INTERNATIONAL STANDARD

ISO 22186

IDF 245

First edition
2020-09

Milk and milk products — Determination of nitrofurazone

Lait et produits laitiers — Détermination de la nitrofurazone

Reference numbers
ISO 22186:2020(E)
IDF 245:2020(E)

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Published in Switzerland

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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.

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).

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. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see www.iso.org/patents).

Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

For an explanation of the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT) see www.iso.org/iso/foreword.html.

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.

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. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see www.iso.org/patents).

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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.

The work was carried out by the ISO/IDF Action Team A13 of the *Standing Committee on Analytical Methods for Additives and Contaminants* under the aegis of its project leaders, Dr J.G. Bendall (NZ) and Dr J.M. Evers (NZ).

Introduction

Nitrofurazone (see Figure 1) is an inhibitory substance that, because of its cancer-causing properties, has been prohibited for use on agricultural animals by many jurisdictions. It is one of the nitrofuran class of inhibitory substances, along with furazolidone, furaltadone and nitrofurantoin, which are similarly prohibited for use on agricultural animals. Whereas analysis of furazolidone, furaltadone and nitrofurantoin may be accomplished through highly specific marker metabolites, in the case of nitrofurazone, its corresponding marker metabolite, semicarbazide, is not specific and can be formed by oxidative pathways in dairy products produced from cows that have not been treated with nitrofurazone. While intact nitrofurazone is not stable in meat products, intact nitrofurazone remains stable in liquid milk and dairy products. This document describes a method for the analysis of nitrofurazone in fluid milk and dairy products.

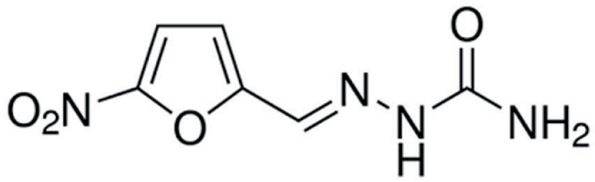

Figure 1 — Chemical structure of nitrofurazone

Milk and milk products — Determination of nitrofurazone

1 Scope

This document specifies a liquid chromatography tandem mass spectrometry (LC–MS/MS) method for the quantification of the inhibitory substance, nitrofurazone, in milk and milk products.

The method has been validated for measuring trace levels of intact nitrofurazone to levels down to 1 ng/g in fluid milk and powdered dairy products on a whole product (i.e. powder) basis. While the method is expected to apply to other dairy matrices, additional validation will be required to demonstrate this.

2 Normative references

There are no normative references in this document.

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

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

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

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

3.1
nitrofurazone concentration
mass fraction of substance determined by the procedure specified in this document

Note 1 to entry: The nitrofurazone concentration is expressed as nanograms per gram of sample (ng/g).

4 Principle

Nitrofurazone is extracted using the QuEChERS protocol in accordance with EN 15662:2018[1] with some modifications.

Liquid milk sample or milk powder sample (first reconstituted with water) is supplemented with $^{13}C^{15}N_2$-nitrofurazone (labelled internal standard) and further extracted with acetonitrile. A liquid–liquid partition is then performed using a mixture of magnesium sulfate ($MgSO_4$) and sodium chloride (NaCl). After centrifugation, the resulting supernatant is cleaned by dispersive solid phase extraction (d-SPE) using a mixture of $MgSO_4$/PSA/C18 sorbents. An aliquot of the extract is evaporated to dryness and reconstituted in methanol before LC–MS/MS analysis in scheduled multiple reaction monitoring (MRM) mode by negative electrospray ionization (ESI).

Positive identification of nitrofurazone in the sample is conducted according to the confirmation criteria defined in EU Commission Decision 2002/657/EC[2]. Quantification is performed by isotopic dilution using $^{13}C^{15}N_2$-nitrofurazone as labelled internal standard. There are two equally acceptable ways to achieve calibration:

a) by the external calibration curve approach;

b) by the matrix-matched calibration curve approach.

5 Reagents and reference substances

5.1 Reagents and materials

All reagents shall be of recognized analytical grade, unless otherwise specified. Water shall be purified to a resistivity of at least 18 MΩ·cm.

5.1.1 Acetonitrile, isocratic grade for LC.

5.1.2 Methanol, isocratic grade for LC.

5.1.3 *N*,*N*-Dimethylformamide (DMF), anhydrous, ≥ 99 %.

