INTERNATIONAL STANDARD

ISO 18465

First edition
2017-01

Microbiology of the food chain — Quantitative determination of emetic toxin (cereulide) using LC-MS/MS

Microbiologie de la chaîne alimentaire — Détermination quantitative de la toxine émétique (céreulide) par CL-SM/SM

Reference number
ISO 18465:2017(E)

ISO copyright office
Ch. de Blandonnet 8 • CP 401
CH-1214 Vernier, Geneva, Switzerland
Tel. +41 22 749 01 11
Fax +41 22 749 09 47
copyright@iso.org
www.iso.org

Contents

Page

Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see www.iso.org/patents).

Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

For an explanation on the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT) see the following URL: www.iso.org/iso/foreword.html.

This document was prepared by the European Committee for Standardization (CEN) Technical Committee CEN/TC 275, *Food Analysis — Horizontal methods*, in collaboration with ISO Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 9, *Microbiology*, in accordance with the agreement on technical cooperation between ISO and CEN (Vienna Agreement).

Introduction

Cereulide, the emetic toxin produced in foods by certain strains of *Bacillus cereus*, is a heat and acid stable toxin that causes nausea and vomiting when ingested. In very rare cases, people can die after ingestion of the toxin. Due to its stability, the toxin may still be present even when *B. cereus* can no longer be detected. The presence of cereulide seems to be linked to starch-rich foods like rice (dishes) and pasta (dishes). However, recent data suggest that the occurrence of food borne outbreaks due to cereulide is more common to foods in general[9]. The toxin has a cyclic structure and consists of in total 12 monomers as a repeat of (D-O-Leucine-D-Alanine-L-O-Valine-L-Valine). Several methods have been developed for the detection and/or quantification of the toxin. Some of these methods are nonspecific bio-assays[3, 4] 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[5, 6, 7, 8]. The chemical methods are more specific for cereulide and have, therefore, been chosen as the starting point for standardization of a method for the quantification of cereulide. Recently, research has been done for the chemodiversity of cereulide. At least 18 cereulide variants were detected by UHPLC-TOFMS and ion-trap MS^n sequencing, among which the previously unknown isocereulides A–G[10].

Microbiology of the food chain — Quantitative determination of emetic toxin (cereulide) using LC-MS/MS

1 Scope

This document describes the quantitative analysis of the emetic toxin cereulide using high performance liquid chromatography (HPLC) or ultra performance liquid chromatography (UHPLC) connected to a tandem mass spectrometer (LC-MS/MS).

This document is applicable to the analysis of the toxin in products intended for human consumption.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 3696, *Water for analytical laboratory use — Specification and test methods*

ISO 1042, *Laboratory glassware — One-mark volumetric flasks*

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

— IEC Electropedia: available at http://www.electropedia.org/

— ISO Online browsing platform: available at http://www.iso.org/obp

3.1
cereulide
toxin cyclo[D-O-Leucine-D-Alanine-L-O-Valine-L-Valine]$_3$ produced by certain strains of the species of *B. cereus*

4 General principle

Cereulide is extracted from the food matrix by shaking the sample with acetonitrile. $^{13}C_6$-Cereulide is used as an internal standard. The components in the solution are separated using HPLC or UHPLC and subsequently detected using tandem mass spectrometry (LC-MS/MS). For MS, the electro spray ionization technique (ESI) is used, using the positive mode. The level of emetic toxin (cereulide) is expressed as µg cereulide/kg product.

5 Reagents

Use only reagents of recognized analytical grade, unless otherwise specified.

5.1 Water, according to ISO 3696.

5.2 Acetonitrile, LC-MS grade.

5.3 Methanol, LC-MS grade.

5.4 Formic acid, 98 % to 100 % pro analyse grade.

5.5 Synthetic $^{13}C_6$-Cereulide.[1)]

5.6 Synthetic Cereulide.[1)]

5.7 Ammonium formate, pro analyse grade.

5.8 Mobile phase A, consisting of 10 mmol/l ammonium formate (5.7) with 0,1 % (v/v) formic acid (5.4) in water (5.1).

5.9 Mobile phase B, consisting of 0,1 % (v/v) formic acid (5.4) in acetonitrile (5.2).

5.10 $^{13}C_6$-Cereulide–stock solution IS-A, ρ = 100 000 ng/ml (in methanol).

