INTERNATIONAL STANDARD

ISO 9233

First edition
1991-09-01

Cheese and cheese rind — Determination of natamycin content — Method by molecular absorption spectrometry and by high-performance liquid chromatography

Fromage et croûtes de fromage — Détermination de la teneur en natamycine — Méthode par spectrométrie d'absorption moléculaire et par chromatographie liquide à haute performance

Reference number
ISO 9233:1991(E)

Foreword

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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.

International Standard ISO 9233 was prepared by Technical Committee ISO/TC 34, *Agricultural food products*, Sub-Committee SC 5, *Milk and milk products*, in collaboration with the International Dairy Federation (IDF) and the Association of Official Analytical Chemists (AOAC) and will also be published by these organizations.

Annex A of this International Standard is for information only.

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Case Postale 56 • CH-1211 Genève 20 • Switzerland

Printed in Switzerland

Cheese and cheese rind — Determination of natamycin content — Method by molecular absorption spectrometry and by high-performance liquid chromatography

1 Scope

This International Standard specifies a method for determining the natamycin content of cheese rind and of the cheese adjacent to the rind.

The limit of detection is 0,5 mg/kg.

The method is suitable to control a maximum tolerance of natamycin on the cheese rind at a content of 1 mg/dm^2 [1)].

The method is also suitable to detect migration of natamycin into the cheese.

2 Normative reference

The following standard contains provisions which, through reference in this text, constitute provisions of this International Standard. At the time of publication, the edition indicated was valid. All standards are subject to revision, and parties to agreements based on this International Standard are encouraged to investigate the possibility of applying the most recent edition of the standard indicated below. Members of IEC and ISO maintain registers of currently valid International Standards.

ISO 707:1985, *Milk and milk products — Methods of sampling*.

3 Definitions

For the purposes of this International Standard, the following definitions apply.

3.1 natamycin content of cheese or cheese rind: The content of natamycin determined by the method specified in this International Standard.

The natamycin content of cheese is expressed in milligrams per kilogram. The natamycin content of cheese rind is expressed in milligrams per square decimetre.

3.2 cheese rind: Outer layer of 5 mm thickness of the cheese, including the cheese coating layer, if present.

4 Principle

Extraction of a known quantity of sample with methanol. Dilution of the extract with water followed by cooling to between − 15 °C and − 20 °C, to precipitate most of the fat, and filtration.

Determination of the natamycin content in the filtrate (after concentration, if necessary) by a spectrometric or high-performance liquid chromatographic (HPLC) method.

5 Reagents and reference substances

All reagents used shall be of recognized analytical grade. The water used shall be distilled or deionized water.

5.1 Methanol.

5.2 Methanol, aqueous solution.

Mix 2 volumes of methanol with 1 volume of water.

5.3 Natamycin, standard solution corresponding to 5 mg of natamycin per litre.

Immediately before use, dissolve in a 100 ml volumetric flask a quantity of a natamycin preparation, of known natamycin content, corresponding

1) This content corresponds to a draft EEC limit for natamycin on cheese rind.

to 50 mg of pure natamycin in the methanol (5.1) and make up to the mark.

In a 50 ml volumetric flask, dilute 5,0 ml of this solution to 50 ml with the aqueous methanol (5.2).

In another 50 ml volumetric flask, dilute 5,0 ml of this diluted solution to 50 ml with the aqueous methanol (5.2).

1 ml of this standard solution contains 5 µg of natamycin. It is necessary that the concentration of this final standard solution be close to that of the test solution as measured in 9.3.1.2. Adjust the final dilution, if required.

5.4 Acetic acid, glacial.

6 Apparatus

Usual laboratory apparatus and, in particular, the following.

6.1 Slicer, or similar apparatus, for cutting portions of cheese rind 5 mm thick and about 3 cm wide (see figure 1 for an example).

