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**Milk and milk products — Determination  
of nitrate content — Method by enzymatic  
reduction and molecular-absorption  
spectrometry after Griess reaction**

*Lait et produits laitiers — Détermination de la teneur en nitrates —  
Méthode par réduction enzymatique et spectrométrie d'absorption  
moléculaire après réaction de Griess*

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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 20541|IDF 197 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.

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## Foreword

**IDF (the International Dairy Federation)** is a non-profit organization representing the dairy sector worldwide. IDF membership comprises National Committees in every member country as well as regional dairy associations having signed a formal agreement on cooperation with IDF. All members of IDF have 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 the IDF National Committees casting a vote.

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.

ISO 20541|IDF 197 was prepared by the International Dairy Federation (IDF) and Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 5, *Milk and milk products*. It is being published jointly by IDF and ISO.

All work was carried out by the Joint ISO-IDF Action Team *Minor compounds* of the Standing Committee on *Minor components and characterization of physical properties* under the aegis of its project leaders, Mr. M. Carl (DE) and Mrs. C. Bäckman (FL).

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# Milk and milk products — Determination of nitrate content — Method by enzymatic reduction and molecular-absorption spectrometry after Griess reaction

**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 International Standard specifies a method for the determination of the nitrate content of milk and milk products by molecular-absorption spectrometry after Griess reaction (preceded by enzymatic reduction).

The method is, in particular, applicable to whole, partly skimmed, skimmed and dried milk, hard, semi-hard and soft cheeses, processed cheese, whey cheese, caseins, caseinates, dried whey and milk protein concentrates.

The method can be used at contents corresponding to a measured concentration in the sample solution (with blank subtracted) of more than 0,2 mg/l.

## 2 Normative references

The following referenced documents are indispensable for the application of this document. For dated references only the edition cited applies. For undated references the last edition of the referenced document (including any amendments) applies.

ISO 565, *Test sieves — Metal wire cloth, perforated metal plate and electroformed sheet — Nominal sizes of openings*

ISO 648, *Laboratory glassware — Single-volume pipettes*

ISO 835, *Laboratory glassware — Graduated pipettes*

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

ISO 3696, *Water for analytical laboratory use — Specification and test methods*

## 3 Terms and definitions

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

### 3.1

#### **nitrite content**

mass fraction of nitrite compounds determined by the procedure specified in this International Standard

### 3.2

#### nitrate content

mass fraction of nitrate compounds determined by the procedure specified in this International Standard

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

## 4 Principle

A test portion is dispersed in warm water. The fat and proteins are removed either by precipitation using Carrez reagents and filtering or by centrifugal ultra-filtration using membrane cones (see Notes 1 and 2). The nitrate is reduced to nitrite in a portion of the filtrate by means of nitrate reductase. A red-violet azo dye is developed, in portions of both the unreduced filtrate (for nitrite) and the reduced solution (for nitrate), by addition of sulfanilamide and *N*-(1-naphthyl)ethylenediamine dihydrochloride, and the absorbance measured at a wavelength of 540 nm (or Hg 546 nm). The nitrite content of the sample and the total nitrite content after reduction of nitrate are calculated by comparing the measured absorbances with those of a set of sodium nitrite calibration solutions. The nitrate content is calculated from the difference between these two contents.

NOTE 1 The two alternative procedures for removal of fat and protein are described in 9.2.1 and 9.2.2.

NOTE 2 For whey powder, whey protein concentrate and similar products, ultra-filtration is used in preference to Carrez precipitation as the latter often leads to turbidity problems and, as a consequence, to poor precision.

NOTE 3 The low endogenous nitrite level is not reported but taken into account in the matrix blank solution.

## 5 Reagents

Unless otherwise specified, use only reagents of recognized analytical grade, free of nitrate and nitrite, and water complying with ISO 3696 grade 3 at least, free from nitrate and nitrite. Water used for preparation of enzyme or coenzyme solutions shall be freshly double-distilled or of equivalent purity.