Weigh 10 mg to the nearest 0,01 mg $^{13}C_6$-Cereulide (5.5) into a glass volumetric flask (6.11) of 100 ml and dissolve, make up to the mark with methanol (5.3). This solution is not corrected for the purity of the compound. Store the solution in the freezer (6.15).

NOTE Cereulide (labelled and non-labelled) stock and standard solutions are extremely stable, meaning over three years when stored in a freezer (6.15)

5.11 $^{13}C_6$-Cereulide–standard solution IS-B, ρ = 1 000 ng/ml (in methanol).

Pipette (6.10) 1 000 µl $^{13}C_6$-Cereulide stock solution IS-A (5.10) in a glass volumetric flask (6.11) of 100 ml, make up to the mark with methanol (5.3) and mix the solution. Store the solution in the freezer (6.15).

5.12 $^{13}C_6$-Cereulide–standard solution IS-C, ρ = 100 ng/ml (in acetonitrile).

Pipette (6.10) 500 µl $^{13}C_6$-Cereulide stock solution IS-A (5.10) in a glass volumetric flask (6.11) of 500 ml, make up to the mark with acetonitrile (5.2) and mix. Store the solution in the freezer (6.15).

5.13 $^{13}C_6$- Cereulide–standard solution IS-D, ρ = 10 ng/ml (in acetonitrile).

Pipette (6.10) 1 000 µl $^{13}C_6$-Cereulide standard solution IS-B (5.11) in a glass volumetric flask (6.11) of 100 ml, make up to the mark with acetonitrile (5.2) and mix. Store the solution in the freezer (6.15).

5.14 Cereulide–stock solution Cer-A, ρ = 100 000 ng/ml (in methanol).

Weigh 5 mg to the nearest 0,01 mg synthetic cereulide (5.6) into a glass volumetric flask (6.11) of 50 ml and dissolve, make up to the mark with methanol (5.3). This solution is not corrected for the purity of the compound. Store the solution in the freezer (6.15).

5.15 Cereulide–standard solution Cer-B, ρ = 100 ng/ml (in acetonitrile).

Pipette (6.10) 500 µl cereulide stock solution A (5.14) in a glass volumetric flask (6.11) of 500 ml, make up to the mark with acetonitrile (5.2) and mix the solution. Store the solution in the freezer (6.15).

1) Chiralix is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

5.16 Cereulide–stock solution Cer-C, ρ = 10 ng/ml (in acetonitrile).

Pipette (6.10) 10 ml cereulide standard solution B (5.15) in a glass volumetric flask (6.11) of 100 ml, make up to the mark with acetonitrile (5.2) and mix the solution. Store the solution in the freezer (6.15).

5.17 Positive control sample or **spiked sample** (level approximately 10 ng/g).

6 Apparatus and equipment

6.1 Tandem mass spectrometer, equipped with ESI interface (in positive mode) and multiple reaction monitoring (MRM) mode.

LC system, pump system (HPLC or UHPLC), degasser, autosampler, column oven.

LC-MS software, suitable of data collection, integration.

6.2 LC column (C-18)

For HPLC, Supelco Discovery®[2] C-18, 100 mm × 2,1 mm, 5 µm or equivalent.

For UHPLC, Waters BEH C-18,[3] 100 mm or 50 mm × 2,1 mm, 1,7 µm or equivalent.

