Evaluation Report
proficiency test

DLA 16/2019

ALM-Verification:

Almond in Cookie-Matrix

5 Samples baked with Almonds
(levels: 2,0 / 10 / 20 / 50 / 100 mg/kg)

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

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EP-Bericht Freigabe *PT-Report Authorization*	Dr. Matthias Besler-Scharf (Technischer Leiter / Technical Manager) *- gezeichnet / signed M. Besler-Scharf* Alexandra Scharf MSc. (QM-Beauftragte / Quality Manager) *- gezeichnet / signed A. Scharf* Datum / Date: 16 December 2019
Unteraufträge *Subcontractors*	Im Rahmen dieser Eignungsprüfung wurden nachstehende Leistungen im Unterauftrag vergeben: Gehaltsprüfung des EP-Parameters, Proteinbestimmung As part of the present proficency test the following services were subcontracted: Quantification of PT-parameter(s), 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.

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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 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 food matrix cookie were provided for qualitative detection and optional quantitative determination of almond. The almond levels of the PT-sample series were in the range from 2,0 mg/kg to 100 mg/kg, whereas the medial level represents the "Action Level" (see Table 1).
The food matrix of sample material was common in commerce butter 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 almond containing samples first cookies were baked (150°C, 30 min) and dried (40°C) using a mixture of raw almonds (further information see below). Afterwards the almond-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 almond containing cookies was added to the basic mixture. The resulting mixture was homogenized again. Afterwards basic mixture was added stepwise (3-5 steps) including homogenization after each step until the total amount of sample material was reached.

For the spiking a mixture of raw and ground almonds from a total of 18 products out of 5 countries (USA, Europe, Australia, Middle East) was used. This mixture of almonds gave a mean recovery rate for almond of about 121 % ± 40 % (n=10) for the samples (matrix: potato powder / maltodextrin) of the PT DLA 14/2019 calculated from different ELISA method results.

Table 1: Composition of DLA-Samples

PT-Sample series	**Level 0** „blank“	**Level 1** 2,0 mg/kg	**Level 2** 10 mg/kg	**Level 3** 20 mg/kg	**Level 4** 50 mg/kg	**Level 5** 100 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	99,8	99,7	99,1	98,3
Cookies (baked 150°C, 30 min) Ingredients: Wheat flour, Sugar, butter, eggs, salt and mixture of raw almonds and further ingredients (maltodextrin, sodium sulfate, silicon dioxide)	-	0,034	0,17	0,34	0,86	1,72
Allergen-Contents	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg
thereof Almonds: - as Almonds* - *with 20,5% protein***	-	**2,01** 0,412	**9,98** 2,05	**20,0** 4,10	**49,7** 10,2	**99,9** 20,5
Extended combined uncertainty (k=2) of almond content (= ± 13 %)		± 0,26	± 1,3	± 2,6	± 6,5	± 13

*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=5,18 for almond 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 almond.
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 (Veratox Almond, n=3). The spiking levels correlated with the ascending mean of results (see Fig. 1). The relative standard deviations (RSD) were in the range of approx. 1,5% to 7% and the recovery rates ranged from 26% to 44% (level 1: estimated < LOD with 113% recovery).

Table 2: Characterization of PT-sample series almond in cookies by ELISA determination (Veratox Almond, n=3).

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	2,01	9,98	20,0	49,7	99,9
Result 1	1,3	2,3	3,9	6,4	13,2	41,2
Result 2	1,3	2,4	4,0	6,5	12,2	44,8
Result 3	1,3	2,1	3,9	6,9	12,9	46,4
Mean [mg/kg]	1,3	2,27	3,93	6,60	12,8	44,1
SD	-	0,15	0,06	0,26	0,51	2,66
RSD [%]	-	6,7	1,5	4,0	4,0	6,0
Recovery [%]	-	113	39	33	26	44

