



Validation of an analytical method for the simultaneous determination of nine intense sweeteners by HPLC-ELSD

Report on the final collaborative trial

Manuela Buchgraber and Andrzej Wasik



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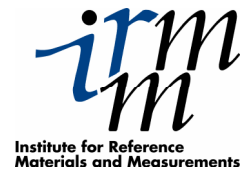
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LIST OF ABBREVIATIONS

ACS-K	acesulfame-K
ALI	alitame
ANOVA	analysis of variance
ASP	aspartame
ASP-ACS-K	aspartame-acesulfame salt
Co	cochran
CYC	cyclamate
DG	double grubbs
DG-JRC	Directorate-General Joint Research Centre
DUL	dulcin
EFSA	European Food Safety Authority
ELSD	evaporative light scattering detection
EU	European Union
Ho _R	HorRAT value
HPLC	high performance liquid chromatography
IRMM	Institute for Reference Materials and Measurements
LOQs	limit of quantification
MS	mean squares
MUD	maximum usable dose
NEO	neotame
NHDC	neohesperidine dihydrochalcone
r	repeatability
R	reproducibility
RSD _r	relative standard deviation of repeatability
RSD _R	relative standard deviation of reproducibility
SAC	saccharin
SCL	sucralose
SD _{BU}	between-units standard deviation
SD _{wU}	within-units standard deviation
SG	single grubbs
SPE	solid phase extraction

S_r

repeatability standard deviation

S_R

reproducibility standard deviation

1 INTRODUCTION

Food additives are substances added intentionally to foodstuffs to perform certain technological functions, for example to colour, to sweeten or to preserve. In the European Union (EU) legislation on food additives is governed by Council Directive 89/107/EEC [1], which is based on the principle that only authorised additives may be used in the manufacture or preparation of foodstuffs. They may only be authorised if there is a technological need for their use, they do not mislead the consumer and they present no hazard to the health of the consumer. Sweeteners form an important class of food additives which are used in an increasingly wide range of food products and beverages. Directive 94/35/EC [2], as amended by Directives 96/83/EC [3] and 2003/115/EC [4], specifically deal with food additives used to impart a sweet taste to foodstuffs. The above mentioned Directives stipulate which sweeteners may be placed on the market for sale to consumers or for use in the production of foodstuffs. Prior to their authorisation, sweeteners are evaluated for their safety by the European Food Safety Authority (EFSA). This can result in being authorised to “quantum satis” level or a maximum usable dose (MUD) or remaining unauthorised. The list of authorised sweeteners is revised regularly by the European Commission in line with the opinion of EFSA, which takes account of the latest scientific advances in the field.

Sweeteners can be classified into two groups, i.e., (i) bulk or (ii) high intensity. Bulk sweeteners are generally carbohydrates such as sucrose, molasses, honey, starch-derived sweeteners, sugar alcohols or tagatose, providing energy (calories) and bulk to food. Their sweetness is similar to sugar, hence used at comparable levels. On the other hand, high-intensity sweeteners possess a sweet taste, but are non-caloric, and provide no bulk to food. They have a greater sweetness than sugar, and are therefore used at lower levels.

At present, eight high-intensity (non-nutritive) sweeteners are included in EU legislation for use in foods, i.e., acesulfame-K (ACS-K), aspartame (ASP),

aspartame-acesulfame salt, cyclamate (CYC), saccharin (SAC), sucralose (SUC), 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.

Due to controversial discussions about their health effects and to ensure proper implementation of existing legislation in order to guarantee consumer safety, EU Member States are required to establish a system of regular surveys to monitor sweetener consumption. To obtain this information robust quantitative methods of analysis are required to measure levels of sweeteners in a broad range of food matrices.

The Institute for Reference Materials and Measurements (IRMM) of the European Commission's Directorate-General Joint Research Centre (DG-JRC) developed a high performance liquid chromatographic method with evaporative light scattering detection (HPLC-ELSD) for the simultaneous identification and quantification of six authorised sweeteners, i.e., ACS-K, ASP, CYC, NHDC, SAC and SCL, and moreover of three non-authorised sweeteners, i.e., neotame (NEO), alitame (ALI) and dulcin (DUL), in beverages, canned or bottled fruits and yoghurts, in a single run. The procedure involves an extraction of the nine sweeteners with a buffer solution, sample clean-up using solid-phase extraction cartridges followed by an HPLC-ELSD analysis. Thaumatin, a group of intensely sweet basic proteins, is primarily used for its flavour modifying properties and not exclusively as a sweetener. Thaumatin, even though belonging to the group of authorised sweeteners in the EU, was not investigated in this study, due to different chemical properties compared to the rest of the authorised sweeteners. Most methods used for the determination of thaumatin involve immunochemical assays and measurement in an enzyme-linked immunosorbent assay reader.

