DLA
Dienstleistung
Lebensmittel
Analytik GbR

**Evaluation Report**
proficiency test

**07/2014**

# Allergens VII:

## Crustaceae and cashew

## in Instant Product

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# Inhalt

# 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 validity of the particular testing method.
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.

# 2. Realisation

## 2.1 Test material

Two PT-samples for the detection of allergens in the range of mg/kg and two spiking material samples (Cashew nuts and crustacean, respectively, potato flour as carrier material) 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 materials are sauce thickener. The basic composition of both sample A and sample B was the same (see table 1). The spiking material (cashew nuts ans crustacean) was added to sample B.
The samples were homogenised and portioned to approximately 25 g.

The composition of the spiking material sample and the amounts of allergens in sample B is given in table 2.

Table 1: Composition of DLA-Samples A and B

| Ingredients | Sample A | Sample B |
|---|---|---|
| sauce thickener<br>Ingredients: starch, lactose, maltodextrin, rice flour, stabilizer: diphosphates, palm oil, milk protein. | 100 g/100g | 93,3 g/100g |
| Ethyl-p-hydroxybenzoate (E 214) | - | 0,1 g/100g |
| Spiking material sample A | - | 5,6 g/100g |
| Spiking material sample B | - | 1,0 g/100g |

Table 2: Added amounts of allergenic ingredients

**Spiking material sample A**
Weighing of Crustacean premix/ spiking material sample 3%)

| No. | row material | ingredients | sample B[1] |
|---|---|---|---|
| 1 | Potato flour | 89,9 g/100 g | 5,0 g/100 g |
| 2 | Crab cream: rapeseed oil, crab meat (Cancer pagurus) 30%, fish roe (codfish, Gadus morhua), pollock (Pollachius virens), pacific cod (Gadus macrocephalus) 11%, salt, sugar, tomato puree, dill, potato flakes, spirit vinegar, preservative: E 211, E 202, spices, flavour, smoke.<br>- as crab meat<br>- as crustacean protein | 10 g/100 g<br>**30.000 mg/kg**<br>**5.580 mg/kg** | 0,56 g/100 g<br>**1.680 mg/kg**<br>**312 mg/kg[2]** |
| 3 | Ethyl-p-hydroxybenzoate (E 214) | 0,1 g/100 g | 5,6 mg/100 g |

* related to total weight of sample B
**[2] according to: Souci et al., Brown shrimp: Protein 18,6 g/100g**

**Dotierungsmaterialprobe B:**
(Weighing of Cashew nut premix/ spiking material sample 5%)

| No. | row material | ingredients | sample B[1] |
|---|---|---|---|
| 1 | Potato flour | 94,9 g/100 g | 0,949 g/100g |
| 2 | Cashew nut puree (100%) | **5,0 g/100 g**<br>**= 50.000 mg/kg** | **0,05 g/100g**<br>**= 500 mg/kg** |
| 3 | Ethyl-p-hydroxybenzoate (E 214) | 0,1 g/100 g | 1 mg/100g |

*** related to total weight of sample B**

*2.1.1 Homogeneity*

Homogeneity of the spiked sample B was checked by 5fold HPLC analysis of ethyl-p-hydroxybenzoate. The resulting standard deviation between the samples of < 4% ensured sufficient homogeneity.

| Probe/ sample | E 214 | | |
|---|---|---|---|
| 1 | 1004 | mg/kg | |
| 2 | 991 | mg/kg | |
| 3 | 1047 | mg/kg | |
| 4 | 1054 | mg/kg | |
| 5 | 1064 | mg/kg | |
| | | | |
| Mittelwert/ mean | 1031,9 | mg/kg | |
| Standardabw./ standard deviation | 32,23 | 3,1 | % |

## 2.2 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 50th week of 2014. The testing method was optional. The tests should be finished at February 6th 2015 the latest.

## 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. cashew nuts or crustacea 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 (a/b) and indication of the related method.
All submitted their results in time. Two participants have submitted two results.

# 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. 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 actually added amount, in comparison to the mean of all results and/or in comparison to the mean of results obtained by a single method.

For ELISA-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 Assigned value

Because the analysed material was no certified reference material the robust mean of the submitted results was used as assigned value X (6). In case the submitted results show hints for bimodal distribution or other reasons for a higher variability the evaluation will be performed additionally with respect to the robust mean of single methods. If possible, this is the standard procedure for the evaluation of ELISA methods for the determination of allergens:

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

Single results giving values outside the measuring range of the participating laboratory were considered for statistical evaluation (e.g. results given as > 25 mg/kg and < 2,5 mg/kg, respectively) when a result indicating „>" is above and a result indicating „<" is below the target range.

## 3.2 Standard deviation

For comparison to the target standard deviation a robust standard deviation ($S^x$) was calculated (6).
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 Outliers

Statistical outliers were determined by Mandel´s-H-Statistic for 95% significance niveau (5). Detected outliers were stated for information only, when z-score was < -2 or > 2.

## 3.4 Target standard deviation

The target standard deviation of the assigned value is determined according to the following methods.

### *3.4.1 General model (Horwitz)*

The relative target standard deviation in % of the assigned value is derived from following equation (Horwitz)

$$\hat{\sigma}_{(\%)} = 2^{(1-0,5\log X)}$$

From the result the target standard deviation is calculated

$$\hat{\sigma} = X * \hat{\sigma}_{(\%)} / 100.$$

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 $\sigma_R$ and the repeatability standard deviation $\sigma_r$ of a precision experiment the between-laboratories standard deviation can be calculated $\sigma_L$ :

$$\sigma_L = \sqrt{(\sigma_R^2 - \sigma_r^2)} \quad .$$

And then, using the number of replicate measurements n, each participant is to perform, the target standard deviation for proficiency assessment is calculated :

$$\hat{\sigma} = \sqrt{(\sigma_L^2 + (\sigma_r^2/n))} \quad .$$

Because in the present proficiency test the number of replicate measurements is n = 1, the reproducibility standard deviation $\sigma_R$ is identical to the target standard deviation $\hat{\sigma}$ .

The following table shows the relative reproducibility standard deviations from proficiency tests of ELISA-methods from German ASU §64 methods (13, 14, 15):

| Method | Parameter | Matrix | Mean values | Relative $\sigma_R$ | Literature |
|---|---|---|---|---|---|
| ELISA | Soy protein | Sausage | 0,36 - 4,07% | 14 - 28% | L 06.00-56 |
| ELISA (Manuf. A) | Peanut | Milk chocolate | 5,9 - 174 mg/kg | 20 - 31% | L 00.00-69 |
| ELISA (Manuf. B) | Peanut | Milk chocolate | 10,1 - 216 mg/kg | 14 - 32% | L 00.00-69 |
| ELISA (Manuf. A) | Peanut | Dark chocolate | 5,7 - 148 mg/kg | 22 - 33% | L 00.00-69 |
| ELISA (Manuf. A) | Hazelnut | Dark chocolate | 1,6 - 16,3 mg/kg | 12 - 33% | L 44.00-7 |
| ELISA (Manuf. A) | Hazelnut | Dark chocolate | 2,4 - 21,3 mg/kg | 14 - 19% | L 44.00-7 |

From these precision data of the ASU §64 methods the calculated relative target standard deviations are in the range of 12 - 33%.