6.2 Fine-slicer, for cutting thin slices of cheese of maximum thickness 1 mm (see figure 2 for an example).

6.3 Grinder or **blender**.

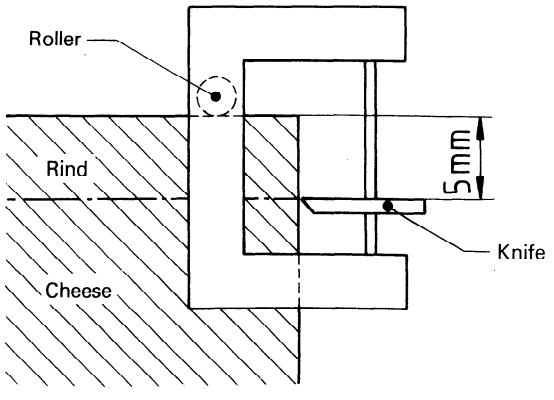


Figure 1 — Example of a slicer for cutting portions of cheese rind 5 mm thick

6.4 Sharp knife, for cutting slices of cheese into small pieces.

6.5 Magnetic stirrer or **shaking machine**.

6.6 Conical flasks, of 100 ml and 200 ml capacity, made of coloured glass and fitted with ground glass stoppers.

6.7 Syringes, disposable, of 10 ml capacity.

6.8 Membrane microfilters, of 0,20 µm and 0,45 µm pore size, resistant to attack by alcoholic solutions.

6.9 Folded filter papers[2], fast speed, of 15 cm diameter.

6.10 Funnel, approximately 7 cm in diameter.

6.11 Freezer, operating in the temperature range − 15 °C to − 20 °C.

6.12 Extraction cartridges type C_{18}[3], to concentrate the filtered extract, if necessary.

6.13 Spectrometer, suitable for recording an ultraviolet (UV) spectrum between 300 nm and 340 nm, equipped with cells of 1 cm optical path length and a recorder.

6.14 Liquid chromatograph, with UV detector capable of measuring at 303 nm and equipped with a recorder and/or integrator.

6.15 Analytical column, of 150 mm length and 4,6 mm internal diameter, type C_8[4], having a particle size of 5 µm.

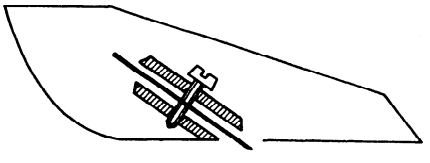


Figure 2 — Example of a fine-slicer for cutting slices of cheese of maximum thickness 1 mm

2) S and S, No. 595 1/2, are examples of suitable products available commercially. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of these products.

3) Sep-pack C_{18} and Waters No. 51910 are examples of suitable products available commercially. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of these products.

4) Lichrosorb RP 8 is an example of a suitable product available commercially. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of this product.

6.16 Guard column, of 100 mm length and 2,1 mm internal diameter, type C_8[5], having a particle size of 30 µm to 40 µm.

7 Sampling

Sampling shall have been carried out in accordance with ISO 707.

A whole cheese, or a segment of a cheese representative of the whole, shall be available to the laboratory.

8 Preparation of the test sample

8.1 Cheese rind

If necessary, cut the laboratory sample into small sections or portions so that the width of the cheese rind is not more than about 3 cm. Remove the whole rind, to a thickness of 5 mm, from all the sections or portions thus obtained using the slicer (6.1).

From the rind obtained, cut a rectangular piece (between 20 cm^2 and 40 cm^2) and determine its surface area, in square centimetres. Determine its mass, in grams.

Grind carefully the whole rind, including the weighed and measured piece, and mix thoroughly. Transfer immediately to a sample jar a quantity of the sample thus prepared.

After preparing each sample, clean all tools which have been in contact with the cheese or cheese rind, first with hot water and then with methanol, and dry thoroughly, for instance using a stream of compressed air.

8.2 Interior of cheese

After removing the rind as described in 8.1, remove a slice of 1 mm maximum thickness from the whole of the outer section of the cheese using the fine-slicer (6.2).

Cut all the slices of cheese into small pieces about 0,5 cm^2 and mix thoroughly. Transfer immediately to a sample jar a quantity of the sample thus prepared.

After preparing each sample, clean all tools which have been in contact with the cheese, first with hot water and then with methanol, and dry thoroughly, for instance using a stream of compressed air.

9 Procedure

9.1 Test portion

In the case of cheese rind, weigh, to the nearest 10 mg, about 10 g of the test sample (8.1) into a 200 ml conical flask (6.6).

In the case of cheese, weigh, to the nearest 10 mg, about 5 g of the test sample (8.2) into a 100 ml conical flask (6.6).

9.2 Preparation of the test solution

In the case of cheese rind, add 100 ml of the methanol (5.1) to the test portion and, in the case of cheese, add 50 ml of the methanol (5.1) to the test portion from a measuring cylinder. Stir the contents of the conical flask for 90 min using a magnetic stirrer (6.5), or shake for 90 min in a shaking machine (6.5).

In the case of cheese rind, add 50 ml of water and, in the case of cheese, add 25 ml of water from a measuring cylinder.