The elaborated method has the advantage that by performing a single analysis using HPLC-ELSD several useful pieces of information can be obtained to be used to control correct labelling by

- (i) proving the absence of three unauthorised sweeteners, i.e., ALI, DUL and NEO,
- (i) proving the absence of six authorised sweeteners, i.e., ACS-K, ASP, CYC, NHDC, SAC and SCL in food products where no sweeteners are labelled,
- (ii) quantifying the amount of six authorised sweeteners, i.e., ACS-K, ASP, CYC, NHDC, SAC and SCL, in case they are labelled on food products and proving that the admixtures are below the given maximum usable dosages as laid down in current EU legislation [2-4].

A substantial in-house testing of the approach [5] formed the basis for the establishment of a draft method protocol (Annex A). On the basis of the in-house validated procedure full method validation by a collaborative trial was carried out. The results of the collaborative trial are presented in this report.

2 METHOD DESCRIPTION

Sweeteners are extracted from test samples with a buffer solution. The extract is cleaned-up by passing through a solid phase extraction (SPE) cartridge, the analytes eluted with methanol, brought to a defined volume with buffer solution and analysed by HPLC with ELSD detection. A detailed description of the method is given in (Annex A)

3 PARTICIPANTS

3.1 Coordination of collaborative trial

European Commission, Directorate-General Joint Research Centre, Institute for Reference Materials and Measurements, Geel (BE)

3.2 Preparation of test samples

European Commission, Directorate-General Joint Research Centre, Institute for Reference Materials and Measurements, Geel (BE)

3.3 Homogeneity testing of test samples

European Commission, Directorate-General Joint Research Centre, Institute for Reference Materials and Measurements, Geel (BE)

3.4 Distribution of test samples

European Commission, Directorate-General Joint Research Centre, Institute for Reference Materials and Measurements, Geel (BE)

3.5 Measurements

- Chemisches- und Veterinäruntersuchungsamt OWL, Bielefeld (DE)
- Chemisches- und Veterinäruntersuchungsamt Stuttgart, Fellbach (DE)
- Faculdade de Farmácia do Porto, Porto (PT)
- Institute for Reference Materials and Measurements, Geel (BE)
- Federal Agency for the Safety of the Food Chain, Liege (BE)
- Landesamt für Verbraucherschutz Sachsen-Anhalt, Halle (DE)
- Südzucker AG Mannheim/Ochsenfurt, Obrigheim (DE)

3.6 Collation and statistical evaluation of results

European Commission, Directorate-General Joint Research Centre, Institute for Reference Materials and Measurements, Geel (BE)

4 TEST SAMPLES

The collaborative testing of a method of analysis requires considerable planning in terms of the design of the trial, the type of matrix or matrices to be analysed, the level of analytes of interest, and the numbers of samples that

are to be included in the trial. Materials are required for which homogeneity and stability of the analytes of interest during the period of the study have to be demonstrated.

The ultimate aim of the study was to provide suitable methodology to be used by individual testing laboratories or enforcement agencies to enforce legislative limits as laid down in current EU legislation [2-4]. Hence, the whole approach was adapted to fit prescribed legal limits, i.e., MUDs for authorised sweeteners as given in Table 1.

Table 1: Present EU limits of all sweeteners for beverages and canned fruits

Sweetener	MUD ⁽¹⁾ for beverages [mg/L]	MUD ⁽¹⁾ for canned fruits [mg/kg]
ACS-K	350	350
ALI ⁽²⁾	-	-
ASP	600	1000
CYC	250	1000
DUL ⁽²⁾	-	-
NEO ⁽²⁾	-	-
NHDC	30	50
SAC	80	200
SCL	300	400

⁽¹⁾ MUD = maximum usable dose according to present EU limits [2-4]

⁽²⁾ unauthorised sweeteners according to present EU limits [2-4]

4.1 Preparation of test samples

Test materials, i.e., 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 usage 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.

