Evaluation Report
proficiency test

DLA ptALM1 (2021)

ALM-Verification:

Peanut in Chocolate

5 Samples containing roasted Peanuts
(Levels 0,50 / 2,5 / 5,0 / 12,5 / 25 mg/kg)

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

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Unteraufträge *Subcontractors*	Im Rahmen dieser Eignungsprüfung wurden nachstehende Leistungen im Unterauftrag vergeben: Proteinbestimmung As part of the present proficency test the following services were subcontracted: protein determination
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.

Contents

1. Introduction

The participation in proficiency testing (PT) 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].

The present PT-format **„Action Level Matrix - ALM Verification"** offers the possibility to prove that the analytical determination method applied by the participating laboratory is capable to reliably detect the allergen content relevant for food labelling by means of a kind of calibration row of 5 samples containing the allergen in a specific food-matrix and a blank sample.
The allergen contents of the PT-sample series vary from 1/10 to 5-fold of the action level, which is normally based on the threshold value dose (VITAL Concept 3.0) or the assessment values of the ALTS/ALS (German Food Expert Committee) (see Table 3). The evaluation of PT-results was performed qualitative in scores from 1-5 (Score 3 = Action Level successfully detected). Quantitative results were given including the recovery rates for information in the report.
Additionally, a quantitative evaluation of the results for the Action Level as well as the Level 5 using z-scores was made for information purposes.

2. Realisation

2.1 Test material

6 PT samples were provided for the qualitative detection and optional quantitative determination of peanuts in the food matrix chocolate. The peanut levels of the PT sample series ranged from 0,5 mg/kg to 25 mg/kg, whereas the medial value represents the "Action Level" (see Table 1).

The food matrix of the test material is a mixture of commercially available dark chocolate (cocoa content: approx. 75-80%). The basic composition was the same for all 6 samples (see Table 1). The basic mixture was homogenized by stirring at 40°C.

Afterwards, the **spiked sample series** was produced as follows: After crushing and homogenization, the spiking material containing peanut was added to an aliquot of the basic matrix and the mixture was homogenized at about 40°C. Then, basic matrix was again added in portions in further steps and in each case homogenized until the total amount was reached.

The 6 PT samples were filled into PE containers in portions of approx. 20 g and sealed in metallized PET foil bags.

For the spiking, a ground mixture of roasted peanuts from a total of 18 single products from 9 countries (USA, Asia, Africa, South America) was used. The mixture of roasted peanuts showed mean recovery rates between 236% and 277% in the spiking level samples of various DLA proficiency tests from 2017 to 2021 for peanuts using various ELISA methods.

Table 1: Composition of DLA-Samples

PT-Sample series	**Level 0** „blank“	**Level 1** 0,5 mg/kg	**Level 2** 2,5 mg/kg	**Level 3** 5 mg/kg	**Level 4** 12,5mg/kg	**Level 5** 25 mg/kg
Ingredients	**g/100 g**	**g/100g**	**g/100g**	**g/100g**	**g/100g**	**g/100g**
Fine dark chocolates (85% and 70% cocoa content, 1:1 mixture) Ingredients: Cocoa mass, sugar, cocoa butter, emulsifier: lecithin (Soya), vanillin Nutrients per 100 g: Fat 46 g, Carbohydrates 27 g, Protein 10 g	100	> 99,9	> 99,9	> 99,9	> 99,9	99,9
further ingredients: *maltodextrin and silicon dioxide*	–	< 0,01	< 0,01	< 0,03	< 0,05	< 0,10
Allergen-Contents	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg
Peanut, roasted milled, mixture (18 products from USA, Asia, Africa, South America) – as peanut* – *thereof 23,2% total protein***	–	0,503 0,117	2,53 0,586	5,04 1,17	12,6 2,92	25,2 5,85
–	–					
Extended combined uncertainty (k=2) of Peanut-content (= ± 12 %)		± 0,060	± 0,30	± 0,60	± 1,5	± 3,0

*Allergen contents as „total food“ as described in column ingredients according to gravimetric mixture
** Protein contents according to laboratory analysis of raw material: 23,2 ± 1,59 %, n=5 (total nitrogen according to Kjeldahl with F=5,46 for peanut protein)

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

Each assigned value, here the spiked allergen-contents, is afflicted with a standard uncertainty. As uncertainties the following factors were considered: protein content of spiking material, mixing homogeneity, homogeneity and stability of peanut (protein).
All uncertainties were expressed in the form of their standard deviations and then added as variances. The square root from the sum of the total variances results in the combined uncertainty “Uc”. Multiplied with the coverage factor k=2 the extended uncertainties of the assigned values "$U(X_{pt})$" are obtained [3, 13, 18-20].

2.1.1 Characterization of the PT sample series

The PT sample series was characterized by ELISA determination (Immunolab Peanut ELISA, n=4). The spiked levels were recorded with good correlation between **spiking** and the **mean** of the results (see Fig. 1). The relative standard deviations (RSD) were in the range of approx. 2,7% to 8,1% and the **recovery** rates at 50% to 63% (level 2-5) and for level 1 at 132% (*values below LOQ).

Tabelle 2: Characterization of the PT sample series peanut in chocolate matrix using ELISA determination (Immunolab peanut, n=4)

PT-Sample	Level 0	Level 1	Level 2	Level 3	Level 4	Level 5
	[mg/kg]	[mg/kg]	[mg/kg]	[mg/kg]	[mg/kg]	[mg/kg]
Spiking	0,0	0,50	2,5	5,0	12,6	25,2
Result 1	<0,2	0,62	1,51	2,64	6,69	16,0
Result 2	<0,2	0,65	1,50	2,48	6,25	14,7
Result 3	<0,2	0,73	1,53	2,52	6,60	17,6
Result 4	<0,2	0,65	1,30	2,53	5,63	15,1
Mean		0,66	1,46	2,54	6,29	15,9
SD	-	0,045	0,108	0,069	0,482	1,29
RSD [%]	-	6,76	7,41	2,73	7,66	8,12
Recovery [%]	-	132	57,7	50,4	50,0	62,8

