Evaluation Report
proficiency test

DLA 17/2019

ALM-Verification:

Egg in Cookie-Matrix

5 Samples baked with Whole Egg Powder (levels: 0,1 / 0,5 / 1,0 / 5,0 / 15 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 at least 5-fold of the action level, which is normally based on the threshold value dose (VITAL Concept 2.0) 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 with the same food matrix of cookies for infants were provided for qualitative detection and optional quantitative determination of egg. The egg levels of the PT-sample series were in the range from 0,1 mg/kg to 15 mg/kg, whereas the medial level represents the "Action Level" (see Table 1).
The food matrix of the sample material was common in commerce cookies. The basic composition was identical for all 6 samples (see Table 1).
After crushing and sieving using an impact mill (mesh 1,5 mm) the basic mixture was homogenized and an aliquot was taken from it as blank sample.

For preparation of the egg containing samples first cookies were baked (150°C, 40 min) and dried (40°C) adding whole egg powder (further information see below). Afterwards the egg-cookies were crushed by a knife mill and homogenized.

Afterwards the **spiked sample series** was produced as follows: After crushing and homogenization an aliquot of the egg containing cookies was added to the basic mixture. The resulting mixture was homogenized again. Afterwards basic mixture was added stepwise (2-3 steps) including homogenization after each step until the total amount of sample material was reached.

The 6 PT-samples were portioned to approximately 20 g in metallized PET film bags.

For the spiking a mixture of whole egg powders from a total of 6 products out of 2 countries (Europe) was used. This mixture of whole egg powders gave a mean recovery rate for egg of about 101 % ± 20 % (n=13) for the samples (matrix: potato powder / maltodextrin) of the PT DLA 15/2019 calculated from different ELISA method results.

Table 1: Composition of DLA-Samples

PT-Sample series	**Level 0** „blank“	**Level 1** 0,1 mg/kg	**Level 2** 0,5 mg/kg	**Level 3** 1,0 mg/kg	**Level 4** 5,0 mg/kg	**Level 5** 15 mg/kg
Ingredients	**g/100 g**	**g/100g**	**g/100g**	**g/100g**	**g/100g**	**g/100g**
Butter Cookies Ingredients: Wheat flour, sugar, butter, barley malt extract, skimmed milk powder, glucose, glucose syrup, raising agent ammonium carbonate, salt, emulsifier lecithin Nutrients per 100 g: Fat 12 g, carbohydrates 76 g, protein 7,1 g	100	>99,9	>99,9	>99,9	>99,9	99,8
Cookies (baked 150°C, 40 min) Ingredients: Wheat flour, Sugar, butter, water, mixture of whole egg powders, salt	-	0,002	0,008	0,017	0,083	0,25
Allergen-Contents	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg
thereof Egg: - as Whole Egg Powder* - *with 46,9% protein***	-	**0,101** 0,047	**0,506** 0,237	**1,01** 0,474	**5,04** 2,36	**15,1** 7,08
Extended combined uncertainty (k=2) of egg content (= ± 11 %)		± 0,011	± 0,056	± 0,11	± 0,55	± 1,7

*Allergen contents as „total food“ as described in column ingredients according to gravimetric mixture
** Protein contents according to laboratory analysis of raw material (total nitrogen according to Kjeldahl with F=6,25 for egg 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 egg 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 (Morinaga ELISA Kit II, n=5). The spiking levels were detectable from level 2 and correlated with the ascending mean of results (see Fig. 1). The relative standard deviations (RSD) were in the range of 7,6% to 36% and the recovery rates ranged from 24% to 32% (level 3: estimated values < LOQ of the test kit).

Table 2: Characterization of PT-sample series as whole egg powder in cookies by ELISA determination (Morinaga ELISA II, n=5).

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	0,10	0,51	1,01	5,0	15,1
Result 1	0,0	<0,2	<0,2	0,30	1,66	3,31
Result 2	0,0	<0,2	<0,2	0,32	1,12	3,86
Result 3	0,0	<0,2	<0,2	0,52	1,33	3,82
Result 4	0,0	<0,2	<0,2	0,24	1,05	3,29
Result 5	0,0	<0,2	<0,2	0,23	1,70	3,65
Mean	0,0	<0,2	<0,2	0,32	1,37	3,59
SD	-	-	-	0,12	0,30	0,27
RSD [%]	-	-	-	36,4	21,9	7,6
Recovery [%]	-	-	-	31,9	27,2	23,7

