SLOVENSKI STANDARD
SIST EN 12856:2000

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Živila - Določevanje K-acesulfama, aspartama in saharina - Metoda tekočinske kromatografije visoke ločljivosti

Foodstuffs - Determination of acesulfame-K, aspartame and saccharin - High performance liquid chromatographic method

Lebensmittel - Bestimmung von Acesulfam-K, Aspartam und Saccharin - Hochleistungsflüssigchromatographisches Verfahren

Produits alimentaires - Dosage de l'acésulfame-K, de l'aspartame et de la saccharine - Méthode par chromatographie liquide haute performance

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67.050	Splošne preskusne in analizne metode za živilske proizvode	General methods of tests and analysis for food products

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English version

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EUROPEAN COMMITTEE FOR STANDARDIZATION
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Central Secretariat: rue de Stassart, 36 B-1050 Brussels

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Foreword

This European Standard has been prepared by Technical Committee CEN/TC 275 "Food analysis - Horizontal methods", the secretariat of which is held by DIN.

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of an identical text or by endorsement, at the latest by October 1999, and conflicting national standards shall be withdrawn at the latest by October 1999.

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 high performance liquid chromatographic (HPLC) method for the determination of acesulfame-K, aspartame and saccharin, see [1], [2] and [3]. It also allows the determination of caffeine, sorbic acid and benzoic acid in foodstuffs. Interlaboratory tests have been carried out with acesulfame-K in marzipan, yogurt, fruit yogurt, orange juice beverage, cola, cream and jam, with aspartame in marzipan, fruit yogurt, orange juice beverage, orange flavoured beverage, cola, jam, and preparation for flan, and with sodium saccharin in marzipan, yogurt, fruit yogurt, orange juice, orange juice beverage, cola, cream and jam.

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 Water for analytical laboratory use - Specification and test methods (ISO 3696:1987)

3 Principle

The sample is extracted or diluted with water. If necessary, the sample solution with the intense sweeteners is purified on a solid phase extraction column or with Carrez reagents. The intense sweeteners in the sample test solution are separated on an HPLC-reversed phase chromatography column and determined spectrometrically at a wavelength of 220 nm.

4 Reagents

During the analysis, unless otherwise stated, use only reagents of recognized analytical grade for HPLC analysis and water of at least grade 1 as defined in EN ISO 3696. When preparing solutions, the purity of the substances shall be taken into account.

4.1 Acetonitrile, for HPLC

4.2 Methanol, for HPLC

4.3 Potassium dihydrogen orthophosphate (KH_2PO_4)

4.4 Dipotassium hydrogen orthophosphate (K_2HPO_4)

4.5 Phosphoric acid, ρ_{20} (H_3PO_4)= 1,71 g/ml, w (H_3PO_4) = 85 % [1])

4.6 Phosphoric acid, w (H_3PO_4) = 5 %

Carefully pipette 6 ml of phosphoric acid (4.5) into a 100 ml volumetric flask, which already contains 80 ml of water. Dilute to the mark with water.

4.7 Carrez solution No 1

Dissolve 15 g potassium hexacyanoferrate (II) ($K_4[Fe(CN)_6] \cdot 3\ H_2O$) in water and dilute to 100 ml.

4.8 Carrez solution No 2

Dissolve 30 g zinc sulfate ($ZnSO_4 \cdot 7\ H_2O$) in water and dilute to 100 ml.

4.9 Phosphate buffer solution I, c (KH_2PO_4) = 0,02 mol/l, pH ≈ 4,3 [2])

Dissolve 2,72 g of potassium dihydrogen orthophosphate (4.3) and 800 ml of water in a 1000 ml beaker. Adjust to pH 4,3 with phosphoric acid (4.6). Transfer the solution to a 1000 ml volumetric flask and dilute to the mark with water.

4.10 Phosphate buffer solution II, c (KH_2PO_4) = 0,0125 mol/l, pH ≈ 3,5

Dissolve 1,70 g of potassium dihydrogen orthophosphate (4.3) and 800 ml of water in a 1000 ml beaker. Adjust to pH 3,5 with phosphoric acid (4.6). Transfer the solutions to a 1000 ml volumetric flask and dilute to the mark with water.

