



Evaluation Report

proficiency test

DLA 14/2019

Response PT Almond:

5 processed Samples Almond (not roasted), Almond (roasted), Marzipan, Almond Spread and Almond Milk

in Potato Powder Matrix

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<i>Status des EP-Bericht</i> <i>Status of PT-Report</i>	<p>Abschlussbericht / Final report (11 November 2019)</p> <p>Gültig ist die jeweils letzte Version/Korrektur des Berichts. Sie ersetzt alle vorangegangenen Versionen. Only the latest version/correction of the report is valid. It replaces all preceding versions.</p>
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<i>Unteraufträge</i> <i>Subcontractors</i>	<p>Falls im Rahmen der Eignungsprüfung eine Prüfung der Gehalte, Homogenität und Stabilität von EP-Parametern durchgeführt wurde, hat DLA diese im Unterauftrag vergeben. In case the analysis of the content, homogeneity and stability of PT-parameters was part of the proficiency test, the determinations were subcontracted by DLA.</p>
<i>Vertraulichkeit</i> <i>Confidentiality</i>	<p>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.</p>

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1. Introduction

The participation in proficiency testing schemes is an essential element of the quality-management-system of every laboratory testing food and feed, cosmetics and food contact materials. The implementation of proficiency tests enables the participating laboratories to prove their own analytical competence under realistic conditions. At the same time they receive valuable data regarding the verification and/or validation of the particular testing method [1, 5].

The purpose of DLA is to offer proficiency tests for selected parameters in concentrations with practical relevance.

Realisation and evaluation of the present proficiency test follows the technical requirements of DIN EN ISO/IEC 17043 (2010) and DIN ISO 13528:2009 / ISO 13528:2015 [2, 3].

The present proficiency test format „**Response PT Allergens**“ includes 5 differently processed samples of an allergen in a simple carrier matrix as well as a “blank sample”. Hereby it offers the possibility to prove that the analytical determination methods used by the participants are suitable to detect the respective processed allergens qualitatively and to determine its quantitative response factors.

In order to ensure comparability of the processed sample material, the allergen contents of the PT sample series were adjusted to approximately the same levels calculated as almond contents. The evaluation of the PT-results was done qualitatively by scores from 1-5 (score 5 = all processings successfully determined). Quantitative results were given including the calculated respective recovery rate (recovery score) for information in the report.

2. Realisation

2.1 Test material

6 PT-samples for qualitative and optionally quantitative determination of almond in unroasted and roasted almonds, marzipan, almond spread and almond milk in potato powder / maltodextrin were provided.

The respective raw materials for the PT sample series were common in commerce partly processed almond products. For each PT-sample 5-19 products of different origin were worked up.

Premixes with contents from approx. 1,0 - 10 % of the regarding allergenic ingredients were produced (s. Tab. 1). For this the products were if necessary air-dried (almond milk 40°C), pre crushed, mixed gravimetrically and homogenized. Afterwards the raw materials were mixed with further ingredients, crushed and homogenized by a ball mill.

The allergen-premixes were added to the carrier matrix of potato powder / maltodextrin (mesh < 500 µm) and homogenized. An aliquot of the carrier matrix was provided as the "blank sample".

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

The contents of almond of the PT-samples were in the range of 29 to 48 mg/kg (see Tab. 1).

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 materials, mixing homogeneity, homogeneity and stability of almond 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, 16-17].

Table 1: Composition of DLA-Samples

PT-Sample series	Sample 1 Almond, raw	Sample 2 Almond milk	Sample 3 Marzipan	Sample 4 Almond roasted	Sample 5 Almond cocoa spread	Sample 6 „blank“
Ingredients	g/100 g	g/100g	g/100g	g/100g	g/100g	g/100g
Potato powder Ingredients: potato, E471, E304, E223, E100 Nutrients per 100 g: Protein 8,3 g, carbohydrates 76 g, fat 0,6 g, salt 0,15 g	75	75	75	75	75	75
Maltodextrin	25	25	25	25	25	25
Allergen-Premixes Ingredients: maltodextrin (45% - 90%), titanium dioxide (<50%), silicon dioxide (<3%), processed allergen products (each 1,0% - 10% almond)	0,039	0,21	0,17	0,043	0,41	-
Allergen-Contents	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg
Almond, raw* Protein 20,5% ** (18 products, 5 countries, USA, Europe, Australia, Southwest Asia)	39,5	-	-	-	-	-
Almond Milk* (3,0% Almonds and water, sugar, salt and other ingredients) Protein 0,626% ** (5 products, Europe, Asia)	-	103 (as dry mass)	-	-	-	-
Marzipan* (62% Almonds and wa- ter, sugar, invert sugar syrup, glucose syrup, alcohol, inver- tase) Protein 13,1% ** (5 products, Europe)	-	-	65,6	-	-	-
Almond, roasted* Protein 21,1 % ** (19 products, 6 countries, USA, Europe, Australia, Southwest Asia)	-	-	-	42,9	-	-
Almond Cocoa Spread* (12% Almonds and other ingredients like skimmed milk powder, whey protein, cocoa, hazelnuts) Total protein 10,3 % *** (5 products, Europe)	-	-	-	-	400	-
- as Almond	39,5	29,7	40,7	42,9	48,1	-
Extended combined uncertainty (k=2) of almond-content (= ± 13 %)	± 5,14	± 3,86	± 5,90	± 5,29	± 6,25	-

*Allergen contents as „total food“ as described in column ingredients according to gravimetric mixture

** Protein contents according to laboratory analysis of raw material mixtures (total nitrogen according to Kjeldahl with F=5,18 for almond protein)

***Protein content calculated according to the declaration of the products (besides almond, other protein sources are included)

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

2.1.1 Homogeneity

The **mixture homogeneity before bottling** was examined 8-fold by **micro-tracer 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 $\geq 5\%$ is equivalent to a good homogeneous mixture and of $\geq 25\%$ to an excellent mixture [14, 15].