* Level 3: values below ELISA kit LOQ

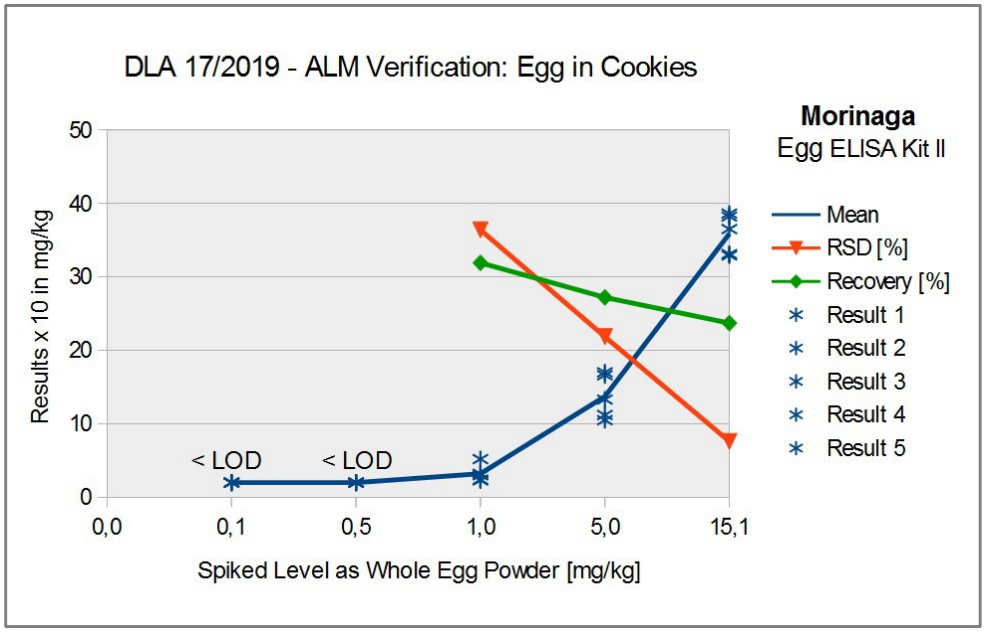


Abb./Fig. 1: ELISA results of PT-sample series as whole egg powder in cookies (Morinaga ELISA Kit II, n=5)), *Note: results x 10, the x-scale is not shown linear to obtain a better recognizability of low values.*

2.1.2 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 1 to 5 showed a probability of 82%, 92%, 99%, 98% and 78%. Additionally particle number results were converted into concentrations, statistically evaluated according to normal distribution and compared to the standard deviation according to Horwitz. For the assessment HorRat values between 0,3 and 1,3 are to be accepted under repeat conditions (measurements within the laboratory) [17]. This gave HorRat values of 0,88, 0,64, 0,54, 0,61 and 0,94 respectively. The results of the microtracer analysis are given in the documentation.

2.1.3 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 PT samples was approx. 0,19 (19,0°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 material (sample 1 to 6) were sent to every participating laboratory in the 48th week of 2019. The testing method was optional. The tests should be finished at January 10th 2020 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 egg as well as a „blank sample" in the matrix cookies.

- *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 1,0 mg/kg egg as whole egg powder can be detected in the processed matrix (= Action Level 1 (VITAL concept 2.0) 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 5.3 Information on the PT)

2.3 Submission of results

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

11 of 13 participants submitted results.

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 [31-34]. 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)[21-23, 32]

Allergen	Threshold dose * (Vital Concept 2.0)	Judgement value ALTS/ALS	Legislative Maximum value for declaration
	mg/kg	mg/kg	mg/kg
Gluten	100	> 80	20 **
Egg (as whole egg powder)	***0,66***	***> 1***	
Peanut	8	> 5	
Soy (as Soy flour)	25	> 20	
Milk (as defatted milk powder)	2,8	> 2,5	
Hazelnut	6,4	> 5	
Cashew	106	> 50	
Almond, Walnut, Pecan, Brazil-Nut, Pistachio, Macadamia	-	> 20	
Sesame, unpeeled	11,8	> 10	
Lupine	100	> 50	
Celery seed	-	> 20	
Mustard seed	1,9	> 5	

* calculated by threshold dose considering an intake of 100 g food [22,23]
** 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 0,1 mg/kg	Level 2 0,5 mg/kg	Level 3 (Action Level) 1,0 mg/kg	Level 4 5,0 mg/kg	Level 5 15 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 [29]. 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 33% - 161% 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 [36-37].

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 [30]. 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 [30].

THE IRMM (Institute for Reference Materials and Measurements) proofed the suitability of five different ELISA-Kits for the determination of peanut [33]. 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} [38-42].

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

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 [28], by the Working Group 12 „Food allergens" of the Technician Committee CEN/TC 275 [25-27], by a international "Food Allergen Working Group" under the leadership of the AOAC Presidential Task Force on Food Allergens [29] and by the Codex Alimentarius Commitee (CAC/GL 74-2010) [24].

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 [24-29]	**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 [24]	**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%.

