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**Milk and milk products — Determination  
of nitrate and nitrite contents —**

Part 3:

**Method using cadmium reduction and flow  
injection analysis with in-line dialysis  
(Routine method)**

*Lait et produits laitiers — Détermination des teneurs en nitrates et en  
nitrites —*

*Partie 3: Méthode par réduction au cadmium et d'analyse par injection avec  
dialyse en ligne (Méthode de routine)*



Reference numbers  
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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 3.

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 part of ISO 14673|IDF 189 may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

International Standard ISO 14673-3|IDF 189-3 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 5, *Milk and milk products*, and the International Dairy Federation (IDF), in collaboration with AOAC International. It is being published jointly by ISO and IDF and separately by AOAC International.

This first edition of ISO 14673-3|IDF 189-3, together with ISO 14673-1|IDF 189-1 and ISO 14673-2|IDF 189-2, cancels and replaces ISO 4099:1984, ISO 6736:1982, ISO 6739:1988, ISO 6740:1985 and ISO 8195:1987, which have been technically revised.

ISO 14673|IDF 189 consists of the following parts, under the general title *Milk and milk products — Determination of nitrate and nitrite contents*:

- Part 1: Method using cadmium reduction and spectrometry
- Part 2: Method using segmented flow analysis (Routine method)
- Part 3: Method using cadmium reduction and flow injection analysis with in-line dialysis (Routine method)

## 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 and AOAC International 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 National Committees casting a vote.

International Standard ISO 14673-3|IDF 189-3 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 5, *Milk and milk products*, and the International Dairy Federation (IDF), in collaboration with AOAC International. It is being published jointly by ISO and IDF and separately by AOAC International.

All work was carried out by the Joint ISO/IDF/AOAC Action Team, *Minerals and minor compounds*, of the Standing Committee on *Minor components characterization of physical properties*, under the aegis of its project leader Mr G. Bråthen (NO).

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# Milk and milk products — Determination of nitrate and nitrite contents —

Part 3:

## Method using cadmium reduction and flow injection analysis with in-line dialysis (Routine method)

**WARNING** — The use of this International Standard may involve hazardous materials, operations and equipment. This standard does not purport to address all the safety problems associated with its use. It is the responsibility of the user of this standard to establish safety and health practices and determine the applicability of regulatory limitations prior to use.

### 1 Scope

This part of ISO 14673|IDF 189 specifies a routine method for the determination of the nitrate and nitrite contents of milk and milk products by cadmium reduction and flow injection analysis (FIA). The method is applicable to hard, semi-hard and soft cheeses of various ages, and processed cheese. The detection limits of the method are 0,5 mg of nitrate ions per kilogram and 1,0 mg of nitrite ions per kilogram, respectively.

The method is also applicable to whey powder, milk powder and milk-based infant food.

NOTE 1 The method closely resembles the FIA method described in reference [2] for the determination of nitrate and nitrite in milk and fluid dairy products. Adaptations were made to allow for the analysis of cheese and to obtain sufficient sensitivity for the determination of low levels of nitrite in cheese and milk-based infant foods.

NOTE 2 For determination of nitrite and nitrate following cadmium reduction, use is made of the same colour reaction as described in ISO 14673-1|IDF 189-1.

### 2 Normative references

The following normative documents contain provisions which, through reference in this text, constitute provisions of this part of ISO 14673|IDF 189. For dated references, subsequent amendments to, or revisions of, any of these publications do not apply. However, parties to agreements based on this part of ISO 14673|IDF 189 are encouraged to investigate the possibility of applying the most recent editions of the normative documents indicated below. For undated references, the latest edition of the normative document referred to applies. Members of ISO and IEC maintain registers of currently valid International Standards.

ISO 1042, *Laboratory glassware — One-mark volumetric flasks*

ISO 14673-1|IDF 189-1, *Milk and milk products — Determination of nitrate and nitrite contents — Part 1: Method using cadmium reduction and spectrometry*

### 3 Terms and definitions

For the purposes of this part of ISO 14673|IDF 189, the following terms and definitions apply.

### 3.1

#### nitrate content

mass fraction of nitrate determined by the procedure specified in this part of ISO 14673 | IDF 189

NOTE The nitrate content is expressed as the mass in milligrams of nitrate ions ( $\text{NO}_3^-$ ) per kilogram of product.

### 3.2

#### nitrite content

mass fraction of nitrite determined by the procedure specified in this part of ISO 14673 | IDF 189

NOTE The nitrite content is expressed as the mass in milligrams of nitrite ions ( $\text{NO}_2^-$ ) per kilogram of product.

