FOOD COMPOSITION AND ADDITIVES

Determination of Nine Intense Sweeteners in Foodstuffs by High-Performance Liquid Chromatography and Evaporative Light-Scattering Detection: Interlaboratory Study

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An interlaboratory trial was conducted to validate an analytical method based on high-performance liquid chromatographic analysis with evaporative light-scattering detection for the simultaneous determination of 9 intense sweeteners, i.e., acesulfame-K, alitame, aspartame, cyclamic acid, dulcin, neotame, neohesperidine dihydrochalcone, saccharin, and sucralose in carbonated and noncarbonated soft drinks and canned or bottled fruits. Seven laboratories participated in the validation study. The majority of the samples fortified with levels close to the limit of quantification had relative standard deviation for reproducibility (RSD_R) values <15%. In most cases, the recovery rates ranged between 90 and 105%, demonstrating satisfactory performance of the method. For samples fortified at levels comparable to the prescribed legal limits stipulated in the current European Union legislation, the method produces acceptably accurate, repeatable, and reproducible results. Trueness, expressed in terms of recovery rates, was demonstrated in most cases by values ranging from 90 to 108%. Comparability of results obtained by individual testing laboratories was good (RSD_R values <10%) for the majority of results. Moreover, HorRat values of <1.1 suggested good performance of the method for all sweeteners and matrixes tested.

Current legislation on food additives in the European Union (EU) is governed by Council Directive 89/107/EEC (1), which is based on the principle that only authorized additives may be used in the manufacture or preparation of foodstuffs. Sweeteners form an important class of food additives, used in an increasingly wide range of food products and beverages. Directive 94/35/EC (2), as amended by Directives 96/83/EC (3), 2003/115/EC (4), and 2006/52/EC (5), specifically deals with food additives used to impart a sweet taste to foodstuffs. These directives stipulate which sweeteners may be placed on the market for sale to consumers or for use in the production of foodstuffs. The European Food Safety Authority evaluates the safety of sweetners, then either authorize usage at a "quantum satis" level or a maximum usable dose (MUD) or denies authorization for use. Currently, 8 high-intensity (non-nutritive) sweeteners are included in EU legislation for use in foods, i.e., acesulfame-K (ACS-K), aspartame (ASP), aspartame-acesulfame (ASP-ACS) salt, cyclamate (CYC), saccharin (SAC), sucralose (SCL), neohesperidine dihydrochalcone (NHDC), and thaumatin. Some of them are synthetic (ACS-K, ASP, ASP-ACS salt, CYC, SAC, SCL), or semi-synthetic (NHDC), while thaumatin occurs naturally (6).

A requirement for proper implementation of existing legislation is the availability of robust quantitative analytical methods to measure levels of sweeteners in a broad range of food matrixes.

The determination of sweeteners has already prompted a great deal of research (7–28). Most of the methods have been developed for individual sweeteners. Relatively few methods have been described for their simultaneous quantification in a single run (29–37). Because most artificial sweeteners are commonly used in combinations, reliable methods that can cover their quantification in a single analysis are needed.

This paper presents the results of an interlaboratory study in which a newly developed high-performance liquid chromatographic method with evaporative light-scattering detection (HPLC-ELSD) for the simultaneous identification and quantification of 6 authorized sweeteners (ACS-K, ASP, CYC, NHDC, SAC, and SCL) and 3 sweeteners not authorized by current EU legislation [neotame (NEO), alitame (ALI), and dulcin (DUL)] in beverages and canned or bottled fruits (38), was ring-trialed to determine its interlaboratory performance. The procedure involves extraction of the 9 sweeteners with a buffer solution, sample cleanup using solid-phase extraction (SPE) cartridges followed by

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Table 1. Test samples used in the interlaboratory study and respective EU limits

	Beverages						Canned fruits					
	Fortified concn, mg/L						Fortified concn, mg/kg					
		Sample						Sample				
Sweetener	MUD[a], mg/L	1[b]	2[c]	3[d]	4[e]	5[f]	MUD[a], mg/kg	6[g]	7[h]	8[i]	9[j]	10[k]
ACS-K	350	0	42.1	282.5	354.2	421.7	350	0	36.5	265.6	338.8	410
ALI[l]	—	0	36.5	80.5	102.6	122.2	—	0	34.6	116.1	145.1	175.5
ASP	600	0	42	485	605	720.3	1000	0	37.3	752.1	967.8	1171.1
CYC	250	0	36.9	239	252.7	300.8	1000	0	32.2	752.6	968.8	1172.3
DUL[l]	—	0	60.7	81.3	101.8	121.1	—	0	50.2	114.3	145.7	176.3
NEO[l]	—	0	37.5	80.5	102.2	121.7	—	0	36.2	118.3	145.4	175.9
NHDC	30	0	36.7	40.2	50.7	60.4	50	0	33.4	37.5	48.9	59.1
SAC	80	0	40.3	65.2	80.9	96.3	200	0	38	150	194	234.8
SCL	300	0	38.9	251.8	302.6		400	0	34.6	313.1	388.2	469.7

[a] MUD = Maximum usable dose according to present EU limits (1–5).
[b] Energy drink, blank.
[c] Energy drink fortified at concentration level close to limits of quantitation (LOQ).
[d] Noncarbonated soft drink fortified at a concentration level of ca 80% of MUDs.
[e] Carbonated soft drink fortified at a concentration level of ca 100% of MUDs.
[f] Carbonated soft drink fortified at a concentration level of ca 120% of MUDs.
[g] Canned cocktail fruits, blank.
[h] Canned cocktail fruits fortified at concentration level close to the LOQ.
[i] Canned pears fortified at a concentration level of ca 75% of MUDs.
[j] Canned pears fortified at a concentration level of ca 100% of MUDs.
[k] Canned pears fortified at a concentration level of ca 115% of MUDs.
[l] Sweeteners not authorized by current EU legislation (1–5).

HPLC-ELSD analysis. The present method has the advantage that a single HPLC-ELSD analysis can yield several useful pieces of information to control correct labeling: (*1*) proving the absence of the 3 sweeteners not authorized by current EU legislation, i.e., ALI, DUL, and NEO; (*2*) proving the absence of the 6 authorized sweeteners, i.e., ACS-K, ASP, CYC, NHDC, SAC, and SCL, in food products where no sweeteners have been declared; (*3*) quantifying the amount of the 6 authorized sweeteners, i.e., ACS-K, ASP, CYC, NHDC, SAC, and SCL, in labeled food products and proving that their levels of addition are below the MUDs as laid down in current EU legislation (1–5).

This interlaboratory study, based on extensive in-house testing of the method (38), demonstrates the method's ability to assess compliance with labeling provisions and its suitability for rapid screening of large numbers of samples for the determination of sweeteners in beverages and canned fruits.

Validation Study

Test Samples

Energy drinks (sugar-sweetened), carbonated soft drinks (sugar-sweetened), soft drinks without carbon dioxide (sugar-sweetened), and canned fruits (cocktail fruits and pears, sugar-sweetened) were purchased in retail stores. Before analysis, each matrix was checked for the absence of the compounds under study to be used as blank samples and for the preparation of fortified test materials. The preparation of the individual test materials is described in detail in ref. 39. The study was designed to meet the requirements of current EU legislation (1–5). Hence, the analysis was adapted to fit the prescribed legal limits, resulting in sample compositions as given in Table 1. For sweeteners not authorized by current EU legislation (ALI, DUL, and NEO), fictitious MUDs were assumed at about 100 mg/L for beverages and about 150 mg/kg for canned fruits. Example chromatograms for test samples 1–5 are given in Figure 1.