4.11 Mobile phase, phosphate buffer and acetonitrile

Add, carefully measured, the required amounts of the selected phosphate buffer to acetonitrile as given in A.5 and mix. Filter through suitable membrane filters, e.g. of pore size 0,45 µm and degas, e.g. for 5 min in an ultrasonic bath. Prepare the mobile phase on the day of use.

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[1]) w is the mass fraction

[2]) c is the substance concentration

4.12 Control solution (optional)

The control solution contains acesulfame-K, sodium saccharin, aspartame, 5-benzyl-3,6-dioxo-2-piperazine acetic acid (diketopiperazine), aspartylphenylalanine, phenylalanine, caffeine, sorbic acid, benzoic acid, theobromine, hydroxymethylfurfural and vanillin.

In a 100 ml volumetric flask, weigh to the nearest 0,1 mg, 30 mg of acesulfame-K, 20 mg of sodium saccharin, 220 mg of aspartame, 60 mg of caffeine, 100 mg of sorbic acid, 100 mg of benzoic acid, 100 mg of vanillin, 10 mg of diketopiperazine, 10 mg of phenylalanine, 10 mg of aspartylphenylalanine, 20 mg of hydroxymethylfurfural and 70 mg of theobromine. Dissolve and dilute to the mark with water.

Pipette 20 ml of this solution into a 100 ml volumetric flask and dilute to the mark with water.

4.13 Stock solution

Weigh, to the nearest 0,1 mg, 100 mg of acesulfame-K, 100 mg of sodium saccharin and 100 mg of aspartame, in the same 100 ml volumetric flask. Dissolve and dilute to the mark with water. This solution contains 1g/l of each sweetener.

4.14 Standard solutions (optional)

4.14.1 Standard solution I

Pipette 10 ml of the stock solution (4.13) into a 100 ml volumetric flask and dilute to the mark with water. This solution contains 100 mg/l of each sweetener.

4.14.2 Standard solution II

Pipette 5 ml of the stock solution (4.13) into a 100 ml volumetric flask and dilute to the mark with water. This solution contains 50 mg/l of each sweetener.

4.14.3 Standard solution III

Pipette 1 ml of the stock solution (4.13) into a 100 ml volumetric flask and dilute to the mark with water. This solution contains 10 mg/l of each sweetener.

5 Apparatus and equipment

Usual laboratory apparatus and, in particular, the following:

5.1 High speed blender, or homogenizer

5.2 Volumetric flasks, of suitable capacities, e.g 1000 ml, 500 ml, and 100 ml

5.3 Beaker, 1000 ml

5.4 Pipettes, of suitable capacities, e.g. 100 ml, 25 ml, 20 ml, 10 ml, 5 ml and 1 ml

5.5 Micropipette, 1000 µl

5.6 Graduated cylinder, 1000 ml

5.7 Fluted filter papers, medium fast, qualitative

5.8 Ultrasonic bath

5.9 Centrifuge with centrifuge tubes of suitable capacity, capable of producing a centrifugal acceleration of at least 1400 *g* at the base of the centrifuge tubes

5.10 Degassing system, for solvents (optional, instead of ultrasonic degassing)

5.11 Membrane filters, of pore size 0,45 µm or smaller

5.12 Filter holder for membrane filters, with suitable syringe

5.13 Solid phase extraction column, with RP C 18 cartridge (optional), e.g. with 500 mg filling

5.14 High performance liquid chromatograph, equipped with an ultraviolet (UV) detector (capable of operating at a wavelength of 220 nm, preferably a diode array detector) and equipped with a recorder and/or integrator which allows the measurement of peak heights and peak areas

5.15 Chromatographic column for HPLC, type reversed phase (RP), e.g. with

- a RP C 18 stationary phase of 3 µm to 10 µm,
- length of 100 mm to 300 mm
- inner diameter of 3 mm to 4 mm
- a guard column, RP C 18 (optional, but usually recommended especially for all solid sample materials).

Performance criterion for suitable separation columns is the baseline resolution of the respective analyte.

Examples for suitable colums or appropriate chromatographic conditions are given in Annex A.