6.3 Centrifuge, capable of a centrifugal force of 1 000*g* to 1 500*g* for 50 ml tubes.

6.4 Centrifuge, capable of a centrifugal force of 10 000*g* to 12 000*g* for 2 ml tubes.

6.5 Centrifuge tubes, (plastic) with closing cap, 2 ml disposable.

6.6 Centrifuge tubes, (glass) with leakage free screw cap 50 ml.

6.7 Horizontal mechanical shaker, capable of holding 50 ml centrifuge tubes.

6.8 Analytical balance, accuracy to the nearest 0,01 mg.

6.9 Grinder, e.g. mixer, blender, cryogenic mixer.

6.10 Calibrated plunger pipettes, ranges from 10 µl to 100 µl, 100 µl to 1 000 µl, and 1 000 µl to 5 000 µl, 2 000 µl to 10 000 µl.

6.11 Glass volumetric flasks, volume of 50 ml, 100 ml and 500 ml according to ISO 1042.

6.12 Glass autosampler vials, with snap/screw cap 2 ml.

6.13 PTFE membrane filters, diameter of 25 mm and 0,45 µm pore size.

6.14 Vortex mixer.

6.15 Freezer, capable of temperatures below −15 °C, preferably below −18 °C.

2) Supelco Discovery® is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

3) Waters BEH C-18 is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

7 Procedure

7.1 Sample preparation

Store the samples (before and during the experiment) in the freezer (6.15) to prevent growth of micro-organisms.

Take (100 ± 25) g of a representative part of the sample and transfer it to a sample jar. In case the amount of sample is limited (for example in case of samples involved in food poisoning), take as much sample as possible and treat identical as the other samples. In this case, a note should be mentioned when reporting the result. Homogenize the sample by grinding (6.9).

Weigh 2,5 g, to the nearest mg (6.8), of the homogenized sample into a centrifuge tube (6.6) and pipette (6.10) 500 µl internal standard solution IS-C (5.12); close the tube with the screw top. Mix (6.14) about 10 s and let the tubes rest for 30 min. Glass tubes should be used especially when solutions are stored for a longer time. For short contact times, plastic tubes can be used as well. Add 29,5 ml acetonitrile (5.2) and close the tube again with the screw top.

Place the tube(s) horizontally on the shaker (6.7) and shake firmly for approximately 1 h. After shaking, centrifuge the tubes for 10 min at 1 000*g* to 1 500*g* (6.3). If the solution is clear without floating particles, no filtration step is necessary. If not, filter the liquid phase using PTFE membrane filters (6.13), or transfer 2 ml in a centrifuge tube (6.5) and centrifuge the solution at 10 000*g* to 12 000*g* (6.4) for 10 min.

Fill an auto sampler vial (6.12) and close the vial with a cap. The samples are now ready for analysis.

7.2 Standard preparation

The standards should be prepared directly in the vials. Pipette (6.10) the volumes as specified in Table 1 with cereulide stock solution Cer-C (5.16), acetonitrile (5.2) (see NOTE) and $^{13}C_6$-cereulide stock solution IS-D (5.13) into a vial (6.12) and close the vial with a cap. Mix (6.14) the solution for 20 s.

NOTE If additional clean up is needed, heptane extraction may be used to reduce the interfering (fatty) components. After adding 29,5 ml acetonitrile, add 10,0 ml heptane and close the tubes with a screw top. Place the tube(s) horizontally on the shaker (6.7) and shake firmly for approximately 1 h. Centrifuge the tubes for 10 min at 1 000*g* to 1 500*g* (6.3). Proceed with the sample preparation with the lower acetonitrile layer.

Table 1 — Preparation of calibration standard solutions

Standard	Cer-C (5.16)	Acetonitrile (5.2)	IS-D (5.13)[a]	Cereulide std concentration
	µl	µl	µl	ng/ml
Standard 0	0	1 000	200	0,00
Standard 1	10	990	200	0,08
Standard 2	50	950	200	0,4
Standard 3	100	900	200	0,8
Standard 4	200	800	200	1,7
Standard 5	500	500	200	4,2
Standard 6	1 000	0	200	8,3

[a] The concentration $^{13}C_6$-Cereulide in the calibration standard solutions is 1,7 ng/ml when adding 200 µl solution IS-D (5.13) to the standards (1 000 µl).