5.1.4 Ammonium acetate, for mass spectrometry.

5.1.5 Natural abundance nitrofurazone, purity ≥ 99 %[1].

5.1.6 $^{13}C^{15}N_2$-Nitrofurazone, purity > 99 %, isotopic purity ≥ 97 %[2].

5.1.7 Magnesium sulfate ($MgSO_4$), anhydrous, purity ≥ 98 %[3].

5.1.8 Sodium chloride (NaCl), purity ≥ 99 %[4].

5.1.9 C-18-sorbent (Octadecylsilyl-modified silica gel), bulk material[5].

5.1.10 Primary secondary amine (PSA) sorbent, bulk material[6].

5.1.11 QuEChERS partition salts[7].

Into each 15 ml polypropylene tube, weigh 4,0 g ± 0,2 g $MgSO_4$ (5.1.7) and 1,00 g ± 0,05 g NaCl (5.1.8).

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1) Nitrofurazone from Sigma Aldrich (PHR1196) is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by either ISO or IDF of this product.

2) $^{13}C^{15}N_2$-Nitrofurazone from Witega (NF019-25) is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by either ISO or IDF of this product.

3) Magnesium sulfate anhydrous from Sigma Aldrich (63136) is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by either ISO or IDF of this product.

4) Sodium chloride from Merck (106404) is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by either ISO or IDF of this product.

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7) Ready-to-use $MgSO_4$–NaCl (4 + 1) salt mixtures from Agilent (Agilent 5982-7550) is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by either ISO or IDF of this product.

5.1.12 Dispersive-SPE (d-SPE) salts[8].

Into each 15 ml polypropylene tube, weigh 1 200 mg ± 20 mg $MgSO_4$ (5.1.7), 400 mg ± 10 mg C-18-sorbent (5.1.9), and 400 mg ± 10 mg PSA-sorbent (5.1.10).

5.2 Reference substances

If necessary, different sized glassware may be substituted for specific volumes listed during the preparation of standard solutions as long as final concentrations are maintained.

5.2.1 Natural abundance nitrofurazone stock solution, mass concentration ρ = 1 mg/ml in *N*,*N*-dimethylformamide (DMF).

Into a 10 ml glass volumetric flask, weigh 10,0 mg ± 0,1 mg natural abundance nitrofurazone (5.1.5). Record the mass to 0,1 mg.

Dissolve and then dilute to volume with DMF (5.1.3). Ensure complete solubility of the solution by vortexing and sonication for at least 5 min.

Aliquot into 2 ml microcentrifuge polypropylene tubes. This avoids reiterated freezing and thawing which could lead to an accelerated degradation of analytes in solution.

Store at −20 °C for up to 1 year protected from light.

Allow warming at room temperature, vortex and sonicate for at least 5 min before use.

5.2.2 Natural abundance nitrofurazone working solution, ρ = 5 µg/ml in methanol.

Into a 10 ml volumetric flask, pipette 50 µl of the natural abundance nitrofurazone stock solution (5.2.1). Dilute to volume with methanol (5.1.2). Mix thoroughly.

Store at −20 °C for up to 1 year protected from light.

Allow warming to room temperature, vortex and sonicate for at least 5 min before use.

5.2.3 Natural abundance nitrofurazone working solution, ρ = 250 ng/ml in methanol.

Into a 10 ml volumetric flask, pipette 500 µl of the natural abundance nitrofurazone working solution (5.2.2). Dilute to volume with methanol. Mix thoroughly.

Store at −20 °C for up to 1 year protected from light.

Allow warming at room temperature, vortex and sonicate for at least 5 min before use.

5.2.4 $^{13}C^{15}N_2$-Nitrofurazone stock solution, ρ = 1 mg/ml in *N*,*N*-dimethylformamide (DMF).

Into a 10 ml glass volumetric flask, weigh 10,0 mg ± 0,1 mg $^{13}C^{15}N_2$-nitrofurazone (5.1.6). Record the mass to 0,1 mg.

Dissolve and then dilute to volume with DMF (5.1.3). Ensure complete solubility of the solution by vortexing and sonication for at least 5 min.

Aliquot into 2 ml microcentrifuge polypropylene tubes. This avoids reiterated freezing and thawing which could lead to an accelerated degradation of analytes in solution.

Store at −20 °C for up to 1 year protected from light.

8) Ready-to-use $MgSO_4$–C18–PSA (3 + 1 + 1) salt mixtures from Agilent (Agilent 5982-5158) is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by either ISO or IDF of this product.