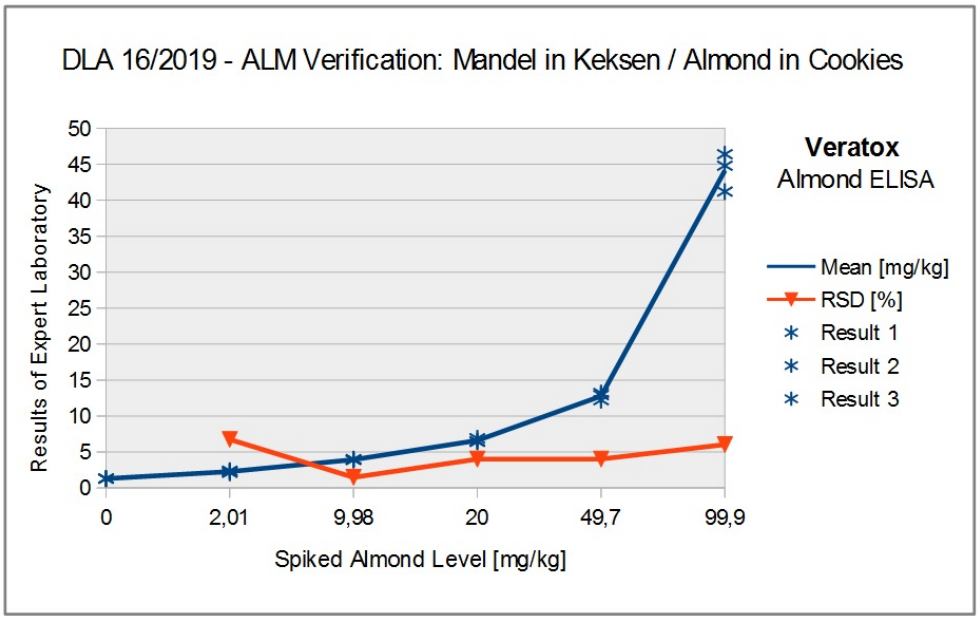


Abb./Fig. 1: ELISA results of PT-sample series almond in cookies (Veratox Almond, n=3), *Note: the x-scale is not shown linear to obtain a better recognizability of low values.*

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 1 to 5 showed a probability of 94%, 17%, 88%, 87% and 79%. 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 0,63, 1,6, 0,92, 0,84 and 0,9 respectively. The value of 1,6 was accepted, because the probability of the Poisson distribution was sufficient. The results of the microtracer analysis are given in the documentation.

2.1.2 Stability

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

The experience with various DLA test materials showed good storage stability with respect to the durability of the sample (spoilage) and the content of the PT parameters for comparable food matrices and water activity (a_W value <0,5).
The a_W value of the PT samples was approx. 0,16 (21,5°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 22nd week of 2019. The testing method was optional. The tests should be finished at July 12th 2019 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 almond as well as a „blank sample" in the matrix of 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 20 mg/kg almond 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 5.2 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 hints about the procedure.
In case participants submitted several results for the same parameter obtained by different methods these results were evaluated with the same evaluation number with a letter as a suffix and indication of the related method.

All 11 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 2,0 mg/kg	Level 2 10 mg/kg	Level 3 (Action Level) 20 mg/kg	Level 4 50 mg/kg	Level 5 100 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 48% - 105% 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} [39-40].

Parameter	Matrix	Mean [mg/kg]	Recov-ery	rob RSD	RSD_r	RSD_R	σpt	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
Brazil Nut	Rice cookie	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 cookie 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 cookie	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 cookie 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 [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.

In the present PT all ELISA results were given as almond, therefore no conversion of results was necessary.

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

Participant	Level 0 „blank“	Level 1 2,0 mg/kg	Level 2 10 mg/kg	Level 3 (Action Level) 20 mg/kg	Level 4 50 mg/kg	Level 5 100 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 – 2,0 mg/kg		Level 2 – 10 mg/kg		Level 3 – 20 mg/kg (Action Level)		Level 4 - 50 mg/kg		Level 5 - 100 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 Almond

4.1.1 Qualitativ: Action Level Matrix-Scores

4.1.1.1 ELISA-Methods

Evaluation number	Level 0 „blank“	Level 1 2,0 mg/kg	Level 2 10 mg/kg	Level 3 (Action Level) 20 mg/kg	Level 4 50 mg/kg	Level 5 100 mg/kg	ALM-Score qualitative	Method	Remarks
	pos/neg	pos/neg	pos/neg	pos/neg	pos/neg	pos/neg	Anzahl erfasster Level 1 - 5		
1	= LOD	positive	positive	positive	positive	positive	5 (100%)	AQ	
3	positive	positive	positive	positive	positive	positive	5 (100%)	AQ	
7	positive	positive	positive	positive	positive	positive	5 (100%)	AQ	
2	negative	positive	positive	positive	positive	positive	5 (100%)	BF	
10	negative	positive	positive	positive	positive	positive	5 (100%)	IL	
4	negative	positive	positive	positive	positive	positive	5 (100%)	RS-F	
9	negative	positive	positive	positive	positive	positive	5 (100%)	RS-F	
6	negative	negative	positive	positive	positive	positive	4 (80%)	VT	
8	negative	> LOD	positive	positive	positive	positive	5 (100%)	VT	
11	< LOQ	positive	positive	positive	positive	positive	5 (100%)	VT	

	Level 0	Level 1	Level 2	Level 3	Level 4	Level 5
Number positivee	2	8	10	10	10	10
Number negativee	6	1	0	0	0	0
Percent positivee	25	89	100	100	100	100
Percent negativee	75	11	0	0	0	0
Consensus value	negative	positive	positive	positive	positive	positive
Spiking	negative	positive	positive	positive	positive	positive

Methods:

AQ = AgraQuant, RomerLabs

BF = MonoTrace ELISA, BioFront Technologies

IL = Immunolab

RS-F= Ridascreen® Fast, R-Biopharm

VT = Veratox, Neogen

Comments:

Level 2 (10 mg/kg) and the Action Level (20 mg/kg) as well as the levels 4 and 5 were successfully detected by all participants. Level 1 was detected by 89% (8) of the participants. The negative result is in agreement with the limit of quantification according to the test kit instructions (VT with 2,5 mg/kg as almond). Using the method AQ positive results were obtained for the blank level 0 in the range of up to 1 mg/kg.