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.

ELISA-Results given as **egg white protein** or **egg protein (egg white and yolk proteins)** were converted to **whole egg powder**. When possible the information provided by the test kit manufacturer was used. A content of 26 % egg white protein and 48 % egg protein in whole egg powder was taken.

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

Participant	Level 0 „blank“	Level 1 0,1 mg/kg	Level 2 0,5 mg/kg	Level 3 (Action Level) 1,0 mg/kg	Level 4 5,0 mg/kg	Level 5 15 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 – 0,1 mg/kg		Level 2 – 0,5 mg/kg		Level 3 – 1,0 mg/kg (Action Level)		Level 4 – 5,0 mg/kg		Level 5 – 15 mg/kg		RR-Score	Method	Remarks
	Result	RR *	Result	RR *	Result	RR *	Result	RR *	Result	RR *	RR *		
	[mg/kg]	[%]	[mg/kg]	[%]	[mg/kg]	[%]	[mg/kg]	[%]	[mg/kg]	[%]	Number in RA**		

* RR = Recovery Rate (RR)

4.1 Proficiency Test Egg

4.1.1 Qualitativ: Action Level Matrix-Scores

4.1.1.1 ELISA-Methods

Evaluation number	Level 0 „Null“	Level 1 0,10 mg/kg	Level 2 0,5 mg/kg	Level 3 (Action Level) 1,0 mg/kg	Level 4 5,0 mg/kg	Level 5 15 mg/kg	ALM-Score qualitative	Method	Remarks
	pos/neg	pos/neg	pos/neg	pos/neg	pos/neg	pos/neg	Number of recorded Level 1 - 6		
8a	negative	negative	negative	negative	negative	negative	0/5 (0%)	AQ	
10	negative	negative	negative	negative	negative	negative	0/5 (0%)	AQ-P	
8b	negative	negative	negative	negative	negative	negative	0/5 (0%)	AQ-P	
5a	negative	negative	negative	negative	negative	negative	0/5 (0%)	BK	
4	negative	negative	negative	negative	positive	positive	2/5 (40%)	MI-II	
7	negative	negative	negative	negative	positive	positive	2/5 (40%)	MI-II	
2	negative	negative	negative	positive	positive	positive	3/5 (60%)	RS	
9	negative	negative	negative	positive	positive	positive	3/5 (60%)	RS	
3a	negative	negative	negative	negative	positive	positive	2/5 (40%)	RS	
6a	negative	negative	negative	negative	positive	positive	2/5 (40%)	RS	
1a	positive	negative	positive	negative	negative	positive	-	RS-F	not rated
3b	negative	negative	negative	negative	negative	negative	0/5 (0%)	RS-F	
5b	negative	negative	negative	negative	negative	negative	0/5 (0%)	RS-F	
6b	negative	negative	negative	negative	negative	negative	0/5 (0%)	RS-F	
1b	positive	negative	positive	negative	negative	negative	-	VT	not rated

	Level 0	Level 1	Level 2	Level 3	Level 4	Level 5
Number positive	2	0	2	2	6	7
Number negative	13	15	13	13	9	8
Percent positive	13	0	13	13	40	47
Percent negative	87	100	87	87	60	53
Consensus value	negative	negative	negative	negative	none	none
Spiking	negative	positive	positive	positive	positive	positive

Methods:

AQ = AgraQuant, RomerLabs

AQ-P = AgraQuant Plus, RomerLabs

BK = BioKits, Neogen

MI-II = Morinaga Institute ELISA II

RS = Ridascreen®, R-Biopharm

RS-F= Ridascreen® Fast, R-Biopharm

VT = Veratox, Neogen

Comments:

The two highest levels 4 (5 mg/kg) and 5 (15 mg/kg) were successfully detected by the participants with the methods MI-II and RS. The action level 3 was detected as positive by two participants with the method RS (both results were at or below the limit of quantification of the method).

With the other ELISA methods no (plausible) positive results were obtained for any of the 5 levels.