The IRMM (Institute for Reference Materials and Measurements) performed an interlaboratory comparison for five different ELISA-test kits for the quantification of peanut (16). The mean values for two matrices were in the concentration range of 0,3 - 16,1 mg/kg and 1,2 - 20,4 mg/kg, respectively. The lowest relative reproducibility standard deviations of the five test kits were for dark chocolate in the range of 20 - 42% and for cookies in the range of 23 – 61%.

A proficiency test (Japan, 2007) for the validation of ElISA-Test-Kits for the determination of crustacean-protein in processed products with 5 food samples have been carried out. The crustacean-protein-content was 8 – 12 mg/kg (22). Recovery rates ranged between 50 – 150%, while the relative reproducibility standard deviation was ≤ 25%. According to the authors the ELISA-Test-Kits fulfilled therefore the current validation criteria for ELISA methods (22).

*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 (6).
Criteria for the level of performance of analytical methods for the quantitative determination of allergens in foods with ELISA were recently elaborated by the Ministry of Health and Welfare (MHLW) in Japan (17), by the working group 12 „Food Allergens" of the technical committee CEN/TC 275 (18, 19) and by an international "Food Allergen Working Group" under the advice of the AOAC Presidential Task Force on Food Allergens (20).

Some of the relevant ELISA validation criteria of the three panels are listed below:

| **Literature** (17, 18, 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) |

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

Based on the currently achievable level of performance of ELISA 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 $\hat{\sigma}$ 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.

## 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 ( $\hat{\sigma}$ ) the result (x) of the participant is deviating from the assigned value (X) (6).
Participants' z-scores were derived as:

$$z = (x - X) / \hat{\sigma} \;;$$

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)*

## 3.6 Quotient $S^x/\hat{\sigma}$

Following the Horrat-value the results of a proficiency-test (PT) can be considered convincing, if the quotient of robust standard deviation and target standard deviation 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 (11).

## 3.7 Standard uncertainty

The assigned value X has a standard uncertainty $u_X$ that depends on the analytical method, differences between the analytical methods used, the test material, the number of participant laboratories and perhaps on other factors. The standard uncertainty $u_X$ for this PT is calculated as follows (6).

$$u_X = 1{,}25 * S^x / \sqrt{(p)}$$

If $u_X \leq 0{,}3 * \hat{\sigma}$ the standard uncertainty of the assigned value needs not to be included in the interpretation of the results of the PT (6). The Quotient $u_X/\hat{\sigma}$ is reported in the characteristics of the test.

## 3.8 Figures

The assigned values 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 (20).

# 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.

ELISA-Results given as crustacean or tropomyosin were converted to crustacean protein. When possible the information supplied by the test kit manufacturer was used. A protein content of 18,6% for crustacean was applied.

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 $X_{ALL}$ | z-Score $X_{Mi}$ | 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:

| | **All Results** *[mg/kg]* | **Method i** *[mg/kg]* |
|---|---|---|
| Assigned value | $X_{ALL}$ | $X_{Method\ i}$ |
| *Number of results* | | |
| Robust mean (X) | | |
| Robust standard deviation ($S^x$) | | |
| Median | | |
| *Target range:* | | |
| Target standard deviation ( $\hat{\sigma}$ ) | | |
| lower limit of target range (X - 2 $\hat{\sigma}$ ) | | |
| upper limit of target range (X + 2 $\hat{\sigma}$ ) | | |
| *Quotient $S^x/\hat{\sigma}$* | | |
| *Standard uncertainty $u_X$* | | |
| *Quotient* $u_X/\hat{\sigma}$ | | |
| *Number of results in the 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 crustaceae

## *4.1.1 ELISA-Results: crustacean protein*

**Qualitative valuation of results: Samples A and B**

| Participant | Result sample A | | result sample B | | Qualitative Valuation | Method | Remarks |
|---|---|---|---|---|---|---|---|
| | pos. / neg. | mg/kg | pos. / neg. | mg/kg | Übereinstimmung mit Konsenswerten | | |
| 1 | negativ | <0,05 | positiv | 0,85 | 2/2 (100%) | EL | * |
| 19 | negative | < 0,020 | positive | 7,2 | 2/2 (100%) | IL | * |
| 7 | negative | < LOQ | positive | 6,08 | 2/2 (100%) | NG | |
| 14 | negative | <2,5 | positive | 4,9 | 2/2 (100%) | NG | * |
| 3 | negative | < 0,2 | positive | 3 | 2/2 (100%) | RB | |
| 4 | negative | | positive | | 2/2 (100%) | RB | |
| 5 | negative | <0.5 | positive | 2,46 | 2/2 (100%) | RB | |
| 6 | negative | < 0,5 | positive | 3,45 | 2/2 (100%) | RB | |
| 9 | negative | < 0,172 | positive | 4 | 2/2 (100%) | RB | |
| 10 | - | / | positive | 1,7 | 1/1 (100%) | RB | |
| 15 | negative | < LOQ | positive | 2,86 | 2/2 (100%) | RB | |
| 18 | negative | <0,5 | positive | 2,54 | 2/2 (100%) | RB | |
| 21 | negative | | positive | 3,73 | 2/2 (100%) | RB | |
| 8 | negative | < LOQ | positive | 5,9 | 2/2 (100%) | RL | |
| 13 | negative | <0.02 | positive | 9,0 | 2/2 (100%) | RL | * |
| 2 | negative | < 0,001 | positive | 6,50 | 2/2 (100%) | TR | * |

| | Probe A | | Probe B | |
|---|---|---|---|---|
| number positive | 0 | | 16 | |
| number negative | 15 | | 0 | |
| procent positive | 0 | | 100 | |
| procent negative | 100 | | 0 | |
| consensus | negative | | positive | |

**** mean calculated by DLA***

**Methods:**

EL = ELISA Systems
IL = Immunolab
NG = Neogen, Veratox
RB = R-Biopharm, Ridascreen®
RL = RomerLabs, AgraQuant
TR = Transia (Nutri Linia)