4.1.1.2 PCR-Methoden

Evaluation number	Level 0 „blank“	Level 1 2,0 mg/kg	Level 2 10 mg/kg	Level 3 (Action Level) 20 mg/kg	Level 4 50 mg/kg	Level 5 100 mg/kg	ALM-Score qualitative	Method	Remarks
	pos/neg	pos/neg	pos/neg	pos/neg	pos/neg	pos/neg	Anzahl erfasster Level 1 - 5		
5	positive	negative	positive	positive	positive	positive	4 (80%)	MS	for Level 1 s. documentation

	Level 0	Level 1	Level 2	Level 3	Level 4	Level 5
Spiking	negative	positive	positive	positive	positive	positive

Methods:

MS = Microsynth

Comments:

One participant has submitted qualitative PCR results. Levels 2 to 5 including the Action Level of 20 mg/kg were successfully detected.
However, the "blank" level was detected as positive, while level 1 was given as negative.

4.1.2 Quantitative: Recovery-Scores

4.1.2.1 ELISA-Results

Evaluation number	Level 1 – 2,0 mg/kg		Level 2 – 10 mg/kg		Level 3 – 20 mg/kg (Action Level)		Level 4 - 50 mg/kg		Level 5 - 100 mg/kg		RR-Score	Method	Remarks
	Result	RR *	Result	RR *	Result	RR *	Result	RR *	Result	RR *			
	[mg/kg]	[%]	[mg/kg]	[%]	[mg/kg]	[%]	[mg/kg]	[%]	[mg/kg]	[%]	Anzahl im AB**		
1	0,65	32	3,13	31	4,88	24	8,08	16	34,8	35	0/5 (0%)	AQ	
3	1,30	**65**	6,30	**63**	8,40	42	27,0	**54**	46,3	46	3/5 (60%)	AQ	
7	1,30	**65**	4,70	47	14,0	**70**	20,0	40	42,0	42	2/5 (40%)	AQ	
2	2,36	**117**	5,70	**57**	7,40	37	18,1	36	58,6	**59**	3/5 (60%)	BF	
10	1,30	**65**	6,00	**60**	9,00	45	21,0	42	52,0	**52**	3/5 (60%)	IL	
4	4,30	214	12,0	**120**	21,0	**105**	49,0	**99**	87,0	**87**	4/5 (80%)	RS-F	
9	4,08	203	9,23	**92**	19,7	**99**	42,9	**86**	99,2	**99**	4/5 (80%)	RS-F	
6	<2.5		4,80	48	7,80	39	15,2	31	56,9	**57**	1/5 (20%)	VT	
8	2,27	**113**	3,93	39	6,60	33	12,8	26	44,1	44	1/5 (20%)	VT	
11	3,55	177	6,12	**61**	9,84	49	22,5	45	55,0	**55**	2/5 (40%)	VT	

RA**	50-150 %	RA**	50-150 %	RA**	50-150 %	RA**	50-150 %	RA**	50-150 %
Number in RA	**5**	Number in RA	**6**	Number in RA	**3**	Number in RA	**3**	Number in RA	**6**
Percent in RA	**56**	Percent in RA	**60**	Percent in RA	**30**	Percent in RA	**30**	Percent in RA	**60**

* Recovery rate 100% Reference value: Almond, s. Page 6

** Acceptance range of AOAC for allergen ELISAs

Methods:

AQ = AgraQuant, RomerLabs

BF = MonoTrace ELISA, BioFront Technologies

IL = Immunolab

RS-F= Ridascreen® Fast, R-Biopharm

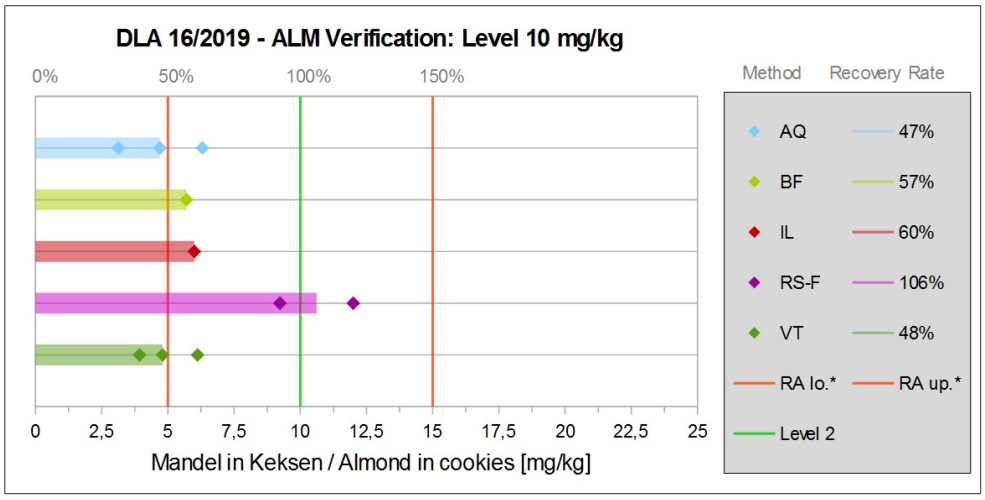
VT = Veratox, Neogen

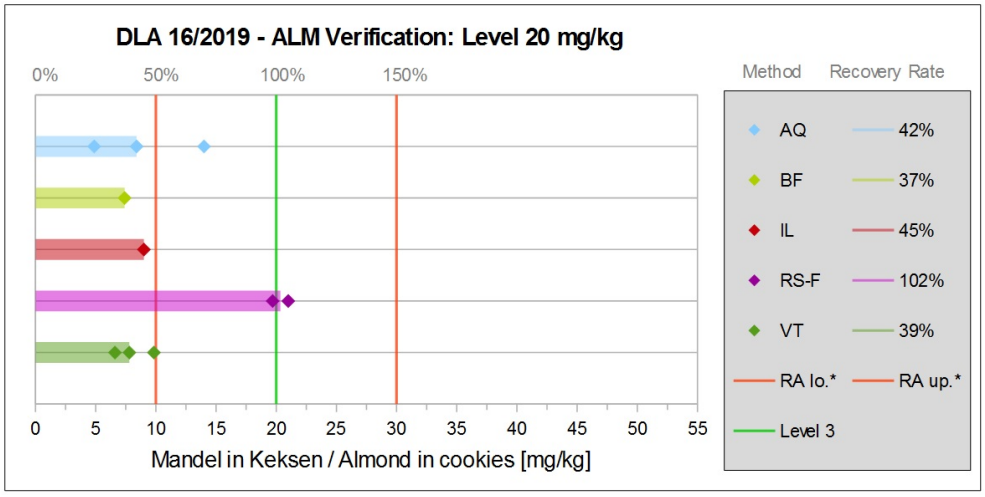
Comments:

For the levels 2 to 5 the recovery rates of the two participants' results of the method RS-F were within the AOAC recommendations of 50-150%. By the other methods recovery rates within the range of acceptance were obtained for up to three levels.

4.1.2.2 PCR-Results

No quantitative PCR results were submitted.





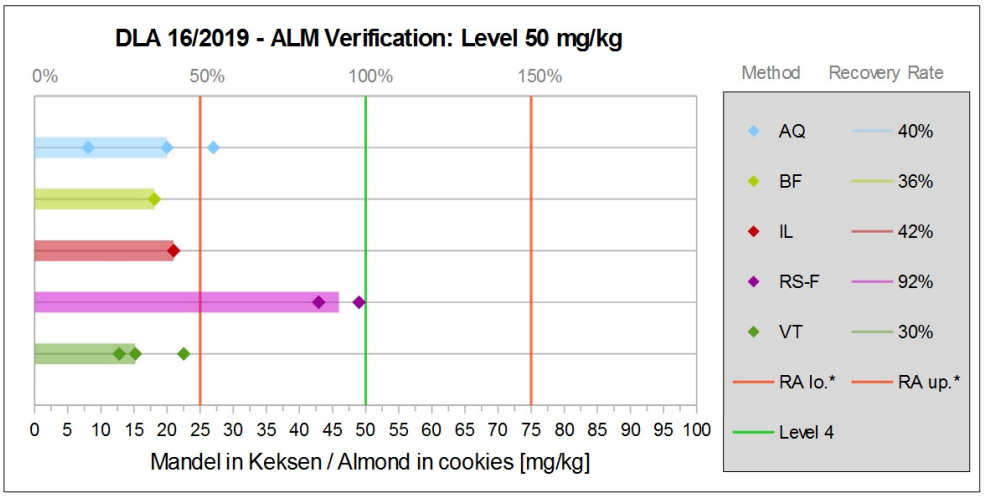


Abb./Fig. 2: Graphs of single results (Level 2-4) separated by methods with corresponding mean recovery rates, lower scale almond content in mg/kg, upper scale recovery rate in % with * range of acceptance from 50% - 150% (* range of acceptance: RA lower limit to RA upper limit)

4.1.3 Informative Data: Statistical characteristics almond

4.1.3.1 ELISA-Methods

Sample: Action Level 20,0 mg/kg

Statistic Data	**All Results°** *[mg/kg]*
Assigned value (X_{pt})	$X_{pt_{ALL}}$
Number of results	8
Number of outliers	0
Mean	8,49
Median	8,10
Robust Mean (X_{pt})	**8,21**
Robust standard deviation (S*)	**2,36**
Target range:	
Target standard deviation σ_{pt}	**2,05**
lower limit of target range	**4,10**
upper limit of target range	**12,3**
Quotient S/σ_{pt}*	*1,2*
Standard uncertainty $U_{(Xpt)}$	*1,04*
Results in the target range	*7*
Percent in the target range	*88*

° *without method RS-F*

Comments on the statistic data:

Assigned value was the robust mean of all results from methods AQ, BF, IL and VT.

