BRITISH STANDARD

BS EN
1139:1995

Method for

Determination of D-isocitric acid content of fruit and vegetable juices: NADPH spectrometric method

The European Standard EN 1139:1994 has the status of a British Standard

UDC 663.81/.82:620.1:543.42:543.477

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This British Standard, having been prepared under the direction of the Consumer Products and Services Sector Board, was published under the authority of the Standards Board and comes into effect on January 1995

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National foreword

This British Standard has been prepared under the direction of the Consumer Products and Services Sector Board and is the English language version of EN 1139:1994 *Fruit and vegetable juices — Enzymatic determination of D-isocitric acid content — NADPH spectrometric method,* published by the European Committee for Standardization (CEN). EN 1139 was produced as a result of international discussions in which the United Kingdom took an active part.

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Summary of pages

This document comprises a front cover, an inside front cover, pages i and ii, the EN title page, pages 2 to 6, an inside back cover and a back cover.

This standard has been updated (see copyright date) and may have had amendments incorporated. This will be indicated in the amendment table on the inside front cover.

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EUROPEAN STANDARD

NORME EUROPÉENNE

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EN 1139

October 1994

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Descriptors: Food products, beverages, fruit and vegetable juices, chemical analysis, determination of content, citric acid, enzymatic methods, spectrophotometric analysis

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 spectrophotométrique par le NADPH

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

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

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

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Foreword

This European Standard was 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 Annex A and Annex B are informative.

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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[a] 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.

[a] Enzyme Commission (EC): Classification system. Enzyme Handbook, Springer, Berlin 1969.

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:

$$\text{D-isocitrate} + \text{NADP} \xrightarrow{\text{ICDH}} \text{2-oxo-glutarate} + \text{NADPH} + \text{C0}_2 + \text{H}^+$$

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

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,* $\rho(BaCl_2.2H_2O)$ = 300 g/l

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

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

Dissolve 125 mg manganese sulfate ($MnSO_4.H_2O$) 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.

[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 products named. Equivalent products may be used if they can be shown to lead the same results.

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5.10 *Tris buffer solution,* pH = 7,4

Dissolve 2,42 g Tris (hydroxymethyl)-aminomethane and 35 g 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.

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, w (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 and 365 nm.

6.4 *Spectral-line photometer,* with mercury lamp 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 sizes 10 μm.

6.7 *Centrifuge,* capable of producing a centrifugal force of 3 000 × 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 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 boiling water-bath with frequent stirring, then after cooling to room temperature transfer quantitively to 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 spectrophotometer, 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 solution. 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.

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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:

$$\rho = \frac{M \times V_1 \times F}{\varepsilon \times \delta \times V_2 \times 1000} \Delta A$$

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;

F is the dilution factor of the sample solution (**7.2.1**);

δ is the light-path of the cuvette, in centimetres;

ε is the extinction coefficient of NADPH:

at 340 nm = 6,3 l $mmol^{-1}cm^{-1}$;

Hg 365 nm = 3,5 l $mmol^{-1}cm^{-1}$;

Hg 334 nm = 6,18 l $mmol^{-1}cm^{-1}$;

If the volumes given in **7.2** are adhered to, the calculation becomes

$$\rho = 3083 \times \frac{\Delta A}{\varepsilon}$$

When using a commercially available combination kit, numerical factor (3083) in the above equation may be 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.

The D-isocitric acid content is reported in milligrams per litre to the nearest milligram.

9 Precision

Details of the interlaboratory test on 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 tests 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 = 2,5 mg/l

9.2 Reproducibility

The absolute difference between two single test results on identical test material reported by two laboratories will exceed the reproducibility value R in not more than 5 % of the cases. The value is:

R = 4,4 mg/l

10 Test report

The test report shall contain the following data:

— all information necessary for the identification of the sample (kind of sample, origin of sample, designation);

— a reference to this European Standard;

— the date and type of sampling procedure (if possible);

— the date of receipt;

— the date of test;

— the test results and units in which they have been expressed;

— whether the repeatability has been verified;

— any particular points observed in the course of the test;

— any operations not specified in the method or regarded as optional, which might have affected the results.

