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# Elaboration and validation of the method for the quantification of the emetic toxin of *Bacillus cereus* as described in EN-ISO 18465 - Microbiology of the food chain – Quantitative determination of emetic toxin (cereulide) using LC-MS/MS

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ABSTRACT

A method for the quantification of the *Bacillus cereus* emetic toxin (cereulide) was developed and validated. The method principle is based on LC-MS as this is the most sensitive and specific method for cereulide. Therefore the study design is different from the microbiological methods validated under this mandate. As the method had to be developed a two stage validation study approach was used. The first stage (pre-study) focussed on the method applicability and the experience of the laboratories with the method. Based on the outcome of the pre-study and comments received during voting at CEN and ISO level a final method was agreed to be used for the second stage the (final) validation of the method. In the final (validation) study samples of cooked rice (both artificially contaminated with cereulide or contaminated with *B. cereus* for production of cereulide in the rice) and 6 other food matrices (fried rice dish, cream pastry with chocolate, hotdog sausage, mini pancakes, vanilla custard and infant formula) were used. All these samples were spiked by the participating laboratories using standard solutions of cereulide supplied by the organising laboratory. The results of the study indicate that the method is fit for purpose. Repeatability values were obtained of 0.6 µg/kg at low level spike (ca. 5 µg/kg) and 7 to 9.6 µg/kg at high level spike (ca. 75 µg/kg). Reproducibility at low spike level ranged from 0.6 to 0.9 µg/kg and from 8.7 to 14.5 µg/kg at high spike level. Recovery from the spiked samples ranged between 96.5% for mini-pancakes to 99.3% for fries rice dish.

## 1. Introduction

*Bacillus cereus* is a food-borne pathogen that causes borne illness by the production of toxins (Kotiranta et al., 2000). The *B. cereus* strains producing cereulide or diarrheal toxins belong to different phylogenetic groups within *B. cereus* (Guinebretière et al., 2008; Ceuppens et al., 2011). There are various diarrheal toxins (NHE, HBL, Cytotoxin-K) that differ in toxicity. The emetic toxin, called cereulide, is a small cyclic peptide which demonstrates high resistance to temperature and pH (Rajkovic et al., 2008). In that respect *B. cereus* is also unique in the fact that both the organism (by spore formation) and toxin are heat stable. The toxin is even more heat resistant than the *B. cereus* spores.

Several cases of foodborne outbreaks linked to cereulide have been reported (Duc et al., 2005; Essen et al., 2000; Pirhonen et al., 2005). In few cases the intoxication can also lead to the death of the patient (e.g. Dierick et al., 2005).

Several methods have been developed for the detection and/or quantification of cereulide. Some of these methods are nonspecific bioassays based on the boar semen motility assay or Hep-2 cell assay (Andersson et al., 1998, 2004; Hughes et al., 1988) and other methods are specifically based on the chemical analysis using liquid chromatography with mass spectrometry (LC-MS/MS) for the detection and quantification of the toxin (Bauer et al., 2010; Biesta-Peters et al., 2010; Häggblom et al., 2002; Jääskeläinen et al., 2003). The chemical methods are more suitable for quantification and more specific for cereulide, and have therefore been chosen as the starting point for standardization of a method for the quantification of cereulide. The method described by Biesta-Peters et al. (2010) was used as a basis. The quantification of the toxin was improved by the use of synthetic ($^{13}C_6$ labelled) cereulide instead of using valinomycine as a substitute for

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cereulide as described previously by Häggblom et al. (2002). Recently, research has been done for the chemodiversity of cereulide. At least 18 cereulide variants were detected by UHPLC-TOFMS (Ultra High Performance Liquid Chromatography - Time Of Flight Mass Spectrometry) and ion-trap MSn sequencing, amongst which the previously unknown isocereulides A–G (Marxen et al., 2015).

