SLOVENSKI STANDARD
SIST EN 12014-5:1999

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Živila - Določevanje vsebnosti nitratov in/ali nitritov - 5. del: Encimska določitev vsebnosti nitratov v hrani za dojenčke in majhne otroke, ki vsebuje zelenjavo

Foodstuffs - Determination of nitrate and/or nitrite content - Part 5: Enzymatic determination of nitrate content of vegetable-containing food for babies and infants

Lebensmittel - Bestimmung des Nitrat- und/oder Nitritgehaltes - Teil 5: Enzymatische Bestimmung des Nitratgehaltes in gemüsehaltiger Säuglings- und Kleinkindernahrung

Produits alimentaires - Détermination de la teneur en nitrates et/ou en nitrites - Partie 5: Détermination enzymatique de la teneur en nitrates des aliments a base de légumes pour bébés et petits enfants

Ta slovenski standard je istoveten z: **EN 12014-5:1997**

ICS:

67.050	Splošne preskusne in analizne metode za živilske proizvode	General methods of tests and analysis for food products
67.080.20	Zelenjava in zelenjavni proizvodi	Vegetables and derived products
67.230	Predpakirana in pripravljena hrana	Prepackaged and prepared foods

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EUROPEAN STANDARD

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Descriptors: food products, infant foods, vegetables, chemical analysis, determination of content, nitrates, nitrites, enzymatic methods

English version

Foodstuffs - Determination of nitrate and/or nitrite content - Part 5: Enzymatic determination of nitrate content of vegetable-containing food for babies and infants

Produits alimentaires - Détermination de la teneur en nitrates et/ou en nitrites - Partie 5: Détermination enzymatique de la teneur en nitrates des aliments à base de légumes pour bébés et petits enfants

Lebensmittel - Bestimmung des Nitrat- und/oder Nitritgehaltes - Teil 5: Enzymatische Bestimmung des Nitratgehaltes in gemüsehaltiger Säuglings- und Kleinkindernahrung

This European Standard was approved by CEN on 1997-02-28. CEN members are bound to comply with the CEN/CENELEC Internal Regulations which stipulate the conditions for giving this European Standard the status of a national standard without any alteration.

Up-to-date lists and bibliographical references concerning such national standards may be obtained on application to the Central Secretariat or to any CEN member.

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CEN

European Committee for Standardization
Comité Européen de Normalisation
Europäisches Komitee für Normung

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Contents

Foreword

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

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

Part 1: General;

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: 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.

This European Standard shall be given the status of a national standard, either by publication of an identical text or by endorsement, at the latest by October 1997 and conflicting national standards shall be withdrawn at the latest by October 1997.

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

1 Scope

This European Standard specifies an enzymatic method for the determination of vegetable-containing food for babies and infants [1], [2]. This method is applicable to nitrate contents in the range of 50 mg/kg to 200 mg/kg

2 Normative References

This European Standard 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 Standard 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

Enzymatic determination in an aqueous sample extract by measuring the amount of NADPH used up in the following reaction:

$$\text{Nitrate} + \text{NADPH} + \text{H}^+ \xrightarrow{\text{nitrate reductase}} \text{Nitrite} + \text{NADP}^+ + \text{H}_2\text{O}$$

where the amount of NADPH used up is equivalent to the quantity of nitrate [3].

4 Reagents

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

4.1 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.

4.2 Carrez solution No 2

Dissolve 300 g of zinc sulfate, $ZnSO_4 \cdot 7\ H_2O$ in water and dilute to 1 000 ml.

4.3 Sodium hydroxide solution, c (NaOH) = 2 mol/l [1])

4.4 Imidazole buffer solution, pH = 7,3 [2])

Dissolve 0,68 g of imidazole ($C_3H_4N_2$) in 80 ml of water, adjust the pH to 7,3 with 2 mol/l hydrochloric acid solution and dilute to 100 ml with water. The solution will be stable for at least 1 year at 4 °C.

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[1]) c is the substance concentration

[2]) These reagents are included in commercially available test kits. If these test kits are used, follow the manufacturer's instructions.

4.5 FAD solution[2])

Prepare a solution containing 0,17 mg of the disodium salt of flavin adenine dinucleotide (FAD-Na_2) per millilitre by using FAD-Na_2 with a content by mass of not less than 88 %. The solution will be stable for at least 1 day if stored at 4 °C.

