SLOVENSKI STANDARD
SIST EN 1138:1996

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Sadni in zelenjavni sokovi - Encimsko ugotavljanje vsebnosti L-jabolčne kisline (L-malata) - NADH spektrometrijska metoda

Fruit and vegetable juices - Enzymatic determination of L-malic acid (L-malate) content - NADH spectrometric method

Frucht- und Gemüsesäfte - Enzymatische Bestimmung des Gehaltes an L-Apfelsäure (L-Malat) - Spektralphotometrische Bestimmung von NADH

Jus de fruits et de légumes - Dosage enzymatique de l'acide L-malique (L-malate) - Méthode spectrométrique par le NADH

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English version

Fruit and vegetables juices - Enzymatic determination of L-malic acid (L-malate) content - NADH spectrometric method

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Frucht- und Gemüsesäfte - Enzymatische Bestimmung des Gehaltes an L-Äpfelsäure (L-Malat) - Spektralphotometrische Bestimmung von NADH

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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of an identical text or by endorsement, at the latest by April 1995, and conflicting national standards shall be withdrawn at the latest by April 1995.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 L-malic acid, present either in the form of the 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) ;
GOT	glutamate-oxaloacetate-transaminase (EC [1] 2.6.1.1) ;
L-MDH	L-malate-dehydrogenase (EC [1] 1.1.1.37) ;
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 and reactions

The method is based on the enzymatic conversion of L-malate to oxaloacetate and spectrometric measurement of the simultaneous reduction of nicotinamide adenine dinucleotide at pH 10,0 (reaction 1) :

$$\text{(1) L-malate} + NAD^+ \xleftrightarrow{\text{L-MDH}} \text{oxaloacetate} + NADH + H^+$$

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

The equilibrium of reaction (1) lies almost completely in the direction of L-malate. However, by trapping the oxaloacetate in a subsequent reaction (2) catalysed by the enzyme GOT in the presence of L-glutamate, the equilibrium can be displaced in favour of oxaloacetate and NADH :

(2) Oxaloacetate + L-glutamate $\xleftrightarrow{\text{GOT}}$ L-asparatate + 2-oxoglutarate

The amount of NADH formed, measured by the increase in absorbance, is equivalent to the amount of L-malic acid.

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 = 10,0

Dissolve 4,75 g glycylglycine and 0,88 g L-glutamic acid in 50 ml water, adjust to pH 10,0 with approximately 4,6 ml sodium hydroxide solution, c (NaOH) = 10 mol/l, and make up to 60 ml with water. The solution is stable for at least three months at 4 °C.

5.3 NAD solution

Dissolve 420 mg NAD in 12 ml water. The solution is stable for at least four weeks at 4 °C.

5.4 GOT enzyme suspension

Glutamate-oxaloacetate transaminase, ρ(GOT) = 2 mg/ml, suspended in a solution of ammonium sulfate, c ($(NH_4)_2SO_4$) = 3,2 mol/l. This results in an enzyme activity of approximately 400 IU/ml with L-aspartate and 2-oxoglutarate as substrates. The suspension is stable for at least one year at 4 °C.

5.5 L-MDH enzyme suspension

L-malate dehydrogenase, l(L-MDH) = 5 mg/ml, suspended in a solution of ammonium sulfate, c ($(NH_4)_2SO_4$) = 3,2 mol/l. This results in an enzyme activity of approximately 6000 IU/ml with oxaloacetate as substrate. The suspension is stable for at least one year 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 accuracy equivalent to 6.1 (alternative 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 334 nm or 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.

Mix cloudy samples well before dilution ; these, and also very strongly coloured samples, may need to be diluted beyond that required by the malate content. Clarify cloudy samples containing very small concentrations of malic acid by prior centrifugation.

Dilute the sample to be examined so that the L-malic acid concentration is between 0,02 g/l and 0,35 g/l. This solution is normally used directly for the determination, even if it is coloured.

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), 0,20 ml NAD solution (5.3), 1,50 ml water and 0,01 ml GOT enzyme suspension (5.4). Mix and after approximately 3 min read the absorbance $(A_1)_{Blank}$ of the solution against air (no cuvette in light-path).

7.2.3 Test sample solution

Pipette into cuvette 1,00 ml buffer solution (5.2), 0,20 ml NAD solution (5.3), 1,40 ml water, 0,01 ml GOT enzyme suspension (5.4) and 0,1 ml test sample. Mix and after approximately 3 min read the absorbance $(A_1)_{Sample}$ of the solution against air (no cuvette in light-path).

If the concentration of L-malic acid in the test sample is less than 0,02 g/l, the test sample volume may be increased to as much as 1,50 ml with a corresponding reduction in the amount of water added, so that the total assay volume remains the same (2,71 ml).

7.2.4 Enzyme reaction and quantification

Start the reaction by addition of 0,01 ml L-MDH enzyme suspension (5.5) to each of the solutions 7.2.2 and 7.2.3. Mix, and on completion of the reaction (about 5 min to 10 min) read the absorbances of the solutions (A_2). Check the completion of reaction by reading again 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 formed (and hence the absorbance difference, ΔA) and the concentration of malic 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 L-malic acid content ρ in g/l 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 L-malic acid = 134,09 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 absorption 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 :

$$\rho = 3{,}647 \times \left(\frac{F \times \Delta A}{\varepsilon} \right)$$

When using a commercially available test-combination kit, the numerical factor (3,647) in the above equation may be different, due to a different total assay volume.

During calculation, take into account any dilution factor and the relationship 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 L-malic acid content in grams per litre to two decimal places.

9 Precision

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

9.1 Repeatability

The absolute difference between two single test results found on identical test material by one operator using the same apparatus within the shortest feasible time interval will exceed the repeatability value r in not more than 5 % of the cases.

The value is :

$$r = 0{,}014 + 0{,}030\ \rho \text{ g/l}$$

where :

ρ is the measured content, calculated as mean value from the two single test results.