SLOVENSKI STANDARD
SIST ENV 12014-3:1999

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Živila - Določevanje vsebnosti nitratov in/ali nitritov - 3. del: Spektrometrijsko določevanje vsebnosti nitratov in nitritov v mesnih proizvodih po encimski redukciji nitratov v nitrite

Foodstuffs - Determination of nitrate and/or nitrite content - Part 3: Spectrometric determination of nitrate and nitrite content of meat products after enzymatic reduction of nitrate to nitrite

Lebensmittel - Bestimmung des Nitrat- und/oder Nitritgehaltes - Teil 3: Spektralphotometrische Bestimmung des Nitrat- und Nitritgehaltes in Fleischerzeugnissen nach enzymatischer Reduktion von Nitrat zu Nitrit

Produits alimentaires - Détermination de la teneur en nitrates et/ou en nitrites - Partie 3: Détermination spectrométrique de la teneur en nitrates et en nitrites des produits carnés apres réduction enzymatique des nitrates en nitrites

Ta slovenski standard je istoveten z: **ENV 12014-3:1998**

ICS:

67.050	Splošne preskusne in analizne metode za živilske proizvode	General methods of tests and analysis for food products
67.120.10	Meso in mesni proizvodi	Meat and meat products

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English version

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EUROPEAN COMMITTEE FOR STANDARDIZATION
COMITÉ EUROPÉEN DE NORMALISATION
EUROPÄISCHES KOMITEE FÜR NORMUNG

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Foreword

This European Prestandard has been prepared by Technical Committee CEN/TC 275 "Food analysis - Horizontal methods", the secretariat of which is held by DIN.

This series "Foodstuffs - Determination of nitrate and/or nitrite content" consist of the following parts:

Part 1: General considerations

Part 2: HPLC/IC method for the determination of nitrate content of vegetables and vegetable products

Part 3: Spectrometric determination of nitrate and nitrite content of meat products after enzymatic reduction of nitrate to nitrite;

Part 4: Ion-exchange chromatographic (IC) method for the determination of nitrate and nitrite content of meat products;

Part 5: Enzymatic determination of nitrate content of vegetable-containing food for babies and infants;

Part 7: Continuous flow method for the determination of nitrate content of vegetables and vegetable products after Cadmium reduction,

According to the CEN/CENELEC Internal Regulations, the national standards organizations of the following countries are bound to announce this European Prestandard: Austria, Belgium, Czech Republic, Denmark, Finland, France, Germany, Greece, Iceland, Ireland, Italy, Luxembourg, Netherlands, Norway, Portugal, Spain, Sweden, Switzerland and the United Kingdom.

1 Scope

This European Prestandard specifies a spectrometric method for the determination of nitrate and nitrite content of meat products and has been validated for a total nitrite and nitrate content of 25 mg/kg as nitrite ion.

NOTE: Experiences have shown that the method is also applicable for total nitrite and nitrate content from 10 mg/kg up to 50 mg/kg as nitrite ion. For further information on applicability, see [1].

2 Normative references

This European Prestandard incorporates by dated or undated reference, provisions from other publications. These normative references are cited at the appropriate places in the text and the publications are listed hereafter. For dated references, subsequent amendments to or revisions of any of these publications apply to this European Prestandard only when incorporated in it by amendment or revision. For undated references the latest edition of the publication referred to applies.

EN 12014-1
Foodstuffs - Determination of nitrate and/or nitrite content - Part 1: General considerations

EN ISO 3696
Water for analytical laboratory use - Specification and test methods (ISO 3696:1987)

3 Principle

Nitrite in an aqueous extract of the analytical sample is treated with sulfanilamide and N-(1-naphthyl)-ethylenediamine dihydrochloride. A red compound is produced which is measured spectrometrically at a wavelength of 540 nm [2].

Nitrate in an aqueous extract of the analytical sample is converted into nitrite by nitrate reductase. Treatment of this nitrite together with the nitrite which is already in the analytical sample with sulfanilamide and N-(1-naphthyl)ethylenediamine dihydrochloride. Photometric measurement of the colour intensity of this to produce a red compound at a wavelength of 540 nm. The nitrate content is calculated from the difference between the spectrometric measurements.

4 Reagents

During the analysis, unless otherwise stated, use only reagents and materials of recognized analytical grade and water of at least grade 3 according to EN ISO 3696. When preparing solutions, the purities of the reagents available shall be taken into account.

