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Milk and milk products — Determination of nitrofurazone

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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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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.

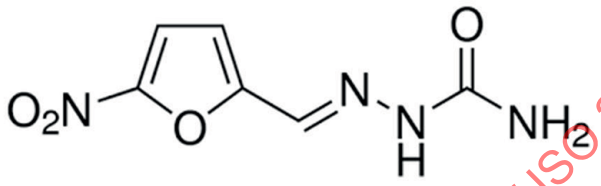

Figure 1 — Chemical structure of nitrofurazone

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.

5) Discovery® DSC-18 SPE Bulk Packing from Sigma Aldrich (52600-U) 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.

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

5.2.5 $^{13}C^{15}N_2$-Nitrofurazone working solution, ρ = 5 µg/ml in methanol.

Into a 10 ml volumetric flask, pipette 50 µl of the $^{13}C^{15}N_2$-nitrofurazone stock solution (5.2.4). Dilute to volume with methanol (5.1.2). 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.

5.2.6 $^{13}C^{15}N_2$-Nitrofurazone working solution, ρ = 250 ng/ml in methanol.

Into a 10 ml volumetric flask, pipette 500 µl of the $^{13}C^{15}N_2$-nitrofurazone working solution (5.2.5). Dilute to volume with methanol (5.1.2). 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.

5.2.7 Standard solutions for calibration by the external calibration curve approach.

Into six separate 5 ml glass volumetric flasks, add both natural abundance nitrofurazone working solution (5.2.3) and $^{13}C^{15}N_2$-nitrofurazone working solution (5.2.6) in the volumes described in Table 1. Dilute to 5 ml volume with methanol (5.1.2).

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.

Table 1 — Standard solutions for external calibration curve

Calibration standard	Volume added solution (5.2.3)	Volume added solution (5.2.6)	Volume to be made up with methanol	Corresponding nitrofurazone concentration ng/ml	Corresponding $^{13}C^{15}N_2$-nitrofurazone concentration ng/ml	Corresponding equivalent-in-sample nitrofurazone concentration ng/g	Corresponding equivalent-in-sample $^{13}C^{15}N_2$-nitrofurazone concentration ng/g
Cal 0	0 µl	200 µl	Dilute to 5 ml	0	10	0	2,5
Cal 1	40 µl	200 µl	Dilute to 5 ml	2	10	0,5	2,5
Cal 2	80 µl	200 µl	Dilute to 5 ml	4	10	1	2,5
Cal 3	160 µl	200 µl	Dilute to 5 ml	8	10	2	2,5
Cal 4	400 µl	200 µl	Dilute to 5 ml	20	10	5	2,5
Cal 5	800 µl	200 µl	Dilute to 5 ml	40	10	10	2,5

5.2.8 Solutions for LC–MS/MS.

5.2.8.1 Mobile phase A, water containing 1 mmol/l ammonium acetate ($NH_4CH_3CO_2$).

Into a weighing boat, weigh 77 mg ± 5 mg ammonium acetate (5.1.4). Transfer the salt into a 1 l bottle, and dilute with water to 1 l volume. Mix well. Store at room temperature for no longer than 1 week.

5.2.8.2 Mobile phase B, methanol.

Use gradient grade methanol for LC (5.1.2).

5.2.8.3 Injection flush port, mixture of water and methanol (1 + 1).

Into a 1 l volumetric flask, add 500 ml of methanol (5.1.2) by means of a measuring cylinder. Dilute to volume with water. Transfer into a LC bottle. Store at room temperature for no longer than 3 months.

6 Apparatus

Standard laboratory apparatus may be used and, in particular, the following.

6.1 Horizontal shaker[9], capable of shaking at 300 r/min.

6.2 Ceramic homogenizer[10], with angled ends that assist with shearing the sample matrix to provide good homogeneity to the extracted sample.

6.3 Nylon syringe filter[11], able to capture fine particles with a high flow rate.

6.4 LC–MS/MS equipment[12].

6.4.1 Gradient pumping system, capable of operating at a speed of 1,0 ml/min.

6.4.2 Autosampler, capable of being temperature controlled at 10 °C and injecting volumes of 5 µl.

7 Sampling

Sampling is not part of the method specified in this document.

It is important that the laboratory receives a sample that is representative and has not been damaged or changed during transport or storage.