*Comments:*

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

## Quantitative evaluation of results: Sample B

| Participants | Crustacean Protein | Z-Score $X_{ALL}$ | Z-Score $X_{RB}$ | Method | Remarks |
|---|---|---|---|---|---|
| | mg/kg | | | | |
| 1 | 0,85 | -3,2 | | EL | * |
| 19 | 7,2 | 2,9 | | IL | * |
| 7 | 6,08 | 1,8 | | NG | |
| 14 | 4,9 | 0,7 | | NG | * |
| 3 | 3 | -1,1 | 0,0 | RB | |
| 4 | | | | RB | |
| 5 | 2,46 | -1,7 | -0,7 | RB | |
| 6 | 3,45 | -0,7 | 0,7 | RB | |
| 9 | 4 | -0,2 | 1,4 | RB | |
| 10 | 1,7 | -2,4 | -1,7 | RB | |
| 15 | 2,86 | -1,3 | -0,1 | RB | |
| 18 | 2,54 | -1,6 | -0,6 | RB | |
| 21 | 3,73 | -0,4 | 1,0 | RB | |
| 8 | 5,9 | 1,6 | | RL | |
| 13 | 9 | 4,6 | | RL | Ourlier $X_{ALL}$, * |
| 2 | 6,5 | 2,2 | | TR | * |

**** Result calculated by DLA***

**Methods:**

EL = ELISA Systems
IL = Immunolab
NG = Neogen, Veratox
RB = R-Biopharm, Ridascreen®
RL = RomerLabs, AgraQuant
TR = Transia (Nutri Linia)

Characteristics: Quantitative evaluation crustacean protein

**Sample B**

| | **All Results** *[mg/kg]* | **Method RB** *[mg/kg]* |
|---|---|---|
| Assigned value | $X_{ALL}$ | $X_{Method\ RB}$ |
| *Number of results* | *15* | *8* |
| Robust mean (X) | 4,2 | 3,0 |
| Robust standard deviation ($S^x$) | 2,4 | 0,85 |
| Median | 3,7 | 2,9 |
| *Target range:* | | |
| Target standard deviation ( $\hat{\sigma}$ ) | 1,0 | 0,74 |
| lower limit of target range (X - 2 $\hat{\sigma}$ ) | 2,1 | 1,5 |
| upper limit of target range (X + 2 $\hat{\sigma}$ ) | 6,3 | 4,5 |
| *Quotient $S^x/\hat{\sigma}$* | *2,3* | *1,2* |
| *Standard uncertainty $u_X$* | *0,76* | *0,38* |
| *Quotient $u_X/\hat{\sigma}$* | *0,73* | *0,51* |
| *Number of results in the target range* | *10 (67%)* | *8 (100%)* |

**Method:**
RS = R-Biopharm, Ridascreen Fast® Crustacean

*Comments:*
*The evaluation of all methods and the evaluation of results from method RB showed a slightly increased or normal variability, respectively. For all results the quotient $S^x/\hat{\sigma}$ was 2,3 and for method RB below 2,0. The comparability of results is given.*

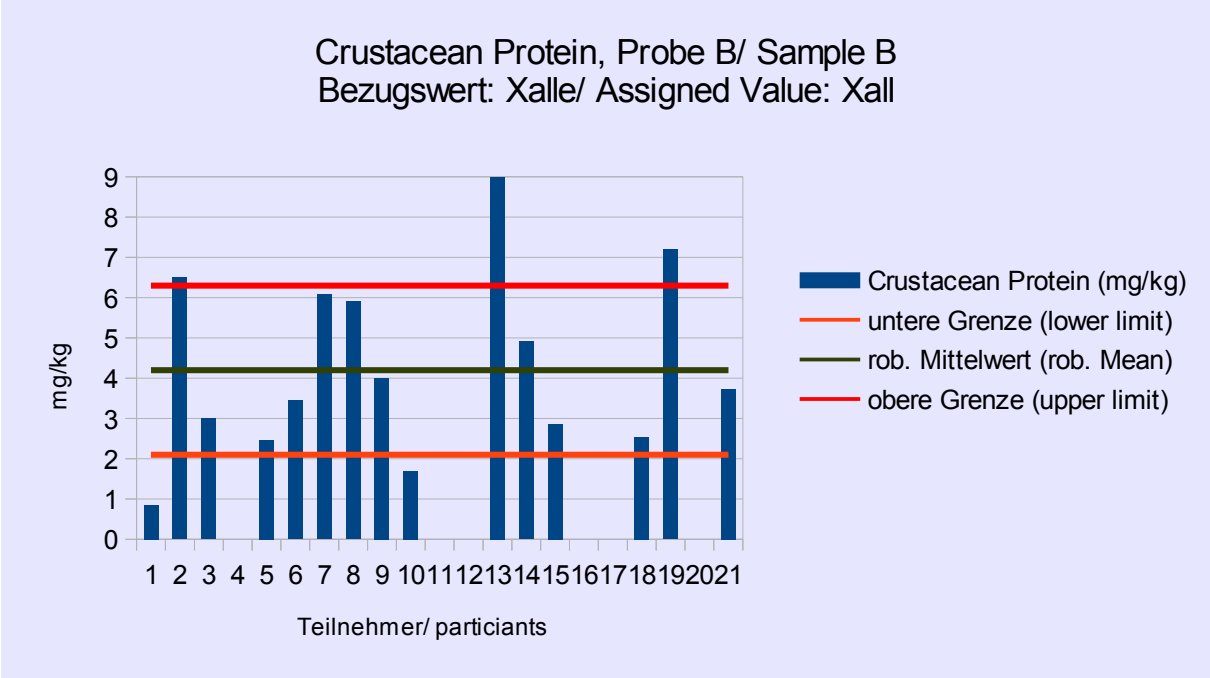


**Fig. 1:** ELISA-Results Crustacean protein, sample B.
Participant 1 = Method EL; 19 = Meth. IL; 7+14 = Meth. NG;
3-6,9,10,,15,18,21 = Meth. RB; 8+13 = Meth. RL; 2 = Meth. TR