Homogeneity

Homogeneity of the test samples was assessed by an internationally agreed procedure (40). From each test material, 6 samples (units) were taken at random from the filling sequence and each sample was split into 2 equal parts (unit subsample). The sweeteners were extracted from each unit subsample and randomly subjected to HPLC analysis using a fully end-capped reversed-phase HPLC column of 250 × 3 mm, 5 μm particle size (Purospher® Star RP-18) from Merck (Darmstadt, Germany). The tests were

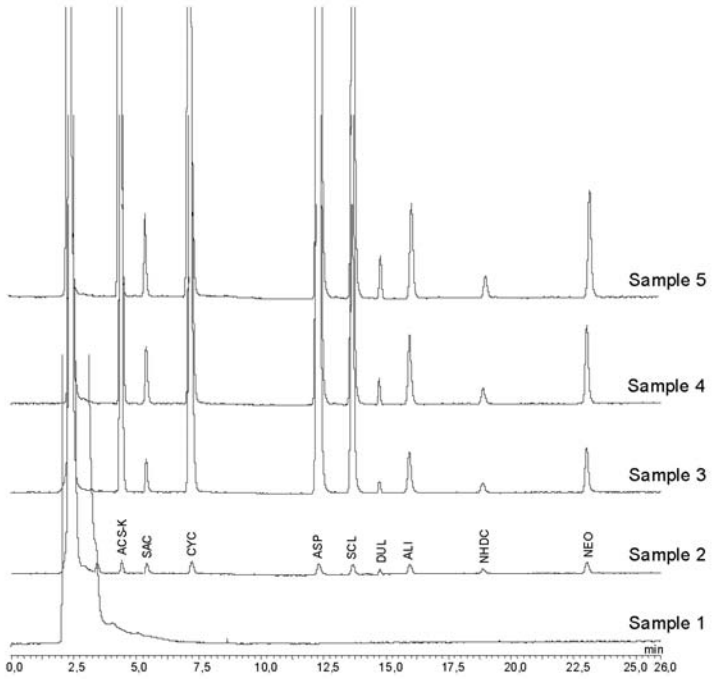

Figure 1. HPLC-ELSD separations of test samples 1–5 using a fully end-capped reversed-phase HPLC column (Purospher® Star RP-18).

performed under repeatability conditions, i.e., the same method on identical test items in the same laboratory by the same operator using the same equipment within a short time scale.

The within- and between-units standard deviations for the contents of ACS-K, ALI, ASP, CYC, DUL, NEO, NHDC, SAC, and SCL were calculated with a one-way analysis of variance (ANOVA) and by applying the *F*-test at the 95%

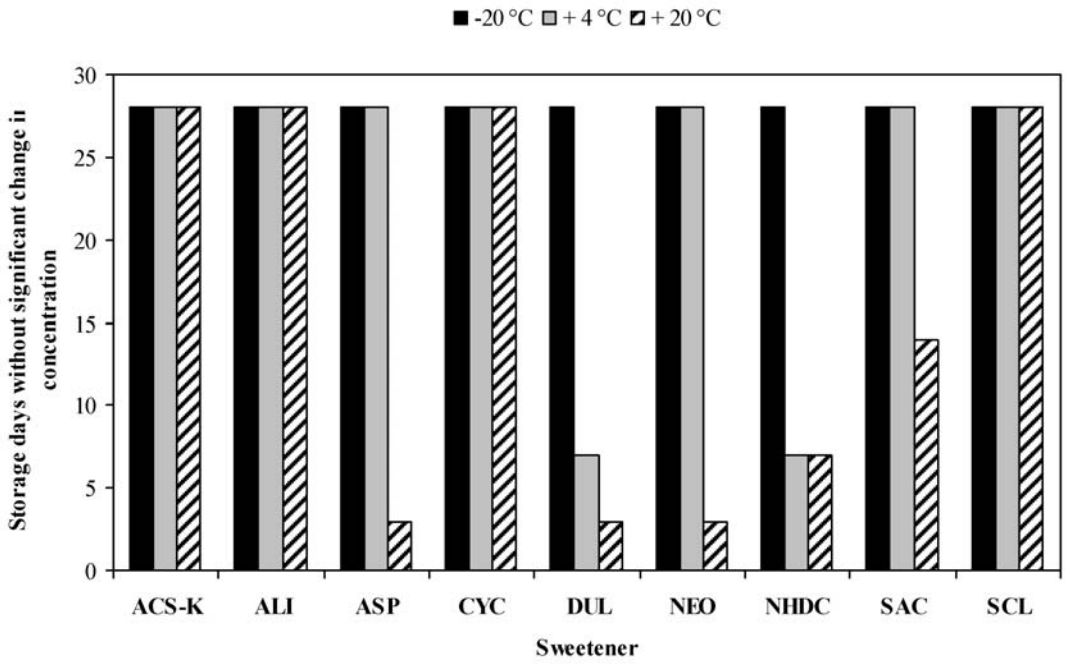

Figure 2. Results of stability study for matrix 1 (beverages).

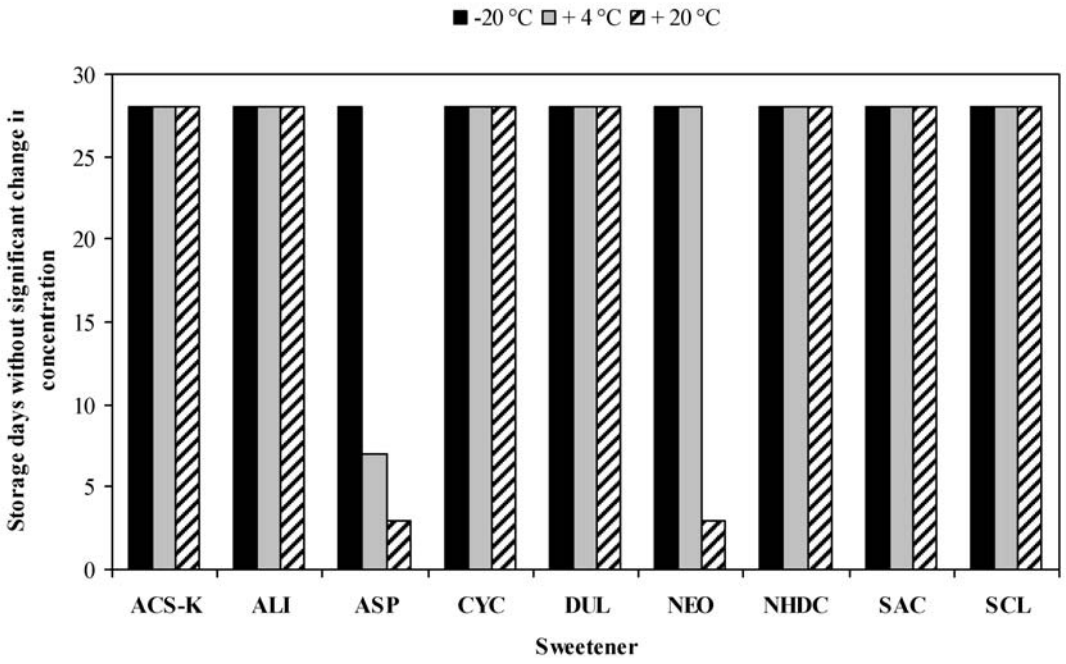

Figure 3. Results of stability study for matrix 2 (canned fruits).

confidence level. The statistical analysis confirmed the homogeneity of the test samples used as test materials for the validation study.

