**Evaluation Report**
proficiency test

**DLA ptAL02 (2021)**

# Allergens II:

# Lupin and Wheat (Gluten)

## in "gluten-free" Crispbread

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

| | |
|---|---|
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| *Unteraufträge*<br>*Subcontractors* | Im Rahmen dieser Eignungsprüfung wurden nachstehende Leistungen im Unterauftrag vergeben: Homogenitätsprüfung der EP-Parameter, Proteinbestimmung<br>As part of the present proficency test the following services were subcontracted: Homogeneity tests of PT-parameter(s), protein determination |
| *Vertraulichkeit*<br>*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.<br>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. |
| *Akkreditierung*<br>*Accreditation*<br><br>ilac-MRA DAkkS Deutsche Akkreditierungsstelle D-EP-21534-01-00 | nach / according to: ISO/IEC 17.043-2010<br><br>Konformitätsbewertung - Allgemeine Anforderungen an Eignungsprüfungen<br>Conformity Assessment - General Requirements for Proficiency Testing<br><br>Die Akkreditierung gilt für den in der Urkundenanlage genannten Umfang.<br>The accreditation is valid for the scope of the annex to the certificate of accreditation |

# Contents

# 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 of the food matrix samples is a customary "gluten-free" crispbread. The basic composition of samples A and B was the same (see table 1).
After crushing and sieving by means of an impact mill (mesh <1,5 mm) the basic mixture was homogenized.

Afterwards the **spiked sample B** was produced as follows:
An additional ingredient was a crispbread baked with the spiking material (195°C, 30 min) containing the allergenic ingredients lupin and wheat (mesh <500 µm). After drying (40°C, 10 h), crushing, sieving (mesh <1,5 mm) and homogenization, this ingredient was added to an aliquot of the basic mixture and the mixture was homogenized. Subsequently, the basic mixture was again added in several steps and homogenized.

For the **spiking level sample**, the allergenic compounds above mentioned were added during a multi-stage addition of potato powder (mesh <500 µm) and homogenization.

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

Table 1: Composition of DLA-Samples

| **Ingredients** | **Probe A** | **Probe B** | **Dotierungs-niveauprobe** |
|---|---|---|---|
| Crispbread, gluten-free<br>Ingredients: potato starch, amaranth flour, sunflower oil, rice flour, fiber from cane sugar, corn flour, millet, sugar, rice sourdough powder (rice flour, water), emulsifier: mono- and diglycerides of fatty acids, yeast, thickener: hydroxypropylmethyl cellulose, rice protein, spices<br>Nutrients per 100 g:<br>Fat 8,0 g, Carbohydrates 73 g, Fiber 7,7 g, Protein 4,0 g, Salt 1,1 g | 100 g/100 g | 94,1 g/100g | - |
| Crispbread (baked 195°C, 30 min)<br>Ingredients: Rice flour, corn flour, millet flour, buckwheat flour, sunflower oil, salt as well as lupin, wheat flour and further ingredients (see below) | - | 5,87 g/100 g | - |
| Potato Powder<br>Ingredients:<br>Potatoes, E471, E304, E223, E100 | - | - | 99,9 g/100 g |
| *Lupin:*<br>- as sweet lupin flour*<br>- thereof 36,6% total protein** | - | <br>46,7 mg/kg<br>17,1 mg/kg | <br>37,3 mg/kg<br>13,7 mg/kg |
| *Wheat:*<br>Wheat flour mixture (21 products from Europe, Asia, USA)<br>- as wheat flour*<br>- thereof 10,1% total protein**<br>- thereof gluten*** | - | <br><br>476 mg/kg<br>48,1 mg/kg<br>41,4 mg/kg | <br><br>238 mg/kg<br>24,0 mg/kg<br>20,7 mg/kg |
| further Ingredients:<br>Maltodextrin, sodium sulfate and silicon dioxide | - | <0,2 g/100 g | <0,2 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 lupin protein and F=5,7 for wheat protein)
*** Protein contents according to literature values (approx. 8,7% gluten in wheat flour [34, 35, 36])

***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 samples B and the spiking level sample showed a probability of 33% and 93%. 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 1,4 and 0,88. The HorRat value of >1,3 was accepted, because the probability was sufficient proof of homogeneity. The results of microtracer analysis are given in the documentation.

**Homogeneity of bottled spiked sample B**

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

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

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

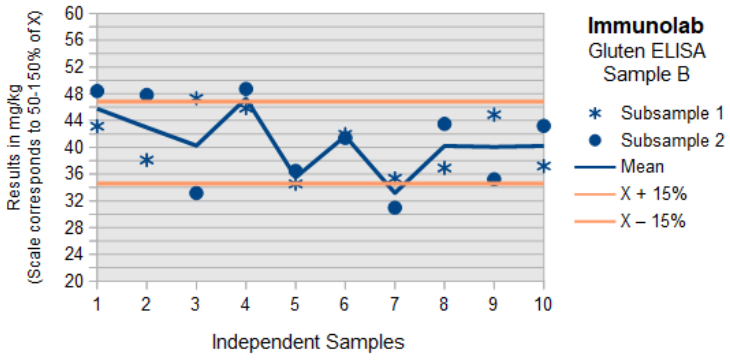
## ELISA-Tests: Homogenität Gluten (Weizen) / Homogeneity Gluten (Wheat)

### *Immunolab Gliadin/Gluten ELISA*

*Sample weights: 1,0 g (0,9 – 1,1 g)*
*Number of replicates: 2*
*Overall result: Gluten 40,7 ± 3,34 mg/kg*

| Sample B | Subsample 1 mg/kg | Subsample 2 mg/kg | Mean mg/kg |
|---|---|---|---|
| 1 | 43,1 | 48,4 | 45,8 |
| 2 | 38,1 | 47,8 | 43,0 |
| 3 | 47,3 | 33,2 | 40,2 |
| 4 | 45,8 | 48,7 | 47,3 |
| 5 | 34,5 | 36,5 | 35,5 |
| 6 | 42,0 | 41,4 | 41,7 |
| 7 | 35,4 | 31,0 | 33,2 |
| 8 | 36,9 | 43,5 | 40,2 |
| 9 | 44,9 | 35,2 | 40,1 |
| 10 | 37,2 | 43,2 | 40,2 |

| | | |
|---|---|---|
| General average X | 40,7 | |
| SD of sample means Sx | 4,21 | 10,4% |
| SD within-samples Sw | 3,63 | 8,9% |
| SD between-samples Ss | 3,34 | 8,2% |

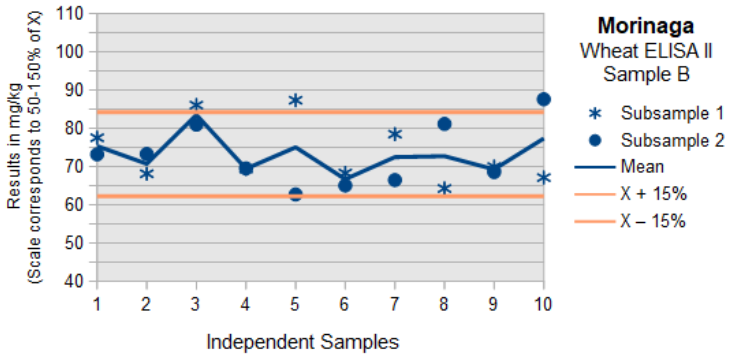



### *Morinaga Wheat ELISA Kit II*

*Sample weights: 1,0 g (0,9 – 1,1 g)*
*Number of replicates: 2*
*Overall result: Wheat protein 73,2 ± 2,04 mg/kg*

| Sample B | Subsample 1 mg/kg | Subsample 2 mg/kg | Mean mg/kg |
|---|---|---|---|
| 1 | 77,5 | 73,2 | 75,3 |
| 2 | 68,1 | 73,3 | 70,7 |
| 3 | 86,0 | 80,9 | 83,5 |
| 4 | 69,3 | 69,5 | 69,4 |
| 5 | 87,3 | 62,7 | 75,0 |
| 6 | 68,3 | 65,0 | 66,7 |
| 7 | 78,4 | 66,4 | 72,4 |
| 8 | 64,2 | 81,1 | 72,7 |
| 9 | 70,0 | 68,5 | 69,2 |
| 10 | 67,1 | 87,6 | 77,3 |

| | | |
|---|---|---|
| General average X | 73,2 | |
| SD of sample means Sx | 4,84 | 6,6% |
| SD within-samples Sw | 6,20 | 8,5% |
| SD between-samples Ss | 2,04 | 2,8% |

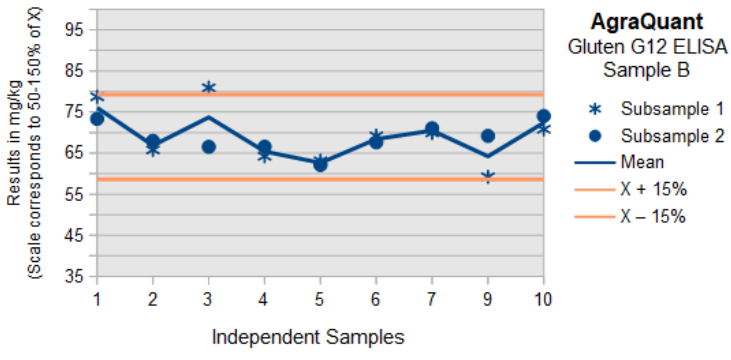



### *AgraQuant Gluten G12 ELISA*

*Sample weights: 1,0 g (0,9 – 1,1 g)*
*Number of replicates: 2*
*Overall result: Gluten 68,9 ± 3,98 mg/kg*

| Sample B | Subsample 1 mg/kg | Subsample 2 mg/kg | Mean mg/kg |
|---|---|---|---|
| 1 | 78,7 | 73,4 | 76,0 |
| 2 | 65,8 | 68,0 | 66,9 |
| 3 | 80,9 | 66,5 | 73,7 |
| 4 | 64,2 | 66,6 | 65,4 |
| 5 | 63,1 | 62,1 | 62,6 |
| 6 | 69,2 | 67,6 | 68,4 |
| 7 | 69,9 | 71,1 | 70,5 |
| 9 | 59,2 | 69,2 | 64,2 |
| 10 | 70,8 | 74,0 | 72,4 |

| | | |
|---|---|---|
| General average X | 68,9 | |
| SD of sample means Sx | 4,57 | 6,6% |
| SD within-samples Sw | 3,16 | 4,6% |
| SD between-samples Ss | 3,98 | 5,8% |



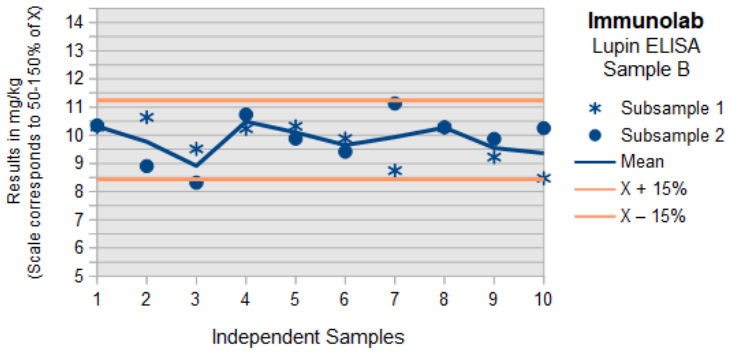
## ELISA-Tests: Homogenität Lupine / Homogeneity Lupin

### *Immunolab Lupin ELISA*

*Sample weights: 1,0 g (0,9 – 1,1 g)*
*Number of replicates: 2*
*Overall result: Lupin 9,34 ± 0,24 mg/kg*

| Sample B | Subsample 1 mg/kg | Subsample 2 mg/kg | Mean mg/kg |
|---|---|---|---|
| 1 | 9,79 | 9,85 | 9,82 |
| 2 | 10,1 | 8,40 | 9,27 |
| 3 | 9,01 | 7,82 | 8,41 |
| 4 | 9,73 | 10,2 | 9,98 |
| 5 | 9,82 | 9,38 | 9,60 |
| 6 | 9,38 | 8,92 | 9,15 |
| 7 | 8,24 | 10,63 | 9,44 |
| 8 | 9,76 | 9,78 | 9,77 |
| 9 | 8,73 | 9,37 | 9,05 |
| 10 | 7,98 | 9,75 | 8,86 |

| | | |
|---|---|---|
| General average X | 9,34 | |
| SD of sample means Sx | 0,485 | 5,2% |
| SD within-samples Sw | 0,599 | 6,4% |
| SD between-samples Ss | 0,236 | 2,5% |

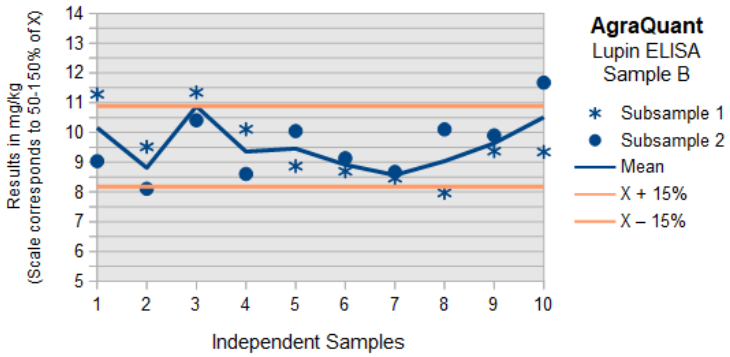



### *AgraQuant Lupin ELISA*

*Sample weights: 1,0 g (0,9 – 1,1 g)*
*Number of replicates: 2*
*Overall result: Lupin 9,03 ± 0,56 mg/kg*

| Sample B | Subsample 1 mg/kg | Subsample 2 mg/kg | Mean mg/kg |
|---|---|---|---|
| 1 | 10,8 | 8,53 | 9,66 |
| 2 | 9,01 | 7,61 | 8,31 |
| 3 | 10,8 | 9,91 | 10,4 |
| 4 | 9,61 | 8,10 | 8,86 |
| 5 | 8,37 | 9,55 | 8,96 |
| 6 | 8,19 | 8,63 | 8,41 |
| 7 | 7,96 | 8,17 | 8,07 |
| 8 | 7,47 | 9,60 | 8,54 |
| 9 | 8,87 | 9,40 | 9,14 |
| 10 | 8,84 | 11,2 | 10,0 |

| | | |
|---|---|---|
| General average X | 9,03 | |
| SD of sample means Sx | 0,766 | 8,5% |
| SD within-samples Sw | 0,743 | 8,2% |
| SD between-samples Ss | 0,558 | 6,2% |



*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 spiking level sample was approx. 0,36 (18,1°C). The stability of the sample material was thus ensured during the investigation period under the specified storage conditions.

