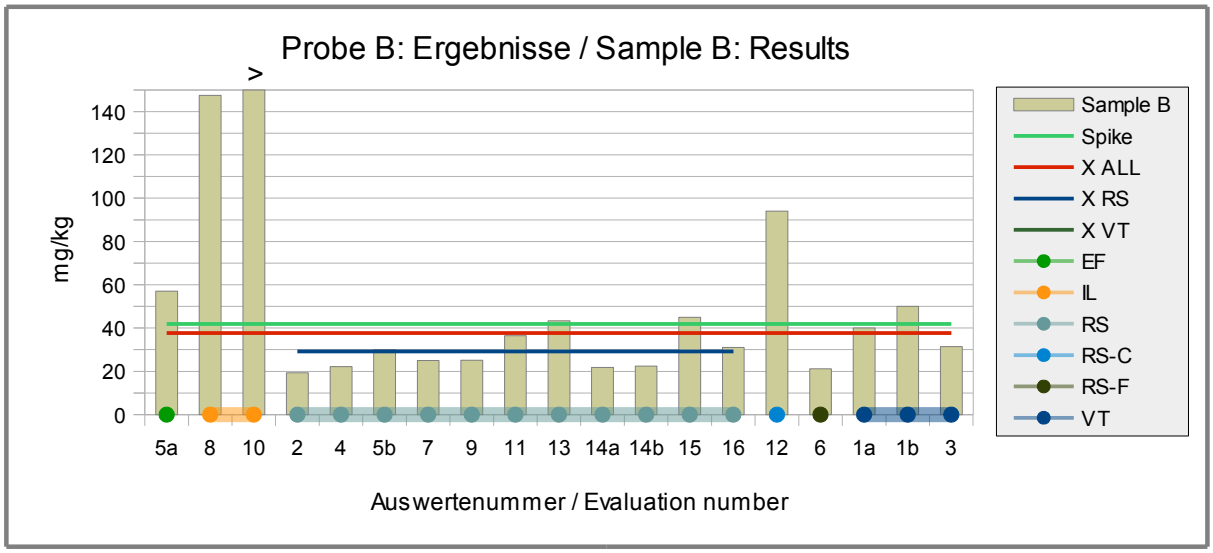

Abb./Fig. 2: ELISA Results Gluten
green line = Spiking level
red line = Assigned value robust mean all results
blue line = Assigned value robust mean results method RS
round symbols = Applied methods (see legend)

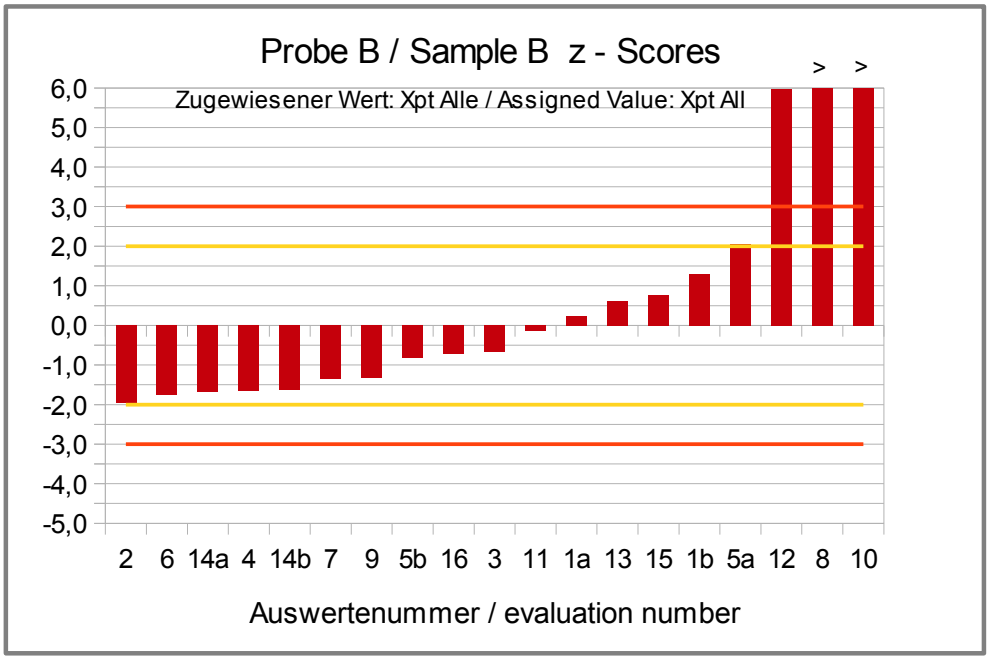

Abb./Fig. 3:
z'-Scores (ELISA Results Gluten)
Assigned value robust mean of all results

