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Milk, milk products and infant formulae — Guidelines for the quantitative determination of melamine and cyanuric acid by LC-MS/MS

Lait, produits laitiers et formules infantiles — Lignes directrices pour la détermination quantitative de la mélamine et de l'acide cyanurique par CL-SM/SM

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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.

International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 2.

The main task of technical committees is to prepare International Standards. Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

In other circumstances, particularly when there is an urgent market requirement for such documents, a technical committee may decide to publish other types of document:

— an ISO Publicly Available Specification (ISO/PAS) represents an agreement between technical experts in an ISO working group and is accepted for publication if it is approved by more than 50 % of the members of the parent committee casting a vote;

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An ISO/PAS or ISO/TS is reviewed after three years in order to decide whether it will be confirmed for a further three years, revised to become an International Standard, or withdrawn. If the ISO/PAS or ISO/TS is confirmed, it is reviewed again after a further three years, at which time it must either be transformed into an International Standard or be withdrawn.

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.

ISO/TS 15495 | IDF/RM 230 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.

Foreword

IDF (the International Dairy Federation) is a non-profit organization representing the dairy sector worldwide. IDF membership comprises National Committees in every member country as well as regional dairy associations having signed a formal agreement on cooperation with IDF. All members of IDF have the right to be represented on the IDF Standing Committees carrying out the technical work. IDF collaborates with ISO in the development of standard methods of analysis and sampling for milk and milk products.

The main task of Standing Committees is to prepare International Standards. Draft International Standards adopted by the Standing Committees are circulated to the National Committees for endorsement prior to publication as an International Standard. Publication as an International Standard requires approval by at least 50 % of IDF National Committees casting a vote.

In other circumstances, particularly when there is an urgent market requirement for such documents, a Standing Committee may decide to publish an other type of normative document which is called by IDF: *Reviewed method*. Such a method represents an agreement between the members of a Standing Committee and is accepted for publication if it is approved by at least 50 % of the committee members casting a vote. A *Reviewed method* is equal to an ISO/PAS or ISO/TS and will, therefore, also be published jointly under ISO conditions.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. IDF shall not be held responsible for identifying any or all such patent rights.

ISO/TS 15495 | IDF/RM 230 was prepared by the International Dairy Federation (IDF) and Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 5, *Milk and milk products*. It is being published jointly by IDF and ISO.

All work was carried out by a Joint Project Group on *Determination of melamine* of the Standing Committee on *Analytical methods for additives and contaminants* under the aegis of its project leaders, Dr S.E. Holroyd (NZ) and Dr T. Delatour (CH).

Milk, milk products and infant formulae — Guidelines for the quantitative determination of melamine and cyanuric acid by LC-MS/MS

1 Scope

This Technical Specification gives guidance for the quantitative determination of melamine and cyanuric acid content in milk, powdered milk products, and infant formulae by electrospray ionization liquid chromatography tandem mass spectrometry (LC-MS/MS).

NOTE Examples of LC-MS/MS methods are given in Annexes A and B.

2 Normative references

The following referenced documents are indispensable for the application 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 3534 (all parts), *Statistics — Vocabulary and symbols*

ISO 5725-1, *Accuracy (trueness and precision) of measurement methods and results — Part 1: General principles and definitions*

3 Terms and definitions

For the purposes of this document, the terms and definitions given in ISO 3534, ISO 5725-1, and the following apply.

3.1
melamine content
mass fraction of substance determined by the procedures specified in this Technical Specification

NOTE The melamine content is expressed in milligrams per kilogram of product.

3.2
cyanuric acid content
mass fraction of substance determined by the procedures specified in this Technical Specification

NOTE The cyanuric content is expressed in milligrams per kilogram of product.

3.3
maximum limit
ML
maximum content of either melamine or cyanuric acid permitted or acceptable in milk, powdered milk products or infant formulae

NOTE The ML can either be a nationally or internationally stated limit or a defined level of concern.

4 Principle

The sample is made homogenous and optionally reconstituted in the case of powdered samples. A suitable solvent is added to the test sample to precipitate proteins from the matrix and to extract melamine and cyanuric acid. After centrifugation, an aliquot of the supernatant is analysed by LC-MS/MS.

For the purposes of this Technical Specification, LC-MS/MS designates any method combining either high-performance liquid chromatography (HPLC) or ultra-performance liquid chromatography (UPLC), with either triple quadrupole or ion-trap mass spectrometric detection. Chromatographic separation is based on hydrophilic interaction liquid chromatography (HILIC) to ensure good separation of melamine and cyanuric acid. Ionization of the substance is accomplished by electrospray ionization (ESI) and detection operates in the selected reaction monitoring (SRM) mode.

NOTE Other ionization techniques with sufficient performance can be used. Other mass analysers can be used as long as they comply with the performance criteria (identification points) in 2002/657/EC (Reference [8]).

Quantification of both melamine and cyanuric acid is based on isotope dilution using stable isotope internal standards for both analytes.

5 Sampling

Sampling is not part of the method specified in this Technical Specification. A recommended sampling method is given in ISO 707 | IDF 50[1].

It is important the laboratory receive a truly representative sample which has not been damaged or changed during transport or storage.

6 Preparation of test sample

The mass of the test sample for analysis or optional reconstitution should be representative of the product and should be appropriate to allow effective extraction of the target analytes.

Isotope-labelled standards should be spiked directly to the dry or wet sample in the first step of analysis.

Concentration of internal standards added should be of the same order of magnitude as that of interest for melamine and cyanuric acid. Extraction may be done with a minimum of 5 ml of extraction solvent per gram of sample material, to give a final percentage mass fraction of organic solvent in the extraction mixture of at least 70 %. Extraction should take place for at least 5 min by either vigorous shaking or a combination of ultrasonification and vortexing. The sample should then be centrifuged under appropriate conditions and filtered through a syringe filter.

Any alternative is acceptable as long as performance criteria and validation match the requirements specified in this Technical Specification.

7 Procedure

7.1 LC-MS/MS analysis — Chromatography

Perform the liquid chromatographic separation using a column dedicated to this purpose.

The minimum acceptable retention time for melamine and cyanuric acid shall be twice the retention time corresponding to the void volume of the column. The retention time in the sample extract shall match that of the calibration standard within a specified retention time window.

The ratio of the relative retention time of the analyte to that of the internal standard shall correspond to that of calibration standards to within ±2,5 %.

7.2 LC-MS/MS analysis — Mass spectrometry

Mass spectrometric detection of melamine and cyanuric acid shall be carried out preferably by employing a triple quadropole LC-MS/MS instrument.

Quantitative determination and identification shall be achieved in SRM mode. When using a triple quadropole instrument for each compound, a minimum of two mass transitions shall be used for the detection of the analyte, while at least one shall be used for the internal standard. However, it is also recommended that two mass transitions be monitored for the internal standard.

The most appropriate transition (most intense) shall be used for quantification (quantifier), and the second one for confirmation (qualifier). The ratio of qualifier intensity to quantifier intensity (Rq/Q) shall be checked and tolerance criteria applied depending on the value of Rq/Q (Table 1).