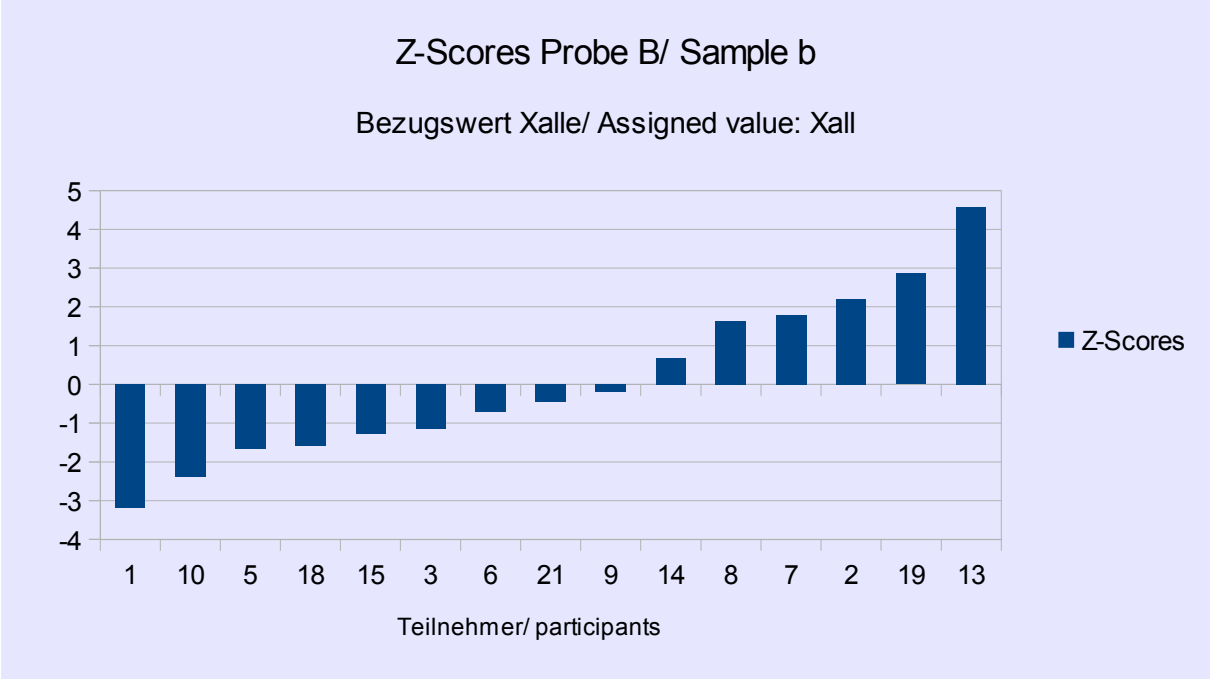


**Fig. 2:** z-Scores (ELISA-Results as crustacean protein) Assigned value robust mean of all results

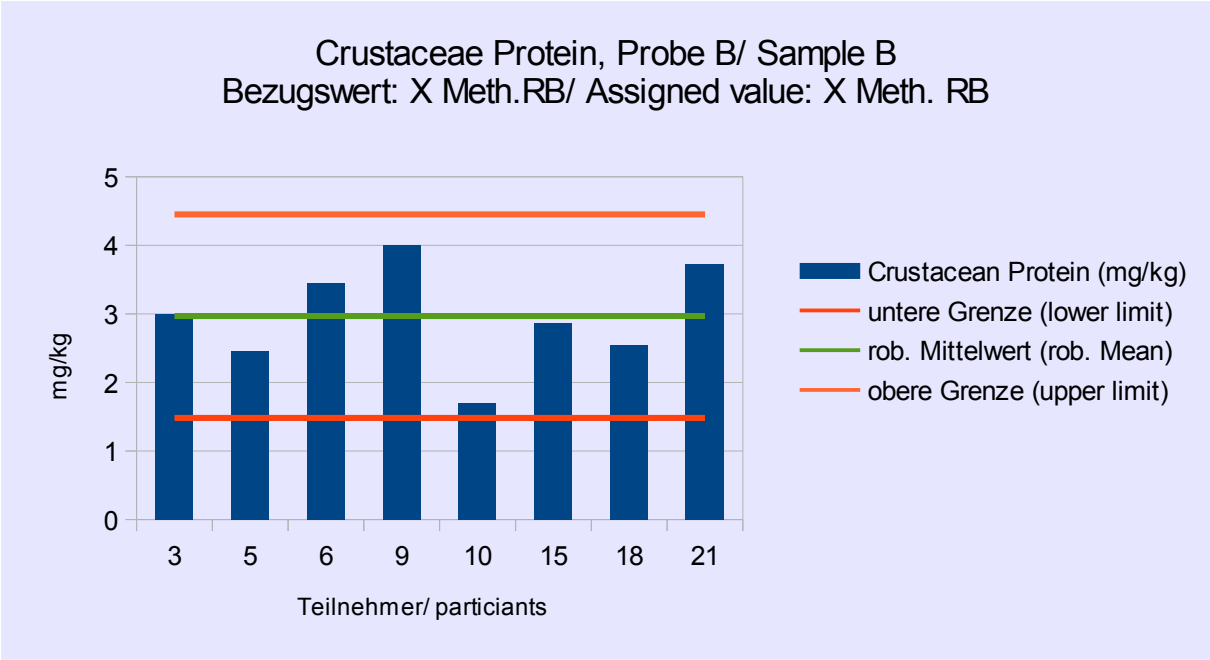


**Fig. 3:** ELISA-Results Crustacean protein, sample B, participants method RB (R-Biopharm, Ridascreen Fast)

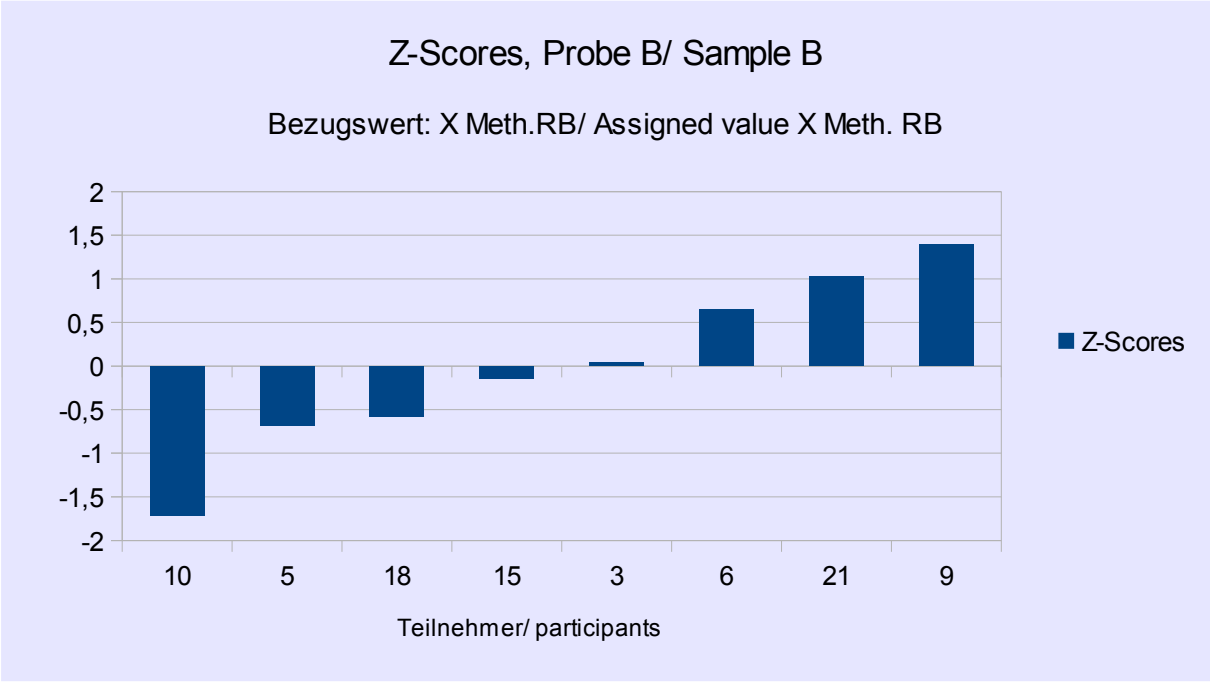


**Fig. 4:** z-Scores (ELISA-Results as crustacean protein) Assigned value robust mean of method RB (R-Biopharm, Ridascreen Fast)