4.1.2 Quantitative: Recovery-Scores

4.1.2.1 ELISA-Results (as whole egg powder)

Evaluation number	Level 1 – 0,10 mg/kg		Level 2 – 0,50 mg/kg		Level 3 – 1,0 mg/kg (Action Level)		Level 4 - 5,0 mg/kg		Level 5 - 15 mg/kg		RR-Score	Method	Remarks
	Result	RR *	Result	RR *	Result	RR *	Result	RR *	Result	RR *	RR *		
	[mg/kg]	[%]	[mg/kg]	[%]	[mg/kg]	[%]	[mg/kg]	[%]	[mg/kg]	[%]	Number in RA **		
8a	<LOD		<LOD		<LOD		<LOD		<LOD			AQ	
10	< LOQ		< LOQ		< LOQ		< LOQ		< LOQ			AQ-P	
8b	<LOD		<LOD		<LOD		<LOD		<LOD			AQ-P	
5a	-		-		-		-		-			BK	
4	-		-		-		1,17	23	4,38	29	0/0 (0%)	MI-II	results converted °
7	<0,64		<0,64		<0,64		1,19	24	6,25	41	0/0 (0%)	MI-II	results converted °
2	< 0,1		< 0,1		< 0,25		0,72	14	2,40	16	0/0 (0%)	RS	
9	<0.25		<0.25		0,25	25	0,70	14	25,0	165	0/0 (0%)	RS	
3a	< 0,25		< 0,25		< 0,25		0,39	7,7	0,84	5,6	0/0 (0%)	RS	
6a	0,03	25	0,084	17	0,11	11	0,60	12	3,31	22	0/0 (0%)	RS	
1a	<0,1		<0,5		<0,1		<0,1		<0,5			RS-F	
3b	< 0,5		< 0,5		< 0,5		< 0,5		< 0,5			RS-F	
5b												RS-F	
6b	< 0,5		< 0,5		< 0,5		< 0,5		< 0,5			RS-F	
1b	<0.6		<2.5		<0.6		<0.6		<2.5			VT	

° calculation p. 15

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

* Recovery rate 100% Reference value: whole egg powder, s. Page 6

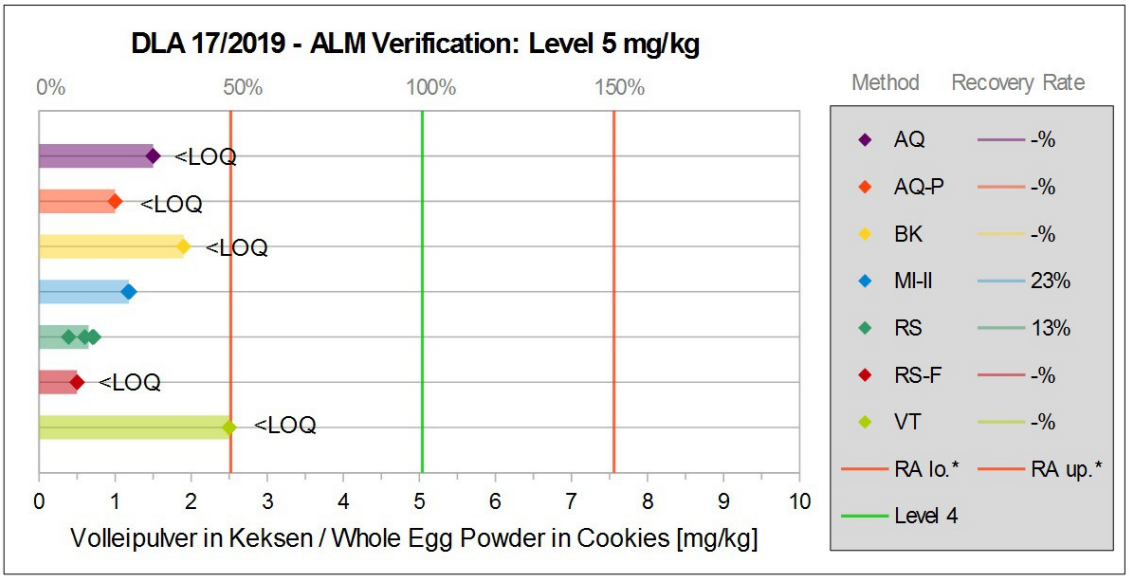
** Acceptance range of AOAC for allergen ELISAs

Methods:

AQ = AgraQuant, RomerLabs
AQ-P = AgraQuant Plus, RomerLabs
BK = BioKits, Neogen
MI-II = Morinaga Institute ELISA II
RS = Ridascreen®, R-Biopharm
RS-F= Ridascreen® Fast, R-Biopharm
VT = Veratox, Neogen

Comments:

For the levels 4 and 5 the recovery rates of the participants' results were between 23% and 41% (method MI-II) and between 6% and 25% (method RS, without result No. 9 for level 5). Therefore they were below the AOAC recommendations of 50-150%.