Table 1 — Maximum permitted relative tolerance for ratio of qualifier intensity to quantifier intensity (Rq/Q)

Rq/Q (% of quantifer)	**Tolerance** (relative in %)
>50	±20
>20 to 50	±25
>10 to 20	±30
⩽10	±50

8 Performance criteria

8.1 General

Each laboratory should validate the implemented method to demonstrate fitness for purpose. The procedure specified in EU Commission Decision 2002/657/EC (Reference [8]) is recommended for validation. Maximum and minimum limits for performance characteristics, as adopted from this procedure, are defined in 8.2 and 8.5.

8.2 Minimum required sensitivity

The required limit of quantification (LoQ) should at least be 10-fold lower than the maximum limit (ML) to ensure reliable quantification at ML.

8.3 Trueness and recovery

Following this guideline, trueness and recovery are typically between 95 % and 105 %. For acceptance of analytical methods, trueness and recovery shall fall between 80 % and 110 %.

8.4 Repeatability

The coefficient of variation of repeatability is typically 3 % to 6 %, with limit <15 %.

8.5 Within-laboratory reproducibility

The coefficient of variation of within-laboratory reproducibility is typically 5 % to 10 %, with limit <15 %.

9 Test report

The test report shall contain at least the following information:

a) all information necessary for the complete identification of the sample;

b) the sampling method used, if known;

c) the test method used, together with reference to this Technical Specification (ISO/TS 15495 | IDF/RM 230:2010);

d) all operating details not specified in this Technical Specification, or regarded as optional, together with details of any incidents which may have influenced the test result(s);

e) the test result(s) obtained;

f) if the repeatability has been checked, the final quoted result obtained.

Annex A
(informative)

Example A — Cow milk and milk-based infant formula — Simultaneous quantitative determination of melamine and cyanuric acid by liquid chromatography electrospray ionization tandem mass spectrometry

A.1 Scope

This annex specifies an in-house validated method for the quantitative determination of melamine and cyanuric acid contents in cow milk (CM) and milk-based powdered infant formula (PIF) by electrospray ionization liquid chromatography tandem mass spectrometry (LC-MS/MS) using the selected reaction monitoring (SRM) mode.

The positive identification of melamine and cyanuric acid in the sample is conducted according to the confirmation criteria defined in EU Commission Decision 2002/657/EC (Reference [8]).

The quantification is performed by an isotopic dilution approach using labelled ($^{13}C_3$,$^{15}N_3$)-melamine and labelled ($^{13}C_3$,$^{15}N_3$)-cyanuric acid as internal standards (ISs). For technical details, validation data and proficiency-test results, see Reference [10].

The method allows an accurate quantification at the following reporting limits and higher:

a) melamine and cyanuric acid contents in CM at levels of 0,05 mg/kg and 0,10 mg/kg, respectively.

b) melamine and cyanuric acid contents in PIF at levels of 0,05 mg/kg and 0,10 mg/kg, respectively.

c) melamine and cyanuric acid contents in PIF at a level of 1,00 mg/kg.

A.2 Terms and definitions

See Clause 3.

A.3 Principle

The analytical procedure encompasses a dilution of the sample in an acetonitrile:water solution, leading to the precipitation of proteins and allowing melamine and cyanuric acid to be extracted at the same time. After centrifugation, the supernatant is analysed by LC-MS/MS in SRM mode, operating in both positive and negative ionization mode. The polarity switching is done within the same run.

A.4 Reagents

Use only reagents of recognized analytical grade, unless otherwise specified, and distilled or demineralized water or water of equivalent purity.

NOTE CAS numbers are given for each reagent.

Before using chemicals, refer to adequate manuals or safety data sheets approved by your local authorities.

A.4.1 Water, for chromatography use (CAS No. 7732-18-5).

A.4.2 Acetonitrile (CH_3CN), gradient grade for liquid chromatography (CAS No. 75-05-8).

A.4.3 Ammonium acetate (CH_3COONH_4), GR for analysis (CAS No. 631-61-8).

A.4.4 Cyanuric acid $(CNOH)_3$, >98 % mass fraction (CAS No. 108-80-5).

A.4.4.1 Cyanuric acid stock standard solution, corresponding to 0,25 mg of cyanuric acid per millilitre.

Weigh, to the nearest 0,1 mg, 63,8 mg of cyanuric acid (A.4.4) into a 250 ml one-mark volumetric flask (A.5.12), making appropriate correction for any impurity as per the certificate of analysis. Dissolve and make up to the mark with water (A.4.1). Sonificate for at least 15 min until complete dissolution.

Store the cyanuric acid stock standard solution at room temperature away from light. Prepare a fresh stock standard solution weekly.

A.4.4.2 Cyanuric acid working solution I, corresponding to 20 µg of cyanuric acid per millilitre.

Pipette 4 ml of the cyanuric acid stock standard solution (A.4.4.1) into a 50 ml one-mark volumetric flask (A.5.12). Make up to the mark with water (A.4.1) and mix. Store the cyanuric acid working solution I at room temperature away from light. Prepare a fresh working solution I weekly.

A.4.4.3 Cyanuric acid working solution II, corresponding to 2 µg of cyanuric acid per millilitre.

Pipette 1 ml of the cyanuric acid working solution I (A.4.4.2) into a 10 ml one-mark volumetric flask (A.5.12). Make up to mark with water (A.4.1) and mix. Store the cyanuric acid working solution II at room temperature away from light. Prepare a fresh working solution II weekly.

A.4.4.4 Cyanuric acid working solution III, corresponding to 200 ng of cyanuric acid per millilitre.

Pipette 0,5 ml of the cyanuric acid working solution I (A.4.4.2) into a 50 ml one-mark volumetric flask (A.5.12). Make up to mark with water (A.4.1) and mix. Store cyanuric acid working solution III at room temperature away from light. Prepare a fresh working solution III weekly.

A.4.5 Labelled cyanuric acid ($^{13}C_3H_3{}^{15}N_3O_3$), isotopic purity: $^{13}C_3 = 99$ %; ring $^{15}N_3 > 98$ %; chemical purity 90 % mass fraction, 100 µg/ml in water – 1,2 ml.

A.4.5.1 Labelled cyanuric acid stock standard solution, corresponding to 100 µg of labelled cyanuric acid per millilitre.

Ready-to-use labelled cyanuric acid stock standard solution is commercially available. Store at room temperature away from light.

Use the same batch of IS for both making the calibration standard solutions in A.6.2 and spiking the extracts as described in the extraction procedure (A.9.1).

A.4.5.2 Labelled cyanuric acid working solution I, corresponding to 20 µg of labelled cyanuric acid per millilitre.

Pipette 1 ml of the labelled cyanuric acid stock standard solution (A.4.5.1) into a 5 ml one-mark volumetric flask (A.5.12). Make up to mark with water (A.4.1) and mix. Store the labelled cyanuric acid working solution I at room temperature away from light.

A.4.5.3 Labelled cyanuric acid working solution II, corresponding to 2 µg of labelled cyanuric acid per millilitre.

Pipette 0,5 ml of the labelled cyanuric acid working solution I (A.4.5.2) into a 5 ml one-mark volumetric flask (A.5.12). Make up to mark with water (A.4.1) and mix. Store the labelled cyanuric acid working solution II at room temperature away from light.

A.4.5.4 Labelled cyanuric acid working solution III, corresponding to 200 ng of labelled cyanuric acid per millilitre.