The European Union, represented by the European Commission, has decided to award a grant for the elaboration of 15 published European Validated Standards (EN) in the field of food hygiene legislation under mandate SA/CEN/ENTR/381/2010-06 Microbiology of Food and animals feeding stuffs (Mandate M/381) (Anon., 2010). The elaboration of the standards was performed by sub-contractors under the mandate M/381, in which The Netherlands Food and Consumer Product Safety Authority (NVWA) was the appointed subcontractor to perform validation work on the standard for quantitative determination of emetic toxin (cereulide) using LC-MS/MS. In order to validate the standards the subcontractors prepared protocols for collaborative studies, prepared samples for the collaborative studies organized amongst interested laboratories, collected and evaluated the results from the collaborative studies and prepared draft European Standards based on scientifically sound and validated methods of analysis.

The validation study was done using different matrices (cooked rice, fried rice dish, cream pastry with chocolate, hotdog sausage, mini pancakes, vanilla custard, and infant formula). Two types of cooked rice samples were used, one that was spiked with cereulide and one that was spiked with *B. cereus* in order to produce cereulide under "natural" conditions. As the method was also under development two inter-laboratory studies were conducted. These studies were carried out in 2012 and 2013.

## 2. Materials and methods

### 2.1. Design of the studies

A pre-study was organized in May 2012 to evaluate the proposed method. The proposed method was based on the internal protocol as used by the project leader of this CEN mandate. The internal protocol was already validated within the laboratory. The participants were supplied with the following standards: cereulide standard (123.8 ng/ml acetonitrile), $^{13}C_6$ Cereulide (internal) standard (103 µg/ml methanol), and standard with unknown concentration to the participants. In addition the following food samples were supplied: cooked rice (samples spiked with cereulide and samples spiked with *B. cereus*), fried rice dish, cream pastry with chocolate, hotdog sausage, mini pancakes and vanilla custard. The food samples were spiked (at 0, 5, 35 and 75 µg/kg) using the supplied standard solutions in each individual laboratory. These samples were tested in duplicate (starting from weighing of the sample). Cooked rice artificially contaminated was supplied at three contamination levels and needed no additional spiking by the participants. The rice samples were tested in 6-fold from weighing of the sample. From the cereulide standard a dilution series was prepared to be used for calibration, the concentration used varied between 0.08 and 8.25 ng/ml. Labelled cereulide was added to all of the weighted sample as an internal standard at 1.72 ng/ml.

The (final) validation study was carried between beginning of September and mid-October 2013. For this a revised protocol was used based on the findings of the Pre-Trail and also comments received on the Committee Draft (CD) of EN-ISO 14685. The same batches of food were tested as in the pre-study and infant formula was added as an additional food matrix. Spiking conditions and number of samples tested were as previously described.

### 2.2. Method under collaborative trail

For the pre-study the participants were asked to use their own equipment and operate the MS system after tuning. The starting conditions were supplied by the NVWA based on their equipment settings. For the HPLC part of the method the conditions used are presented in Table 1. Most sample extracts can be analysed using an isocratic method. When a sample contains a lot of egg, components of the egg will be present in the sample extract. These components have longer elution times and may disturb the subsequent analyses. Therefore a gradient elution method was proposed to be used for samples containing eggs. The conditions used for isocratic and gradient elution are also presented in Table 1. For the MS the general conditions given were as described in Table 2.

The method used for the final validation study was based on the CD version of the ISO 18465. In line with the pre-study the use of LC and MS equipment were left open to the laboratory for the (final) validation study. In Table 3 a summary is presented of the conditions that were fixed and that were left open for the validation study.

The MS parameters vary depending on the instrument/manufacturer and shall be obtained by tuning the instrument before analysis.