* Level 1: values below LOQ

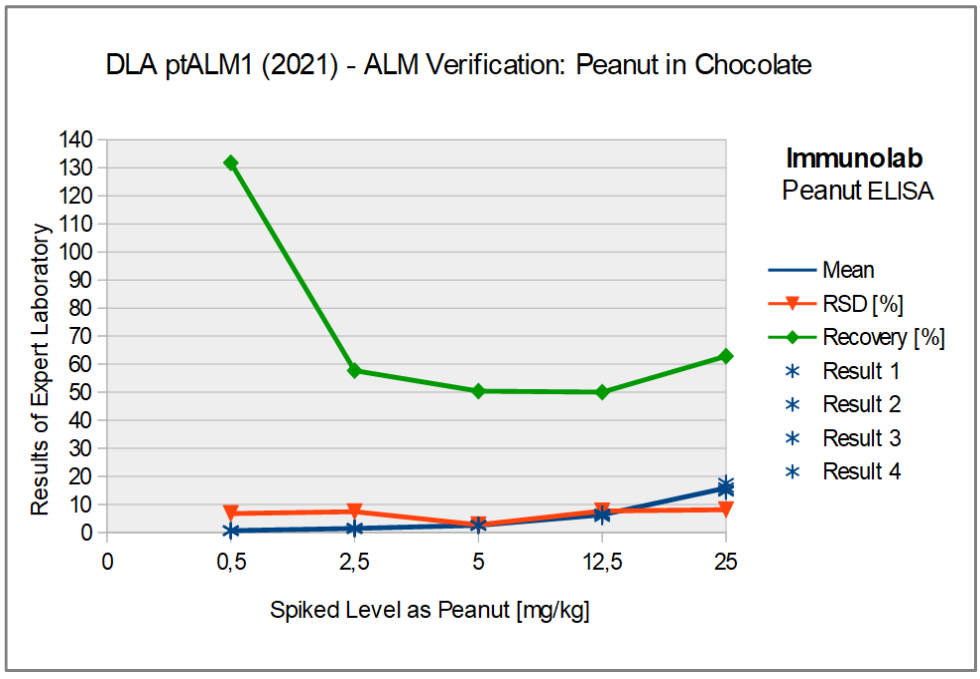


Abb./Fig. 1: Presentation of ELISA results of the PT sample series peanut in chocolate matrix (Immunolab peanut, n=4), Note: the x-axis is not shown linearly to better identify the low levels.

2.1.2 Stability

The food matrix sample material is dark chocolate, which can be kept for years due to its low water content. Experience has shown that the storage stability or shelf life of the samples (microbial spoilage) is therefore guaranteed during the test period under the specified storage conditions.

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).

2.2 Sample shipment and information to the test

The portions of test material (sample 1 to 6) were sent to every participating laboratory in the 46th week of 2021. The testing method was optional. The tests should be finished at January 14th 2022 the latest. With the cover letter along with the sample shipment, the following information was given to participants:

The proficiency test Action Level Matrix (ALM) – Verification consists of five different samples with specified contents of peanut as well as a „blank sample" in the matrix chocolate.

- *The 6 samples are numbered in a random order.*
- *It is to be proven qualitatively by any suitable method that the so-called „Action Level" of 5 mg/kg peanut can be detected in the processed matrix (= Action Level 1 (VITAL concept 3.0) and judgement value of the German Commission ALTS/ALS).*
- *If possible, the indication of quantitative results is desirable in order to compare them with the levels of addition.*

Please note the attached information on the proficiency test.
(see documentation, section 5.2 Information on the PT)

2.3 Submission of results

The participants submitted their results in standard forms, which have been sent by email or were available on our website. On one hand the results given as positive/negative and on the other hand the indicated results of the allergenic ingredients e.g. total food item or protein in mg/kg were evaluated.
Queried and documented were the indicated results and details of the test methods like specificity, limit of quantification, test kit manufacturer and remarks 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.
Of 8 participants, 7 submitted results for at least one method.

3. Evaluation

Different ELISA-methods for the determination of allergens in foods are using different antibodies, which are usually calibrated with different reference materials and may utilize differing extraction methods. Among others this can induce different results of the analyte content [32-35]. Furthermore, matrix- and/or processing of samples can have a strong impact on the detectability of allergens by ELISA and/or PCR methods.

In the present PT the allergenic ingredient was provided in an especially processed food matrix in a kind of a calibration line with concentrations in the range of the so called Action Level. The allergen content here referred to as the "Action Level" is highlighted by colour in Table 3.

The participant results were evaluated qualitatively with an Action Level Matrix Score (*ALM-Score*) which indicates the number of successfully detected concentration levels.
The quantitative results were evaluated with a Recovery-Score (*RR-Score*) which indicates the number of results with a recovery rate in the range of 50 - 150% of the spiking level.

Table 3: Threshold doses, judgement values and legislative maximum values.(Highlighted by colour: Action Level in the present PT)[21-24, 33]

Allergen	Threshold dose * (Vital Concept 3.0, 2019)		Judgement value ALTS/ALS (2020)		Legislative Maximum value for declaration
	Protein mg/kg	Food mg/kg	Protein mg/kg	Food mg/kg	mg/kg
Egg (as whole egg powder)	2	4,4	> 2	> 4,4	
Milk (as defatted milk powder)	2	5,6	> 2	> 5	
Fish (Finfish, fresh)	13	65	> 13	> 50 (steamed)	
Crustaceans (Shrimps, cooked)	250	1100	> 250	> 2100 (Shrimps, cooked)	
Peanut	2	8	> 2	**> 5**	
Lupin	26	65	> 26	> 50	
Soy (as Soyflour)	5	13	> 5	> 10	
Cashew / Pistachio	0,5	2,6	> 1	> 5	
Hazelnut and other Tree Nuts (Almond, Brazil Nut, Macadamia)	1	6,4 (4-10)	> 1	> 5	
Walnut / Pecan	0,3		> 1	> 5	
Celery Seed	0,5	-	> 0,5	> 10	
Mustard Seed	0,5	1,9	> 0,5	> 2	
Sesame, unpeeled	1	5,9	> 1	> 5	
Wheat	7	70	> 7	> 100 (>5 Gluten)	20 (Gluten)**

* calculated by threshold dose considering an intake of 100 g food, protein contents from [22] or nutritional tables Souci/Fachmann/Kraut [22,23, 24]
** Maximum value for declaration as „gluten free" according to EU-VO 828/2014 [21]

3.1 Action Level Matrix Score (ALM-Score)

The qualitative valuation of each participant's results was performed with the so called ALM-Scores from 1-5 considering the number of "positive" or "negative" results matching the spiking of the PT-sample series (see Tab. 4). An ALM-Score from > 3 indicates a successful detection of the Action Level.
The results of the matrix sample Level 0 were not evaluated if the participant result is in accordance with ≥75% positive or negative results of participants (consensus value) or if the result is below the limit of quantification of the used method.