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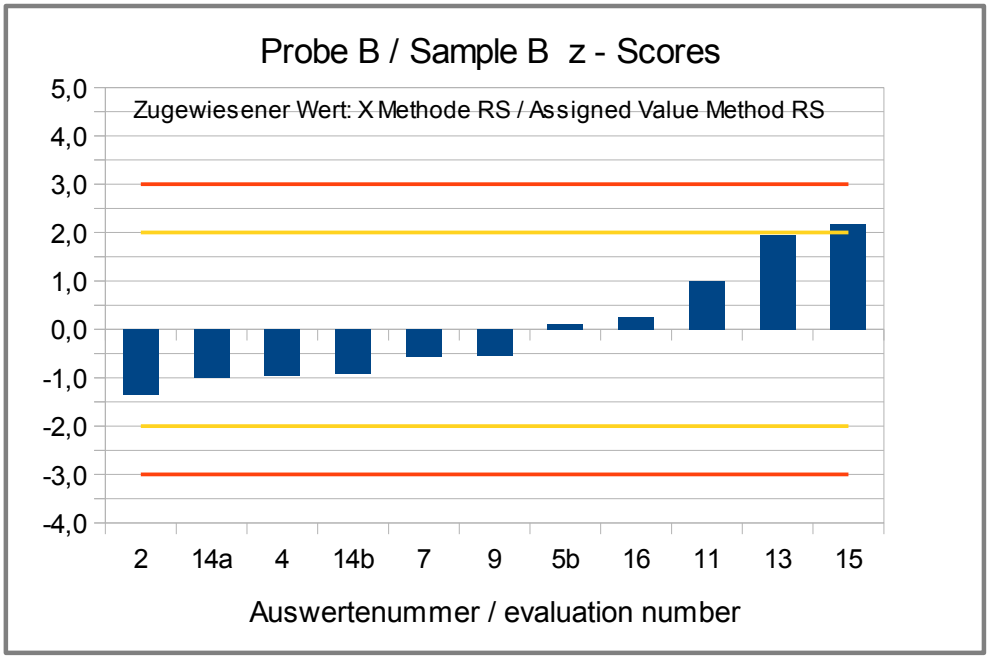

Abb./Fig. 4:
z-Scores for information (ELISA Results Gluten)
Assigned value robust mean of method RS (R-Biopharm, Ridascreen)

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Recovery Rates ELISA for Gluten:

Evaluation number	Sample B	Recovery rate*	Method	Remarks
	[mg/kg]	[%]		
5a	57,0	**136**	EF	
8	148	352	IL	
10	415	991	IL	
2	19,3	46	RS	
4	22,2	**53**	RS	
5b	30,0	**72**	RS	
7	25,0	**60**	RS	
9	25,2	**60**	RS	
11	36,5	**87**	RS	
13	43,4	**104**	RS	
14a	21,8	**52**	RS	
14b	22,4	**53**	RS	
15	45,0	**107**	RS	
16	31,0	**74**	RS	
12	94,0	224	RS-C	
6	21,1	**50**	RS-F	
1a	40,0	**95**	VT	
1b	50,0	**119**	VT	
3	31,4	**75**	VT	

RA**	50-150 %
Number in RA	**15**
Percent in RA	**79**

Methods:
EF-R5 = SensiSpec Ingezim Gluten R5, Eurofins
IL = Immunolab
RS = Ridascreen®, R-Biopharm
RS-C = Ridascreen® competitive, R-Biopharm
RS-F= Ridascreen® Fast, R-Biopharm
VT-R5 = Veratox, Neogen

* Recovery rate 100% relative size: gluten, s. Page 5
** Range of acceptance of AOAC for allergen ELISAS

Comments:
For the spiked food matrix sample B 79% (15) of the recovery rates by ELISA methods were within the range of the AOAC-recommendation of 50-150%.

