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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 Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 5, *Milk and milk products*, and the International Dairy Federation (IDF). It is being published jointly by ISO and IDF.

Any feedback or questions on this document should be directed to the user’s national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

IDF (the International Dairy Federation) is a non-profit private sector organization representing the interests of various stakeholders in dairying at the global level. IDF members are organized in National Committees, which are national associations composed of representatives of dairy-related national interest groups including dairy farmers, dairy processing industry, dairy suppliers, academics and governments/food control authorities.

ISO and IDF collaborate closely on all matters of standardization relating to methods of analysis and sampling for milk and milk products. Since 2001, ISO and IDF jointly publish their International Standards using the logos and reference numbers of both organizations.

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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.

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

3 Terms and definitions

No terms and definitions are listed in this document.

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

- ISO Online browsing platform: available at https://www.iso.org/obp
- IEC Electropedia: available at https://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.

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).

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

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.

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[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.

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.

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

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.

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

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.

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*.

6.8 Standard micro test tubes, 1,5 ml.

6.9 Vials, for LC.

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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[2] TSKgel is the trademark of a product supplied by Tosoh 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.

[3] BEH is the trademark of a product supplied by Waters 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.

7 Procedure

7.1 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, for example, liquid nitrogen after cutting in small cubes of ≤1 cm.

7.2 Extraction

7.2.1 General

The following samples shall be included in each series:

- calibration solutions;
- procedure blank ($n = 1$);
- (certified) reference material at an 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. A sample matrix can be used where previous analysis showed that no cyanuric acid (CYA) and melamine (MEL) were present in the sample above the LOQ. The sample amount used for extraction is 1 g of blank sample. Deviations in sample mass shall be taken into account in Formula (5) (m = sample mass in g).

The calibration curve is used for identification and quantification. 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 %.

7.2.2 Extraction procedure

Weigh, to the nearest 0,01 g, 1 g of sample into a disposable tube (6.1). Add 250 µl of ^{13}C melamine stock solution (5.2.4) and 250 µl of ^{13}C cyanuric acid stock solution (5.2.5). Incubate for at least 1 h at ambient temperature. Add 5 ml of hot water (96 °C to 100 °C) and shake manually for 30 s to obtain a slurry. Add 5 ml of acetonitrile (5.1.2) and shake again. Mix the slurry with 30 ml of acetonitrile (5.1.2) and 10 ml of water. Shake well for 5 min and centrifuge for 10 min at 3 400g. Transfer 1,5 ml of the extract into a standard micro test tube (6.8) and centrifuge for 10 min at 7 800g. Transfer from the upper layer 1 000 µl into a LC vial (6.9) and add 50 µl of benzoguanamine standard solution II (5.3.6). Minor changes to the above protocol are allowed provided that it can be demonstrated that it will lead to the same results.

NOTE The melamine-cyanuric acid complexes can remain intact during extraction, but they dissociate as a result from formic acid in the eluent.

7.3 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.

8 System settings

8.1 HPLC parameters

The gradient parameters given 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 8.3.

Table 2 — Gradient parameters HPLC

Calibration solution number	Time min	Flow ml/min	Mobile phase A %	Mobile phase B %
1	Initial	0,25	10	90
2	8,00	0,25	10	90
3	13,00	0,25	65	35
4	14,00	0,25	90	10
5	15,00	0,25	90	10
6	15,50	0,25	10	90
7	25,00	0,25	10	90

8.2 HPLC-MS/MS parameters

During the validation of the method, a mass spectrometer of the type Micromass™ [4)] Quattro Ultima was used, and this is what the instrumental settings listed in Table 3 refer to. Other instruments from several other vendors were also used. More information is given in Reference [2]. The mass spectrometer is operated in alternating mode for positive and negative ions in three different windows. Window 1 is for the positive ions (benzoguanamine), window 2 is for the negative ions (cyanuric acid) and window 3 is for the positive ions (melamine). The window defining retention times are determined before each series by analysing a calibration solution of melamine and cyanuric acid, first in the positive mode and then in the negative mode for the duration of the LC gradient.