**Table 1**
LC (isocratic and gradient elution) Conditions for the quantification of cereulide used in the pre-study.

| LC conditions | | | |
|---|---|---|---|
| Column | Supelco Discovery RP-C18, 100 mm * 2.1 mm, size 5 µm | | |
| Mobile phase | A: 100% Acetonitril (LCMS grade)<br>B: 10 mMol Ammonium formiate 1% trifluoroacetic acid (TFA)-solution in water | | |
| Time (min) | Flow (ml/min) | A (%) | B (%) |
| Isocratic elution conditions | | | |
| 0.00 | 0.200 | 10 | 90 |
| 16 | 0.200 | 10 | 90 |
| Gradient elution conditions | | | |
| 0.00 | 0.200 | 10 | 90 |
| 12.00 | 0.200 | 10 | 90 |
| 14.00 | 0.200 | 0 | 100 |
| 30.00 | 0.200 | 0 | 100 |
| 35.00 | 0.200 | 10 | 90 |
| 40.00 | 0.200 | 10 | 90 |
| Injection volume | 20 µl | | |
| Concentration range[a] | 0.08–8.25 ng/ml (0.4 pg–40 pg) | | |

[a] Cereulide concentration range for which the method can accurately measure the concentration without further concentration or dilution.

**Table 2**
General MS conditions for the quantification of cereulide suggested to be used in the pre-study.

| Ionisation technic | ESI (electrospray ionisation), positive |
|---|---|
| MS run time | 14 min |
| Delay | 0 min |
| Divert valve | 0–3 min to waste; 3–13 min to source, 13–14 min to waste |
| Scan area MS and MS/MS | 1000.00–1200.00 *m/z* |
| Probe-position | 3D |
| Datatype | Centroid |
| Parent-ion $^{13}C_6$ labelled Cereulide – Internal Standard ($[M + NH4^+]^+$) | 1176.7 (quanticiation, screen area ± 0.5 m/z) |
| Parent-ion Cereulide ($[M + NH4^+]^+$) | 1170.7 (quanticiation, screen area ± 0.5 *m/z*) |
| Capillary temperature | 350 °C |
| Sheath gas | 30 |
| Aux/sweep | 4 |
| Source voltage kV | 5.30 |
| Source current µA | 80.00 |
| Capillary voltage V | 48.00 |
| Tube lens offset V | 31.00 |

**Table 3**
LC-MS conditions that were fixed and left open for the participants for conducting the quantification of cereulide in the final validation study.

| Fixed conditions | Left open conditions |
|---|---|
| - Extraction fluid (acetonitrile)<br>- Column RP-18<br>- Use of internal standard $^{13}C_6$-Cereulide.<br>- Eluents composition (Mobile phase A: 10 mMol Ammoniumformate with 0.1% Formic acid in water; Mobile phase B: Acetonitrile with 0.1% Formic acid)<br>- Use of glass tools | - Heptane clean-up<br>- Column dimensions<br>- UPLC or HPLC<br>- Transitions<br>- Isocratic or gradient elution<br>- Filtration or centrifugation extracts<br>- Type MS (Quadrupole/ Q-trap/Ion-Trap)<br>- Flow rate LC<br>- Injection Volume |

**Table 4**
General MS conditions and tuning parameters (based on a Waters® Micromass®4 Quattro Premier) for the quantification of cereulide as presented in ISO 18465.

| Ionisation | ESI + |
|---|---|
| Capillary voltage | 3.5 kV |
| Cone voltage | 65 V |
| Extractor | 5 V |
| RF lens | 0 V |
| Source temp. | 120 °C |
| Desolvation temp | 500 °C |
| Desolvation gas flow | 1200 (l/h) |
| Cone gas flow | 100 (l/h) |
| LM Resolution 1 | 15.0 |
| HM Resolution 1 | 15.0 |
| Ion energy 1 | 0.5 |
| Entrance | 0 |
| Collision | 74 |
| Exit | 1.0 |
| LM Resolution 2 | 15.0 |
| HM Resolution 2 | 15.0 |
| Ion energy 2 | 2.0 |
| Multiplier | 650 |
| API gas | On |
| Col gas | On |
| Mass Range calibration | 50–1300 m/z |