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4.1.2 PCR-Results: Durum Wheat/ 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		
9	negative		positive		2/2 (100%)	SFA	
11	positive		positive		1/2 (50%)	SFA	Sample A: positive < LOQ (1 mg/kg)
1a	negative		positive		2/2 (100%)	div	
1b	negative		positive		2/2 (100%)	div	
4	negative		positive		2/2 (100%)	div	
16	negative		positive		2/2 (100%)	div	

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

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

Comments:
The consensus value is in qualitative agreement with the spiking of sample B. For sample A one positive result was obtained with the method SFA (Sure Food Allergen).

Quantitative valuation of PCR-results: Sample B

A quantitative evaluation of results was not carried out because there were no quantitative results.

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4.1.3 Further 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		
16	negative		positive		2/2 (100%)	RQ	

	Sample A		Sample B	
Spiking	negative		positive	

Methods:
RQ = RIDA®QUICK (Lateral Flow), R-Biopharm

Comments:
The results of the participant are in qualitative agreement with the spiking of sample B.

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5. Documentation

5.1 Details by the participants

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

5.1.1 ELISA: Gluten

Meth. Abr.	Evaluation number	Date of Analysis	Result Sample A		Result Sample B		NWG / LOD *	BG / LOQ *	MU*	quantitative Result given as	Method
		day/month	qualitative	mg/kg	qualitative	mg/kg	mg/kg	mg/kg	%	e.g. food /protein	ELISA Test-Kit+Manufacturer
EF	5a	30.07.19	negative	<3,12	positive	57	3,12	3,12		Gluten	SENSISpec Ingezim Gluten R5 30.GLU.K2, Eurofins
IL	8	04.07.19	negative	<0,8	positive	147,5				Gluten	Immunolab Gliadin/Gluten ELISA
IL	10	16.07.19	positive	63,27	positive	415,3	0,3	2		Gluten	Immunolab Gliadin/Gluten ELISA
RS	2	23.08.19	negative	<3	positive	19,27	3	3	31,94	Gluten	Ridascreen® Gliadin R7001, R-Biopharm
RS	4	25.07.19	negative		positive	22,2	5	5	23	Gluten	Ridascreen® Gliadin R7001, R-Biopharm
RS	5b	03.07.19	negative	<5	positive	30	3	5		Gluten	Ridascreen® Gliadin R7001, R-Biopharm
RS	7	25.07.19	negative	<3	positive	25	1,5	3	95	Gluten	Ridascreen® Gliadin R7001, R-Biopharm
RS	9		negative		positive	25,17	3	5		Gluten	Ridascreen® Gliadin R7001, R-Biopharm
RS	11	07.08.19	negative		positive	36,5	3	5	30	Gluten	Ridascreen® Gliadin R7001, R-Biopharm
RS	13	10.07.19	dd	<5,00	-	43,4				Gluten	Ridascreen® Gliadin R7001, R-Biopharm
RS	14a	08.07.	negative	< 5,0	positive	21,8	1	5		Gluten	Ridascreen® Gliadin R7001, R-Biopharm
RS	14b	08.07.	negative	< 5,0	positive	22,4	1	5		Gluten	Ridascreen® Gliadin R7001, R-Biopharm
RS	15	11.07.19	negative		positive	45	5	5	43	Gluten	Ridascreen® Gliadin R7001, R-Biopharm
RS	16	08.07.19	-	< BG	-	31	1	5	10	Gluten	Ridascreen® Gliadin R7001, R-Biopharm
RS-C	12	26.07.19	negative	< 10	positive	94				Gluten	Ridascreen® Gliadin competitive R7021, R-Biopharm
RS-F	6	21.08.19	negative	<10	positive	21,14	1	10		Gluten	Ridascreen® FAST Gliadin R7002, R-Biopharm
VT	1a	23.7./27.8.19	negative	<5	positive	40	5	5		Gluten	Veratox Gliadin R5, Neogen
VT	1b	23./24.07.19	negative	<5	positive	50	5	5		Gluten	Veratox Gliadin R5, Neogen
VT	3a	22.08.19	Positive	12,2	Positive	35,6	2	3,73	12,9	Gluten	Veratox Gliadin R5, Neogen
VT	3a	22.08.19	Positive	12,6	Positive	27,2	2	3,73	12,9	Gluten	Veratox Gliadin R5, Neogen

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

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Continuation ELISA Gluten:

Meth. Abr.	Evaluation number	Specifity	Remarks to the Method (Extraction and Determination)	Method accredited ISO/IEC 17025	Further Remarks
		Antibody	e.g. Extraction Solution / Time / Temperature	yes/no	
EF	5a	R5 Mendez, recognizes Prolamine from w heat, rye and barley	according to manufacturer's instructions	no	
IL	8				
IL	10		40%ethanol /5min/22C	yes	
RS	2	As Per Kit Instructions	As Per Kit Instructions	Yes	
RS	4	R5 (against Prolamine from w heat, rye, barley)		yes	
RS	5b	R5 Mendez, recognizes Prolamine from w heat, rye and barley	according to manufacturer's instructions	yes	
RS	7		Cocktail Solution r-biopharm	yes	
RS	9			yes	
RS	11		according to Manual	yes	Specificity: Prolamins from w heat (gliadin), rye and barley
RS	13	R5	Cocktail (patented) R7006 used for sample preparation, evaluations softw are version 1.106.0.0240beta	no	1. proficiency test for validation ELISA
RS	14a	according to manufacturer's instructions w ith Cocktail solution/Ethanol	yes		
RS	14b	according to manufacturer's instructions w ith skimmed milk pow der Cocktail solution/Ethanol	yes		
RS	15	R5 Antibody	according to manual	yes	
RS	16	monoclonal R5.Antibody	Cocktail (patent -R7006)/ according to kit manufacturer's manual	yes (according to DIN EN ISO/IEC 17025:2005)	
RS-C	12	R5	60% ethanol + fish gelatin / 20 min / room temperature	yes	AR results
RS-F	6	Peroxidase coupled R5 Antibody	Rida Extraction Solution (colorless) Art. Nr R7098 / Method according to R-biopharm's instructions	no	
VT	1a	Gliadin R5	Protocol for heat-treated samples	yes	
VT	1b	Gliadin R5	Protocol for not heat-treated samples	yes	
VT	3a	R5	0,25 g + 2,5 mL renaturing cocktail solution (w / extraction additive); incubation at 50°C, 40 min; final volume 10 mL; dilution 1:12,5.		
VT	3a	R6	0,25 g + 2,5 mL renaturing cocktail solution (w / extraction additive); incubation at 50°C, 40 min; final volume 10 mL; dilution 1:12,5.		

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5.1.2 PCR: Wheat/ Gluten

Meth. Abr.	Evaluation number	Date of Analysis	Result Sample A		Result Sample B		NWG / LOD *	BG / LOQ *	MU*	quantitative Result given as	Method
		day/month	qualitative	mg/kg	qualitative	mg/kg	mg/kg	mg/kg	%	e.g. food /protein	PCR Test-Kit+Manufacturer
SFA	9		negative		positive		0,4			Gluten	Sure Food ALLERGEN, R-Biopharm / Congen
SFA	11	04.07.19	positive		positive		0,4	1	30	gluten containing cereal	Sure Food ALLERGEN, R-Biopharm / Congen
div	1a	15.08.19	negative		positive					Weizen-DNA	realtime PCR
div	1b	15.08.19	negative		positive					Weizen-DNA	realtime PCR
div	4		negative		positive					Please select!	Alary et al. 2002. Quantification of common wheat adulteration of durum wheat pasta using real-time quantitative Polymerase chain reaction. Cereal Chem 79 (4) 553-558
div	16	15.07.19	negative		positive		-	-	-	Weizen-DNA	ALARY et al. 2002

* NWG Nachw eisgrenze / BG Bestimmungsgrenze

* LOD limit of detection / LOQ limit of quantitation

* MU Messunsicherheit / MU measurement uncertainty

Meth. Abr.	Evaluation number	Specifity	Remarks to the Method (Extraction and Determination)	Method accredited ISO/IEC 17025	Further Remarks
		Target-Sequence / -DNA	e.g. Extraction / enzymes / clean-up / real time PCR / gel electrophoresis / cycles	yes/no	
SFA	9			no	
SFA	11	Wheat like spelt and Khorasan-w heat, rye, barley, oat	SureFood Prep Advanced Protokoll 1	yes	the result for sample A w as below LOQ but above LOD Artikelnr.: S3606
div	1a	Gliadin gene		yes	LOD: 10-20 DNA-copies
div	1b	HMW-Glutenin gene		no	LOD: 10-20 DNA-copies
div	4				
div	16	Lipid-Transfer protein gene (ltp)	CTAB precipitation, QIAgen PCR Purification Kit, Real Time PCR	yes (according to DIN EN ISO/IEC 17025:2005)	