Reanalyse the sample from start when a result is outside the calibration range because of its high concentration, weighing less of the sample <2,5 g.

If a limited amount of sample is available, use an equivalent ratio of internal standard and solutions.

The final concentration of $^{13}C_6$-Cereulide (IS) in the sample extract and standards is ρ = 1,7 ng/ml.

The concentrations in Table 1 depend on the exact weighed amounts of cereulide Cer-A (5.14) and $^{13}C_6$-Cereulide IS-A (5.10)

7.3 LC-MS analysis

7.3.1 LC conditions

Any suitable LC system can be used. Parameters, flow, retention time and gradients are instrument/type column/manufacturer dependent and shall be determined by optimizing the LC system. Both isocratic and gradient elution methods can be used. The parameters displayed below can be used as initial conditions for optimization.

Injection volume:	1 µl to 20 µl
Column:	C-18 HPLC or UHPLC column (6.2)
Column oven:	30 °C to 50 °C
Autosampler tray temperature:	5 °C to 10 °C
Mobile phase A:	10 mmol ammonium formate (5.7) with 0,1 % (v/v) formic acid in water (5.1)
Mobile phase B:	Acetonitrile (5.2) with 0,1 % (v/v) formic acid (5.4)

7.3.2 MS conditions and tuning parameters

The MS parameters can vary; they are instrument/manufacturer dependent and shall be obtained by tuning the instrument before analysis. The parameters displayed below can be used as initial conditions for optimization; examples displayed are from a Waters® Micromass®[4] Quattro Premier.

Ionization:	ESI +
Capillary voltage:	3,5 kV
Cone voltage:	65 V
Extractor:	5 V
RF lens:	0 V
Source temperature:	120 °C
Desolvation temperature:	500 °C
Desolvation gas flow:	1 200 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

4) Waters® Micromass® is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

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 to 1 300 m/z

7.3.3 Transitions (multiple reaction monitoring, MRM)

The transitions mentioned can deviate depending on the mass spectrometer. They are recommended but other transitions can also be used. A list of possible transitions is available in Annex B.

Table 2 — Cereulide quantification MRM $(M+NH_4)^+$: 1 170,7 Precursor ion

	Cone voltage V	Dwell s	Collision energy eV
First product ion trace 1 170,7 > 314,4 Quantification ion (most abundant transition)	65	0,3	74
Second product ion trace 1 170,7 > 499,4 Qualification ion (second most abundant transition)	65	0,3	56

Table 3 — $^{13}C_6$-Cereulide quantification MRM $(M+NH_4)^+$: 1 176,7 Precursor ion

	Cone voltage V	Dwell s	Collision energy eV
First product ion trace 1 176,7 > 172,2 Internal standard quantification ion (most abundant transition)	65	0,3	90
Second product ion trace 1 176,7 > 315,4 Qualification ion (second most abundant transition)	65	0,3	74

8 Calculation

A calibration line is made from the results from the cereulide standards as specified in Table 1 (calibration range). The calibration line shall be forced through the origin. The calculation method shall be set as an internal standard method, and allows quantification of the samples which are within the range.

Concentration cereulide (ng/ml) is calculated with the integration software; a response factor X should be used because of the internal standard method. *X* is calculated with the formula of the calibration line, as given in Formula (1):

$$\text{response factor X} = \frac{\text{Area Cereulide} \times \text{Conc. IS}}{\text{Area IS} \times \text{Conc. Cereulide}} \quad (1)$$

The cereulide level in the sample can be calculated using Formula (2):

$$\text{cereulide conc. sample}\left(\mu g/kg\right) = \frac{X \times \text{Volume}\left(ml\right)}{\text{Weight sample}\left(g\right)} \quad (2)$$

The ion ratio in standards and samples should be calculated using Formula (3):

$$\text{ratio}\left(\%\right) = \frac{\text{Abundance daughter 2 (Qualifier ion)}}{\text{Abundance daughter 1 (Qualifier ion)}} \quad (3)$$

Limit of detection (LOD): The calculated concentration in the solution (or the concentration recalculated back to the original sample) when the signal of the second product ion is three times the background noise (i.e. signal to noise ratio of the second product ion equals 3).