**5.1 Sodium hydroxide solution**,  $c(\text{NaOH}) = 1 \text{ mol/l}$ .

**5.2 Sodium chloride solution**,  $c(\text{NaCl}) = 0,9 \text{ g/100 ml}$ .

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

**5.4 Hydrochloric acid solution**,  $c(\text{HCl}) = 2 \text{ mol/l}$ .

Carefully add 160 ml of hydrochloric acid (5.3) to about 700 ml of water in a 1 000 ml one-mark volumetric flask (6.4) while regularly swirling the contents. Cool the contents to room temperature. Dilute to the mark with water and mix carefully.

**5.5 Carrez reagents**, as follows:

**5.5.1 Carrez reagent I:** Potassium hexacyanoferrate(II) solution,  $c(\text{K}_4[\text{Fe}(\text{CN})_6] \cdot 3\text{H}_2\text{O}) = 150 \text{ g/l}$ .

Dissolve 15,0 g of potassium hexacyanoferrate(II) trihydrate in water in a 100 ml one-mark volumetric flask (6.4). Dilute to the mark with water and mix.

**5.5.2 Carrez reagent II:** Zinc sulfate solution,  $c(\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}) = 300 \text{ g/l}$ .

Dissolve 30,0 g of zinc sulfate heptahydrate in water in a 100 ml one-mark volumetric flask (6.4). Dilute to the mark with water and mix.

## 5.6 Standard solutions, as follows:

### 5.6.1 Sodium nitrite ( $\text{NaNO}_2$ ) stock solution.

Accurately weigh ( $75,0 \pm 0,1$ ) mg of pre-dried (at  $102^\circ\text{C}$  for 2 h) sodium nitrite into a 100 ml one-mark volumetric flask. Dissolve it in a suitable amount of water. Dilute to the mark with water and mix. The nitrite stock solution obtained contains 500 mg of nitrite per litre.

Prepare calibration solutions by diluting the stock solution with water to give several solutions with different nitrite concentrations in the range from 0,05 mg/l to 5,0 mg/l.

When stored at room temperature, the sodium nitrite stock solution remains stable for 1 day.

### 5.6.2 Potassium nitrate ( $\text{KNO}_3$ ) stock solution.

Accurately weigh ( $81,5 \pm 0,1$ ) mg of pre-dried (at  $102^\circ\text{C}$  for 2 h) potassium nitrate into a 100 ml one-mark volumetric flask. Dissolve it in a suitable amount of water. Dilute to the 100 ml mark with water and mix. The obtained nitrate stock solution contains 500 mg of nitrate per litre.

Prepare calibration solutions by diluting the stock solution with water to give several solutions with different nitrate concentrations in the range from 0,05 mg/l to 5,0 mg/l.

When stored at  $4^\circ\text{C}$ , the potassium nitrate stock solution remains stable for 1 week.

## 5.7 Potassium phosphate buffer solution, pH = 7,5.

Accurately weigh ( $57,6 \pm 0,1$ ) mg of dipotassium hydrogen phosphate ( $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$ ) into a 100 ml one-mark volumetric flask. Dissolve it in a suitable amount of water. Dilute to the mark with water and mix.

Accurately weigh ( $17,0 \pm 0,1$ ) mg of potassium dihydrogen phosphate ( $\text{KH}_2\text{PO}_4$ ) into a 50 ml one-mark volumetric flask. Dissolve it in a suitable amount of water. Dilute to the mark with water and mix.

Using the pH-measurement unit (6.18), adjust the pH of the dipotassium hydrogen phosphate solution to pH 7,5 by addition of potassium dihydrogen phosphate solution.

When stored at  $4^\circ\text{C}$ , the potassium phosphate buffer solution remains stable for 2 weeks.

## 5.8 NADPH/FAD solution.

Weigh accurately ( $5,6 \pm 0,1$ ) mg of 3-nicotinamide-adenine dinucleotide phosphate (reduced form), tetrasodium salt ( $\beta\text{-NADPH-Na}_4$ , with a mass fraction of at least 98 %), and ( $80,0 \pm 0,1$ ) mg flavine-adenine dinucleotide, disodium salt ( $\text{FAD-Na}_2$ , with a mass fraction of at least 88 %), into a 25 ml one-mark volumetric flask.