***Recovery Rates for crustacean protein:***
***Spiking Material Sample and Sample B***

| Participant | Spiking material | Revorery | Sample B | Recovery | Method | Remarks |
|---|---|---|---|---|---|---|
| | mg/kg | % | mg/kg | % | | |
| 1 | 18,5 | 0,33 | 0,85 | 0,27 | EL | * |
| 10b | 0,065 | 0,001 | - | - | EL | |
| 19 | 120 | 2,15 | 7,2 | 2,31 | IL | * |
| 7 | > 2,28 | > 0,04 | 6,08 | 1,95 | NG | |
| 14 | 100,2 | 1,8 | 4,9 | 1,57 | NG | * |
| 3 | - | - | 3 | 0,96 | RB | |
| 4 | - | - | | | RB | |
| 5 | 56,79 | 1,02 | 2,46 | 0,79 | RB | |
| 6 | - | - | 3,45 | 1,11 | RB | |
| 9 | 94 | 1,68 | 4 | 1,28 | RB | |
| 10a | > 13,5 | > 0,24 | 1,7 | 0,54 | RB | |
| 15 | 73,8 | 1,32 | 2,86 | 0,92 | RB | |
| 18 | 116 | 2,08 | 2,54 | 0,81 | RB | |
| 21 | 11,92 | 0,21 | 3,73 | 1,2 | RB | |
| 8 | 104 | 1,86 | 5,9 | 1,89 | RL | |
| 13 | - | - | 9 | 2,88 | RL | Outlier $X_{ALL}$, * |
| 2 | - | - | 6,5 | 2,08 | TR | * |

| RA*[1] | 50 – 150 % | RA*[1] | 50 – 150 % |
|---|---|---|---|
| Number in RA | 0 | Number in RA | 0 |
| | | | |
| Percent in RA | 0 | Percent in RA | 0 |

**** Result calculated by DLA***
***[1] Range of acceptance to AOAC for allergen ELISAs**

**Methods:**

EL = ELISA Systems
IL = Immunolab
NG = Neogen, Veratox
RB = R-Biopharm, Ridascreen®
RL = RomerLabs, AgraQuant
TR = Transia (Nutri Linia)

*Comments:*

*For the spiking material sample and for sample B no participant obtained a recovery rate within the range of the AOAC-recommendation of 50-150%.*

*Taking **into account that the** results are performed by 6 different test kit producers from participants in different (European and not European) countries and that the results were obtained in a narrow range between 1,5 until 6,5 , it seems probable that the crustacean protein from the crab cream has been highly processed and significantly inactivated.*

### *4.1.2 PCR-Results: Crustacean-DNA*

| Participant | Sample A | | Sample B | | Qualitative Valuation | Method |
|---|---|---|---|---|---|---|
| | pos. / neg. | mg/kg | pos. / neg. | mg/kg | mg /kg | |
| 17 | negative | | positive | | 2/2 (100%) | 4L |
| 4 | negative | | positive | | 2/2 (100%) | Co |
| 9 | negative | <5 | positive | >5 | 2/2 (100%) | Co |
| 10 | negative | / | positive | / | 2/2 (100%) | Co |
| 16 | negative | ≤ 0,4 | positive | ≤ 0,4 | 2/2 (100%) | Co |
| 20 | negative | | positive | | 2/2 (100%) | Co |
| 11 | negative | | positive | | 2/2 (100%) | div |

| | Probe A | | Probe B |
|---|---|---|---|
| **Number positive** | **0** | | **7** |
| **Number negative** | **7** | | **0** |
| **Percent positive** | **0** | | **100** |
| **Percent negative** | **100** | | **0** |

**Methods:**
4L = 4LAB Diagnostics Srl
Co = Congen/ r-Biopharm, SureFood® Allergen Crustaceans
div = not indicated / other method

*Comments:*
*For the detection of Crustacean-DNA by PCR 100% positive results for sample B and 100% negative results for sample A were obtained.*

***Recovery Rates for Peanut:***
***(Spiking Material Sample and Sample B)***

*Because there were no quantitative results a statistical evaluation was not done.*

## 4.2 Proficiency Test Cashew nuts

### *4.2.1 ELISA-Results: Cashew nuts*

**Qualitative valuation of results: Samples A and B**

| Participant | Sample A | | Sample B | | Qualitative Valuation | Method | Remarks |
|---|---|---|---|---|---|---|---|
| | pos. / neg. | mg/kg | pos. / neg. | mg/kg | | | |
| 19 | negative | < 2 | positive | 12,5 | 2/2 (100%) | IL | |
| 1 | negative | <2 | positive | 880 | 2/2 (100%) | RL | |
| 5 | negative | <2 | positive | 339,58 | 2/2 (100%) | RL | |
| 7 | negative | < LOQ | positive | 2526 | 2/2 (100%) | RL | |
| 12 | negative | < 2.0 | positive | 1910 | 2/2 (100%) | RL | |
| 13 | negative | <2 | positive | >600 | 2/2 (100%) | RL | |

| | Probe A | | Probe B | |
|---|---|---|---|---|
| Number positive | 0 | | 6 | |
| Number negative | 6 | | 0 | |
| Percent positive | 0 | | 100 | |
| Percent negative | 100 | | 0 | |
| Consensus | negative | | positive | |

**Methods:**
IL = Immunolab RL = RomerLabs, AgraQuant

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

## Quantitative valuation of results: Sample B

| Participants | Cashew-nüsse | Z-Score $X_{ALL}$ | Method | Remarks |
|---|---|---|---|---|
| | mg/kg | | | |
| 19 | 12,5 | | IL | |
| 1 | 880 | | RL | |
| 5 | 339,58 | | RL | |
| 7 | 2526 | | RL | |
| 12 | 1910 | | RL | |
| 13 | >600 | | RL | |

**Methods:**
IL = Immunolab RL = RomerLabs, AgraQuant

Characteristics: Quantitative evaluation cashew nuts

**Sample B**

| | **All Results** *[mg/kg]* |
|---|---|
| Assigned value | $X_{ALL}$ |
| *Number of results* | *5* |
| Robust mean (X) | 1130 |
| Robust standard deviation ($S^x$) | 1200 |
| Median | 880 |
| *Target range:* | |
| Target standard deviation ( $\hat{\sigma}$ ) | |
| lower limit of target range (X - 2 $\hat{\sigma}$ ) | |
| upper limit of target range (X + 2 $\hat{\sigma}$ ) | |
| *Quotient $S^x/\hat{\sigma}$* | |
| *Standard uncertainty $u_X$* | |
| *Quotient* $u_X/\hat{\sigma}$ | |
| *Number of results in the target range* | |

Because of the low number of the quantitative results and a relatively high variance an statistical evaluation was not done.