Powdered samples shall be homogenized before taking test portions. Transfer the whole sample into a container of capacity about twice that of the laboratory sample volume. Close the container immediately. Mix thoroughly by repeatedly shaking and inverting the container. Alternatively, mix well the powdered laboratory sample directly into its original container by means of a spoon before taking a test portion.

9) IKA Labortechnik KS 501 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.

10) QuEChERS Ceramic Homogenizers from Agilent (Agilent 5982-9313) are 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 these products.

11) Syringe filter type Millex Nylon, 0,45 µm, 13 mm 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.

12) Agilent ZORBAX Rapid Resolution High Throughput (RRHT) SB-Phenyl column packed with 1,8 µm particles, 100 mm × 4,6 mm; Phenomenex SecurityGuard™ C_{18} cartridge; Agilent 1290 Infinity Binary LC system; and AB Sciex 5500 triple quadrupole mass spectrometer with a turbo-spray ESI interface are all examples of suitable products 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 these products.

8 Procedure

8.1 Test portion

8.1.1 Liquid milk

Into 50 ml polypropylene tubes, weigh 10,00 g ± 0,10 g of fluid milk.

Add 100 µl of the $^{13}C^{15}N_2$-nitrofurazone working solution (5.2.6). Mix thoroughly. This spike corresponds to 2,5 ng/g equivalent-in-sample concentration.

8.1.2 Powdered dairy products

Into 50 ml polypropylene tubes, weigh 2,50 g ± 0,10 g of powdered dairy sample.

Add 25 µl of the $^{13}C^{15}N_2$-nitrofurazone working solution (5.2.6). This spike corresponds to 2,5 ng/g equivalent-in-sample concentration. Mix thoroughly and make sure that the spiked volume is totally absorbed by the matrix. Wait for 5 min. Then add 10 ml water. Shake vigorously by hand until the whole sample is completely dispersed in the solution. Add a ceramic homogenizer.

8.2 Extraction procedure

Add 10 ml acetonitrile (5.1.1) to the spiked sample in polypropylene tubes (see 8.1.1 or 8.1.2) and shake by hand for a few seconds.

Shake on a horizontal shaker at 300 r/min for 10 min.

Add the QuEChERS salt mixture (5.1.11) to each tube. Close the tube and immediately hand-shake the resulting slurry by inversion, or by vortexing, to prevent any lump formation.

Shake on a horizontal shaker at 300 r/min for 10 min.

Centrifuge at 4 000*g* at room temperature for 5 min.

Transfer the supernatant (7 ml) into a 15 ml polypropylene tube already containing d-SPE salts (5.1.12). Close the tube and immediately hand-shake by inversion, or by vortexing, to prevent any lump formation.

Shake on a horizontal shaker at 300 r/min for 10 min.

Centrifuge at 4 000*g* at room temperature for 5 min.

Transfer the supernatant of the extract (1 ml for a fluid milk sample, or 4 ml for a powdered dairy product sample) into a fresh 15 ml polypropylene tube and evaporate to dryness under a gentle stream of nitrogen at 40 °C ± 2 °C.

Dissolve the residue in 250 µl methanol (5.1.2). Vortex for a few seconds, sonicate for at least 1 min and vortex again.

Filter the solution through a 0,45 µm nylon syringe filter (6.3) into a HPLC vial for further LC–MS/MS analysis.

8.3 Reagent blank

In order to control any contamination during the sample workup, a reagent blank shall be analysed along with each series of routine samples. Proceed exactly as described in 8.1 and 8.2 without adding any test portion.

8.4 Positive quality control

8.4.1 General

It is recommended to include a positive quality control in each series, using a sample previously demonstrated to be free of nitrofurazone.

8.4.2 Liquid milk

Into a 50 ml polypropylene tube, weigh 10,00 g ± 0,10 g fluid milk.

Add 40 µl of natural abundance nitrofurazone working solution (5.2.3). Mix thoroughly. This spike corresponds to 1,0 ng/g equivalent-in-sample concentration.

Add 100 µl of $^{13}C^{15}N_2$-nitrofurazone working solution (5.2.6). Mix thoroughly. This spike corresponds to 2,5 ng/g equivalent-in-sample concentration.

Follow the procedure as described in 8.2.

8.4.3 Powdered dairy products

Into 50 ml polypropylene tubes, weigh 2,50 g ± 0,10 g of powdered dairy sample.

