SLOVENSKI STANDARD
SIST EN 1137:1996

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Sadni in zelenjavni sokovi - Encimsko ugotavljanje vsebnosti citronske kisline (citrata) - NADH spektrometrijska metoda

Fruit and vegetable juices - Enzymatic determination of citric acid (citrate) content - NADH spectrometric method

Frucht- und Gemüsesäfte - Enzymatische Bestimmung des Gehaltes an Citronensäure (Citrat) - Spektralphotometrische Bestimmung von NADH

Jus de fruits et de légumes - Dosage enzymatique de l'acide citrique (citrate) - Méthode spectrométrique par le NADH

Ta slovenski standard je istoveten z: **EN 1137:1994**

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English version

Fruit and vegetable juices - Enzymatic determination of citric acid (citrate) content - NADH spectrometric method

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CEN

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

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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of an identical text or by endorsement, at the latest by April 1995, and conflicting national standards shall be withdrawn at the latest by April 1995.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 a method for the enzymatic determination of the total content of citric acid, present either in the form of free acid or its salts, 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 :

NAD	β-Nicotinamide-adenine-dinucleotide ;
NADH	β-Nicotinamide-adenine-dinucleotide, reduced form ;
CL	Citrate lyase (EC [1] 4.1.3.6) ;
MDH	Malate dehydrogenase (EC [1] 1.1.1.37) ;
LDH	Lactate dehydrogenase (EC [1] 1.1.1.27) ;
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.

4 Principle

The method is based on the enzymatic conversion of citrate to oxaloacetate. Pyruvate is formed by spontaneous decarboxylation of oxaloacetate. The subsequent enzymatic reduction of both oxaloacetate and pyruvate by the reduced form of nicotinamide adenine dinucleotide is followed spectrometrically. The total amount of NADH used, measured by the decrease in absorbance, is equivalent to the amount of citric acid.

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

4.1 Reactions

The following enzymatic reactions take place at pH = 7,8 :

(1) Citrate $\xrightarrow{CL}$ oxaloacetate + acetate

(2) Oxaloacetate + NADH + H^+ $\xrightarrow{MDH}$ L-malate + NAD^+

(3) Pyruvate + NADH + H^+ $\xrightarrow{LDH}$ L-lactate + NAD^+

5 Reagents

5.1 General

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

5.2 Glycylglycine buffer, pH = 7,8

Dissolve 7,13 g glycylglycine in 70 ml water, adjust to pH 7,8 with about 13 ml sodium hydroxide, (c(NaOH) = 5 mol/l), add 10 ml zinc chloride solution, ρ ($ZnCl_2$) = 0,8 g/l and dilute to 100 ml with water. The buffer is stable for at least four weeks at 4 °C.

5.3 NADH solution

Dissolve 30 mg reduced β-nicotinamide-adenine-dinucleotide, disodium salt (β-NADH-Na_2) and 60 mg sodium hydrogen carbonate ($NaHCO_3$) in 6 ml water. The solution is stable for at least four weeks at 4 °C.

5.4 MDH/LDH enzyme suspension

Mix 0,1 ml malate dehydrogenase, ρ (MDH) = 5 mg/ml, approximately 6000 IU/ml with oxaloacetate as substrate, 0,4 ml ammonium sulfate solution, c ($(NH_4)_2SO_4$) = 3,2 mol/l and 0,5 ml lactate dehydrogenase, ρ (LDH) = 5 mg/ml, approximately 2750 IU/ml with pyruvate as substrate. The suspension is stable for at least one year at 4 °C.

5.5 CL enzyme suspension

Dissolve 168 mg lyophilisate (5 mg enzyme protein) of citrate lyase (approximately 1,25 IU/ml with citrate as substrate) in 1 ml of ice-cold water. The suspension is stable for at least one week at 4 °C and for at least four weeks when frozen.

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 accuracy equivalent to 6.1 (alternative to 6.1) e.g. positive displacement capillary pipettes.

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

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

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

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. In this case, the relative density shall be indicated. 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.

Dilute the sample to be examined so that the citric acid concentration is between 0,02 g/l and 0,4 g/l. This solution is used directly for the determination, even if it is coloured. Mix cloudy juices well and dilute them ; they, and also very strongly coloured juices, may need to be diluted beyond that required by the citrate content.

7.2 Test procedure

7.2.1 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 Blank test solution

Pipette into cuvette 1,00 ml buffer solution (5.2), 0,1 ml NADH solution (5.3), 2,00 ml water and 0,02 ml MDH/LDH enzyme suspension (5.4). Mix, read the absorbance $(A_1)_{Blank}$ of the solution against air (no cuvette in light path) after approximately 5 min.

7.2.3 Test sample solution

Pipette into cuvette 1,00 ml buffer solution (5.2), 0,1 ml NADH solution (5.3), 0,20 ml of the test sample, 1,80 ml water and 0,02 ml MDH/LDH enzyme suspension (5.4). Mix, read the absorbance $(A_1)_{Sample}$ of the solution against air (no cuvette in light path) after approximately 5 min.

If the initial absorbance value is too high, (A1 > 1,000) start a new determination, beginning at 7.2.2 and using a reduced concentration of NADH. The capacity of the test is affected by this measure, so that the concentration range of the test sample shall also be reduced.

If the citrate concentration of the sample solution is less than 0,02 g/l, the sample volume to be pipetted into the cuvette can be increased to as much as 2,0 ml. In this case, the volume of water to be added is correspondingly reduced so that both blank and sample cuvettes contain the same total volume. The volume of sample solution (V_2) in the calculation (see clause 8) shall then be altered accordingly.

7.2.4 Enzyme reaction and quantification

Start the reaction by the addition of 0,02 ml CL enzyme suspension (5.5) to each of the solutions 7.2.2 and 7.2.3. Mix, and after the reaction is complete (about 5 min to 10 min) read the absorbance (A_2) of the solutions against air (no cuvette in light path). Check the completion of the reaction by reading after a further 2 min.

8 Calculation

According to the reactions on which this determination is based, there is a linear proportionality between the amount of NADH used (and hence the absorbance difference, ΔA) and the concentration of citric acid.

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

The calculation of the concentration of a substance in dilute solution by absorptiometric measurement is based on the Beer-Lambert law.

The citric acid content, ρ, in grams per litre of sample, 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 citric acid (anhydrous) = 192,1 grams per mole ;
V_1	is the total volume of solution in cuvette in millilitres ;
V_2	is the volume of sample solution added to cuvette in millilitres ;
F	is the dilution factor of sample solution ;
δ	is the light path of cuvette in centimetres ;
ε	is the extinction coefficient of NADH ; at 340 nm = 6,3 l mmol^{-1} cm^{-1} ; 365 nm = 3,4 l mmol^{-1} cm^{-1} ; 334 nm = 6,18 l mmol^{-1} cm^{-1}.

If the volumes given in (7.2.3) are not altered , then :

$$\rho = 3{,}016 \times \left(\frac{F \times \Delta A}{\varepsilon}\right)$$

When using a commercially available test-combination kit, the numerical factor (3,016) of the above equation is different, due to a different total assay volume (V_1).

During calculation, take into account any dilution factor and the relation of the value to mass or volume. If a concentrated product has been diluted to single strength, report the relative density of the single strength sample.

Report the citric acid concentration in grams per litre to two decimal places.