**Table 5**
Results of cereulide levels obtained and the variation from the pre-study data in *B. cereus* and cereulide spiked cooked rice samples (mean of six samples per laboratory).

| | *B. cereus* spiked (µg/kg) | Cereulide spiked (µg/kg) |
|---|---|---|
| Lab-1 | 24.72 ± 1.06[a] | 10.70 ± 0.64[a] |
| Lab-2 | 29.82 ± 1.00 | 12.57 ± 0.28 |
| Lab-3 | 21.67 ± 0.69 | 9.71 ± 0.42 |
| Lab-4 | 27.48 ± 0.79 | 11.22 ± 0.23 |
| Lab-5 | 27.99 ± 1.22 | 12.08 ± 0.93 |
| Lab-6 | 27.38 ± 0.48 | 11.86 ± 1.09 |
| Lab-7 | 26.31 ± 0.85 | 11.26 ± 0.64 |
| Mean | 26.48 | 11.34 |
| $s_R$[b] | 2.75 | 1.13 |
| $RSD_R$[c] | 10.40 | 9.98 |

[a] Standard deviation of repeatability (within a laboratory).
[b] $s_R$ = standard deviation of reproducibility (over all laboratories).
[c] $RSD_R$ = relative standard deviation.

The participants were supplied with a protocol with conditions for testing based on a Waters® Micromass®4 Quattro Premier system. The MS conditions for this system are presented in Table 4.

Cereulide was quantified based on the fragments (daughters) produced in the MS system of the parent ion (being the $NH_4^+$ adduct of cereulide with molecular mass of 1170.7). The commonly found most abundant daughters were of molecular mass of 314.4 and 499.4. Other daughter could be used as well, a list of possible daughters is presented in the standard 18,465 (Anon., 2016). The established daughters with the highest abundance depend on the extent of the applied collision energy and MS settings.

### 2.3. Preparation of test materials

The standards for synthetic cereulide (CXP080113-058) and for synthetic $^{13}C_6$-labelled cereulide (CXP120105) were produced by Chiralix (The Netherlands) as described in Biesta-Peters et al. (2010). The cereulide standard was dissolved in acetonitrile and for the labelled cereulide in methanol, both standards were stored at −18 °C.

Rice samples spiked with *B. cereus* and incubated for production of cereulide (so called *B. cereus* spiked samples) were obtained as follows. *Bacillus cereus* F4810/72 was grown overnight at 30 °C in BHI. A diluted suspension from this culture was prepared at approximately 1000 cfu/ml. Each of 10 portions of 100 g of cooked rice was spiked with 2.5 ml of the dilution. The inoculum was homogenised in the rice using a stomacher bag and mixing it by hand. Each bag was incubated at 21 °C for 32 h. After incubation the samples were mixed manually and homogenised using liquid nitrogen (cryogenic homogenisation). This process was repeated three times. After homogenisation the samples were packed in small portions (ca 10 g) and stored at −18 °C.

To obtain cereulide spiked rice samples, rice was cooked and spiked with the cereulide standard to obtain the desired concentrations.

Afterwards the spiked rice was mixed manually and homogenised in three steps using liquid nitrogen (cryogenic homogenisation). After homogenisation the samples were packed in small portions (ca 10 g) and stored at −18 °C.

The other food samples used in the study were fried rice dish, cream pastry with chocolate, hotdog sausage, mini pancakes, vanilla custard and infant formula (this last matrix was only used in the final validation study). The food samples came from local retailers and were from a single batch of the same product. Samples were homogenised before storage by using liquid nitrogen (cryogenic homogenisation). The obtained powders were stored at −18 °C until use.