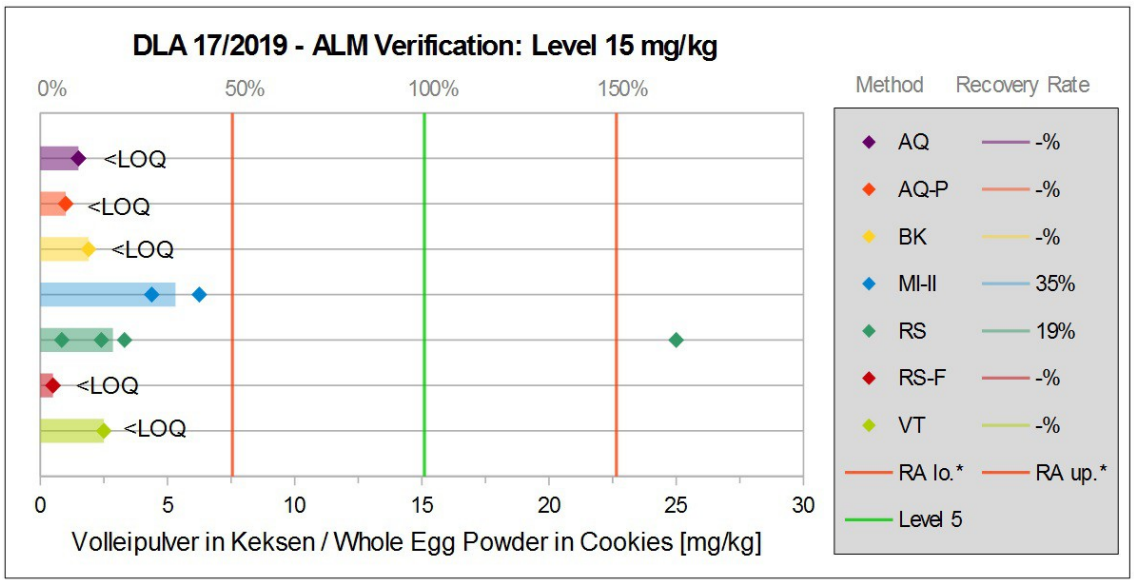


Abb./Fig. 2: Graphs of single results (Level 4+5) separated by methods with corresponding mean recovery rates, lower scale egg content as whole egg powder in mg/kg, upper scale recovery rate in % with * range of acceptance from 50% - 150% (* range of acceptance: RA lower limit to RA upper limit)

4.1.3 Informative Data: Statistical characteristics egg

4.1.3.1 ELISA-Methods (as whole egg powder)

Sample: Level 5,0 mg/kg

Statistic Data	**All Results°** *[mg/kg]*
Assigned value (X_{pt})	$X_{pt_{ALL}}$
Number of results	6
Number of outliers	0
Mean	0,795
Median	0,710
Robust Mean (X_{pt})	**0,795**
Robust standard deviation (S*)	**0,364**
Target range:	
Target standard deviation σ_{pt}	**0,199**
lower limit of target range	**0,397**
upper limit of target range	**1,19**
Quotient S/σ_{pt}*	*1,8*
Standard uncertainty $U_{(Xpt)}$	*0,186*
Results in the target range	*5*
Percent in the target range	*83*

° methods MI-II and RS

Comments on the statistic data:

Since at least 5 results were not available for any of the methods, the assigned value was taken from the robust mean of the results from both methods MI-II and RS.

The calculation of the z-scores was based on a target standard deviation of 25% (see Fig. 3, p. 19).

All data are for information only.

Note: It should be noted that the results for the two methods suggest a different response. The comparability of the results is therefore limited.