Whenever interferences are identified with a diode array detector or by measurement at a second wavelength, an alternative chromatographic condition shall be chosen.

6 Procedure

6.1 Preparation of the sample test solution

6.1.1 Clear liquid products (e.g. lemonades, cola, beverages)

Dilute 20 ml of the sample in a 100 ml volumetric flask with water. Filter the solution through a membrane filter of pore size 0,45 µm before injection.

6.1.2 Cloudy liquid products (e.g. juices, flavoured milk drinks)

Dilute 20 ml of the homogenized sample in a 100 ml volumetric flask with 50 ml water, add 2 ml of Carrez solution No 1 (4.7), mix and add 2 ml of Carrez solution No 2 (4.8). Shake vigorously and allow the solution to stand at room temperature for 10 min. Dilute to the mark with water. Filter through a fluted filter paper discarding the first 10 ml of the filtrate. Pass the filtrate through a membrane filter of pore size 0,45 µm before injection.

To make allowance for the volume of any precipitate, if the fat free insoluble matter in the sample volume (here 20 ml) exceeds approximately 3 g, it is advisable to centrifuge the clarified sample mixture for 10 min at at least 1400 *g* before filtering it quantitatively into the 100 ml volumetric flask. Wash the settled matter twice with water and centrifuge again, collect each of the supernatants in the 100 ml volumetric flask and then dilute the solution to the mark with water. This procedure may also be followed when the amount of insoluble matter is less than 3 g.

6.1.3 Jams, preserves, marmalades and related products (except fruit curds)

Weigh, to the nearest 1 mg, about 20 g of homogenized sample into a 100 ml volumetric flask. Add about 60 ml of water and place the flask in an ultrasonic bath at 40 °C for 20 min. The temperature shall not exceed 40 °C since aspartame can be degraded.

Cool the solution to room temperature. Add 2 ml of Carrez solution No 1 (4.7), mix and than add 2 ml of Carrez solution No 2 (4.8). Shake vigorously and allow the solution to stand at room temperature for 10 min. Dilute to the mark with water. Filter the solution through a fluted filter paper discarding the first 10 ml of the filtrate. Pass the filtrate through a membrane filter of pore size 0,45 µm before injection.

To make allowance for the volume of any precipitate, if the fat free insoluble matter in the initial sample mass exceeds approximately 3 g, it is advisable to centrifuge the clarified sample mixture for 10 min at at least 1400 *g* before filtering it quantitatively into the 100 ml volumetric flask. Wash the settled matter twice with water and centrifuge again, collect each of the supernatants in the 100 ml volumetric flask and then dilute the solution to the mark with water. This procedure may also be followed when the amount of insoluble matter is less than 3 g.

6.1.4 Semi solid and solid products (e.g. curd cheese desserts, yoghurt products, delicatessen salads except custard powder)

Weigh, to the nearest 1 mg, about 10 g to 20 g of the thoroughly homogenized sample into a 100 ml volumetric flask. Add about 50 ml of water and place the volumetric flask in the ultrasonic bath at 40 °C for 20 min. The temperature shall not exceed 40 °C since aspartame can be degraded.

Cool the solution to room temperature. Add 2 ml of Carrez solution No 1 (4.7), mix, add 2 ml of Carrez solution No 2 (4.8). Shake vigorously and allow the solution to stand at room temperature for 10 min. Dilute to the mark with water. Filter through a fluted filter paper discarding the first 10 ml of the filtrate.

In the case of very complex matrices, additional purification using the solid phase extraction column (5.13) may be necessary to protect the separating column, since colourings, flavourings and fat cannot be separated by Carrez clarification. In this case, add 2 ml of the clarified filtrate to the cartridge, previously activated with 3 ml of methanol (4.2) and 20 ml of water and elute with about 20 ml of mobile phase (4.11). Pass the filtrate through a membrane filter of pore size 0,45 µm before injection.

To make allowance for the volume of any precipitate, if the fat free insoluble matter in the initial sample mass exceeds approximately 3 g, it is advisable to centrifuge the clarified sample mixture for 10 min at at least 1400 *g* before filtering it quantitatively into the 100 ml volumetric flask. Wash the settled matter twice with water and centrifuge again, collect each of the supernatants in the 100 ml volumetric flask and then dilute the solution to the mark with water. This procedure may also be followed when the amount of insoluble matter is less than 3 g.

