SLOVENSKI STANDARD
SIST-TS CEN/TS 15606:2009

01-december-2009

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

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

Lebensmittel - Bestimmung von Acesulfam-K, Aspartam, Neohesperidin-Dihydrochalcon und Saccharin - Hochleistungs-flüssigchromatographisches Verfahren

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

Ta slovenski standard je istoveten z: **CEN/TS 15606:2009**

ICS:

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

SIST-TS CEN/TS 15606:2009 **en,fr,de**

TECHNICAL SPECIFICATION

SPÉCIFICATION TECHNIQUE

TECHNISCHE SPEZIFIKATION

CEN/TS 15606

September 2009

ICS 67.050

English Version

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

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

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

This Technical Specification (CEN/TS) was approved by CEN on 20 June 2009 for provisional application.

The period of validity of this CEN/TS is limited initially to three years. After two years the members of CEN will be requested to submit their comments, particularly on the question whether the CEN/TS can be converted into a European Standard.

CEN members are required to announce the existence of this CEN/TS in the same way as for an EN and to make the CEN/TS available promptly at national level in an appropriate form. It is permissible to keep conflicting national standards in force (in parallel to the CEN/TS) until the final decision about the possible conversion of the CEN/TS into an EN is reached.

CEN members are the national standards bodies of Austria, Belgium, Bulgaria, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Iceland, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, Netherlands, Norway, Poland, Portugal, Romania, Slovakia, Slovenia, Spain, Sweden, Switzerland and United Kingdom.

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

Management Centre: Avenue Marnix 17, B-1000 Brussels

Ref. No. CEN/TS 15606:2009: E

Contents

Page

Foreword

This document (CEN/TS 15606:2009) has been prepared by Technical Committee CEN/TC 275 “Food analysis - Horizontal methods”, the secretariat of which is held by DIN.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. CEN [and/or CENELEC] shall not be held responsible for identifying any or all such patent rights.

According to the CEN/CENELEC Internal Regulations, the national standards organizations of the following countries are bound to announce this Technical Specification: Austria, Belgium, Bulgaria, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Iceland, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, Netherlands, Norway, Poland, Portugal, Romania, Slovakia, Slovenia, Spain, Sweden, Switzerland and the United Kingdom.

1 Scope

This Technical Specification (CEN/TS 15606:2009) specifies a high performance liquid chromatographic (HPLC) method with UV-detection for the determination of acesulfame-K, aspartame, neohesperidine-dihydrochalcone and saccharin in foodstuffs. The method has been fully validated [1] for the dialysis procedure through collaborative trial (see 8.2, 8.3 and Annex C), according to the IUPAC Harmonised Protocol [2], on the following analyte matrix combinations:

— acesulfame-K (from 86 mg/l to 331 mg/l) and aspartame (from 97 mg/kg to 610 mg/l)in water-based drink, fruit-based drink, cheesecake with biscuit base, canned soup and instant chocolate drink

— saccharin (from 70 mg/l to 97 mg/kg) in water-based drink, fruit-based drink, cheesecake with biscuit base and canned soup;

— neohesperidine-dihydrochalcone (from 27 mg/l to 57 mg/kg)in water-based drink, fruit-based drink and canned soup.

2 Normative references

The following referenced documents are indispensable for the application of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) 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 with Carrez reagents. Alternatively, solid samples are slurried in NaCl/HCl solution and the sweeteners extracted by dialysis using cellulose acetate (molecular weight cut-off of approximately 12 000). The purified extract is analysed by ion-pair reversed-phase HPLC with UV detection.

4 Reagents

4.1 General

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

4.2 Methanol, HPLC grade.

4.3 Hydrochloric acid, $w(HCl) \approx 36\ \%$ [1].

4.4 Phosphoric acid, $c(H_3PO_4) = 5$ mol/l [2].

---

[1] w is the mass fraction.

[2] c is the substance concentration.