Pipette 0,5 ml of the labelled cyanuric acid working solution I (A.4.5.2) into a 50 ml one-mark volumetric flask (A.5.12). Make up to mark with water (A.4.1) and mix. Store the labelled cyanuric acid working solution III at room temperature away from light.

A.4.6 Melamine ($C_3H_6N_6$), >99 % mass fraction (CAS No. 108-78-1).

A.4.6.1 Melamine stock standard solution, corresponding to 0,25 mg of melamine per millilitre.

Weigh, to the nearest 0,1 mg, 62,5 mg of melamine (A.4.6) into a 250 ml one-mark volumetric flask (A.5.12). Dissolve and make up to the mark with water (A.4.1). Sonificate for at least 15 min until complete dissolution. Store at room temperature away from light. Prepare a fresh stock solution weekly.

A.4.6.2 Melamine working solution I, corresponding to 20 µg of melamine per millilitre.

Pipette 4 ml of the melamine stock standard solution (A.4.6.1) into a 50 ml one-mark volumetric flask (A.5.12). Make up to mark with water (A.4.1) and mix. Store the melamine working solution I at room temperature away from light. Prepare a fresh working solution I weekly.

A.4.6.3 Melamine working solution II, corresponding to 2 µg of melamine per millilitre.

Pipette 1 ml of the melamine working solution I (A.4.6.2) into a 10 ml one-mark volumetric flask (A.5.12). Make up to mark with water (A.4.1) and mix. Store the melamine working solution II at room temperature away from light. Prepare a fresh working solution II weekly.

A.4.6.4 Melamine working solution III, corresponding to 200 ng of melamine per millilitre.

Pipette 0,5 ml of the melamine working solution I (A.4.6.2) into a 50 ml one-mark volumetric flask (A.5.12). Make up to mark with water (A.4.1) and mix. Store the melamine working solution III at room temperature away from light. Prepare a fresh working solution weekly.

A.4.7 Labelled melamine [$^{13}C_3N_3(^{15}NH_2)_3$], isotopic purity: $^{13}C_3 = 99$ %; amino- $^{15}N_3 = 98$ %; chemical purity ⩾98 % mass fraction, 100 µg/ml water –1,2 ml, e.g. CNLM-8150-1.2[1)].

A.4.7.1 Labelled melamine stock solution, corresponding to 10 µg of labelled melamine per millilitre.

Labelled melamine stock solution is available commercially. Store at room temperature away from light. Use the same batch of the IS for both making the calibration solutions in A.6.2 and spiking the extracts as described in the extraction procedure (A.9.1).

A.4.7.2 Labelled melamine working solution I, corresponding to 20 µg of labelled melamine per millilitre.

Pipette 1 ml of the labelled melamine stock solution (A.4.7.1) into a 5 ml one-mark volumetric flask (A.5.12). Make up to mark with water (A.4.1) and mix. Store the labelled melamine working solution I at room temperature away from light.

A.4.7.3 Labelled melamine working solution II, corresponding to 2 µg of labelled melamine per millilitre.

Pipette 0,5 ml of the labelled melamine working solution I (A.4.7.2) into a 5 ml one-mark volumetric flask (A.5.12). Make up to mark with water (A.4.1) and mix. Store the labelled melamine working solution II at room temperature away from light.

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1) CNLM-8150-1.2 is the trade name of a product supplied by Cambridge Isotope Laboratories. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO or IDF of this product. Equivalent products may be used if they can be shown to lead to the same results.

A.4.7.4 Labelled melamine working solution III, corresponding to 200 ng of labelled melamine per millilitre.

Pipette 0,5 ml of the labelled melamine working solution I (A.4.7.2) into a 50 ml one-mark volumetric flask (A.5.12). Make up to mark with water (A.4.1) and mix. Store the labelled melamine working solution III at room temperature away from light.

A.5 Apparatus

Glassware (e.g. volumetric flasks, A.5.12) used for the preparation of the standard stock and working solutions can be contaminated, especially with melamine, and should be cleaned by the following procedure:

a) use warm tap water and laboratory detergent for manual washing of glassware;

b) manually clean glassware using a laboratory brush;

c) thoroughly rinse all glassware including stoppers with warm tap water followed by three rinses with distilled water;

d) rinse the glassware with ethanol and allow the glassware to dry in air.

Use usual laboratory apparatus and, in particular, the following.

A.5.1 Conical tubes, polypropylene, of capacity 50 ml.

A.5.2 Centrifuge, equipped with a rotor, adapted for tubes of 50 ml, with acceleration of 4 000*g*.

A.5.3 Volumetric pipettes, of capacities 0,5 ml, 1 ml, and 4 ml, ISO 835[2], class A (or AS).

A.5.4 Automatic micropipette, ISO 8655-2[6], accurate, precise, and calibratable by the user.

A.5.5 Nylon syringe filter, of porosity 0,22 µm.

A.5.6 Degassing system (if not part of the LC system).

A.5.7 Analytical balance, capable of being read to the nearest 0,1 mg.

A.5.8 Sonification device.

A.5.9 Vials.

A.5.10 LC-MS/MS equipment.

HPLC Agilent 1100[2)] coupled to a 4000 QTrap[3)] triple stage quadrupole mass spectrometer equipped with a TurboIon Spray® ionization source[3)].

2) HPLC Agilent 1100 is the trade name of a product supplied by Agilent. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO or IDF of this products. Equivalent products may be used if they can be shown to lead to the same results.

3) 4000 QTrap and Turbulon Spray are trade names of products supplied by Applied Biosystems. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO or IDF of these products. Equivalent products may be used if they can be shown to lead to the same results.

A.5.11 HPLC Column, TSKgel Amide-80[4)], diameter 2,0 mm, length 250 mm, 5 µm ID.

A.5.12 One-mark volumetric flasks, of capacities 5 ml, 10 ml, 50 ml, 100 ml, 250 ml, and 1 000 ml, ISO 1042[3], class A.

A.6 Preparation of reagents

A.6.1 Acetonitrile and water solution (ratio 70+30)

Transfer using measuring cylinders, 70 ml of acetonitrile (A.4.2) and 30 ml of water (A.4.1) into a 100 ml one-mark volumetric flask (A.5.12) and mix. Store at room temperature for no longer than 1 week.

A.6.2 Calibration curves for melamine and cyanuric acid

A.6.2.1 Melamine and cyanuric acid standard solutions (0 mg/l to 2,00 mg/l) with corresponding ISs (at 1,00 mg/l level)

Prepare separately five mixed standard solutions, each in a 5 ml one-mark volumetric flask (A.5.12) in a concentration range from 0 mg/l to 2,00 mg/l and the corresponding ISs added at a 1,00 mg/l level as shown in Table A.1.

Store the five standard solutions obtained at 4 °C away from light.

