DLA
Dienstleistung
Lebensmittel
Analytik GbR

Evaluation Report
proficiency test

09/2016

Allergens IX:

Casein and Egg White Proteins

in Wine

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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].

2. Realisation

2.1 Test material

Two PT-samples for the detection of allergens in the range of mg/kg and one spiking material sample were provided for analysis. The spiking material sample contains the respective allergenic ingredients in the range of 1-10 % and was added to the spiked PT-sample. The results of the spiking material sample should give the possibility of a comparison with the spiked sample in respect to the detectability of the allergens with and without the influence of matrix and / or food processing.

The test material is a common in commerce white wine "riesling" (Mosel, German quality wine). The basic composition of both sample A and sample B was the same (see table 1). The pH value of the the wine was adjusted to pH 7-8 in order to stabilize the allergens in solution. The spiking material sample containing the allergenic ingredients skimmed milk powder and egg white proteins was added to sample B.
The composition of the spiking material sample and the amounts of allergens in sample B is given in table 2.

After homogenisation the samples were portioned to approximately 200 mL in PE-bottles with screw lock.

Table 1: Composition of DLA-Samples

Ingredients	Sample A	Sample B
White Wine Riesling Labelling: Riesling slight sharp, German quality wine, Mosel, contains sulfites, 10.0 % vol Pre-treatment: pH adjusted with sodium carbonate solution to pH 7-8	100 g/100g	99,6 g/100g
Spiking material sample	-	0,449 g/100g

Table 2: Added amounts of allergenic ingredients

Ingredients	Spiking material sample	Amounts in Sample B
Glucose	81,1 %	0,364 %
Fructose	8,11 %	0,036 %
Milk: Ingredients: Skimmed milk (pasteurized, spray dried) - as Skimmed milk powder - thereof Total protein* - thereof Casein*	 82500 mg/kg (= 8,25%) 27200 mg/kg 21800 mg/kg	 370 mg/kg 122 mg/kg 98 mg/kg
Egg White Powder (fining agent) Ingredients: Hen's egg white (pasteurized, spray dried) - thereof Egg white protein* - thereof Lysozyme*	24800 mg/kg (= 2,48%) 19840 mg/kg 694 mg/kg	111 mg/kg 89 mg/kg 3,1 mg/kg

* Protein content calculated according to labeling/specification/literature

2.1.1 Homogeneity

Homogeneity of the spiking material sample and spiked sample B was checked by ELISA-test for egg white proteins (fig. 1). The resulting standard deviations between the samples of < 15% ensured sufficient homogeneity [14, 15, 18, 19]. In case the criterion for sufficient homogeneity of the test items is not fulfilled the impact on the target standard deviation will be verified. If necessary the evaluation of results will be done considering the standard uncertainty of the assigned value (s. 3.8 and 3.11) [3].

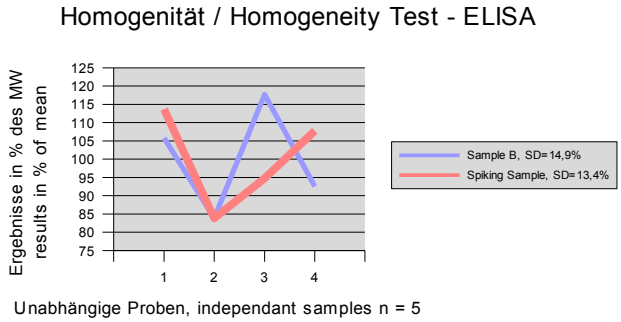

Fig. 1: Testing of homogeneity of DLA-sample B and spiking material sample. Results are given in percent of the arithmetic mean

2.2 Sample shipment and information to the test

The portions of test material (sample A and sample B as well as the spiking material sample) were sent to every participating laboratory in the 8th week of 2016. The testing method was optional. The tests should be finished at April 8th 2016 the latest.

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

Important Note: The pH-value of the wine samples A and B was adjusted with a sodium carbonate solution to pH 7-8, in order to stabilize the allergens in solution/suspension. Before analysis we recommend to shake the wine samples gently.

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. casein and egg white protein in mg/kg were evaluated.
Queried and documented were the indicated results and details of the test methods like specifity, 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 participants submitted their results in time.

3. Evaluation

Different ELISA-methods for the determination of allergens in foods are eventually using different antibodies, are usually calibrated with different reference materials and may utilize differing extraction methods. Among others this can induce different results of the content of the analyte [21, 22, 23, 24]. It is for this reason that we contrast the results of the present proficiency test with several assigned values. Thereby it is possible to evaluate each single result in comparison to the mean of all results and/or in comparison to the mean of results obtained by a single method. For comparison the actually added amount is plotted in the figures of the results.

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

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

3.1 Consensus value from participants (assigned value)

The robust mean of the submitted results was used as assigned value (X_{pt}) („consensus value from participants") providing a normal distribution. The calculation was done according to algorithm A as described in annex C of ISO 13528 [3].
The condition is that the majority of the participants' results show a normal distribution or are distributed unimodal and symmetrically. To this end, an examination of the distribution is carried out, inter alia, using the kernel density estimate [3, 12].

In case there are indications for sources of higher variability such as a bimodal distribution of results, a cause analysis is performed. Frequently different analytical methods may cause an anomaly in results' distribution. If this is the case, separate evaluations with own assigned values (X_{pti}) are made whenever possible.

If possible, this is the standard procedure for the evaluation of ELISA methods for the determination of allergens:

i) ***Robust mean of all results*** - $X_{pt_{ALL}}$
ii) ***Robust mean of single methods*** - $X_{pt_{METHOD\ i}}$
with at least 5 quantitative results given.

Single results giving values outside the measuring range of the participating laboratory or given as „0" are not considered for statistical evaluation (e.g. results given as > 25 mg/kg and < 2,5 mg/kg, respectively) [3].

3.2 Robust standard deviation

For comparison to the target standard deviation σ_{pt} (standard deviation for proficiency assessment) a robust standard deviation (S^x) was calculated. The calculation was done according to algorithm A as described in annex C of ISO 13528 [3].

The following robust standard deviations were considered:

i) ***Robust standard deviation of all results*** - S^x_{ALL}
ii) ***Robust standard deviation of single methods*** - $S^x_{METHOD\ i}$
with at least 5 quantitative results given.

3.3 Exclusion of results and outliers

Before statistical evaluation obvious blunders, such as those with incorrect units, decimal point errors, and results for a another proficiency test item can be removed from the data set [2]. All results should be given at least with 2 significant digits. Specifying 3 significant digits is usually sufficient.

Results obtained by different analytical methods causing an increased variability and/or a bi- or multimodal distribution of results, are treated separately or could be excluded in case of too few numbers of results. For this results are checked by kernel density estimation [3, 12].

Results are identified as outliers by the use of robust statistics. If a value deviates from the robust mean by more than 3 times the robust standard deviation, it is classified as an outlier [3]. Detected outliers are stated for information only, when z-score are < -2 or > 2. Due to the use of robust statistics outliers are not excluded, provided that no other reasons are present [3].