4.1 **Bromothymol blue solution** (indicator solution)

Dissolve 0,2 g of bromothymol blue in 100 ml of ethanol (96 % by mass).

4.2 **Sodium hydroxide solution** c (NaOH) ≈ 1 mol/l [1])

4.3 **Carrez solution No. 1**

Dissolve 150 g of potassium hexacyanoferrate(II), $K_4[Fe(CN)_6] \cdot 3\ H_2O$ in water and dilute to 1 000 ml. Store the solution in a brown bottle and replace it every two months.

4.4 **Carrez solution No. 2**

Dissolve 230 g of zinc acetate, $Zn(CH_3COO)_2 \cdot 2\ H_2O$, in water and dilute to 1 000 ml.

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[1]) c is the substance concentration

4.5 Reagents for the enzymatic determination of nitrate[2])

4.5.1 Imidazole buffer solution, pH = 7,8

Dissolve 0,68 g of imidazole in an 100 ml volumetric flask in 80 ml of water, adjust the pH to 7,8 with hydrochloric acid (c (HCl) = 2 mol/l) and dilute to the mark with water.

4.5.2 Solution of tetrasodium salt of reduced ß-nicotinamide adenine dinucleotide phosphate (ß-NADPH-Na_4, mass fraction of at least 98 %)

Dissolve 125 mg of reduced ß-nicotinamide adenine dinucleotide phosphate and 2,5 mg of disodium salt of flavin adenin dinucleotide (mass fraction of at least 88%) in 50 ml of the imidazole buffer solution (4.5.1). Prepare this solution immediately before use.

4.5.3 Nitrate reductase buffer solution

Dissolve 11 mg of nitrate reductase lyophilisate from aspergillus sp. (EC 1.6.6.2, approximately 0,4 U/mg[3]) in 10 ml of imidazole buffer solution (4.5.1).

The solution will be stable for about 14 days if kept at 4 °C.

4.6 Colour reagents

4.6.1 Colour reagent No. 1

Dissolve 8 g of sulfanilamide in 500 ml of water while heating on a water bath. Cool the solution to room temperature and filter if necessary. Add 330 ml of concentrated hydrochloric acid while stirring continuously and dilute the solution to 1 000 ml with water. The solution will be stable for several weeks at room temperature.

4.6.2 Colour reagent No. 2

Dissolve 0,330 g of N-(1-naphthyl)ethylenediamine dihydrochloride in 250 ml of water. Keep the solution in a brown bottle and replace it every week.

4.6.3 Colour reagent mixture

Prepare the required amount of colour reagent mixture by mixing equal parts by volume of colour reagents No. 1 (4.6.1) and No. 2 (4.6.2) on the day of use.

4.7 Sodium nitrite solutions

It is recommended to prepare sodium nitrite reference solutions on the day of use. Sodium nitrite is a hygroscopic substance.

4.7.1 Sodium nitrite stock solution, ρ ($NaNO_2$) = 400 mg/l

Dissolve 200 mg of sodium nitrite weighed to the nearest 0,1 mg in water and dilute to 500 ml.

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[2]) Also the test kit for the enzymatic determination of nitrate from Boehringer, Mannheim may be used. This information is given for the convenience of users of this European Prestandard and does not constitute an endorsement by CEN of the product named. Equivalent products may be used if they can be shown to lead to the same results.

[3]) U, This unit (often called the International unit or prestandard unit) is defined as the amount of enzyme which catalyses the transformation of 1 μmol substrate per minute under standard conditions.

4.7.2 **Diluted sodium nitrite stock solution** ρ ($NaNO_2$) = 20 mg/l

Pipette 25 ml of the sodium nitrite stock solution (4.7.1) into a 500 ml volumetric flask and dilute to the mark with water.

4.7.3 **Standard sodium nitrite solutions**

Pipette 10 ml, 20 ml, 30 ml and 40 ml, respectively, of the diluted stock solution (4.7.2) containg 0,13 mg, 0,27 mg, 0,40 mg, and 0,53 mg, respectively, of nitrite ion into a series of 200 ml volumetric flasks, adding 0,2 ml of bromothymol blue solution (4.1) and titrate with sodium hydroxide solution (4.2) until the colour changes to blue (amount required: about two drops).

