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**Cheese, cheese rind and processed  
cheese — Determination of natamycin  
content —**

Part 2:

**High-performance liquid  
chromatographic method for cheese,  
cheese rind and processed cheese**

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*Fromage, croûte de fromage et fromages fondus — Détermination de la  
teneur en natamycine —*

<https://standards.iteh.ai/standards/ISO/9233-2:2007>  
*Partie 2: Méthode par chromatographie liquide à haute performance  
pour fromage, croûte de fromage et fromages fondus*



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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](mailto:copyright@iso.org)  
Web [www.iso.org](http://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](mailto:info@fil-idf.org)  
Web [www.fil-idf.org](http://www.fil-idf.org)

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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.

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 9233-2|IDF 140-2 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 5, *Milk and milk products* and the International Dairy Federation (IDF) and is being published jointly by ISO and IDF.

This first edition of ISO 9233-2|IDF 140-2, together with ISO 9233-1|IDF 140-1, cancels and replaces the first edition of ISO 9233:1991, which has been technically revised.

ISO 9233|IDF 140 consists of the following parts, under the general title *Cheese, cheese rind and processed cheese — Determination of natamycin content*:

- *Part 1: Molecular absorption spectrometric method for cheese rind*
- *Part 2: High-performance liquid chromatographic method for cheese, cheese rind and processed cheese*

## Foreword

**IDF (the International Dairy Federation)** is a worldwide federation of the dairy sector with a National Committee in every member country. Every National Committee has 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.

Draft International Standards adopted by the Action Teams and Standing Committees are circulated to the National Committees for voting. Publication as an International Standard requires approval by at least 50% of IDF National Committees casting a vote.

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ISO 9233-2|IDF 140-2 was prepared by the International Dairy Federation (IDF) and Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 5, *Milk and milk products* and is being published jointly by IDF and ISO.

All work was carried out by the Joint ISO-IDF Action Team on *Selected food additives and vitamins* of the Standing Committee on *Analytical methods for additives and contaminants* under the aegis of its project leader, Mr. M. Carl (DE).

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# Cheese, cheese rind and processed cheese — Determination of natamycin content —

## Part 2: High-performance liquid chromatographic method for cheese, cheese rind and processed cheese

### 1 Scope

This part of ISO 9233|IDF 140 specifies a method for the determination of natamycin mass fraction in cheese, cheese rind and processed cheese of above 0,5 mg/kg and of the surface-area-related natamycin mass in cheese rind of above 0,03 mg/dm<sup>2</sup>.

### 2 Terms and definitions

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

**2.1 natamycin content** <https://standards.iteh.ai/catalog/standards/sist/8a78cflf-4d4c-453b-bc7b-1c7a2704498a/iso-9233-2-2007>  
mass fraction of substances determined by the procedure specified in this part of ISO 9233|IDF 140

NOTE The natamycin content is expressed in milligrams per kilogram.

**2.2 surface-area-related natamycin mass in cheese rind**  
surface-area-related mass of substances determined by the procedure specified in this part of ISO 9233|IDF 140

NOTE The surface-area-related natamycin mass is expressed in milligrams of natamycin per square decimetre of cheese rind.

**2.3 cheese rind**  
outer layer of the cheese of thickness 5 mm, excluding the coating layer, if present.

### 3 Principle

A known quantity of sample is extracted with methanol. The extract is diluted with water followed by cooling to between –15 °C and –20 °C to precipitate most of the fat, followed by filtration. The natamycin content or surface-area-related natamycin mass is determined in the filtrate (after concentration, if necessary) by high-performance liquid chromatography (HPLC).

## 4 Reagents

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

**4.1 Methanol** (CH<sub>3</sub>OH).

**4.2 Methanol**, aqueous solution.

Mix 2 volumes of methanol (4.1) with 1 volume of water.

**4.3 Natamycin standard solutions.**

**4.3.1 Natamycin standard stock solution**, of concentration 500 mg/l.

Immediately before use, dissolve in methanol (4.1) a quantity of a natamycin preparation of known natamycin content, corresponding to 50 mg of pure natamycin (C<sub>33</sub>H<sub>47</sub>NO<sub>13</sub>), in a 100 ml one-mark volumetric flask (5.1). Make up to the mark with water and mix.

**4.3.2 Natamycin standard working solution**, of concentration 5 mg/l.

Pipette 5,0 ml of the natamycin standard stock solution (4.3.1) in a 50 ml one-mark volumetric flask (5.1). Dilute to the mark with aqueous methanol (4.2) and mix.