3.4 Target standard deviation (for proficiency assessment)

The target standard deviation of the assigned value σ_{pt} (= standard deviation for proficiency assessment) can be determined according to the following methods.
In the present PT the target standard deviation was determined according to 3.4.3 value by perception.

3.4.1 General model (Horwitz)

Based on statistical characteristics obtained in numerous PTs for different parameters and methods Horwitz has derived a general model for estimating the reproducibility standard deviation σ_R [6]. Later the model was modified by Thompson for certain concentration ranges [10]. The reproducibility standard deviation σ_R can be applied as the relative target standard deviation σ_{pt} in % of the assigned values and calculated according to the following equations [3]. For this the assigned value X_{pt} is used for the concentration c.

Equations	**Range of concentrations**	**corresponds to**
$\sigma_R = 0{,}22c$	$c < 1{,}2 \times 10^{-7}$	< 120 µg/kg
$\sigma_R = 0{,}02c^{0{,}8495}$	$1{,}2 \times 10^{-7} \leq c \leq 0{,}138$	≥ 120 µg/kg
$\sigma_R = 0{,}01c^{0{,}5}$	$c > 0{,}138$	> 13,8 g/100g

with c = mass content of analyte (as relative size, e.g. 1 mg/kg = 1 ppm = 10^{-6} kg/kg)

The target standard deviation according to Horwitz is currently not achievable by ELISA-methods for values in the mg/kg range and was therefore not considered for evaluation.

3.4.2 Value by precision experiment

Using the reproducibility standard deviation σ_R and the repeatability standard deviation σ_r of a precision experiment (collaborative trial or proficiency test) the target standard deviation σ_{pt} can be derived considering the number of replicate measurements m of participants in the present PT [3]:

$$\sigma_{pt} = \sqrt{\sigma_R^2 - \sigma_r^2\left(m - 1/m\right)}$$

Because in the present proficiency test the number of replicate measurements is n = 1, the reproducibility standard deviation σ_R is identical to the target standard deviation σ_{pt}.

Table 2: Relative repeatability standard deviations (RSD_r) and relative reproducibility standard deviations (RSD_R) from precision experiments [26, 27, 29]

Parameter	**Matrix**	**Mean**	**RSD_r**	**RSD_R**	**Method / Literature**
Caseinate	White wines	0,057 – 0,78 mg/L	-	35,1 – 90,0 %	ELISA [26]
Caseinate	White wines	1,4 – 3,0 mg/L	-	20,3 – 29,4 %	ELISA [26]
Caseinate	White wines	6,3 – 6,8 mg/L	-	12,1 – 21,4 %	ELISA [26]
Egg white proteins	Red wines	1,0 – 1,4 mg/L	23,0 - 27,6 %	30,6 – 32,9 %	ELISA [27]
Egg white proteins	Red wines	3,5 – 4,2 mg/L	14,7 – 19,3 %	26,2 – 31,1 %	ELISA [27]
Egg white proteins	Red wines	5,9 – 6,9 mg/L	12,5 – 16,5 %	20,1 – 25,7 %	ELISA [27]
Casein	Red wines	1,02 mg/L	11,7 %	19,4 %	ELISA [29]
Casein	Red wines	5,6 – 8,5 mg/L	14,7 – 24,0 %	24,8 – 35,6 %	ELISA [29]
Casein	White wines	0,12 -0,80 mg/L	9,1 - 35,0 %	13,7 - 53,8 %	ELISA [29]
Casein	White wines	4,1 – 5,5 mg/L	10,8 – 13,6 %	16,7 – 18,3 %	ELISA [29]
Egg white proteins	Red wines	0,26 mg/L	55,5 %	67,5 %	ELISA [29]
Egg white proteins	Red wines	1,1 – 7,6 mg/L	10,3 – 12,3 %	13,2 – 21,3 %	ELISA [29]
Egg white proteins	White wines	0,59 mg/L	37,4 %	52,1 %	ELISA [29]
Egg white proteins	White wines	3,6 – 6,5 mg/L	11,1 – 17,3 %	17,2 – 22,1 %	ELISA [29]

The precision data in table 2 were obtained in collaborative trials with spiked wine samples by ELISA testkit methods, some of them modified [26, 27, 29]. Depending on the allergen amount relative reproducibility standard deviations were 12 – 36 % in the range of > 1 mg/L and 14 - 90 % in the range of < 1 mg/L.

3.4.3 Value by perception

The target standard deviation for proficiency assessment can be set at a value that corresponds to the level of performance that the coordinator would wish laboratories to be able to achieve [3].
Criteria for the level of performance of analytical methods for the quantitative determination of allergens in foods were recently elaborated e.g. by the Ministry of Health and Welfare (MHLW) in Japan [18], by the working group 12 „Food Allergens" of the technical committee CEN/TC 275 [15-17], by an international "Food Allergen Working Group" under the advice of the AOAC Presidential Task Force on Food Allergens [19] and by the Codex Alimentarius Committee (CAC/GL 74-2010) [14].

Some of the relevant ELISA and PCR validation criteria of the mentioned panels are listed in tables 3 and 4, respectively.

Table 3: ELISA-Validation

Literature [14-20]	**Recovery rate**	**Repeatability standard deviation**	**Reproducibility standard deviation**
MHLW 2006	50 - 150%		≤ 25%
CEN 2009		≤ 20%	
AOAC 2010	50 - 150%	6,9 - 34,4% (a)	19,5 - 57,2 (a)
CAC 2010	70 - 120%	≤ 25%	≤ 35%

(a) = Example from an hypothetical proficiency scheme in the range of 0,5 - 5 mg/kg

Table 4: PCR-Validation

Literature [14]	**Recovery rate**	**Repeatability standard deviation**	**Reproducibility standard deviation**
CAC 2010	± 25% (a)	≤ 25%	≤ 35%

(a) = Trueness / Richtigkeit

Based on the currently achievable level of performance of ELISA and PCR methods for the quantitative determination of allergens in foods, which could be deduced from the data of precision experiments and from validation criteria, we set a relative target standard deviation σ_{pt} of 25%.
This target standard deviation was applied for the statistical evaluation of the results by z-score and was used for all assigned values mentioned in 3.1.

Legal requirements and maximum level recommendations

The labeling of allergens is settled by the regulation of food information for consumers (EU 1169/2011). Especially for wine requirements for labeling of the use of allergen-containing fining agents during winemaking is given in the Implementing Regulation EU 579/2012. Besides sulfite fining agents from milk and egg have to be labeled, if they are detectable in the wine.
Based on data obtained by collaborative studies the International Organisation of Vine and Wine (OIV) settled a limit of detection of

≤ 0,25 mg/L and a limit of quantification of ≤ 0,5 mg/L as criteria for the quantification of casein from milk and albumin and/or lysozyme from egg in wine [28].