Table A.1 — Melamine and cyanuric acid standard mixtures 1 to 5

	Standard solution				
	1	2	3	4	5
Melamine working solution II (A.4.6.3), µl	0	25	50	75	100
Cyanuric acid working solution II (A.4.4.3), µl	0	25	50	75	100
Labelled melamine working solution II (A.4.7.3), µl	50	50	50	50	50
Labelled cyanuric acid working solution II (A.4.5.3), µl	50	50	50	50	50
Acetonitrile-water solution (A.6.1), µl	4 900	4 850	4 800	4 750	4 700
This corresponds to:					
Concentration of total analytes, ng/ml	0	10	20	30	40
Concentration of total IS, ng/ml	20	20	20	20	20
Content of analytes (equivalent in sample), mg/kg	0,00	0,50	1,00	1,50	2,00
Content of ISs (equivalent in sample), mg/kg	1,00	1,00	1,00	1,00	1,00

A.6.2.2 Standard solutions for melamine (0 mg/l to 0,20 mg/l) and cyanuric acid (0 mg/l to 0,30 mg/l) with corresponding ISs (at 0,10 mg/l level)

Prepare separately five mixed standard solutions, each in a 5 ml one-mark volumetric flask (A.5.12), in a concentration range for melamine from 0 mg/l to 0,20 mg/l and for cyanuric acid from 0 mg/l to 0,30 mg/l with the corresponding IS for both analytes added at a 0,10 mg/l level as shown in Table A.2.

Store the standard solutions obtained at 4 °C away from light.

4) TSKgel Amide-80 is the trade name of a product supplied by Tosoh Bioscience. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO or IDF of this product. Equivalent products may be used if they can be shown to lead to the same results.

Table A.2 — Melamine and cyanuric acid standard mixtures 6 to 10

	Standard solution				
	6	7	8	9	10
Melamine working solution III (A.4.6.4), µl	0	25	50	75	100
Cyanuric acid working solution III (A.4.4.4), µl	0	50	75	100	150
Labelled melamine working solution III (A.4.7.4), µl	50	50	50	50	50
Labelled cyanuric acid working solution III (A.4.5.4), µl	50	50	50	50	50
Acetonitrile-water solution (A.6.1), µl	4 900	4 825	4 775	4 725	4 650
This corresponds to:					
Concentration of melamine, ng/ml	0	1	2	3	4
Concentration of cyanuric acid, ng/ml	0	2	3	4	6
Concentration of IS (melamine or cyanuric acid), ng/ml	2	2	2	2	2
Content of melamine (equivalent in sample), mg/kg	0,00	0,05	0,10	0,15	0,20
Content of cyanuric acid (equivalent in sample), mg/kg	0,00	0,10	0,15	0,20	0,30
Content of IS (melamine and cyanuric acid, equivalent in sample), mg/kg	0,10	0,10	0,10	0,10	0,10

A.6.3 Solutions for liquid chromatography

A.6.3.1 **Mobile phase A**: aqueous ammonium acetate solution, 10 mmol/l.

Transfer 0,77 g of ammonium acetate (A.4.3) into a 1 000ml one-mark volumetric flask (A.5.12). Dissolve with ~50 ml of water (A.4.1). Make up to the mark with water and mix. Filter the solution over a 0,22 µm filter.

A.6.3.2 **Mobile phase B**: acetonitrile.

Use HPLC grade acetonitrile (A.4.2).

A.7 Sampling

Sampling is not part of the method specified in this annex of this Technical Specification. A recommended sampling method is given in ISO 707 | IDF 50[1].

It is important the laboratory receive a truly representative sample which has not been damaged or changed during transport or storage.

Store raw milk at 4 °C or frozen, if the analysis cannot be conducted immediately upon receipt.

A.8 Preparation of test samples

A.8.1 Milk-based powdered infant formula

Arrange for a homogenous laboratory sample by transferring it into a sample container of capacity about twice that of the laboratory sample volume. Close the container immediately.

Mix thoroughly by repeatedly shaking and inverting the container.

A.8.2 Cow milk

Bring the test sample to room temperature. Ensure that the sample is homogenous before weighing the test portion by gently inverting it a number of times.

A.9 Procedure

A.9.1 Extraction procedure

Weigh, to the nearest 0,01 g, 1 g of CM (A.8.2) or PIF (A.8.1) into a 50 ml conical tube (A.5.1).

a) For up to 2 mg/kg content fraction levels of melamine and cyanuric acid with both ISs added at the 1 mg/kg level, spike the test portion with 50 µl of labelled melamine working solution I (A.4.7.2) and 50 µl of labelled cyanuric acid working solution I (A.4.5.2). Mix thoroughly until the spiked volumes are totally absorbed by the matrix.

b) For up to 0,2 mg/kg content levels of melamine and up to 0,3 mg/kg content levels of cyanuric acid with both ISs added at the 0,1 mg/kg level, spike the test portion with 50 µl of labelled melamine working solution II (A.4.7.3) and with 50 µl of labelled cyanuric acid working solution II (A.4.5.3). Mix thoroughly until the spiked volumes are totally absorbed by the matrix.

Then add 5 ml of water (A.4.1) to the conical tube as prepared in either a) or b). Mix, ensuring no clumps remain in the sample. Add 5 ml of acetonitrile (A.4.2). Shake vigorously for at least 1 min.

Add 30 ml of acetonitrile (A.4.2) and 10 ml of water (A.4.1). Shake vigorously for at least 5 min, ensuring no clumps remain in the sample. Ideally, use an automated shaker. Centrifuge the obtained solution at 4 000g at room temperature for 10 min to obtain a clear supernatant.

If the supernatant is not clear, transfer an aliquot into another tube and centrifuge again at 8 500g at room temperature for 10 min using a benchtop centrifuge.

Transfer between 0,5 ml and 1 ml of the clear supernatant into an HPLC vial. Proceed with LC-MS/MS analysis (A.9.3).

A.9.2 Reagent blank

In order to compensate, if necessary, for any contamination with melamine and cyanuric acid during the sample workup, use a reagent blank made without adding the matrix. Analyse the reagent blank along with each series of routine samples.

A.9.3 LC-MS/MS analysis

A.9.3.1 HPLC conditions

Mobile phase A:	Ammonium acetate solution (A.6.3.1)
Mobile phase B:	Acetonitrile (A.6.3.2).
Injection volume:	5 µl
Column:	TOSOH TSKgel Amide-80, diameter 2,0 mm, length 250 mm, internal diameter 5 µm
Temperature:	Room temperature
Flow rate:	0,25 ml/min
Gradient:	LC gradient as described in Table A.3
Diverter valve:	HPLC flow is directed into the MS detector for 3,0 min to 13,5 min

Table A.3 — LC gradient used for the analysis of melamine and cyanuric acid

Time min	A %	B %
0	10	90
8	10	90
13	65	35
14	90	10
15	90	10
15,5	10	90
25	10	90

Under these conditions, the retention time of cyanuric acid is ~6,4 min and that of melamine is ~11,5 min (see Figures A.1 to A.3).

A.9.3.2 MS parameters

MS parameters are obtained by syringe-infusing at a flow rate of 5 µl/min separately melamine working solution I (A.4.6.2) and cyanuric acid working solution I (A.4.4.2), both with concentration of 20 µg/ml, into the HPLC flow at 0,25 ml/min using a T connector. The HPLC flow is constituted of 50 % mobile phase A (A.6.3.1) and 50 % mobile phase B (A.6.3.2). Tables A.4 to A.6 give the instrumental operating conditions for the analysis of melamine and cyanuric acid.

