SLOVENSKI STANDARD
SIST EN 16858:2017

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Živila - Določevanje melamina in cianurinske kisline v živilih s tekočinsko kromatografijo in tandemsko masno spektrometrijo (LC-MS/MS)

Foodstuffs - Determination of melamine and cyanuric acid in foodstuffs by liquid chromatography and tandem mass spectrometry (LC-MS/MS)

Lebensmittel - Bestimmung von Melamin und Cyanursäure in Lebensmitteln mit Flüssigchromatographie und Tandem-Massenspektrometrie (LC-MS/MS)

Produits alimentaires - Détermination de la teneur en mélamine et en acide cyanurique dans les produits alimentaires par chromatographie en phase liquide couplée à la spectrométrie de masse en tandem (CL-SM/SM)

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67.050	Splošne preskusne in analizne metode za živilske proizvode	General methods of tests and analysis for food products

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Foodstuffs - Determination of melamine and cyanuric acid in foodstuffs by liquid chromatography and tandem mass spectrometry (LC-MS/MS)

Produits alimentaires - Détermination de la teneur en mélamine et en acide cyanurique dans les produits alimentaires par chromatographie en phase liquide couplée à la spectrométrie de masse en tandem (CL-SM/SM)

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This European Standard was approved by CEN on 10 April 2017.

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EUROPEAN COMMITTEE FOR STANDARDIZATION
COMITÉ EUROPÉEN DE NORMALISATION
EUROPÄISCHES KOMITEE FÜR NORMUNG

CEN-CENELEC Management Centre: Avenue Marnix 17, B-1000 Brussels

Contents

Page

European foreword

This document (EN 16858:2017) has been prepared by Technical Committee CEN/TC 275 "Food analysis - Horizontal methods", the secretariat of which is held by DIN.

This European Standard shall be given the status of a national standard, either by publication of an identical text or by endorsement, at the latest by November 2017, and conflicting national standards shall be withdrawn at the latest by November 2017.

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1 Scope

This European Standard specifies a method for the determination of melamine and cyanuric acid in foodstuffs with liquid chromatography in combination with tandem mass spectrometry. The method has been validated in an interlaboratory study via the analysis of spiked samples of milk based infant formula, soy based infant formula, milk powder, whole milk, soy drink and milk chocolate ranging from 0,71 mg/kg to 1,43 mg/kg for melamine and 0,57 mg/kg to 1,45 mg/kg for cyanuric acid. The limits of quantification (LOQ) for melamine and cyanuric acid in food are 0,05 mg/kg and 0,25 mg/kg, respectively. The upper limit of the working range is up to 10 mg/kg for melamine and up to 25 mg/kg for cyanuric acid.

2 Normative references

The following documents, in whole or in part, are normatively referenced in this document and are indispensable for its application. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

EN ISO 3696:1995, *Water for analytical laboratory use — Specification and test methods (ISO 3696:1987)*

3 Principle

A test portion of the homogenous food sample is fortified with ^{13}C labelled internal standards (melamine and cyanuric acid). After incubation for at least one hour, water is added to the sample and after shaking, the slurry is dissolved in a mixture of acetonitrile and water. The sample is shaken and centrifuged. After separation of supernatant from sediments benzoguanamine is added as a recovery standard. An aliquot of the aqueous supernatant is injected into a LC-MS/MS system. The triple quadrupole mass spectrometer is coupled either to high performance liquid chromatography (HPLC) or to ultra performance liquid chromatography (UHPLC). Chromatography is based on hydrophilic interaction liquid chromatography (HILIC). Ionization is achieved by electrospray ionization (ESI) in multiple reaction monitoring (MRM).

4 Reagents

Use only reagents of recognized analytical grade and water complying with grade 1 of EN ISO 3696:1995, unless otherwise specified. Use only reagents with purity suitable for melamine and cyanuric acid analysis. Check the purity of the reagents and reference materials (e.g. standard solutions) by performing a blank test under the same conditions as used in the method. The chromatogram shall not show any interfering impurity at the retention time of compounds of interest.

4.1 Formic acid, mass fraction is 98 % to 100 % (CAS 64-18-6).

4.2 Acetonitrile, HPLC gradient grade (CAS 75-05-8).

4.3 Ammonium acetate, mass fraction approximately 98 % (CAS 631-61-8).

4.4 Methanol, Ultra LC-MS grade (CAS 67-56-1).

4.5 Melamine, mass fraction ≥ 99 %, solid, (CAS 108-78-1).

4.6 Cyanuric acid, solid, (CAS 108-80-5).

4.7 Benzoguanamine, solid, (CAS 91-76-9).

4.8 ^{13}C Melamine, $^{13}C_3$ (99 %), Amino-$^{15}N_3$ (98 %), solution with mass concentration ρ = 1 000 µg/ml.