3.5 z-Score

To assess the results of the participants the z-score is used. It indicates about which multiple of the target standard deviation (σ_{pt}) the result (x_i) of the participant is deviating from the assigned value (X_{pt}) [3].
Participants' z-scores are derived from:

$$z_i = \frac{\left(x_i - x_{pt}\right)}{\sigma_{pt}}$$

The requirements for the analytical performance are generally considered as fulfilled if

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

For information the z-scores below are calculated with a target standard deviation of 25%:

i) ***z-Score*** - z_{ALL} *(with respect to all methods)*
ii) ***z-Score*** - $z_{METHOD\ i}$ *(with respect to single methods)*

3.5.1 Warning and action signals

In accordance with the norm ISO 13528 it is recommended that a result that gives rise to a *z*-score above 3,0 or below −3,0, shall be considered to give an "action signal" [3]. Likewise, a *z*-score above 2,0 or below −2,0 shall be considered to give a "warning signal". A single "action signal", or "warning signal" in two successive PT-rounds, shall be taken as evidence that an anomaly has occurred which requires investigation. For example a fault isolation or a root cause analysis through the examination of transmission error or an error in the calculation, in the trueness and precision must be performed and if necessary appropriate corrective measures should be applied [3].

In the figures of z-scores DLA gives the limits of warning and action signals as yellow and red lines respectively. According to ISO 13528 the signals are valid only in case of a number of ≥ 10 results [3].

3.6 Quotient S*/σ_{pt}

Following the HorRat-value the results of a proficiency-test (PT) can be considered convincing, if the quotient of robust standard deviation S* and target standard deviation σ_{pt} does not exceed the value of 2.
A value > 2 means an insufficient precision, i.e. the analytical method is too variable, or the variation between the test participants is higher than estimated. Thus the comparability of the results is not given [3].

3.7 Standard uncertainty of the assigned value

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

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

If $U_{(Xpt)} \leq 0{,}3\ \sigma_{pt}$ the standard uncertainty of the assigned value needs not to be included in the interpretation of the results of the PT [3]. Values exceeding 0,3 imply, that the target standard deviation could be too low with respect to the standard uncertainty of the assigned value. The Quotient $U_{(Xpt)}/\sigma_{pt}$ is reported in the characteristics of the test.

3.8 Figures

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

3.9 Recovery rates: Spiking

For the results of the spiking material sample and the spiked sample recovery rates were calculated with respect to the known content of added allergens. The related values of added allergens are given in 2.1 test material in table 2. As a range of acceptance RA for valuating participant's results the range of 50 - 150% for the recovery rates of allergen-ELISAs proposed by the AOAC was used [19]. For quantitative PCR determinations we use the same range of acceptance.

4. Results

All following tables are anonymized. With the delivering of the evaluation-report the participants are informed about their individual evaluation-number.
The following result sections are structured equally for the allergenic components. First all results for a certain analyte are reported together for sample A and afterwards for sample B.

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

ELISA-results, which were given as total milk protein, were converted into casein, when available with respect to the instructions of the test kit manufacturers. The original results are given in the documentation.
A content of 80% casein in total milk protein was assumed.

ELISA-results, which were given as whole egg powder or ovalbumin, were converted into egg white proteins, when available with respect to the instructions of the test kit manufacturers. The original results are given in the documentation.
A content of 26% egg white proteins in whole egg powder was assumed. For ovalbumin a cross-reactivity to egg white proteins of 75% was taken according to test-kit instructions (Immunolab).

Evaluation was done separately for ELISA and PCR-techniques. The results were grouped according to the applied methods (e.g. test-kits) and sorted chronologically according to the evaluation-number of the participants.

Results were valuated qualitatively with respect to the percentages of positive and negative results, respectively. If there are ≥ 75 % positive or negative results, a consensus result is determined for each sample. Each participant result is valuated qualitatively with respect to the consensus value. The valuation was given as a percentage of results in agreement with the consensus values.

When there are at least 5 quantitative results for all methods or for single methods a statistical evaluation was done.

In cases when a statistical evaluation of the quantitative values was done the result table was given as indicated below:

Evaluation number	Result	Result	z-Score Xpt_{ALL}	z-Score $Xpt_{M\,i}$	Method	Remarks
	pos/neg	[mg/kg]	X All	X Method i		

The statistical evaluation of results for each parameter was calculated in cases where at least 50% results were positive and at least 5 quantitative values were given:

Characteristics	**All Results** *[mg/kg]*	**Method i** *[mg/kg]*
Assigned value (X_{pt})	$\boldsymbol{X_{pt_{ALL}}}$	$\boldsymbol{X_{pt_{METHOD\ i}}}$
Number of results		
Number of outliers		
Median		
Robust mean (X_{pt})		
Robust standard deviation (S*)		
Target data:		
Target standard deviation σ_{pt}		
lower limit of target range (X_{pt} - 2σ_{pt})		
upper limit of target range (X_{pt} + 2σ_{pt})		
Quotient S*/σ_{pt}		
Standard uncertainty $U_{(Xpt)}$		
Quotient $U_{(Xpt)}/\sigma_{pt}$		
Number of results in target range		
Percent in target range		

After that the recovery rates of the results for the spiking sample and the spiked sample are reported. The number of results within the range of acceptance of 50-150% is given.

4.1 Proficiency Test Casein

4.1.1 ELISA-Results: Casein

Qualitative valuation of results: Samples A and B

Evaluation number	Sample A	Sample A	Sample B	Sample B	Qualitative Valuation	Method	Remarks
	pos/neg	[mg/kg]	pos/neg	[mg/kg]	Agreement with consensus value		
1	negative	< 0.1	positive		2/2 (100%)	AQ	
5	negative	< 0,1	positive	78	2/2 (100%)	AQ	
7	negative	<0.2	positive	>6	2/2 (100%)	AQ	
13	negative	<0,2	positive	73	2/2 (100%)	AQ	
8	negative	<0,5	positive	24	2/2 (100%)	IL	
15	negative	<0.1	positive	36	2/2 (100%)	IL	
3	negative		positive	1,84	2/2 (100%)	RS1	
4	negative		positive	2,1	2/2 (100%)	RS1	
6	negative	<0,5	positive	3,2	2/2 (100%)	RS1	
9	negative	<0.5	positive	1,59	2/2 (100%)	RS1	
10	negative		positive	1,7	2/2 (100%)	RS1	
11	negative	<0.24	positive	2,2	2/2 (100%)	RS1	
12	negative	n.n.	positive	26,1	2/2 (100%)	RS2	Result converted *

* calculation see p. 14

	Sample A		Sample B	
Number positive	0		13	
Number negative	13		0	
Percent positive	0		100	
Percent negative	100		0	
Consensus value	negative		positive	

Methods:

AQ = AgraQuant Casein, RomerLabs
IL = Immunolab Casein
RS1 = Ridascreen Fast Casein, R-Biopharm
RS2 = Ridascreen Fast Milk, R-Biopharm

Comments:

There were 100% negative results for sample A and 100% positive results for sample B by the ELISA-methods. The consensus values are in qualitative agreement with the spiking of sample B.