Add 4 ml each of Carrez solution No. 1 (4.3) and No. 2 (4.4), dilute to the mark with water, thoroughly mix the contents of the flask and filter through a fluted filter paper. Discard the first 20 ml of each of the filtrates.

4.8 **Potassium nitrate solutions**

It is recommended to prepare potassium nitrate solutions on the day of use.

4.8.1 **Potassium nitrate stock solution**, ρ (KNO_3) = 600 mg/l [4])

Dissolve 300 mg of potassium nitrate weighed to the nearest 0,1 mg in water in a 500 ml volumetric flask and dilute the solution to the mark with water.

4.8.2 **Diluted potassium nitrate stock solution**, ρ (KNO_3) = 30 mg/l

Pipette 25 ml of the potassium nitrate stock solution (4.8.1) into a 500 ml volumetric flask and dilute to the mark with water.

4.8.3 **Potassium nitrate standard solutions**

Pipette 10 ml, 20 ml, 30 ml and 40 ml, respectively, of the diluted stock solution (4.8.2), corresponding to 0,13 mg 0,27 mg, 0,40 mg, and 0,53 mg, respectively, of nitrite ion into a series of 200 ml volumetric flasks, add 0,2 ml of bromothymol blue solution (4.1) and titrate with sodium hydroxide solution (4.2) until the colour changes to blue (amount required: about two drops).

Add 4 ml each of Carrez solution No. 1 (4.3) and No. 2 (4.4), dilute to the mark with water, thoroughly mix the contents of the flask and filter through a fluted filter paper. Discard the first 20 ml of each of the filtrates.

5 **Apparatus and equipment**

Usual laboratory apparatus and, in particular, the following

5.1 **Homogenizing equipment**, mechanical or electrical, capable of homogenizing the test sample, this includes a high-speed rotational cutter, or a mincer fitted with a plate with holes not exceeding 4,5 mm in diameter.

5.2 **Water bath**, capable of being maintained at 100 °C

5.3 **Fluted filter papers**, nitrate and nitrite free

5.4 **pH meter**, with pH measuring cell (glass and reference electrodes)

5.5 **Spectrometer**, for carrying out measurements at a wavelength of 540 nm, with suitable cells, made of glass or plastics which have no significant optical absorption at a wavelength of 540 nm.

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[4]) ρ is the mass concentration

6 Procedure

6.1 Preparation of the sample solution

Homogenize the laboratory sample with the appropriate equipment (5.1) Take care that the temperature of the sample material does not rise above 25 °C. If a mincer is used, pass the sample at least twice through the equipment. Weigh, to the nearest 10 mg, 10 g of the homogenized sample into e.g. a wide neck conical flask, add about 50 ml of water and homogenize for 30 s to 60 s. Rinse the shaft of the homogenizer into the flask with 50 ml of hot water, then add 0,2 ml of bromothymol blue solution (4.1), titrate the extract with sodium hydroxide solution (4.2) until the colour changes from yellow to bluish green/greenish grey and then heat for 15 min in a boiling water bath (5.2). During this process shake repeatedly and dissolve any lumps formed with a homogenizer.

NOTE: In order to become familiar with the various shades of colour in adjusting the pH while learning the method, titrate a few sample extracts using the indicator and then measure the pH. For uncooked meat products the pH should not exceed 8,5 since otherwise it may not be possible to clarify the solutions by filtering after adding the Carrez reagent. In the case of simmered or boiled meat products, the danger of this happening is much less and the pH may be allowed to rise to about 9,5. If a pH meter is used, adjust the pH value to 8,0 to 8,5.

In the case of boiled sausages, the (instantaneous) colour change to bluish green when adjusting the pH is readily detected. On the other hand, this change is usually sluggish in the case of extracts of uncooked sausages and the shade of colour is greyish green. In the case of boiled sausages containing blood, a similar shade of colour is usually observed. Even in the case of extracts having a strong natural colour, the colour change can be recognized very readily with a little practice despite the fact that the shade of colour is frequently not the (theoretically) expected one because the colour of the extract is superimposed on that of the indicator.

In the case of extracts of uncooked sausages, a reversal of the colour change is sometimes observed after a certain time. In such cases, readjust the pH by adding a little sodium hydroxide solution.