The calculation of the z-scores was based on a target standard deviation of 25% (see Fig. 3, p. 23).

All data are for information only.

Note: The two results of method RS-F were not considered, because they gave a separate peak in the kernel density estimation. The robust mean of all methods is not suitable for evaluation of these results.

Sample: Level 10 mg/kg

Statistic Data	**All Results°** *[mg/kg]*
Assigned value (X_{pt})	$X_{pt_{ALL}}$
Number of results	8
Number of outliers	0
Mean	5,09
Median	5,25
Robust Mean (X_{pt})	**5,09**
Robust standard deviation (S*)	**1,29**
Target range:	
Target standard deviation σ_{pt}	**1,27**
lower limit of target range	**2,54**
upper limit of target range	**7,63**
Quotient S/σ_{pt}*	*1,0*
Standard uncertainty $U_{(Xpt)}$	*0,57*
Results in the target range	*8*
Percent in the target range	*100*

° *without method RS-F*

Comments on the statistic data:

Assigned value was the robust mean of all results from methods AQ, BF, IL and VT.

The calculation of the z-scores was based on a target standard deviation of 25% (see Fig. 4, p. 23).

All data are for information only.

Note: The two results of method RS-F were not considered, because they gave a separate peak in the kernel density estimation. The robust mean of all methods is not suitable for evaluation of these results.

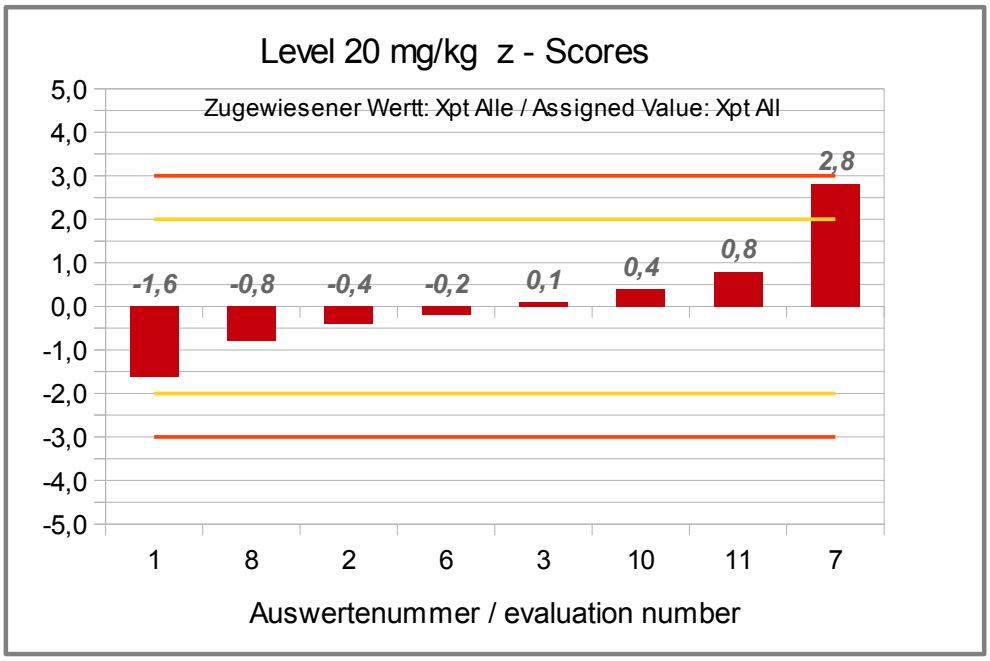


Abb./Fig. 3:
z-Scores action level 20,0 mg/kg (ELISA-results as almond)
Assigned value: median of all results