Quantitative valuation of results: Sample B

Evaluation number	Casein	z-Score Xpt_{ALL}	z-Score Xpt_{RS1}	Method	Remarks
	[mg/kg]				
1				AQ	
5	78			AQ	
7	>6			AQ	
13	73			AQ	
8	24			IL	
15	36			IL	
3	1,84		-0,4	RS1	
4	2,1		0,1	RS1	
6	3,2		2,3	RS1	
9	1,59		-0,9	RS1	
10	1,7		-0,6	RS1	
11	2,2		0,3	RS1	
12	26,1			RS2	Result converted *

* calculation see p. 14

Methods:

AQ = AgraQuant Casein, RomerLabs
IL = Immunolab Casein
RS1 = Ridascreen Fast Casein, R-Biopharm
RS2 = Ridascreen Fast Milk, R-Biopharm

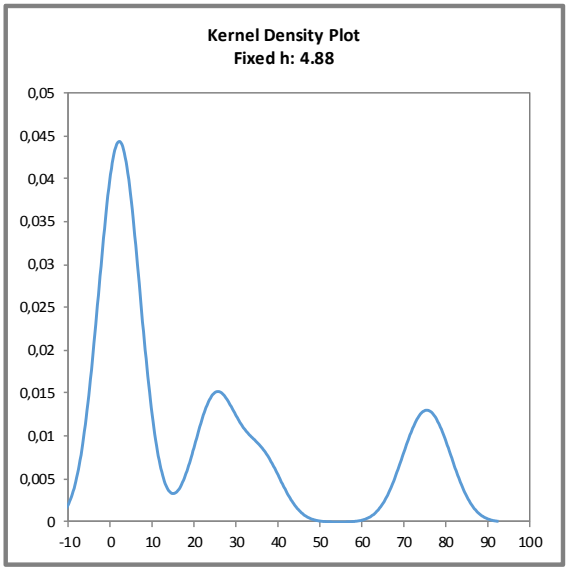

Fig. 2: Kernel Density Plot of all ELISA-results casein
(with h = σpt of Xpt_{ALL})

Comments:
The kernel density estimation shows a trimodal distribution due to differences of the applied methods: 1. method RS1, 2. method IL and RS2 and 3. method AQ (s. fig. 2).

Characteristics: Quantitative evaluation Casein

Sample B

Characteristics	**All Results** *[mg/kg]*	**Method RS1** *[mg/kg]*
Assigned value (X_{pt})	$X_{pt_{ALL}}$	$X_{pt_{METHOD\ RS1}}$
Number of results	*11*	*6*
Number of outliers	*-*	*0*
Median	3,20	1,97
Robust mean (X_{pt})	19,5	2,03
Robust standard deviation (S*)	25,6	0,476
Target data:		
Target standard deviation σ_{pt}		0,507
lower limit of target range (X_{pt} - 2σ_{pt})		1,01
upper limit of target range (X_{pt} + 2σ_{pt})		3,04
Quotient S/σ_{pt}*		*0,94*
Standard uncertainty $U_{(Xpt)}$		*0,243*
Quotient $U_{(Xpt)}/\sigma_{pt}$		*0,48*
Number of results in target range		*5*
Percent in target range		*83%*

Method:
RS1 = R-Biopharm, Ridascreen Fast®

Comments to the statistical characteristics:

The evaluation of all methods showed a multimodal distribution of results depending on testkit methods (see fig. 2). Therefore an evaluation of results across the methods was not performed.
The evaluation of results from method RS1 showed a low variability. The quotient S/σ_{pt} was clearly below 2,0. The robust standard deviation is in the range of established values for the reproducibility standard deviation of the applied method (see 3.4.2 value by precision experiments and 3.4.3 value by perception). The comparability of results is given.*

The robust mean of the evaluation of method RS1 was approximately 2% of the spiking level of casein to sample B and below the recommendations for the applied method (s. 3.4.3 and "Recovery rates of Casein" p. 21).

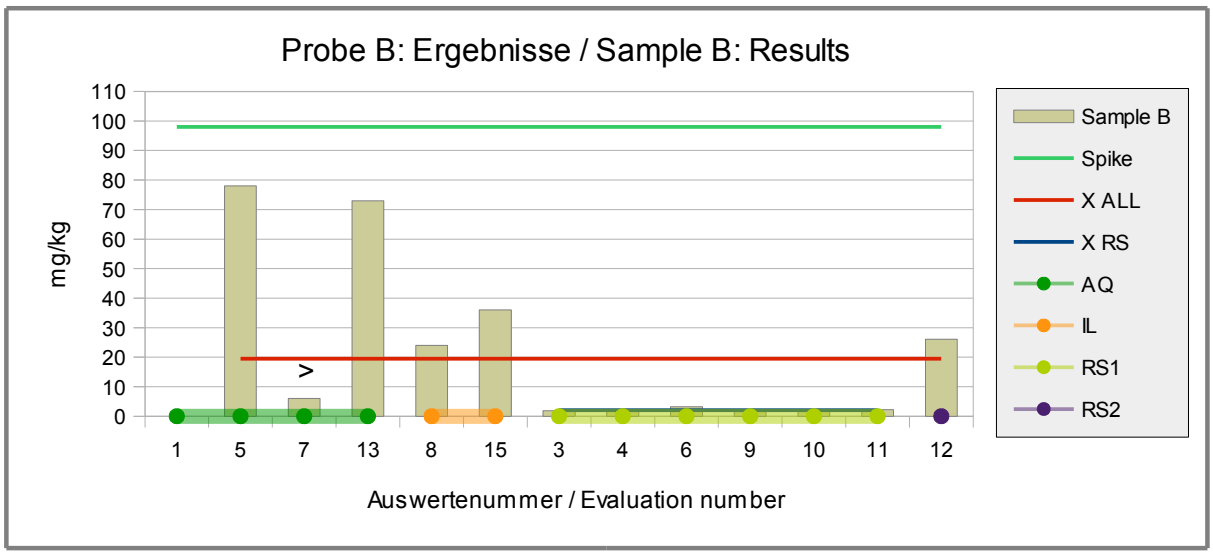

Fig. 3a: ELISA-Results Casein
green line = Spiking level
red line = Assigned value robust mean all results
blue line = Assigned value robust mean results method RS1
round symbols = Applied methods (see legend)

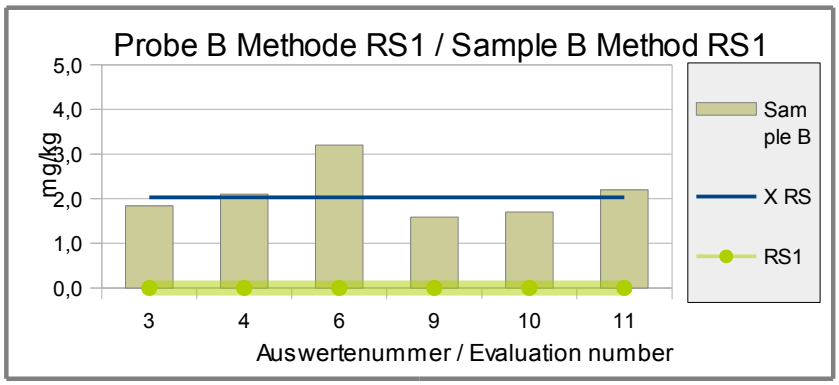

Fig. 3b: ELISA-Results Casein – Zoom Method RS1
blue line = Assigned value robust mean results method RS1
round symbols = Applied methods (see legend)