4.5 Sodium hydroxide, $c(NaOH) = 5$ mol/l.

4.6 Saccharin, $w(C_7H_5NO_3S)$: 98,0 % to 101,0 %. or $w(C_7H_4NNaO_3S \times 2H_2O)$: 99,0 % to 101,0 %.

4.7 Neohesperidine-dihydrochalcone, $w(C_{28}H_{34}O_{15}) \geq 99{,}0$ %.

4.8 Acesulfame-K, $w(C_4H_4KNO_4S) \geq 99{,}0$ %.

4.9 Aspartame, $w(C_{14}H_{18}N_2O_5) \geq 98$ %.

4.10 Sodium chloride

4.11 Tetrapropylammonium hydrogensulphate (TPrA-HSO_4)

4.12 HPLC mobile phases

4.12.1 Mobile phase 1 (5 % methanol in water)

Pipette (5.4) 50 ml of methanol (4.2) into a 1 l volumetric flask (5.2). Make up to the mark with water. Add 0,24 g ± 0,01 g of TPrA-HSO_4 (4.11) and shake. Adjust to pH 3,5 with drop-wise addition of either 5 mol/l sodium hydroxide (4.5) or 5 mol/l phosphoric acid (4.4).

4.12.2 Mobile phase 2 (75 % methanol in water)

Transfer 750 ml methanol (4.2) from a measuring cylinder into a 1 l volumetric flask (5.2) and make up to the mark with water. Add 0,24 g ± 0,01 g TPrA-HSO_4 (4.11) and shake. Adjust to pH 3,5 with drop-wise addition of either a 5 mol/l sodium hydroxide (4.5) or 5 mol/l phosphoric acid (4.4).

4.12.3 Isocratic elution mobile phase (15 % methanol in water)

Transfer 150 ml of methanol (4.2) from a measuring cylinder into a 1 l volumetric flask (5.2) and make up to the mark with water. Add 0,24 g ± 0,01 g of TPrA-HSO_4 (4.11) and shake. Adjust to pH 3,5 with drop-wise addition of either 5 mol/l of sodium hydroxide (4.5) or 5 mol/l of phosphoric acid (4.4).

Filter (5.5) the solutions and sonicate (5.9) for 30 min.

4.13 Dialysing solution, 1,71 mol/l NaCl and 0,01 mol/l HCl, optional.

Dissolve 100 g of NaCl (4.10) and 1 ml of concentrated HCl (4.3) to 1 l in a volumetric flask (5.2) with water.

4.14 Dialysate solution, 0,01 mol/l HCl, optional.

Dissolve 1 ml of concentrated HCl (4.3) to 1 l in a volumetric flask (5.2) with water.

4.15 Stock solutions of acesulfame-K, aspartame, saccharin and neohesperidine-dihydrochalcone

Prepare 1 000 mg/l solutions of acesulfame-K (4.8) and aspartame (4.9) in water. Prepare a solution of saccharin (4.6) equivalent to 1 000 mg/l free imide (e.g. 1 126 mg/l of anhydrous sodium salt) in water and a 1 000 mg/l solution of neohesperidine-dihydrochalcone (4.7) in a mixture of 50 % methanol (4.2) and 50 % water.

4.16 Mixed calibration solutions containing acesulfame-K, aspartame, saccharin and neohesperidine-dihydrochalcone

From the stock solutions (4.15) prepare a series of mixed standards containing the sweeteners at concentrations equivalent to a maximum level of 150 % of the appropriate limit (for example, those given in Commission Directives 94/35/EC and 96/83/EC, as amended) whilst taking the dilution steps within the procedure into account.

EXAMPLE Calibration standards for measurement of sweeteners in a dairy-based dessert.

The present EU limits for the four sweeteners in dairy-based desserts are:

- acesulfame-K 350 mg/kg;
- saccharin 100 mg/kg (free imide);
- aspartame 1 000 mg/kg;
- neohesperidine dihydrochalcone 50 mg/kg.

150 % of the limits are:

- acesulfame K 525 mg/kg;
- saccharin 150 mg/kg (free imide);
- aspartame 1 500 mg/kg;
- neohesperidine-dihydrochalcone 75 mg/kg.

Sweeteners from 20 g of sample are extracted in 200 ml of dialysate (10:1 dilution). Thus, calibration should be carried out for extracts containing up to the following concentrations of sweetener:

- acesulfame-K 50 mg/l;
- saccharin 15 mg/l (free imide);
- aspartame 150 mg/l;
- neohesperidine-dihydrochalcone 7 mg/l.

For a seven point calibration, take the volumes as given in Table 1 from the 1 000 mg/l stock solutions (4.15) and make up to 50 ml with water in volumetric flasks (5.2).