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Annex A (informative)
Bibliography

[1] Determination of D-isocitric acid, enzymatic method: No 54, 1984.

— In: Analyses [Collection]/International Federation of Fruit Juice Producers.

— Loose-leaf edition, as of 1989.- Zug: Swiss Fruit Union.

[2] Untersuchung von Lebensmitteln: Bestimmung von D-Isocitronensäure in Fruchtsäften: L31.00-9, 1984-11 [Food Analysis: Determination of D-isocitric acid in fruit juices: L31.00-9, 1984-11] — *in: Amtliche Sammlung von Untersuchungsverfahren nach§35 LMBG: Verfahren zur Probenahme und Untersuchung von Lebensmitteln, Tabakerzeugnissen, kosmetischen Mitteln und Bedarfsgegenständen/Bundesgesundheitsamt [In: Collection of official methods under article 35 of the German Federal Foods Act: Methods of sampling and analysis of foods, tobacco products, cosmetics and commodity goods/Federal Health Office]*

— Loseblattausgabe, Stand 31.12.1991, Bd.l. [Loose-leaf edition, as of 1991-12-31, Vol.l.]

— Berlin, Köln: Beuth Verlag GmbH.

[3] S.Wallrauch und G.Greiner Flüssiges Obst, 1977, vol. 44, p. 241–245. Bestimmung der D-Isocitronensäure in Fruchtsäften und alkoholfreien Erfrischungsgetränken. [Determination of D-isocitric acid in fruit juices and nonalcoholic soft drinks].

Annex B (informative)
Statistical results of the interlaboratory test

In accordance with ISO 5725:1986, the following parameters have been defined in an interlaboratory test. (For literature pertaining to the method see Annex A). The test was conducted by the Max von Pettenkofer Institute of the Federal Health Office, Food Chemistry Department, Berlin, BRD:

Year of inter-laboratory test 1982
number of laboratories 28 and 12
number of samples 3

Table 1

Sample	A	B	C
Number of laboratories retained after eliminating outliers	22	21	10
Number of outliers (Laboratories)	6	7	2
Number of accepted results	118	108	50
Mean value ($\bar{x}$) (mg/l)	57	82	90
Repeatability standard deviation (s_r) (mg/l)	0,8657	0,9561	0,8224
Repeatability relative standard deviation (RSD_r) [%]	1,52	1,16	0,86
Repeatability limit (r) (mg/l)	2,4	2,7	2,3
Reproductibility standard deviation (s_R) (mg/l)	1,5718	1,4406	1,7981
Reproducibility relative standard deviation (RSD_R) [%]	2,76	1,76	1,87
Reproducibility limit (R) (mg/l)	4,4	4,0	5,0

Sample types:
A blackcurrant nectar I
B Orange juice
C black currant nectar II

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National annex NA (informative)
Committees responsible

The United Kingdom participation in the preparation of this European Standard was entrusted by the Consumer Products and Services Sector Board to Technical Committee AW/21, upon which the following bodies were represented:

British Food Manufacturing Industries Research Association

British Fruit Juice Importers' Association

British Retail Consortium

British Soft Drinks Association Limited

Campden Food and Drink Research Association

Department of Trade and Industry (Laboratory of the Government Chemist)

Ministry of Agriculture, Fisheries and Food

Royal Society of Chemistry

National annex NB (informative)
Cross-references

Publication referred to	Corresponding British Standard
ISO 3696:1987	BS 3978:1987 *Specification for water for laboratory use*
ISO 5725:1986	BS 5497 *Precision of test methods* Part 1:1987 *Guide for the determination of repeatability and reproducibility for a standard test method by inter-laboratory tests*

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