Optimization of the MS transition parameters can differ depending on the triple quadrupole mass spectrometer characteristics. The parameters shall be determined by infusion of melamine and cyanuric acid and optimizing the cone energy and the collision energy for the transition.

Table 3 — Source and analyser parameters for MS/MS

Source	(ES-)	(ES+)
Capillary (kV)	3,00	2,70
Cone (V)	25	35
Source temperature (°C)	120	120
Desolvation temperature (°C)	350	350
Cone gas flow (l/h)	190 to 193	190 to 193
Desolvation gas flow (l/h)	560 to 565	560 to 565
Collision cell pressure (Pa)	approximately 0,26	approximately 0,26

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4) Micromass is the trademark of a product supplied by Waters 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.

Table 3 *(continued)*

Source	**(ES-)**	**(ES+)**
Analyser		
Low mass (LM) 1 resolution	12,5	14,0
High mass (HM) 1 resolution	12,5	14,0
Ion energy 1 (V)	1,0	1,0
LM 2 resolution	12,5	13,0
HM 2 resolution	12,5	13,0
Ion energy 2 (V)	1,0	1,0
Multiplier (V)	650	650

The parameters given in Table 4 and Table 5 have shown to give successful results. Depending on the HPLC-MS/MS system, the parameters might need to be slightly adjusted. Alternatively, a UHPLC system can be used, see 8.3.

Table 4 — MRM parameters for MS/MS

Analyte	Transition	Ionization mode	Dwell time s	Inter channel delay s	Interscan time s	Cone voltage V	Collision energy eV
BENZ IS	188 > 85 188 > 104	ES+	0,2 0,2	0,01 0,01	0,1 0,1	35 35	20 25
CYA	128 > 85 128 > 42	ES-	0,2 0,2	0,01 0,01	0,1 0,1	25 25	10 15
^{13}C CYA IS	134 > 89 134 > 44	ES-	0,2 0,2	0,01 0,01	0,1 0,1	25 25	10 15
MEL	127 > 85 127 > 68	ES+	0,2 0,2	0,01 0,01	0,1 0,1	35 35	20 30
^{13}C MEL IS	133 > 89 133 > 71	ES+	0,2 0,2	0,01 0,01	0,1 0,1	35 35	20 30

Key

^{13}C CYA IS: ^{13}C cyanuric acid

^{13}C MEL IS: ^{13}C melamine

BENZ: benzoguanamine

Table 5 — HPLC parameters

Injection volume (µl)	5
Column temperature (°C)	40
Solvent delay start 1 (min)	0
Solvent delay end 1 (min)	1,5
Solvent delay start 2 (min)	15
Solvent delay end 2 (min)	23
Time MRM ES- (min)	4 to 8,5
Time MRM ES+ (min)	1,5 to 4, 8,5 to 15
Mobile phase A	water with 10 mmol/l ammonium acetate (5.4.2)
Mobile phase B	acetonitrile (5.4.3)
Column	TSKgel® Amide-80 HILIC column (6.11)

8.3 UHPLC parameters

The gradient parameters given in Table 6 have shown to give successful results. Depending on the UHPLC-MS/MS system, the parameters might need to be slightly adjusted. Alternatively, a HPLC system can be used, see 8.1.

Table 6 — Gradient parameters UHPLC (split ratio 1:1)

Calibration solution number	**Time** min	**Flow** ml/min	**Mobile phase A** %	**Mobile phase B** %
1	Initial	0,7	10	90
2	1,00	0,7	10	90
3	3,20	0,7	6,0	94
4	3,50	0,7	50	50
5	5,00	0,7	50	50
6	5,20	0,7	0	100
7	9,00	0,7	0	100

8.4 UHPLC-MS/MS parameters

During the validation of the method, a mass spectrometer of the type Micromass™ 4) Quattro Ultima was used, and this is what the instrumental settings listed in Table 7 refer to. The mass spectrometer is operated in alternating mode for positive and negative ions in two different windows. Window 1 is for the negative ions (cyanuric acid) and window 2 is for the positive ions (melamine and benzoguanamine). The window defining retention times are determined before each series by analysing a calibration solution of melamine and cyanuric acid, first in the positive mode and after that in the negative mode for the time of the gradient.