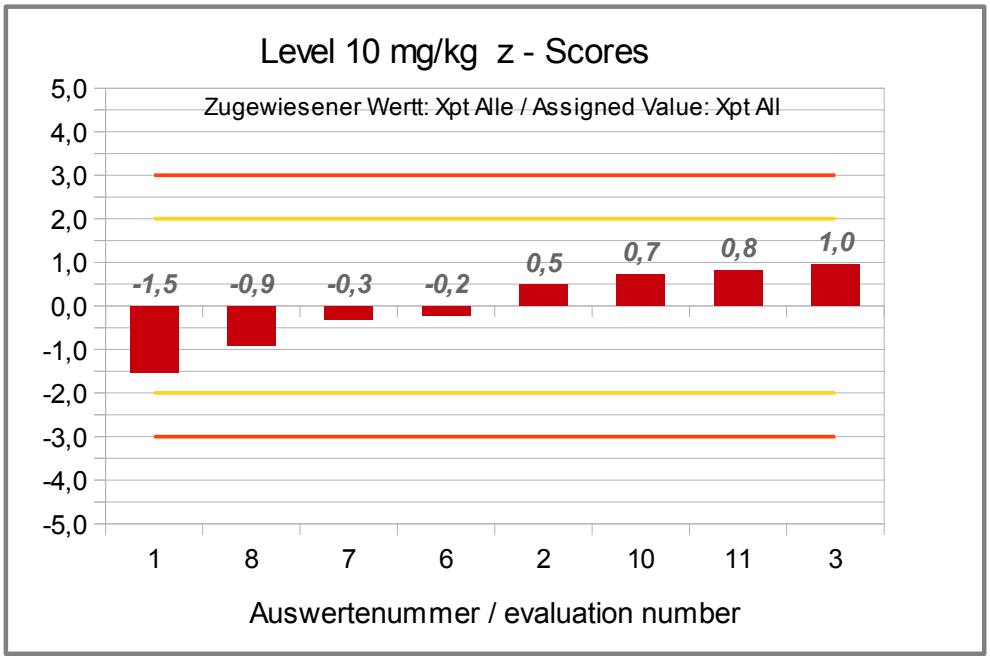


Abb./Fig. 4:
z-Scores level 10 mg/kg (ELISA-results as almond)
Assigned value: robust mean (alg. A) of all results

4.1.3.2 PCR-Methods

There were no quantitative results by PCR methods submitted.

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 10 mg/kg		Result Sample 2 Level 20 mg/kg		Result Sample 3 Level 100 mg/kg		Result Sample 4 Level 2,0 mg/kg		Result Sample 5 Level 50mg/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	1	08.07.19	-	3,13	-	4,88	-	34,75	-	0,65	-	8,08	-	0,2	0,2	0,4		Almond	AgraQuant ELISA Almond COKAL0748, RomerLabs
AQ	3	18.06.19	positive	6,3	positive	8,4	positive	46,3	positive	1,3	positive	27	positive	0,6	0,2	0,4	50	Almond	AgraQuant ELISA Almond COKAL0748, RomerLabs
AQ	7	06.06.19	positive	4,7	positive	14	positive	42	positive	1,3	positive	20	positive	1	0,2	0,4	40	Almond	AgraQuant ELISA Almond COKAL0748, RomerLabs
BF	2	12.07.19	positive	5,7	positive	7,4	positive	58,6	positive	2,36	positive	18,1	negative	0	0,15	1		Almond	MonoTrace Almond ELISA kit, BioFront Technologies
IL	10	12.06.19	positive	6	positive	9	positive	52	positive	1,3	positive	21	negative	<0,4				Almond	Immunolab Almond ELISA
RS-F	4	21.06.19	positive	12	positive	21	positive	87	positive	4,3	positive	49	negative	<2,5	1,7	1,5		Almond	Ridascreen® FAST Almond R6901, R-Biopharm
RS-F	9	17.06.	positive	9,23	positive	19,7	positive	99,2	positive	4,08	positive	42,9	negative	< 2,5	0,1	2,5		Almond	Ridascreen® FAST Almond R6901, R-Biopharm
VT	6	26.06.19	positive	4,8	-		positive	56,9	negative	<2.5	-		-		2,5	2,5		Almond	Veratox Almond, Neogen
VT	6	05.07.19	-		positive	7,8	-		-		positive	15,2	negative	<2.5	2,5	2,5		Almond	Veratox Almond, Neogen
VT	8	17.06.19	-	3,93	-	6,60	positive	44,13	-	2,27	-	12,77	-	1,30	2,5	2,5		Almond	Veratox Almond, Neogen
VT	11	19/6/2019	-	6,12	-	9,84	-	55,03	-	3,55	-	22,52	-	BLQ		2,5		Results are expressed as ppm of total almond	Veratox Almond, Neogen

* 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	
AQ	1			yes\	
AQ	3	Almond	Additive added	yes	
AQ	7	Almond	aqueous buffer, w ater bath 60 ° C for 15 minutes	no	
BF	2	monoclonal antibody-based kit	1:10 extraction ratio, 10 minutes at 60C	no	
IL	10				
RS-F	4	Almond proteins	as per kit instructions	yes	
RS-F	9		as per kit instructions w ith skimmed milk pow der	yes	Standards further diluted, therefore for sample 6 the result of <1,25 could be possibly given!
VT	6		PBS/15/60C	Yes	
VT	8				
VT	11	Not known	125 mL PBS, 15 minutes, 60 C shaker w aterbath	no	Read on Biotek reader. R2 = 0.997. Sample # 3 w as diluted 1:5 to obtain result.