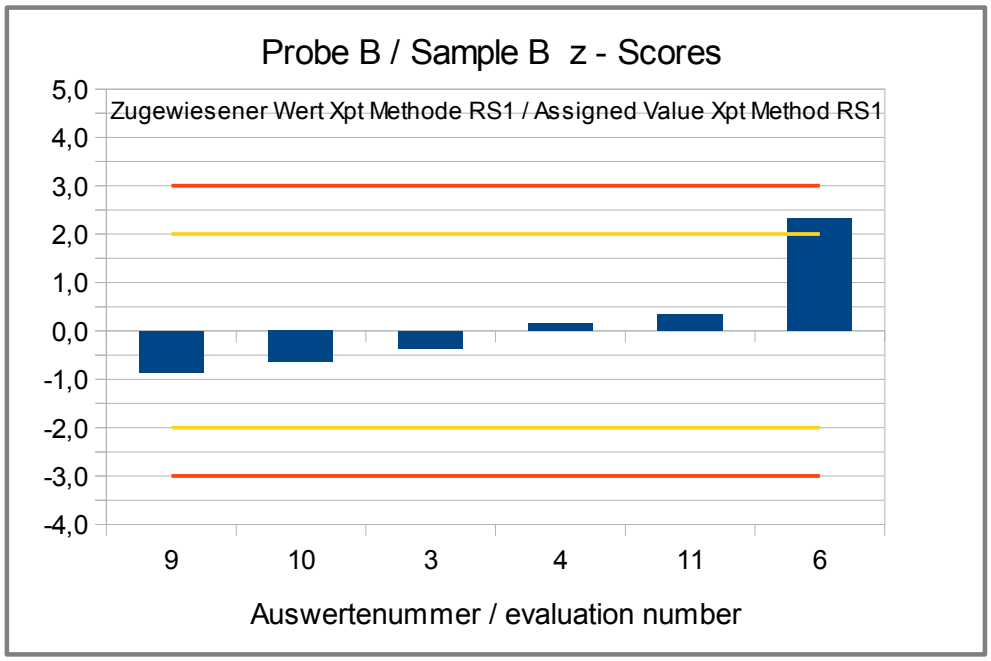

Fig. 4: z-Scores (ELISA-Results as Casein)
Assigned value robust mean of method RS1 (R-Biopharm, Ridascreen)

Recovery Rates for Casein:
Spiking Material Sample and Sample B

Evaluation number	Spiking material	Recovery rate	Sample B	Recovery rate	Method	Remarks
	[mg/kg]	[%]	[mg/kg]	[%]		
1					AQ	
5	48900	224	78	**80**	AQ	
7	28500	**131**	>6		AQ	
13	42000	193	73	**74**	AQ	
8	363454	1667	24	24	IL	
15	15500	**71**	36	37	IL	
3	11714	**54**	1,84	1,9	RS1	
4			2,1	2,1	RS1	
6			3,2	3,3	RS1	
9	20815	**95**	1,59	1,6	RS1	
10	41690	191	1,7	1,7	RS1	
11			2,2	2,2	RS1	
12	19500	**89**	26,1	27	RS2	Result converted *

* calculation see p. 14

RA*	50-150 %	RA*	50-150 %
Number in RA	**5**	Number in RA	**2**
Percent in RA	**56**	Percent in RA	**18**

* Range of acceptance of AOAC for allergen ELISAS

Recovery rate
100% relative size:
Casein, s. page 4

Methods:

AQ = AgraQuant Casein, RomerLabs
IL = Immunolab Casein
RS1 = Ridascreen Fast Casein, R-Biopharm
RS2 = Ridascreen Fast Milk, R-Biopharm

Comments:
For the spiking material sample 56% of the participants obtained a recovery rate within the range of the AOAC-recommendation of 50-150%. For the wine-sample B produced with the spiking material sample 2 of the recovery rates (method AQ) were in the range of acceptance.

4.2 Proficiency Test Egg White Protein

4.2.1 ELISA-Results: Egg White Protein, total

Qualitative valuation of results: Samples A and B

Evaluation number	Sample A	Sample A	Sample B	Sample B	Qualitative Valuation	Method	Remarks
	pos/neg	[mg/kg]	pos/neg	[mg/kg]	Agreement with consensus value		
7	negative	<0.4	positive	>8	2/2 (100%)	BC	
5	negative	< 0,05	positive	101	2/2 (100%)	IL1	
8	negative	<0,5	positive	63	2/2 (100%)	IL1	
15a	negative	< 0.2	positive	50	2/2 (100%)	IL1	
15b	negative	< 0.017	positive	40	2/2 (100%)	IL2	Result converted *
1	negative	< 0.065	positive	49,1	2/2 (100%)	RS	Result converted *
3	negative		positive	39,74	2/2 (100%)	RS	Result converted *
4	negative		positive	46,4	2/2 (100%)	RS	Result converted *
6	negative	<0,13	positive	46,1	2/2 (100%)	RS	Result converted *
9	negative	<0.13	positive	42,31	2/2 (100%)	RS	
10	negative		positive	60,8	2/2 (100%)	RS	
11	negative	<0.1	positive	>10	2/2 (100%)	RS	
12	negative	n.n.	positive	66,8	2/2 (100%)	RS	Result converted *
13	negative	<0,13	positive	65	2/2 (100%)	RS	Result converted *
14	negative	-	positive	88,4	2/2 (100%)	RS	Result converted *
2	negative		positive	113,66	2/2 (100%)	TC	

* calculation see p. 14

	Sample A		Sample B	
Number positive	0		16	
Number negative	16		0	
Percent positive	0		100	
Percent negative	100		0	
Consensus value	negative		positive	

Methods:

BC = BioCheck
IL1 = Immunolab Egg White Protein
IL2 = Immunolab Ovalbumin
RS = Ridascreen®, R-Biopharm
TC = Tecna

Comments:

There were 100% negative results for sample A and 100% positive results for sample B by the ELISA-methods. The consensus values are in qualitative agreement with the spiking of sample B.