Table 4: Evaluation of results using ALM-Scores

Level 0 „blank“	Level 1 0,5 mg/kg	Level 2 2,5 mg/kg	Level 3 (Action Level) 5 mg/kg	Level 4 12,5 mg/kg	Level 5 25 mg/kg	ALM-Score qualitative	Detection Action Level
pos/neg	pos/neg	pos/neg	pos/neg	pos/neg	pos/neg	Number of detected Levels 1 - 5	
negative	negative	negative	negative	negative	positive	1 (20%)	not successful
negative	negative	negative	negative	positive	positive	2 (40%)	not successful
negative	negative	negative	positive	positive	positive	3 (60%)	successful
negative	negative	positive	positive	positive	positive	4 (80%)	successful
negative	positive	positive	positive	positive	positive	5 (100%)	successful

3.2 Recovery-Score (RR-Score)

The evaluation of the quantitative participant results for the spiked PT-samples was done by recovery scores (*RR-Scores*) which are related to the number of recovery rates in the range of acceptance. The RR-Scores are calculated by counting the number of results in the range of acceptance (s. below) per number of quantitatively determined samples. Further the percentage is given in the brackets behind.
The recovery rates were calculated considering the content of spiked allergen (level of addition). The reference values are calculated from the values for Level 1 to 5 given in section 2.1 Sample material, Table 1. As range of acceptance RA for the evaluation of the participant results the range of the AOAC-recommendation of 50-150% for allergen-ELISAs was used [30]. This range was also used in the present PT for quantitative PCR-results.
Only exact quantitative results were considered. Single results outside the given measuring range (e.g. indicated with > 25 mg/kg or < 2,5 mg/kg) or indicated with "0" were not considered.

The given recovery rates enable inter alia an assessment of matrix and/or processing influences.

3.2.1 Recovery rates by precision experiments

In ring trials of ASU §64 methods recovery rates in the range from 57% - 119% were obtained by ELISA methods and 39 – 113% for PCR methods, depending on matrix or processing and concentration (s. Table 5a and 5b). The given target standard deviation σ_{pt} was calculated for a number of m = 2 repeated measurements.

Table 5a: ELISA-Methods – Recovery rates and precision data from chosen precision experiments [37-38]

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

The Working Group on Prolamin Analysis and Toxicity (WGPAT)performed ring trials for validation of two commercial ELISA-Kits for determination of gluten using monoclonal R5 antibodies [31]. 12 food samples with gliadin contents in the range of 0 - 168 mg/kg were analysed by 20 laboratories. The obtained recovery rates were in the range between 65 and 110%, the relative repeatability standard deviation was between 13 – 25% (1. method) and 11 - 22% (2. method) and the relative reproducibility standard deviation between 23 - 47 % (1. method) and 25 - 33% (2. method). The authors concludes that both ELISA-Kits fulfil the validation criteria for ELISA methods [31].

THE IRMM (Institute for Reference Materials and Measurements) proofed the suitability of five different ELISA-Kits for the determination of peanut [34]. The mean values were in the concentration range of 0,3 - 16,1 mg/kg and/or 1,2 - 20,4 mg/kg. The smallest relative reproducibility standard deviation for each Kit was obtained for dark chocolate at 20 - 42% and cookies at 23 - 61%.

Table 5b: PCR-Methods - Relative repeated standard deviation (RSD_r) and relative reproducibility standard deviation (RSD_R) according to chosen evaluation from experiments by precision and the resulting target standard deviation σ_{pt} [48, 41-43]

Parameter	Matrix	Mean [mg/kg]	Recov-ery	rob RSD	RSD_r	RSD_R	σ_{pt}	Method / Literature
Peanut	Rice biscuits	23,4 5,19	113 % 99,7 %	15,6% 15,0%	11,6% 14,7%	14,4% 18,1%	11,8% 14,8%	rt-PCR ASU 00.00-169
Peanut	Wheat biscuits (DLA)	1,97	39,3 %	16,2%	16,0%	19,5%	15,8%	rt-PCR ASU 00.00-169
Peanut	Milk powder Boiled sausage	3,66 2,44	73,2 % 49,4 %	15,8% 15,6%	12,8% 11,9%	14,8% 15,9%	11,7% 13,5%	rt-PCR ASU 00.00-169
Almond	Rice biscuits	105,2 18,0 10,5	105 % 90 % 105 %	-	19,3% 44,0% 32,0%	27,5% 49,1% 38,8%	23,9% 38,0% 31,5%	rt-PCR ASU 18.00-20
Almond	Wheat biscuits Sauce powder	114,3 88,1	94,6 % 88,1 %	-	22,1% 43,9%	41,8% 43,1%	38,8% - %	rt-PCR ASU 18.00-20
Almond	Rice biscuits	109 21,3 12,3	109 % 107 % 121 %	-	17,6% 35,8% 32,0%	32,8% 45,0% 47,8%	30,3% 37,2% 42,1%	rt-PCR multiplex ASU 18.00-22
Almond	Wheat biscuits Sauce powder	120,7 112	98,2 % 94,1 %	-	15,7% 36,2%	32,5% 42,8%	30,5% 34,3%	rt-PCR multiplex ASU 18.00-22
Brazil nut	Rice biscuits	89,1 17,3 9,8	89,1 % 86,5 % 98 %	-	34,1% 36,2% 40,2%	34,4% 38,2% 41,8%	24,5% 28,4% 30,6%	rt-PCR ASU 18.00-21
Brazil nut	Wheat biscuits Sauce powder	80,8 42,6	65,7 % 42,6 %	-	25,6% 27,5%	36,4% 39,7%	31,6% 34,6%	rt-PCR ASU 18.00-21
Brazil nut	Rice biscuits	96,6 14,2	96,6 % 71 %	-	16,8% 54,2%	31,8% 56,5%	29,5% 41,5%	rt-PCR multiplex ASU 18.00-22
Brazil nut	Wheat biscuits Sauce powder	76,5 48,4	62,2 % 48,4 %	-	15,6% 34,4%	35,8% 37,5%	34,1% 28,5%	rt-PCR multiplex ASU 18.00-22

3.2.2 Values by perception

Requirements to the performance of analysis methods for quantitative determination of allergens in food were compiled for example from the Ministry of Health and Welfare (MHLW) in Japan [29], by the Working Group 12 „Food allergens" of the Technician Committee CEN/TC 275 [26-28], by a international "Food Allergen Working Group" under the leadership of the AOAC Presidential Task Force on Food Allergens [30] and by the Codex Alimentarius Commitee (CAC/GL 74-2010) [25].

The following relevant ELISA and/or PCR validation criteria of the committees are given in Table 6 and 7.

Table 6: ELISA validation criteria

Literature [25-30]	**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 hypothetical ring trail in the concentration range of 0,5 - 5 mg/kg

Table 7: PCR validation criteria

Literature [25]	**Recovery Rate**	**Repeatability Standard Deviation**	**Reproducibility Standard Deviation**
CAC 2010	± 25% (a)	≤ 25%	≤ 35%

(a) = Trueness / Richtigkeit

Due to the current performance of ELISA and PCR methods for quantitative determination of allergens in food, which can be derived from precision data by experiments and from validation criteria mentioned above, a common relative target standard deviation (σ_{pt} value) from 25% was defined. The recovery rate was set to 50-150%.

3.3 z-Score (Spiking Levels)

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}), here the spiking levels [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

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

The z-scores were calculated according with the target standard deviation of 25% (see 3.2.2).

3.4 z'-Score (Spiking Levels)

The z'-score can be used for the valuation of the results of the participants, in cases the standard uncertainty has to be considered. 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 (σ_{pt}) and the standard uncertainty ($U_{(X_{pt})}$) [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

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

4. Results

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

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 harmonizes participants' results giving different specifications (e.g. as protein or as allergenic food) as far as possible.

