SLOVENSKI STANDARD
SIST EN 1139:1996

01-avgust-1996

Sadni in zelenjavni sokovi - Encimsko ugotavljanje vsebnosti D-isocitronske kisline - NADH spektrometrijska metoda

Fruit and vegetable juices - Enzymatic determination of D-isocitric acid content - NADPH spectrometric method

Frucht- und Gemüsesäfte - Enzymatische Bestimmung des Gehaltes an D-Isocitronensäure - Spektralphotometrische Bestimmung von NADPH

Jus de fruits et de légumes - Dosage enzymatique de l'acide D-isocitrique - Méthode spectrométrique par le NADPH

Ta slovenski standard je istoveten z: **EN 1139:1994**

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English version

Fruit and vegetable juices - Enzymatic determination of D-isocitric acid content - NADPH spectrometric method

Jus de fruits et de légumes - Dosage enzymatique de l'acide D-isocitrique - Méthode spectrométrique par le NADPH

Frucht- und Gemüsesäfte - Enzymatische Bestimmung des Gehaltes an D-Isocitronensäure - Spektralphotometrische Bestimmung von NADPH

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CEN

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

Central Secretariat: rue de Stassart,36 B-1050 Brussels

Foreword

This European Standard has been prepared by the Technical Committee CEN/TC 174 "Fruit and vegetable juices - Methods of analysis", the secretariat of which is held by AFNOR.

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of an identical text or by endorsement, at the latest by April 1995, and conflicting national standards shall be withdrawn at the latest by April 1995.

Annexes designated "informative" are given only for information. In this standard annexes A and B are informative.

According to the CEN/CENELEC Internal Regulations, 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, United Kingdom.

1 Scope

This european standard specifies an enzymatic method for the determination of the total content of D-isocitric acid, present either in the form of the free acid or its salts including esters and lactones in fruit and vegetable juices and related products.

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.

ISO 5725:1986	Precision of test methods - Determination of repeatability and reproducibility for a standard test-method by inter-laboratory tests
ISO 3696:1987	Water for analytical laboratory use - Specification and test methods

3 Symbols and abbreviations

For the purposes of this standard, the following symbols and abbreviations apply :

ICDH	Isocitrate dehydrogenase (EC [1] 1.1.1.42) ;
NADP	β-Nicotinamide-adenine-dinucleotide-phosphate ;
NADPH	β-Nicotinamide-adenine-dinucleotide-phosphate, reduced ;
IU	1 International Unit (IU) of enzyme activity catalyzes the conversion of 1 µmol of substrate per minute at 25 °C under standard conditions ;
c	Substance concentration ;
ρ	Mass concentration ;
ω	Mass fraction ;
g	Acceleration due to gravity at the surface of the earth.

4 Principle

D-Isocitric acid is isolated from the test sample via its barium salt and is determined enzymatically. In this method D-isocitrate is oxidatively decarboxylated to 2-oxo-glutarate by NADP in the presence of the enzyme ICDH :

$$\text{D-isocitrate} + \text{NADP} \xrightarrow{\text{ICDH}} \text{2-oxo-glutarate} + \text{NADPH} + CO_2 + H^+$$

The amount of NADPH formed (measured by the increase in absorbance) is equivalent to the amount of D-isocitrate present.

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[1] Enzyme Commission (EC) : Classification System. Enzyme Handbook, Springer, Berlin 1969.

5 Reagents

Use only reagents of recognized analytical grade and only water in accordance with at least grade 3 of ISO 3696:1987.

5.1 Activated charcoal, Clarocarbon G® [1)]

5.2 Hydrochloric acid, c (HCl) = approximately 4 mol/l.

5.3 Sodium hydroxide solution, c (NaOH) = approximately 4 mol/l.

5.4 Ammonia solution, ω (NH_3) = 25 g/100 g.

5.5 Acetone

5.6 Barium chloride solution, $\rho(BaCl_2 \cdot 2H_2O)$ = 300 g/l.

Dissolve 30 g barium chloride ($BaCl_2 \cdot 2H_2O$) in water and dilute to 100 ml.