The microtracer analysis of the present PT samples 1 to 5 showed a probability of 85%, 85%, 90%, 51% and 100%. 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 0,8, 0,7, 0,7, 1,0 and 0,5 respectively. 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. 0,32 (21,0°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

One portion of the test material (sample 1 to 6) were sent to every participating laboratory in the 16th week of 2019. The testing method was optional. The tests should be finished at May 31st 2019 the latest.

With the cover letter along with the sample shipment the following information was given to participants:

There are 5 different samples with similar contents of the allergenic parameter Almond, which is differently processed, contained in a simple carrier matrix as well as a "blank"-sample (carrier matrix).

- The samples 1-5 are numbered in a random order. They contain Almond (not roasted), Almond (roasted), Marzipan, Almond Spread and Almond Milk.*
- Please give all your quantitative results as total Almond, if possible indicate the underlying total protein content in Almonds.*
- Possible conversion factors for processed Almond products are queried separately in the result submission file.*

*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 in mg/kg were evaluated.

Queried and documented were the indicated results and details of the test methods like specificity, 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 12 participants submitted the results in time.

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 [26-29, 40]. 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 five different processed products containing the allergen almond, almond (raw), almond (roasted), marzipan, almond spread and almond milk, were provided to determine the qualitative detectability and to determine the response of the used quantitative methods.

The participant results were evaluated *qualitatively* with a score from 1-5 indicating the number of successfully detected processed products. 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.

3.1 Qualitative Score

The qualitative valuation of each participant's results was performed with Scores from 1-5 considering the number of "positive" or "negative" results matching the **spiking of the PT-sample series** (see Tab. 2). A Score from 5 indicates, that all processed products were detected successfully.

The results of the matrix sample no. 6 ("blank"-sample) were not evaluated if the participant result is in accordance with $\geq 75\%$ positive or negative results of participants (consensus value) or if the result is below the limit of quantification of the used method.

Table 2: Evaluation of results using qualitative Scores

Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Score	Suitability
Almond, raw	Almond milk	Marzipan	Almond, roasted	Almond Cocoa Spread	„blank“	qualitative	qualitative
pos/neg	pos/neg	pos/neg	pos/neg	pos/neg	pos/neg	number of detected Samples 1 - 5	
negative	negative	negative	negative	negative	negative	0 (0%)	not successful
negative	negative	negative	negative	positive	negative	1 (20%)	1 product group
negative	negative	negative	positive	positive	negative	2 (40%)	2 product groups
negative	negative	positive	positive	positive	negative	3 (60%)	3 product groups
negative	positive	positive	positive	positive	negative	4 (80%)	4 product groups
positive	positive	positive	positive	positive	negative	5 (100%)	5 product groups

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 the spiked allergen (level of addition). The reference values are calculated from the values for samples 1 to 5 given in section 2.1 Sample material in 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-EL-ISAs was used [21]. This range was also used in the present PT for quantitative PCR- and LC/MS-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 experiment

In ring trials of ASU §64 methods recovery rates in the range from 57% - 119% were obtained by ELISA methods and 43% - 121% for PCR methods, depending on matrix or processing and concentration (s. Table 3a and 3b). The given target standard deviation σ_{pt} was calculated for a number of $m = 2$ repeated measurements.

Table 3a: ELISA-Methods - Recovery rates and precision data from selected precision experiments[33-34].

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

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) proved 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 3b: PCR-Methods - Relative repeatability standard deviations (RSD_r) and relative reproducibility standard deviations (RSD_R) according to selected evaluations from experiments by precision and the resulting target standard deviation σ_{pt} [35-37].

Parameter	Matrix	Mean [mg/kg]	Recovery	rob RSD_r	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
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 [25], by the Working Group 12 „Food allergens“ of the Technician Committee CEN/TC 275 [22-24], by a international "Food Allergen Working Group" under the leadership of the AOAC Presidential Task Force on Food Allergens [26] and by the Codex Alimentarius Committee (CAC/GL 74-2010) [21].

The following relevant ELISA and/or PCR validation criteria of the committees are given in Table 4 and 5.

Table 4: ELISA validation criteria

Literature [21-26]	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 5: PCR validation criteria

Literature [20]	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.

Evaluation was done separately for ELISA- (and Lateral Flow), PCR- and LC/MS methods.

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.

The **comparability of quantitative result specification** was given as all ELISA, PCR and LC/MS results were reported as almond. A conversion of the results was not required.

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

Evaluation number	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6 „blank“	Score qualitative	Method	Remarks
	pos/neg	pos/neg	pos/neg	pos/neg	pos/neg	pos/neg	number of detected Samples 1 - 5		

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

Evaluation number	Sample 1		Sample 2		Sample 3		Sample 4		Sample 5		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**		

* Recovery Rate

4.1 Proficiency Test Processed Almond Products

4.1.1 Qualitative Scores: ELISA-Methods

Evaluation number	Sample 1 Almond, raw	Sample 2 Almond milk	Sample 3 Marzipan	Sample 4 Almond, roasted	Sample 5 Almond Cocoa Spread	Sample 6 „blank“	Score qualitative	Method	Remarks
	pos/neg	pos/neg	pos/neg	pos/neg	pos/neg	pos/neg	Number of detected samples 1 - 5		
8	positive	negative	positive	positive	positive	negative	4 (80%)	AQ	
9b	positive	negative	positive	positive	positive	negative	4 (80%)	AQ	
10	positive	negative	positive	positive	positive	negative	4 (80%)	AQ	
2a	positive	negative	positive	positive	positive	negative	4 (80%)	AQ-P	corresp. to method ELISAFast; Sample 2 positive <LOD
2b	positive	negative	positive	positive	positive	negative	4 (80%)	AS	Sample 2 positive < LOD
1	positive	positive	positive	positive	positive	negative	5 (100%)	BF	
12	positive	negative	positive	positive	positive	negative	4 (80%)	IL	
7	positive	negative	positive	positive	positive	negative	4 (80%)	RS-F	
9a	positive	negative	positive	positive	positive	negative	4 (80%)	RS-F	
3	positive	positive	positive	positive	positive	negative	5 (100%)	VT	
4	positive	positive	positive	positive	positive	negative	5 (100%)	VT	