Stability Study

To determine proper storage and transport conditions for the individual sweeteners in the respective test materials, a stability study was performed using an isochronous study design (41). It is based on storing the samples at different temperatures for different time intervals; at the end of the study, all measurements are done simultaneously. The stability of the spiked test materials was tested at –20, 4, and 20°C for 3 days, and 1, 2, and 4 weeks. A reference sample was kept at –70°C. At the onset of the study, all samples were stored at –70°C, at which their stability was supposed to be good. For each storage temperature studied, samples were moved from the reference temperature to the studied storage temperature at different times. At the defined end time, samples were immediately analyzed along with the reference samples, which were kept for the entire study at –70°C. The results of the reference samples were used as starting values. The storage days, for which no changes in the absolute concentration were observed, are given for the individual matrixes and storage temperatures in Figures 2 and 3.

In beverages, 6 sweeteners were stable up to 4 weeks, independent of the storage temperature. Only ASP, NEO, and NHDC were less stable compounds, i.e., ASP degraded at 20°C after only 3 days, DUL was stable up to 7 days at 4°C and up to 3 days at 20°C, and NEO showed a fast degradation at 20°C, whereas it was stable up to 4 weeks at 4°C and –20°C.

In canned fruits, almost all sweeteners were stable up to 4 weeks, independent of the storage temperature. Only NEO and ASP were less stable compounds, i.e., ASP degraded at 4°C after 7 days and at 20°C after only 3 days. NEO showed a fast degradation at 20°C, whereas it was stable up to 7 weeks at 4°C and –20°C.

Consequently, after preparation, all test samples were refrigerated at –70°C. All test samples were packed into insulated boxes, along with cooling bags, and sent by courier mail to the participants. Upon receipt of the test samples (<24 h in all cases), the participants were requested to store the test samples immediately in a freezer (–20°C) until use. Samples had to be analyzed within 3 weeks, ensuring proper stability of all compounds.

Design of the Validation Study

Ten laboratories from 5 countries, with experience in HPLC-ELSD analysis, were contacted to participate in the study.

A pretrial was organized to allow the individual laboratories to implement the proposed method. They received a training set of 2 test samples with known concentrations of all 9 sweeteners, i.e., one beverage with a low concentration and one with a high concentration of all 9 sweeteners, which could be used for optimization purposes and demonstration of a correctly functioning chromatographic system. Out of the 10 laboratories contacted, 8 submitted results; however, the data set of one laboratory had to be excluded from the technical and statistical evaluation of the study results because the data set was incomplete and not acquired following the method protocol and study guidelines.

For the interlaboratory study the participants received a shipment containing 20 containers of test samples, i.e., every sample provided as blind duplicate, labeled randomly, and each containing a test portion of approximately 10 g. Additionally, the participants were provided with a set of crystalline reference substances for calibration purposes.

Table 2. Method parameters varied by individual laboratories in the interlaboratory study

	Laboratory						
Parameter	1	2	3	4	5	6	7
SPE characteristics							
Brand name	Chromabond	Chromabond	Bakerbond spe	Chromabond	Chromabond	Chromabond	Chromabond
Stationary phase	C18 endcapped	C18 endcapped	C18	C18 endcapped	C18 endcapped	C18 endcapped	C18 endcapped
Capacity, mL/mg	6/1000	6/1000	3/500	6/1000	6/1000	6/1000	6/1000
HPLC apparatus							
Manufacturer	Agilent	Jasco	Shimadzu	Dionex	Jasco	Varian	Dionex
Column characteristics							
Brand name	Purospher Star	Purospher Star	Purospher Star	Nucleodur	Purospher Star	Purospher Star	Purospher Star
Stationary phase	RP-C18 endcapped	RP-C18 endcapped	RP-C18 endcapped	C-18ec Pyramid	RP-C18 endcapped	RP-C18 endcapped	RP-C18 endcapped
Length, mm	250	250	250	250	250	250	250
id, mm	3	3	3	3	3	3	3
Particle size, μm	5	5	5	5	5	5	5
HPLC mobile phase							
Mobile phase A composition, (v/v/v)	Methanol–buffer solution–acetone (69 + 24 + 7)	Methanol–buffer solution–acetone (69 + 24 + 7)	Methanol–buffer solution–acetone (69 + 24 + 7)	Methanol–buffer solution–acetone (69 + 24 + 7)	Methanol–buffer solution–acetone (69 + 24 + 7)	Methanol–buffer solution–acetone (69 + 24 + 7)	Methanol–buffer solution–acetone (69 + 24 + 7)
Mobile phase B composition, (v/v/v)	Methanol–buffer solution–acetone (11 + 82 + 7)	Methanol–buffer solution–acetone (11 + 82 + 7)	Methanol–buffer solution–acetone (11 + 82 + 7)	Methanol–buffer solution–acetone (11 + 82 + 7)	Methanol–buffer solution–acetone (11 + 82 + 7)	Methanol–buffer solution–acetone (11 + 82 + 7)	Methanol–buffer solution–acetone (11 + 82 + 7)
Flow rate, mL/min	0.5	0.5	0.5	0.5	0.6	0.55	0.5
HPLC separation mode							
Gradient program, min - mobile phase A %	0 min - 100% A; 4 min - 100% A; 11 min - 47% A; 23 min - 2% A; 24 min - 2% A; 26 min - 100% A	0 min - 5% A; 10 min - 60% A; 30 min - 95% A; 31 min - 95% A; 32 min - 5% A; 45 min - 5% A	0 min - 0% A; 15 min - 100% A; 18 min - 100% A; 20 min - 0% A; 35 min - 0% A	0 min - 0% A; 4 min - 0% A; 11 min - 53% A; 23 min - 100% A; 24 min - 100% A; 26 min - 0% A; 36 min - 0% A	0 min - 0% A; 4 min - 0% A; 11 min - 53% A; 21 min - 100% A; 23 min - 100% A; 25 min - 0% A; 31 min - 0% A	0 min - 0% A; 4 min - 0% A; 11 min - 53% A; 23 min - 100% A; 24 min - 100% A; 26 min - 0% A; 36 min - 0% A	0 min - 0% A; 4 min - 0% A; 11 min - 53% A; 23 min - 100% A; 24 min - 100% A; 26 min - 0% A; 36 min - 0% A
HPLC injection mode							
Manual/automatic	Automatic	Automatic	Automatic	Automatic	Automatic	Automatic	Automatic
ELSD conditions							
Manufacturer	Sedex 85, Sedere	Varex MKIII, Alltech	ELSD-LT II, Shimadzu	Sedex, Sedere	Sedex 75, Sedere	ELSD 2000ES, Alltech	ELSD 2000ES, Alltech
Drift tube temp., °C	40	90	50	43	45	85	85
Nitrogen/air, pressure/flow	Nitrogen 3.2 bar	Nitrogen 2.5 L/min	Air 3 bar	Nitrogen 3.5 bar	Air 2.5 bar	Nitrogen 2.5 L/min	Nitrogen 2.5 L/min
Gain	7	1	9	10	2	1	1