Immediately place the conical flask in the freezer (6.11) and allow it to stand for about 60 min.

Filter the cold extract through a folded filter paper (6.9), discarding the first 5 ml of the filtrate.

NOTE 1 The filtration has to 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 (6.7) and filter through a microfilter of 0,45 µm pore size and then through a microfilter of 0,20 µm pore size (6.8).

The minimum amount of filtrate required is 3 ml for direct spectrometric measurement (9.3.1), 20 µl per injection for direct chromatographic measurement (9.3.2), and 25 ml and 50 ml for 5 times and 10 times concentration respectively (9.3.3).

Proceed in accordance with 9.3.1, 9.3.2 or 9.3.3 as appropriate.

9.3 Determination

9.3.1 Spectrometric measurement

9.3.1.1 Natamycin standard solution

Record the spectrum of the natamycin standard solution (5.3) in the range from 300 nm to 340 nm. Measure the absorbance at the maximum of about

5) Perisorb RP 8 is an example of a suitable product available commercially. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of this product.

317 nm, at the minimum of about 311 nm and at 329 nm exactly. Use the aqueous methanol (5.2) as a blank.

For an example of the spectrum of a standard solution, see figure 3.

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

The exact position of the maximum at 317 nm and the minimum at 311 nm may be slightly shifted, owing to variations in the calibration of the apparatus. Always use the actual maximum and minimum values.

9.3.1.2 Test solution

Record the spectrum of the test solution (9.2) in the range 300 nm to 340 nm. Measure the absorbance at the maximum of about 317 nm, at the minimum of about 311 nm, and at 329 nm exactly. Use the aqueous methanol (5.2) as a blank.

If the natamycin content of the sample is so low that detection is impossible or almost impossible (signal-to-noise ratio less than 3) but determination is nevertheless still required, proceed in accordance with 9.3.3.

For examples of spectra of test solutions, see figure 4.

NOTE 2 The presence of spices, particularly pepper, in the cheese can interfere with the result. This will be obvious by distortion of the absorbance graph, as shown in figure 4.

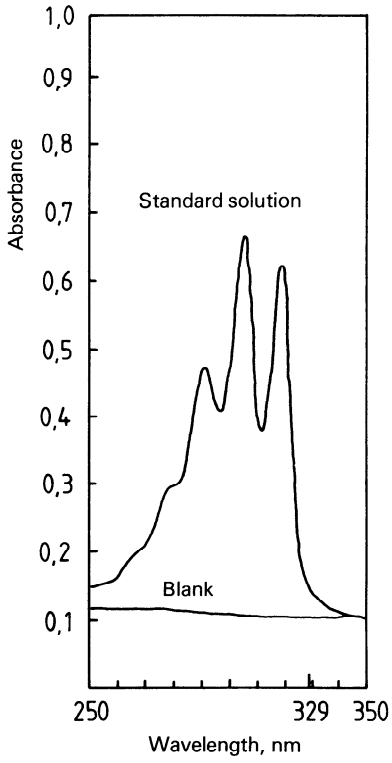
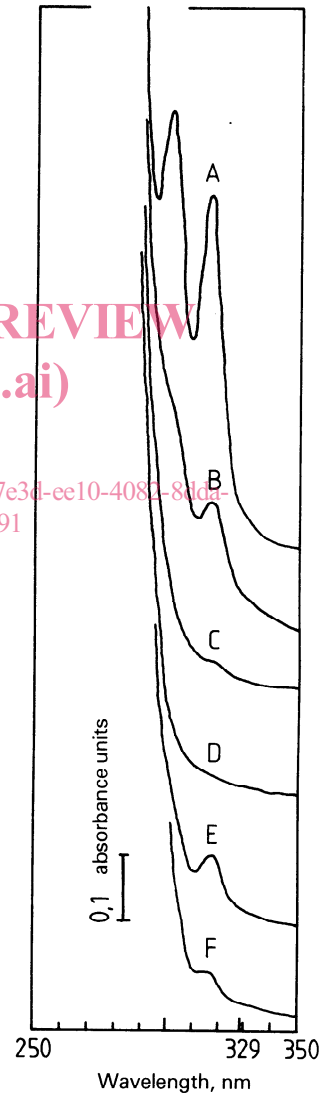


Figure 3 — Example of a spectrum of a natamycin standard solution and a blank



A Cheese rind, natamycin content 61 mg/kg
B Cheese rind, natamycin content 15 mg/kg
C Cheese, natamycin content 1,7 mg/kg
D Cheese, natamycin content 0,3 mg/kg
E Cheese, as C, after concentration ×5
F Cheese, as D, after concentration ×10