5.7 Sodium sulfate solution ρ (Na_2SO_4) = 71 g/l.

Dissolve71 g sodium sulfate (Na_2SO_4) in water and dilute to 1 litre.

5.8 Manganese sulfate solution, c ($MnSO_4$) = approximately 0,075 mol/l.

Dissolve125 mg manganese sulfate ($MnSO_4 \cdot H_2O$) in 10 ml water. This solution is stable for at least 6 months at room temperature.

5.9 Tris buffer solution, pH = 7,0.

Dissolve 2,42 g Tris (hydroxymethyl)-aminomethane and 35 mg ethylenediamine tetra-acetic acid disodium salt (dihydrate) in 80 ml water, acidify to pH 7,0 with hydrochloric acid (5.2) and dilute with water to 100 ml. This buffer solution keeps for at least 1 year at + 4 °C.

5.10 Tris buffer solution, pH = 7,4.

Dissolve 2,42 g Tris (hydroxymethyl)-aminomethane and 35 mg ethylenediamine tetra-acetic acid disodium salt (dihydrate) in 80 ml water, acidify to pH 7,4 with hydrochloric acid (5.2) and dilute with water to 100 ml. This buffer solution keeps for at least 1 year at + 4 °C.

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1) Clarocarbon G® is the trade-name of a product supplied by Merck, Bundesrepublik Deutschland. This information is given for the convenience of users of this standard 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.

5.11 NADP solution

Dissolve 50 mg β-nicotinamide adenine dinucleotide phosphate-disodium salt (NADP-Na_2) in 5 ml water. This solution keeps for at least 4 weeks at + 4 °C.

5.12 ICDH enzyme solution

Dissolve Isocitrate-dehydrogenase from pig's heart, ρ(ICDH) = 10 mg/ml, (approximately 20 IU/ml), in glycerol solution, r (glycerol) = 50 g/100 g . This solution is stable for approx. 6 months at + 4 °C.

6 Apparatus

Usual laboratory apparatus and, in particular, the following :

6.1 Enzyme test pipettes, graduated along the stem only, with long ungraduated delivery tip.

6.2 Pipettes, with an accuracy equivalent to (6.1) e.g. positive displacement capillary pipettes.

6.3 Cuvettes, made of glass or plastic, of 10 mm optical path length, and which do not have significant absorption at 334, 340 or 365 nm.

6.4 Spectral-line photometer, with mercury lamp and filters for measuring at 365 nm or 334 nm.

6.5 Spectrometer, (variable wavelength) for measuring at 340 nm (alternative to 6.4).

6.6 Pleated filter paper, pore size 10 µm.

6.7 Centrifuge, capable of producing a centrifugal force of 3000 x g at the base of the centrifuge tubes (6.8) (the value of g is fixed, for the purpose of this standard, at 9,81 $m.sec^{-2}$).

6.8 Centrifuge tubes, 100 ml capacity.

7 Procedure

7.1 Preparation of the test sample

Normally products shall not be pretreated and their analysis by this method shall be on a volumetric basis, results being expressed per litre of sample. The analysis of concentrated products may also be carried out on a volumetric basis, after dilution to a known relative density. Based on a weighed sample and taking the dilution factor for analysis into account, the results may also be expressed per kilogram of product. In products with high viscosity and/or very high content of cells (for example pulp), determination on the basis of a weighed test sample is the usual procedure.

7.2 Determination

7.2.1 Isolation of D-isocitrate from the test sample

Treat 10 ml of test sample with 5 ml sodium hydroxide solution (5.3) in a 100 ml centrifuge tube (6.8) and allow to stand for 10 min at room temperature (approximately 20 °C to 25 °C).