	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6
Number positive	11	3	11	11	11	0
Number negative	0	8	0	0	0	11
Percent positive	100	27	100	100	100	0
Percent negative	0	73	0	0	0	100
Consensus value	positive	none	positive	positive	positive	negative
Spiking	positive	positive	positive	positive	positive	negative

Methods:

AQ = AgraQuant, RomerLabs
 AQ-P = AgraQuant Plus, RomerLabs
 AS = AgraStrip (Lateral Flow), RomerLabs
 BF = MonoTrace ELISA, BioFront Technologies
 IL = Immunolab
 RS-F= Ridascreeen® Fast, R-Biopharm
 VT = Veratox, Neogen

Comments:

For the samples 1 and 3 to 5 consensus values of 100% positive results were obtained by the ELISA-methods. For the processed sample 2 (almond milk) predominantly negative results but no consensus value of ≥ 75% were obtained.

4.1.2 Qualitative Scores: PCR-Methods

Evaluation number	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Score qualitative	Method	Remarks
	Almond, raw	Almond milk	Marzipan	Almond, roasted	Almond Cocoa Spread	„blank“			
	pos/neg	pos/neg	pos/neg	pos/neg	pos/neg	pos/neg	Number of detected samples 1 - 5		
7	positive	positive	positive	negative	positive	negative	4 (80%)	ASU	Sample 2 traces
10b	positive	positive	positive	negative	positive	negative	4 (80%)	ASU	
11	positive	positive	positive	negative	positive	negative	4 (80%)	ASU	
5a	positive	positive	positive	positive	positive	negative	5 (100%)	GI	
5b	positive	positive	positive	positive	positive	negative	5 (100%)	GI-3	
6	negative	negative	positive	negative	positive	negative	2 (40%)	SFA	Sample 1 slightly positive
10a	positive	positive	positive	positive	positive	negative	5 (100%)	SFA	

	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6
Number positive	6	6	7	3	7	0
Number negative	1	1	0	4	0	7
Percent positive	86	86	100	43	100	0
Percent negative	14	14	0	57	0	100
Consensus value	positive	positive	positive	none	positive	negative
Spiking	positive	positive	positive	positive	positive	negative

Methods:

ASU = ASU §64 Methode/method

GI = GEN-IAL First Allergen

GI-3= GEN-IAL First Allergen Triplex Nut

SFA = Sure Food Allergen, R-Biopharm / Congen

Comments:

For samples 3 (marzipan) and 5 (almond cocoa spread) consensus values of 100% positive results were obtained with PCR-methods. For the samples 1 (raw almond) and 2 (almond milk) consensus values of 86% positive results were obtained.

For sample 4 (almond, roasted) no consensus value of $\geq 75\%$ was obtained.

4.1.3 Qualitative Scores: LC-MS/MS-Methods

Evaluation number	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Score qualitative	Method	Remarks
	Almond, raw	Almond milk	Marzipan	Almond, roasted	Almond Cocoa Spread	„blank“			
	pos/neg	pos/neg	pos/neg	pos/neg	pos/neg	pos/neg	Number of detected samples 1 - 5		
6b	positive	positive	positive	positive	positive	negative	5 (100%)	LC-MS/MS	

	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6
Spiking	positive	positive	positive	positive	positive	negative

Methods:

LC-MS/MS = liquid chromatography

-Mass spectrometry

Comments:

For all products (samples 1 to 5) the participant obtained positive results using a LC/MS-method.

4.1.4 Quantitative: ELISA-Methods Recovery Rates-Scores (RR-Scores)

Evaluation number	Sample 1 Almond, raw		Sample 2 Almond milk		Sample 3 Marzipan		Sample 4 Almond, roasted		Sample 5 Almond Cocoa Spread		RR-Score RR *	Method	Remarks
	Result [mg/kg]	RR *	Result [mg/kg]	RR *	Result [mg/kg]	RR *	Result [mg/kg]	RR *	Result [mg/kg]	RR *			
8	35,7	90	<LOD		38,8	95	25,8	60	22,1	46	3/5 (60%)	AQ	
9b	38,6	98	< 0,4		42,9	105	32,4	76	19,3	40	3/5 (60%)	AQ	
10	32,9	83			35,1	86	26,6	62	17,0	35	3/5 (60%)	AQ	
2a	67,0	170	<LOD		69,0	170	44,0	103	39,0	81	2/5 (40%)	AQ-P	corresponds to method ELISA Fast
2b												AS	
1	40,4	102	1,10	3,7	48,2	118	40,1	93	33,8	70	4/5 (80%)	BF	
12	35,8	91	0		36,9	91	24,7	58	16,5	34	3/5 (60%)	IL	
7	78,0	197	<2,5		110	270	57,0	133	49,0	102	2/5 (40%)	RS-F	
9a	60,0	152	< 2,5		66,5	163	35,9	84	33,8	70	2/5 (40%)	RS-F	
3	35,9	91	8,10	27	40,1	99	25,9	60	21,6	45	3/5 (60%)	VT	
4	53,0	134	20,0	67	56,0	138	39,0	91	40,0	83	5/5 (100%)	VT	
RA**		50-150 %	RA**		50-150 %	RA**		50-150 %	RA**		50-150 %		
Number in RA		7	Number in RA		1	Number in RA		7	Number in RA		10	Number in RA 5	
Percent in RA		70	Percent in RA		33	Percent in RA		70	Percent in RA		100	Percent in RA 50	

