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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

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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.

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.

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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. Backman (FL).

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 (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(NaOH) = 1 mol/l.

5.2 Sodium chloride solution, c(NaCl) = 0,9 g/100 ml.

5.3 Hydrochloric acid, ρ_{20}(HCl) = 1,19 g/ml.

5.4 Hydrochloric acid solution, c(HCl) = 2 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, $c(K_4[Fe(CN)_6].3H_2O)$ = 150 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, $c(ZnSO_4.7H_2O)$ = 300 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 ($NaNO_2$) stock solution.

Accurately weigh (75,0 ± 0,1) mg of pre-dried (at 102 °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 (KNO_3) stock solution.

Accurately weigh (81,5 ± 0,1) mg of pre-dried (at 102 °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 °C, the potassium nitrate stock solution remains stable for 1 week.

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

Accurately weigh (57,6 ± 0,1) mg of dipotassium hydrogen phosphate ($K_2HPO_4.3H_2O$) 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 ± 0,1) mg of potassium dihydrogen phosphate (KH_2PO_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 phospate solution to pH 7,5 by addition of potassium dihydrogen phosphate solution.

When stored at 4 °C, the potassium phosphate buffer solution remains stable for 2 weeks.

5.8 NADPH/FAD solution.

Weigh accurately (5,6 ± 0,1) mg of 3-nicotinamide-adenine dinucleotide phosphate (reduced form), tetrasodium salt (β-NADPH-Na_4, with a mass fraction of at least 98 %), and (80,0 ± 0,1) mg flavine-adenine dinucleotide, disodium salt (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 °C, the nitrate reductase solution remains stable for 2 weeks.

5.10 Colour reagents, as follows:

5.10.1 Colour reagent solution I: Sulfanilamide ($NH_2C_6H_4SO_2NH_2$).

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 ($C_{10}H_7NHCH_2CH_2NH_2.2HCl$).

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 ± 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.