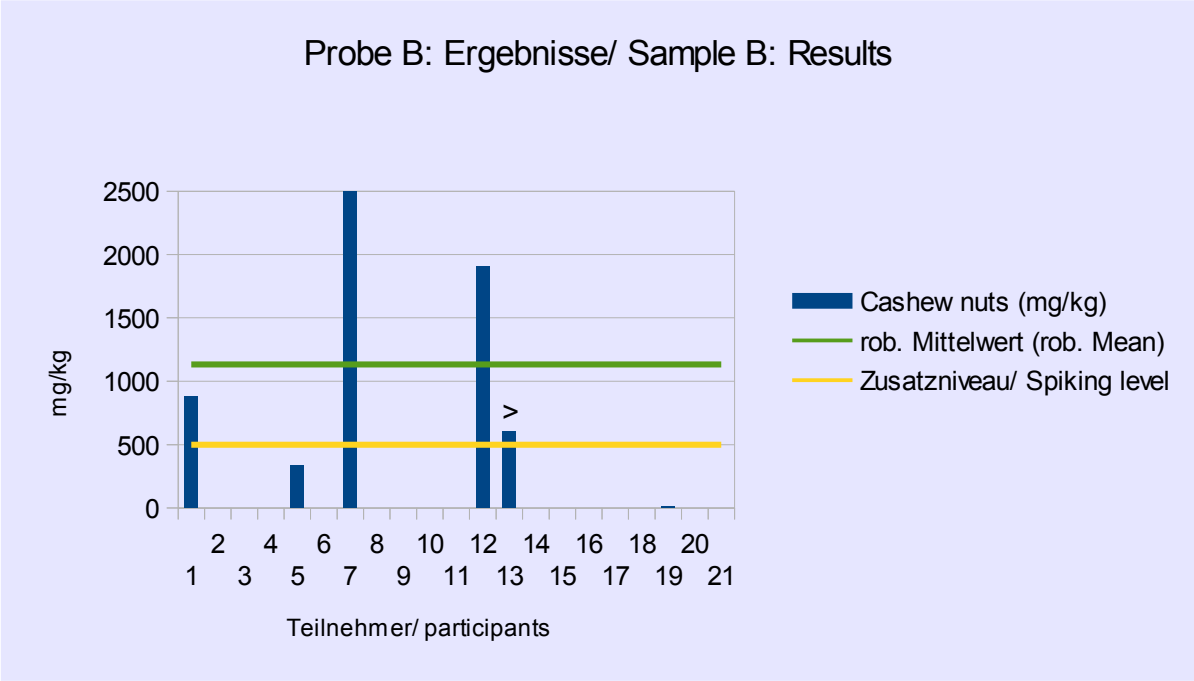


**Fig. 1:** ELISA-Results Cashew nuts, sample B.
Participant 19 = Method IL; 1+5+7+12+13 = Meth. RL.

***Recovery Rates for Cashew nuts:***
***Spiking Material Sample and Sample B***

| Participants | Spiking material | Recovery | Sample B | Recovery | Method | Remarks |
|---|---|---|---|---|---|---|
| | mg /kg | % | mg/kg | % | | |
| 19 | 56000 | 112 | 12,5 | 2,5 | IL | |
| 1 | 70000 | 140 | 880 | 176 | RL | |
| 5 | 32597 | 65,2 | 339,58 | 67,9 | RL | |
| 7 | >25 | (> 0,05) | 2526 | 505 | RL | |
| 12 | 275.000 | 550 | 1910 | 382 | RL | |
| 13 | | - | >600 | (> 120) | RL | |

| RA* | 50-150% | RA* | 50-150% |
|---|---|---|---|
| Number in RA | 3 | Number in RA | 1 |
| | | | |
| Percent in AB | 75 | Percent in RA | 20 |

*** Range of acceptance to AOAC for allergen ELISAs**

**Methods:**
AIL = Immunolab RL = RomerLabs, AgraQuant

*Comments:*
*For the spiking material sample 3 participants obtained a recovery rate within the range of the AOAC-recommendation of 50-150%. For sample B produced with the spiking material sample one of the recovery rates were in the range of acceptance.*

### *4.2.2 PCR-Results: Cashew nuts DNA*

| Participants | Sample A | Sample A | Sample B | Sample B | Qualtative Valuation | Mehod |
|---|---|---|---|---|---|---|
| | pos / neg | mg/kg | pos / neg | mg/kg | Übereinstimmung mit Konsenswert | |
| 10 | negative | / | positive | / | 2/2 (100%) | Co |
| 16 | negative | ≤ 0,4 | positive | ≤ 0,4 | 2/2 (100%) | Co |
| 1 | negative | - | positive | - | 2/2 (100%) | div |
| 9 | negative | <10 | positive | >10 | 2/2 (100%) | div |
| 11 | negative | | positive | | 2/2 (100%) | div |
| 20 | negative | | positive | | 2/2 (100%) | div |
| 14 | negative | | positive | | 2/2 (100%) | Ge |
| 3 | negative | | positive | | 2/2 (100%) | QG |

| | Sample A | | Sample B |
|---|---|---|---|
| Number positive | 0 | | 8 |
| Number negative | 8 | | 0 |
| Percent positive | 0 | | 100 |
| Percent negative | 100 | | 0 |
| Consensus | negative | | positive |

**Methods:**
Co = Congen/ r-Biopharm, Sure Food Allergen
div = not indicated / other method
Ge = Generon, Specialfinder Cashew
QG = Qiagen, Quanti Fast®

*Comments:*
*There were 100% positive results for sample B and 100% negative results for sample A by the PCR-methods. The results are in qualitative agreement with the spiking of sample B.*

**Quantitative valuation of results: Sample B**

*Because there were no quantitative results a statistical evaluation was not done.*

***Recovery Rates for Cashew nuts:***

*Because there were no quantitative results a statistical evaluation was not done.*

### *4.2.3 Further results: Cashew nuts*

| Participant | Sample A | | Sample B | | Spiking material | | Results given as |
|---|---|---|---|---|---|---|---|
| | pos. / neg. | mg/kg | pos. / neg. | mg/kg | pos. / neg. | mg /kg | e.g. food / protein |
| 8 | negative | - | positive | - | posistive | - | Protein |

Notes to Methods*:*
- *BioAvid Lateral Flow Test for Cashew nuts*
- 5g sample/45mL water (with 0.4g NaCl) -> extracted at 25 C° for 5 min.
- Remark: Spiking Sample estimated at >1000 mg/kg

