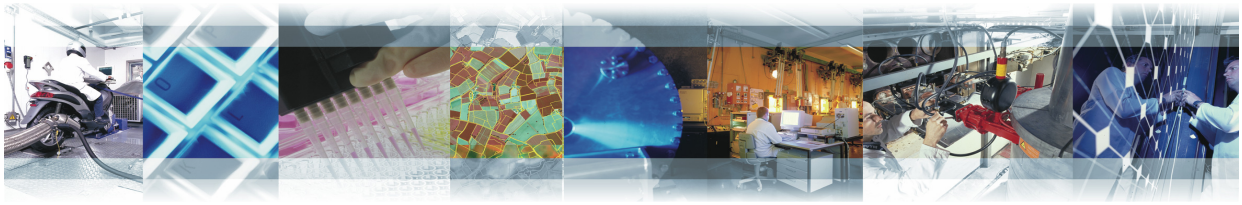

Validation of an analytical method for the simultaneous determination of nine intense sweeteners by HPLC-ELSD

Report on the final collaborative trial

Manuela Buchgraber and Andrzej Wasik

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

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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

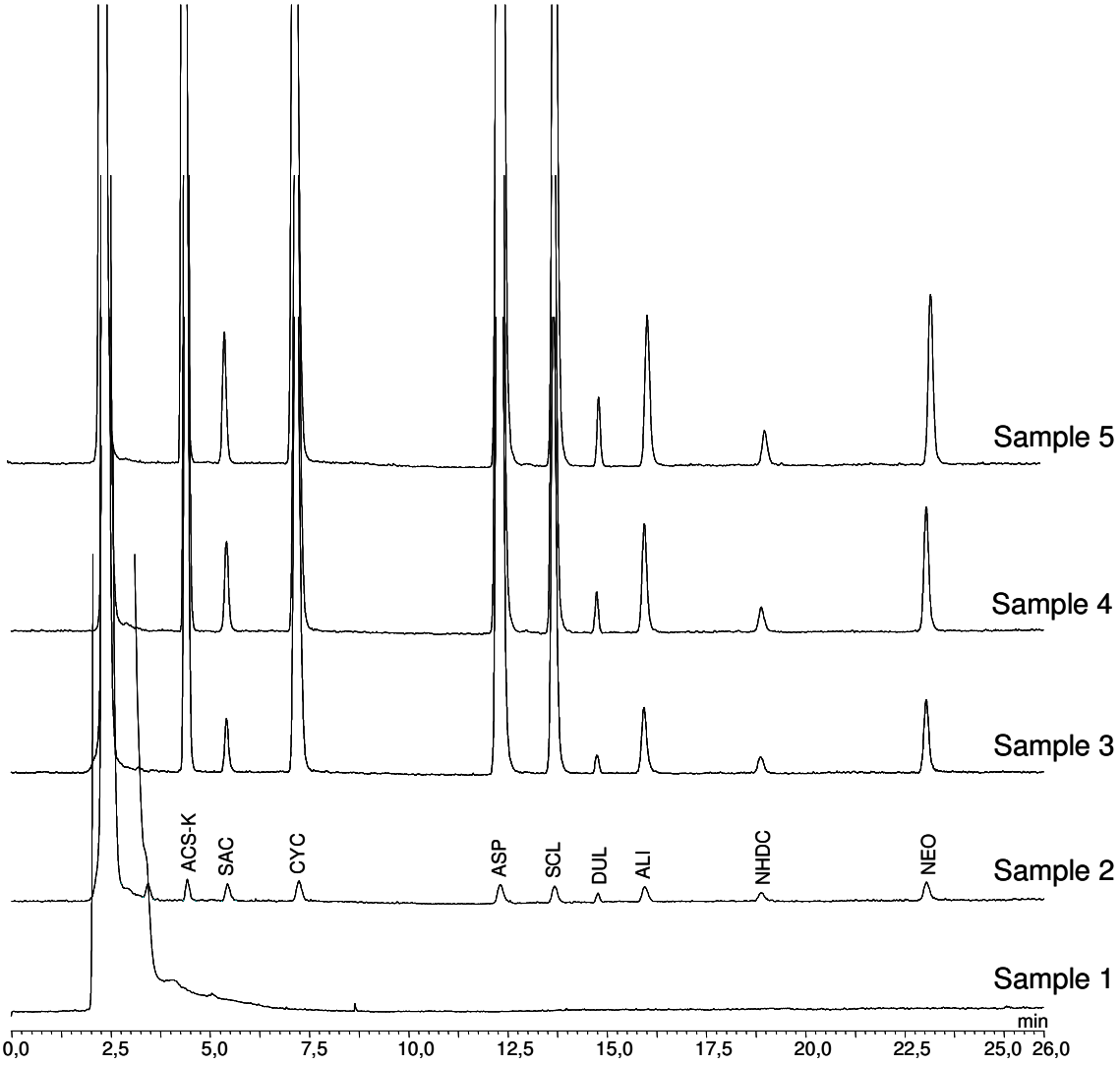

Figure 1. HPLC-ELSD separations of test samples 1-5 using a fully end-capped reversed phase HPLC column of 250 mm x 3 mm, 5 µm dimensions (Purospher® Star RP-18) from Merck (Darmstadt, Germany)

4.3 Homogeneity study

Homogeneity of the test samples was assessed by internationally agreed procedures [6]. From each test sample six sample containers (units) were taken from the sequence and the content of the container split into two equal parts (unit sub-sample). The sweeteners were extracted from each unit sub-sample and randomly subjected to HPLC analysis using a fully end-capped reversed phase HPLC columns of 250 mm x 3 mm, 5 µm dimensions (Purospher® Star RP-18) from Merck (Darmstadt, Germany). The tests were carried out 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 individual results obtained for the duplicate set of values for each sample (replicate A and B) are given in Tables B 1-8 (Annex B).

The within- and between-units standard deviations for the contents of ACS-K, ALI, ASP, CYC, DUL, NEO, NHDC, SAC and SCL were calculated by using one-way analysis of variance (ANOVA) applying the F-test at the 95 % confidence level. The between-units standard deviation (SD_{BU}) was used as an estimate of the inhomogeneity between-units and the within-units standard deviation (SD_{WU}) as an estimate of the combined effects of the repeatability of the method and the possible within-unit inhomogeneity.

All tests (Tables 4-5) confirmed that the between-units inhomogeneity was insignificant ($P > 0.05$). Therefore, the homogeneity of the test samples was considered sufficient to be used as test materials for the validation study.

Table 5. Statistical results of homogeneity study for beverages obtained by ANOVA

Sample	Sweetener	Mean [mg/L]	SD_{BU}	SD_{WU}	P-value	F	$F<F_{critical}$
2	**ACS-K**	40.6	0.36	0.87	0.36	1.35	yes
3	**ACS-K**	281.1	(1)	6.66	1.00	0.06	yes
4	**ACS-K**	338.5	1.72	3.25	0.30	1.56	yes
5	**ACS-K**	393.5	3.70	4.99	0.20	2.10	yes
2	**SAC**	37.2	(1)	0.96	0.65	0.70	yes
3	**SAC**	62.2	(1)	1.66	1.00	0.05	yes
4	**SAC**	75.2	0.58	1.00	0.28	1.67	yes
5	**SAC**	90.9	(1)	1.57	0.83	0.40	yes
2	**CYC**	33.0	(1)	0.69	0.85	0.37	yes
3	**CYC**	259.4	(1)	6.37	0.93	0.24	yes
4	**CYC**	266.5	2.87	3.00	0.12	2.82	yes
5	**CYC**	316.0	1.41	3.21	0.35	1.38	yes
2	**ASP**	43.2	0.84	1.08	0.18	2.19	yes
3	**ASP**	501.5	(1)	12.7	1.00	0.03	yes
4	**ASP**	604.9	6.83	6.43	0.09	3.25	yes
5	**ASP**	710.9	1.82	6.91	0.43	1.14	yes
2	**SCL**	41.5	(1)	1.59	0.66	0.67	yes
3	**SCL**	255.6	(1)	6.38	0.99	0.07	yes
4	**SCL**	293.7	2.98	2.89	0.10	3.13	yes
5	**SCL**	348.8	2.31	3.43	0.23	1.91	yes
2	**DUL**	57.8	(1)	2.09	0.90	0.29	yes
3	**DUL**	82.2	(1)	2.68	0.67	0.66	yes
4	**DUL**	98.4	1.50	1.66	0.13	2.65	yes
5	**DUL**	117.4	1.09	1.50	0.20	2.04	yes
2	**ALI**	34.8	(1)	1.11	0.65	0.69	yes
3	**ALI**	78.3	1.41	1.79	0.18	2.24	yes
4	**ALI**	96.3	0.67	0.98	0.22	1.94	yes
5	**ALI**	115.5	(1)	1.48	0.84	0.40	yes
2	**NHDC**	30.2	(1)	1.39	0.82	0.42	yes
3	**NHDC**	44.5	0.78	0.81	0.12	2.85	yes
4	**NHDC**	51.7	(1)	1.61	0.81	0.44	yes
5	**NHDC**	60.2	0.52	1.02	0.31	1.51	yes
2	**NEO**	40.0	0.06	0.91	0.49	1.01	yes
3	**NEO**	80.7	(1)	1.08	0.68	0.63	yes
4	**NEO**	101.4	1.11	1.79	0.25	1.76	yes
5	**NEO**	119.8	(1)	1.76	0.54	0.88	yes

(1) Mean squares $(MS)_{BU} < MS_{WU}$

Table 6. Statistical results of homogeneity study for canned fruits obtained by ANOVA

Sample	Sweetener	Mean [mg/kg]	SD_{BU}	SD_{WU}	P-value	F	$F<F_{critical}$
7	**ACS-K**	40.8	2.28	1.85	0.06	4.02	yes
8	**ACS-K**	271.6	2.10	2.39	0.14	2.54	yes
9	**ACS-K**	338.8	4.28	3.74	0.07	3.61	yes
10	**ACS-K**	401.8	1.99	3.67	0.29	1.59	yes
7	**SAC**	56.7	[1]	2.06	0.89	0.31	yes
8	**SAC**	163.9	[1]	2.29	0.54	0.89	yes
9	**SAC**	204.4	1.61	2.77	0.27	1.67	yes
10	**SAC**	243.7	[1]	4.20	0.84	0.38	yes
7	**CYC**	28.2	[1]	2.52	0.90	0.29	yes
8	**CYC**	774.2	0.54	5.91	0.48	1.02	yes
9	**CYC**	947.5	[1]	20.48	0.51	0.96	yes
10	**CYC**	1104.3	4.53	10.17	0.34	1.40	yes
7	**ASP**	40.0	[1]	1.17	0.55	0.87	yes
8	**ASP**	769.9	2.13	4.94	0.35	1.37	yes
9	**ASP**	972.6	8.62	13.74	0.25	1.79	yes
10	**ASP**	1168.7	2.43	12.33	0.46	1.08	yes
7	**SCL**	39.1	[1]	1.75	0.76	0.51	yes
8	**SCL**	311.7	1.62	2.90	0.29	1.62	yes
9	**SCL**	388.4	4.16	5.42	0.19	2.18	yes
10	**SCL**	476.2	[1]	8.57	0.53	0.92	yes
7	**DUL**	50.5	[1]	3.92	0.68	0.63	yes
8	**DUL**	114.7	1.24	1.94	0.24	1.82	yes
9	**DUL**	143.6	[1]	4.11	0.84	0.39	yes
10	**DUL**	176.6	[1]	6.82	0.71	0.59	yes
7	**ALI**	36.7	[1]	1.11	0.65	0.69	yes
8	**ALI**	111.7	1.19	1.11	0.09	3.30	yes
9	**ALI**	140.8	1.60	1.85	0.15	2.50	yes
10	**ALI**	173.7	[1]	4.29	0.66	0.67	yes
7	**NHDC**	34.6	[1]	1.39	0.82	0.43	yes
8	**NHDC**	38.1	[1]	1.77	0.97	0.15	yes
9	**NHDC**	50.3	[1]	3.32	0.75	0.53	yes
10	**NHDC**	57.8	[1]	4.78	0.84	0.39	yes
7	**NEO**	40.0	[1]	2.02	0.51	0.96	yes
8	**NEO**	117.3	1.09	2.76	0.37	1.31	yes
9	**NEO**	145.8	2.94	2.35	0.06	4.12	yes
10	**NEO**	180.5	[1]	4.22	0.95	0.19	yes

[1] Mean squares $(MS)_{BU} < MS_{WU}$

4.4 Stability study

In order to gain knowledge about proper storage conditions for the individual sweeteners in the respective test materials a stability study was carried out using an isochronous measurement design [10]. It is based on a storage design of the samples at different temperatures for different time intervals allowing all measurements to be done at the same time, i.e., at the end of the study. The stability of the spiked test materials was tested at -20 °C, 4 °C and +20 °C for the following time periods, i.e., 3 days, 1, 2, and 4 weeks. A reference sample was kept at -70 °C. At the beginning all samples were stored at -70 °C at which their stability was supposed to be good. For each of the storage temperatures studied, samples were moved from the reference temperature to the corresponding studied storage temperatures at different times. At the defined end time the samples were immediately analysed along with the reference samples, which were kept for the entire study at -70 °C, the results of the latter being used as a starting value. The storage days, where no changes in the absolute concentration were observed, are given for the individual matrices and storage temperatures in Figures 2-3.

In beverages six sweeteners were stable up to four weeks independent of the storage temperature. Only ASP, NEO and NHDC were recognized as less stable compounds, i.e., ASP degraded at +20 °C already after three 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 four weeks at +4 °C and -20 °C.

In canned fruits almost all sweeteners were stable up to four weeks independent of the storage temperature. Only NEO and ASP were recognized as less stable compounds, i.e., ASP degraded at +4 °C after seven days and at +20 °C already after three days, and NEO showed a fast degradation at +20 °C, whereas it was stable up to seven weeks at +4 °C and -20 °C.

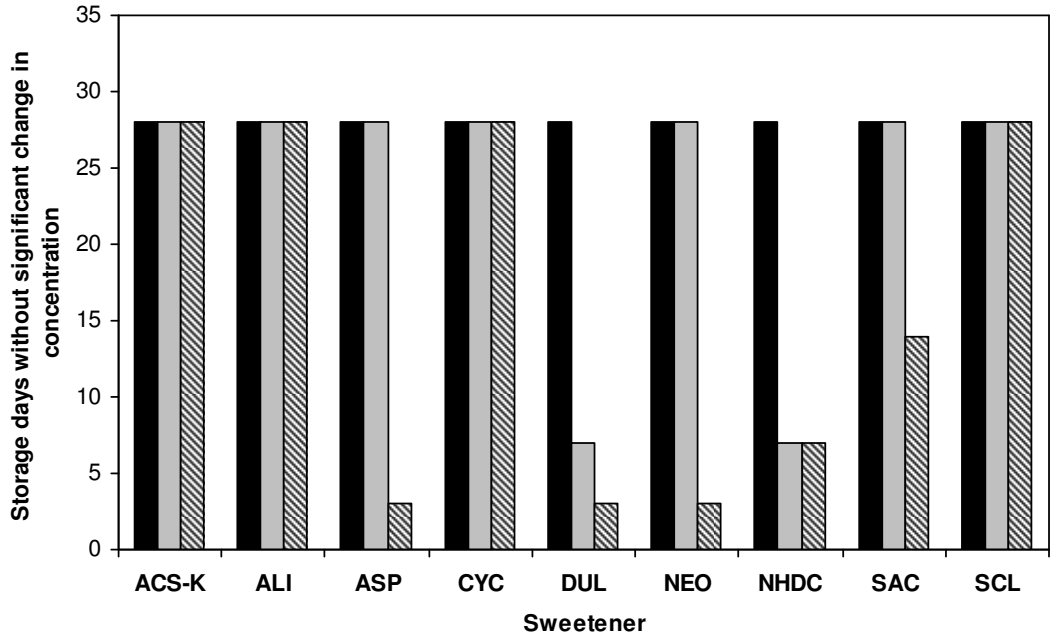

Figure 2. Results of stability study matrix 1 – beverages (reference sample stored at -70 °C)

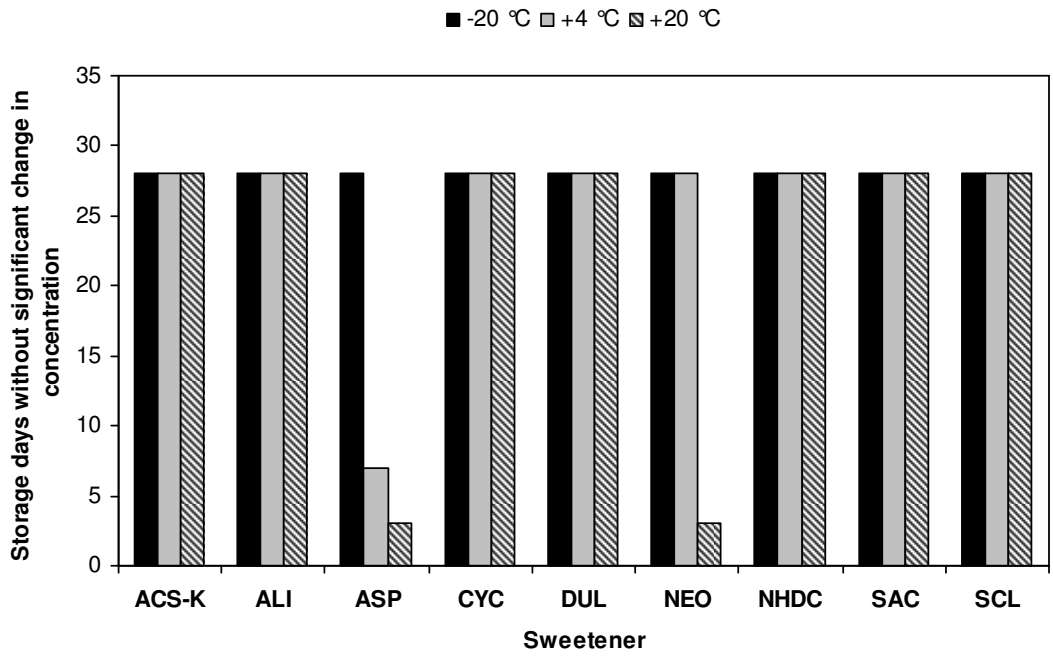

Figure 3. Results of stability study matrix 2 – canned fruits (reference sample stored at -70 °C)

Consequently, all test samples were refrigerated at -70 °C after preparation pending dispatch to the participants. Before dispatching 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 after at least 24 hours the participants were requested to store the test samples immediately in a freezer (-20 °C) until usage. The samples had to be analysed within the following three weeks, ensuring proper stability of all compounds.

5 DESIGN OF THE COLLABORATIVE TRIAL

Ten laboratories, located in five different countries, with experience in HPLC-ELSD analysis were contacted to participate in the study.

A pre-trial was organised to allow the individual laboratories to implement the proposed method. They received a training set of two test samples with given composition of all nine sweeteners, i.e., one beverage with a low concentration and one with a high concentration of all nine sweeteners, which could be used for optimisation purposes and demonstration of a correctly functioning chromatographic system. Out of the ten laboratories contacted, eight laboratories 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 was not acquired under conditions as laid down in the method protocol and study guidelines.

For the collaborative trial the participants were provided with a method protocol (Annex A), collaborative study guidelines (Annex C), the test samples (Tables 1-2), and standards to prepare own sets of calibration solutions (Table 3). 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.

5.1 Methods used by individual laboratories

A brief outline of the HPLC-ELSD methods used by the participants is given in Table D 1 (Annex D). The applied methods differed with respect to the SPE cartridges (Chromabond® and Bakerbond®) used, the HPLC columns (Purospher® Star and Nucleodur®), the HPLC gradients, and the ELSD brands along with the drift tube temperature, gain and nitrogen or air flow.

5.2 Analysis of test samples

The ten test samples, which were provided as blind duplicates, had to be analysed once (in total 20 analyses) under conditions as described in the provided method protocol (Annex A).

Calibration graphs of the individual sweeteners had to be determined as described in the method protocol (Annex A) before the analysis of the first test sample and after analysis of the last test sample.

A flow-scheme detailing the handling of the samples is given in the collaborative study guidelines (Annex C).

5.3 Reporting of results

The results were reported by using an electronic reporting sheet (MS Excel® format) which was provided by the coordinator. The following information had to be filled into the evaluation sheet by the participants:

- applied method conditions such as column type, instrument, etc.
- concentration and peak area of the calibration solutions for the construction of the calibration equations
- intercept and the slope obtained for the individual calibration equations
- sample code (as given on the sample label), the used sample mass, etc.
- obtained peak areas of all nine sweeteners
- any observations the labs considered as important

6 RESULTS OF COLLABORATIVE TRIAL

6.1 Technical evaluation through pre-trial

The results of the individual laboratories participating in the pre-trial 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 seven laboratories were accepted for the final collaborative trial by demonstrating a correctly functioning chromatographic system

6.2 Statistical evaluation of submitted results

The individual results of the collaborative trial as submitted by the participants are listed in Tables E 1-8 (Annex E). Graphs of the plotted laboratory means and the corresponding laboratory ranges of all sweeteners and each test sample are shown in Figures F 1-72 (Annex F). Additionally, the graphs are highlighting the data sets from individual laboratories that have been rejected for statistical reasons.

All data sets were subjected to statistical tests by procedures described in the internationally agreed *Protocol for the Design, Conduct and Interpretation of Method Performance Studies* [7], using the Cochran (Co) test to identify outlying variances, and the single Grubbs (SG) and double Grubbs (DG) tests to detect outlying data set averages.

Calculations for repeatability (r) and reproducibility (R) as defined by the protocol [7] were carried out on those results remaining after removal of outliers. The precision data obtained in the collaborative trial were compared with "predicted" levels of precision obtained from the Horwitz equation, i.e., 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., $Ho_R = RSD_R$(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 1 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 in terms of bias and analyte recovery.

The results for the individual sweeteners are given in Tables G 1-9 (Annex G).

6.2.1 Blank samples

Two samples, i.e., sample 1 and 6, were provided a blank samples, to be used to demonstrate the ability to prove the absence of all nine sweeteners. The outcome was evaluated in terms of the number of "correct", "false positive" and "false negative" results. The efficiency of the method, i.e., the percentage of correctly classified samples, was 100 %. Both samples were classified correctly by all laboratories.

6.2.2 Acesulfame-K

The relative standard deviations for repeatability (RSD_r) and reproducibility (RSD_R) for concentration levels around the MUDs were in case of beverages (sample 3-5) <6 % and for canned fruits (samples 8-10) <5 %. The obtained results are in close agreement with results given in a European Standard for a standardised method for the simultaneous determination of ACS-K, ASP and SAC by HPLC and spectrophotometrical detection at a wavelength of 220 nm [8]. Precision figures obtained for test samples (sample 2 and 7) with lower levels, i.e., close to the LOQs, were higher but still in an acceptable range. Results from one 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 %.

6.2.3 Alitame

For ALI, belonging to the group of non authorised sweeteners, data from seven laboratories resulted in most cases in RSD_R values of <4.5 %. Only sample 2, 3 and 7 showed higher RSD_R values 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, i.e., from 85 to 122 %, than for canned fruits (samples 7-10), i.e., from 97 to 104 %.

6.2.4 Aspartame

The obtained overall mean concentrations for ASP were in close agreement with the true concentrations, expressed by recovery rates between 90 and 100 %. Results from lab 3 were removed for sample 2, 7 and 10, from lab 5 for sample 5, and from lab 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 are highly comparable with values given in the European Standard [8]. 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.

6.2.5 Cyclamate

Results from laboratory 3 for sample 8 and from laboratory 5 for sample 10 were removed as Cochran outliers. For concentration levels around the legal limits, the RSD_R values were less than 6.2 %. The values are comparable to values given in a European standard [9] 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 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.

6.2.6 Dulcin

DUL, a non-authorised sweetener, 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 as non-compliant. For the rest of the results no data 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 %.

6.2.7 Neotame

Neotame, belonging to the group of unauthorised sweeteners, was tested between concentration levels of 35 to 175 mg/kg. All data sets were used for the statistical evaluation of the results. 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.

6.2.8 Neohesperidine dihydrochalcone

The RSD_R values obtained for NHDC were higher than for the rest of the sweeteners. 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. The obtained 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, i.e., an energy drink spiked with a lower NHDC amount; the RSD_R value was close to 30 %, the HorRAT value above 2.0 and the recovery rate <90 %.

6.2.9 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 %. At lower admixtures, in case of sample 2 the recovery rate was just below 90 % and in case of sample 7 rose to 116 %. Results from laboratory 6 obtained for sample 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 standardised method [8]. 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 interlaboratory precision. For the rest of the samples the HorRAT values were between 0.5 and 1.2.

6.2.10 Sucralose

In case of SCL, none of the results submitted by the seven laboratories were removed for statistical reasons. Precision measures, expressed as RSD_r and RSD_R, for concentration levels around the MUDs were in case of beverages (samples 3-5) <6 % and for canned fruits (samples 8-10) <3 %. The highest RSD_R value of 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 resulting recovery rates ranging from 93 to 102 %.

6.3 Summary of statistical evaluation

A brief overview on the performance characteristics of the method for all nine sweeteners is given in Table 7. The results are split into two categories, i.e., results obtained (i) for samples fortified with very low sweetener amounts (close to the limit of quantifications), and (ii) for samples fortified with sweetener amounts around the prescribed legal limits (+/- 20 % of the MUDs). For the three unauthorised sweeteners, where consequently no legal limits are available, fictitious MUDs were chosen, i.e., 100 mg/L for beverages and of 150 mg/kg for canned fruits.

Table 7. Summary of statistical evaluation for all nine sweeteners

Sweetener	Matrix	Low levels (sample 2 and 7)		
		Recovery [%]	RSD_R [%]	HorRAT
ACS-K	1 [3]	90.9	10.9	1.2
	2 [4]	105.1	14.8	1.6
ALI [1]	1 [3]	85.3	9.5	1.0
	2 [4]	104.2	9.7	1.0
ASP	1 [3]	90.7	16.0	1.7
	2 [4]	99.9	9.7	1.0
CYC	1 [3]	76.8	20.6	2.1 [2]
	2 [4]	85.2	17.9	1.8
DUL [1]	1 [3]	90.6	6.1	0.7
	2 [4]	99.3	8.6	1.0
NEO [1]	1 [3]	100.1	6.4	0.7
	2 [4]	103.0	5.9	0.6
NHDC	1 [3]	85.5	28.5	3.0 [2]
	2 [4]	105.6	12.4	1.3
SAC	1 [3]	89.8	11.1	1.2
	2 [4]	116.7	19.0	2.1 [2]
SCL	1 [3]	94.7	14.2	1.5
	2 [4]	102.1	10.9	1.2
Sweetener	Matrix	Levels around MUDs [+/- 20 %] (samples 3-5 and 8-10)		
		Recovery [%], ranges	RSD_R [%], ranges	HorRAT, ranges
ACS-K	1 [3]	90.9 - 94.4	5.0 - 6.2	0.8 - 0.9
	2 [4]	95.3 - 97.6	4.5 - 4.9	0.7 - 0.7
ALI [1]	1 [3]	85.8 - 93.7	2.7 - 10.9	0.3 - 1.3
	2 [4]	97.9 - 99.8	3.1 - 4.3	0.4 - 0.6
ASP	1 [3]	96.7 - 100.0	3.4 - 6.9	0.6 - 1.1
	2 [4]	95.6 - 98.4	2.8 - 4.0	0.5 - 0.7
CYC	1 [3]	101.6 - 104.1	5.0 - 6.2	0.7 - 0.9
	2 [4]	93.9 - 99.6	3.4 - 4.1	0.6 - 0.8
DUL [1]	1 [3]	94.0 - 98.0	4.6 - 4.9	0.6 - 0.7
	2 [4]	97.3 - 97.9	3.1 - 4.3	0.4 - 0.5
NEO [1]	1 [3]	94.7 - 96.8	4.5 - 5.9	0.6 - 0.7
	2 [4]	96.7 - 98.7	4.5 - 5.4	0.6 - 0.7
NHDC	1 [3]	98.2 - 106.4	8.7 - 15.6	1.0 - 1.7
	2 [4]	100.4 - 108.0	6.6 - 11.5	0.7 - 1.3
SAC	1 [3]	91.0 - 92.1	4.6 - 6.6	0.5 - 0.8
	2 [4]	99.7 - 101.3	6.4 - 7.0	0.9 - 1.0
SCL	1 [3]	93.5 - 97.3	3.8 - 5.7	0.6 - 0.8
	2 [4]	97.7 - 98.4	2.1 - 2.8	0.3 - 0.4

[1] unauthorised sweeteners according to current EU legislation
[2] indication of unsatisfactory interlaboratory precision
[3] 1 = beverages
[4] 2 = canned fruits

For samples fortified with very low sweetener amounts only in three cases HorRAT values >2 were observed, i.e., CYC and NHDC in beverages and

SAC in canned fruits. For the majority of the samples the RSD_R values remained below 15 % and in most cases the recovery rates ranged between 90 and 105 % demonstrating satisfactory performance of the method to be used to prove the absence either of unauthorised sweeteners or authorised sweeteners, which are not labelled.

For samples with sweetener admixtures around the prescribed legal limits it could be demonstrated that the defined method protocol produces acceptably accurate, repeatable, and reproducible results, offering an important measure to control correct labelling around the legal limits for six authorised sweeteners. Trueness, expressed in terms of recovery rates, was demonstrated in most cases by values ranging from 90 to 108 %. 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 less than 1.1 suggested for all sweeteners and matrices tested good performance of the method.

7 CONCLUSIONS

Validated analytical methods are those that have been subjected to collaborative trial assessment and for which performance characteristics such as trueness, repeatability (r) and reproducibility (R) have been determined. The objective of the performed collaborative trial, i.e., to demonstrate that the defined method protocol produces acceptably accurate, repeatable and reproducible results when applied by individual laboratories, was accomplished.

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 of synthetic and semi-synthetic high intensity sweeteners by

(ii) proving the absence of three unauthorised sweeteners, i.e., ALI, DUL and NEO,

(iii) 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,

(iv) 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].

The validated method described here offers an important measure to assess compliance with labelling provisions and is suitable for a rapid screening of large numbers of samples to determine six authorised and three unauthorised sweeteners in beverages and canned fruits.

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ANNEX A – METHOD PROTOCOL

Foodstuffs - Simultaneous Determination of Multiple Sweeteners by High Performance Liquid Chromatography with Evaporative Light Scattering Detection

Scope

This draft standard specifies a high performance liquid chromatographic method with evaporative light scattering detection (HPLC-ELSD) for the simultaneous determination of multiple sweeteners, i.e., acesulfame-K (ACS-K), alitame (ALI), aspartame (ASP), cyclamic acid (CYC), dulcin (DUL), neotame (NEO), neohesperidine dihydrochalcone (NHDC), saccharin (SAC) and sucralose (SCL), in the following food matrices: water-based flavoured drinks and canned or bottled fruits.

Principle

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.