Dissolve them in a suitable amount of potassium phosphate buffer solution (5.7). Dilute to the mark with the buffer solution (5.7) and mix.

Freshly prepare the NADPH/FAD solution immediately before use.

## 5.9 Nitrate reductase (NR) solution.

Weigh 65 mg of nitrate reductase (NR) from *Aspergillus niger* (EC 1.6.6.2, lyophilizate containing approximately 0,4 U/mg) into a 10 ml measuring tube. Add 5 ml of water and mix.

When stored at  $4^\circ\text{C}$ , the nitrate reductase solution remains stable for 2 weeks.

**5.10 Colour reagents**, as follows:

**5.10.1 Colour reagent solution I:** Sulfanilamide ( $\text{NH}_2\text{C}_6\text{H}_4\text{SO}_2\text{NH}_2$ ).

Weigh 400 mg of sulfanilamide into a 50 ml one-mark volumetric flask (6.4). Dissolve it, heating on a water-bath if necessary, in hydrochloric acid solution (5.4).

Cool the solution to room temperature. Dilute to the mark with the hydrochloric acid solution (5.4) and mix. If necessary, filter the reagent solution thus obtained.

When stored at 4 °C, colour reagent solution I remains stable for 4 weeks.

**5.10.2 Colour reagent solution II:** *N*-(1-Naphthyl)ethylenediamine dihydrochloride ( $\text{C}_{10}\text{H}_7\text{NHCH}_2\text{CH}_2\text{NH}_2 \cdot 2\text{HCl}$ ).

Weigh 50 mg of *N*-(1-naphthyl)ethylenediamine dihydrochloride into a 50 ml one-mark volumetric flask (6.4). Dissolve it in a suitable amount of water.

Dilute to the 50 ml mark with water and mix. If necessary, filter the solution thus obtained.

When stored at 4 °C, colour reagent solution II remains stable for 4 weeks.

**5.11** Reagent kits are also commercially available. Carefully follow the instructions of this International Standard when using such kits (in particular in the case of 5.8).

## **6 Apparatus**

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

Usual laboratory equipment and, in particular, the following:

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

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

**6.3 Conical flasks**, of capacities 100 ml, 500 ml and 1 000 ml, with ground-glass stoppers.

**6.4 One-mark volumetric flasks**, of nominal capacities 25 ml, 50 ml, 100 ml and 1 000 ml, complying with the requirements of ISO 1042, class A.

**6.5 Pipettes**, capable of delivering 1 ml, 2 ml, 5 ml and 10 ml, respectively, complying with the requirements of ISO 648, class A, or ISO 835. Where appropriate, burettes may be used instead of pipettes.

**6.6 Graduated pipettes**, of the partial-delivery type, as used in enzyme tests.

**6.7 Graduated cylinders**, of capacities 20 ml and 50 ml.

**6.8 Beakers**, of capacities 20 ml and 50 ml.

**6.9 Centrifuge**, with cooling device, capable of centrifuging cups (6.10) and membrane cones (6.21) with a centrifugal acceleration of 3 000*g*.

**6.10 Centrifuge cups**, of diameter 15 mm.

**6.11 Membrane filter**, of pore size 0,45 µm, for use with a syringe.

**6.12 Glass funnel**, of suitable diameter.

**6.13 Spectrometer**, suitable for measuring absorbance at a wavelength of 540 nm, or **spectral line photometer** with a mercury lamp and filter, suitable for measuring absorbance at a wavelength of 546 nm.

**6.14 Optical cells**, semi-micro type (disposable or glass cuvettes), of optical path length 1 cm.

**6.15 Grinding device**, suitable for grinding the test sample, if necessary. To avoid loss of moisture, the device shall not produce undue heat.

**6.16 Test sieve**, of woven wire cloth, of diameter 200 mm, with openings of nominal size 500 µm and a receiver complying with the requirements of ISO 565.