Table 7 — Source and analyser parameters for MS/MS

Source	**(ES-)**	**(ES+)**
Capillary (kV)	3,00	2,70
Cone (V)	30	35
Source temperature (°C)	120	120
Desolvation temperature (°C)	350	350
Cone gas flow (l/h)	190 to 193	190 to 193
Desolvation gas flow (l/h)	560 to 565	560 to 565
Collision cell pressure (Pa)	approximately 0,26	approximately 0,26
Analyser		
LM 1 resolution	12,5	15,0
HM 1 resolution	12,5	15,0
Ion energy 1 (V)	1,0	1,0
LM 2 resolution	12,5	15,0
HM 2 resolution	12,5	15,0
Ion energy 2 (V)	1,0	1,0
Multiplier (V)	650	650

The parameters given in Table 8 and Table 9 have shown to give successful results.

Table 8 — MRM parameters for MS/MS

Analyte	**Transition**	**Ionization mode**	**Dwell time** s	**Inter channel delay** **s**	**Interscan time** s	**Cone** V	**Collision energy** eV
CYA	128 > 85	ES-	0,05	0,01	0,03	25	10
	128 > 42		0,05	0,01	0,03	25	15
^{13}C CYA IS	134 > 89	ES-	0,05	0,01	0,03	25	10
	134 > 44		0,05	0,01	0,03	25	15
MEL	127 > 85	ES+	0,05	0,01	0,03	35	20
	127 > 68		0,05	0,01	0,03	35	30
^{13}C MEL IS	133 > 89	ES+	0,05	0,01	0,03	35	20
	133 > 71		0,05	0,01	0,03	35	30
BENZ IS	188 > 85	ES+	0,2	0,01	0,03	35	20
	188 > 104		0,2	0,01	0,03	35	25

Key

^{13}C CYA IS: ^{13}C cyanuric acid

^{13}C MEL IS: ^{13}C melamine

BENZ: benzoguanamine

Table 9 — UHPLC parameters

Injection volume (µl)	5
Column temperature (°C)	40
Time MRM ES- (min)	0 to 2
Time MRM ES+ (min)	2 to 10
Mobile phase A	mixture of water and formic acid (5.4.4)
Mobile phase B	mixture of acetonitrile and water with ammonium acetate (5.4.5)
Column	UHPLC® BEH HILIC column (6.12)

8.5 Preparation of the LC-MS/MS system and samples

8.5.1 Tuning of the LC-MS/MS system

Check the LC-MS/MS every two weeks for the correct mass calibration according to the directions provided by the manufacturer. Calibrate the system only when there is a difference observed between the measured and the theoretical mass.

8.5.2 Checking of the instrument settings

Inject 5 µl of a solution of a medium concentration and check the peak shape and retention times for melamine and cyanuric acid.

8.5.3 Checking of the sensitivity of the system

Inject 5 µl of a solution of the lowest concentration. The signal to noise ratio of melamine of transition 127 > 85 and of cyanuric acid of transition 128 > 85 shall be at least 9. If not, take appropriate action, e.g. by cleaning the cone or by changing the analytical column (bad peak shape).

8.5.4 Sample list

Analyse in each series the following samples:

- calibration solutions;
- procedure blank ($n = 1$);
- (certified) reference material at an appropriate level or a home-made reference sample;
- all samples;
- calibration solution number 3, 4 or 5 (see Table 1).

9 Calculation and expression of results

9.1 General

The calculation of results is performed generally within quantification software, often supplied with the LC-MS/MS instrument. In order to perform an accurate calculation, several aspects of the data need to be evaluated, as listed below.