Homogeneity of samples was only analysed for the *B. cereus* spiked rice by checking 10 samples in duplicate using the LC-MS/MS method as described. The results were analysed using Analysis of Variance (ANOVA). The stability of the spiked cooked rice samples was determined by analysing several samples in duplicate between June 2011 and November 2013 using the LC-MS/MS method as described. The results were analysed, like the homogeneity of the samples, using Analysis of Variance (ANOVA).

### 2.4. Statistical analysis of the data

The pre-trail data were summarised per matrix, spiking level and laboratory. The mean, standard deviation ($s$) and relative standard deviation (RSD) were calculated for this using MS-Excel®.

The data of the final validation study were evaluated using Excel® ANOVA with single factor (concentration or recovery of cereulide). After elimination of outlying results the remaining data were used to calculate the following parameters per matrix: Residual Standard Deviation ($RSD_r$ per lab and for all labs), Repeatability (r per lab and for all labs), Reproducibility (R), Limit of Detection (L.O.D.), Limit of Quantification (L.O.Q.), Linearity (Correlation Coefficient) and Recovery. The L.O.D. is calculated as the mean of the blank samples plus 3,3 times the standard deviation of the blank samples. The L.O.Q. is calculated as 2 times the L.O.D.

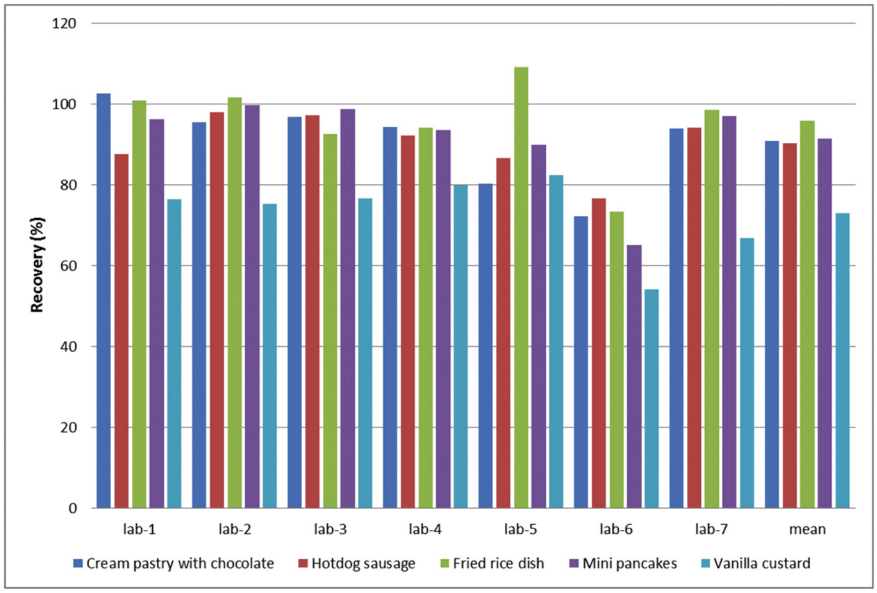


**Fig. 1.** Recovery of cereulide (%) per laboratory and per matrix.

**Table 6**
Overview of the LC-MS equipment used by the participants in the validation study.

| Lab | MS-system | | | LC-system | |
|---|---|---|---|---|---|
| | Instrument | Type | Software | Instrument | Type |
| 1 | Waters | Quattro Premier | Masslynx | Waters | Alliance 2696 |
| 2 | AB Sciex | API 3200 (QqQ) | Analyst | Agilent | Agilent 1200 series |
| 3 | Waters | Xevo TQ-S | Masslynx | Waters | Acquity UPLC CI.1 |
| 4 | Waters | Xevo TQ-S | Masslynx | Waters | Acquity |
| 5 | Agilent | 6490 Triple Quad | Mass Hunter | Agilent | Agilent 1290 Infinity |
| 6 | Waters | Xevo TQ-S | Masslynx | Waters | Acquity UPLC |
| 7 | Waters | Quattro Ultima | Masslynx | Waters | Acquity |
| 8 | AB Sciex | AB Sciex 5500 | Analyst | Waters | Acquity UPLC |
| 9 | Waters | Xevo TQ-S | Masslynx | Waters | Acquity SDS |
| 10 | Waters | Xevo TQ-S | Masslynx | Waters | Acquity I-Class |