Cool to room temperature and transfer the contents of the flask quantitatively to a 200 ml volumetric flask and add 4 ml each of Carrez solutions No. 1 (4.3) and No. 2 (4.4), shaking after each addition. Then dilute to the mark with water, mix thoroughly and filter through a fluted filter paper (5.3), discarding the first 20 ml of the filtrate. The clear residual filtrate is used for the determination (sample solution).

6.2 Preparation of the calibration graphs

6.2.1 Calibration graph for the nitrite content

Mix 2,0 ml of water for the blank and 2,0 ml of each of the standard sodium nitrite solutions (4.7.3) with 1,0 ml of water and 3,0 ml of the colour reagent mixture (4.6.3) in a test tube, shake and store the solution in the dark at room temperature.

After 30 min, measure the absorbance values of each solution at a wavelength of 540 nm in a spectrometer against water.

Plot the absorbance values obtained for the four sodium nitrite solutions (4.7.3) against the corresponding absolute amounts of nitrite-ions (in 200 ml solution), respectively, in milligrams of nitrite ion.

6.2.2 Calibration graph for the nitrate content

Introduce 0,2 ml of the NADPH-solution (4.5.2), 2,0 ml of water for the blank or 2,0 ml of each of the standard potassium nitrate solutions (4.8.3) respectively and 0,8 ml of nitrate reductase buffer solution (4.5.3) into a test tube, mix thoroughly and allow to stand for 1 h at room temperature. Then add 3,0 ml of the colour reagent mixture (4.6.3), shake and store the solution in the dark at room temperature.

After 30 min, measure the absorbance values of each solution at a wavelength of 540 nm in a spectrometer (5.5) against water.

Plot the absorbance values obtained for the four potassium nitrate standard solutions (4.8.3) against the corresponding absolute amounts of total nitrite/nitrate, (in 200 ml solution), in milligrams of nitrite ion.

6.3 Determination of nitrite content

Mix 2,0 ml of the sample solution (6.1) in a cell with 1,0 ml of water and 3,0 ml of the colour reagent mixture (4.6.3) and allow to stand in the dark at room temperature.

After 30 min, measure the absorption A_{NO_2} in a spectrometer at a wavelength of 540 nm against water.

6.4 Determination of the total nitrite/nitrate content

Introduce 0,2 ml of the NADPH-solution (4.5.2), 2,0 ml of sample solution (6.1) and 0,8 ml of nitrate reductase buffer solution (4.5.3) into a test tube, mix thoroughly and allow to stand for 1 h at room temperature. Then add 3,0 ml of the colour reagent mixture (4.6.3), shake and store the solution in the dark at room temperature.

After 30 min, measure the absorption $A_{NO_2+NO_3}$ at a wavelength of 540 nm in a spectrometer against water.

7 Calculation

7.1 Calculation of the nitrite content

Read off the absolute amount of nitrite, x_{NO_2}, corresponding to the absorption value A_{NO_2} from 6.3 determining nitrite without reduction step from the nitrite calibration graph from 6.2.1.

Calculate the mass fraction, w_{NO_2}, in milligrams per kilogram of nitrite ion in the sample using equation (1):

$$w_{NO_2} = \frac{x_{NO_2} \times 1000}{m} \qquad (1)$$

where:

x_{NO_2} is the absolute amount of nitrite (in 200 ml solution) without reduction step, in milligrams, read off from the calibration graph from 6.2.1;

m is the mass of the test portion, in 200 ml of the sample test solution, in grams.

7.2 Calculation of the total nitrite/nitrate content

Read off the amount of the absorption value $A_{NO_2+NO_3}$ from 6.4 determining total nitrate/nitrate with reduction step from the nitrate calibration graph from 6.2.2 as nitrite ion.

Calculate the mass fraction, $w_{NO_2+NO_3}$, in milligrams per kilogram, of total nitrate/nitrite as nitrite ion in the sample using equation (2):

$$w_{NO_2+NO_3} = \frac{x_{NO_2+NO_3} \times 1000}{m} \qquad (2)$$

where:

$x_{NO_2+NO_3}$ is the absolute amount of total nitrate/nitrite (in 200 ml of solution) expressed as nitrite ion after the reduction step, in milligrams, read off from the calibration graph from 6.2.2;

m is the mass of the test portion, in 200 ml of the sample solution, in grams.