Add 10 µl of natural abundance nitrofurazone working solution (5.2.3). Mix thoroughly. This spike corresponds to 1,0 ng/g equivalent-in-sample concentration.

Add 25 µl $^{13}C^{15}N_2$-nitrofurazone working solution (5.2.6). This spike corresponds to 2,5 ng/g equivalent-in-sample concentration.

Mix thoroughly and make sure that the spiked volumes are totally absorbed by the matrix. Wait for 5 min.

Add 10 ml water. Shake vigorously by hand until the whole sample is completely dispersed in the solution. Add a ceramic homogenizer.

Follow the procedure as described in 8.2.

8.4.4 Calculations

Calculate the internal-standard–corrected recovery (R_{corr}) of the positive QC (quality control) sample using Formula (1):

$$R_{corr} = \frac{\rho_T}{\rho_{Spiked}} \times 100 \quad (1)$$

where

ρ_T is the total concentration of the compound measured in the spiked sample (ng/g);

ρ_{Spiked} is the concentration of the compound spiked in the sample (ng/g) (i.e. 1,0 ng/g).

Recoveries should be within the 70 % to 110 % range during routine analysis according to 2002/657/EC requirements[2].

8.5 Matrix-matched calibration curves

8.5.1 General

In order to overcome the effect of compounds in the samples causing interference, either through signal enhancement or suppression, quantification may be achieved by the matrix-matched calibration

approach, using a set of matrix-matched calibration standards. This is an equally acceptable alternative to the external calibration approach that uses an external calibration curve (5.2.7).

8.5.2 Matrix-matched calibration samples for fluid milk

Prepare a set of calibration standards using seven separate 50 ml polypropylene tubes. To each tube, weigh in 10,00 g ± 0,10 g fluid milk (previously demonstrated to be free of nitrofurazone).

Then add natural abundance nitrofurazone working solution (5.2.3) at volumes of 0 µl, 20 µl, 40 µl, 80 µl, 200 µl, 400 µl and 800 µl to the seven different tubes, to give equivalent-in-sample concentrations of 0 ng/g, 0,5 ng/g, 1 ng/g, 2 ng/g, 5 ng/g, 10 ng/g and 20 ng/g. Mix each tube thoroughly.

Then to each tube add 100 µl $^{13}C^{15}N_2$-nitrofurazone working solution (5.2.6). Mix each tube thoroughly. This spike corresponds to 2,5 ng/g equivalent-in-sample concentration.

Follow the procedure as described in 8.2.

8.5.3 Matrix-matched calibration samples for powdered dairy products

Prepare a set of calibration standards using seven separate 50 ml polypropylene tubes. To each tube, weigh in 2,50 g ± 0,10 g powdered dairy sample (previously demonstrated to be free of nitrofurazone).

Then add natural abundance nitrofurazone working solution (5.2.3) at volumes of 0 µl, 5 µl, 10 µl, 20 µl, 50 µl, 100 µl and 200 µl to the seven different tubes, to give equivalent-in-sample concentrations of 0 ng/g, 0,5 ng/g, 1 ng/g, 2 ng/g, 5 ng/g, 10 ng/g and 20 ng/g. Mix each tube thoroughly.

Then to each tube add 25 µl $^{13}C^{15}N_2$-nitrofurazone working solution (5.2.6). Mix each tube thoroughly. This spike corresponds to 2,5 ng/g equivalent-in-sample concentration.

Mix thoroughly and make sure that the spiked volumes are totally absorbed by the matrix. Wait for 5 min.

Add 10 ml water. Shake vigorously by hand until the whole sample is completely dispersed in the solution. Add a ceramic homogenizer.

Follow the procedure as described in 8.2.

8.6 LC–MS/MS conditions

8.6.1 General

Due to slight differences between LC–MS/MS instruments, even of the same model from the same manufacturer, slight modifications to the conditions listed below may be necessary to achieve optimal performances. Operating the chromatographic system with different conditions (including different temperatures, with a different column type, or other differing conditions) will not necessarily result in false or less sensitive results, but will require that any effects of such changes be thoroughly validated.

8.6.2 Chromatographic conditions

Suggested conditions for the chromatographic separation are as follows:

— Mobile phase A: Aqueous solution of ammonium acetate ($NH_4CH_3CO_2$) (5.2.8.1).

— Mobile phase B: Methanol (5.1.2).

— Injection volume: 5 µl.

— Column oven temp: 60 °C.