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5.1.3 Further results: Gluten

Meth. Abr.	Evaluation number	Date of Analysis	Result Sample A		Result Sample B		NWG / LOD *	BG / LOQ *	MU*	quantitative Result given as	Method
		day/month	qualitative	mg/kg	qualitative	mg/kg	mg/kg	mg/kg	%	e.g. food /protein	Test-Kit+Manufacturer/ Literature
RQ	16	04.07.19	negative		positive		approx. 6,3			Gluten	RIDA® QUICK Gliadin R7003, R-Biopharm

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

Meth. Abr.	Evaluation number	Specifity	Remarks to the Method (Extraction and Determination)	Method accredited ISO/IEC 17025	Further Remarks
RQ	16	monoclonal R5.Antibody	Cocktail (patent -R7006)/ according to kit manufacturer's manual	yes (according to DIN EN ISO/IEC 17025:2005)	

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5.2 Homogeneity

5.2.1 Mixture homogeneity before bottling

Microtracer Homogeneity Test
DLA 10-2019 Sample B

Weight whole sample	2,80	kg
Microtracer	FSS-rot lake	
Particle size	75 – 300	µm
Weight per particle	2,0	µg
Addition of tracer	21,3	mg/kg

Result of analysis

Sample	Weight [g]	Particle number	Particles [mg/kg]
1	5,00	18	7,2
2	5,07	18	7,1
3	5,15	22	8,5
4	5,00	24	9,6
5	5,37	18	6,7
6	4,80	20	8,3
7	5,09	15	5,9
8	5,09	22	8,6

Poisson distribution		
Number of samples	8	
Degree of freedom	7	
Mean	19,7	Particles
Standard deviation	3,10	Particles
χ^2 (CHI-Quadrat)	3,42	
Probability	**84**	%
Recovery rate	36	%

Normal distribution		
Number of samples	8	
Mean	7,8	mg/kg
Standard deviation	1,22	mg/kg
rel. Standard deviaton	15,8	%
Horwitz standard deviation	11,8	%
HorRat-value	**1,3**	
Recovery rate	36	%

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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 10-2019***
PT name	***Allergens X: Gluten from Durum Wheat in „gluten-free“ Noodles***
Sample matrix (processing)	***Samples A + B:*** *"Gluten-free" Noodles/ ingredients: Maize flour, emulgator: mono and diglycerides of fatty acids and allergenic food Durum Wheat noodles (one of both samples)*
Number of samples and sample amount	*2 different Samples A + B: 25 g each*
Storage	*Samples A + B: room temperature (long term cooled 2 - 10°C)*
Intentional use	*Laboratory use only (quality control samples)*
Parameter	*qualitative + quantitative: Gluten / Wheat (protein / DNA)* *Samples A + B: < 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. From* ***Samples A + B*** *the* ***total sample amount*** *should be* ***homogenized*** *each.*
Result sheet	*One result each should be determined for Samples A and B.* *The results should be filled in the result submission file.*
Units	*mg/kg*
Number of digits	*at least 2*
Result submission	*The result submission file should be sent by e-mail to:* ***pt@dla-lvu.de***
Deadline	***the latest August 23rd 2019***
Evaluation report	*The evaluation report is expected to be completed 6 weeks after deadline of result submission and sent as PDF file by e-mail.*
Coordinator and contact person of PT	*Matthias Besler-Scharf PhD*

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

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6. Index of participant laboratories in alphabetical order

Teilnehmer / Participant	Ort / Town	Land / Country
		Germany
		ITALY
		SWITZERLAND
		Germany
		ITALY
		Germany
		Germany
		Germany
		Germany
		Germany
		Germany
		ARGENTINA
		GREAT BRITAIN
		GREECE
		AUSTRIA
		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.]

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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
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17. AOAC Official Methods of Analysis: Guidelines for Standard Method Performance Requirements, Appendix F, p. 2, AOAC Int (2016)
18. Codex Alimentarius Commission (2010) - Guidelines on performance criteria and validation of methods for detection, identification and quantification of specific DNA sequences and specific proteins in foods, CAC/GL 74-2010
19. DIN EN ISO 15633-1:2009; Nachweis von Lebensmittelallergenen mit immunologischen Verfahren - Teil 1: Allgemeine Betrachtungen /

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