# 5. Documentation

Details by the participants

## 5.1 ELISA: Crustacean protein

*Primary data*

| Participant | Result sample A | | Result sample B | | Result spiking sample | | Result given as | Meth. Abr. | Test-Kit or Literature |
|---|---|---|---|---|---|---|---|---|---|
| | pos. / neg. | mg/kg | pos. / neg. | mg/kg | pos. / neg. | mg/kg | e.g.food / protein | | |
| 1 | negative | <0,05 | positive | 0,17 | positive | 3,7 | Tropomyosin from Crustacean | EL | ELISA / ELISA Systems / Crustacean Tropomyosin Residue ESCRURD-48 |
| 10b | - | / | - | / | + | 0,065 ppm | Crustacea Protein | EL | ELISA / ELISA Systems / Crustacean Residue Assay (ESCRURD-48) |
| 19 | negative | < 0,020 | positive | 1,44 | positive | 24 | Tropomyosin Penaeus spp. | IL | Immunolab Crustacean ELISA |
| 7 | negative | < LOQ | positive | 6,075 | positive | >2.28 | Crustacea Protein | NG | Veratox for Crustacea Allergen, Neogen |
| 14 | negative | <2,5 | positive | 26,42 | positive | 538,5 | Crustacea | NG | Veratox for Crustacea Allergen |
| 3 | negative | < 0,2 | positive | 3 | | | Protein | RB | ELISA/r-biopharm/RIDASCREEN® Fast Crustacean |
| 4 | negative | | positive | | positive | | | RB | Ridascreen fast Crustacean |
| 5 | negative | <0.5 | positive | 2,46 | positive | 56,79 | Food | RB | R-Biopharm Ridascreen FAST Crustacean |
| 6 | negative | < 0,5 | positive | 3,45 | n.d. | n.d. | Crustacea Protein | RB | R-7302 R-biopharm /Batch 12184 |
| 9 | negative | < 0,172 | positive | 4 | positive | 94 | Protein | RB | RIDASCREEN®FAST Crustacean |
| 10a | - | / | + | 1.7ppm | + | >13.5ppm | Crustacea Protein | RB | Ridascreen Fast Crustaceans (R7302), r-Biopharm |
| 15 | negative | < LOQ | positive | 2,86 | positive | 73,8 | Protein | RB | r-Biopharm AG R 7302 Fast Crustacean |
| 18 | | <0,5 | | 2,54 | | 116 | Protein | RB | RIDASCREEN FAST Crustacean, Art. No:R7302 |
| 21 | negative | | positive | 3,73 | positive | 11,92 | Protein | RB | ELISA/ridascreen fast crustacean/R7302 |
| 8 | negative | < LOQ | positive | 5,9 | positive | 104 | Protein | RL | Romer AgraQuant Crustacea |
| 13 | negative | <0.02 | positive | 1,8 | | | Tropomyosin | RL | AgraQuant Crustacea |
| 2 | negative | < 0,001 | positive | 1,30 | | | Tropomyosin | TR | NutriLinia (Transia) |

**Methods:**

EL = ELISA Systems
IL = Immunolab
NG = Neogen, Veratox
RB = R-Biopharm, Ridascreen®
RL = RomerLabs, AgraQuant
TR = Transia (Nutri Linia)

*Other details to the Methods*

| Participant | Meth. Abr. | Remarks to the method (Extraction and determination) | further remarks |
|---|---|---|---|
| | | e.g. extraction solution / time / temperature | |
| 1 | EL | As per Kit Instructions | |
| 10b | EL | | |
| 19 | IL | Results in Tropomyosin could by means of the table of the added manual converted in the corresponding raw material. | |
| 12 | (NG) | | Not done. The kit that we use for the analysis (Neogen Biokits Shellfish assay kit 902076K) is no longer on the market. |
| 7 | NG | Extraction: PBS / 15 min at 30C in shaking waterbath / centrifugation<br>Determination: 4 parameter curve | |
| 14 | NG | According to Manual | |
| 3 | RB | Extraction and determination according to the manual/Antibody react specific with Crustacean protein. | |
| 4 | RB | 20 min sample preparation, 30 min test analysis, antibody specific to detect crustacean proteins | |
| 5 | RB | As per Kit Instructions | |
| 6 | RB | 10 Min. 60°C | |
| 9 | RB | | |
| 10a | RB | | |
| 15 | RB | According to the manual with extraction buffer | |
| 18 | RB | Allergen extraction buffer (1:10) / 10 minutes / 60°C | Detects particles in the samples. It shows not homogeneous |
| 21 | RB | included in the kit/as provided by the kit/specifity 100% | |
| 8 | RL | 1g sample/20mL diluted extraction buffer (from kit) -> extracted at 40C for 15min. | Sample B result based on 1/10 dilution; Spiking Sample result based on 1/100 dilution |
| 13 | RL | | |
| 2 | TR | | |

**Methods:**

EL = ELISA Systems
IL = Immunolab
NG = Neogen, Veratox
RB = R-Biopharm, Ridascreen®
RL = RomerLabs, AgraQuant
TR = Transia (Nutri Linia)

## 5.2 ELISA: Cashew nuts

*Primary data*

| Participant | Sample A | | Sample B | | Result spiking sample | | Result given as | Meth. Abr. | Test Kit or literature |
|---|---|---|---|---|---|---|---|---|---|
| | pos. / neg. | mg/kg | pos. / neg. | mg/kg | pos. / neg. | mg /kg | e.g. food / Protein | | |
| 19 | negative | < 2 | positive | 12,5 | positive | 56000 | total Cashew nuts | IL | Immunolab Cashew ELISA |
| 1 | negative | <2 | positive | 880 | positive | 70000 | Cashew nuts | RL | ELISA / Romer Labs / AgraQuant Cashew Assay COKAL 3148 |
| 5 | negative | <2 | positive | 339,58 | positive | 32597 | food | RL | Romer Labs Agraquant Cashew |
| 7 | negative | <LOQ | positive | 2526 | positive | >25 | total Cashew nuts | RL | AgraQuant FAST Cashew, Romer Labs |
| 12 | negative | < 2.0 | positive | 1910 | positive | 275.000 | Cashew nuts | RL | Agraquant Cashew test kit, Romer labs, Lot: CW 1001-1212, Exp.date 09 Nov 2013 |
| 13 | negative | <2 | positive | >600 | | | Cashew nuts | RL | AgraQuant Cashew Nut |