After adding 5 ml hydrochloric acid (5.2) dilute the solution to 25 ml with water. Then add consecutively 2 ml ammonia solution (5.4), 3 ml barium chloride solution (5.6) and 20 ml acetone (5.5). Mix thoroughly with a glass rod. Allow to stand for 10 min and then centrifuge (6.7) for about 5 min.

Carefully decant the supernatant solution and add 20 ml sodium sulfate solution (5.7) to the precipitate in the centrifuge tube and stir with a glass rod. Dissolve the clumped precipitate by heating for 10 min in a boiling water-bath with frequent stirring, then after cooling to room temperature transfer quantitatively to a 50 ml graduated flask with tris-buffer solution (5.9) and make up to the mark.

Transfer the contents of the graduated flask to a conical flask containing 1 g activated charcoal (5.1), allow to stand 5 min and then filter through a pleated filter paper (6.6). The clear, colourless filtrate is used for the enzymatic determination of isocitrate (7.2.2).

7.2.2 Enzymatic determination of D-isocitrate

7.2.2.1 General

The determination shall normally be carried out at constant temperature, between 20 °C and 25 °C. A constant temperature in the range 25 °C to 37 °C may also be used, providing equivalent results are obtained.

The absorption maximum of NADH is at 340 nm. When using a variable wavelength spectrometer, measure at the absorption maximum only. When using a mercury vapour lamp, spectral-line photometer, measure at a wavelength of 334 nm or 365 nm.

Do not use single-mark transfer pipettes for pipetting the solutions. Solutions of enzyme, coenzyme and buffer may be added from suitable automatic pipettes. Enzyme test pipettes (6.1) or their equivalent (6.2) shall be used for pipetting the sample solution.

The determination may also be carried out using a commercially available test-combination kit.

If the substance to be determined is available in a suitably pure form, it is recommended to include it as a standard solution.

7.2.2.2 Blank test solution

Pipette into cuvette (6.3) 3,0 ml buffer solution (5.10), 0,1 ml manganese sulfate solution (5.8) and 0,1 ml NADP solution (5.11). Mix, after about 3 minutes, read the absorbance (A_{1Blank}) against air (no cuvette in the light path).

7.2.2.3 Test sample solution

Pipette into cuvette (6.3) 2,0 ml buffer solution (5.10), 0,1 ml manganese sulfate solution (5.8), 0,1 ml NADP solution (5.11) and 1,00 ml test sample (from 7.2.2). Mix, after about 3 minutes, read the absorbance ($A_{1Sample}$) against air (no cuvette in the light path).

7.2.2.4 Enzyme reaction and quantification

Start the reaction by the addition of 0,01 ml ICDH enzyme solution (5.12) to each of the solutions 7.2.2.2 and 7.2.2.3. Mix, wait until the reaction has stopped (5-10 min) and read the absorbances (A_2) of the solutions against air. If the reaction has not stopped after 10 min, continue to read the absorbance at 5 min intervals until the absorbance increases at a constant rate and extrapolate back to A_2 at the time when the enzyme solution (ICDH) was added.

8 Calculation

According to the reaction on which the determination is based, there is a linear proportionality between the amount of NADPH formed (and therefore the absorbance difference) and the concentration of D-isocitric acid :

$$\Delta A = (A_2 - A_1)_{Sample} - (A_2 - A_1)_{Blank}$$

The calculation of the concentration of a substance in dilute solution by absorptiometric measurement is based on the Beer - Lambert law. The D-Isocitric acid content ρ of the sample in milligrams per litre is calculated from the following equation :

$$\rho = \frac{M \times V_1 \times F}{\varepsilon \times \delta \times V_2 \times 1\,000} \times \Delta A$$

where :

M	is the molecular mass of D-Isocitric acid = 192.1 grams per mole ;
V_1	is the total volume of the test solution within the cuvette, in millilitres ;
V_2	is the volume of sample solution used in preparing the test solution, in millilitres ;