## 4 Principle

4.1 A test portion is suspended in warm extraction buffer. Fat is separated by centrifuging and rapid cooling. Analyses are made of small portions of the de-fatted solution by flow injection analysis (FIA). In-line dialysis is used to remove protein and remaining fat. The nitrate ions are reduced to nitrite ions by cadmium. The nitrite ions are reacted with sulfanilamide and *N*-1-naphthyl ethylenediamine dihydrochloride to give a red-coloured azo dye. The colour is measured in a flow cell at maximum absorption of the dye at 540 nm with reference to the absorption measured at 620 nm.

4.2 The nitrite and nitrate contents of the test sample are calculated with reference to the measured absorbances for a series of standard solutions of nitrite and nitrate, respectively. If the nitrite content exceeds 0,5 mg/kg, or exceeds 10 % of the nitrate content, correction of the nitrate content is made by subtracting the nitrite content from the obtained nitrate results.

## 5 Reagents

Use only reagents of recognized analytical grade unless otherwise specified.

5.1 **Water**, distilled or deionized, or water of equivalent purity, free from nitrate and nitrite ions.

5.2 **Cadmium reduction column**, for example Aquatec-Tecator <sup>1)</sup>.

5.3 **Extraction buffer solution or carrier solution (C2).**

Dissolve 26,6 g of ammonium chloride ( $\text{NH}_4\text{Cl}$ ) in 800 ml water in a 1 000 ml conical flask. By adding concentrated ammonia, adjust the pH to 8,5. Dilute to 1 000 ml with water and mix.

5.4 **Hydrochloric acid (HCl)**, ( $\rho_{20} = 1,19$  g/ml).

5.5 **Reagent solution (R1).**

Dissolve 5,0 g sulfanilamide ( $\text{NH}_2\text{C}_6\text{H}_4\text{SO}_2\text{NH}_2$ ) in a mixture of 300 ml water and 26 ml of hydrochloric acid (5.4) in a 500 ml volumetric flask (6.3). Dilute to the mark with water and mix.

5.6 **Reagent solution (R2).**

Dissolve 0,5 g of *N*-1-naphthyl ethylenediamine dihydrochloride ( $\text{C}_{10}\text{H}_7\text{NHCH}_2\text{CH}_2\text{NH}_2 \cdot 2\text{HCl}$ ) in water in a 500 ml volumetric flask (6.3). Dilute to the mark with water and mix.

The solution may be stored for up to 1 week in a well-stoppered brown bottle in a refrigerator.

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1) Aquatec-Tecator is an example of a suitable product available commercially. This information is given for the convenience of users of this part of ISO 14673 | IDF 189 and does not constitute an endorsement by ISO of this product.

**5.7 Regeneration solution**,  $c(\text{HCl}) = 0,1 \text{ mol/l}$ .

Dilute 80 ml of hydrochloric acid (5.4) with water to 1 litre and mix.

**5.8 Sodium nitrate standard solution**,  $c(\text{NO}_3^-) = 1\,000 \text{ mg/l}$ .

Before use, dry sodium nitrate ( $\text{NaNO}_3$ ) to constant mass at  $110\text{ }^\circ\text{C}$  to  $120\text{ }^\circ\text{C}$ . Dissolve 137,1 mg of the dry  $\text{NaNO}_3$  in water in a 100 ml volumetric flask (6.3). Dilute to the mark with water and mix.

**5.9 Sodium nitrate calibration standard solutions.**

Prepare the sodium nitrate calibration standard solution on the day of use. Pipette amounts of 25  $\mu\text{g/l}$ , 50  $\mu\text{g/l}$ , 100  $\mu\text{g/l}$ , 150  $\mu\text{g/l}$  and 250  $\mu\text{g/l}$  of sodium nitrate standard solution (5.8) into separate 50 ml volumetric flasks (6.3). Dilute to the mark with extraction buffer solution (5.3) to obtain sodium nitrate calibration standards solutions corresponding to 0,50 mg/l, 1,00 mg/l, 2,00 mg/l, 3,00 mg/l and 5,00 mg/l nitrate ions respectively.

**5.10 Sodium nitrite standard solution**,  $c(\text{NO}_2^-) = 1\,000 \text{ mg/l}$ .

Before use, dry the sodium nitrite ( $\text{NaNO}_2$ ) to constant mass at  $110\text{ }^\circ\text{C}$  to  $120\text{ }^\circ\text{C}$ . Prepare the sodium nitrite standard solution on the day of use. Dissolve 150,0 mg of the dry  $\text{NaNO}_2$  in water in a 100 ml volumetric flask (6.3). Dilute to the mark with water and mix.