## 2.2 Sample shipment and information to the test

The portions of test materials sample A, B and the spiking level sample were sent to every participating laboratory in the 9th week of 2021. The testing method was optional. The tests should be finished at 30th April 2021 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 Lupin and Wheat (Gluten) in the range of mg/kg in the matrix of „gluten-free" crispbread. 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 and should be analysed like a normal sample.*

*Please note the attached information on the proficiency test.*
(see documentation, section 5.4 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.

14 participants submitted at least one result.

# 3. Evaluation

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

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

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

## 3.1 Consensus value from participants (assigned value)

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

If possible, this is the standard procedure for the evaluation of methods 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 < 2,5 mg/kg, respectively) [3].

## 3.2 Robust standard deviation

For comparison to the target standard deviation $\sigma_{pt}$ (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 - $S^x_{ALL}$***
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, 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.

## 3.4 Target standard deviation (for proficiency assessment)

The target standard deviation of the assigned value $\sigma_{pt}$ (= 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 $\sigma_R$ [6]. Later the model was modified by Thompson for certain concentration ranges [10]. The reproducibility standard deviation $\sigma_R$ can be applied as the relative target standard deviation $\sigma_{pt}$ 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 $\sigma_R$ and the repeatability standard deviation $\sigma_r$ of a precision experiment (collaborative trial or proficiency test) the target standard deviation $\sigma_{pt}$ 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 $\sigma_{pt}$ were calculated for a number of m = 2 replicate measurements. With a number of m = 1 replicate measurements the reproducibility standard deviation $\sigma_R$ is identical to the target standard deviation $\sigma_{pt}$.

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 $\sigma_{pt}$ [30-31]

| Parameter | Matrix | Mean [mg/kg] | Recovery | rob RSD | $RSD_r$ | $RSD_R$ | $\sigma_{pt}$ | Method / Literature |
|---|---|---|---|---|---|---|---|---|
| Peanut | Milk chocolate | 173,7<br>33,8<br>5,9 | 87 %<br>85 %<br>59 % | -<br>-<br>- | 8,8%<br>5,2%<br>7,8% | 31%<br>20%<br>31% | 30,4%<br>19,7%<br>30,5% | ELISA Manuf. A<br>ASU 00.00-69 |
| Peanut | Milk chocolate | 215,7<br>40,1<br>10,1 | 108 %<br>100 %<br>101 % | -<br>-<br>- | 5,9%<br>7,2%<br>7,3% | 32%<br>14%<br>16% | 31,7%<br>13,0%<br>15,1% | ELISA Manuf. B<br>ASU 00.00-69 |
| Peanut | Dark chocolate | 148,2<br>30,9<br>5,7 | 74 %<br>77 %<br>57 % | -<br>-<br>- | 6,0%<br>13%<br>6,1% | 22%<br>25%<br>33% | 21,6%<br>23,2%<br>32,7% | ELISA Manuf. A<br>ASU 00.00-69 |
| Hazelnut | Dark chocolate | 16,3<br>7,56<br>3,73<br>1,62 | 81 %<br>76 %<br>75 %<br>81 % | -<br>-<br>-<br>- | 4,7%<br>8,9%<br>13%<br>15% | 12%<br>15%<br>24%<br>33% | 11,5%<br>13,6%<br>22,2%<br>31,2% | ELISA Manuf. A<br>ASU 44.00-7 |
| Hazelnut | Dark chocolate | 21,3<br>10,7<br>4,69<br>2,37 | 106 %<br>107 %<br>94 %<br>119 % | -<br>-<br>-<br>- | 7,1%<br>11%<br>11%<br>9,3% | 14%<br>19%<br>17%<br>17% | 13,1%<br>17,3%<br>15,1%<br>16,4% | ELISA Manuf. B<br>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 21 – 45% 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%.

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 $\sigma_{pt}$ [32,33]

| **Parameter** | **Matrix** | **Mean [mg/kg]** | **Recov-ery** | **rob RSD** | **$RSD_r$** | **$RSD_R$** | **$\sigma_{pt}$** | **Method / Literature** |
|---|---|---|---|---|---|---|---|---|
| Lupin | Rice cookies | 102<br>17,4<br>9,5 | 102 %<br>87 %<br>95 % | - | 14,6%<br>26,5%<br>39,1% | 23,0%<br>33,1%<br>42,6% | 20,6%<br>27,3%<br>32,3% | rt-PCR multiplex<br>ASU 18.00-22 |
| Lupin | Wheat cookies<br>Sauce powder | 80,8<br>53,6 | 64,1 %<br>53,6 % | - | 10,5%<br>23,9% | 29,5%<br>48,0% | 28,6%<br>44,8% | rt-PCR multiplex<br>ASU 18.00-22 |
| Wheat + Rye | Boiled saus-age (100°C, 60 min) | 96,1 | 120 % | - | 21,3% | 35,4% | 32,0% | rt-PCR<br>ASU 08.00-66 |
| Wheat + Rye | Sausage, autoclaved | 74,9 | 11,0 % | - | 24,6% | 32,7% | 27,7% | rt-PCR<br>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 [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 $\sigma_{pt}$ 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 ($\sigma_{pt}$) 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].

## 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 to the square root of quadrat sum of the target standard deviation ($\sigma_{pt}$) and the standard uncertainty ($U_{(Xpt)}$) [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 $\sigma_{pt}$'.

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*/$\sigma_{pt}$

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 $\sigma_{pt}$ does not exceed the value of 2.
A value > 2 means an insufficient precision, i.e. the analytical method is too variable, or the variation between the test participants is higher than estimated. Thus the comparability of the results is not given [3].

## 3.8 Standard uncertainty and traceability

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

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

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

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

## 3.10 Recovery rates: Spiking

For the results of the spiking level sample and the spiked sample recovery rates were calculated with respect to the known content of added allergens. The related values of added allergens are given in 2.1 test material in table 1. As a range of acceptance RA for valuating participant's results the range of 50 - 150% for the recovery rates of allergen-ELISAs proposed by the AOAC was used [23]. For quantitative PCR or LC/MS determinations we use the same range of acceptance.
The corresponding z-scores were calculated according to 3.5 with the target standard deviation of 25% (see 3.4.3).

# 4. Results

All following tables are anonymized. With the delivering of the evaluation report the participants are informed about their individual evaluation number.
Evaluation was done separately for ELISA and PCR-techniques. The results were grouped according to the applied methods (e.g. test kits) and sorted chronologically according to the evaluation number of the participants.
The following result sections are structured equally for the allergenic components. First all results of ELISA or PCR methods for a certain parameter are reported for samples A and B (qualitative / possibly quantitative) and afterwards for the spiking level sample (quantitative). The recovery rates of results for the spiking level sample and the spiked sample A or B are reported then.

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

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

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

In the present PT all ELISA-results for gluten were given as **gluten**, therefore no conversion was necessary. Quantitative PCR-results were submitted as wheat or gluten-containing cereals and evaluated as such.

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 ($X_{pt}$) | $\boldsymbol{X_{pt_{ALL}}}$ | $\boldsymbol{X_{pt_{METHOD\ i}}}$ |
| *Number of results* | | |
| *Number of outliers* | | |
| Mean | | |
| Median | | |
| Robust mean ($X_{pt}$) | | |
| Robust standard deviation (S*) | | |
| *Target data°:* | | |
| Target standard deviation $\sigma_{pt}$ *or* $\sigma_{pt}'$ | | |
| 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*/$\sigma_{pt}$ *or* S*/$\sigma_{pt}'$ | | |
| *Standard uncertainty* $U_{(X_{pt})}$ | | |
| *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.

# 4.1 Proficiency Test Lupin

## *4.1.1 ELISA Results: Lupin (as Lupin 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 | | |
| 6 | negative | <LOQ | positive | 2,56 | 2/2 (100%) | AQ | result converted ° |
| 2 | negative | < 2 | positive | 4,16 | 2/2 (100%) | EZ | result converted ° |
| 3 | negative | <0,6 | positive | 3,40 | 2/2 (100%) | RS-F | |
| 9 | negative | < 1,0 | positive | 4,80 | 2/2 (100%) | RS-F | |
| 12 | negative | <1 | positive | 3,81 | 2/2 (100%) | RS-F | |
| 14 | negative | <1 | positive | 7,00 | 2/2 (100%) | RS-F | |
| 5 | negative | <2 | positive | 2,75 | 2/2 (100%) | SP | result converted ° |
| 13 | negative | <2 | positive | 4,03 | 2/2 (100%) | SP | result converted ° |

° calculation p. 19

| | Sample A | | Sample B | |
|---|---|---|---|---|
| Number positive | 0 | | 8 | |
| Number negative | 8 | | 0 | |
| Percent positive | 0 | | 100 | |
| Percent negative | 100 | | 0 | |
| Consensus value | negative | | positive | |

**Methods:**

AQ = AgraQuant, RomerLabs

EZ = EZ plate

RS-F= Ridascreen® Fast, R-Biopharm

SP = SensiSpec ELISA Kit, Eurofins

*Comments:*

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

## Quantitative valuation of ELISA-results: Sample B

| Evaluation number | Lupin protein | z-Score $Xpt_{ALL}$ | Method | Remarks |
|---|---|---|---|---|
| | [mg/kg] | | | |
| 6 | 2,56 | -1,4 | AQ | result converted ° |
| 2 | 4,16 | 0,28 | EZ | result converted ° |
| 3 | 3,40 | -0,50 | RS-F | |
| 9 | 4,80 | 0,93 | RS-F | |
| 12 | 3,81 | -0,08 | RS-F | |
| 14 | 7,00 | 3,2 | RS-F | |
| 5 | 2,75 | -1,2 | SP | result converted ° |
| 13 | 4,03 | 0,14 | SP | result converted ° |

° calculation p. 19

**Methods:**

AQ = AgraQuant, RomerLabs

EZ = EZ plate

RS-F= Ridascreen® Fast, R-Biopharm

SP = SensiSpec ELISA Kit, Eurofins

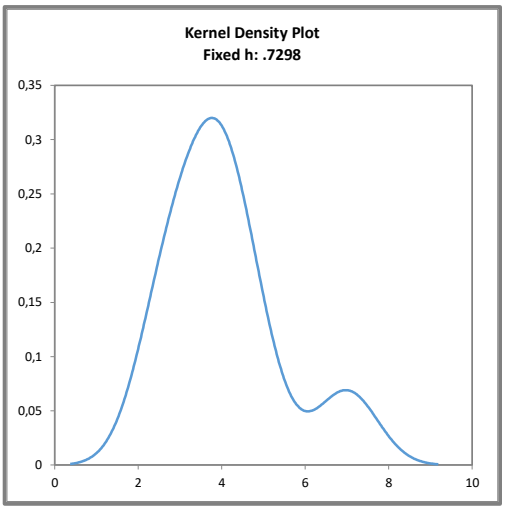



**Abb. / Fig. 1:**
Kerndichte-Schätzung aller ELISA-Ergebnisse (mit h = 0,75 x $\sigma pt$ von $Xpt_{ALL}$)

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

*Comments:*
The kernel density estimation shows almost a symmetrical distribution of results with a smaller side peak at approx. 7 mg/kg due a single result outside the target range.

