SLOVENSKI STANDARD
SIST EN 15911:2011

01-marec-2011

Živila - Simultano določevanje devetih sladil z metodo tekočinske kromatografije visoke ločljivosti in z detekcijo disperzije svetlobe (ELS)

Foodstuffs - Simultaneous determination of nine sweeteners by high performance liquid chromatography and evaporative light scattering detection

Lebensmittel - Gleichzeitige Bestimmung von neun Süßungsmitteln mit Hochleistungs-Flüssigchromatographie und Verdampfungs-Lichtstreu-Detektion

Denrées alimentaires - Dosage simultané de neuf édulcorants par chromatographie liquide haute performance et détection à diffusion de lumière par évaporation

Ta slovenski standard je istoveten z: **EN 15911:2010**

ICS:

67.180.10 Sladkor in sladkorni izdelki Sugar and sugar products

SIST EN 15911:2011 **en,fr,de**

EUROPEAN STANDARD

NORME EUROPÉENNE

EUROPÄISCHE NORM

EN 15911

October 2010

ICS 67.180.10

English Version

Foodstuffs - Simultaneous determination of nine sweeteners by high performance liquid chromatography and evaporative light scattering detection

Denrées alimentaires - Détermination simultanée de neuf édulcorants par chromatographie liquide haute performance et détection à diffusion de lumière

Lebensmittel - Gleichzeitige Bestimmung von neun Süßungsmitteln mit Hochleistungs-Flüssigchromatographie und Verdampfungs-Lichtstreu-Detektion

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COMITÉ EUROPÉEN DE NORMALISATION
EUROPÄISCHES KOMITEE FÜR NORMUNG

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Foreword

This document (EN 15911:2010) 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of an identical text or by endorsement, at the latest by April 2011, and conflicting national standards shall be withdrawn at the latest by April 2011.

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1 Scope

This European Standard specifies a method for the simultaneous determination of nine sweeteners in beverages and canned or bottled fruits by high performance liquid chromatography (HPLC) with evaporative light scattering detection (HPLC-ELSD). This method has been validated in an interlaboratory study via the analysis of spiked samples on the following matrices:

— acesulfame-K (ACS-K) (from 38,3 mg/l to 383,5 mg/l) in beverages and (from 38,4 mg/kg to 391,3 mg/kg) in canned fruits;

— alitame (ALI) (from 31,1 mg/l to 114,5 mg/l) in beverages and (from 36 mg/kg to 175,2 mg/kg) in canned fruits;

— aspartame (ASP) (from 38,1 mg/l to 702 mg/l) in beverages and (from 37,2 mg/kg to 1 120,2 mg/kg) in canned fruits;

— cyclamic acid (CYC) (from 28,3 mg/l to 307,2 mg/l) in beverages and (from 27,5 mg/kg to 1 100,6 mg/kg) in canned fruits;

— dulcin (DUL) (from 55,0 mg/l to 115,1 mg/l) in beverages and (from 49,8 mg/kg to 172,6 mg/kg) in canned fruits;

— neotame (NEO) (from 37,6 mg/l to 115,3 mg/l) in beverages and (from 37,3 mg/kg to 173,7 mg/kg) in canned fruits;

— neohesperidine dihydrochalcone (NHDC) (from 31,4 mg/l to 59,3 mg/l) in beverages and (from 35,3 mg/kg to 59,3 mg/kg) in canned fruits;

— saccharin (SAC) (from 36,2 mg/l to 87,6 mg/l) in beverages and (from 44,3 mg/kg to 235,3 mg/kg) in canned fruits;

— sucralose (SCL) (from 36,8 mg/l to 346,8 mg/l) in beverages and (from 35,3 mg/kg to 462,4 mg/kg) in canned fruits.

For further information on the validation see Clause 8 and Annex C.

NOTE The method has been fully validated [1] through collaborative trial, according to the IUPAC Harmonised Protocol [2], on analyte-matrix combinations of all nine sweeteners in beverages and canned or bottled fruits.

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 3696:1995, *Water for analytical laboratory use — Specification and test methods (ISO 3696:1987)*

3 Principle

The procedure involves extraction of the nine sweeteners with a buffer solution, sample clean-up using solid-phase extraction cartridges followed by HPLC-ELSD analysis.

4 Reagents

During the analysis, unless otherwise stated, use only reagents of recognised analytical grade for HPLC analysis and water of at least grade 1 as defined in EN ISO 3696:1995. When preparing solutions, the purity of the substances shall be taken into account.