**6.17 Magnetic stirrer**.

**6.18 pH-measurement unit**, consisting of a pH-meter and glass/reference electrodes, capable of measuring at 20 °C.

**6.19 Water bath**, with shaking facility, capable of operating at  $(70 \pm 0,5)$  °C.

**6.20 Hotplate**.

**6.21 Membrane cones**, MWCO 5 000 D, capacity 4 ml, for centrifugal ultra-filtration of the sample solution.

## 7 Sampling

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

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

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

## 8 Preparation of test sample

### 8.1 Dried milk, dried whey and milk protein concentrates

Transfer the test sample to a sample container (6.2) of capacity about twice the volume of the test sample. Close the container immediately. Mix the test sample thoroughly by repeatedly shaking and inverting the container.

### 8.2 Caseins and caseinates

**8.2.1** Thoroughly mix the test sample, if necessary after transferring all of it to a sample container (6.2) of suitable capacity, by repeatedly shaking and inverting the container.

**8.2.2** Transfer 50 g of the test sample to a test sieve (6.16). If the 50 g portion completely, or nearly completely, passes through the sieve, pass the whole mixed test sample (see 8.2.1) through the sieve. If the test sample does not pass completely through the sieve, use the grinding device (6.15) to ensure that it does.

Immediately transfer the entire sieved test sample to a sample container (6.2). Mix thoroughly in the closed container. During these operations, take precautions to avoid any change in the water content of the product.

After the test sample has been prepared, proceed with the preparation of the test portion (see 9.1) as soon as possible.

### 8.3 Cheese

**8.3.1** Prior to the analysis, remove any rind or mouldy surface layer from the test sample so as to provide a test sample representative of the cheese as it is usually consumed.

**8.3.2** Grind the test sample by means of a suitable device (6.15). Mix the ground mass quickly and, if possible, grind a second time and again mix thoroughly. Clean the grinding device after grinding each sample. If the test sample cannot be ground, mix it thoroughly by intensive stirring and kneading.

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

**8.3.4** Do not use ground cheese which shows mould growth or is beginning to deteriorate.

### 8.4 Whey cheese

Prepare the test sample as specified in 8.3.2.

## 9 Procedure

### 9.1 Preparation of the test portion

#### 9.1.1 Milk

Weigh out, to the nearest 0,1 mg, approximately 10 g to 15 g of test sample. Transfer the test portion quantitatively to a 100 ml conical flask (6.3). Add progressively 50 ml of boiling water.

Shake the mixture in a water bath (6.19), maintained at 70 °C, for 15 min.

#### 9.1.2 Dried milk, dried whey, caseins, caseinates and milk protein concentrates

Weigh out, to the nearest 0,1 mg, approximately 2,0 g to 2,5 g of test sample (see 8.1 or 8.2). Transfer the test portion quantitatively to a 100 ml conical flask (6.3). Add progressively 50 ml of boiling water.

Shake the mixture in a water bath (6.19), maintained at 70 °C, for 15 min.

#### 9.1.3 Cheese, processed cheese and whey cheese

Weigh out, to the nearest 0,1 mg, approximately 3 g of test sample (see 8.3 or 8.4). Carefully mix the test portion with 15 ml of water, using e.g. a glass rod, to obtain a lump-free mixture. Transfer the mixture quantitatively to a 100 ml conical flask (6.3). Add progressively 30 ml of water at 70 °C.

Shake the mixture in a water bath (6.19), maintained at 70 °C, for 15 min.

### 9.2 Removal of fat and protein

#### 9.2.1 By Carrez precipitation and filtration

Cool the prepared test portion (see 9.1.1, 9.1.2 or 9.1.3) to room temperature. Add, in the following order, while swirling or stirring on the magnetic stirrer (6.17) thoroughly during and after each addition, 5 ml of Carrez reagent I (5.5.1) and 5 ml of Carrez reagent II (5.5.2). Adjust the pH-value to  $8,0 \pm 0,1$  using sodium hydroxide solution (5.1).