4.6 NADPH solution[2])

Prepare a solution of 5,6 mg of tetrasodium salt of reduced ß-nicotinamide adenine dinucleotide phosphate (ß-NADPH-Na_4) per millilitre by using ß-NADPH-Na_4 with a content by mass of not less than 98 %. The solution will be stable for at least 1 day if stored at 4 °C but for only 2 h at room temperature.

4.7 Nitrate reductase solution[2])

Dissolve 65 mg of freeze-dried nitrate reductase having an activity of about 0,4 U/mg [3]) and obtained from Aspergillus species (EC 1.6.6.2) [4] in 5 ml of water. The solution will be stable for at least 2 weeks if stored at 4 °C.

5 Apparatus and equipment

Usual laboratory apparatus and, in particular, the following

5.1 Homogenizer (disperser, electric mixer or similar equipment).

Ensure that the equipment ensures thorough dispersion or homogenization of the sample and does not release heavy metals which would inhibit the enzymes used.

5.2 Combined hot plate and **magnetic stirrer**

5.3 Fluted filter paper.

5.4 Graduated pipettes for enzymatic analysis, set for partial discharge, in 0,05 ml, 0,1 ml, 1 ml and 2 ml, or **piston-type pipettes.**

5.5 Glass or plastics cells having an optical path length of 1 cm and no significant absorption at wavelengths of 334 nm, 340 nm or 365 nm.

5.6 Spectrometer, for measurement at a wavelength of 340 nm, with a spectral bandwidth of not more than ± 5 nm.

NOTE: Spectral-line filter photometers fitted with a mercury vapour lamp, for measurement at 365 nm or 334 nm, may also be used.

5.7 Centrifuge, capable of producing a centrifugal acceleration of 2 900 *g* at the base of centrifuge tubes (5.8), optional.

5.8 Centrifuge tubes, of suitable capacity

5.9 Refrigerator, capable of being maintained at 4 °C

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[2]) see footnote on page 3

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

6 Procedure

6.1 Sample preparation

Bring sample material which has been stored in a cool place to room temperature. Mix the entire contents of the container in a suitable mixer (5.1), if necessary grinding and homogenizing it. Ensure that the temperature of the sample does not exceed 60 °C.

For fluid samples, if necessary, dilute the sample to be analysed until the nitrate concentration is 30 mg/l to 300 mg/l. The resultant sample solution can be used for the determination without further preparation even if it is coloured, a volume (V_2) of 0,1 ml being taken for the test.

6.2 Determination

6.2.1 Sample solution preparation

Weigh out about 5 g or another suitable amount of the sample prepared as specified in 6.1 to the nearest milligram into a 100 ml conical flask, and add 50 ml of boiling water. Cover the flask with a watch glass, place it in a boiling water bath (e.g. glass beaker) for 20 min to 30 min, stirring to prevent the formation of lumps (see 5.2).

Prevent overheating the bottom of the conical flask by avoiding direct contact with the bottom of the water bath.

Remove the magnetic stirrer, add 5,0 ml of Carrez solution No 1 (4.1), and shake the mixture for 5 s to 10 s. Then add 5,0 ml of Carrez solution No 2 (4.2) and shake again for 5 s to 10 s. Adjust the pH of the mixture to a value of 8 to 9 (but not greater than 9) with sodium hydroxide solution (4.3) and shake again.

Cool the contents of the conical flask to room temperature and filter quantitatively into a 100 ml volumetric flask, V_1, rinsing out the last portions with water. Dilute to the mark with water.

If the initial sample mass is high, for example more than 10 g, it is advisable to centrifuge the clarified sample mixture for 10 min at 2 900 *g* before filtering it quantitatively into the 100 ml volumetric flask. Wash the settled matter twice with water and centrifuge again, collect each of the supernatants in the 100 ml volumetric flask and then dilute the solution to the mark with water.

Shake the volumetric flask and place it in a refrigerator (5.9) at approximately 4 °C for 30 min to allow any fat to separate. Then filter the contents of the flask through a fluted filter paper (5.3), discarding the first few millilitres of the filtrate.

Collect the filtrate (sample solution) in a conical flask having a ground glass stopper. Use the sample solution to determine nitrate content employing 1,0 ml (V_2) in the test.

Any slight cloudiness will not normally interfere with the determination.

6.2.2 Measurement of the enzymatic reaction

Carry out the determination at about 20 °C.