**5.11 Sodium nitrite standard solution**,  $c(\text{NO}_2^-) = 50,0 \text{ mg/l}$ .

Pipette 5,00 ml of the sodium nitrite standard solution (5.10) in a 100 ml volumetric flask. Dilute to the mark with water and mix.

**5.12 Sodium nitrite calibration standard solution.**

Shortly before use, pipette amounts of 25  $\mu\text{l}$ , 50  $\mu\text{l}$ , 100  $\mu\text{l}$ , 200  $\mu\text{l}$  and 400  $\mu\text{l}$  of the sodium nitrite standard solution (5.11) into separate 50 ml volumetric flasks (6.3). Dilute to the mark with extraction buffer solution (5.3) to obtain sodium nitrite calibration standard solutions corresponding to 0,025 mg/l, 0,050 mg/l, 0,100 mg/l, 0,200 mg/l and 0,400 mg/l nitrite ions respectively. Mix well.

**5.13 Sodium nitrite reference solution**,  $c(\text{NO}_2^-) = 1,48 \text{ mg/l}$ .

Pipette 1 480  $\mu\text{l}$  of the sodium nitrite standard solution (5.11) in a 50 ml volumetric flask (6.3). Dilute to the mark with extraction buffer solution (5.3) and mix. Prepare the nitrite reference solution shortly before use.

## 6 Apparatus

Clean all glassware thoroughly and rinse with distilled water to ensure that it is free from nitrate and nitrite ions.

Usual laboratory equipment and, in particular, the following.

**6.1 Analytical balance**, capable of weighing to the nearest 1 mg, with a readability of 0,1 mg.

**6.2 Sample container**, provided with an airtight lid.

**6.3 Volumetric flasks**, of nominal capacity 50 ml, 100 ml and 500 ml respectively, complying with the requirements of ISO 1042, class B.

**6.4 Pipettes**, capable of delivering 25  $\mu\text{l}$ , 50  $\mu\text{l}$ , 100  $\mu\text{l}$ , 150  $\mu\text{l}$ , 200  $\mu\text{l}$ , 250  $\mu\text{l}$ , 400  $\mu\text{l}$  and 1 480  $\mu\text{l}$  respectively (semi-automatic pipettes).

**6.5 Grinding device**, appropriate for grinding the test sample, if necessary. To avoid loss of moisture, the device should not produce undue heat. A hammer mill shall not be used.

**6.6 Laboratory mixer or homogenizer** (e.g. an Ultra-turrax<sup>2)</sup>), with glass containers of capacity 250 ml or 400 ml, suitable for suspending test portions of cheese.

**6.7 Table centrifuge**, with centrifuge tubes of capacity 50 ml, capable of centrifuging at 1 500 g.

**6.8 Flow injection analyser (FIA)** (e.g. a Tecator<sup>2)</sup>), with a Fiastar analyser, controller, dialysis module, sampler, provided with sample cups of 4 ml, with specific instrument control and evaluation software, nitrite/nitrate manifold (see Figure 1), a sample loop volume of 200 µl, two mixing coils of length 60 cm and of diameter 0,7 mm, connecting tubings and pump tubings providing the flow rates given in Table 1.

Other flow injection analysers may use other procedures. The instructions of the manufacturer, therefore, should be strictly followed.

**Table 1 — Flow rates**

Values in millilitres per minute

Solution	Flow rate for nitrate	Flow rate for nitrite
Sample/wash	2	2
Carrier C1 (water) (5.1)	2	1,2
Carrier C2 (5.3)	2	1,2
Reagent R1 (5.5)	0,6	0,4
Reagent R2 (5.6)	0,6	0,4

**6.9 Matrix spectrophotometer** (e.g. SKALAR<sup>2)</sup>), equipped with interference filter 540 nm and interference filter 620 nm, a flow cell of path length 5 cm and of volume 40 µl.

**6.10 FIA program parameters for nitrite.**

**6.10.1 Program parameters**

Evaluation	peak height
Calibration equation	linear
Number of standards	5
FIA-pattern collection time/cycle time.	120 s

**6.10.2 Timing parameters**

Window for peak maximum	30 s to 50 s
Signal filtering maximum	2 s
Window for baseline detection	0 to 10 s
Injection period	0 to 60 s
Sample loop wash	60 s to 80 s
Sample loop filling	80 s to 120 s

2) These products are examples of a suitable products available commercially. This information is given for the convenience of users of this part of ISO 14673 | IDF 189 and does not constitute an endorsement by ISO of these products.