Table 3. Preparation of calibration standard solutions

	Preparation of mixed stock standard solution, μg/mL										
Mixed stock standard	ACS-K	ALI	ASP	CYC-Na	CYC[a]	DUL	NEO	NHDC	SAC-Na·2H$_2$O	SAC[a]	SCL
Mass (mg) weighed into 500 mL volumetric flask[b]	45	25	125	140[c]	–	25	25	15	35[d]	—	50
Final concn of sweetener *i* in mixed stock standard, μg/mL	90	50	250	—	249.42	50	50	30	53.17	100	

Preparation of individual calibration standard solutions			
Calibration standard solution	Volume volumetric flask, mL	Volume mixed stock standard, mL	Volume buffer solution, mL
1	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

Concn of sweetener *i* (μg/mL) in the individual calibration standard solutions									
Calibration standard solution	ACS-K	ALI	ASP	CYC	DUL	NEO	NDHC	SAC	SCL
1	90	50	250	249.4	50	50	30	53.2	100
2	72	40	200	199.5	40	40	24	42.5	80
3	54	30	150	149.7	30	30	18	31.9	60
4	36	20	100	99.8	20	20	12	21.3	40
5	18	10	50	49.9	10	10	6	10.6	20
6	10.8	6	30	29.9	6.0[e]	6	3.6[e]	6.4	12
7	5.4	3.0[e]	15	15	3.0[e]	3.0[e]	1.8[e]	3.2[e]	6
8	2.7[e]	1.5[e]	7.5	7.5	1.5[e]	1.5[e]	0.9[e]	1.6[e]	3.0[e]

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

[b] First weigh into 100 mL volumetric flask, dissolve in approximately 50 mL methanol–water (1 + 1) mixture, and then transfer quantitatively into 500 mL volumetric flask.

[c] Equivalent to 124.71 mg free cyclamic acid; conversion factor to calculate mass of free cyclamic acid = 0.8908; $m_{CYC} = 0.8908 \times m_{CYC\text{-}Na}$.

[d] Equivalent to 26.58 mg free saccharin; conversion factor to calculate mass of free saccharin = 0.7595; $m_{SAC} = 0.7595 \times m_{SAC\text{-}Na\cdot 2H2O}$.

[e] The concentration level might be below the limit of quantitation. 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 7-point calibration is performed, ignoring the result obtained for calibration solution 8. The results can differ from laboratory to laboratory.

Participants were also provided with a method protocol, collaborative study guidelines, and an electronic evaluation and reporting sheet (MS Excel® format). The 10 test samples, which were provided as blind duplicates, had to be analyzed once (in total 20 analyses) under conditions described in the provided method protocol. Calibration graphs of the individual sweeteners had to be determined as described in the method protocol before analysis of the first test sample and after analysis of the last test sample.

The collaborators were requested to follow the method protocol exactly. However, the HPLC-ELSD method gave some freedom to choose procedural parameters (e.g., LC apparatus, ELSD apparatus, column type, etc.) within certain limits. A brief outline of the HPLC-ELSD methods used by the participants is given in Table 2. The applied methods differed with respect to the SPE cartridges (Chromabond®, Macherey-Nagel, Düren, Germany; and Bakerbond®, Krackeler Scientific, Inc., Albany, NY), the LC columns (Purospher Star, Merck; and Nucleodur®, Macherey-Nagel), the LC gradients, and the ELSD brands, along with the drift tube temperature, gain, and nitrogen or air flow.

METHOD

Scope

The method is specified for the determination of 9 intense sweeteners, ACS-K, ALI, ASP, CYC, DUL, NHDC, NEO, SAC, and SCL, in beverages and canned or bottled fruits.

Principle

Sweeteners are extracted from a known quantity of test sample with a buffer solution. The extract is cleaned up by passing through a SPE cartridge, the analytes are eluted with methanol, brought to a defined volume with buffer solution, and analyzed by HPLC-ELSD.

Reagents

Use only reagents of recognized analytical grade, unless otherwise stated.

(**a**) *ACS-K*.—With a mass fraction of at least 99.0% (Fluka, Hannover, Germany).

(**b**) *ALI*.—With a mass fraction of at least 99.0% (Finechemie Co., Chongqing, People's Republic of China).

(**c**) *ASP*.—With a mass fraction of at least 99.0% (Supelco, Taufenkirchen, Germany).

(**d**) *DUL*.—With a mass fraction of at least 95.0%.

(**e**) *NEO*.—With a mass fraction of at least 99.0% (LGC Promochem, Teddington, UK).

(**f**) *NHDC*.—With a mass fraction of at least 95.0% (Sigma-Aldrich, Steinheim, Germany).

(**g**) *SAC sodium salt dihydrate*.—With a mass fraction of at least 99.0% (Sigma-Aldrich).

(**h**) *Sodium-CYC*.—With a mass fraction of at least 99.0% (Supelco).

(**i**) *SCL*.—With a mass fraction of at least 99.0% (LGC Promochem).

(**j**) *Formic acid*.—Purity >98%.

(**k**) *Water*.—LC grade.

(**l**) *Triethylamine*.—Purity >99.5%.

(**m**) *Methanol*.—LC grade.

(**n**) *Acetone*.—LC grade.

(**o**) *Buffer solution (pH 4.5)*.—Dissolve 4 mL formic acid in 5 L water. Adjust to pH 4.5 with ca 12.5 mL triethylamine.

(**p**) *LC mobile phase A*.—Methanol–buffer solution–acetone (69 + 24 + 7, v/v/v). Mix 690 mL methanol with 240 mL buffer solution and 70 mL acetone. Degas by sonication for 10 min.

(**q**) *LC mobile phase B*.—Methanol–buffer solution–acetone (11 + 82 + 7, v/v/v). Mix 110 mL methanol with 820 mL buffer solution and 70 mL acetone. Degas by sonication for 10 min.

(**r**) *Mixed stock standard solution*.—Prepare a mixed stock standard solution of all 9 sweeteners (ACS-K, ALI, ASP, CYC-Na, DUL, NEO, NHDC, SAC-Na, and SCL) by weighing the given masses of the individual sweetener standards (Table 3) into a 100 mL beaker and dissolving them in ca 50 mL methanol–water (1 + 1). Transfer the obtained solution quantitatively into a 500 mL volumetric flask and make up to the mark with the buffer solution. Mix thoroughly by sonication.

(**s**) *Calibration standard solutions*.—From the mixed stock standard solution, prepare a series of calibration 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 MUD as specified in current EU legislation (1–5), while taking the dilution steps within the procedure into account. For sweeteners not authorized by current EU legislation (ALI, DUL, and NEO), fictitious MUDs were assumed at ca 100 mg/L for beverages and ca 150 mg/kg for canned fruits. Pipet appropriate volumes (Table 3) from the mixed stock standard solution into appropriate volumetric flasks (10–50 mL), make up to the mark with buffer solution, and shake thoroughly. Table 3 details the concentration of sweetener *i* in each calibration standard.

Apparatus

(**a**) *Common laboratory glassware, such as graduated cylinders, volumetric pipets, glass beakers, etc*.

(**b**) *Analytical balance*.—Capable of weighing to 0.01 mg.

(**c**) *Laboratory balance*.—Capable of weighing to 0.01 g.

(**d**) *Positive displacement pipet, or equivalent*.—Capable of delivering 1–10 mL (variable volume).

(**e**) *Volumetric flasks*.—10, 25, 50, 100, and 500 mL capacity.

(**f**) *Centrifuge tubes*.—Polypropylene, 50 mL capacity.