- The calculations (9.2 to 9.4) will be based on peak area (not peak height).
- The retention times of all compounds of interest are checked against the pre-set retention times in the data processing method. If necessary, retention times are modified in the data processing method to allow automatic identification of compounds and processing of data. After processing of the data, every result is manually checked for correct integration. Retention times will be evaluated against the criteria in 9.3.
- The coefficient of variation of the relative response for melamine and cyanuric acid shall be less than 5 % over the calibration range. An averaged relative response factor shall be used for each compound.
- The coefficient of variation for ^{13}C MEL IS and ^{13}C CYA IS shall be less than 30 %.

9.2 Calibration criteria

Use at least a five-point calibration curve and calculate the correlation coefficient r^2. It is recommended not to force through zero.

The sample results shall fit within the calibrated concentration range. When a result is outside of the calibrated concentration range, the sample shall be appropriately diluted and reanalysed until it fits within the range.

9.3 Identification criteria

Melamine and cyanuric acid are identified on retention time, mass transition and mass transition ratio. The relative intensities of the detected mass transitions (based on peak area), expressed as a percentage of the intensity of the most intense mass transition, shall correspond to those of the calibration solution, measured under the same conditions within the following tolerances (see Reference [1]), as given in Table 6.

Table 6 — Relative tolerances

Relative intensity % of base peak	Relative tolerances
>50 %	±20 %
>20 % to 50 %	±25 %
>10 % to 20 %	±30 %
<10 %	±50 %

For each sample, the ratio of the retention time of melamine and cyanuric acid to that of the respective ^{13}C analogues shall correspond to the ratio of the retention time of melamine and cyanuric acid in the calibration solution at a tolerance of ±2,0 %.

9.4 Recovery

It shall be stressed that recovery is only monitored for the performance of the method. Recovery is for information only and recovery factors are not applied for correction of results.

The recovery is estimated from the ratio of the relative response factors of the stable isotope labelled internal standards (^{13}C MEL IS and ^{13}C CYA IS).

Calculate the recovery, R_{rec}, in %, for the used internal standard (^{13}C MEL IS) using Formulae (1) and (2):

$$R_{\text{rec}} = \frac{F_{\text{rmsa}}}{F_{\text{rmst}}} \times 100 \qquad (1)$$

$$F_{\text{rm}} = \frac{A_{\text{ism}} \times \rho_{\text{rs}}}{A_{\text{rs}} \times \rho_{\text{ism}}} \qquad (2)$$

where

F_{rmsa} is the relative response factor (F_{rm}) of mass labelled internal standard in the sample;

F_{rmst} is the relative response factor (F_{rm}) of mass labelled internal standard in the calibration solution;

A_{ism} is the area of the most intense ion of ^{13}C MEL IS in the sample;

A_{rs} is the area of the most intense ion of BENZ in the sample;

ρ_{ism} is the mass concentration of ^{13}C MEL IS, in µg/ml;

ρ_{rs} is the mass concentration of BENZ, in µg/ml.

The collected data make it possible to check a series of recovery values.

a) The matrix suppression or enhancement can be calculated from the area for BENZ in the sample extract compared to the average BENZ area of the calibration levels. The matrix suppression or enhancement should be between 60 % and 150 %.

b) The recovery percentage of the internal standard ^{13}C MEL IS in a sample can be calculated as follows. Firstly, calculate the relative area of ^{13}C MEL IS by determining the ratio between the areas of ^{13}C MEL IS and BENZ IS of the sample of interest. Then calculate the ratio for ^{13}C MEL IS and BENZ IS in a calibration solution. Finally, divide the calculated ^{13}C MEL IS/BENZ ratio in the sample by the ^{13}C MEL/BENZ ratio in the calibration solution and multiply by 100 %.

NOTE In specific cases, for example if an interferant from can lining is present in the sample, BENZ is not a suitable internal standard for recovery and can lead to an underestimation of recovery. An alternative triazine internal standard can be used, such as acetoguanamine, after being tested for applicability.

Evaluate the recovery for ^{13}C CYA IS following the same procedure as described above.

The trueness can be determined by analysis of a (certified) reference material at an appropriate level or a home-made reference sample (see 7.2.1). Additionally, the trueness may be determined by comparing the calculated result for MEL and CYA in a fortified blank sample with the theoretical value (fortified amount), expressed as a percentage. The trueness should be between 80 % and 110 %.

