SLOVENSKI STANDARD
SIST EN 12138:1998

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Sadni in zelenjavni sokovi - Encimatsko določevanje D-jabolčne kisline - NAD spektrometrijska metoda

Fruit and vegetable juices - Enzymatic determination of D-malic acid content - NAD spectrometric method

Frucht- und Gemüsesäfte - Enzymatische Bestimmung des Gehaltes an D-Äpfelsäure - Spektralphotometrische Bestimmung von NAD

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

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67.160.20	Brezalkoholne pijače	Non-alcoholic beverages

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English version

Fruit and vegetable juices - Enzymatic determination of D-malic acid content - NAD spectrometric method

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

Frucht- und Gemüsesäfte - Enzymatische Bestimmung des Gehaltes an D-Äpfelsäure - Spektralphotometrische Bestimmung von NAD

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

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

Contents

Foreword

This European Standard has been prepared by 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of an identical text or by endorsement, at the latest by March 1998, and conflicting national standards shall be withdrawn at the latest by March 1998.

According to the CEN/CENELEC Internal Regulations, the national standards organizations of the following countries are bound to implement this European Standard: 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 Standard specifies an enzymatic method for the determination of the total content of D-malic acid, 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.

EN ISO 3696:1995	Water for analytical laboratory use - Specification and test methods (ISO 3696:1987)
ISO 5725:1986	Precision of test methods - Determination of repeatability and reproducibility for a standard test method by inter-laboratory tests

3 Symbols and abbreviations

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

D-MDH	D-Malate dehydrogenase decarboxyl
NAD	ß-Nicotinamide-adenine-dinucleotide
IU	1 International Unit (IU) of enzyme activity catalyses the conversion of 1 µmol of substance per minute at 25 °C under standard conditions
c	Substance concentration
ρ	Mass concentration

4 Principle

D-malic acid (D-malate) is oxidized by ß-nicotinamide-adenine-dinucleotide (NAD) in the presence of D-malate dehydrogenase decarboxyl (D-MDH) to oxaloacetate which then decomposes to give pyruvate and carbonic acid.

$$\text{D-malate} + NAD^+ \xrightleftharpoons{\text{D-MDH}} \text{pyruvate} + CO_2 + NADH + H^+$$

The amount of NADH formed (measured by the increase in the ultraviolet absorbance of the solution) is equivalent to the amount of D-malate present.

The D-malate dehydrogenase is not 100 % specific to D-malic acid. It reacts with L-tartaric acid at a much lower rate. If it is necessary to assay D-malic acid in the presence of high concentrations of tartaric acid, as in wine, the sample has to be pre-treated to remove the tartaric acid.

5 Reagents

5.1 General

Use only reagents of recognized analytical grade and only water in accordance with at least grade 3 of EN ISO 3696:1995.

NOTE : The test can also be carried out using single reagents.

5.2 Test kit combination

The Boehringer Mannheim test kit[1)] contains :

5.2.1 Bottle 1 with approximately 30 ml of solution consisting of HEPES-buffer (4-(2-hydroxyethyl)-1-piperazine ethansulfonic acid), pH = 9,0 and stabilizers ;

5.2.2 Bottle 2 with approximately 210 mg of lyophilized NAD ;

5.2.3 Three bottles with lyophilized D-MDH, approximately 8 IU each ;

5.3 Calcium hydroxide

5.4 Ethanol approximately 98 %

5.5 Potassium hydroxide

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 equivalent accuracy to that of 6.1 e.g. positive displacement capillary pipettes.

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

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1) Boehringer Mannheim is an example of a suitable product available commercially. This information is given for the convenience of users of this standard and does not constitute an endorsement by CEN of this product.

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

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

6.6 Volumetric flasks, of 50 ml capacity.

6.7 Membrane filter, of pore size 0,2 µm.

7 Procedure

7.1 Sample preparation

7.1.1 Normal preparation

Normally products shall not be pre-treated, however dilution may be necessary 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 a high viscosity and/or a very high content of cells (for example pulp), a determination on the basis of a weighed test sample is the usual procedure.

Clarify cloudy samples containing low concentrations of D-malic acid by prior centrifugation or membrane filtration through a 0,2 µm filter (6.7).

7.1.2 Preparation of the test samples containing high levels of tartaric acid

Take 25 ml of juice and mix with 125 mg of calcium hydroxide (5.3) and 5 ml of ethanol (5.4) (approximatively 98 %) for 2 min. Adjust the pH of the solution to between 7 and 8 with potassium hydroxide (5.5) ; transfer quantitatively into a 50 ml volumetric flask (6.6) and dilute to the mark. Clarify solution (as given 7.1.1) and use colourless clear solution in enzyme assay.

7.2 Test procedure

7.2.1 General

The determination shall be carried out between 20 °C and 25 °C or a constant temperature in the range of 25 °C to 37 °C providing equivalent results are obtained.

The absorption maximum of NADH is at a wavelength of 340 nm. When using a variable wavelength spectrometer (6.5), measure at the absorption maximum only. When using a mercury vapour lamp, spectral-line photometer (6.4), 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.

It is recommended to include D-malic acid as a standard solution in this assay.

7.2.2 Blank test solution

Pipette into the cuvette 1,00 ml of the HEPES buffer (5.2.1), 0,10 ml of the NAD solution (5.2.2) and 1,80 ml of water. Mix and after 6 min, read the absorbance (A_{1Blank}) against air (with no cuvette in the lightpath).

7.2.3 Test sample solution

Pipette into the cuvette 1,00 ml of the HEPES buffer (5.2.1), 0,10 ml of the NAD solution (5.2.2), 1,70 ml of water and 0,10 ml of sample solution. Mix and after 6 min, read the absorbance ($A_{1Sample}$) against air (with no cuvette in the lightpath).

7.2.4 Enzyme reaction and quantification

Start the reaction by the addition of 0,05 ml of D-MDH solution (5.2.3) to each of the solutions given in 7.2.2 and 7.2.3. Mix and wait until the reaction has stopped (approximately 20 min) and read the absorbances (A_2) of the solutions against air. If the reaction has not stopped after 20 min, continue to read the absorbances at 5 min intervals until it increases at a constant rate and extrapolate this back to the time when the final enzyme solution D-MDH was added. (This process allows for side reactions and, the so called "creep" reaction, which is discribed in more detail in Annex B).

8 Calculation

According to the reaction on which the determination is based, there is a linear proportionality between the amount of NADH formed (and therefore the absorbance difference) and the concentration of D-malic acid, as given in the following equation :

$$\Delta A = (A_2 - A_1)_{Sample} - (A_2 - A_1)_{Blank} \qquad (1)$$

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

$$\rho_{(\text{D-malic acid})} = \frac{M \cdot V}{\epsilon \cdot \delta \cdot v \cdot 1000} \cdot \Delta A \qquad (2)$$

where :

M is the molecular mass of D-malic acid (134,09 grams per mole) ;

V is the total volume of the test solution within the cuvette, in millilitres ;

v is sample volume, in millilitres ;

δ is the lightpath of the cuvette in centimetres ;