Limit of quantification (LOQ): The calculated concentration in the solution (or the concentration recalculated back to the original sample) when the signal of the second product ion is six times the background noise (i.e. signal to noise ratio of the second product ion equals 6).

9 Quality controls

Check if both product ions from cereulide in a sample exist and meet the LOD-LOQ requirement. If not, there is no positive identification or measurement possible.

Check if the ion ratio from the cereulide product ions (Rs) in a sample meets the criteria for conformation of identity as established in Reference [2]. For a positive identification, the ratio of product ions in a sample shall lie within a certain range, which is established by the average ratio of the product ions in the standards/reference (Rr) and a required tolerance interval. This interval is dependent on the level of the average ratio in the standards/references.

Ratio (Rr) in Reference > 50 %: Positive identification, ratio sample (Rs) lies within Rr ± 20 %.

Ratio (Rr) in Reference > 20 % to 50 %: Positive identification, ratio sample (Rs) lies within Rr ± 25 %.

Ratio (Rr) in Reference > 10 % to 20 %: Positive identification, ratio sample (Rs) lies within Rr ± 30 %.

Ratio (Rr) in Reference < 10 %: Positive identification, ratio sample (Rs) lies within Rr ± 50 %.

The change in retention times during a set of measurements shall not exceed the average retention time of the measurements of the standard ±2,5 %.

Investigate for every series of samples the positive sample control (5.17) and check if the results correspond with the preset criteria (e.g. Shewart control chart/Recovery chart).

Check if the area of the peak of the second daughter of standard 1 is at least six times the background noise (≥LOQ) (S/N ratio ≥ 6).

Check if the correlation coefficient (r) of the linear calibration line is at least 0,995.

Standard 6 (Table 1) shall be measured every 10 samples to check the trend in the signal of the device (declining peak area). The decline of the peak area shall be less than 10 %.

If one of the above checks does not meet the quality requirements, try to find out why or what went wrong. If necessary, reanalyse the samples.

10 Precision

10.1 General

Details on the interlaboratory study of the precision of the method are summarized in Annex A. The values derived from the interlaboratory study are not applicable to analyte concentration ranges and/or matrices other than those given in Annex A.

10.2 Repeatability

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.

Table 4 — Repeatability values per matrix and level of cereulide

Repeatability		
Matrix	$\bar{x}$ (µg/kg)	***r*** (µg/kg)
natural contaminated cooked rice		
level 1	25,0	3,5
level 2	78,1	8,0
spiked cooked rice		
level 1	22,5	2,2
fried rice		
level 1	4,9	0,5
level 2	34,4	3,3
level 3	73,7	7,0
cream pastry with chocolate		
level 1	4,8	0,6
level 2	33,5	4,5
level 3	71,7	9,6
hotdog sausage		
level 1	4,9	0,6
level 2	33,9	3,9
level 3	72,8	8,4
mini pancakes		
level 1	4,8	0,6
level 2	33,4	4,1
level 3	71,6	8,7
vanilla custard		
level 1	4,8	0,6
level 2	33,6	4,5
level 3	71,9	9,5
infant formula		

Table 4 *(continued)*

level 1	4,8	0,6
level 2	33,9	4,5
level 3	72,7	9,7

10.3 Reproducibility

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 5 — Reproducibility values per matrix and level of cereulide

