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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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ISO copyright office
Case postale 56 • CH-1211 Geneva 20
Tel. + 41 22 749 01 11
Fax + 41 22 749 09 47
E-mail copyright@iso.org
Web www.iso.org

International Dairy Federation
Diamant Building • Boulevard Auguste Reyers 80 • B-1030 Brussels
Tel. + 32 2 733 98 88
Fax + 32 2 733 04 13
E-mail info@fil-idf.org
Web www.fil-idf.org

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Contents

Page

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;

— an ISO Technical Specification (ISO/TS) represents an agreement between the members of a technical committee and is accepted for publication if it is approved by 2/3 of the members of the committee casting a vote.

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.