Reagents, solutions and standards

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

3.1 **Acesulfame-K** (adequate purity).

3.2 **Alitame** (adequate purity).

3.3 **Aspartame** (adequate purity).

3.4 **Dulcin** (adequate purity).

3.5 **Neotame** (adequate purity).

3.6 **Neohesperidine dihydrochalcone** (adequate purity).

3.7 **Saccharin, sodium salt dihydrate** (adequate purity).

3.8 **Sodium cyclamate** (adequate purity).

3.9 **Sucralose** (adequate purity).

3.10 **Formic acid** (puriss. p.a. ~ 98 %).

3.11 Water (HPLC grade).

3.12 Triethylamine (puriss. p.a. > 99.5 %).

3.13 Methanol (HPLC grade).

3.14 Acetone (HPLC grade).

3.15 Buffer solution (pH = 4.5).

Dissolve 4 mL of formic acid (3.10) in 5 L of water (3.11). Adjust to pH 4.5 with ca. 12.5 mL triethylamine (3.12).

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

Mix 690 mL of methanol (3.13) with 240 mL of buffer solution (3.15) and with 70 mL of acetone (3.14). Degas by sonication for 10 minutes.

3.17 HPLC Mobile phase B, methanol - buffer solution – acetone 11:82:7 (v/v/v)

Mix 110 mL of methanol (3.13) with 820 mL of buffer solution (3.15) and with 70 mL of acetone (3.14). Degas by sonication for 10 minutes.

3.18 Mixed stock standard solution, ACS-K, ALI, ASP, CYC-Na, DUL, NEO, NHDC, SAC-Na and SCL; $c_{(\text{sweetener i})}$ ~ 30 - 250 µg/mL

Prepare a mixed stock standard solution of all nine sweeteners by weighing in the given masses of the individual sweetener standards (Table 1) first into a 100 mL volumetric flask and dissolving them in approximately 50 mL of a methanol:water (1:1) mixture 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 (3.15). Mix thoroughly by sonication until complete dissolution.

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.

Note: The final concentrations of the individual sweeteners (µg/mL) in the mixed stock standard solution have to be calculated by using the actual weighed masses.

Table 1. Masses of individual standards for preparation of mixed stock standard solution

Standard	Mass [mg] weighed in 500 mL volumetric flask [3]	Final 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) **Cyclamic acid (CYC) (free acid)**	140 [1] –	– 249.42
Dulcin (DUL)	25	50
Neotame (NEO)	25	50
Neohesperidine dihydrochalcone (NHDC)	15	30
Saccharin, sodium salt dihydrate (SAC-Na·2H$_2$O) **Saccharin (SAC) (free imide)**	35 [2] –	– 53.17
Sucralose (SCL)	50	100

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

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

[3] first weigh in into 100 mL volumetric flask, dissolve in approximately 50 mL of a methanol:water (1:1) mixture and then transfer quantitatively into 500 mL volumetric flask

3.19 Calibration standard solutions

From the mixed stock standard solution (3.18) 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 120 % of the given limits, such as those in Commission Directives 94/35/EC as amended by Directives 96/83/EC and 2003/115/EC (see Table 2), whilst taking the dilution steps within the procedure into account (see Table 3).

Table 2: Present EU limits for the nine sweeteners in water-based drinks and canned fruits

Sweetener	MUD [1] for water-based drinks [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 dosage according to present EU limits

(2) unauthorised sweeteners according to present EU limits

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 water-based drinks. In case only the latter matrix is analysed the calibration series can be fitted to the MUDs of water-based drinks.

Pipette the following volumes (see Table 3) from the mixed stock standard solution (3.18) into appropriate volumetric flasks (10 - 50 mL) and make up to the mark with buffer solution (3.15) and shake thoroughly.

Table 3. Preparation of series of calibration standard solutions

Calibration solution	Volume of volumetric flask [mL]	Volume taken from mixed stock standard solution (3.18) [mL]	Volume taken from buffer solution (3.15) [mL]
1 (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

(1) undiluted mixed stock standard solution (3.18)

Table 4 details the concentration of sweetener i in each calibration standard following preparation described in Table 3.

Table 4. Concentration of the sweetener i in the individual calibration standard solutions

	Calibration solution							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Sweetener	**µg/mL**	**µg/mL**	**µg/mL**	**µg/mL**	**µg/mL**	**µg/mL**	**µg/mL**	**µg/mL**
ACS-K	90.0	72.0	54.0	36.0	18.0	10.8	5.4	2.7 (1)
ALI	50.0	40.0	30.0	20.0	10.0	6.0	3.0 (1)	1.5 (1)
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 (1)	3.0 (1)	1.5 (1)
NEO	50.0	40.0	30.0	20.0	10.0	6.0	3.0 (1)	1.5 (1)
NHDC	30.0	24.0	18.0	12.0	6.0	3.6 (1)	1.8 (1)	0.9 (1)
SAC	53.2	42.5	31.9	21.3	10.6	6.4	3.2 (1)	1.6 (1)
SCL	100.0	80.0	60.0	40.0	20.0	12.0	6.0	3.0 (1)

(1) 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. The results can differ from laboratory to laboratory.

1 4 Apparatus and equipment

Usual laboratory apparatus and, in particular, the following:

4.1 Common laboratory glassware, such as graduated cylinders, volumetric pipettes, etc.

4.2 Analytical balance, capable of weighing to 0.01 mg.

4.3 Laboratory balance, capable of weighing to 0.01 g.

4.4. Positive displacement pipette, or equivalent**,** capable of delivering 1-10 mL (variable volume).

4.5 Volumetric flasks, of 10 mL, 25 mL, 50 mL, 100 mL and 500 mL capacity.

4.6 Centrifuge tubes, polypropylene, 50 mL capacity.

4.7 Graduated test tubes, 5 mL capacity.

4.8 Food blender, suitable for homogenisation of food samples (e.g. Grindomix GM200, Retsch).

4.9 Ultrasonic bath.

4.10 Centrifuge, capable of maintaining 4000 rpm.

4.11 SPE Vacuum system, or equivalent.

4.12 Equipment for solvent evaporation.

4.13 pH meter.

4.14 C18 SPE cartridges, such as Chromabond® C18ec, 6 mL/1000 mg (Macherey-Nagel, or equivalent).

4.15 Reversed phase HPLC column C-18, allowing sufficient separation of all nine sweeteners. Column dimensions of 250 mm x 3 mm I.D., fully end capped stationary phase with particles of size 5 µm. Suitable columns are:

- Purospher® STAR RP-18 end capped, 250 x 3 mm, 5 µm particle size (Merck)
- Nucleodur C-18ec Pyramid, 250 x 3 mm, 5 µm particle size (Macherey-Nagel)
- Zorbax Extend-C18, 250 x 3 mm, 5 µm particle size (Agilent)

4.16 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 (e.g. Alltech ELS 2000ES or equivalent).

4.17 Data acquisition and analysis software.

2 5 Sampling

Sampling is not part of this method.

3 6 Procedure

6.1 Preparation of test sample

Comminute the entire test sample to give a homogenous suspension (4.8). Liquid samples may be subjected directly to the extraction procedure.

6.2 Extraction and clean-up

6.2.1 Weigh ca. 5 g (M1, recorded to 2 decimal places) of the homogenised test sample (6.1) into a volumetric flask of 50 mL (V1). Make up to the mark with buffer solution (3.15), mix thoroughly by hand to obtain a homogeneous suspension and sonicate (4.9) for 15 min.

6.2.2 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 water-based soft drinks), this step can be ignored.

6.2.3 Condition the SPE cartridges (4.14) applying 3 mL methanol (3.13) 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).

6.2.4 Equilibrate the SPE cartridges applying 2 mL of buffer solution (3.15) 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 two times.

6.2.5 Load the SPE cartridges with 5 mL of sample extract (V2 first loading), i.e., the supernatant from the centrifuge tubes (6.2.2), 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 (V2 in total 10 mL).

6.2.6 Wash the SPE cartridges with 3 mL of buffer solution (3.15) 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).

6.2.7 Elute the sweeteners from the SPE cartridges applying 2 mL of methanol (3.13) and collecting 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 of methanol (3.13) and elute it subsequently to the same 5 mL test tube using the same vacuum conditions but this time letting the SPE cartridges run dry.

Note: Avoid in all steps (6.2.1 to 6.2.7) that the sorbent bed runs dry with the only exception of the last step, i.e., second elution of analytes (6.2.7).

6.2.8 Evaporate the solvent from the methanolic SPE extract to 2.5 mL under a stream of nitrogen at ambient temperature.

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

6.2.9 Fill the graduated test tube containing the SPE extract (6.2.8) up to the 5 mL mark with buffer solution (3.15) (V3). Mix thoroughly and transfer the content into a suitable HPLC vial and analyse by HPLC.

6.3 HPLC conditions

Establish suitable HPLC conditions to meet the predefined performance criteria (8.2). The separation and quantification have proven to be satisfactory if the following experimental conditions are followed:

- Column: see 4.15
- Column temperature: ambient temperature
- Injection volume: 10 µL
- Mobile phase: see 3.16 and 3.17
- Mobile phase flow rate: 0.5 mL/min
- Separation mode: gradient
- Gradient program:

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

- 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

Note: The given detector parameters are applicable to the Alltech ELS 2000ES system. Alternative ELSD systems may be used provided the same results are obtained as indicated in 8.2.

6.4 HPLC sequence

The sequence of injection can be performed in single, double or triple injection according to the needs and has to include:

- 8 calibration standard solutions differing in concentration level (3.19)
- test sample(s)
- after every 20th test sample an extra series of calibration standard solutions shall be analysed (3.19).

Note: In case of a screening analysis, the sequence of injection can be different from the sequence mentioned above.

6.5 Construction of calibration graph

Analyse the eight calibration standard solutions (3.19, Table 3) using HPLC conditions identical to those used for the test samples (6.3), 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) (Figure 1). Fit a straight line to the results. 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.

Use the resulting function (y = b1x + b0) to calculate the concentration of sweetener i in the measured solution (where *b1* is the value of the slope of the linear function and *b0* is the value where the calibration function intercepts the y-axis).

Note: The calibration graphs of the nine sweeteners can differ in the number of calibration points used (3.19, see Table 4), e.g., ACS-K (seven point calibration), ALI (six point calibration), ASP (eight point calibration), CYC (eight point calibration), DUL (five point calibration), NEO (six point calibration), NHDC (five point calibration), SAC (six point calibration), SCL (seven point calibration). Examples of the individual calibration graphs of all nine sweeteners are given in Figures A 1 - A 9 (Annex A).

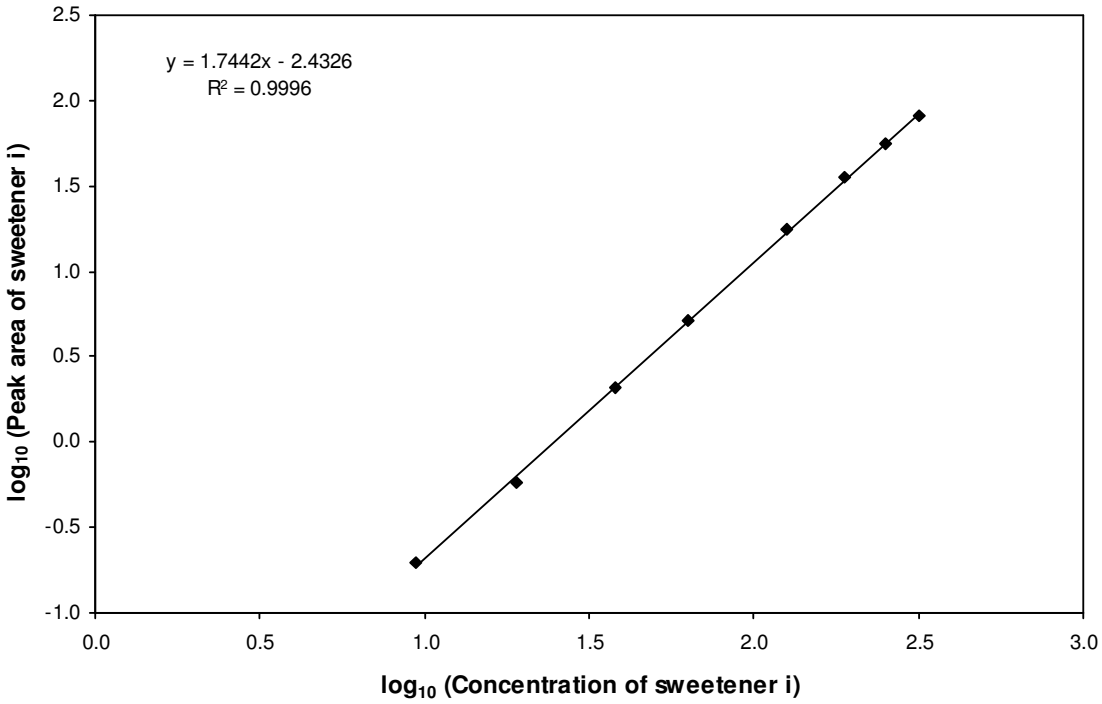

Figure 1. Example of calibration graph for sweetener i, for which *b0* results in -2.4326 and *b1* in 1.7442

6.6 HPLC analysis of test sample

Analyse 10 µL of the sample extract solution (6.2.9).

6.7 Interpretation of chromatographic data

6.7.1 Identify the individual sweeteners in the test samples by comparison of the retention time of sweeteners observed during the analysis of standard solutions analysed in the same batch as samples with the retention time of compounds eluted during the analysis of the test samples. The elution order of the individual sweeteners together with the retention times are given in an example chromatogram in Figure B 1 (Annex B).

6.7.2 Measure the peak area response (Ri) observed for sweetener i in each solution. In case 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, the test sample solution is diluted with buffer solution (3.15) and the diluted extract re-analysed.

4 7 Calculation of results

Quantitative determination of sweetener i is carried out by integration of the peak area i (R_i) (6.7.2) obtained from the analysis of the injected SPE extract (6.6). Use the resulting calibration function, i.e., y = b1x + b0 (6.5) to calculate the concentration of sweetener i (C_{1i}) in the measured sample extract solution using equation 1 and 2.

Equation 1. $$\log_{10} C_{1i} = \frac{(\log_{10} R_i) - b0_i}{b1_i}$$

Equation 2. $$C_{1i}\,[\mu g/g] = 10^{(\log_{10} C_{1i})}$$

where

R_i	is the peak area response (6.7.2) for sweetener i
$b0_i$	is the intercept of the calibration line (6.5) for sweetener i
$b1_i$	is the slope of the calibration line (6.5) for sweetener i
C_{1i}	is the concentration of sweetener i in the SPE extract [µg/mL]

Calculate the concentration of sweetener i in the test sample according to equation 3.

Equation 3. $$C_{2i}\left[\frac{\mu g}{g}\right] = \frac{C_{1i} \text{ x } V_1 \text{ x } V_3}{M_1 \text{ x } V_2}\left[\frac{\mu g \text{ x mL x mL}}{mL \text{ x g x mL}}\right]$$

where

C_{1i}	is the concentration of sweetener i in the SPE extract [µg/g] (as determined in Equation 2)
C_{2i}	is the concentration of sweetener i in the sample [µg/g]
M_1	is the mass of the sample taken for extraction [g], i.e., 5 g (6.2.1)
V_1	is the total volume of the sample solution [mL], i.e., 50 mL (6.2.1)
V_2	is the volume of the sample solution loaded onto the SPE cartridge [mL], i.e., 10 mL (6.2.5)
V_3	is the final volume of the SPE extract [mL], i.e., 5 mL (6.2.9)

5 8 Procedural requirements

8.1 General

The details of the chromatographic procedure depend, among other factors, on equipment, type of column, means of injection of the test solution, 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.

8.2 System suitability test – Resolution of separation system

The HPLC-ELSD system shall be capable of separating all nine sweeteners from each other with at least baseline separation. This requirement can be proven by using calibration solution 1 (3.19) as shown in Figure B 1 (Annex B).

Moreover, the system shall be capable of separating all nine 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-Y-lactone and sorbitol, etc. are removed throughout the SPE clean-up. A commonly encountered critical pair is alitame (unauthorised sweetener) and quinine, which is not removed by the SPE clean-up.

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 flow) must be optimized.

NOTE: Some performance characteristics of the method derived from the in-house validation are given in Annex C.

6 ANNEX A

(informative)