Reproducibility		
Matrix	$\bar{x}$ (µg/kg)	***R*** (µg/kg)
natural contaminated cooked rice		
level 1	25,0	5,8
level 2	78,1	13,1
spiked cooked rice		
level 1	22,5	2,8
fried rice		
level 1	4,9	0,6
level 2	34,4	4,4
level 3	73,7	9,4
cream pastry with chocolate		
level 1	4,8	1,0
level 2	33,5	6,8
level 3	71,7	14,5
hotdog sausage		
level 1	4,9	0,6
level 2	33,9	4,1
level 3	72,8	8,7
mini pancakes		
level 1	4,8	0,7
level 2	33,4	5,1
level 3	71,6	11,0
vanilla custard		
level 1	4,8	0,9
level 2	33,6	6,5
level 3	71,9	14,0
infant formula		
level 1	4,8	0,9
level 2	33,9	6,2
level 3	72,7	13,4

Annex A
(informative)

Results of the interlaboratory study

An international interlaboratory study involving 11 laboratories in nine countries was carried out on cooked rice, fried rice, cream pastry with chocolate, mini-pancakes, hotdog sausage, vanilla custard and infant formulae. The food samples were each tested at three different levels of spiking, except for the cooked rice, tested as naturally contaminated cooked rice (*B. cereus* was inoculating in the cooked rice and incubating under conditions that cereulide was produced). The study was organized in 2013 by the Netherlands Food and Consumer Product Safety Authority (NVWA). In accordance with ISO 5725-2:1994, the following parameters were calculated to give the precision data shown in Tables A.1 to A.3.

Table A.1 — Results of the data analysis for cooked rice (naturally contaminated and spiked)

	Cooked rice (naturally contaminated and spiked)		
	Naturally contaminated[a] (medium level)	Naturally contaminated[a] (high level)	Spiked
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 r: ($r = 2{,}8 \times s_r$), (µg/kg)	3,5	8,0	2,2
Repeatability limit 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 R: ($r = 2{,}8 \times s_R$), (µg/kg)	5,8	13,1	2,8
Reproducibility limit R: ($r = 2{,}8 \times C_{V,R}$), (%)	23,3	16,7	12,4
Recovery	—	—	—

[a] The naturally contaminated cooked rice was obtained by inoculating *B. cereus* in the rice and incubating the rice for the production of cereulide.

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

Table A.2 — Results of the data analysis for spiked fried rice, cream pastry with chocolate and hotdog sausage

	Fried rice	Cream pastry with chocolate	Hotdog sausage
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	6
Working range calibration line in food products (µg/kg)	1 to 99	1 to 99	1 to 99
Mean value $\bar{x}$ (µg/kg)			
— low concentration	4,9	4,8	4,9
— medium concentration	34,4	33,5	33,9
— high concentration	73,7	71,7	72,8
Coefficient of variation of repeatability $C_{V,r}$ (%)	3,4	4,8	4,1
Repeatability limit r: ($r = 2{,}8 \times C_{V,r}$), (%)	9,5	13,4	11,6
Coefficient of variation of reproducibility $C_{V,R}$ (%)	4,6	7,2	4,3
Reproducibility limit R: ($r = 2{,}8 \times C_{V,R}$), (%)	12,8	20,2	12,0
Recovery	99,3	96,7	97,7

Table A.3 — Results of the data analysis for spiked mini-pancakes, vanilla custard and infant formula

	Mini-pancakes	Vanilla custard	Infant formula
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	6
Working range calibration line in food products (µg/kg)	1 to 99	1 to 99	1 to 99
Mean value $\bar{x}$ (µg/kg)			
— low concentration	4,8	4,8	4,8
— medium concentration	33,4	33,6	33,9
— high concentration	71,6	71,9	72,7
Coefficient of variation of repeatability $C_{V,r}$ (%)	4,3	4,7	4,8
Repeatability limit r: ($r = 2{,}8 \times C_{V,r}$), (%)	12,2	13,3	13,4
Coefficient of variation of reproducibility $C_{V,R}$ (%)	5,5	6,9	6,6
Reproducibility limit R: ($r = 2{,}8 \times C_{V,R}$), (%)	15,4	19,4	18,4
Recovery	96,5	96,6	98,1