In the present PT, one result was given as **peanut protein** and converted into the **whole food item peanut** using the experimentally determined protein content of the raw materials for roasted peanuts (cf. Tab. 1, p. 6). All other ELISA and PCR results were reported as peanut, so no conversions were required.

The qualitative results are presented in the corresponding evaluation table as indicated below:

Participant	Level 0 „blank“	Level 1 0,5 mg/kg	Level 2 2,5 mg/kg	Level 3 (Action Level) 5 mg/kg	Level 4 12,5 mg/kg	Level 5 25 mg/kg	ALM-Score qualitative	Method	Remarks
	pos/neg	pos/neg	pos/neg	pos/neg	pos/neg	pos/neg	Number of detected Levels 1 - 5		

In cases when quantitative values were submitted, the result table are given as indicated below:

Participant	Level 1 – 0,5 mg/kg			Level 2 – 2,5 mg/kg			Level 3 – 5,0 mg/kg (Action Level)			Level 4 – 12,5 mg/kg			Level 5 – 25 mg/kg			RR-Score	Method	Remarks
	Result	RR *		Result	RR *		Result	RR *		Result	RR *		Result	RR *		RR *		
	[mg/kg]	[%]	[Z_{WFR}]	[mg/kg]	[%]	[Z_{WFR}]	[mg/kg]	[%]	[Z_{WFR}]	[mg/kg]	[%]	[Z_{WFR}]	[mg/kg]	[%]	[Z_{WFR}]	Number in RA**		

* RR = Recovery Rate

4.1 Proficiency Test Peanut

4.1.1 Qualitative: Action Level Matrix-Scores

4.1.1.1 ELISA-Methods

Evaluation number	Level 0 „blank“	Level 1 0,5 mg/kg	Level 2 2,5 mg/kg	Level 3 (Action Level) 5,0 mg/kg	Level 4 12,5 mg/kg	Level 5 25 mg/kg	ALM-Score qualitative	Method	Remarks
	pos/neg	pos/neg	pos/neg	pos/neg	pos/neg	pos/neg	Number of recorded Level 1 – 5		
7	negative	positive	positive	positive	positive	positive	5 (100%)	BF	
1	negative	negative	negative	positive	positive	positive	3 (60%)	BK	
2	negative	negative	positive	positive	positive	positive	4 (80%)	MI-II	
4	negative	positive	positive	positive	positive	positive	5 (100%)	RS	
5	negative	positive	positive	positive	positive	positive	5 (100%)	RS	Level 1+2: classification by DLA
3	negative	positive	positive	positive	positive	positive	5 (100%)	SP	

	Level 0	Level 1	Level 2	Level 3	Level 4	Level 5
Number positive	0	4	5	6	6	6
Number negative	6	2	1	0	0	0
Percent positive	0	67	83	100	100	100
Percent negative	100	33	17	0	0	0
Consensus value	negative	no	positive	positive	positive	positive
Spiking	negative	positive	positive	positive	positive	positive

Methods:

BF = MonoTrace ELISA, BioFront Technologies

BK = BioKits, Neogen

MI-II = Morinaga Institute ELISA Kit II

RS = Ridascreen®, R-Biopharm

SP = SensiSpec ELISA Kit, Eurofins

Comments:

All participants detected the action level of 5 mg/kg. Also, for level 2 (1/2 of the action level) 83% positive results were still obtained. The lowest level of 0,5 mg/kg (1/10 of the action level) was still detected as positive by 4 participants (methods BF, RS and SP). This value is within the range or below the limits of quantification (LOQ) of the methods.

4.1.1.2 PCR-Methods

Evaluation number	Level 0 „blank“	Level 1 0,5 mg/kg	Level 2 2,5 mg/kg	Level 3 (Action Level) 5,0 mg/kg	Level 4 12,5 mg/kg	Level 5 25 mg/kg	ALM-Score qualitative	Method	Remarks
	pos/neg	pos/neg	pos/neg	pos/neg	pos/neg	pos/neg	Number of recorded Level 1 – 5		
2	negative	positive	positive	positive	positive	positive	5 (100%)	ASU	
5	negative	negative	negative	positive	positive	positive	3 (60%)	SFA	
6a	negative	positive	positive	positive	positive	positive	5 (100%)	div	
6b	negative	negative	negative	positive	positive	positive	3 (60%)	div	

	Level 0	Level 1	Level 2	Level 3	Level 4	Level 5
Number positive	0	2	2	4	4	4
Number negative	4	2	2	0	0	0
Percent positive	0	50	50	100	100	100
Percent negative	100	50	50	0	0	0
Consensus value	negative	none	none	positive	positive	positive
Spiking	negative	positive	positive	positive	positive	positive

Methods:

ASU = ASU §64 Methode/method

SFA = Sure Food ALLERGEN, R-Biopharm / Congen

div = not indicated / other method

Comments:

All participants detected the action level of 5 mg/kg. For level 2 (1/2 of the action level) and level 1 (1/10 of the action level), 50% positive results were still obtained. These low levels are within the range or below the limits of detection (LOD) of the methods.

4.1.2 Quantitative: Recovery Scores and z-Scores

4.1.2.1 ELISA-Results

Evaluation number	Level 1 – 0,50 mg/kg			Level 2 – 2,5 mg/kg			Level 3 – 5,0 mg/kg (Action Level)			Level 4 – 12,5 mg/kg			Level 5 – 25 mg/kg			RR-Score	Method	Remarks
	Result	RR *		Result	RR *		Result	RR *		Result	RR *		Result	RR *		RR *		
	[mg/kg]	[%]	[Z_{RR}]	[mg/kg]	[%]	[Z_{RR}]	[mg/kg]	[%]	[Z_{RR}]	[mg/kg]	[%]	[Z_{RR}]	[mg/kg]	[%]	[Z_{RR}]	Number in RA**		
7	0,44	**88**	-0,50	2,30	**91**	-0,36	5,06	**100**	0,01	11,7	**93**	-0,27	21,9	**87**	-0,53	5/5 (100%)	BF	
1	<1			<1			2,30	46	-2,2	7,10	**56**	-1,7	16,5	**65**	-1,4	2/3 (67%)	BK	
2	<0,86			3,41	**135**	1,4	6,90	**137**	1,5	17,7	**140**	1,6	33,2	**132**	1,3	4/4 (100%)	MI-II	Result converted °
4	0,99	197	3,9	4,17	165	2,6	8,45	168	2,7	18,9	**150**	2,0	39,7	157	2,3	1/5 (20%)	RS	
5	0,75	**149**	2,0	2,53	**100**	0,00	8,04	159	2,4	17,7	**141**	1,6	41,5	165	2,6	3/5 (60%)	RS	
3	0,30	**59**	-1,7	1,41	**56**	-1,8	2,79	**55**	-1,8	8,84	**70**	-1,2	24,1	**96**	-0,18	5/5 (100%)	SP	