Calibration graphs of individual sweeteners

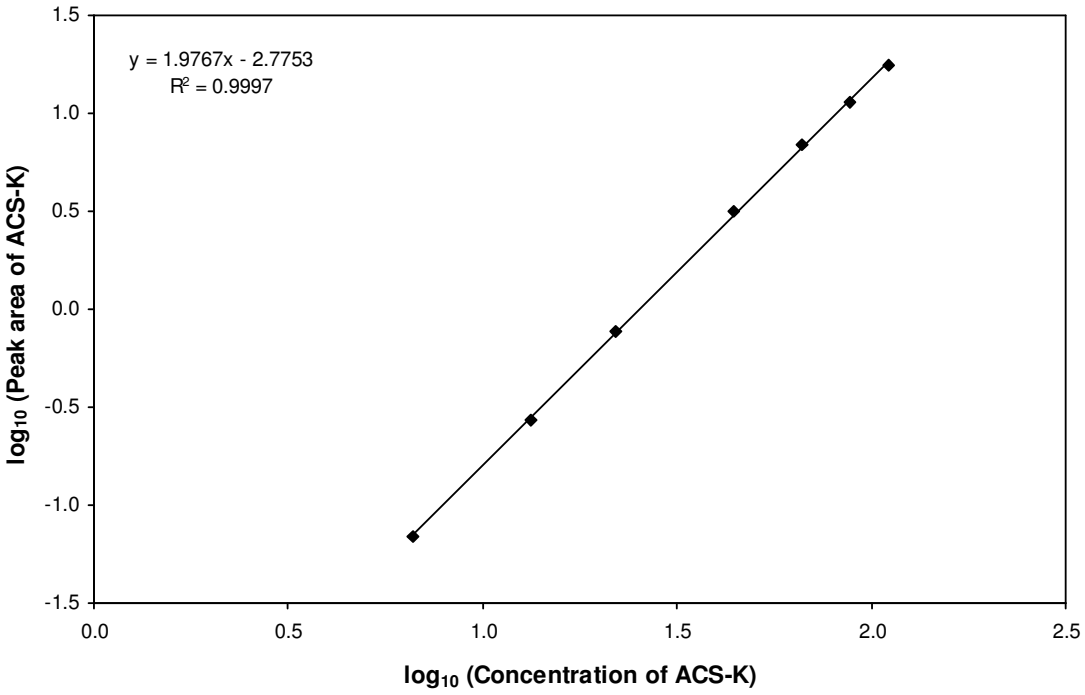

Figure A 1. Seven point calibration graph of ACS-K

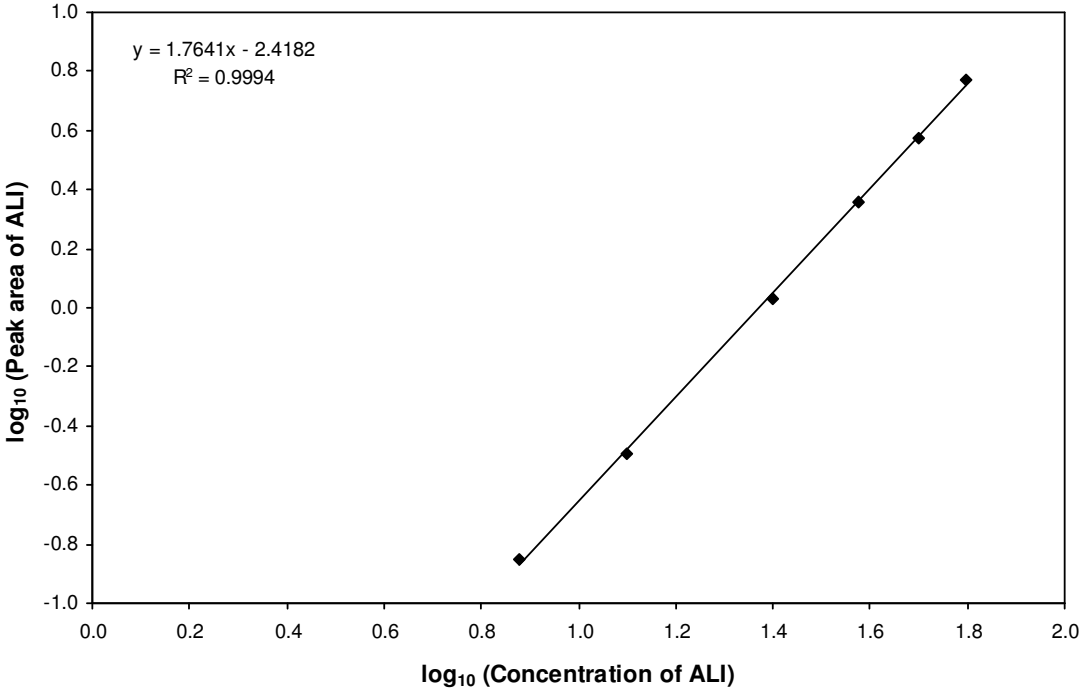

Figure A 2. Six point calibration graph of ALI

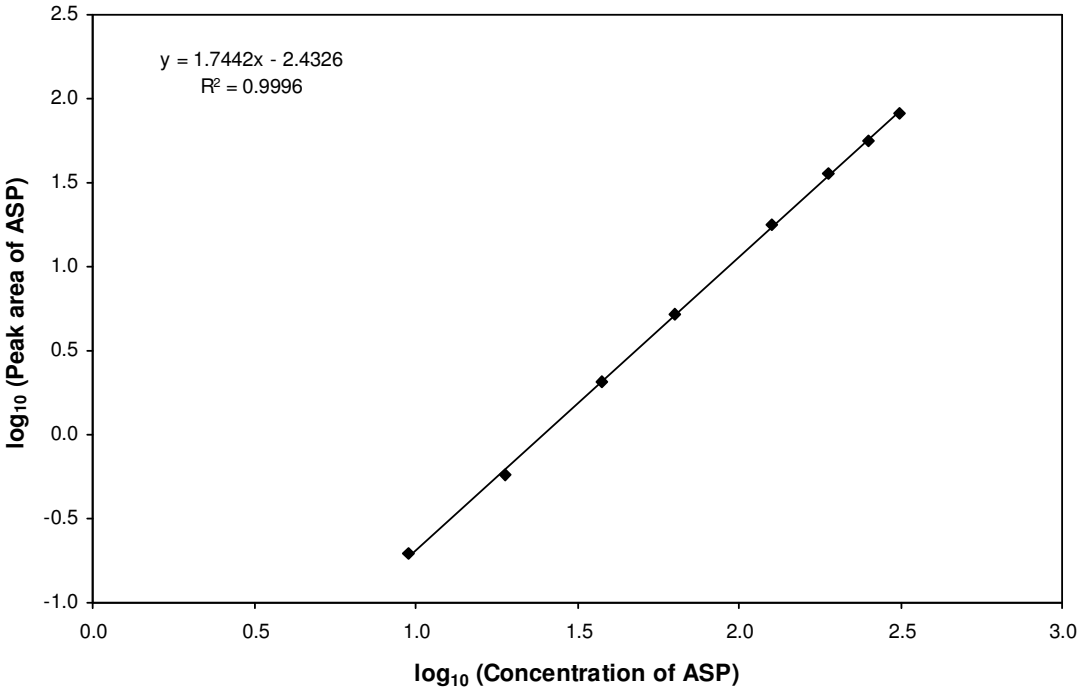

Figure A 3. Eight point calibration graph of ASP

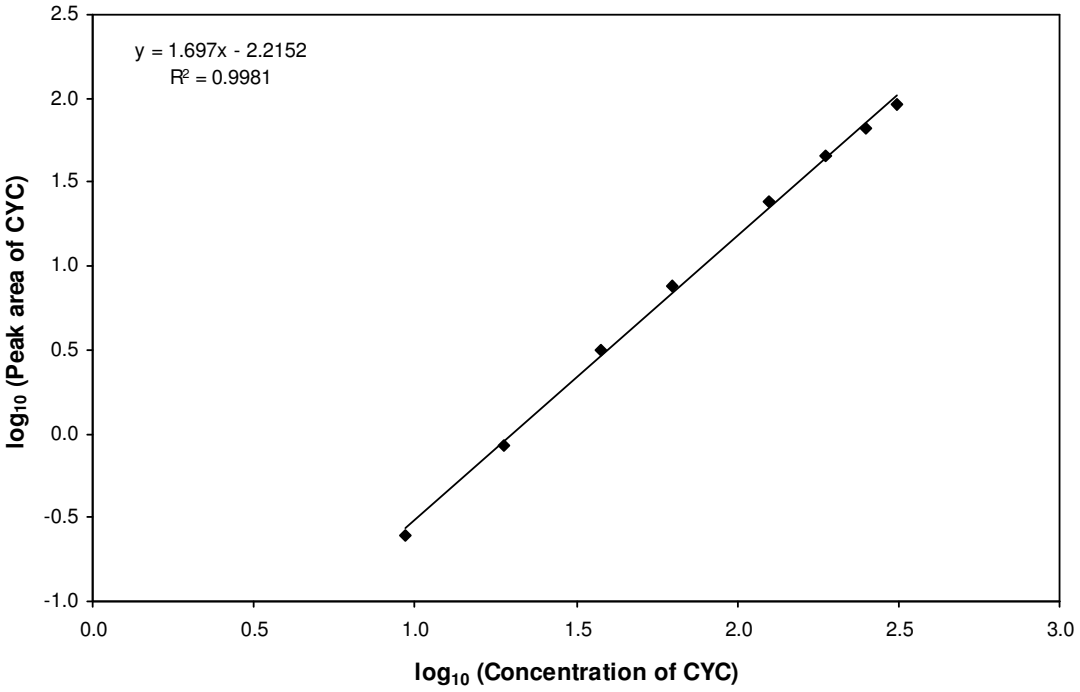

Figure A 4. Eight point calibration graph of CYC

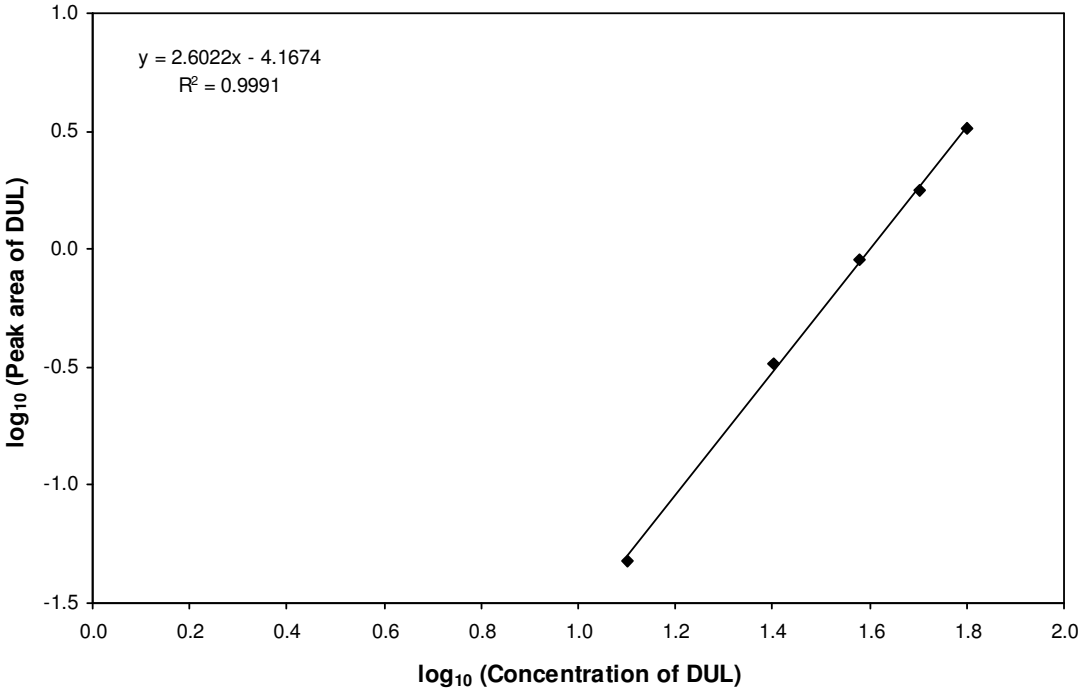

Figure A 5. Five point calibration graph of DUL

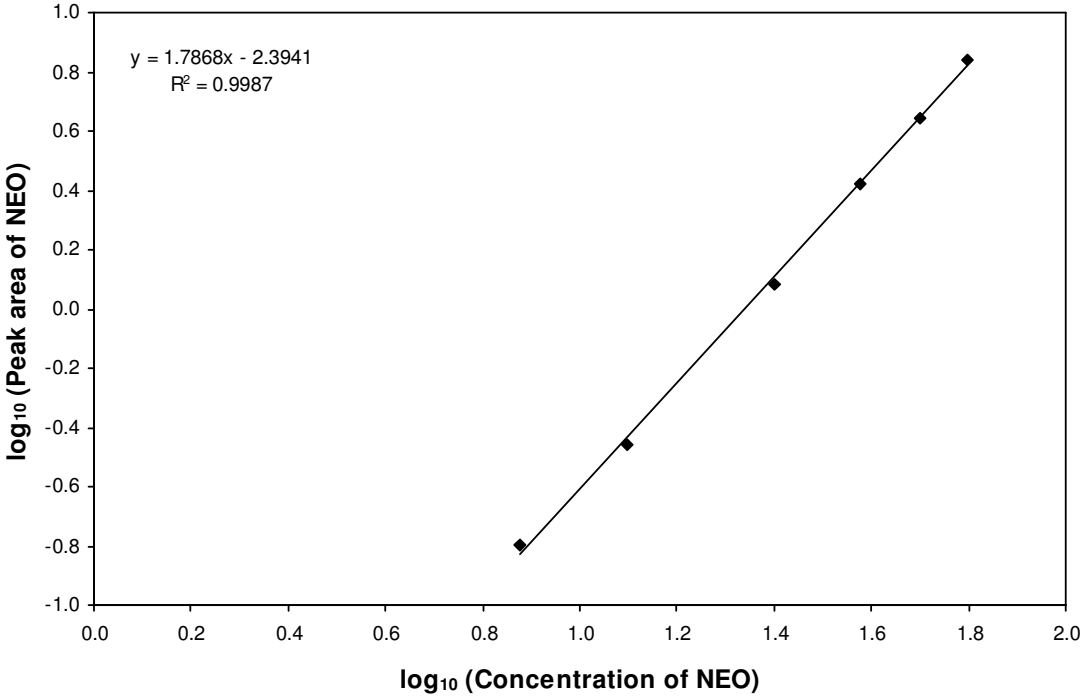

Figure A 6. Six point calibration graph of NEO

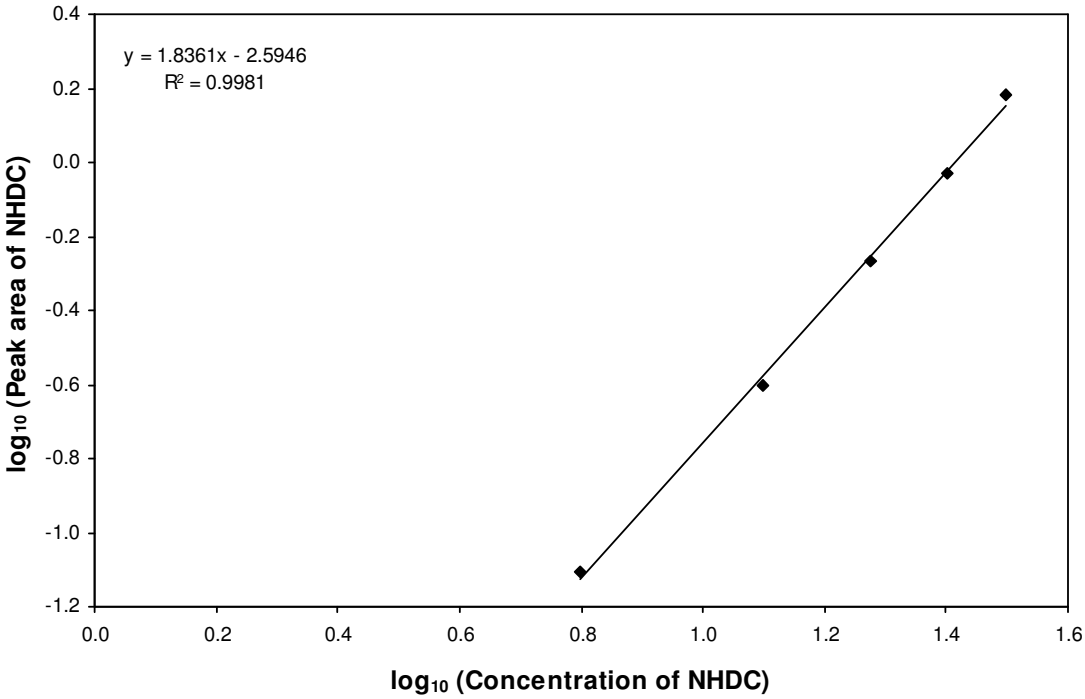

Figure A 7. Five point calibration graph of NHDC

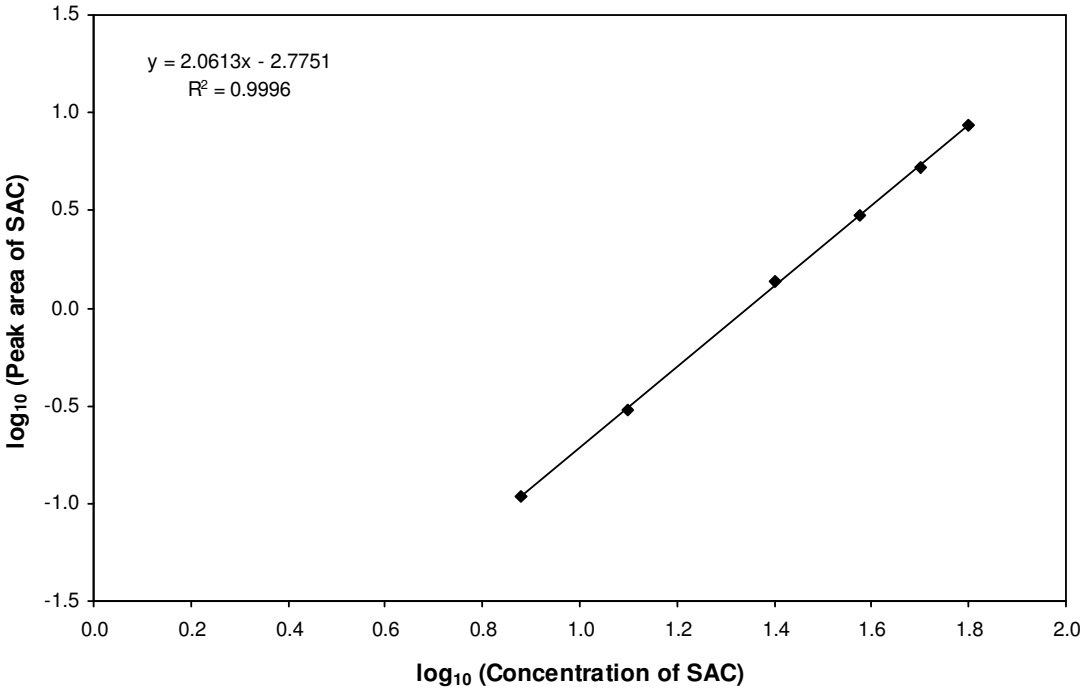

Figure A 8. Six point calibration graph of SAC

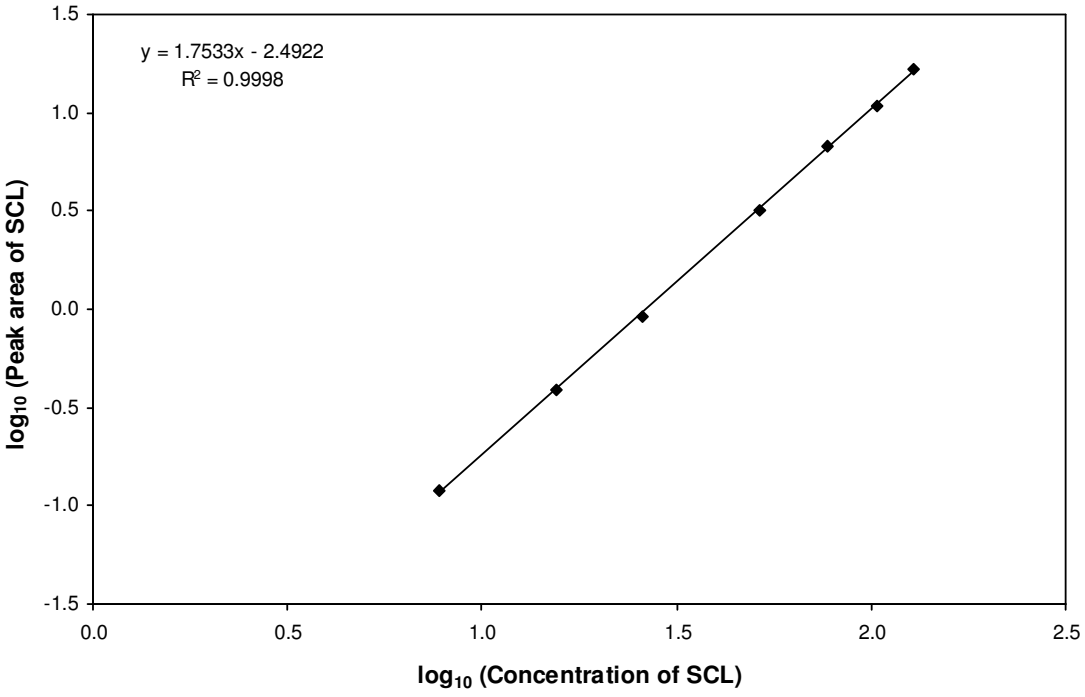

Figure A 9. Seven point calibration graph of SCL

7 ANNEX B

(informative)

Typical chromatogram for calibration standard

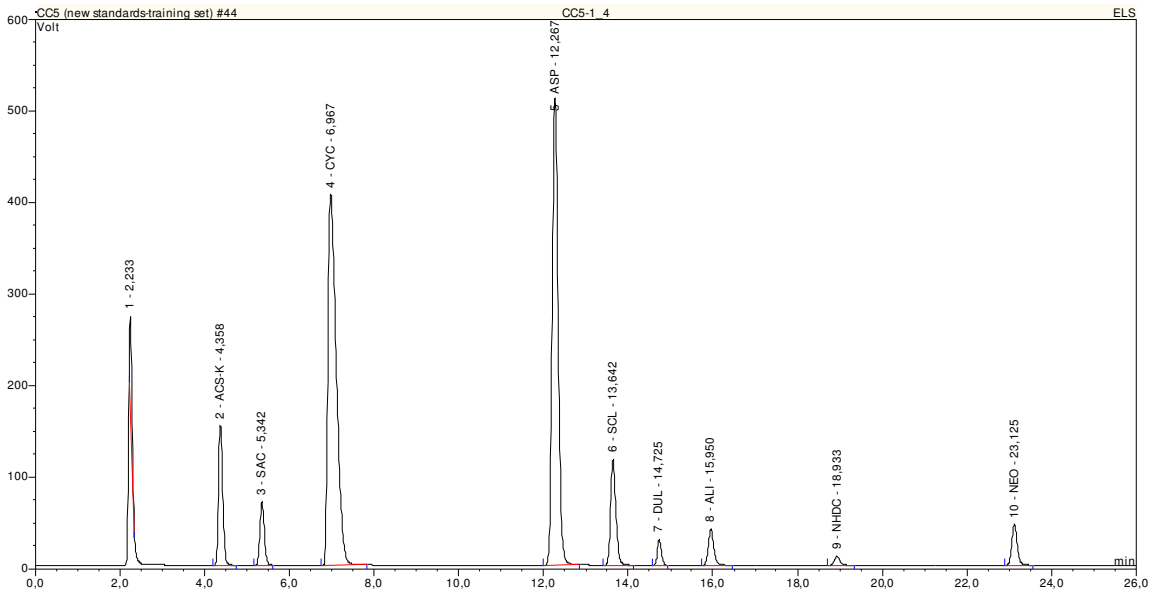

Figure B 1. Chromatographic separation of all nine sweeteners obtained by analysis of calibration solution 1 (3.19)

8 ANNEX C

(informative)

Performance characteristics of method based on in-house validation

Table C 1. Performance characteristics for water-based drinks

	ACS-K	ALI [2]	ASP	CYC	DUL [2]	NEO [2]	NHDC	SAC	SCL
MUD [1] [mg/L]	350	-	600	250	-	-	30	80	300
LOD [mg/L]	13	13	14	13	31	13	15	14	13
LOQ [mg/L]	29	26	27	27	49	26	29	30	26
Recovery [%] [3]	95-102	93-97	94-98	101-103	90-94	92-96	95-109	103-105	94-98
RSD_r [%] [4]	2.8	2.6	2.7	2.7	2.2	2.6	3.6	2.6	2.9

[1] MUD = Maximum usable dose according to present EU legislation

[2] UA = unauthorized sweeteners according to present EU legislation

[3] range from three different concentration levels

[4] three replicates

Table C 2. Performance characteristics for canned fruits

	ACS-K	ALI [2]	ASP	CYC	DUL [2]	NEO [2]	NHDC	SAC	SCL
MUD [1] [mg/kg]	350	-	1000	1000	-	-	50	200	400
LOD [mg/kg]	13	13	13	13	30	13	11	13	13
LOQ [mg/kg]	29	27	26	26	43	26	25	26	26
Recovery [%] [3]	100-104	94-97	93-96	99-101	93-96	93-96	80-85	102-106	95-99
RSD_r [%] [4]	2.4	3.8	4.2	2.4	2.6	2.2	5.7	2.8	4

[1] MUD = Maximum usable dose according to present EU legislation

[2] UA = unauthorized sweeteners according to present EU legislation

[3] range from three different concentration levels

[4] three replicates

ANNEX B – HOMOGENEITY DATA

Table B 1. Individual sweetener data obtained for homogeneity study for selected units of test sample 2

Unit	Replicate	Beverages - Sample 2								
		ACS-K	SAC	CYC	ASP	SCL	DUL	ALI	NHDC	NEO
1	A	40.9	38.0	32.8	43.9	42.7	58.7	32.8	30.4	40.4
	B	39.7	37.5	33.4	41.8	40.3	57.4	34.4	29.6	38.9
2	A	39.6	38.0	32.8	44.7	40.2	59.1	34.7	27.3	39.6
	B	40.0	36.6	32.8	42.2	40.5	57.3	33.7	26.2	39.4
3	A	40.8	35.7	32.7	41.5	39.3	58.9	33.5	26.8	39.5
	B	39.1	36.7	32.4	41.3	42.8	58.2	33.7	27.8	40.7
4	A	40.4	35.9	33.6	45.4	43.4	58.8	35.8	32.3	39.9
	B	41.6	38.6	32.8	43.8	40.8	58.3	35.8	32.0	42.0
5	A	42.5	38.0	31.7	42.7	44.1	58.9	36.5	31.9	40.2
	B	40.8	37.4	33.8	43.0	41.8	55.4	36.8	32.8	41.2
6	A	41.1	37.1	33.6	43.6	41.6	59.5	35.3	33.3	39.1
	B	40.6	37.0	33.1	44.6	40.7	53.6	34.8	31.7	39.7

Table B 2. Individual sweetener data obtained for homogeneity study for selected units of test sample 3

Unit	Replicate	Beverages - Sample 3								
		ACS-K	SAC	CYC	ASP	SCL	DUL	ALI	NHDC	NEO
1	A	282.1	62.5	258.8	501.8	258.0	84.0	76.8	44.9	79.8
	B	280.8	38.5	261.3	500.4	256.4	83.3	76.5	45.1	80.4
2	A	290.6	64.4	266.2	517.5	263.0	86.6	78.5	45.3	81.6
	B	275.6	60.2	249.5	485.6	245.7	81.2	74.2	44.4	79.8
3	A	271.9	60.1	249.8	485.9	247.9	79.6	74.7	42.2	79.0
	B	289.0	63.3	263.2	515.6	261.1	86.6	78.7	43.3	81.1
4	A	281.3	62.8	257.8	504.3	256.4	81.8	81.7	44.5	80.3
	B	278.4	61.7	262.2	500.9	253.9	79.5	79.6	45.9	81.1
5	A	281.0	62.7	264.1	506.7	258.1	81.7	79.1	44.1	82.2
	B	281.6	62.2	261.8	501.4	255.5	80.0	79.2	43.9	79.9
6	A	281.6	61.6	259.0	500.4	255.5	81.0	80.0	46.2	81.5
	B	279.7	63.1	258.5	497.8	255.1	80.7	79.9	44.3	81.8

Table B 3. Individual sweetener data obtained for homogeneity study for selected units of test sample 4

Unit	Replicate	Beverages - Sample 4								
		ACS-K	SAC	CYC	ASP	SCL	DUL	ALI	NHDC	NEO
1	A	342.9	75.4	271.2	615.3	297.5	98.1	97.9	49.6	101.1
	B	337.6	75.8	266.3	607.6	295.7	98.3	96.6	54.2	104.1
2	A	340.1	75.9	273.4	616.5	299.0	101.0	96.3	52.1	104.0
	B	341.6	76.2	269.8	615.0	299.0	101.0	97.9	52.9	103.5
3	A	336.2	73.4	261.7	596.1	290.0	97.9	94.9	51.7	99.3
	B	343.9	74.5	267.2	614.1	298.3	101.3	95.2	50.7	103.8
4	A	335.7	73.8	264.7	598.3	291.5	97.6	97.0	52.0	101.0
	B	331.3	75.2	258.8	588.2	287.2	92.9	94.3	51.4	98.4
5	A	340.2	77.2	268.4	606.8	293.8	98.4	97.2	53.7	101.7
	B	339.3	75.1	267.3	603.7	291.8	98.1	97.1	50.8	100.5
6	A	338.6	75.7	265.7	598.8	289.6	98.5	95.4	50.7	99.3
	B	334.5	73.7	263.5	598.4	291.7	98.2	95.9	50.2	99.6

Table B 4. Individual sweetener data obtained for homogeneity study for selected units of test sample 5

Unit	Replicate	Beverages - Sample 5								
		ACS-K	SAC	CYC	ASP	SCL	DUL	ALI	NHDC	NEO
1	A	394.7	91.4	316.1	708.3	348.5	118.2	114.9	60.2	119.2
	B	394.1	89.9	312.3	708.6	348.5	116.6	114.5	60.2	118.9
2	A	397.9	90.4	318.2	716.1	354.8	119.7	117.0	61.6	121.9
	B	396.7	89.4	316.7	710.8	350.1	118.5	115.0	59.3	119.2
3	A	406.2	91.5	323.3	729.1	356.1	118.5	116.3	59.6	122.1
	B	396.3	90.5	317.3	711.1	348.0	120.0	115.2	57.7	118.7
4	A	392.4	90.1	317.0	709.3	348.8	116.5	115.4	60.2	118.8
	B	387.8	91.9	314.2	704.0	345.9	114.8	116.2	60.7	119.4
5	A	389.2	91.9	315.4	709.0	349.9	116.4	115.5	61.9	121.5
	B	390.9	92.3	316.5	711.9	348.2	116.6	116.6	60.8	121.2
6	A	381.3	88.6	308.7	699.5	340.1	114.1	112.4	59.6	116.2
	B	394.5	93.3	316.6	713.1	346.6	118.4	116.7	61.1	120.3

Table B 5. Individual sweetener data obtained for homogeneity study for selected units of test sample 7

Unit	Replicate	Canned fruits - Sample 7								
		ACS-K	SAC	CYC	ASP	SCL	DUL	ALI	NHDC	NEO
1	A	40.9	56.9	26.7	40.2	38.4	48.7	36.7	34.1	41.6
	B	46.9	58.5	31.6	39.3	36.9	53.2	35.9	33.8	40.8
2	A	42.0	55.6	28.1	40.2	40.8	56.3	37.0	34.4	38.8
	B	41.7	58.6	27.7	39.1	37.0	46.7	37.2	36.3	41.6
3	A	38.9	58.7	26.0	41.2	38.9	47.2	36.7	33.3	41.1
	B	38.2	53.6	29.1	40.6	41.3	51.2	38.2	36.7	42.0
4	A	43.5	57.4	32.2	37.1	40.1	56.6	38.7	35.0	42.5
	B	41.6	54.1	27.0	40.4	39.2	50.2	36.1	34.0	37.8
5	A	41.0	57.1	25.2	39.7	40.5	47.1	36.7	34.3	39.5
	B	40.6	57.4	29.0	41.4	36.8	46.9	35.3	33.2	35.8
6	A	36.5	56.6	27.9	39.9	39.2	49.3	37.1	36.2	40.1
	B	37.2	55.3	28.0	40.3	39.7	53.2	35.2	33.8	38.5

Table B 6. Individual sweetener data obtained for homogeneity study for selected units of test sample 8

Unit	Replicate	Canned fruits - Sample 8								
		ACS-K	SAC	CYC	ASP	SCL	DUL	ALI	NHDC	NEO
1	A	277.7	167.5	788.7	777.0	312.5	115.8	111.1	38.8	115.4
	B	273.0	163.2	772.9	770.0	311.1	114.4	111.1	36.3	115.8
2	A	272.4	167.3	774.9	776.7	318.6	119.3	115.0	39.7	123.3
	B	270.1	162.3	770.6	761.9	310.6	115.6	112.8	37.8	116.8
3	A	269.7	164.1	768.0	768.4	311.9	117.2	112.3	39.6	121.2
	B	268.6	167.3	768.4	765.6	309.6	114.1	111.4	36.8	117.5
4	A	269.2	164.0	775.7	765.7	306.2	112.0	108.6	39.0	112.6
	B	271.9	161.1	776.5	762.4	308.1	112.4	109.3	37.8	115.6
5	A	271.8	163.0	778.1	773.9	313.2	115.5	110.9	38.6	116.1
	B	266.1	162.9	766.7	776.4	311.7	114.1	111.9	36.7	119.0
6	A	274.1	161.6	777.1	769.6	311.1	111.1	111.2	35.8	115.3
	B	274.7	162.9	772.8	770.7	316.0	115.4	114.0	39.7	119.4

Table B 7. Individual sweetener data obtained for homogeneity study for selected units of test sample 9

Unit	Replicate	Canned fruits - Sample 9								
		ACS-K	SAC	CYC	ASP	SCL	DUL	ALI	NHDC	NEO
1	A	342.7	205.7	954.2	971.4	385.4	138.9	138.0	48.0	148.3
	B	343.7	203.7	962.9	981.1	390.0	148.8	140.4	51.4	144.9
2	A	338.1	203.3	948.9	962.7	379.1	144.3	136.5	44.3	136.7
	B	329.2	196.1	926.0	949.4	376.8	142.2	138.5	50.8	142.2
3	A	339.8	209.2	956.1	984.9	397.4	146.7	143.3	51.0	147.8
	B	335.3	204.6	931.2	953.2	383.0	142.2	140.7	50.6	147.5
4	A	339.2	203.1	935.9	964.7	387.4	142.4	140.7	46.0	143.6
	B	344.7	206.9	984.3	994.5	391.6	138.2	141.7	54.3	146.1
5	A	335.4	204.2	944.8	967.3	385.2	141.9	139.2	51.5	146.4
	B	329.3	203.4	906.9	957.4	395.1	149.6	144.0	49.9	148.2
6	A	344.3	205.9	962.7	992.9	394.6	145.1	143.2	54.1	146.8
	B	343.9	206.4	956.2	991.8	395.3	142.9	143.1	51.6	150.8

Table B 8. Individual sweetener data obtained for homogeneity study for selected units of test sample 10

Unit	Replicate	Canned fruits - Sample 10								
		ACS-K	SAC	CYC	ASP	SCL	DUL	ALI	NHDC	NEO
1	A	394.9	247.9	1082.2	1145.2	465.2	165.0	169.6	50.9	176.7
	B	402.3	241.5	1108.3	1167.5	476.0	180.8	173.4	57.6	181.8
2	A	404.5	243.5	1115.6	1183.0	477.9	179.5	175.3	61.7	180.0
	B	407.2	243.9	1115.6	1175.6	478.1	175.6	175.5	57.3	181.8
3	A	404.3	243.6	1103.8	1166.0	466.8	170.2	168.1	52.9	176.2
	B	406.4	244.6	1121.9	1190.5	486.9	182.0	178.8	63.4	186.8
4	A	396.2	233.9	1093.0	1154.9	463.5	169.9	167.4	52.1	175.9
	B	399.9	246.3	1098.8	1171.6	480.1	181.9	176.0	61.8	183.2
5	A	397.0	246.1	1094.8	1170.8	482.6	181.6	175.9	61.0	182.4
	B	405.7	242.9	1108.4	1170.7	490.5	185.2	179.7	59.4	182.7
6	A	400.5	244.1	1103.2	1154.5	471.1	174.2	171.2	58.1	177.5
	B	402.9	246.4	1106.3	1174.3	475.8	173.8	173.1	57.5	181.7

ANNEX C – COLLABORATIVE STUDY GUIDELINES

1 Objective

To validate a high performance liquid chromatographic method with evaporative light scattering detection (HPLC-ELSD) for the simultaneous determination of acesulfame-K (ACS-K), alitame (ALI), aspartame (ASP), cyclamic acid (CYC), dulcin (DUL), neotame (NEO), neohesperidine dihydrochalcone (NHDC), saccharin (SAC) and sucralose (SCL) in water-based flavoured drinks and canned or bottled fruits.

2 Samples

The shipment contains 20 ampoules of test samples, i.e.,

- five test samples for water-based flavoured drinks provided as blind duplicates, and
- five test samples for canned fruits provided as blind duplicates,

each containing a test portion of approximately 10 g. The samples are labelled randomly.

Additionally,

- nine ampoules containing the individual sweetener standards in amounts, as given in Table 1,

are provided for calibration purposes.

Table 1. Amounts of sweeteners provided for calibration purposes

Sweetener: labelled as	Amounts provided [mg]
Acesulfame-K	ca. 100
Alitame	ca. 60
Aspartame	ca. 300
Sodium cyclamate	ca. 300
Dulcin	ca. 100
Neotame	ca. 60
Neohesperidine dihydrochalcone	ca. 100
Saccharin, sodium salt dihydrate	ca. 100
Sucralose	ca. 150

NOTE: Upon receipt of the test samples store them immediately in a freezer (-20 °C) until usage.

3 Method

Participants have to apply the attached *"Standard Operation Procedure (SOP) – Draft Version"* (***20070205 CT SOP.pdf***) to perform the analyses.

4 Sample work-up

4.1 System suitability check

Use "*Calibration solution 1*" (as described in the SOP) to check the resolution power of the applied HPLC-ELSD system.

NOTE: Proceed with the analyses of the test samples only if the system suitability criteria are fulfilled as laid down in the SOP. Operating conditions may be changed to obtain optimum separation.

4.2 Preparation of calibration graphs

Use the provided sweetener standards (Table 1) to prepare the mixed stock standard solution as described in 3.18 of the SOP. Continue to prepare the individual calibration solutions as laid down in the SOP.

NOTE: The individual sweetener standards are provided in amounts to allow at least preparation of two independent mixed stock standard solutions.

4.3 Analyses of test samples

Treat the test samples as laid down in the SOP. Each test sample shall be analysed once (**in total 20 analyses**). The samples shall be analysed in random order.

NOTE: Take the test sample ampoules out of the freezer and let them unfreeze at room temperature. After complete melting shake the ampoules thoroughly to obtain homogenous test solutions. In case of the canned fruit samples take special care and make sure that (i) no phase separation occurs, and (ii) homogenous distribution of the individual sweeteners is guaranteed by shaking them thoroughly.

Calibration graphs of the individual sweeteners have to be determined before the analysis of the first test sample and after analysis of the last test sample.

A flow-scheme detailing the handling of the test samples is given below:

Design of the system suitability check

- Optimise resolution using "*Calibration solution 1*"

Proceed with analyses of test samples only if system suitability criteria are met!

↓

Analyses of test samples

- Analyse the eight calibration solutions (Injection a; 8 injections)
- **Analyse the 20 test samples (20 injections)**

Note: It is up to you to decide if you prefer to make a double injection of each sample (40 injections)! In case you make a double injection from each sample solution, calculate the average peak area and enter the averaged value into the electronic reporting sheet.

- Analyse the eight calibration solutions (Injection b; 8 injections)
- Report and calculate results using the electronic reporting sheet

5 Reporting of results

Use the provided electronic reporting sheet (MS Excel®; "***20070205 CT Electronic reporting.xls"***) to report and calculate the final results as follows:

- Report applied method conditions such as column type, instrument, etc., in ***"Method conditions"***

- Report *"Concentration"* and *"Peak area"* of the calibration solutions for the construction of the calibration graph of sweetener i in ***"Calibration graph i"***

- Report the obtained data for the test samples in ***"Analyses of test samples"*** as follows:
  - Report the *"intercept b0"* and the *"slope b1"* obtained for the individual calibration graphs
  - Report the "*Sample code (as given on the sample label*)" and the used *"Sample mass", "*$Volume_{total}$*", "*$Volume_{SPE\ loading}$*"* and the *"*$Volume_{SPE\ extract}$*"*

- Report the obtained *"Peak area"* of all nine sweeteners.

- Report any observations you consider as important in ***"Remarks"***.

NOTE: The electronic reporting sheet has been password protected in order to avoid any modifications of its structure. You are only allowed to enter data in the yellow-marked cells. All necessary calculations will be done automatically.

Submit the electronic reporting sheet by e-mail to the following address:

manuela.buchgraber@ec.europa.eu

Additionally, send hard copies of all chromatograms and integrator print outs to the following address:

Dr Manuela Buchgraber
Food Safety and Quality Unit
European Commission; DG Joint Research Centre
Institute for Reference Materials and Measurements (IRMM)
Retieseweg 111
B-2440 Geel (Belgium)

Deadline for submission of results: 28 February 2007

6 General remarks

- Make at least one practice run on your own samples to familiarise yourself with the procedure so that you can avoid errors in manipulations.

- On receipt of the samples store them in the freezer until analysis.

- Follow the method you have chosen in detail; do not insert minor modifications.