*Recovery rate 100% Reference value: Almond, see page 6

** Acceptance range of AOAC for allergen ELISAs

Methods:

AQ = AgraQuant, RomerLabs

AQ-P = AgraQuant Plus, RomerLabs

AS = AgraStrip (Lateral Flow), RomerLabs

BF = MonoTrace ELISA, BioFront Technologies

IL = Immunolab

RS-F= Ridascreen® Fast, R-Biopharm

VT = Veratox, Neogen

Comments:

For sample 4 (almond, roasted) 100% of the recovery rates of the ELISA methods were in the range of acceptance of 50-150%. For raw almond (sample 1) and marzipan (sample 3) 70% of the recovery rates were in the range of acceptance. For sample 2 (almond milk) and sample 5 (almond cocoa spread), a lower response was observed. For sample 5 50% of the results were in the range of acceptance and 50% were below the range of acceptance. For sample 2 one recovery rate was in the range of acceptance and two were below.

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4.1.5 Quantitative: PCR-Methods Recovery Rates-Scores (RR-Scores)

Auswertenummer	Sample 1 Almond, raw		Sample 2 Almond milk		Sample 3 Marzipan		Sample 4 Almond, roasted		Sample 5 Almond Cocoa Spread		RR-Score RR *	Method	Remarks		
	Result	RR *	Result	RR *	Result	RR *	Result	RR *	Result	RR *					
	[mg/kg]	[%]	[mg/kg]	[%]	[mg/kg]	[%]	[mg/kg]	[%]	[mg/kg]	[%]				Number in RA**	
7												ASU			
10b												ASU			
11	73,0	185	<20		46,0	113	<5		30,0	62	2/4 (50%)	ASU	Sample 2 not evaluated		
5a												GI			
5b												GI-3			
6	< 0,4		< 0,4		0,740	1,8	< 0,4		0,670	1,4	0/5 (0%)	SFA			
10a												SFA			
RA**		50-150 %		RA**		50-150 %		RA**		50-150 %					
Number in RA		0		Number in RA		0		Number in RA		0		Number in RA		1	
Percent in RA		0		Percent in RA		-		Percent in RA		50		Percent in RA		-	
Percent in RA		0		Percent in RA		-		Percent in RA		50		Percent in RA		50	

Methods:

ASU = ASU §64 Methode/method

GI = GEN-IAL First Allergen

GI-3= GEN-IAL First Allergen Triplex Nut

SFA = Sure Food Allergen, R-Biopharm / Congen

*Recovery rate 100% Reference value: Almond, see page 6

** Acceptance range of AOAC for allergen ELISAs

Comments:

Two participants have determined quantitative results using PCR methods. One participant has received recovery rates in the range of acceptance of 50-150% for sample 5 (almond cocoa spread) and sample 3 (marzipan). For raw almonds (Sample 1), a result was above the range of acceptance. For sample 2 (almond milk) and sample 4 (roasted almonds) no results above the detection limits were obtained.

Note: The result of <20 mg/kg for sample 2 was not valuated for the WFR score because this could be within or outside the acceptance range.

4.1.6 Quantitative: LC/MS-Methods Recovery Rates-Scores (RR-Scores)

Auswertenummer	Sample 1 Almond, raw		Sample 2 Almond milk		Sample 3 Marzipan		Sample 4 Almond, roasted		Sample 5 Almond Cocoa Spread		RR-Score	Method	Remarks
	Result	RR *	Result	RR *	Result	RR *	Result	RR *	Result	RR *			
	[mg/kg]	[%]	[mg/kg]	[%]	[mg/kg]	[%]	[mg/kg]	[%]	[mg/kg]	[%]	Number in RA **		
6	72,0	182	27,1	91	80,8	199	103	239	120	250	0/5 (0%)	LC-MS/MS	
	RA**	50-150 %	RA**	50-150 %	RA**	50-150 %	RA**	50-150 %	RA**	50-150 %			
	Number in RA	0	Number in RA	1	Number in RA	0	Number in RA	0	Number in RA	0			
	Percent in RA	0	Percent in RA	100	Percent in RA	0	Percent in RA	0	Percent in RA	0			

Methods:

LC-MS/MS = liquid chromatography
-Mass spectrometry

*Recovery rate 100% Reference value: Almond, see page 6

** Acceptance range of AOAC for allergen ELISAs

Comments:

For almond milk (sample 2) the participant obtained with a LC-MS/MS method a recovery rate in the range of acceptance of 50-150%. The recovery rates for raw almonds (sample 1), marzipan (sample 3), roasted almonds (sample 4) and almond cocoa spread (sample 5) were well above the range of acceptance, respectively.