6.1.5 Custard powder

Weigh, to the nearest 1 mg, about 10 g of the sample into a 500 ml volumetric flask. Add about 400 ml of water and proceed as described above, i.e. add 6 ml of Carrez solution No 1 (4.7), mix, add 6 ml of Carrez solution No 2 (4.8) for clarification.

To make allowance for the volume of any precipitate, if the fat free insoluble matter in the initial sample mass exceeds approximately 3 g, it is advisable to centrifuge the clarified sample mixture for 10 min at at least 1400 *g* before filtering it quantitatively into the 500 ml volumetric flask. Wash the settled matter twice with water and centrifuge again, collect each of the supernatants in the 500 ml volumetric flask and then dilute the solution to the mark with water. This procedure may also be followed when the amount of insoluble matter is less than 3 g.

6.2 Identification

Identify the intense sweeteners in the sample solution by comparing the retention times of the analyte concerned in the sample solution with that of the standard substance, or by simultaneous injection of the standard solution and the

sample test solution, or by adding the standard solution to the sample test solution and recording an absorption curve in the relevant wavelength range.

Inject equal volumes of the sample test and standard solutions. Intervals between successive injections of the standard solutions should not be less than 15 min. To minimize the risk that substances eluted from earlier injections will be confused with components from subsequent samples, successive injections of the sample test solutions should be made at intervals sufficiently long.

In case of possible interferences washing of the columns is recommended. A suitable mobile phase for washing would have the following composition: 50 parts per volume of mobile phase (4.11)+ 50 parts per volume of acetonitrile.

Suitable chromatographic conditions for identification are described in Annex A.

Specimen chromatograms are given for information in Annex B.

6.3 Determination

For the determination by the external standard method, integrate the peak areas or determine the peak heights and compare the results with the corresponding values for the standard substance with the nearest peak area/height, or use a calibration graph.

To prepare a calibration graph, inject a suitable amount of standard solutions of appropriate mass concentrations. Plot the peak heights or peak areas of the standard solutions against the corresponding mass concentrations in milligrams per litre. Check the linearity of the calibration graph.

Alternatively, the calibration may also be evaluated mathematically by the regression. Check the linearity of the regression graph.

Suitable chromatographic conditions for determination are described in Annex A.

7 Calculation

7.1 External standard method

Calculate the mass fraction, w, expressed in milligrams per kilogram, or the mass concentration, ρ, in milligrams per litre, of the intense sweetener concerned, using equation (1):

$$w \text{ or } \rho = \frac{A_1 \times V_1 \times m_1 \times F}{A_2 \times V_2 \times m_0} \times 1000 \qquad (1)$$

where:

A_1 is the peak area of the intense sweetener concerned obtained with the sample test solution;
A_2 is the peak area of the intense sweetener concerned obtained with the standard solution;
V_1 is the volume of the sample solution, in millilitres (here: 100 ml or 500 ml);
V_2 is the volume of the standard solution, in millilitres (here: 100 ml);
m_1 is the mass of the intense sweetener concerned in the standard solution (V_2), in milligrams;
m_0 is the mass of the test portion, in grams or millilitres;
F is the dilution factor for the purification method used (e.g. column clarification = 10; Carrez clarification = 1).

7.2 Calibration graph

Calculate the mass fraction, w, expressed in milligrams per kilogram, or the mass concentration, ρ, in milligrams per litre, of the intense sweetener concerned, using equation (2):

$$w \text{ or } \rho = \frac{C \times F \times V_1}{m_0} \qquad (2)$$

where:

C is the amount of the intense sweetener concerned in the sample test solution, read off from the calibration graph, in milligrams per litre or milligrams per kilogram;

F, V_1, m_0 see equation (1).

7.3 Expression of results

Report the result for sodium saccharin or saccharin as free imide, for acesulfame-K and for aspartame without a decimal place.

NOTE: The conversion factor from sodium saccharin to saccharin as free imide is 0,7593.

8 Precision

8.1 General

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

8.2 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 limit r in not more than 5 % of the cases.