° Calculation see p.16

RA**	50-150 %	RA**	50-150 %	RA**	50-150 %	RA**	50-150 %	RA**	50-150 %
Number in RA	3	Number in RA	4	Number in RA	3	Number in RA	6	Number in RA	4
Percent in RA	75	Percent in RA	80	Percent in RA	50	Percent in RA	100	Percent in RA	67

* Recovery rate 100% relative size: peanut, s. Page 6

** Range of acceptance of AOAC for allergen ELISAs

Methods:

BF = MonoTrace ELISA, BioFront Technologies
BK = BioKits, Neogen
MI-II = Morinaga Institute ELISA Kit II
RS = Ridascreen®, R-Biopharm
SP = SensiSpec ELISA Kit, Eurofins

Comments:

50% to 100% of the recovery rates of the quantitative participant results were in the acceptance range of 50-150%. With three methods, recovery rates within the range of acceptance were obtained for all determinable levels (presentation of levels 2-4 in Fig. 2).

4.1.2.2 PCR-Results

Evaluation number	Level 1 – 0,50 mg/kg			Level 2 – 2,5 mg/kg			Level 3 – 5,0 mg/kg (Action Level)			Level 4 – 12,5 mg/kg			Level 5 – 25 mg/kg			RR-Score	Method	Remarks
	Result	RR *		Result	RR *		Result	RR *		Result	RR *		Result	RR *		RR *		
	[mg/kg]	[%]	[Z_{RR}]	[mg/kg]	[%]	[Z_{RR}]	[mg/kg]	[%]	[Z_{RR}]	[mg/kg]	[%]	[Z_{RR}]	[mg/kg]	[%]	[Z_{RR}]	Number in RA **		
2																	ASU	
5	0,27	**53,7**	-1,9	3,04	**120**	0,81	7,11	**141**	1,6	15	**119**	0,77	24,0	**95,2**	-0,19	5/5 (100%)	SFA	
6a	<2.5			3,00	**119**	0,75	5,3	**105**	0,20	12	**95**	-0,18	25,0	**99,1**	-0,03	4/4 (100%)	div	
6b	<5			<5			<20			<20			25,0	**99,1**	-0,03	1/1 (100%)	div	
	RA**	50-150 %		RA**	50-150 %		RA**	50-150 %		RA**	50-150 %		RA**	50-150 %				
	Number in RA	1		Number in RA	2		Number in RA	2		Number in RA	2		Number in RA	3				
	Percent in RA	100		Percent in RA	100		Percent in RA	100		Percent in RA	100		Percent in RA	100				

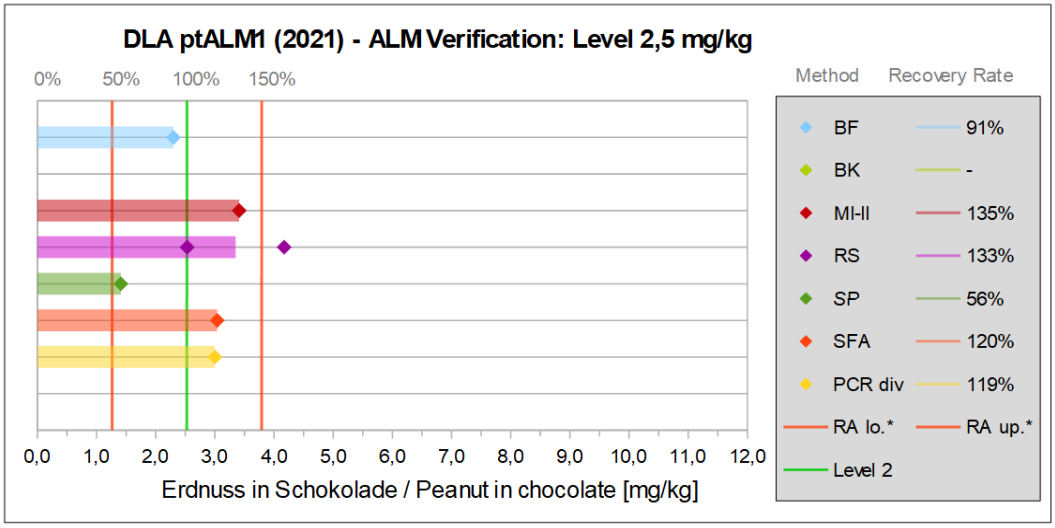
Methods:
ASU = ASU §64 Methode/method
SFA = Sure Food ALLERGEN, R-Biopharm / Congen
div = not indicated / other method

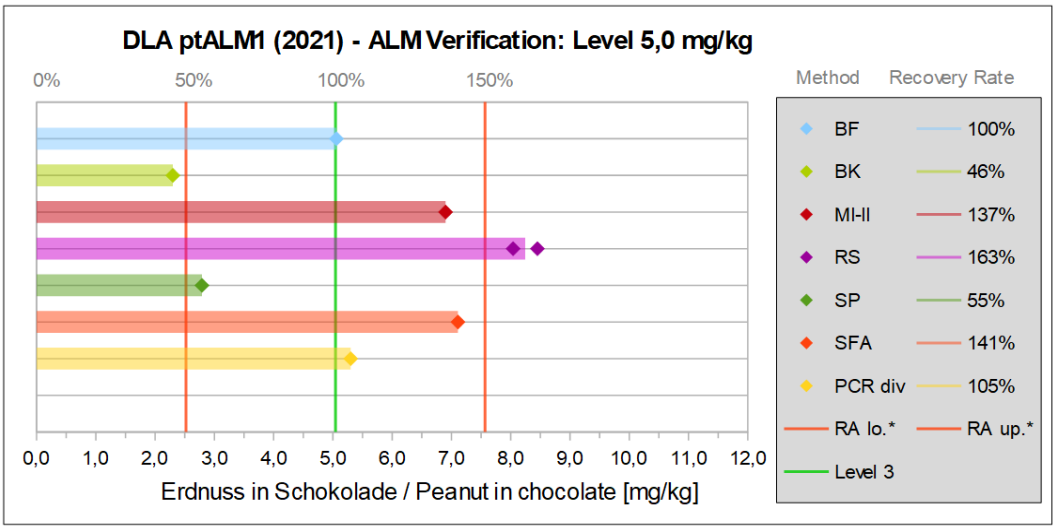
* Recovery rate 100% relative size: peanut, s. Page 6

** Range of acceptance of AOAC for allergen ELISAs

Comments:

Using PCR, two participants provided quantitative results for at least one level. All recovery rates were within the acceptance range of 50-150% (presentation of levels 2-4 in Fig. 2).