The absorption maximum of NADPH occurs at a wavelength of 340 nm. If a spectrometer (5.6) is used, carry out the measurements at the absorption maximum, only, but if a spectralline filter photometer fitted with a mercury vapour lamp is used, work at a wavelength of 365 nm or 334 nm.

Table 1: Pipetting plan

Fluid pipetted into the cells	Blank	Test solution
Buffer solution (4.4) FAD solution (4.5) NADPH solution (4.6) Sample solution (see 6.2.1) Water	1,00 ml 0,05 ml 0,10 ml - 2,00 ml	1,00 ml 0,05 ml 0,10 ml 1,00 ml 1,00 ml
Mix, and after about 5 min measure the absorbances (A_1) with no cell (5.5) in the reference path, then start the reaction by adding the following		
Nitrate reductase solution (4.7)	0,05 ml	0,05 ml
Mix and, after 40 min, measure the absorbances of the solutions in quick succession, with no cell in the reference path (A_2). Repeat this measurement after a further 20 min (A_3).		

The change (decrease) in the absorbance of the test solution $(A_1 - A_2)_{\text{test solution}}$ should be between 0,1 and 0,5. If the change in the absorbance is too small, the volume of the sample solution pipetted into the cell may be increased (up to 2,00 ml). In such cases, reduce the quantity of water used so that the cells for the test solution and the blank contain the same total volume. If the change in absorbance is too great, dilute the sample solution. If such steps are necessary, allow for the change in the dilution factor (F) in the calculation.

Only one blank per series is necessary.

7 Calculation

In the reaction which forms the basis of this determination there is a linear relationship between the quantity of NADPH used up, and consequently the change in absorbance, and the nitrate concentration.

Calculate the change in absorbance of the blank, ΔA_{blank}, by using equation (1):

$$\Delta A_{blank} = (A_1 - A_2)_{blank} - 2 \cdot (A_2 - A_3)_{blank} \quad \text{... (1)}$$

Calculate the change in absorbance of the test solution, $\Delta A_{\text{test solution}}$, by using equation (2):

$$\Delta A_{test\ solution} = (A_1 - A_2)_{test\ solution} - 2 \cdot (A_2 - A_3)_{test\ solution} \quad \text{... (2)}$$

Calculate the change in absorbance, ΔA, allowing for the blank value by using equation (3):

$$\Delta A = \Delta A_{test\ solution} - \Delta A_{blank} \quad \text{... (3)}$$

The calculation of the concentration of a substance in a diluted solution using photometric absorption measurements is based on the Beer-Lambert law.

Calculate the mass fraction w, of nitrate, in milligrams per kilogram of sample, by using equation 4:

$$w = \frac{\Delta A \cdot M \cdot V_1 \cdot V_3 \cdot F \cdot 1000}{\epsilon \cdot \delta \cdot V_2 \cdot m} \quad \ldots (4)$$

where:

ΔA is the change in absorbance according to equation (3)

M is the relative molecular mass of nitrate ion (M = 62,0 g/mol);

V_1 is the total volume, in millilitres, of sample solution (6.2.1) (here: V_1 = 100 ml);

V_2 is the volume, in millilitres, of sample solution used to prepare the test solution (6.2.2) (here: V_2 = 1,00 ml);

V_3 is the total volume, in millilitres, of test solution (6.2.2) (here: V_3 = 3,20 ml);

F is the dilution factor (here: F = 1);

m is the initial sample mass, in grams;

ϵ is the molar absorptivity of NADPH,
i.e. $6{,}3 \times 10^3$ l · mol^{-1} · cm^{-1} at 340 nm,
$3{,}5 \times 10^3$ l · mol^{-1} · cm^{-1} at 365 nm, (Hg)
$6{,}18 \times 10^3$ l · mol^{-1} · cm^{-1} at 334 nm, (Hg);

δ is the optical path length, in centimetres, of the cell (here: 1 cm).

If the pipetting plan (see table 1) is adhered to and the sample solution is not diluted, then calculate the mass fraction w, of nitrate, in milligrams per kilogram of sample, by using equation 5:

$$w = \frac{\Delta A \cdot 19\,840\,000}{\epsilon \cdot m} \quad \ldots (5)$$

where:

ΔA see equation (3);

ϵ, m see equation (4).

Report the result in milligrams per kilogram or milligrams per litre without decimal places and according to current legislation.

8 Precision

Details of the interlaboratory test of the method are summarized in annex A. The values derived from the interlaboratory test may not be applicable to other analyte concentration ranges and matrices other than given in annex A.