ANNEX D – APPLIED METHODS

Table D 1. Method conditions applied by individual laboratories

	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7
SPE characteristics							
- brand name	Chromabond®	Chromabond®	Bakerbond spe®	Chromabond®	Chromabond®	Chromabond®	Chromabond®
- stationary phase	C18ec	C18ec	C18	C18ec	C18ec	C18ec	C18ec
- 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
- i.d. [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 %]	0min - 100% A; 4min - 100% A; 11min - 47% A; 23min - 2% A; 24min -2% A; 26min -100% A	0min - 5% A; 10min - 60% A; 30min - 95% A; 31min - 95 % A; 32min - 5% A; 45min - 5% A	0min - 0% A; 15min - 100% A; 18min - 100 % A; 20min - 0% A; 35min - 0% A	0min - 0% A; 4min - 0% A; 11min - 53% A; 23min - 100% A; 24min - 100 % A; 26min - 0% A; 36min - 0% A	0min - 0% A; 4min - 0% A; 11min - 53% A; 21min - 100% A; 23min - 100 % A; 25min - 0% A; 31min - 0% A	0min - 0% A; 4min - 0% A; 11min - 53% A; 23min - 100% A; 24min - 100 % A; 26min - 0% A; 36min - 0% A	0min - 0% A; 4min - 0% A; 11min - 53% A; 23min - 100% A; 24min - 100 % A; 26min - 0% A; 36min - 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	ELS 2000ES, Alltech
- drift tube temperature [°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

ANNEX E – SUBMITTED DATA

Table E 1. Results accepted on technical grounds for sample 2

Lab	Replicate	ACS-K	ALI	ASP	CYC	DUL	NEO	NHDC	SAC	SCL
		[mg/L]								
1	A	35.6	28.4	36.1	22.7	55.1	36.2	30.6	35.9	39.7
	B	34.8	32.6	38.7	24.1	56.4	37.5	32.5	34.6	39.2
2	A	36.6	30.9	35.8	29.3	53.8	39.4	44.0	37.1	36.0
	B	36.0	30.6	36.1	28.6	56.4	39.1	38.8	36.6	35.4
3	A	43.3	29.7	66.8	36.3	56.4	36.6	17.4	35.5	40.2
	B	39.2	25.9	60.7	35.9	56.4	37.5	18.9	34.9	38.5
4	A	38.1	26.6	37.4	24.8	46.8	33.5	30.5	33.8	34.7
	B	41.2	30.6	37.2	25.8	50.3	34.9	27.1	38.0	36.7
5	A	29.7	32.7	28.0	18.5	53.8	35.8	25.7	28.6	27.0
	B	35.5	36.8	29.9	21.1	53.3	35.3	27.1	30.9	25.4
6	A	46.0	32.6	48.7	33.2	58.4	41.5	48.4	42.2	41.4
	B	40.1	31.5	43.5	30.0	56.6	39.7	38.1	43.6	38.2
7	A	40.9	32.8	43.9	32.8	58.7	40.4	30.4	38.0	42.7
	B	39.7	34.4	41.8	33.4	57.4	38.9	29.6	37.5	40.3

Table E 2. Results accepted on technical grounds for sample 3

Lab	Replicate	ACS-K	ALI	ASP	CYC	DUL	NEO	NHDC	SAC	SCL
		[mg/L]								
1	A	265.6	68.3	485.2	242.2	79.4	76.9	41.8	61.1	247.7
	B	264.6	67.9	482.6	245.1	81.1	74.7	42.1	60.6	248.3
2	A	259.3	73.3	512.9	269.3	85.5	84.6	55.1	61.8	255.8
	B	258.1	65.4	489.8	248.9	82.2	81.4	54.6	57.8	245.5
3	A	281.9	76.3	533.4	261.4	82.0	76.5	43.0	60.6	253.2
	B	292.7	79.2	545.1	257.9	79.2	77.1	42.1	61.3	253.2
4	A	247.4	60.7	433.2	231.5	69.9	69.1	36.5	59.3	225.5
	B	264.9	62.8	452.6	242.9	76.9	70.4	37.8	63.0	234.0
5	A	244.8	64.5	467.3	225.9	80.7	83.4	36.9	56.1	237.7
	B	243.9	70.1	454.5	220.6	74.2	78.3	31.3	54.4	235.9
6	A	277.6	60.2	465.0	256.8	80.1	78.2	43.9	78.5	242.3
	B	268.5	59.8	461.9	255.9	81.8	78.3	46.3	60.6	238.7
7	A	281.0	79.1	506.7	264.1	81.7	82.2	44.1	62.7	258.1
	B	281.6	79.2	501.4	261.8	80.0	79.9	43.9	62.2	255.5

Table E 3. Results accepted on technical grounds for sample 4

Lab	Replicate	ACS-K	ALI	ASP	CYC	DUL	NEO	NHDC	SAC	SCL
		[mg/L]								
1	A	339.1	97.1	589.8	257.3	94.7	95.7	50.2	74.9	296.0
	B	327.0	97.2	595.2	259.3	94.3	96.4	50.6	74.1	291.9
2	A	328.5	97.7	602.5	261.1	98.4	101.7	55.9	78.4	291.7
	B	317.9	97.7	597.0	258.9	101.5	101.4	61.6	73.3	289.4
3	A	303.2	95.0	616.2	255.3	95.4	96.3	48.8	68.8	281.4
	B	330.3	102.5	617.5	243.8	95.5	91.6	49.2	68.4	276.9
4	A	315.6	91.9	546.9	257.6	92.7	92.3	45.6	74.6	270.1
	B	310.1	93.6	558.4	254.5	92.5	89.1	46.8	74.0	275.3
5	A	295.8	96.9	542.8	232.3	85.8	99.0	48.5	69.0	253.1
	B	291.0	96.0	530.2	230.4	86.9	92.2	45.6	67.6	252.6
6	A	359.9	96.4	580.4	273.3	99.1	98.9	52.9	75.9	295.2
	B	336.8	93.4	579.3	268.8	100.8	98.8	52.9	85.6	289.8
7	A	340.1	96.3	616.5	273.4	101.0	104.0	52.1	75.9	299.0
	B	341.6	97.9	615.0	269.8	101.0	103.5	52.9	76.2	299.0

Table E 4. Results accepted on technical grounds for sample 5

Lab	Replicate	ACS-K	ALI	ASP	CYC	DUL	NEO	NHDC	SAC	SCL
		[mg/L]								
1	A	385.8	116.7	701.7	303.4	112.1	115.4	58.9	89.5	343.6
	B	393.4	116.0	695.7	307.5	113.9	112.5	57.9	89.5	348.8
2	A	379.6	118.9	695.9	312.5	120.1	119.2	63.2	94.5	346.7
	B	378.4	119.3	699.9	314.1	120.7	121.4	68.5	92.6	349.3
3	A	379.4	117.6	738.8	307.2	110.9	111.2	57.1	80.9	334.8
	B	370.9	113.6	729.7	297.6	114.2	111.9	59.1	80.5	330.7
4	A	375.8	107.8	655.0	298.8	109.0	104.4	52.3	89.2	326.4
	B	368.9	107.1	671.1	307.4	109.6	110.5	56.3	89.6	334.2
5	A	373.0	113.6	671.7	289.6	112.2	113.0	53.1	83.0	373.5
	B	344.4	110.4	619.4	273.0	108.2	117.0	53.0	80.8	346.9
6	A	408.3	115.4	708.2	329.0	122.5	116.6	61.6	87.1	356.0
	B	421.8	117.6	711.0	331.7	122.8	123.0	68.3	95.9	366.9
7	A	394.7	114.9	708.3	316.1	118.2	119.2	60.2	91.4	348.5
	B	394.1	114.5	708.6	312.3	116.6	118.9	60.2	89.9	348.5

Table E 5. Results accepted on technical grounds for sample 7

Lab	Replicate	ACS-K	ALI	ASP	CYC	DUL	NEO	NHDC	SAC	SCL
		[mg/kg]								
1	A	31.0	39.7	34.8	23.1	53.4	36.6	35.1	45.4	37.4
	B	30.1	39.3	39.3	20.0	52.0	36.7	35.8	44.9	38.8
2	A	39.8	36.0	35.4	27.1	48.5	39.6	42.9	35.4	35.8
	B	39.7	35.5	34.2	26.9	48.8	39.6	42.3	37.1	34.9
3	A	40.6	37.0	60.5	32.6	54.3	37.3	36.1	36.2	37.4
	B	45.2	35.0	54.6	33.6	53.6	36.8	31.8	36.2	37.5
4	A	36.0	33.0	35.1	23.0	42.7	32.5	31.6	43.8	32.1
	B	43.8	39.5	39.8	34.7	50.1	36.0	34.0	48.8	34.5
5	A	29.0	38.6	29.2	19.5	47.7	36.0	32.4	34.0	25.8
	B	32.9	27.6	39.6	28.5	43.6	37.9	26.5	40.7	31.6
6	A	42.9	34.8	39.3	33.0	NC [(1)]	36.3	36.8	51.9	37.0
	B	42.6	34.2	40.9	26.5	NC [(1)]	36.8	38.3	52.2	33.2
7	A	42.0	37.0	40.2	28.1	56.3	38.8	34.4	55.6	40.8
	B	41.7	37.2	39.1	27.7	46.7	41.6	36.3	58.6	37.0

[(1)] NC = non compliant (no data were reported)

Table E 6. Results accepted on technical grounds for sample 8

Lab	Replicate	ACS-K	ALI	ASP	CYC	DUL	NEO	NHDC	SAC	SCL
		[mg/kg]								
1	A	253.9	114.4	758.2	784.6	109.5	120.2	37.7	153.3	308.9
	B	259.4	114.4	750.2	769.8	108.9	115.4	37.6	156.6	307.7
2	A	253.4	115.6	732.8	743.2	115.7	121.5	49.0	146.7	303.7
	B	257.7	117.0	742.4	753.2	116.5	122.6	49.3	150.1	308.6
3	A	240.5	112.2	751.6	701.0	106.7	106.4	38.4	133.9	293.1
	B	267.3	118.7	794.3	753.7	113.7	116.6	38.5	142.6	312.1
4	A	262.7	110.4	710.1	717.6	106.2	105.5	37.3	153.2	296.8
	B	249.5	105.8	692.0	707.3	105.2	105.9	36.8	150.4	288.6
5	A	237.2	119.0	706.6	706.9	104.1	121.5	45.2	136.9	303.7
	B	251.5	115.8	738.4	718.8	111.7	118.3	27.8	145.9	317.9
6	A	239.9	119.0	722.3	710.6	104.3	121.5	35.2	140.0	308.6
	B	273.9	109.3	712.8	773.2	109.0	116.0	43.7	161.8	304.2
7	A	272.4	115.0	776.7	774.9	119.3	123.3	39.7	167.3	318.6
	B	270.1	112.8	761.9	770.6	115.6	116.8	37.8	162.3	310.6

Table E 7. Results accepted on technical grounds for sample 9

Lab	Replicate	ACS-K	ALI	ASP	CYC	DUL	NEO	NHDC	SAC	SCL
		[mg/kg]								
1	A	330.1	142.7	956.4	982.5	143.1	142.5	49.7	202.1	385.9
	B	327.5	144.7	968.6	975.9	143.9	144.5	48.7	203.4	385.0
2	A	327.5	146.2	924.1	930.6	147.7	146.7	56.6	192.9	379.9
	B	330.6	147.7	924.8	939.7	150.1	146.7	50.8	195.0	382.9
3	A	296.8	140.7	953.0	871.3	135.9	127.3	49.8	171.0	368.9
	B	302.5	145.3	947.5	871.4	141.0	130.6	49.8	174.6	372.9
4	A	320.0	135.1	895.3	889.5	134.3	133.2	46.9	190.4	368.5
	B	332.1	145.0	975.8	923.5	146.2	138.5	45.5	197.2	396.1
5	A	311.1	143.9	932.0	868.1	137.8	145.6	44.3	179.4	368.6
	B	309.0	146.6	927.8	871.8	138.3	144.9	48.0	178.4	377.7
6	A	325.5	134.2	905.6	931.5	138.1	133.4	50.8	198.8	367.6
	B	361.5	136.6	946.1	971.4	139.7	136.5	51.1	212.6	379.6
7	A	344.3	143.2	992.9	962.7	145.1	146.8	54.1	205.9	394.6
	B	343.9	143.1	991.8	956.2	142.9	150.8	51.6	206.4	395.3

Table E 8. Results accepted on technical grounds for sample 10

Lab	Replicate	ACS-K	ALI	ASP	CYC	DUL	NEO	NHDC	SAC	SCL
		[mg/kg]								
1	A	392.1	176.8	1152.1	1143.8	170.9	173.6	55.5	241.0	466.4
	B	396.1	176.2	1135.4	1143.0	170.6	171.3	57.3	242.4	467.3
2	A	384.4	178.2	1093.0	1104.7	184.8	180.0	73.0	226.5	458.0
	B	385.1	179.1	1089.8	1094.9	180.0	177.0	67.0	227.0	453.9
3	A	409.6	188.1	1196.2	1091.2	173.0	174.4	61.3	233.5	463.6
	B	375.4	178.8	1127.8	1057.7	173.8	169.6	61.2	223.7	446.4
4	A	370.9	173.0	1080.9	1035.1	168.0	163.8	53.4	223.3	462.6
	B	367.9	170.8	1098.8	1058.9	168.7	164.6	54.1	223.5	455.7
5	A	389.2	188.2	1125.8	1134.0	174.0	189.0	59.0	228.6	473.1
	B	370.1	169.2	1129.7	1041.6	169.7	176.6	56.5	214.0	459.0
6	A	425.4	170.7	1121.1	1140.1	172.0	171.7	61.0	269.1	473.9
	B	409.3	159.6	1086.9	1128.1	162.7	160.9	55.8	251.2	446.8
7	A	400.5	171.2	1154.5	1103.2	174.2	177.5	58.1	244.1	471.1
	B	402.9	173.1	1174.3	1106.3	173.8	181.7	57.5	246.4	475.8