— Flow rate: 0,9 ml/min.

— Needle wash: In flush port for 15 s using water–methanol (1 + 1) (5.2.8.3).

— Diverter valve: LC flow is directed into the MS detector between 3,5 min and 5 min.

— Autosampler temp: 10 °C.

Suggested conditions for the chromatographic separation are as follows:

a) start with a gradient of A/B 90:10 and continue for 1 min;

b) move the gradient to 40:60 over 3 min;

c) then move the gradient to 10:90 over 0,2 min;

d) hold the gradient at 10:90 for 1,3 min;

e) step the gradient back to A/B 90:10 over 0,3 min;

f) re-equilibrate with A/B 90:10 for 2,7 min.

8.6.3 MS/MS conditions

MS parameters are obtained by injecting, via syringe, standard solutions at 250 ng/ml (5.2.3 and 5.2.6) along with the LC flow at 0,9 ml/min using a T-connector (syringe flow rate of 10 µl/min). The LC flow is constituted with 50 % A (5.2.8.1) and 50 % B (5.2.8.2).

Acquisition is performed in negative ionization mode by monitoring at least two transition reactions per compound. Suggested conditions for source parameters and individual compounds parameters are given in Tables 2 and 3.

Table 2 — Suggested conditions for source parameters

Source parameter	Conditions
Ionization type	Electrospray (ESI)
Polarity	Negative ionization
Ion spray voltage	–2 500 V
Source block temperature	600 °C
Curtain gas	172 kPa (25 psi)
Ion source gas 1 (GS1)	276 kPa (40 psi)
Ion source gas 2 (GS2)	345 kPa (50 psi)
Dwell time	25 ms

Table 3 — Suggested conditions for individual compound parameters

Q1 Mass Da	Q3 Mass Da	ID	DP	EP	CE	CXP	Peak area ratio ± limit %
197,1	80,1	Nitrofurazone_1	−20	−10	−14	−10	1,16 ± 20
197,1	124,1	Nitrofurazone_2	−20	−10	−12	−10	
200,1	82,1	IS_1	−20	−10	−13	−10	0,94 ± 20
200,1	126,1	IS_2	−20	−10	−12	−10	

Key
DP declustering potential
EP entrance potential
CE collision energy
CXP collision exit potential

8.7 Sequence set-up

Before routine analysis, allow both LC and mass spectrometer systems to equilibrate at the initial LC conditions (i.e. 90 % A at 900 µl/min, oven temperature at 60 °C). Ensure that the system pressure is stable and that there are no leaks.

Inject solutions in the following order:

a) Methanol solution (5.1.2) (as blank solvent), twice, in order to stabilize the LC column.

b) Calibration solutions. Either external calibration solutions (5.2.7) or matrix-matched calibration solutions (8.5.2 and 8.5.3).

c) Methanol solution (5.1.2) (as blank solvent), to check for absence of carry-over.

d) Extract solutions (see 8.2).

e) Inject a methanol solution (5.1.2) (as blank solvent) after each eight to 10 extract injections to check for any carry-over or instrument issue(s).

f) Calibration solutions (5.2.7) at the end of the batch to check the stability of the instrument along the sequence.

9 Calculation and expression of results

9.1 General

Quantification is performed by the isotopic dilution approach using $^{13}C^{15}N_2$-nitrofurazone as the labelled internal standard.

9.2 Peak integration

As shown in Annex A, a chromatogram of nitrofurazone shows two non-resolved peaks. These are attributed to *cis* and *trans* isomeric forms due to photochemical isomerization of the imine bond. To compensate for potential *cis*/*trans* peak ratio changes, depending on the matrix or analytical conditions, the two peaks shall be integrated together for both external calibration solutions and matrix extracts.

9.3 Calibration curves

9.3.1 External calibration curve from external calibration standards

Draw the external calibration curve by plotting ratios of the instrumental peak area for natural abundance nitrofurazone to $^{13}C^{15}N_2$-nitrofurazone (= y-axis) against the ratio of aliquoted natural abundance nitrofurazone to $^{13}C^{15}N_2$-nitrofurazone (= x-axis) for the external calibration solutions (5.2.7). Calculate the slope and the intercept by linear regression.

Check the linearity of the external calibration curve:

— regression coefficient, R^2, should be higher than 0,99;

— relative standard deviation of the average of response factors (= y/x) should be < 15 %.