Transfer the suspension quantitatively to a 100 ml one-mark volumetric flask (6.4). Dilute to the mark with water and mix carefully. Transfer an aliquot to a centrifuge cup (6.10) and place the cup in the centrifuge (6.9). Centrifuge at a centrifugal acceleration of 3 000g at 20 °C for 15 min.

Rinse a membrane filter (6.11) with 5 ml of sodium chloride solution (5.2) and subsequently with 5 ml of water. Filter the clear supernatant liquid obtained from the centrifugation through the cleaned membrane filter (6.11). Discard the first few millilitres and use the rest of the filtrate for the determination (see 9.4).

It is essential to obtain a clear filtrate within the time specified. If it is not obtained (for example, if well-matured cheeses are being analysed), use a larger volume of each precipitation reagent (5.5.1 and 5.5.2) and reduce the volume of hot water used in 9.1 accordingly.

### 9.2.2 By centrifugal ultra-filtration

Instead of precipitation of fat and protein by Carrez reagents I and II (see 9.2.1), ultra-filtration of the test portion by membrane cones in a centrifuge can be used to obtain a clear filtrate for the determination (see 9.4).

Cool the test portion (see 9.1.1, 9.1.2 or 9.1.3) to room temperature. Adjust the pH-value to  $8,0 \pm 0,1$ . Transfer the suspension quantitatively to a 100 ml one-mark volumetric flask (6.4). Dilute to the mark with water and mix carefully.

Rinse a membrane cone (6.21) with 4 ml of sodium chloride solution (5.2) and subsequently with 4 ml of water. Transfer an aliquot to the cleaned membrane cone and place the cone in the centrifuge (6.9). Centrifuge at a centrifugal acceleration of 3 000g at 20 °C for 20 min.

NOTE 1 Membrane cones for use in a centrifuge are commercially available, e.g. the Vivaspin<sup>1)</sup> 4 ml concentrator with a polyethersulfone membrane and a molecular mass cut-off of 5 000 D is a particularly suitable product.

NOTE 2 It is not necessary to filter all the test portion suspension in the membrane cone. Pre-filtering of the sample with 5 µm membrane filters (6.11) can be used to avoid clogging of the membrane and to speed up the filtration.

## 9.3 Reagent blank test

Carry out a reagent blank test in parallel with the determination (see 9.4). Prepare the reagent blank solution as described in 9.1 and 9.2, but replacing the test portion in 9.1 by an equal volume of water.

## 9.4 Determination

Using a spectrometer (6.13) at a wavelength of 540 nm or Hg 546 nm and semi-micro optical cells (6.14), carry out the determination at between 20 °C and 25 °C.

Before transferring the sample solution or reagent blank solution, rinse the pipette with sample solution or reagent blank solution, respectively.

For pipetting of the reagent solutions, piston pipettes may be used. For pipetting of sample and reagent blank solutions, use graduated pipettes of the type used in enzyme tests (6.6).

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1) Vivaspin® is the name of a suitable product available commercially. This information is given for the convenience of the users of this International Standard but does not constitute an endorsement by either ISO or IDF of the product named.

For the nitrate and nitrite (total nitrite) determination, proceed in accordance with the scheme in Table 1.

**Table 1 — Nitrate and nitrite (total nitrite) procedure**

| Operation                                                                                                                                                                                                                                                                 | Reagent blank solution<br>ml | Test portion solution<br>ml |
|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------|-----------------------------|
| Pipette into the optical cell:                                                                                                                                                                                                                                            |                              |                             |
| Test portion solution (see 9.2.1 or 9.2.2)                                                                                                                                                                                                                                | —                            | 0,500                       |
| Reagent blank solution (see 9.3)                                                                                                                                                                                                                                          | 0,500                        | —                           |
| NADPH/FAD solution (5.8)                                                                                                                                                                                                                                                  | 0,250                        | 0,250                       |
| Nitrate reductase solution (5.9)                                                                                                                                                                                                                                          | 0,020                        | 0,020                       |
| Mix, e.g. using a spatula or by swirling after sealing <sup>a</sup> , incubate for 30 min at room temperature, then add:                                                                                                                                                  |                              |                             |
| Colour reagent solution I (5.10.1)                                                                                                                                                                                                                                        | 0,250                        | 0,250                       |
| Colour reagent solution II (5.10.2)                                                                                                                                                                                                                                       | 0,250                        | 0,250                       |
| Mix, e.g. using a spatula, or by swirling after sealing. Allow the cuvette to stand in the dark at room temperature for 15 min.                                                                                                                                           |                              |                             |
| Read off the absorbance, $A_{(ni+na)s}$ and $A_{(ni+na)bl}$ , against air (no cuvette in the reference light beam) or against water. If the value of the absorbance exceeds 1,7, dilute the sample solution and consider the dilution factor when calculating the result. |                              |                             |
| <sup>a</sup> Sealing of the optical cell can be done using e.g. Parafilm <sup>2)</sup> .                                                                                                                                                                                  |                              |                             |