4.1 Acesulfame-K, with a mass fraction w of at least 99,0 %.

4.2 Alitame, $w \geq 99{,}0$ %.

4.3 Aspartame, $w \geq 99{,}0$ %.

4.4 Dulcin, for HPLC.

4.5 Neotame, $w \geq 99{,}0$ %.

4.6 Neohesperidine dihydrochalcone, $w \geq 95{,}0$ %.

4.7 Saccharin, sodium salt dihydrate, $w \geq 98{,}0$ %.

4.8 Sodium cyclamate, $w \geq 99{,}0$ %.

4.9 Sucralose, $w \geq 99{,}0$ %.

4.10 Formic acid, HCOOH for HPLC.

4.11 Triethylamine, $(C_2H_5)_3N$, $w \geq 99{,}5$ %.

4.12 Methanol, for HPLC.

4.13 Acetone, for HPLC.

4.14 Buffer solution (pH = 4,5).

Dissolve 4 ml of formic acid (4.10) in 5 l of water. Adjust to pH 4,5 with approximately 12,5 ml triethylamine (4.11).

4.15 HPLC mobile phase A, methanol/buffer solution/acetone 69:24:7 (v/v/v).

Mix 690 ml of methanol (4.12) with 240 ml of buffer solution (4.14) and with 70 ml of acetone (4.13). Degas by sonication for 10 min.

4.16 HPLC Mobile phase B, methanol/buffer solution/acetone 11:82:7 (v/v/v).

Mix 110 ml of methanol (4.12) with 820 ml of buffer solution (4.14) and with 70 ml of acetone (4.13). Degas by sonication for 10 min.

4.17 Mixed stock solution, containing ACS-K, ALI, ASP, CYC-Na, DUL, NEO, NHDC, SAC-Na and SCL; mass concentration ρ(sweetener i) = 30 µg/ml to 250 µg/ml.

Prepare a mixed stock solution of all nine sweeteners by weighing the given masses of the individual sweetener standards (Table 1) first into a 100 ml beaker and dissolving them in 50 ml of methanol/water (1:1) until complete dissolution. Then transfer the obtained solution quantitatively into a 500 ml volumetric flask and make up to the mark with the buffer solution (4.14). Mix thoroughly by sonication until complete dissolution.

Table 1 — Masses of individual standards for preparation of mixed stock solution

Standard	Mass weighed into 500 ml volumetric flask [c] mg	Final mass concentration of sweetener *i* in mixed stock standard µg/ml
Acesulfame-K (ACS-K)	45	90
Alitame (ALI)	25	50
Aspartame (ASP)	125	250
Sodium cyclamate (CYC-Na)	140 [a]	–
Cyclamic acid (CYC) (free acid)	–	249,42
Dulcin (DUL)	25	50
Neotame (NEO)	25	50
Neohesperidine dihydrochalcone (NHDC)	15	30
Saccharin, sodium salt dihydrate (SAC-Na·2H_2O)	35 [b]	–
Saccharin (SAC) (free imide)	–	53,17
Sucralose (SCL)	50	100

[a] Equivalent to 124,71 mg free cyclamic acid; conversion factor to calculate mass of free cyclamic acid = 0,890 8; $m_{CYC} = 0{,}890\ 8 \times m_{CYC\text{-}Na}$.

[b] Equivalent to 26,58 mg free saccharin; conversion factor to calculate mass of free saccharin = 0,759 5; $m_{SAC} = 0{,}759\ 5 \times m_{SAC\text{-}Na\cdot 2H2O}$.

[c] First weigh into 100 ml beaker, dissolve in 50 ml of a methanol:water (1:1) mixture and then transfer quantitatively into 500 ml volumetric flask.

NOTE In case of cyclamic acid and saccharin, their sodium salts are used, since they are either not available in free form or poorly soluble.

The final concentrations of the individual sweeteners in micrograms per millilitre in the mixed stock solution have to be calculated by using the actually weighed masses.

4.18 Standard solutions.

From the mixed stock solution (4.17) prepare a series of standard solutions containing the sweeteners at levels fitting appropriate limits, e.g. the highest concentration of the calibration shall be at least equivalent to 125 % of the given limits, such as those in Commission Directives [3], [4], [5] (see Table D.1), whilst taking the dilution steps within the procedure into account (see Table 2). For sweeteners not authorised by the current EU legislation (ALI, DUL and NEO) fictitious maximum usable dosages (MUD) are assumed at approximately 200 mg/l or 200 mg/kg.