**Methoden:**
IL = Immunolab RL = Romerlabs, AgraQuant

*Other details to the methods*

| Participant | Meth. Abr. | Remarks for the Method (Extraction and determination) | further remarks |
|---|---|---|---|
| | | z.B. Extraction solution / time / temperature | |
| 19 | IL | | |
| 1 | RL | as per Kit instruction | |
| 5 | RL | as per Kit instruction | Large variation on results for cashew noted |
| 7 | RL | Extraction: Additives supplied by kit / 80-100C water, shaking for 15 sec / centrifugation , Determination: 4 parameter curve | |
| 12 | RL | Extraction buffer provided with the kit. 1g sample/20 ml preheated extraction buffer. Incubation 15 minutes. | The test kit had expired but a reference material that was analysed simoultaneoulsy showed results similar to when the test kit had not expired. |
| 13 | RL | | |

**Methods:**
IL = Immunolab RL = Romerlabs, AgraQuant

## *5.3 PCR: Crustacean DNA*

*Primary data*

| Participant | Sample A | | Sample B | | Spiking Sample | | Result given as | Meth. Abr. | Test Kit or Literature |
|---|---|---|---|---|---|---|---|---|---|
| | pos. / neg. | mg/kg | pos. / neg. | mg/kg | pos. / neg. | mg /kg | z.B.Lebensmittel / Protein | | |
| 17 | negative | | positive | | positive | | Crustacean DNA | 4L | 4LAB Diagnostics Srl |
| 4 | negative | | positive | | positive | | | Co | Surefood PREP Allergen, SureFood Allergen Crustaceans |
| 9 | negative | <5 | positive | >5 | positive | >5 | food | Co | SureFood® Allergen Crustaceans Real Time PCR |
| 10 | negative | / | positive | / | positive | / | Crustacean DNA | Co | Sure Food Allergen , Congen / r-Biopharm |
| 16 | negative | ≤ 0,4 | positive | ≤ 0,4 | positive | ≤ 0,4 | Crustacean DNA | Co | SureFood ALLERGEN Crustaceans, r-biopharm S3112 |
| 20 | negative | | positive | | positive | | | Co | Congen, SureFood Allergen Crustaceans |
| 11 | negative | | positive | | | | | div | Test Kit |

**Methods:**

4L = 4LAB Diagnostics Srl
Co = Congen/ r-Biopharm, SureFood® Allergen Crustaceans
div = not indicated / other method

*Other Remarks to the Methods*

| Participant | Meth. Abr. | Remarks to the method (Extraction and determination) | further remarks |
|---|---|---|---|
| | | | |
| 17 | 4L | Extraction DNA and real time PCR with probe | |
| 4 | Co | Real time PCR: Proteinase K, spin filter; Cycles 35, FAM-TAMRA Fluorescence Detection Setup | |
| 9 | Co | | |
| 10 | Co | Internal method | |
| 16 | Co | DNA extraction according to foodproof Magnetic Preparation Kit III, biotecon S40013L; detection according to kit r-biopharm kit | |
| 20 | Co | Wizard, Real Time PCR, 35 cycles | |
| 11 | div | RealtimePCR | LOD 5 gene copies |

**Methods:**

4L = 4LAB Diagnostics Srl
Co = Congen/ r-Biopharm, SureFood® Allergen Crustaceans
div = not indicated / other method

## *5.4 PCR: Cashew nuts DNA*

*Primary data*

| Participant | Sample A | | Sample B | | Spiking Sample | | Result given as | Meth. Abr. | Test Kit or Literature |
|---|---|---|---|---|---|---|---|---|---|
| | pos. / neg. | mg/kg | pos. / neg. | mg/kg | pos. / neg. | mg /kg | e.g.food / protein | | Methode / Anbieter / Artikel |
| 10 | - | / | + | / | + | / | Cashew nut DNA | Co | Sure Food Allergen , Congen / r-Biopharm |
| 16 | negative | ≤ 0,4 | positive | ≤ 0,4 | positive | ≤ 0,4 | Cashew nut DNA | Co | SureFood ALLERGEN Cashew, r-biopharm S3115 |
| 1 | negative | - | positive | - | positive | - | | div | End-point PCR / intern method/ - |
| 9 | negative | <10 | positive | >10 | positive | >10 | food | div | „Detection of cashew nut DNA in spiked baked goods using a Real-Time Polymerase Chain Reaction method", J.L. Brzezenski, Journal of AOAC International Vol. 89. No. 4, 2006, 1035-1038. |
| 11 | negative | | positive | | | | | div | Literature |
| 20 | negative | | positive | | negative | | | div | Food Anal. Methods (2008) 1:136-143 DOI 10.1007/s12161-008-9023-6 „Detection of Cashew Nut in Foods by a Specific Real-time PCR Method", Alexandra Ehlert & Christine Hupfer & Anja Demmel & Karl-Heinz Engel & Ulrich Busch |
| 14 | negative | | positive | | positive | | Cashew nut DNA | Ge | SPECIALfinder Cashew Assay-Generon |
| 3 | negative | | positive | | | | Cashew nut Gen Ana 03 | QG | 5xQuantiFast® Pathogen PCR Fa.Qiagen Primer/Sonde: eurofins/mwg/ operon. method according to Ehlert et al 2008 |

**Methods:**
Co = Congen/ r-Biopharm, Sure Food Allergen
div = not indicated / other method
Ge = Generon, Specialfinder Cashew
QG = Qiagen, Quanti Fast®

*Other Remarks to the Methods*

| Participant | Meth. Abr. | Remarks to the method (Extraction and determination) | Further Remarks |
|---|---|---|---|
| | | | |
| 10 | Co | Internal method | |
| 16 | Co | DNA Extraction according to „Foodproof Magnetic Preparation" Kit III, biotecon S40013L; Detection according to Test-Kit r-biopharm | |
| 1 | div | CTAB Lyse / Proteinase K /Promega Wizard / End-point PCR 45 cycles / Agarose Gel electrophoresis | |
| 9 | div | | |
| 11 | div | Real time PCR | LOQ 5 Gen copies |
| 20 | div | Wizard, Real Time PCR, 45 cycles | |
| 14 | Ge | Real time PCR | |
| 3 | QG | Extraction: Dneasy Rmericon Food Kit/ Proteinase K/ Real Time PCR/ 45 cycles | |

**Methods:**
Co = Congen/ r-Biopharm, Sure Food Allergen
div = not indicated / other method
Ge = Generon, Specialfinder Cashew
QG = Qiagen, Quanti Fast®

## 6. Index of participant laboratories

| Teilnehmer/ participant | Ort/ town | Land/ country |
|---|---|---|
| | | Spain |
| | | Spain |
| | | Germany |
| | | France |
| | | Canada |
| | | Cyprus |
| | | Germany |
| | | Italy |
| | | Germany |
| | | Italy |
| | | Italy |
| | | Italy |
| | | Germany |
| | | Sweden |
| | | ENGLAND |
| | | Germany |
| | | Germany |
| | | ENGLAND |
| | | Sweden |
| | | Netherlands |
| | | Canada |
| | | |

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

# 7. Index of references


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