Table A.4 — Typical MS parameters for melamine

Parameter	Applied Biosystems Sciex 4000 QTrap
Ionization type	Electrospray (ESI)
Spray voltage	3,5 kV
Polarity	Positive ionization
Source block temperature	500 °C
Gas	Curtain gas: 69 kPa (10 psi) Ion source gas 1: 207 kPa (30 psi) Ion source gas 2: 207 kPa (30 psi)
Declustering potential (DP)	60 V
Entrance potential (EP)	10 V
Collision exit potential (CXP)	15 V
CID gas pressure (SRM)	0,8 Pa (6 mTorr), nitrogen
Peak width (for each transition)	0,5 Th
Resolution	Unit (both quadrupoles)
Scan time (for each transition)	100 ms
Negative/positive switching at T	8 min

Table A.5 — Typical MS parameters for cyanuric acid

Parameter	Applied Biosystems Sciex 4000 QTrap
Ionization type	Electrospray (ESI)
Spray voltage	−3,5 kV
Polarity	Negative ionization
Source block temperature	500 °C
Gas	Curtain gas: 69 kPa (10 psi) Ion source gas 1: 207 kPa (30 psi) Ion source gas 2: 207 kPa (30 psi)
Declustering potential (DP)	−40 V
Entrance potential (EP)	−10 V
Collision exit potential (CXP)	−15 V
CID gas pressure (SRM)	0,8 Pa (6 mTorr), nitrogen
Peak width (for each transition)	0,7 Th
Resolution	Unit (both quadrupoles)
Scan time (for each transition)	100 ms
Negative/positive switching at T	8 min

Table A.6 — Transition reactions monitored for the analysis of melamine and cyanuric acid and their corresponding IS and peak area ratios along with their limit of acceptance according to EU Commission Decision 2002/657/EC (Reference [8])

Analyte	Transition reactions (m/z)[a] for:		Peak area ratio ± limit (%)
	Quantification	Analyte confirmation	
Melamine	127,0 → 85,1 (26)	127,0 → 68,0 (45)	0,28 ± 25
Labelled melamine [$^{13}C_3N_3(^{15}NH_2)_3$] (IS)	133,0 → 89,1 (26)	133,0 → 71,1 (45)	0,19 ± 25
Cyanuric acid	128,0 → 42,1 (30)	128,0 → 85,2 (14)	0,55 ± 20
Labelled cyanuric acid ($^{13}C_3H_3{}^{15}N_3O_3$) (IS)	134,0 → 44,1 (30)	134,0 → 88,9 (14)	0,52 ± 20

[a] Collision energies, in electron volts, are given in parentheses.

NOTE Depending on the MS detector used, the peak area ratios from the diagnostic transition reactions can differ from those given in Table A.6.

A.9.3.3 Instrument check tests

Ensure that the LC-MS/MS apparatus is working in such conditions that the method remains fit for purpose. This involves injecting of a low concentration calibrant (e.g. standard solution 7, Table A.2) to check whether the sensitivity of the instrument is adequate.

A.9.4 Calibration procedure and sample analyses

Melamine and cyanuric acid are quantified by means of external calibrations. Inject the standard solutions (A.6.2.1 or A.6.2.2) along with each series of routine samples.

A.9.5 Confirmation

Melamine and cyanuric acid are considered to be positively identified in the sample when all confirmation criteria a) to c), as defined in EU Commission Decision 2002/657/EC (Reference [8]), are fulfilled.

a) A signal is visible at the two diagnostic transition reactions selected for each analyte and at the two diagnostic transition reactions selected for its corresponding IS. The signal-to-noise ratio for each diagnostic ion shall be $\geqslant$3:1.

b) The ratio of the chromatographic retention time of the analyte to that of the corresponding IS, i.e. the relative retention time of the analyte, corresponds to that of the averaged retention time of the calibration solutions within a ±2,5 % tolerance.

c) The peak area ratio from the different transition reactions recorded for each analyte is within the tolerances fixed by the EU Commission Decision 2002/657/EC criteria (Reference [8]), as shown in Table A.6.

A.10 Calculation and expression of results

A.10.1 Calculations

Appropriate software enables the construction of a customized template, which then serves to check the linearity of calibration curves, to calculate the peak area ratios obtained from the different transition reactions (as defined in Table A.6), to confirm the presence or absence of the analytes, and to give the final results, expressed in milligrams per kilogram.

NOTE The calibration curve is of the type:

$$\alpha = bw + a$$

where

α is an area ratio;

b is the slope;

w is a mass fraction;

a is the intercept.

Calculate the analyte content, w_a, in milligrams per kilogram (where the analyte is melamine, $w_a = w_m$; where it is cyanuric acid, $w_a = w_c$), using the following equation:

$$w_a = \frac{(A_a / A_{IS}) - a}{b} \times \frac{m_{IS}}{m_a \times 1\,000}$$

where

A_a is the peak area of the analyte in the sample;

A_{IS} is the peak area of the IS in the sample;

a is the intercept of the regression line for the transition reaction used for quantification;

b is the slope of the regression line for the transition reaction used for quantification;

m_{IS} is the mass, in nanograms, of IS added to the test portion (A.9.1);

m_a is the mass, in grams, of the test portion (A.9.1).

A.10.2 Expression of results

Express the results for the analyte(s) to two decimal places.

A.11 Performance characteristics

A.11.1 Linearity

The ranges of linearity tested are listed against a) and b).

a) Melamine: 0 mg/kg to 0,20 mg/kg and 0 mg/kg to 2,00 mg/kg (content equivalent in samples) respectively.

b) Cyanuric acid: 0 mg/kg to 0,30 mg/kg and 0 mg/kg to 2,00 mg/kg (content equivalent in samples) respectively.

The calibration follows a linear model, the residuals are evenly distributed, and the response factors are stable in the mass fraction ranges considered.

A.11.2 Limits of detection

The LoDs (signal-to-noise ratio equals 3) are 0,03 mg/kg for melamine and 0,05 mg/kg for cyanuric acid, respectively, in both CM and PIF.

A.11.3 Limits of quantitation

The LoQs are 0,05 mg/kg for melamine and 0,10 mg/kg for cyanuric acid, respectively, in both CM and PIF.

A.11.4 Decision limits and detection capabilities in PIF

Assuming a maximum limit (ML) of 1 mg/kg in PIF, the decision limits, w_{CC_α}, and detection capabilities, w_{CC_β}, are as listed against a) and b).

a) For melamine: w_{CC_α} = 1,03 mg/kg; w_{CC_β} = 1,05 mg/kg.

b) For cyanuric acid: w_{CC_α} = 1,04 mg/kg; w_{CC_β} = 1,09 mg/kg.

A.11.5 Recovery

For both melamine and cyanuric acid, internal standard-corrected recoveries were in the range 99 % to 116 %.

A.11.6 Precision

The values for relative repeatability limit and relative within-laboratory reproducibility limits were obtained from small interlaboratory studies based on ISO 5725-1 and ISO 5725-2[5], but not fulfilling all requirements.

A.11.6.1 Relative repeatability limit

Typical relative repeatability values obtained using the procedure described in this method are given in Table A.7.

Table A.7 — Typical relative repeatability values

Analyte	Matrix	Relative repeatability value %	Content range mg/kg
Melamine	CM	6	0,05 to 0,15
	PIF	12	0,05 to 0,15
	PIF	4	0,5 to 1,5
Cyanuric acid	CM	8	0,1 to 0,2
	PIF	11	0,1 to 0,2
	PIF	5	0,5 to 1,5

A.11.6.2 Relative within-laboratory reproducibility limit

Typical relative within-laboratory reproducibility values obtained using the procedure described in this method are given in Table A.8.