Quantitative valuation of results: Sample B

Evaluation number	Egg White Protein	z-Score Xpt_{ALL}	z-Score Xpt_{RS}	Method	Remarks
	[mg/kg]				
7	>8			BC	
5	101	2,7		IL1	
8	63	0,2		IL1	
15a	50	-0,7		IL1	
15b	40	-1,3		IL2	Result converted *
1	49,1	-0,7	-0,4	RS	Result converted *
3	39,74	-1,4	-1,1	RS	Result converted *
4	46,4	-0,9	-0,6	RS	Result converted *
6	46,1	-0,9	-0,6	RS	Result converted *
9	42,31	-1,2	-0,9	RS	
10	60,8	0,0	0,4	RS	
11	>10			RS	
12	66,8	0,4	0,9	RS	Result converted *
13	65	0,3	0,7	RS	Result converted *
14	88,4	1,9	2,5	RS	Result converted *
2	113,66	3,5		TC	

* calculation see p. 14

Methods:

BC = BioCheck
IL1 = Immunolab Egg White Protein
IL2 = Immunolab Ovalbumin
RS = Ridascreen®, R-Biopharm
TC = Tecna

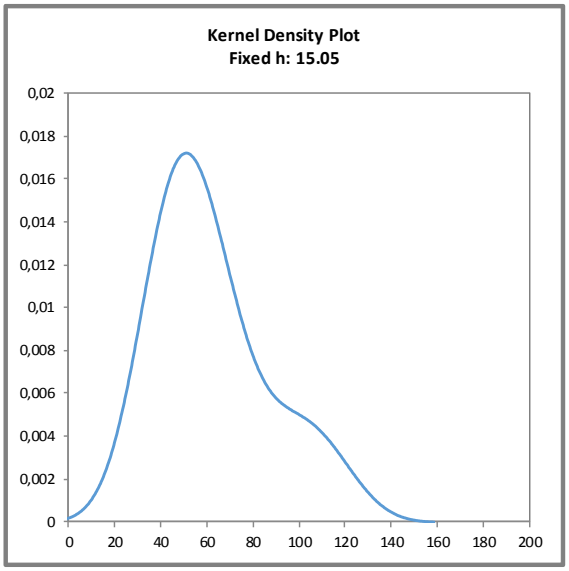

Fig. 5: Kernel Density Plot of all ELISA-results egg white protein (with h = σpt of Xpt_{ALL})

Comment:
The kernel density estimation shows nearly a normal distribution of results with a shoulder at approximately 100-115 mg/kg (method IL) (s. fig. 5).

Characteristics: Quantitative evaluation Gluten

Sample B

Characteristics	**All Results** *[mg/kg]*	**Methode RS** *[mg/kg]*
Assigned value (X_{pt})	$X_{pt_{ALL}}$	$X_{pt_{METHOD\ RS}}$
Number of results	*14*	*9*
Number of outliers	*0*	*0*
Median	55,4	49,1
Robust mean (X_{pt})	60,2	54,8
Robust standard deviation (S*)	21,7	14,6
Target data:		
Target standard deviation σ_{pt}	15,1	13,7
lower limit of target range (X_{pt} - 2σ_{pt})	30,1	27,4
upper limit of target range (X_{pt} + 2σ_{pt})	90,3	82,1
Quotient S/σ_{pt}*	*1,4*	*1,1*
Standard uncertainty $U_{(Xpt)}$	*7,24*	*6,07*
Quotient $U_{(Xpt)}/\sigma_{pt}$	*0,48*	*0,44*
Number of results in target range	*12*	*8*
Percent in target range	*86%*	*89%*

Method:
RS = R-Biopharm, Ridascreen®

Comments to the statistical characteristics:

The evaluation of all methods and the evaluation of results from method RS showed a normal to low variability, respectively. The quotients S/σ_{pt} were clearly below 2,0. The robust standard deviation is in the range of established values for the reproducibility standard deviation of the applied methods (see 3.4.2 value by precision experiments and 3.4.3 value by perception). The comparability of results is given. This conclusion is limited for the evaluation across the methods, because there are only a few results each for the methods IL and TC.*

The robust means of the evaluations were with 68% and 62% of the spiking level of egg white protein to sample B within the recommendations for the applied methods (s. 3.4.3 and "Recovery rates of Egg White Protein" p.27).

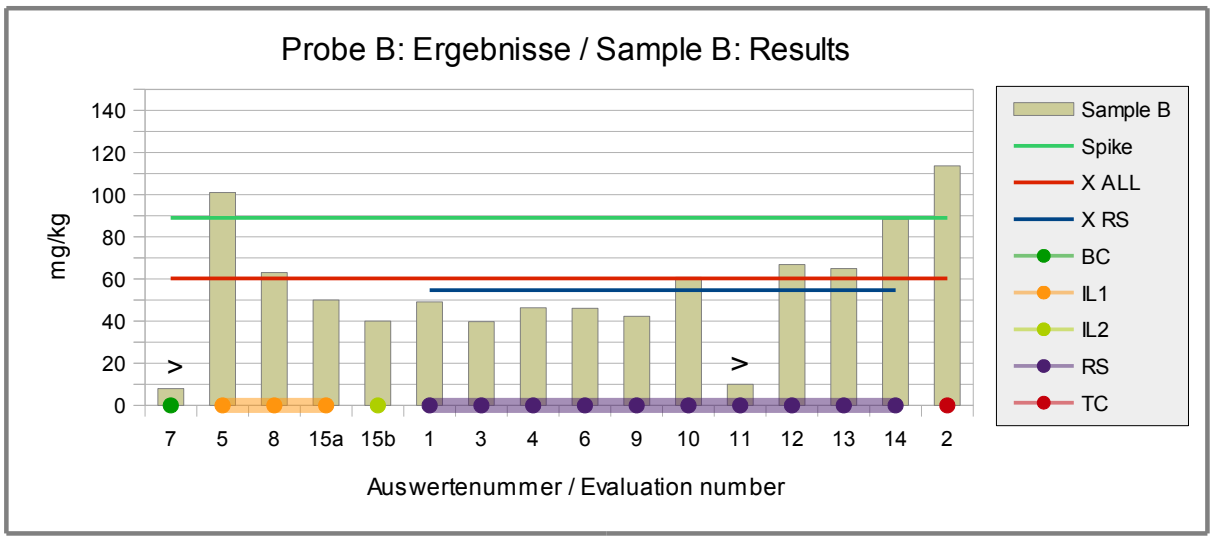

Fig. 6: ELISA-Results Egg White Protein
green line = Spiking level
red line = Assigned value robust mean all results
blue line = Assigned value robust mean results method RS
round symbols = Applied methods (see legend)