Characteristics: Quantitative evaluation ELISA Lupin Protein

**Sample B**

| **Statistic Data** | **All Results** *[mg/kg]* |
|---|---|
| Assigned value ($X_{pt}$) | $\boldsymbol{X_{pt_{ALL}}}$ |
| *Number of results* | 8 |
| *Number of outliers* | 0 |
| Mean | 4,06 |
| Median | 3,92 |
| **Robust Mean ($X_{pt}$)** | **3,89** |
| **Robust standard deviation (S*)** | **1,15** |
| *Target range:* | |
| **Target standard deviation $\sigma_{pt}$** | **0,97** |
| **lower limit of target range** | **1,95** |
| **upper limit of target range** | **5,84** |
| *Quotient S*/$\sigma_{pt}$* | *1,2* |
| *Standard uncertainty $U_{(Xpt)}$* | *0,509* |
| *Results in the target range* | *7* |
| *Percent in the target range* | *88* |

*Comments to the statistical characteristics and assigned values:*

*The kernel density estimation showed an almost symmetrical distribution with no clear method-dependent differences.*

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

*The robust mean of the evaluation was 23% ($X_{ALL}$) of the spiking level of lupin to sample B and thus below the range of the recommendations for the applied methods (s. 3.4.3 and "Recovery rates of lupin protein" p.28).*

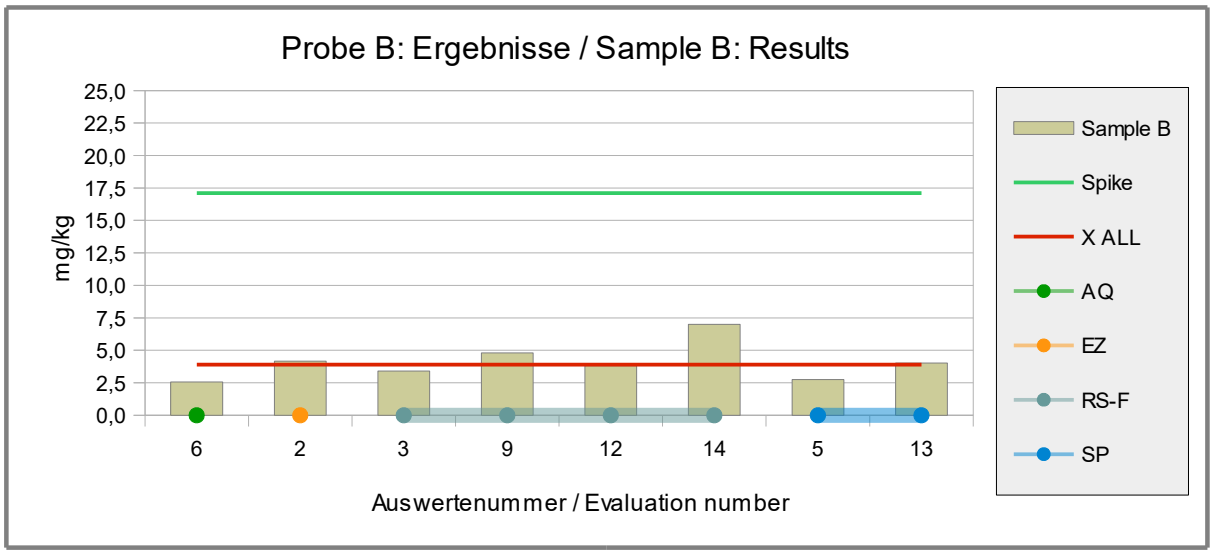



**Abb./Fig. 2:** ELISA Results Lupin Protein
green line = Spiking level
red line = Assigned value robust mean all results
round symbols = Applied methods (see legend)

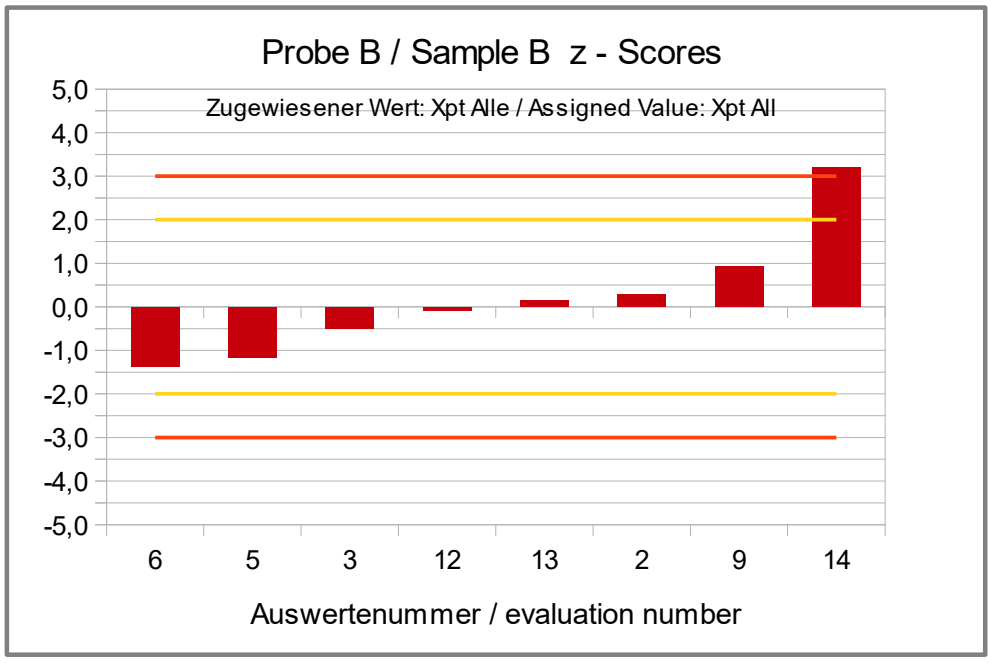



**Abb./Fig. 3:**
z-Scores (ELISA Results Lupin Protein)
Assigned value robust mean of all results

## Quantitative valuation of results: Spiking level sample

| Evaluation number | Lupin protein | z-Score $Xpt_{ALL}$ | Method | Remarks |
|---|---|---|---|---|
| | [mg/kg] | | | |
| 6 | 18,3 | -0,29 | AQ | result converted ° |
| 2 | 22,7 | 0,60 | EZ | result converted ° |
| 3 | 10,1 | -2,0 | RS-F | |
| 9 | 15,4 | -0,88 | RS-F | |
| 12 | 19,5 | -0,04 | RS-F | |
| 14 | 23,0 | 0,66 | RS-F | |
| 5 | 22,0 | 0,46 | SP | result converted ° |
| 13 | 23,8 | 0,83 | SP | result converted ° |

° calculation p. 19

**Methods:**

AQ = AgraQuant, RomerLabs

EZ = EZ plate

RS-F= Ridascreen® Fast, R-Biopharm

SP = SensiSpec ELISA Kit, Eurofins

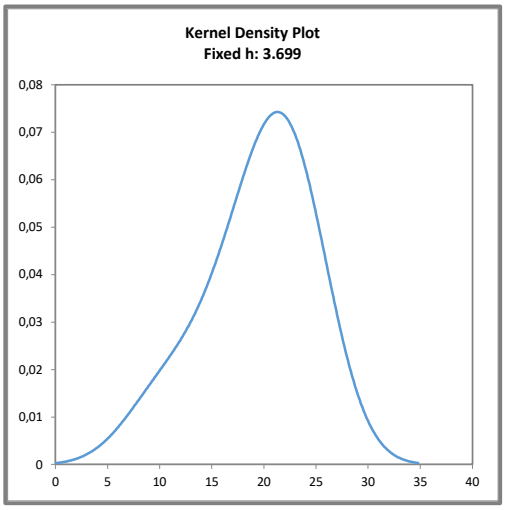



**Abb. / Fig. 4:**
Kerndichte-Schätzung aller ELISA-Ergebnisse (mit h = 0,75 x $\sigma pt$ von $Xpt_{ALL}$)

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

*Comments:*
The kernel density estimation shows nearly a symmetrical distribution of results with a slight shoulder at approx. 10 mg/kg.

Characteristics: Quantitative evaluation ELISA Lupin Protein

**Spiking level sample**

| **Statistic Data** | **All Results** *[mg/kg]* |
|---|---|
| Assigned value ($X_{pt}$) | $X_{pt_{ALL}}$ |
| *Number of results* | 8 |
| *Number of outliers* | 0 |
| Mean | 19,4 |
| Median | 20,8 |
| **Robust Mean ($X_{pt}$)** | **19,7** |
| **Robust standard deviation (S*)** | **4,41** |
| *Target range:* | |
| **Target standard deviation $\sigma_{pt}$** | **4,93** |
| **lower limit of target range** | **9,86** |
| **upper limit of target range** | **29,59** |
| *Quotient S*/$\sigma_{pt}$* | *0,89* |
| *Standard uncertainty $U_{(Xpt)}$* | *1,95* |
| *Results in the target range* | *8* |
| *Percent in the target range* | *100* |

*Comments to the statistical characteristics and assigned values:*

*The kernel density estimation showed an almost symmetrical distribution with no clear method-dependent differences.*

*The evaluation of all methods showed a low variability of results. The quotient S*/$\sigma_{pt}$ was below 1,0. The robust standard deviations are in the range of established values for the reproducibility standard deviation of the applied methods (see 3.4.2 value by precision experiments and 3.4.3 value by perception). The comparability of results is given. This conclusion is limited for the evaluation across the methods, because there were only a few results for some methods.*

*The robust mean of the evaluation was 144% ($X_{ALL}$) of the spiking level of lupin to the spiking level sample and thus within the range of the re-commendations for the applied methods (s. 3.4.3 and "Recovery rates of lupin protein" p.28).*

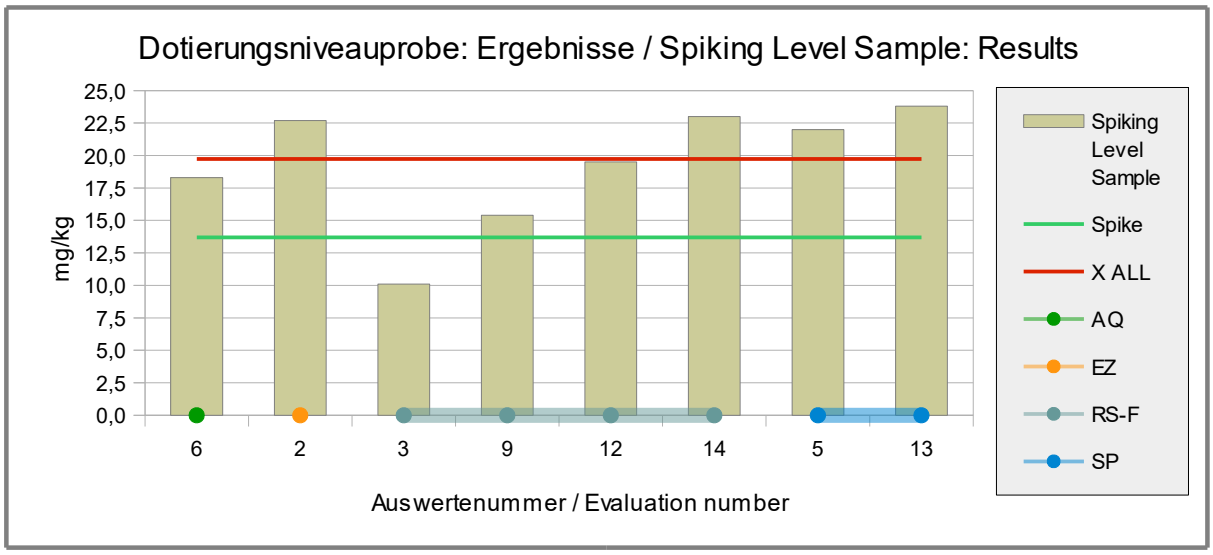



**Abb./Fig. 5:** ELISA Results Lupin Protein
green line = Spiking level
red line = Assigned value robust mean all results
round symbols = Applied methods (see legend)

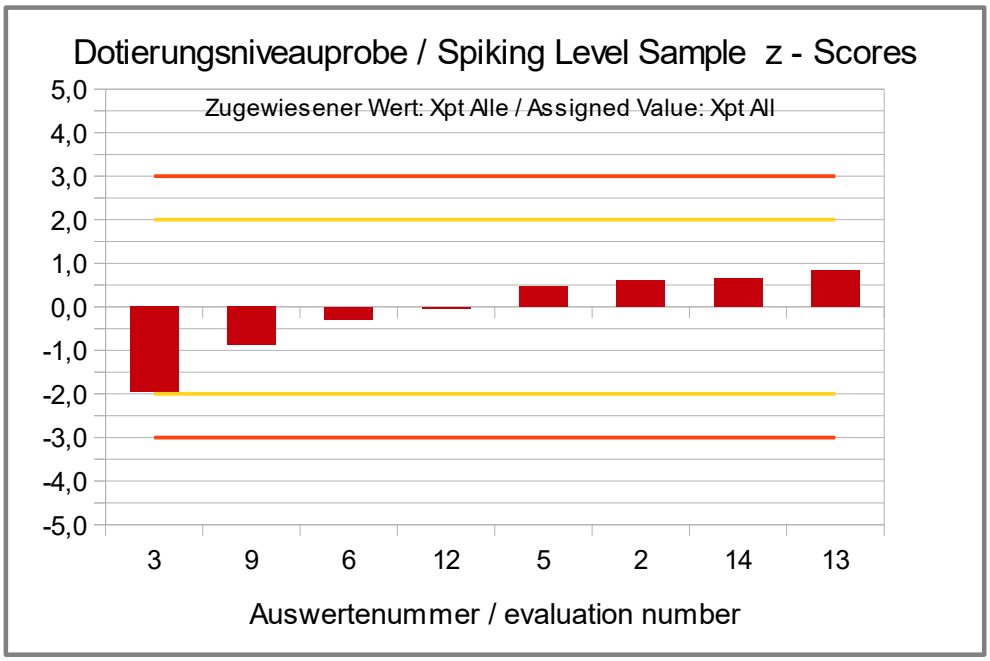



**Abb./Fig. 6:**
z'-Scores (ELISA Results Lupin Protein)
Assigned value robust mean of all results

## Recovery Rates with z-Scores ELISA for Lupin: Spiking Level Sample and Sample B

| Evaluation number | Spiking Level Sample | Recovery rate* | | Sample B | Recovery rate* | | Method | Remarks |
|---|---|---|---|---|---|---|---|---|
| | [mg/kg] | [%] | [$Z_{RR}$] | [mg/kg] | [%] | [$Z_{RR}$] | | |
| 6 | 18,3 | **134** | 1,3 | 2,56 | 15 | -3,4 | AQ | result converted ° |
| 2 | 22,7 | 166 | 2,6 | 4,16 | 24 | -3,0 | EZ | result converted ° |
| 3 | 10,1 | **74** | -1,1 | 3,40 | 20 | -3,2 | RS-F | |
| 9 | 15,4 | **112** | 0,50 | 4,80 | 28 | -2,9 | RS-F | |
| 12 | 19,5 | **142** | 1,7 | 3,81 | 22 | -3,1 | RS-F | |
| 14 | 23,0 | 168 | 2,7 | 7,00 | 41 | -2,4 | RS-F | |
| 5 | 22,0 | 161 | 2,4 | 2,75 | 16 | -3,4 | SP | result converted ° |
| 13 | 23,8 | 174 | 2,9 | 4,03 | 24 | -3,1 | SP | result converted ° |