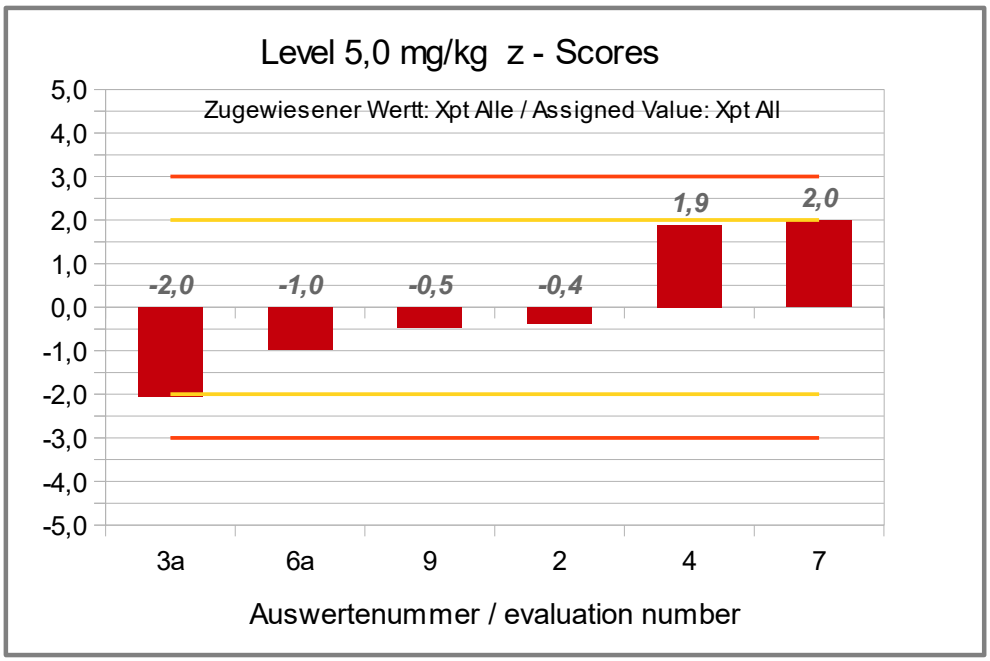


Abb./Fig. 3:
z-Scores action level 5,0 mg/kg (ELISA-results as whole egg powder)
Assigned value: robust mean of all results (Alg. A)

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-Methods

Meth. Abbr.	Evaluation number	Date of Analysis	Result Sample 1 Level 0,5 mg/kg		Result Sample 2 Level 0,1 mg/kg		Result Sample 3 Level 5,0 mg/kg		Result Sample 4 Level 1,0 mg/kg		Result Sample 5 Level 15mg/kg		Result Sample 6 „blank“		NWG / LOD *	BG / LOQ *	MU*	Quantitative 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	%	e.g. Food / Protein	Test-Kit + Provider
AQ	8a	20.12.19	negative	<LOD	negative	<LOD	negative	<LOD	negative	<LOD	negative	<LOD	negative	<LOD	0,05	0,4		Egg white protein, total	AgraQuant ELISA Egg White COKAL0848, RomerLabs
AQ-P	10	28.11.19	negative	< LOQ	negative	< LOQ	negative	< LOQ	negative	< LOQ	negative	< LOQ	negative	< LOQ	0,5	1		Whole Egg Powder	AgraQuant Plus ELISA Egg COKAL1848F, RomerLabs
AQ-P	8b	18.12.19	negative	<LOD	negative	<LOD	negative	<LOD	negative	<LOD	negative	<LOD	negative	<LOD	0,5	1		Whole Egg Powder	AgraQuant Plus ELISA Egg COKAL1848F, RomerLabs
BK	5a	19.12.19	negative		negative		negative		negative		negative		negative		0,5	0,5		Egg white protein, total	BioKits Egg Assay Kit, Neogen
MI-II	4	20/12 und 06/01	negative		negative		positive	0,56	negative		positive	2,1	negative		0,3	0,3		egg protein	Morinaga Egg (Ovalbumin) ELISA Kit II (M2111)
MI-II	7	06.12.19	negative	<0,31	negative	<0,31	positive	0,57	negative	<0,31	positive	3	negative	<0,31	0,31	0,31		Whole egg protein	Morinaga Egg (Ovalbumin) ELISA Kit II (M2111)
RS	2	10.01.20	negative	< 0,1	negative	< 0,1	positive	0,72	positive	< 0,25	positive	2,4	negative	< 0,1	0,1	0,25		Whole Egg Powder	RIDASCREEN Egg R6411
RS	9	09.01.20	negative	<0.25	negative	<0.25	positive	0.7	positive	0.25	positive	25	negative	<0.25	0.13	0.25	30%	Whole Egg Powder	Ridascreen® Egg; R6411, R-Biopharm
RS	3a	06.01.20	negative	< 0,25	negative	< 0,25	positive	0,388	negative	< 0,25	positive	0,844	negative	< 0,25		< 0,25		Whole Egg Powder	RIDASCREEN Egg R6411, R-Biopharm
RS	6a	18/12	negative	0,084	negative	0,025	positive	0,601	negative	0,108	positive	3,31	negative	0,016	0,13	0,25		Whole Egg Powder	Ridascreen® Egg R6411, R-Biopharm
RS-F	1a	07.01.20	positive	<0.5	negative	<0.1	negative	<0.1	negative	<0.1	positive	<0.5	positive	<0.5	0,1	0,5		Whole Egg Powder	Ridascreen® FAST Egg Protein R6402, R-Biopharm
RS-F	3b	10.12.19	negative	< 0,5	negative	< 0,5	negaitv	< 0,5	negative	< 0,5	negative	< 0,5	negative	< 0,5		< 0,5		Whole Egg Powder	RIDASCREEN FAST Egg Protein R6402, R-Biopharm
RS-F	5b	19.12.19	negative		negative		negative		negative		negative		negative		0,5	0,5		Whole Egg Powder	Ridascreen® FAST Egg Protein R6402, R-Biopharm
RS-F	6b	09/12	negative	< 0,5	negative	< 0,5	negative	< 0,5	negative	< 0,5	negative	< 0,5	negative	< 0,5	0,1	0,5		Whole Egg Powder	Ridascreen® FAST Egg Protein R6402, R-Biopharm
VT	1b	19.12.19	positive	<2.5	negative	<0.6	negative	<0.6	negative	<0.6	negative	<2.5	positive	<2.5	0,6	2,5		Whole Egg Powder	Veratox Egg Allergen, Neogen