Figure 4 — Examples of spectra of various test solutions

9.3.2 High-performance liquid chromatographic measurement

9.3.2.1 Adjustment of the liquid chromatograph

(6.14, 6.15, 6.16)

The following chromatographic conditions are recommended:

mobile phase: methanol/water/acetic acid, 60 + 40 + 5 ($V/V/V$)

flow: 1 ml/min

detector set: 303 nm, 0,005 absorbance units, full scale

recorder: 10 mV

theoretical (typical) plate count: 1 500 minimum

NOTE 3 When a column other than that given as an example (6.15) is used, the methanol-to-water ratio may have to be adjusted. The relative amount of acetic acid (5.4) specified, however, is essential to keep the absorption 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 (9.3.2.2).

9.3.2.2 Calibration graph

Pipette 1 ml, 2 ml, 4 ml, 6 ml and 8 ml of the standard natamycin solution (5.3) into a series of 50 ml volumetric flasks and fill to the mark with the aqueous methanol (5.2).

These solutions contain 0,1 µg/ml, 0,2 µg/ml, 0,4 µg/ml, 0,6 µg/ml and 0,8 µg/ml respectively of natamycin. Inject, in turn, 20 µl of each of these solutions and determine the area or height of the peak obtained.

Plot the values of peak area or peak height for each solution on the ordinate against the natamycin concentration, in micrograms per millilitre, on the abscissa. For an example of a high-performance liquid chromatogram of a standard solution, see figure 5.

NOTE 4 The presence of spices, particularly pepper, in the cheese can interfere with the determination in that a peak may be produced in the chromatogram which has the same retention time as that corresponding to natamycin.

Separation can be achieved by means of gradient elution or by the isocratic use of an alternative mobile phase.

9.3.2.3 Test solution

Inject 20 µl of the test solution (9.2). Measure the area or the height of the peak having the same retention time as the standard natamycin solutions.

Carry out the measurement as rapidly as possible.

If the peak area or peak height of the test solution is so low that interpolation on the calibration graph is impossible or almost impossible but determination is nevertheless still required, proceed in accordance with 9.3.3.

For examples of high-performance liquid chromatograms of test solutions, see figure 6.

9.3.3 Concentration of the filtered extract, for determination of low natamycin contents

9.3.3.1 Concentration

Decide whether a concentration of about 5 times or about 10 times is desired. Base this decision on the data found in accordance with 9.3.1.2 or 9.3.2.3 and on the required detection limit.

Pipette 25 ml or 50 ml (for 5 or 10 times concentration respectively) of the test solution (9.2) into a beaker. Add respectively 50 ml or 100 ml of water and mix.

Activate an extraction cartridge type C_{18} [3] (6.12) using 3 ml to 5 ml of the methanol (5.1), then wash with 10 ml of water.

Pass the diluted test solution through the cartridge at a speed of about 25 ml/min with the aid of a syringe (6.7).

Rinse the cartridge with 10 ml of water with the aid of a syringe (6.7).

Elute the natamycin with 3 ml of the methanol (5.1) with the aid of a syringe (6.7).

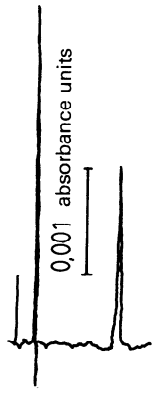
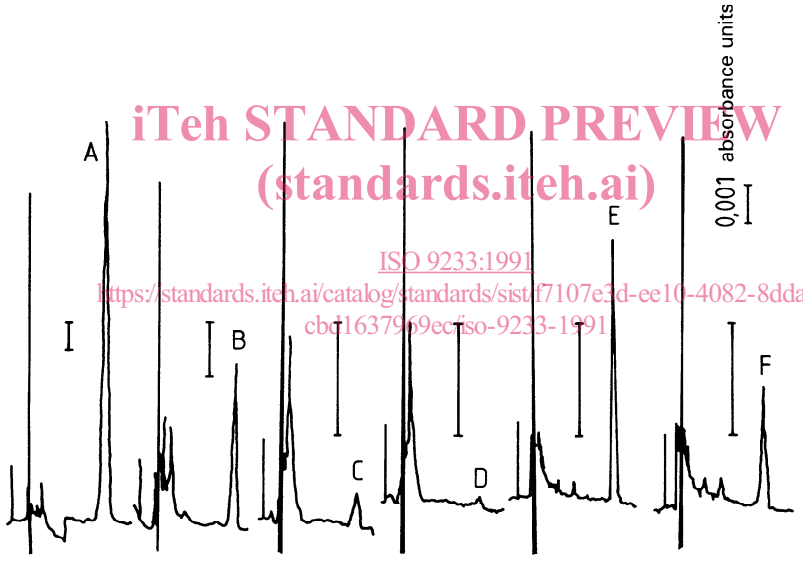