Table A.8 — Typical relative within-laboratory reproducibility values

Analyte	Matrix	Relative within-laboratory reproducibility %	Content range mg/kg
Melamine	CM	13	0,05 to 0,15
	PIF	15	0,05 to 0,15
	PIF	6	0,5 to 1,5
Cyanuric acid	CM	10	0,1 to 0,2
	PIF	31	0,1 to 0,2
	PIF	13	0,5 to 1,5

A.12 Sample figures

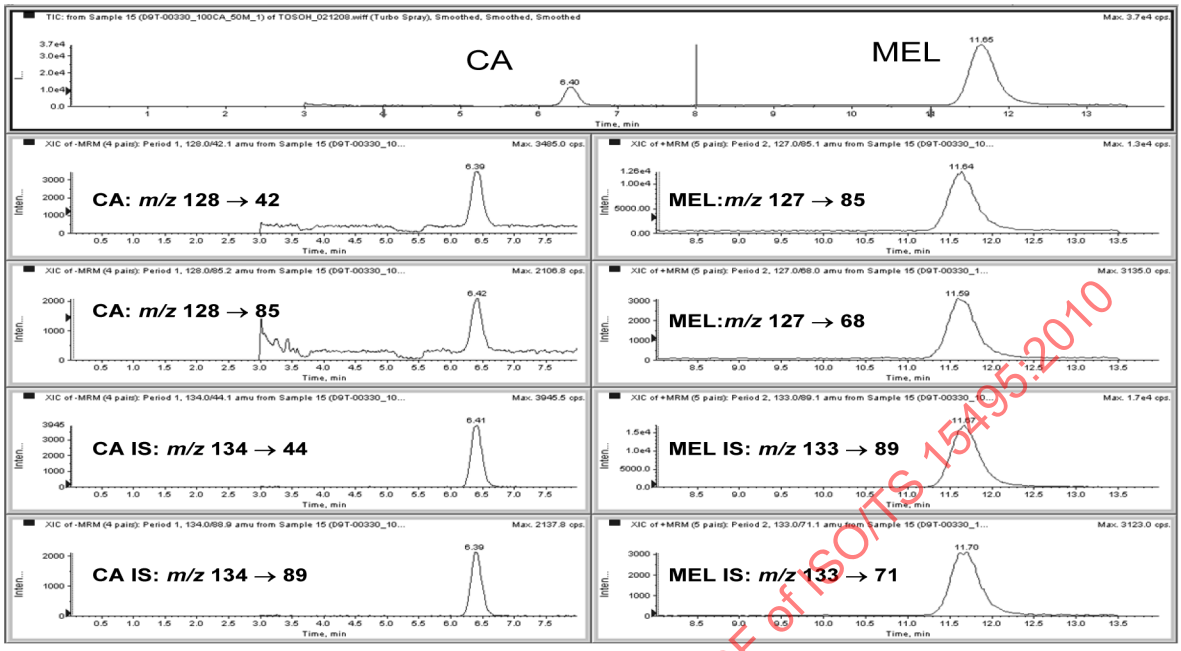

Figure A.1 — Example of LC-MS/MS chromatograms of melamine (MEL) and cyanuric acid (CA) from an extract of spiked cow milk with spiking levels: MEL = 0,05 mg/kg (IS = 0,1 mg/kg); CA = 0,10 mg/kg (IS = 0,1 mg/kg)

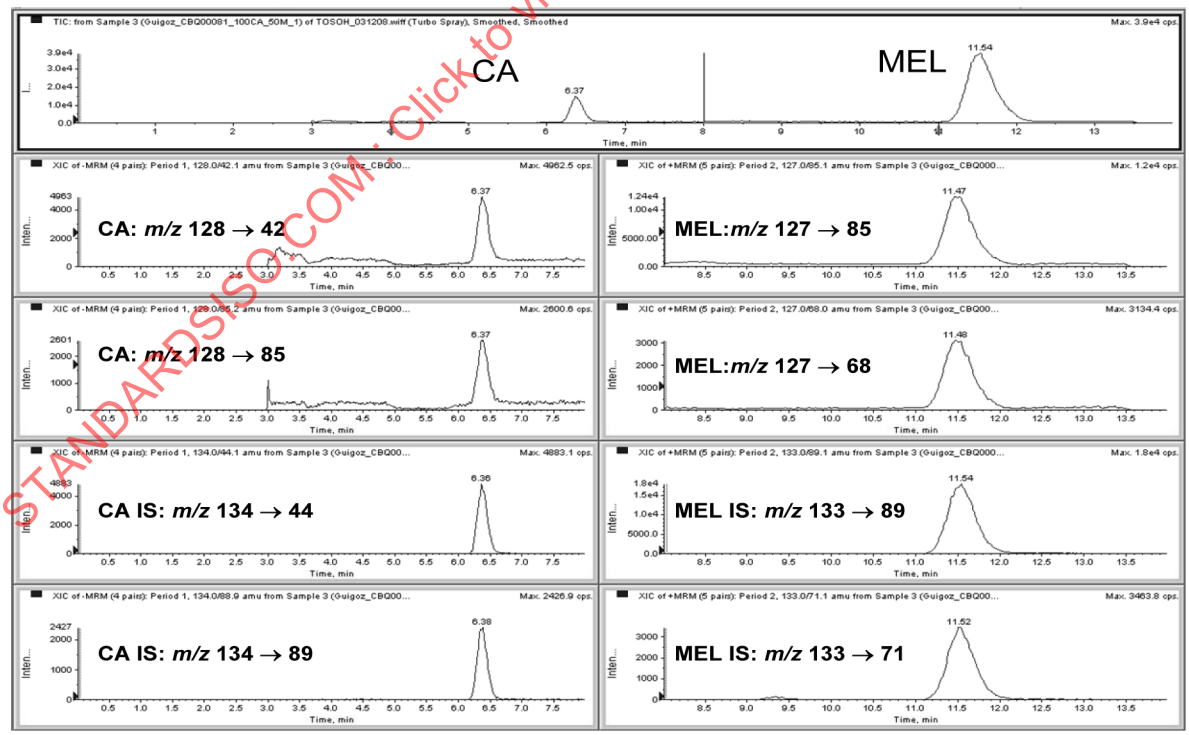

Figure A.2 — Example of LC-MS/MS chromatograms of melamine (MEL) and cyanuric acid (CA) from an extract of spiked milk-based powdered infant formula with spiking levels: MEL 0,05 mg/kg (IS 0,1 mg/kg); CA 0,10 mg/kg (IS 0,1 mg/kg)