The user of the standard has to check whether the limits in Table D.1 are still valid. If not, the mass concentration of the standard substance in the calibration solution shall be adjusted to meet the current MUDs.

NOTE The present procedure is simplified by preparing one calibration series for both food matrices. The described calibration series is fitted to canned fruits as the MUDs for canned fruits are in some cases higher than the MUDs for beverages. In case only the latter matrix is analysed the calibration series can be fitted to the MUDs of beverages.

Pipette the following volumes (see Table 2) from the mixed stock solution (4.17) into appropriate volumetric flasks (10 ml to 50 ml) and make up to the mark with buffer solution (4.14) and shake thoroughly.

Table 2 — Preparation of series of standard solutions

Calibration solution	Volume of volumetric flask ml	Volume taken from mixed stock solution (4.17) ml	Volume taken from buffer solution (4.14) ml
1[a]	10	10	0
2	10	8	2
3	10	6	4
4	10	4	6
5	10	2	8
6	25	3	22
7	50	3	47
8	50	1,5	48,5

[a] Undiluted mixed stock solution (4.17).

Table 3 details the concentration of sweetener *i* in each calibration standard following preparation described in Table 2.

If not all of the sweeteners covered by this standard are the subject of analysis in routine use of the method, when applied to a particular set of samples consideration may be given to reduce the levels of the calibration solutions used for those samples.

Table 3 — Concentration of sweetener *i* in the individual standard solutions

Sweetener	Calibration solution mg/ml							
	1	2	3	4	5	6	7	8
ACS-K	90,0	72,0	54,0	36,0	18,0	10,8	5,4	2,7 [a]
ALI	50,0	40,0	30,0	20,0	10,0	6,0	3,0 [a]	1,5 [a]
ASP	250,0	200,0	150,0	100,0	50,0	30,0	15,0	7,5
CYC	249,4	199,5	149,7	99,8	49,9	29,9	15,0	7,5
DUL	50,0	40,0	30,0	20,0	10,0	6,0 [a]	3,0 [a]	1,5 [a]
NEO	50,0	40,0	30,0	20,0	10,0	6,0	3,0 [a]	1,5 [a]
NHDC	30,0	24,0	18,0	12,0	6,0	3,6 [a]	1,8 [a]	0,9 [a]
SAC	53,2	42,5	31,9	21,3	10,6	6,4	3,2 [a]	1,6 [a]
SCL	100,0	80,0	60,0	40,0	20,0	12,0	6,0	3,0 [a]

[a] The concentration level might be below the limit of quantification (LOQ). If yes, the result obtained by HPLC analysis is not included in the construction of the calibration graph, e.g. in case of ACS-K a seven point calibration is performed, ignoring the result obtained for calibration solution 8.

5 Apparatus and equipment

Usual laboratory apparatus and, in particular, the following:

5.1 Common laboratory glassware, such as graduated cylinders, volumetric pipettes, glass beakers.

5.2 Analytical balance, capable of weighing to 0,01 mg.

5.3 Laboratory balance, capable of weighing to 0,01 g.

5.4 Positive displacement pipette, or equivalent, capable of delivering 1 ml to 10 ml (variable volume).

5.5 Volumetric flasks, of suitable capacity, e.g. 10 ml, 25 ml, 50 ml, 100 ml and 500 ml.

5.6 Centrifuge tubes, made of polypropylene, of suitable capacity, e.g. 50 ml.

5.7 Graduated test tubes, of suitable capacity, e.g. 5 ml.

5.8 Food blender, suitable for homogenisation of food samples.

5.9 Ultrasonic bath.

5.10 Centrifuge, capable of maintaining 4 000 min^{-1}.

5.11 SPE Vacuum system, or equivalent.

5.12 Equipment for solvent evaporation.

5.13 pH meter.

5.14 C_{18} SPE cartridges.

5.15 Analytical reverse phase column, fully end-capped, allowing sufficient separation of all nine sweeteners.

E.g. with:

— an RP C 18 stationary phase of 5 µm;

— a length of 250 mm;

— internal diameter of 3 mm.

5.16 HPLC system, equipped with a binary pump capable of maintaining a flow rate of 0,5 ml/min, preferably an automatic injection system, and an evaporative light scattering detector.

Other detection systems such as MS as substitute for ELSD or UV and DAD when substances do absorb in the UV region can also be used provided that the equivalent performance characteristics can be obtained.

5.17 Data acquisition and analysis software.

6 Procedure

6.1 General

Comminute the entire test sample to give a homogenous suspension (5.8). Liquid samples can be subjected directly to the extraction procedure.

