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Milk, milk products and infant formula — Determination of melamine and cyanuric acid by liquid chromatography and tandem mass spectrometry (LC-MS/MS)

Lait, produits laitiers et formule infantile — Détermination de la teneur en mélamine et en acide cyanurique par chromatographie en phase liquide couplée à la spectrométrie de masse en tandem (CL-SM/SM)

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Foreword

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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).

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This document was prepared by the IDF *Standing Committee on Analytical Methods for Additives and Contaminants* and ISO Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 5, *Milk and milk products*. It is being published jointly by ISO and IDF.

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Milk, milk products and infant formula — Determination of melamine and cyanuric acid by liquid chromatography and tandem mass spectrometry (LC-MS/MS)

1 Scope

This document specifies a method for the determination of melamine and cyanuric acid with liquid chromatography in combination with tandem mass spectrometry (LC-MS/MS). 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 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.

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

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3 Terms and definitions

No terms and definitions are listed in this document.

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

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

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

4 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 1 h, 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 the 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 high 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).

5 Reagents

5.1 List of reagents

Use only reagents of recognized analytical grade and water conforming to grade 1 of ISO 3696, unless otherwise specified. Use only reagents with purity suitable for melamine and cyanuric acid analysis.

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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.

5.1.1 Formic acid, mass fraction of 98 % to 100 % (CAS RN®[1)] 64-18-6).

5.1.2 Acetonitrile, HPLC gradient grade (CAS RN® 75-05-8).

5.1.3 Ammonium acetate, mass fraction of approximately 98 % (CAS RN® 631-61-8).

5.1.4 Methanol, Ultra LC-MS grade (CAS RN® 67-56-1).

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

5.1.6 Cyanuric acid, solid (CAS RN® 108-80-5).

5.1.7 Benzoguanamine, solid (CAS RN® 91-76-9).

5.1.8 ^{13}C melamine, $^{13}C_3$ (99 %), Amino-$^{15}N_3$ (98 %), solution with mass concentration ρ = 1 000 µg/ml. Other isotope marked melamine/cyanuric acid solutions can be used if they lead to similar results; this would need to be appropriately validated.

EXAMPLE 1,3,5-Triazine-2,4,6-triamine-13C3 (melamine), 1,3,5-Triazine-2,4,6-triol-13C3, 2,4,6-Trihydroxy-1,3,5-triazine-13C (cyanuric acid).

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5.1.9 ^{13}C cyanuric acid, $^{13}C_3$ (99 %), $^{15}N_3$ (98 %), solution with ρ = 1 000 µg/ml.

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NOTE Alternatives can be used as per 5.1.8.

5.2 Preparation of stock solutions

5.2.1 Melamine stock solution, ρ = 1 000 µg/ml.

Weigh, to the nearest 0,1 mg, approximately 100 mg of melamine (5.1.5) into a 100 ml glass flask (6.2) 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.

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

Weigh, to the nearest 0,1 mg, approximately 100 mg of cyanuric acid (5.1.6) into a 100 ml glass flask (6.2) 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.

5.2.3 Benzoguanamine stock solution, ρ = 1 000 µg/ml.

Weigh, to the nearest 0,1 mg, approximately 100 mg of benzoguanamine (5.1.7) into a 100 ml glass flask (6.2) and add by weighing an amount of methanol (5.1.4) 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.

1) CAS Registry Number® (CAS RN®) is the trademark of a product supplied by CAS corporation. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO or IDF of the product named. Equivalent products may be used if they can be shown to lead to the same results.

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5.2.4 ^{13}C melamine stock solution, ρ = 20 µg/ml.

Pipette 1,0 ml of ^{13}C melamine (5.1.8) into a 50 ml volumetric flask (6.3). 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 (6.2). The solution is stable under these conditions during at least 1 year.

5.2.5 ^{13}C cyanuric acid stock solution, ρ = 20 µg/ml.

Pipette 1,0 ml of ^{13}C cyanuric acid (5.1.9) into a 50 ml volumetric flask (6.3). 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 (6.2). The solution is stable under these conditions during at least 1 year.

5.3 Preparation of standard solutions

5.3.1 Melamine standard solution I, ρ = 20 µg/ml.

Pipette 1,0 ml of the melamine stock solution (5.2.1) into a 50 ml volumetric flask (6.3). Make up to the mark with the dilution solution (5.4.6) and mix. The concentration is 20 µg/ml. Transfer this solution into a 100 ml glass flask (6.2) and store it in a refrigerator at 4 °C (±3 °C). The solution is stable under these conditions during at least 1 month.

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

Pipette 1,0 ml of the cyanuric acid stock solution (5.2.2) into a 50 ml volumetric flask (6.3). Make up to the mark with the dilution solution (5.4.6) and mix. The concentration is 20 µg/ml. Transfer this solution into a 100 ml glass flask (6.2) and store it in a refrigerator at 4 °C (±3 °C). The solution is stable under these conditions during at least 1 month.

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

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Pipette 1,0 ml of the melamine standard solution I (5.3.1) into a 100 ml volumetric flask (6.3). Make up to the mark with the dilution solution (5.4.6) and mix. The concentration is 0,2 µg/ml. Transfer this solution into a 100 ml glass flask (6.2) and store it in a refrigerator at 4 °C (±3 °C). The solution is stable under these conditions during at least 1 month.