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

Continuation details by participants:

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	
AQ	8a			yes	
AQ-P	10		Water (60°C) / 1 minut	no	
AQ-P	8b			no	
BK	5a	Ovomucoid (Gal d1)		yes	
MI-II	4		Short Time Extraction Method	yes	
MI-II	7	recognizes the egg white protein ovalbumin	according to manufacturer's instructions	yes	
RS	2		according to manual		
RS	9	Egg white protein (Ovalbumin; Ovomukoid)	Allergen extraction buffer with egg extractor, additive and skim-med milk; 10 min / 60 ° C	no	processed matrix
RS	3a		Protein extraction according to manufacturer's instructions 9.2	no	
RS	6a	Antibodies detect egg white proteins ovalbumin and ovomucoid (also heated samples)	Sample extraction for processed/ heated samples; 1 g sample + 0.5 g skimmed milk powder/ 1 ml egg extractor + 19 ml allergen extraction buffer with additive; 10 min at 60 ° C	no	Qualitative assessment according to Action Level 1 of 1 ppm whole egg powder Sample 3 weakly positive
RS-F	1a		according to kit instructions with the addition of casein		Result for samples 1 + 6 in the area of the technical limit of detection
RS-F	3b		according to the manufacturer's instructions	yes	after dilution of the standard 0.25 mg/kg to 0.05 mg/kg, sample 3 = 0.061 mg/kg, sample 5 = 0.166 mg/kg
RS-F	5b	Ovalbumin and Ovomucoid		yes	
RS-F	6b	Antibodies detect egg white proteins ovalbumin and ovomucoid	1 g sample/ 20 ml allergen extraction buffer; 10 min at 60 ° C	no	Qualitative assessment according to Action Level 1 of 1 ppm whole egg powder
VT	1b		according to kit instructions		Result for samples 1 + 6 in the area of the technical limit of detection

5.2 Homogeneity

5.2.1 Mixture homogeneity before bottling

Microtracer Homogeneity Test
DLA 17-2019 Sample 1

Weight whole sample	1,00	kg
Microtracer	FSS-rot lake	
Particle size	75 – 300	µm
Weight per particle	2,0	µg
Addition of tracer	25,3	mg/kg

Result of analysis

Sample	Weight [g]	Particle number	Particles [mg/kg]
1	5,04	65	25,8
2	5,10	81	31,8
3	5,15	73	28,3
4	5,15	75	29,1
5	4,96	63	25,4
6	5,03	73	29,0
7	4,71	72	30,6
8	5,07	81	32,0

Poisson distribution		
Number of samples	8	
Degree of freedom	7	
Mean	72,9	Particles
Standard deviation	6,19	Particles
χ^2 (CHI-Quadrat)	3,68	
Probability	**82**	%
Recovery rate	115	%

Normal distribution		
Number of samples	8	
Mean	29,0	mg/kg
Standard deviation	2,46	mg/kg
rel. Standard deviaton	8,50	%
Horwitz standard deviation	9,64	%
HorRat-value	**0,88**	
Recovery rate	115	%

Microtracer Homogeneity Test
DLA 17-2019 Sample 2

Weight whole sample	1,00	kg
Microtracer	FSS-rot lake	
Particle size	75 – 300	µm
Weight per particle	2,0	µg
Addition of tracer	36,0	mg/kg

Result of analysis

Sample	Weight [g]	Particle number	Particles [mg/kg]
1	4,83	106	43,9
2	5,09	117	46,0
3	5,09	110	43,2
4	4,89	104	42,5
5	4,82	95	39,4
6	4,97	115	46,3
7	4,95	100	40,4
8	5,07	104	41,0

Poisson distribution		
Number of samples	8	
Degree of freedom	7	
Mean	106,3	Particles
Standard deviation	6,22	Particles
χ^2 (CHI-Quadrat)	2,54	
Probability	**92**	%
Recovery rate	119	%

Normal distribution		
Number of samples	8	
Mean	42,8	mg/kg
Standard deviation	2,50	mg/kg
rel. Standard deviaton	5,85	%
Horwitz standard deviation	9,09	%
HorRat-value	**0,64**	
Recovery rate	119	%

Microtracer Homogeneity Test
DLA 17-2019 Sample 3

Weight whole sample	1,40	kg
Microtracer	FSS-rot lake	
Particle size	75 – 300	µm
Weight per particle	2,0	µg
Addition of tracer	21,8	mg/kg