### 6.10.3 Calibration check

Tolerance	± 5 %
Repeat after (samples)	10 samples
Standard used	0,100 mg/l NO <sub>2</sub> <sup>-</sup>

### 6.11 FIA program parameters for nitrate.

#### 6.11.1 Program parameters

Evaluation	peak height
Calibration equation	linear
Number of standards	5
FIA-pattern collection time/cycle time	60 s

#### 6.11.2 Timing parameters

Window for peak maximum	25 s to 40 s
Signal filtering time	1 s
Window for baseline detection	0 to 15 s
Injection period	0 to 20 s
Sample loop wash	20 s to 35 s
Sample loop filling	35 s to 60 s

#### 6.11.3 Calibration check

Tolerance	± 5 %
Repeat after (samples)	10 samples
Standard used	2,00 mg/l NO <sub>3</sub> <sup>-</sup>

## 7 Sampling

Sampling is not part of the method specified in this part of ISO 14673 | IDF 189. A recommended sampling method is given in ISO 707.

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

Store the test sample in such a way that deterioration and change in composition are prevented.

## 8 Preparation of test sample

### 8.1 Cheese

**8.1.1** Prior to analysis, remove the rind or mouldy surface layer of the test sample in such a way as to obtain a sample representative of the cheese as it is usually consumed.

**8.1.2** Grind the test sample by means of an appropriate grinding device (6.5). Mix the ground mass quickly and, if possible, grind a second time and again mix thoroughly. Clean the device after grinding of each sample. If the test sample cannot be ground, mix it thoroughly by intensive stirring and kneading.

**8.1.3** As soon as possible after grinding, transfer the test sample to a sample container (6.2) to await the determination which, preferably, should be carried out without delay. If a delay is unavoidable, take all precautions to ensure proper conservation of the test sample and to prevent preservation of moisture on the inside surface of the container.

**8.1.4** Do not examine ground cheese showing unwanted mould growth or beginning to deteriorate.

## **9 Procedure**

### **9.1 Checking the reducing capacity of the cadmium column**

**9.1.1** Check the reducing capacity of the cadmium column at the beginning and at the end of a series of determinations and at least twice a day.

**9.1.2** Prepare the FIA system for operation according to 9.6.1.

**9.1.3** Fill a cup with sodium nitrite reference solution (5.13).

**9.1.4** Fill another cup with the sodium nitrate calibration standard solution (5.9).

**9.1.5** Analyse the sodium nitrate calibration standard solution (9.1.4) and the sodium nitrite reference solution (9.1.3). Divide the peak height found for nitrate by the peak height for nitrite and multiply by 100 to obtain the percentage reducing capacity of the cadmium column. If the reducing capacity is less than 95 %, regenerate the column (see 9.2).

### **9.2 Regeneration of the cadmium column**

Regenerate the cadmium column at the end of each day after use, or more frequently if the check of its reducing capacity (9.1.5) indicates a loss of efficiency.

Disable pump flows for carrier solution C2 (5.3) and reagents R1 (5.5) and R2 (5.6). Unscrew the tubing at the inlet of the dialysis module connecting the injector to the dialysis module. Run water (5.1) used as carrier solution C1 through the injector until the system is filled. Connect the cadmium column to the outlet of the injector. Start the pump again and make three to five injections of reagent solution R2 (5.6) followed by the sodium nitrate standard solution (5.8). Wash the column by passage of carrier solution C2 (5.3).

### **9.3 Test portion**

#### **9.3.1 Cheese and dried milk products**

Weigh, to the nearest 1 mg, 2,5 g of the test sample (8.1.2) into a 50 ml centrifuge tube (6.7).

#### **9.3.2 Liquid milk products**

Weigh, to the nearest 1 mg, 10 g of the test sample (8.1.2) into a 50 ml centrifuge tube (6.7).

### **9.4 Extraction**

**9.4.1** Add 24 ml of extraction buffer (5.3), preheated to a temperature of 50 °C to 55 °C, to the test portion (9.3) in the 50 ml centrifuge tube. Mix with the homogenizer for about 3 min until the test portion is well suspended.