° calculation p. 19

| RA** | 50-150 % | RA** | 50-150 % |
|---|---|---|---|
| Number in RA | 4 | Number in RA | 0 |
| | | | |
| Percent in RA | 50 | Percent in RA | 0 |
| | | | |

**Methods:**
AQ = AgraQuant, RomerLabs
EZ = EZ plate
RS-F= Ridascreen® Fast, R-Biopharm
SP = SensiSpec ELISA Kit, Eurofins

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

** Range of acceptance of AOAC for allergen ELISAS

*Comments:*
*50% (4) of the participants obtained for the spiking level sample a recovery rate by ELISA methods within the range of the AOAC-recommendation of 50-150%. For the processed spiked food matrix sample B all of the recovery rates were below the range of acceptance.*
*The related z-scores are based on the target standard deviation of 25%.*

### *4.1.2 PCR Results: Lupin*

**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 | | |
| 5 | negative | | positive | | 2/2 (100%) | ASU | |
| 1 | negative | NA | positive | NA | 2/2 (100%) | SFA-ID | |
| 3 | negative | | positive | | 2/2 (100%) | SFA-ID | |
| 9 | negative | | positive | | 2/2 (100%) | SFA-ID | |
| 12 | negative | <1 | positive | 26,7 | 2/2 (100%) | SFA-ID | |
| 11 | negative | | positive | | 2/2 (100%) | div | |
| 14 | negative | | positive | | 2/2 (100%) | div | |

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

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

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

**Quantitative Valuation PCR: Sample B**

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

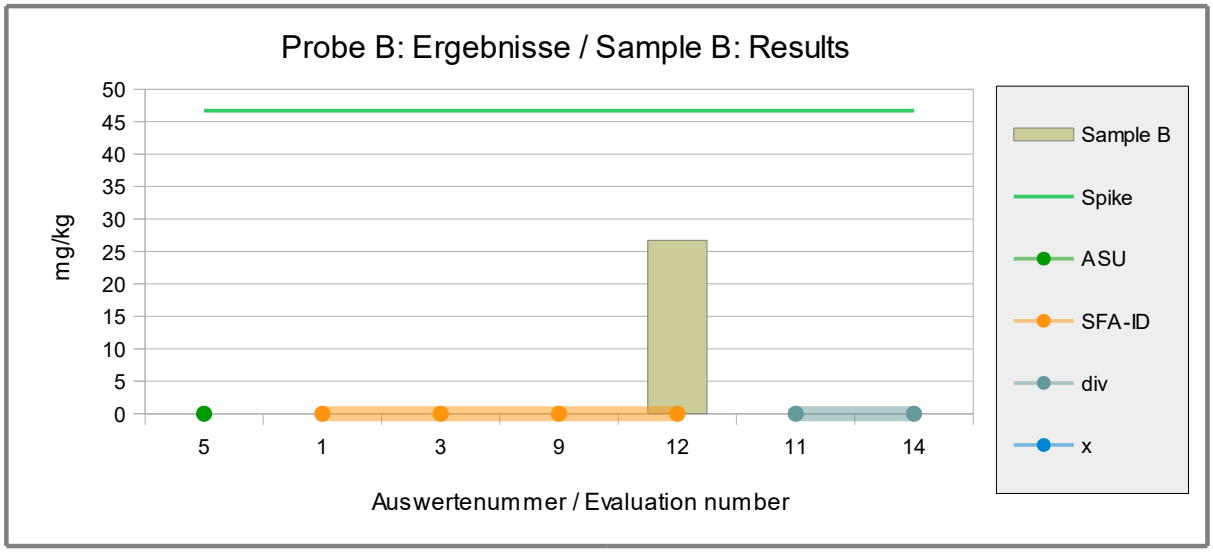



**Abb./Fig. 7:** PCR Results Lupin
green line = Spiking level
round symbols = Applied methods (see legend)

## Qualitative Valuation PCR: Spiking Level Sample

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

| Evaluation number | Lupin | Lupin | z-Score $Xpt_{ALL}$ | Method | Remarks |
|---|---|---|---|---|---|
| | pos/neg | [mg/kg] | | | |
| 5 | positive | | | ASU | |
| 1 | positive | NA | | SFA-ID | |
| 3 | positive | | | SFA-ID | |
| 9 | positive | | | SFA-ID | |
| 12 | positive | 29,3 | | SFA-ID | |
| 11 | positive | | | div | |
| 14 | positive | | | div | |

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

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

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

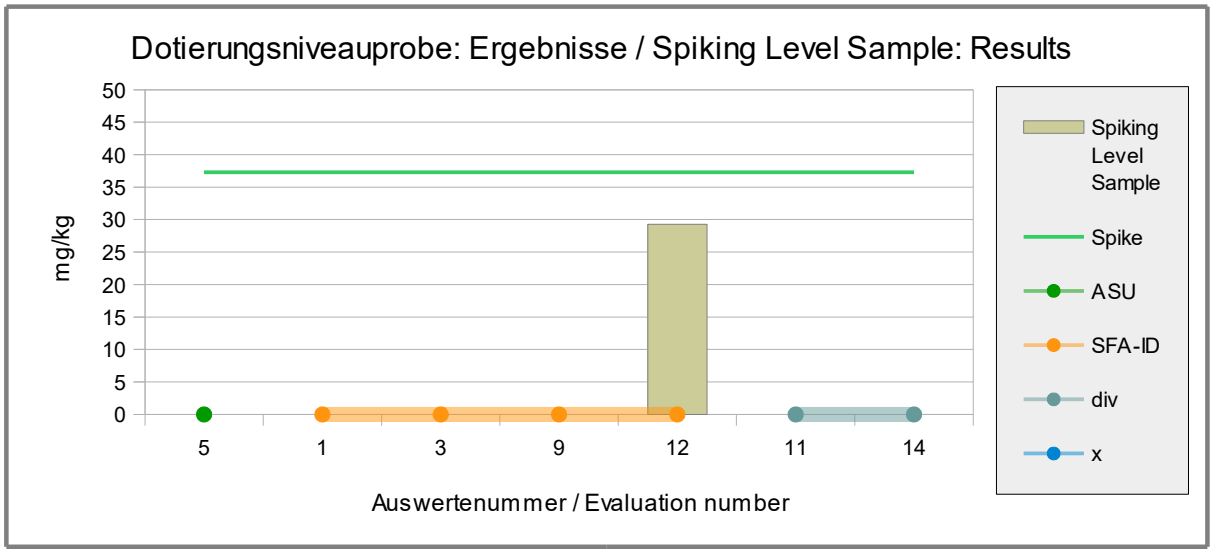



**Abb./Fig. 8:** PCR Results Lupin
green line = Spiking level
round symbols = Applied methods (see legend)

## *Recovery Rates with z-Scores PCR for Lupin: Spiking Material Sample and Sample B*

| Evaluation number | Spiking Level Sample | Recovery rate* | | Sample B | Recovery rate* | | Method | Remarks |
|---|---|---|---|---|---|---|---|---|
| | [mg/kg] | [%] | [$Z_{RR}$] | [mg/kg] | [%] | [$Z_{RR}$] | | |
| 5 | | | | | | | ASU | |
| 1 | NA | | | NA | | | SFA-ID | |
| 3 | | | | | | | SFA-ID | |
| 9 | | | | | | | SFA-ID | |
| 12 | 29,3 | **78** | -0,86 | 26,7 | **57** | -1,7 | SFA-ID | |
| 11 | | | | | | | div | |
| 14 | | | | | | | div | |

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

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

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

** Range of acceptance of AOAC for allergen ELISAS

*Comments:*
*One participant determined quantitative results by PCR. For the spiking level sample as well as the processed spiked food matrix sample B the recovery rates were within the AOAC-recommendation of 50-150%.*
*The related z-scores are based on the target standard deviation of 25%.*

# 4.2 Proficiency test Wheat (Gluten)

## *4.2.1 ELISA Results: Gluten*

### Qualitative valuation of results: Samples A and B

| Evaluation number | Sample A | Sample A | Sample B | Sample B | Qualitative Valuation | Method | Remarks |
|---|---|---|---|---|---|---|---|
| | pos/neg | [mg/kg] | pos/neg | [mg/kg] | Agreement with consensus value | | |
| 6 | positive | <LOQ | positive | 65,0 | 1/2 (50%) | AQ-G12 | |
| 13 | negative | < 4 | positive | 42,0 | 2/2 (100%) | IL | |
| 1 | negative | NA | positive | 58,4 | 2/2 (100%) | RS | |
| 2 | negative | < 5 | positive | 69,2 | 2/2 (100%) | RS | |
| 3 | negative | < 5 | positive | > 80 | 2/2 (100%) | RS | |
| 4 | negative | < 5,0 | positive | 52,0 | 2/2 (100%) | RS | |
| 5a | negative | < 5 | positive | 58,0 | 2/2 (100%) | RS | |
| 7 | negative | < 5,0 | positive | 53,9 | 2/2 (100%) | RS | |
| 12 | negative | < 3 | positive | 67,7 | 2/2 (100%) | RS | |
| 8 | negative | < 5,0 | positive | 59,2 | 2/2 (100%) | RS-F | |
| 10 | negative | < 10 | positive | 27,7 | 2/2 (100%) | RS-F | |
| 5b | negative | < 3,12 | positive | 46,0 | 2/2 (100%) | SP-R5 | |
| 14 | negative | < 5 | positive | 72,0 | 2/2 (100%) | VT-R5 | |

| | Sample A | | Sample B | |
|---|---|---|---|---|
| Number positive | 1 | | 13 | |
| Number negative | 12 | | 0 | |
| Percent positive | 8 | | 100 | |
| Percent negative | 92 | | 0 | |
| Consensus value | negative | | positive | |

**Methods:**
AQ-G12 = AgraQuant, RomerLabs
IL = Immunolab
RS = Ridascreen®, R-Biopharm
RS-F= Ridascreen® Fast, R-Biopharm
SP-R5 = SensiSpec Ingezim Gluten R5, Eurofins
VT-R5 = Veratox, Neogen

*Comments:*
*The consensus values are in qualitative agreement with the spiking of sample B.*
*One positive result for sample A was obtained by method AQ-G12 (Romer Labs) below the limit of quantification.*

## Quantitative valuation of results: Sample B

| Evaluation number | Gluten | z-Score $Xpt_{ALL}$ | z-Score $Xpt_{RS}$ | Method | Remarks |
|---|---|---|---|---|---|
| | [mg/kg] | | | | |
| 6 | 65,0 | 0,58 | | AQ-G12 | |
| 13 | 42,0 | -1,0 | | IL | |
| 1 | 58,4 | 0,11 | 0,01 | RS | |
| 2 | 69,2 | 0,87 | 0,06 | RS | |
| 3 | > 80 | | | RS | |
| 4 | 52,0 | -0,34 | -0,02 | RS | |
| 5a | 58,0 | 0,08 | 0,01 | RS | |
| 7 | 53,9 | -0,20 | -0,01 | RS | |
| 12 | 67,7 | 0,77 | 0,05 | RS | |
| 8 | 59,2 | 0,17 | | RS-F | |
| 10 | 27,7 | -2,1 | | RS-F | |
| 5b | 46,0 | -0,76 | | SP-R5 | |
| 14 | 72,0 | 1,1 | | VT-R5 | |

**Methods:**
AQ-G12 = AgraQuant, RomerLabs
IL = Immunolab
RS = Ridascreen®, R-Biopharm
RS-F= Ridascreen® Fast, R-Biopharm
SP-R5 = SensiSpec Ingezim Gluten R5, Eurofins
VT-R5 = Veratox, Neogen

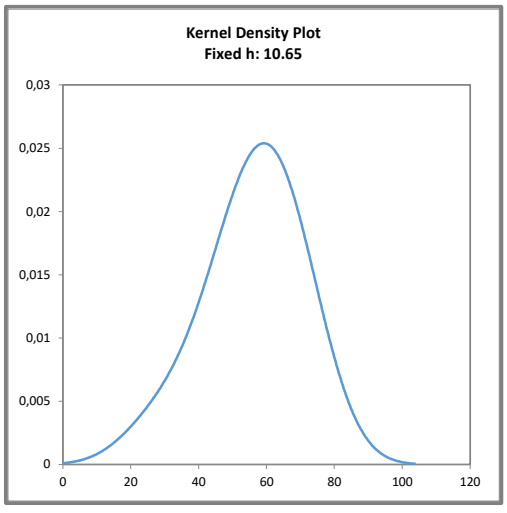



**Abb. / Fig. 9:**
Kerndichte-Schätzung aller ELISA-Ergebnisse (mit h = 0,75 x $\sigma pt$ von $Xpt_{ALL}$)

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

*Comments:*
The kernel density estimation shows nearly a symmetrical distribution of results.