(**g**) *Graduated test tubes*.—5 mL capacity.

(**h**) *Food blender*.—Suitable for homogenization of food samples (e.g., Grindomix GM200, Retsch, Haan, Germany).

(**i**) *Ultrasonic bath*.

(**j**) *Centrifuge*.—Capable of maintaining 4000 rpm.

(**k**) *SPE vacuum system*.

(**l**) *Equipment for solvent evaporation*.

Table 4. HPLC gradient program

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

(**m**) *pH meter*.

(**n**) *C18 SPE cartridges*.—Chromabond® C18ec, 6 mL/1000 mg (Macherey-Nagel), or equivalent.

(**o**) *Fully end-capped reversed-phase HPLC analytical columns*.—250 × 3 mm, particle size 5 μm, allowing sufficient separation of all 9 sweeteners. Suitable columns are Zorbax Extend-C18 (Agilent Technologies, Santa Clara, CA); Purospher® Star RP-18 (Merck); Nucleodur C18 Pyramid (Macherey-Nagel); Nucleodur® C8 Gravity (Macherey-Nagel).

(**p**) *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 (e.g., Alltech ELSD 2000ES or equivalent, Deefield, IL).

(**q**) *Data acquisition and analysis software*.

Preparation of Test Sample

Comminute the entire test sample to give a homogenous suspension. Liquid samples may be subjected directly to the extraction procedure.

Extraction and Cleanup

(**a**) Weigh ca 5 g (M_1, recorded to 2 decimal places) of the homogenized test sample into a 50 mL volumetric flask (V_1). Make up to the mark with buffer solution, mix thoroughly by hand to obtain a homogeneous suspension, and sonicate for 15 min.

(**b**) Transfer the obtained suspension to a 50 mL centrifuge tube. Centrifuge at 4000 rpm for 10 min.

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

(**c**) Condition the SPE cartridges with 3 mL methanol and let it pass through using a slight vacuum resulting in a flow rate of 1–2 mL/min. Make sure that a small portion of methanol remains above the sorbent bed (1 mm).

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

(**e**) Load the SPE cartridges with 5 mL of sample extract (V_2 first loading), i.e., the supernatant from (**b**), and let it pass through using a slight vacuum resulting in a flow rate of 1–2 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).

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

(**g**) Elute the sweeteners from the SPE cartridges with 2 mL methanol and collect the eluate in a 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 methanol, and elute it subsequently to the same 5 mL test tube using the same vacuum conditions, but this time letting the SPE cartridge run dry.

Note: Avoid in all steps that the sorbent bed runs dry, with the exception of the last step, i.e., second elution of analytes.

(**h**) Evaporate the solvent from the methanolic SPE extract to 3 mL under a stream of nitrogen at ambient temperature.

Note: Avoid temperatures above 40°C because aspartame can degrade.

(**i**) Fill the graduated test tube containing the SPE extract up to the 5 mL mark with buffer solution (V_3). Mix thoroughly and transfer the contents into a suitable HPLC vial and analyze by HPLC.

HPLC Conditions

Establish suitable HPLC conditions to meet the predefined procedural requirements. The separation and quantification have proven to be satisfactory if the following experimental conditions are followed: column, *see Apparatus* (**o**); column temperature, ambient; injection volume, 10 μL; mobile phase, *see Reagents* (**p**) and (**q**); mobile phase flow rate, 0.5 mL/min; separation mode, gradient; gradient program *see* Table 4; detector, ELSD; ELSD drift tube temperature, 85°C; ELSD nitrogen flow, 2.5 L/min; ELSD gain, 1; ELSD impactor, off.

Note: The given detector parameters are applicable to the Alltech ELSD 2000ES system. Alternative ELSD systems and experimental conditions, used in an interlaboratory study, are listed in Table 2. HPLC and ELSD operating conditions may be changed to obtain optimum separation.

Construction of Calibration Graph

Analyze the 8 calibration standard solutions (Table 3) using HPLC conditions identical to those used for the test samples, i.e., inject 10 μL of each solution into the HPLC system. Construct a calibration chart for each sweetener *i* from the results of the analysis of the standard solutions. Plot the obtained peak area as $\log_{10}$(Peak area *i*) (*y*-axis) against the $\log_{10}$(Concentration *i*) (*x*-axis). Fit a straight line ($y = a + bx$) to the results, where b is the value of the slope of the linear function and a is the value where the calibration function intercepts the *y*-axis. If the results of the analyses of the standard solutions are linear, the calibration line may be used to calculate the concentration of sweetener *i* in the sample extract.

HPLC Analysis of Test Sample

Analyze 10 μL of the sample extract solution.

Interpretation of Chromatographic Data

(**a**) To identify the individual sweeteners in the test samples, compare retention times of compounds eluted during the analysis of standard solutions with the retention times of compounds eluted during the analysis of test samples. The elution order of individual sweeteners and their retention times are shown in Figure 1.

(**b**) Measure the peak area response (R_i) observed for sweetener *i* in each solution. If the peak area of sweetener *i* in the chromatogram of the test sample solution exceeds the area of the respective sweetener peak in the chromatogram obtained for the calibration standard solution with the highest concentration, dilute the test sample solution with buffer solution and reanalyze the diluted extract.

Calculations

An individual sweetener *i* is quantitatively determined by integration of the peak area *i* (R_i) obtained from the analysis of the injected SPE extract. The resulting calibration function $y = bx + a$ is used to calculate the concentration of sweetener *i* (C_{1i}) in the measured sample extract solution using Equations 1 and 2:

$$\log_{10} C_{1i} = \frac{\left(\log_{10} R_i\right) - a_i}{b_i} \qquad (1)$$

$$C_{1i},\ \mu g / mL = 10^{(\log_{10} C_{1i})} \qquad (2)$$

where R_i = peak area response for sweetener *i*; a_i = intercept of the calibration line for sweetener *i*; b_i = slope of the calibration line for sweetener *i*; and C_{1i} = concentration of sweetener *i* in the SPE extract (μg/mL).

The mass fraction of sweetener *i* in the test sample is calculated according to Equation 3.

$$C_{2i} = \left[\frac{\mu g}{g}\right] \frac{C_{1i} \times V_1 \times V_3}{M_1 \times V_2} \left[\frac{\mu g \times mL \times mL}{mL \times g \times mL}\right] \qquad (3)$$

where C_{1i} = concentration of sweetener *i* in the SPE extract (μg/mL; as determined in Equation 2); C_{2i} = mass fraction of sweetener *i* in the sample (μg/g); M_1 = mass of the sample taken for extraction (g), i.e., 5 g; V_1 = total volume of the sample solution (mL), i.e., 50 mL; V_2 = volume of the sample solution loaded onto the SPE cartridge (mL), i.e., 10 mL; and V_3 = final volume of the SPE extract (mL), i.e., 5 mL.

Procedural Requirements

(**a**) *HPLC system*.—The chromatographic analysis depends on equipment, type, age, and supplier of the column, sample size, and detector. Different columns may be used, and injection volumes may be varied, if the requirements of the system suitability tests are met.

(**b**) *System suitability test/resolution of separation system*.—The HPLC-ELSD system shall be capable of separating all 9 sweeteners from each other with at least baseline separation. Moreover, the system shall be capable of separating all 9 sweeteners from other components of the matrix. Many matrix components—such as sodium benzoate, sorbic acid, citric acid, phosphoric acid, malic acid, ascorbic acid, glutamic acid, sucrose, glucose, fructose, lactose, caffeine, taurine, D-glucurono-γ-lactone, and sorbitol, etc.—are removed throughout the SPE cleanup. A commonly encountered critical pair is alitame (unauthorized sweetener) and quinine, which is not removed by the SPE cleanup.