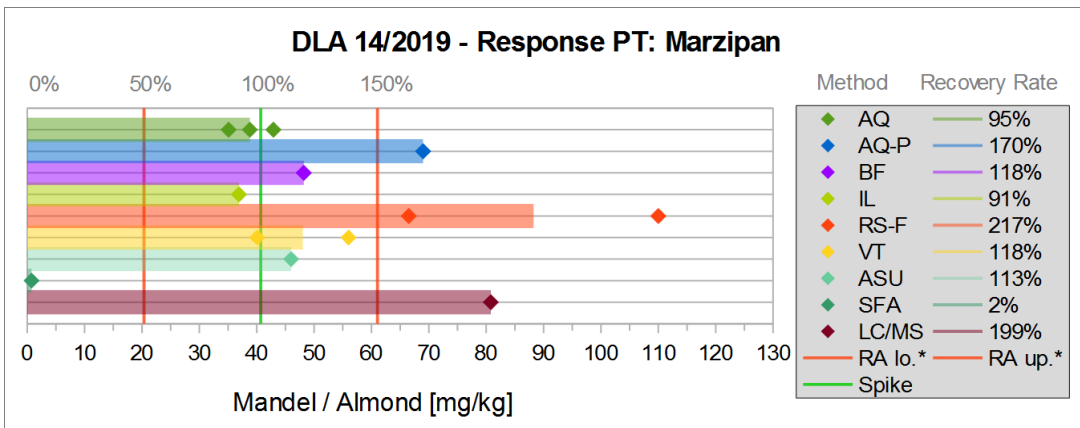
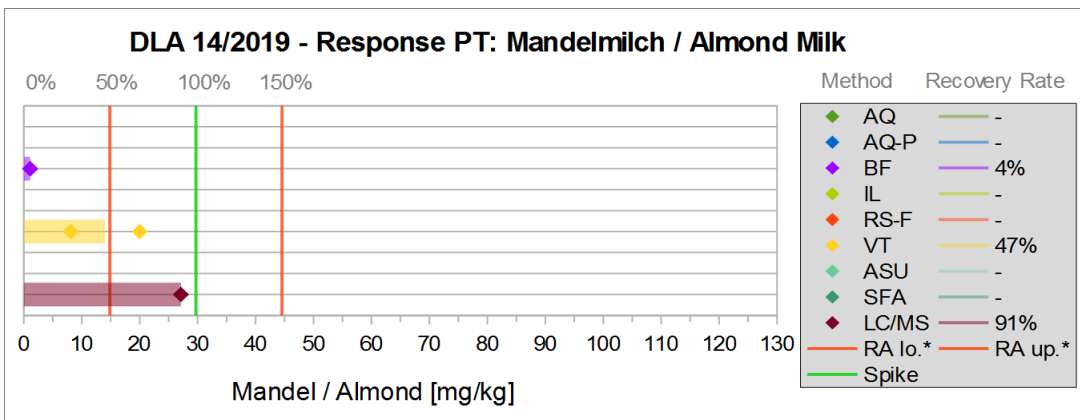
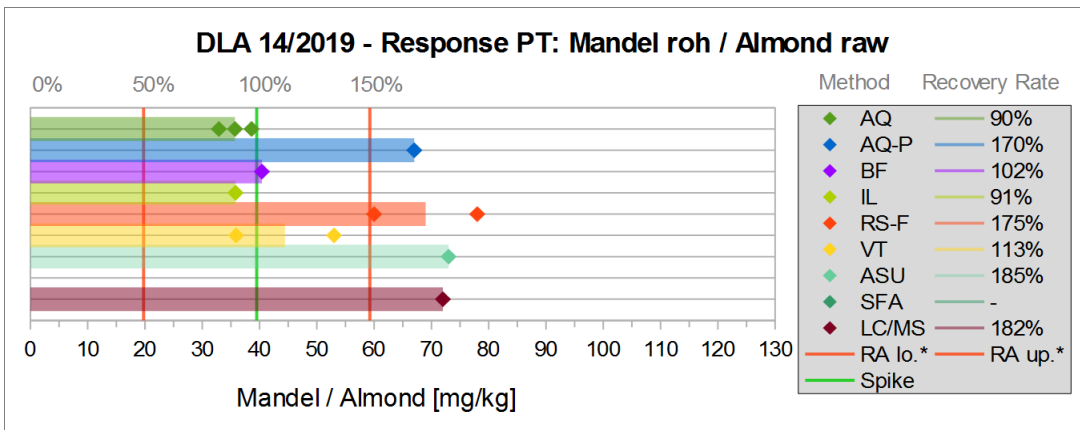


Abb./Fig. 1: Graphs of single results (Samples 1-3) separated by methods with corresponding mean recovery rates, lower scale almond content in mg/kg, upper scale recovery rate in %, with * range of acceptance from 50% - 150% (* range of acceptance: RA lower limit to RA upper limit)

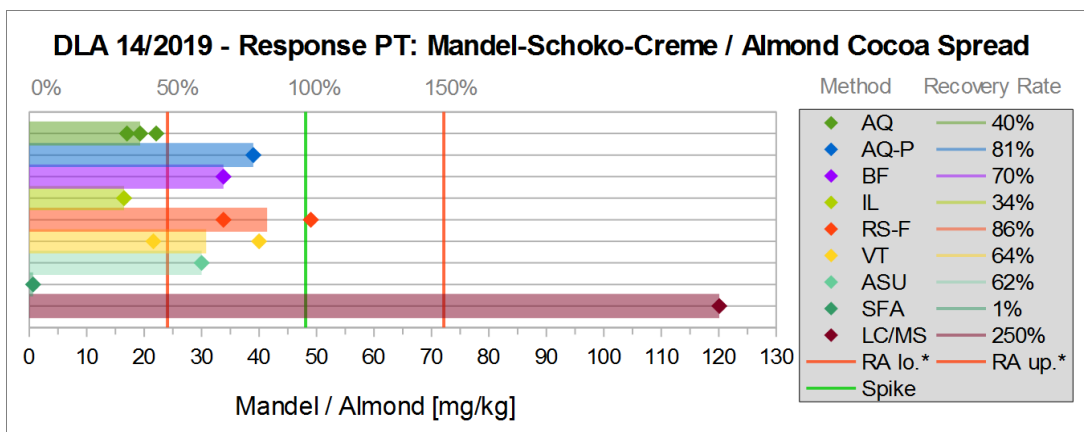
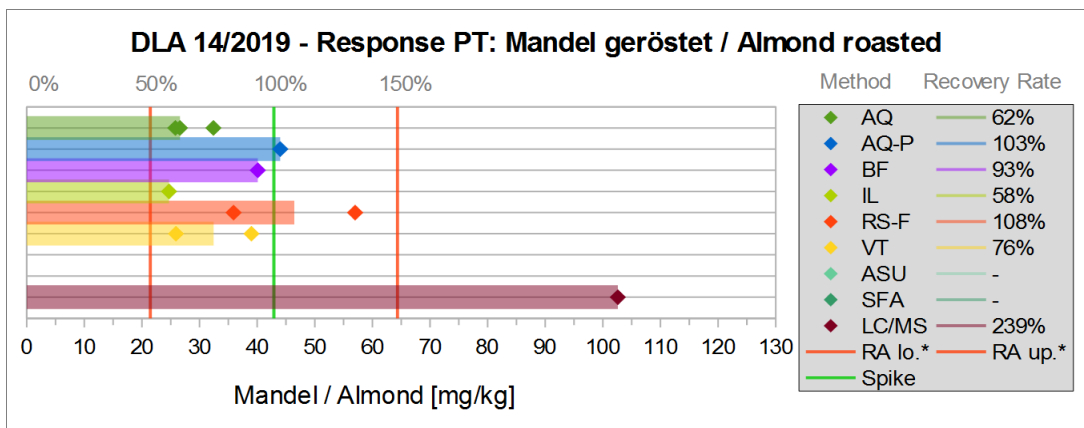


