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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

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Foreword

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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.

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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

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