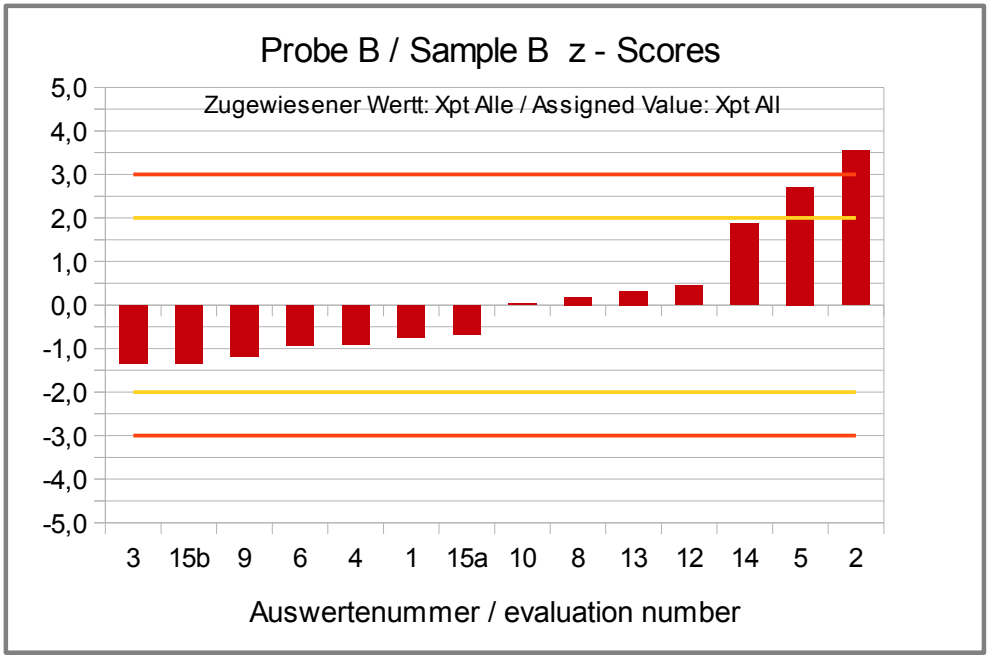

Fig. 7: z-Scores (ELISA-Results as Egg White Protein)
Assigned value robust mean of all results

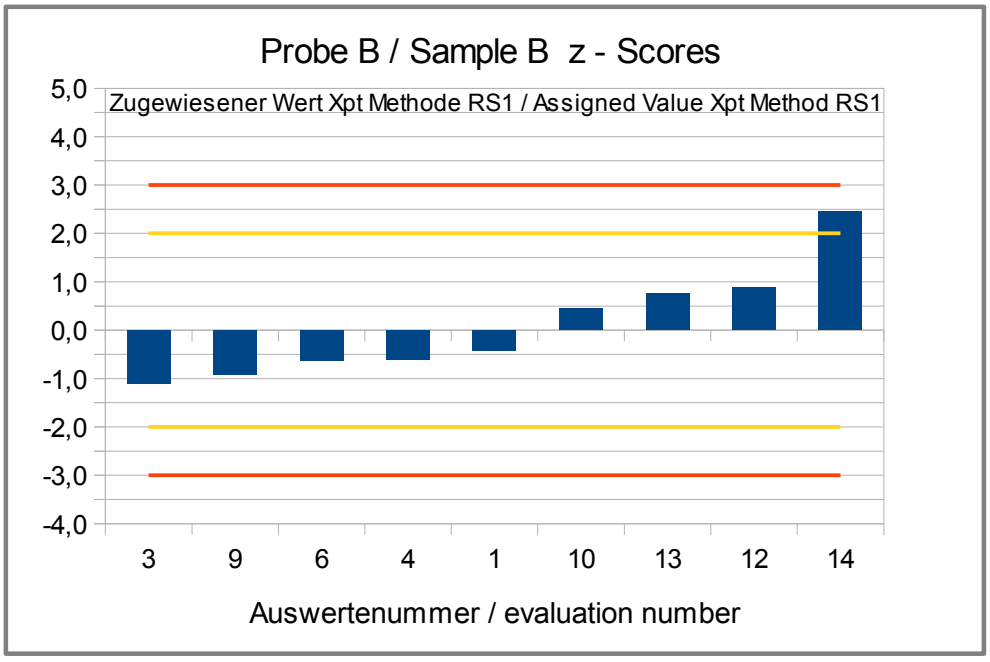

Fig. 8: z-Scores (ELISA-Results as Egg White Protein)
Assigned value robust mean of method RS
(R-Biopharm, Ridascreen)

Recovery Rates for Egg White Protein:
Spiking Material Sample and Sample B

Evaluation number	Spiking material	Recovery rate	Sample B	Recovery rate	Method	Remarks
	[mg/kg]	[%]	[mg/kg]	[%]		
7	8500	43	>8		BC	
5	21600	**109**	101	**113**	IL1	
8	378476	1908	63	**71**	IL1	
15a	16700	**84**	50	**56**	IL1	
15b	11500	**58**	40	45	IL2	Result converted *
1			49,1	**55**	RS	Result converted *
3	20255	**102**	39,74	45	RS	Result converted *
4			46,4	**52**	RS	Result converted *
6			46,1	**52**	RS	Result converted *
9	9820	49	42,31	48	RS	
10	16660	**84**	60,8	**68**	RS	
11			>10		RS	
12	12250	**62**	66,8	**75**	RS	Result converted *
13	13500	**68**	65	**73**	RS	Result converted *
14	14000	**71**	88,4	**99**	RS	Result converted *
2	14600	**74**	113,66	**128**	TC	

* calculation see p. 14

RA*	50-150 %	RA*	50-150 %
Number in RA	**9**	Number in RA	**11**
Percent in RA	**75**	Percent in RA	**79**

* Range of acceptance of AOAC for allergen ELISAS

Recovery rate
100% relative size:
Egg White Protein, s. page 4

Methods:

BC = BioCheck
IL1 = Immunolab Egg White Protein
IL2 = Immunolab Ovalbumin
RS = Ridascreen®, R-Biopharm
TC = Tecna

Comments:

For the spiking material sample 75% of the participants obtained a recovery rate within the range of the AOAC-recommendation of 50-150%. For the wine-sample B produced with the spiking material sample 79% of the recovery rates were in the range of acceptance.

4.1.2 ELISA-Results: Lysozyme

Qualitative valuation of results: Samples A and B

Evaluation number	Sample A	Sample A	Sample B	Sample B	Qualitative Valuation	Method	Remarks
	pos/neg	[mg/kg]	pos/neg	[mg/kg]	Agreement with consensus value		
15	negative	< 0.013	positive	0,7	-	IL	

Methods:
IL = Immunolab

Comments:
There was only one result submitted for the detection of lysozyme by ELISA. It is in qualitative agreement with the spiking of sample B.

Recovery Rates for Lysozyme:
Spiking Material Sample and Sample B

Evaluation number	Spiking material	Recovery rate	Sample B	Recovery rate	Method	Remarks
	[mg/kg]	[%]	[mg/kg]	[%]		
15	160	23	0,7	23	IL	

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

* Range of acceptance of AOAC for allergen ELISAS

Recovery rate
100% relative size:
Lysozyme, s. page 4

Comments:
The recovery rates of the participants' results were 23% for both the spiking material sample and the wine-sample B produced with the spiking material sample.