Note: In case of failure, the chromatographic conditions (e.g., sample volume injected, mobile phase rate, gradient program, etc.) or the ELSD conditions (e.g., drift tube temperature, nitrogen/air flow) must be optimized.

Results and Discussion

The results of the individual laboratories participating in the pretrial were examined with respect to separation efficiency, relative standard deviation of repeatability (RSD_r), and analyte recoveries. Based on the technical evaluation of the submitted data sets, 7 laboratories were accepted for the final interlaboratory study by demonstrating a correctly functioning chromatographic system.

All data sets were subjected to statistical tests described in the *Protocol for the Design, Conduct and Interpretation of Method Performance Studies* (42), using the Cochran test to identify outlying variances, and the single and double Grubbs tests to detect outlying data set averages. Details of the submitted data are summarized in a comprehensive report (39).

Calculations for repeatability (r) and reproducibility (R), as defined by the protocol (42), were performed on those results remaining after removal of outliers. The precision data obtained in the interlaboratory study were compared with "predicted" levels of precision obtained from the Horwitz equation:

$$\text{Predicted } RSD_R = 2C^{-0.15}$$

where C is the measured concentration of analyte in the sample expressed as a decimal fraction. The HorRat value, i.e., the ratio RSD_R (measured)/predicted RSD_R (Horwitz), gives a comparison of the actual precision measured with the precision predicted by the Horwitz equation. The calculated HorRat values can be used as a performance parameter indicating the acceptability of the precision of a method. A HorRat value of <2 usually indicates satisfactory interlaboratory precision, whereas a value >2 usually indicates unsatisfactory performance of the method.

Moreover, the trueness of the analytical method was assessed from recovery assays, by comparing the known concentration with the found concentration. The performance characteristics for the individual sweeteners are given in Table 5.

Blank Samples

Samples 1 and 6 were blank samples, used to assess the method's ability to prove the absence of all 9 sweeteners.

Table 5. Method performance characteristics for ACS-K, ALI, and ASP[a]

	ACS-K								ALI								ASP							
Parameter	2	3	4	5	7	8	9	10	2	3	4	5	7	8	9	10	2	3	4	5	7	8	9	10
No. laboratories	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7
No. outliers	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	1	1	0	2	1
Identity of outlying labs							6										3			5	3		4, 6	3
Reason for removal							Co[b]										SG[b]			Co	SG		Co	Co
No. of accepted labs	7	7	7	7	7	7	6	7	7	7	7	7	7	7	7	7	6	7	7	6	6	7	5	6
Mean value, mg/L or mg/kg	38.3	266.6	324.1	383.5	38.4	259.2	323.0	391.3	31.1	69.1	96.4	114.5	36.0	113.7	142.5	175.2	38.1	485.1	584.8	702.0	37.2	739.8	951.9	1120.2
True value, mg/L or mg/kg	42.1	282.5	354.2	421.7	36.5	265.6	338.8	410.0	36.5	80.5	102.6	122.2	34.6	116.1	145.1	175.5	42.0	485.0	605.0	720.3	37.3	752.1	967.8	1171.1
Bias, mg/L or mg/kg	3.8	15.9	30.2	38.2	–1.9	6.4	15.9	18.7	–5.4	11.4	6.2	7.6	–1.5	2.4	2.5	0.3	3.9	–0.1	20.2	18.3	0.0	12.3	1 5.9	51.0
Recovery, %	90.9	94.4	91.5	90.9	105.1	97.6	95.3	95.4	85.3	85.8	93.9	93.7	104.2	97.9	98.3	99.8	90.7	100.0	96.7	97.5	99.9	98.4	98.4	95.6
Repeatability standard deviation (s_r), mg/L or mg/kg	2.6	6.0	10.6	9.2	2.7	9.1	4.1	11.4	2.2	2.8	2.3	1.5	3.5	2.5	3.1	6.4	1.9	9.5	5.0	5.8	3.6	16.5	4.5	13.5
Repeatability relative standard deviation (RSD_r), %	6.9	2.3	3.3	2.4	6.9	3.5	1.3	2.9	7.1	4.0	2.3	1.3	9.7	2.2	2.2	3.7	4.9	1.9	0.9	0.8	9.7	2.2	0.5	1.2
Repeatability limit (r), mg/L or mg/kg	7.4	16.9	29.7	25.7	7.4	25.6	11.5	32.0	6.2	7.7	6.3	4.3	9.7	6.9	8.8	18.0	5.2	26.5	14.1	16.2	10.1	46.3	12.5	37.8
Reproducibility standard deviation s_R, mg/L or mg/kg	4.2	15.6	20.1	19.3	5.7	12.7	16.0	17.5	3.0	7.5	2.6	3.9	3.5	3.8	4.4	7.5	6.1	33.3	30.9	23.5	3.6	29.3	27.5	31.7
Reproducibility relative standard deviation (RSD_R), %	10.9	5.9	6.2	5.0	14.8	4.9	4.9	4.5	9.5	10.9	2.7	3.4	9.7	3.3	3.1	4.3	16.0	6.9	5.3	3.4	9.7	4.0	2.9	2.8
Reproducibility limit (R), mg/L or mg/kg	11.6	43.8	56.2	54.0	15.9	35.5	44.8	49.1	8.3	21.1	7.2	11.0	9.7	10.6	12.3	21.1	17.1	93.3	86.6	65.9	10.1	82.0	77.1	88.8
HorRat value = RSD_R/predicted RSD_R[c]	1.2	0.9	0.9	0.8	1.6	0.7	0.7	0.7	1.0	1.3	0.3	0.4	1.0	0.4	0.4	0.6	1.7	1.1	0.9	0.6	1.0	0.7	0.5	0.5

[a] Year of collaborative trial 2007.

[b] Co = Cochran; SG = Single Grubbs.

[c] Predicted $RSD_R = 2C^{-0.15}$; C = estimated mean concentration.

Results were evaluated in terms of the number of "correct," "false-positive," and "false-negative" results. The percentage of correctly classified samples was 100%. Both samples were classified correctly by all laboratories.

Acesulfame-K

The RSD_r and RSD_R values for concentration levels around the MUDs were <6% for beverages (samples 3–5) and <5% for canned fruits (samples 8–10). These results (Table 5) were in close agreement with the results from a standardized method for the simultaneous determination of ACS-K, ASP, and SAC by HPLC and spectrophotometrical detection at a wavelength of 220 nm (43). Precision figures obtained for test samples (samples 2 and 7) with lower levels, i.e., close to the limit of quantitation (LOQ), were higher but still in an acceptable range. Results from laboratory 6 were removed as Cochran outliers. The calculated HorRat values ranged from 0.7 to 1.6, demonstrating an acceptable performance of the method independent of concentration level and type of matrix. Recovery rates were between 90 and 105%.

Alitame

For ALI, belonging to the group of nonauthorized sweeteners, data from 7 laboratories in most cases yielded RSD_R values of <4.5% (Table 5). Samples 2, 3, and 7 showed higher RSD_R values of around 10%, which were still in the expected range. The obtained HorRat values, ranging from 0.4 to 1.0, confirmed satisfactory interlaboratory precision. The recovery rates of the analyte obtained for beverages (samples 2–5) showed a higher spread, from 85 to 122%, than for canned fruits (samples 7–10), from 97 to 104%.