Pipette 5,0 ml of the thus diluted solution into another 50 ml one-mark volumetric flask (5.1). Dilute to the mark with aqueous methanol (4.2) and mix. The concentration of this natamycin standard working solution is 5 µg/ml.

This concentration shall be close to that of the test solution measured in 8.3.3. Adjust the standard working dilution by pipetting and diluting another quantity, if required.

**4.4 Acetic acid** (CH<sub>3</sub>CO<sub>2</sub>H), glacial.

## 5 Apparatus

Usual laboratory equipment and, in particular, the following.

**5.1 One-mark volumetric flasks**, of capacities 50 ml and 100 ml.

**5.2 Slicer or similar apparatus**, capable of cutting cheese portions of thickness 5 mm and of width about 30 mm (Figure A.1 shows an example).

**5.3 Fine slicer**, capable of cutting thin cheese slices of maximum thickness 1 mm (Figure A.2 shows an example).

**5.4 Grinder or blender.**

**5.5 Sharp knife**, capable of cutting cheese slices into small pieces.

**5.6 Magnetic stirrer or shaking machine.**

**5.7 Conical flasks**, of capacities 100 ml and 200 ml, made of coloured glass and fitted with ground-glass stoppers.

**5.8 Syringes**, disposable, of capacity 10 ml.



- 5.9 Membrane microfilters**, of pore size 0,20 µm and 0,45 µm, resistant to attack by alcoholic solutions.
- 5.10 Folded paper filters**, fast speed, of diameter 150 mm. [e.g. S and S, No. 595 1/2<sup>1</sup>].
- 5.11 Funnel**, of diameter approximately 70 mm.
- 5.12 Freezer**, capable of freezing at a temperature of between –15 °C and –20 °C.
- 5.13 Extraction cartridges**, to concentrate the filtered extract, if necessary [e.g. Sep-pack C18<sup>1</sup>) or Waters No. 51910<sup>1</sup>].
- 5.14 Liquid chromatograph**, with UV detector, capable of measuring at 303 nm and equipped with a recorder and/or integrator.
- 5.15 Analytical column**, of length 150 mm, of internal diameter 4,6 mm, type C8, having a particle size of 5 µm [e.g. Lichrosorb RP8<sup>1</sup>].
- 5.16 Guard column**, of length 100 mm, of internal diameter 2,1 mm, type C8, having a particle size of 30 µm to 40 µm [e.g. Perisorb RP8<sup>1</sup>].
- 5.17 Sample jar**, of suitable capacity.

## 6 Sampling

A representative sample should have been sent to the laboratory. It should not have been damaged or changed during transport or storage.

Sampling is not part of the method specified in this part of ISO 9233|IDF 140. A recommended sampling method is given in ISO 707|IDF 50.

The laboratory sample shall be a whole cheese, or a segment of a cheese representative of the whole.

## 7 Preparation of test sample

### 7.1 Cheese rind

If necessary, cut the test sample into sectors or smaller portions so that the width of the cheese rind is not more than about 30 mm. Using the slicer (5.2), remove the whole rind from all obtained sectors or portions by slicing off a maximum thickness of 5 mm.

From the rind obtained, cut, using a sharp knife (5.5), a rectangular piece of area between 2 dm<sup>2</sup> and 4 dm<sup>2</sup>. Determine its surface area, in square decimetres, and its mass, in kilograms.

Grind (5.4) carefully the whole rind, including the weighed and measured piece, and mix thoroughly. Immediately transfer a quantity of the sample thus prepared to a sample jar (5.17).

After preparing each test sample, clean all tools that have been in contact with the sample with hot water and then with methanol (4.1). Dry all tools thoroughly, e.g. by using a stream of compressed air.

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1) Example of a suitable product available commercially. This information is given for the convenience of users of this part of ISO 9233|IDF 140 and does not constitute an endorsement by ISO or by IDF of this product.

## 7.2 Cheese interior and processed cheese

After removing the rind (7.1), use the fine slicer (5.3) to remove a slice of maximum thickness 1 mm from the whole of the outer section of the test sample.

Cut all sample slices into small pieces of about 50 mm<sup>2</sup> and mix thoroughly. Immediately transfer a quantity of the sample thus prepared to a sample jar (5.17).

After preparing each test sample, clean all tools that have been in contact with the test sample with hot water and then with methanol (4.1). Dry all tools thoroughly, e.g. by using a stream of compressed air.