5. Documentation

Details by the participants

5.1 ELISA: Casein

Primary data

Evaluation number	Result Sample A		Result Sample B		Result Spiking Sample		quantitative Result given as	Method	Meth. Abr.
	qualitative	mg/kg	qualitative	mg/kg	qualitative	mg/kg	e.g. food / food protein	Test-Kit + Manufacturer	
1	negative	< 0.1	positive		-		Casein	AgraQuant Casein (COKAL1200), RomerLabs	AQ
5	negative	< 0,1	positive	78	positive	48900	Casein	AgraQuant Casein (COKAL1200), RomerLabs	AQ
7	-	<0.2	-	>6	-	28500	Casein	AgraQuant Egg (COKAL0848), RomerLabs	AQ
13	negative	<0,2	positive	73	positive	42000	Casein	AgraQuant Casein (COKAL1200), RomerLabs	AQ
8	negative	<0,5	positive	24	qualitative	363454	Casein	Immunolab Casein ELISA	IL
15	negative	<0.1	positive	36	positive	15500	Casein	Immunolab Casein ELISA	IL
3	negative		positive	1,839	positive	11714	Casein	Ridascreen Fast Casein (R4612), r-Biopharm	RS1
4	negative		positive	2,1	positive		Casein	Ridascreen Fast Casein (R4612), r-Biopharm	RS1
6	-	<0,5	-	3,2	-		Casein	Ridascreen Fast Casein (R4612), r-Biopharm	RS1
9	negative	<0.5	positive	1,59	positive	20815	Casein	Ridascreen Fast Casein (R4612), r-Biopharm	RS1
10	negative		positive	1,7	positive	41690	Casein	Ridascreen Fast Casein (R4612), r-Biopharm	RS1
11	negative	<0.24	positive	2,2	-		Given as	Ridascreen Fast Casein (R4612), r-Biopharm	RS1
12	-	n.n.	-	32,6	-	24381	total milk protein	Ridascreen Fast Milch (R4652), r-Biopharm	RS2

Methods:

AQ = AgraQuant Casein, RomerLabs
IL = Immunolab Casein
RS1 = Ridascreen Fast Casein, R-Biopharm
RS2 = Ridascreen Fast Milk, R-Biopharm

Other details to the Methods

Evaluation number	Meth. Abr.	Specifity	Remarks to the Method (Extraction and Determination)	Further Remarks
		Antibody	e.g. Extraction Solution / Time / Temperature	
1	AQ		Sample preparation according to Wine-Application Note	
5	AQ	Casein	Sample A 1:10 (v/v) extracted with extraction buffer; spiking material sample only diluted with extraction buffer and extracted; all at room temperature	At dilution of spiking with alcoholic solution and water and extraction afterwards with buffer clearly lower values were found
7	AQ	Casein	Aqueous Buffer Heated to 60°C	Proteins precipitated out of solution generating inconsistent results
13	AQ			
8	IL	Anti-Casein		
15	IL			
3	RS1			
4	RS1		as per Kit Instructions	
6	RS1		Extraction buffer diluted/ 10min/60°C	
9	RS1	casein from cow, sheep, goat and buffalo milk	As per Kit Instructions	
10	RS1			
11	RS1			
12	RS2			

5.2 ELISA: Egg White Proteins including Lysozyme

Primary data

Evaluation number	Result Sample A		Result Sample B		Result Spiking Sample		quantitative Result given as	Method	Meth. Abr.
	qualitative	mg/kg	qualitative	mg/kg	qualitative	mg/kg	e.g. food / food protein	Test-Kit + Manufacturer	
7	-	<0.4	-	>8	-	8500	Egg white proteins, total	EggCheck - BioCheck	BC
5	negative	< 0,05	positive	101	positive	21600	Egg white proteins, total	Immunolab OVALBUMIN ELISA	IL1
8	negative	<0,5	positive	63	qualitative	378476	Egg white proteins, total	Immunolab Eiklar ELISA	IL1
15a	negative	< 0.2	positive	50	positive	16700	Egg white proteins, total	Immunolab Eiklar ELISA	IL1
15b	negative	< 0.013	positive	30	positive	8600	Ovalbumin	Immunolab Ovalbumin ELISA	IL2
1	negative	< 0.25	positive	189	-		Whole egg powder	Ridascreen Fast Ei (R4602), r-Biopharm	RS
3	negative		positive	152,83	positive	77904	Whole egg powder	Ridascreen Fast Ei (R4602), r-Biopharm	RS
4	negative		positive	178,5	positive		Whole egg powder	Ridascreen Fast Ei (R4602), r-Biopharm	RS
6	-	<0,5	-	177,3	-		Whole egg powder	Ridascreen Fast Ei (R4602), r-Biopharm	RS
9	negative	<0.13	positive	42,31	positive	9820	Egg white proteins, total	Ridascreen Fast Ei (R4602), r-Biopharm	RS
10	negative		positive	60,8	positive	16660	Egg white proteins, total	Ridascreen Fast Ei (R4602), r-Biopharm	RS
11	negative	<0.1	positive	>10	-		Given as	Ridascreen Fast Ei (R4602), r-Biopharm	RS
12	-	n.n.	-	257	-	47110	Whole egg powder	Ridascreen Fast Ei (R4602), r-Biopharm	RS
13	negative	<0,5	positive	250	positive	52000	Whole egg powder	Ridascreen Fast Ei (R6402), r-Biopharm	RS
14	negative	-	positive	340	positive	54000	Whole egg powder	Ridascreen Fast Ei (R6402), r-Biopharm	RS
2	negative		positive	113,66	positive	14600	Egg white proteins, total	other: TECNA I'SCREEN EGG	TC
15	negative	< 0.013	positive	0.7	positive	160	Lysozyme	Immunolab Lysozym ELISA	IL3

Methods:

BC = BioCheck
IL1 = Immunolab Eiklarprotein
IL2 = Immunolab Ovalbumin
IL3 = Immunolab Lysozym
RS = Ridascreen®, R-Biopharm
TC = Tecna

Other details to the methods

Evaluation number	Meth. Abr.	Specifity	Remarks to the Method (Extraction and Determination)	Further Remarks
		Antibody	e.g. Extraction Solution / Time / Temperature	
7	BC	Egg white proteins, total	Aqueous Buffer Heated to 60°C	Proteins precipitated out of solution generating inconsistent results
5	IL1	OVALBUMIN	Conversion of Ovalbumin to egg white protein (dry) by factor of 1,85; spiking material sample only diluted and extracted by extraction buffer; all at room temperature	At dilution of spiking with alcoholic solution and water and extraction afterwards with buffer clearly lower values were found
8	IL1	Anti-ovomucoid antibodies		
15a	IL1			
15b	IL2			
1	RS		Sample preparation according to Wine-Application Note	
3	RS			
4	RS		as per kit instructions	
6	RS		Extraction buffer diluted/ 10min/60°C	
9	RS	Ovalbumin and Ovomucoid	As per kit instructions - converted result to report as Egg White Protein as described in the kit instructions	
10	RS		Given as egg white protein, whole egg powder would be: Sample B: 231 mg/kg; Spiking Material: 63350 mg/kg	
11	RS			
12	RS			
13	RS			
14	RS	Egg white proteins, total	-	-
2	TC	Egg white proteins, total	10ml extraction solution/15' time/60°C	Sample B and Spiked sample needed to be diluted to produce a result in the range of the kit.
15	IL3			

6. Index of participant laboratories

Teilnehmer / Participant	Ort / Town	Land / Country
		UNITED KINGDOM
		SWITZERLAND
		SPAIN
		Germany
		Germany
		Germany
		FRANCE
		Germany
		Germany
		SWITZERLAND
		ITALY
		Germany
		Germany
		NETHERLANDS
		UNITED KINGDOM

[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
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