6.2 Preparation of test sample

6.2.1 Step 1

Weigh approximately 5 g (M_1, recorded to two decimal places) of the homogenised test sample (6.1) into a volumetric flask of 50 ml (V_1). Make up to the mark with buffer solution (4.14), mix thoroughly by hand to obtain a homogeneous suspension and sonicate (5.9) for 15 min.

6.2.2 Step 2

Transfer the obtained suspension to a 50 ml centrifuge tube. Centrifuge at 4 000 min^{-1} for 10 min.

NOTE In case the test sample gives a clear solution (e.g. some beverages), this step can be ignored.

6.3 Solid phase extraction

6.3.1 Step 1

Condition the cartridges (5.14) by applying 3 ml of methanol (4.12) and let it pass through using a slight vacuum resulting in a flow rate of 1 ml/min to 2 ml/min. Make sure that a small portion of methanol remains above the sorbent bed (1 mm).

6.3.2 Step 2

Equilibrate the cartridges (5.14) by applying 2 ml of buffer solution (4.14) and let it pass through using a slight vacuum resulting in a flow rate of 1 ml/min to 2 ml/min. Make sure that a small portion of buffer solution remains above the sorbent bed (1 mm). Repeat the procedure two times.

6.3.3 Step 3

Load the cartridges (5.14) with 5 ml of sample extract (V_2 first loading), i.e. the supernatant from (6.2.2), and let it pass through using a slight vacuum resulting in a flow rate of 1 ml/min to 2 ml/min. Make sure that a small portion remains above the sorbent bed (1 mm). Repeat the procedure once more (V_2 in total 10 ml).

6.3.4 Step 4

Wash the cartridges (5.14) with 3 ml of buffer solution (4.14) and let it pass through using a slight vacuum resulting in a flow rate of 1 ml/min to 2 ml/min. Make sure that a small portion of buffer solution remains above the sorbent bed (1 mm).

6.3.5 Step 5

Elute the sweeteners from the cartridges (5.14) by applying 2 ml of methanol (4.12) and collecting the eluate in a graduated 5 ml test tube. Use a slight vacuum to obtain a flow rate of 1 ml/min. Make sure that a small portion of methanol remains above the sorbent bed (1 mm). Wait 10 min before applying a second portion of 2 ml of methanol and elute it subsequently to the same 5 ml test tube using the same vacuum conditions but this time letting the cartridges (5.14) run dry.

Avoid in all steps (6.2.1 to 6.3.5) that the sorbent bed runs dry with the only exception of the last step, i.e. second elution of analytes (6.3.5).

6.3.6 Step 6

Evaporate the solvent from the methanolic SPE extract to 3 ml under a stream of nitrogen at ambient temperature.

Temperatures above 40 °C have to be avoided, since aspartame can degrade.

6.3.7 Step 7

Fill the graduated test tube containing the SPE extract (6.3.6) up to the 5 ml mark with buffer solution (4.14) (V_3). Mix thoroughly and transfer the content into a suitable HPLC vial and analyse by HPLC.

6.4 HPLC conditions

Establish suitable HPLC conditions to meet the predefined performance criteria (6.5). The separation and quantification have proven to be satisfactory using the following experimental conditions and HPLC gradient conditions as outlined in Table 4:

- Column: see 5.15;
- Column temperature: ambient temperature;
- Injection volume: 10 µl;
- Mobile phase: see 4.15 and 4.16;
- Separation mode: gradient;
- Detector: evaporative light scattering detector (ELSD);
- ELSD drift tube temperature: 85 °C;
- ELSD nitrogen flow: 2,5 l/min;
- ELSD gain: 1;
- ELSD impactor: Off.

Table 4 — gradient analysis by HPLC, Flow rate 0,5 ml/min

Time min	Mobile phase A %	Mobile phase B %
0	0	100
4	0	100
11	53	47
23	100	0
24	100	0
26	0	100
36	0	100

NOTE The given detector parameters are applicable to the Alltech ELSD 2000ES system[1)]. Alternative ELSD systems and experimental conditions, used in an inter-laboratory study, are listed in Annex A. HPLC and ELSD operating conditions can be changed to obtain optimum separation.

6.5 System suitability test – Resolution of separation system

The details of the chromatographic procedure depend, among other factors, on equipment, type, age, and supplier of the column, sample size and detector. Different columns can be used, and injection volumes can be varied, if the requirements of the system suitability tests are met.

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