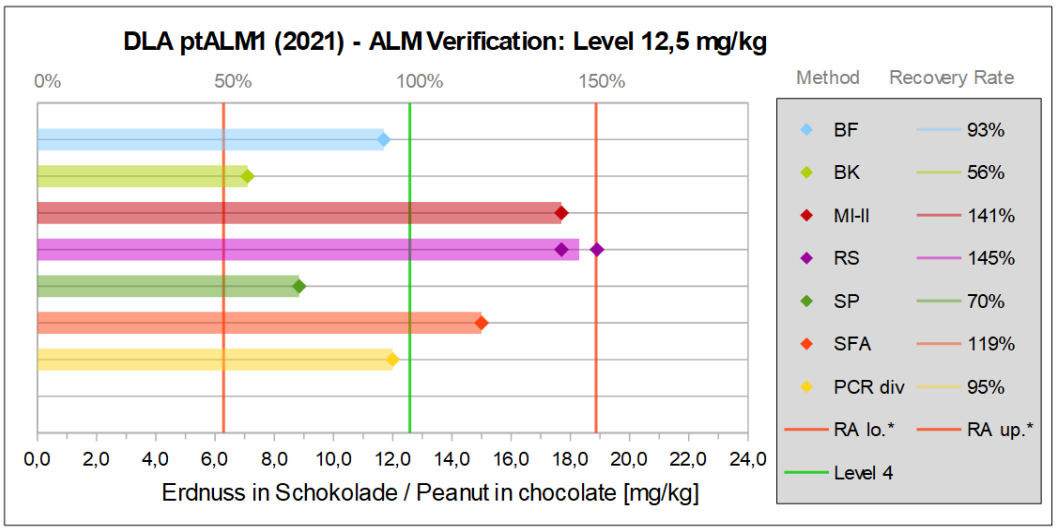


Abb./Fig. 2: Presentation of single results (Level 2-4 separated according to methods with indication of the average recovery rate), lower scale peanut content in mg/kg, upper scale % recovery rate in % with * acceptance range of 50% - 150% (* range of acceptance: RA lower limit to RA upper limit)

4.1.3 Informative Data: Statistical characteristics Peanut

4.1.3.1 ELISA- and PCR-Methods

Sample: Action Level 5,0 mg/kg

Statistic Data	**All Results** *[mg/kg]*
Assigned value (X_{pt})	$X_{pt_{ALL}}$
Number of results	8
Number of outliers	0
Mean	5,74
Median	6,10
Robust Mean (X_{pt})	**5,74**
Robust standard deviation (S*)	**2,61**
Target range:	
Target standard deviation σ_{pt}	**1,44**
lower limit of target range	**2,87**
upper limit of target range	**8,61**
Quotient S/σ_{pt}*	*1,8*
Standard uncertainty $U_{(Xpt)}$	*1,15*
Results in the target range	*6*
Percent in the target range	*75*

Comments on the statistic data:

The robust mean of the results of all methods including PCR methods was used as the assigned value.

The calculation of the z-scores was based on a target standard deviation of 25% (see Fig. 3, p. 22).

All data are for information only.

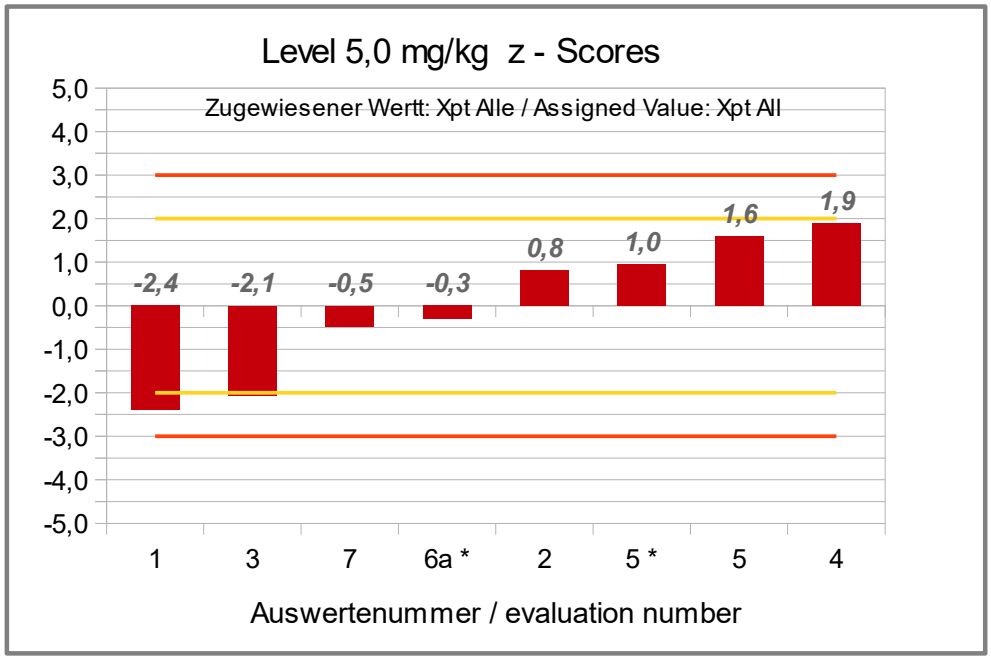


Abb./Fig. 3:
z-Scores Action Level 5,0 mg/kg (ELISA- and PCR-Results)
Assigned value: robust mean of all results
* PCR-Results

Sample: Level 12,5 mg/kg

Statistic Data	**All Results** *[mg/kg]*
Assigned value (X_{pt})	$X_{pt_{ALL}}$
Number of results	8
Number of outliers	0
Mean	13,6
Median	13,5
Robust Mean (X_{pt})	**13,6**
Robust standard deviation (S*)	**4,97**
Target range:	
Target standard deviation σ_{pt}	**3,40**
lower limit of target range	**6,81**
upper limit of target range	**20,4**
Quotient S/σ_{pt}*	*1,5*
Standard uncertainty $U_{(Xpt)}$	*2,20*
Results in the target range	*8*
Percent in the target range	*100*

Comments on the statistic data:

The robust mean of the results of all methods including PCR methods was used as the assigned value.

The calculation of the z-scores was based on a target standard deviation of 25% (see Fig. 4, p. 24).

All data are for information only.