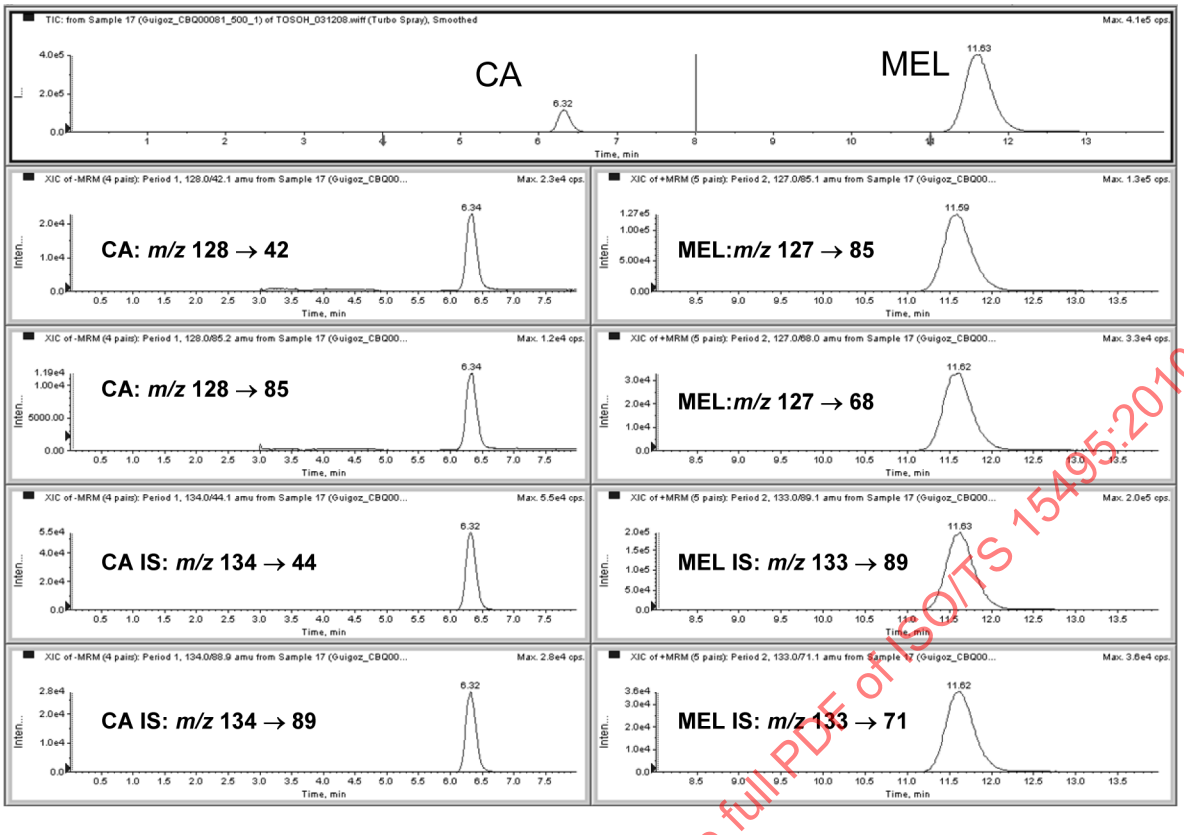

Figure A.3 — Example of LC-MS/MS chromatograms of melamine (MEL) and cyanuric acid (CA) from an extract of spiked milk-based powdered infant formula with spiking levels: MEL 0,50 mg/kg (IS 1,0 mg/kg); CA 0,50 mg/kg (IS 1,0 mg/kg)

A.13 Test report

The test report of method A should contain at least the following information:

a) all information necessary for the complete identification of the sample;

b) the sampling method used, if known;

c) the test method used, together with reference to this annex of this Technical Specification (ISO/TS 15495 | IDF/RM 230:2010, Annex A);

d) all operating details not specified in this annex of this Technical Specification, or regarded as optional, together with details of any incidents which may have influenced the test result(s);

e) the test result(s) obtained;

f) if the repeatability has been checked, the final quoted result obtained.

Annex B
(informative)

Example B — Quantitative analysis of melamine and cyanuric acid in milk-based infant formula and cow milk by liquid chromatography using linear ion-trap mass spectrometry

B.1 Scope

This annex of this Technical Specification specifies a method involving the determination of melamine and cyanuric acid in milk-based infant formula and cow milk by the use of a linear ion-trap tandem mass spectrometry (LC-MS/MS) method with an electrospray ion source and in selected reaction monitoring (SRM) mode. Quantification is performed by the isotopic dilution approach using labelled ($^{13}C_3$, $^{15}N_3$)-melamine and labelled ($^{13}C_3$, $^{15}N_3$)-cyanuric acid as internal standards (ISs).

B.2 Terms and definitions

See Clause 3.

B.3 Principle

Melamine and cyanuric acid are simultaneously extracted in acetonitrile while achieving protein precipitation. After centrifugation, the supernatant is considered for subsequent analysis by ion-trap LC-MS/MS in SRM mode. Melamine and cyanuric acid are analysed separately in positive and negative mode respectively.

B.4 Reagents

Use only reagents of recognized analytical grade, unless otherwise specified, and distilled or demineralized water or water of equivalent purity.

NOTE CAS numbers are given for each reagent.

Before using chemicals, refer to adequate manuals or safety data sheets approved by your local authorities.

B.4.1 Water, for chromatography (CAS No. 7732-18-5).

B.4.2 Acetonitrile (CH_3CN), gradient grade for liquid chromatography (CAS No. 75-05-8).

B.4.3 Ammonium acetate (CH_3COONH_4), GR for analysis (CAS No. 631-61-8).

B.4.4 Formic acid (CH_2O_2) **solution**, corresponding to 1 % volume fraction (CAS No. 64-18-6).

Pipette 10 ml formic acid (CAS No. 64-18-6, ACS grade) into a 1 000 ml one-mark-volumetric flask (B.5.8). Make up to the mark with water (B.4.1) and mix.

B.4.5 Cyanuric acid [$(CNOH)_3$], >98 % mass fraction (CAS No. 108-80-5).

B.4.5.1 Cyanuric acid stock standard solution, corresponding to 0,1 mg of cyanuric acid per millilitre.

Weigh, to the nearest 0,1 mg, 20 mg of cyanuric acid (B.4.5) into a 200 ml one-mark volumetric flask (B.5.8). Dissolve and make up to the mark with formic acid solution (B.4.4). Sonificate for 10 min until complete dissolution.

The cyanuric acid stock standard solution can be stored for up to 10 days at 4 °C. Prepare a fresh stock standard solution every 10 days.

B.4.5.2 Cyanuric acid working solution, corresponding to 10 µg of cyanuric acid per millilitre.

Pipette 10 ml of the cyanuric acid stock standard solution (B.4.5.1) into a 100 ml one-mark volumetric flask (B.5.8). Make up to the mark with formic acid solution (B.4.4). Sonificate for at least 10 min until complete dissolution.

The cyanuric acid working solution can be stored for up to 10 days at 4 °C. Prepare a fresh stock standard solution every 10 days.

B.4.6 Labelled cyanuric acid ($^{13}C_3H_3{}^{15}N_3O_3$), isotopic purity: $^{13}C_3 = 99$ %; ring $^{15}N_3 > 98$ %; chemical purity 90 % mass fraction, 100 µg/ml in water – 1,2 ml.

B.4.6.1 Labelled cyanuric stock standard solution, corresponding to 100 µg of labelled cyanuric acid per millilitre.

The labelled cyanuric stock standard solution can be purchased ready to use from Cambridge Isotope Laboratories[5)] (purity 90 % mass fraction). Store the stock standard solution at room temperature away from light.

B.4.6.2 Labelled cyanuric acid working solution, corresponding to 10 µg labelled cyanuric acid per millilitre.