ANNEX F – MEAN & RANGE PLOTS

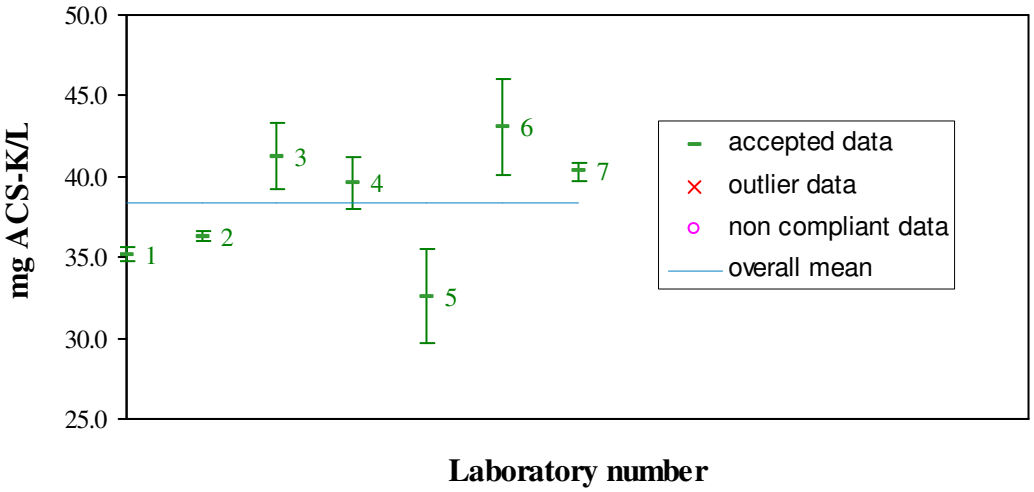

Figure F 1. Laboratory means and ranges of determined ACS-K amounts in sample 2

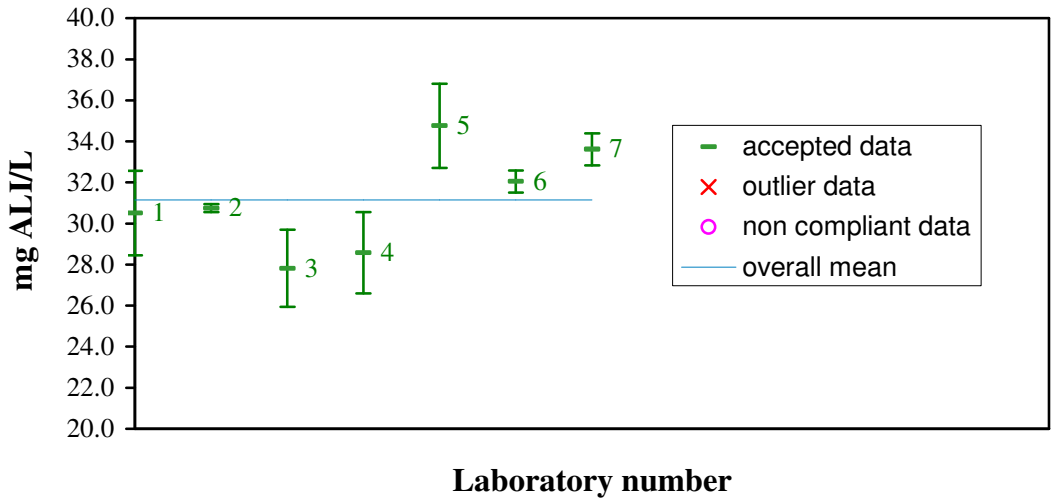

Figure F 2. Laboratory means and ranges of determined ALI amounts in sample 2

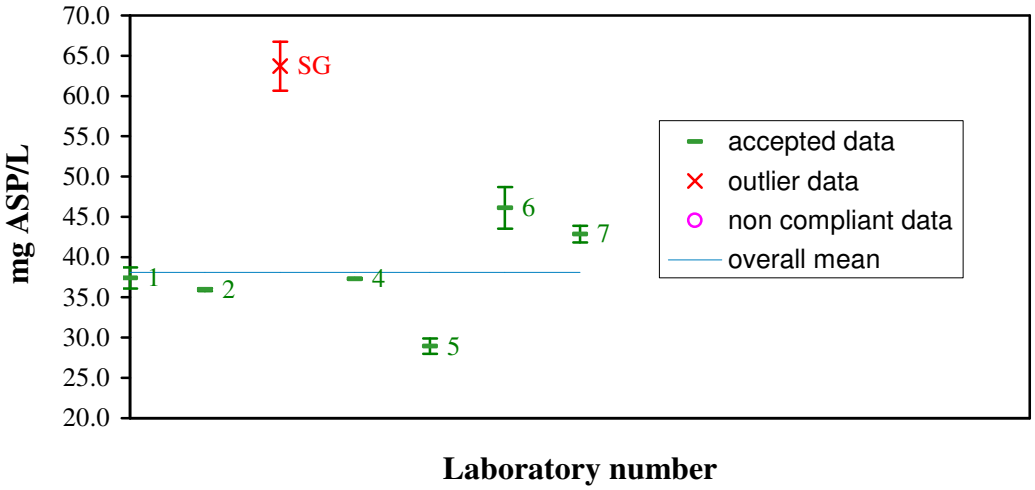

Figure F 3. Laboratory means and ranges of determined ASP amounts in sample 2

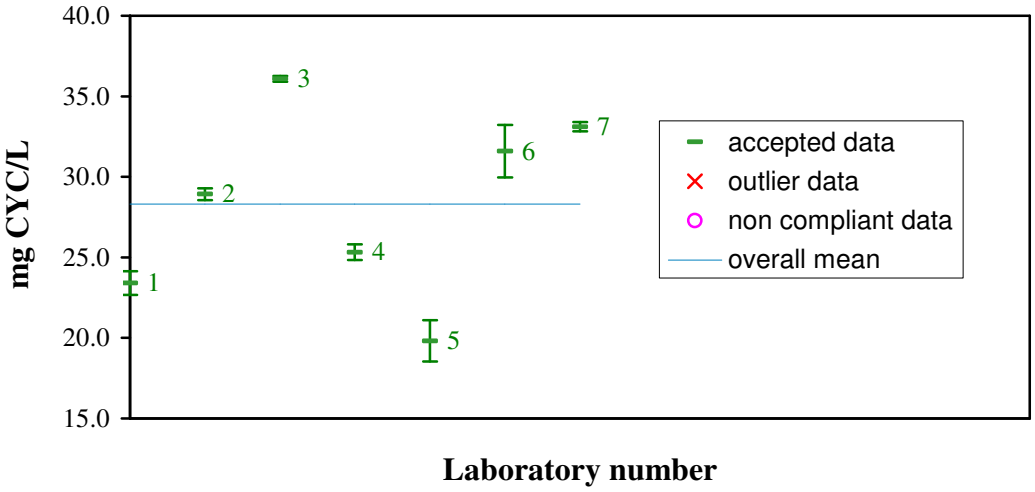

Figure F 4. Laboratory means and ranges of determined CYC amounts in sample 2

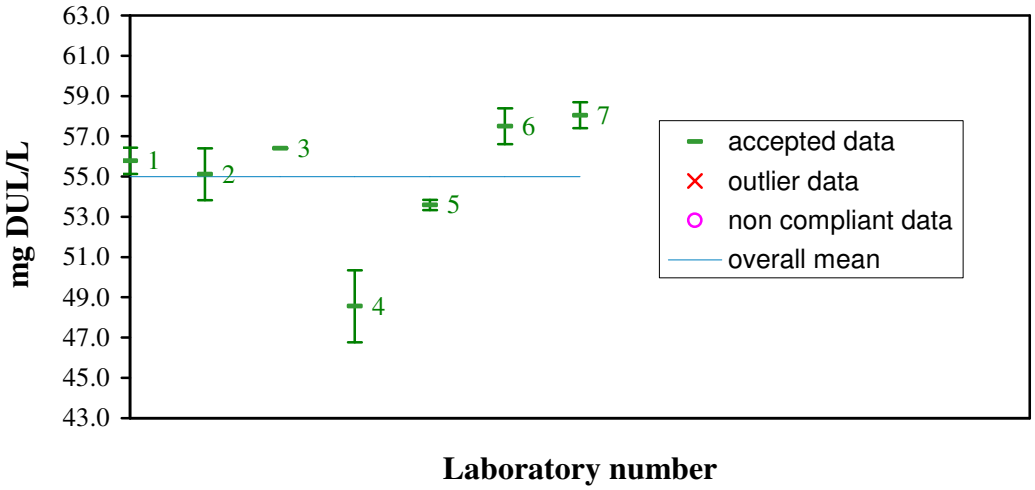

Figure F 5. Laboratory means and ranges of determined DUL amounts in sample 2

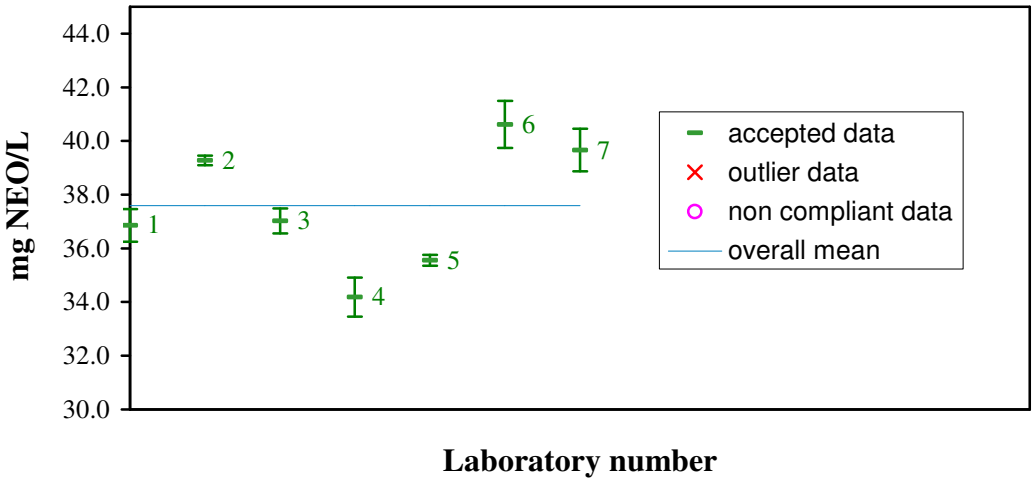

Figure F 6. Laboratory means and ranges of determined NEO amounts in sample 2

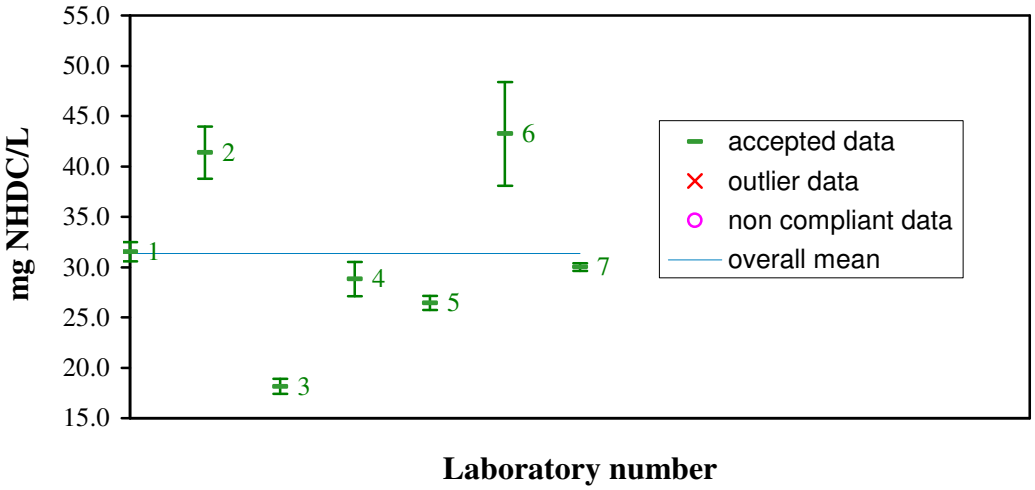

Figure F 7. Laboratory means and ranges of determined NHDC amounts in sample 2

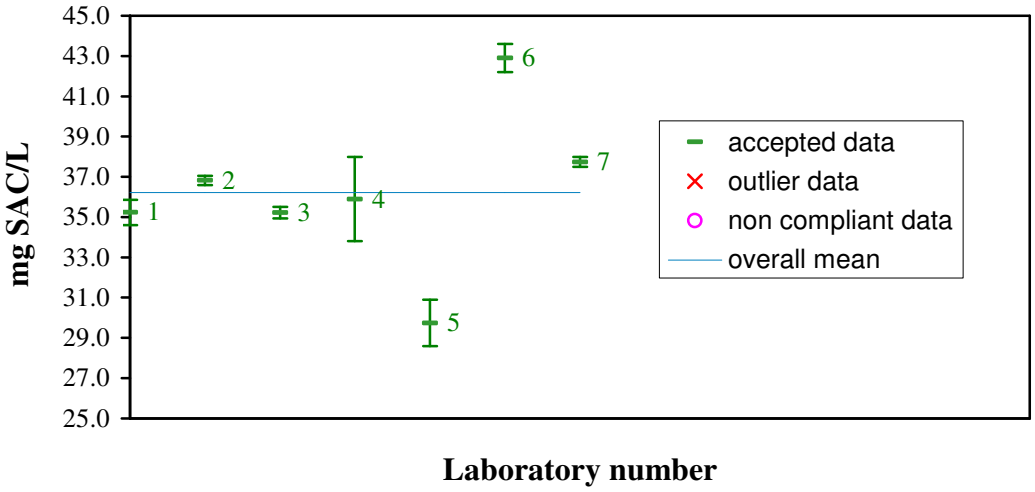

Figure F 8. Laboratory means and ranges of determined SAC amounts in sample 2

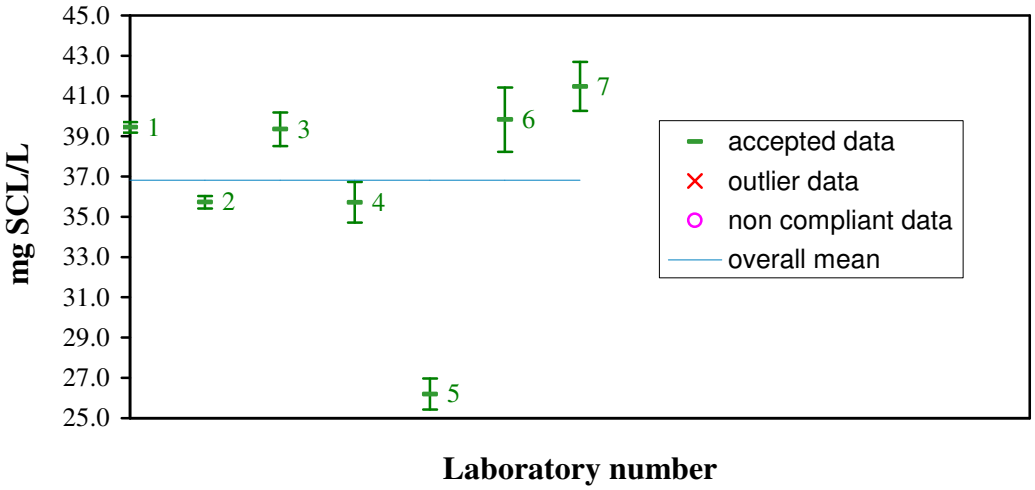

Figure F 9. Laboratory means and ranges of determined SCL amounts in sample 2

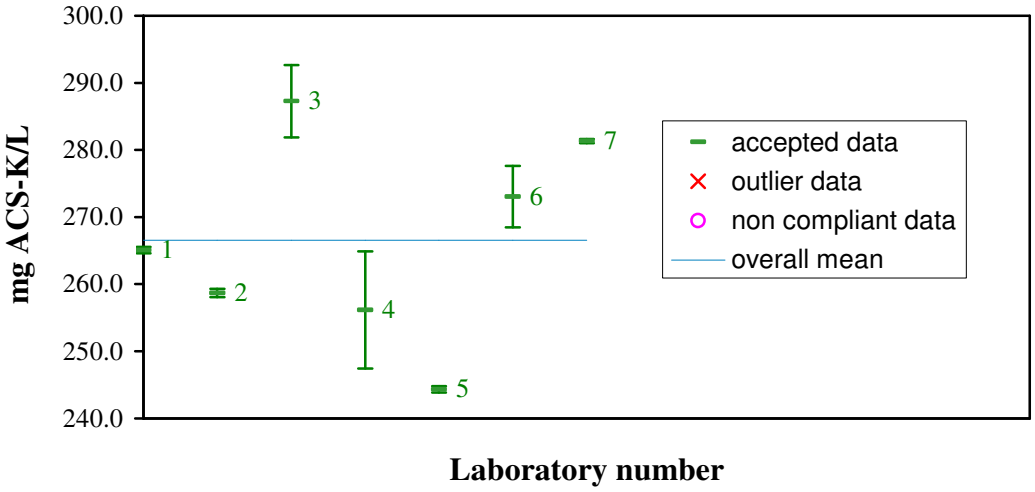

Figure F 10. Laboratory means and ranges of determined ACS-K amounts in sample 3

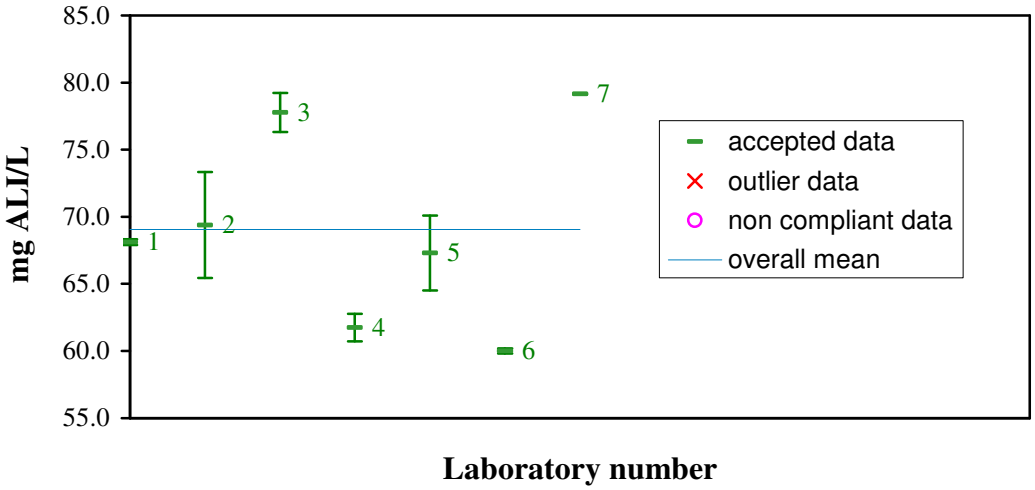

Figure F 11. Laboratory means and ranges of determined ALI amounts in sample 3

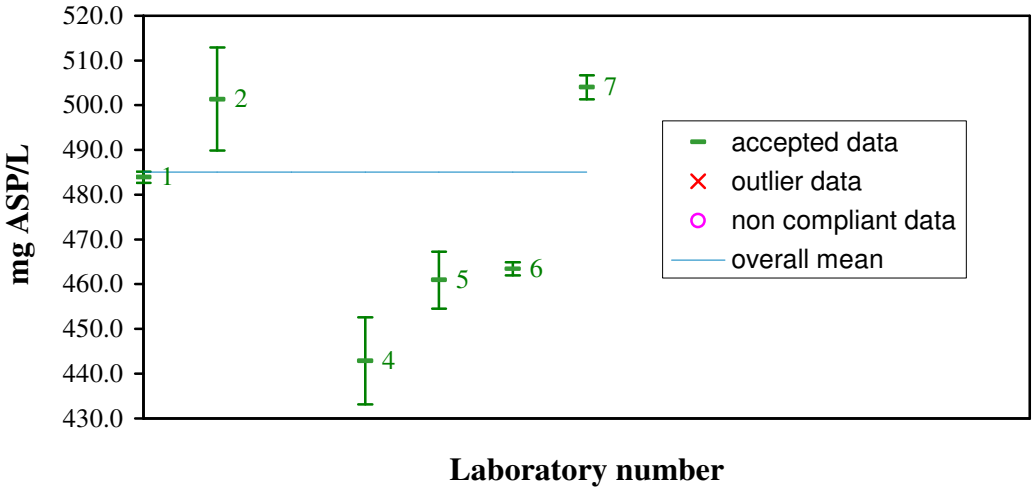

Figure F 12. Laboratory means and ranges of determined ASP amounts in sample 3

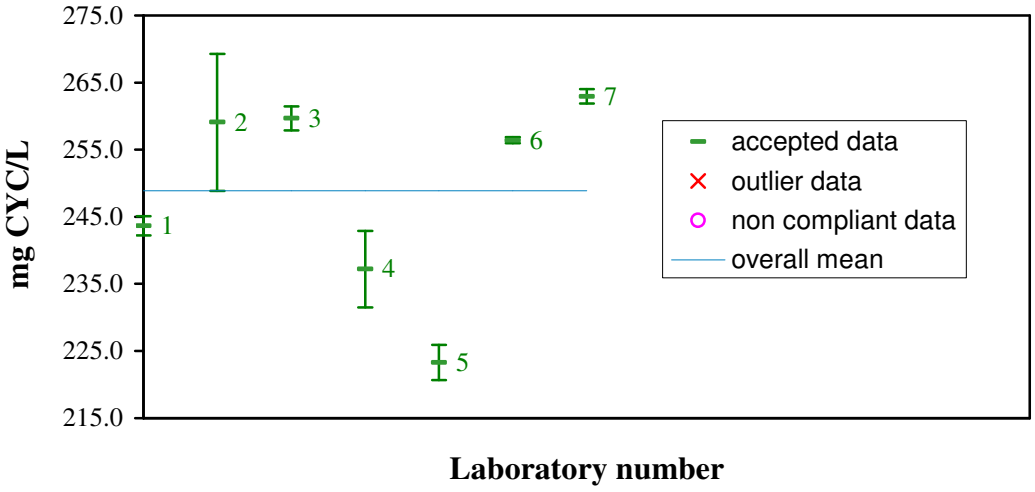

Figure F 13. Laboratory means and ranges of determined CYC amounts in sample 3

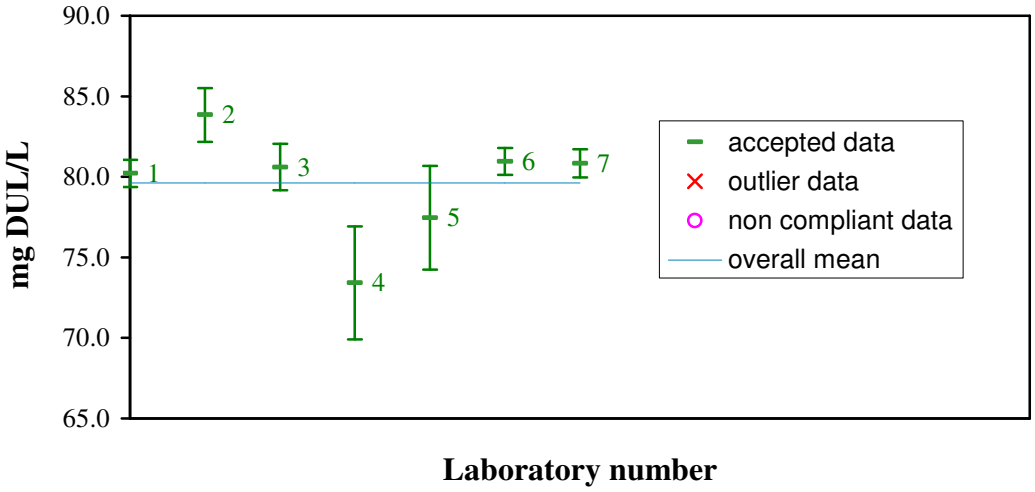

Figure F 14. Laboratory means and ranges of determined DUL amounts in sample 3

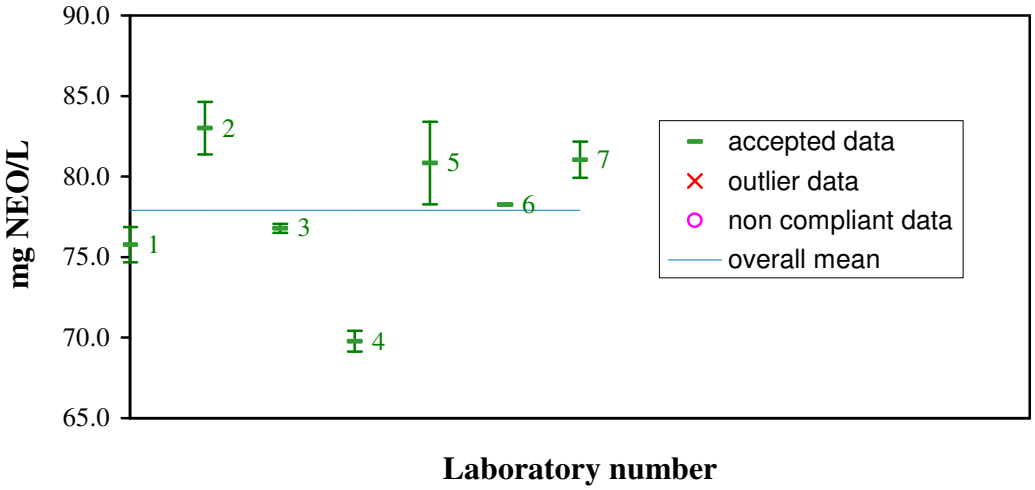

Figure F 15. Laboratory means and ranges of determined NEO amounts in sample 3

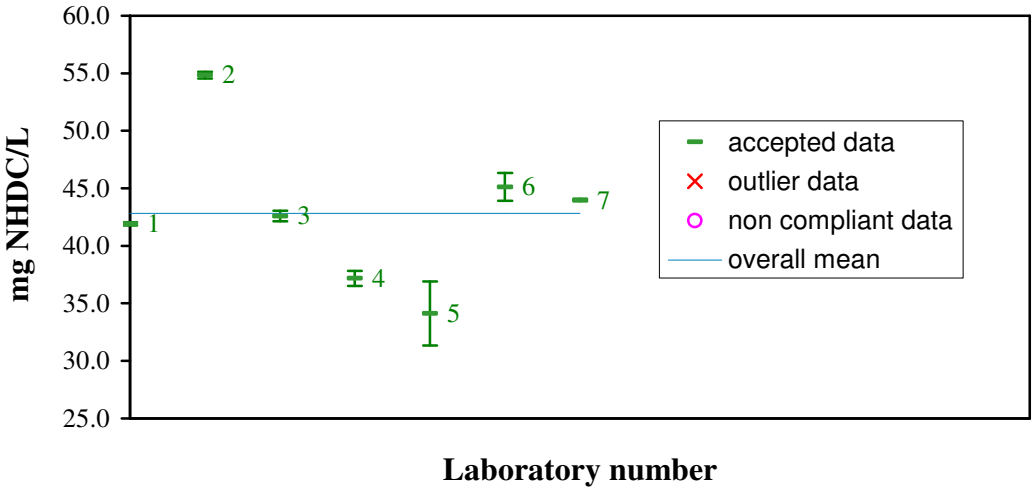

Figure F 16. Laboratory means and ranges of determined NHDC amounts in sample 3

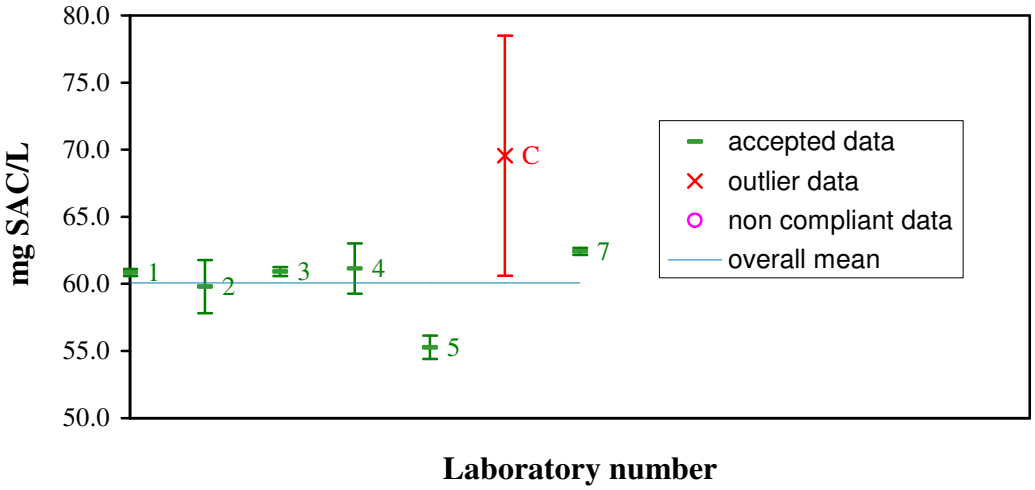

Figure F 17. Laboratory means and ranges of determined SAC amounts in sample 3

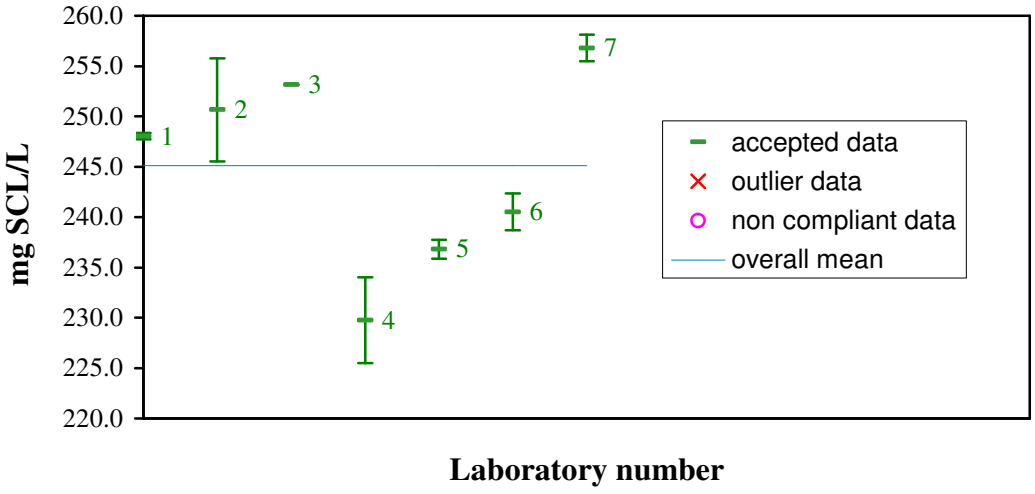

Figure F 18. Laboratory means and ranges of determined SCL amounts in sample 3

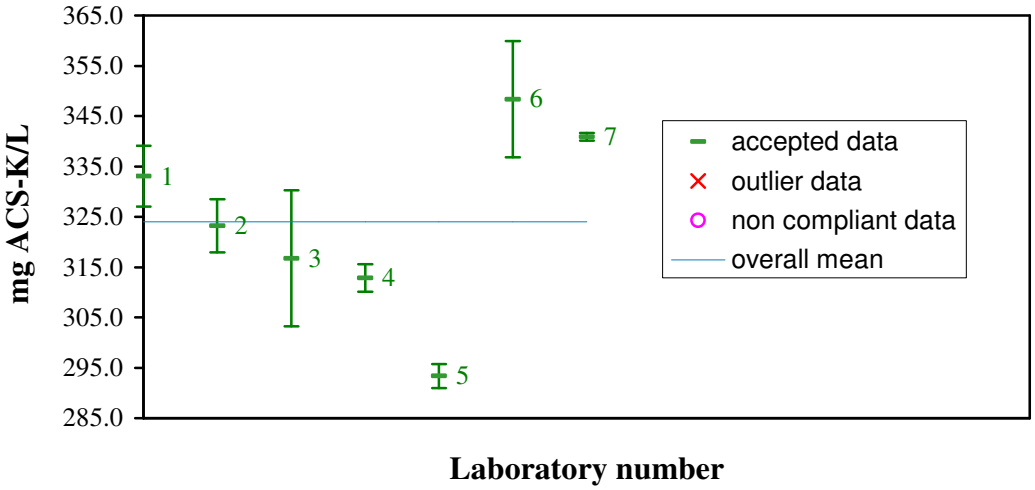

Figure F 19. Laboratory means and ranges of determined ACS-K amounts in sample 4

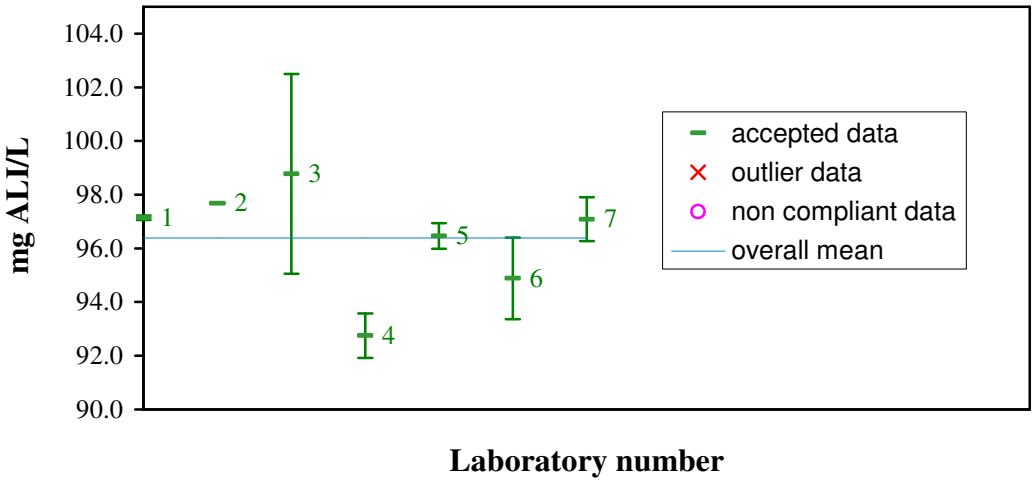

Figure F 20. Laboratory means and ranges of determined ALI amounts in sample 4

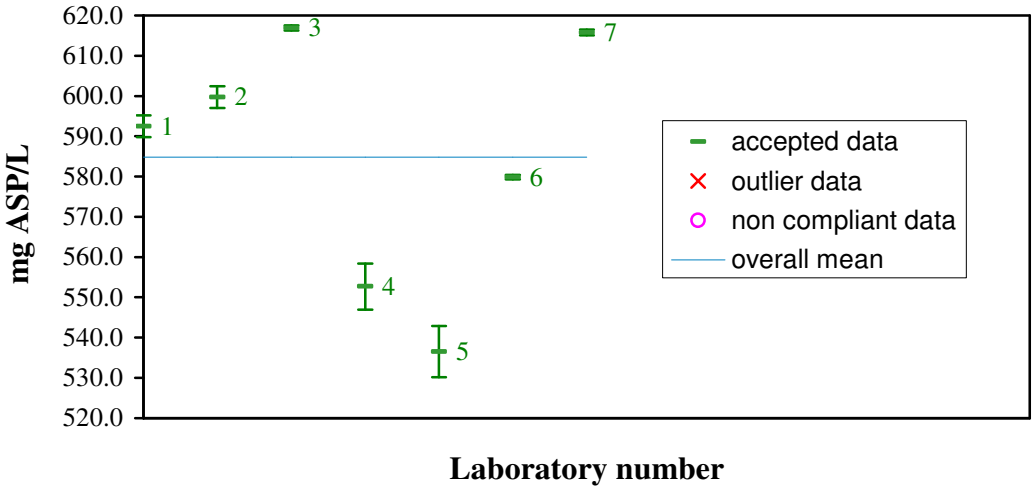

Figure F 21. Laboratory means and ranges of determined ASP amounts in sample 4

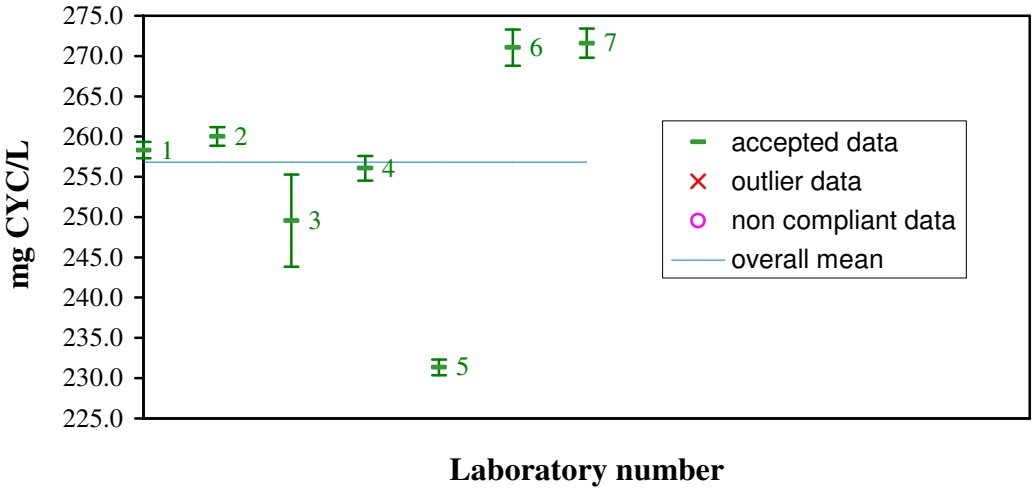