Table 1 — Preparation of the calibration graph

	Acesulfame-K ml	**Saccharin** µl	**Aspartame** ml	**Neohesperidine-dihydrochalcone** µl
1	0,25	75	0,75	50
2	0,50	150	1,50	100
3	0,75	225	2,25	150
4	1,00	300	3,00	200
5	1,50	450	4,50	250
6	2,00	600	6,00	300
7	2,50	750	7,50	350

Table 2 details the concentration of sweeteners in each calibration standard following dilution:

Table 2 — Concentration of sweeteners in each calibration solution

	Acesulfame-K mg/l	**Saccharin (free imide)** mg/l	**Aspartame** mg/l	**Neohesperidine-dihydrochalcone** mg/l
1	5	1,5	15	1
2	10	3	30	2
3	15	4,5	45	3
4	20	6	60	4
5	30	9	90	5
6	40	12	120	6
7	50	15	150	7

4.17 Blank and fortified samples for determination of recovery

If the sample preparation is carried out by dialysis, a homogenised sample of the same type as the test sample should be prepared. Divide the sample into two portions. Add a known quantity of sweeteners to one portion (gravimetrically as solid or volumetrically in solution as appropriate).

4.18 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.

4.19 Carrez solution No 2

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

5 Apparatus and equipment

5.1 General

Usual laboratory equipment and glassware. The following items are also required.

5.2 Volumetric flasks, e.g. 50 ml and smaller.

5.3 HPLC vials, clear, 2 ml.

5.4 Pipette, 50 ml and smaller.

5.5 Mobile phase glass filter equipment

5.6 Screw cap bottles, 250 ml.

5.7 Glass funnels

5.8 Homogeniser, Ultra Turrax ® or equivalent.

5.9 Ultrasonic bath

5.10 Magnetic stirrer, multi-place preferred (or roller mixer, or some other means of mixing the dialysate).

5.11 Mini-magnets

5.12 Membrane syringe filters, 13 mm, 0,45 µm (PVDF or equivalent).

5.13 Mobile phase filters, diameter 4,8 cm, pore size 0,45 µm.

5.14 Dialysis tubing, cellulose acetate, planar width 4,8 cm; molecular weight cut-off approximately 12 000, optional.

5.15 pH meter

5.16 High performance liquid chromatograph, equipped with an analytical column (5.17), a pump system (gradient capability), an autosampler, an ultraviolet (UV) detector (capable of operating at a wavelength of 220 nm and 280 nm, preferably a diode array detector) and equipped with a recorder and/or integrator which allows the measurement of peak heights and peak areas.

5.17 HPLC-reversed phase column, e.g. ODS3 10 µm; 250 mm x 3,2 mm or equivalent (10 µm particle size 250 mm x 3,2 mm).

NOTE Column dimensions other than those specified in this Technical Specification can be used (e.g. of diameter 3,0 mm to 4,6 mm, length 100 mm to 250 mm). Separation parameters (flow, injection volume) should be adapted to guarantee equivalent results.

5.18 HPLC System suitability

Sweeteners acesulfame K, saccharin, aspartame and neohesperidine-dihydrochalcone should be resolved (R_s = 1,3) from each other and from other components of the matrix. Two commonly encountered 'critical pairs' are (a) acesulfame-K and theobromine; and (b) aspartame and caffeine.

6 Procedure

6.1 Sample preparation without dialysis

6.1.1 Clear liquid products such as lemonades, cola or 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 such as juices or flavoured milk drinks

Dilute 20 ml of the homogenised sample in a 100 ml volumetric flask with 50 ml water, add 2 ml of Carrez solution No 1 (4.18), mix and add 2 ml of Carrez solution No 2 (4.19). 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 injecting the sample test solution into the HPLC system.

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 1 400 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 homogenised 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 (4.18), mix and then add 2 ml of Carrez solution No 2 (4.19). 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 injecting the sample test solution into the HPLC system.

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 a centrifugal acceleration of at least 1 400 *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 such as curd cheese desserts, yoghurt products or delicacy 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 (4.18), mix, add 2 ml of Carrez solution No 2 (4.19). 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 (e.g. C18-SPE-Cartridges) 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 (4.12). Pass the filtrate through a membrane filter of pore size 0,45 µm before injecting the sample test solution into the HPLC system.

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 1 400 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 (4.18), mix, add 6 ml of Carrez solution No 2 (4.19) 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 1 400 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.