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* | 12 | 6 |
| *Number of outliers* | 0 | 0 |
| Mean | 55,9 | 59,9 |
| Median | 58,2 | 58,2 |
| **Robust Mean ($X_{pt}$)** | **56,8** | **59,9** |
| **Robust standard deviation (S*)** | **12,3** | **8,05** |
| *Target range:* | | |
| **Target standard deviation $\sigma_{pt}$** | **14,2** | **15,0** |
| **lower limit of target range** | **28,4** | **29,9** |
| **upper limit of target range** | **85,2** | **89,8** |
| *Quotient S*/$\sigma_{pt}$* | *0,86* | *0,54* |
| *Standard uncertainty $U_{(Xpt)}$* | *4,42* | *4,11* |
| *Results in the target range* | *11* | *6* |
| *Percent in the target range* | *92* | *100* |

**Methods:**
RS = R-Biopharm, Ridascreen®

*Comments to the statistical characteristics and assigned values:*

*The kernel density estimation showed an almost symmetrical distribution with no clear method-dependent differences.*

*The evaluations of all methods and of method RS showed low variabilities of results. The quotients S*/$\sigma_{pt}$ were below 1,0. The robust standard deviations are in the range of established values for the reproducibility standard deviation of the applied methods (see 3.4.2 value by precision experiments and 3.4.3 value by perception). The comparability of results is given. This conclusion is limited for the evaluation across the methods, because there were only a few results for some methods.*

*The robust means of the evaluations were 137% ($X_{ALL}$) and 145% ($X_{RS}$) of the spiking level of gluten to sample B and thus within the recommendations for the applied methods (s. 3.4.3 and "recovery rates for gluten", p.41).*

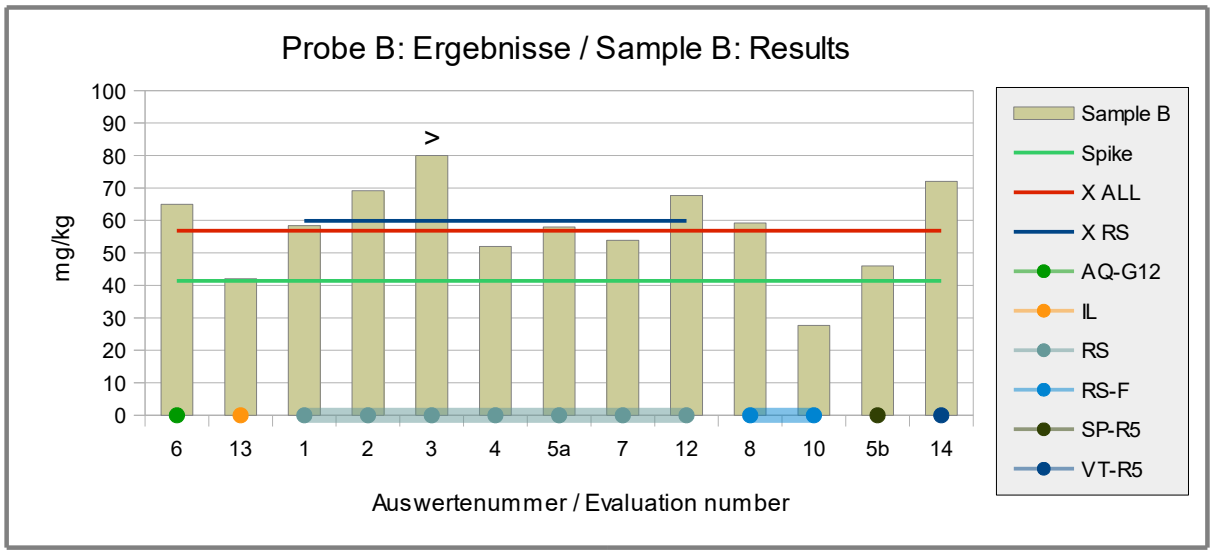



**Abb./Fig. 10:** ELISA Results Gluten
green line = Spiking level (Spike)
red line = Assigned value robust mean all results
blue line = Assigned value robust mean method RS
round symbols = Applied methods (see legend)

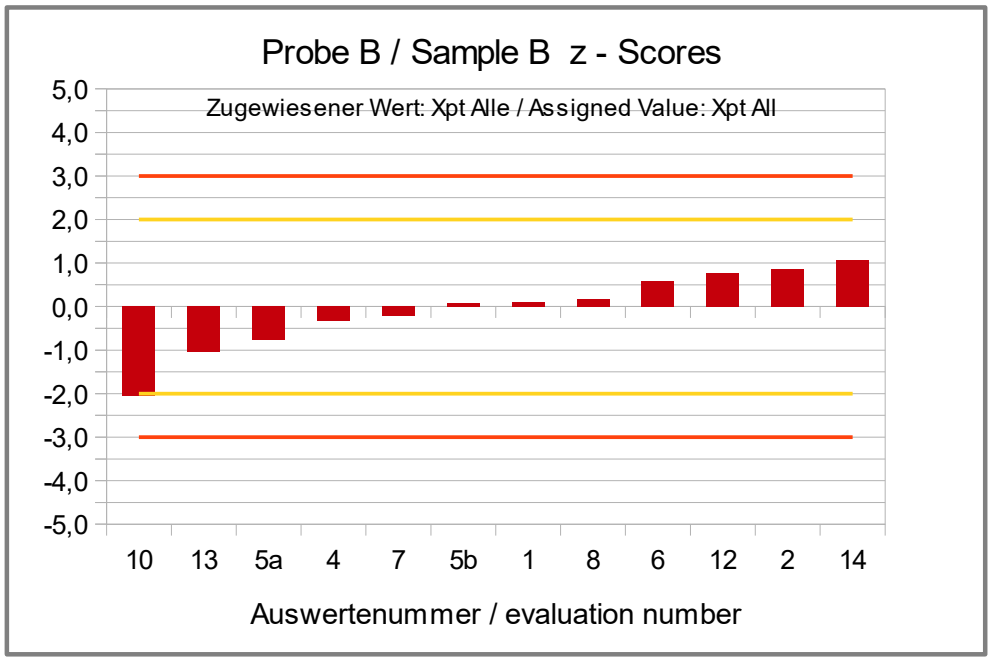



**Abb./Fig. 11:**
z-Scores (ELISA Results Gluten) Assigned value robust mean of all results

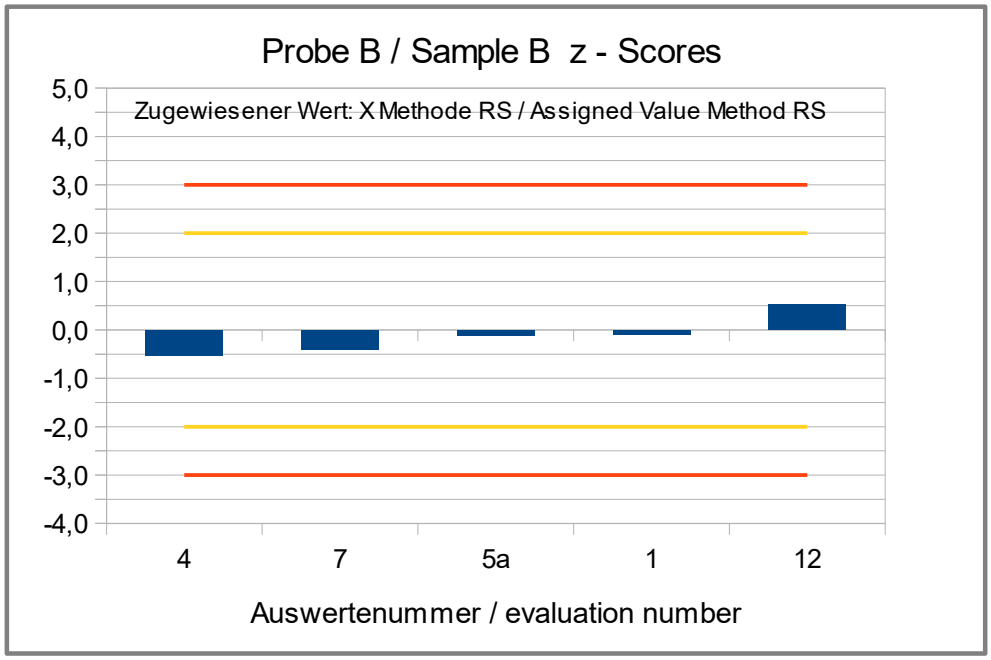



**Abb./Fig. 12:**
z-Scores (ELISA Results Gluten) Assigned value robust mean of results method RS (R-Biopharm, Ridascreen)

## Quantitative valuation of ELISA results: Spiking level sample

| Evaluation number | Gluten | z-Score $Xpt_{ALL}$ | z-Score $Xpt_{RS}$ | Method | Remarks |
|---|---|---|---|---|---|
| | [mg/kg] | | | | |
| 6 | 23,0 | -0,93 | | AQ-G12 | |
| 13 | 46,0 | 2,1 | | IL | |
| 1 | 19,5 | -1,4 | -0,19 | RS | |
| 2 | 34,1 | 0,55 | 0,07 | RS | |
| 3 | | | | RS | |
| 4 | 32,7 | 0,37 | 0,05 | RS | |
| 5a | 32,0 | 0,27 | 0,04 | RS | |
| 7 | 25,5 | -0,60 | -0,08 | RS | |
| 12 | 35,8 | 0,77 | 0,10 | RS | |
| 8 | 23,6 | -0,85 | | RS-F | |
| 10 | 15,8 | -1,9 | | RS-F | |
| 5b | 28,0 | -0,26 | | SP-R5 | |
| 14 | 44,0 | 1,9 | | VT-R5 | |

**Methods:**
AQ-G12 = AgraQuant, RomerLabs
IL = Immunolab
RS = Ridascreen®, R-Biopharm
RS-F= Ridascreen® Fast, R-Biopharm
SP-R5 = SensiSpec Ingezim Gluten R5, Eurofins
VT-R5 = Veratox, Neogen

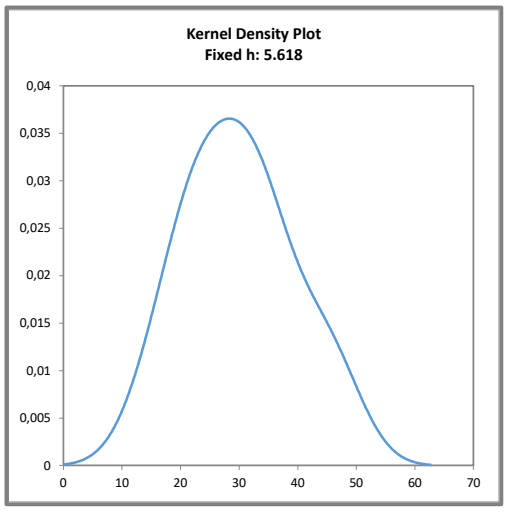



**Abb. / Fig. 13:**
Kerndichte-Schätzung aller ELISA-Ergebnisse (mit h = 0,75 x $\sigma pt$ von $Xpt_{ALL}$)

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

*Comments:*
The kernel density estimation shows nearly a symmetrical distribution of results.