4.9 ^{13}C Cyanuric acid, $^{13}C_3$ (99 %), $^{15}N_3$ (98 %), solution with ρ = 1 000 µg/ml.

4.10 Preparation of stock solutions

4.10.1 Melamine stock solution, ρ = 1 000 µg/ml

Weigh, to the nearest 0,01 mg, approximately 100 mg of melamine (4.5) into a 100 ml glass flask (5.2) and add by weighing an amount of water to achieve a concentration of 1 000 µg/ml. Store the solution in a refrigerator at 4 °C (±3 °C). The solution is stable under these conditions during at least 1 year if the mass is carefully monitored.

4.10.2 Cyanuric acid stock solution, ρ = 1 000 µg/ml

Weigh, to the nearest 0,01 mg, approximately 100 mg of cyanuric acid (4.6) into a 100 ml glass flask (5.2) and add by weighing an amount of water to achieve a concentration of 1 000 µg/ml. Store the solution in a refrigerator at 4 °C (±3 °C). The solution is stable under these conditions during at least 1 year if the mass is carefully monitored.

4.10.3 Benzoguanamine stock solution, ρ = 1 000 µg/ml

Weigh, to the nearest 0,01 mg, approximately 100 mg of benzoguanamine (4.7) into a 100 ml glass flask (5.2) and add by weighing an amount of methanol (4.4) to achieve a concentration of 1 000 µg/ml. Store the solution in a refrigerator at 4 °C (±3 °C). The solution is stable under these conditions during at least 1 year if the mass is carefully monitored.

4.10.4 ^{13}C Melamine stock solution, ρ = 20 µg/ml

Pipette 1,0 ml of ^{13}C melamine (4.8) into a 50 ml volumetric flask (5.3). Make up to the mark with water and mix. The final concentration is 20 µg/ml. Transfer this solution into a 100 ml glass flask (5.2). The solution is stable under these conditions during at least 1 year if the mass is carefully monitored.

4.10.5 ^{13}C Cyanuric acid stock solution, ρ = 20 µg/ml

Pipette 1,0 ml of ^{13}C cyanuric acid (4.9) into a 50 ml volumetric flask (5.3). Make up to the mark with water and mix. The final concentration is 20 µg/ml. Transfer this solution into a 100 ml glass flask (5.2). The solution is stable under these conditions during at least 1 year if the mass is carefully monitored.

4.11 Preparation of standard solutions

4.11.1 Melamine standard solution I, ρ = 20 µg/ml

Pipette 1,0 ml of the melamine stock solution (4.10.1) into a 50 ml volumetric flask (5.3). Make up to the mark with dilution solution (4.16) and mix. The concentration is 20 µg/ml. Transfer this solution into a 100 ml glass flask (5.2) and store it in a refrigerator at 4 °C (±3 °C). The solution is stable under these conditions during at least 1 month if the mass is carefully monitored.

4.11.2 Cyanuric acid standard solution I, ρ = 20 µg/ml

Pipette 1,0 ml of the cyanuric acid stock solution (4.10.2) into a 50 ml volumetric flask (5.3). Make up to the mark with dilution solution (4.16) and mix. The concentration is 20 µg/ml. Transfer this solution into a 100 ml glass flask (5.2) and store it in a refrigerator at 4 °C (±3 °C). The solution is stable under these conditions during at least 1 month if the mass is carefully monitored.

4.11.3 Melamine standard solution II, ρ = 0,2 µg/ml

Pipette 1,0 ml of the melamine standard solution I (4.11.1) into a 100 ml volumetric flask (5.3). Make up to the mark with dilution solution (4.16) and mix. The concentration is 0,2 µg/ml. Transfer this solution into a 100 ml glass flask (5.2) and store it in a refrigerator at 4 °C (±3 °C). The solution is stable under these conditions during at least 1 month if the mass is carefully monitored.