9.5 Calculation of results

Calculate the relative response factor, F_{rn}, for native compounds from the calibration curve, using Formula (3):

$$F_{\mathrm{rn}} = \frac{A_{\mathrm{xmc}} \times \rho_{\mathrm{ismc}}}{\rho_x \times A_{\mathrm{ismc}}} \tag{3}$$

where

A_{xmc} is the area of melamine and cyanuric acid;

A_{ismc} is the area of ^{13}C melamine and ^{13}C cyanuric acid;

ρ_{ismc} is the mass concentration of ^{13}C melamine and ^{13}C cyanuric acid, in µg/ml;

ρ_{x} is the mass concentration of native compound, in µg/ml.

Consequently, calculate the averaged relative response factor, $\overline{F_{\mathrm{rn}}}$, using Formula (4):

$$\overline{F_{\mathrm{rn}}} = \frac{1}{a} \times \sum_{i=1}^{a} F_{\mathrm{rn}} i(b) \tag{4}$$

where

a is the number of standards (concentration levels);

b is the native compound;

i is the calibration level.

Calculate the mass fraction, w_{x}, of the compound of interest using Formula (5);

$$w_x = \frac{A_{\mathrm{x}} \times \rho_{\mathrm{is}}}{A_{\mathrm{is}} \times F \times \overline{F_{\mathrm{rn}}}} \tag{5}$$

where

w_{x} is the mass fraction of melamine and cyanuric acid, in mg/kg;

A_{x} is the area of melamine and cyanuric acid in sample extracts;

A_{is} is the area of ^{13}C melamine and ^{13}C cyanuric acid in sample extracts;

ρ_{is} is the mass concentration of injected labelled internal standard, in µg/ml;

F is the calculation factor from concentration (in µg/ml) to content on sample basis (in mg/kg) = m/v, where v = the final volume, in ml, and m = the test portion, in g.

10 Precision data

10.1 General

Details of the interlaboratory test of the precision of the method are summarized in Annex A. The values derived from the interlaboratory test may not be 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 material 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.

The values for melamine are:

$\bar{x}$ = 1,26 mg/kg r = 0,183 mg/kg (infant formula milk-based)

$\bar{x}$ = 0,73 mg/kg r = 0,367 mg/kg (infant formula soy-based)

$\bar{x}$ = 1,04 mg/kg r = 0,136 mg/kg (milk powder)

$\bar{x}$ = 1,43 mg/kg r = 0,126 mg/kg (whole milk)

$\bar{x}$ = 1,06 mg/kg r = 0,435 mg/kg (soy drink)

$\bar{x}$ = 0,71 mg/kg r = 0,354 mg/kg (milk chocolate)

The values for cyanuric acid are:

$\bar{x}$ = 0,83 mg/kg r = 0,175 mg/kg (infant formula milk-based)

$\bar{x}$ = 0,87 mg/kg r = 0,292 mg/kg (infant formula soy-based)

$\bar{x}$ = 1,45 mg/kg r = 0,375 mg/kg (milk powder)

$\bar{x}$ = 0,57 mg/kg r = 0,104 mg/kg (whole milk)

$\bar{x}$ = 0,91 mg/kg r = 0,365 mg/kg (soy drink)

$\bar{x}$ = 0,96 mg/kg r = 0,365 mg/kg (milk chocolate)

10.3 Reproducibility

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

The values for melamine are:

$\bar{x}$ = 1,26 mg/kg R = 0,680 mg/kg (infant formula milk-based)

$\bar{x}$ = 0,73 mg/kg R = 0,626 mg/kg (infant formula soy-based)

$\bar{x}$ = 1,04 mg/kg R = 0,647 mg/kg (milk powder)

$\bar{x}$ = 1,43 mg/kg R = 0,453 mg/kg (whole milk)

$\bar{x}$ = 1,06 mg/kg R = 0,657 mg/kg (soy drink)

$\bar{x}$ = 0,71 mg/kg R = 0,609 mg/kg (milk chocolate)