5.1.2 PCR-Methods

Meth. Abbr.	Evaluation number	Date of Analysis	Result Sample 1 Level 10 mg/kg		Result Sample 2 Level 20 mg/kg		Result Sample 3 Level 100 mg/kg		Result Sample 4 Level 2,0 mg/kg		Result Sample 5 Level 50mg/kg		Result Sample 6 „blank“		NWG / LOD *	BG / LOQ *	MU*	Quantitative Result given as	Method
			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
MS	5		pos	2	pos	20	pos	50	neg*		pos	100	pos	10	0,005%Mandel DNA		50% at LOD	Almond/Food item	Microsynth Primer/Sonden + Qiagen MasterMix

* NWG Nachweisgrenze / BG Bestimmungsgrenze

* LOD limit of detection / LOQ limit of quantitation

* MU Messunsicherheit / MU measurement uncertainty

Continuation details by participants: PCR-Methods

Method Abk.	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	
MS	5	PruA1 Gene	DNA Extraction with Proteinase K, Clean Up with Chloroform and Columns /Amplif. with RealTIme PCR 45 Cycles	yes	Allergen analytics is performed qualitatively. Real Time PCR determination includes always a rel standard curve from fresh material, used for valuation of the complete determination and the measured values. Here the quantitative concentrations in ppm* were assigned by the measured Ct values. Result sample 4: in one approach the amplification was detected only in the 40th cycle (after PCR inhibition was removed)

5.2 Homogeneity

5.2.1 Mixture homogeneity before bottling

Microtracer Homogeneity Test
DLA 16-2019 Sample 1

Weight whole sample	1,01	kg
Microtracer	FSS-rot lake	
Particle size	75 – 300	µm
Weight per particle	2,0	µg
Addition of tracer	26,8	mg/kg

Result of analysis

Sample	Weight [g]	Particle number	Particles [mg/kg]
1	5,02	90	35,9
2	5,04	93	36,9
3	5,05	102	40,4
4	4,99	95	38,1
5	5,05	93	36,8
6	5,00	101	40,4
7	4,99	86	34,5
8	5,01	89	35,5

Poisson distribution		
Number of samples	8	
Degree of freedom	7	
Mean	93,6	Particles
Standard deviation	5,48	Particles
χ^2 (CHI-Quadrat)	2,25	
Probability	**94**	%
Recovery rate	139	%

Normal distribution		
Number of samples	8	
Mean	37,3	mg/kg
Standard deviation	2,18	mg/kg
rel. Standard deviaton	5,86	%
Horwitz standard deviation	9,28	%
HorRat-value	**0,63**	
Recovery rate	139	%

Microtracer Homogeneity Test
DLA 16-2019 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	29,9	mg/kg

Result of analysis

Sample	Weight [g]	Particle number	Particles [mg/kg]
1	4,99	63	25,3
2	5,02	52	20,7
3	5,05	53	21,0
4	5,02	77	30,7
5	5,03	75	29,8
6	4,99	54	21,6
7	5,00	61	24,4
8	5,05	64	25,3

Poisson distribution		
Number of samples	8	
Degree of freedom	7	
Mean	62,4	Particles
Standard deviation	9,56	Particles
χ^2 (CHI-Quadrat)	10,3	
Probability	**17**	%
Recovery rate	83	%

Normal distribution		
Number of samples	8	
Mean	24,9	mg/kg
Standard deviation	3,81	mg/kg
rel. Standard deviaton	15,3	%
Horwitz standard deviation	9,86	%
HorRat-value	**1,6**	
Recovery rate	83	%

Microtracer Homogeneity Test
DLA 16-2019 Sample 3

Weight whole sample	1,35	kg
Microtracer	FSS-rot lake	
Particle size	75 – 300	µm
Weight per particle	2,0	µg
Addition of tracer	28,2	mg/kg

Result of analysis

Sample	Weight [g]	Particle number	Particles [mg/kg]
1	5,03	51	20,3
2	5,05	48	19,0
3	5,00	56	22,4
4	5,03	48	19,1
5	5,02	57	22,7
6	4,98	45	18,1
7	4,99	44	17,6
8	5,01	50	20,0

Poisson distribution		
Number of samples	8	
Degree of freedom	7	
Mean	49,9	Particles
Standard deviation	4,66	Particles
χ^2 (CHI-Quadrat)	3,05	
Probability	**88**	%
Recovery rate	71	%

Normal distribution		
Number of samples	8	
Mean	19,9	mg/kg
Standard deviation	1,86	mg/kg
rel. Standard deviaton	9,35	%
Horwitz standard deviation	10,2	%
HorRat-value	**0,92**	
Recovery rate	71	%

Microtracer Homogeneity Test
DLA 16-2019 Sample 4

Weight whole sample	1,00	kg
Microtracer	FSS-rot lake	
Particle size	75 – 300	µm
Weight per particle	2,0	µg
Addition of tracer	22,3	mg/kg

Result of analysis

Sample	Weight [g]	Particle number	Particles [mg/kg]
1	5,02	60	23,9
2	4,97	70	28,2
3	4,99	72	28,9
4	5,06	61	24,1
5	5,02	73	29,1
6	4,99	63	25,3
7	4,99	61	24,4
8	5,00	65	26,0