For the nitrite determination, proceed in accordance with the scheme in Table 2.

**Table 2 — Nitrite procedure (matrix blank, see Clause 4, Note 3)**

| Operation                                                                                                                                                                                                                                                       | Reagent blank solution<br>ml | Test portion solution<br>ml |
|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------|-----------------------------|
| Pipette into the optical cell:                                                                                                                                                                                                                                  |                              |                             |
| Test portion solution (9.2.1 or 9.2.2)                                                                                                                                                                                                                          | —                            | 0,500                       |
| Reagent blank solution (9.3)                                                                                                                                                                                                                                    | 0,500                        | —                           |
| Water                                                                                                                                                                                                                                                           | 0,270                        | 0,270                       |
| Mix, e.g. using a spatula or by swirling after sealing <sup>a</sup> , then add:                                                                                                                                                                                 |                              |                             |
| Colour reagent solution I (5.10.1)                                                                                                                                                                                                                              | 0,250                        | 0,250                       |
| Colour reagent solution II (5.10.2)                                                                                                                                                                                                                             | 0,250                        | 0,250                       |
| Mix, e.g. using a spatula or by swirling after sealing. Allow the cuvette to stand in the dark at room temperature for 15 min.                                                                                                                                  |                              |                             |
| Read off the absorbance, $A_{nis}$ and $A_{nibl}$ , against air (no cuvette in the reference light beam) or against water. If the value of the absorbance exceeds 1,7, dilute the sample solution and consider the dilution factor when calculating the result. |                              |                             |
| <sup>a</sup> Sealing of the optical cell can be done using e.g. Parafilm <sup>2)</sup> .                                                                                                                                                                        |                              |                             |

The determination can also be carried out in normal macrocuvettes. In this case, the volumes of sample, reagent blank and reagents have to be adjusted accordingly.

2) Parafilm<sup>®</sup> is the name of a suitable product available commercially. This information is given for the convenience of the users of this International Standard but does not constitute an endorsement by either ISO or IDF of the product named.

## 9.5 Calibration

Prepare calibration graphs using the calibration solutions prepared in accordance with 5.6.1 and 5.6.2, plotting each absorbance obtained by the procedure described in 9.4 against the corresponding nitrite or nitrate concentration, respectively, in milligrams per litre.

## 10 Calculation and expression of results

### 10.1 Nitrite (matrix blank) content (see Clause 4, Note 3)

#### 10.1.1 Calculation

Using the calibration graph prepared using the solutions prepared in 5.6.1, read or calculate from the absorbance difference  $\Delta A_{ni} = A_{nis} - A_{nibl}$  (see Table 2) the corresponding nitrite (matrix blank) concentration of the sample solution,  $c_{ni}$ . Calculate the nitrite (matrix blank) content of the sample,  $w_{ni}$ , using the following equation:

$$w_{ni} = c_{ni} \times V \times \frac{d}{m}$$

where

$w_{ni}$  is the nitrite content of the sample, in milligrams of nitrite per kilogram;

$c_{ni}$  is the concentration, read from the calibration graph, corresponding to the measured absorbance of the test portion solution, in milligrams of nitrite ion per litre (see 9.5);

$m$  is the mass, in grams, of the test portion (see 9.1);

$V$  is the volume, in millilitres, of the test solution (see 9.2.1 or 9.2.2) (in this case,  $V = 100$  ml);

$d$  is the dilution factor (if no dilution was carried out,  $d = 1$ ).