Abb./Fig. 2: Graphs of single results (Samples 4-5) separated by methods with corresponding mean recovery rates, lower scale almond content in mg/kg, upper scale recovery rate in %, with * range of acceptance from 50% - 150% (* range of acceptance: RA lower limit to RA upper limit)

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

Method Abr.	Evaluation Number	Date of Analysis	Result Sample 1		Result Sample 2		Result Sample 3		Result Sample 4		Result Sample 5		Result Sample 6		NWG / LOD *	BG / LOQ *	MU*	Specification of quantitative result as
			qualitative	mg/kg	qualitative	mg/kg	qualitative	mg/kg	qualitative	mg/kg	qualitative	mg/kg	qualitative	mg/kg	mg/kg	mg/kg	mg/kg	preferred as almond
AQ	8	06.05.19	positive	35,7	negative	<LOD	positive	38,8	positive	25,8	positive	22,1	negative	<LOD	0,2	0,4		Almond
AQ	9b	08.05.	positive	38,6	negative	< 0,4	positive	42,9	positive	32,4	positive	19,3	negative	< 0,4	0,2	0,4		Almond
AQ	10	02.05.19	positive	32,9	negative		positive	35,1	positive	26,6	positive	17	negative		0,4	0,4	0,16	Almond
AQ-P	2a		positive	67	negative	<LOD	positive	69	positive	44	positive	39	negative	<LOD	1	1		Almond
AS	2b		positive		negative		positive		positive		positive		negative		2			Almond
BF	1	31.05.19	positive	40,4	positive	1,1	positive	48,2	positive	40,1	positive	33,8	negative	0	0,12	1		Almond
IL	12	02.05.19	positive	35,8	negative	0	positive	36,9	positive	24,7	positive	16,5	negative	0				Almond
RS-F	7	06.05.19	positive	78	negative	<2,5	positive	110	positive	57	positive	49	negative	<2,5	1,7	2,5		Almond
RS-F	9a	30.04.	positive	60	negative	< 2,5	positive	66,5	positive	35,9	positive	33,8	negative	< 2,5	0,1	2,5		Almond
VT	3	06.05.19	positive	35,9	positive	8,1	positive	40,1	positive	25,9	positive	21,6	negative	<2.5	2,5	2,5		Almond
VT	4	17.05.2019 + 24.05.2019	positive	53	positive	20	positive	56	positive	39	positive	40	negative	<2.5	2,5	2,5	40.5% (20, 8, 20, 20, 20, 20 mg/kg)	Almond

* NWG Nachweisgrenze / BG Bestimmungsgrenze

* LOD limit of detection / LOQ limit of quantitation

* MU Messunsicherheit / MU measurement uncertainty

Continuation details by participants: ELISA-Methods

Method Abr.	Evaluation Number	Method	Specificity	Total protein content in almond (According to method prescription)	Conversion for processed almond	Remarks to the Method (Extraction and Determination)	Method accredited to ISO / IEC 17025	Further remarks
		Test-Kit + Provider	Antibody	%	Recalculation from X to Y (factor or %)	e.g. Extraction solution / time / temperature	yes/no	
AQ	8	AgraQuant ELISA Almond COKAL0748, RomerLabs					yes	
AQ	9b	AgraQuant ELISA Almond COKAL0748, RomerLabs				according to manufacturer's instructions	no	
AQ	10	AgraQuant ELISA Almond COKAL0748, RomerLabs		21,15		according to manual	yes	
AQ-P	2a	ELISAFast® Almond					yes	Sample 2 just <LOD
AS	2b	AgraStrip® Almond					yes	Sample 2 weakly positive, but <LOD
BF	1	MonoTrace Almond ELISA kit, BioFront Technologies	monoclonal antibody-based kit			1:20 extraction ratio, 10 minutes at 60C	No	
IL	12	Immunolab Almond ELISA						
RS-F	7	Ridascreen® FAST Almond R6901, R-Biopharm	recognizes almond protein	approx. 25		according to manufacturer's instructions	yes	
RS-F	9a	Ridascreen® FAST Almond R6901, R-Biopharm				according to manufacturer's instructions, mw ith skimmed milk powder	yes	
VT	3	Veratox Almond, Neogen				PBS/15 minutes/60C 4 parameter	Yes	
VT	4	Veratox Almond, Neogen	Almond protein (not specified by provider)	It is not declared in Instructions, nor Validation Report for Veratox for Almond Allergen (Neogen item 8440)	Not declared	As per kit instructions	NO	Samples 1, 2, 3, 4 5 initially obtained results over the upper limit for the kit Additional dilution was used in order to obtain values (1-1/20; 2-1/5; 3-1/10;4-1/10; 5-1/10)