Figure F 22. Laboratory means and ranges of determined CYC amounts in sample 4

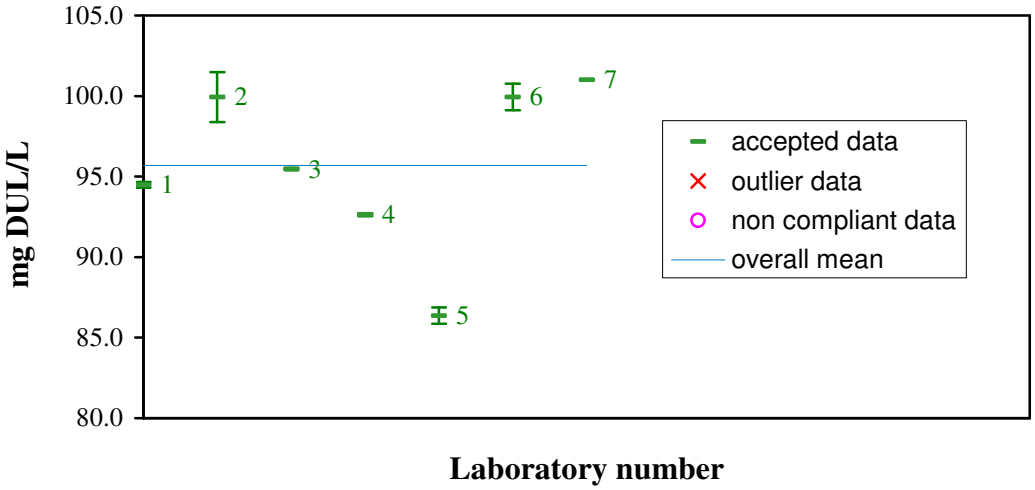

Figure F 23. Laboratory means and ranges of determined DUL amounts in sample 4

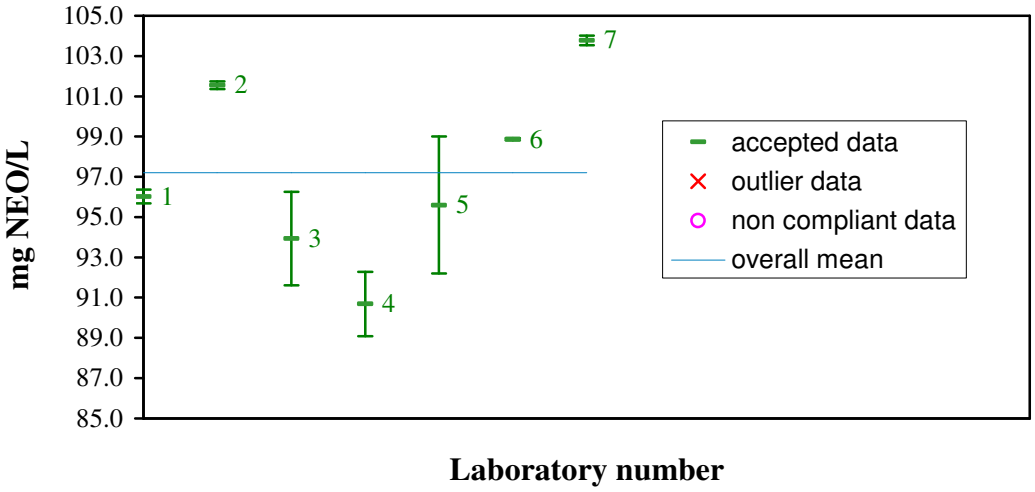

Figure F 24. Laboratory means and ranges of determined NEO amounts in sample 4

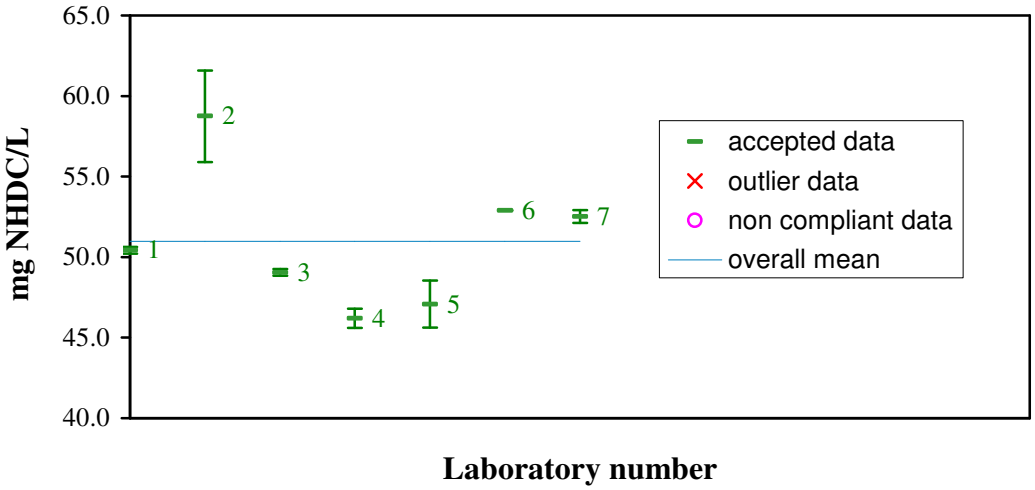

Figure F 25. Laboratory means and ranges of determined NHDC amounts in sample 4

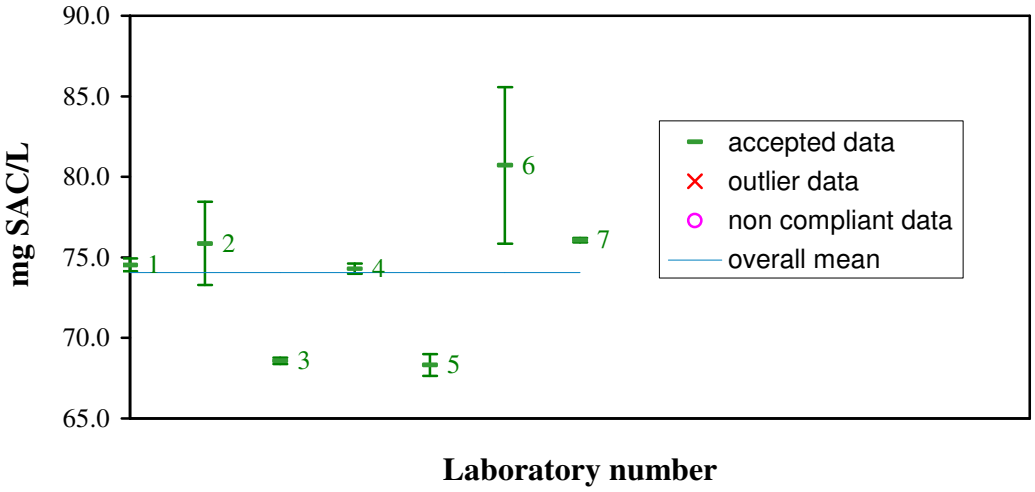

Figure F 26. Laboratory means and ranges of determined SAC amounts in sample 4

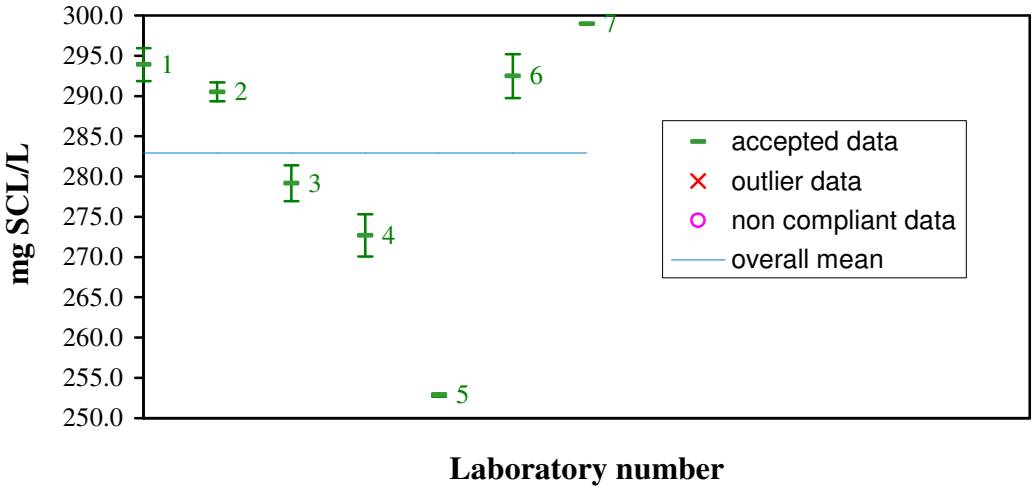

Figure F 27. Laboratory means and ranges of determined SCL amounts in sample 4

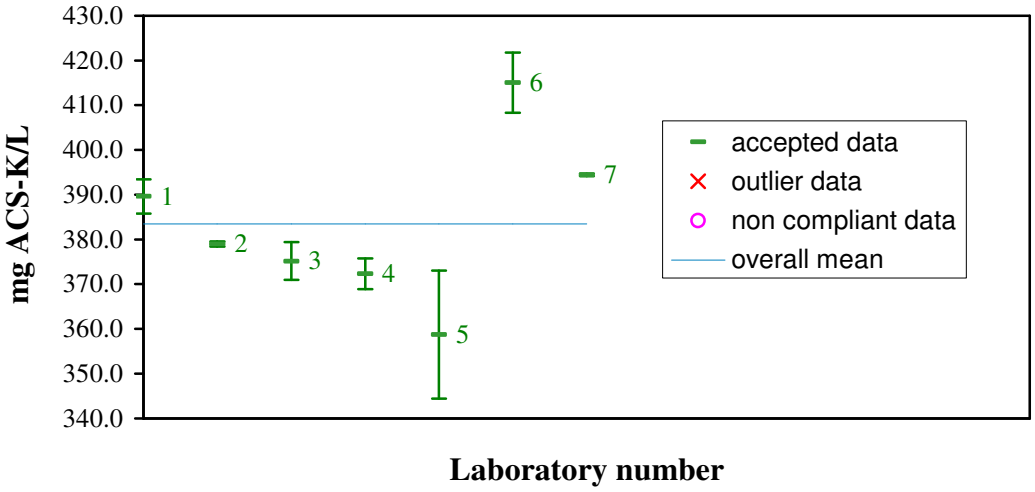

Figure F 28. Laboratory means and ranges of determined ACS-K amounts in sample 5

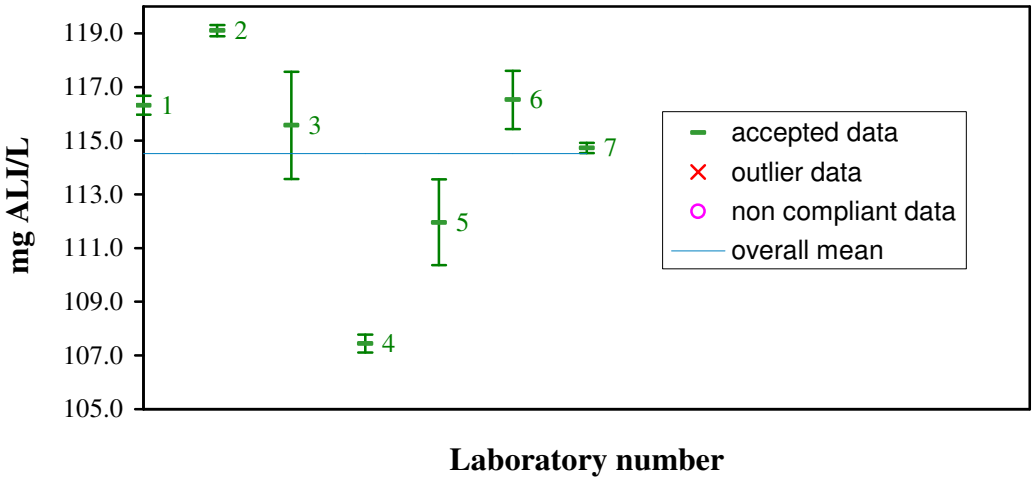

Figure F 29. Laboratory means and ranges of determined ALI amounts in sample 5

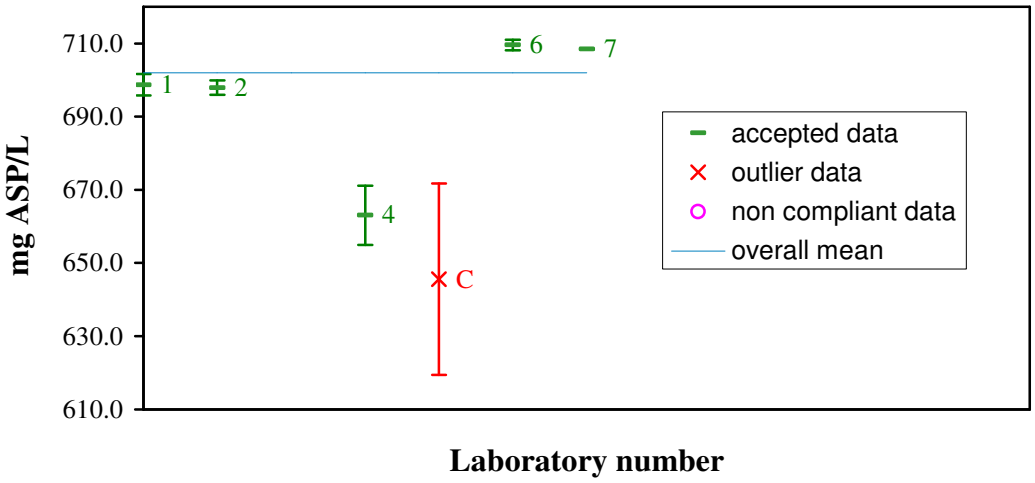

Figure F 30. Laboratory means and ranges of determined ASP amounts in sample 5

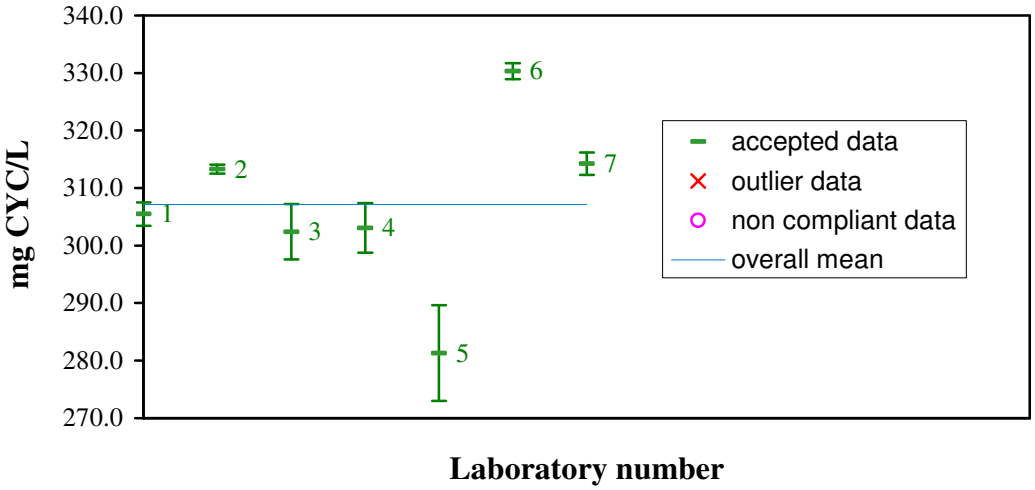

Figure F 31. Laboratory means and ranges of determined CYC amounts in sample 5

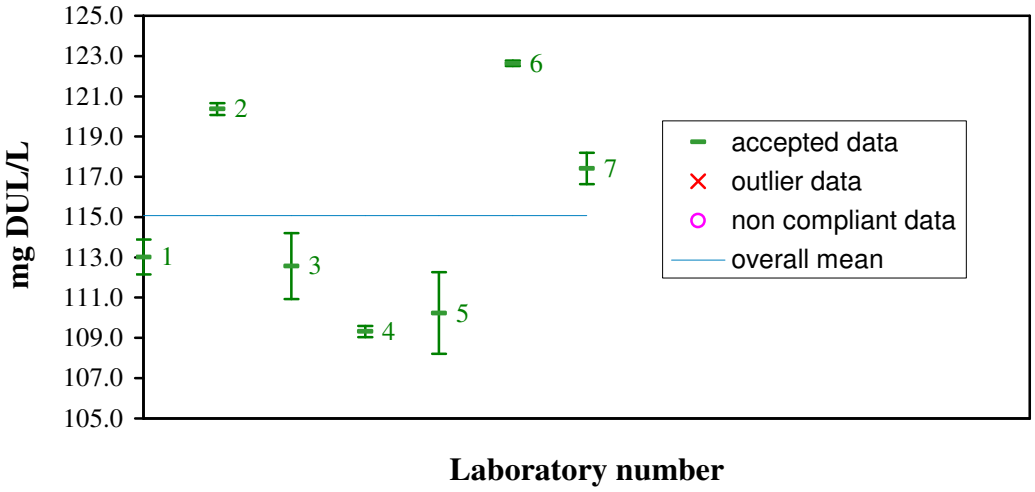

Figure F 32. Laboratory means and ranges of determined DUL amounts in sample 5

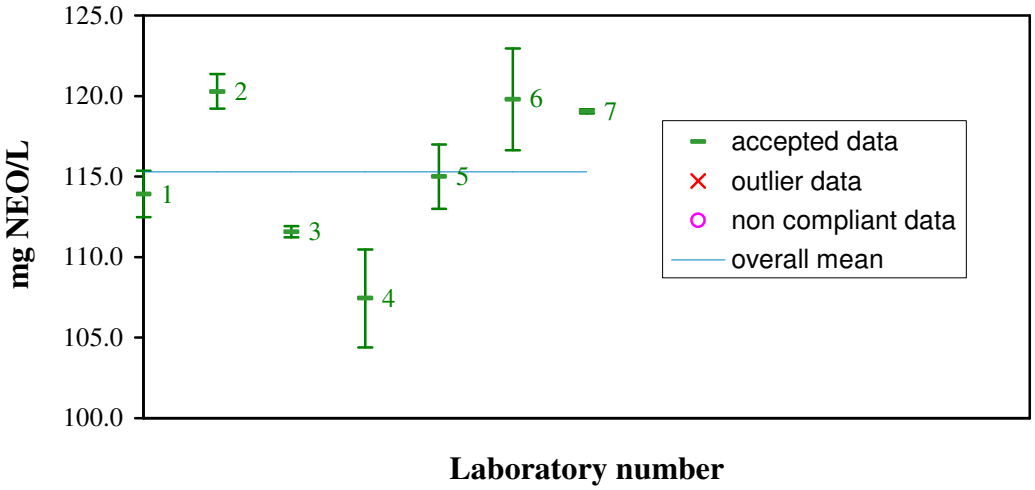

Figure F 33. Laboratory means and ranges of determined NEO amounts in sample 5

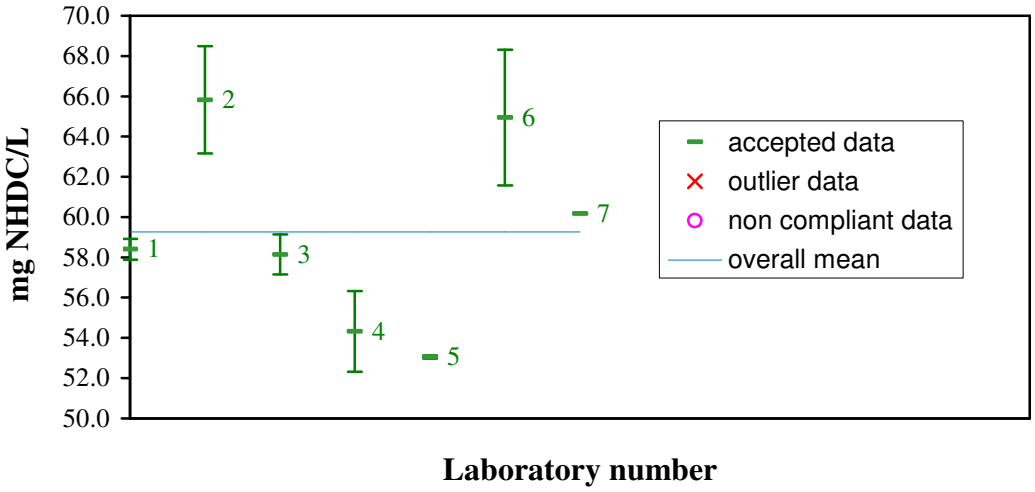

Figure F 34. Laboratory means and ranges of determined NHDC amounts in sample 5

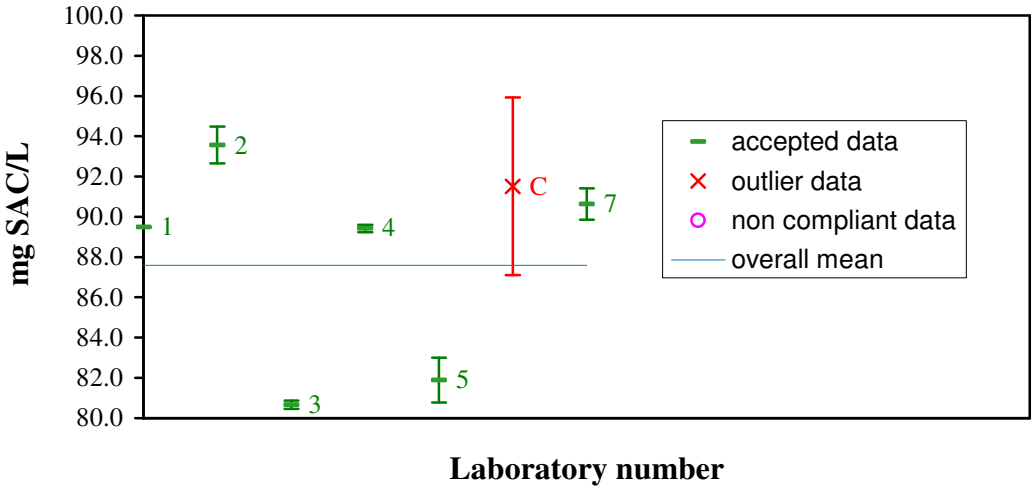

Figure F 35. Laboratory means and ranges of determined SAC amounts in sample 5

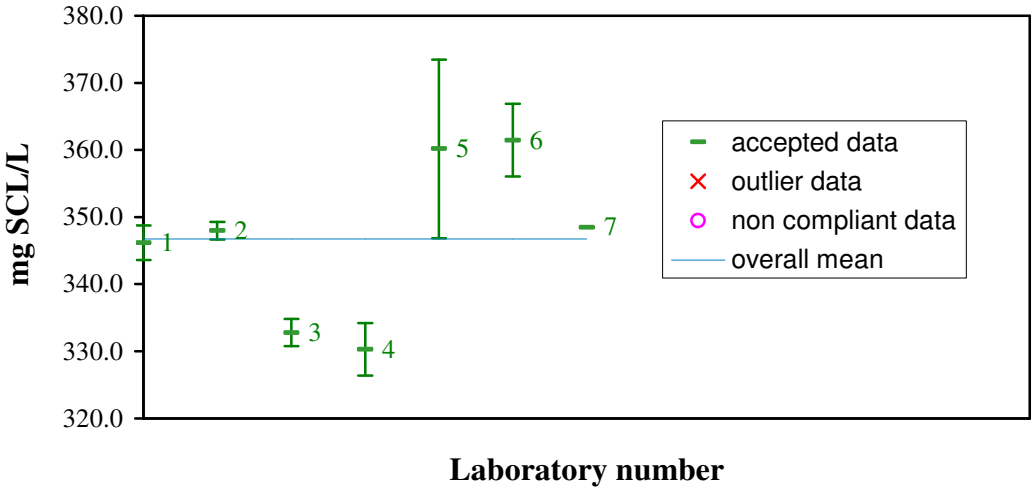

Figure F 36. Laboratory means and ranges of determined SCL amounts in sample 5

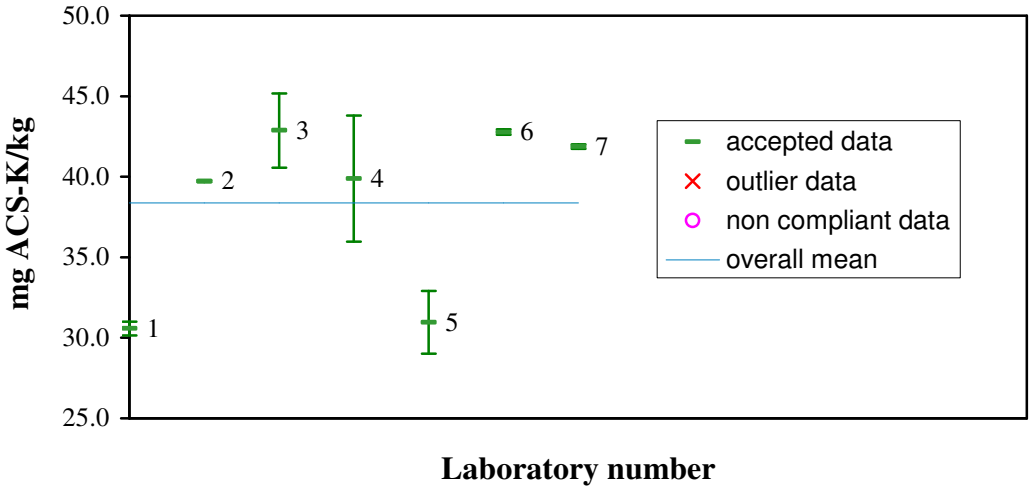

Figure F 37. Laboratory means and ranges of determined ACS-K amounts in sample 7

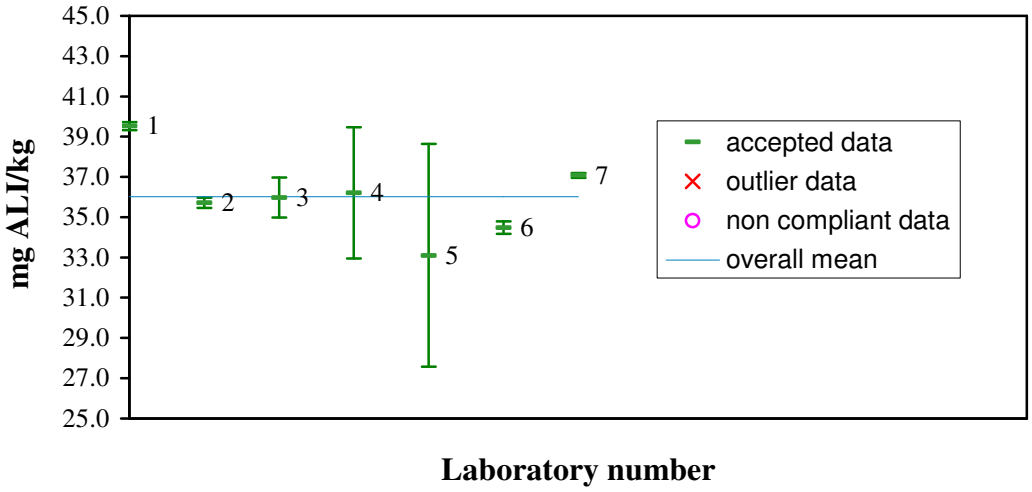

Figure F 38. Laboratory means and ranges of determined ALI amounts in sample 7

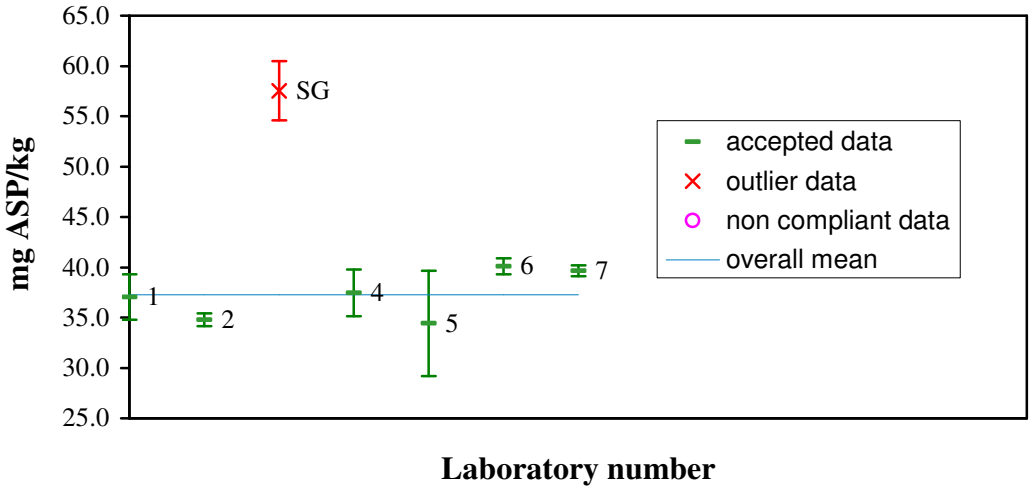

Figure F 39. Laboratory means and ranges of determined ASP amounts in sample 7

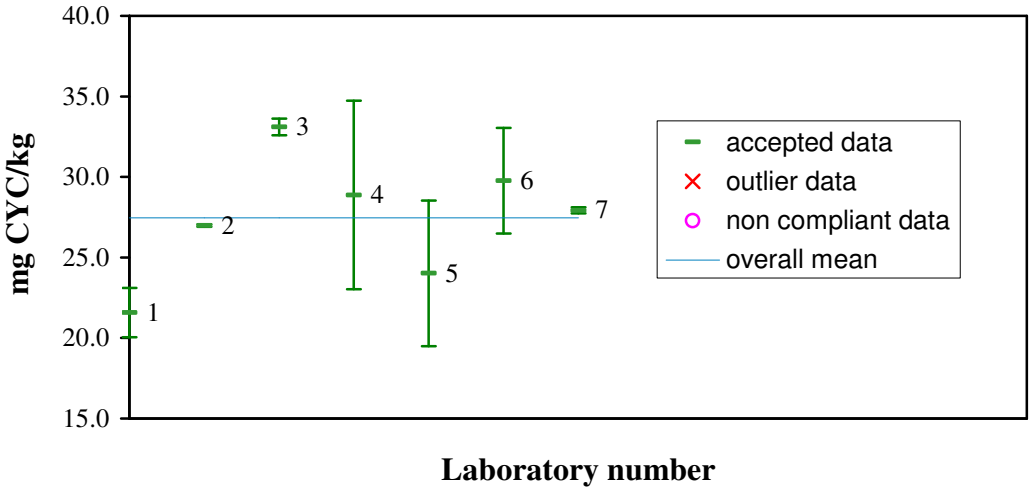

Figure F 40. Laboratory means and ranges of determined CYC amounts in sample 7

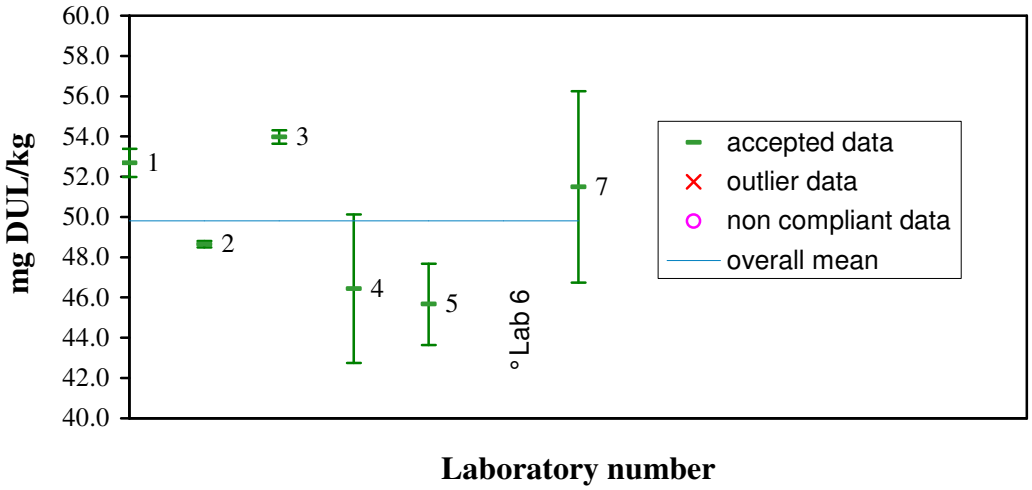

Figure F 41. Laboratory means and ranges of determined DUL amounts in sample 7

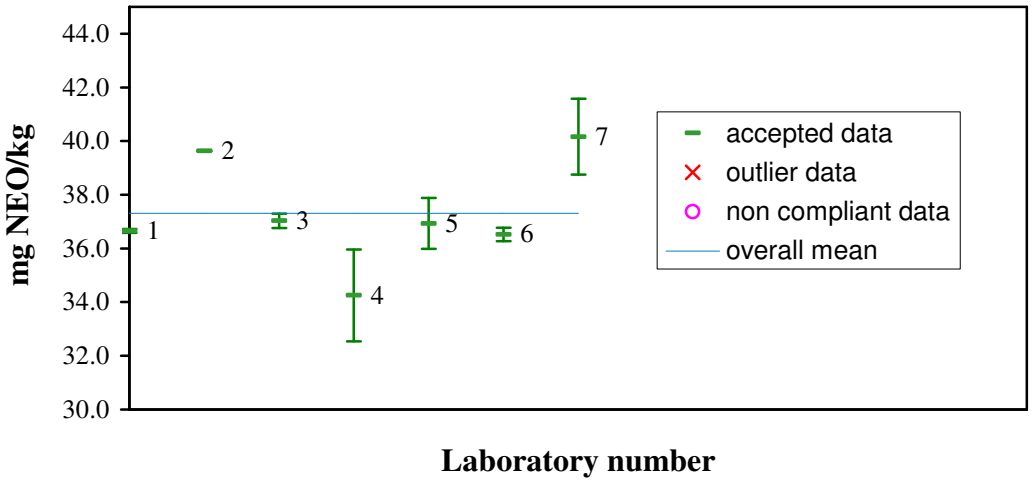

Figure F 42. Laboratory means and ranges of determined NEO amounts in sample 7

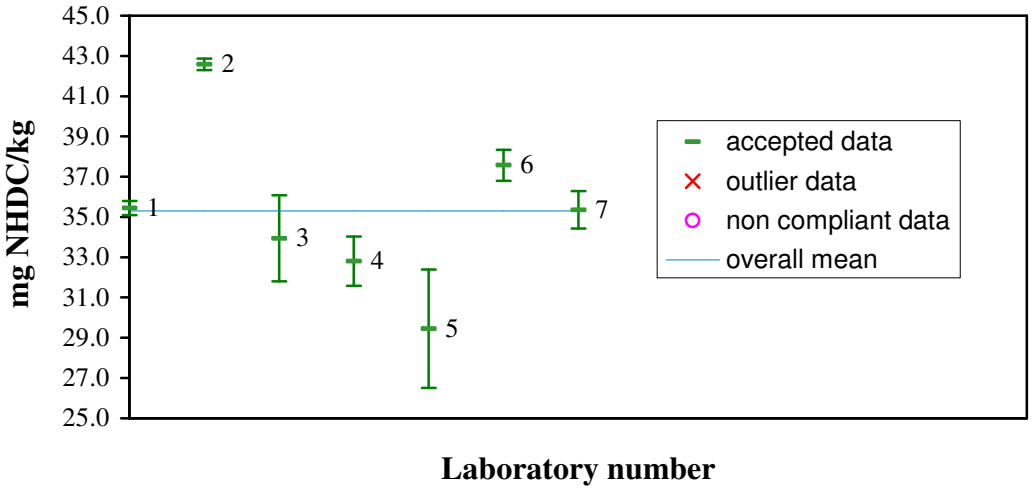