Poisson distribution		
Number of samples	8	
Degree of freedom	7	
Mean	65,6	Particles
Standard deviation	5,42	Particles
χ^2 (CHI-Quadrat)	3,14	
Probability	**87**	%
Recovery rate	118	%

Normal distribution		
Number of samples	8	
Mean	26,2	mg/kg
Standard deviation	2,17	mg/kg
rel. Standard deviaton	8,26	%
Horwitz standard deviation	9,79	%
HorRat-value	**0,84**	
Recovery rate	118	%

Microtracer Homogeneity Test
DLA 16-2019 Sample 5

Weight whole sample	1,01	kg
Microtracer	FSS-rot lake	
Particle size	75 – 300	µm
Weight per particle	2,0	µg
Addition of tracer	22,9	mg/kg

Result of analysis

Sample	Weight [g]	Particle number	Particles [mg/kg]
1	5,02	68	27,1
2	5,04	57	22,6
3	5,06	63	24,9
4	4,99	59	23,6
5	5,01	58	23,2
6	5,05	63	25,0
7	4,97	63	25,4
8	4,96	48	19,4

Poisson distribution		
Number of samples	8	
Degree of freedom	7	
Mean	59,9	Particles
Standard deviation	5,79	Particles
χ^2 (CHI-Quadrat)	3,92	
Probability	**79**	%
Recovery rate	104	%

Normal distribution		
Number of samples	8	
Mean	23,9	mg/kg
Standard deviation	2,31	mg/kg
rel. Standard deviaton	9,67	%
Horwitz standard deviation	9,92	%
HorRat-value	**0,97**	
Recovery rate	104	%

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 16-2019***
PT name	***ALM - Verification Almond: 5 samples containing almonds in cookie matrix*** *(and a "blank sample")*
Sample matrix (processing)	***Samples 1-6:*** *Butter Cookies (baked at appr. 150°C)/ ingredients: Wheat flour, sugar, butter, barley malt extract, skimmed milk powder, glucose, glucose syrup, baking agent ammonium carbonate, salt, emulsifier lecithins, other food additives, egg and almonds (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):* *Almond / Almond protein / DNA* *Levels (almond): 2,0 / 10 / 20 / 50 / 100 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 July 12th 2019.***
Evaluation report	*The evaluation report is expected to be completed 6 weeks after deadline of result submission and sent as PDF file by e-mail.*
Coordinator and contact person of PT	*Matthias Besler-Scharf PhD*

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

6. Index of participant laboratories in alphabetical order

Teilnehmer / Participant	Ort / Town	Land / Country
		Germany
		USA
		Germany
		Germany
		AUSTRIA
		Scotland, UK
		Scotland, UK
		USA
		AUSTRIA
		AUSTRIA
		USA

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

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

7. Index of references

1. DIN EN ISO/IEC 17025:2005; Allgemeine Anforderungen an die Kompetenz von Prüf- und Kalibrierlaboratorien / General requirements for the competence of testing and calibration laboratories
2. DIN EN ISO/IEC 17043:2010; Konformitätsbewertung – Allgemeine Anforderungen an Eignungsprüfungen / Conformity assessment – General requirements for proficiency testing
3. ISO 13528:2015 & DIN ISO 13528:2009; Statistische Verfahren für Eignungsprüfungen durch Ringversuche / Statistical methods for use in proficiency testing by interlaboratory comparisons
4. ASU §64 LFGB: Planung und statistische Auswertung von Ringversuchen zur Methodenvalidierung / DIN ISO 5725 series part 1, 2 and 6 Accuracy (trueness and precision) of measurement methods and results
5. Verordnung / Regulation 882/2004/EU; Verordnung über über amtliche Kontrollen zur Überprüfung der Einhaltung des Lebensmittel- und Futtermittelrechts sowie der Bestimmungen über Tiergesundheit und Tierschutz / Regulation on official controls performed to ensure the verification of compliance with feed and food law, animal health and animal welfare rules
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18. EN ISO/IEC 17034:2016; Konformitätsbewertung - Allgemeine Anforderungen an die Kompetenz von Referenzmaterialherstellern / General requirements for the competence of reference material producers
19. ISO Guide 34:2000; General requirements for the competence of reference material producers
20. DAkkS 71 SD 1/4 016; Ermittlung und Angabe der Messunsicherheit nach Forderungen der DIN EN ISO/IEC 17025 (2011) [Estimation and indication of the measurement uncertainty]
21. Durchführungsverordnung der Kommission/ Commission Implementing Regulation EU 828/2014; über die Anforderungen an die Bereitstellung von Informationen 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
22. Taylor et al. (2014) Establishment of reference doses for residues of allergenic foods: report of the VITAL Expert Panel, Food Chem Toxicol 63: 9-17

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25. DIN EN ISO 15633-1:2009; Nachweis von Lebensmittelallergenen mit immunologischen Verfahren - Teil 1: Allgemeine Betrachtungen / Foodstuffs - Detection of food allergens by immunological methods - Part 1: General considerations
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