Pipette 1,1 ml of labelled cyanuric acid stock standard solution (B.4.6.1) into a 10 ml one-mark volumetric flask (B.5.8). Make up to the mark with formic acid solution (B.4.4) and mix.

The labelled cyanuric acid working solution is used as IS. Store the solution at 4 °C.

B.4.7 Melamine ($C_3H_6N_6$), >98 % mass fraction (CAS No. 108-78-1).

B.4.7.1 Melamine stock standard solution, corresponding to 0,1 mg melamine per millilitre.

Weigh, to the nearest 0,1 mg, 20 mg of melamine (B.4.7) into a 200 ml one-mark volumetric flask (B.5.8). Dissolve and make up to the mark with formic acid solution (B.4.4). Sonificate for at least 10 min until complete dissolution.

The melamine stock standard solution can be stored for up to 10 days at 4 °C. Prepare a fresh stock standard solution every 10 days.

B.4.7.2 Melamine working solution I, corresponding to 10 µg of melamine per millilitre.

Pipette 10 ml of melamine stock standard solution (B.4.7.1) into a 100 ml one-mark volumetric flask (B.5.8). Make up to the mark with formic acid solution (B.4.4). Sonificate for at least 10 min until complete dissolution.

Melamine working solution I can be stored for up to 10 days at 4 °C. Prepare a fresh working solution I every 10 days.

B.4.7.3 Melamine working solution II, corresponding to 1 µg of melamine per millilitre.

5) Cambridge Isotope Laboratories is an example of a suitable supplier. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO and IDF of this supplier.

Pipette 1 ml of the melamine working solution I (B.4.7.2) into a 10 ml one-mark volumetric flask (B.5.8). Make up to the mark with formic acid solution (B.4.4). Sonificate for at least 10 min until complete dissolution.

Melamine working solution II can be stored for up to 10 days at 4 °C. Prepare a fresh working solution II every 10 days.

B.4.8 Labelled melamine [$^{13}C_3N_3(^{15}NH_2)_3$], isotopic purity: $^{13}C_3 = 99$ %; amino-$^{15}N_3 = 98$ %; chemical purity $\geqslant$98 % mass fraction, 100 µg/ml in water – 1,2 ml.

B.4.8.1 Labelled melamine stock standard solution, corresponding to 100 µg of labelled melamine per millilitre.

The isotope stock solution is commercially available. Store the stock standard solution at room temperature away from light.

B.4.8.2 Labelled melamine working solution, corresponding to 10 µg of labelled melamine per millilitre.

Pipette 1 ml of labelled melamine stock standard solution (B.4.8.1) into a 10 ml one-mark volumetric flask (B.5.8). Make up to the mark with formic acid solution (B.4.4).

The labelled melamine working solution is used as IS. Store the solution at 4 °C.

B.5 Apparatus

Glassware (e.g. volumetric flasks, B.5.8) used for the preparation of the stock standard and working solutions can be contaminated, especially with melamine. All glassware, therefore, should be cleaned with a laboratory detergent, rinsed with distilled water, then dried in air.

Use usual laboratory apparatus and, in particular, the following.

B.5.1 Centrifuge tubes, conical, polypropylene, of capacity 50 ml, e.g. 21008-169[6].

B.5.2 Centrifuge, with rotor adapted for 50 ml tubes and radial acceleration, 4 000g, e.g. RC6[7].

B.5.3 HPLC Column, ZIC-HILIC[8], 2,1 mm × 150 mm, 5 µm, 20 nm.

B.5.4 Plastic syringes, of capacity 3 ml, Norm-Ject[6].

B.5.5 Syringe filters, Acrodisc 13 mm, 0,2 µm nylon membrane, e.g. 28143-985[6].

B.5.6 HPLC Surveyor MS[9] **pump** coupled to a **LTQ-XL**[9] **linear ion-trap mass spectrometer** equipped with an **electrospray ion source**.

6) 21008-169, Norm-Ject (53548-014), and 281343-985 are trade names of products supplied by VWR. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO or IDF of these products. Equivalent products may be used if they can be shown to lead to the same results.

7) RC6 is the trade name of a product supplied by Sorvall. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO or IDF of this product. Equivalent products may be used if they can be shown to lead to the same results.

8) ZIC-HILIC is the trade name of a product supplied by Merck available from the Nest Group, article Q150454. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO or IDF of this product. Equivalent products may be used if they can be shown to lead to the same results.

9) Surveyor MS and LTQ-XL are trade names of products supplied by ThermoElectron. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO or IDF of these products. Equivalent products may be used if they can be shown to lead to the same results.

B.5.7 Analytical balance, capable of being read to the nearest 0,1 mg.

B.5.8 One-mark volumetric flasks, of capacities 10 ml, 100 ml, 200 ml, and 1 000 ml, ISO 1042[3], class A.

B.6 Preparation of reagents

B.6.1 Calibration curve for melamine and cyanuric acid

B.6.1.1 Standard solutions for melamine (0 mg/l to 5 mg/l) with corresponding melamine IS (at 100 mg/l level)

Prepare the melamine standard solutions in six separate 10 ml one-mark volumetric flasks (B.5.8) as described in Table B.1. Store the melamine standard solutions at 4 °C.

Table B.1 — Melamine standard solutions

	Standard solution					
	1	2	3	4	5	6
Melamine working solution I (B.4.7.2), µl	0	0	100	200	450	700
Melamine working solution II (B.4.7.3), µl	0	150	0	0	0	0
Labelled melamine working solution (IS) (B.4.8.2), µl	150	150	150	150	150	150
Acetonitrile (B.4.2), µl	9 850	9 700	9 750	9 650	9 400	9 150
This corresponds to:						
Concentration of melamine, ng/ml	0	15	100	200	450	700
Concentration of labelled melamine (IS), ng/ml	150	150	150	150	150	150
Content of melamine (equivalent in sample), mg/kg	0,00	0,10	0,66	1,33	3,00	4,66
Content of melamine IS (equivalent in sample), mg/kg	1,00	1,00	1,00	1,00	1,00	1,00

B.6.1.2 Standard solutions for cyanuric acid (0 mg/l to 5 mg/l) with corresponding cyanuric acid IS (at 0,66 mg/l level)

Prepare the mixed cyanuric acid standard solutions in six separate 10 ml one-mark volumetric flasks (B.5.8) as described in Table B.2. Store the cyanuric acid standard solutions at 4 °C.

Table B.2 — Cyanuric acid standard solutions

	Standard solution					
	1	2	3	4	5	6
Cyanuric acid working solution (B.4.5.2), µl	0	25	100	200	550	700
Labelled cyanuric acid working solution IS (B.4.6.2), µl	100	100	100	100	100	100
Acetonitrile (B.4.2), µl	9 900	9 875	9 800	9 700	9 350	9 200
This corresponds to:						
Concentration of cyanuric acid, ng/ml	0	25	100	200	550	700
Concentration of cyanuric acid IS, ng/ml	100	100	100	100	100	100
Content of cyanuric acid (equivalent in sample), mg/kg	0,00	0,17	0,66	1,33	3,66	4,66
Content of cyanuric acid IS (equivalent in sample), mg/kg	0,66	0,66	0,66	0,66	0,66	0,66