Figure F 43. Laboratory means and ranges of determined NHDC amounts in sample 7

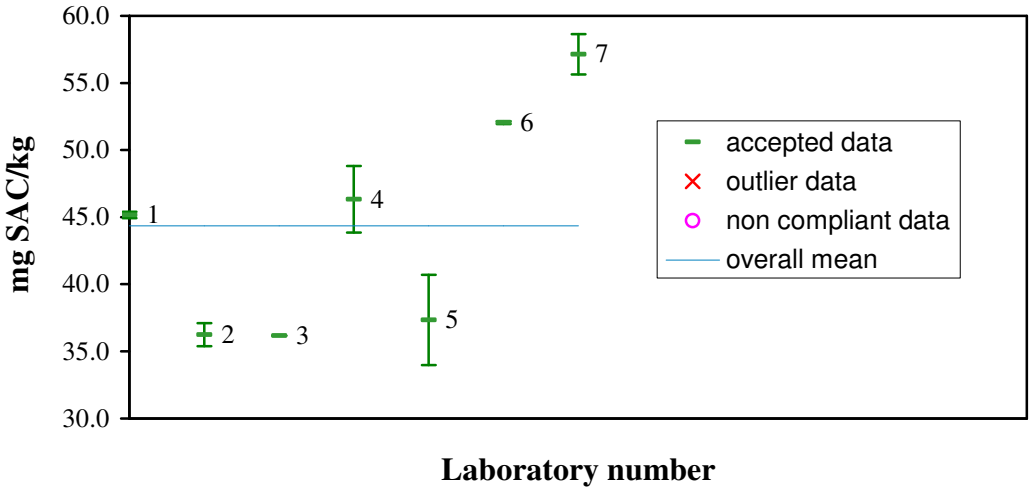

Figure F 44. Laboratory means and ranges of determined SAC amounts in sample 7

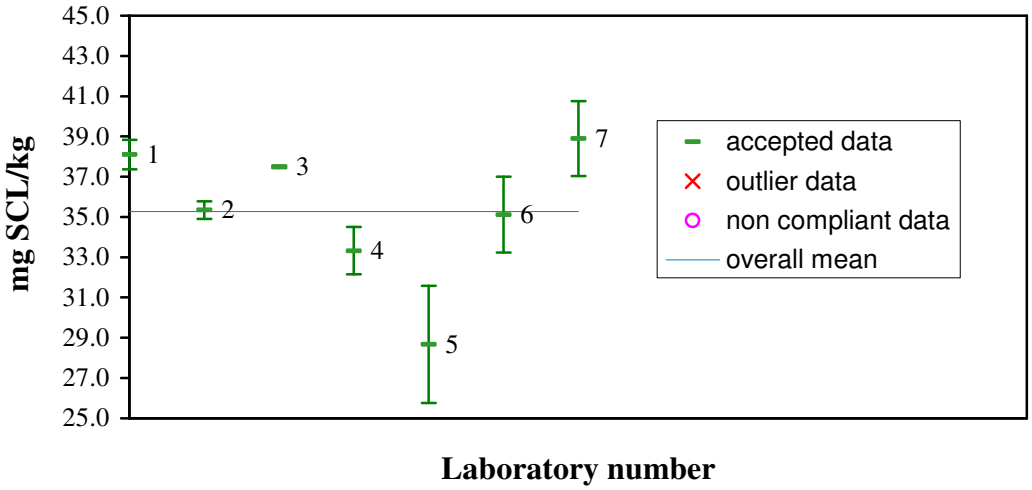

Figure F 45. Laboratory means and ranges of determined SCL amounts in sample 7

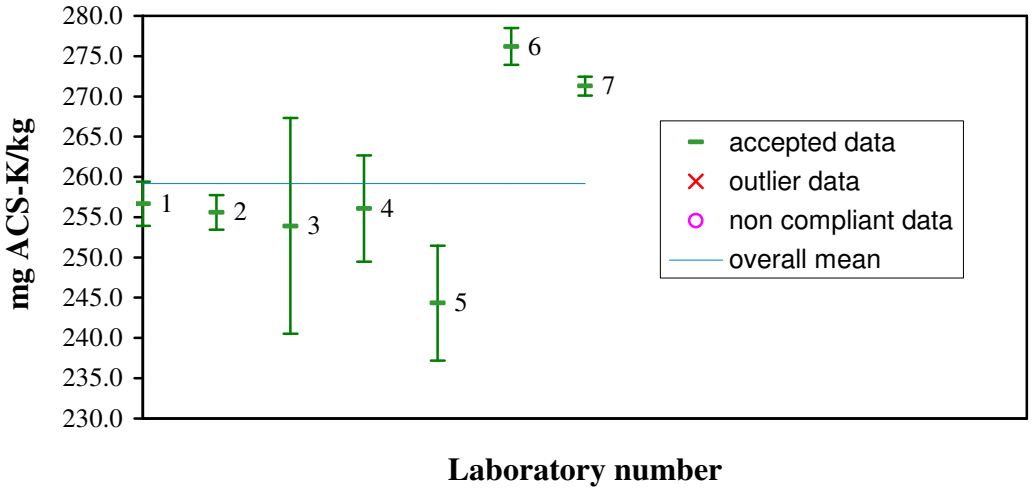

Figure F 46. Laboratory means and ranges of determined ACS-K amounts in sample 8

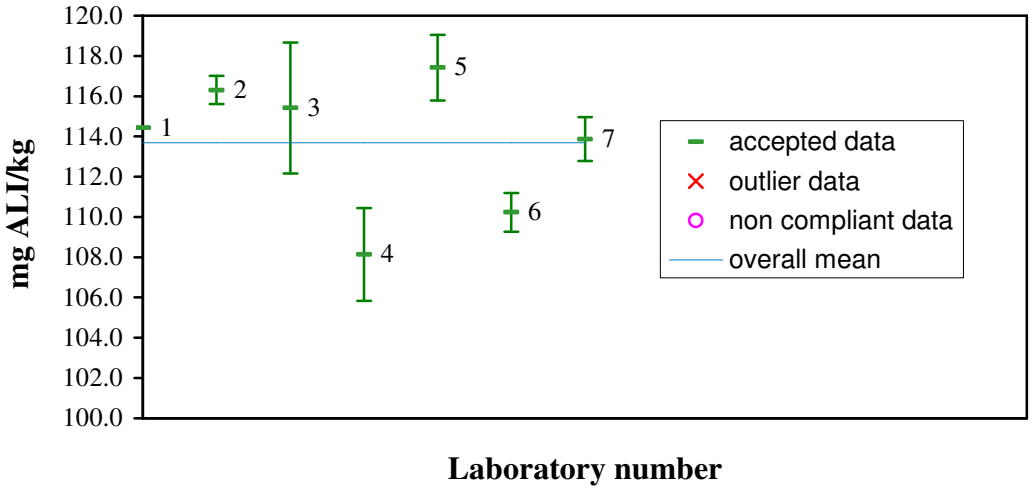

Figure F 47. Laboratory means and ranges of determined ALI amounts in sample 8

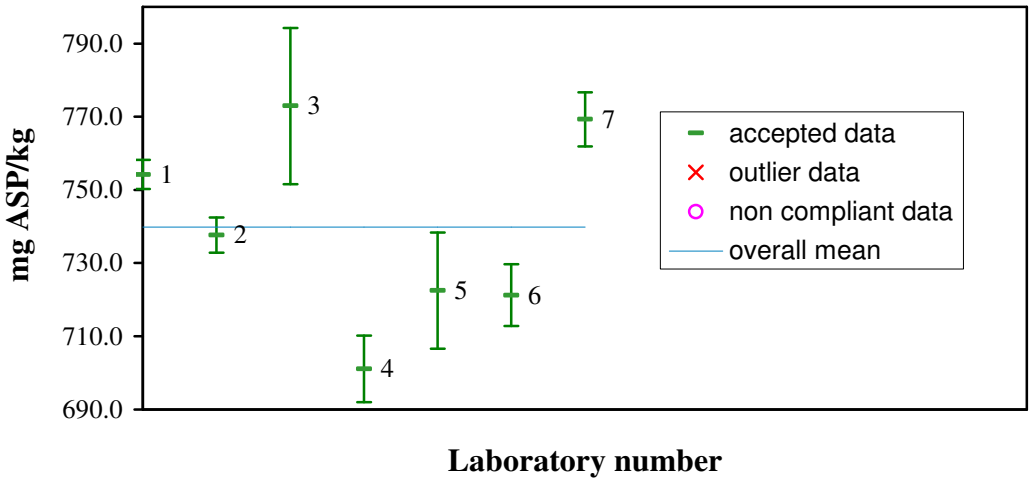

Figure F 48. Laboratory means and ranges of determined ASP amounts in sample 8

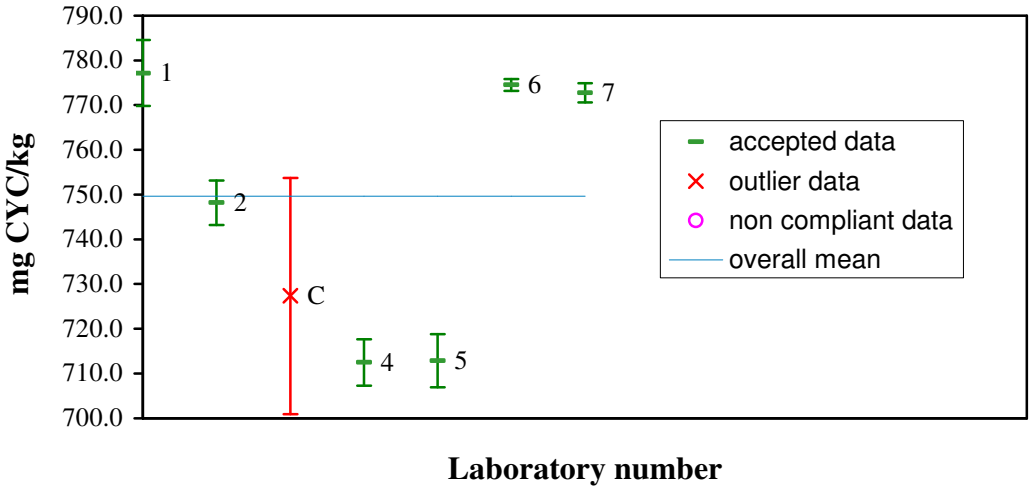

Figure F 49. Laboratory means and ranges of determined CYC amounts in sample 8

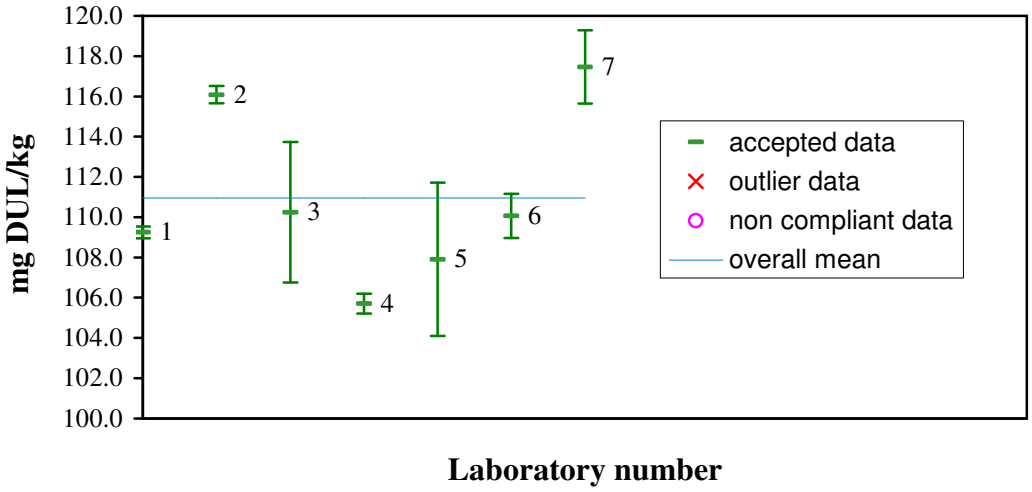

Figure F 50. Laboratory means and ranges of determined DUL amounts in sample 8

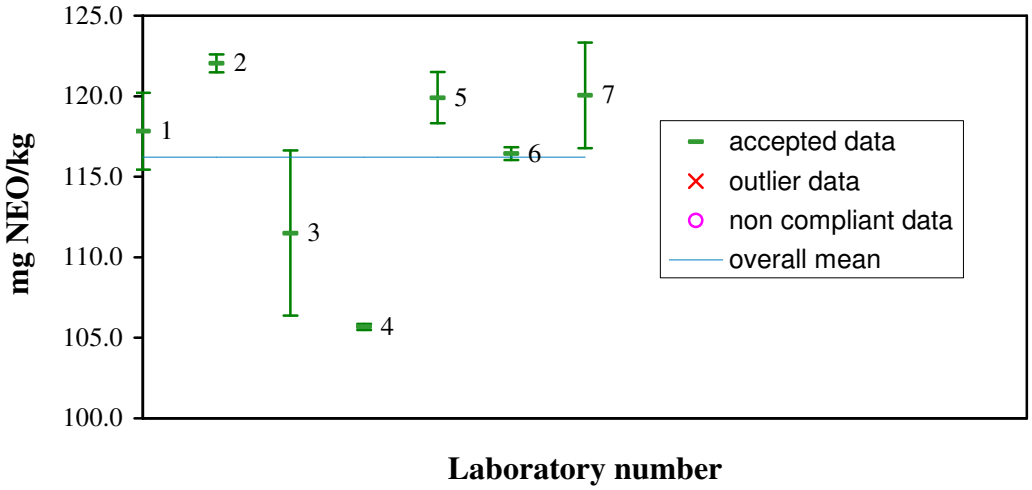

Figure F 51. Laboratory means and ranges of determined NEO amounts in sample 8

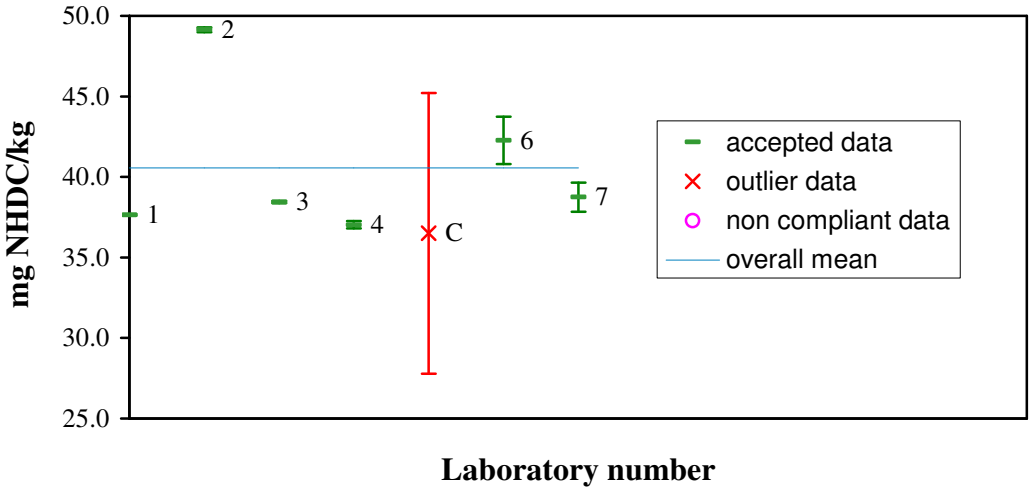

Figure F 52. Laboratory means and ranges of determined NHDC amounts in sample 8

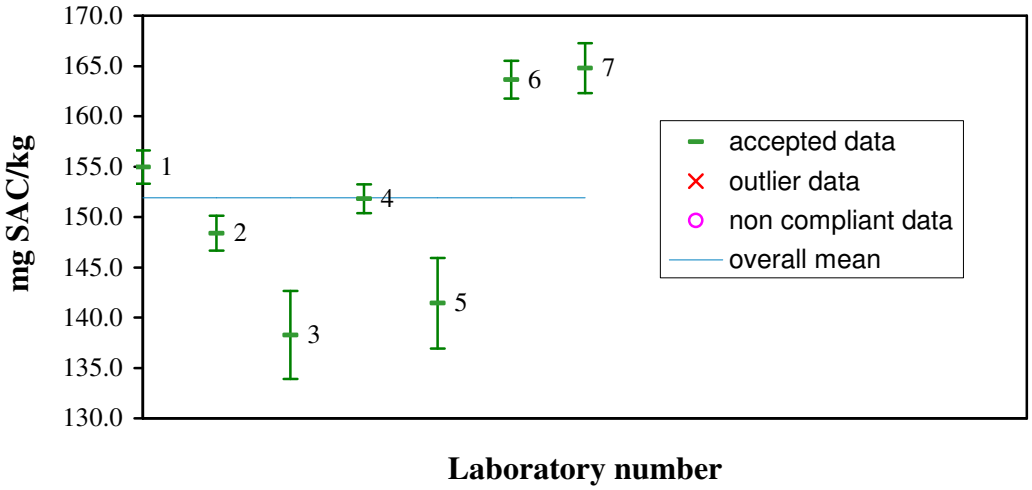

Figure F 53. Laboratory means and ranges of determined SAC amounts in sample 8

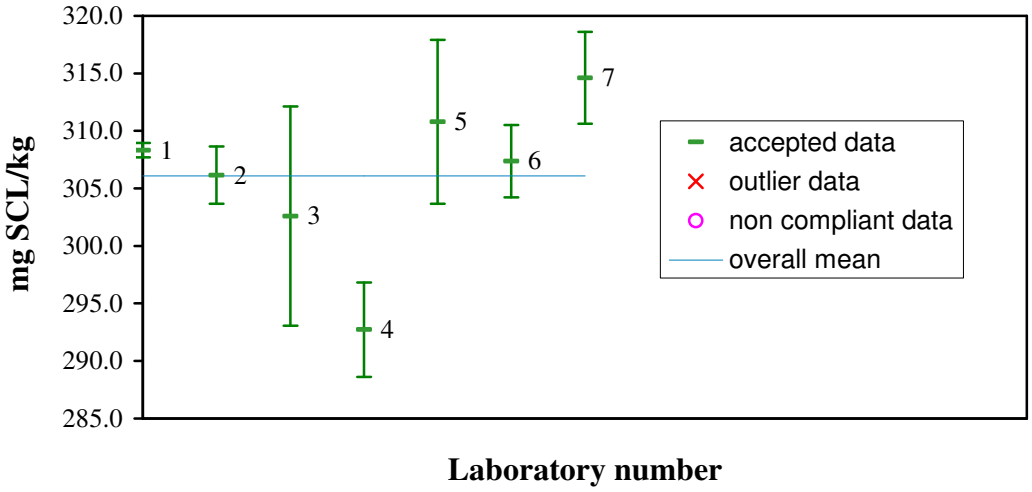

Figure F 54. Laboratory means and ranges of determined SCL amounts in sample 8

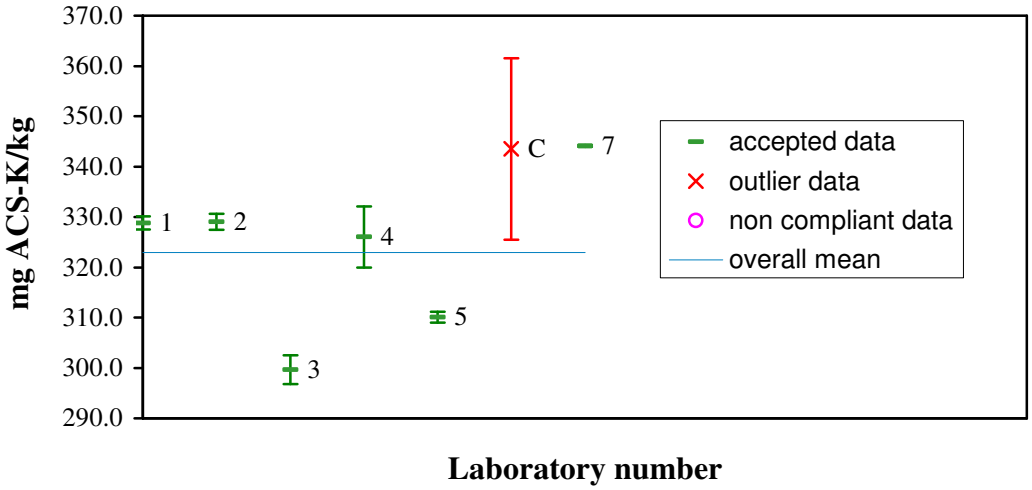

Figure F 55. Laboratory means and ranges of determined ACS-K amounts in sample 9

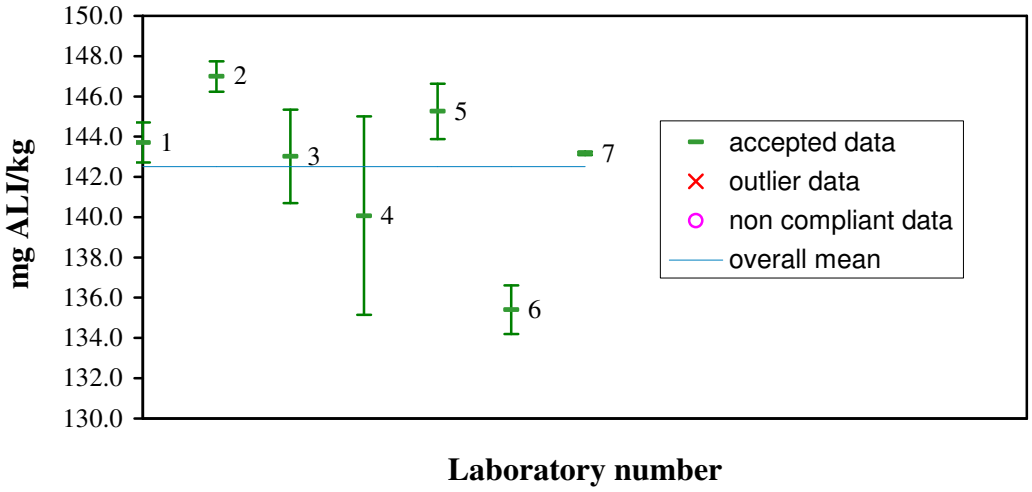

Figure F 56. Laboratory means and ranges of determined ALI amounts in sample 9

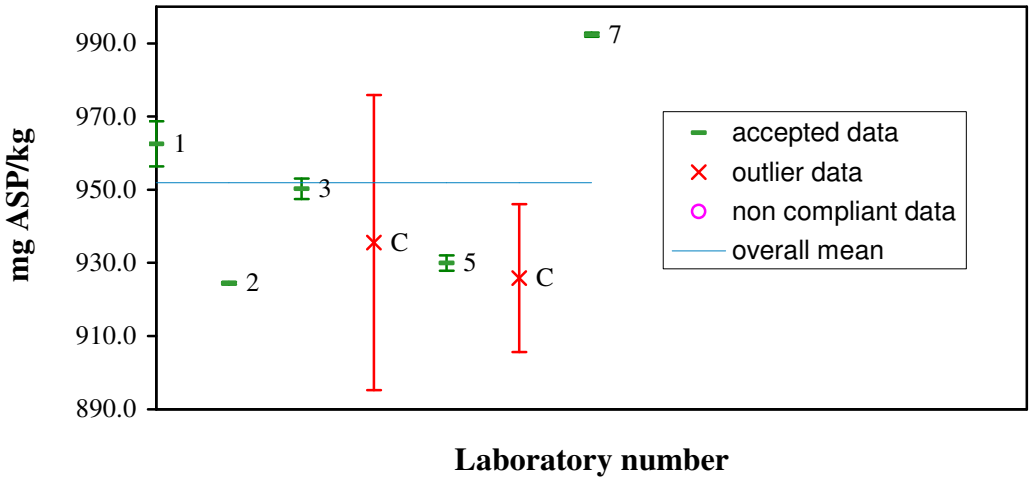

Figure F 57. Laboratory means and ranges of determined ASP amounts in sample 9

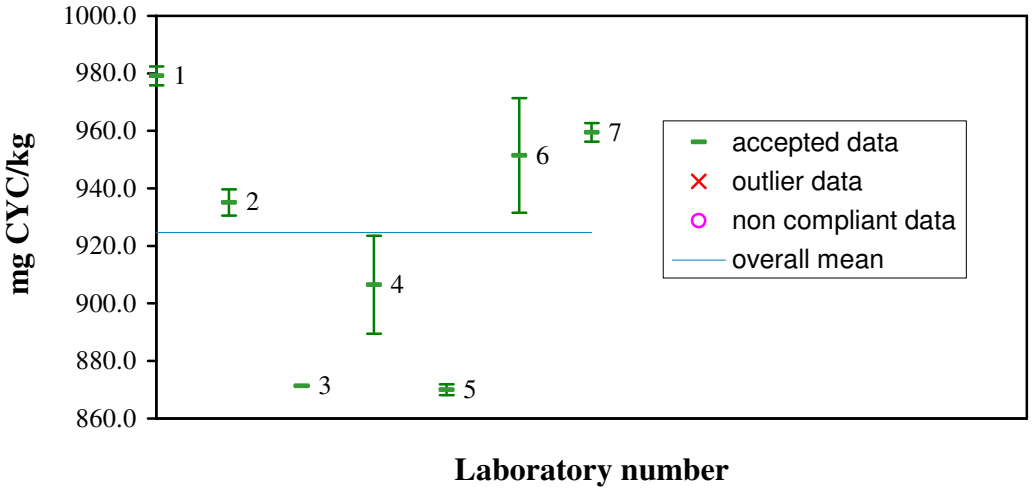

Figure F 58. Laboratory means and ranges of determined CYC amounts in sample 9

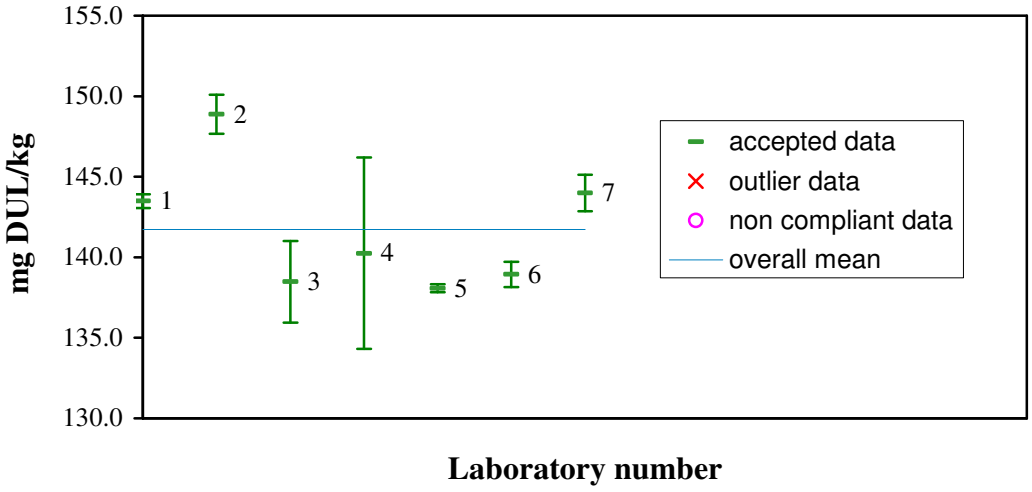

Figure F 59. Laboratory means and ranges of determined DUL amounts in sample 9

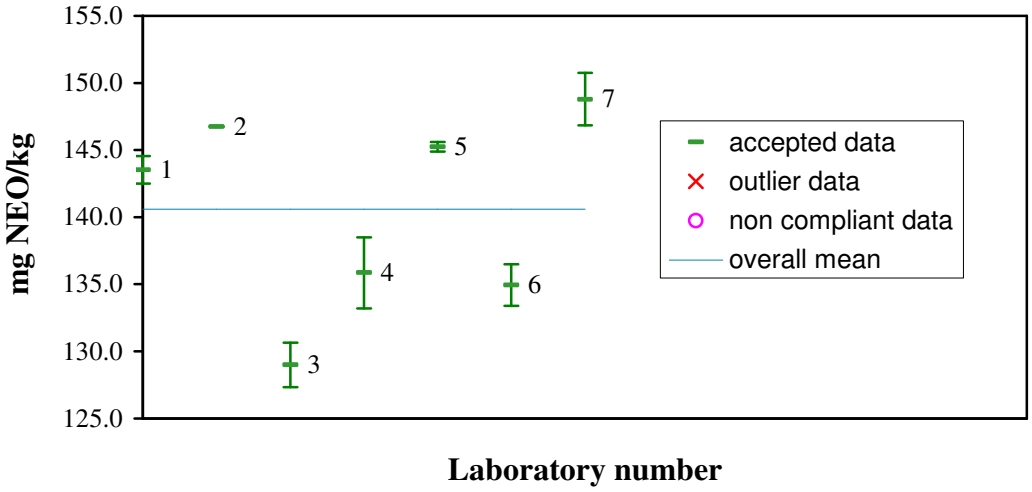

Figure F 60. Laboratory means and ranges of determined NEO amounts in sample 9

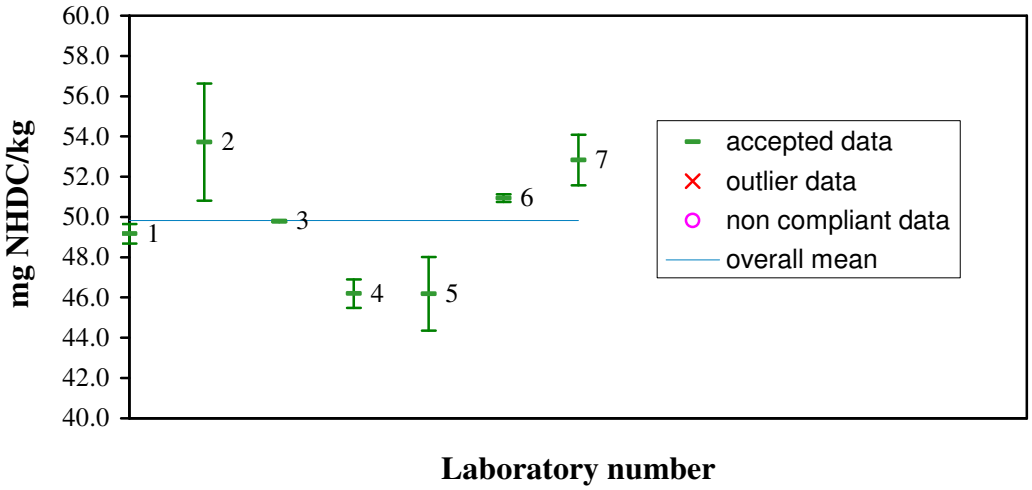

Figure F 61. Laboratory means and ranges of determined NHDC amounts in sample 9

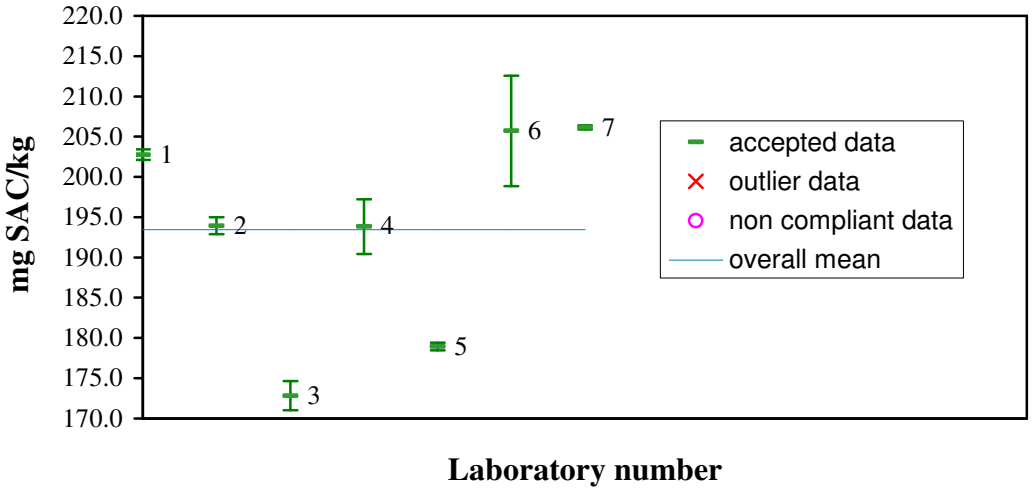

Figure F 62. Laboratory means and ranges of determined SAC amounts in sample 9

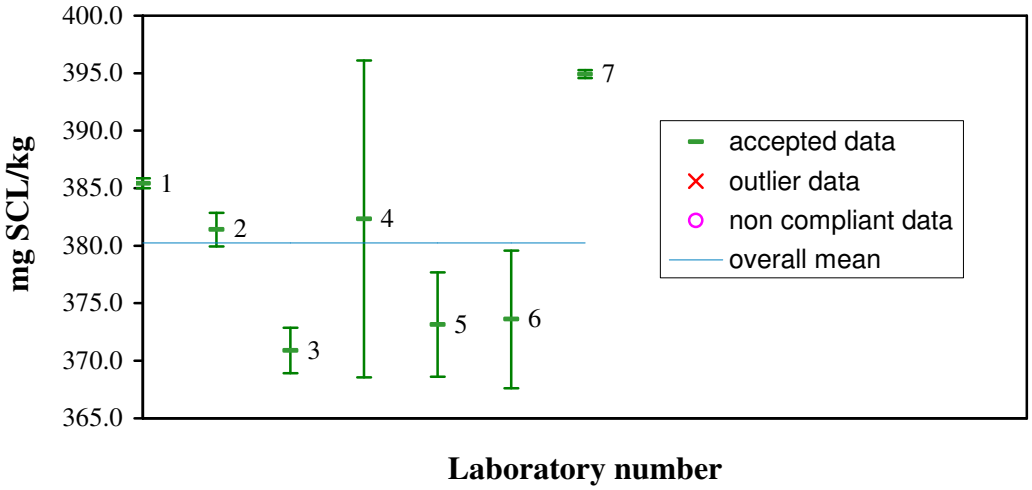

Figure F 63. Laboratory means and ranges of determined SCL amounts in sample 9

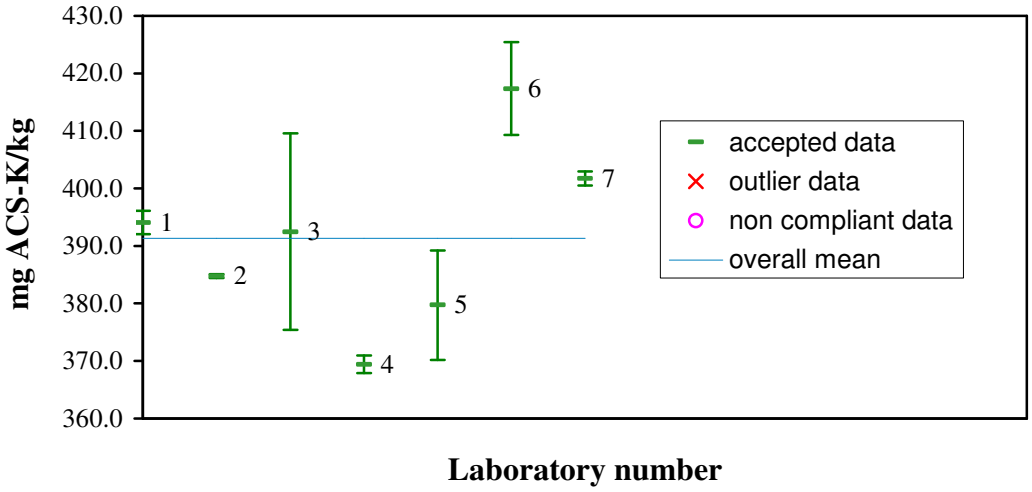

Figure F 64. Laboratory means and ranges of determined ACS-K amounts in sample 10