Figure 5 — Example of a high-performance liquid chromatogram of a standard solution containing 0,5 µg/ml of natamycin



A Cheese rind, natamycin content 61 mg/kg
B Cheese rind, natamycin content 15 mg/kg
C Cheese, natamycin content 1,7 mg/kg
D Cheese, natamycin content 0,3 mg/kg
E Cheese, as C, after concentration ×5
F Cheese, as D, after concentration ×10

Figure 6 — Examples of high-performance liquid chromatograms of various test solutions

9.3.3.2 Spectrometric measurement

Add 1,5 ml of water to the eluate (9.3.3.1) and mix.

Take the solution in a syringe (6.7) and filter through a microfilter of 0,45 µm pore size, and then through a microfilter of 0,20 µm pore size (6.8), into a cell.

Proceed as in 9.3.1.2.

9.3.3.3 High-performance liquid chromatographic measurement

Dilute the eluate (9.3.3.1) to 5 ml with the methanol (5.1).

Proceed as in 9.3.2.3.

10 Expression of results

10.1 Spectrometric method

Calculate the natamycin content, w_s, of the sample using equation (1):

$$w_s = \frac{V \times A_s}{m_t \times A_N} \times c_N \quad \ldots (1)$$

where

w_s is the natamycin content of the sample, in milligrams per kilogram;

c_N is the natamycin concentration of the natamycin standard solution, in micrograms per millilitre;

A_N is the net absorbance of the natamycin standard solution at about 317 nm;

A_s is the net absorbance of the test solution at about 317 nm;

m_t is the mass of the test portion (9.1), in grams;

V is the total volume of the test solution (9.2), in millilitres.

A_N can be taken from the UV spectrum of the standard solution (see, for example, figure 3), using the straight line between the absorbance at about 311 nm and that at 329 nm as a baseline, or can be calculated from equation (2):

$$A_N = (A_1)_N - \frac{2}{3}(A_2)_N - \frac{1}{3}(A_{329})_N \quad \ldots (2)$$

where

$(A_1)_N$ is the maximum absorbance at about 317 nm;

$(A_2)_N$ is the minimum absorbance at about 311 nm;

$(A_{329})_N$ is the absorbance at 329 nm.

A_s can be taken from the UV spectrum of the test solution (see, for example, figure 7), using the straight line between the absorbance at about 311 nm and that at 329 nm as a baseline, or can be calculated from equation (3):

$$A_s = (A_1)_s - \frac{2}{3}(A_2)_s - \frac{1}{3}(A_{329})_s \quad \ldots (3)$$

where

$(A_1)_s$ is the maximum absorbance at about 317 nm;

$(A_2)_s$ is the minimum absorbance at about 311 nm;

$(A_{329})_s$ is the absorbance at 329 nm.

In the case of the test solution representing the cheese sampled below the rind, the value w_s gives the natamycin content resulting from migration of natamycin into the cheese.

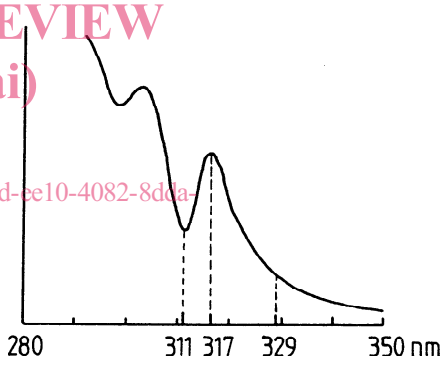


Figure 7 — UV spectrum of a sample containing natamycin

In the case of the test solution representing the cheese rind, calculate the natamycin content on the surface of the cheese rind, w'_s, expressed in milligrams per square decimetre, from w_s using equation (4):

$$w'_s = \frac{w_s \times m_r}{A} \quad \ldots (4)$$

where

w_s is the natamycin content of the sample, in milligrams per kilogram;

A is the area of the weighed piece of cheese rind (8.1), in square decimetres;

m_r is the mass of the weighed piece of cheese rind (8.1), in kilograms.