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* | 12 | 6 |
| *Number of outliers* | 0 | 0 |
| Mean | 30,0 | 29,9 |
| Median | 30,0 | 32,4 |
| **Robust Mean ($X_{pt}$)** | **30,0** | **30,0** |
| **Robust standard deviation (S*)** | **10,4** | **6,94** |
| *Target range:* | | |
| **Target standard deviation $\sigma_{pt}$** | **7,49** | **7,5** |
| **lower limit of target range** | **15,0** | **15,0** |
| **upper limit of target range** | **44,9** | **44,9** |
| *Quotient S*/$\sigma_{pt}$* | *1,4* | *0,93* |
| *Standard uncertainty $U_{(Xpt)}$* | *3,75* | *3,54* |
| *Results in the target range* | *11* | *6* |
| *Percent in the target range* | *92* | *100* |

**Methods:**
RS = R-Biopharm, Ridascreen®

*Comments to the statistical characteristics and assigned values:*

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

*The evaluation of results of all methods as well as the results of method RS showed a normal and low variability of results, respectively. The quotients S*/$\sigma_{pt}$ were below 2,0 and 1,0. The robust standard deviations are 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 145% ($X_{ALL}$) and 145% ($X_{RS}$) 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", p.41).*

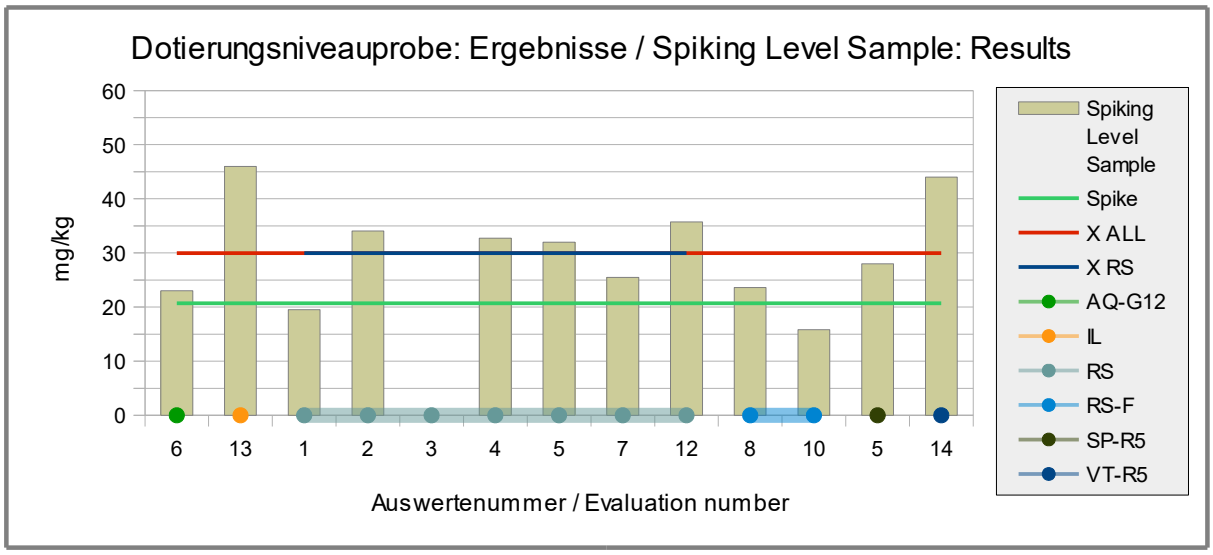



**Abb./Fig. 14:** ELISA Results Gluten
green line = Spiking level (Spike)
red line = Assigned value robust mean all results
blue line = Assigned value robust mean method RS
round symbols = Applied methods (see legend)

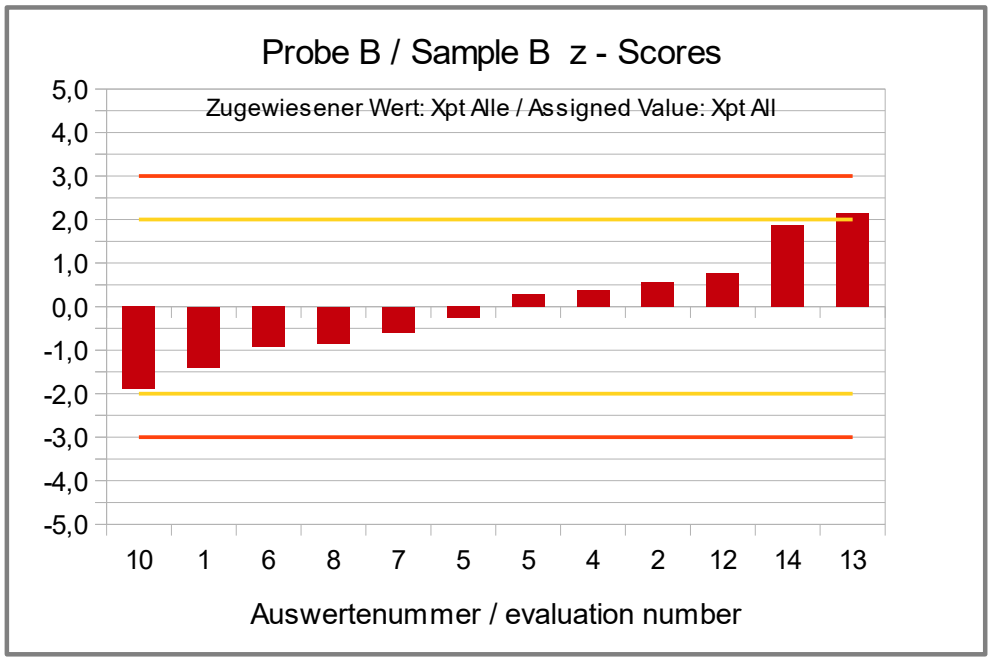



**Abb./Fig. 15:**
z-Scores (ELISA Results Gluten) Assigned value robust mean of all results

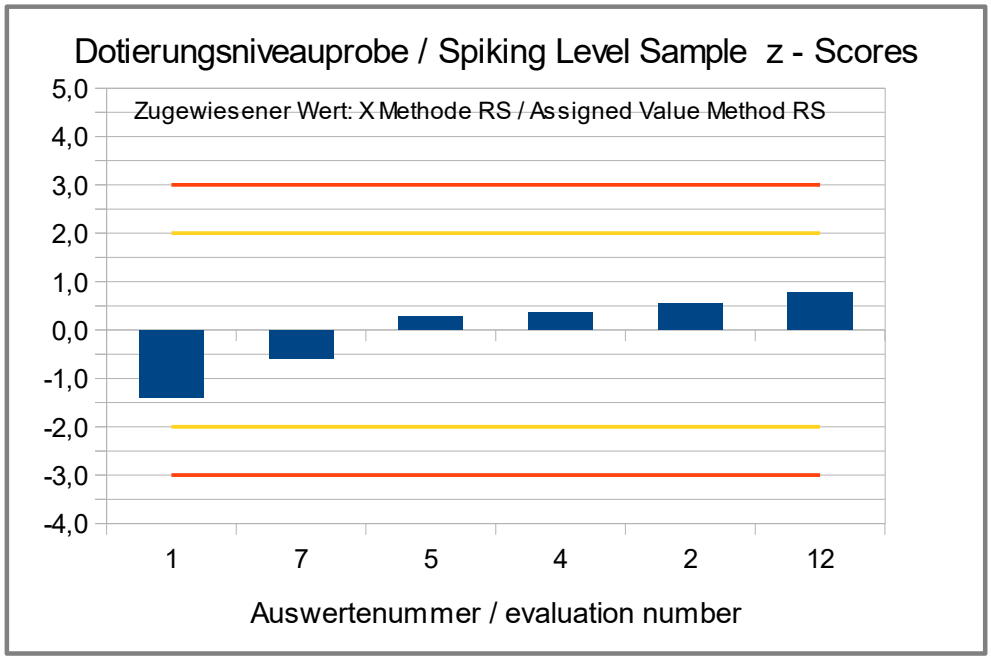



**Abb./Fig. 16:**
z-Scores (ELISA Results Gluten) Assigned value robust mean of results method RS (R-Biopharm, Ridascreen)

## *Recovery Rates with z-Scores for Gluten: Spiking level sample and Sample B*

| Evaluation number | Spiking Level Sample | Recovery rate* | | Sample B | Recovery rate* | | Method | Remarks |
|---|---|---|---|---|---|---|---|---|
| | [mg/kg] | [%] | [$Z_{RR}$] | [mg/kg] | [%] | [$Z_{RR}$] | | |
| 6 | 23,0 | **111** | 0,44 | 65,0 | 157 | 2,3 | AQ-G12 | |
| 13 | 46,0 | 222 | 4,9 | 42,0 | **101** | 0,06 | IL | |
| 1 | 19,5 | **94** | -0,23 | 58,4 | **141** | 1,6 | RS | |
| 2 | 34,1 | 165 | 2,6 | 69,2 | 167 | 2,7 | RS | |
| 3 | | | | 80,0 | | | RS | |
| 4 | 32,7 | 158 | 2,3 | 52,0 | **126** | 1,0 | RS | |
| 5a | 32,0 | 155 | 2,2 | 58,0 | **140** | 1,6 | RS | |
| 7 | 25,5 | **123** | 0,93 | 53,9 | **130** | 1,2 | RS | |
| 12 | 35,8 | 173 | 2,9 | 67,7 | 164 | 2,5 | RS | |
| 8 | 23,6 | **114** | 0,56 | 59,2 | **143** | 1,7 | RS-F | |
| 10 | 15,8 | **76** | -0,94 | 27,7 | **67** | -1,3 | RS-F | |
| 5b | 28,0 | **135** | 1,4 | 46,0 | **111** | 0,44 | SP-R5 | |
| 14 | 44,0 | 213 | 4,5 | 72,0 | 174 | 3,0 | VT-R5 | |

| RA** | 50-150 % | RA** | 50-150 % |
|---|---|---|---|
| Number in RA | 6 | Number in RA | 8 |
| | | | |
| Percent in RA | 50 | Percent in RA | 67 |
| | | | |

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

** Range of acceptance of AOAC for allergen ELISAS

**Methods:**

AQ-G12 = AgraQuant, RomerLabs

IL = Immunolab

RS = Ridascreen®, R-Biopharm

RS-F= Ridascreen® Fast, R-Biopharm

SP-R5 = SensiSpec Ingezim Gluten R5, Eurofins

VT-R5 = Veratox, Neogen

*Comments:*

*For the spiking level sample 50% (6) of the participants obtained a recovery rate within the range of the AOAC-recommendation of 50-150%. For the processed spiked food matrix sample B 67% (8) of the obtained recovery rates were within the recommended range.*

*The related z-scores are based on the target standard deviation of 25%.*

### *4.2.2 PCR Results: Gluten-containing Cereals (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 | | |
| 3 | negative | | positive | | 2/2 (100%) | SFA-ID | |
| 12a | negative | <10 | positive | 447 | 2/2 (100%) | SFA-ID | Gluten-containing cereals |
| 12b | negative | <10 | positive | 543 | 2/2 (100%) | SFA-4p | Wheat |
| 14a | negative | | positive | | 2/2 (100%) | div | |
| 14b | negative | | positive | | 2/2 (100%) | div | |

| | Sample A | | Sample B | |
|---|---|---|---|---|
| Number positive | 0 | | 5 | |
| Number negative | 5 | | 0 | |
| Percent positive | 0 | | 100 | |
| Percent negative | 100 | | 0 | |
| Consensus value | negative | | positive | |

**Methods:**
SFA-ID = Sure Food Allergen ID, R-Biopharm / Congen
SFA-4p = Sure Food Allergen 4plex, R-Biopharm / Congen
div = keine genaue Angabe / andere Methode
div = not indicated / other method

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

**Quantitative Valuation PCR: Sample B**

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

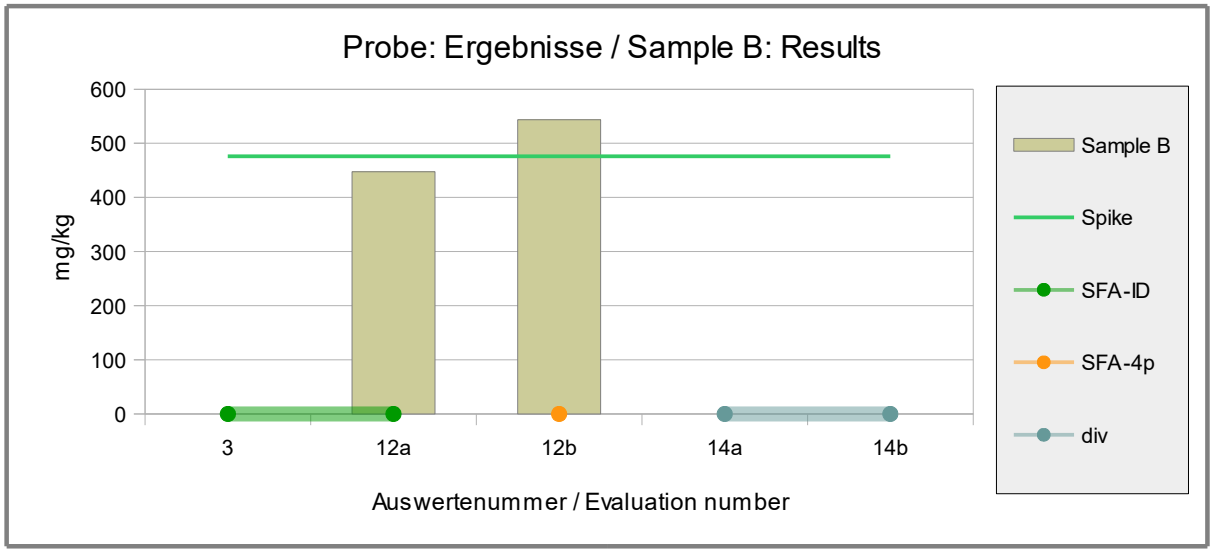



**Abb./Fig. 17:** PCR Results Gluten-containing Cereals (Wheat)
green line = Spiking level
round symbols = Applied methods (see legend)

## Qualitative Valuation PCR: Spiking Level Sample

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

| Evaluation number | Gluten-cont. Cereals | Gluten-cont. Cereals | z-Score $Xpt_{ALL}$ | Methods | Remarks |
|---|---|---|---|---|---|
| | pos/neg | [mg/kg] | | | |
| 3 | positive | | | SFA-ID | |
| 12a | positive | 363 | | SFA-ID | Gluten-containing cereals |
| 12b | positive | 519 | | SFA-4p | Wheat |
| 14a | positive | | | div | |
| 14b | positive | | | div | |

| | |
|---|---|
| Number positive | 5 |
| Number negative | 0 |
| Percent positive | 100 |
| Percent negative | 0 |
| Consensus value | positive |

**Methoden:**

SFA-ID = Sure Food Allergen ID, R-Biopharm / Congen
SFA-4p = Sure Food Allergen 4plex, R-Biopharm / Con
div = keine genaue Angabe / andere Methode
div = not indicated / other method