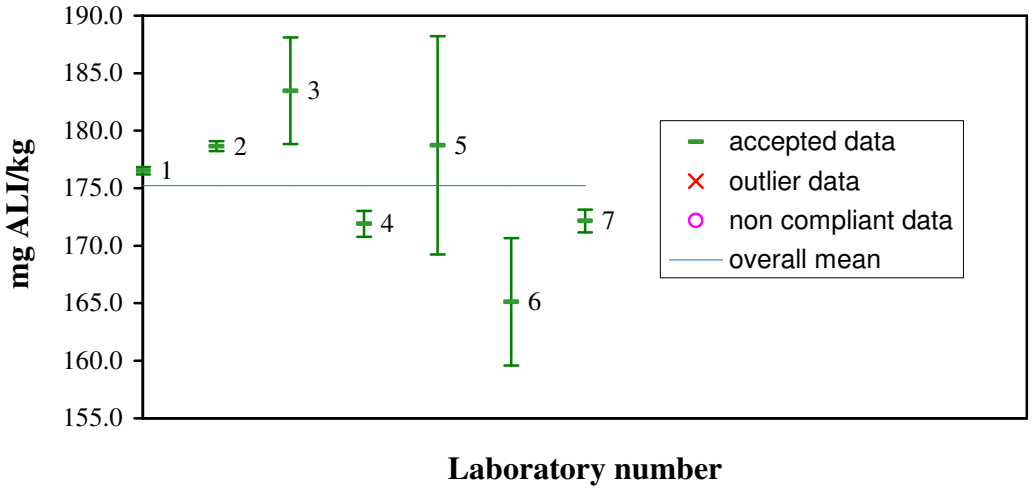

Figure F 65. Laboratory means and ranges of determined ALI amounts in sample 10

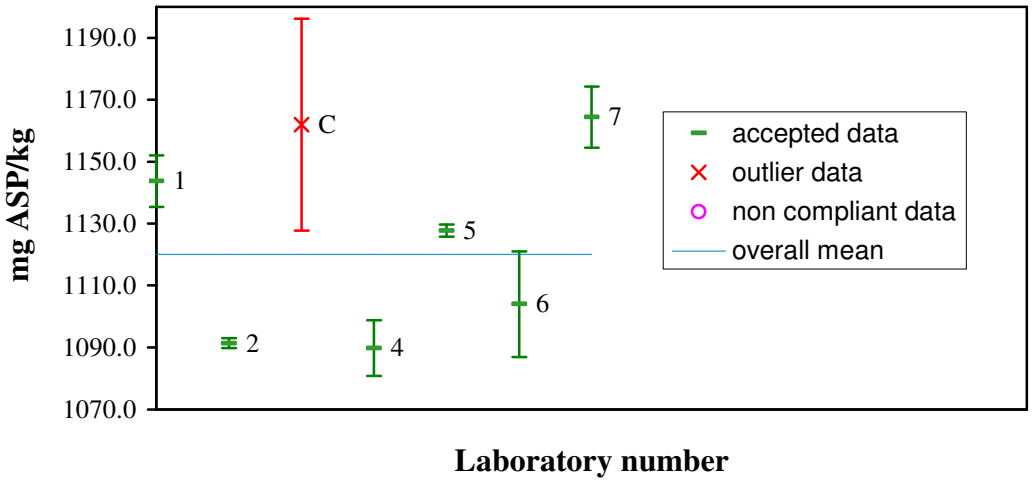

Figure F 66. Laboratory means and ranges of determined ASP amounts in sample 10

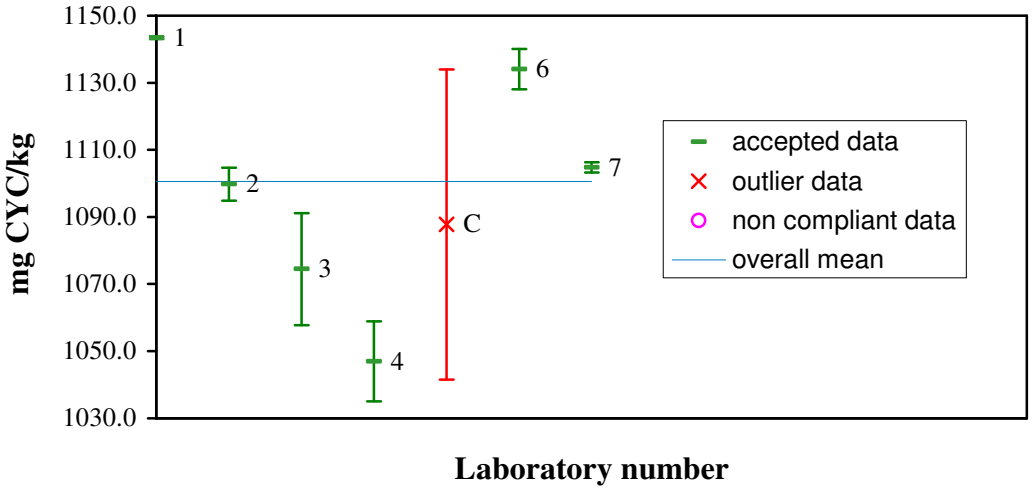

Figure F 67. Laboratory means and ranges of determined CYC amounts in sample 10

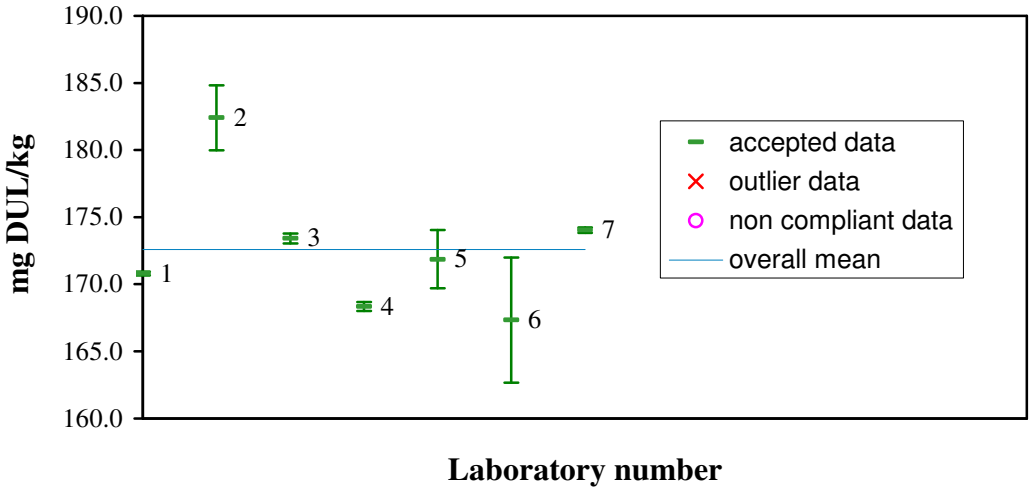

Figure F 68. Laboratory means and ranges of determined DUL amounts in sample 10

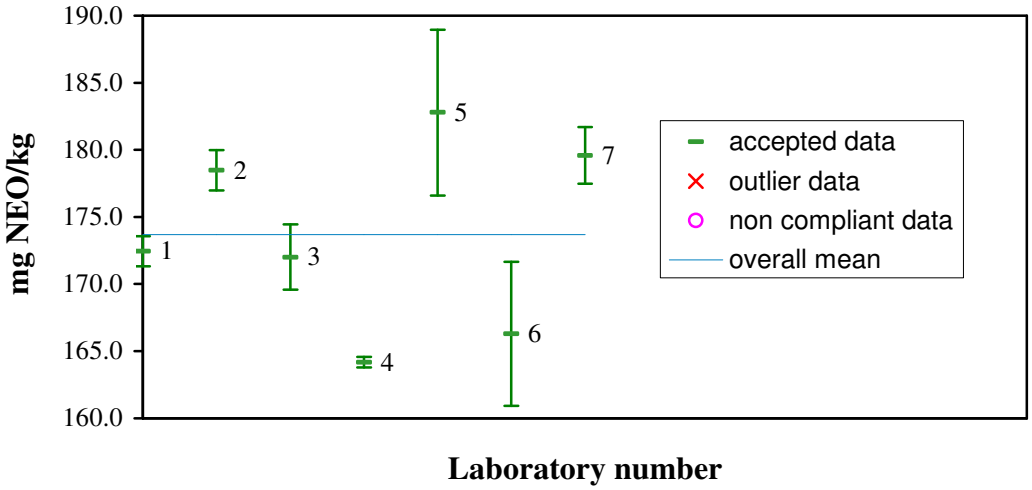

Figure F 69. Laboratory means and ranges of determined NEO amounts in sample 10

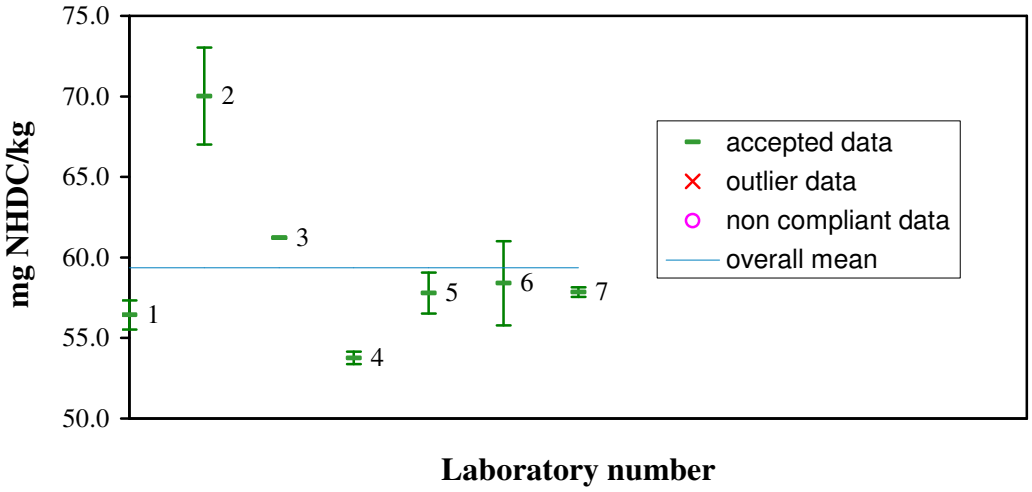

Figure F 70. Laboratory means and ranges of determined NHDC amounts in sample 10

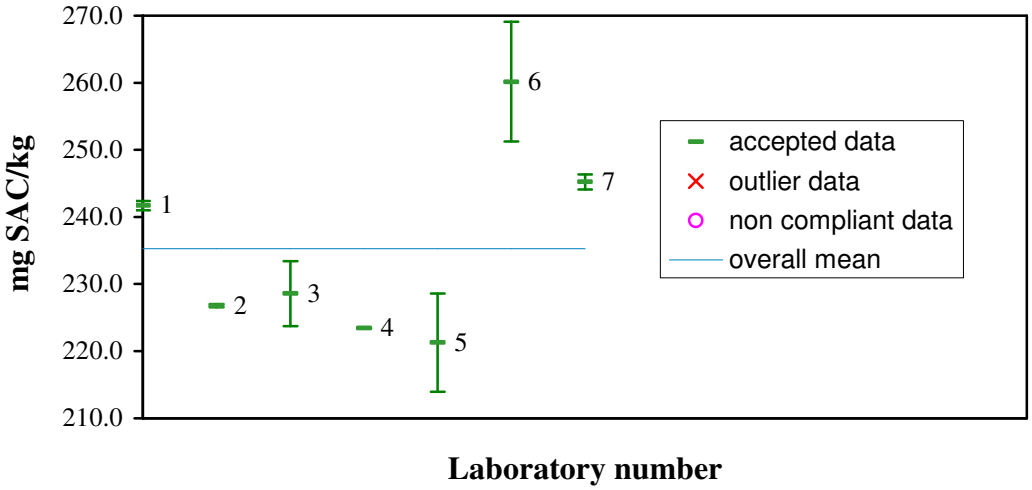

Figure F 71. Laboratory means and ranges of determined SAC amounts in sample 10

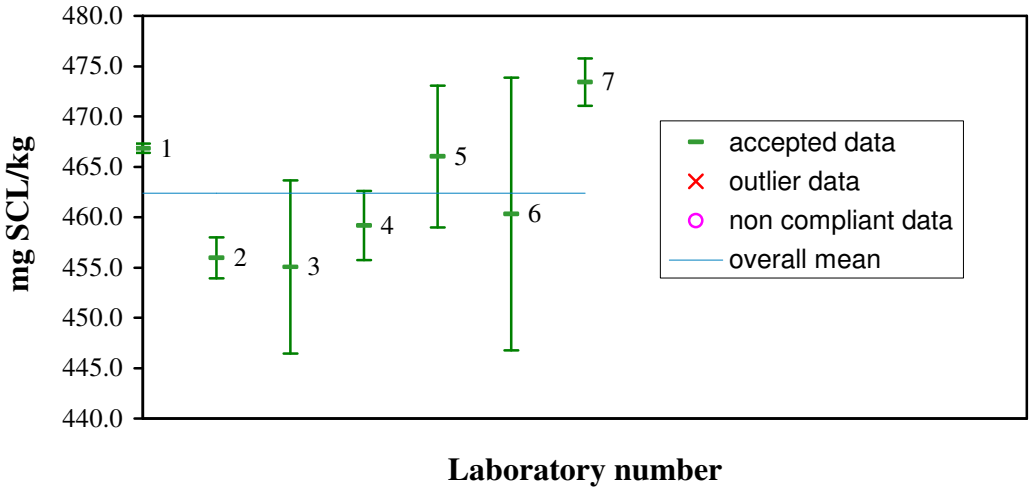

Figure F 72. Laboratory means and ranges of determined SCL amounts in sample 10

ANNEX F – STATISTICALLY EVALUATED RESULTS

Table G 1. Statistical evaluation of ACS-K amounts accepted on technical and statistical grounds

Sweetener	**ACS-K**			
Year of collaborative trial	**2007**			
Sample (Beverages)	**2**	**3**	**4**	**5**
Number of laboratories	7	7	7	7
Number of outliers	0	0	0	0
Identity of outlying laboratories				
Reason for removal				
Number of accepted laboratories	7	7	7	7
Mean value [mg/L]	38.3	266.6	324.1	383.5
True value [mg/L]	42.1	282.5	354.2	421.7
Recovery [%]	90.9	94.4	91.5	90.9
Repeatability standard deviation s_r [mg/L]	2.6	6.0	10.6	9.2
Repeatability relative standard deviation RSD_r [%]	6.9	2.3	3.3	2.4
Repeatability limit r [mg/L]	7.4	16.9	29.7	25.7
Reproducibility standard deviation s_R [mg/L]	4.2	15.6	20.1	19.3
Reproducibility relative standard deviation RSD_R [%]	10.9	5.9	6.2	5.0
Reproducibility limit R [mg/L]	11.6	43.8	56.2	54.0
HorRAT value = RSD_R/predicted RSD_R (1)	1.2	0.9	0.9	0.8
Sample (Canned fruits)	**7**	**8**	**9**	**10**
Number of laboratories	7	7	7	7
Number of outliers	0	0	1	0
Identity of outlying laboratories			6	
Reason for removal			Co (2)	
Number of accepted laboratories	7	7	6	7
Mean value [mg/kg]	38.4	259.2	323.0	391.3
True value [mg/kg]	36.5	265.6	338.8	410.0
Recovery [%]	105.1	97.6	95.3	95.4
Repeatability standard deviation s_r [mg/kg]	2.7	9.1	4.1	11.4
Repeatability relative standard deviation RSD_r [%]	6.9	3.5	1.3	2.9
Repeatability limit r [mg/kg]	7.4	25.6	11.5	32.0
Reproducibility standard deviation s_R [mg/kg]	5.7	12.7	16.0	17.5
Reproducibility relative standard deviation RSD_R [%]	14.8	4.9	4.9	4.5
Reproducibility limit R [mg/kg]	15.9	35.5	44.8	49.1
HorRAT value = RSD_R/predicted RSD_R (1)	1.6	0.7	0.7	0.7

(1) predicted $RSD_R = 2C^{-0.15}$; C = estimated mean concentration; (2) Co = Cochran

Table G 2. Statistical evaluation of ALI amounts accepted on technical and statistical grounds

Sweetener	**ALI**			
Year of collaborative trial	**2007**			
Sample (Beverages)	**2**	**3**	**4**	**5**
Number of laboratories	7	7	7	7
Number of outliers	0	0	0	0
Identity of outlying laboratories				
Reason for removal				
Number of accepted laboratories	7	7	7	7
Mean value [mg/L]	31.1	69.1	96.4	114.5
True value [mg/L]	36.5	80.5	102.6	122.2
Recovery [%]	85.3	85.8	93.9	93.7
Repeatability standard deviation s_r [mg/L]	2.2	2.8	2.3	1.5
Repeatability relative standard deviation RSD_r [%]	7.1	4.0	2.3	1.3
Repeatability limit r [mg/L]	6.2	7.7	6.3	4.3
Reproducibility standard deviation s_R [mg/L]	3.0	7.5	2.6	3.9
Reproducibility relative standard deviation RSD_R [%]	9.5	10.9	2.7	3.4
Reproducibility limit R [mg/L]	8.3	21.1	7.2	11.0
HorRAT value = RSD_R/predicted RSD_R [(1)]	1.0	1.3	0.3	0.4
Sample (Canned fruits)	**7**	**8**	**9**	**10**
Number of laboratories	7	7	7	7
Number of outliers	0	0	0	0
Identity of outlying laboratories				
Reason for removal				
Number of accepted laboratories	7	7	7	7
Mean value [mg/kg]	36.0	113.7	142.5	175.2
True value [mg/kg]	34.6	116.1	145.1	175.5
Recovery [%]	104.2	97.9	98.3	99.8
Repeatability standard deviation s_r [mg/kg]	3.5	2.5	3.1	6.4
Repeatability relative standard deviation RSD_r [%]	9.7	2.2	2.2	3.7
Repeatability limit r [mg/kg]	9.7	6.9	8.8	18.0
Reproducibility standard deviation s_R [mg/kg]	3.5	3.8	4.4	7.5
Reproducibility relative standard deviation RSD_R [%]	9.7	3.3	3.1	4.3
Reproducibility limit R [mg/kg]	9.7	10.6	12.3	21.1
HorRAT value = RSD_R/predicted RSD_R [(1)]	1.0	0.4	0.4	0.6

[(1)] predicted $RSD_R = 2C^{-0.15}$; C = estimated mean concentration

Table G 3. Statistical evaluation of ASP amounts accepted on technical and statistical grounds

Sweetener	**ASP**			
Year of collaborative trial	**2007**			
Sample (Beverages)	**2**	**3**	**4**	**5**
Number of laboratories	7	7	7	7
Number of outliers	1	0	0	1
Identity of outlying laboratories	3			5
Reason for removal	SG [(3)]			Co [(2)]
Number of accepted laboratories	6	7	7	6
Mean value [mg/L]	38.1	485.1	584.8	702.0
True value [mg/L]	42.0	485.0	605.0	720.3
Recovery [%]	90.7	100.0	96.7	97.5
Repeatability standard deviation s_r [mg/L]	1.9	9.5	5.0	5.8
Repeatability relative standard deviation RSD_r [%]	4.9	1.9	0.9	0.8
Repeatability limit r [mg/L]	5.2	26.5	14.1	16.2
Reproducibility standard deviation s_R [mg/L]	6.1	33.3	30.9	23.5
Reproducibility relative standard deviation RSD_R [%]	16.0	6.9	5.3	3.4
Reproducibility limit R [mg/L]	17.1	93.3	86.6	65.9
HorRAT value = RSD_R/predicted RSD_R [(1)]	1.7	1.1	0.9	0.6
Sample (Canned fruits)	**7**	**8**	**9**	**10**
Number of laboratories	7	7	7	7
Number of outliers	1	0	2	1
Identity of outlying laboratories	3		4, 6	3
Reason for removal	SG [(3)]		Co [(2)]	Co [(2)]
Number of accepted laboratories	6	7	5	6
Mean value [mg/kg]	37.2	739.8	951.9	1120.2
True value [mg/kg]	37.3	752.1	967.8	1171.1
Recovery [%]	99.9	98.4	98.4	95.6
Repeatability standard deviation s_r [mg/kg]	3.6	16.5	4.5	13.5
Repeatability relative standard deviation RSD_r [%]	9.7	2.2	0.5	1.2
Repeatability limit r [mg/kg]	10.1	46.3	12.5	37.8
Reproducibility standard deviation s_R [mg/kg]	3.6	29.3	27.5	31.7
Reproducibility relative standard deviation RSD_R [%]	9.7	4.0	2.9	2.8
Reproducibility limit R [mg/kg]	10.1	82.0	77.1	88.8
HorRAT value = RSD_R/predicted RSD_R [(1)]	1.0	0.7	0.5	0.5

[(1)] predicted $RSD_R = 2C^{-0.15}$; C = estimated mean concentration; [(2)] Co = Cochran; [(3)] SG = Single Grubbs

Table G 4. Statistical evaluation of CYC amounts accepted on technical and statistical grounds

Sweetener	**CYC**			
Year of collaborative trial	**2007**			
Sample (Beverages)	**2**	**3**	**4**	**5**
Number of laboratories	7	7	7	7
Number of outliers	0	0	0	0
Identity of outlying laboratories				
Reason for removal				
Number of accepted laboratories	7	7	7	7
Mean value [mg/L]	28.3	248.9	256.8	307.2
True value [mg/L]	36.9	239.0	252.7	300.8
Recovery [%]	76.8	104.1	101.6	102.1
Repeatability standard deviation s_r [mg/L]	1.2	6.6	3.6	5.9
Repeatability relative standard deviation RSD_r [%]	4.4	2.6	1.4	1.9
Repeatability limit r [mg/L]	3.5	18.4	10.2	16.5
Reproducibility standard deviation s_R [mg/L]	5.8	15.4	14.0	15.5
Reproducibility relative standard deviation RSD_R [%]	20.6	6.2	5.5	5.0
Reproducibility limit R [mg/L]	16.3	43.1	39.2	43.4
HorRAT value = RSD_R/predicted RSD_R [(1)]	2.1	0.9	0.8	0.7
Sample (Canned fruits)	**7**	**8**	**9**	**10**
Number of laboratories	7	7	7	7
Number of outliers	0	1	0	1
Identity of outlying laboratories		3		5
Reason for removal		Co [(2)]		Co [(2)]
Number of accepted laboratories	7	6	7	6
Mean value [mg/kg]	27.5	749.7	924.7	1100.6
True value [mg/kg]	32.2	752.6	968.8	1172.3
Recovery [%]	85.2	99.6	95.5	93.9
Repeatability standard deviation s_r [mg/kg]	4.4	7.0	14.5	12.7
Repeatability relative standard deviation RSD_r [%]	16.1	0.9	1.6	1.2
Repeatability limit r [mg/kg]	12.4	19.6	40.5	35.6
Reproducibility standard deviation s_R [mg/kg]	4.9	30.9	44.4	37.2
Reproducibility relative standard deviation RSD_R [%]	17.9	4.1	4.8	3.4
Reproducibility limit R [mg/kg]	13.7	86.5	124.2	104.3
HorRAT value = RSD_R/predicted RSD_R [(1)]	1.8	0.7	0.8	0.6

[(1)] predicted $RSD_R = 2C^{-0.15}$; C = estimated mean concentration; [(2)] Co = Cochran

Table G 5. Statistical evaluation of DUL amounts accepted on technical and statistical grounds

Sweetener	**DUL**			
Year of collaborative trial	**2007**			
Sample (Beverages)	**2**	**3**	**4**	**5**
Number of laboratories	7	7	7	7
Number of outliers	0	0	0	0
Identity of outlying laboratories				
Reason for removal				
Number of accepted laboratories	7	7	7	7
Mean value [mg/L]	55.0	79.6	95.7	115.1
True value [mg/L]	60.7	81.3	101.8	121.1
Recovery [%]	90.6	98.0	94.0	95.0
Repeatability standard deviation s_r [mg/L]	1.4	2.9	1.0	1.5
Repeatability relative standard deviation RSD_r [%]	2.5	3.7	1.0	1.3
Repeatability limit r [mg/L]	3.8	8.2	2.8	4.3
Reproducibility standard deviation s_R [mg/L]	3.3	3.9	5.2	5.2
Reproducibility relative standard deviation RSD_R [%]	6.1	4.9	5.5	4.6
Reproducibility limit R [mg/L]	9.4	10.9	14.7	14.7
HorRAT value = RSD_R/predicted RSD_R [(1)]	0.7	0.6	0.7	0.6
Sample (Canned fruits)	**7**	**8**	**9**	**10**
Number of laboratories	7	7	7	7
Number of outliers	1	0	0	0
Identity of outlying laboratories	6			
Reason for removal	NC [(2)]			
Number of accepted laboratories	6	7	7	7
Mean value [mg/kg]	49.8	111.0	141.7	172.6
True value [mg/kg]	50.2	114.3	145.7	176.3
Recovery [%]	99.3	97.0	97.3	97.9
Repeatability standard deviation s_r [mg/kg]	3.7	3.0	3.6	3.1
Repeatability relative standard deviation RSD_r [%]	7.4	2.7	2.5	1.8
Repeatability limit r [mg/kg]	10.3	8.4	10.1	8.6
Reproducibility standard deviation s_R [mg/kg]	4.3	4.8	4.7	5.4
Reproducibility relative standard deviation RSD_R [%]	8.6	4.3	3.3	3.1
Reproducibility limit R [mg/kg]	12.0	13.4	13.1	15.2
HorRAT value = RSD_R/predicted RSD_R [(1)]	1.0	0.5	0.4	0.4

[(1)] predicted $RSD_R = 2C^{-0.15}$; C = estimated mean concentration; [(2)] NC = Non compliant data

Table G 6. Statistical evaluation of NEO amounts accepted on technical and statistical grounds

Sweetener	**NEO**			
Year of collaborative trial	**2007**			
Sample (Beverages)	**2**	**3**	**4**	**5**
Number of laboratories	7	7	7	7
Number of outliers	0	0	0	0
Identity of outlying laboratories				
Reason for removal				
Number of accepted laboratories	7	7	7	7
Mean value [mg/L]	37.6	77.9	97.2	115.3
True value [mg/L]	37.5	80.5	102.2	121.7
Recovery [%]	100.1	96.8	95.1	94.7
Repeatability standard deviation s_r [mg/L]	0.9	1.9	2.4	2.8
Repeatability relative standard deviation RSD_r [%]	2.3	2.4	2.4	2.4
Repeatability limit r [mg/L]	2.4	5.2	6.7	7.7
Reproducibility standard deviation s_R [mg/L]	2.4	4.6	4.8	5.2
Reproducibility relative standard deviation RSD_R [%]	6.4	5.9	5.0	4.5
Reproducibility limit R [mg/L]	6.8	12.9	13.5	14.4
HorRAT value = RSD_R/predicted RSD_R (1)	0.7	0.7	0.6	0.6
Sample (Canned fruits)	**7**	**8**	**9**	**10**
Number of laboratories	7	7	7	7
Number of outliers	0	0	0	0
Identity of outlying laboratories				
Reason for removal				
Number of accepted laboratories	7	7	7	7
Mean value [mg/kg]	37.3	116.2	140.6	173.7
True value [mg/kg]	36.2	118.3	145.4	175.9
Recovery [%]	103.0	98.2	96.7	98.7
Repeatability standard deviation s_r [mg/kg]	1.3	3.6	2.2	4.8
Repeatability relative standard deviation RSD_r [%]	3.5	3.1	1.6	2.8
Repeatability limit r [mg/kg]	3.6	10.1	6.2	13.5
Reproducibility standard deviation s_R [mg/kg]	2.2	6.3	7.5	7.7
Reproducibility relative standard deviation RSD_R [%]	5.9	5.4	5.3	4.5
Reproducibility limit R [mg/kg]	6.2	17.6	21.1	21.7
HorRAT value = RSD_R/predicted RSD_R (1)	0.6	0.7	0.7	0.6

(1) predicted $RSD_R = 2C^{-0.15}$; C = estimated mean concentration

Table G 7. Statistical evaluation of NHDC amounts accepted on technical and statistical grounds

Sweetener	**NHDC**			
Year of collaborative trial	**2007**			
Sample (Beverages)	**2**	**3**	**4**	**5**
Number of laboratories	7	7	7	7
Number of outliers	0	0	0	0
Identity of outlying laboratories				
Reason for removal				
Number of accepted laboratories	7	7	7	7
Mean value [mg/L]	31.4	42.8	51.0	59.3
True value [mg/L]	36.7	40.2	50.7	60.4
Recovery [%]	85.5	106.4	100.5	98.2
Repeatability standard deviation s_r [mg/L]	3.3	1.7	1.8	2.6
Repeatability relative standard deviation RSD_r [%]	10.6	3.9	3.5	4.4
Repeatability limit r [mg/L]	9.3	4.7	4.9	7.3
Reproducibility standard deviation s_R [mg/L]	9.0	6.7	4.4	5.2
Reproducibility relative standard deviation RSD_R [%]	28.5	15.6	8.7	8.8
Reproducibility limit R [mg/L]	25.1	18.7	12.4	14.5
HorRAT value = RSD_R/predicted RSD_R [(1)]	3.0	1.7	1.0	1.0
Sample (Canned fruits)	**7**	**8**	**9**	**10**
Number of laboratories	7	7	7	7
Number of outliers	0	1	0	0
Identity of outlying laboratories		5		
Reason for removal		Co [(2)]		
Number of accepted laboratories	7	6	7	7
Mean value [mg/kg]	35.3	40.5	49.8	59.3
True value [mg/kg]	33.4	37.5	48.9	59.1
Recovery [%]	105.6	108.0	102.0	100.4
Repeatability standard deviation s_r [mg/kg]	2.2	1.0	2.0	2.3
Repeatability relative standard deviation RSD_r [%]	6.1	2.5	4.0	3.9
Repeatability limit r [mg/kg]	6.1	2.8	5.6	6.5
Reproducibility standard deviation s_R [mg/kg]	4.4	4.6	3.3	5.5
Reproducibility relative standard deviation RSD_R [%]	12.4	11.5	6.6	9.2
Reproducibility limit R [mg/kg]	12.2	13.0	9.2	15.3
HorRAT value = RSD_R/predicted RSD_R [(1)]	1.3	1.3	0.7	1.1

[(1)] predicted $RSD_R = 2C^{-0.15}$; C = estimated mean concentration; [(2)] Co = Cochran

Table G 8. Statistical evaluation of SAC amounts accepted on technical and statistical grounds

Sweetener	**SAC**			
Year of collaborative trial	**2007**			
Sample (Beverages)	**2**	**3**	**4**	**5**
Number of laboratories	7	7	7	7
Number of outliers	0	1	0	1
Identity of outlying laboratories		6		6
Reason for removal		Co [(2)]		Co [(2)]
Number of accepted laboratories	7	6	7	6
Mean value [mg/L]	36.2	60.1	74.1	87.6
True value [mg/L]	40.3	65.2	80.9	96.3
Recovery [%]	89.8	92.1	91.5	91.0
Repeatability standard deviation s_r [mg/L]	1.4	1.7	3.0	1.0
Repeatability relative standard deviation RSD_r [%]	3.8	2.8	4.0	1.1
Repeatability limit r [mg/L]	3.9	4.7	8.3	2.7
Reproducibility standard deviation s_R [mg/L]	4.0	2.8	4.9	5.2
Reproducibility relative standard deviation RSD_R [%]	11.1	4.6	6.6	5.9
Reproducibility limit R [mg/L]	11.3	7.7	13.6	14.5
HorRAT value = RSD_R/predicted RSD_R [(1)]	1.2	0.5	0.8	0.7
Sample (Canned fruits)	**7**	**8**	**9**	**10**
Number of laboratories	7	7	7	7
Number of outliers	0	0	0	0
Identity of outlying laboratories				
Reason for removal				
Number of accepted laboratories	7	7	7	7
Mean value [mg/kg]	44.3	151.9	193.4	235.3
True value [mg/kg]	38.0	150.0	194.0	234.8
Recovery [%]	116.7	101.3	99.7	100.2
Repeatability standard deviation s_r [mg/kg]	2.4	4.0	4.3	6.7
Repeatability relative standard deviation RSD_r [%]	5.5	2.7	2.2	2.9
Repeatability limit r [mg/kg]	6.8	11.3	12.0	18.8
Reproducibility standard deviation s_R [mg/kg]	8.4	10.6	13.5	15.0
Reproducibility relative standard deviation RSD_R [%]	19.0	7.0	7.0	6.4
Reproducibility limit R [mg/kg]	23.6	29.6	37.7	42.0
HorRAT value = RSD_R/predicted RSD_R [(1)]	2.1	0.9	1.0	0.9

[(1)] predicted $RSD_R = 2C^{-0.15}$; C = estimated mean concentration; [(2)] Co = Cochran

Table G 9. Statistical evaluation of SCL amounts accepted on technical and statistical grounds

Sweetener	**SCL**			
Year of collaborative trial	**2007**			
Sample (Beverages)	**2**	**3**	**4**	**5**
Number of laboratories	7	7	7	7
Number of outliers	0	0	0	0
Identity of outlying laboratories				
Reason for removal				
Number of accepted laboratories	7	7	7	7
Mean value [mg/L]	36.8	245.1	282.9	346.8
True value [mg/L]	38.9	251.8	302.6	360.3
Recovery [%]	94.7	97.3	93.5	96.3
Repeatability standard deviation s_r [mg/L]	1.4	3.8	2.7	8.2
Repeatability relative standard deviation RSD_r [%]	3.7	1.5	0.9	2.4
Repeatability limit r [mg/L]	3.8	10.6	7.4	22.9
Reproducibility standard deviation s_R [mg/L]	5.2	10.1	16.2	13.3
Reproducibility relative standard deviation RSD_R [%]	14.2	4.1	5.7	3.8
Reproducibility limit R [mg/L]	14.7	28.2	45.3	37.4
HorRAT value = RSD_R/predicted RSD_R [(1)]	1.5	0.6	0.8	0.6
Sample (Canned fruits)	**7**	**8**	**9**	**10**
Number of laboratories	7	7	7	7
Number of outliers	0	0	0	0
Identity of outlying laboratories				
Reason for removal				
Number of accepted laboratories	7	7	7	7
Mean value [mg/kg]	35.3	306.1	380.2	462.4
True value [mg/kg]	34.6	313.1	388.2	469.7
Recovery [%]	102.1	97.7	98.0	98.4
Repeatability standard deviation s_r [mg/kg]	2.2	7.4	8.5	9.7
Repeatability relative standard deviation RSD_r [%]	6.3	2.4	2.2	2.1
Repeatability limit r [mg/kg]	6.3	20.6	23.8	27.1
Reproducibility standard deviation s_R [mg/kg]	3.8	8.7	10.4	9.7
Reproducibility relative standard deviation RSD_R [%]	10.9	2.8	2.7	2.1
Reproducibility limit R [mg/kg]	10.8	24.4	29.1	27.1
HorRAT value = RSD_R/predicted RSD_R [(1)]	1.2	0.4	0.4	0.3

[(1)] predicted $RSD_R = 2C^{-0.15}$; C = estimated mean concentration

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Abstract

A collaborative trial was conducted to validate an analytical method for the simultaneous determination of nine intense sweeteners, i.e., acesulfame-K, alitame, aspartame, cyclamic acid, dulcin, neotame, neohesperidine dihydrochalcone, saccharin and sucralose in carbonated and non-carbonated soft drinks, and canned or bottled fruits. 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. Trueness, expressed in terms of recovery rates, was demonstrated in most cases by values ranging from 90 to 108 %. 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 less than 1.1 suggested for all sweeteners and matrices tested good performance of the method.

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