5.1.2 PCR-Methods

Method Abr.	Evalu- ation Number	Date of Analysis	Result Sample 1		Result Sample 2		Result Sample 3		Result Sample 4		Result Sample 5		Result Sample 6		NWG / LOD *	BG / LOQ *	MU*	Specification of quantita- tive result as
			qualitative	mg/kg	qualitative	mg/kg	qualitative	mg/kg	qualitative	mg/kg	qualitative	mg/kg	qualitative	mg/kg				
		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	preferred as almond
ASU	7	08.05.19	positive		positive		positive		negative		positive		negative		40			Almond-DNA
ASU	10b	29.04.19	positive		positive		positive		negative		positive		negative					Almond-DNA
ASU	11		positive	73	positive	<20	positive	46	negative	<5	positive	30	negative	<5	5	20	50	Almond
GI	5a	24.05.19	positive		positive		positive		positive		positive		negative		5			Please select!
GI-3	5b	28.05.19	positive		positive		positive		positive		positive		negative		5			Please select!
SFA	6		negative	< 0,4	negative	< 0,4	positive	0,74	negative	< 0,4	positive	0,67	negative	< 0,4	0,4	1	40	Almond
SFA	10a	30.04.19	positive		positive		positive		positive		positive		negative					Almond-DNA

* NWG Nachweisgrenze / BG Bestimmungsgrenze

* LOD limit of detection / LOQ limit of quantitation

* MU Messunsicherheit / MU measurement uncertainty

Continuation details by participants: PCR-Methods

Method Abr.	Evaluation Number	Method	Specificity	Total protein content in almond (According to method prescription)	Conversion for processed almond	Remarks to the Method (Extraction and Determination)	Method accredited to ISO / IEC 17025	Further remarks
		Test-Kit + Provider	Target sequence / DNA	%	Recalculation from X to Y (factor or %)	e.g. Extraction / Enzyme / Clean-Up / Real Time PCR / Gel Electrophoresis / Cycles	yes/no	
ASU	7	ASU §64 Methode/method				§64 LFGB L 18.00-20:2014-08	yes	Sample 2: Traces at the LOD
ASU	10b	ASU §64 Methode/method				Extraction with the Macherey & Nagel NucleoSpin Food Kit	yes	
ASU	11	ASU §64 Methode/method	PRU AV1-Gen; 129bp			CTAB-precipitation method, s. e.g. ASU L 18.00-22	yes	Calibration/ Quantification using matrix standards, spiked material: almond, defatted
GI	5a	First-Almond (GEN-IAL)				First-DNA all tissue Kit	yes	
GI-3	5b	First-Allergen Triplex Nut II (GEN-IAL)				First-DNA all tissue Kit	yes	
SFA	6	Sure Food Allergen, R-Biopharm / Congen				It. Manual Prep Advanced P1	yes	Sample 1 weakly positive, ct>35, according to manual judged as negative
SFA	10a	Sure Food Allergen, R-Biopharm / Congen				Extraction with the Macherey & Nagel NucleoSpin Food Kit		Method not yet verified.

5.1.3 LC/MS-Methods

Method Abr.	Evaluation Number	Date of Analysis	Result Sample 1		Result Sample 2		Result Sample 3		Result Sample 4		Result Sample 5		Result Sample 6		NWG / LOD *	BG / LOQ *	MU*	Specification of quantitative result as
			qualitative	mg/kg	qualitative	mg/kg	qualitative	mg/kg	qualitative	mg/kg	qualitative	mg/kg	qualitative	mg/kg	mg/kg	mg/kg	mg/kg	preferred as almond
LC-MS/MS	6b	11./12. Jun 19	positive	72	positive	27,1	positive	80,8	positive	102,6	positive	120,1	negative		S/N > 3	20	0,4	Almond

* NWG Nachweisgrenze / BG Bestimmungsgrenze

* LOD limit of detection / LOQ limit of quantitation

* MU Messunsicherheit / MU measurement uncertainty

Method Abr.	Evaluation Number	Method	Specificity	Total protein content in hazelnut (According to method prescription)	Conversion for processed hazelnut	Remarks to the Method (Extraction and Determination)	Method accredited to ISO / IEC 17025	Further remarks
		Test-Kit + Provider + Literature						
LC-MS/MS	6b	LC-MS/MS	Specific peptides			Protein extraction followed by enzymatic digestion	yes	

5.2 Homogeneity

5.2.1 Mixture homogeneity before bottling

Microtracer Homogeneity Test

DLA 14-2019 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	34,7	mg/kg

Result of analysis

Sample	Weight [g]	Particle number	Particles [mg/kg]
1	5,05	79	31,3
2	5,04	70	27,8
3	5,02	72	28,7
4	5,03	71	28,2
5	5,03	84	33,4
6	5,01	83	33,1
7	5,03	71	28,2
8	5,00	81	32,4

Poisson distribution

Number of samples	8	
Degree of freedom	7	
Mean	76,4	Particles
Standard deviation	6,04	Particles
χ^2 (CHI-Quadrat)	3,35	
Probability	85	%
Recovery rate	88	%

Normal distribution

Number of samples	8	
Mean	30,4	mg/kg
Standard deviation	2,40	mg/kg
rel. Standard deviation	7,9	%
Horwitz standard deviation	9,6	%
HorRat-value	0,8	
Recovery rate	88	%

Microtracer Homogeneity Test

DLA 14-2019 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	35,3	mg/kg

Result of analysis

Sample	Weight [g]	Particle number	Particles [mg/kg]
1	5,02	112	44,6
2	5,03	108	42,9
3	4,99	103	41,3
4	5,03	119	47,3
5	5,03	101	40,2
6	5,06	117	46,2
7	5,00	119	47,6
8	5,01	104	41,5