**9.4.2** Centrifuge the test solution in tube (9.4.1) at about 1 500 g for 5 min.

**9.4.3** Place the centrifuge tube in a mixture of water and ice for 15 min to cool the test solution.

**9.4.4** Withdraw with a pipette (6.4) the de-fatted test solution from underneath the fat layer in the centrifuge tube. Fill the cups of the FIA, for both the nitrite and nitrate determinations, with the obtained de-fatted test solution.

## 9.5 Determination of nitrite content

**9.5.1** Install the manifold of the flow injection analyser (FIA) according to the scheme in Figure 1. Use the pump tubings of the FIA (6.8) for the determination of nitrite while leaving out the cadmium reduction column (5.2). Connect the bottles with both reagent solutions R1 (5.5) and R2 (5.6) and carrier solution C2 (5.3). Start the pumps of the FIA to flush the system for 5 min to 10 min.

**9.5.2** Load the analyser with the FIA-program for nitrite (6.10). Run the sodium nitrite calibration standard solutions (5.12) to calibrate the system, followed by the de-fatted test solution (9.4.4).

**9.5.3** Check the calibration, both at the end of a series and after each group of 10 test solutions, by analysing the sodium nitrite calibration standard solution of 0,100 mg/l nitrite ions (5.12).

## 9.6 Determination of nitrate content

**9.6.1** Install the manifold of the flow injection analyser (FIA) according to the scheme in Figure 1. Use the pump tubings of the FIA (6.8) for the determination of nitrate while using the cadmium reduction column (5.2). Connect the bottles with both reagent solutions R1 (5.5) and R2 (5.6) and carrier solution C2 (5.3). Start the pumps of the FIA to fill the system with the liquids. Stop the pumps and install the cadmium reduction column (5.2). Start the pumps again to flush the system for 5 min to 10 min. Check the reducing capacity of the cadmium column (9.1).

**9.6.2** Load the analyser with the FIA-program for nitrate (6.11). Run the sodium nitrate calibration standard solutions (5.9), to calibrate the system, followed by the de-fatted test solution (9.4.4).

**9.6.3** Check the calibration, both at the end of a series and after each group of 10 test solutions, by analysing the sodium nitrate calibration standard solution of 2,00 mg/l nitrate ions (5.9).

## 10 Calculation and expression of results

### 10.1 Nitrite content

#### 10.1.1 Calculation of nitrite content

Calculate the nitrite content of the test sample,  $\omega_{N1}$ , using the following equation:

$$\omega_{N1} = \frac{25}{m} \times c_{N1}$$

where

$\omega_{N1}$  is the nitrite content of the sample, in milligrams of nitrite ions per kilogram;

$c_{N1}$  is the numerical value of the concentration, read from the calibration graph, corresponding to the measured absorbance of the test solution (9.5.2), in micrograms of nitrite ions per litre;

$m$  is the mass of the test portion (9.3), in grams.

#### 10.1.2 Expression of results

Express the results to one decimal place.

## 10.2 Nitrate content

### 10.2.1 Calculation of nitrate content

Calculate the nitrate content of the sample,  $\omega_{N2}$ , using the following equation:

$$\omega_{N2} = \frac{25}{m} \times c_{N2}$$

where

$\omega_{N2}$  is the nitrate content of the sample, in milligrams of nitrate ions per kilogram;

$c_{N2}$  is the numerical value of the concentration, read from the calibration graph, corresponding to the measured absorbance of the test solution (9.6.2), in micrograms of nitrate ions per litre;

$m$  is the mass of the test portion (9.3), in grams.

### 10.2.2 Calculation of the corrected nitrate content

Calculate the corrected nitrate content of the sample,  $\omega_{NC}$ , using the following equation:

$$\omega_{NC} = (\omega_{N2} - \omega_{N1}) \frac{62}{46}$$

where  $\omega_{NC}$  is the nitrate content of the test sample corrected for its nitrite content, in milligrams of nitrate ions per kilogram.

### 10.2.3 Expression of results

Express the results to whole numbers.

## 11 Precision

### 11.1 General

The values for repeatability and reproducibility limits are expressed for the 95 % probability level and may not be applicable to concentration ranges and matrices other than those given.

### 11.2 Nitrite content

#### 11.2.1 Repeatability

The absolute 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, will in not more than 5 % of cases be greater than 0,5 mg/kg of product.

#### 11.2.2 Reproducibility

The absolute difference between two single test results, obtained using the same method on identical test material in different laboratories with different operators using different equipment, will in not more than 5 % of cases be greater than 1,0 mg/kg of product.