## 3. Results and discussion

### 3.1. Stability and homogeneity of the samples

As this study was not targeting a micro-organism itself but a metabolite of it (cereulide), it was not possible to apply the general document drafted for carrying out stability and homogeneity studies. Cereulide is known to be very stable even under extreme temperature conditions, including factors like pH and presence of fats (Rajkovic et al., 2008). All matrices, except for the infant formula, were homogenised by milling them using liquid nitrogen. The obtained powder were regarded as homogenous as for the powdered infant formula. No further checks were done. The homogeneity of samples was only checked for the *B. cereus* spiked rice by checking 10 samples in duplicate and it was concluded to be homogeneous (data not shown).

Stability of the samples focussed on the stability of the spiked cooked rice matrix. The results of the samples obtained between June 2011 and November 2013 were used to check the level of cereulide in the samples and therewith the stability. Results indicated that the level of cereulide in the samples remained stable over the period tested.

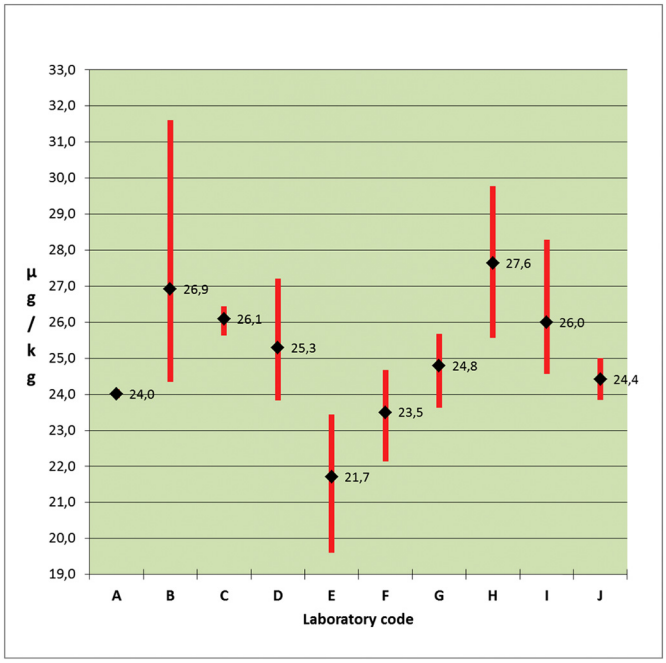


**Fig. 2.** Variation of results between and within laboratories for *B. cereus* spiked rice sample A (after elimination of data from 1 laboratory).

The matrices used for both the pre-validation and the validation study were boxed and shipped on dry ice to guarantee stability.

### 3.2. Results of the pre-study

Seven laboratories participated in the pre-study. The measured concentration of cereulide in the foods and topics related to the method and the protocol for conducting the study were discussed with the participants during a meeting held in October 2012. A summary of the results is presented in Table 5 (recovery from the spiked food samples) and Fig. 1 (concentration in cereulide and *B. cereus* spiked cooked rice).