9.3.2 Matrix-matched calibration curve from matrix-matched calibration standards

Draw the matrix-matched calibration curve by plotting ratios of the instrumental peak area for natural abundance nitrofurazone to $^{13}C^{15}N_2$-nitrofurazone (= y-axis) against the ratio of aliquoted natural abundance nitrofurazone to $^{13}C^{15}N_2$-nitrofurazone (= x-axis) for the matrix-matched calibration solutions (see 8.5.2 and 8.5.3). Calculate the slopes and the intercept by linear regression.

Check the linearity of the matrix-matched calibration curves:

— regression coefficient, R^2, should be higher than 0,99;

— relative standard deviation of the average of response factors (= y/x) should be < 15 %.

9.4 Identification and confirmation

Nitrofurazone is identified and confirmed when the following criteria are fulfilled as described by the 2002/657/EC document[2]:

a) a signal is visible at the two diagnostic transition reactions for both natural abundance nitrofurazone and $^{13}C^{15}N_2$-nitrofurazone;

b) the ratio of the chromatographic retention time of nitrofurazone to that of the internal standard, i.e. the relative retention time of nitrofurazone, shall correspond to that of the external calibration solution at a tolerance of ±2,5 %;

c) the variability of ion ratios for the different transition reactions recorded for both natural abundance nitrofurazone and $^{13}C^{15}N_2$-nitrofurazone shall be within ±20 % (see 8.5.3).

9.5 Calculation using external calibration curve

Calculate the mass fraction, *w*, of nitrofurazone in nanograms per gram of sample (ng/g), using Formula (2):

$$w = \frac{\left(\frac{A_a}{A_{is}}\right) - I}{S} \times \frac{m_{is}}{m_a} \qquad (2)$$

where

A_a is the peak area of nitrofurazone in the sample (transition reaction used for quantification);

A_i is the peak area of $^{13}C^{15}N_2$-nitrofurazone in the sample (transition reaction used for quantification);

I is the intercept of the regression line (see 9.3.1) for the transition reaction used for quantification;

S is the slope of the regression line (see 9.3.1) for the transition reaction used for quantification;

m_{is} is the mass of $^{13}C^{15}N_2$-nitrofurazone added to the test portion, in ng (i.e. 25 ng for fluid milk, 6,25 ng for powdered dairy products);

m_a is the mass of the test portion, in g.

9.6 Calculation using matrix-matched calibration curve

Calculate the mass fraction, w, of nitrofurazone in nanograms per gram of sample (ng/g), using Formula (3):

$$w = \frac{\left(\frac{A_a}{A_{is}}\right) - I}{S} \times \frac{m_{is}}{m_a} \quad (3)$$

where

A_a is the peak area of nitrofurazone in the sample (transition reaction used for quantification);

A_{is} is the peak area of $^{13}C^{15}N_2$-nitrofurazone in the sample (transition reaction used for quantification);

I is the intercept of the regression line (see 9.3.2) for the transition reaction used for quantification;

S is the slope of the regression line (see 9.3.2) for the transition reaction used for quantification;

m_{is} is the mass of $^{13}C^{15}N_2$-nitrofurazone added to the test portion, in ng (i.e. 25 ng for fluid milk, 6,25 ng for powdered dairy products);

m_a is the mass of the test portion, in g.

9.7 Expression of results

Express the results in ng/g to two significant figures.

10 Precision

10.1 General

Details of the interlaboratory test of the precision of the method are given in Annex B. The values derived from the interlaboratory test may not be applicable to analyte concentration ranges and/or matrices other than those given in Annex B.

10.2 Repeatability

For the range 0,1 ng/g to 5 ng/g of nitrofurazone, and irrespective of the type of dairy product matrices described in Annex B, on 95 % of occasions the difference between two independent single test results, obtained using the same method on identical test material in the same laboratory by the same operator using the same equipment within a short interval of time, are expected to agree within 13 % and 16 % of their average, by the external calibration approach and matrix-matched calibration approach, respectively. These values correspond to the relative repeatability limits, *r*, at 95 % confidence levels, obtained from linear fittings of s_r with concentration from all nitrofurazone levels and all dairy product matrices described in Annex B.