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

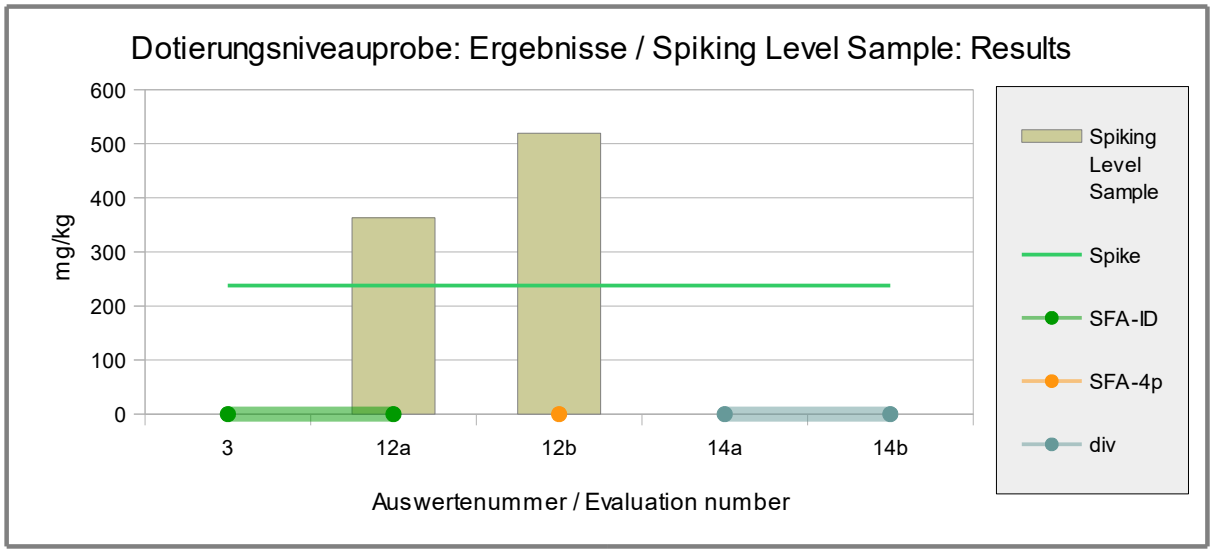



**Abb./Fig. 18:** PCR Results Gluten-containing Cereals (Wheat)
green line = Spiking level
round symbols = Applied methods (see legend)

## *Recovery Rates with z-Scores PCR for Gluten-containing Cereals (Wheat): Spiking Material Sample and Sample B*

| Evaluation number | Spiking Level Sample | Recovery rate* | | Sample B | Recovery rate* | | Method | Remarks |
|---|---|---|---|---|---|---|---|---|
| | [mg/kg] | [%] | [$Z_{RR}$] | [mg/kg] | [%] | [$Z_{RR}$] | | |
| 3 | | | | | | | SFA-ID | |
| 12a | 363 | 153 | 2,1 | 447 | **94** | -0,24 | SFA-ID | Gluten-containing cereals |
| 12b | 519 | 218 | 4,7 | 543 | **114** | 0,57 | SFA-4p | Wheat |
| 14a | | | | | | | div | |
| 14b | | | | | | | div | |

| RA** | 50-150 % | RA** | 50-150 % |
|---|---|---|---|
| Number in RA | **0** | Number in RA | **2** |
| | | | |
| Percent in RA | **0** | Percent in RA | **100** |
| | | | |

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

** Range of acceptance of AOAC for allergen ELISAS

**Methods:**

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

SFA-4p = Sure Food Allergen 4plex, R-Biopharm / Congen

div = keine genaue Angabe / andere Methode

div = not indicated / other method

*Comments:*

*Two participants determined quantitative results by PCR. For the processed spiked food matrix sample B, the recovery rates were within the range of the AOAC-recommendation of 50-150%. For the spiking level sample the recovery rates were slightly and clearly above the recommendation, respectively.*

*The related z-scores are based on the target standard deviation of 25%.*

## 4.3 Participant z-Scores: overview table

***Z-Scores for the assigned values from participants results (consensus values)***

| Evaluation number | ELISA Lupinprotein: **Xpt** (div. Methods) | | ELISA Gluten: **Xpt** (div. Methods) | | ELISA Gluten: **Xpt** (Method: RS) | |
|---|---|---|---|---|---|---|
| | Sample B | Spiking Level Sample | Sample B | Spiking Level Sample | Sample B | Spiking Level Sample |
| 1 | | | 0,11 | -1,4 | 0,01 | -0,19 |
| 2 | 0,28 | 0,60 | 0,87 | 0,55 | 0,06 | 0,07 |
| 3 | -0,50 | -2,0 | | | | |
| 4 | | | -0,34 | 0,37 | -0,02 | 0,05 |
| 5a | -1,2 | 0,46 | 0,08 | 0,27 | 0,01 | 0,04 |
| 5b | | | -0,76 | -0,26 | | |
| 6 | -1,4 | -0,29 | 0,58 | -0,60 | | |
| 7 | | | -0,20 | | -0,01 | -0,08 |
| 8 | | | 0,17 | -0,85 | | |
| 9 | 0,93 | -0,88 | | | | |
| 10 | | | -2,1 | -1,9 | | |
| 11 | | | | | | |
| 12 | -0,08 | -0,04 | 0,77 | 0,77 | 0,05 | 0,10 |
| 13 | 0,14 | 0,83 | -1,0 | 2,1 | | |
| 14 | 3,2 | 0,66 | 1,10 | 1,9 | | |

**Methods:** RS = Ridascreen®, R-Biopharm

Bewertung des z-Scores / valuation of z-score (DIN ISO 13528:2009-01):
*-2 ≤ z-score ≤ 2 erfolgreich / successful (in green)*
*-2 > z-score > 2 „Warnsignal“ / warning signal (in yellow)*
*-3 > z-score > 3 „Eingriffssignal“ / action signal (in red)*

***Z-Scores for the assigned values from spiking level (recovery rates)***

| Evaluation number | ELISA Lupinprotein | | ELISA Gluten | | PCR Lupin | | PCR Gluten-containing Cereals (Wheat) | |
|---|---|---|---|---|---|---|---|---|
| | Sample B | Spiking Level Sample | Sample B | Spiking Level Sample | Sample B | Spiking Level Sample | Sample B | Spiking Level Sample |
| 1 | | | 1,6 | -0,23 | | | | |
| 2 | -3,0 | 2,6 | 2,7 | 2,6 | | | | |
| 3 | -3,2 | -1,1 | | | | | | |
| 4 | | | 1,0 | 2,3 | | | | |
| 5/5a | -3,4 | 2,4 | 1,6 | 2,2 | | | | |
| 5b | | | 0,44 | 1,4 | | | | |
| 6 | -3,4 | 1,3 | 2,3 | 0,44 | | | | |
| 7 | | | 1,2 | 0,93 | | | | |
| 8 | | | 1,7 | 0,56 | | | | |
| 9 | -2,9 | 0,50 | | | | | | |
| 10 | | | -1,3 | -0,94 | | | | |
| 11 | | | | | | | | |
| 12/12a | -3,1 | 1,7 | 2,5 | 2,9 | -1,7 | -0,86 | -0,24 | 2,1 |
| 12b | | | | | | | 0,57 | 4,7 |
| 13 | -3,1 | 2,9 | 0,06 | 4,9 | | | | |
| 14 | -2,4 | 2,7 | 3,0 | 4,5 | | | | |

Bewertung des z-Scores / valuation of z-score (DIN ISO 13528:2009-01):
*-2 ≤ z-score ≤ 2 erfolgreich / successful (in green)*
*-2 > z-score > 2 „Warnsignal“ / warning signal (in yellow)*
*-3 > z-score > 3 „Eingriffssignal“ / action signal (in red)*

# 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: Lupin*

| Meth. Abr. | Evaluation number | Date of analysis | Result Sample A | | Result Sample B | | Result Spiking Sample | | NWG / LOD * | BG / LOQ * | MU* | quantitative Result given as | Method |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Day/Month | qualitative | mg/kg | qualitative | mg/kg | qualitative | mg/kg | mg/kg | mg/kg | % | | Test-Kit + Manufacturer |
| AQ | 6 | 20.04.21 | negative | <LOQ | positive | 7 | positive | 50 | 0,2 | 2 | 50 | Lupin | AgraQuant ELISA Lupin COKAL1548, RomerLabs |
| EZ | 2 | 06.04.21 | negative | < 2 | positive | 11,36 | positive | 62 | | 2 | | Lupin | other: please fill in! |
| RS-F | 3 | | negative | <0,6 | positive | 3,4 | positive | 10,1 | | 0,6 | | Lupin protein | 4.2 Kit RIDASCREEN®FAST Lupine Art. No. R6102 |
| RS-F | 9 | 06.04.21 | - | < 1,0 | - | 4,8 | - | 15,4 | 0,7 | 1 | 31 | Lupin protein | Ridascreen Fast Lupine R6102 |
| RS-F | 12 | 12.03.21 | negative | <1 | positive | 3,81 | positive | 19,51 | 1 | 1 | 27,32 | Lupin protein | other: please fill in! |
| RS-F | 14 | 30.04.21, 11.05.21 | negative | <1 | positive | 7 | positive | 23 | 1 | 1 | | Lupin protein | Ridascreen Fast Lupine, r-Biopharm |
| SP | 5 | 19.03.21 | negative | <2 | positive | 7,5 | positive | 60 | 1,5 | 2 | | Lupin | SensiSpec ELISA Lupin, Eurofins |
| SP | 13 | 05.03.21 | negative | <2 | positive | 11 | positive | 65 | 0,2 | 2 | | Lupin | SensiSpec ELISA Lupin, Eurofins |

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

| Meth. Abr. | Evaluation no. | Specifity | Remarks to the Method (Extraction and Determination) | Method Accredited ISO/IEC 17025 | Further Remarks |
|---|---|---|---|---|---|
| | | Antibody | e.g. Extraction Solution / Time / Temperature | yes/no | |
| AQ | 6 | | | no | |
| EZ | 2 | | 1g in Extraction buffer (supplied by the kit) / 15 minutes / 60°C | YES | Kit used: EZ PLATE LUPIN 2-30 ppm |
| RS-F | 3 | | | no | |
| RS-F | 9 | | | yes | lotto 24310 |
| RS-F | 12 | As per kit instructions | As per kit instructions | Yes | Kit - Ridascreen FAST Lupine Kit |
| RS-F | 14 | | | yes | |
| SP | 5 | detects lupinproteins | As per kit instructions | yes | |
| SP | 13 | | | | |

## *5.1.2 ELISA: Gluten*

| Meth. Abr. | Evaluation number | Date of analysis | Result Sample A | | Result Sample B | | Result Spiking Sample | | NWG / LOD * | BG / LOQ * | MU* | quantitative Result given as | Method |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Day/Month | qualitative | mg/kg | qualitative | mg/kg | qualitative | mg/kg | mg/kg | mg/kg | % | | Test-Kit + Manufacturer |
| AQ-G12 | 6 | 21.04.21 | positive | <LOQ | positive | 65 | positive | 23 | 2 | 4 | 50 | Gluten | AgraQuant ELISA Gluten G12 COKAL0200, RomerLabs |
| IL | 13 | 05.03.21 | negative | <4 | positive | 42 | positive | 46 | 0,6 | 4 | | Gluten | Immunolab Gliadin/Gluten ELISA |
| RS | 1 | | negative | NA | positive | 58,4 | positive | 19,5 | 3 | 10 | | Gluten | Ridascreen® Gliadin R7001, R-Biopharm |
| RS | 2 | 22.03.21 | negative | < 5 | positive | 69,18 | positive | 34,07 | | 5 | | Gluten | Ridascreen® Gliadin R7001, R-Biopharm |
| RS | 3 | | negative | < 5 | positive | > 80 | - | | | 5 | | Gluten | Ridascreen® Gliadin R7001, R-Biopharm |
| RS | 4 | 09.04.21 | - | <5,00 | - | 51,96 | - | 32,73 | 1 | 5 | | Gluten | Ridascreen® Gliadin R7001, R-Biopharm |
| RS | 5a | 11.03.21 | negative | <5 | positive | 58 | positive | 32 | 3 | 5 | | Gluten | Ridascreen® Gliadin R7001, R-Biopharm |
| RS | 7 | | - | <5,0 | - | 53,9 | - | 25,5 | 1 | 5 | | Gluten | Ridascreen® Gliadin R7001, R-Biopharm |
| RS | 12 | 09.03.21 | negative | <3 | positive | 67,7 | positive | 35,75 | 3 | 3 | 30,6 | Gluten | Ridascreen® Gliadin R7001, R-Biopharm |
| RS-F | 8 | | - | <5,0 | - | 59,2 | - | 23,6 | 1 | 5 | | Gluten | Ridascreen® FAST Gliadin R7002, R-Biopharm |
| RS-F | 10 | 09.04.21 | negative | <10,00 | positive | 27,68 | positive | 15,82 | 1 | 10 | | Gluten | Ridascreen® FAST Gliadin R7002, R-Biopharm |
| SP-R5 | 5b | 09.03.21 | negative | <3,12 | positive | 46 | positive | 28 | 3,12 | 3,12 | | Gluten | SENSISpec Ingezim Gluten R5 30.GLU.K2, Eurofins |
| VT-R5 | 14 | 27.04.21, 05.05.21, 07.05.21 | negative | <5 | positive | 72 | positive | 44 | 5 | 5 | | Gluten | Veratox Gliadin R5, Neogen |