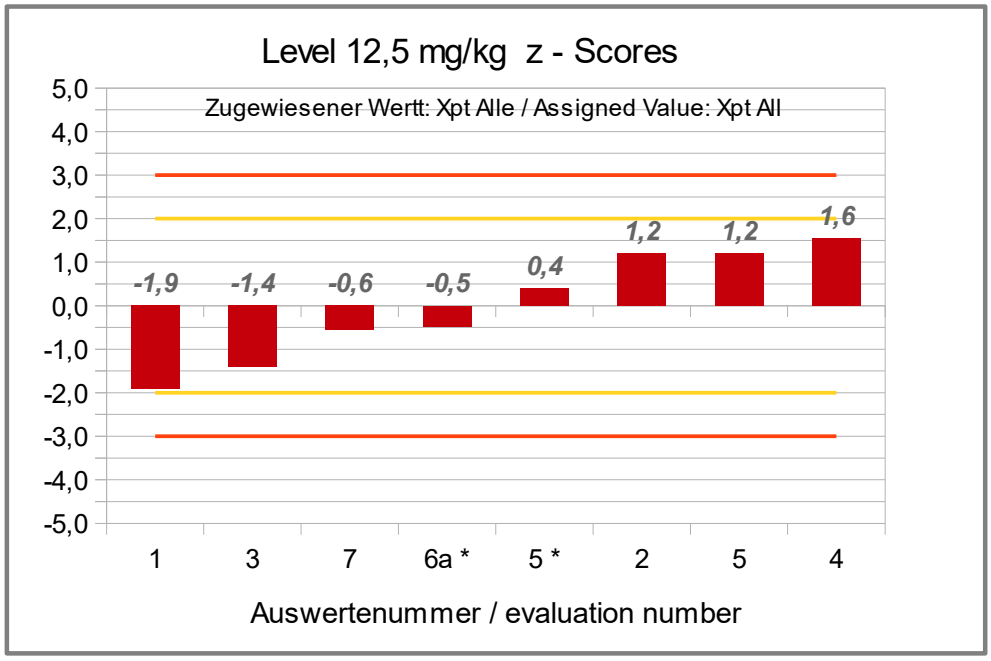


Abb./Fig. 4:
z-Scores Level 12,5 mg/kg (ELISA- and PCR-Results)
Assigned value: robust mean of all results
* PCR-Results

4.2 Participant z-Scores: overview table

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

Evaluation number	ELISA Peanut					PCR Peanut				
	Level 1	Level 2	Level 3	Level 4	Level 5	Level 1	Level 2	Level 3	Level 4	Level 5
1			-2,2	-1,7	-1,4					
2		1,4	1,5	1,6	1,3					
3	-1,7	-1,8	-1,8	-1,2	-0,18					
4	3,9	2,6	2,7	2,0	2,3					
5	2,0	0,00	2,4	1,6	2,6	-1,9	0,81	1,6	0,77	-0,19
6a							0,75	0,20	-0,18	-0,03
6b										-0,03
7	-0,50	-0,36	0,01	-0,27	-0,53					

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 and Lateral Flow Methods

Meth. Abbr.	Evaluation number	Date of Analysis	Result Sample 1 Level 5,0 mg/kg		Result Sample 2 Level 0,50 mg/kg		Result Sample 3 Level 25 mg/kg		Result Sample 4 Level 12,5 mg/kg		Result Sample 5 Level 2,5 mg/kg		Result Sample 6 Blank		NWG / LOD *	BG / LOQ *	MU*	Quantitativee Result given as	Method
		Day/Month	qualitative	mg/kg	qualitative	mg/kg	qualitative	mg/kg	qualitative	mg/kg	qualitative	mg/kg	qualitative	mg/kg	mg/kg	mg/kg	mg/kg	e.g. Food / Protein	Test-Kit + Provider
BF	7	08 March	positive	5,06	positive	0,44	positive	21,9	positive	11,72	positive	2,3	negative	0	0,12	1		Peanut	MonoTrace Peanut ELISA kit, BioFront Technologies
BK	1	08.12.2021	positive	2,3	negative	<1	positive	16,5	positive	7,1	negative	<1	negative	<1		1		Peanut	BioKits Peanut Assay Kit, Neogen
MI-II	2	9.12.	positive	1,6	negative	<0,2	positive	7,7	positive	4,1	positive	0,79	negative	<0,2	0,2	0,2		Peanut protein	Morinaga Peanut Sensitive ELISA Kit II (Art. Nr. M2120)
RS	4	12.01.22	positive	8,45	positive	0,99	positive	39,7	positive	18,9	positive	4,17	negative		0,08	0,75	--	Peanut	RIDASCREEN Peanut R6811, R-Biopharm
RS	5	10.12.	positive	8,04	negative	0,75	positive	41,5	positive	17,7	negative	2,53	negative	< 0,75	0,08	0,75		Peanut	other: please select!
SP	3	07.01.22	positive	2,786	positive	0,295	positive	24,113	positive	8,838	positive	1,405	negative	<0.2	0.1	1		Peanut	Eurofins SensiSpec Peanut ELISA Kit

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

Continuation details by participants: ELISA-Methods

Method Abbr.	Evaluation number	Specificity	Remarks to the Method (Extraction and Determination)	Method accred. accord. ISO/IEC 17025	Further remarks
		Antibody	e.g. Extraction solution / Time / Temperature	yes/no	
BF	7	monoclonal antibody-based kit	1:10 extraction ratio, 10 minutes at 60C	yes	
BK	1		following manufacturer instructions scale down weigth 1g.	yes	
MI-II	2	detects peanut proteins	according to manufacturer information	yes	
RS	4	peanut proteins	according to Testkit /10 min /60°C	no	
RS	5		according to manufacturer instructions	yes	Ridascreen Peanut R6811, r-biopharm; Sample 2 and 5: assessed as negative according to action level 5,0 (0,75 or 2,53 mg/kg positively detected)
SP	3				application of extraction additive

5.1.2 PCR-Methods

Meth. Abbr.	Evaluation number	Date of Analysis	Result Sample 1 Level 5,0 mg/kg		Result Sample 2 Level 0,50 mg/kg		Result Sample 3 Level 25 mg/kg		Result Sample 4 Level 12,5 mg/kg		Result Sample 5 Level 2,5 mg/kg		Result Sample 6 Blank		NWG / LOD *	BG / LOQ *	MU*	Quantitativee Result given as	Method
		Day/Month	qualitative	mg/kg	qualitative	mg/kg	qualitative	mg/kg	qualitative	mg/kg	qualitative	mg/kg	qualitative	mg/kg	mg/kg	mg/kg	mg/kg	e.g. Food / Protein	Test-Kit + Provider
ASU	2	27.12.	positive		positive		positive		positive		positive		negative		5			Peanut DNA	ASU §64 Methode/method
SFA	5	27.12.	positive	7,11	negative	0,27	positive	24	positive	15	negative	3,04	negative	< 1,0	0,4	1		Peanut	Sure Food ALLERGEN, R-Biopharm / Congen
div	6a	23.12.21	positive	5,3	positive	<2.5	positive	25	positive	12	positive	3	negative	< 0.5	0,5	2,5		Peanut-DNA	Selection PCR Methods
div	6b	24.12.21	positive	<20	negative	<5	positive	25	positive	<20	negative	<5	negative	<5	5	20		Peanut-DNA	Selection PCR Methods

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

Continuation details by participants: PCR-Methods

Method Abbr.	Evaluation number	Specificity	Remarks to the Method (Extraction and Determination)	Method accred. accord. ISO/IEC 17025	Further remarks
		Target-Sequence / -DNA	e.g. Extraction / enzymes / clean-up / real time PCR / gel electrophoresis / cycles	yes/no	
ASU	2		CTAB / Proteinase K / Rnase A / Promega Maxw ell / Realtime PCR / 45 cycles	yes	§ 64 LFGB L 00.00-169:2019-07, sample 2: traces at the detection limit
SFA	5		Purification w ith SureFood Prep Advanced, additionally w ith tw ice the amount of lysis buffer and elution buffer	no	Results from both processings used
div	6a	MT-ATP6			
div	6b	Ara h 2			