4.11.4 Cyanuric acid standard solution II, ρ = 0,2 µg/ml

Pipette 1,0 ml of the cyanuric acid standard solution I (4.11.2) into a 100 ml volumetric flask (5.3). Make up to the mark with dilution solution (4.16) and mix. The concentration is 0,2 µg/ml. Transfer this solution into a 100 ml glass flask (5.2) and store it in a refrigerator at 4 °C (±3 °C). The solution is stable under these conditions during at least 1 month if the mass is carefully controlled.

4.11.5 Benzoguanamine standard solution I, ρ = 20 µg/ml

Pipette 1,0 ml of the benzoguanamine stock solution (4.10.3) into a 50 ml volumetric flask (5.3). Make up to the mark with dilution solution (4.16) and mix. The concentration is 20 µg/ml. Transfer this solution into a 100 ml glass flask (5.2) and store it in a refrigerator at 4 °C (±3 °C). The solution is stable under these conditions during at least 1 month if the mass is carefully monitored.

4.11.6 Benzoguanamine standard solution II, ρ = 2 µg/ml

Pipette 1,0 ml of the benzoguanamine standard solution I (4.11.5) into a 10 ml volumetric flask (5.3). Make up to the mark with dilution solution (4.16) and mix. The concentration is 2 µg/ml. Transfer this solution into a 20 ml glass flask (5.2) and store it in a refrigerator at 4 °C (±3 °C). The solution is stable under these conditions during at least 1 month if the mass is carefully controlled.

4.11.7 ^{13}C Melamine and ^{13}C cyanuric acid standard solution, ρ = 2 µg/ml

Pipette 0,5 ml of the ^{13}C melamine stock solution (4.10.4) and 0,5 ml of the ^{13}C cyanuric acid stock solution (4.10.5) into a 5 ml volumetric flask (5.3). Make up to the mark with water and mix. The concentrations for both compounds is 2 µg/ml. Transfer this solution into a 20 ml glass flask (5.2) and store it in a refrigerator at 4 °C (±3 °C). The solution is stable under these conditions during at least 1 month if the mass is carefully monitored.

4.11.8 Preparation of calibration solutions

Pipette the volumes as indicated in Table 1 into a 10 ml volumetric flask (5.3) and make up to the mark with the dilution solution (4.16).

Table 1 — Preparation of the calibration solutions

	Calibration solution number						
	1	2	3	4	5	6	7
Melamine standard solution I (4.11.1), ml	0	0	0	0	0	0,05	0,25
Cyanuric acid standard solution I (4.11.2), ml	0	0	0	0	0	0,05	0,25
Melamine standard solution II (4.11.3), ml	0	0,05	0,25	1,25	2,5	0	0
Cyanuric acid standard solution II (4.11.4), ml	0	0,05	0,25	1,25	2,5	0	0
Benzoguanamine standard solution II (4.11.6), ml	0,5	0,5	0,5	0,5	0,5	0,5	0,5
^{13}C Melamine – ^{13}C cyanuric acid standard solution (4.11.7), ml	0,5	0,5	0,5	0,5	0,5	0,5	0,5
Concentration ^{13}C melamine – ^{13}C cyanuric acid, µg/ml	0,1	0,1	0,1	0,1	0,1	0,1	0,1
Concentration native melamine – cyanuric acid, µg/ml	0	0,001	0,005	0,025	0,05	0,1	0,5

4.12 Mobile phase A for HPLC, substance concentration *c*(ammonium acetate) = 10 mmol/l

Dissolve 0,77 g of ammonium acetate (4.3) in 1 000 ml of water.

4.13 Mobile phase B for HPLC

Acetonitrile (4.2).

4.14 Mobile phase A for UHPLC

Mix 3 parts per volume of formic acid (4.1) with 97 parts per volume of water.

4.15 Mobile phase B for UHPLC, *c*(ammonium acetate) = 20 mmol/l in a mixture of 3 parts per volume of water and 97 parts per volume of acetonitrile.

Dissolve 1,54 g of ammonium acetate (4.3) in 30 ml of water. Add 970 ml of acetonitrile (4.2) to the mixture and shake firmly. The turbid mixture will clear overnight.

NOTE The optimal choice for the mobile phases A and B may depend on the instrument configuration (see 5.10 to 5.12), in more particular the type of column used. Equivalent products may be used if they can be shown to lead to the same results.

4.16 Dilution solution

Transfer, using measuring cylinders, 70 ml of acetonitrile (4.2) and 30 ml of water into a 100 ml volumetric flask (5.3) and mix. Store at room temperature for no longer than one month.