#### 10.1.2 Expression of results

Express the test result to one decimal place.

### 10.2 Total nitrite/nitrate content

#### 10.2.1 Calculation

Using the calibration graph prepared using the solutions prepared in 5.6.2, read or calculate from the absorbance difference  $\Delta A_{ni+na} = A_{(ni+na)s} - A_{(ni+na)bl}$  (see Table 1) the corresponding nitrite and nitrate (total nitrite) concentration of the sample solution,  $c_{ni+na}$ .

Calculate the nitrite + nitrate (total nitrite) content of the sample,  $w_{ni+na}$ , using the following equation:

$$w_{ni+na} = c_{ni+na} \times V \times \frac{d}{m}$$

where

$w_{ni+na}$  is the nitrite + nitrate (total nitrite) content of the sample, in milligrams of nitrite per kilogram;

$c_{ni+na}$  is the concentration, read from the calibration graph, corresponding to the measured absorbance of the test portion solution, in milligrams of nitrite ion per litre (see 9.5);

$m$ ,  $V$  and  $d$  are as defined in 10.1.1.

### 10.2.2 Expression of results

Express the test result to one decimal place.

## 10.3 Nitrate content

### 10.3.1 Calculation

Calculate the nitrate content,  $w_{na}$ , in milligrams per kilogram, of the sample using the following equation:

$$w_{na} = 1,35 \times (w_{ni+na} - w_{ni})$$

where 1,35 is the ratio of the molecular masses of the nitrate and nitrite ions.

### 10.3.2 Expression of results

Express the result to the nearest whole number.

### 10.3.3 Reducing capacity

Within each series of measurements, check the reducing capacity by comparing results obtained using the nitrate standard solutions (see 5.6.2) with the corresponding nitrite standard solutions (5.6.1), taking into account the ratio of the molecular masses.

The reducing capacity shall be at least 95 %.

## 11 Precision

### 11.1 Interlaboratory testing

The values derived from interlaboratory tests may not be applicable to content ranges and matrices other than those used in the tests.

The values of the repeatability and reproducibility were derived from the results of interlaboratory tests carried out in accordance with ISO 5725-2.

The repeatability and reproducibility limits for nitrate were derived from a German study carried out in 1998 and an international study carried out in 2004, both in accordance with ISO 5725-2. The data which resulted are given in Annex A. No figures have been determined for nitrite.

### 11.2 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 the following:

|                                                                                                                                                        |                           |
|--------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------|
| for whey powder, whey protein concentrates and similar products, using Carrez precipitation and filtration at contents between 40 mg/kg and 160 mg/kg: | 30 mg/kg;                 |
| for other products, using Carrez precipitation/filtration:                                                                                             | $(4 + 0,07w_{na})$ mg/kg; |
| for all products, using centrifugal ultra-filtration,                                                                                                  | $(6 + 0,1w_{na})$ mg/kg.  |

### 11.3 Reproducibility

The absolute 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, will in not more than 5 % of cases be greater than the following:

|                                                                                                                                                        |                           |
|--------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------|
| for whey powder, whey protein concentrates and similar products, using Carrez precipitation and filtration at contents between 40 mg/kg and 160 mg/kg: | 41 mg/kg;                 |
| for other products, using Carrez precipitation/filtration:                                                                                             | $(6 + 0,2w_{na})$ mg/kg;  |
| for all products, using centrifugal ultra-filtration:                                                                                                  | $(10 + 0,1w_{na})$ mg/kg. |

### 12 Test report

The test report shall include at least the following information:

- all information required for complete identification of the sample;
- the sampling method used, if known;
- the method used, with reference to this International Standard;
- all operating details not specified in this International Standard, or regarded as optional, together with details of any incidents which may have influenced the result(s);
- the test result(s) obtained or, if the repeatability has been checked, the final quoted results obtained.