The values for acesulfame-K are:

marzipan	$\bar{x}$ =	256,6 mg/kg	r =	52,0 mg/kg
fruit yogurt	$\bar{x}$ =	230,8 mg/kg	r =	21,8 mg/kg
orange juice beverage	$\bar{x}$ =	172 mg/l	r =	5,8 mg/l
jam	$\bar{x}$ =	60 mg/kg	r =	8 mg/kg
orange juice beverage	$\bar{x}$ =	370 mg/l	r =	30 mg/l
cola	$\bar{x}$ =	351 mg/l	r =	20 mg/l
cream	$\bar{x}$ =	316 mg/kg	r =	15 mg/kg
yogurt	$\bar{x}$ =	264 mg/kg	r =	35 mg/kg
orange juice	$\bar{x}$ =	24,3 mg/kg	r =	6 mg/kg

The values for aspartame are:

marzipan	$\bar{x}$ =	845,2 mg/kg	r =	41,2 mg/kg
fruit yogurt	$\bar{x}$ =	468 mg/kg	r =	29,9 mg/kg
orange juice beverage	$\bar{x}$ =	308 mg/l	r =	14,2 mg/l
cola	$\bar{x}$ =	270,7 mg/l	r =	10,7 mg/l
cola	$\bar{x}$ =	185 mg/l	r =	11 mg/l
orange flavoured beverage	$\bar{x}$ =	301 mg/l	r =	25 mg/l
jam	$\bar{x}$ =	26 mg/kg	r =	13 mg/kg
preparation for flan	$\bar{x}$ =	3100 mg/kg	r =	600 mg/kg

The values for sodium saccharin are:

marzipan	$\bar{x}$ =	228 mg/kg	r =	28,2 mg/kg
fruit yogurt	$\bar{x}$ =	116 mg/kg	r =	7,7 mg/kg
orange juice beverage	$\bar{x}$ =	50,8 mg/l	r =	3,4 mg/l
cola	$\bar{x}$ =	75 mg/l	r =	4 mg/l
jam	$\bar{x}$ =	60 mg/kg	r =	5 mg/kg
orange juice beverage	$\bar{x}$ =	82 mg/l	r =	6 mg/l
cola	$\bar{x}$ =	64,9 mg/l	r =	5 mg/l
cream	$\bar{x}$ =	68,4 mg/kg	r =	15 mg/kg
yogurt	$\bar{x}$ =	71,4 mg/kg	r =	25 mg/kg
orange juice	$\bar{x}$ =	16,1 mg/kg	r =	6 mg/kg

8.3 Reproducibility

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

The values for acesulfame-K are:

marzipan	$\bar{x}$ =	256,6 mg/kg	R =	79,6 mg/kg
fruit yogurt	$\bar{x}$ =	230,8 mg/kg	R =	64,7 mg/kg
orange juice beverage	$\bar{x}$ =	172 mg/l	R =	14,3 mg/l
jam	$\bar{x}$ =	60 mg/kg	R =	30 mg/kg
orange juice beverage	$\bar{x}$ =	370 mg/l	R =	66 mg/l
cola	$\bar{x}$ =	351 mg/l	R =	55 mg/l
cream	$\bar{x}$ =	316 mg/kg	R =	138 mg/kg
yogurt	$\bar{x}$ =	264 mg/kg	R =	133 mg/kg
orange juice	$\bar{x}$ =	24,3 mg/kg	R =	34 mg/kg

The values for aspartame are:

marzipan	$\bar{x}$ =	845,2 mg/kg	R =	165,7 mg/kg
fruit yogourt	$\bar{x}$ =	468 mg/kg	R =	108,6 mg/kg
orange juice beverage	$\bar{x}$ =	308 mg/l	R =	104,2 mg/l
cola	$\bar{x}$ =	270,7 mg/l	R =	41,5 mg/l
cola	$\bar{x}$ =	185 mg/l	R =	38 mg/l
orange flavoured beverage	$\bar{x}$ =	301 mg/l	R =	88 mg/l
jam	$\bar{x}$ =	26 mg/kg	R =	20 mg/kg
preparation for flan	$\bar{x}$ =	3100 mg/kg	R =	2300 mg/kg