5 Apparatus

All technical descriptions are examples of possible system setups and parameters and shall be scaled or adopted to the user's equipment. Usual laboratory glassware and equipment and, in particular, the following.

5.1 Disposable polypropylene carbonate tube, of approximately 50 ml.

5.2 Glass flask, with volume of 20 ml and 100 ml.

5.3 One-mark volumetric flask, with volumes of 5 ml, 10 ml, 50 ml and 100 ml.

5.4 Shaking machine, adjustable from 0 strokes/min to 300 strokes/min.

5.5 Ultra sonication bath.

5.6 Centrifuge, with the capability to centrifuge 50 ml tubes (see 5.1) and maximum *g* force of at least 4 000 *g*.

5.7 Centrifuge, with the capability to centrifuge standard micro test tubes (see 5.8) and a maximum *g* force of at least 8 000 *g*.

5.8 Standard micro test tubes, 1,5 ml.

5.9 Vials, for LC.

5.10 Liquid chromatograph triple quadrupole mass spectrometer consisting of:

5.10.1 Pump system, capable of delivering a gradient at the required flow.

5.10.2 Injector, capable to inject 5 µl.

5.11 TSKgel® Amide-80[1] **HILIC column,** length of 100 mm, internal diameter of 3,0 mm and particle size of 3 µm.

5.12 UHPLC® BEH (bridged ethyl hybrid)[1] **HILIC column,** length of 150 mm, internal diameter of 2,1 mm and particle size of 1,7 µm.

6 Procedure

6.1 Sample preparation

Warm all milk based liquid products, e.g. full fat milk, skimmed milk, raw milk and chocolate milk to 40 °C and shake gently.

If necessary, mill all dry products based on milk, soy or wheat, e.g. milk powder, infant formula and biscuits, to a particle size of < 1 mm.

Mill all milk or cocoa based products, e.g. chocolate, candy and sweets (toffee), cryogenically using e.g. liquid nitrogen after cutting in small cubes of ≤ 1 cm.

6.2 Extraction

6.2.1 General

The following samples shall be included in each series:

— calibration solutions;

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[1] This is an example of a suitable product available commercially. This information is given for the convenience of users of this European Standard and does not constitute an endorsement by CEN of this product. Equivalent products may be used if they can be shown to lead to the same results.

— procedure blank (n = 1);

— (certified) reference material at appropriate level or a home made reference sample;

— all samples;

— calibration solution number 3, 4 or 5 (see Table 1).

The procedure blank shall be free of contaminants at or above the limits of quantification. The sample amount used for extraction is 1 g. Deviations in sample mass shall be taken into account in Formula (5) in 8.5 (m = sample mass in g).

The calibration curve is used for identification and quantification and calibration solution 3, 4 or 5 after the series is used for control of the stability. The areas of melamine and cyanuric acid obtained with this standard shall be equal to the areas obtained with the same standard in the calibration curve with a maximum deviation of 10 %.

6.2.2 Extraction procedure

Weigh, to the nearest 0,01 g, 1 g of sample into a disposable tube (5.1). Add 250 µl of ^{13}C melamine stock solution (4.10.4) and 250 µl of ^{13}C cyanuric acid stock solution (4.10.5). Incubate for at least 1 h at ambient temperature. Add 5 ml of water and shake manually for 30 s to obtain a slurry. Add 5 ml of acetonitrile (4.2) and shake again. Mix the slurry with 30 ml acetonitrile (4.2) and 10 ml of hot water (96 °C to 100 °C). Shake well for 5 min and centrifuge for 10 min at 3 400 *g*. Transfer 1,5 ml of the extract into a standard micro test tube (5.8) and centrifuge for 10 min at 7 800 *g*. Transfer from the upper layer 1 000 µl into a LC vial (5.9) and add 50 µl of benzoguanamine standard solution II (4.11.6).

6.3 Determination

Inject 5 µl of the calibration solutions (1 to 7, see Table 1) and 5 µl of sample extracts. Identify melamine and cyanuric acid peaks on the basis of retention time, transitions and ion ratio. Determine the amount of melamine and cyanuric acid by comparing the area of the sample peaks with those of the known amount of the corresponding melamine and cyanuric acid peaks in the calibration solutions. Calibration is based on the isotope dilution principle.

7 System settings

7.1 HPLC parameters

The gradient parameters as in Table 2 have shown to give successful results. Depending on the LC-MS/MS system, the parameters might need to be slightly adjusted. Alternatively, a UHPLC system can be used, see 7.3.