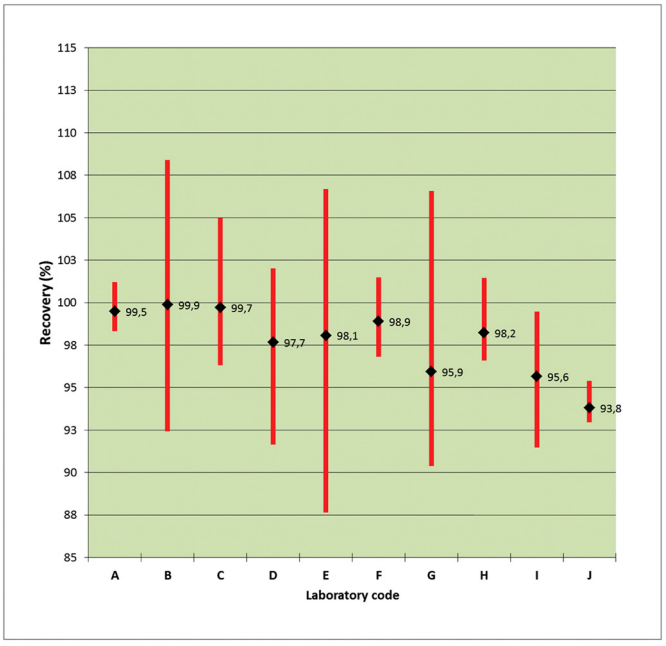


**Fig. 3.** Variation of results between and within laboratories for cereulide in hot dog sausage (after elimination of data from 1 laboratory).

### 3.3. Results of the interlaboratory study

Eleven laboratories participated in the final validation study. The results were discussed in a meeting with the participant in December 2013. In Table 6 an overview is given of the equipment used by the participants.

The evaluation of the data indicated that one laboratory had consistently lower results than the other labs and their results for all food matrices were outside the confidence interval at 99% according to the Youden plot (data not shown). During the technical discussion on the results of that laboratory it was noticed that it had problems with the stability of the MS-system even though an exact explanation for this could not be found. It was decided not to use the data from this laboratory in the calculation of the performance characteristics of the method. So for the final calculations the results from 10 laboratories remained.

Fig. 2 presents an overview of the variation in results within and between laboratories for *B. cereus* spiked rice, Fig. 3 presents the results for hot dog sausage. The L.O.Q. in the samples was, except for one lab, below 1 μg/kg. The linearity (regression coefficient) of the calibration curve was in all laboratories higher than 0.9985.

### 3.4. Performance assessment

The calculated performance characteristics of the method are presented in Table 7 for the cereulide and *B. cereus* spiked cooked rice samples and in Table 8 for the other food samples.

The repeatability limit ranged between 9.5% (fried rice dish) and 14% (*B. cereus* spiked cooked rice at low level). The reproducibility limits ranged between 12.0% (hot dog sausage) and 23.3% (*B. cereus* spiked cooked rice at low level). No systematic differences could be observed in the repeatability and reproducibility limits between the food samples spiked by the participants and the (*B. cereus* or cereulide) spiked cooked rice samples. The recovery from the spiked food samples varied between 96.5% for mini-pancakes to 99.3% for fried rice dish. This was calculated on the duplicate results at three levels of contamination.

## 4. Conclusion

From the validation study it can be concluded that the method is validated for a broad range of foods (in total 7 different matrices were included in the study). Most of the matrices contained starch in the products as starch rich products are frequently found in cases of food poisoning. The method is quite flexible with respect to the use of equipment needed as can be seen in the validation study as well. This can lead to differences in sensitivity of the method but in most cases the L.O.Q. of the method was below 1 μg/kg in the samples.

**Table 7**
Performance characteristics of the method for cereulide quantification in naturally and spiked cooked rice samples.