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

*Continuation ELISA Gluten:*

| Meth. Abr. | Evaluation no. | Specifity | Remarks to the Method (Extraction and Determination) | Method Accredited ISO/IEC 17025 | Further Remarks |
|---|---|---|---|---|---|
| | | Antibody | e.g. Extraction Solution / Time / Temperature | yes/no | |
| AQ-G12 | 6 | | Sample A w as <LOQ w e got indicative 3 mg/kg | yes | |
| IL | 13 | | | | |
| RS | 1 | | | | |
| RS | 2 | | 0,25 g in Cocktail solution (supplied by the kit) / 40 minutes / 50°C | YES | |
| RS | 3 | | | yes | |
| RS | 4 | R5 | | yes | Sample B: Mean from single results 55,20 and 56,35 and 44,33 mg Gluten/kg. |
| RS | 5a | R5 antibody from Mendez detects prolamins (Gliadins) from w heat, rye and barley | As per kit instructions | yes | |
| RS | 7 | R 5 | 1 g Sample + 10 ml Eco Cocktail -> 10 min at 50°C // 10 min cooling // + 30 ml Ethanol 80% -> 10 min shaking // 5 min centrifugation // w ith dilution buffer diluted // 0,1 ml for test | no | ECO Coktail used (not the patened) benutzt. |
| RS | 12 | As per kit instructions | As per kit instructions | Yes | |
| RS-F | 8 | R 5 | 1 g Sample + 10 ml Eco Cocktail -> 10 min at 50°C // 10 min cooling // + 30 ml Ethanol 80% -> 10 min shaking // 5 min centrifugation // w ith dilution buffer diluted // 0,1 ml for test | no | ECO Coktail used (not the patened) benutzt. |
| RS-F | 10 | Peroxidase-labeled R5 Antibody | Rida Extraction Solution (colorless) Art. Nr R7098 / Methode nach Arbeitsanw eisung von R-biopharm | no | |
| SP-R5 | 5b | R5 antibody from Mendez detects prolamins (Gliadins) from w heat, rye and barley | As per kit instructions | yes | |
| VT-R5 | 14 | GliadinR5 | | yes | calculation of Gluten performed as 2fold value of Gliadin |

### *5.1.3 PCR: Lupin*

| Meth. Abr. | Evaluation no. | Date of Analysis | Result Sample A | | Result Sample B | | Result Spiking Sample | | NWG / LOD * | BG / LOQ * | MU* | quantitative Result given as | Method |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | day/month | qualitative | mg/kg | qualitative | mg/kg | qualitative | mg/kg | mg/kg | mg/kg | % | | Test-Kit+Manufacturer |
| ASU | 5 | 08.04.21 | negative | | positive | | positive | | | | | Lupin-DNA | ASU §64 Methode/method |
| SFA-ID | 1 | | negative | NA | positive | NA | positive | NA | 0,4 | | | Please select! | Sure Food Allergen ID, R-Biopharm / Congen |
| SFA-ID | 3 | | negative | | positive | | positive | | 0,4 | | | Lupin-DNA | Sure Food Allergen ID, R-Biopharm / Congen |
| SFA-ID | 9 | 31.03.21 | negative | | positive | | positive | | 0,4 | | | Lupin-DNA | Sure Food Allergen ID, R-Biopharm / Congen |
| SFA-ID | 12 | 11.03.21 | negative | <1 | positive | 26,72 | positive | 29,28 | 1 | 1 | 40 | Lupin | Sure Food Allergen ID, R-Biopharm / Congen |
| div | 11 | 28.04.21 | negative | | positive | | positive | | 100 | | | Please select! | IN HOUSE QAULITAtive METHOD |
| div | 14 | 30.04.21; | negative | | positive | | positive | | | | | Lupin-DNA | inhouse method |

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

| Meth. Abr. | Evaluation no. | Specifity | Remarks to the Method (Extraction and Determination) | Method Accredited ISO/IEC 17025 | Further Remarks |
|---|---|---|---|---|---|
| | | Antibody | e.g. Extraction Solution / Time / Temperature | yes/no | |
| ASU | 5 | | CTAB / Proteinas K / RNase A / Promega Maxwell / Real-Time PCR / 45 Cycles | | |
| SFA-ID | 1 | | | | |
| SFA-ID | 3 | | | yes | |
| SFA-ID | 9 | | | yes | cod. Kit S3611 lotto 22220 |
| SFA-ID | 12 | As per kit instructions | As per kit instructions | No | |
| div | 11 | L1PR10.1A (Ypro10.1a) | phenolchloroform exatraction, qiagen dneasy kit, end point PCR, 45 cycles, | yes | |
| div | 14 | | | | |

## *5.1.4 PCR: Gluten-containing Cereals (Wheat)*

| Meth. Abr. | Evaluation no. | Date of Analysis | Result Sample A | | Result Sample B | | Result Spiking Sample | | NWG / LOD * | BG / LOQ * | MU* | quantitative Result given as | Method |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | day/month | qualitative | mg/kg | qualitative | mg/kg | qualitative | mg/kg | mg/kg | mg/kg | % | e.g. food /protein | Test-Kit+Manufacturer |
| SFA-ID | 3 | | negative | | positive | | positive | | 0,4 | | | Gluten | Sure Food Allergen ID, R-Biopharm / Congen |
| SFA-ID | 12a | 10.03.21 | negative | <10 | positive | 447,49 | positive | 363,22 | 10 | 10 | 40 | Gluten-containing Cereals | Sure Food Allergen ID, R-Biopharm / Congen |
| SFA-4p | 12b | 10.03.21 | negative | <10 | positive | 543,46 | positive | 519,41 | 10 | 10 | 40 | Wheat | Sure Food Allergen 4plex, R-Biopharm / Congen |
| div | 14a | 30.04.21; 03.05.21 | negative | | positive | | positive | | | | | Wheat-DNA and other cereals with gliadin gene | inhouse method |
| div | 14b | 03.05.21; | negative | | positive | | positive | | | | | Wheat, Spelt, Kamut, Rye DNA | inhouse method |

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

| Meth. Abr. | Evaluation no. | Specifity | Remarks to the Method (Extraction and Determination) | Method Accredited ISO/IEC 17025 | Further Remarks |
|---|---|---|---|---|---|
| | | Antibody | e.g. Extraction Solution / Time / Temperature | yes/no | |
| SFA-ID | 3 | | | yes | |
| SFA-ID | 12a | As per kit instructions | As per kit instructions | No | Using Q40 Batch 25290 |
| SFA-4p | 12b | As per kit instructions | As per kit instructions | No | Using Q40 Batch 25290 |
| div | 14a | | | | |
| div | 14b | | | | |

## 5.2 Homogeneity

### 5.2.1 Mixture homogeneity before bottling

**Microtracer Homogeneity Test**
**DLA ptAL02 Sample B**

| | | |
|---|---|---|
| Weight whole sample | 2,34 | kg |
| Microtracer | FSS-rot lake | |
| Particle size | 75 – 300 | µm |
| Weight per particle | 2,0 | µg |
| Addition of tracer | 26,9 | mg/kg |

**Result of analysis**

| Sample | Weight [g] | Particle number | Particles [mg/kg] |
|---|---|---|---|
| 1 | 5,04 | 49 | 19,4 |
| 2 | 5,00 | 54 | 21,6 |
| 3 | 4,99 | 56 | 22,4 |
| 4 | 4,97 | 45 | 18,1 |
| 5 | 4,98 | 51 | 20,5 |
| 6 | 4,99 | 67 | 26,9 |
| 7 | 4,98 | 49 | 19,7 |
| 8 | 4,98 | 65 | 26,1 |

| **Poisson distribution** | | |
|---|---|---|
| Number of samples | 8 | |
| Degree of freedom | 7 | |
| Mean | 54,5 | Particles |
| Standard deviation | 7,89 | Particles |
| $\chi^2$ (CHI-Quadrat) | 7,99 | |
| **Probability** | **33** | % |
| Recovery rate | 81 | % |

| **Normal distribution** | | |
|---|---|---|
| Number of samples | 8 | |
| Mean | 21,8 | mg/kg |
| Standard deviation | 3,16 | mg/kg |
| rel. Standard deviaton | 14,5 | % |
| Horwitz standard deviation | 10,1 | % |
| **HorRat-value** | **1,4** | |
| Recovery rate | 81 | % |

**Microtracer Homogeneity Test**
**DLA ptAL02 Spiking Level Sample**

| | | |
|---|---|---|
| Weight whole sample | 1,50 | kg |
| Microtracer | FSS-rot lake | |
| Particle size | 75 – 300 | µm |
| Weight per particle | 2,0 | µg |
| Addition of tracer | 18,9 | mg/kg |

**Result of analysis**

| Sample | Weight [g] | Particle number | Particles [mg/kg] |
|---|---|---|---|
| 1 | 5,00 | 45 | 18,0 |
| 2 | 4,99 | 40 | 16,0 |
| 3 | 5,00 | 41 | 16,4 |
| 4 | 4,98 | 38 | 15,3 |
| 5 | 4,98 | 49 | 19,7 |
| 6 | 5,02 | 40 | 15,9 |
| 7 | 5,01 | 47 | 18,8 |
| 8 | 4,95 | 45 | 18,2 |

| **Poisson distribution** | | |
|---|---|---|
| Number of samples | 8 | |
| Degree of freedom | 7 | |
| Mean | 43,1 | Particles |
| Standard deviation | 3,95 | Particles |
| $\chi^2$ (CHI-Quadrat) | 2,53 | |
| **Probability** | **93** | % |
| Recovery rate | 91 | % |

| **Normal distribution** | | |
|---|---|---|
| Number of samples | 8 | |
| Mean | 17,3 | mg/kg |
| Standard deviation | 1,58 | mg/kg |
| rel. Standard deviaton | 9,15 | % |
| Horwitz standard deviation | 10,4 | % |
| **HorRat-value** | **0,88** | |
| Recovery rate | 91 | % |

## 5.3 Information on the Proficiency Test (PT)

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

| | |
|---|---|
| *PT number* | ***ptAL02- 2021*** |
| *PT name* | ***Allergens II: Lupin and Wheat (Gluten) in „gluten-free“ Pastry with "Spiking Level Sample“*** |
| *Sample matrix (processing)* | ***Samples A + B:***<br>*Crisp bread (baked) / Ingredients: potato starch, amaranth flour, sunflower oil, rice flour, fiber from cane sugar, corn flour, millet, sugar, rice sourdough powder (rice flour, water), emulsifier: mono- and diglycerides of fatty acids, yeast, thickener: hydroxypropylmethyl cellulose, rice protein, spices , other food additives and allergenic foods (one of both samples)*<br>***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*<br>*+ 1 Spiking Level Sample: 15 g* |
| *Storage* | *Samples A, B + Spiking Level Sample:*<br>*room temperature (PT period), cooled 2 - 10°C (long term)* |
| *Intentional use* | *Laboratory use only (quality control samples)* |
| *Parameter* | *qualitative + quantitative:*<br>*Lupin (Lupin protein, DNA), Wheat (gluten, DNA)*<br>*Samples A + B: < 500 mg/kg*<br>*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.*<br>*In general we recommend to homogenize a representative sample amount before analysis according to good laboratory practice, especially in case of low sample weights. Preferably, the total sample amount is homogenized.* |
| *Result sheet* | *One result each should be determined for Samples A and B and the Spiking Level Sample.*<br>*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:*<br>***pt@dla-lvu.de*** |
| *Last Deadline* | ***the latest April 30th 2021*** |
| *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.

# 6. Index of participant laboratories in alphabetical order

| Teilnehmer / Participant | Ort / Town | Land / Country |
|---|---|---|
| | | SWITZERLAND |
| | | ITALY |
| | | Germany |
| | | ITALY |
| | | Germany |
| | | FRANCE |
| | | Germany |
| | | Germany |
| | | ITALY |
| | | GREAT BRITAIN |
| | | AUSTRIA |
| | | GREAT BRITAIN |
| | | 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.]*

# 7. Index of references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2. DIN EN ISO/IEC 17043:2010; Konformitätsbewertung – Allgemeine Anforderungen an Eignungsprüfungen / Conformity assessment – General requirements for proficiency testing
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4. ASU §64 LFGB: Planung und statistische Auswertung von Ringversuchen zur Methoden-validierung / DIN ISO 5725 series part 1, 2 and 6 Accuracy (trueness and preci-sion) of measurement methods and results
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20. DIN EN ISO 15634-1:2009; Nachweis von Lebensmittelallergenen mit molekularbiologischen Verfahren - Teil 1: Allgemeine Betrachtungen / Foodstuffs - Detection of food allergens by molecular biological methods - Part 1: General considerations
21. DIN EN ISO 15842:2010 Lebensmittel – Nachweis von Lebensmittelallergenen – Allgemeine Betrachtungen und Validierung von Verfahren / Foodstuffs - Detection of food allergens - General considerations and validation of methods
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34. Durchführungsverordnung der Kommission/ Commission Implementing Regulation EU 828/2014; über die Anforderungen an die Bereitstellung von Information-en für Verbraucher über das Nichtvorhandensein oder das reduzierte Vorhandensein von Gluten in Lebensmitteln / on the requirements for the provision of information to consumers on the absence or reduced presence of gluten in food
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36. Köhler & Andersen (2014) Analyse von Glutengehalten in Getreide und getreidehaltigen Produkten, Tabellenwerk zum Nährstoffgehalt von Lebens-mitteln 3.1.5.1, Deutsche Forschungsanstalt für Lebensmittelchemie Leibniz Institut Jahresbericht 2014 [Analysis of gluten contents in cereals and cereal products, nutrient tables of foods]