Result of analysis

Sample	Weight [g]	Particle number	Particles [mg/kg]
1	4,97	80	32,2
2	5,01	70	27,9
3	5,00	74	29,6
4	5,02	75	29,9
5	5,01	72	28,7
6	4,91	78	31,8
7	5,12	79	30,9
8	5,04	72	28,6

Poisson distribution		
Number of samples	8	
Degree of freedom	7	
Mean	75,0	Particles
Standard deviation	3,87	Particles
χ^2 (CHI-Quadrat)	1,40	
Probability	**99**	%
Recovery rate	137	%

Normal distribution		
Number of samples	8	
Mean	29,9	mg/kg
Standard deviation	1,54	mg/kg
rel. Standard deviaton	5,16	%
Horwitz standard deviation	9,59	%
HorRat-value	**0,54**	
Recovery rate	137	%

Microtracer Homogeneity Test
DLA 17-2019 Sample 4

Weight whole sample	1,20	kg
Microtracer	FSS-rot lake	
Particle size	75 – 300	µm
Weight per particle	2,0	µg
Addition of tracer	22,8	mg/kg

Result of analysis

Sample	Weight [g]	Particle number	Particles [mg/kg]
1	5,02	62	24,7
2	4,97	66	26,6
3	4,87	70	28,7
4	5,03	67	26,6
5	5,06	71	28,1
6	4,89	62	25,4
7	5,07	71	28,0
8	5,03	62	24,7

Poisson distribution		
Number of samples	8	
Degree of freedom	7	
Mean	66,4	Particles
Standard deviation	3,97	Particles
χ^2 (CHI-Quadrat)	1,66	
Probability	**98**	%
Recovery rate	117	%

Normal distribution		
Number of samples	8	
Mean	26,6	mg/kg
Standard deviation	1,59	mg/kg
rel. Standard deviaton	5,98	%
Horwitz standard deviation	9,77	%
HorRat-value	**0,61**	
Recovery rate	117	%

Microtracer Homogeneity Test
DLA 17-2019 Sample 5

Weight whole sample	1,60	kg
Microtracer	FSS-rot lake	
Particle size	75 – 300	µm
Weight per particle	2,0	µg
Addition of tracer	27,2	mg/kg

Result of analysis

Sample	Weight [g]	Particle number	Particles [mg/kg]
1	5,03	66	26,2
2	5,17	83	32,1
3	4,98	68	27,3
4	5,12	62	24,2
5	4,92	69	28,0
6	5,08	63	24,8
7	4,96	64	25,8
8	5,03	66	26,2

Poisson distribution		
Number of samples	8	
Degree of freedom	7	
Mean	67,6	Particles
Standard deviation	6,19	Particles
χ^2 (CHI-Quadrat)	3,96	
Probability	**78**	%
Recovery rate	99	%

Normal distribution		
Number of samples	8	
Mean	26,8	mg/kg
Standard deviation	2,46	mg/kg
rel. Standard deviaton	9,15	%
Horwitz standard deviation	9,75	%
HorRat-value	**0,94**	
Recovery rate	99	%

5.3 Information on the Proficiency Test (PT)

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

PT number	***DLA 17-2019***
PT name	***ALM-Verification Egg: 5 Samples containing Whole Egg Powder in Infant-Cookies Matrix*** *(and a "blank sample")*
Sample matrix (processing)	***Samples 1-6:*** *Cookies (baked at appr. 150°C)/ ingredients: Wheat flour, sugar, butter, barley malt extract, glucose syrup, baking agent ammonium carbonate, salt, whole milk powder, emulsifier lecithins, other food additives and egg (except "blank sample")*
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): Egg / eggprotein* *Levels (as whole egg powder): 0,1 / 0,5 / 1,0 / 5,0 / 15 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 10th 2020***
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
		Germany
		SWITZERLAND
		Germany
		Germany
		Germany
		Germany
		Germany
		Germany
		Germany
		AUSTRIA
		AUSTRIA
		USA
		Germany

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

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

7. Index of references

1. DIN EN ISO/IEC 17025:2005; Allgemeine Anforderungen an die Kompetenz von Prüf- und Kalibrierlaboratorien / General requirements for the competence of testing and calibration laboratories
2. DIN EN ISO/IEC 17043:2010; Konformitätsbewertung – Allgemeine Anforderungen an Eignungsprüfungen / Conformity assessment – General requirements for proficiency testing
3. ISO 13528:2015 & DIN ISO 13528:2009; Statistische Verfahren für Eignungsprüfungen durch Ringversuche / Statistical methods for use in proficiency testing by interlaboratory comparisons
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39. ASU §64 LFGB L 18.00-22 Untersuchung von Lebenmitteln - 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]ASU §64 LFGB L 16.01-9 Untersuchung von Lebenmitteln - 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]
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