## 8 Procedure

### 8.1 Test portion

#### 8.1.1 Cheese rind

Weigh, to the nearest 10 mg, approximately 10,00 g of test sample (7.1) into a 200 ml conical flask (5.7).

#### 8.1.2 Cheese interior and processed cheese

Weigh, to the nearest 10 mg, approximately 5,00 g of test sample (7.2) into a 100 ml conical flask (5.7).

### 8.2 Preparation of test solution (standards.iteh.ai)

#### 8.2.1 Cheese rind

[ISO 9233-2:2007](https://standards.iteh.ai/catalog/standards/sist/8a78cflf-4d4c-453b-bc7b-1c7a2704498a/iso-9233-2-2007)

##### 8.2.1.1 Initial steps

<https://standards.iteh.ai/catalog/standards/sist/8a78cflf-4d4c-453b-bc7b-1c7a2704498a/iso-9233-2-2007>

Add 100 ml of methanol (4.1) to the test portion in the conical flask (8.1.1). Stir the contents of the conical flask for 90 min with a magnetic stirrer (5.6) or shake for 90 min in a shaking machine (5.6).

Add 50 ml water. Immediately place the conical flask in the freezer (5.12) for about 60 min.

##### 8.2.1.2 Filtration

Filter the cold extract through a folded filter paper (5.10) while discarding the first 5 ml of filtrate. The filtration should be carried out while the suspension is still cold to avoid dissolution of the fat and consequently turbid filtrates.

Bring the filtrate to room temperature. Take a portion of the filtrate in a syringe (5.8). Filter through a membrane microfilter of pore size 0,45 µm (5.9) and then through a membrane microfilter of pore size 0,20 µm (5.9).

The minimum amount of test solution (filtrate) required is 20 µl per injection for direct chromatographic measurement (8.3.4), and 25 ml or 50 ml for measurement at 5 or 10 times concentration (8.3.5), respectively.

#### 8.2.2 Cheese interior and processed cheese

##### 8.2.2.1 Initial steps

Use a measuring cylinder to add 50 ml of methanol (4.1) to the test portion in the conical flask (8.1.2). Stir the contents of the conical flask for 90 min with a magnetic stirrer (5.6) or shake for 90 min in a shaking machine (5.6).

Use a measuring cylinder to add 25 ml of water. Immediately place the conical flask in the freezer (5.12) for about 60 min.

#### 8.2.2.2 Filtration

Filter the solution as described in 8.2.1.2.

### 8.3 Determination

#### 8.3.1 Determination and detection limits

The laboratory applying the method shall establish the limits of detection and determination under its own instrumental conditions using recognized calculation methods to verify that natamycin can be determined down to levels of 0,5 mg/kg and 0,03 mg/dm<sup>2</sup>.

#### 8.3.2 Adjustment of the liquid chromatograph (5.14)

The following chromatographic conditions are recommended:

Mobile phase:	Methanol (4.1):water:acetic acid (4.4) — 12:8:1 (parts by volume)
Flow:	1 ml/min
Detector set:	303 nm, 0,005 absorbance units, full scale
Recorder:	10 mV
Theoretical (typical) plate count:	1 500 minimum

When a column other than that given as an example (5.15) is used, adjust the methanol:water ratio. The relative amount of acetic acid (4.4) to methanol specified, however, is essential to keep the absorbance maximum at 303 nm.

Before each series of samples, a standard with a known natamycin content shall be injected to determine the retention time and to check the calibration graph (8.3.3).

Because natamycin is unstable in aqueous methanol, carry out the measurement as rapidly as possible.

#### 8.3.3 Calibration graph

Pipette 1 ml, 2 ml, 4 ml, 6 ml and 8 ml, respectively, of natamycin standard working solution (4.3.2) into a series of 50 ml one-mark volumetric flasks (5.1). Make up to the mark with aqueous methanol (4.2) and mix.

The calibration solutions thus obtained contain 0,1 µg/ml, 0,2 µg/ml, 0,4 µg/ml, 0,6 µg/ml, and 0,8 µg/ml of natamycin respectively. Inject, in turn, 20 µl of each standard solution on to the column. Determine the area or height of the peak obtained.

Plot the peak area or peak height obtained for each solution on the ordinate against the natamycin concentration, in micrograms per millilitre, on the abscissa. A specimen HPL chromatogram of a standard solution is shown in Figure A.3.

#### 8.3.4 Test solution

Inject 20 µl of the test solution (8.2.1.2 or 8.2.2.2). Measure the area or the height of the peak having the same retention time as the natamycin calibration solutions.

Carry out the measurement as rapidly as possible.