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

Pipette 1,0 ml of the cyanuric acid standard solution I (5.3.2) into a 100 ml volumetric flask (6.3). Make up to the mark with the dilution solution (5.4.6) and mix. The concentration is 0,2 µg/ml. Transfer this solution into a 100 ml glass flask (6.2) and store it in a refrigerator at 4 °C (±3 °C). The solution is stable under these conditions during at least 1 month.

5.3.5 Benzoguanamine standard solution I, ρ = 20 µg/ml.

Pipette 1,0 ml of the benzoguanamine stock solution (5.2.3) into a 50 ml volumetric flask (6.3). Make up to the mark with the dilution solution (5.4.6) and mix. The concentration is 20 µg/ml. Transfer this solution into a 100 ml glass flask (6.2) and store it in a refrigerator at 4 °C (±3 °C). The solution is stable under these conditions during at least 1 month.

5.3.6 Benzoguanamine standard solution II, ρ = 2 µg/ml.

Pipette 1,0 ml of the benzoguanamine standard solution I (5.3.5) into a 10 ml volumetric flask (6.3). Make up to the mark with the dilution solution (5.4.6) and mix. The concentration is 2 µg/ml. Transfer this solution into a 20 ml glass flask (6.2) and store it in a refrigerator at 4 °C (±3 °C). The solution is stable under these conditions during at least 1 month.

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5.3.7 ^{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 (5.2.4) and 0,5 ml of the ^{13}C cyanuric acid stock solution (5.2.5) into a 5 ml volumetric flask (6.3). Make up to the mark with water and mix. The concentration for both compounds is 2 µg/ml. Transfer this solution into a 20 ml glass flask (6.2) 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.

5.4 Preparation of calibration solutions

5.4.1 Pipette the volumes given in Table 1 into a 10 ml volumetric flask (6.3) and make up to the mark with the dilution solution (5.4.6).

Table 1 — Preparation of the calibration solutions

Calibration solution	Calibration solution number						
	1	2	3	4	5	6	7
Melamine standard solution I (5.3.1), in ml	0	0	0	0	0	0,05	0,25
Cyanuric acid standard solution I (5.3.2), in ml	0	0	0	0	0	0,05	0,25
Melamine standard solution II (5.3.3), in ml	0	0,05	0,25	1,25	2,5	0	0
Cyanuric acid standard solution II (5.3.4), in ml	0	0,05	0,25	1,25	2,5	0	0
Benzoguanamine standard solution II (5.3.6), in ml	0,5	0,5	0,5	0,5	0,5	0,5	0,5
^{13}C melamine and ^{13}C cyanuric acid standard solution (5.3.7), in ml	0,5	0,5	0,5	0,5	0,5	0,5	0,5
Concentration ^{13}C melamine – ^{13}C cyanuric acid, in µg/ml	0,1	0,1	0,1	0,1	0,1	0,1	0,1
Concentration native melamine – cyanuric acid, in µg/ml	0	0,001	0,005	0,025	0,05	0,1	0,5

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

Dissolve 0,77 g of ammonium acetate (5.1.3) in 1 000 ml of water.

5.4.3 Mobile phase B for HPLC.

Acetonitrile (5.1.2).

5.4.4 Mobile phase A for UHPLC.

Mix 3 parts per volume of formic acid (5.1.1) with 97 parts per volume of water.

5.4.5 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 (5.1.3) in 30 ml of water. Add 970 ml of acetonitrile (5.1.2) to the mixture and shake firmly. The turbid mixture will clear overnight.

The optimal choice for the mobile phases A and B can depend on the instrument configuration (see 6.10 to 6.12), particularly the type of column used. Equivalent products may be used if they can be shown to lead to the same results.

5.4.6 Dilution solution.

Transfer, using measuring cylinders, 70 ml of acetonitrile (5.1.2) and 30 ml of water into a 100 ml volumetric flask (6.3) and mix. Store at room temperature for no longer than 1 month.

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6 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 shall be used.

6.1 Disposable polypropylene carbonate tube, of approximately 50 ml.

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

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

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

6.5 Ultra sonication bath.

6.6 Centrifuge, with the capability to centrifuge 50 ml tubes (6.1) and a maximum *g* force of at least 4 000*g*.

6.7 Centrifuge, with the capability to centrifuge standard micro test tubes (6.8), chilled and a maximum *g* force of at least 6 000*g*.

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6.8 Standard micro test tubes, 1,5 ml.

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6.9 Vials, for LC.

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6.10 Liquid chromatograph triple quadrupole mass spectrometer consisting of:

a) **a pump system,** capable of delivering a gradient at the required flow;

b) **an injector,** capable of injecting 5 µl.

6.11 TSKgel® Amide-80[2] **HILIC column,** length of 100 mm, internal diameter of 3,0 mm and particle size of 3 µm.

6.12 UHPLC BEH™ (bridged ethyl hybrid)[3] **HILIC column,** length of 150 mm, internal diameter of 2,1 mm and particle size of 1,7 µm.

6.13 Liquid nitrogen.

6.14 Milling equipment, capable of milling to a final particle size of <300 µm.

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