5.2 Information on the Proficiency Test (PT)

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

PT number	***DLA ptALM1 - 2021***
PT name	***ALM-Verification Peanut: 5 Samples containing roasted Peanuts in Chocolate-Matrix (levels: 0,50 / 2,5 / 5,0 / 12,5 / 25 mg/kg)*** *(and a "blank sample")*
Sample matrix (processing)	***Samples 1-6:*** *Chocolate approx. 78%/ ingredients: Cocoa mass, sugar, cocoa butter, emulsifier: lecithins, vanilla extract other food additives and the allergenic food peanut*
Number of samples and sample amount	*5 different Samples: 20 g each* *+ 1 „blank sample" : 20 g*
Storage	*Samples : room temperature (long term 2 - 10°C)*
Intentional use	*Laboratory use only (quality control samples)*
Parameter	*qualitative (optional: quantitative):* *Peanut (Peanut protein / DNA)* *Levels: 0,50 / 2,5 / 5,0 / 12,5 / 25 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. Preferably the total sample amount should be homogenized.*
Result sheet	*One qualitative (and optional quantitative) result each should be determined for Samples 1-6.* *The results should be filled in the result submission file.*
Units	*positive / negative (optional: 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 January 14th 2022***
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
		AUSTRIA
		Germany
		USA
		SWITZERLAND
		CANADA
		Germany
		Germany
		USA
		ITALY

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

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

7. Index of references

1. DIN EN ISO/IEC 17025:2005; Allgemeine Anforderungen an die Kompetenz von Prüf- und Kalibrierlaboratorien / General requirements for the competence of testing and calibration laboratories
2. DIN EN ISO/IEC 17043:2010; Konformitätsbewertung – Allgemeine Anforderungen an Eignungsprüfungen / Conformity assessment – General requirements for proficiency testing
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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
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36. ASU §64 LFGB L 06.00-56 Bestimmung von Sojaprotein in Fleisch und Fleischerzeugnissen Enzymimmunologisches Verfahren (2007) [Determination of soyprotein in meat and meat products by enzyme immunoassay]
37. ASU §64 LFGB L 00.00-69 Bestimmung von Erdnuss-Kontaminationen in Lebensmitteln mittels ELISA im Mikrotiterplattensystem (2003) [Foodstuffs, determination of peanut contamintions in foodstuffs by ELISA in microtiterplates]
38. ASU §64 LFGB L 44.00-7 Bestimmung von Haselnuss-Kontaminationen in Schokolade und Schokoladenwaren mittels ELISA im Mikrotiterplattensystem (2006) [Food-stuffs, determination of hazelnut contamintions in chocolate and chocolate products by ELISA in microtiterplates]
39. ASU §64 LFGB L 16.01-9 Untersuchung von Lebensmitteln - Bestimmung von Soja (Glycine max) in Getreidemehl mittels real-time PCR (2016) [Foodstuffs, determination of soya (Glycine max) in cereal flour by real-time PCR]
40. ASU §64 LFGB L 18.00-19 Untersuchung von Lebensmitteln - Nachweis und Bestimmung von Sesam (Sesamum indicum) in Reis- und Weizenkeksen sowie in Soßenpulver mittels real-time PCR (2014) [Foodstuffs, detection and determination of sesame (Sesamum indicum) in rice and wheat cookies and sauce powders by PCR]
41. ASU §64 LFGB L 18.00-20 Untersuchung von Lebensmitteln - Nachweis und Bestimmung von Mandel (Prunus dulcis) in Reis- und Weizenkeksen sowie in Soßenpulver mittels real-time PCR (2014) [Foodstuffs, detection and determination of almond (Prunus dulcis) in rice and wheat cookies and sauce powders by PCR]

42. ASU §64 LFGB L 18.00-21 Untersuchung von Lebensmitteln - Nachweis und Bestimmung von Paranuss (Bertholletia exceisa) in Reis- und Weizenkeksen sowe in Soßenpulver mittels real-time PCR (2014) [Foodstuffs, detection and determination of brazil nut (Bertholletia exceisa) in rice and wheat cookies and sauce powders by PCR]
43. ASU §64 LFGB L 18.00-22 Untersuchung von Lebensmitteln - Simultaner Nachweis und Bestimmung von Lupine, Mandel, Paranuss und Sesam in Reis- und Weizenkeksen sowie Soßenpulver mittels real-time PCR (2014) [Foodstuffs, simultaneous detection and determination of lupin, almond, brazil nut and sesame in rice and wheat cookies and sauce powders by PCR]
44. ASU §64 LFGB L 08.00-59 Untersuchung von Lebensmitteln - Nachweis und Bestimmung von Senf (Sinapis alba) sowie Soja (Glycine max) in Brühwürsten mittels real-time PCR (2013) [Foodstuffs, detection and determination of mustard (Sinapis alba) and soya (Glycine max) in boiled sausages by real-time PCR]
45. ASU §64 LFGB L 08.00-64 Untersuchung von Lebensmitteln - Nachweis und Bestimmung von von schwarzem Senf (Brassica nigra L.) und braunem Senf (Brassica juncea L.) in Brühwurst mittels real-time PCR (2016) [Foodstuffs, detection and determination of black mustard (Brassica nigra L.) and brown mustard (Brassica juncea L.) in boiled sausages by real-time PCR]
46. ASU §64 LFGB L 08.00-65 Untersuchung von Lebensmitteln - Simultaner Nachweis und Bestimmung von schwarzem Senf (Brassica nigra L.), braunem Senf (Brassica juncea L.), weißem Senf (Sinapis alba), Sellerie (Apium graveolens) und Soja (Glycine max) in Brühwurst mittels real-time PCR (2017) [Foodstuffs, simultaneous detection and determination of black mustard (Brassica nigra L.), brown mustard (Brassica juncea L.), white mustard (Sinapis alba), celery (Apium graveolens) and soya (Glycine max) in boiled sausages by real-time PCR]
47. ASU §64 LFGB L 08.00-66 Untersuchung von Lebensmitteln - Nachweis und Bestimmung von Weizen (Triticum L.) und Roggen (Secale cereale) in Brühwurst mittels real-time PCR (2016) [Foodstuffs, detection and determination of wheat (Triticum L.) and rye (Secale cereale) in boiled sausages by real-time PCR]
48. ASU §64 LFGB L 00.00-169 Untersuchung von Lebensmitteln - Nachweis und Bestimmung von Erdnuss in Lebensmitteln mittels real-time PCR (2019) [Foodstuffs, detection and determination of peanut in foods by real-time PCR]