10.3 Reproducibility

For the range 0,1 ng/g to 5 ng/g of nitrofurazone, and irrespective of the type of dairy product matrices described in Annex B, on 95 % of occasions the difference between two independent single test results, obtained using the same method on identical test material in different laboratories with different operators using different equipment, are expected to agree within 26 % and 27 % of their average, by the external calibration approach and matrix-matched calibration approach, respectively. These values correspond to the relative reproducibility limits, *R*, at 95 % confidence levels, obtained from linear fittings of s_R with concentration from all nitrofurazone levels and all dairy product matrices described in Annex B.

11 Test report

The test report shall contain the following information:

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

b) the sampling method used, if known;

c) the test method used, with reference to this document, i.e. ISO 22186 | IDF 245:2020;

d) the test result(s) obtained, including a reference to whether the results were calculated by either the external calibration curve approach (see 9.5) or the matrix-matched calibration curve approach (see 9.6);

e) whether there were any deviations from the procedure;

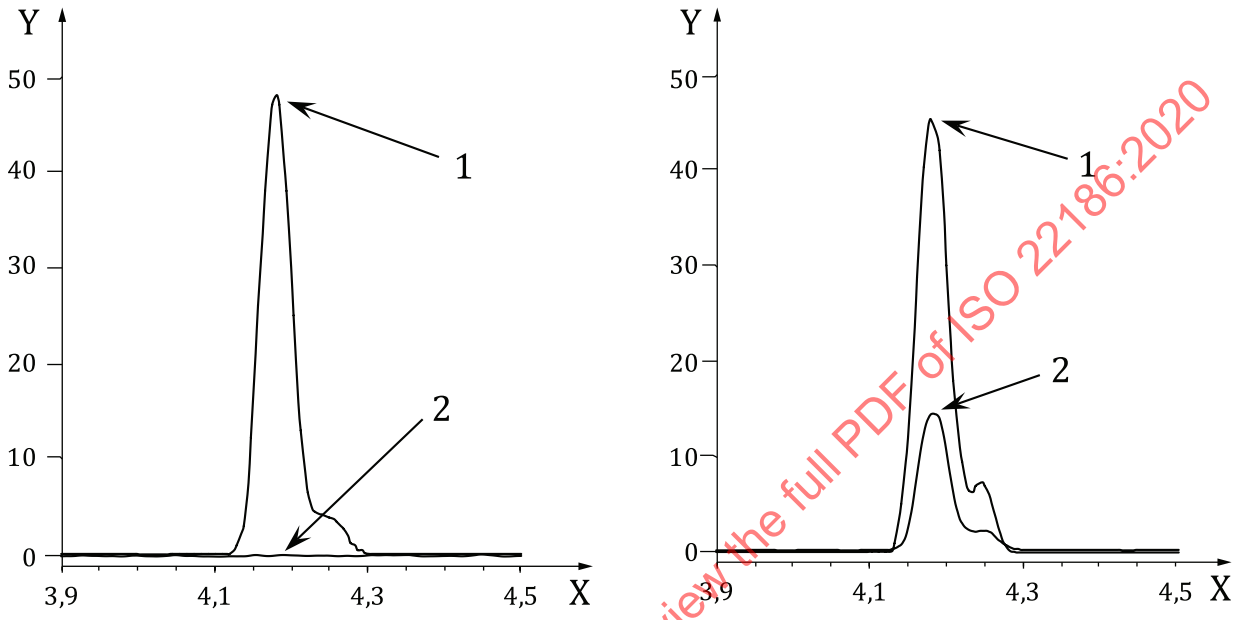
f) whether there were any unusual features observed during the analysis;

g) details of any incident that could have influenced the test result(s);

h) the date of the test.

Annex A
(informative)

Examples of chromatograms



a) Sample containing no added natural abundance nitrofurazone and $^{13}C^{15}N_2$-nitrofurazone internal standard (2,5 ng/g)

b) Sample containing added natural abundance nitrofurazone (1 ng/g spike) and $^{13}C^{15}N_2$-nitrofurazone internal standard (2,5 ng/g)

Key

X time, min
Y ion intensity (× 1 000)
1 4,18 min, *m/z* 200,1 → 82,1 transition
2 4,18 min, *m/z* 197,1 → 80,1 transition

NOTE The overlaid traces for the *m/z* 197,1 → 80,1 and *m/z* 200,1 → 82,1 transitions show natural abundance- and $^{13}C^{15}N_2$-nitrofurazone internal standard, respectively.

Figure A.1 — LC-MS/MS chromatograms of fluid milk samples