Aspartame

The obtained overall mean concentrations for ASP were in close agreement with the true concentrations, expressed by recovery rates between 90 and 100% (Table 5). Results from laboratory 3 were removed for samples 2, 7, and 10, from laboratory 5 for sample 5, and from laboratories 4 and 6 for sample 9. The RSD_R values for beverages (samples 3–5) determined around the prescribed legal limits for ASP were <7%, and for canned fruits (samples 8–10) <4%. The obtained values were comparable with values given in the European Standard (43). Even though the RSD_R value for ASP at a very low concentration level (sample 2) rose to 16%, the resulting HorRat value of 1.7 still suggested good performance of the method.

Cyclamate

Results from laboratory 3 for sample 8 and from laboratory 5 for sample 10 were removed as Cochran outliers (Table 6). For concentration levels around the legal limits, the RSD_R values were <6.2%. The values are comparable to values given in a European Standard (44) for the determination of cyclamate in foodstuffs by HPLC. Acceptability of the method is demonstrated through HorRat values ranging from 0.6 to 0.9 and recovery rates ranging from 93 to 104%. At low concentration levels, the RSD_R for sample 2 rose to 20%, resulting in a HorRat value of 2.1, which indicated unsatisfactory performance of the method. In case of canned fruits (sample 7), even though the RSD_R was close to 18%, the HorRat value still suggested acceptable performance.

Dulcin

DUL, a sweetener not authorized by current EU legislation, was tested for concentration levels between 50 to 175 mg/kg. Only one laboratory (6) did not report data for sample 7 and was, therefore, considered noncompliant (Table 6). No other results were excluded for statistical reasons. Independent of sample type or concentration level, the performance of the method was very good, expressed in terms of RSD_R values of <8%, HorRat values of <1.0, and recovery rates between 90 to 100%.

Neotame

Neotame, belonging to the group of unauthorized sweeteners, was tested at concentration levels of 35–175 mg/kg. All data sets were used for the statistical evaluation of the results (Table 6). A similar outcome was observed as for DUL. RSD_R values ranging from 4.5 to 6.4%, HorRat values <0.7, and recovery rates between 95 and 103% suggested good performance of the method, independent of matrix type or fortified level.

Neohesperidine Dihydrochalcone

The RSD_R values obtained for NHDC were higher than for the rest of the sweeteners (Table 7). At concentration levels around the legal limits, the RSD_R values ranged from 6.6 to 15.6%. However, the calculated HorRat values, ranging from 0.7 to 1.7, suggested acceptable interlaboratory precision. Recovery rates at those levels were between 98 and 108%. The same results were obtained for canned fruits fortified with a lower level of NHDC (sample 7), whereas the performance of the method was unsatisfactory for sample 2, an energy drink spiked with a lower NHDC amount; the RSD_R value was close to 30%, the HorRat value >2.0, and the recovery rate <90%.

Saccharin

The obtained overall mean concentrations for SAC at higher concentration levels were in close agreement with the true concentrations, expressed by recovery rates between 91 and 102% (Table 7). At lower admixtures, in case of sample 2, the recovery rate was just below 90% and in case of sample 7, it rose to 116%. Results from laboratory 6 obtained for samples 3 and 5 showed a higher variation between blind duplicates than the rest of the laboratories, and were removed as Cochran outliers. The RSD_R values obtained for levels around the legal limits demonstrated good interlaboratory precision. RSD_R values of <7% obtained in this study were lower compared to reproducibility measures given in a standardized method (43). Only for sample 7 (canned fruits fortified with low SAC amounts), a calculated HorRat value of 2.1 indicated a poor performance of the method in terms of

Table 6. Method performance characteristics for CYC, DUL, and NEO[a]

	CYC								DUL								NEO							
Parameter	2	3	4	5	7	8	9	10	2	3	4	5	7	8	9	10	2	3	4	5	7	8	9	10
No. labs	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7
No. outliers	0	0	0	0	0	1	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
Identity of outlying labs						3		5					6											
Reason for removal						Co[b]		Co					NC[c]											
No. accepted labs	7	7	7	7	7	6	7	6	7	7	7	7	6	7	7	7	7	7	7	7	7	7	7	7
Mean value, mg/mL or mg/kg	28.3	248.9	256.8	307.2	27.5	749.7	924.7	1100.6	55.0	79.6	95.7	115.1	49.8	111.0	141.7	172.6	37.6	77.9	97.2	115.3	37.3	116.2	140.6	173.7
True value, mg/kg	36.9	239.0	252.7	300.8	32.2	752.6	968.8	1172.3	60.7	81.3	101.8	121.1	50.2	114.3	145.7	176.3	37.5	80.5	102.2	121.7	36.2	118.3	145.4	175.9
Bias, mg/mL or mg/kg	8.6	–9.9	–4.2	–6.3	4.8	2.9	44.0	71.7	5.7	1.7	6.1	6.1	0.3	3.4	3.9	3.7	–0.1	2.5	5.0	6.4	–1.1	2.1	4.8	2.2
Recovery, %	76.8	104.1	101.6	102.1	85.2	99.6	95.5	93.9	90.6	98.0	94.0	95.0	99.3	97.0	97.3	97.9	100.1	96.8	95.1	94.7	100.3	98.2	96.7	98.7
Repeatability standard deviation (s_r), mg/mL or mg/kg	1.2	6.6	3.6	5.9	4.4	7.0	14.5	12.7	1.4	2.9	1.0	1.5	3.7	3.0	3.6	3.1	0.9	1.9	2.4	2.8	1.3	3.6	2.2	4.8
Repeatability relative standard deviation (RSD_r), %	4.4	2.6	1.4	1.9	16.1	0.9	1.6	1.2	2.5	3.7	1.0	1.3	7.4	2.7	2.5	1.8	2.3	2.4	2.4	2.4	3.5	3.1	1.6	2.8
Repeatability limit (r), mg/mL or mg/kg	3.5	18.4	10.2	16.5	12.4	19.6	40.5	35.6	3.8	8.2	2.8	4.3	10.3	8.4	10.1	8.6	2.4	5.2	6.7	7.7	3.6	10.1	6.2	13.5
Reproducibility standard deviation (s_R), mg/mL or mg/kg	5.8	15.4	14.0	15.5	4.9	30.9	44.4	37.2	3.3	3.9	5.2	5.2	4.3	4.8	4.7	5.4	2.4	4.6	4.8	5.2	2.2	6.3	7.5	7.7
Reproducibility relative standard deviation (RSD_R), %	20.6	6.2	5.5	5.0	17.9	4.1	4.8	3.4	6.1	4.9	5.5	4.6	8.6	4.3	3.3	3.1	6.4	5.9	5.0	4.5	5.9	5.4	5.3	4.5
Reproducibility limit (R), mg/mL or mg/kg	16.3	43.1	39.2	43.4	13.7	86.5	124.2	104.3	9.4	10.9	14.7	14.7	12.0	13.4	13.1	15.2	6.8	12.9	13.5	14.4	6.2	17.6	21.1	21.7
HorRat value = RSD_R/predicted RSD_R[d]	2.1	0.9	0.8	0.7	1.8	0.7	0.8	0.6	0.7	0.6	0.7	0.6	1.0	0.5	0.4	0.4	0.7	0.7	0.6	0.6	0.6	0.7	0.7	0.6

[a] Year of collaborative trial 2007.
[b] Co = Cochran.
[c] NC = Noncompliant data.
[d] Predicted $RSD_R = 2C^{-0.15}$; C = estimated mean concentration.