| Matrix | *B. cereus* spiked[a] | | Cereulide spiked |
|---|---|---|---|
| | (25.0 μg/kg) | (78.1 μg/kg) | (22.5 μg/kg) |
| Number of participating collaborators | 11 | 11 | 11 |
| Number of collaborators retained after evaluation of the data | 10 | 10 | 10 |
| Number of samples | 6 | 6 | 6 |
| Number of samples retained after evaluation of the data | 6 | 6[b] | 6 |
| Mean value $\bar{x}$ (μg/kg) | 25.0 | 78.1 | 22.5 |
| Repeatability standard deviation $s_r$ (μg/kg) | 1.2 | 2.9 | 0.8 |
| Coefficient of variation of repeatability $C_{V,r}$ (%) | 5 | 3.7 | 3.4 |
| Repeatability limit[c] $r$: ($r = 2.8 \times s_r$), (μg/kg) | 3.5 | 8.0 | 2.2 |
| Repeatability limit[c] $r$: ($r = 2.8 \times C_{V,r}$), (%) | 14 | 10.3 | 9.6 |
| Reproducibility standard deviation $s_R$ (μg/kg) | 2.1 | 4.7 | 1.0 |
| Coefficient of variation of reproducibility $C_{V,R}$ (%) | 8.3 | 6.0 | 4.4 |
| Reproducibility limit[d] $R$: ($R = 2.8 \times s_R$), (μg/kg) | 5.8 | 13.1 | 2.8 |
| Reproducibility limit[d] $R$: ($R = 2.8 \times C_{V,R}$), (%) | 23.3 | 16.7 | 12.4 |
| Recovery (%) | – | – | – |

[a] The *B. cereus* spiked cooked rice was obtained by inoculating *B. cereus* in the rice and incubating the rice for the production of cereulide under "natural" conditions.

[b] One of the participants reported 5 results instead of 6.

[c] The absolute difference between two single test results found on identical test materials by one operator using the same apparatus within the shortest feasible time interval will exceed the repeatability limit $r$ in not more than 5% of the cases.

[d] The absolute difference between two single test results found on identical test materials reported by two laboratories will exceed the reproducibility limit $R$ in not more than 5% of the cases.

**Table 8**
Performance characteristics of the method for the quantification of cereulide in various food samples.

| Matrix | Fried rice | Cream pastry with chocolate | Hotdog sausage | Mini pancakes | Vanilla custard | Infant formula |
|---|---|---|---|---|---|---|
| Number of participating collaborators | 11 | 11 | 11 | 11 | 11 | 11 |
| Number of collaborators retained after evaluation of the data | 10 | 10 | 10 | 10 | 10 | 10 |
| Number of samples | 6 | 6 | 6 | 6 | 6 | 6 |
| Number of samples retained after evaluation of the data | 6 | 6 | 6 | 6 | 6 | 6 |
| Working range calibration line in food products (μg/kg) | 1 to 99 | 1 to 99 | 1 to 99 | 1 to 99 | 1 to 99 | 1 to 99 |
| Mean value low $\bar{x}$ (μg/kg) | 4.9 | 4.8 | 4.9 | 4.8 | 4.8 | 4.8 |
| Mean value mid $\bar{x}$ (μg/kg) | 34.4 | 33.5 | 33.9 | 33.4 | 33.6 | 33.9 |
| Mean value high $\bar{x}$ (μg/kg) | 73.7 | 71.7 | 72.8 | 71.6 | 71.9 | 72.7 |
| Coefficient of variation of repeatability $C_{V,r}$ (%) | 3.4 | 4.8 | 4.1 | 4.3 | 4.7 | 4.8 |
| Repeatability limit[a] $r$: ($r = 2.8 \times C_{V,r}$), (%) | 9.5 | 13.4 | 11.6 | 12.2 | 13.3 | 13.4 |
| Coefficient of variation of reproducibility $C_{V,R}$ (%) | 4.6 | 7.2 | 4.3 | 5.5 | 6.9 | 6.6 |
| Reproducibility limit[b] $R$: ($R = 2.8 \times C_{V,R}$), (%) | 12.8 | 20.2 | 12.0 | 15.4 | 19.4 | 18.4 |
| Recovery (%) | 99.3 | 96.7 | 97.7 | 96.5 | 96.6 | 98.1 |

[a] The absolute difference between two single test results found on identical test materials by one operator using the same apparatus within the shortest feasible time interval will exceed the repeatability limit $r$ in not more than 5% of the cases.

[b] The absolute difference between two single test results found on identical test materials reported by two laboratories will exceed the reproducibility limit $R$ in not more than 5% of the cases.

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