Poisson distribution

Number of samples	8	
Degree of freedom	7	
Mean	110,4	Particles
Standard deviation	7,28	Particles
χ^2 (CHI-Quadrat)	3,36	
Probability	85	%
Recovery rate	125	%

Normal distribution

Number of samples	8	
Mean	44,0	mg/kg
Standard deviation	2,90	mg/kg
rel. Standard deviation	6,6	%
Horwitz standard deviation	9,1	%
HorRat-value	0,7	
Recovery rate	125	%

Microtracer Homogeneity Test**DLA 14-2019 Sample 3**

Weight whole sample	1,00	kg
Microtracer	FSS-rot lake	
Particle size	75 – 300	µm
Weight per particle	2,0	µg
Addition of tracer	28,0	mg/kg

Result of analysis

Sample	Weight [g]	Particle number	Particles [mg/kg]
1	5,06	78	30,8
2	4,97	80	32,2
3	5,02	76	30,3
4	5,06	78	30,8
5	5,02	85	33,9
6	5,03	89	35,4
7	5,00	89	35,6
8	5,00	75	30,0

Poisson distribution

Number of samples	8	
Degree of freedom	7	
Mean	81,3	Particles
Standard deviation	5,73	Particles
χ^2 (CHI-Quadrat)	2,83	
Probability	90	%
Recovery rate	116	%

Normal distribution

Number of samples	8	
Mean	32,4	mg/kg
Standard deviation	2,28	mg/kg
rel. Standard deviation	7,06	%
Horwitz standard deviation	9,48	%
HorRat-value	0,74	
Recovery rate	116	%

Microtracer Homogeneity Test**DLA 14-2019 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	27,8	mg/kg

Result of analysis

Sample	Weight [g]	Particle number	Particles [mg/kg]
1	5,02	104	41,4
2	5,01	104	41,5
3	5,00	108	43,2
4	5,09	90	35,4
5	5,05	112	44,4
6	4,99	85	34,1
7	5,05	108	42,8
8	5,03	107	42,5

Poisson distribution

Number of samples	8	
Degree of freedom	7	
Mean	102,3	Particles
Standard deviation	9,55	Particles
χ^2 (CHI-Quadrat)	6,25	
Probability	51	%
Recovery rate	146	%

Normal distribution

Number of samples	8	
Mean	40,7	mg/kg
Standard deviation	3,80	mg/kg
rel. Standard deviation	9,34	%
Horwitz standard deviation	9,16	%
HorRat-value	1,0	
Recovery rate	146	%

Microtracer Homogeneity Test

DLA 14-2019 Sample 5

Weight whole sample	1,00	kg
Microtracer	FSS-rot lake	
Particle size	75 – 300	µm
Weight per particle	2,0	µg
Addition of tracer	21,2	mg/kg

Result of analysis

Sample	Weight [g]	Particle number	Particles [mg/kg]
1	5,02	54	21,5
2	4,97	50	20,1
3	5,05	47	18,6
4	5,01	52	20,8
5	5,00	51	20,4
6	5,09	56	22,0
7	5,05	51	20,2
8	5,03	50	19,9

Poisson distribution

Number of samples	8	
Degree of freedom	7	
Mean	51,4	Particles
Standard deviation	2,60	Particles
χ^2 (CHI-Quadrat)	0,92	
Probability	100	%
Recovery rate	96	%

Normal distribution

Number of samples	8	
Mean	20,4	mg/kg
Standard deviation	1,04	mg/kg
rel. Standard deviaton	5,07	%
Horwitz standard deviation	10,2	%
HorRat-value	0,50	
Recovery rate	96	%

5.3 Information on the Proficiency Test (PT)

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

<i>PT number</i>	DLA 14-2019
<i>PT name</i>	Response PT Almond: Processed Samples Almond (not roasted), Almond (roasted), Marzipan, Almond Spread and Almond Milk in Potato Powder Matrix (levels: 25 - 150 mg/kg)
<i>Sample matrix (processing)</i>	Samples 1-6: Carrier matrix / ingredients: potato powder (approx. 75%), maltodextrin (approx. 25%) and other food additives and allergenic foods (only samples 1-5)
<i>Number of samples and sample amount</i>	5 different Samples: 20 g each + 1 "Blank" Sample: 20 g
<i>Storage</i>	Samples 1-6: room temperature (long term cooled 2 - 10°C)
<i>Intentional use</i>	Laboratory use only (quality control samples)
<i>Parameter</i>	qualitative + quantitative: Almond / Almond Protein / DNA from Almond, (not roasted), Almond (roasted), Marzipan, Almond Spread and Almond Milk Samples 1-5: approx. 25 - 150 mg/kg (as total almond)
<i>Methods of analysis</i>	Analytical methods are optional
<i>Notes to analysis</i>	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. It is the best to homogenize the whole sample.
<i>Result sheet</i>	One result each should be determined for Samples 1 - 6 and the The results should be filled in the result submission file. In case of several determinations the mean.
<i>Units</i>	mg/kg
<i>Number of digits</i>	at least 2
<i>Result submission</i>	The result submission file should be sent by e-mail to: pt@dla-lvu.de
<i>Deadline</i>	the latest <u>May 31st 2019</u>
<i>Evaluation report</i>	The evaluation report is expected to be completed 6 weeks after deadline of result submission and sent as PDF file by e-mail.
<i>Coordinator and contact person of PT</i>	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
		SPAIN
		Germany
		Germany
		Germany
		Germany
		Germany
		SCOTLAND
		AUSTRIA

[Die Adressdaten der Teilnehmer wurden für die allgemeine Veröffentlichung des Auswertebereichs 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 Methodvalidierung / DIN ISO 5725 series part 1, 2 and 6 Accuracy (trueness and precision) of measurement methods and results
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