Table 7. Method performance characteristics for NHDC, SAC, and SCL[a]

	NHDC								SAC								SCL							
Parameter	2	3	4	5	7	8	9	10	2	3	4	5	7	8	9	10	2	3	4	5	7	8	9	10
No. labs	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7
No. outliers	0	0	0	0	0	1	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0
Identity of outlying labs						5				6		6												
Reason for removal						Co[b]				Co		Co												
No. accepted labs	7	7	7	7	7	6	7	7	7	6	7	6	7	7	7	7	7	7	7	7	7	7	7	7
Mean value, mg/mL or mg/kg	31.4	42.8	51.0	59.3	35.3	40.5	49.8	59.3	36.2	60.1	74.1	87.6	44.3	151.9	193.4	235.3	36.8	245.1	282.9	346.8	35.3	306.1	380.2	462.4
True value, mg/mL or mg/kg	36.7	40.2	50.7	60.4	33.4	37.5	48.9	59.1	40.3	65.2	80.9	96.3	38.0	150.0	194.0	234.8	38.9	251.8	302.6	360.3	34.6	313.1	388.2	469.7
Bias, mg/mL or mg/kg	5.3	–2.6	–0.3	1.1	–1.9	–3.0	–1.0	–0.2	4.1	5.1	6.9	8.7	–6.4	–1.9	0.6	–0.6	2.1	6.7	19.7	13.5	–0.7	7.0	7.9	7.3
Recovery, %	85.5	106.4	100.5	98.2	105.6	108.0	102.0	100.4	89.8	92.1	91.5	91.0	116.7	101.3	99.7	100.2	94.7	97.3	93.5	96.3	102.1	97.7	98.0	98.4
Repeatability standard deviation (s_r), mg/mL or mg/kg	3.3	1.7	1.8	2.6	2.2	1.0	2.0	2.3	1.4	1.7	3.0	1.0	2.4	4.0	4.3	6.7	1.4	3.8	2.7	8.2	2.2	7.4	8.5	9.7
Repeatability relative standard deviation (RSD_r), %	10.6	3.9	3.5	4.4	6.1	2.5	4.0	3.9	3.8	2.8	4.0	1.1	5.5	2.7	2.2	2.9	3.7	1.5	0.9	2.4	6.3	2.4	2.2	2.1
Repeatability limit (r), mg/mL or mg/kg	9.3	4.7	4.9	7.3	6.1	2.8	5.6	6.5	3.9	4.7	8.3	2.7	6.8	11.3	12.0	18.8	3.8	10.6	7.4	22.9	6.3	20.6	23.8	27.1
Reproducibility standard deviation s_R, mg/mL or mg/kg	9.0	6.7	4.4	5.2	4.4	4.6	3.3	5.5	4.0	2.8	4.9	5.2	8.4	10.6	13.5	15.0	5.2	10.1	16.2	13.3	3.8	8.7	10.4	9.7
Reproducibility relative standard deviation (RSD_R), %	28.5	15.6	8.7	8.8	12.4	11.5	6.6	9.2	11.1	4.6	6.6	5.9	19.0	7.0	7.0	6.4	14.2	4.1	5.7	3.8	10.9	2.8	2.7	2.1
Reproducibility limit (R), mg/mL or mg/kg	25.1	18.7	12.4	14.5	12.2	13.0	9.2	15.3	11.3	7.7	13.6	14.5	23.6	29.6	37.7	42.0	14.7	28.2	45.3	37.4	10.8	24.4	29.1	27.1
HorRat value = RSD_R/predicted RSD_R[c]	3.0	1.7	1.0	1.0	1.3	1.3	0.7	1.1	1.2	0.5	0.8	0.7	2.1	0.9	1.0	0.9	1.5	0.6	0.8	0.6	1.2	0.4	0.4	0.3

[a] Year of collaborative trial 2007.

[b] Co = Cochran.

[c] Predicted $RSD_R = 2C^{-0.15}$; C = estimated mean concentration.

Table 8. Summary of method performance characteristics for all 9 sweeteners

		LOQs[a]		MUDs[b]	
Sweetener	Sample	RSD_r, %	RSD_R, %	RSD_r, %	RSD_R, %
ACS-K	BEV[c]	6.9	10.9	3.3	6.2
	CAN[d]	6.9	14.8	2.9	4.5
ALI	BEV	7.1	9.5	4.0	10.9
	CAN	9.7	9.7	3.7	4.3
ASP	BEV	4.9	16.0	1.9	6.9
	CAN	9.7	9.7	1.2	2.8
CYC	BEV	4.4	20.6	2.6	6.2
	CAN	16.1	17.9	1.6	4.8
DUL	BEV	2.5	6.1	1.0	5.5
	CAN	7.4	8.6	1.8	3.1
NEO	BEV	2.3	6.4	2.4	5.9
	CAN	3.5	5.9	1.6	5.3
NHDC	BEV	10.6	28.5	3.9	15.6
	CAN	6.1	12.4	2.5	11.5
SAC	BEV	3.8	11.1	4.0	6.6
	CAN	5.5	19.0	2.9	6.4
SCL	BEV	3.7	14.2	0.9	5.7
	CAN	6.3	10.9	2.2	2.7

[a] Fortified levels close to limit of quantitation.
[b] Fortified levels close to MUDs according to current EU legislation.
[c] BEV = Beverages.
[d] CAN = Canned fruits.

interlaboratory precision. For the rest of the samples, the HorRat values were between 0.5 and 1.2.

Sucralose

For SCL, no results were removed for statistical reasons. Precision measures, expressed as RSD_r and RSD_R, for concentration levels around the MUDs were <6% for beverages (samples 3–5) and <3% for canned fruits (samples 8–10; Table 7). The highest RSD_R value (14%) was obtained for sample 2, spiked with a very low amount of SCL. However, as for the rest of the samples, the obtained HorRat value still indicated satisfactory interlaboratory precision. Acceptability of the method in terms of trueness was demonstrated by recovery rates ranging from 93 to 102%.

Conclusions

An overview on the performance characteristics of the method for all 9 sweeteners is given in Table 8. The results are split into 2 categories: results obtained for samples fortified with very low sweetener amounts (close to the LOQs), and those for samples fortified with sweetener amounts around the prescribed legal limits (±20% of the MUDs).

For samples fortified with very low sweetener amounts, the majority of the obtained RSD_R values remained below 15%, demonstrating satisfactory performance of the method.

For samples fortified at levels around the MUDs, no correlation between concentrations and obtained precision data could be observed. Therefore, as a conservative estimate, the highest RSD_R values obtained were adopted as repeatability figures. Even so, it could be demonstrated that the defined method protocol produces acceptably accurate, repeatable, and reproducible results. High comparability of results obtained by individual testing laboratories was ensured by RSD_R values <10% for the majority of results. Moreover, HorRat values of <1.1 for all sweeteners and matrixes tested suggested good performance of the method.

The interlaboratory study demonstrated that the present method produces acceptably accurate, repeatable, and reproducible results when performed by individual laboratories. The validated method described here offers an important measure to assess compliance with labeling provisions and is suitable for rapid screening of large numbers

of samples to determine 6 authorized and 3 unauthorized sweeteners in beverages and canned fruits.

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