Before usage the beverages were sonicated and the canned fruits were homogenised using a food blender and an Ultraturrax. The individual test samples were prepared by weighing appropriate amounts of pure standards

(half of the amounts as given in Tables 2-3) into 500 mL glass bottles, adding ca. 500 g of homogenised test materials and mixing its content for 6 hours using a Turbula mixer.

Subsequently, from each test material 50 containers were filled with a test portion of approximately ten grams and refrigerated at -70 °C. The design was set up in a way to meet the requirements to control legal limits for synthetic and semi-synthetic high-intensity sweeteners, i.e., sample 1 and 6 = blank, sample 2 and 7 = close to limit of quantification, sample 3 and 8 = ca. 75 - 80 % of MUDs; sample 4 and 9 = ca. MUDs, and sample 5 and 10 = ca. 115-120 % of MUDs. For unauthorised sweeteners (ALI, DUL and NEO) fictitious MUDs were assumed at ca. 100 mg/L for beverages and ca. 150 mg/kg for canned fruits.

Example chromatograms for test samples 1-5 are given in Figure 1.

Table 2. Beverages fortified with different concentration levels of all nine sweeteners

	Beverages				
	Sample 1⁽¹⁾	Sample 2⁽²⁾	Sample 3⁽³⁾	Sample 4⁽⁴⁾	Sample 5⁽⁵⁾
Sweetener	Fortified concentration in [mg/L]				
ACS-K	0	42.1	282.5	354.2	421.7
ALI	0	36.5	80.5	102.6	122.2
ASP	0	42.0	485.0	605.0	720.3
CYC	0	36.9	239.0	252.7	300.8
DUL	0	60.7	81.3	101.8	121.1
NEO	0	37.5	80.5	102.2	121.7
NHDC	0	36.7	40.2	50.7	60.4
SAC	0	40.3	65.2	80.9	96.3
SCL	0	38.9	251.8	302.6	360.3

⁽¹⁾ Energy drink - blank; ⁽²⁾ energy drink fortified at concentration level close to the limit of quantification (LOQs); ⁽³⁾ non-carbonated soft drink fortified at a concentration level of ca. 80 % of MUDs; ⁽⁴⁾ carbonated soft drink fortified at a concentration level of ca. 100 % of MUDs; ⁽⁵⁾ carbonated soft drink fortified at a concentration level of ca. 120 % of MUDs

Table 3. Canned fruits fortified with different concentration levels of all nine sweeteners

	Canned fruits				
	Sample 6⁽¹⁾	Sample 7⁽²⁾	Sample 8⁽³⁾	Sample 9⁽⁴⁾	Sample 10⁽⁵⁾
Sweetener	Fortified concentration in [mg/kg]				
ACS-K	0	36.5	265.6	338.8	410.0
ALI	0	34.6	116.1	145.1	175.5
ASP	0	37.3	752.1	967.8	1171.1
CYC	0	32.2	752.6	968.8	1172.3
DUL	0	50.2	114.3	145.7	176.3
NEO	0	36.2	118.3	145.4	175.9
NHDC	0	33.4	37.5	48.9	59.1
SAC	0	38.0	150.0	194.0	234.8
SCL	0	34.6	313.1	388.2	469.7

⁽¹⁾ Canned cocktail fruits - blank; ⁽²⁾ canned cocktail fruits fortified at concentration level close to the limit of quantification; ⁽³⁾ canned pears fortified at a concentration level of ca. 75 % of MUDs; ⁽⁴⁾ canned pears fortified at a concentration level of ca. 100 % of MUDs; ⁽⁵⁾ canned pears fortified at a concentration level of ca. 115 % of MUDs

4.2 Shipment of test samples

The participants received a shipment containing 20 containers of test samples, i.e., five test samples of different beverages (Table 2), and five test samples of various canned fruits (Table 3), all of them provided as blind duplicates, labelled randomly, and each containing a test portion of approximately ten grams.

Additionally, nine ampoules containing the individual sweetener standards in amounts, as given in Table 4, were provided for calibration purposes.

Table 4. Amounts of sweeteners provided for calibration purposes

Sweetener	Amounts provided [mg]
ACS-K	ca. 100
ALI	ca. 60
ASP	ca. 300
CYC	ca. 300
DUL	ca. 100
NEO	ca. 60
NHDC	ca. 100
SAC	ca. 100
SCL	ca. 150

